PHARMACOKINETIC STUDY AND ASSESSMENT OF CYTOKINE EXPRESSION LEVEL ELICITED BY A NOVEL *Acinetobacter baumannii* PHAGE IN MOUSE MODEL

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RECOMMENDATION

This is to certify that **Mr. Madhav Regmi** has successfully completed his dissertation work entitled **"PHARMACOKINETIC STUDY AND ASSESSMENT OF CYTOKINE EXPRESSION LEVEL ELICITED BY A NOVEL *Acinetobacter baumannii* PHAGE IN MOUSE MODEL"** under our supervision.

This thesis work was performed for the partial fulfillment for award of Master of Science in Biotechnology under the course code BT 621. The result presented here is his original findings. We, hereby, recommend this thesis for final evaluation.

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CERTIFICATE OF EVALUATION

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Dedicated to My Parents

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ACRONYMS

ABR	: Antibiotic Resistant Bacteria
AST	: Antibiotic Susceptibility Test
BLAST	: Basic Local Alignment Search Tool
CDBT	: Central Department of Biotechnology
CFU	: Colony Forming Units
DDCT	: Delta Delta Cycle Threshold
DLAA	: Double Layer Agar Plaque Assay
DNA	: Deoxy Ribonucleic Acid
dsDNA	: Double Stranded Deoxy Ribonucleic Acid
dsRNA	: Double Stranded Deoxy Ribonucleic Acid
EDTA	: Ethylene Di-amine Tetra acetic acid
EPA	: Environmental Protection Agency
FDA	: Food and Drug Administration
gDNA	: genomic Deoxy Ribonucleic Acid
GRAS	: Generally Recognized as Safe
ICTV	: International Committee for Taxonomy of Viruses
KDa	: Kilodalton
Kbp	: Kilo Base Pair
LB	: Luria Bertani Broth
MDR	: Multi Drug Resistant
MEGA	: Molecular Evolutionary Genetic Analysis
mm	: Millimeter
MOI	: Multiplicity of Infection
MRSA	: Methicillin-Resistant Staphylococcus Aureus
NCBI	: National Center of Biotechnology Information
ng	: Nanogram

nm	: Nanometer
OD	: Optical Density
PBS	: Phosphate Buffer Saline
PCR	: Polymerase Chain Reaction
PFU	: Plaque Forming Units
RNA	: Ribo Nucleic Acid
Rpm	: Revolutions per Minute
SDS-PAGE	: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SM	: Sodium Chloride and Magnesium Sulfate
ssDNA	: Single Stranded Deoxy Ribonucleic Acid
ssRNA	: Single Stranded Ribonucleic Acid
TSA	: Tryptic Soya Agar
UPGMA	: Unweighted Pair Group Method with Arithmetic mean

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ABSTRACT

Pharmacokinetic study and assessment of cytokine expression level elicited by a novel *Acinetobacter baumannii* phage in mouse model

Introduction: Emergence of antimicrobial drug resistance in pathogenic bacteria has become critical problem worldwide. *Acinetobacter baumannii* is one of the major pathogens causing various nosocomial infections. It is listed as is a Priority-1 critical pathogen for the research and development of new antimicrobial agents by WHO in 2017. Use of bacteriophage to control antibiotic resistant infections in human is being explored in Europe and America in recent years. However, data about the dose and the timing required to ensure a successful treatment remain mostly empirical. Effective bacteriophage therapy requires awareness of various novel kinetic phenomena not known in conventional drug treatments. This study aimed to isolate lytic bacteriophage, characterize physiochemically and molecularly. Furthermore, we investigated pharmacokinetics and assessed pro-inflammatory cytokine expression level elicited by the phage in the mouse model.

Methodology: Six different strains of multidrug resistant *Acinetobacter baumannii* were collected from the hospital and they were molecularly identified by sequencing 16S rRNA gene. Carbapenem resistance gene blaNDM-1 was amplified by PCR. Sewage samples were collected from different rivers of Kathmandu valley and bacteriophage were isolated following Double Agar Assay method. Among the isolated bacteriophages one potent phage was characterized morphologically and physiochemically. Burst size was obtained from one step growth curve. Whole genome sequence analysis was done using illumina platform. Pharmacokinetics of bacteriophage was studied in mice model and cytokine expression in mice during the application of phage (200 µl, 1.02×10^8 PFU/ml) was calculated using Delta Delta Cycle Threshold method.

Results: Bacterial strains were molecularly confirmed to be *Acinetobacter baumannii*. The 16S rRNA gene segments were submitted to NCBI gene bank and accession number were received. The host (A56) was found carbapenem resistant and blaNDM-1 positive. Total four lytic phages against *Acinetobacter baumannii* were isolated from different sewage samples, one of the most potent phages (phage TU_A56) was morphologically and physiochemically characterized. Burst size of the phage was found to be 304 virions per bacterium. Similarly, the phage was found to be stable up to 60°C and wide range of pH 3-12, with optimal pH 7 and showed multiple host range. Transmission electron micrograph revealed icosahedral symmetry with a short tail and the phage belonged to order Caudovirales and family Myoviridae. From whole genome analysis of phage, it was evident that the phage genome is

free of toxic genes. The PHASTER annotation of the genome showed 478 intact proteins and 20 incomplete proteins. The GC content of phage genome was found to be 49.5 % and the circular genome map created by PHASTER showed the genome size of 12310 kbp. From the pharmacokinetic study the phage elimination rate constant was found to be 0.18339 per hour and 0.233064 per hour, the volume of distribution was found to be 15.81357 ml and 12.78292 ml and elimination half-life was found 3.7803 hour and 2.973437 hour for oral and intraperitoneal dose respectively. The phage distribution was found to be rapid in IP route (within four hours of administration) as compared to oral route which took 8 hours to disseminate inside mouse body parts (Liver Kidney Spleen Blood). Phage clearance occurred gradually after 4 hours and 8 hours of IP and oral administration respectively. The phage was completely cleared after48 hours and 72 hours of IP and oral administration respectively. The pro inflammatory cytokine IL-6 was expressed up to 0.47-fold in case of oral dose whereas 0.27-fold in case of IP dose and TNFα gene could not be amplified by reverse transcription.

Conclusion: The bacteriophage (phage TU_A56) showed effective lytic capability, multiple host range and stability in wide range of temperature and pH. The phage genome was free of toxic gene. The phage did not cause significant increase in IL-6 expression in both oral and IP dose. Moreover, the phage persisted inside the mice body minimum of 48 hours and maximum of 72 hours after single IP and oral dose respectively. Thus, we can conclude that the phage TU_A56 is a potent candidate for phage therapy. The pharmacokinetics study and Cytokine expression analysis helps in understanding the different dimensions of phage therapy.

Keywords: Phage Therapy, Multidrug resistant, *Acinetobacter baumannii*, cytokine expression, whole genome sequencing.

Chapter One
INTRODUCTION

1.1. Background

Emergence of antimicrobial drug resistance in pathogenic bacteria has become critical problem worldwide. A growing number of infections are becoming harder to treat as the antibiotics used to treat them have become less effective due to the resistance mechanism developed by bacteria. In developing countries like Nepal, the result of antibiotics resistance is even more catastrophic because of the lack of knowledge of patients, unguided prophylactic use of antibiotics and use of antibiotic in animal husbandry (Ayukekbong, Ntemgwa *et al.*, 2017). Before 19th century infectious disease was the main cause of death all over the world. After the discovery of antibiotic in 1928 by Fleming, the death due to infectious disease decreased and the life expectancy of people increased. The extensive and haphazard use of antibiotics has led to the condition where most of the antibiotics discovered till date no longer can kill the pathogen due to the mechanism developed by the pathogen to either tolerate or to resist the effect of these agents. As a result the infectious disease is once again on the top second list of cause of death all over the world (Martens & Demain, 2017). The number of antibiotic resistant pathogens is increasing day by day where as the number of new antibiotics discovered is very few as the new drug discovery is a complex process which can take up 12-15 years and costs about $1billion.(Hughes, Rees *et al.*, 2011; Ventola, 2015). So, the multidrug resistant superbugs are spreading all over the world.

Nearly 17 million people die annually due to the bacterial infections around the globe. In US only around 2 million people get infected with antibiotic resistant pathogen and among them 23000 die eventually (Martens & Demain, 2017). If the condition of a developed nation like the United States is such worse, we can imagine what is the condition of developing country like Nepal. In a study done in western Nepal the prevalence of MRSA among the patient was found 69.7%. Among 112 MRSA isolates, 45 (41%) were multi-drug resistant, conferring resistance to common antibiotics such as penicillin, amoxicillin, ampicillin, cotrimoxazole, and cephalexin. (Tiwari, Das *et al.*, 2009). In another study 41% of total 217 isolates were found multi drug resistant. The most prevalent organism were *E. coli* and ESBL producing *Citrobacter spp.* (Baral, Neupane *et al.*, 2012).

It is an undeniable fact that AMR is increasing continuously with the time, so everyone should be concerned about the consequences. Antibiotic resistance is not the result of actions of a single person or a single sector. Public, farmers, healthcare professionals, government and industries all are responsible for this situation in their own way. WHO has published

Prescription for action for every group responsible to minimize the spread of antimicrobial resistance. WHO in 2015 has started the Global Antimicrobial Resistance Surveillance System (GLASS) to support the global action plan on antimicrobial resistance. Despite all the efforts antibiotic resistance is on its peak and what we are doing is not enough to mitigate its effect. In scenario where WHO has already stated that **"The world is running out of antibiotics",** a fast cheap and reliable alternative to antibiotics is current necessity. Scientist from different sector are trying different alternatives such as herbal medicine, essential oils, heavy metals(Low, Kenward *et al.*, 2017), genetic engineering and bacteriophages to solve the problem of antimicrobial resistance. Among all the alternative option, bacteriophage has the longest history of being used an antimicrobial agent. In fact, bacteriophage was the only treatment available against the bacterial infection before the discovery of antibiotics. So, bacteriophage can be the potent solution to battle antibiotics resistance as phages are the natural predators of pathogenic bacteria and kill even MDR ones and are specific towards the certain species alike the antibiotics.

1.1.1. Bacteriophage

As the name "Bacteriophage" suggests "bacteria "and the Greek word" phagein" to devour, they are the type of virus that eat or lyse the bacteria specifically. Bacteriophage are obligatory parasite of bacteria and they need a host bacterium to replicate itself. It was discovered independently by Frederick Twort, a medically trained bacteriologist from England in 1915, and Félix d'Hérelle a French-Canadian microbiologist two years later in 1917. The name "bacteriophage" was also proposed by d'Herelle himself (Duckworth, 1976). Structurally, phages are made up of an outer protein coat, Nucleic acid (either DNA or RNA but never both) and a tail with structures such as; collar, sheath, baseplate, tail fiber and pin. Generally, the bacteriophages are named after their host because they are specific to one or limited number of bacterial host strains. Bacteriophages also called as phage are ubiquitous organism, probably the largest biomass on earth with an estimated total number of 10^{32} bacteriophages on the planet (Wittebole, De Roock *et al.*, 2014). Phages are believed to have a great role in ecology. In most of the environment they are high in number then

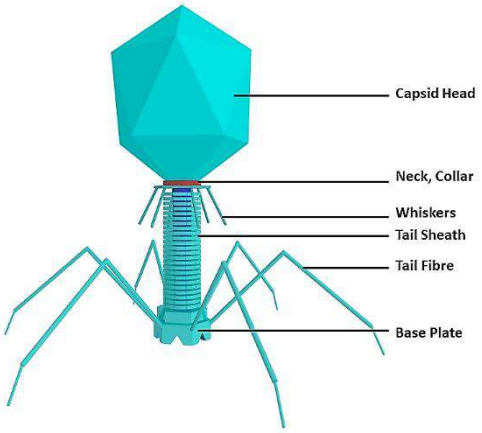

Figure: 1. 1 Schematic diagram of Schematic diagram of bacteriophage

bacteria. Phage help in shaping the community of bacteria by killing, transforming the metabolic ability via gene transfer and by terminating the bacterial blooms.

Generally, phage consists of nucleic acid molecule also called as genome surrounded by coat known as capsid or head. The nucleic acid can be either DNA or RNA but not both and it can exist in various forms such as single stranded and double stranded. The head or capsid is made up of morphological subunits called protomers. Many but not all phages have tails, length of tail varies among family of phages. Phages have contractile sheath which surrounds tail and contracts during infection of the bacterium in some phages. Some phages also have a base plate and one or more tail fibers are attached to the tail end. Phages are specific to one or a limited number of bacterial host strains thus, they are generally named after the bacteria group, strain, or species they infect. For example, the phage that infect the bacterium Escherichia coli are called coliphage in general named as T "even" phages T2, T4 and T6 etc. Most of the phages are tailed bacteriophage (Order: Caudovirales), accounting for 96% of all phages present on earth and are the oldest known virus group (Ackermann, 2009). The size of most phages ranges from 22 nm – 200 nm in length. The largest bacteriophage known bacteriophage T4 is about 200 nm long and about 80 – 100 nm wide. Tailed bacteriophages with genomes larger than 200 kbp are classified as Jumbo phages (Yuan & Gao, 2017).

1.1.2. Bacteriophage History

The English chemist Ernest Hankin in 1896 had made the early presumptions of lytic activity of phage present in the water of Ganges river in his article “The bactericidal action of waters of Jumna and Ganges on the cholera microbe”(Stephen T Abedon, Thomas-Abedon *et al.*, 2011). He mentioned that a heat-sensitive unknown substance which passed through fine porcelain filters was responsible for preventing the spread of cholera epidemics. About two decades after the Hankin’s finding, Frederick Twort, a medically trained bacteriologist from England in 1915 explained the interesting glassy transformation of the micrococcus colony. He summarized that the cause of glassy transformation was an infectious, filterable agent that killed bacteria and, in the process, multiplied itself. Unfortunately, Twort's paper went un-recognized until 26 March 1921(5 years, 3 months, and 22 days). Despite his observation, he couldn’t state that the glassy transformation was due to the virus of bacteria. In 1910 when d’Herelle was in Mexico there was locust invasion, the Indians reported to d'Herelle that in a certain place the ground was scattered with the carcasses of these insects. After examination he found out that it was the death of insects was caused by the coccobacilli in diarrheal liquid. When he cultured the bacteria, he observed circular clear areas which he named as '*taches vierges*' (**clear spots**), these spots were two or three millimeters in

diameter, flecking the cultures grown on agar. He couldn't see anything in the slide prepared from the clear spot on the plate so he generalized that the agent which caused the formation of the clear spots must be able to pass a porcelain filter of the Chamberland type, which will retain all bacteria. Later, in March 1915, during world war-I, while searching for a solution to locusts in Tunisia d'Herelle re-observed the phenomena, to investigate their significance he showed them to Charles Nicolle, the director of the Pasteur Institute, he suggested that it might be the sign of a filtrable virus carried by Coccobacilli (Duckworth, 1976). D'Herelle presented his findings in September 1917 during the meeting of the Academy of Sciences and they were published immediately. According to his reminiscence, the name "bacteriophage" was also proposed by d'Herelle himself. (Sulakvelidze, Alavidze *et al.*, 2001).

1.1.3. Bacteriophage Life cycle and classification

Life Cycle

Like any other viruses, phages are also obligatory parasites and need a host cellular machinery to reproduce. Based on the mode of replication, the phages are categorized into **lytic** and **lysogenic phage**. The first step in the replication process is attachment of phage particle to its host specifically via receptors present in host cell surface, this process gives a bacteriophage it's host specificity. This process is followed by the injection of the phage genome inside the host cell called as penetration. The following process slightly differs among the lytic and lysogenic phages. In case of **lytic phages**, after the injection of phage genomes, it takes over the cellular machinery of the host bacterium to express its genes and synthesizes viral proteins essential for the replication process and structural proteins (apsid protein) (Ofir & Sorek, 2018). After the synthesis of all the required proteins, the nucleic acid is assembled into a capsid forming new progeny phages and the virions emerge from the host cell by rupturing it with the help of phage

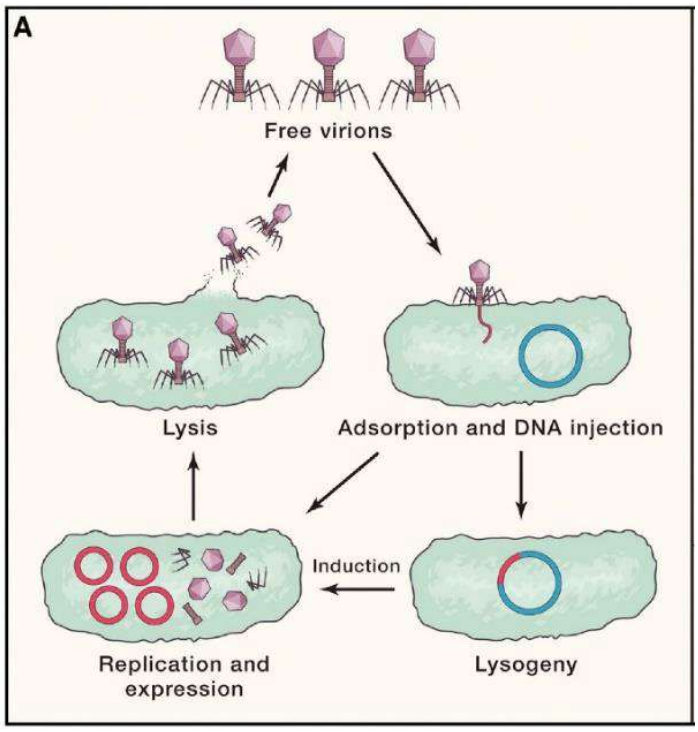

Figure: 1. 2 Phage life cycle

(Ofir & Sorek, 2018)

proteins called lysin. Whereas in case of **lysogenic phage**, after the injection of phage genome into a host cell, it gets incorporated into the host genome and rest as a prophage until its lysogenic conversion and the state is called lysogeny. Usually the lysogenic conversion occurs when the host encounters stress. The phage genome multiply along with the host genome and the daughter host cells are also capable of producing phage upon lysogenic conversion. Lysis of the bacterial cell after phage maturation occurs by several methods depending on the type of phage involved. Double stranded DNA containing phages produce endolysin an enzyme which digests peptidoglycan in the cell wall; it reaches the cell wall by the action of a second protein called holin which permeabilizes the cell membrane. Some phages also contain genes which code for two alternative lysis proteins which are thought to interfere with the cell membrane or wall. Single stranded DNA containing phages possess only one lysis protein which is thought to interrupt peptidoglycan synthesis. Filamentous phages (Inoviridae) can emerge from the infected bacterial cells through phage encoded channels in the bacterial cell wall. This means lysis does not take place and although the bacterium survives this process the growth rate is usually reduced (Wang, Smith *et al.*, 2000).

Classification of Bacteriophage

The latest scheme of classification of bacteriophage is given by the International Committee on Taxonomy of Viruses (ICTV). The ICTV provides data on virus taxonomy through the master species list (MSL), The ICTV provides data on virus taxonomy through the master species list (MSL), which currently lists 7 orders, 112 families, 610 genera and 3,704 species(M. J. Adams, Lefkowitz *et al.*, 2016). This system classifies bacteriophage according to the type of nucleic acid and virion morphology. More than 40 criteria are used for phage differentiation into genera and species (Novik, Ladutska *et al.*, 2011) .

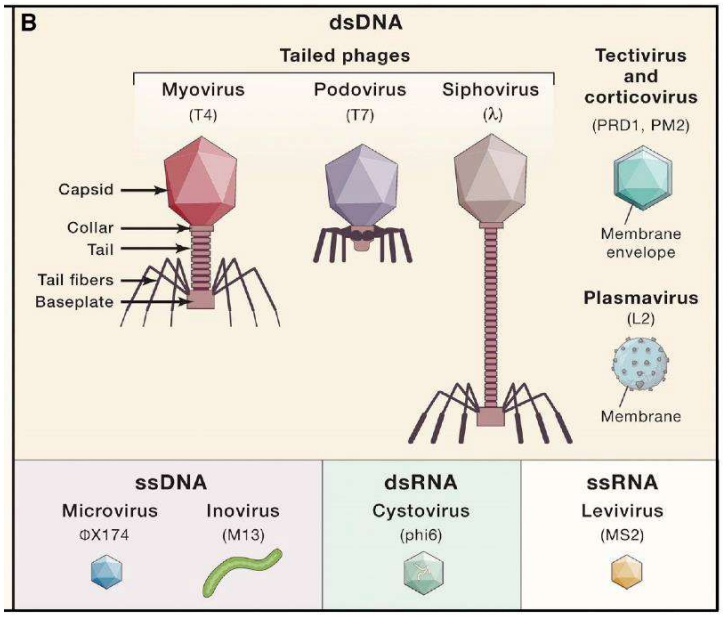

Figure: 1. 3 Phage taxonomy based on morphology and genome composition. A representative type phage for each taxonomical group is in parenthesis.

(Ofir & Sorek, 2018)

Generally, the parameters based on which phages are classified are genome type and the morphology of their virion, among which the former one gives more insights into the distinguishing features of phages. The phages containing larger genome are dsDNA phages and are member of virus order Caudovirales. Similarly, the phages having comparatively smaller ssDNA fall under family Microviridae. Likewise, phages having smaller ssRNA genome are member of family Leviviridae.

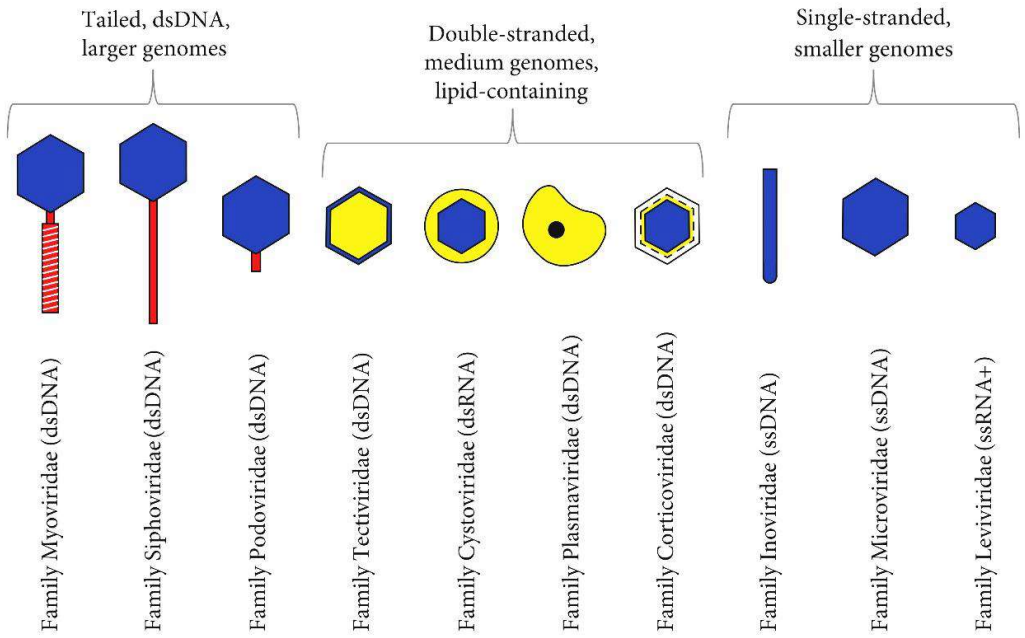

Figure: 1. 4 Bacteriophage families, morphologies, genome types, and relative genome sizes (Hyman & Abedon, 2012)

1.1.4. Phage Therapy

The term Phage Therapy (PT) is used to describe the use of bacteriophage in treatment of bacterial infection as a therapeutic agent. Bacteriophages are natural predators of bacteria, they kill bacteria in course of their replication. Phage therapy exploits this lytic nature of bacteriophage in treatment of bacterial infection. Bacteriophages has been used in the treatment of infection since their discovery. At first, the efficacy of bacteriophage as a drug was tested in animals such as pigs, cows and chickens. When the phage showed promising result in animal, scientist started treating humans using bacteriophage and the term phage therapy originated (Summers, 2012). Phage had been used to treat dysentery in a 12 years old boy and the infection among German and Japanese soldiers (Summers, 2001).

After successful treatment of various infections in human, the phage preparations were prepared and sold commercially by various companies such as Eli Lilly during 19th century (Sulakvelidze et al., 2001). Despite the commercial availability of phage preparation, the phage therapy was not admired from the beginning. This was due to the lack of information about the bacteriophage itself, the inconsistent result it produced in the treatment and the lack of reproducibility (Wittebole et al., 2014).After the Fleming's historic discovery of penicillin, antibiotics became popular due to their broad spectrum of killing and greater potency and phage therapy was neglected. However, the emergence of multi-drug resistant bacterial pathogens and their continuous spread in recent years has brought an urgency to search for a fast, reliable and economic alternative to antibiotics (Sulakvelidze, 2011). In recent years the phage therapy is gaining popularity due to AMR. Scientists are trying to implement the advancements of technology such as genetic engineering to give phage therapy a new perspective and to overcome the problems It had in past. Genetically engineered phage and purified phage endolysin had shown a remarkable effect against multidrug resistance pathogen which give a new direction to the phage therapy.

1.1.5. *Acinetobacter baumannii*

Acinetobacter baumannii is a gram-negative, strictly aerobic, nonfermenting, non-fastidious, nonmotile, catalase-positive, oxidase-negative bacterium. It is responsible for 2 – 10% of all Gram-negative hospital acquired infection (Joly-Guillou, 2005). According to Antibiotic-resistant priority pathogens list published by who on 27 February 2017, *A. baumannii* is a Priority-1 critical pathogen for R&D of new drug. It has emerged as an important nosocomial pathogen in last decade (Neonakis, Spandidos *et al.*, 2011). It causes skin and soft tissue infections, wound infections, urinary tract infections and secondary meningitis, Ventilator associated pneumonia and bloodstream infections among which Ventilator associated pneumonia and bloodstream infection are most common (Antunes, Visca *et al.*, 2014).

Acinetobacter is a diverse group of organisms that includes 17 named species and 15 species that have no valid names. *Acinetobacter baumannii* and the closely related species 3 and 13TU are the clinically important species. Some *A. baumannii* strains that are circulating in hospitals multidrug resistant (MDR) and can spread epidemically. A recent manifestation of MDR *A. baumannii* is its association with wound infection in severely injured soldiers. *A. baumannii* has an extraordinary capability to develop resistance against various antimicrobial agents and many resistance mechanisms have been identified in this organism. The treatment of *A. baumannii* infections is often obstruct by the multidrug resistance of the causative pathogen, Combination therapy is the last alternative in such cases (Dijkshoorn, Nemec *et al.*, 2007).

1.2. Rationale of The Study

Growing number of antimicrobial drug resistance in pathogenic bacteria has become critical problem worldwide. Multidrug resistant superbugs are leading cause of death in today's world. *Acinetobacter baumannii* is one of the major pathogens causing hospital acquired infections. It is listed as is a Priority-1 critical pathogen for R&D of new drug by WHO in 2017. To kill this nasty superbug and prevent spread of antimicrobial drug resistance among pathogens, fast, economic and reliable alternative is necessary.

Among all other alternatives, phages are the agent holding a history of killing several pathogens and saving lives since their discovery. In past, the use of phage as antimicrobial was limited due to the lack of knowledge and sophisticated instruments to study more about bacteriophage. With the advancement of technology in science, and the vigorous research in the field pf phage therapy, most of the doubts are clear now. So, the phage holds immense possibility in the battle against antimicrobial resistance.

In this study, we aimed to isolate bacteriophage against multi drug resistance *Acinetobacter baumannii*, characterize, pharmacokinetic study and assessment of pro inflammatory cytokines expression in mice model. This will be the first study in case of Nepal to my best knowledge. This study will indeed help to carry out further animal trials of phage therapy.

1.3. Objectives

1.3.1. General Objectives

Isolation, characterization and pharmacokinetic study of lytic bacteriophage against multi drug resistant *Acinetobacter baumannii* and assessment of pro-inflammatory cytokine expression level elicited by the phage in the mouse model.

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1.3.2. Specific Objectives

- Molecular identification of carbapenem resistant strains of the *Acinetobacter baumannii* by 16S rRNA gene sequencing and amplification of blaNDM gene.
- Isolation and purification of bacteriophage against MDR strains of *Acinetobacter baumannii* from different sewage samples of Kathmandu Valley.
- Morphological, physiochemical and molecular characterization of the phage.
- Assess the presence or absence of toxic genes by the whole genome sequence analysis of the phage.
- Pharmacokinetics of the phage in mouse model.
- Assessment of pro-inflammatory cytokine expression in mouse after oral and IP administration of phage.

1.4. Research Hypothesis

1.4.1. Null Hypothesis

H_0 1 = The rate of absorption, distribution and clearance of phage in mice is same in oral and IP dose.

H_0 2 = There is no significant difference in expression of cytokine (IL-6 and TNFα) level elicited by bacteriophages in phage treated and control mice.

1.4.2. Alternative Hypothesis

H_1 1 = The rate of absorption, distribution and clearance of phage is different in oral and IP dose.

H_1 2 = There is significant difference in expression of cytokine (IL-6 and TNFα) level elicited by bacteriophages in phage treated and control mice.

Chapter Two

LITERATURE REVIEW

2.1. Multi Drug Resistant *Acinetobacter baumannii*

Acinetobacter baumannii is a Gram-negative bacterium that is responsible for infections associated with high morbidity and mortality. *Acinetobacter baumannii* is the most common nosocomial human pathogen of the genus, and it is notorious for its ability to thrive desiccation and occurs in the environment, which favors transmission in healthcare settings (Wong, Nielsen *et al.*, 2017). CDC's National Healthcare Safety Network in 2009–2010 reported that *A. baumannii* was responsible for 1.8% of all healthcare-associated infections (HAIs). It is estimated that there are annual 1 million cases of *Acinetobacter* infections (Sievert, Ricks *et al.*, 2013) . Huge number of MDR Acinetobacter species were reported in Nepal in a study conducted by Mishra *et. al., those Acinetobacter* species consisted *Acinetobacter calcoaceticus baumannii* complex. It was found that 95% of *Acinetobacter* isolates were MDR, and 12.9% were ESBL-producer (Mishra, Rijal *et al.*, 2013). The Centers for Disease Control and Prevention (CDC) stated that multidrug resistant (MDR) *Acinetobacter* infection is a serious threat and there are 7300 MDR *Acinetobacter* infections in the United States per year, which cause 500 deaths (CDC, 2013). The most common *A. baumannii* infection are healthcare-associated pneumonia and bacteremia; however, other well-known infection types include skin and soft tissue infections, wound infections, urinary tract infections and secondary meningitis, Ventilator associated pneumonia and bloodstream infections among which Ventilator associated pneumonia and bloodstream infection are most common (Antunes et al., 2014).

There are numerous virulence properties of *Acinetobacter* spp. which include capsular polysaccharide enabling immune evasion, which allows for a high density of bacteria that causes lipopolysaccharide Toll-like receptor 4-mediated sepsis. However, the major factor for the consequences due to *Acinetobacter* infection is antibiotic resistance, which complicates effective drug selection process hindering the timely treatment (Munoz-Price & Weinstein, 2008). *Acinetobacter* has low intrinsic permeability to drugs due to reduced outer membrane porins, and it contains a major genomic island coding resistance which contains 45 resistance genes, enabling resistance to multiple classes of antibiotics (Wong et al., 2017). β-lactam antibiotics are drug of choice for *A. baumannii*, and β-lactam resistance (penicillins, cephalosporins and carbapenems) is caused by multiple mechanisms such as chromosomal mediated and plasmid or transposon encoded. Combination of Broad-spectrum β-lactamases along with active efflux systems, low outer membrane permeability, the absence of Penicillin

Binding Protein-2, and transposon-mediated aminoglycoside, sulfonamide, tetracycline resistance mechanisms have resulted in large number multidrug resistant bacterial populations increasingly being reported all over the world (Blackwell, Hamidian *et al.*, 2016; Vila, Martí *et al.*, 2007; Wong et al., 2017).

2.2. Bacteriophage History

The English chemist Ernest Hankin in 1896 had made the early presumptions of lytic activity of phage present in the water of Ganges river in his article “The bactericidal action of waters of Jumna and Ganges on the cholera microbe”(Stephen T Abedon, Thomas-Abedon, et al., 2011). He mentioned that a heat-sensitive unknown substance which passed through fine porcelain filters was responsible for preventing the spread of cholera epidemics. About two decades after the Hankin’s finding, Frederick Twort, a medically trained bacteriologist from England in 1915 explained the interesting glassy transformation of the micrococcus colony. He summarized that the cause of glassy transformation was an infectious, filterable agent that killed bacteria and, in the process, multiplied itself. Unfortunately, Twort's paper went un-recognized until 26 March 1921(5 years, 3 months, and 22 days). Despite his observation, he couldn’t state that the glassy transformation was due to the virus of bacteria. In 1910 when d’Herelle was in Mexico there was locust invasion, the Indians reported to d'Herelle that in a certain place the ground was scattered with the carcasses of these insects. After examination he found out that it was the death of insects was caused by the coccobacilli in diarrheal liquid. When he cultured the bacteria, he observed circular clear areas which he named as '*taches vierges*' (**clear spots**), these spots were two or three millimeters in diameter, flecking the cultures grown on agar. He couldn’t see anything in the slide prepared from the clear spot on the plate so he generalized that the agent which caused the formation of the clear spots must be able to pass a porcelain filter of the Chamberland type, which will retain all bacteria. Later, in March 1915, during world war-I, while searching for a solution to locusts in Tunisia d'Herelle re-observed the phenomena, to investigate their significance he showed them to Charles Nicolle, the director of the Pasteur Institute, he suggested that it might be the sign of a filtrable virus carried by Coccobacilli (Duckworth, 1976). D'Herelle presented his findings in September 1917 during the meeting of the Academy of Sciences and they were published immediately. According to his reminiscence, the name “bacteriophage” was also proposed by d'Herelle himself. (Sulakvelidze et al., 2001).

2.3. Ancient History of Phage Therapy

Before the golden era of antibiotics, phages were believed to be used to treat several bacterial infections (Diseases). It is apparent that Phage was the only agent used to cure bacterial infections in 1920s (Wittebole et al., 2014). Soon after the discovery of bacteriophage, d'Herrel began testing the efficacy of phage preparations in animals such as guinea pigs and rabbits (Duckworth, 1976). He used phages to treat dysentery in a 12-year-old boy. Probably, this was the very first successful human trial of phage therapy. This experiment was performed in Hospital des Enfants-Malades in Paris in 1919 under the supervision of Professor Victor-Henri Hutinel, who was the hospital's chief of pediatrics. D'Herelle, Hutinel, and several hospital interns had to ingest the phage preparation to confirm its safety before administering it to the dysentery patient. The boy fully recovered in couples of days after a single dose of antidysentery phage preparation (Lobocka & Szybalski, 2012). Furthermore, during World War II, phages were used to treat infection of German and Japanese armies (Summers, 2012). D'Herelle mentioned in his book about the commercial production of phage for the treatment of cholera in India (Kazhal & Iftimovich, 1968). About five D'Herelle, Hutinel's phage preparations namely Bacte´-coli-phage, Bacte´-rhino-phage, Bacte´-intesti-phage, Bacte´-pyo-phage, and Bacte´-staphy-phage against various disease were available commercially and was marketed by a company wich later became L'Ore´al(Sulakvelidze et al., 2001; Summers, 1999). In 1924 the Oswaldo Cruz Institute in Rio de Janeiro, Brazil, initiated the production of phage as the medication for dysentery in latin American countries, the phage preparations were also sent to hospital around Brazil too (Dublanchet & Bourne, 2007).In the 1940s, the Company Eli Lilly (Indianapolis, IN) produced phage therapeutics against bacterial pathogens such as *staphylococci, streptococci, Escherichia coli*.(Sulakvelidze et al., 2001).

Despite the availability of phage preparation commercially, the concept of use of phage (a virus) to treat diseases was not welcoming from the beginning. The criticism at the very beginning was due to the lack of information about the bacteriophage itself, the inconsistent result it produced in the treatment and the lack of reproducibility in published papers (Wittebole et al., 2014).

With the discovery of antibiotics that provided broad spectrum and greater potency, interest in phage therapy decreased gradually. However, the emergence of multi-drug resistant bacterial pathogens and their continuous spread in recent years has brought urgency to develop an alternative to antibiotics and bacteriophage seems to regaining its leverage. Nowadays, scientists are coming up with new and advanced approaches to phage therapy (Sulakvelidze, 2011).

2.4. Revitalization of Phage Therapy

Nowadays, phage research is mainly focused on cell line infection protection and phage rescue in animal model. A study conducted in Hela cell line and BALB/c mice showed that the page possesses no cytotoxicity and the phage was safe to use in vivo. The Hela cell were successfully rescued from the pan drug resistant *Acinetobacter baumannii* infection and the survival rate was similar as negative control even when the phage is applied after 2 hours of bacterial infection. Similarly, BALB/c mice were also accelerated the healing from localized infection of multi drug resistant *Acinetobacter baumannii*. When a mice challenged with *P. aeruginosa* developed gut-derived sepsis, oral administration of phage saved 66.7% subject from mortality compared to 0% in control group (Watanabe, Matsumoto *et al.*, 2007). Another study carried out in hamster model infected with *Clostridium difficile* a single dose of phage showed reduced colonization of *C. difficile* after 36 hours of administration of phage in 11 of 12 mice whereas control hamster receiving *C. difficile* and treated with clindamycin died within 96 hours (Nale, Spencer *et al.*, 2015). Another study in mice model infected with O25b:H4-ST131 *E. coli* strain shows the shows the similar kind of result (Pouillot, Chomton *et al.*, 2012). In a study it is also reported that phage administration restores the antibiotic sensitivity in multi drug resistant *P. aeruginosa*(Chan, Sistrom *et al.*, 2016). In addition, group of mice treated with phage survived the systemic infection of multidrug resistant *Acinetobacter baumannii* whereas untreated group die within one day of infection(Yin, Huang *et al.*, 2017). These studies suggest that the phage therapy can be used not only for the pathogenic bacterial infection but also for the multi drug resistant bacterial infections.

A randomized control trial (RCT) with phage therapy in 42 patients with chronic venous leg ulcers showed that the bacteriophage is not associated any kind of harmful effect(Rhoads, Wolcott *et al.*, 2009). In another study, among 151 cases of *Staphylococcal* infection, 75% of the patient with ulcerated varicose vein showed positive result and patient with gastrointestinal infection showed 100% positive result (Adebayo, Gabriel-Ajobiewe *et al.*, 2017). Another study in 24 patients with chronic otitis caused by antibiotic-resistant *P. aeruginosa*, the result showed that the phage therapy showed efficacy and safety, *P. aeruginosa* infection is hard to treat due to the formation of biofilm.(Wright, Hawkins *et al.*, 2009)

A recent case study in a 68 old diabetic patient with a necrotizing pancreatitis complicated by MDR *A. baumannii* infected pancreatic pseudocyst, who was in comma for several weeks when received phage therapy as a cocktail of phages, woke up from coma after two days and recovered fully in 245 days and discharged he even returned to his work after recovery(Schooley, Biswas *et al.*, 2017).

Phage therapy is not only limited to the animal model, many health care centers provide the phage therapy to the patient infected with multidrug resistant superbugs where antibiotics no longer function. Phage therapy center in Georgia has been treating patient with chronic, difficult, antibiotic-resistant bacterial infections that do not respond to conventional antibiotic therapies for over 80 years and has succeed in 95% of the cases.(Stephen T. Abedon, 2018)

After a successful treatment of a 68 years old patient with MDR Acinetobacter baumannii infection University of California San Diego School of Medicine in collaboration with national research institutions and private industry has established North America's first Center for Innovative Phage Applications and Therapeutics (IPATH) (News Medical Life Sciences, 2018).

In recent years there are a lot of phage-based companies are established. Some of them are emphasizing pre-clinical phage therapy research and development, some provide phage mediated bio control, some manufacture enzybiotics and some are well established Phage Therapy centers which provide patient phage therapy treatment (Stephen T. Abedon, 2018).

The above-mentioned research has justified most of the problem of phage therapy such as the application of the phage therapy in systemic infection, persistence of the phage in vivo. In concern of safety, FDA also has approved phage therapy as last line of treatment. But still the phage therapy is not accepted and adopted worldwide due to few drawbacks such as narrow host range of phages and possibility of phage mediated horizontal gene transfer. Advancements in biotechnology have further expanded the promising aspects of Phage Therapy such as bio engineered phages and the recombinant phage endolysin.(Lin, Koskella *et al.*, 2017).

Isolation, cloning and expression of holin and lysin gene of *Actinomyces naeslundii* Phage Av-1 was successfully carried out and the expression protein showed the lytic activity in lysoplate (Delisle, Barcak *et al.*, 2006). In a similar study *Acinetobacter baumannii* Bacteriophage ØABP-01 Endolysin (LysABP-01) was cloned and expressed which showed the enhance lysis in combination with colistin (Thummeepak, Kitti *et al.*, 2016). In another study, the lysin protein LysAB2 P3 derived from the phage endolysin gene cloning rescued mouse infected with lethal dose of *A. baumannii* (5×10^8 CFU) with 60% rate of survival.

After reviewing the research done so far in the field of phage and phage therapy It can be said that in the era where people are dying helplessly due to the infection of MDR superbugs, bacteriophage is a bright ray of hope. It is fast and cheap than antibiotic discovery. It can also be a personalized therapy. Phage can be used to treat any type of infection either it is localized or systemic. The doubts people had in past has been cleared by the recent research

and world is slowly attracted towards the phage therapy. It is obvious that people still have some insecurity using live virus as a drug and doubt on horizontal gene transfer. The recent studies in genetically modified phage and the phage derived endolysin will solve all the problems in phage therapy and it is the ultimate goal of phage therapy (Ryan, Gorman *et al.*, 2011).

2.5. Advantages of Phage Therapy over Antibiotics

Bacteriophages are highly specific in nature; thus, they are unlikely to disturb normal flora in the same manner as current antibiotics (Skurnik & Strauch, 2006). Phages are Self-multiplying, self-limiting and Target specifically and possess no any serious side effects. Higher concentration of antibiotics is required to inactivate or eradicate the biofilm cells as the biofilm ages (Anwar, Strap *et al*. 1992). In case of phage, initial dose is usually low (Donlan, 2009). Phages do not discriminate between multidrug resistant bacteria and antibiotics susceptible bacteria that's why are very useful against antibiotics resistant bacteria. Phages are effective against most resistant bacteria and they have ability to clear biofilms. Many studies showed that biofilms are significantly more resistant to chemical antibiotics than planktonic bacterial cells (Stephen T Abedon, García *et al.*, 2017). Bacteria cause antibiotics resistance usually due to the mutation, but in case of phage mutation rate is higher than that of bacteria so can respond fast to phage-resistant bacteria. Although resistance to phage has been reported, it is claimed that this can be avoided by using a 'phage cocktail' (O'flynn, Coffey *et al.*, 2006). Furthermore, there are studies that suggest phages do not elicit antibody production in the blood stream (Mattey & Spencer, 2008).

Problem with bacteriophages

Endotoxins: Phage progeny are released after the lysis host cell during the lytic replication cycle. During cell lysis, lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria is released. Lipopolysaccharide acts as an endotoxin and if they are present in high concentration then they can trigger a coagulation cascade, modify hemodynamics, invoke fever, endotoxic shock, and hypotension (Boratyński, Syper *et al.*, 2004). Purifying phage preparation using chromatography and ultrafiltration can produce endotoxin-free preparation (Boratyński et al., 2004; Matsuda, Freeman *et al.*, 2005).

Phage specificity: Although the specificity of phage for its host is an advantage, it can be the limiting factor for phage therapy and biocontrol. There are several options to circumvent this problem: using phage with broad host range (Ross, Ward *et al.*, 2016) using host range mutant bacteriophage or using a mixture of different phages (Chan, Abedon *et al.*, 2013).

Bacteriophage resistance: Development of phage-resistant mutation can make the phage therapy unproductive. Phage-resistance mechanisms encoded by bacteria (bacterial resistance) serve to limit phage host range. It is found that the rate of developing resistance to phages is approximately 10-fold lower than that to antibiotics. However, using phage cocktail (a mixture of phages) that uses different cell receptors can restrain rise of phage resistance (Tanji, Shimada *et al.*, 2004).

Only obligate lytic phages can be used for therapeutic propose. The phage must be able to lyse the host bacterial cell Instead of integrating its genome in bacterial DNA to be usable for phage therapy. Temperate phages play a major role in the exchange of genetic material between different bacterial strain and hence they are considered unsuitable for phage therapy.

2.6. Whole Genome Sequencing

After the discovery double helix structure of DNA by American biologist James Watson and English physicist Francis Crick in the 1950s tremendous work in the field of molecular biology has been done(Pray, 2008). It was difficult to understand the complexity and diversity of genomic material in different organism and its role in health and disease without genome sequencing technologies. There were very limited technologies to be able to do sequencing of the genome in the past and the technologies available were also very slow and costly. The Human Genome Project was started in 1990 with the goal of sequencing and identifying all three billion human genomes, finding the genetic cause of disease and then developing treatments. It is also considered as a megaproject because the human genome has approximately 3.3 billion base pairs. With the sequence in hand, the next step was to identify the genetic variants that increase the risk for common diseases like cancer and diabetes. With the invention of high throughput screening in the mid-2000s the cost of whole genome sequencing dropped 50,000-fold(Kircher & Kelso, 2010).

NGS technologies are evolving continuously over the past decade and have incorporated many revolutionary innovations to deal with the complexities of genomes. These advancements in sequencing technologies are able to give long genome reads and they have brought the cost of sequencing a human genome down to around US$1,000(Goodwin et al., 2016).

Despite all these advancements there are some limitations of next generation sequencing. NGS platforms provide vast arrays of data, but the associated error rates (~0.1–15%) are higher and the read lengths generally shorter (35–700 bp for short-read approaches) than that of traditional Sanger sequencing platforms. NGS also requires careful examination of the

result specially for variant discovery and clinical applications. Although, the long-read sequencing technologies overcome one of the limitations of the NGS, it is still comparatively expensive than other platforms which is limiting its worldwide adaptation. NGS is bringing some alternative technologies that can carry out similar task but at low cost to compete with other technologies(Goodwin et al., 2016).

2.7. Illumina Sequencing

It has become clear that genomes are highly complex molecule with many long repetitive elements, copy number alterations and structural diversities that are relevant to evolutionary history, adaptation and disease. However, many of these complex elements are so long that short-read paired-end technologies are incapable of resolving them. Long-read sequencing provides several kilobases long reads, allowing for the resolution of these large structural features. Such long reads can cover complex or repetitive regions with a single continuous read, thus eliminating ambiguity in the positions or size of genomic elements. Long reads can also be useful for transcriptomic research, as they can span entire mRNA transcripts, enabling scientists to identify the accurate connectivity of exons, introns and gene isoforms. Currently, there are two main types of long-read technologies: single-molecule real-time sequencing approaches and synthetic approaches. The Illumina sequencing falls under the synthetic approach.

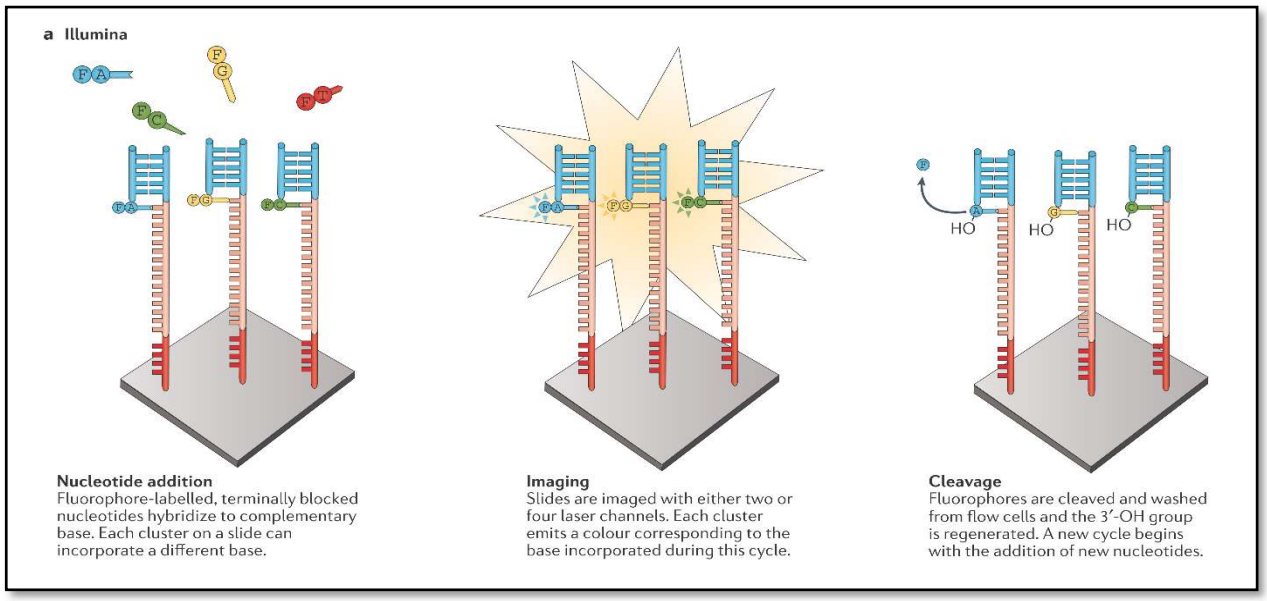


Figure: 2. 1 Different steps involved in Illumina Sequencing (Goodwin, McPherson *et al.*, 2016)

2.8. Cytokine Expression Analysis by Real Time PCR

Real time Polymerase Chain Reaction also known as Quantitative PCR(Q-PCR) is the method by which the PCR product can be determined in real time as it is being amplified and is very useful for investigating gene expression. Quantifying gene expression levels has become an important tool in molecular biological laboratories. By measuring the amount of cellular RNA (particularly mRNA), one can determine to what extent the gene of interest is being expressed. The expression levels of genes may change dramatically in various experimental conditions. Some examples of quantitative gene expression studies include: validation of the extent of transcription of a gene, to study the difference in expression of a gene in the experimental condition compared to the normal state, change in gene expression when the subject or the cells are exposed to a chemical substance (e.g., drug, toxin or hormone) (Schmittgen & Livak, 2008)

Real-time RT-PCR is a powerful tool for the detection of gene expression level. The quantitative endpoint for real-time PCR is the threshold cycle (CT). The threshold cycle can be defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold. By presenting data as the CT value, it can be insured that the PCR is in the log phase of amplification. The numerical value of the CT is inversely related to the amount of amplicon in the reaction mixture. (i.e., the lower the CT value, the higher the amount of amplicon). (Livak & Schmittgen, 2001)

There are various methods of interpreting Real Time PCR data among them most common are absolute quantification and relative quantification. Absolute expression provides the exact copy number following transformation of the data via a standard curve where as in relative quantification, the real-time PCR data is presented relative to another internal control gene. Absolute quantification is required when exact quantity of amplicon is desired, for example, viral load in a sample. The drawback of absolute quantification includes the increased effort to generate standard curves. Moreover, it is often unnecessary to present absolute copy number and relative expression will sufficient. For example, if there is an increase in the expression of a gene from 10,000 to 50,000 copies per cell, reporting the data as a fivefold increase in gene expression is sufficient. (Livak & Schmittgen, 2001)

A number of methods have been developed over the years to calculate the relative gene expression. The efficiency correction method is used to calculate the relative expression ratio from the real-time PCR efficiency and the CT value. Real-time PCR data has also been analyzed using sigmoidal curve fitting methods. It fits the data obtained from Real Time PCR to an

empirical equation which gives the prediction of the PCR efficiency and an approximate value of the initial copy number of the amplicon. Another method of analyzing quantitative real-time PCR data is the comparative CT method which is also known as the $2^{-\Delta\Delta Ct}$ method. This method makes various speculations, including that the efficiency of the PCR is close to 1 and the PCR efficiency of the target gene is near to the internal control gene.(Livak & Schmittgen, 2001)

Every method of analyzing relative real time PCR data have its own advantages and disadvantages. Merit of the efficiency correction method is that the efficiency of PCR of the target and internal control genes are included in the equation so the differences in the efficiency between target and internal control will be taken for the calculation. The sigmoidal curve fitting models have the plus point that there is no need to calculate the PCR efficiency by separate experiment and it is estimated during the analysis. The use of nonlinear regression analysis for the calculations is the drawback of the simulated kinetic model. Moreover, recent study showed the problem in fitting curve where the fluorescence signal is hidden in noise and this may account for the false estimations of amplification efficiency. Ease of use and the ability to present data as 'fold change' in expression are the advantages of the comparative CT method.

$$Fold\ change = 2^{-\Delta\Delta Ct}$$

$2^{-\Delta\Delta Ct}$ = [(C T gene of interest -C T internal control) sample A - (C T gene of interest- C T internal control) sample B]

This equation may be used to compare expression of a gene in two different samples (sample A and sample B); each sample is related to an internal control gene. Sample A may be the experimental sample and sample B, the untreated control (Jain, Nijhawan *et al.*, 2006).

2.9. Proinflammatory Cytokines

Cytokines are mediators of host responses to infection, inflammation, immune responses, and trauma. Some cytokines may make disease worse called as proinflammatory cytokines, whereas others help to reduce inflammation and promote healing called as anti-inflammatory cytokines. Tumor necrosis factor (TNFα) Interleukin (IL)-6 and are proinflammatory cytokines, and when they are expressed in abnormal amount, they produce fever, inflammation, tissue destruction, and shock in some cases (Stenvinkel, Ketteler *et al.*, 2005). The expression of IL-6 during infection is reported from 6-24-fold during inflammation (R. Berti, A. Williams *et al.*, 2002).

2.10. Pharmacokinetics and Pharmacodynamics of Bacteriophage Therapy

Abundant data can be found related to phage therapy but when it comes to pharmacology of phage in animal model, there is a limited number of data available. Pharmacology mainly deals with the drug and body interactions, it can be further subdivided into pharmacokinetics-body's impact on drug- and pharmacodynamics-drugs impact on the body. Pharmacokinetics deals with drug's ability to reach desirable concentrations in the locality of targeted tissues, as it is necessary to bring about the desirable effect of drug. The pharmacokinetics focuses into absorption, distribution, metabolism and excretion of drug. Absorption is drug dissemination into the blood stream. From the blood stream the drug is transported to the body tissues this process is known as distribution. Once the drug reaches the target tissues, it is metabolized and modified into its active form, the last step is excretion of drugs from the body. All these processes of pharmacokinetics play their role in both increasing and decreasing drug concentration. For instance, both absorption and distribution result in the decrease in drug concentration resulting in the drug dilution, which on the other hand increases drug density in the particular body organ or tissue (Stephen T Abedon, Kuhl *et al.*, 2011).

Based on the pharmacokinetics of drug different methods of drug delivery are adopted. The route of administration is selected taking consideration of several factors such as target tissue, sensitivity of drug to body enzymes, drug in take routine, patient's convenience and so on. In phage therapy, metabolism may work two ways; either inactivation of phage particles by patient's immune system or activation followed by replication of phages. Furthermore, excretion results in decreased drug concentration, on the other hand , it can lead to increase in phage concentration in organs like kidney and bladder, which can be therapeutically beneficial (Vandenheuvel, Lavigne *et al.*, 2015).

The success of phage therapy relies on ample phage concentration in the locality of the target to eradicate pathogen bacteria from the body. The increase in the number of phages up to adequate level can be achieved by two means. One with in situ replication, also called as active treatment, or as a result of pharmacologically conventional dosing, or the passive treatment. The administration of phage should be in such dose which compensate the mechanism of phage reduction. Thus, the goal is to achieve minimum phage concentration at the target organ or site, which is enough for the reduction of the bacterial cells to a desired level. Moreover, the phage preparations should be purified to remove bacterial debris and

bacterial components, including endo-toxins particularly when phages are to be delivered directly to an animal's systemic circulation (Chan et al., 2013).

The study done so far suggests that the phage when administered orally can reach to blood stream of animal 2-4 hour after the single dose. Phages are found in internal organ such as liver spleen and kidney in approximately 10 hours of dose. Study done in 1986 showed that the phage can persist up to several days in the subject's body (Jassim & Limoges, 2014). Sulakvelidze *et.al*. in 2001 suggested that the human immune may recognize phage as foreign particle and elicit immune response(Sulakvelidze et al., 2001). Neutralizing antibodies have been reported in pharmacokinetic studies after 10-12 days of administration of phages in mice model experiment and further study on phage pharmacokinetics is necessary (Kutter, De Vos *et al.*, 2010).

2.11. Phage as a Food Preservative

Besides the phage therapy for curing localized and systemic infections, phage can be used to kill the food spoilage bacteria and food borne pathogens. *Listeria monocytogenes* a major concern to food industry as it is the causative agent of listeriosis in human. FDA (Food and Drug Administration) approved the use and the preparation of bacteriophages generally recognized as safe (GRAS) as food additives for bio control of this pathogen. FDA approved Phage biocontrol of L. monocytogenes was available in 2006 as ListShield™ (Strydom & Witthuhn, 2015). Other similar products such as ListexTM that targets *L. monocytogenes* (Carlton, Noordman *et al.*, 2005), ECP 100TM (Ecoshield™) targeting *E. coli* O157:H7 in ground beef, fruits, and vegetables and SALMONELEX™ targeting Salmonella are the branded phage preparation available as a food biocontrol (CHATAIN-LY, 2014). Similarly, various studies show promising result such as biocontrol of *campylobacter spp*. in chicken and beef. Elimination of *Salmonella spp* in raw meat, chocolate, vegetables. Control of *Staphylococcus aureus* in cheese. Biocontrol of *Cronobacter sakazakii* in infant formula and so on(Kazi & Annapure, 2016).

2.12. Bacteriophage Based Pathogen Detection

Detection of pathogen using phage exploits its narrow host range property, host phage interaction, initial recognition and attachment property of phage while other take advantage of the insertion and expression of phage genome inside the host cell. Phage mediated assays are more efficient, sensitive, and faster than conventional plaque assay method (Ripp, 2009).

There are several techniques of detection of pathogen using phage;

1. Reporter Phage

A reporter phage carries an easily detectable signal which indicates the presence of number of the pathogen inside the cell. Generally, a reporter phage consists of a gene capable of producing a reporter protein (bioluminescent, fluorescent, or chemiluminescent) when expressed inside the host. The gene is incorporated directly inside the phage genome, after infection the gene is transferred into host cell along with the phage genome and gets expressed subsequently yielding the signal molecules. The reporter genes used frequently are green fluorescent protein (gfp) reporter gene, Lux Reporter gene, Luc Reporter gene, LacZ Reporter gene and so on (Ripp, 2009).

2. Labeled Phage

Alike the detection by reporter gene after infection, the labelled phage exploits the initial attachment property of phage to its host cell. The phage is labelled with a fluorescent dye and capable of detecting the presence of the pathogenic host upon attachment (Ripp, 2009).

3. Phage display

Phage display is another popular application of bacteriophage. In this technique a gene segment of interest is incorporated in the capsid protein coding gene as a fusion protein, because of which the portion of foreign protein is displayed on the capsid of the phage. Such type of phage is useful in determining the binding site for the protein or to screen the ligand and to study receptor binding sites (Ripp, 2009).

Chapter Three

MATERIALS AND METHODS

3.1. Bacterial (host) Sample Collection

The eight putative strains of MDR *Acinetobacter baumannii* samples were collected from National Public Health Laboratory. The samples were transported to the Central Department of Biotechnology in a NA tube and the samples were streaked onto MA.

3.2. Antibiotic Sensitivity Test (AST) / Antibiogram Assay

Since collected bacteria were clinical samples, AST was performed again to re-confirm that the strains were MDR. Antibiotics used were of a different generation and various classes such as Quinolone, Aminoglycosides and β-lactam.

Table: 3. **1** List of antibiotics used

SN	ANTIBIOTICS	CODE
1	Gentamycin (aminoglycoside)	GEN 10
2	Ofloxacin (Quinolone 2nd generation)	OF 30
3	Piperacillin (penicillin 4th generation)	PIT 100
4	Nalidixic acid (synthetic drug)	NA 30
5	Ceftazidime (cephalosphorin 3rd generation)	CAZ 30
6	Cefotaxime (cephalosphorin 3rd generation)	CTX 30
7	Meropenem (Carbapenem)	MRP 10
8	Imipenem (Carbapenem)	IMP 10

3.3. Bacterial Genomic DNA Extraction

At first, 1ml of bacterial culture was centrifuged at 12000 rpm for 2 minutes and pellet was collected. After that 567 µL of TE buffer was added to the pellet and resuspended by short vortexing. Thirty microliters of 10% SDS and 3 µL of a 20 mg/mL solution of proteinase K were added. Then, it was mixed and incubated for 1 hour at 37°C. After incubation, 100 µL of 5 M NaCl was added and mixed. Afterwards 80 µL of a CTAB/NaCl solution (0.7 M NaCl, 10% CTAB) was added. This solution was incubated at 65°C for 10 min. After incubation, an equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed. It was centrifuged for 5 min, and the aqueous solution was transferred carefully (avoiding transferring interface) to a new tube. Again, it was centrifuged at 14,000 rpm for 5 min and supernatant was transferred to a fresh tube. The first extraction was repeated (chloroform: isoamyl alcohol alone). After that 0.6 volume of isopropanol was added and mixed gently until the DNA precipitates. Centrifugation was done and isopropanol was removed. One microliter of 70% ethanol was added to wash the salt away from the DNA. Again, Centrifugation was done, and the ethanol was aspirated, and the DNA was left for drying on the bench top at room temperature. The pellet was resuspended in 50 µL of TE buffer and was kept at 4°C.Then the band of the genomic DNA was visualized in 0.8% Agarose gel electrophoresis.

3.4. Molecular Characterization of Host

The 16S rRNA gene segment (1.5kb) of 6 clinical sample of putative *Acinetobacter baumannii* strains were amplified by PCR. Aliquots (5µl) of the amplified products were analyzed by electrophoresis on 1 % agarose gels in Tris Acetate EDTA (TAE) buffer. After that the gel was observed on UV transilluminator and image was taken. The PCR products were then exported to Xceleris Laboratory for sequencing. The forward and reverse sequences obtained after sequencing were aligned and the contig was prepared using BioEdit program. The bacterial identity was confirmed by comparing the sequences obtained with the gene sequences available in the Genbank database using Basic Local Alignment Search Tool (BLAST) software at NCBI (http://blast.ncbi. nlm.nih.gov). The sequences were submitted in the Genbank database and accession number was received. Phylogenetic Tree was constructed using MEGA-X software. The set of primer used and PCR condition is given in table below.

Table: 3. 2 16S rRNA Primer Details

Gene	Primer	Sequence	TM	Amplicon length(bp)
16S rRNA	Forward	AGAGTTTGATCMTGGCTCAG	56.4	1500
	Reverse	CGGTTACCTTGTTACGACTT	56.4	

Table: 3. 3 PCR Condition

S.N.	Steps	Temperature (°C)	Time
1	Enzyme Activation	95	2 minutes
2	Initial Denaturation	95	30 seconds
3	Annealing	55	30 seconds
4	Extension	72	2.5 minutes
5	Final extension	72	5 minutes
6	Final hold	4	∞

3.5. Amplification of Antimicrobial Resistance Coding Gene by PCR

The New Delhi metallo-β-lactamase (NDM) gene (869bp) was amplified using the following set of primers and condition.

Table: 3. 4 New Delhi metallo-β-lactamase (NDM) gene primer details

Gene	Primer	Sequence	TM	Amplicon length(bp)
bla(NDM)	Forward	AATGCTGAATAAAAGGAAAA	47.6	869
	Reverse	GGCAGATTGGGGGTGA	51.8	

Table: 3. 5 PCR Condition

S.N.	Steps	Temperature (°C)	Time
1	Enzyme Activation	95	2 minutes
2	Initial Denaturation	95	30 seconds
3	Annealing	56.7 decrease 0.5 per cycle	30 seconds
4	Extension	72	90 seconds
5	step (2-4) 14 cycle		
6	Denaturation	95	30 seconds
7	Annealing	49.7	30 seconds
8	Extension	72	90 seconds
9	step (6-8) 19 cycle		
10	Final Extension	72	5 minutes
11	Final hold	4	∞

3.6. Phylogenetic Tree Construction

The phylogenetic tree was constructed by aligning sequence with ClustalW algorithm and software used was MEGA-X.

3.7. Sewage Sample Collection and Processing

Sewage samples were collected from different sites of rivers in Kathmandu valley. The most polluted site was chosen for the better chance of obtaining phage. The sample was collected in a 50 ml falcon tube and transported to the Central Department of Biotechnology. After collection, the sample were processed by subjecting to centrifugation at 4100 rpm for 30 minutes. The centrifuged sewage sample was then filtered through a 0.22µm syringe filter and the filtrate was collected in a sterile Falcon tube and stored at 4°C for further analysis.

Table: 3. 6 Sample Collection Site		
SN	Name of sample collection site	GPS co-ordinates
1	Balkhu 1	27.68232, 85.29894
2	Balkhu 2	27.68447, 85.2998
3	Balkhu 3	27.6836, 85.29946
4	Teku 1	27.69789, 85.30214
5	Teku 2	27.69546, 85.30025
6	Pashupati 1	27.70913, 85.34865
7	Pashupati 2	27.70718, 85.34862
8	Kirtipur 1	27.6786, 85.27297
9	Kirtipur 2	27.679, 85.26932
10	Kirtipur 3	27.68285, 85.2743
11	Gongabu 1	27.73707, 85.30836

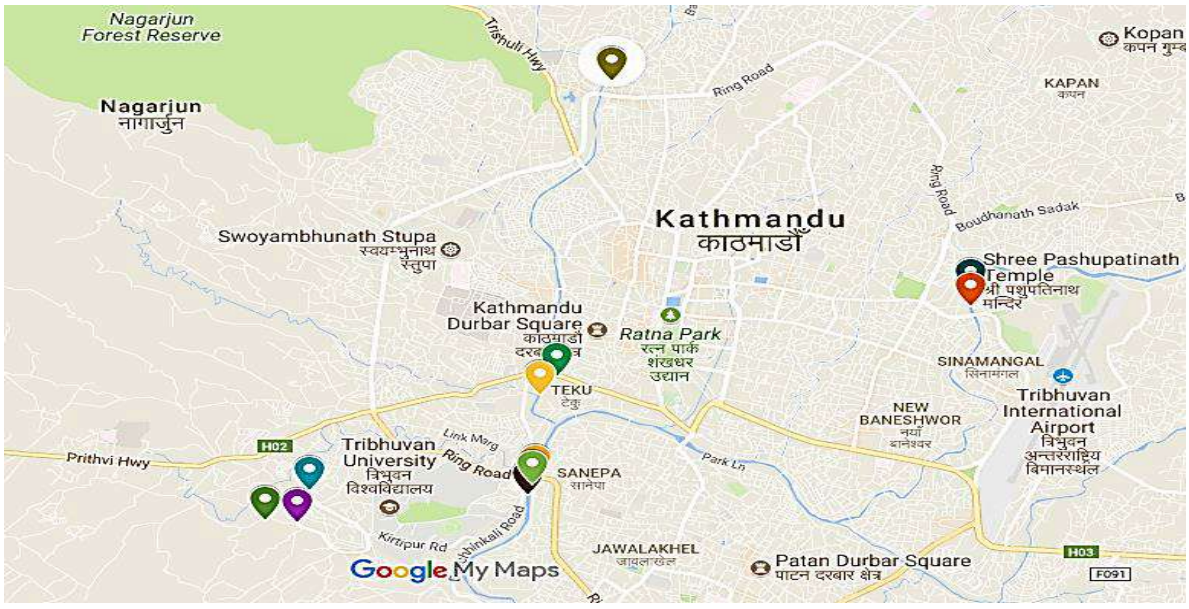


Figure: 3. 1 Sample collection sites on google map.

3.8. Bacteriophage isolation

The Isolation of bacteriophage was done using standard double-layer agar assay (DLAA)(Swanstrom & Adams, 1951).

Double Layer Agar Assay (DLLA method)

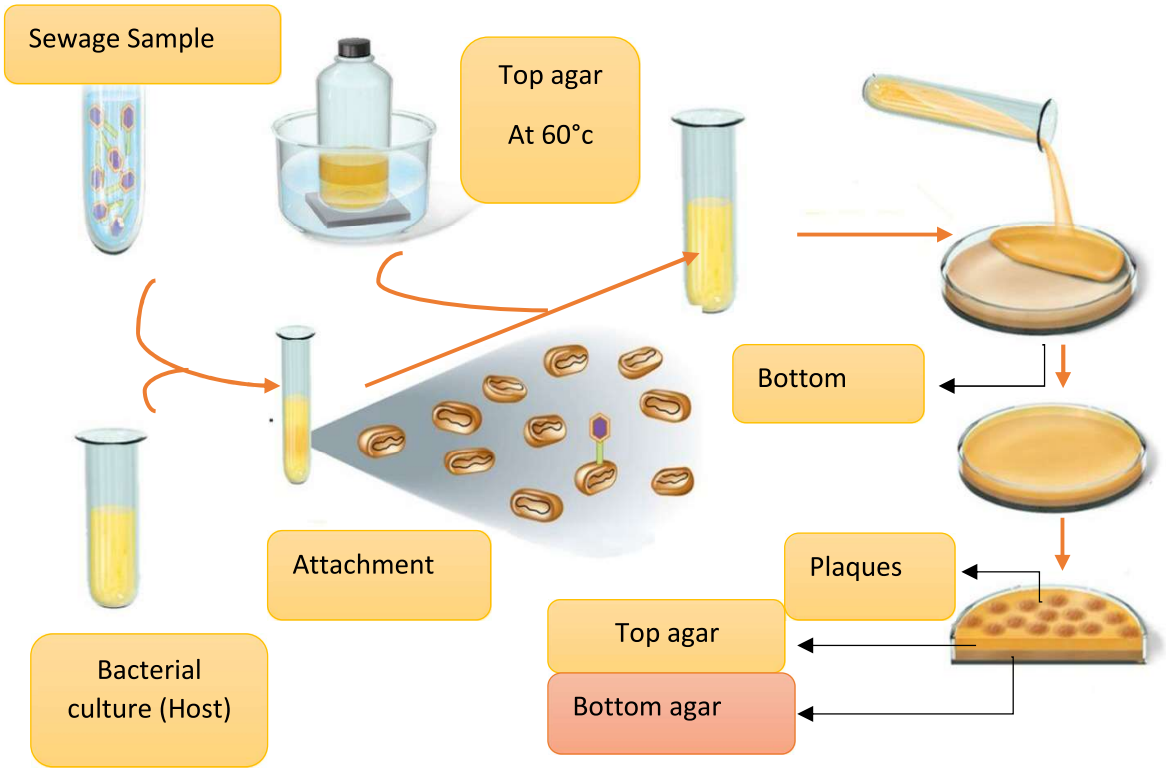


Figure: 3. 2 DLAA technique flow chart.

Double Layer Agar Assay (DLAA) is the standard method for isolation of phages from environmental sources like sewage water, dairy waste, animal waste, soil et cetera. In this method, a thin layer hard agar plate of Tryptic soy agar plate (1.5%) was prepared and simultaneously soft agar (0.5% TSA) was prepared and kept at 55°C. In the mixture of 1ml processed filtrate and 100µl of host bacteria, 3 ml of semisolid agar was added and poured onto the 1.5% TSA plate and spread uniformly. The 1.5% agar forms bottom layer and a mixture of few phage particles present in the sample and a very large number of host cells in a soft agar forms the top layer. When the plates are incubated, susceptible bacterial cells multiply rapidly and produce a lawn of confluent growth on the medium. The phage particle present in the sample adsorbs to a susceptible cell, penetrates the cell, replicates and release

new virions which subsequently infect other bacteria in culture. This cycle continues until large numbers of bacteria have been lysed. Due to the destruction of the cell, single, circular, non-turbid areas called plaques in the bacterial lawn, where there is no growth of bacteria because the phage progeny originating from single virus particles have multiplied sufficiently to kill bacteria over an easily visible area. Eventually, the plaque becomes large enough to be visible to our naked eye.

3.9. Clonal purification of Phage

The initial plaques formed in the Petri dish may be of different phages, to study the morphological, physical and molecular character of phage, it should be in pure form. The plaque-streak assay was used to purify populations of phages from the sample with putatively heterogeneous populations. This technique is very similar to streaking for single bacterial colonies. First, a single isolated plaque was touched with a sterile loop and continuously streaked on 1.5% TSA. The host bacterial culture was mixed with 3ml of semisolid agar and poured in the streaked Petri dish form the ending point and plate was incubated inverted overnight at 37°c after solidification of agar. Three subsequent rounds of streaking were performed for the purification.

3.10. Preparation of phage lysates

Phage lysates were prepared by amplifying the phage in petri dish. Ten TSA plates were streaked with a single plaque using continuous streaking technique and allowed to dry. After that, 100µl bacterial culture was mixed with 3 ml of semisolid agar and poured into the streaked plate. The plates were allowed to solidify and were incubated overnight at 37°c. After incubation, 4 ml of SM buffer was poured on the plates containing plaques. The petri dishes were agitated in a shaker for 2 hours at 100 rpm. After that, the SM buffer along with the top agar was transferred to a falcon tube and vortexed for about 15 minutes. The vortexed solution was then subjected to centrifugation at 4100 rpm for 20 minutes. The supernatant was filtered through a 0.22µm syringe filter and the filtrate was collected in a sterile Falcon tube and stored at 4°C for further analysis.

3.11. Determination of phage titer

Phage lysate was serially diluted up to 10^{-15} dilution using SM buffer. Nine hundred microlitre of SM buffer was aliquoted in eppendorf tubes (labelled 10^{-1} to 10^{-15}) and 100 microlitre of phage was mixed with 900 microlitre of SM buffer in 10^{-1} eppendorf tube. Subsequently 100 microlitre of phage was transferred to the next dilution labelled eppendorf tube and mixed well. DLAA was performed using 1 ml of serially diluted lysate from each dilution. The plates

were incubated inverted overnight at 37°c and the pfu per ml was calculated using the formula given below.

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

3.12. Host Range Analysis

Host range of purified phage stock was determined by spot assay. For this, a lawn culture of 40 hosts bacteria were prepared by mixing 100µl of log phase bacterial culture with 3 ml of soft agar. The mixture was then poured into the 1.5% TSA plates and allowed to set. After the solidification of agar, 5µl of phage stock (10^8) was poured over the lawn culture and the plates were incubated inverted overnight.

3.13. Protein Profiling by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analysis of phage protein by direct heating method and acetone precipitation method was done using following two methods.

3.14. Acetone Precipitation

Two hundred microliter phage stock (10^{-8} dilution) was mixed with 800 microliter of cold acetone(-20°C) and vortexed. After vortex the sample was incubated for 60 minutes at (-20°C). After the incubation the sample was centrifuged at 13000 rpm for 10 minutes. The supernatant was discarded carefully and the pellet was air dried for 30 minutes. After that the pallet was dissolved in 25 microliter of phosphate buffer saline. SDS-PAGE was carried out according to (He, 2011). Briefly, 25 µl of samples were added to 25 µl of 2 × Laemmli buffer and boiled for 10 mins. Samples were then loaded to 10 % PAGEr™ precast gels and electrophoresed with tris-glycine buffer. Five µl of protein marker with 1 µl of loading dye was also loaded after boiling for 5-10 mins. After electrophoresis the gels were stained with Coomassie Brilliant Blue R-250 (CBB) for overnight and then bands were visualized after adding destaining solution (Sillankorva, 2009).

3.15. Direct heating Method

For Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis (SDS- PAGE) separation, purified 25 µl Pseudomonas phages were mixed with equal volume of 2x sample buffer (0.125 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 2%-mercaptoethanol (-ME), 0.02% bromophenol blue) heated in a boiling water bath for 3-5 minutes (Sen & Ghosh, 2005). Samples were then

loaded to 10 % PAGEr^{TM} precast gels and electrophoresed with tris-glycine buffer. SDS-PAGE was carried out according to Laemmli. After electrophoresis the gels were stained with Coomassie Brilliant blue R-250 (CBB) for overnight and then bands were visualized after adding destaining solution (Kumari, Harjai *et al.*, 2009).

3.16. Transmission Electron Microscopy (TEM)

TEM analysis of on potent phage was done. The phage was selected based on lytic activity. Stock having phage titer 3.2×10^8 was sent to Sophisticated Instrumentation Centre for Applied Research & Testing (SICART), Gujarat, India in cold chain for TEM analysis. The phage lysates were fixed with fixative (2.5% glutaraldehyde and 2% paraformaldehyde which was prepared in 0.1M sodium phosphate buffer (pH 7.2)). Equal volume of phage lysate and fixative were added, mixed and left overnight for fixation. On following day, the fixed phages were subjected to high-speed centrifugation (35,000×g) for 3 hours. After that lower portion of phage lysate were processed for TEM viewing 10 µL fixed phage lysate (per sample) was deposited on separate 300 mesh carbon-coated copper grid and after 2 minutes excess phage lysate was soaked-off using blotting paper. The copper grid was then flooded with 2% (w/v) uranyl acetate (pH 4.5) and after 2 minutes, excess stain was soaked-off with blotting paper. The copper grid was dried and finally examined in JEM-2100F Transmission Electron Microscope (JEOL, USA, 200 KV) under various magnifications. TEM micrographs were processed using ImageJ 1.52a (https://imagej.nih.gov/ij) for determining tail size (width and length) and size of phage capsid / head. Three readings of head and tail (width and length) were recorded and mean value was calculated to minimize the error.

3.17. In-vitro Lysis of Host

Log phase culture of *Acinetobacter baumannii* (OD 0.15) was infected with the phage stock at different Multiplicity of Infection (MOI) (0.5, 1, 5 and 10) and optical density was measured at the interval of 1 hour, 2 hour and 3 hour and the graph of OD versus time was plotted.

3.18. Physiochemical Characterization of Bacteriophage

Response of phages on exposure to varying physiochemical condition is considered as a key model for understanding the ability of the organism under question to adapt to novel environments. To use phage as antibacterial agent, phage should be stable in different physiological parameter such as temperature and P^H. So, P^H and thermal stability of phage was tested in laboratory following protocol of (Jin, Li *et al.*, 2012).

3.19. pH Stability

The pH of the LB was adjusted with 1 M HCl and 0.5 M NaOH to obtain a pH ranging from 1-14. A total of 100 microliter of bacteriophage suspension (10^{-6} stock dilution,390pfu/ml) was poured into 1.5 ml of pH-adjusted medium. After 1-hour incubation at 37°C, the surviving phage particles were counted immediately using the DLAA method. And the graph of pfu against time was plotted.

3.20. Thermal Stability

To check thermal stability of phage, 1 milliliter phage stock (10^{-7} stock dilution, 46 pfu/ml) was aliquoted in Eppendorf tubes and incubated at 25°C, 37°C, 50°C, 60°C, 70°C and 80°C for 10, 20, 30, 40, 50 and 60 minutes for each temperature group. DLAA was performed after the incubation to quantify the viable phage particle. And the graph of pfu against time was plotted.

3.21. One Step Growth Curve Analysis and Burst Size Determination

One step growth curve analysis was performed according to (Jin et al., 2012). Thousand microliters of bacterial culture at an OD600 of 0.15 (7.0×10^7 CFU/ml) was mixed with 100 microliter phage stock at 3.2×10^8 (0.5 MOI approx.) and Eppendorf tubes were incubated at 37°C. One Eppendorf tube was withdrawn after each 10 minutes and the phage particles were counted by DLAA and curve was drawn time versus plaque. Bacterial viable counts were determined before the bacteria were mixed with the phage and were assessed periodically. The burst size was calculated using given formula.

$$B = \frac{\Delta V}{|\Delta B|} = \frac{V_e - V_0}{|B_e - B_0|}$$

Where, B is the burst size, ΔV represents changes in the viral number, ΔB represents changes in the bacterial number, V_e is the viral number at the end of the experiment, V_0 is the viral number at the beginning of the experiment, B_e is the bacterial number at the end of the experiment, and B_0 is the bacterial number at the beginning of the experiment.(Jiang, Kellogg *et al.*, 1998)

3.22. Whole Genome Sequencing.

One phage sample (phage TU_A56) was exported to Xcelris Genomics (Ahmedabad, India) in dry ice for Next Generation Sequencing. Whole genome sequencing of phages was performed on Illumina HiSeq 2000/2500 platform under project ID: 913.

3.23. Isolation, Qualitative and Quantitative Analysis of gDNA:

Genomic DNA was isolated from the phage sample using Norgen Phage DNA Isolation kit (Cat. 46800, 46850). Quality of gDNA sample was checked on 1% agarose gel (loaded 3 µl) for the single intact band. The gel was run at 110V for 30 mins. One microlitre of each sample was loaded in Nanodrop 8000 for determining A260/280 ratio and 1 µl of each sample was used for determining concentration using Qubit® 2.0 Fluorometer.

3.24. Preparation of Library

The paired-end sequencing library was prepared using Truseq Nano DNA Library prep kit. The library preparation process was initiated with 200 ng g-DNA. The g-DNA was mechanically sheared into smaller fragments by The Covaris® M220 Focused-ultrasonicator™ followed by continuous step of end-repair where an 'A' is added to the 3' ends making the DNA fragments ready for adapter ligation. Following this step, platform-specific adapters are ligated to both ends of the DNA fragments. These adapters contain sequences essential for binding dual-barcoded libraries to a flow cell for sequencing, allowing for PCR amplification of adapter-ligated fragments, and binding standard Illumina sequencing primers. To ensure maximum yields from limited amounts of starting material, a high-fidelity amplification step was performed using HiFi PCR Master Mix.

3.25. Quantity and Quality Check (QC) of Library on Bioanalyzer

The amplified library was analyzed in Bioanalyzer 2100 (Agilent Technologies) using High Sensitivity (HS) DNA chip as per manufacturer's instructions.

3.26. Cluster Generation and Sequencing

After obtaining the Qubit concentration for the library and the mean peak size from Bioanalyzer profile, library was loaded onto illumina platform for cluster generation and sequencing. Paired-End sequencing allows the template fragments to be sequenced in both the forward and reverse directions. The library molecules bind to complementary adapter oligos on paired end flow cell. The adapters were designed to allow selective cleavage of the forward strands after re-synthesis of the reverse strand during sequencing. The copied reverse strand was then used to sequence from the opposite end of the fragment.

3.27. Genome Sequencing Data Analysis

The whole genome sequence file of phage was sent to us by Xcelris Genomics lab as fasta file (.fas) file. The fasta file had the 11 thousand scaffolds. The genome file was first edited using SnapGene viewer 4.2.4. tool. After that the species distribution was determined using BLAST+. BLASTn was performed to find best species match in the NCBI database. Similarly, BLASTx was performed to find the protein present in the phage and to determine whether there is presence of toxic protein or not.

After that the genome was visualized and annotated using online phage genome annotation tool PHASTER (http://phaster.ca/) (Arndt, Grant *et al.*, 2016). A linear phage genome was constructed, ORF were determined and the putative genes were annotated using NCBI as reference database.

Phylogenetic tree of tail protein was constructed using MEGA-X taking the protein sequences of another *Acinetobacter baumannii* phages present in NCBI database.

The whole genome analysis annotation is a highly specialized and time demanding task, so the genome annotation is still going on. The partial submission of the phage genome has been done in NCBI database and the submission will be completed as soon as the genome gets fully annotated.

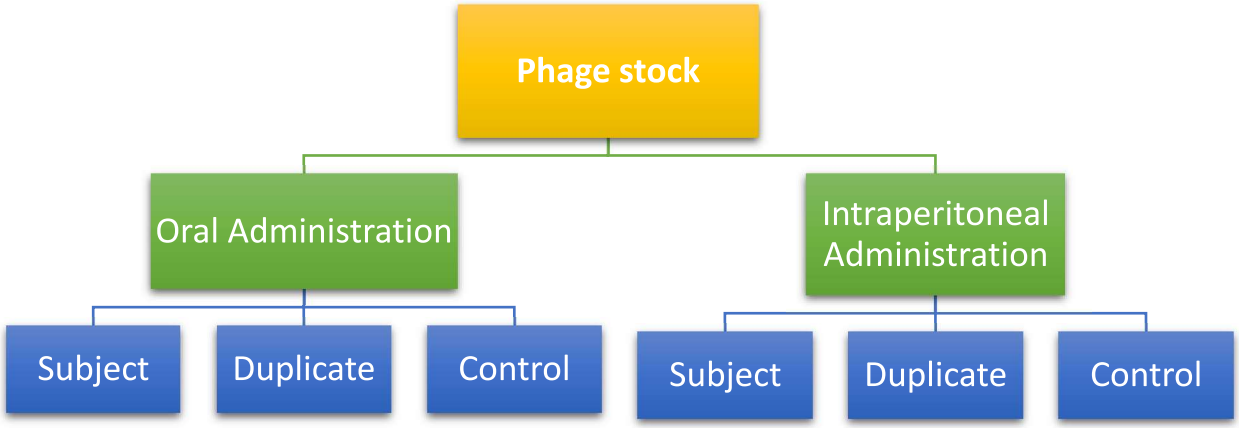
3.28. Pharmacokinetics Study of Phage TU_A56 in Mouse Model

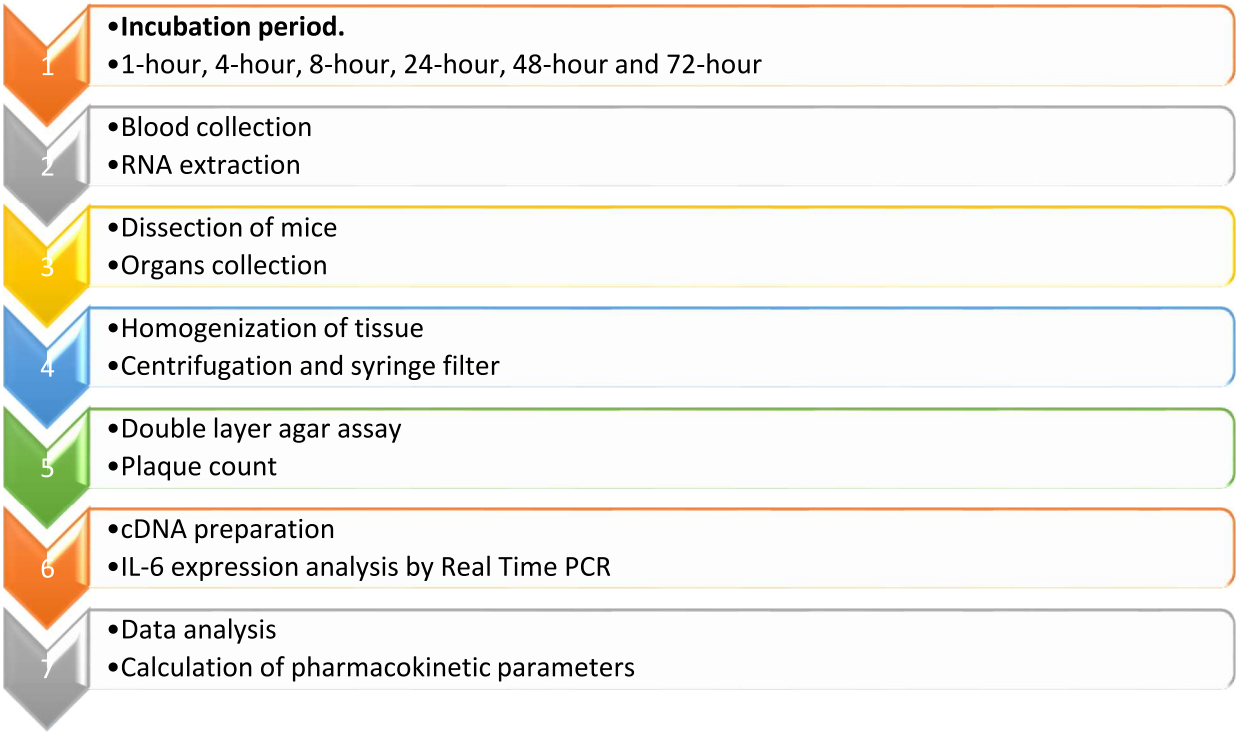
Pharmacokinetics of the Phage TU_A56 was studied in mice model according to the modified protocol (Gill, Pacan *et al.*, 2006; Sarhan, Ibrahim *et al.*, 2017). Thirty-six Female BALB/c mice of age 6 weeks were used in the experiment. Two hundred microliter phage stock of phage titer 1.02×10^8 was administered orally with the help of a steel gavage tube and similarly 200 microliters of phage was administered intraperitoneally. Each group of mice (oral and IP) had two experimental subject and one negative control. SM buffer was used as negative control. Blood sample was collected in EDTA tubes after 1-hour, 4-hour, 8-hour, 24-hour, 48-hour and 72-hour after the administration of phage. Chloroform was used to anesthetize mice during the blood collection. After the blood collection the mice were killed by cervical dislocation and was dissected to isolate the internal organs such as kidney liver and spleen. RNA was extracted from the blood collected using Direct-zol RNA Kit and the serum was also separated and stored for further study.

The organs were weighed and homogenized in mortar and pestle with 2 ml of SM buffer and the suspension were collected in Eppendorf tube. The suspension was subjected to

centrifugation at 12000 rpm for 15 minutes and the supernatant was filtered using syringe filter to avoid bacterial cells. After that the filtrate was then mixed with 100 microliter of bacterial culture (OD_{600}=0.15) and allowed for attachment for minutes. The sample were mixed with 3ml 0.6% TSA and the mixture was poured over the 1.5% TSA. The plates were incubated at 37°C overnight and the plaques were observed and different pharmacokinetic parameters were calculated. Plaque forming unit per ml and pfu/gram were calculated for blood and organ tissues respectively and the graph of pfu/ml versus time was plotted using Microsoft Excel.

Flow chart





3.29. Cytokine Expression Analysis by Real Time PCR

Comparative CT method was used to calculate the relative expression ratio from the real-time PCR efficiency and the CT value. Protocol from (Jain et al., 2006; Livak & Schmittgen, 2001) was followed. It is also known as the $2^{-\Delta\Delta Ct}$ (DDCT) method. cDNA was prepared Bio-Rad iScript™ cDNA Synthesis Kit and the real time PCR was performed with the Bio-rad SYBR® Green Master Mix(2X). Three sets of primer of IL-6, TNFα and β actin were used among them β actin was used for the baseline control of the gene expression. The following sets of primers and the PCR condition were used in the experiment.

Table: 3. 7 Mouse Cytokine Primers

S.N.	Gene	Primer (Forward)	Primer (Reverse)
1	IL-6	5′-GAGGATACCACTCCCAACAGACC-3′	5′-AAGTGCATCATCGTTGTTCATACA-3′
2	TNFα	5′-CATCTTCTCAAAATTCGAGTGACAA-3′	5′-TGGGAGTAGACAAGGTACAACCC-3′
3	β actin	5′-CTGTCCCTGTATGCCTCTG-3′	5′-ATGTCACGCACGATTTCC-3′

Table: 3. 8 Real Time PCR Condition

S.N.	Steps	Temperature (°C)	Time
1	Enzyme Activation	95	10 minutes
2	Initial Denaturation	94	15 seconds
3	Annealing	55	30 seconds
4	Extension	72	1 minute
5	Melt curve	85	10 minutes
Total Cycles = 45			

The fold of IL-6 expression was calculated using DDCT method($Fold\ change = 2^{-\Delta\Delta Ct}$)

$2^{-\Delta\Delta Ct}$ = [(C T gene of interest -C T internal control) sample A - (C T gene of interest- C T internal control) sample B](Schmittgen & Livak, 2008).And the graph of expression of IL-6 versus time was plotted.

Chapter Four

RESULTS AND DISCUSSIONS

4.1. Antibiotic Sensitivity Test (AST)/ Antibiogram Assay

Among six strains of *Acinetobacter baumannii*, all showed resistance against eight different antibiotics used in Kirby Bauer Disk diffusion test (Hudzicki, 2009).

Table: 4. 1 Antibiotic susceptibility Test (AST) to confirm MDR Acinetobacter baumannii

SN	Bacterial Sample	GEN 10	OF 30	PIT 100	NA 30	CAZ 30	CTX 30	MRP 10	IMP 10
1	A1								
2	A4								
3	A5								
4	A6								
5	A8								
6	A6								

Red, green, and yellow colors in boxes indicate resistant, sensitive, and intermediate respectively.

Figure: 4. 1 Antibiotic susceptibility test by Kirby Bauer disc-diffusion method. A, B, C, D, E, F showing AST of strain A1, A4, A5, A8, A6, and A56, respectively. The bacterial isolates were considered as MDR since they were resistant to more than one antibiotic. The clear zones indicate zone of lysis of bacteria.

The antibiotics used were of a different generation and various classes such as Quinolone aminoglycosides and β-lactam hence the bacteria were MDR. We performed only disk test method to check the susceptibility of the bacteria toward antibiotics.

4.2. Bacterial Genomic DNA Extraction

Genomic DNA of 6 Bacterial sample extracted using Ctab/NaCl method analysed in 0.8% agarose gel showed smiley band when observed in UV transilluminator. Smiley band of gDNA is visible in the figure below.

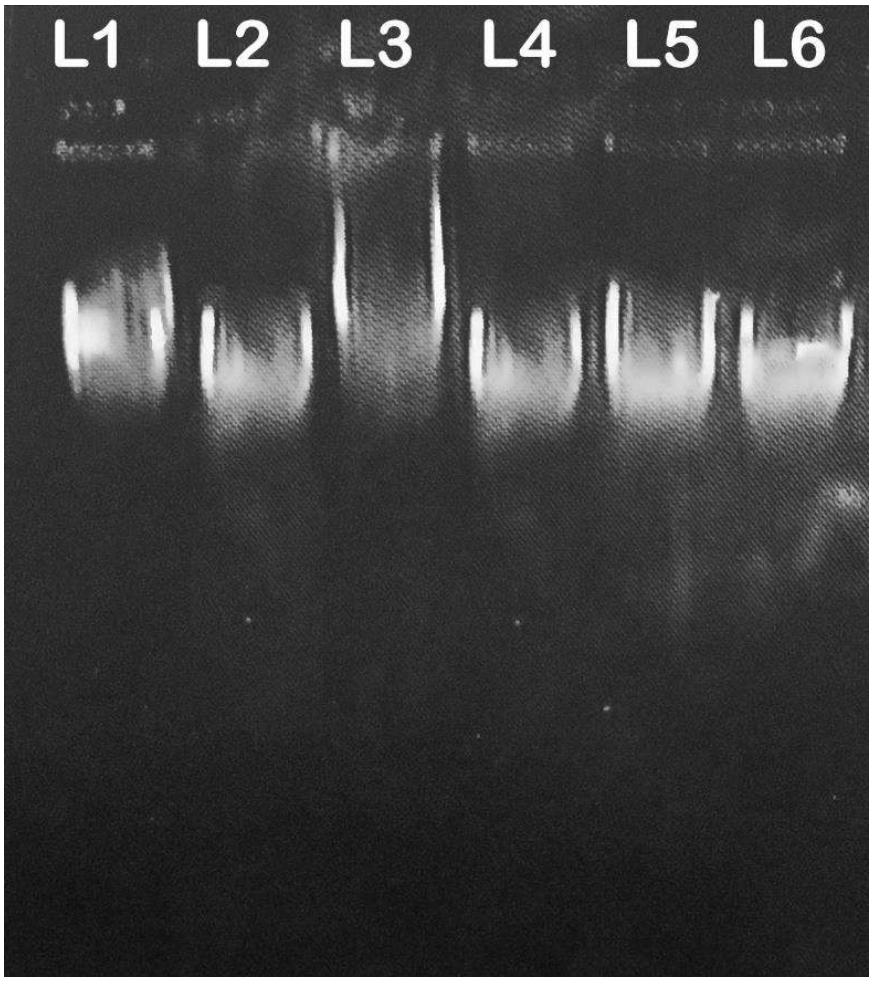


Figure: 4. 2 Genomic DNA of Host bacteria on UV transilluminator. Lane 1: A56, lane 2: A1, lane 3: A3, lane 4: A4, lane 5: A5, lane 6: A6, lane 7: A7 and lane 8: A8 respectively.

4.3. Molecular Identification of Host

4.3.1. PCR Amplification of 16S rRNA Gene

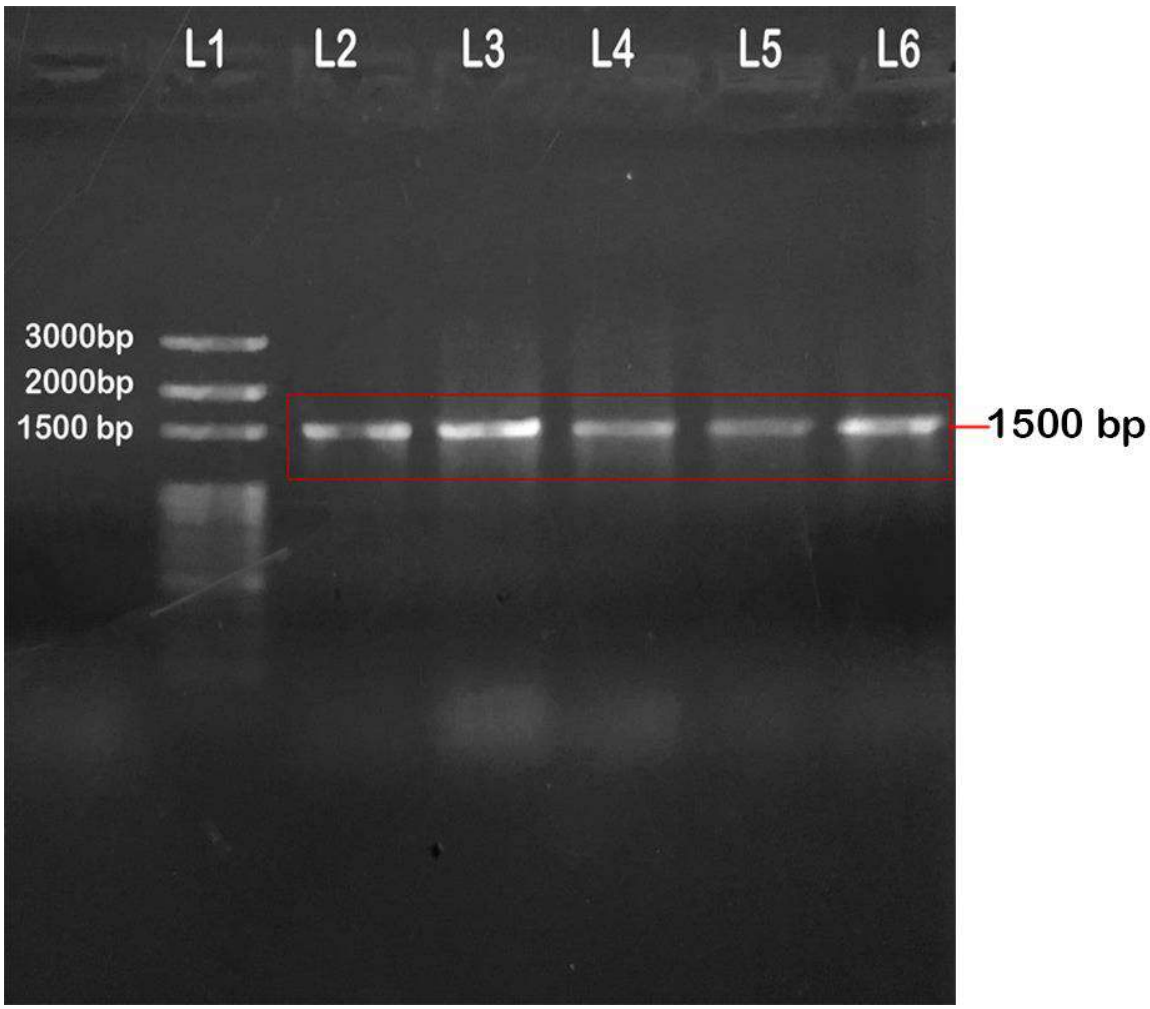
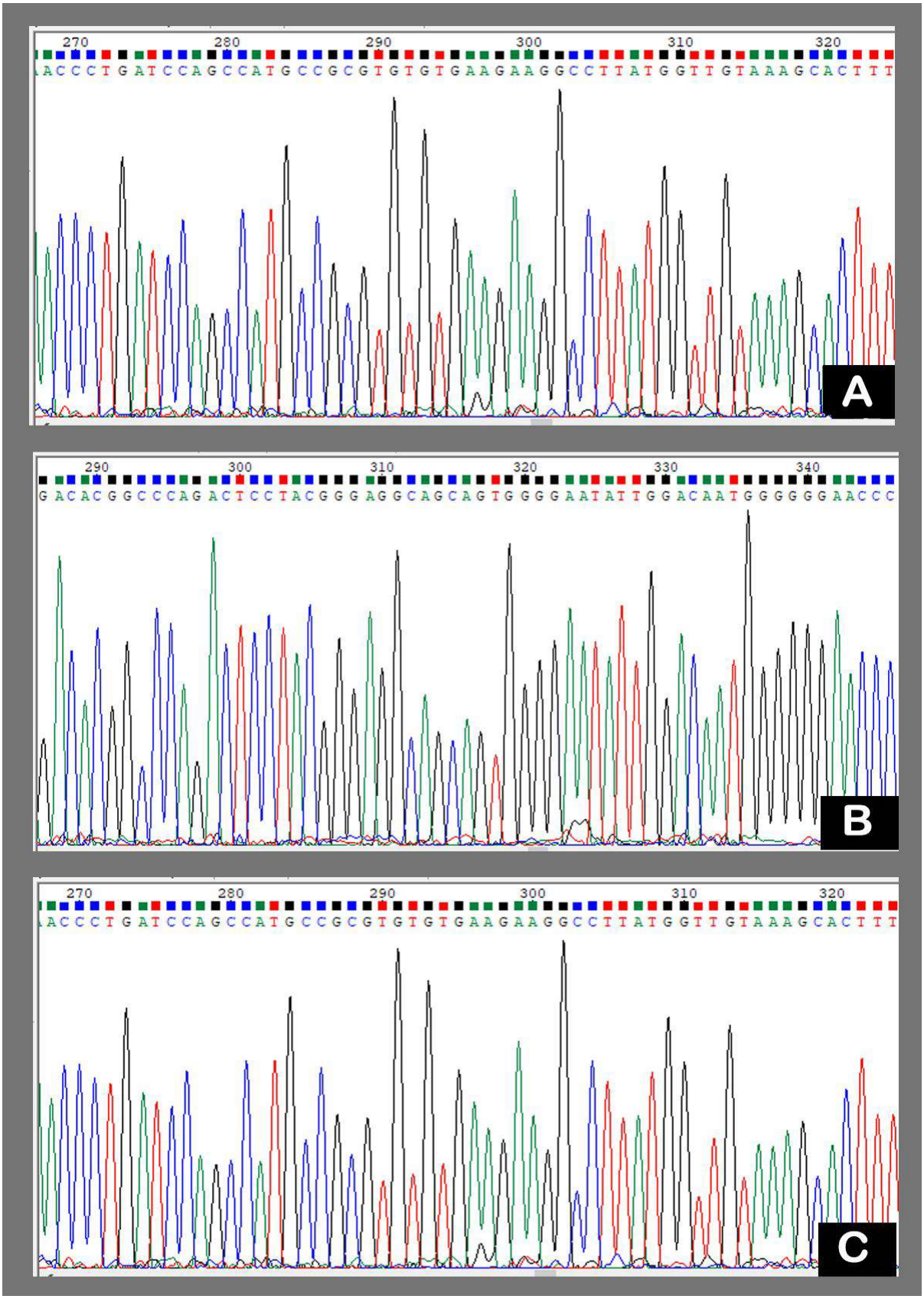


Figure: 4. 3 Amplification of 16S rRNA gene segment by PCR. Lane 1, 2, 3, 4, 5, represent the 100 bp DNA ladder ample A56, A2, A4, A5, A6, A7 and A8 respectively. All sample have distinct band of 1500 bp PCR product of 16S rRNA gene.

The 16S rRNA gene sequences of six strains *Acinetobacter baumannii* were received. The sequences when viewed using chromas software showed chromatogram as follows.


270
280
290
300
310
320
A
290
300
310
320
330
340
B
270
280
290
300
310
320
C

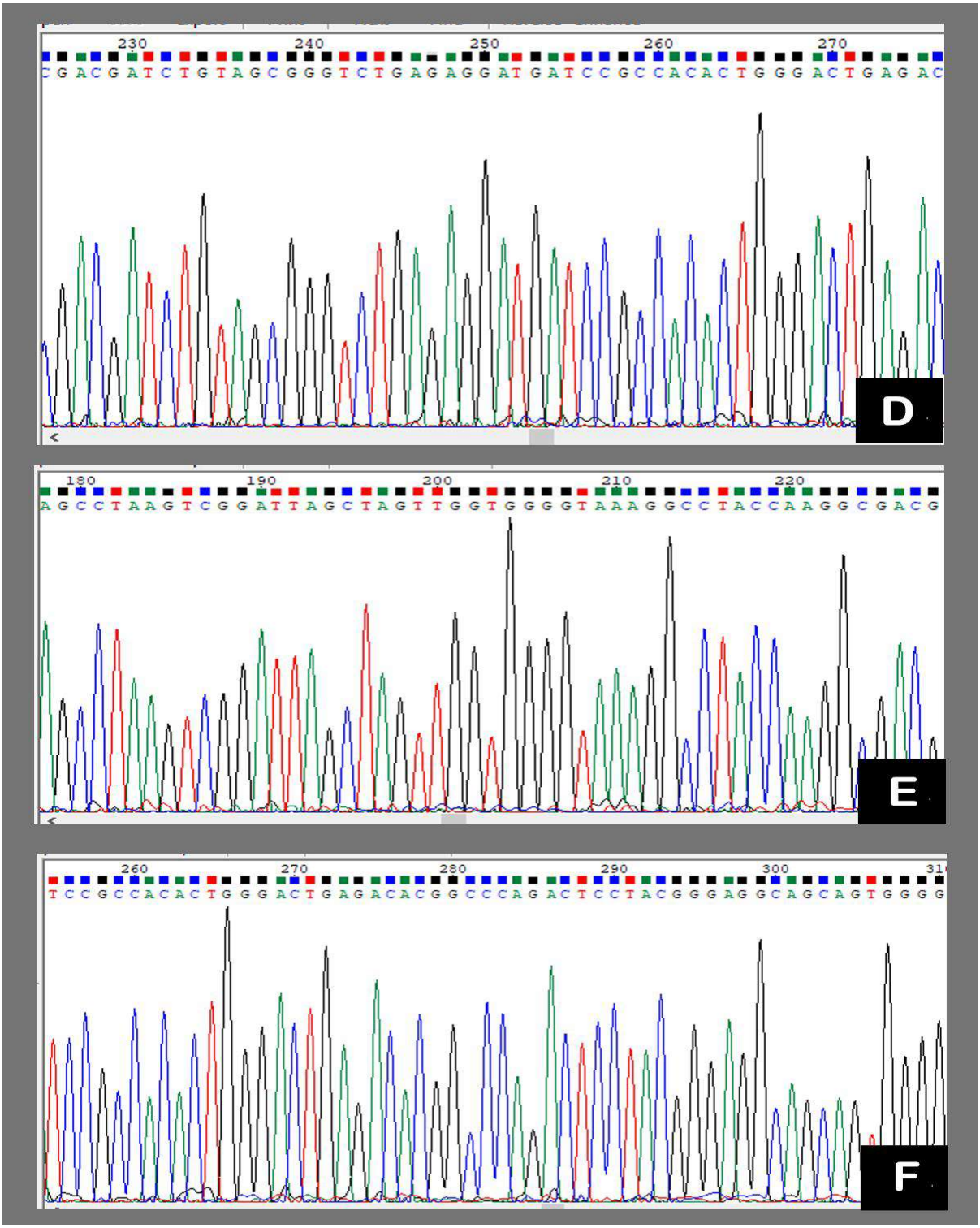


Figure: 4. 4 Chromatogram file viewed on Chromas software. Fig A, B, C, D, E and F represents chromatogram of sample A56, A1, A4, A5, A8, A6 respectively. The quality of bases can be determined by the color on the top of each peak. Lesser the noise higher the base call and higher the quality of base and vice versa.

Blast result of all six sample showed 99 percent match with the *Acinetobacter baumannii* stains. The gene sequences were submitted to NCBI under following accession number.

Table: 4. 2 Bacterial strains and their accession number

S.N.	Strain Name	Accession Number
1	*Acinetobacter baumannii* strain A1	MH806384
2	*Acinetobacter baumannii* strain A4	MH880097
3	*Acinetobacter baumannii* strain A5	MH819505
4	*Acinetobacter baumannii* strain A6	MH880098
5	*Acinetobacter baumannii* strain A8	MH807822
6	*Acinetobacter baumannii* strain A56	MH806378

4.3.2. Phylogenetic Tree Construction

The Maximum likelihood phylogenetic tree constructed by aligning sequences with ClustalW algorithm using MEGA-X program showed that among 6 samples, A1 and A8 were closely related A4, A5, and A6 were closely related but A56 was distantly related to the other five samples. And the close ancestor of all samples was *Acinetobacter baumannii* strain BR52 2H. A phylogenetic tree is an estimation of the evolutionary relationships among taxa (or sequences) and their hypothetical common ancestors (Nei & Kumar). Generally phylogenetic trees are built from molecular data: DNA or protein sequences. Traditionally the aim of most molecular phylogenetic trees was to estimate the relationships among the species represented by those sequences, but nowadays the purposes have expanded to include understanding the relationships among the sequences themselves without regard to the host species (Hall, Pikis *et al.*, 2009), and elucidating mechanisms that lead to microbial outbreaks among many others. phylogenetic tree construction requires four distinct steps: (Step 1) identify and acquire a set of homologous DNA or protein sequences, (Step 2) align those sequences, (Step 3) estimate a tree from the aligned sequences, and (Step 4) present that tree in such a way as to clearly convey the relevant information to others. In our case the homologous sequences were taken from the NCBI gane bank. And the sequence alignment was done

by ClustalW algorithm using MEGA-X. Maximum likelihood tree was constructed from the alignment.

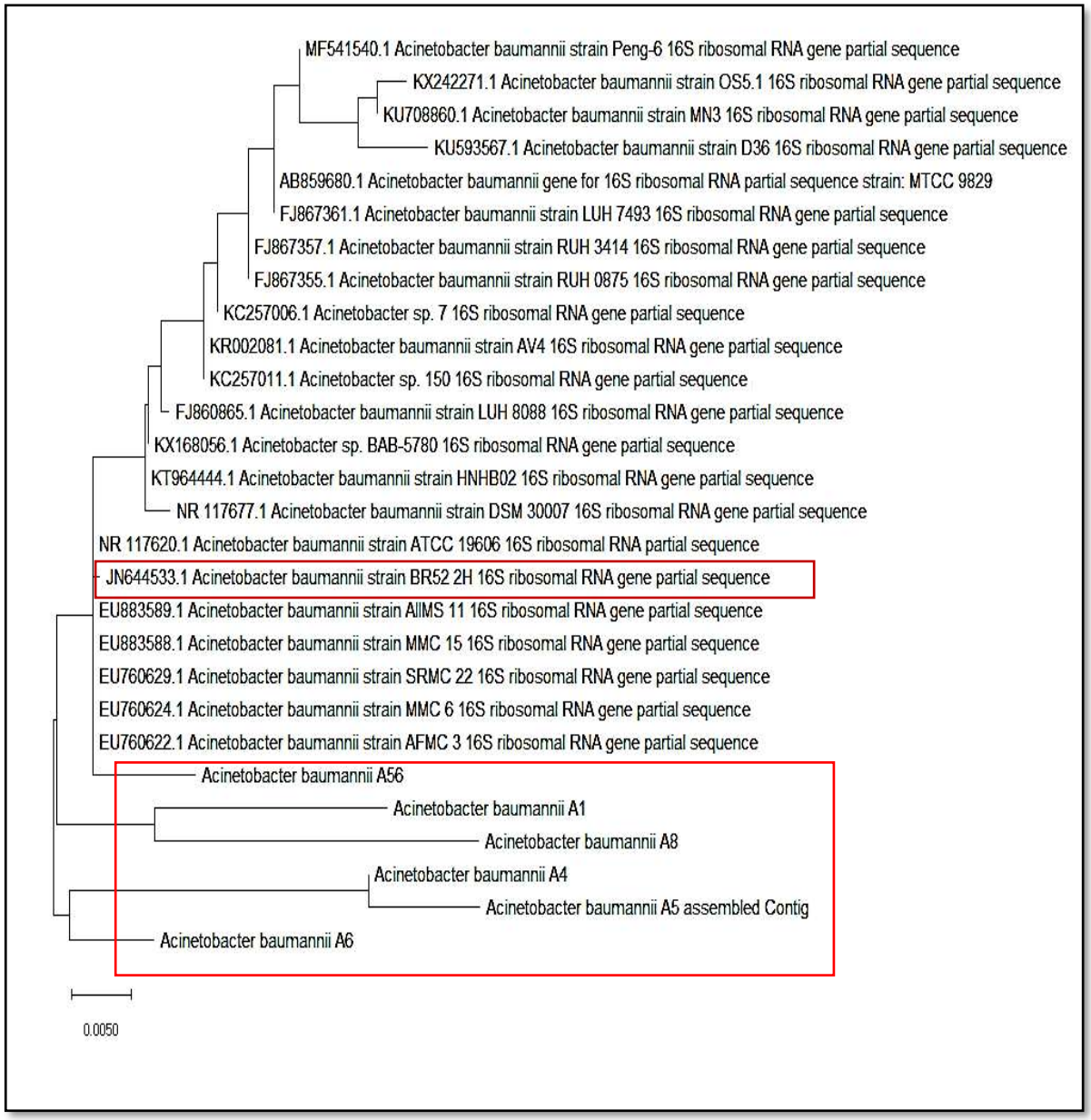


Figure: 4. 5 Molecular Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Kumar, Stecher *et al.*, 2016). Phylogenetic tree of 16S rRNA gene showed close ancestral relationship of sample A56, A1, A4, A5, A8 and A6 with *Acinetobacter baumannii* strain BR52.

4.4. Amplification of full length Metallo-beta-lactamase gene, bla (NDM-1) by PCR

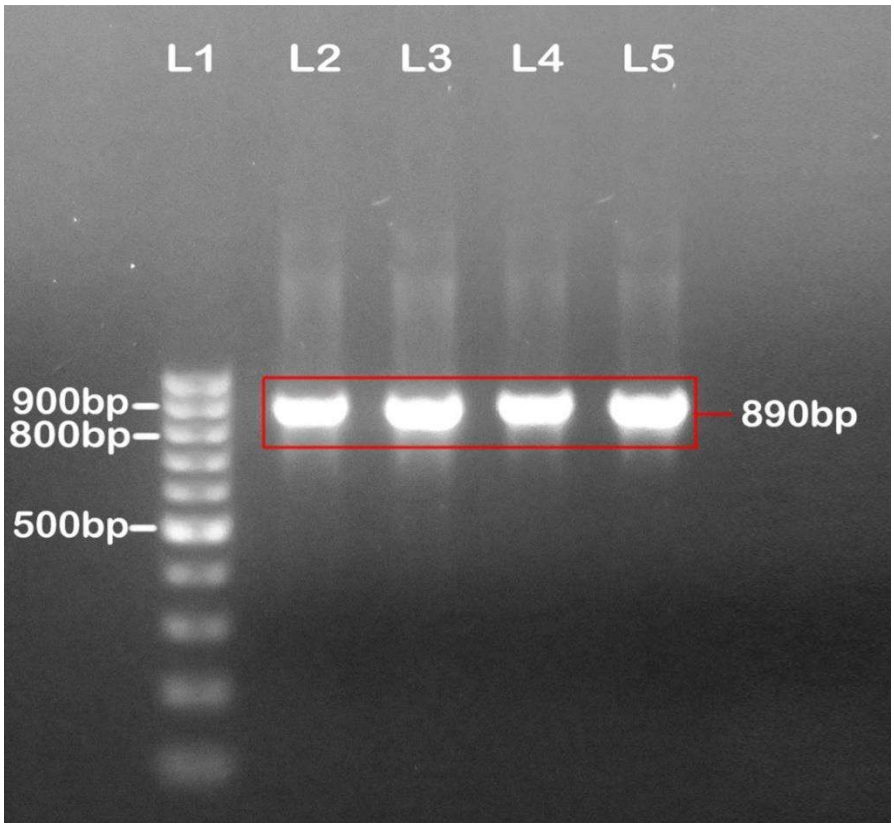


Figure: 4. 6 PCR amplification of full length metallo-beta-lactamase gene, bla (NDM-1). L1 100 bp DNA ladder, L2-L5 890bp PCR product of sample A1, A8, A5 and A56 respectively.

In our study we found out that the sample we have taken are causing chromosomal mediated drug resistance due to newly emerged New Delhi metallo-β-lactamase (NDM) (chromosomal).

In recent studies of plasmid mediated multidrug resistance *Acinetobacter* are mainly due to the expression of class D carbapenemases, called oxacillinases particularly OXA, Metallo-β-lactamases (MBL) encoding genes, including the VIM, IMP. Recently emerged New Delhi metallo-β-lactamase (NDM), have also been increasingly reported in *Acinetobacter* spp. isolated from different parts of the world (Tzouvelekis, Markogiannakis *et al.*, 2012). In our study we found out that our samples were causing multidrug resistance by chromosomal New Delhi metallo-β-lactamase (NDM) gene.

PHAGE SCREENING AND MANIPULATIONS

4.5. Isolation of Bacteriophage by DLAA Method

All together four bacteriophages were isolated against four strains of multi drug resistant *Acinetobacter baumannii* among six stains collected from hospital. Although the sewage/water sample was collected from various places of Kathmandu, we were able to isolate the bacteriophages from the water sample of Balkhu and Teku. This may be because the samples from Blakhu and Teku were more polluted than other samples. The initial plates contained mixed population of phages showing variation in plaque morphology and lysis. Out of six samples we were not able to isolate the phage against two strains of *Acinetobacter baumannii*. The reason behind this may be the strains were migrated to Nepal with the human host from another country and the rivers of Kathmandu does not contain phages against them or may be due to the small sample size.

Table: 4. 3 Table showing Phage and their site of isolation.

SN	Name of sample collection site	GPS Co-ordinates	Host A1	Host A4	Host A5	Host A6	Host A8	Host A56
1	Balkhu 1	27.68232, 85.29894	**-**	**-**	**-**	**-**	**-**	**+**
2	Balkhu 2	27.68447, 85.2998	**+**	**-**	**-**	**-**	**-**	NA
3	Balkhu 3	27.6836, 85.29946	NA	**-**	**-**	**-**	**-**	NA
4	Teku 1	27.69789, 85.30214	NA	**-**	**-**	**-**	**+**	NA
5	Teku 2	27.69546, 85.30025	NA	**-**	**+**	**-**	NA	NA
6	Pashupati 1	27.70913, 85.34865	NA	**-**	NA	**-**	NA	NA

Note: In this table, '+' denotes presence of phage in the sample, '-' denotes absence of phage in the sample and 'NA' denotes not tested.

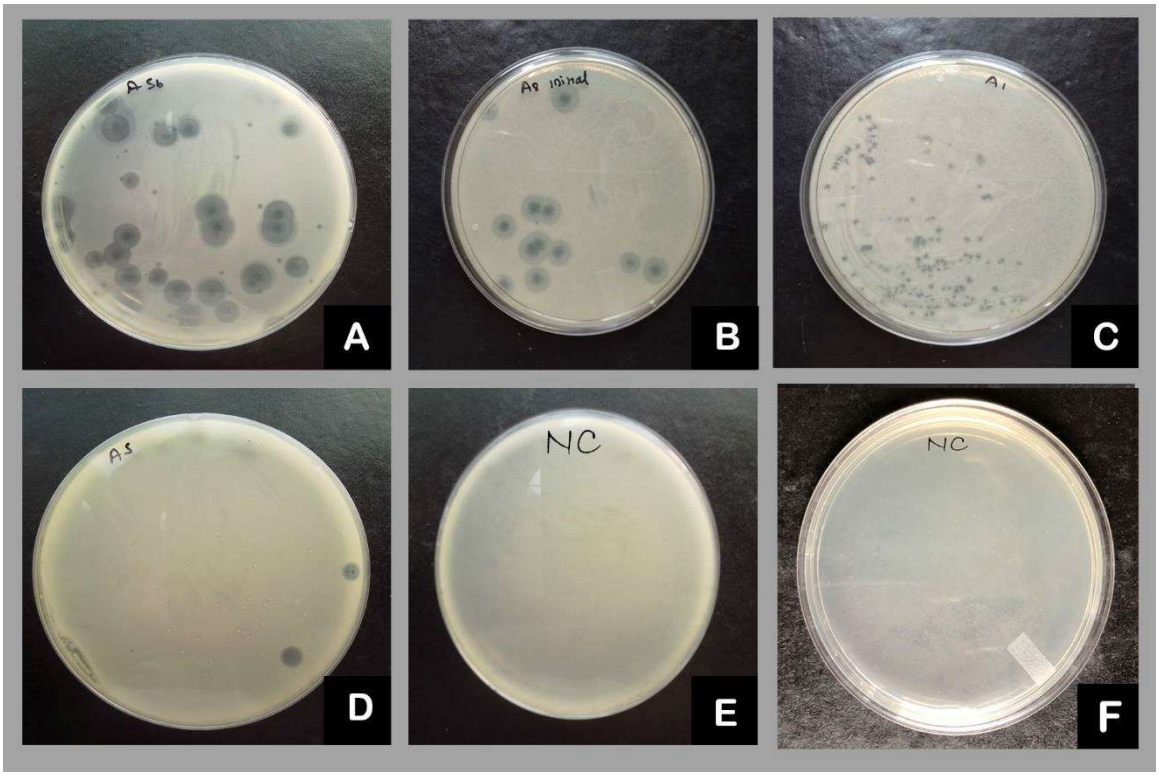


Figure: 4. 7 Initial plaques of bacteriophage observed in DLAA plates. A: Mixed plaques observed against host sample A56 in initial screening plate (pin head and Bulls eye). B: Bull's eye plaque observed against host sample A8 in initial plate. C: Pin head plaque observed against host sample A1. D: Bull's eye plaque observed against host sample A5. E: Negative control (bacteria only) F: Negative control (water sample only)

Different types of plaques were isolated from various water sample. The plaque morphology observed were small pin headed clear plaque and large bull's eye plaque and the most dominant was Bull's eyes plaque. Among numerous plaques single clear plaque was selected for further processing. There were different sized and different morphological plaques which indicates the presence of more than one type of phage in the water sample. The size of the plaque is proportional to the efficiency of adsorption, the length of the latent period, and the burst size of the phage. A diversity of plaque sizes can result if the phage infects cells at different times during the bacterial growth phase that is phage that adsorb early make larger plaques than those that adsorb later. And another fact is that the physical size of phage also influences the overall size of plaque it produces. As smaller phage has less physical size and it diffuses more easily and quickly through semi-solid agar in plaque assay plate in order to produce a zone of lysis. Whereas larger phages face difficulty in diffusion and hence formed small size plaque. And another fact is that A slowly proliferating phage, one which yields a low number of progeny phage, will more likely to produce a smaller plaque compared to quickly proliferating phage.

A clear plaque refers that the host is completely susceptible to the phage which is formed in our three phages. Bull's eye plaque form predominantly because phage-induced bacterial lysis is less efficient or complete later during plaque development than it is early on during plaque development. Decreasing lytic efficiency can be a consequence of aging of the bacterial lawn, associated increases in the size of microcolonies making up the bacterial lawn, or because of less general phenomena such as the lysis inhibition phenotype seen with T-even phages. The halo around the plaque indicates that decapsulation of the bacterial host cell by phage produced soluble enzyme such as depolymerase. The hazy ring suggests that phage produced a depolymerase enzyme that defused through the agar layer and degraded the bacterial capsular polysaccharide (CPS) into different oligosaccharide components. Early studies showed that certain Klebsiella pneumoniae bacteriophages produced depolymerase during phage proliferation and released the enzyme from infected bacteria that targeted another bacteria's CPS (M. H. Adams & Park, 1956)

Phages were isolated following double agar assay. It helps 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 we use SM (sodium chloride and magnesium sulphate) buffer. The gelatin present in SM buffer helps to stabilize the phage particle while storage. Chloroform maintains the sterility of phage stock by hindering bacterial growth without causing any harm to phage. The visualization of visible plaques denotes the positive result for phage isolation. Plaques are clear zones formed in a lawn of cells due to lysis by phage. The phages were isolated in high titre without enrichment which indicates that the rivers of Kathmandu valley are heavily polluted with hospital waste because *Acinetobacter baumannii* is a pathogen normally causing tissue infection. And another major concern is that there is presence of multidrug resistant pathogenic bacteria in river flowing around Kathmandu valley which may results in multidrug resistant pathogen infection in people who encounter the river water. In our study, four bacteriophages against four different samples of *Acinetobacter baumannii* were isolated. The high phage titre was found to be in Phage TU_A56 and Phage TU_A1 bacteria from balkhu sample during initial isolation.

4.6. Clonal Purification of Bacteriophage

The initial bacteriophage isolated plate may contain diverse population of phage as we may not be able to see them with our naked eyes. To study the morphology, physiochemical characteristics genome sequence we need to purify the phage to be sure that there is a single phage strain in our phage stock.

The bacteriophage was purified using continuous streaking technique. Progeny plaques originated from a single mother plaque were used to prepare phage stock. And the research was carried out from the phage stock. Phage stock of four phage was prepared and stored at 4°C for further research.

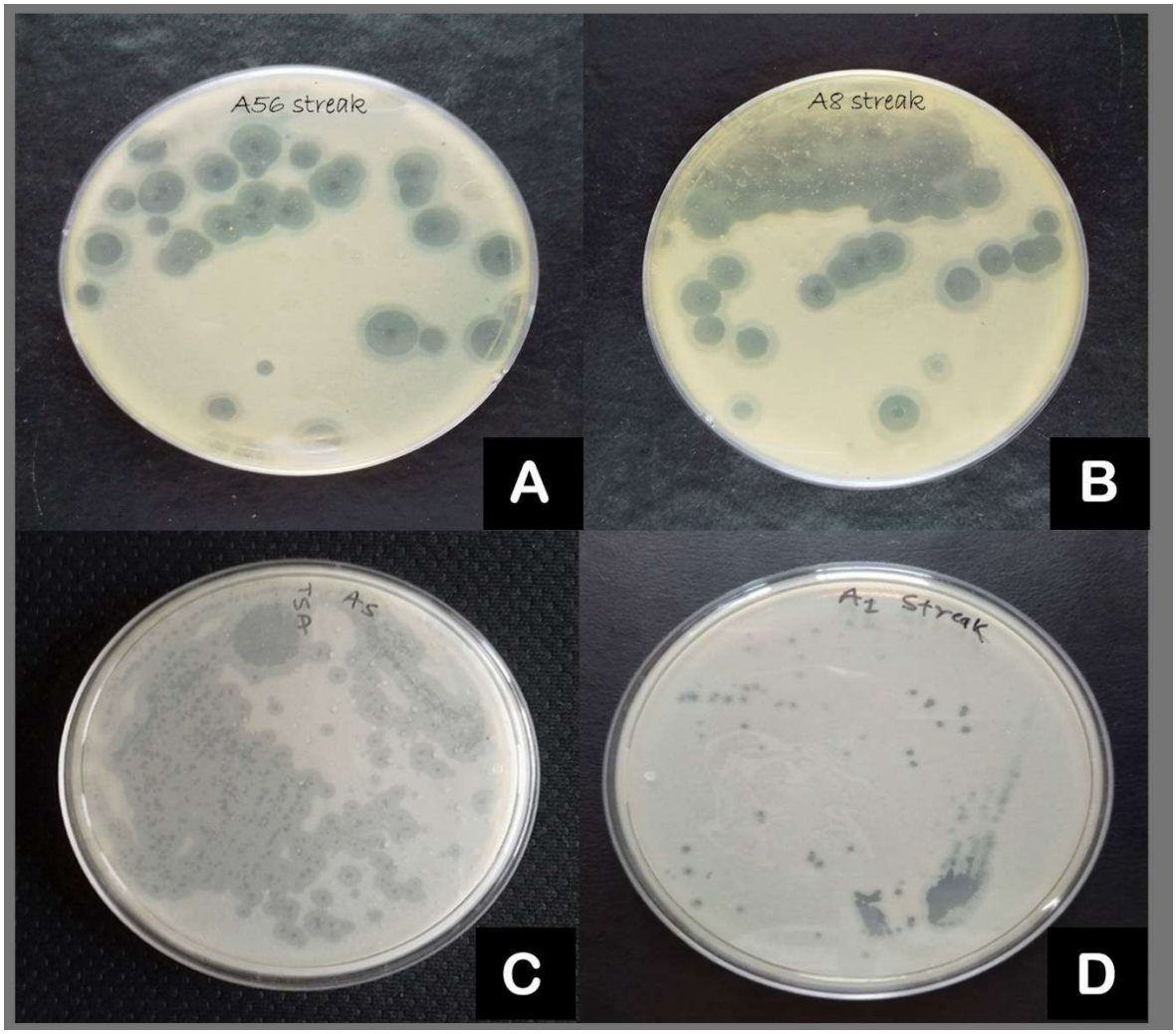


Figure: 4. 8 Purification of phage by continuous streaking method. A, B, C and D Shows the isolated plaque obtained after streaking the single plaque on TSA media.

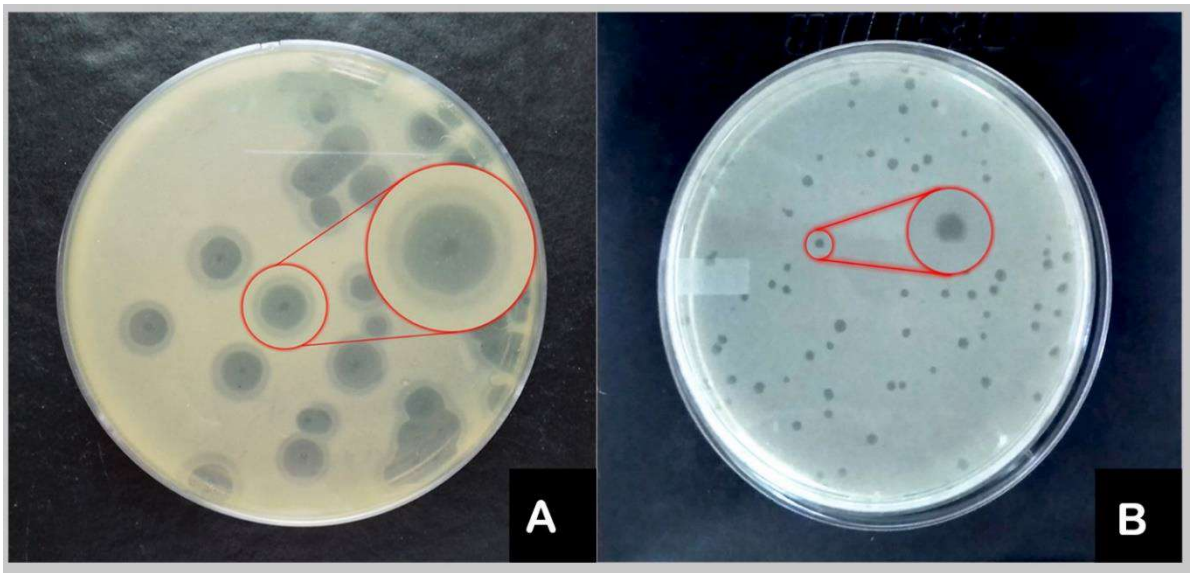


Figure: 4. 9 Zoomed view of Bull's eye Plaque and Pin head type plaque. A: 50% zoomed view of Bull's eye plaque showing complete lysis at the center and incomplete lysis at the periphery, B: 50% zoomed view of Pin head plaque showing complete lysis in small area.

Bull's eye plaque form predominantly because phage-induced bacterial lysis is less efficient or incomplete during late phase of plaque development than in its early phase. Decreasing lytic efficiency can be a consequence of aging of the bacterial lawn, associated increases in the size of colonies making up the bacterial lawn, or because of less general phenomena such as the lysis inhibition phenotype seen with T7 phages (www.archaealviruses.org). Different types of bacteriophages found in each plate based on the formation of plaques with different degree of transparency and sizes are listed in table below.

Table: 4. 4 Plaque morphology

S.N.	**Name of Phage**	**Plaque morphology**	**Lysis**
1	TUphage_A1	Pin head	Clear
2	TUphage_A5	Bull's eye	Clear center with turbid periphery
3	TUphage_A8	Bull's eye	Clear center with turbid periphery
4	TUphage_A56	Bull's eye	Clear center with turbid periphery

4.7. Determination of Phage Titer

Countable plaques were observed in dilution 10^{-14}, 10^{-8} ,10^{-4} and 10^{-5} in the DLAA plates of A56, A8, A1 and A5 respectively. Phage titer was calculated using formula given below and found to be 3.7×10^{15}, 2.7×10^{6}, 1.1×10^{9} and 3.9×10^{5} for phage stock A56, A8, A1 and A5 respectively.

$$\mathbf{Pfu/ml} = \frac{\mathbf{Number\ of\ plaques\ observed}}{\mathbf{Diilution} \times \mathbf{Volume\ of\ sample}}$$

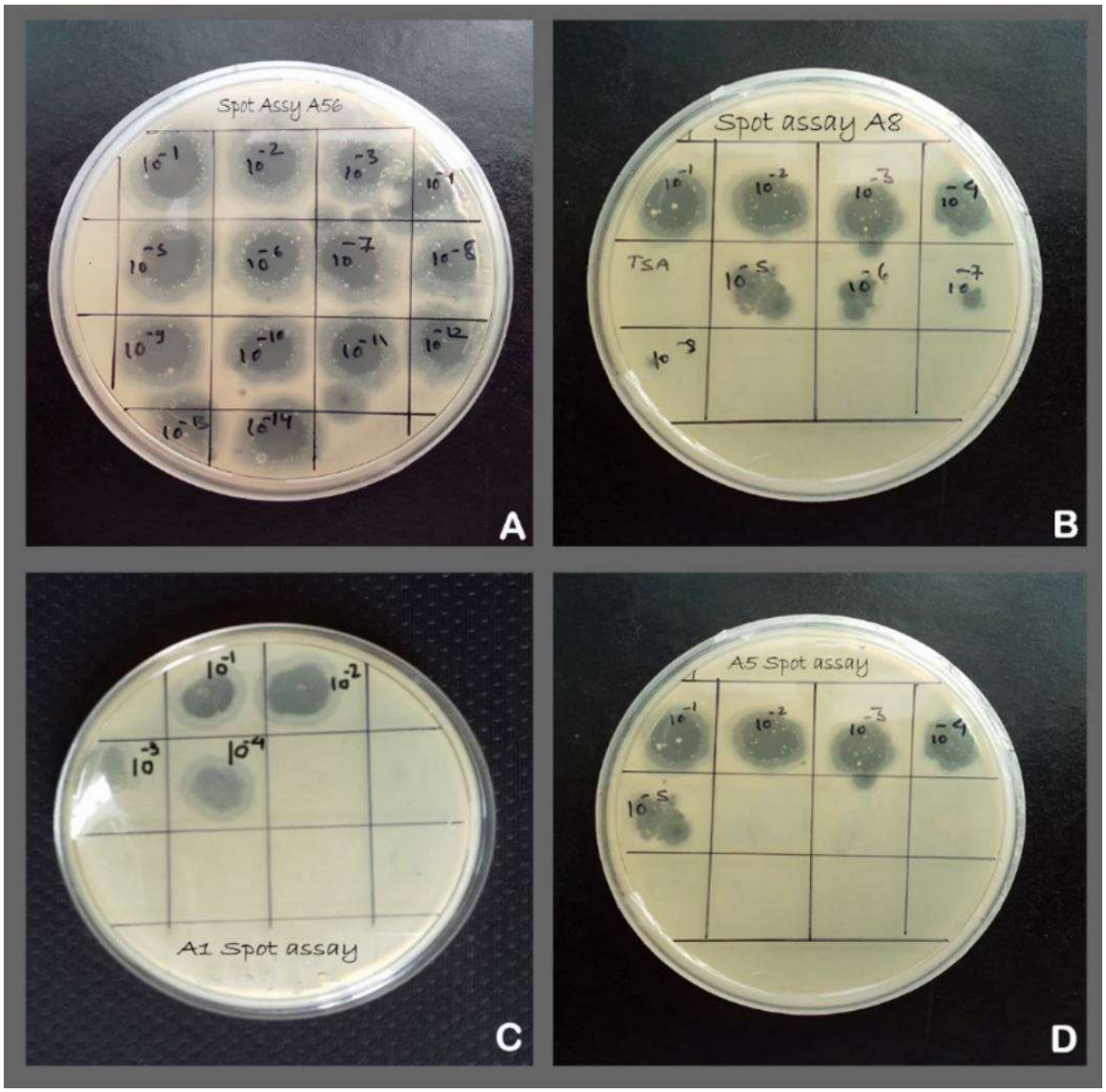


Figure: 4. 10 Spot Assay of phage stock showing lysis of bacterial lawn culture by different dilutions of phage stock. A, B, C and D are spot assay of phage stock A56, A8, A1 and A5, phage stocks showed lysis up to 10-14, 10-8 ,10-4 and 10-5 respectively.

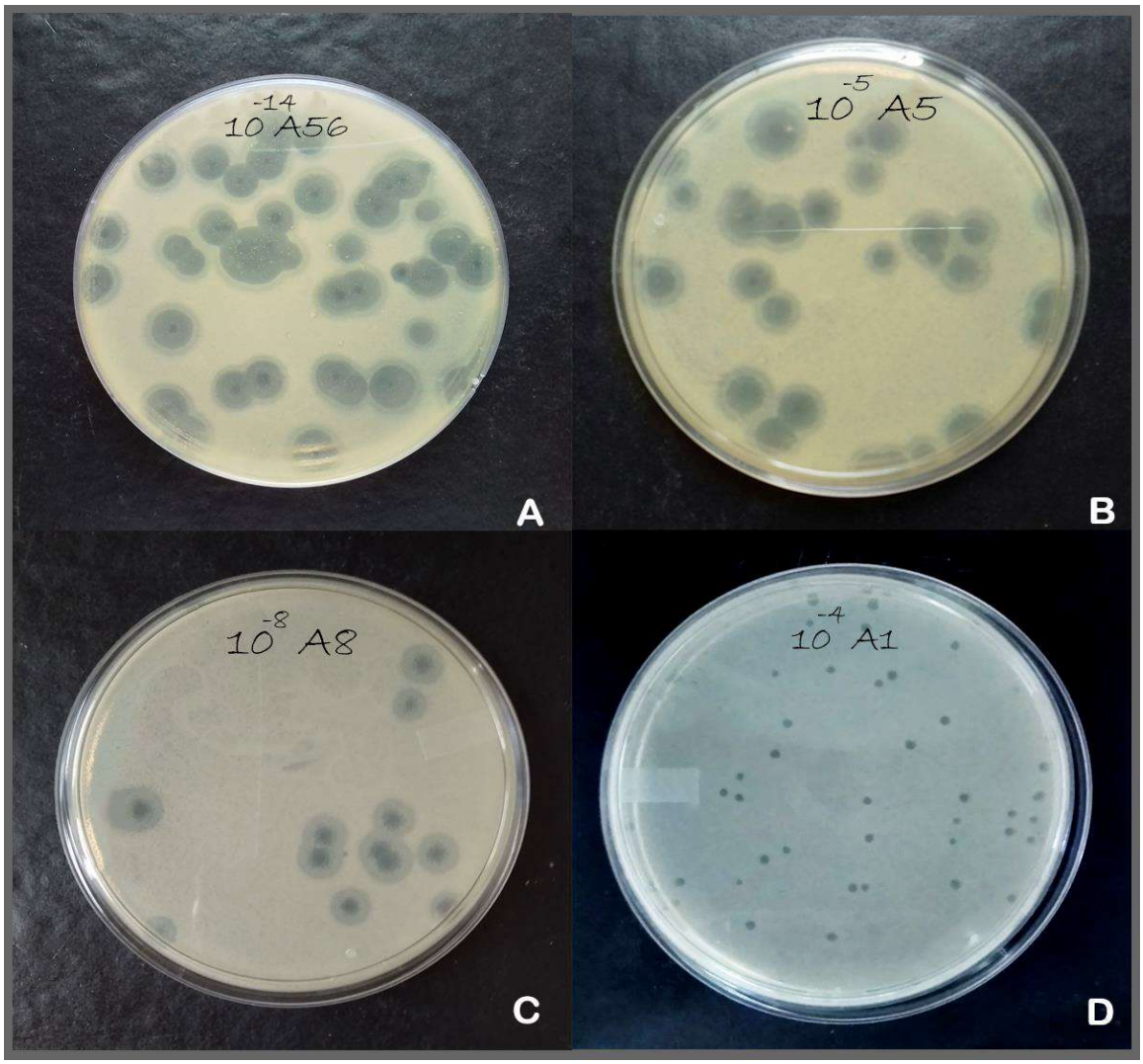


Figure: 4. 11 DLAA plates showed countable plaques in different dilutions. A: TU Phage_A56 stock showed 39 Bull's eye plaques in 10^{-14} dilution. B: TU Phage_A5 stock showed 27 Bull's eye plaques in 10^{-5} dilution. C: TU Phage_A8 stock showed 11Bull's eye plaques in 10^{-8} dilution and E: TU Phage_A1 stock showed 37 Pin head plaques in 10^{-4} dilution.

Table: 4. 5 Phage titer in different dilution

S.N.	Name of phage	Dilution	$\text{Pfu/ml} = \frac{\text{Number of plaques observed}}{\text{Diilution} \times \text{Volume of sample}}$
1	PhageA56	10^{-14}	3.7×10^{15}
2	PhageA5	10^{-8}	2.7×10^{6}
3	PhageA8	10^{-4}	1.1×10^{9}
4	PhageA1	10^{-5}	3.9×10^{5}

For the experiments such as whole genome sequencing and TEM, high phage titer is required so the PhageA56 was amplified up to 10^{14}. We did not perform WGS and TEM for other phages so their titer is kept low because if we had increased our titer, we had to dilute the stock for other experiments.

4.8. Host Range Analysis

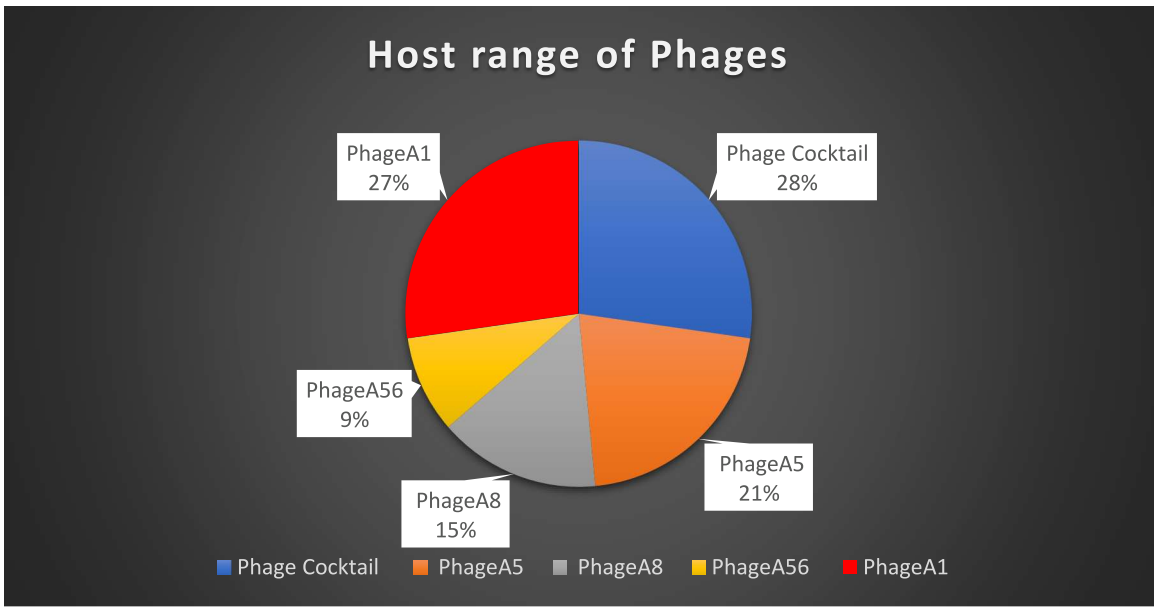


Figure: 4. 12 Pie chart showing the host range percentage of different phage and phage cocktail. Phage cocktail shows the highest host range among all phages with 28% of total host killed, whereas the phageA56 has least host range with 9% of total host killed. PhageA1 seems to be the phage with highest host range of 27% percent and phagA5 and PhageA8 fall in middle with 21% and 15% host range among killed host.

Host range is one of the important characteristics of bacteriophage for therapeutics use. The wider the host range of the phage more it is likely to kill broad range of multidrug resistant bacteria. Spot assay is a quick way to check whether a phage can infect a bacterium or not. A small drop or "spot" of phage stock was placed onto a plate inoculated with the bacterium. All spot tests were repeated in triplicate to confirm results of phage lysis rather than bacteriocin induced lysis. From this study, it was found that, each phage had a distinct host range, with no individual phage being able to lyse all strains. Phage cocktail showed the highest host range among all phages with 28% of total host killed, whereas the phageA56 had least host range with 9% of total host killed. PhageA1 seems to be the phage with highest host range of 27% percent and phagA5 and PhageA8 fall in middle with 21% and 15% host range among killed host.

4.9. Protein Profiling by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Profiling of PhageA56 protein was done by SDS PAGE. Two distinct bands of PhageA56 were observed having size of approximately 87KDa and 55KDa. The size of band was calculated using GelAnalyzer software. The larger sized band could be assigned to the major capsid protein and another smaller sized band can be assumed as structural proteins; head-tail connector protein, capsid assembly protein, internal virion protein.

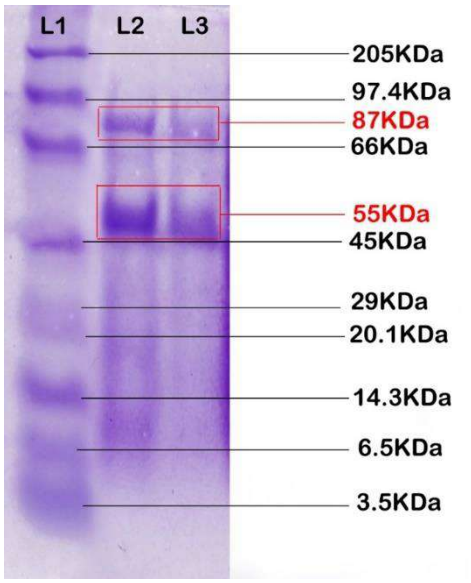


Figure: 4. 13 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. L1: Protein marker L2: Acetone precipitated phage sample showing two distinct bands of phage protein having size approximately 87KDa and 55KDa. L3: Heated phage sample showing two faint band of size approximately 87KDa and 55KDa.

On analyzing phage proteins bands, clear bands were formed on lane 2 and lane While on lane 3 were faint. The clear bands were formed by acetone precipitation method and faint bands were formed from direct heating method. Thus, we can conclude that phage proteins ruptured on precipitation and on heating at 100°C. Both methods can be used for analysis of phage proteins but according to my study acetone precipitation method was better than direct heating method.

4.10. Transmission Electron microscopy TEM

Out of four isolated phages, TEM of only one phage was done due to tha lack of resources. Transmission Electron Micrograph revealed that the phageA56 belonged to order Caudovirales as being a tailed phage. The phage was classified according to the ICTV guideline. The dsDNA tailed phage having tail falls under order Caudovirales, which accounts about total 96% of phage observed till date. T4 phage is a well-studied classic example of order Caudovirales and family Myoviridae. The order caudovirales has four families namely, Ackermannviridae, Myoviridae, Siphoviridae, and Podoviridae. Among four families, Myoviridae has six sub families; Eucampyvirinae, Ounavirinae, Peduovirinae Spounavirinae Tevenvirinae and Vequintavirinae; each of these subfamilies contains two genera. According to ICTB there are total 11 genera with unidentified family in Myoviridae due to lack of enough data.

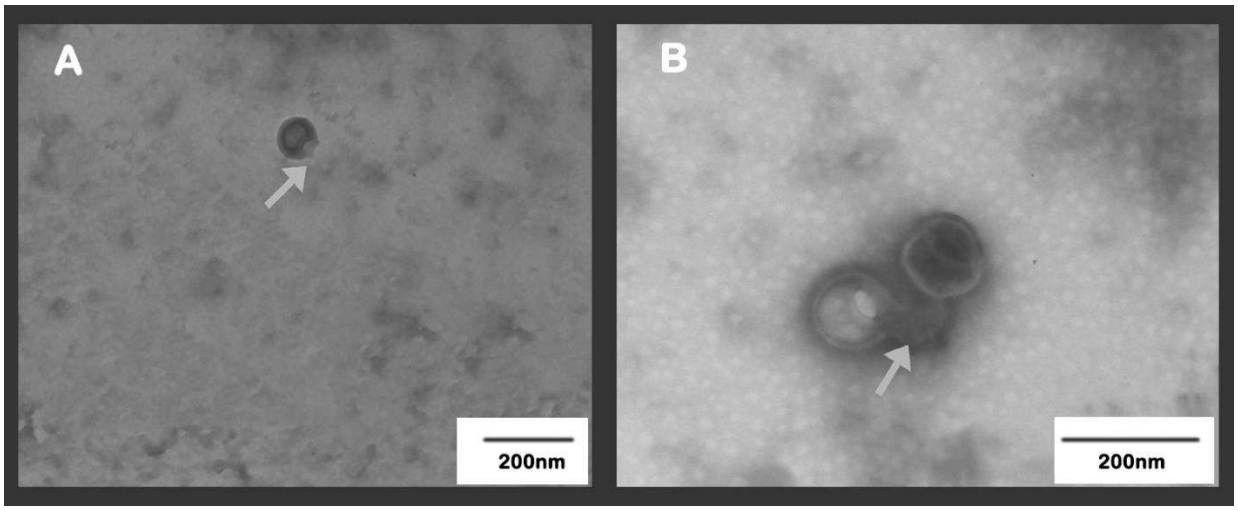


Figure: 4. 14 Transmission Electron Microscopy (TEM) of selected phages (negatively stained). The micrograph reveals that PhageA56 was tailed phage (Order: Caudovirales) A and B showing short tail and icosahedral symmetry of phageA56.

Since we do not have TEM facility in our laboratory or even in our country, we had to send our sample to Sophisticated Instrumentation Centre for Applied Research & Testing (SICART), Gujarat, India. The images we received were not as we expected and that is why the tail in our phage was not clearly visible.

Table: 4. 6 Classification of phage according to ICTV current guidelines based on transmission electron micrograph.

S.N.	Phage Name	Capsid (nm*)	Tail(nm*)		Shape	Putative order/ Family
			Length	Width		
1	PhageA56	72.9	56	24	Icosahedral	Caudovirales/ Myoviridae
***nm= Nanometer**						

As reported by the ICTV guideline the main criteria for the classification of bacteriophage is capsid size, capsid symmetry, absence or presence of tail, tail length and the genome size. In the table above, we can see that our phage has icosahedral capsid of size 72.9nm and short tail of size 56×24nm. Electron micrograph data correlates to data reported by (Comeau, Tremblay *et al.*, 2012). He has described dwarf Myoviruses having capsid size ranging from 55 to 75 nm and their tail size ranging from 62 to 115 nm. from this data we can speculate that or phage (PhageA56) falls under Order Caudovirales and family Myoviridae. Furthermore, to confirm the family of the PhageA56 we did nucleotide blast of the structural protein coding tail fiber gene and the bast hit matched with the phage of Myoviridae family.

4.11. In Vltro lysis of host cell

Lysis of *Acinetobacter baumannii* cells by phage was determined by comparing the optical density (OD_{600}) of bacterial culture with bacteriophage at different MOI with the growth of bacterial culture free of bacteriophage. PhageA56 showed efficient reduction of the bacterial cells in the liquid culture. The optimum lysis was observed at 5 MOI. The multiplicity of infection or MOI is the ratio of phage to bacteria. For example, when referring to a bacterial culture inoculated with virus particles, the multiplicity of infection or MOI is the ratio of the number of virus particles to the number of bacteria present in a defined space. Here the optimum MOI is 5 means that optimum lysis occurs at the ratio 1 phage: 5 bacteria.

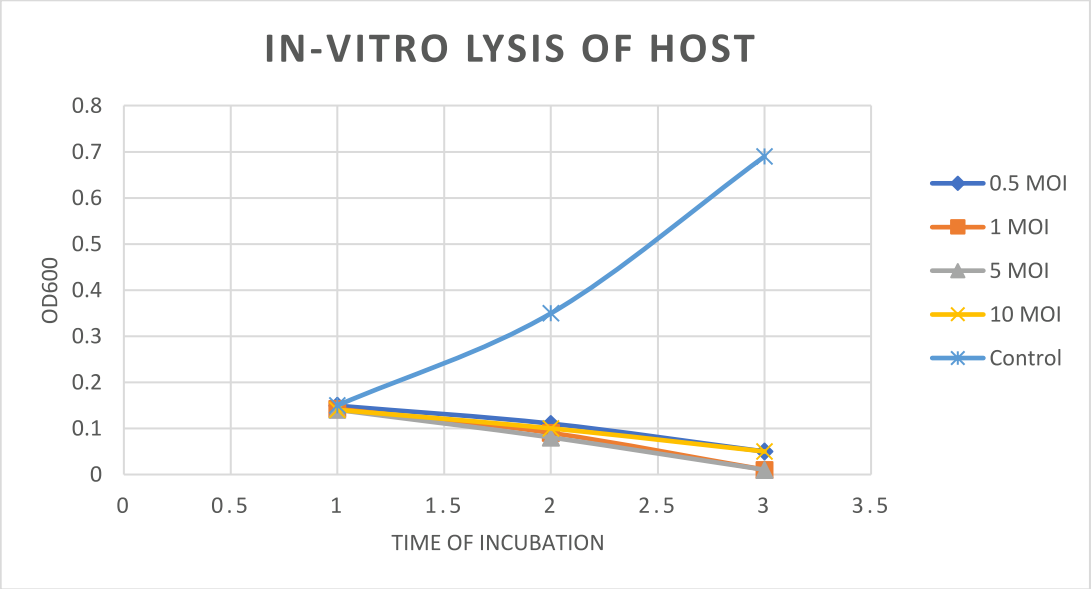


Figure: 4. 15 In-vitro lysis of bacteria by phage. The lysis of cell is measured by spectrophotometer. The decrease in optical density of a culture medium over the time indicates the lysis of bacterial cell. The control graph shows the culture having only bacteria and the other graph show the phage and bacteria culture in different MOI. The optimum lysis can be seen in 5 MOI.

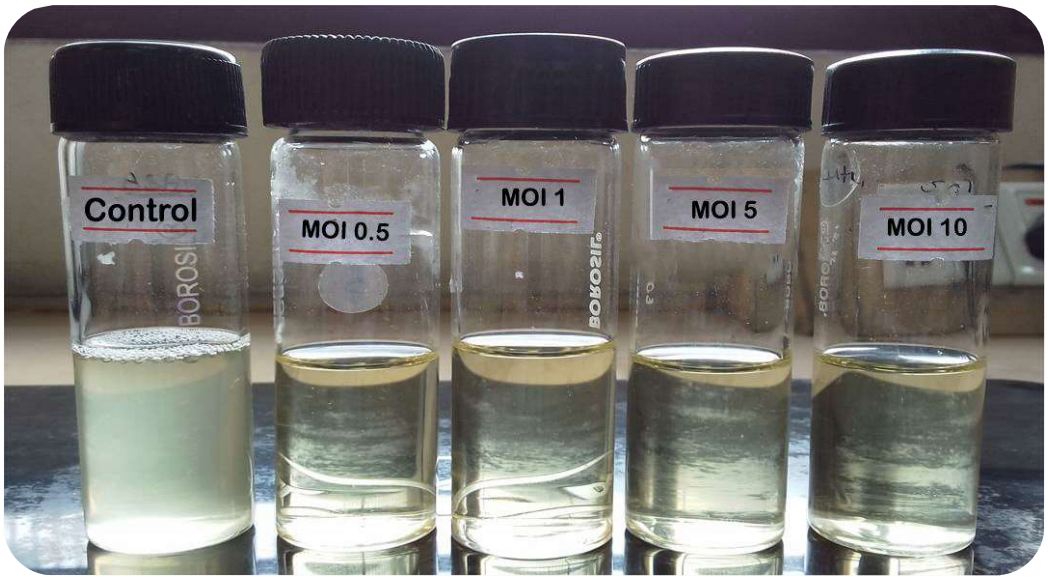


Figure: 4. 16 In-vitro lysis of bacterial cell by Phage. Control has only bacterial culture and is turbid due to bacterial growth and other tubes has phage and Bacteria in different MOI showing clear culture due to lysis of bacteria by phage.

4.12. Physiochemical Characterization

4.12.1. P^H Stability

Acidity or alkalinity of the environment a crucial factor in phage survivability. pH stability test showed that PhageA56 is stable in pH 3 to pH 11, and most stable at pH 4-8 during one hour of incubation. Phage showed most efficient lytic activity at pH 7 showing 39 plaques in DLAA plates (39 pfu/ml). On the other hand, phage lost its lytic activity in pH below 3 and above 12. From this result it can be concluded that PhageA56 is stable at wide range of P^H.

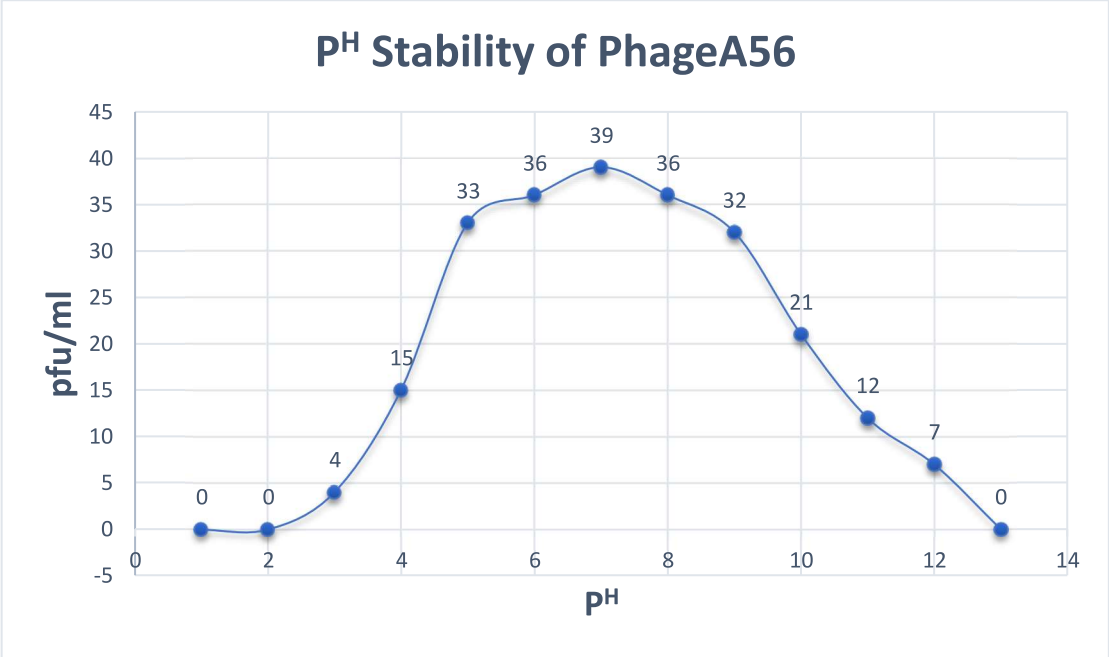


Figure: 4. 17 pH stability of PhageA56. Stability of PhageA56 in pH ranging from pH2 to pH 12 was checked by pre-incubating the phage suspensions at pH levels 2-13 at 37˚c for 1 hour. PhageA56 was stable in pH ranging from 3-12 and it could not survive at pH below pH 3 and pH above pH 12

It was shown that hydrogen ion concentration influences phage aggregation. For example, MS2 phages showed significant ability to aggregate when the pH was less than or equal to the phage isoelectric point (PI = 3.9) (Jończyk, Kłak *et al.*, 2011). Their aggregates could be up to 6 µm in diameter. This may cause a decline in phage count and an easier elimination of aggregates through their adsorption on membranes than single virions. (Rahimi-Midani, Lee *et al.*, 2018). At the higher and lower P^H, agar could not set properly and the plaque morphology was also changed.

4.12.2. Thermal Stability

Stability of phages on exposure to varying temperatures is considered as a key model for understanding the ability of the organism to adapt into novel environments. Temperature plays a fundamental role in attachment, penetration, multiplication, and the length of the latent period of phages (in the case of lysogenic phages). It determines the occurrence, viability, and storage of bacteriophages. The results showed that the PhageA56 was surprisingly stable at 60°C for 40 minutes though the titer decreased from 46 pfu/ml to 10 pfu/ml. The phage was not stable above 70°C as no plaques were observed after 10 minutes of incubation at 70°C and 80°C. Temperature plays a fundamental role in attachment, penetration, multiplication, and the length of the latent period of phages (in the case of lysogenic phages). It determines the occurrence, viability, and storage of bacteriophages. The effect of different temperature and Ph showed that phageA56 was able to withstand exposure to temperatures upto 60˚C for 40 minutes, without affecting phage viability. However, activity of phage was lost at 70˚C. Here in experiment we used dry heat. From our study we found that the phages were stable more in moist heat compared to dry heat.

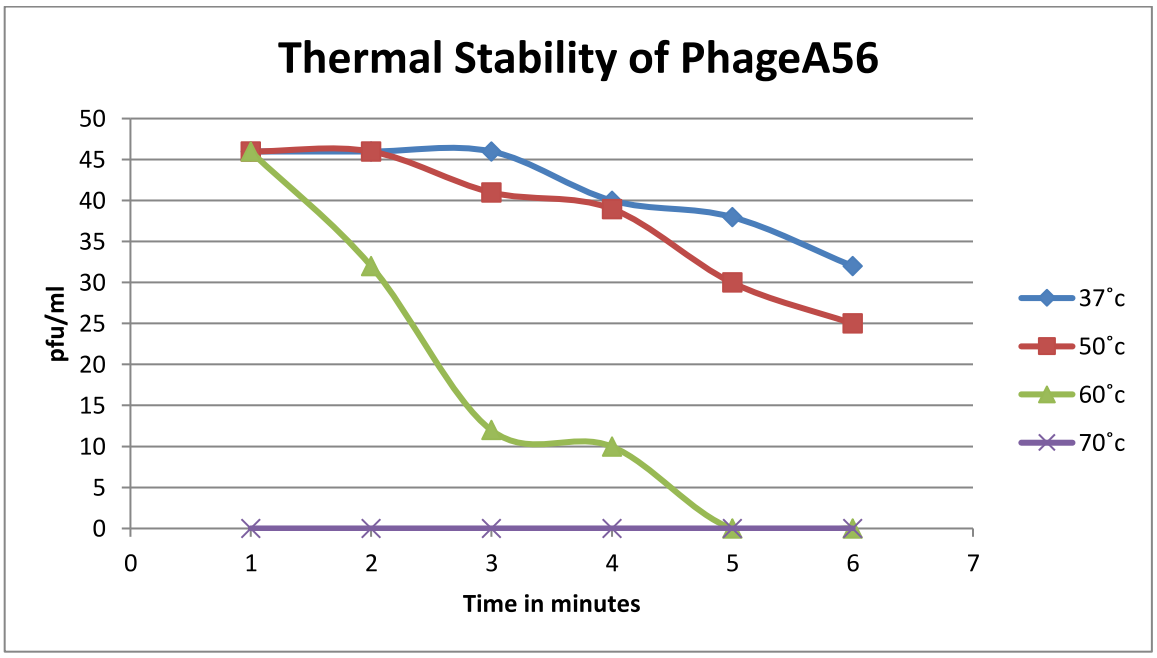


Figure: 4. 18 Thermal stability of PhageA56. Temperature stability of phage A56 was determined at different temperature in different time of incubations. Phage was found to be stable even at 60˚c up to 40 minutes. The phage was not stable at 70 ˚C and above. This result showed that infectivity of phage decreases at the temperature increases.

4.13. One step growth curve and Burst size

Life cycle and the different phages in the replication of the bacteriophage was studied by one step growth curve experiment. From this study we determined different stages of virus growth curve such as latent period, raise period and burst size. Our result showed that the latent period of phageA56 was of 30 minute and the burst size was found to be 304 virions per bacterium. The phageA56 has a short latent period meaning it can lyse the bacterial cell rapidly.

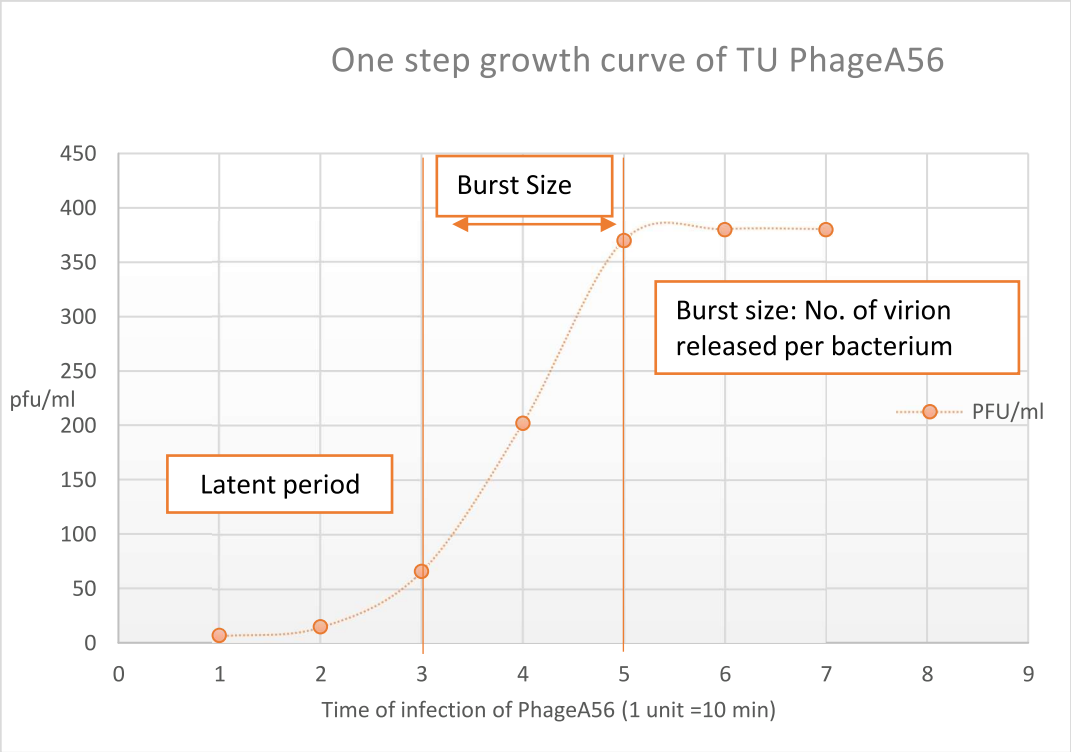


Figure: 4. 19 One-step growth curve of PhageA56: The latent period of phage was of thirty minutes and the burst size was found to be 304 virion per bacterium.

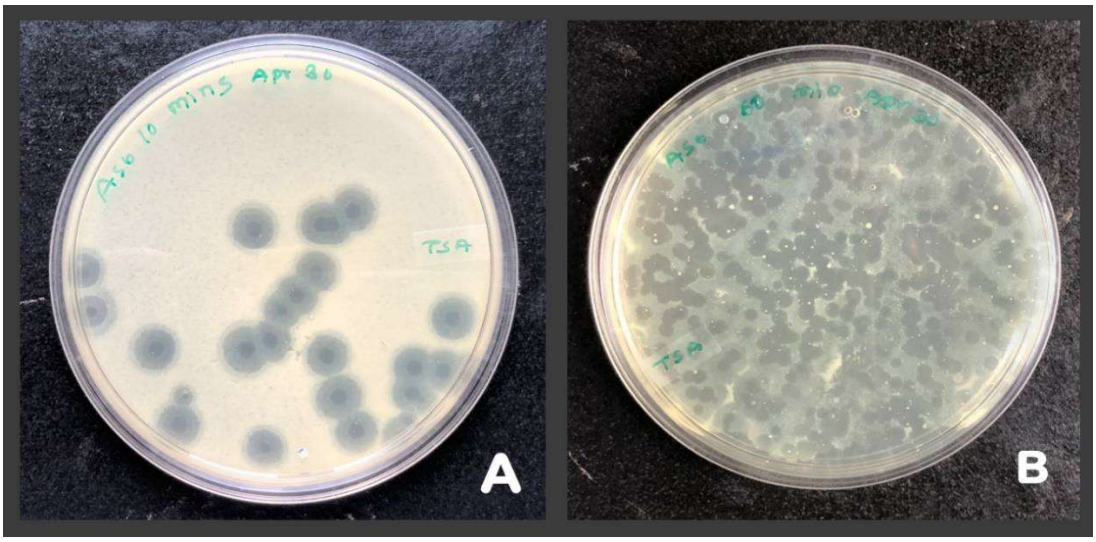


Figure: 4. 20 Burst size of PhageA56. A. Number of plaques at the beginning (10 minutes). B. Number of plaques after the release of progeny (60 min).

Burst size of a bacteriophage is the number of virions released after the lysis of an infected bacterium. The burst size of a phage depends upon the various factors such as nutritional availability in a bacterial culture and functions of phage-coded proteins such as polymerases and other regulatory proteins essential for replication; inherently low efficiency of these proteins may not allow the phage to make full use of bacterial machinery and consequently the burst size may be limited (Gadagkar & Gopinathan, 1980). In a study (Gadagkar & Gopinathan, 1980) found that the burst size of a phage is inversely correlated with multiplicity of infection and our finding supports this finding as our phage has a large burst size of 304 virions per bacterium and optimum MOI 5. The latent period of a phage infection is a time in which phage synthesize early protein needed for replication (nucleic acid synthesis), coat protein and other structural proteins and in the raise period the phage assembles all the protein to make a mature virion. And after the assembly the phage is released by lysing the host bacterial cell.

4.14. Whole Genome Sequencing

4.15. Isolation, Qualitative and Quantitative Analysis of gDNA:

The agarose gel image of phage genomic DNA isolated using Norgen Phage DNA Isolation kit showed a clear distinct band corresponding to HindIII marker's 23130bp band. The concentration of the DNA was found 243ng/µl as recorded by Qubit® 2.0 Fluorometer. The $A_{260/280}$ ratio was found to be 1.8 which eliminates possibility of protein and RNA contamination in the DNA sample. The yield of the genomic DNA was 9.7µg which was enough for the WGS so the sample was processed further.

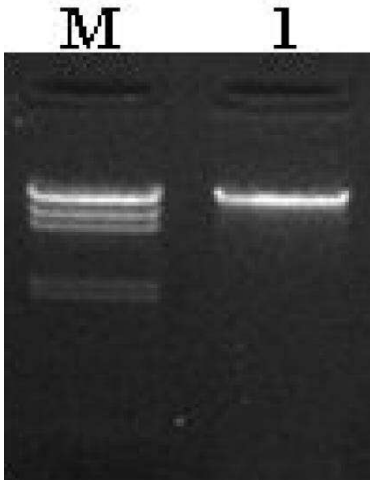
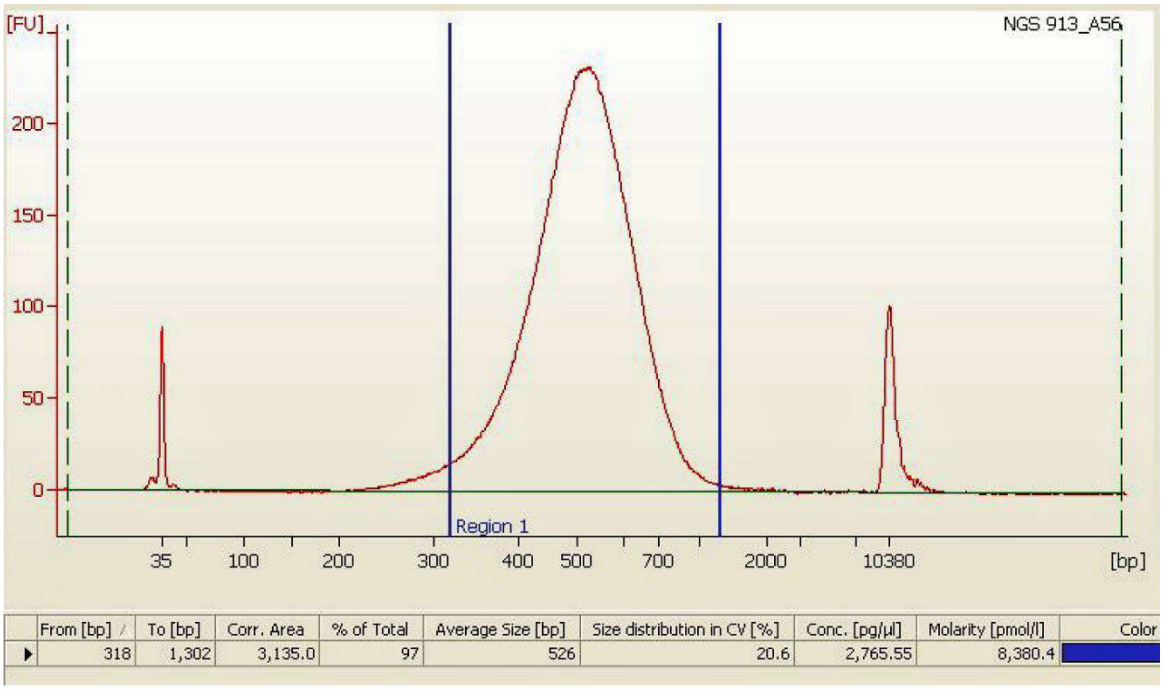


Figure: 4. 21 Quality control of phage DNA on 1% agarose gel. Lane M: HindIII marker, Lane 1: Phage genomic DNA of 23130kb.

4.16. Bioanalyzer Profiles of library Loaded in Agilent DNA HS chip

The libraries were prepared from gDNA samples of Phage TU_A56using Illumina TruSeq Nano DNA HT Library Sample Preparation Kit. The mean sizes of the fragmented libraries were 526 bp. The libraries were then sequenced (2x150 bp chemistry) to generate around 1.5 GB data.



From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
318	1,302	3,135.0	97	526	20.6	2,765.55	8,380.4	

Figure: 4. 22 Bio-analyzer profiles of library loaded in Agilent DNA HS chip. The average size of fragmented library of Phage TU_A56 was 526.

4.17. Bioinformatics Analysis of NGS Data

4.18. Data generation

The paired end data was generated on Illumina platform (2 X 150 chemistry) for the sample and the statistics of high-quality data are as follows:

Table: 4. 7 Read data statistics for the sample Phage TU_A56

S.N.	File name	Total reads	Total base	Data in GB
1	A56_Phage Stock R1	35704688	5341192151	5.34
2	A56_Phage Stock R2			

4.19. De novo Assembly

De novo assembly from all the high-quality reads was carried out using CLC genomics workbench v6.0 which is a powerful de-bruijn graph-based assembler. The statistical elements of the assembled genome were calculated using in-house PERL and they are given below.

Table: 4. 8 Statistical elements of genome assembly

Detail	Data
No. of scaffolds	11832
No. of contigs	13330
Total genome length including gaps (in bps)	12310343
Total genome length without gaps (in bps)	12229690
Average scaffold size (in bps)	1040
Scaffold N50	110760
Maximum scaffold size	689639

4.20. Genome Annotation

Nucleotide blast was performed from the assembled fasta sequence against NCBI nucleotide database using BLAST+. Figure below shows the blast hit result for the phage TU_A56. Top hit species distribution was plotted based on the number of hits obtained for different organisms via blastx annotation. Here, all the organisms including phage and non-phage have been considered.

It was observed from the blastx result that the sample phage TU_A56 has maximum genome similarity with Acinetobacter Phage YMC13/03/R2096(40 hits) so it can be understood that our phage was closely related to Acinetobacter phage. The phage genome also showed similarities with other phages like Pseudomonas phage and Klebsiella phage. As the species distribution showed similarity with other species phages, we have annotated the phage genome based on result obtained from PHASTER.

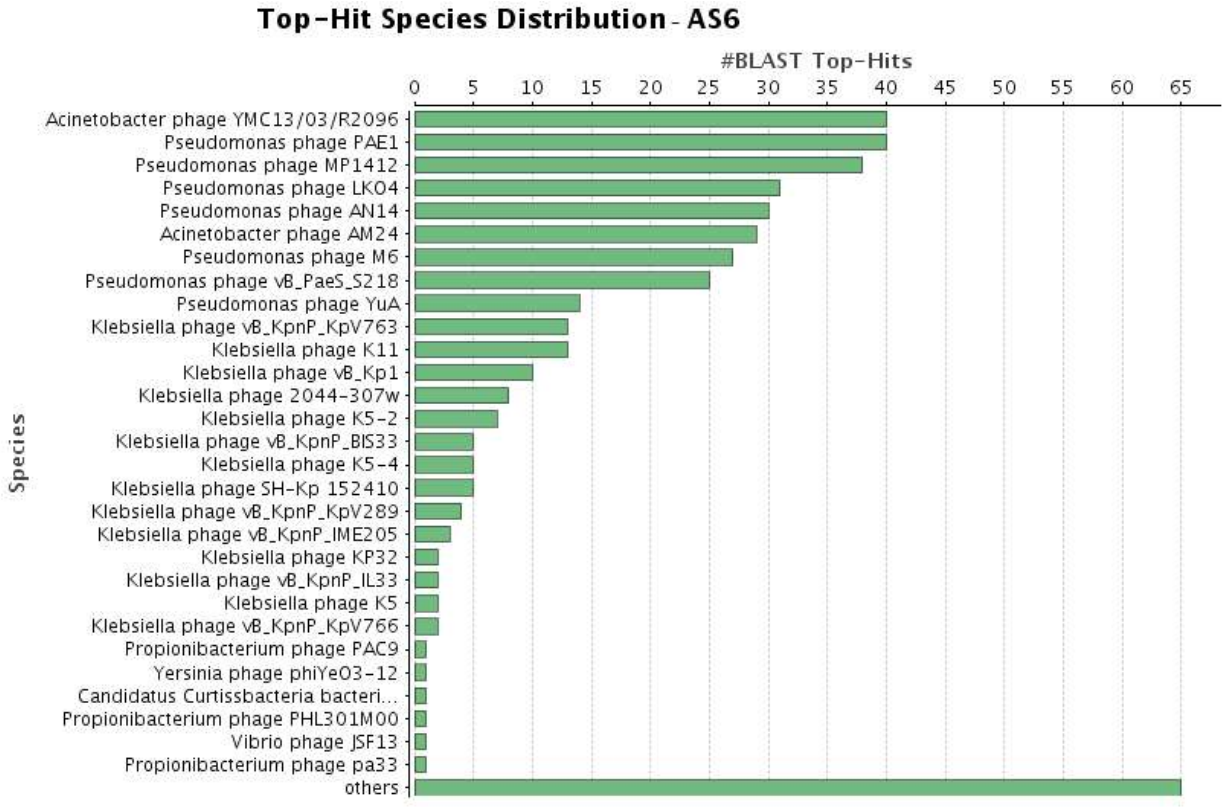


Figure: 4. 23 Top hit species distribution for sample A56. The X-axis represents the count of hits obtained from blastx functional annotations.

The blast result shows Acinetobacter baumannii phage in the top of the list means that our phage has highest resemblance with that phage. The genome also shows somewhat similarity with the other phages such as pseudomonas phage Klebsiella phage Yersinia phage, vibrio phage and others.

4.21. PHASTER Annotation

PHASTR (PHAge Search Tool Enhanced Release) is an advance form of PHAST which is a web server for the rapid identification and annotation of phage sequences. It is a curated phage database, widely used for the characterization and gene prediction of phages.

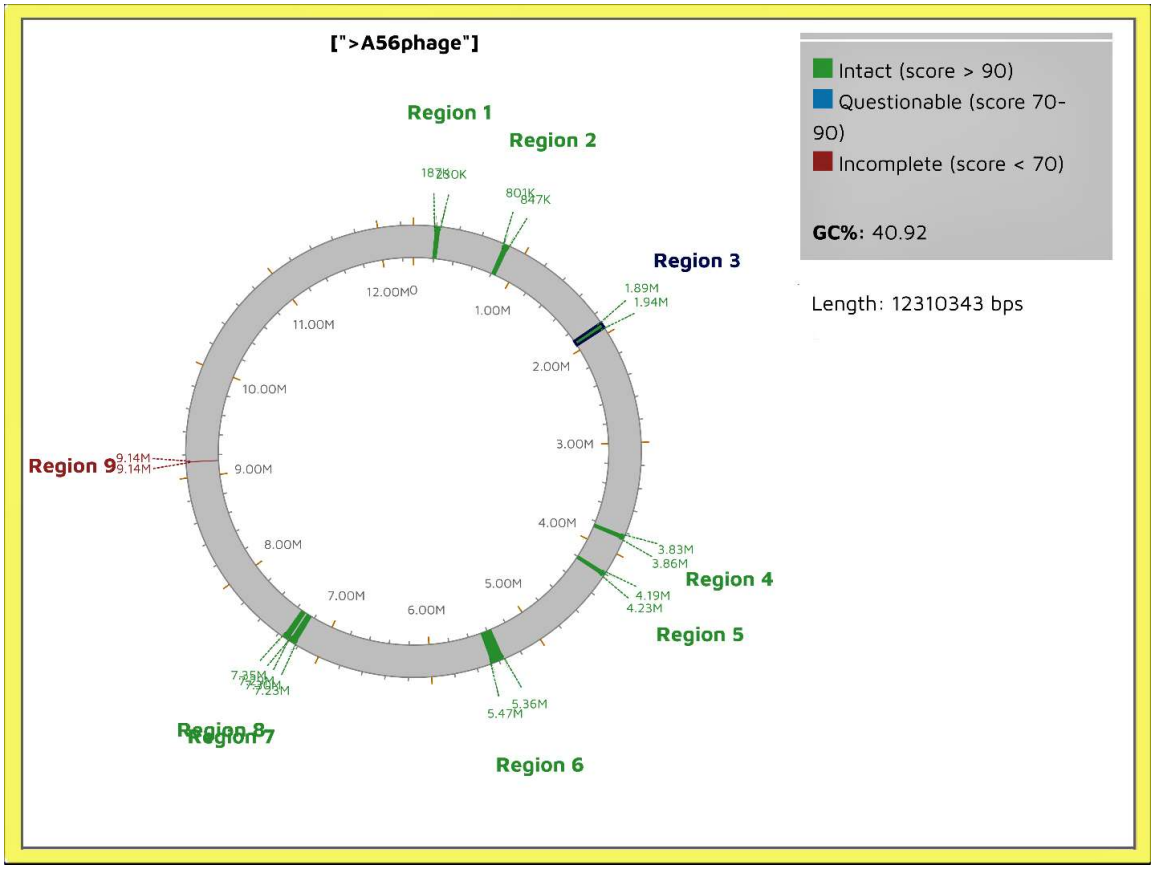


Figure: 4. 24 PHASTER generated circular genome map. The length of whole genome is 12310343 bps and GC content of region 3 is 40.92%. Circular map shows 6 regions of genome and their positions that has annotated genes.

The average size of a bacteriophage ranges from *Leuconostoc* phage L5 (2,435bp) to *Pseudomonas* phage 201phi2−1 (316,674b) (Hendrix, 2003). The genome size shown by PHASTER is a raw data size and contains many un-annotated regions in the genome. The GC content of region 1 of the genome is 40.9%. Overall GC content was found to be 49.5%.

Figure: 4. 25 Figure showing the different protein coding gene map. Different Genes/proteins lysis protein, terminase, coat protein, tail shaft protein, fiber protein, plate protein and their location in a genome. Different proteins are denoted by different colors.

The circular genome map created by PHASTER showed the 12310 kbp genome. PHASTER annotation showed 478 intact proteins and 20 incomplete proteins. The presence of proteins coding regions in the phage genome such as lysis protein, terminase, coat protein, tail shaft protein, fiber protein, plate protein and so on was also observed. The presence of lysis protein coding gene confirms that our phage is a lytic phage. Moreover, the presence of gene coding for tail shaft verifies that our phage is a tailed phage. In the image above, hypothetical proteins are not shown to make image clear and readable, there were several hypothetical proteins which are not fully studied and their function is not fully known.

PHASTER annotation revealed that the genome has no similarity with bacterial proteins which denotes that the Phage TU_A56 does not carry any virulent genes of host bacteria which rules out the possibility of transfer of virulent gene to the human or other bacteria when used as drug. This makes our phage a potential candidate for phage therapy.

4.22. Phylogenetic Tree of Phage tail Protein

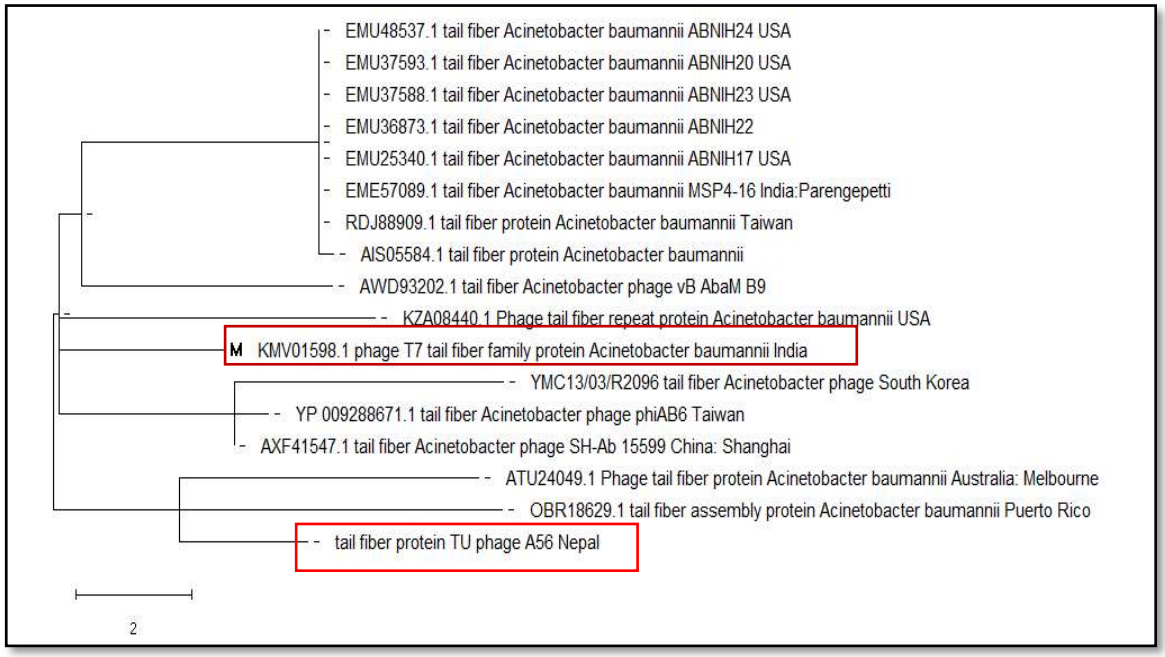


Figure: 4. 26 Molecular Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. Evolutionary analyses were conducted in MEGA X. The phylogenetic tree of tail fiber protein showed that the maximum likely ancestor of our phage was T7 family phage from India.

Phage tail protein phylogenetic tree showed that the maximum likely ancestor of our phage was T7 family phage from India and the phage is closely related to phage of Myoviridae family.

4.23. Pharmacokinetics Study in Mice Model

Different pharmacokinetic parameters were calculated using Microsoft excel from the graph of number of plaques forming unit per ml of blood was plotted against the time. The elimination rate constant, elimination half-life, clearance and volume of distribution of phage in the mouse body were calculated from the graph for both oral and intraperitoneal administration.

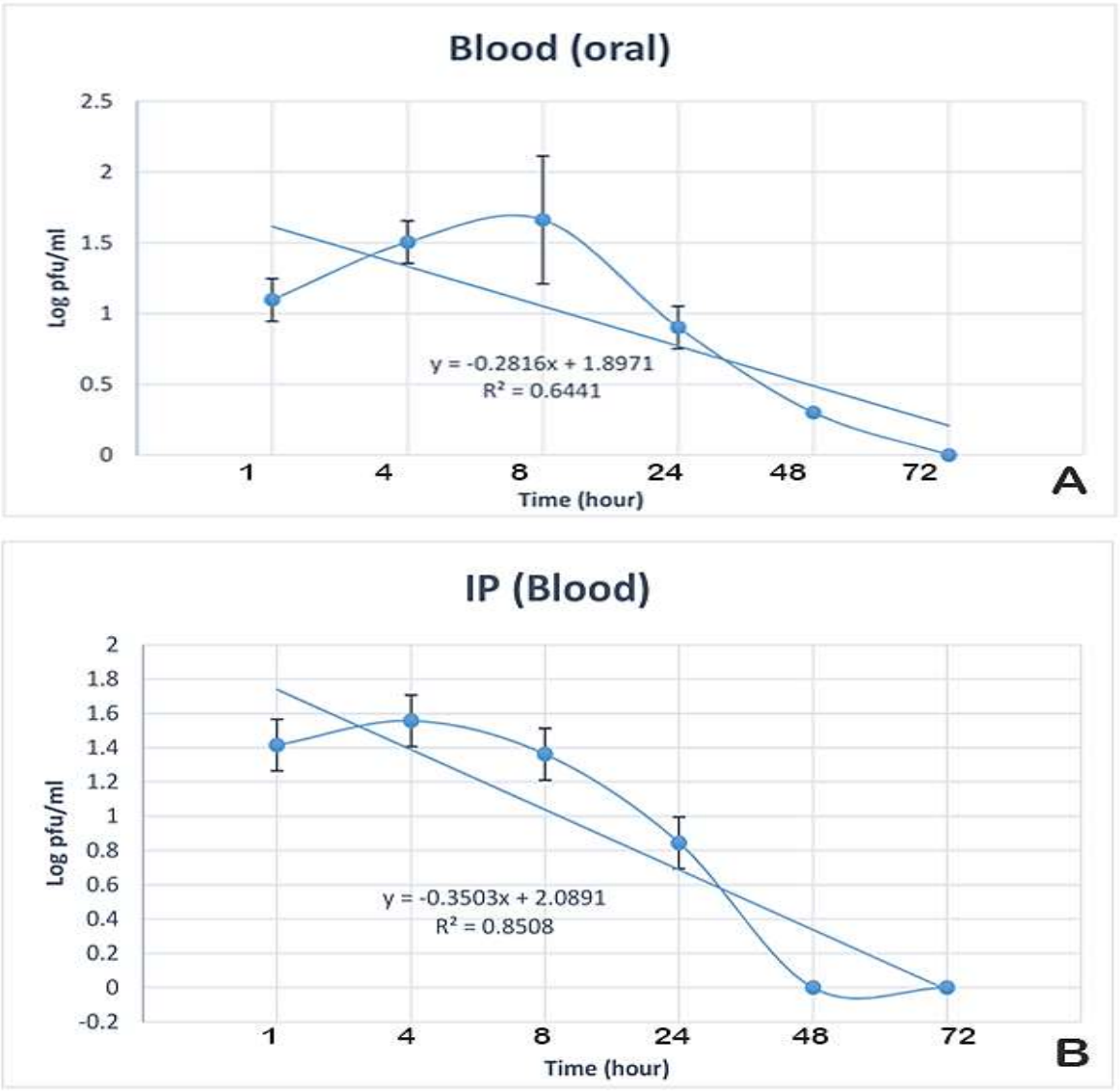


Figure: 4. 27 Semi log plot of PFU/ml Versus Time (hour) after oral and IP administration A and B respectively. The graph shows the gradual increase in the concentration of the phage in blood of mice up to the 8 hours of the oral dose of phage whereas 4 hours in case of IP dose. The concentration of phage in the bloodstream is decreasing after 8hrs and 4hrs for oral dose and IP dose.

Since the dose is given orally and intraperitoneally, the pharmacokinetics follows first order kinetics(Leon Shargel, 2016)

From one compartment model,

The volume of distribution (V_D) is given by:

$$V_D = \frac{D_0}{C_0}$$

Concentration of phage at 0-time (C_0), elimination rate constant (k) and Elimination half-life ($t_{1/2}$) is given by:

$$C{=}C0\ e{-}k1tlogC_t = \log C_0 - \frac{k_1}{2.303}t$$

$$log\frac{C_0}{2} = logC_0 - \frac{K}{2.303}t$$

$$-log2 = \frac{K}{2.303}t$$

$$t_{1/2} = \frac{2.303log2}{k}$$

$$t_{1/2} = \frac{0.693}{k}$$

Clearance (CL) is given by:

$$Cl = kV_D$$

Where, V_D is volume of distribution, D_0 is given dose, C_0 is concentration of phage at 0 time, k_1 is elimination rate constant and $t_{1/2}$ is elimination half-life.

Different pharmacokinetics parameters were calculated using formula listed above and values are tabulated in table 4.9.

Table: 4. 9 Pharmacokinetics Parameters of Phage TU_A56

S.N.	Pharmacokinetics parameters	Oral	IP
1	Concentration of phage at 0-time (C_0)	12.64736pfu/ml	15.64588pfu/ml
2	Elimination rate constant (k)	0.18339 per hr	0.233064 per hr
3	Volume of distribution (V_D)	15.81357ml	12.78292ml
4	Elimination half-life($t_{1/2}$)	3.7803hr	2.973437hr
5	Clearance (CL)	2.898925ml/hr	2.979233ml/hr

The elimination rate constant is defined as the fraction of phage in a mice that is eliminated per unit of time, e.g., fraction/h. Here the elimination constant for oral administration is found to be 0.18339. It means that 0.18339 fraction of the actual phage concentration is eliminated per hour. Similarly, for IP administration the elimination rate constant is slightly higher i.e. 0.233064. This data suggests that the rate of elimination is higher in case of IP administration than that of oral administration.

The volume of distribution V_D of a phage represents the degree to which a drug is distributed in body tissue of mice, higher V_D indicates a greater amount of tissue distribution. A V_D may be greater than the total volume of body water (Joanne Lymn, 2007), and would indicate that the drug is highly distributed into tissue. Average weight of our mice was 18 gram and A mice body has 66% water (Cizek, 1954). So, our mice contained 11 gram or 11ml of water (Say) and oral dose has 15ml V_D similarly IP has 12ml V_D it means that our phage is highly distributed among the tissue of the mice. And the distribution is better in oral dose than in IP dose. Elimination half-life can be defined as the time taken for the phage concentration in the body to be reduced by 50%. Here the elimination half-life is 3.7hr and 2.9hr for oral and IP dose respectively. It means that when the phage is given orally after about 4hr half of the phages are eliminated similarly in case of IP dose half of phages are eliminated after 3hrs of the dosing. Clearance (CL) is the amount of phage removed from the whole body of mice per hour. The rate of clearance is 2.8ml/hr in case of oral dosing whereas it is 2.9ml/hr in case of IP dosing.

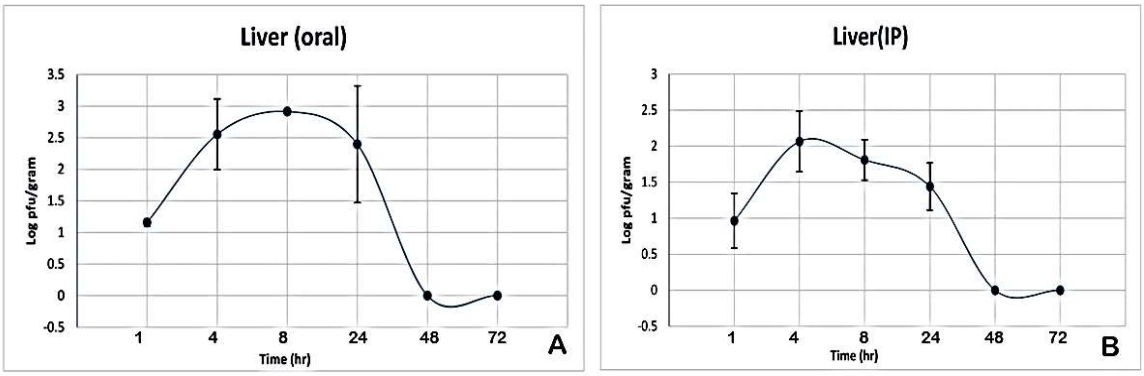


Figure: 4. 28 Log pfu/gram versus Time. A. Log pfu/gram in liver at different time after oral administration. B. Log pfu/gram in liver at different time after administration. The phage persisted in liver of mouse up to 72 hours of administration via both oral and IP route. Highest concentration of phage was found after 8 hours and 4 hours after oral and IP administration respectively.

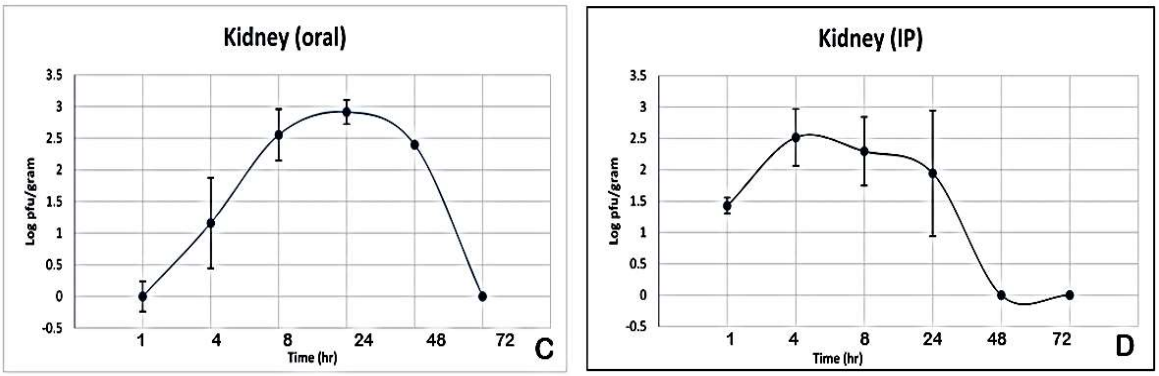


Figure: 4. 29 Log pfu/gram versus Time. C. Log pfu/gram in kidney at different time after oral administration. D. Log pfu/gram in kidney at different time after IP administration. The phage persisted in liver of mouse up to 72 hours and 48 hours after oral and Ip administration respectively. Highest concentration of phage was found after 24 hours and 8 hours after the oral and IP administration respectively.

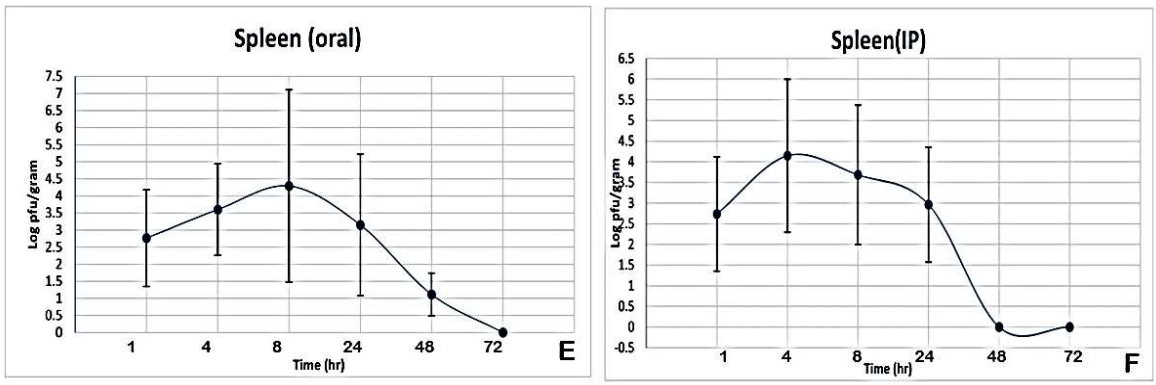


Figure: 4. 30 Log pfu/gram versus Time. E. Log pfu/gram in spleen at different time after oral administration. F. Log pfu/gram in spleen at different time after IP administration. The phage persisted in liver of mouse up to 72 hours and 48 hours after oral and Ip administration respectively. Highest concentration of phage was found after 8 hours after the oral and IP administration.

Pharmacokinetic study showed that after inoculation of the phage in mouse, concentration of phage in the organs rose gradually up to 8 hours in oral dose and 4 hours in IP dose. That was the time taken for the distribution of phage all over the body of mice. Highest titre of phage was found in spleen in 8hrs (4.29 log pfu/gram) and 4hrs (4.144 log pfu/gram) after oral and IP dose respectively.

The trend showed that for oral dose the phage distribution occurs up to 8hours and the titer decreases gradually due to the elimination of phage. Whereas, for IP dose the distribution occurred up to about 4 hours and the titer gradually decreases due to elimination of phage. The phage when administered orally was able cross gut wall and migrated to peripheral blood system (phagemia) and different organs of the body, which implies that our phage was resistant to gastric juices and can cross the tissue barrier this phenomenon was also described by (Cerveny, DePaola *et al.*, 2002). This phenomenon was also mentioned later by (Górski & Weber-Dabrowska, 2005). Who concluded that some phages may not only stay within the gut lumen but also cross the intestinal wall in a process like bacterial translocation. Although the processes controlling the viral translocation remain unknown, it was suggested that phage passage is determined by various factors, including phage concentration, specific sequences within the phage capsid proteins interacting with enterocyte receptors, and phage interactions with gut immune cells. After Ip dose phage distribution took place up 4 hours and after 4 hours phage clearance took place and phage were cleared after 48 hours of administration in all three organs.

In our study, phage reached a high titer in spleen after the eight hour and 4 hour following oral and IP administration respectively. In contrast, phage reached the highest titer after IV injection relatively faster than oral administration. Some researchers explained same phenomenon (Cerveny et al., 2002; Keller & Engley Jr, 1958; Oliveira, Sereno *et al.*, 2009) which supports our findings. They reported that orally administered phages can reach the peripheral blood and migrated to the infection sites. It is suggested that Gastrointestinal administration for systemic phage delivery results in highly variable phage counts in the bloodstream, making the clinical importance of this administration route questionable (Wolochow, Hildebrand *et al.*, 1966). Bacteriophage are cleared by organs of the reticuloendothelial system such as the spleen, liver and other filtering organs (Merril, Biswas *et al.*, 1996). The persistence of bacteriophages in the spleen seems to be significant. In our experiment also, phages were present in highest concentration and lasted long among all other organs. Similar result was observed by (Uhr & Weissmann, 1965) in rabbits. It was also stated that in mice that bacteriophages were rapidly phagocytized by the liver Kupffer's cells and that this clearance pathway seemed to be the

vital one. The presence of bacteriophages in urine which indicates that bacteriophages are also able to pass through the renal filter. The phenomena was reported in human by (Hildebrand & Wolochow, 1962), mice (Wolochow et al., 1966) and rabbits (Schultz & Neva, 1965). Furthermore, phage titre in kidneys could be higher than their concentration in plasma, (Schultz & Neva, 1965) demonstrated that phages could be observed in urine samples when phage doses passes the limit of 10^9 PFU/ mouse and phage plasma concentration was higher than 10^5 PFU/ml. The role of the kidneys in the clearance of bacteriophages was also observed in fish: phages were detected in fish kidney even a month after a single administration (Russell, Taylor *et al.*, 1976).

4.24. Cytokine Expression Analysis by Real Time PCR

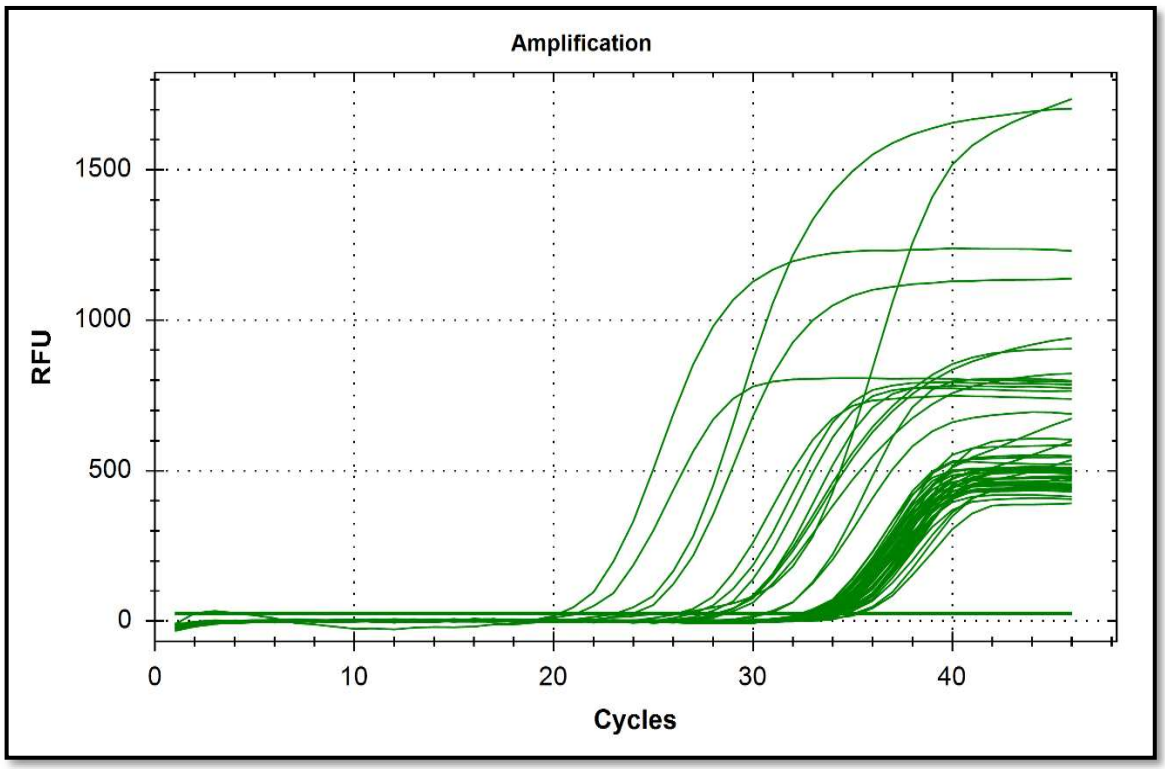


Figure: 4. 31 Real time PCR curve showing RFU versus cycle. The house keeping gene beta actin gene is amplified from around 20 cycle while IL-6 is amplified around 35 cycle of the PCR

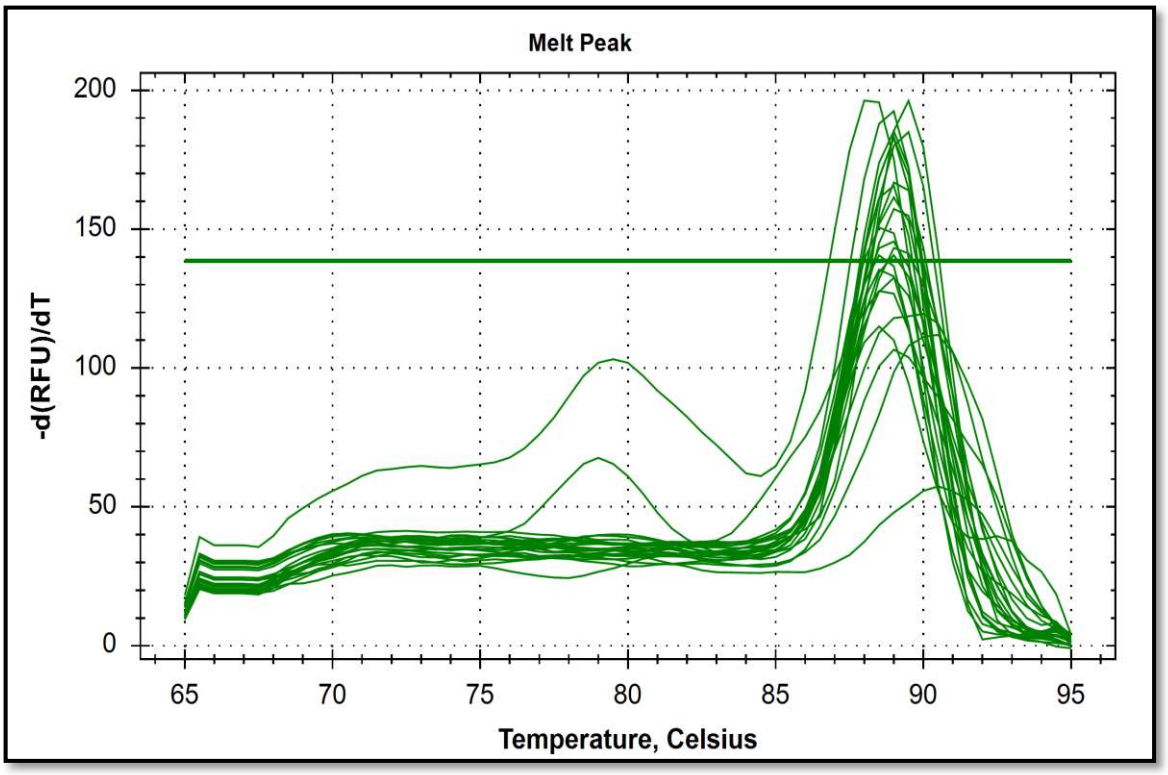


Figure: 4. 32 Melt Curve: melt curve shows single peak around 88°C which indicates the absence of non-specific amplification.

Figure: 4. 33 Time versus fold increment of IL-6 expression. The yellow line depicts fold increment after IP administration of bacteriophage and the blue line shows the slightly higher fold increment after oral administration.

Interleukin-6 (IL-6) expression analysis by DDCT method taking beta actin as a baseline control for the gene expression is shown in graph below. There is negligible fold increment in IL-6 expression. In oral administration the expression of iL-6 was high in comparison to the IP administration. However, the level of expression is below 0. 5-fold which is not the

significant increase in the Expression. During inflammation 1.5-fold (modest) to 25-fold IL-6 expression occurs in human (R. Berti, A. J. Williams *et al.*, 2002; Park, Lee *et al.*, 2010). Since our aim is to use phage as a therapeutic, the insignificant increase in the IL-6 expression is a good sign. From the result It could be said that the phage is not causing inflammation and harm to mice.

Il-6 is a pro inflammatory cytokine cells and an anti-inflammatory myokine. A pro inflammatory cytokine is a type of signaling molecule released by immune cells like T-helper and macrophages and plays an important role in mediating the host immune response. Transient and immediate expression of IL-6 occurs in response to environmental stress factors such as infections and tissue injuries (Tanaka, Narazaki *et al.*, 2014). This expression triggers an alarm signal and activates host defense mechanisms against stress. The small increase in the expression of IL-6 may be due to the stress developed in mouse during the experiment. We could not amplify the gene coding TNFα. This may be due to various reasons, our study couldn't find the exact reason behind that.

Chapter Five

SUMMARY

Eight multi drug resistant Acinetobacter baumannii were collected form NPHL and molecular identification of the bacteria was done by sequencing the 16S rRNA gene segment. All bacteria showed 99% similarity with *Acinetobacter baumannii* when performed BLAST in NCBI database. The gene segments were submitted to NCBI GenBank. After confirmation of the host four bacteriophages against four strains of MDR Acinetobacter baumannii were isolated from the water sample of rivers of Kathmandu valley. All isolated phages were not only specific to their primary host but also showed host range in other strains of the same species. One of the most potent phage TU_A56 showing wide host range and clear lytic properties was selected for further research

Protein profiling of phage TU-A56 showed two distinct bands of 55kDa and 87kDa in SDS-PAGE by acetone precipitation method, those bands were assumed to be tail and capsid protein respectively. The TEM micrograph and the blast result showed that the phage TU_A56 belonged to order Caudovirales family Myoviridae. The in vitro lysis of the host bacteria by phage showed optimum lysis at 5 MOI. The phage had burst size of 304 virions per bacterium. Whole genome sequence analysis showed genome size of phage 12.310343 Mbp which is the raw size of partially annotated genome. The PHASTER annotation of the genome showed 478 intact proteins and 20 incomplete proteins. The GC content of phage genome was found to be 49.5 %. The PHASTER annotation did not showed any toxic genes of bacteria inside the phage genomes which makes it a potent candidate for phage therapy.

The pharmacokinetics study of the phage in mice model showed that the phage was first disseminated from the site of administration to the whole body and gradually cleared by reticuloendothelial system of mice. When the phages (1.02×10^8 PFU/ml) administrated orally, the phage titer in the body organs gradually rose up to the 8 hour and after that the phage titer started to decrease. Phage is cleared completely after 72 hours of the single oral dose. The phage persisted longest in spleen up to 72 hours. Similarly, when the phage was given intraperitoneally, phage disseminated faster than oral dose. After 4 hour of IP administration the phage showed highest titer in different body organ such as blood, kidney, spleen and liver. After 4 hours the phage titer gradually decreased from the body parts due to clearance.

Cytokine expression analysis by Realtime PCR showed that the IL-6 expression in the mouse was 0.2-fold in IP administration whereas 0.4-fold in case of oral administration as compared to beta actin gene expression which is not the significant level of expression to cause harm and inflammation to the mice. We could not amplify the gene coding TNFα this may be due to the fact that there was no mRNA present in mice blood coding for TNFα but we could not confirm the result as we lack positive results for TNFα.

Chapter Six

CONCLUSION

Total four phage against four multidrug resistant *Acinetobacter baumannii* strains were isolated which were lytic in nature. Out of four one potent phage was characterized physiologically and morphologically. The phage TU_A56 was found to be stable up to 60°C and wide range of pH (3-12) and had multiple host range. Transmission electron micrograph revealed a short tail and icosahedral symmetry and the phage belonged to order Caudovirales and family Myoviridae. The phage was found to have optimum MOI 5 and burst size 304 virions per bacterium. Whole enome analysis confirmed that the phage was free of virulent genes of bacterial origin which ruled out possibility of virulent gene transfer from phage to bacterial strain making it an excellent candidate for therapeutic use. Additionally, lysin proteins is also predicted in in our phage, which may be separately used as an antibiotic in lysin therapy.

The pharmacokinetics study of phage in mice model showed that the rate of absorption, distribution and clearance of phage was different in oral and IP dose ie ($p > 0.05$). Thus, we rejected the null hypothesis 1 and accepted the alternative hypothesis which states “The rate of absorption, distribution and clearance of phage is different in oral and IP dose”.

Similarly, there was no significant difference in expression of cytokine (IL-6 and TNFα) level elicited by bacteriophages in phage treated and untreated mice. Thus, we rejected the null hypothesis 2 and accepted the alternative “There is significant difference in expression of cytokine (IL-6 and TNFα) level elicited by bacteriophages in phage treated and untreated mice.”.

This study was done for the first time in Nepal which helps to improve our understanding of the phage characterization, propagation, adsorption as well as it gives an insight into the pharmacokinetics and pharmacodynamics of phage.

LIMITATIONS OF THE STUDY

1. Small sample size: Bacteriophage only four strains of MDR *Acinetobacter baumannii* were isolated and among them only one was further studied due to the lack of time and resources
2. Sophisticated Instruments: lack of sophisticated instruments like TEM we had to export our sample to abroad, we could not get our TEM result as expected. We were unable to concentrate our phage due to lack of ultracentrifugation.
3. Lack of experience and expertise: Whole genome sequence analysis of bacteriophage is a time demanding and task and requires expertise in bioinformatics, due to lack of both we were unable to fully annotate whole genome of our phage.
4. We could not perform the animal trial (mouse rescue) dude to the limited time frame and lack of resources.

RECOMMENDATIONS

- The pharmacokinetics of bacteriophage in mouse model and assessment of cytokine expression level after administration of phage gave a new dimension to phage research, and has provide enough ground work for further phage therapy experiment in animal model.
- Extensive study of the phage genome sequencing to know the genetic diversity, phage host interaction is necessary to accelerate the journey of phage therapy.
- Molecular cloning and expression of the endolysin protein from the phage genome could be a potent antimicrobial agent.
- Experimental phage therapy to treat infection caused by the MDR bacteria in mice model
- Study on different dose concentration of the phage.
- Extensive pharmacodynamic study also should be done.
- Recombinant lysin protein is another potential aspect of phage therapy.

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APPENDIX

Table: Host Range Analysis of Phages						
S.N.	Bacterial strain	Phage A1	Phage A5	Phage A8	Phage A56	Cocktail
1	A1	++	++	-	-	++
2	A3	-	-	-	-	-
3	A5	-	++	-	-	++
4	A6	-	-	-	-	-
5	A7	-	-	-	-	-
6	A8	++	++	++	++	++
7	A10	-	-	-	-	-
8	A11	-	-	-	-	-
9	A12	++	+	-	-	++
10	A13	-	-	-	-	-
11	A14	-	-	-	-	-
12	A15	-	-	-	-	-
13	A17	-	-	-	-	-
14	A19	-	-	-	-	-
15	A21	-	-	-	-	-

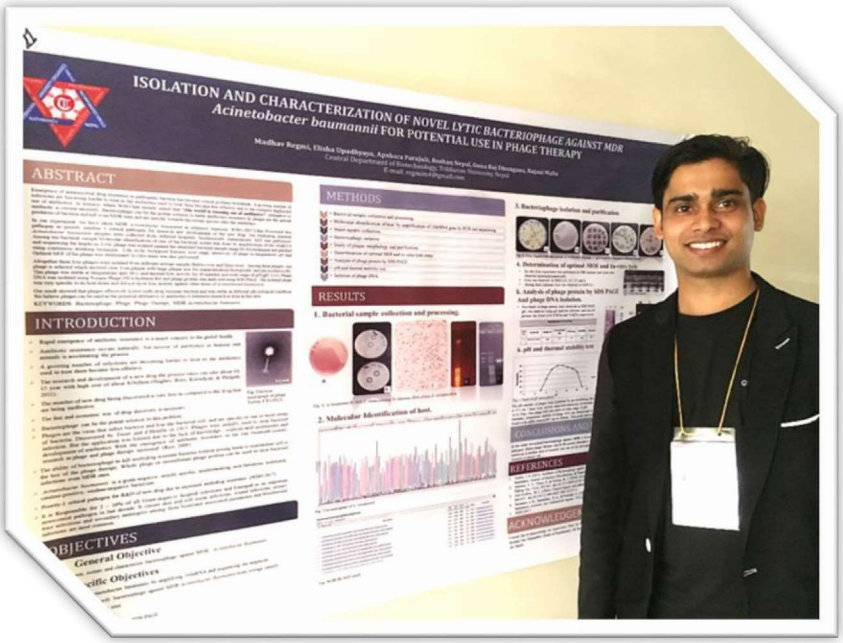
16	A22	-	-	-	-	-
17	A23	-	-	-	-	-
18	A24	-	-	-	-	-
19	A26	-	-	-	-	-
20	A30	-	-	-	-	-
21	A32	-	-	-	-	-
22	A34	++	++	++	-	++
23	A35	-	-	-	-	-
24	A38	-	-	-	-	-
25	A39	-	-	-	-	-
26	A43	-	-	-	-	-
27	A44	++	-	-	-	++
28	A47	-	-	-	-	-
29	A50	-	-	-	-	-
30	A53	-	-	-	-	-
31	A54	+	-	-	-	+
32	A56	++	-	++	++	++
33	A58	-	-	-	-	-
34	A59	++	++	++	++	++
35	A61	-	-	-	-	-
36	A62	-	-	-	-	-

37	A67	-	-	-	-	-
38	A70	++	++	++	-	++
39	A74	-	-	-	-	-
40	A77	-	-	-	-	-

Note: '++' indicates clear lysis, '+' indicates partial lysis and '–' indicates no lysis at all.

Some Glimpses

Me working during pharmacokinetics experiment



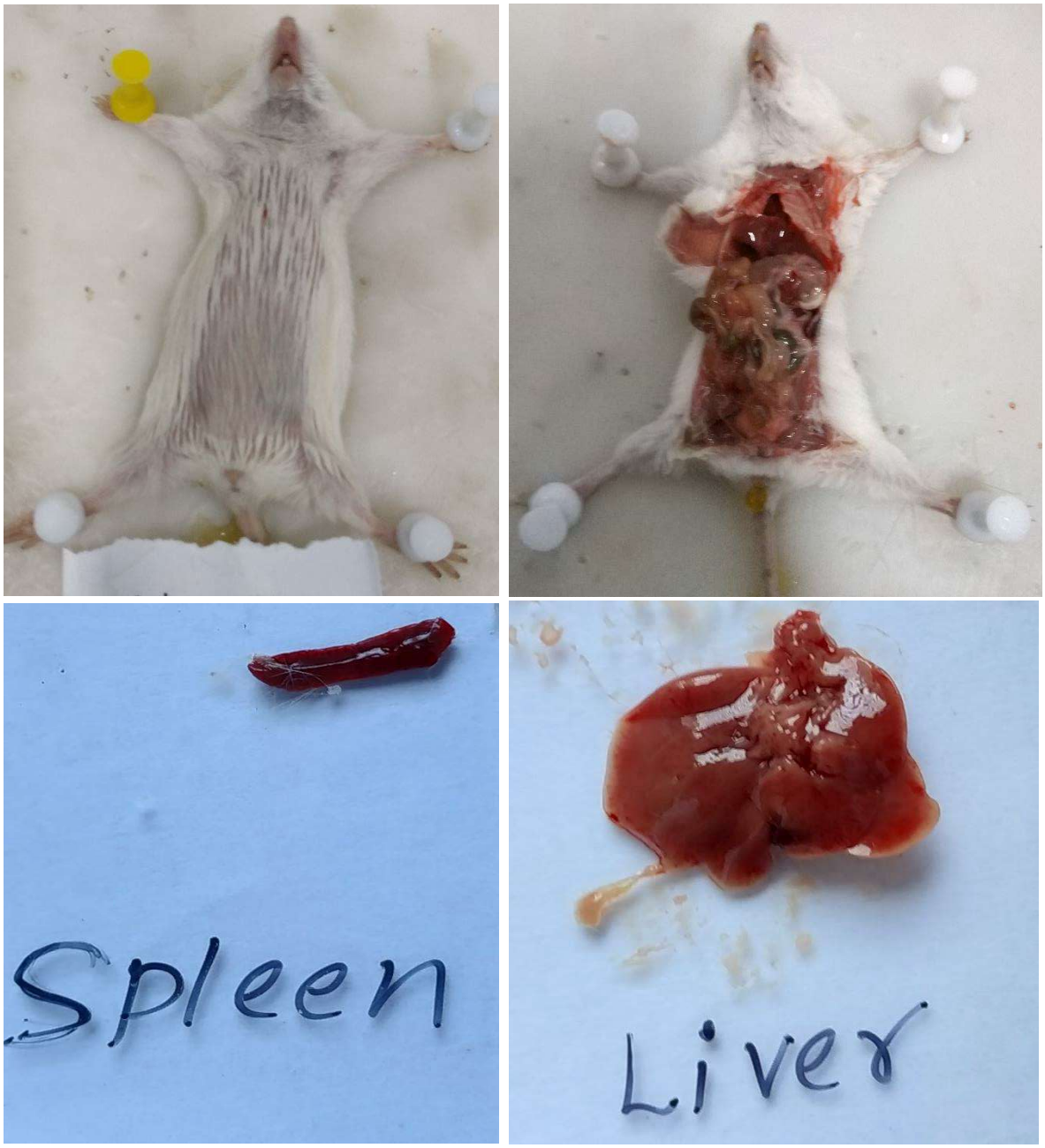
Poster presentation at NAST on World DNA Day 2017

Welcome Farewell programme

Phage team working during pharmacokinetics experiment

Prof. Malla warded by NAST

Discussion about phage bank



Mice dissection during experiment