CHARACTERIZATION, WHOLE GENOME ANALYSIS AND APPLICATION OF BACTERIOPHAGES TO CONTROL BIOFILM PRODUCED BY CARBAPENEM RESISTANT BACTERIA PRIORITIZED BY WORLD HEALTH ORGANIZATION

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DEDICATIONS

MY BELOVED PARENTS

MY SUPERVISORS

& all those people around the globe who are fighting against the serious bacterialdiseases and hope this will bring glorious effort for PHAGE THERAPY.

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ACRONYMS

µg	:	microgram [one billionth (1×10^{-9}) of a kilogram]
µl	:	microliter [one millionth (1×10^{-6}) of a liter]
ABR	:	Anti-Bacterial Resistance
AMR	:	Antimicrobial Resistance
AST	:	Antibiotic Sensitivity Test
ATCC	:	American Type Culture Collection
BALB/c	:	Bagg ALBino/genotype 'c'
BAM	:	Bacteriophage Adherence to Mucus
BIM/s	:	Bacteriophage Induced Mutant/s
BLAST	:	Basic Local Alignment Search Tool
BLASTN	:	Basic Local Alignment Search Tool – Nucleotide
bp	:	base pairs
CDBT	:	Central Department of Biotechnology
CDC	:	Centers for Disease Control and Prevention
CDS	:	Coding DNA Sequence
CF	:	Cystis Fibrosis
CFU	:	Colony Forming Unit
CNS	:	Central Nervous System
CPS	:	Capsular Polysaccharide
CRE	:	Carbapenem Resistant Enterobacteriaceae
CRISPR	:	Clustered Regularly Interspaced Short PalindromicRepeats
CRKP	:	Carbapenem Resistant klebsiella pneumoniae
CTAB	:	Cetyl trimethylammonium bromide
DLA	:	Double Layer Agar
DLAA	:	Double Layer Agar Assay
DNA	:	Deoxyribonucleic acid

DNA HT library: Deoxyribonucleic Acid High-Throughput library

dsDNA : double stranded Deoxyribonucleic Acid

EARS : European Antimicrobial Resistance Surveillance

ECDC : European Centre for Disease Prevention and Control

ELISA : Enzyme linked immunosorbent assay

EOP : Efficiency of Plating

EPS : Exo Polysaccharides

ESBL : Extended Spectrum Beta Lactamase

FDA : Food and Drug Administration

GB : Giga Bytes

GC content : Guanine – Cytosine content

gDNA : genomic Deoxyribonucleic Acid

GPS : Global Positioning Service

GRAS : Generally Recognized As Safe

HCAI : Health Care Associated Infection

i.p : intraperitoneal

ICTV : International Committee on Taxonomy of Viruses

IMP : Imipenam

IOM : Institute of Medicine

JNU : Jawaharlal Nehru University

Kbp : Kilo base pairs

kDa : kiloDalton

KPC : Carbapenemases producing Klebsiella pneumoniae

LB : Luria Bertani

Log : logarithm

LPS : Lipopolysaccharide

MBL : Metallo Beta Lactamase

MCP : Major Coat Protein

MDR : Multidrug Resistance

MEGA : Molecular Evolutrionary Genetic Analaysis

MHA : Muller Hington Agar

MHR : Multiple Host Range

ml : milliliter

MOI : Multiplicity of Infection

mRNA : messenger RNA

MRSA : Methicillin Resistant Staphylococcus aureus

MTB : *Mycobacterium Tubercuosis*

MTP : Major Tail Protein

MW : Molecular Weight

NA : Nutrient Agar

NCBI : National Center for Biotechnology Information

NDM : New Delhi metallo-beta-lactamase

NGS : Next Generation Sequencing

NIH : National Institute of Health

nm : nanometer [one billionth (1×10-9) of a meter]

NPHL : National Public Health Laboratory

OD : Optical Density

ORF : Open Reading Frame

OXA : Oxacillinase

PBP : Penicillin Binding Protein

PBS : Phosphate Buffer Saline

PCR : Polymerase Chain Reaction

PDR : Pan Drug Resistant

PEG : Polyethylene glycol

Pfu : plaque forming unit

PHASTER : Phage Search Tool Enhanced Release

PT	: Phage Therapy
RBP	: Receptor Binding Protein
RNA	: Ribonucleic acid
rRNA	: ribosomal Ribonucleic Acid
SDS-PAGE	: Sodium dodecyl sulfate-polyacrylamide gelelectrophoresis
SM buffer	: Sodium Magnesium buffer
ss DNA	: single stranded Deoxyribonucleic Acid
TAE	: Tris-Acetate EDTA buffer.
TE	: Tris-Chloride EDTA buffer.
TEM	: Transmission Electron Microscopy
tRNA	: transfer Ribonucleic Acid
TSA	: Tryptic Soya Agar
TSB	: Tryptic Soya Broth
TU	: Tribhuvan University
UF	: Ultra Filtration
UPGMA	: Unweighted Pair Group Method with Airthmetic Mean
USA	: United States of America
UTI	: Urinary Tract Infections
UV	: Ultraviolet
VAP	: Ventilator Associated Pneumonia
VRE	: Vancomycin Resistant Enterococci
VRSA	: Vancomycin Resistant Staphylococcus aureus
WGS	: Whole Genome Sequencing
WHO	: World Health Organization
XDR	: Extensively Drug Resistant
ZOI	: Zone of Inhibition
μm	: Micrometer

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ABSTRACT

"Characterization, Whole Genome Analysis and Application of Bacteriophages to Control Biofilm Produced by Carbapenem Resistant Bacteria Prioritized byWorld Health Organization (WHO)". Carbapenem-resistant enterobacteriaceae are among the most prioritized critical pathogens according to WHO. Their emergence and spread have now become a major public health concern creating serious problem in treatment of infectious diseases. Carbapenem-resistant enterobacteriaceae includes *Klebsiella pneumoniae* producing KPC- type carbapenemase, *Escherichia coli* and *Pseudomonas aeruginosa* producing ESBL. The use of bacteriophages for killing bacteria has drawn recent attention, which has potential as alternative to antibiotics. The phages are referred as bio-control agent as they can kill multi-drug resistant strains in the environment. In this study, we aimed to isolate bacteriophage against carbapenemase-producing *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and characterize for potential phage therapy as an alternative to antibiotics.Eight different strains from host bacteria (*K. pneumoniae* and *P. aeruginosa*) were collected and Molecular identification of bacterial isolates was confirmed by amplification of the 16srRNA, blaKPC, blaNDM genes.Lytic phages were isolated against those host strains through double-layer agar assay method. Phages were purified by successively sub-culturing single plaque & standard spot assay. Among the isolated bacteriophages one potent phage was characterized morphologically and physiochemically .Life cycle, biological features, multiple host range, sensitivity of phage to temperature and pH was determined. Transmission electron microscopy was done for morphological identification. Sequencing of most potent lytic phages was done by illumina platform.The phages were used to control the biofilm formation using microtiter dish method. Host bacteria were confirmed as *Klebsiella pneumoniae* from 16SrRNA sequencing and gene sequence was submitted to NCBI.Twenty one lytic phages against carbapenem resistant bacteria and the Bull's eyes plaques are found in Klebsiella phage and in clear lytic plaques in case of Pseudomonas phage.Almost all phages showed wide type of host range except PhageTU-Kleb27 which was found to be of host specific. Klebsiella phage were found to interspecies and Pseudomonas phages were limited with its species only.Klebsiella phage cocktail showed interspecies host range (in *Acinetobacter baumannii*).Among all the phages, only two most potent phages was characterized morphologically and physiochemically.Both the phages showed stability upto 70˚c optimal temperature being 37˚c and stable in wide range of pHoptimal being at pH 7.TEM image revealed that PhageTU-Kleb27 was found to be of podoviridae with short tail and PhageTU-P53 was found to be of siphoviridae with long tail.The burst size of PhageTU-Kleb27 was found to be 20 phages per bacterium with latent period 20 minutes and 160pfu/ml was found to be incase of PhageTU-Kleb27 with latent period of 20 minutes. Whole genome sequencing revealed that there is no nay virulence gene.The phages showed significant reduction in biofilm formation after phage treatment.Our result showed that phages showed effective lytic capability, multiple host range and stability in wide range of temperature and pH with no any toxic gene in its genome and ability to clear biofilm which can possibly be used in therapeutics and as professed in scientific world.

Keyword: Bacteriophage, TEM, Biofilms and Phage Therapy, Carbapenem resistant.

CHAPTER ONE
INTRODUCTION

1.1. Background

Antimicrobial resistance is currently a global threat spreading all over the world. As, latest report of WHO confirms that due to the widespread antibiotic resistance, the world is running out of antibiotics leading us to the post antibiotic era (WHO ,2017).Antimicrobial resistance also known as drug resistance is the capability of microorganisms such as bacteria, fungi, virus and even parasites to grow and multiply in spite of the exposure to various antimicrobial agents that are used to treat them effectively. Eventually, leading to persistent infections which may spread to others. As a matter of fact, antimicrobial resistance could lead to a time in which antibiotics would no longer work.Also, common infections and minor injuries that are easy to treat at the current stage could again become fatal. Nowadays, Antimicrobial resistance is rising dangerously at high level. The efficiency of antibiotics against pathogens has gradually decreased. Now, the simple infections have become hard to treat with available antibiotics. The emergence of antibiotic-resistant pathogenic bacteria is an increasing health hazard. The European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC), create a standardized international terminology which describes different pattern of acquired resistance, and bacteria are classified as Multidrug-resistant (MDR), Extensively Drug- Resistant (XDR) and Pandrug-Resistant (PDR).MDR is defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories; XDR is defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories. Initially, XDR was used to describe extensively drug-resistant *Mycobacterium tuberculosis* (XDR MTB). Similarly, PDR: 'pan' means 'all'; pandrug resistant means resistant to all approved commercially available antimicrobials (Magiorakos et al., 2012). Now, the term 'superbugs' are used for the microbes with enhanced morbidity and mortality due to multiple mutations endowing high levels of resistance to the antibiotic classes recommended for treatment (Davies, 2010) . Currently, the most notorious superbug is the Gram-positive organisms, Methicillin-resistant *Staphylococcus aureus*.Similarly, carbapenem-resistant Enterobacteriaceae (CRE), ESBL-producing Enterobacteriaceae (ESBL), Vancomycin-resistnat Enterococcus (VRE), Multidrug-resistant *Pseudomonas aeruginosa* and Multidrug resistant *Acinetobacter* are referred as 'superbugs.'

Bacterial resistance to antibiotics is both natural and inevitable (Ventola, 2015). There are various intrinsic factors inside the microbes considered as biological factor such as mutation, horizontal gene transfer, phenotypic changes etc (Hawkey, 1998) and main mechanisms confers by carbapenem resistant bacteria are efflux pump Beta-lactamases, efflux systems, changes in outer membrane proteins (permeability barriers) and changes

in penicillin-binding proteins (Codjoe & Donkor, 2017; Tenover, 2006). During microbial reproduction mutations can occur. In the course of time this can lead to the induction of genes that make the organism resistant to antimicrobial agent under exposure. Moreover, genes with drug resistant quality can be transferred between the microbes that lead to the further increase in resistance to various other microorganisms. Likewise, microorganisms can also alter their components to become resistant to preferred antimicrobial agents. Mutations in ribosomal protein genes leading to antibiotics resistance have a number of extraribosomal effects (mistranslation, temperature sensitivity, phage propagation, etc) that influence cell function. Different selective pressures may lead to mutations that coincidentally confer a level of antibiotic resistance (Davies, 2010). An antibiotic resistance phenotype does not necessarily occur solely in response to antibiotic selection. The misuse and overuse of broad spectrum antibiotics also accelerates the resistance of antimicrobials agents (Fair & Tor, 2014). The most common example of antimicrobial resistance is Methicillin resistant *Staphylococcus aureus*, it is a potentially fatal bacterial infection prevalent in hospitals and Carbapenem-resistant enterobacteriaceae (CRE) .The resistance developed in such a manner that even carbapenem group (beta lactamase) group cannot inhibit the bacterial infections, carbapenem-resistant Klebsiella pneumonia (CRKP) rates have been dramatically increasing worldwide over the past 10 years. Production of various carbapenemases is a key mechanism mediating the emergence of CRE eg: *Klebsiella pneumoniae* carbapenemase (KPC), encoded by bla KPC gene, bla NDM, bla IMP, blaVIM and blaOXA-48(Netikul & Kiratisin, 2015). It has added serious challenge to current antibiotic crisis era, while the resistance to colistin (last resort treatment for life-threatening infections caused by Enterobacteriaceae) has recently been detected in several countries and regions, making infection untreatable (Granata & Petrosillo, 2017). In February 2017, WHO publishes the list of Antibiotic-resistant 'Priority Pathogens' for research and development of new antibiotics, in which Carbapenem-resistant enterobacteriaceae (CRE) are categorized as most critical group (list at Appendix). These bacteria have induced abilities to find a new way to resist treatment and can pass along genetic material that allows other bacteria to become drug-resistant as well. According to WHO, drug-resistant bacteria are estimated to cause 700,000 deaths each year. If no action is taken, they are expected to kill 10 million people annually by 2050 AD (O'neill, 2018). Antibiotic resistant infections are already widespread across the globe and the rate of morbidity and mortality due to antibiotic resistant bacteria infections is increasing in both developed and developing country. Each year in the U.S. at least 2 million people get an antibiotic-resistant infection and at least 23,000 people die. The consequence of antibiotic resistant is much more worsening in case of developing countries. The gross abuse of antimicrobials facilitates the emergence of resistance in developing countries, by their availability over the counter, without prescription and through unregulated supply chains. It is well known that any use of antimicrobials however appropriate and justified, contributes to the development of resistance, but widespread unnecessary and excessive use makes the situation worse. A systemic

review on antibiotic resistance in developing countries showed that mortality was associated with resistant bacteria, statistical significance was evident with an odds ratio (OR) 2.828 (95% CI, 2.231-3.584, p=0.000).Similarly, ESKAPE pathogens were associated with the highest risk of mortality and with high statistical significance (OR 3.217, 95% CIs; 2.395-4.321;p=0.001) in developing countries(Founou et al., 2017). Among all the developing countries, Nepal is not the exception in the global issue of antibiotic resistance. The study done in tertiary care university hospital in Kathmandu, Nepal, Nearly, 96% of the Gram negative bacterial isolates causing nosocomial infections were found multidrug resistant (Parajuli et al., 2017). In developing countries, another cause of antimicrobial resistance is non-human use of antimicrobials that is they are used to prevent and treat disease in animals, used as growth promoters in animal breeding and as additives in plant agriculture. The use of antibiotics to animals has great consequences to both animal and human health resulting the transfer of resistance gene from animal to human by different means (consumption of food, direct contact with food producing animals or environmental spread). The use of antimicrobials drugs in health care, agriculture or industrial settings exerts a selection pressure, which can favor the survival of resistant bacteria within microbial communities. It is clear that the situation today is more complex, antibiotic resistance seems inevitable. So, what strategy should be taken to prevent or atleast delay this process. From past years many health sectors experts have been proposed solutions to control strictly on consumption of antibiotics by all major international health groups (eg: WHO, CDC). There is an urgent need to investigate alterative treatment options as only the few antibiotics are left and new antibiotics have not yet been discovered. Between 1983 and 1987, 16 new antibiotics were approved by the US FDA, but this has dropped gradually, until 2008–2016, when six were approved (Clark, 2015; B. Li & Webster, 2018). The compounds which were easiest to identify, purify and commercialize have already been discovered making the isolation of new compounds riskier and less profitable. There is a challenge to discover new drugs due to lengthy, complex, and costly process and problem in target identification and toxicity of the lead molecule.

There is an urgent need for investment in research to investigate alternative therapy to tackle global-antibiotic-crisis. Thus, antibiotics and their alternatives are prudence required. With the failure of conventional antibiotics treatment becoming a global crisis, now the scientific world is looking increasingly to novel approaches, either reduce the selection for antibiotic resistance or to replace the antibiotic altogether. They are herbal remedies, bacteriocins, and predatory bacteria-Bdellovibrio bacteriovorus (BALOs), Bacteriophages, antibiotic peptides, gene editing enzymes (clustered regularly interspaced short palindromic repeats - CRISPR), metals (silver, copper, gold). Each approach has its own benefits and costs. The most useful replacement for antibiotics is, like antibiotic, natural agent (virus) that inhibits or kills bacteria that is Bacteriophage. The application of lytic phages to kill pathogenic bacteria is called phage therapy. The emergence of antibiotics resistance pushes scientists to go back to the long forgotten

cure i.e, Bacteriophage therapy (Matsuzaki et al., 2005). Bacteriophages are known as bacterial parasites whose life is completely associated with bacteria and found abundant in nature. They invade bacterial cells and disrupt bacterial metabolism and cause bacterium lysis. As natural killers of bacteria, phages are nowadays regaining attention due to alarming widespread emergence antimicrobial resistant. Thus, in this present scenario of an alarming antibiotic crisis, phages can be used as an effective alternative to antibiotics. Phage has been focused in this intensive study to establish their infection process against carbapenem resistant clinical isolates and morphological characteristics, as it was thought that they would have been likely candidates for bacterial infection therapy as an alternative to antibiotics and it could be the novel strategy for preventing or delaying the biofilm growth of MDR bacteria. Almost decade before the discovery of penicillin, the practice of phage therapy was being developed, they are used at the treatment against pathogens such as *Shigella dysenteriae* in 1911 (Lin et al., 2017a) .From the conventional method of use of naturally occurring phage to infect and lyse bacteria has been now well developed to use of biotechnological advanced phage therapeutics such as Phage-derived lytic proteins, bioengineered phages. There are several clinical trials are ongoing for phage therapy research and one FDA approved phage therapy product is on the market which is used to kill virulent food-borne pathogens (Lin et al., 2017a). From this research, we can get understanding on phages and their bacterial host, which could open the door for phage therapy.

1.1.1 Bacteriophage

Bacteriophages are viruses that kill Bacteria and found everywhere in the nature. Bacteriophage was discovered independently by Frederick Twort (1915) and Felix D'Herelle (1917) (Duckworth,1976).Bacteriophages or "phages" are viruses of prokaryotes (Abendon,2009) .Viruses are a group of biological entities with a genome consisting either of DNA or RNA and encapsulated in a protein coat (capsid) (Weinbauer, 2004).Most of the bacteriophages are highly host specific and have the ability to proliferate inside bacterial cell and it affects fast growing prokaryotic phylotypes more than slow growing ones (Baudoux etal., 2008). Phages have an estimated population size of 1030 or more in environment (Chibani-chennoufn et al., 2004) . In this "new age of phage research" (Chibani-chennoufi et al., 2004) the beneficial part is that bacteriophages are abundant in the environment and they influence the biosphere extensively (Howard-varona et al., 2017). Phages have been a topic of interest to scientists as tools to understand fundamental molecular biology, as vectors of horizontal gene transfer and drivers of bacterial evolution, as sources of diagnostic and genetic tools and as novel therapeutic agents (Clokie et al., 2011).

Phages basically consists of nucleic acid molecule (genome) surrounded by coat known as capsid or head. The genetic material of phages consists of single-stranded or double-stranded RNA or DNA and ranges from the 3.5 kb of the ssRNA phage MS2 to the 500 kb of the dsDNA Bacillus phage G. The head or capsid is made up of morphological subunits

called protomers. The head or capsid forms the protective container for the condensed bacteriophage chromosome and the formation of this closed shell for genome packaging requires the recruitment and organization of multiple copies of protein subunits. It is estimated that the pressure within the capsid is as much as 20 atmospheres (Cordova et al., 2003) the size of the phage capsid is correlated to the size of the genome being packaged. All members of the Caudovirales have capsids with icosahedral (20 sides/12 vertices) symmetry or prolate derivatives thereof, providing a characteristic appearance under the electron microscope. Three gene products are critical for capsid formation; the portal, scaffold and MCP (Major coat protein). Several other factors, including accessory proteins, chaperones and proteases are also required (Huang et al., 2011). Many but all phages do not have tails, length of tail varies and a contractile sheath which contracts during infection of the bacterium, may surrounds tail. The tail functions to facilitate adsorption and attachment of the phage to the host cell surface and provide a conduit for genome ejection. The tail is based upon three main components: the tape measure protein, the major tail protein (MTP) and the tail terminator protein (E. S. Miller et al., 2003). In some phages (T4), a base plate and one or more tail fibers are attached to the tail end as shown in figure (1.1). Not all phages have base plates and tail fibers. The detail structure of T4 bacteriophage with capsid, tail and tail fibers is shown in below figure (Figure 1.1).

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, 2003; Ackermann, 1998)

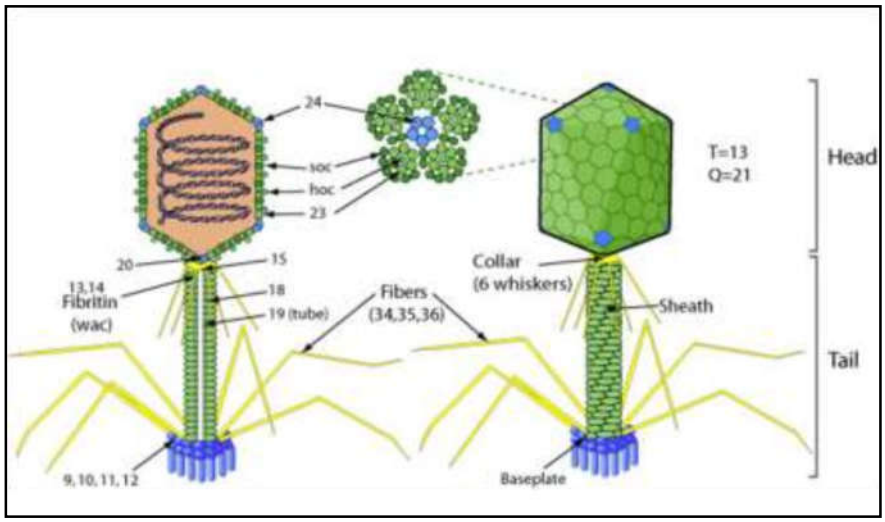


Figure 1. 1: Detailed structure of T4 bacteriophage

It contains Capsid head, tail and long tail fibers.The capsid head contains linear double-stranded DNA and two non-essential outer capsid proteins, Hoc and Soc, which decorate the capsid surface.It has 6 long tail fibers attached to the baseplates which help for the attachment of the phage on to the surface of host cell during the process of adsorption (Rao & Black, 2010a). Figure adapted from www.viralzone.expasy.org

The size of most phages range from 22nm – 200nm in length.The largest bacteriophage known as T4 is about 200 nm long and about 80 – 100 nm wide (Rao & Black, 2010b). Tailed bacteriophages with genomes larger than 200 kbp are classified as Jumbo phages (Yuan &Gao, 2017). The largest phage in terms of capsid size and DNA content is *Bacillus megaterium*, phage G of myovirus from Poland. It has a head of 160 nm in diameter and contains a DNA of 497,513 bp (Ackermann et al., 2014). Single stranded RNA phages (family Leviviridae) are the smallest phages of all with genome size of 3.5kb to 10.5kb.Phages are classified by the International Committee on Taxonomy of Viruses (ICTV) according to morphology and nucleic acid. They are classified into 13 order based on; Tailed bacteriophages, order Caudovirales (lat. Cauda meaning tail), Non-tailed phages, DNA-containing bacteriophages with polyhedral capsids, RNA-containing bacteriophages with polyhedral capsids, Filamentous bacteriophages, Pleomorphic bacteriophages. The caudovirales are classified into three families: the Myoviridae (long contractile tail, eg: Enterobacteria phage T4), the Siphoviridae (long non- contractile tail, eg: Enterobacteria phage T5) and the Podoviridae (short non-contractile tail, eg: Enterobacteria phage T7) (Hern et al., 2014).The diagrammatic representation of Myoviridae is shown in below figure .

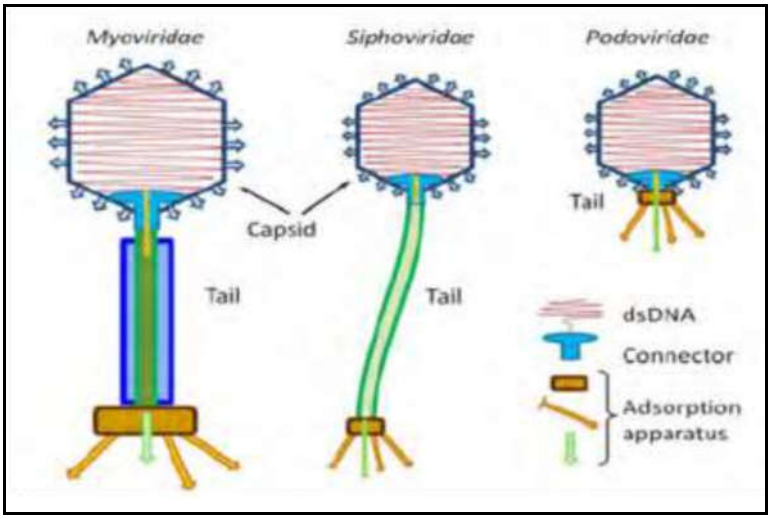


Figure 1. 2: Classification of Tailed Phage Family (Caudovirales order).

The Caudovirales order of bacteriophages is characterized by dsDNA genomes and by the common overall organization of the virus particles characterized by a capsid and a tail.

1.1.2 Classification of Bacteriophages

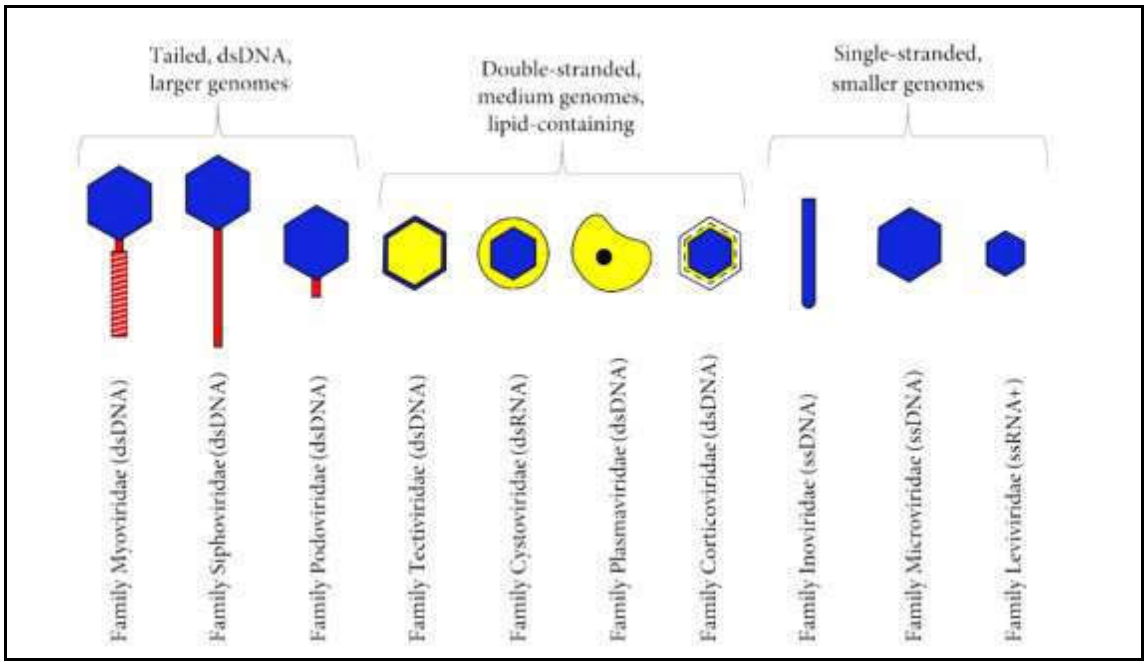


Figure 1. 3: Classification of Bacteriophages: Bacteriophage families, morphologies, genome types

In figure, Blue coloration indicates capsids, red indicates tails, and yellow refers to lipids.Figure adapted from (Hyman & Abendon, 2011).

Phages are enormously diverse and vary from one another based on structural, physiochemical, and biological properties. Classification of viruses is based on several factors such as their host preference, viral Morphology, genome type and auxiliary structures such as tails or envelopes. In 1933, Burnet showed that enterobacterial phages represent a heterogeneous group and they may be differentiated by the following criteria: particle size defined during filtration, serological properties, host range and storage stability (Burnet, 1933). Later Ruska was the first to apply electron microscopy for viral taxonomic studies. As a result, he proposed classification scheme comprising three morphological phage types. And then the taxonomic classification of bacteriophage is coordinated by an International Committee on Taxonomy of Viruses (ICTV) in 1973. In 1971, the ICTV issued its first report which included six phage “genera”: T-even phages, l, lipid phage PM2, the ϕ Xgroup, "filamentous phage", and the "ribophage group". Groups were listed with type species and properties. The ICTV taxonomic system requires visualization of the phage structure using electron microscopy. The discovery of the electron microscope in the 1940s enabled the identification of different phage morphologies; phages can be tailed, polyhedral, and filamentous or pleomorphic, and some have lipid or lipoprotein envelopes. At least 5,360 tailed and 179 cubic, filamentous, and pleomorphic bacterial viruses have been examined in the electron microscope since the introduction of negative staining in 1959 (Hans-Ackermann, 2011). Up-to-date classification scheme encompasses 1 order, 13 families and 34 genera of bacteriophages. The families are distinguished taking into

account the type of nucleic acid and virion morphology. Over 40 criteria are engaged for phage differentiation into genera and species. The different classes of tailed phage are Podoviridae, Siphoviridae and Myoviridae and the nontailed virions are filamentous (family Inoviridae), spherical (family Cystoviridae), or pleomorphic family Plasmaviridae), while the rest are icosahedral, in many cases resembling tailed phage heads but without any tail which is shown in above figure (Figure1.3)

1.1.3 Bacteriophage Abundance in Environment

Bacteriophages occur everywhere in the biosphere that play major ecological role and considered to be the most prominent biological entities on the planet with an estimated population size of 10^{30} or more (Chibani-chennoufi et al., 2004). Simply the phages are found where their host aside. When no appropriate hosts are present, many phages can maintain their ability to infect for decades, unless damaged by external agents. Phages have colonized in all environments and ecological niches including different forbidding habitat such as island waters, volcanic hot springs, salt water, soil, cold water (Clokie et al., 2011).

Estimated 10^{30}–10^{32} phages exist in the biosphere which is 10-fold higher than the total number of bacterial cells (Abendon et al, 2011) and an estimated 10^{23} phage infections occur per second. Every fourty eight hours, phages destroy about half the bacteria in the world, a dynamic process that occurs in all ecosystems (Clokie et al., 2011).

Their population densities are estimated to be 10^{9} per gram of soil (Williamson, 2010), 107 per ml of seawater (Wommack & Colwell, 2000), and 10^{31} in planet (Whitman et al., 1998).There are approximately 10 times as many viruses as the combined number of all cellular organisms, and most viruses are bacteriophages (phages), viruses that infect bacteria (Aziz et al., 2015). Marine water is one of the major resources of bacteriophage and several studies indicate a greater Variance in overall phage prevalence in these ecosystems, sea water is one of the major reservoirs. The prevalence of Bacteriophage in freshwater is higher than in marine water. The abundance of viruses varies strongly in different environments and is related to bacterial abundance or activity. Phages are widely distributed in locations populated by bacterial hosts, such as soil or the intestine of animals. They are also an inherent element of the human microbiome, and therefore they are well tolerated when used in phage therapy (Reyes et al, 2012) .The human gut is estimated to contain 10^{15} phages, and majority of phages are found to be temperate rather than obligatory lytic (Dalmasso, Hill & Ross,2014). One of the densest natural sources for phages and other viruses is sea water, where up to 9×10^{8} virions per milliliter have been found in microbial mats at the surface. Up to 70% of marine bacteria may be infected by phages. It is estimated that there are 5 to 10 viruses for each bacteria (Weinbauer, 2004).

The environment is a unique source of all types of phages, offering the possibility to isolate them for therapeutic purposes (Shukla et al., 2014).The isolation of phages from

environmental sources is more often possible when the sample contains the target bacteria and phages against human bacterial pathogens that are found most probably in sewage sample of hospital vicinity area. The new virulent phages can be isolated from the sewage water sources, stream water .Now it is suggested that the richest source of the most effective therapeutic phages is the material from patients who recovered from an infection isolated phages from sewage.

1.1.4 Phage Impact on Bacterial Population

From the literature studies on marine biodiversity have shown that bacteriophages influence their host bacterial organism in a density-dependent manner (Ventura et al., 2011). This is in harmony with "kill-the-winner" model where the predation is directed towards "winner" (abundant) bacterial population in that environment (Rodriguez-Valera et al., 2009). Barr et al. (2013) documented that phages adhered to mucus (BAM) of the human gut can provide an innate protection to the underlying epithelial tissue from outside pathogens (Barr et al., 2013). Moreover, the presence of prophage in bacteria residing in gut flora provides a competitive advantage to the host which makes the pathogenic organism hard to out compete commensal organism hence maintaining the stability of human gut microbiome (Ventura et al., 2011).The phages in gut play active role in human gut immunity and metabolism by forming part of the mucus-associated microbiome, where they may serve as a first line of defense against invading bacteria.

1.1.5 Life cycle of Bacteriophage and Mechanism of Lysis of Bacteria by Bacteriophage

Like any other virus, phages also require the help of specific host for their multiplication. In the absence of a host they remain as inert particles with their genetic material safely packed inside the protective protein covering. Phages carry their genetic information either in the form of DNA or RNA. Adsorption of phage on to the host is the first and the most important step in phage multiplication and it is via their tail tips so that they recognize the receptor on the host surface. Most phages are highly specific for their receptors and the receptor material on host cell can either be carbohydrate, protein, lipopolysaccharides (LPS) or flagella. Some phages require a cluster of one specific receptor for proper adsorption. Whereas, for some phage adsorption stage requires different stage involving the different set of receptors such as T4-like phages (Guttman et al., 2004).This attachment is weak and reversible and is followed by irreversible binding to a bacterium, mediated by one or more of the components of the base plate. The phage genome is introduced into the bacterial cytoplasm (called penetration) either by the contraction of the sheath or by weakening the components of the bacterial envelope by digestive enzymes. Entrance of the phage genome into the cell starts the infection period and the productive cycle ends either with phage-induced bacterial destruction (lysis) or with an extruding or budding of maturing phage virions (chronic release) across the bacterial cell envelope (in a manner that does not lead to immediate

bacterial death) (Fischetti et al., 2006). Infections that result in phage release can be described as productive. Alternatively, phage infections can enter a latent state (lysogeny or pseudolysogeny) or can fail to infect successfully (phage restriction or abortive infection). The most common types of life style are depicted in the Figure 1.4.

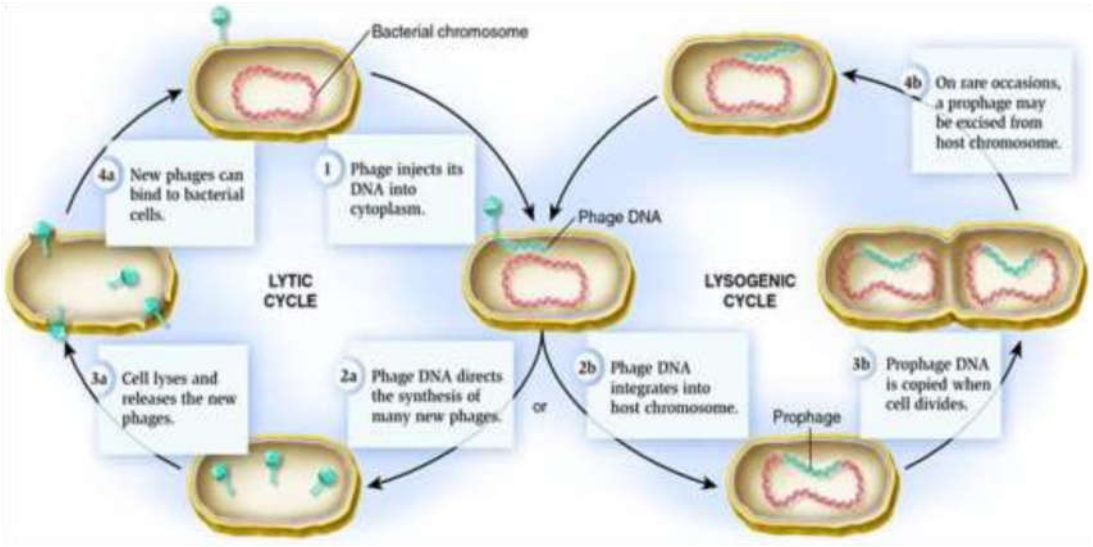


Figure 1. 4: Overall process involved in lytic and lysogenic life cycle of Bacteriophages

Bacteriophages may have a lytic cycle or a lysogenic cycle, and a few viruses are capable of carrying out both. The bacteriophage recognizes the receptors on the host cell membrane surface and adsorbs using its tail and tail fiber proteins. This is then followed by internalization of the phage genome after which the cycle proceeds to either the lysogenic or lytic replication cycles. During lytic cycle, phages infect and lyse the host cell, whereas lysogenic, is characterised by integration of the phage DNA into the host cell genome. Under certain conditions the prophage excises from the bacterial chromosome and initiates the lytic cycle.

1.1.6 Lytic cycle

The lytic lifestyle was studied and characterized in the first half of the 20th century, just over two decades after the discovery of bacteriophages. It was found that there were distinctive steps in the infection process of a phage; adsorption of the phage to the host cell, multiplication of new phage progeny, and finally lysis of the bacterial cell to release the new phages. The lytic lifecycle is very short often only minutes long (Ackermann, 1998). Entrance of the phage genome into the cell begins the infection period. The first stage is eclipse period during which infectious phage particles cannot be found either inside or outside the bacterial cell. The lytic life cycle results in the destruction of the host cell (Guttman et al., 2004). Several steps occur inside the host cell which include gene expression, genome replication and morphogenesis – i.e. the formation of the capsids (and tails) and the packaging of the genomes into the capsids .The phage nucleic acid takes over the host biosynthetic machinery, whereby phage specified mRNAs and

proteins are made. There is an orderly expression of phage directed macromolecular synthesis. Early mRNA codes for early proteins that are necessary for phage DNA synthesis and for shutting off host DNA, RNA and protein biosynthesis. In some cases the early proteins actually degrade the host chromosome. The late proteins are the structural proteins that comprise the phage as well as the proteins needed for lyses of the bacterial cell. Synthesis of late mRNA and late proteins takes place only after phage DNA synthesis. Once this is over, phage nucleic acid and structural proteins are assembled and accumulated within the cell which is followed finally by the release of hundreds of new phages by bacterial cell lysis.

1.1.7 Lysogenic infection

Lysogenic cycle is another phage proliferation technique. Here, new phage particles are not immediately produced and the host cell is not destroyed. In the lysogenic cycle, the phage genome is integrated into the bacterial genome by transposition or site-specific recombination or resides as a plasmid within the host cell cytoplasm and it multiplies cooperatively with the host bacteria without destroying it, and consequently the phage genome is passed to all bacterial progeny by cell division. Bacteria that integrate the phage genome into their own are known as lysogens; they are resistant to infection by phages that are genetically related to previously lysogenized phages (Campbell, 1996). Some lysogenic phages have toxic genes in their genome. In lysogenic relationship lytic genes are inhibited by the phage gene product termed a repressor which binds to a particular operator site on the phage DNA, shutting off transcription of almost all phage genes except that of the repressor. Due to lytic inhibition, the phage gene will remain integrated into the host chromosome which is called a prophage and will replicate along with the host replication process (Little, 2005). Prophages remain dormant until the lytic cycle is induced by number of physical or chemical agents, such as mitomycin C, hydrogen peroxide, UVradiation, temperature and pressure (Paul & Jiang, 1996; Weinbauer & Suttle, 1999). This process is called induction.

1.1.8 Pseudolysogenic infection

Pseudolysogeny is a form of phage-host cell interaction, where the nucleic acid of the phage resides within the bacterial cell in an unstable, inactive state. Pseudolysogeny occurs mainly when host is exposed to starvation (Williamson et al., 2001). When proper nutrients are added, the pseudolysogenic state is resolved. Pseudolysogeny provides potential to enhance bacteriophage survival, as the phage genome is protected from environmental conditions by ‘sheltering’ within the host cell (R. V. Miller, 2001) so that phage can maintain themselves in the large numbers observed in what would appear to be hostile habitats (Hennes et al., 1995; Paul et al., 2002).

1.1.9 Chronic infection

A chronically infecting phage can release progeny into the extracellular environment without terminating its infection, i.e. phages are slowly and continuously shed or

extruded across the host cell envelope without destroying either the infected bacterium or the phage multiplication rather than being released as a single burst event (Abedon, 2008). The intracellular chronic cycle has been best described for filamentous phages of *E. coli* (Paepe etal., 2010; Dybvig et al., 1985). The extrusion process driven by phage-encoded proteins located in the cell envelope preserves the integrity of the cell membrane of the host (Snyder and Champness, 2003; Volkova et al., 2014).

1.1.10 Mechanism of lysis

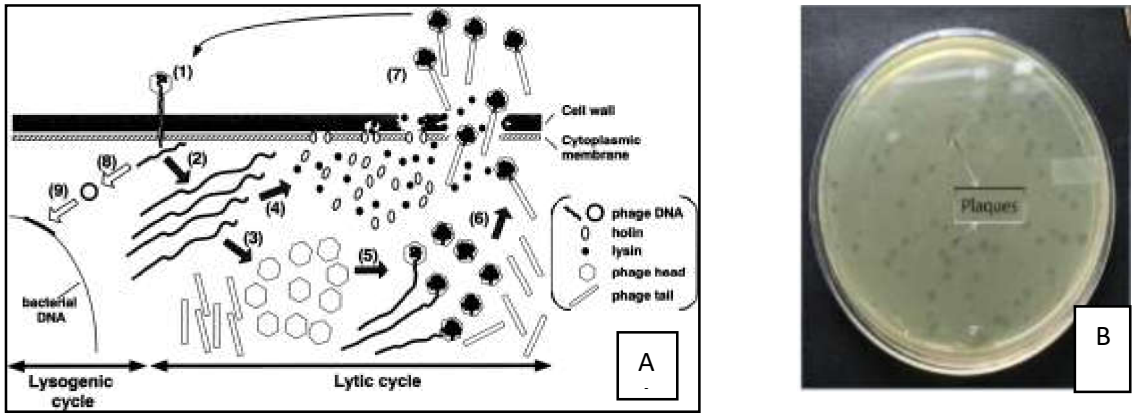


Figure 1. 5: Schematic illustration of phage-induced bacteriolysis

(1) Adsorption and DNA injection (2) DNA replication (3) production of head and tail (4) synthesis of holin and lysin (5) DNA packaging (6) completion of phage particle (7) disruption of the cell wall and release of the progeny (8) circularization of phage DNA (9) integration of the phage DNA into the host genome (Figure adapted from (Matsuzaki et al., 2005)) Fig B: Lysis of bacteria bacteria forming plaques on bacterial lawn.

The progeny phage production requires compromising the structural integrity of the murein sacculus, which forms a tough, dynamic meshwork that surrounds and confers the shape on the cell (Höltje & Glauner, 1990).It is carried out by the coordinated action of two proteins, holin and endolysin (lysin) encoded by the phage genome. Holin proteins form a ―hole‖ in the cell membrane, enabling lysin to reach the outer peptidoglycan layers (R Young, 1992). Lysin is a peptidoglycan-degrading enzyme (peptidoglycan hydrolase). At least four different muralytic activities are found in phage endolysins: transglycosidases and lysozymes that attack the glycosidic bonds linking the amino-sugars in the cell wall, amidases and endopeptidases that attack the amide and peptide bonds of the oligopeptide cross-linking chains(R Young, 1992). Although the endolysins, like the T4 E lysozyme, the T7 gp 3.5 amidase and the lamda R transglycosylase are small globular proteins, the recently identified endolysins possess multiple muralytic activities and greater molecular masses≥60kDa (Navarre et al., 1999). The mechanism of bacteriolysis by phage is depicted in figure (Figure 1.6 A).

Filamentous phage particles do not form in the cytoplasm; rather they are continually extruded or secreted across the bacterial membranes as they are assembled, without

causing cell lysis. They do not kill their host and the infected cells continue to grow and divide indefinitely while producing phage (Rakonjac, 2012).

Some small phages such as ΦX174 and Qβ, which have single stranded DNA and RNA respectively, do not have the genes for holin or lysin proteins (Bernhardt et al., 2002). They accomplish lysis by producing a protein that inhibits cell wall biosynthesis. Their inhibitory gene products are known as ―protein antibiotics‖. The ΦX174 gene product, gpE inhibits MraY. Review of literature which catalyzes the formation of the first lipid-linked murein precursor while Qβ gpA2 inhibits MurA, which catalyzes the first step in the murein biosynthesis pathway (Thomas et al., 2001).

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 which permeabilises the cell membrane called holin. 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 (Young et al., 2000).

Latent period (the time taken from initial infection to lysis) duration can be affected by host density with lower concentrations of bacteria leading to a longer period of time before lysis. A compromise has to be achieved because a shorter latent period leads to a smaller burst size. When bacterial hosts are plentiful a smaller burst size is less important as there are more hosts to infect, therefore plenty more progeny can be produced. When hosts are scarce the latent period gradually evolves to become longer enabling many more progeny to be produced increasing the possibility that more bacterial cells will be encountered and infected (Abedon et al., 2001).

Lytic infection or the lysis of bacteria is indicated by the formation of a zone of clearing called plaque (Figure 1.5 B).Plaques are clear zones formed in a lawn of bacteria due to lysis by phage. Plaques which are zones of bacterial lysis can be seen in figure which is caused by phage action and appear as circular zones of lysis on lawns of bacterial cells.The period encompassing phage adsorption to genome replication, production of progeny virions and lysis of the host cell is described as the latent period. The duration from adsorption to the time of formation of progeny virions, when intracellular virions can be detected by plaque assay after prematurely lysing the host cell, is described as the eclipse phase (Adams , 1959; Ellis & Delbrück, 1939) .

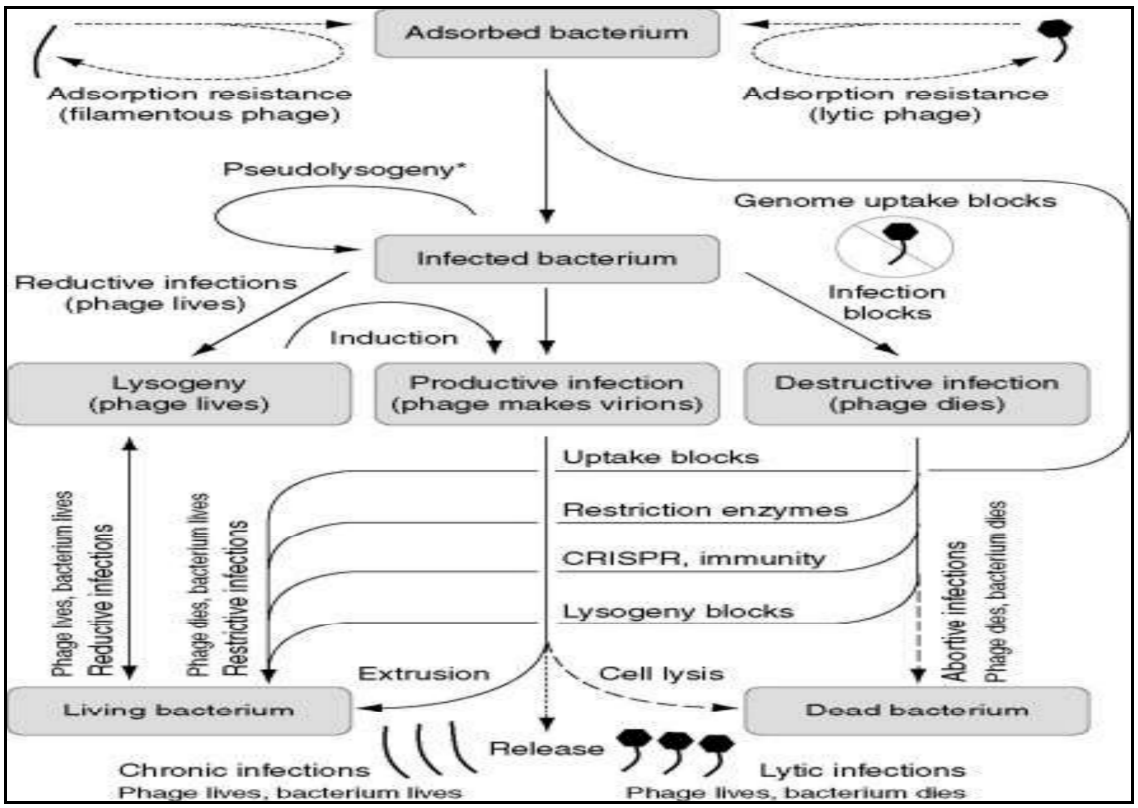


Figure 1. 6: Schematic representation of the possible outcomes of infections of bacteria by filamentous and tailed bacteriophages.

The diagram shows the interactions between phage and host and the survival outcome for each Adsorption to and release from bacterial cells are shown as short dashed lines. Infections leading to death of the bacterial cell are shown as long dashed lines. (Figure source (Abedon et al., 2010).

An infection event which results in either integration or replication of the virion genome may be termed a successful infection. A productive infection is one which results in the assembly and release of progeny virions competent for subsequent infection of host bacteria. Many things can go wrong in the course of the viral life cycle such that productive infection is never achieved ie: Infection does not always result in the death of the host cell and survival of the bacteriophage which is shown in figure (Figure 1.6). The plaque formation is a clear indication of productive phage infection. There are several bacterial mechanisms of phage resistance which include adsorption resistance, which results in reduced interaction between phage and bacterium, another mechanism is 'restriction,' where bacteria live but phages die and abortive infections, where both phage and bacterium die (Abedon et al., 2010). Adsorption resistance includes loss of phage receptor molecules on hosts as well as physical barriers hiding receptor molecules (e.g., capsules). Bacteria have evolved a number of resistance mechanisms including preventing uptake of the phage genome, superinfection immunity, clustered regularly inter-spaced palindromic repeats (CRISPR) and restriction modification systems (Labrie et al, 2010) . Phage-resistance mechanisms encoded by bacteria (bacterial resistance) serve to limit phage host range of bacteriophage.

1.1.11 Antimicrobial Resistance- a global issue

Antimicrobial resistance is an ability of microbes to grow in the presence of a chemical (drug) that would normally kill them or limit their growth. It can affect anyone of any age, in any geographical location. Antibiotic resistance is one of the biggest threats to global health, food security, and development. According to CDC (Central for Disease Control and Prevention) even in U.S.A, each year at least 2 million people get an antibiotic-resistant infection and at least 23,000 people die and this rate is significantly higher in developing country. Many studies have demonstrated that a large proportion of international travelers acquire resistant bacteria during visits in areas with a high prevalence of resistant bacteria (Woerther et al., 2013).The unusual resistance had spread to other patients. The CDC has found that out of 5776 people , one in four had a gene that helped spread its resistance, while 221 contained an especially rare resistance gene Enterobacteriaceae, that could produce an enzyme capable of breaking down common antibiotics. The germs that had begun to evolve from 2001 will be becoming more resistant to carbapenems and other antibiotic drugs. These carbapenem-resistant Enterobacteriaceae, named as "nightmare bacteria" by the CDC spread rapidly in the US and around the globe (CDC, 2018).The first reported antibiotic used in livestock led to antibiotic-resistant bacteria was in 1951; here Starr and Reynolds (1951) described streptomycin resistance in generic intestinal bacteria from turkeys that were fed with antibiotic. The new resistance mechanisms are emerging and spreading all around the globe in an unprecedented way, threatening our ability to treat common infectious diseases. Across the world, third and fourth generation cephalosporin resistant *Escherichia Coli* are becoming difficult to treat due to their ability to secrete extended-spectrum β-lactamase (ESBLs). In 2013, 17 out of 22 European countries reported 85% to 100% of ESBL positive E. coli isolates (European Antimicrobial Resistance Surveillance Network, EARS-Net, 2014). In 2009 and 2010, 28% of the *E. coli* families (Enterobacteriacae) from 11 Asian countries were reported to be ESBL producers, and their resistance to third- and fourth-generation cephalosporins ranged between 26% and 50%. Fighting this threat is a public health priority that requires a collaborative global approach across sectors. Similarly in Nepal, as in many countries, antibiotic resistance bacterial infections are the primary concern causing increased morbidity and mortality rate. In laboratory based study among the patients of intensive care unit of Tribhuvan University Teaching Hospital, Kathmandu, Nepal out of 135 patients suspected of healthcare-associated infection (HCAI), Extremely high level of drug resistance was found to be 95.8% along with the production of β-lactamases (ESBL; 43.7%, AmpC; 27.5%), MBL; 50.2% and KPC; 4.2% (Parajuli et al., 2017).

Unfortunately, the antibiotics which are ‘miracle drugs’ of the 20th century now led to extreme crisis of antibiotic resistance threats across the globe. The antibiotic resistance genes, now being abundantly available in the environment and that are highly encouraged by the overuse and misuse of antibiotics are decreasing the effectiveness of these infection fighters. It becomes more challenging to ‘defeat’ microbes with

antibiotics (Spellberg & Gilbert, 2014). There is no 'endgame'; resistance is inevitable (Spellberg, 2014). In 2011, WHO declared "combat drug resistance: no action today, no cure tomorrow. To prescribe a powerful new antibiotic is easy, to protect it is hard. This problem will gradually become worse unless we fundamentally change the way our society deals with the discovery, development, use, and protection of these life-saving drugs (antibiotics). It requires the collaboration of all relevant sectors, especially the government, and those involved in both human and animal health, public, farmer, healthcare personals and all the pharmaceutical companies to tackle the antibiotic resistance from infection to prevention with the knowledge of how to use and stop misuse of antibiotics. Recently, antimicrobial Stewardship Programs encourage rational prescribing of antibiotics and remind healthcare workers – and their patients – about consequences of antibiotic over-use and misuse which promote judicious use of antimicrobials to combat antimicrobial resistance (Leuthner & Doernb, 2013).

1.1.12 Carbapenem Resistance Enterobacteriaceae

Carbapenems are potent member of the β-lactam family of antimicrobials that are structurally related to the penicillins. The most commonly used carbapenem antimicrobials are imipenem, meropenem, doripenem, ertapenem, panipenem and biapenem. Carbapenems are considered as one of the most reliable drugs due to its unique structure of a carbapenem coupled to a β-lactam ring which confers protection against most β lactamases such as metallo-β-lactamase (MBL) as well as extended spectrum β-lactamases .In recent time the emergence and spread of resistance even to these antibiotics constitute a major public health concern. Carbapenem-resistant Enterobacteriaceae (CRE) is Gram-negative bacteria that are resistant to the carbapenem class of antibiotics. They are resistant because they produce an enzyme called a carbapenemase that disables the drug molecule.It occurs mainly among Gram-negative pathogens such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, *Escherichia coli* etc .This may be intrinsic or mediated by transferable carbapenemase-encoding genes.

The molecular classification is based on the conserved and distinguishing amino acid motifs and divides β-lactamases into classes, A, B, C, and D. The enzymes Classes A, C, and D include enzymes that hydrolyze their substrates by forming an acyl enzyme through an active site serine, whereas class B β-lactamases are metalloenzymes that utilize at least one active-site zinc ion to facilitate β-lactam hydrolysis (Bush & Jacoby, 2010) .Genes encoding for β-lactamases are generally termed bla genes which is followed by their different enzymes like bla KPC, blaNDM. The resistance to carbapenems in some species is intrinsic and in some bacteria it is acquired by mutational events or gene acquisition via horizontal gene transfer (Meletis, 2015). Carbapenem resistance genes are mediated via one of two distinct genetic mechanisms, a mutation in the bacterial chromosome or by a transmissible element; either a plasmid or a transposon (Codjoe & Donkor, 2017).

1.1.13 *Klebsiella pneumoniae*

Klebsiella pneumoniae is a Gram-negative, rod-shaped non-motile bacillus from the genus Klebsiella and family Enterobacteriaceae. *K. pneumoniae* is facultatively anaerobic, oxidase-negative, and produces acid and gas from lactose ie.lactose fermenter. Klebsiella pneumoniae, is a member of the group of ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) that are capable of escaping the bactericidal action of antibiotics and are more prone to develop antimicrobial resistance (AMR) (Pendleton et al., 2013). It is an enteric bacterium, noted in the intestinal tract of 5% of healthy humans (Hsu et al., 2015) .It can cause different types of healthcare-associated infections, including pneumonia, bloodstream infections, wound or surgical site infections and meningitis. K. pneumoniae infections are typically "nosocomial" infections. It is the third most frequently isolated microorganism in blood cultures from sepsis patients that can cause severe epidemic and endemic nosocomial infections (Wu and Li, 2015). Virulent *K. pneumoniae* is encapsulated and it appears as quite opaque and mucoid when growing on agar and avirulent *K.pneumoniae* has no capsules appear smaller and translucent on similar agar surfaces.*K.Pneumoniae isolates* can be roughly classified into two pathotypes: opportunistic *K. pneumoniae*, which are often multidrug-resistant (mdrKp), and hypervirulent *K. pneumoniae* (hvKp) which are able to infect healthy individuals and cause invasive infections including pyogenic liver abscess.

The pathogenicity of *K. pneumoniae* is multifactorial including capsular serotype, lipopolysaccharide, iron-scavenging systems, and fimbrial and nonfimbrial adhesions urease, adhesins, outer-membrane proteins and biofilms (Table 1.1). *K. pneumoniae* is an important Biofilm-forming organism responsible for a wide range of infections. Because of the collective threat of several virulence factors, *K. pneumoniae* has attained superbug status and is one of the most common antibiotic-resistant bacteria (Li, 2015b).*K. pneumoniae* is found to be developing rapid resistance against various antimicrobial agents, including broad-spectrum cephalosporins and β-lactams. Though there are several mechanisms that lead to antibiotic resistance, the emergence of Klebsiella pneumoniae carbapenemase (KPC) and the New Delhi Metallo-beta-lactamase (NDM)(Kumarasamy et al., 2010) poses some serious threat especially in nosocomial infections (Daikos and Markogiannakis, 2011).Recent global surveillance data from Europe, North and South America, and Asia revealed that the frequency of ESBL-producing *K. pneumoniae* was 7.5 to 44% (Reinert et al., 2007). As the prevalence of ESBL-producing isolates increased, carbapenems were used to treat serious infections caused by ESBL-producing K. pneumoniae. However, carbapenem-resistant *K. pneumoniae* (CRKP) rates also have been dramatically increasing worldwide over the past 10 years (Karumidze et al., 2013).

Table 1. 1: Virulence Factor of *Klebsiella pneumoniae*

Name	Activity in Virulence
Capsular polysaccharides	Evade phagocytosis
LPS	Evade serum killing factors
Adherence factors	Adhere to host surface
Siderophores	Acquire iron
Urease	Allow growth in urinary trac
Antibiotic resistance	Tolerate antibiotics
Outer-membrane proteins	Resist antibiotics
Biofilms	Survive in harsh host

1.1.14 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram negative aerobic rod-shaped bacterium that produces a distinctive blue/green pigment and an exopolysaccharide known as alginate. It is an opportunistic pathogen of animals and humans. There is little data on the prevalence of *P. aeruginosa* in the environment although it is known to exist in soil and various categories of water, and is a well known plant pathogen. The majority of the studies of *P aeruginosa* have concentrated on its implication in human disease as it can cause infections of varying severity affecting many different organs. This bacterium is the most often found pathogen leading to the respiratory tract of cystic fibrosis (CF). It is responsible for a wide range of human diseases, such as septicaemia, pneumonia, and several other kinds of infection. Another important aspect of *P. aeruginosa* as a pathogen is its resistance to many antibiotics making it very hard to treat (Todar, 2005). *P. aeruginosa* possesses all of the major bacterial resistance mechanisms, alginate reduces antibiotic diffusion, efflux pumps remove antibiotics from the bacterial cell, enzymes such as beta-lactamases degrade certain antibiotics, and mutations in various genes affect antibiotic targets (lambert, 2002).

A well known characteristic of *Pseudomonas aeruginosa* strains is their ability to produce various pigments, indeed this used to be the method of identifying different strains. The usual blue/green pigment (pyocyanine) and yellow/green fluorescent pigment (pyoverdine) can be replaced by a brown (pyomelanin) or red (pyorubin) pigment or an unpigmented variant. Only *P. aeruginosa* is capable of producing water soluble pigment; pyocyanin (blue-green) which distinguish them from other *Pseudomonas* species. Among all the pigments, derivatives of pyocyanin and pyochelin, act as a virulence factor which is a siderophore that is produced under low-iron condition to sequester iron from the environment for growth of the pathogen (Figure 1.7). It could play a role in invasion if it extracts iron from the host to permit bacterial

growth in a relatively iron-limited environment. They have distinctive grapelike odor due to production of amino acetophenone and have ability to grow up to 42˚C. Another characteristic features are containing single polar flagellum, non spore forming, capsulated "polysaccharide capsule", aerobic, oxidase and catalase positive. Colorization, odor and colonies morphologies help in the preliminary identification of *P. aeruginosa*.

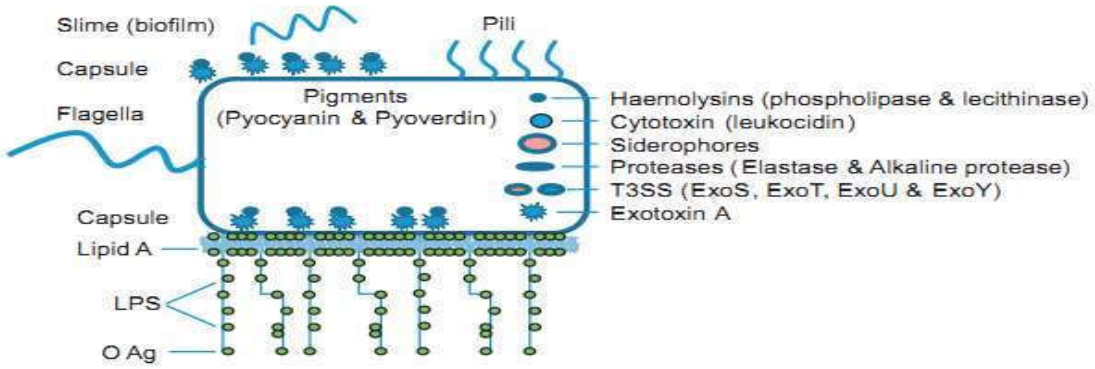


Figure 1. 7: Virulence factors of *Pseudomonas aeruginosa*

This bacterium possesses abundant virulence factors causing a wide range of diseases, such as Pneumonia, chronic lung infections, septicaemia, urinary tract infections etc. The relative location in the bacterial cell is indicated in figure.It contains flagellum, pilus, LPS and capsule as structural virulence factor .Similarly it contains exopolysaccharide biofilm and different proteases, toxins, haemolysins and siderophores(Pyocyanin pigment) as extracellular factor and various types of exotoxins and exoenzymes .Figure adapted from (Li, 2015a)

1.1.15 Bacteriophage Application

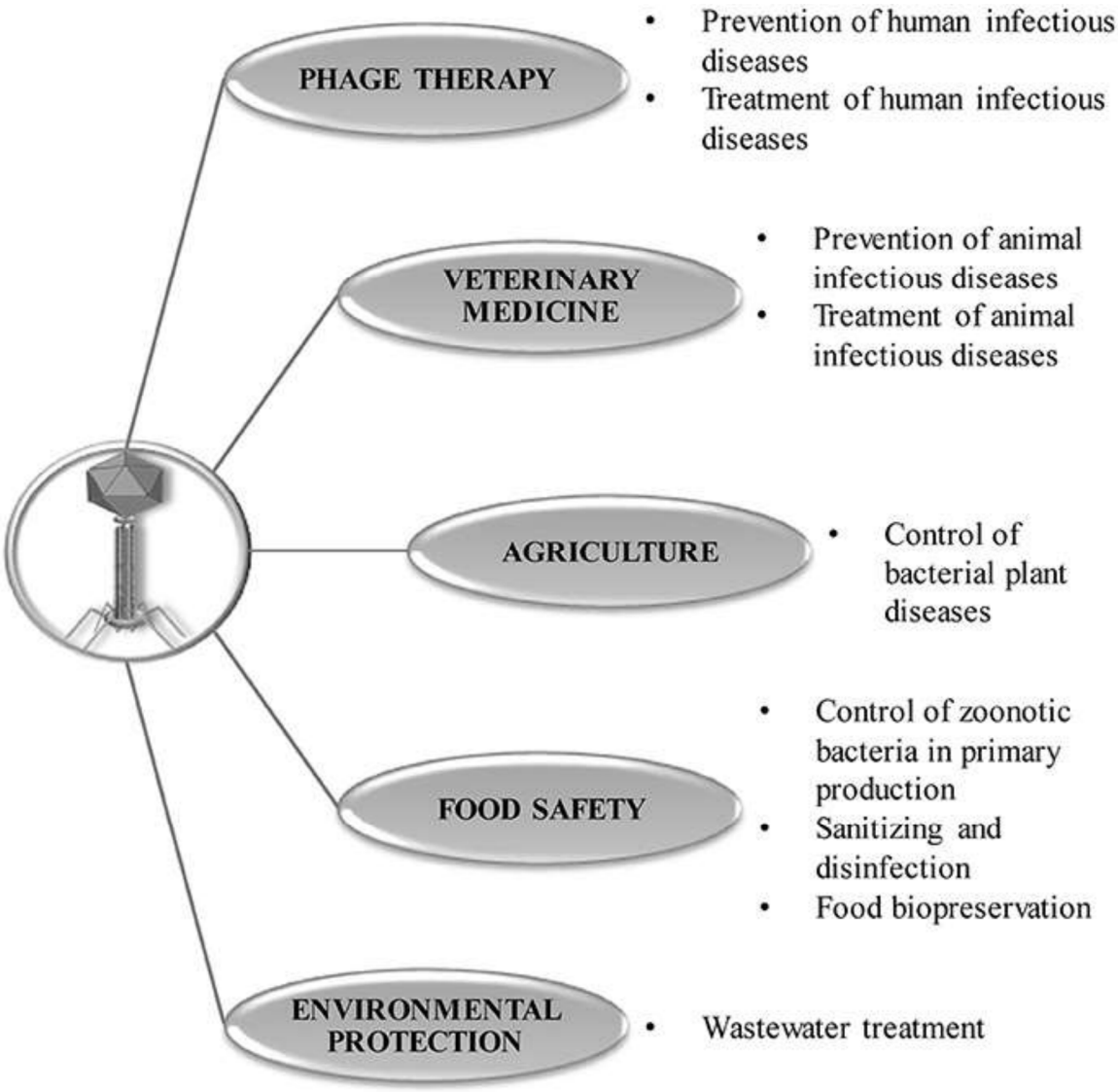


Figure 1. 8: Application of Bacteriophages:

Showing application of phages in various fields' industrial application, Biodetection application, Therapeutics application, Veterinary application, Biocontrol application.

The rise of antibiotics resistance has rekindled interest in the development of alternative antimicrobial agent (Jassim & Limoges, 2014). Bacteriophages are now again reconsidered as an antimicrobial tool ,one of the best possible option in the therapeutics use of specific phage particles that target bacterial pathogens. In the early twentieth century, a poor understanding of the biological mechanism of phage activity and subsequent discovery and general application of broad-spectrum antibiotics was acclaimed; as in the late 1930s and 1940s, interest in the therapeutic use of bacteriophage declined and for many years was only considered as a research tool in molecular biology (Clark & March, 2006). Now, Bacteriophage has drawn attention to scientific community to rediscover of phage therapy in modern medicine in this antibiotic crisis situation. Continuous research on phage and its understanding explores

the application of bacteriophage in diverse field such as food safety, agriculture, veterinary applications, environment sanitation, industry and clinical diagnostic application such as detection and typing of bacteria in human infection (Figure 1.8). Phages are not only used in humans to treat infections , however they are also being developed for environmental prophylaxis; for example to get rid of the pathogens in the environment and animals that could contaminate food supplies, to control infections in poultry production (Wernicki et al., 2017) for the treatment of fish pathogens in aquaculture (Richards, 2014). Conventionally, phage therapy relies on the use of naturally-occurring phages to infect and lyse bacteria at the site of infection. Now, with the biotechnological advances and availability of necessary molecular tools and techniques, Phages are also used as a delivery vehicle for gene therapy, as a biocontrol agent, uses in the development of phage-derived vaccine and in phage display technique and use of bioengineered phages and purified phage lytic proteins in phage therapy.

1.1.16 Phage Therapy

Phage therapy is the therapeutic use of lytic bacteriophages to treat pathogenic bacterial infections. Phages have been used in the treatment of infections in plant, animal and human beings with varying degree of success (Sulakvelidze & Alavidze, 2001). After the discovery of phage in 1919, d'Herelle and his colleagues in Paris had begun using bacteriophages in a therapeutic way, launching the "Phage Therapy" to treat four children from bacterial dysentery (Criscuolo, et al, 2017). In 1921, Bruynoghe and Maisin published the first paper describing the efficacy of bacteriophages in the treatment of a staphylococcal skin infection: they injected the phage preparation around surgically opened lesions and the infection regressed within 24–48 hours (Sulakvelidze &Alavidze, 2001).Later on ,Bacteriophage therapy rapidly developed globally and attracted the attention of pharmaceutical companies and independently, the Russian and German army physicians, who started using phages to treat soldiers during the time of second world war (Linden & Jones, 2012). In the United States during the 1940s commercialization of phage therapy was undertaken by Eli Lilly and Company (Dubey et al., 2016). The discovery of broad range antibiotics played the major role in declining the interest of producing phage commercially at that time. However, phage therapy was continued to be offered in the Eliava Institute and later by others also such as the Hirszfeld Institute of Immunology and Experimental Therapy in Wroclaw, Poland (Deresinski, 2009) and in those early days extensive research and development on phage therapy was done in Russia, Georgia and Poland .There are various success stories available about potential use of bacteriophage and its effectiveness in human patient in Eastern Europe. Human safety trials have also been performed successfully which was undertaken on Staphage Lysate by Delmont Laboratories (USA) (Jassim & Limoges, 2014) .According to the National Insitutes of Health (NIH,USA), Phages are innovative components that may be used to combat with microbial resistance (NIH,2014).The use of bacteriophages in phage therapy is based on their ability to recognize, adsorb and multiply only within the bacterial cell and causes its lysis (Burrowes et al., 2011).For

therapeutic uses, obligatory lytic bacteriophages are highly desirable, because they result in rapid killing of their target host cell, bacteriophage numbers increase rapidly and transduction is rare and similarly, well-described and characterized phages are preferred one for phage therapy. Phages host specificity acts as an advantage since it is less likely to interfere with the natural flora of host. Nowadays, research has been performed on using of phage lytic enzymes rather than using whole phage (Fischetti, 2005) or using genetically modified non-lytic phage to deliver bactericidal protein-encoding DNA to pathogenic bacteria (Westwater et al., 2003). Innovations in the gene editing tool CRISPR/Cas9 have created novel opportunities for phage therapy. One example of which is the use of bioengineered phage to deliver a CRISPR/Cas9 system to disrupt antibiotic resistance genes and destroy antibiotic resistance plasmid (Lin et al., 2017b). These phages may be applied in hospital to reduce frequency and spread of antibiotic resistance genes.

Advantages of using phage therapy over antibiotics

Due to unique properties of phages, there are various advantages of phage therapy over the use of chemical antibiotics. Phages are being emphasized these days due to its ability to kill multidrug resistant bacteria and even it can clear biofilm produced by bacteria ,once they lysed bacteria they cannot regain their viability (Loc-Carrillo & Abedon, 2011).Some of the advantages are given below on the basis of phage properties:

- Bacteriophages are highly specific, thus 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.
- In case of phage, initial dose of phage can be low because the viruses self-propagate compared to antibiotics (Donlan, 2009). It is termed as auto “dosing” because the phages themselves contribute to establishing the phage dose.
- No correlation between phage and antibiotics resistance. The development of resistance of bacteria to antibiotics does not parallel to the development of phage resistance in bacteria (Allen et al, 2017).
- Phages are effective against most resistant bacteria and even to mature form of biofilm. Many studies showed that biofilms are significantly more resistant to chemical antibiotics than planktonic bacterial cells Phages have ability to clear biofilms (Harper et al., 2014).
- There are studies that suggest phages do not increase the antibody level in the blood stream (Chhibber & Kumari, 2008).
- Phages mutate at a higher rate than bacteria and are able to respond fast to possible phage-resistant bacteria (Bhardwaj, 2014).
- Bacteriophages are environmentally friendly and are based on natural selection, isolating and identifying bacteria in a very rapid process compared to new

antibiotic development, which may take several years, may cost millions of dollars for clinical trials, and may also notbe very cost effective.

- Although resistance to phage has been reported, it is claimed that this can be avoided by using a 'phage cocktail' (O'Flynn et al., 2004). Furthermore phages have co-evoled with bacteria and thus it is claimed to be easier to overcome phage resistance (Abedon, 2009; Abedon, 2009(1)).

Drawbacks associated with the use of bacteriophages

- **Endotoxins**: Phage multiplication using host cell is a primary step for phage production. During cell lysis, lipopolysaccharide, 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 (Dabrowska et al., 2004). Purifying phage preparation using chromatography and ultrafiltration can produce endotoxin-free preparation (Boratyński et al., 2004).
- **Phage specificity**: The specificity of phage for its host, although 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 et al., 2016), using host range mutant bacteriophage (Flaherty et al., 2001) or using a mixture of different phages (Chan et al., 2013).
- **Phage inactivation**: Phage inactivation by human serum can pose a limitation in phage therapy.
- **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 (Labrie et al., 2010; Tanji et al., 2004).
- Not all phages can be used for therapeutic propose. Only obligate lytic phages that lyse the bacterial cell directly instead of integrating its genome in bacterial DNA (temperate) are usable for phage therapy. Temperate phages play a major role in the exchange of genetic material between different bacterial strains. Besided obligate lytic property fully characterized phage with its thermal stability, efficacy and fully sequenced phage devoid of any virulence gene is needed for phage therapy (Loc-Carrillo & Abedon, 2011).

1.2. Current Studies

Phage therapy, the use of phages to cure bacterial infections, has received much attention in recent years due to the emergence and rapid spread of antibiotics resistance. Almost decade before the discovery of penicillin, the practice of phage

therapy was being developed, they were used at the treatment against pathogens such as *Shigella dysenteriae* in 1911 (Lin et al., 2017a).

In recent years, possibility of phage therapy has been increased due to the scientific advancement of biotechnological tools such as phage-derived lytic proteins, bioengineered phages etc. Current research on the use of phages and their lytic proteins specifically, against multidrug-resistant bacterial infections, suggests that phage therapy has the potential to be used as either an alternative or a supplement to antibiotic treatments. With advancements in Bioinformatics analysis, phages characterization is done by examining the phage genome sequencing so that the phage biology is fully understood. Nowadays, its application has been broadening in diversified field like in various agricultural settings to improve the safety of food and to control food borne diseases as well as to reduce the use of antibiotics in livestock similarly phage therapy has shown success in treating infections in livestock, plants, aqua-cultured fish and human (Kutter & Sulakvelidze, 2004). Now the researcher are interested in various biotechnological application of phages and they are used in the potential of bacteriophage particles in human infections (bacteriophage therapy), nanocages for gene delivery, food biopreservation and safety, bio-control of plant pathogens, phage display, bacterial biosensing devices, vaccines and vaccine carriers, biofilm and bacterial growth control, surface disinfection, corrosion control.

In context of Nepal, as antibiotic resistance is raising tremendously it has become a major public health concern however, unfortunately no any productive work regarding the alternative to antibiotics has not been done yet and very few study were published about Bacteriophages. In this study, we aimed to show a way for the possibility of implementation of phage therapy in Nepal. In this study we focused on the isolation of bacteriophages against carbapenem resistant bacteria and its potential application as therapeutic agents via understanding the phage biology of this particular phage through whole genome sequencing.

1.3. Objectives

1.3.1 General Objectives

- Isolation, characterization and whole genome analysis of potential phages and asses its use to control bofilm produced by carbapenem resistant bacteria.

1.3.2 Specific Objectives

- To screen, isolate and purify lytic bacteriophage against carbapenem resistant bacteria from holy rivers of Kathmandu valley.
- To determine phage-host range using different types of multidrug-resistant bacteria
- To study protein profile of phage protein by SDS-PAGE
- To identify the phages morphology by transmission electron microscopy (TEM)
- To determine growth characteristics and physiochemical properties of most potent bacteriophage.
- To analyze the phage genome through Whole genome sequencing using Illumina platform.
- To apply the bacteriophage as antimicrobial agent for the disruption of the boflim produced by carbapenem resistant bacteria.

1.4. Research Hypothesis

In the age of multidrug resistance, could phage therapy be the effective alternative to antibiotics we have been searching for?

Null Hypothesis

There are no bacteriophages in the water of Kathmandu valley which can lyse the carbapenem resistant human pathogenic bacteria and cannot be as used to control the biofilm produced by them.

Alternative Hypothesis

The lytic bacteriophages are found in the water of Kathmanadu valley which can lyse the carbapenem resistant human pathogenic bacteria as well as used to control the biofilm produced by them.

1.5. Rationale

The increasing numbers of multidrug resistant bacteria to existing antibiotics add the serious challenge to the human health. Diseases that were easily treatable few years back are now difficult to treat or even some are untreatable with the similar therapeutic agent. Bacteria are evolving more rapidly with unusual spread of resistance gene resulting to the outbreak of superbugs. The fact is that resistance genes of last resort antibiotics have been spreading worldwide. The emergence of carbapenem resistant pathogens *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* have created massive problems in treating patients in hospitals as WHO also priotrized these pathogens as critical group for Research and Development of new drug. This draws a special attention to an urgent need to co-ordinate a global effort in the search of alternative improved treatment. Hence, to mitigate this antibiotic crisis the strong candidate to the alternative of antibiotics is Bacteriophage, a living drug. Recent findings also support the potential of phages as alternatives to antibiotics. Phages are an 'intelligent' drug which multiplies at the site of the infection until there are no more bacteria. In past history, due to lack of knowledge on basic phage biology and their molecular organization has led to some clinical failures but now huge number of research with advancements of biotechnological tools, phage characterization is done through sequencing which helps to understand the phage biology showing great possibility of phage therapy to tackle against antibiotic crisis. There are various success stories of phage therapy to treat a patient near death from a multidrug-resistant bacterium.

In context of Nepal, as antibiotic resistance is raising tremendously it has become a major public health concern however, unfortunately no any productive work regarding the alternative to antibiotics has not been done yet and very few study were published about Bacteriophages. This study aims to explore, identify and characterize various lytic bacteriophages to determine their therapeutic potential as an antimicrobial agent. At first potential bacteriophages (lytic phages) are isolated, screened and characterized against MDR carbapenem resistant *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* from sewage water (polluted water) of Kathmandu valley where they are most likely to be found. Thus, we want to explore the way for the possibility of phage mediated biocontrol as well as implementation of phage therapy by characterizing locally available bacteriophage, when all available antibiotics are failed. Knowledge on the phage structure, understanding of their infection process and lysis mechanism through sequencing, its host range, physiochemical stability and possibility to use as antibacterial agent for disruption of biofilm would definitely allow the design of potential phages that can control the antibiotic resistant bacterial infections.

CHAPTER TWO

LITERATURE REVIEW

2.1. History of Bacteriophage Discovery

Bacteriophages are thought to be the most ancient entities on earth able to infect almost all Bacteria(Clokie et al., 2011).For the first time, in 1896 Ernest Hankin (a British bacteriologist) reported for the presence of antibacterial activity (against *Vibrio cholerae*) in the waters of the Ganges and Yamuna rivers in India (Wittebole et al., 2014).He used porcelain filters to strain the river water, removing bacteria and larger organisms while retaining a suspension that could kill *Vibrio cholera* for limiting the spread of cholera epidemics in the area. He summarized that this might in some way be responsible for the reduced number of cases of gastrointestinal infection, particularly cholera, in those villages close to the river (Abendon & Mazure, 2011). In 1898, Gamaleya (a Russian bacteriologist) observed a similar phenomenon while working with *Bacillus subtilis*. Two decades later, in 1915 another British bacteriologist, Frederick Twort, found a bacteria-killing agent "glassy transformation" while working with *Micrococcus* cultures, although he hesitated to hypothesize that it was a virus. Twort did not pursue this finding due to financial difficulties. After two years, bacteriophages were "officially" discovered by Felix d'Herelle, a French-Canadian microbiologist at the Institute Pasteur in Paris. 1910s, French-Canadian microbiologist Felix d'Herelle was testing fecal filtrates from soldiers infected with Shigella , he saw kill zones on the culture plates, he proposed it was "ultravirus" that caused lysis of bacterial cell in liquid media and created clear patches on the bacterial lawn which he primarily called it taches, then taches verges and later plaques and he coined the term "bacteriophage," from the Greek word 'phagein' which means "to eat," or 'to devour' to describe the disease-fighting agents. Many scientists accepted the independent discovery of bacteriophages made by Twort and d'Herelle and referred to it as the 'Twort-d'Herelle phenomenon', and later the 'Bacteriophage phenomenon'.

2.2. History of Phage Therapy

Almost a decade before the discovery of penicillin, the practice of phage therapy was being developed as a treatment for bacterial infection. From 1920 to 1940, phage therapy was extensively used to treat various infectious diseases (Dublanchet & Fruciano, 2008). Now, Phage therapy is widely being reconsidered as an alternative to antibiotics due to decrease in effectiveness of available antibiotics along with the increase in antibiotic resistance pathogens. Early attempt to use phages therapeutically resulted with the number of successes. Notable examples are the use of phages to treat cholera, dysentery and typhoid fever; although many other studies were performed at the same time.The potential of phages as a therapeutic agent was first identified and put into test by Felix d'Herelle. Soon after Félix d'Herelle discovered bacteriophages in association with diarroheal illnesses, he speculated that phages were responsible for the

usual recovery from such disease through their antibacterial action in vivo. Furthermore, he proposed to actively employ laboratory-produced phages as both prophylactic and therapeutic agents against bacterial infections. From its first field trials as a prophylactic against avian typhosis (*Salmonella gallinarum*) in rural France in 1919 to its widespread use in humans in the pre-antibiotic 1930s.He used phages to successfully treat a 12-year-old boy with severe dysentery. He also succeeded in treating three other patients with the same disease (Sulakvelidze & Alavidze, 2001). Similarly, review describes the first use of intravenous bacteriophage, which was used in the treatment of cholera by Asheshov in India (Abedon et al., 2011). D'Herelle produced the first commercial phage cocktails products—BactéColi-Phage, Bacté-Intesti-Phage, Bacté-Dysentérie-Phage, BactéPyo-Phage and Bacté-Rhino-Phage as therapy for infections and were marketed by the Societé Francaise de Teintures Inoffensives pour Cheveux (Safe hair dye company of France; now L'Oréal) (Abedon et al., 2011; Sulakvelidze & Alavidze, 2001). One 1931 trial of phage therapy as a treatment for cholera in the Punjab region of India involved a cohort of 118 control subjects and 73 experimental subjects that received phage treatment; d'Herelle observed a 90% reduction in mortality with 74 lethal outcomes in the control group and only 5 in the experimental group. Lang et al.48 reported the use of bacteriophage in seven patients with chronic orthopedic infections with resistant organisms. They were able to cure two cases of hip prostheses infected with gram-negative bacteria (after removal of the prostheses), one case of tibial osteomyelitis due to Proteus, Staphylococcus aureus, and one case of septic non-union of the femur due to pan-resistant Providencia. La Médicine in 1936 was devoted to phage therapy. Its individual reviews provided detail data on the treatment of such conditions as typhoid fever, acute colitis, peritonitis, prostate and urinary tract infections, furunculosis, sepsis and otolaryngology. Mikeladze described the treatment of 21 patients with typhoid fever with bacteriophage, using 10 mL of lysate for three to five consecutive days. There has been long and apparently successful history of phage therapy in Georgia and other parts of the Soviet Union however only few primary publication in English-language journals. The history of Phage therapy was extensively reviewd in many literatures, in which phages were used to treat several bacterial infections. In, Georgia phage therapy center was founded in the 1930s by George Eliava, in association with Félix d'Hérelle is now the Eliava Institute in Tbilisi. Specifically dedicated to phage therapy and this institute still exist today. Phages from the Eliava institute have been used to treat hundreds of thousands, if not millions of people, and at its height it employed 1200 people and produced 2 tons of phage per week .Thousands of patients have been treated with phages in Poland, particularly in association with the Hirszfeld Institute of Immunology and Experimental Therapy in Wrocńaw, which was founded in 1954. Since 2005, the institute itself has had a phage therapy center dedicated especially to treating antibiotic-resistance infections. In, US One of the first studies of subcutaneous phage administration was carried out at the Michigan Department of Health, where 208 patients were treated which were infected with chronic furunculosis; 78% of them were found with no occurring infections for at least 6 months after treatment however, only

3% showed no improvement (Larkum, 1929). In US during 1930s, several therapeutic phages were produced and Eli Lilly company was established for commercial phage production, which produced sterile-filtered phage-lysates (Staphylo-lysate, Colo-lysate, Ento-lysate, Neiso-lysate) and the same preparations in a water-soluble jelly base (e.g., Staphylo-jel) for treating abscesses, purulent wounds, vaginitis, mastoiditis and respiratory infections. Unfortunately, they found that all of them had problems with quality control, stability and establishment of efficacy. The majority of studies of phage therapy on humans came from only two institutions: the Hirszfeld Institute in Wroclaw (Poland) and theEliava Institute in T'blisi (Georgia).

Phage therapy has a long history, though for most of that history this approach has been neglected by the English-speaking western world. In the early days, there was a poor understanding of the biological nature of phages and lack of understanding led to a poor efficacy of phage products. Despite the initial successes briefly described above, phage therapy failed to become widely accepted. As a result, phage therapy was widely dismissed by most of western medicine after the introduction of pharmaceutical antibiotics in the 1940's. Initial exaggerated claims, the lack of controlled experiments and lack of documentation also played an important role in the abandonment of this method (Thiel, 2004).The exception to this is in the former Soviet Union and Eastern Europe where clinical phage therapy has been used extensively to treat antibiotic-resistant infections caused by a range of infectious bacteria such as *Staphylococcus, Pseudomonas*, *Klebsiella*, and *E. coli*. Nowadays, due to the looming antibiotic crisis there is now a renewed interest in phages so a number of companies are producing therapeutic phage products using novel and advanced technology.

2.3. Recent advancement inPhage Therapy

After several years of abandonment, the use of bacteriophages (phages) for killing bacteria has now gained a recent attention and appraisal. This has led to a vast innovation in phage research, varied fields, with impressive outcomes in revitalization of phage therapy with advance technology.Phage research costs have decreased significantly due to advancements in technology. Although being lifesaving therapeutics, one of the biggest challenges relates to regulations and policy surrounding clinical use and implementation beyond compassionate cases.Some major phage studies during 100 years of phage research are depicited in below figure (Figure 2.2).

With safety trails completed in 1959, Staphylococcal phage lysate (SPL) was licensed for human therapeutic usage and was successfully administered using different routes (intranasal by aerosol, topically, orally, subcutaneously and also intravenously) (Golkar et al., 2014). From late 1950s to early 1960s, overall phage therapy efficacy was demonstrated in clinical trial. Among 607 patients, all of whom had failed to respond to conventional treatment by antibiotics, were treated by phage therapy. The results were reportedly good: 80% of the patients recovered, 18% improved, and only 2% exhibited no changes. Furthermore, no side effects were also reported(Slopek et al.,1985).There is

productive result on clinical trials in which Rhoads and colleagues reported on safety in a small phase I trial in patients with venous leg ulcers and reported no adverse events with the administration of phages(Rhoads et al., 2014).Wright et al. demonstrated efficacy and safety of anti-Pseudomonal phages against late stage recurrent otitis which was dominated by *MDR-P.aeruginosa*.number of clinical trials have been registered This was summarized in given figure (Figure 2.1).(www.globalclinicaltrialdata.com).

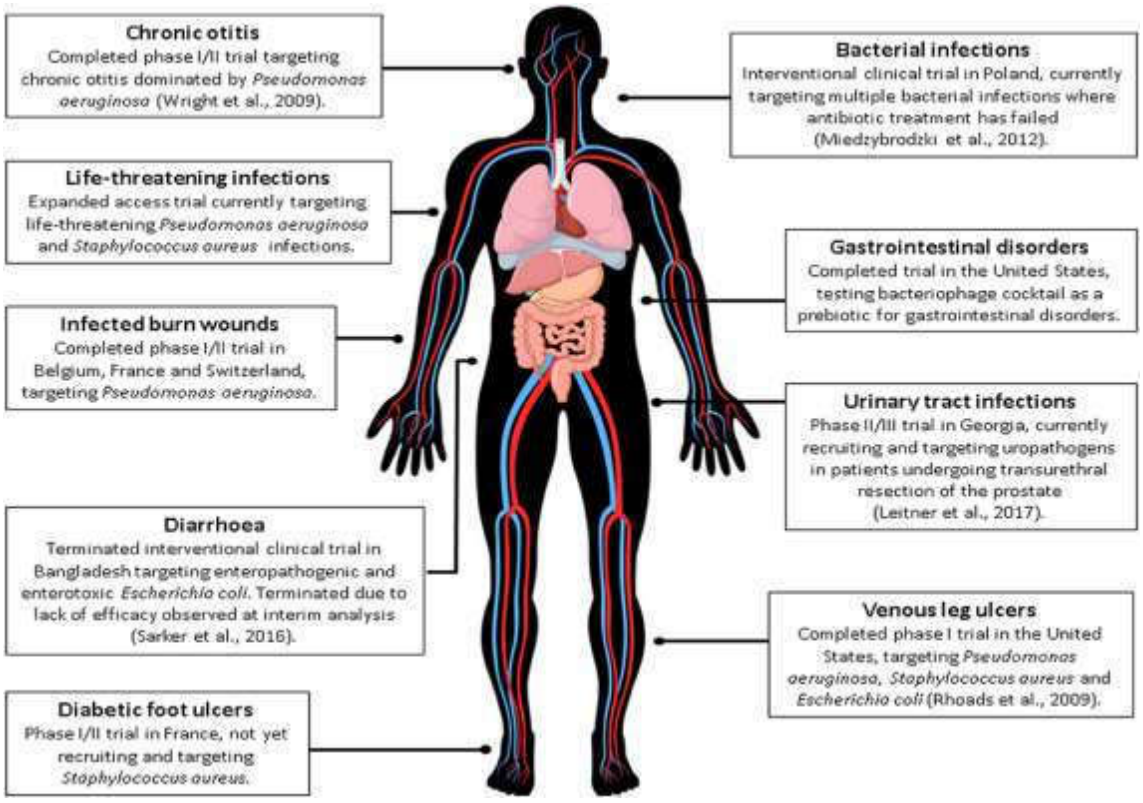


Figure 2. 1: Summary of human phage therapy trials and the range of target sites/ infections. Source: www.clinical trials.com Figure adapted from (Furfaro, Payne, & Chang, 2018)

When inoculated into mice simultaneously with *S. aureus* A170 (10^8CFU/mouse), phage (10^9 PFU) rescued 97% of the mice; when applied to nonlethal (5×10^6 CFU/mouse) 10-day infections, the phage also fully cleared the bacteria. The phage MSa, delivered inside macrophages by *S. aureus*, kills the intracellular staphylococci in vivo and in vitro. The phage can also prevent abscess formation and reduce the bacterial load and weight of abscesses. These results suggest a potential use of the phage for the control of both local and systemic human *S. aureus* infections (Salvatore et al., 2007). Phage interactions with animals in general and human beings in particular have been comprehensively reviewed, and there have been no reports of significant adverse reactions despite their long history of administration to humans.

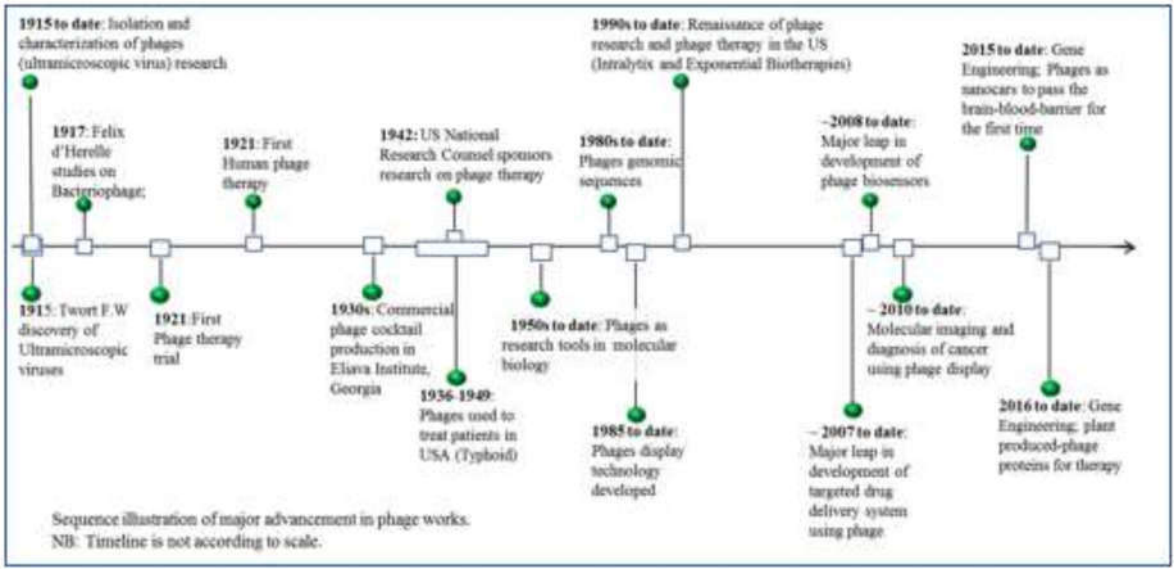


Figure 2. 2: Timeline of some major phage studies.Some major phage accomplishments during the first 100 years of phage research. I: 1915-1939 Discovery of phage and some crude attempts are phage therapy, II: 1940-1960 Phage physiology and molecular genetics; antibiotics replace phage therapy, III: 1961-2000 Phage applications and earnest efforts at phage therapy and IV: 2001-cur Intense phage therapy applications and vast unexpected applications.

Studies indicated that a single dose of the *P. aeruginosa* phage cocktail significantly decrease the mortality of thermally injured, *P. aeruginosa*-infected mice (from 6% survival without treatment to 22 to 87% survival with treatment) and that the route of administration was particularly important to the efficacy of the treatment, with the i.p. route providing the most significant (87%) protection (McVay et al., 2007).First randomized controlled trial (RCT) provided evidence on phage therapy in patients with MDR PA-related chronic otitis (Wright et al., 2009) and CF MDR PA is a typical candidate for phage therapy,.

Phages induced non neutralizing antibodies and were active 2 weeks after experimental infection of mice; phage-resistant bacteria were avirulent and short lived in vivo. More importantly, phage-resistant bacteria were excellent vaccines, protecting against lethal doses of heterologous S. enterica serovars (Capparelli et al., 2009).

Only a very few studies used human cell lines to determine the safety and immune responses of phages. The immunogenicity of 4 phages of Caudovirales order, were checked in PBMC and immortalized cell lines (HT-2 and Caco-2 intestinal epithelial cells). When exposed to high phage concentrations (10^9 PFU/well), cytokine driven inflammatory responses were induced from all cell types. Although phages appeared to inhibit the growth of intestinal epithelial cell lines, they also appear to be non-cytotoxic (Mirzaei et al., 2016) .The cell line study on the dynamics of phage-bacteria interactions revealed novel facts of phage biology, showing that phage can reduce *C. difcile* numbers more efectively in the presence of HT-29 cells than in vitro (Shan et al., 2018). The safety and efficacy of therapeutic phage treatment of *C. difcile* infections have also previously studied using hamster, artifcial gut, and more recently the insect *Galleria mellonella*

models, suggested that phage treatment appeared to be safe and efective; hamster data showed that they responded positively to phage treatment, and artifcial gut model data showed that *C. difcile* numbers and toxin levels were reduced with minimal disruption to commensal bacteria (Meader et al., 2010).

Miernikiewicz et al. performed an extensive study of the immunological efects of phage T4 and its head surface proteins.They found that phage T4 and its surface proteins gp23*, gp24*, Hoc and Soc did not affect production of the inflammatory cytokine and ROS production (Miernikiewicz et al., 2013). Recently Majewska et al followed the antibody production (i.e. IgM, IgG and secretory IgA) afer oral application of phage T4 to mice. However, the orally applied phage T4 induced anti-phage antibodies only after a combination of long exposure times (i.e. IgG day 36 and IgA day 79) and high doses (Matysiak et al., 2015).

Another case report showed therapeutic application of phage OMKO1 to treat a chronic *P. aeruginosa* infection of an aortic Dacron graft with associated aorto-cutaneous fistula. In which 78 years old man with recurrent infection as treated with OMK01 phage, it appears to have been effective at biofilm reduction on prosthetic graft material, contributing to eradicate *P. aeruginosa* infection (Chan et al., 2018).

The gene expression profile of peripheral blood monocytes from six donors for twelve immunity-related genes (i.e. CD14, CXCL1, CXCL5, IL1A, IL1B, IL1RN, IL6, IL10, LYZ, SOCS3, TGFBI and TNFA) was studied which were induced by *Staphylococcus aureus* phage ISP and four *Pseudomonas aeruginosa* phages (i.e. PNM, LUZ19, 14-1 and GE-vB_Pae-Kakheti25). The phages were able to induce clear and reproducible immune responses. Immune response was shown to be endotoxin-independent and predominantly anti-infammatory (Belleghem et al., 2017).

Another study proved that phages can aid in the killing of phagocytosed *S. aureus*. The authors showed that phage particles absorbed to the bacterial surface have an impact on the killing of engulfed *S. aureus* inside phagocytic cells (e.g. macrophages) by 38.7%. Furthermore, it has been indicated that phages are able to interact with human mucosal surfaces and form a non-host derived immune barrier (Matysiak et al., 2017).

The understanding of phage therapy kinetics is essential when live viruses are used therapeutically to control bacterial infections. Pharmacodynamics describes a drug's impact on the body while pharmacokinetics describes the body's impact on drugs. Another way of considering these terms is that pharmacodynamics is a description of both the positive and negative consequences of drugs reaching certain densities in the body while pharmacokinetics is concerned with the ability of drugs to reach and sustain those densities. Pharmacokinetics accounts for a drug’s ability to reach sufficient concentrations in the locality of targeted tissues, as it is very important to bring about the pharmacodynamic effects of the drug. This pharmacokinetics summarizes into absorption, distribution, metabolism and excretion (Qadir et al., 2018). There are a very

few publications that emphasize on the pharmacokinetics of phage therapy. The pharmacokinetic study of the *Staphylococcus aureus* specific bacteriophage in healthy rabbit first time in Iraq showed that phage was rapidly absorbed through gut wall and highly distributed. Phage reached a high titer in blood after the two hour following oral administration. In contrast, phage reached the highest titer after IV injection within the few minutes (Sarhan et al., 2017).

Similar study emphasizes the use of bacteriophage for the treatment of multidrug resistant *P. aeruginosa*. *P. aeruginosa* was used to induce septicemia in streptozotocin (STZ) induced diabetic and nondiabetic mice by intraperitoneal (i.p.) injection of 3×10^8 CFU, resulting in a fatal bacteremia within 48 hrs (Shivshetty et al., 2014).Survival rates of nearly 100% in groups given phage therapy concurrent with XDRAB at different multiplicities of infection. In mice that received phage therapy after a 1-hour delay, the survival rate decreased to about 50%. The bacterial load in the blood decreased from 10^8 to $10^2$and 10^3 colony-forming units (CFU)/mL in the concurrent treatment group. In the phage therapy group, the levels of the cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), were low at 3 hours after infection (Chen et al., 2018).

Fischetti and coworkers showed the new aspect of phage therapy using a bacteriophage lytic enzyme to prevent bacterial infection and they coined the term `enzybiotics' for these proteins (lysine proteins), describing both their enzymatic and antibacterial properties (Fischetti et al., 2002).It was the first study done to investigate the prophylactic use of a phage encoded lysin in an in vivo model system for prevention and elimination of upper respiratory colonization of mice by group A streptococci. At the end of a bacteriophage lytic cycle in a sensitive bacterial host, all double-stranded DNA bacteriophages produce a lytic system that consists of a holin and at least one peptidoglycan hydrolase, or ‘‘lysin’’, capable of degrading the bacterial cell wall. Typically, the holin is expressed in the late stages of phage infection forming a pore in the cell membrane allowing the lysin(s) to gain access to the cell wall peptidoglycan resulting in release of progeny phage (Young, 1992). Lysin, added to sensitive organisms in the absence of bacteriophage, lyses the cell wall producing a phenomenon known as ‘‘lysis from without”. Mathias and his team reviewed all the detail of endolysin including lytic activity and potential as antimicrobials highlights bacteriophage endolysin as novel antimicrobials (Schmelcher et al., 2012). Similarly, Tiwari and team mentioned Enzybiotics as a new weapon in the Army of antimicrobials (Tiwari et al., 2014). Now, the researcher approaches the construction of recombinant endolysin derivatives and the development of novel delivery strategies for various applications, such as the production of endolysins in lactic acid bacteria and their conjugation to nanoparticles (Kashani et al., 2018).To enhance the therapeutic activity and to avoid the development of resistance, multiple endolysins may be used in combination with antibiotics to treat bacterial infections. The presence of endolysin has advantages of usage related to bacterial resistance. This is well explained in the theory of phage-bacteria co-evolution, which postulates that to ensure phage multiplication and survival in the environment, phage

and its endolysins were naturally selected, which difficult bacterial resistance (Torres-Barceló, 2018).Thus due to presence of endolysin it makes the probability of the bacteria acquiring mechanisms of resistance is low. Two putative phage lysin genes (ply) from the clostridial phages phiCP39O and phiCP26F were cloned and expressed in *Escherichia coli* and were capable of lysing both parental phage host strains of *C. perfringens* as well as other strains of the bacterium in spot and turbidity reduction assays (Wang et al., 2011). One study showed an engineered endolysin enzyme Ply187AN-KSH3b with increased lytic activity, a fusion of the endopeptidase domain of the staphylococcal Ply187 lysin (Ply187AN) with the SH3b CBD of another staphylococcal phage endolysin, LysK and construct displayed >10-fold-higher staphylo lytic than that of Ply187AN and was also more active than LysK in multiple activity assays (Mao et al., 2013). In another study, phage ST79 of *Burkholderia pseudomallei* endolysin gene identified as peptidase M15A was cloned, expressed, purified and evaluate its potential to lyse pathogenic bacteria which showed a broad spectrum against Gram-negative bacteria PG or, in combination with an antibiotic the same way as combined drug methodology (Khakhum et al., 2016).

Apart from isolating naturally occurring phages, there is possibility of modified phages which is devoid of undesired properties with improved therapeutic potential. One of the finding is that phage of dual activity antitumor and bactericidal without an observable reduction in antimicrobial activity (Dabrowska et al., 2014) .Another modification of phage , ie: PEGylated phage caused a decrease in the level of cytokines such as IFN-gamma and IL-6 in both the non-immunized and phage immunized mice (Kim et al., 2008).The modified phages particles were characterized by a longer half-life in the organisms, which may be of practical importance in enhancing the effectiveness of phage therapy.

Unlike classic phage therapy, which uses one or more types of phages to infect and lyse specific bacterial strains, new approach is using these specialized viruses to supply CRISPR/Cas to rid bacteria of antibiotic-resistance plasmids in the environment before the microbes are able to infect a host. The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins have evolved in prokaryotes to protect against phage attack and undesired plasmid replication by targeting foreign DNA or RNA the viruses evolve diverse anti-CRISPR proteins to fight back. In recent days, scientists are interested to use phages for delivering a programmable DNA nuclease, clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated (Cas), to reverse antibiotic resistance and eliminate the transfer of resistance between strains. The CRISPR system is now used to attack resistance genes using phage as a vehicle. In Tel Aviv University, Israel Yosef and his team used the CRISPR-Cas system to destroy specific DNAs that confer antibiotic resistance and to concurrently confer a selective advantage to antibiotic sensitive bacteria by virtue of resistance to lytic phages (Yosef et al., 2015).The anti-CRISPR operon was found in the same genomic position in a variety of related Pseudomonas phages. A total of five distinct anti-CRISPR protein families that inhibit the type I-F

system and four protein families that inhibit the type I-E system were identified (Maxwell, 2016). The discovery of anti-CRISPRs provides a lamp, highlighting the way to regulate the genome editing activities of CRISPR-Cas9. By utilizing the anti-CRISPRs from phages, we hope to develop a braking system for gene editing, to make sure that therapies based on gene editing can be fully controlled (Zhu et al., 2018).

The recent success story of phage therapy is a treatment of Tom Patterson, PhD, a 69-year-old professor in the Department of Psychiatry at UC San Diego School of Medicine, who was comatose and near death due to superbug infections of MDR *Acinetobacter baumanni* where every antibiotic had failed to treat.Tom's wife, Steffanie Strathdee, PhD, chief of the Division of Global Public Health in the Department of Medicine and other Physicians and scientists at UC San Diego Health, with many collaborators, used an experimental bacteriophage therapy giving intravenous saved by a cocktail of phages purified from sewage for the first time in US to treat systemic infections. This miraculous recovery of a patient might be the catalyst for developing the remedies to the growing global threat of antimicrobial resistance (Lafee & Bushman, 2017).

Pivotal studies on genomic technologies theorized that genomic engineered phages, genetically manipulated to modify their host range and induce specific depolymerase biofilm-degrading enzymes, interact better than their naturally-occurring species to lyse antibiotic-resistant bacteria hosts (Edgar et al., 2012; Lu & Collins, 2007; Pei & Lamas-Samanamud, 2014; Pires et al., 2016; Salmond & Fineran, 2015).

Despite the fact that phage therapy is not yet approved by FDA, phages have already been used to save lives in experimental treatments. Inspite of increasing attack of superbugs, phage therapy with the advancement of modern genomic technologies, establishment of phage therapy center and various clinical trials has been creating a better aspect towards modern therapeutics as potential alternative to antibiotics. If antibiotic crisis rose even largely, we may hear more success stories of phage therapy like above mentioned in near future. As suggested, personalized phage therapy is probably more effective than preporter preparations, as it is based on tailored preparations which can be adjusted to changing bacterial pathogens (Mattila et al., 2015). It can be said that with different types of modifications in phages, open new possibilities in the phage therapy.

2.4. Phage Cocktail Therapy Polyphage Therapy

Strain and species specificity of bacteriophage have several advantage along with some limitations. By targeting a single pathogen, phage therapy could be less effective against certain infections which are colonized by more than one type of bacteria. In recent time investigation is going on in the use of phage cocktails to broaden the spectrum of activity of therapeutic phage formulations.The multiple phages with a diversity of host ranges are often combined into mixtures called 'phage cocktails' and the application of phage cocktails, in phage therapy that is, therapy involving the simultaneous use of more than

one phage type is called polyphage therapy (Chan & Abedon, 2012). The narrowness of phage host ranges with in a few strain,now can be broaden its lytic spectrum with the use of phage cocktail.Several studied shown that two phages or mixture of phages may collectively kill the bacterial population more rapidly or more completely than either phage alone (Schmerer et al., 2014). The phage cocktail has shown advantageous effect to prevent the occurrence of cross-resistance, and, based on this phenomenon, a bacterium which is resistant to one phage may remain sensitive to another, and cocktails that contain phages using different receptors for binding to bacteria may be a better one.Besides the phage cocktail approach, modifying isolation procedures and growth conditions can give the phages with much broader host ranges (Ross et al., 2016). For example, phage Mu is polyvalent, which is able to infect species of *Escherichia, Citrobacter, Shigella, Enterobacter*, and *Erwinia*. Moreover, over 700 strains of *Staphylococcus aureus* can be lysed by a single phage (Roach & Debarbieux, 2017). Formulations of phage mixtures (cocktails) may also provide a solution to achieve mid-spectrum phage therapies(Malik et al., 2017).The phage cocktail is used not only in treating infections but it is also used in preventing infections as prophylactic treatment.In one study, three virulent phage cocktail (ICP cocktail) were used in two animal model of cholera pathogenesis (infant mouse and rabbit models) which successfully kill *V. cholerae* in vitro, prevent colonization of the infant mouse and preclude the onset of cholera-like diarrhoea in the infant rabbit (Yen et al., 2017). Athough there is difficulty in regulatory procedures and characterization of each phage for safety and effectively used of phage cocktail, is gaining attention to kill all possible target bacteria by developing phage bank.

2.5. Therapy using Klebsiella phage

Due to the increasing prominence of multi-drug resistant *Klebsiella pneumoniae* bacteria and the spread of resistant genes, bacteriophages are being explored as an alternative treatment option.*K.pneumoniae*specific virulent phages.Bacteriophages special for *K.pneumoniae* are also highly abundant in natural environments just as well as its host. Numerous studies highlighted the invitro and invivo potential of therapeutics klebsiella phage.

Chhibber et al.delivered phage via an intraperitoneal route to treat experimental lobar pneumonia that was induced by *K. pneumoniae* in mice and had a great efficacy (Chhibber et al., 2008). Cao et al administrated intranasal phage to treat *Klebsiella pneumonia*e lung infection in mice resulting in protection against lethal infection and lower inflammatory cytokines levels in the lung (Cao et al., 2015). Similarly, in a burn wound mouse model of *k pneumoniae* infection, topical phage application resulted in a significant reduction in mortality In this case treatment was shown to be effective even when phage administration was delayed for up to 18 h after bacterial inoculation (Kumari et al., 2011).Likewise liposopme loaded phage cocktail enhanced bacterial clearance and rate of healing (Chadha et al., 2017). Similarly, in another study using an

intragastric infection model, a single dose of ϕNK5 (klebsiellaphage) was able to inhibit *K. pneumoniae*-induced liver injury, bacteremia, and cytokine production (Hung et al., 2011). In latest research, φBO1E phage was able to protect larvae from death following infection with KPC-KP strains of clade II of CG258 in *Galleria mellonella* infection model (D'Andrea et al., 2017b). Another emerging aspect of klebsiella phage includes for the prevention of the biofilm formation.Depolymerase producing *K pneumoniae* phage in combination with iron antagonizing agents, showed ability to eradicate early biofilm of *k pneumoniae* a promising preventive strategy (Chhibber et al., 2013). Likewise, ZCKP1 phage against MDR *K pneumoniae* of foot wound of a diabetic patient showed the ability to reduce bacterial counts and biofilm biomass (Taha et al., 2018). Many studied have addressed the efficacy of phage therapy in invivo and invitro model, still there is much further work required for applying into human. Although bacteriophage therapy has been successfully used to treat various diseases in both animals and humans, there is no report associated with phage therapy of *K. pneumoniae*infections in human.This positive result with efficacy of potential agent of phage therapy, of years of invitro and invivo studies in different model has opened the door to human clinical trial to combat this carbapenem resistant critical pathogen *Klebsiella pneumonia*e infections.

2.6. Applications of Bacteriophages

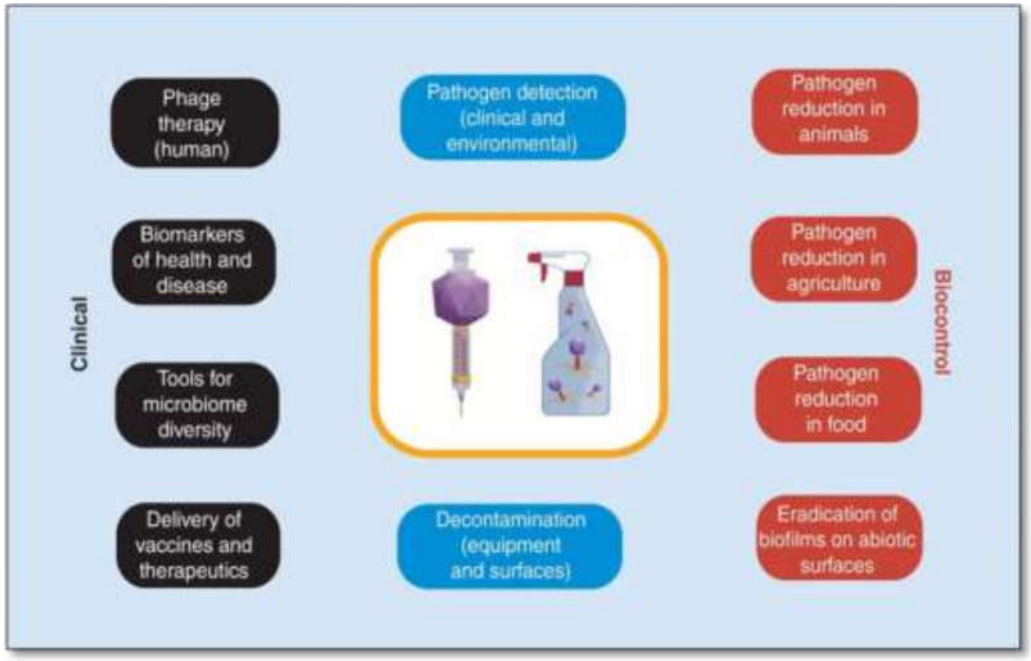


Figure 2. 3: Application of bacteriophage as biocontrol, biodetection , biomarkers, pathogen detection and decontamination in clinical and food and agriculture sector.

Bacteriophages found a variety of applications in biology and medicine due to their intrinsic properties such as high stability under a variety of harsh environmental conditions (including exposure to nucleolytic and proteolytic enzymes desiccation, high temperatures and wide range of pH, the feasibility of large-scale production and manipulation. Other important advantages of bacteriophages that make them attractive are as follows; being a non-pathogenic and non-replicating vehicle in eukaryotic cells do

not cause recombination events because of the nonexistence of eukaryote-related sequences (or homologous sequences) in their genome , and do not carry antibiotic resistance genes (Clark and March, 2004).

2.7. Bacteriopahge based diagnostics

2.7.1 Phage Display: In phage display method, a gene encoding a protein of interest is inserted into a phage coat protein gene which ultimately expressed on the surface of bacteriophage or display on outside containing the gene for the protein inside which provides a physical linkage between peptides (i.e., the phenotype), which are displayed on the surface of a bacteriophage particle and the encoding DNA (genotype) (Clark and March, 2006). Phage display was first described by George P Smith in 1985, introducing a method to identify a gene as a medium of presenting polypeptides on the surface of lysogenic filamentous bacteriophages against which he had raised antibodies. Using this technique a library can be generated up to 10^{11} different variants and screened to isolate proteins or peptide with particular application. It can be used to isolate protein with high affinity that can act as a diagnostic tool in detection of pathogen or agents posing a biological threat (Petrenko and Vodyanoy, 2003). Phage-display library can also help in identifying the protein with enhanced enzymatic activity by screening a library of proteins with a randomly altered active site (Fernandez-Gacio et al., 2003). Phage display is also used on a wide range of applications in different research area, including epitope mapping, identification of new receptors & ligands, in vitro protein evolution, drug discovery, invitro diagnostic, and antibody production and so on. Hence it has now become one of the most powerful and widely used laboratory techniques for the study of protein-protein, protein-peptide and protein-DNA interactions. Another phage-display technique has been used to treat cocaine addiction in a rodent model. Here, antibody fragments are expressed on the phage surface , when phages are administrated nasally they travel to the central nervous system (CNS) and bound with the cocaine molecule effectively preventing the action of cocaine on the brain (Dickerson et al., 2005). Similarly, several therapeutic antibodies that are currently either approved or in clinical trials have been developed by phage display technology. This phage display is now being widely used and given much attention as the Nobel Prize in Chemistry for 2018 was awarded to Sir Gregory P. Winter from MRC Laboratory of Molecular Biology, Cambridge, UK for the phage display of peptides and antibodies which are supposed to comeback against autoimmune disease and can also be used to cure metastatic cancer.

2.7.2 Phages as vaccine delivery vehicle:

Bacteriophages have been used as transport for vaccine delivery in two ways: one is directly vaccinating with phages expressing vaccine antigens on their surface and another is by incorporating a DNA vaccine expression cassette into phage genome and using the phage particle to deliver that DNA cassette (Clark and March, 2004). In phage-display vaccination method, the target antigen can either be generated by

transcriptional fusion to coat protein or by artificially conjugating antigen protein to the phage surface which enables broad range antigen display ability (Molenaar et al., 2002). It has been demonstrated that unmodified phages deliver DNA vaccine more efficiently compared to standard DNA vaccine procedure as phage coat protein protects the DNA vaccine more efficiently and shows greater antibody response (Clark and March, 2006).

2.7.3 Phage Typing:

The specificity of phages for bacterial cells enables them to be used as a diagnostics tool for detection of bacterial species and typing of the bacterial cell. For these purposes, several methods can be employed such as delivery of reporter gene (e.g. lux or green fluorescent protein) using phages that would be expressed after successful infection of target bacteria (Funatsu et al., 2002; Kodikara et al., 1991). Another method involves detecting specific absorption of phage that had fluorescent dye covalently attached to its surface (Goodridge et al., 1999; Hennes et al., 1995). Detection of the cellular components that are released after bacterial lysis caused by phages specific to those bacteria, such as adenylate kinase provides an alternative way for identifying pathogenic bacteria (Corbitt et al., 2000). Phage amplification assay is another technique that has been extensively used to detect virulent bacteria such as Salmonella, Pseudomonas, Mycobacterium tuberculosis, E.coli, Campylobacter and Listeria species (Barry et al., 1996).

2.8. Whole genome sequencing – Next generation sequencing of Bacteriophage

Whole genome sequencing (WGS) refers to the construction of the complete nucleotide sequence of a genome. After the discovery of double helix DNA by Watson and Crick in 1990 , understanding of complex structure of organism sequencing technology was established .The first ever complete genome sequenced was of the bacteriophage ØX74 in 1977 which contains only 5,368 base pairs genome (Sanger et al., 1977) followed by sequencing of the dsDNA phage lambda T7 in the early 1980s. After the breakthrough sequence of the respective phage, in 1990 Human genome project was launched to sequence all 3 billion letters of a human genome and was completed in 2006 prior to its time.

Recently, next-generation sequencing (NGS) technology has allowed a massive increase in capacity to sequence genome at relatively low cost and in a short time frame with high throughput in form of short reads. Next Generation Sequencing (NGS) technologies refer to the shotgun sequencing methods, such as 454, Illumina, SOLiD and Ion Torrent, which have replaced the first-generation, single-molecule sequencing technologies such as Sanger. Early genome-sequencing studies mainly focused on small genomes. Now the novel sequencing technologies enable description of an increasing number of phage genomes, a critical piece of information to understand their life cycle, phage-host interactions, and evolution. Phages have smaller genome than their bacterial hosts, yet

there are currently fewer fully sequenced phages than bacterial genomes (Garneau et al., 2017). In the last ten years, the number of bacterial and phage genomes has increased dramatically, coinciding with the decreasing cost-per-base of sequencing. Due to unique feature of bacteriophage genome, they are occasionally hard to be sequenced (Klumpp et al., 2012) which are listed below.

- Phages are not self-replicating and they do rely on their host macromolecular machinery for their replication and growth hence isolation of phage genomic material completely devoid of host genetic material involves extensive purification steps.
- Sometimes phage preparations are associated with host debris and cellular membrane fraction that contaminate the genomic material and interfere with subsequent steps of DNA sequencing.
- Lytic phages have notoriously highly methylated genomes because bacteria possess restriction-modification systems to safeguard the integrity of their genomes from invading DNAs.
- Some phage genomes are notoriously rich in extreme GC content different from that of their host that may possess problem for PCR and sequencing.
- Phage genomes contain extremely long direct or inverted repeats and terminal redundancies that are problematic for assembly of the whole-genome sequence from the reads.
- Regions of uneven sequence depth along the length of the genome, when amplifying or generating libraries via random-priming method may cause problem for many of the common assembly algorithm as the program assume that the uneven coverage is due to repeats or contamination, resulting in artificially poor assemblies.
- Almost 80% of the genome sequences in the genome online database (GOLD) are unfinished draft sequences. For bacteria and other organisms, complete genome finishing may not be a requisite for many applications, but for small genomes such as bacteriophages, finishing the genome sequencing is essential to obtain a complete understanding of their biology ,Whereas in bacterial and human genomics, mapping of reads to a finished reference genome can be a powerful analytical tool not only for genome assembly, however also for the discovery of genetic variations such as insertions/deletions (indels) and single nucleotide polymorphisms (SNPs), in phage genomics this is commonly not feasible due to the absence of a reference genome for any given phage.

One of the most important features of bacteriophage genomes is their apparent mosaic structure; each genome can be considered as a unique combination of modules that are exchangeable among the population. Due to the mosaic nature, even closely related phages are highly divergent, enabling reference mapping a useless effort. The size of the modules, their rates of exchange, and the phage genomes carrying them all vary with phages of different virion morphology, size, and host-range all participants in an orgy of

recombination (Hatfull, 2009; Roger et al., 1999). This mosaicism is not only unique to the phage population however, is also prevalent in bacteria, where genes are acquired by horizontal genetic exchange (predominantly transduction, transformation and conjugation).

Despite all the challenges mentioned above, next generation sequencing platforms offer the best opportunity for whole-genome sequencing of phages which helps to understand phage-bacteria interactions during phage infection cycle.The sequencing is also used to taxonomically classify phages in orders,families,subfamilies ,genus and species.Further, phage genome analysis allow easy identification of phages lacking integration machinery, virulence gene ,toxic gene which will facilitate safe and effective use of phages as biocontrol agents.

To date, approximately 8,300 complete bacteriophage genomes have been sequenced (Ha & Denver, 2018). Whole-genome sequencing has become mandatory for regulatory approval of any healthcare or food-industry application of phage or phage products however researchers have to face diificulty in sequencing platforms, assembly options and the massive amounts of data they produce. Researchers deal with large amounts of raw data with limited bioinformatics skill. Meanwhile, the results of NGS sequencing runs can easily add up to several hundred gigabases of raw sequence and large assemblies files which are also hard to deal with. A reliable, redundant data storage option is mandatory to ensure data safety and consistency. Also, powerful computer workstations and computing grids are needed for data processing and result visualization.

2.8.1 Illumina sequencing

Illumina is now becomes the dominant vendor of high-throughput DNA sequencing machine.Illumina used “sequencing-by-synthesis” technology.The Illumina NGS workflows include four basic steps library preparation, cluster generation, sequencing and data analysis.The Illumina technology delivers various amounts of data, which is generally bulky for the typical laboratory workstation computer to assemble; necessitating the use of high throughput computing grids or cloud services. It generates number of short sequencing reads (i.e., up to 6 billion paired-end reads, equaling 600 Gb of sequence information in one ten day run using 2 flow cells). Illumina sequencing generates a large amount of data but with very short read lengths (35-100bp) and it may be sometime problematic when phage genomes contain repetitive sequence stretches (Klumpp et al., 2012).

2.9. Bacterial biofilms

Biofilms were first observed after the microscope were discovered in which Dutch researcher Antoine van Leuvenhoek (1632-1723) observed Bofilm when examining the “scuff” from his teeth using a self constructed microscope capable of enlargements of up to five hundred times(Costerton et al., 1999). In nature, many species of bacteria, fungi,

protozoa and algae form biofilms. Biofilm is a multicellular community composed of prokaryotic and/or eukaryotic cells embedded in a matrix composed, at least partially, of material synthesized by the sessile cells in the community. Bacterial biofilms are formed by communities that are embedded in a self-produced matrix of extracellular polymeric substances (EPS).The EPS enable bacteria to defend against their adverse environment, thus assuming increased microbial resistance to antimicrobials(Flemming et al., 2016). Importantly, bacteria in biofilms exhibit a set of 'emergent properties' that differ substantially from free-living bacterial cells. Common biofilm producing bacteria are including gram-positive (e.g. *Bacillus* spp, *Listeria monocytogenes*, *Staphylococcus* spp, and lactic acid bacteria, including *Lactobacillus plantarum* and *Lactococcus lactis*) and gram-negative species (e.g. *Escherichiacoli*, or *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*.Biofilms can be of monolayer or multilayer depending on the interaction between the surface and constituent cells (Karatan & Watnick, 2009). The structure of the biofilm is dependent on many factors: the component microrganisms, their physiological state, the physical environment including turbulent or linear low conditions and the surface to which the cells are attached.

Stages of biofilm formation

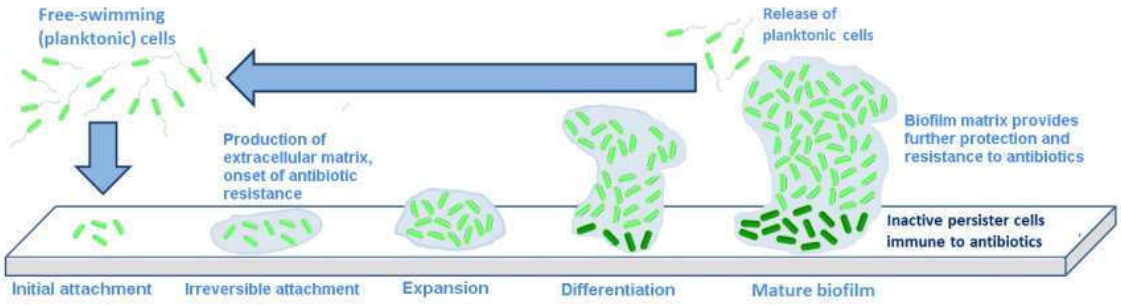


Figure 2. 4: Biofilm formation steps, from attachment of single cells, maturation of the biofilm and release through cluster detachment and seeding dispersal.Stages of biofilm development:

(1) Initial attachment (2) irreversible attachment (3) replication (4) maturation and (5) dispersion (Figure adapted from (Abedon, 2011).

The stages of bacterial biofilm formation are shown in above figure 2.3, from attachment to biofilm formation in motile organism of *Pseudomonas aeruginosa*. However non-motile organism S. aureus also formed biofilm. Bacterial biofilms are regularly observed at the site of persistent infections (Myllymaa et al., 2013) adhered to tissue surfaces suggesting that biofilms are associated with virulence and chronic infection. It is found that formation of bacterial biofilms is crucial in the pathogenesis of many clinically important infections and is difficult to eradicate because they exhibit resistance to antimicrobial treatments. Biofilms tolerate 100 to 1000-fold higher levels of antimicrobial agents than planktonic cultures and, although several strategies are adopted to prevent biofilm formation and their removal, biofilms do persist in a wide range of industrial surfaces (Flemming, 2008). The mechanism of biofilm formation is

not completely understood but biofilm formation is important for the pathogenesis of K. pneumoniae, especially for antibiotic resistant plasmids caring strains (Hennequin et al., 2012). Type 3 fimbria was characterized as an accessory for enabling biofilm formation on biotic and abiotic surfaces (Murphy et al., 2013). The impact of biofilms on health and the economic consequences on Industry has promoted the development of different approaches to control or remove biofilm formation.

2.10. Biofilm-phage interaction and Use of Bacteriophages to control biofilm

It has often been assumed that biofilms confer resistance to bacteriophages, due to the impermeability of the biofilm matrix. However, although they are far larger than chemical antibiotics, bacteriophages are still far smaller than their bacterial hosts, and many bacteriophages can not infect bacteria within biofilms. The first and crucial step in phage infection is the adsorption of phages to the receptors of the target bacteria. The EPS matrix, in which bacteria are embedded in, can constitute a problem for phages, as it needs to be penetrated so that phages can reach and adsorb to the specific receptors located on the target host's surface. However, it has been reported that phages are well capable of penetrating through the EPS matrix by diffusion or due to the presence of phage associated enzymes. These enzymes have the role of destroying the matrix so that the phages can get in contact with lipopolysaccharides, outer membrane proteins or other receptors necessary for the start of the host infection (Abedon, 2011). Bacteriophages act differently on bacteria contained within biofilms than any chemical antibiotics. There are at least four mechanisms which are given in below Figure 2.4.

Studies have been carried out of phage interactions with biofilms involving such bacteria as *Pseudomonas aeruginosa*, *E. coli*, *Listeria monocytogenes*, *Enterobacter agglomerans*, and *Staphylococcus aureus.* Doolittle et al. (1995; 1996) demonstrated lytic infection of *E. coli* biofilms by bacteriophage T4 and used fluorescent probes to track the interactions of the phage with the biofilms. Hughes (1998), exploring biofilm bacteria from a food processing factory, showed that *E. agglomerans* phage SF153b has a polysaccharide depolymerase that can disrupt biofilms through exopolysaccharide (EPS) degradation even when a phage mutation blocks cell infection and lysis (Hughes et al., 1996). Despite the ability phages have in reducing the host cells present in biofilms, there are several factors which can influence the lytic performance of phages (example: a change in temperature, growth media, flow, the EPS matrix, among other parameters and lead to a decreased phage killing of their target hosts in biofilms. Also, the metabolic state of the hosts in biofilms poses a problem for phage treatment as exponentially growing cells are faster attacked than cells at the later growth phases. Along with natural bacteriophages phage derived lytic proteisn are used as antimicrobial agent to control biofilm formation. From various literature review it is seen that phages are able to clear or control the various stage of biofilm and the use of phage as antimicrobial agent are still evolving although phages are non-toxic and environment friendly still

more research should be done on purification, propagation and application of phage and phage proteins and regulatory framework on phage application should be established which nourished the growing stage of phage therapy.

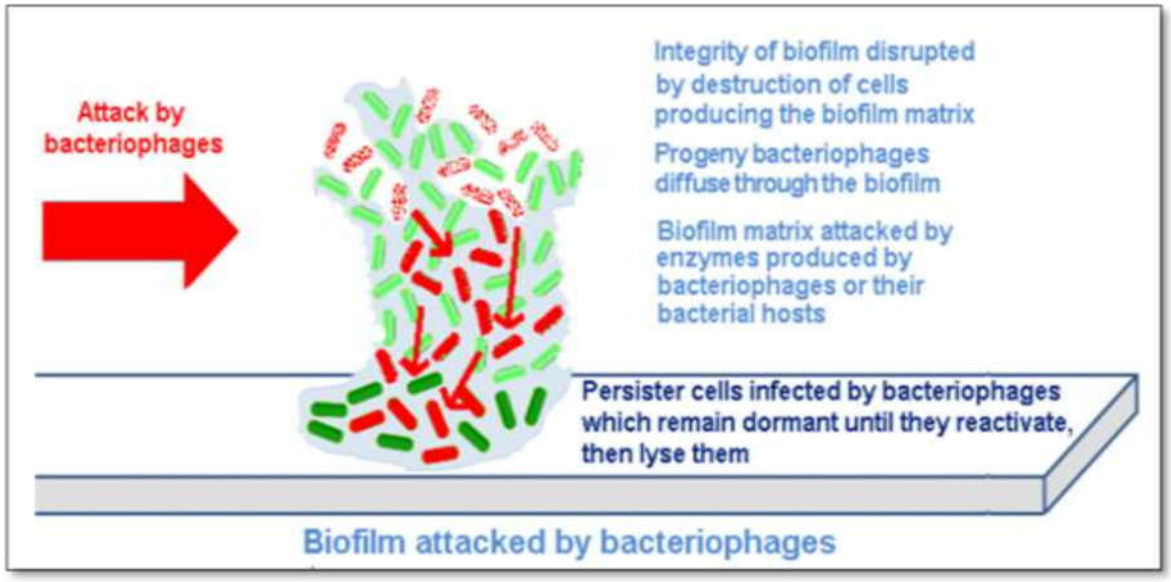


Figure 2. 5: Mechanism of destruction of a biofilm by bacteriophages

CHAPTER THREE

MATERIALS AND METHODS

3.1. Preparation of Media and Solutions

For the growth of bacteria Luria-Bertani broth was used. For Antibiotic susceptibility test, Muller Hinton Agar (himedia) was used. Likewise, Tryptic soya broth - TSB (HiMedia, India) was used for phage isolation, purification and amplification. Agar was separately added to TSB in appropriate concentration whenever required. SM buffer (Sodium chloride and Magnesium sulfate(SM) Buffer) were used as phage buffer.

3.2. Bacterial Strain collection and culture

All the multidrug resistance bacterial strain used in the research work were collected from Institute of Medicine (IOM), Maharajgunj and Nepal Public Health Laboratory (NPHL),Teku. Bacteria samples were streaked on sterile Nutrient agar plate and Macconcky agar plate and incubated at 37˚c for 24 hour. Before each experiment, bacterial samples were freshly subcultured and 3-hour cultures (active-log phase) were used. For the preservation of bacteria glycerol stock were prepared and were stored at -80˚c. A single colony was grown in Luria Bertani(LB) broth at 37˚c for overnight , another day pellet was collected and 700µl of autoclaved 50% glycerol and 300µl of autoclaved LB broth was added and kept in 37˚c for overnight and next day stored at 4˚c and then at -80˚c. Bacteria include *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*.

Table 3. 1: Bacteria used and their code name

SN	Bacteria	Code name
1	*Klebsiella pneumonia*	K27
2	*Klebsiella pneumonia*	NK
3	*Klebsiella pneumonia*	K41
4	*Klebsiella pneumonia*	K52
5	*Klebsiella pneumonia*	K57
6	*Pseudomonas aeruginosa*	P19
7	*Pseudomonas aeruginosa*	P43
8	*Pseudomonas aeruginosa*	P57

3.3. Antibiotic Susceptibility Test

All of the collected bacterial samples were multidrug resistant bacteria confirmed by hospital and it was further reconfirmed in our laboratory by using widely accepted Kirby-Bauer disc diffusion technique for antibiotic susceptibility test. The antiobiotics used were 6mm diameter and results were interpreted as sensitive(S), intermediate (I) and resistant(R) according to manufactures guideline of antibiotics.

In this study, different classes of antibiotics were used such as: beta lactam, carbapenem groups, aminoglycosides, fluoroquinolones etc. For AST, single colony of bacteria was picked and overnight grown then it was diluted upto optical density compared to 0.5 MacFarland standards. Then, lawn of bacterial was prepared by using sterile cotton swab and plates were left for few minutes to air dry. Now, selected antibiotics were kept properly above the lawn culture maintaining the distance with each other. The plates were then incubated in 37˚c for 24 hour and after incubation, clear zone for inhibition of bacterial growth was observed and this was referred as Zone of inhibition. The zone of inhibition was measured using scale and Bacterial strains were then classified (as sensitive, intermediate, resistant) according to the company's guidelines of antibiotics. ATCC strain of *Pseudomonas aeruginosa* was used as reference strain for AST test.

3.4. Molecular Detection of Bacteria by 16SrRNA Gene Amplification and sequencing

Clinical isolates of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were further confirmed by sequencing the 16S rRNA gene.

3.5. Genomic DNA extraction of bacterial strain

At first, 1ml of bacterial culture was centrifuged at 12000rpm for 2minutes and then pellets were collected and excess of media was removed. Then, 567 µL of TE buffer was added to the pelleted cells and the pellets were resuspended by repeated pipetting .Furthermore, 30 µL 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. Moreover, 80 µL of a CTAB/NaCl solution (0.7 M NaCl, 10% CTAB) was added. The solution was then incubated at 65°C for 10 mins. After incubation, an equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed then it was centrifuged for 5 min for 12000rpm and the aqueous solution was transferred to a new tube. Again, it was centrifuged at 14,000 rpm for 5 min and supernatant was transferred to a new tube. Well, the first extraction was repeated again (chloroform: isoamyl alcohol alone). Then, 0.6 volume of isopropanol was added and was mixed gently till the DNA got precipitated. Further, Centrifugation was done and isopropanol was removed 1 ml of 70% ethanol was then added to wash the salt away from the DNA. Centrifugation was done one more time, and the ethanol was discarded. The pellet was resuspended in 50-100 µL of TE buffer and was kept at 4°C.Finally, agarose gel electrophoresis was done in 0.8% agarose and genomic DNA bands were visualized.

3.6. PCR amplification of 16SrRNA gene

Molecular identification of the host bacteria was done by PCR using a primer pair for 16S rDNA. A portion of the 16S rRNA gene (1.5kb) was amplified from the genomic DNA.The primers details and PCR conditions were given in below table (Table 3.2 and 3.3).

Aliquots (5µl) of the amplified products were analyzed by electrophoresis on 1 % agarose gels in Tris Acetate EDTA (TAE) buffer. Gel was stained with ethidium bromide (0.5 mg/ml) for 20 minutes and viewed on a UV transilluminator and image captured with the help of Gel Doc system.

Then, Products after PCR amplification were subjected to Xceleris Laboratory for sequencing. Nucleotide sequences of the PCR amplicon of 16S rRNA gene were done by Sanger sequencing. The forward and reverse sequences obtained after sequencing were aligned and the contig was prepared using BioEdit programme .The identity was determined by comparing the sequences obtained with the gene sequences available in the Genbank database using Basic Local Alignment Search Tool (BLAST) software at NCBI site.(http://blast.ncbi. nlm.nih.gov).The sequence was deposited in the Genbank database and accession number was received.confirmed by sequencing the 16S rRNA gene.

Table 3. 2: Primers used to amplify 16SrRNA gene

Primer name	Tm	Sequence	Amplicon length(bp)
27F	56.4	AGAGTTTGATCMTGGCTCAG	1500
1492R	56.4	CGGTTACCTTGTTACGACTT	

Table 3. 3: PCR condition of 16SrRNA gene amplification

S.N.	Steps	Temperature (°C)	Time	Cycle
1	Enzyme Activation	95	2 minutes	
2	Initial Denaturation	95	30 seconds	
3	Annealing	55	30 seconds	
4	Extension	72	2.5 minutes	35
5	Final extension	72	5 minutes	
6	Final hold	4	∞	

3.7. Molecular amplification of resistance gene (blandm, blakpc etc)

PCR of blaNDM gene (fulllength 869) was done in following programme (Table 3.5) using following set of primer (Table 3.4).

Table 3. 4: Primers used to amplify blaNDM gene

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

Table 3. 5: PCR condition of amplification of blaNDM gene.

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
8	Extension	72	90 seconds
9	step (6-8) 19 cycle		
10	Final Extension	72	5 minutes
11	Final hold	4	∞

3.8. Water sample collection

Water samples were collected from different river of Kathmandu valley. Samples were taken from Kalanki River, Balkhu River, Teku River, Basundhara sample and Sali nadi. While collecting the sample stacked water was preferred rather than the running water. During water sample collection the sample water at the site was mixed thoroughly before collection. 100 ml water was collected in sterile tubes and the tubes were then centrifuged to 4000 rpm for 30 minutes inorder to remove unwanted contaminants and other bacterial cell debris. Then, with the help of membrane filter of size 0.22µm (PES Filter Media, Whatman™) syringe filter was done to remove other unwanted materials. The filtrate was collected in sterile falcon tubes. Now, this processed filtrate was used as phage source in experiment.

Figure 3. 1: Sample collection site

Fig A: Sample collection site at Balkhu near Bayodha hospital. Fig B: Sample collection site of Balkhu

3.9. Bacteriophage Isolation

Bacteriophage isolation was done by standard technique of Double Layer Agar Assay (DLLA) method (Adams, 1951). One ml of processed water sample was taken is sterile tube and 100µl (1-2 drops) of log phase bacterial growth (which was grown in lb broth) was added on it and allowed to remain for attachment. The mixture was left atleast for 5 minutes without any disturbance for attachment of phage and host bacteria. Then, 3ml of semisolid Tryptic soya broth (0.5% agar at 50˚c) was added to tubes and with gently shaking then further poured into the already prepared tryptic soya agar plate(1%agar).After solidification of the media then plates were incubated in the incubator at 37˚c for 24 hour.

Next day, after incubation presence/absence of plaque was observed. Those plate which had clear, round plaque those are selected for further processing. For the negative control, only the bacterial culture was added to semisolid media and poured into TSA plate and incubated.Plate with positive result, which showed lytic activity, were observed for plaque morphology (size, colour, turbitidity).

Double Layer Agar Assay (DLLA method)

Double Layer Agar Assay (DLAA) is the standard method for isolation of phages from environmental sources like sewage water, dairy waste, animal waste, soil etc. The double-layer assay provides significantly greater counts than other methods of virus quantification given by Adamas (Adams , 1959; Adams, 1951). A thin layer hard agar plate of Tryptic soya agar plate (1%) was prepared and meanwhile soft agar of TSB (0.5%) was prepared. In the mixture of 1ml processed filtrate and 100µl of host bacteria, 3ml of semisolid was added and poured into the TSA (1% agar) plate. The hard agar serves as a base layer (to form gel), and a mixture of few phage particles (diluted stock) and a very large number of host cells in a soft agar forms the upper overlay. When the plates are incubated, susceptible bacterial cells multiply rapidly and produce a lawn of confluent growth on the medium. When one phage particle adsorbs to a susceptible cell, penetrates the cell, replicates and release new phage particles which infect other

bacteria in the vicinity of the initial host cell. The growth or spread of the new viruses is then restricted or limited to the neighboring cells by the gel. This cycle is repeated until large numbers of bacteria have been destroyed. The destroyed cells produce 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. Thus phage infection results clear or translucent zone, termed a plaque through double layer agar assay. Eventually the plaque becomes too large to be visible to our naked eye. The infectious phage unit is thus termed a plaque-forming unit (pfu).The visualization of individual plaques permits far more than mere enumeration. It is the basis for the isolation of phages, their characterization by plaque morphology (clear versus turbid lysis, size of plaque, presence/absence of a halo), and the isolation of phage mutants.

3.10. Purification of Phage: Phage Streak Protocol

There might be more than one type of phage in initial isolated plates so for further processing and characterization the phage should be pure. Analogous to bacteria, each phage plaque is presumed to be clonal, having originated from a single virion. The picking and subculturing of plaques ensures that a phage population is descended from a single virion and is therefore clonal, or “pure”. Streaking the phage on bacterial lawns is an efficient way of isolating single plaques from sample putatively heterogeneous populations(Chase & Bradley, 2011). For streaking, at first the clear plaques ware selected and labeled in the petridish. The sterile wooden stick was taken and touched the center of the putative phage and gently streaked back and forth across the top third of the agar plate without lifting the stick from the agar. The starting line and ending line of streaking was marked. Now after streaking, 3ml of TSB (0.5%) was taken in sterile tubes and 100µl of active log phase host bacteria was added. The mixture was carefully poured to TSA plate slowly onto the most dilute (marked end line) of the streaked plate. It was allowed to spread across the plate from the most dilute point to the more concentrated area by gently tilting or tapping the plate. Then, the plates were allowed to solidify and incubated at 37˚C for 24 hour. The day after, the plaques were observed on the streaked line. Three round of streaking was done from single plaque inorder to obtain pure isolated single plaque morphology. The negative control plates were prepared by making phage streak line and overlay by only TSB (0.5%) agar without host bacteria.

3.11. Preparation of Phage Lysate

Phage amplification was done in TSA plates through streaking of single plaque in several plates and phage stocks were prepared by extraction of phage solution from those streaked plates. From the pure isolated plaque, streaking was done in several plates and after overnight incubation. The plaques were observed in streaking line and then the upper layer of top agar where plaque remains were collected by scarping use of buds in falcon tubes. Then, SM buffer was added in the ration of 5ml per plate. Now the solution

was mixed with vigorous vortexing and then centrifugation was done for 20 to 30 minutes at 4000rpm.Now the supernatant was collected and syringe filter was done to avoid any bacterial debris. Finally the pure phage stock was prepared and phage titer was determined by serial dilution of phage and DLAA method as mentioned above. At, a single time normally 50ml phage solutions were collected with average concentration upto 10^{10} to 10^{12} pfu/ml. In some cases, stock was prepared with DLAA plate in which selected plaque was carefully cut-out using pipette tip and immediately dissolved in 5mL of SM buffer. The tube was kept in minimum 2 hour of shaking by chloroform (50µl/ml) extraction. After centrifugation at 4000 rpm; supernatant was filtered through 0.22 syringe filter.

3.12. Phage titre assay: spot assay and DLLA method

This is the basis for determining the concentration of phage particles in a given solution and conducting successive rounds of purification of an individual phage. Only one, completely isolated plaque per plate was selected for further study .The filtrate was serially diluted up to 10^{10} using sterile tips and changing the tips in each dilution. Now, for spot assay, the grid was drawn onto the bottom of the agar plate and was labelled with designations for phage dilutions from 10^{1} to 10^{10} and a negative control was taken as SM buffer only. At, first bacterial lawn was prepared, 3ml of semisolid and 100 µl of active log phase host bacteria were mixed and the mixture was spread uniformly to the labeled plate. Then, the plate was allowed to complete dry. Now, 5 µl of all phages dilutions prepared were transferred aseptically onto the corresponding blocks on the grid. The droplets were allowed to soak into the agar until no apparent liquid remains on the agar. The plates were inverted and placed into the 37ºC incubator for 24 hour. After incubation, plaques or clear zone was observed.

3.13. Titration of phage

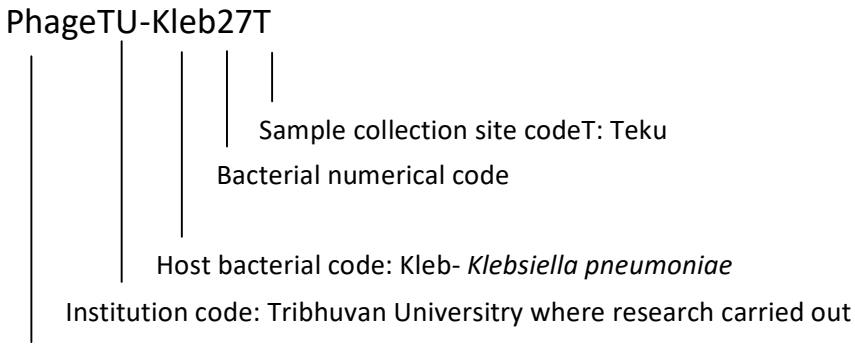
After spot assay, the highest titre was selected which showed clear lysis and DLAA was performed for all dilutions using same bacteria as host from which phage were initially isolated. For DLLA, 1ml of serially diluted phage sample and 100µl host bacteria was mixed and kept for attachment for 5mins and DLLA was done After a series of dilutions and plating, the number of plaques were counted on each plate and number of plaque-forming units per milliliter of original phage sample was calculate. After overnight incubation, the plates were checked for plaque formation and plate with an individually distinguishable plaque was selected for phage titre determination. Counted plaque number was used to determine the titre of stock solution using this formula:

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

3.14. Bacteriophage naming

The most important rule of bacteriophage naming is “do not use an existing name.” The current approach to bacteriophage naming is a tripartite construct consisting of the bacterial host genus name, the word “phage”, and a unique identifier, for example “Escherichia phage T4”. Since the first two components of this naming construct are not unique, the third component is critical to the usability of the name. Its seems easy for Providing an appropriate name for a bacteriophage but the effects might be long-lasting in databases and in official taxon names so we must follow bacteriophage naming guidelines(Adriaenssens & Brister, 2017). We had followed conventional naming approach where Phage is named such that a word ‘phage or a symbol Ø’ and is followed by Tribhuvan University (TU), where the research was carried out and bacterial code (two uppercase letters if genus and species are known and three sentence case letters code if only genus is known) along with numerical serial code assigned to each strains and finally ending with an uppercase letter code that represents water/sewage sample collection site. We used underscores to separate parts of the designation (e.g., Phage TU-kleb27), but these underscores cannot be carried over into official taxon names.

Example:



3.15. Host Range Analysis: spot assay

3.16. Intra host range analysis

Host range analysis was done by spot assay. Different strains of *Klebsiella pneumoniae* were taken from CDBT and IOM which were reported as carbapenem resistant bacteria. At first all the bacteria were grown aseptically to the active log phase.Three milliliters of 0.5 % warm (50ºC) top agar was mixed with 60-70µl of each bacterial culture in different sterile test-tube and poured on separate fresh solid Tryptic soya agar plate (TSA) with proper labeling (Verma et al., 2009). There is five different type of klebsiella phage named as Phage TU- Kleb NK ,Phage TU-Kleb27 ,Phage TU-Kleb52, PhageTU-Kleb57 and one is cocktail of klebsiella phage another one is SM buffer taken as negative control for the experiment and the grid were marked in plate for each phage.After solidification of

the top agar layer, 5 µl of the high titre phage stock (10^8-10^{10}) was spotted on the corresponding marked line and allowed to dry.The plates were incubated for 24 hours at 37˚C and checked for the presence of bacterial lysis. Similarly, host range of pseudomonas phage also tested with 20 different bacteria and by 3 different phages.

3.17. Interspecies host range analysis

Phage cocktails were prepared by mixing the selected phages of each host group in equal volume having phage lysate titre >10^6 pfu/ml. For selected phages, Klebsiella phage cocktail was prepared by mixing 5 different selected phages (Phage TU- Kleb NK, Phage TU-Kleb27, PhageTU-Kleb41, Phage TU-Kleb52 and PhageTU-Kleb57). Likewise Pseudomonas phage cocktail was the mixture of 3 different pseudomonas phages (PhageTU-P19, PhageTU-P43 and PhageTU-P53). 88 Different strain of *Pseudomonas ,Acinetobacter, Klebsiella* and *Salmonella* spp were collected for multi host range analysis. All the single phages and mixed cocktail of all phages were tested on each bacterium as in above mention method.The positive results tests were repeated three times for the reproducibility of the result.

3.18. Storage of phage solution: Infection of host with phage and recovery of phage

Phage solution for long term storage was maintained as glycerol stock. The working solutions of phage were kept in -4˚c and it works for about 6 month or more without any fluctuation in concentration of phage. For long term storage there is problem in reviving of both phage and hosts. Therefore, we designed the storage protocol for both host bacteria and phage simultaneously. At, first bacteria was grown in log phase in 5ml fresh LB media. Then 1 ml of phage solution of high titre was added and incubated at 37˚C prior to the bacterial generation time normally we use 20min for *Klebsiella* in order to trap the phage particle while adsorbing on the host surface and to stop the infection process. After incubation, it was centrifuged at 4000rpm for 10 minutes and supernatant was discarded and pellet was collected. The pellet was dissolved with 1ml SM buffer and serial dilution and DLLA was done for checking the infection of phage. For storage, 1ml of 50% autoclaved glycerol were mixed with that 1 ml dissolved pellet and stored in 4˚c in first day and then -20˚c .The revivility and infection of phage and host in every 6 month interval.To recover the phage from the infected cells the whole content of the vial(1ml) was melted at room temperature and aliquots or dilutions were mixed with 2ml fresh enrichment media (LB) and it was incubated in shaking incubator at 37˚c for 3 hours.Then the contents were centrifuged to 3000rpm for 5min top collect supernatant.Then the DLAA was done done for plaque observation.

3.19. Characterization of phage

Klebsiella pneumoniae specific lytic phage (PhageTU-Kleb27) and *Pseudomonas aeruginosa* specific lytic phage (PhageTU-P53) which poses excellent and consistent bacterial cell lysis capacity were chosen for further characterization.

3.20. Protein profiling by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS- PAGE)

SDS of protein was done by Acetone precipitation method and direct heating method.Analysis of phage proteins by SDS-PAGE were done from entire phage particles. Sodium dodecyl sulfate polyacrylamide gel electrophoresis wasperformed as described by Laemmli (Lämmli, 1970)

3.21. Acetone Precipitation method

500µl purified phage solution was precipitated with 4 volumes of ice-cold acetone for1hr30 mins, supernatant was decanted and pellet was air dried, resuspended in100µl PBS buffer (8 g l-1 NaCl, 0.2 g l-1 KCl, 0.2 g l-1 KH_2PO_4, 1.44 g l-1 $Na_2HPO_4 \times 2H_2O$, pH7.5).

3.22. SDS

The concentrated phage particles were collected from acetone precipitation method. Now 15 µl of each concentrated and high titer phage(10^{10} pfu/ml) of different 5 klebsiella phage sample were , mixed with the 25 µl sample buffer (62.5 mM Tris HCl, 2% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.001% (w/v) bromophenol blue) and heated in a boiling water bath for 10 min. Polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). Discontinuous sodium dodecyl sulphate (SDS) gel electrophoresis was carried out on slabs of 12% acrylamide and electrophoresed with Tris-glycine buffer. After electrophoresis, the gels were stained with Bio-Safe Coomassie Stain and visualized the band after treating with destaining solution. The marker of Genii was used as standard protein and used to measure the size of proteins of phage.

3.23. Morphological analysis by Transmission Electron Microscopy (TEM)

The most potent lytic phage was selected for Transmission Electron Microscopy. The phage titres were maintained above 10^8 to 10^{10}pfu/ml. Phage lysates were transported to Advanced Instrumentation Research Facility – Jawaharlal Nehru University (AIRF-JNU), New Delhi, India in cold chain. Transmission electron microscopy (TEM) of bacteriophages was performed using the method described by Ackermann (Ackermann, 2009).Phage lysates were fixed with fixative (2.5%gluteraldehyde and 2%paraformaldehyde, prepared in 0.7M sodium phosphate buffer (pH 7.2). For fixation, equal volume of phage lysate and fixative were added, mixed and left overnight. Next

day, the fixed phages were subjected to high-speed centrifugation (35,000g) for 3 hours.Pellets of phages was deposited on separate 300 mesh carbon-coated copper grid and then flooded with 2%(w/v) uranyl acetate (pH 4.5) and after 2 min excess stain was soaked-off with blotting paper. The copper grid was dried and examined in Transmission electron microscope. Phage morphology was observed from the micrographs. The Micrograph obtained from TEM was analyzed through 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 taken. The bacteriophage isolate was assigned to a respective family in accordance with the recommended guidelines of the International Committee on Taxonomy of Viruses (ICTV), based upon examination of virion particle morphology.

3.24. Determination of optimal multiplicity of infection

Multiplicity of infection (MOI) is the ratio of phage particles to host bacteria. It is calculated by dividing the number of phage added (volume in ml x PFU/ml) by the number of bacteria added (volume in ml x colony forming units/ml). Optimal MOI was determined according to Lu et al. (2003). Briefly, bacteria were infected at different MOI (0.01, 0.1, 0.5, 1, 5 and 10 PFU/ml) and were incubated at 37 ˚C for one hour. At the end of the incubation period, the mixture was centrifuged at 8000 g for 10 minutes and supernatant was passed through 0.22 µm membrane filter (Millipore, USA). The lysate was then assayed to determine the phage titre employing the double agar overlay method described previously. Phage-free cultures (containing only bacterial host) and host-free cultures (containing only phage) were used as controls.The MOI giving maximum yield was considered as optimal MOI**.**

3.25. Influence of physical parameters on phage viability

Physical parameters have a critical role in maintaining phage viability. Hence the effect of different physical parameters like pH and temperature on phage viability was studied.

3.26. Effect of temperature on phage viability

Stability of PhageTU-Kleb27 and PhageTU-P53 at different temperatures was determined by diluting phage particles to a final concentration of 10^8 PFU/ml in a final volume of 1 ml of SM buffer. The aliquots were incubated at 25°C (control), 37 °C, 40 °C, 50 °C ,60 °C, 70˚c and 80˚C for 10, 20, 40 and 60 minutes respectively (D'Andrea et al., 2017). After incubation at higher temperature, these phage containing tubes were immediately placed in ice. The samples were assayed using double layer agar method with log phase bacteria to determine the number of surviving plaque PFU. The counts of surviving phage were expressed as PFU/ ml and plotted against temperature values.

3.27. Effect of pH on phage viability

pH stability test was done according to (Han et al., 2014) with a slight modification. A pH range of pH 2 to pH 12 was prepared by adjusting the pH of fresh Luria bertanni broth. 1M HCl was added drop by drop to achieve desired acidic pH and 1M NaOH was used to achieve desired basic pH. 300µl of phage suspension of high titre (10^8pfu/ml for PhageTU-P53 and 10^5pfu/ml for PhageTU-kleb27) was mixed with 700µl of pH adjusted medium final solution making 1ml and incubated for 3 hours at 37˚C. Phage suspension having pH 7 was used as a control. After the incubation period, phage titer was determined by double layer agar method against host bacteria *Klebsiella pneumonia* (K27). Similar process was done in Phage TU-P53 and phage titer was determined by double layer agar method against host bacteria *Pseudomonas aeruginosa* (P53). The counts of surviving phage were expressed as PFU/ ml and plotted against pH values.

3.28. One step growth curve of bacteriophage

One step growth curves were studied to determine the infection potential of each phage .It generally takes 1 hour to complete the cycle of phage infection. Host bacteria, *Klebsiella pneumoniae* (K27) and *Pseudomonas aeruginosa* (P53) was grown on nutrient broth and optical density of the culture was adjusted (OD 0.3 at 600 nm) to reach active logarithmic phase to give a 10^8 cfu/ml .One step growth curve analysis was performed by mixing 1000 microliter of bacterial culture with 100 microliter phage stock (10^5 pfu/ml of k27 phage and 10^{10} pfu/ml of phage P53) and incubating the eppendorf tubes at 37˚C. One Eppendorf tubes was withdrawn after each 10 minutes for a total duration of 60 min and the phage particles were counted by DLAA method. Bacterial viable counts were determined before the bacteria were mixed with the phage and were assessed periodically. The burst size was calculated using formula

$$B = \frac{\Delta V}{|\Delta B|} = \frac{V_e - V_0}{|B_e - B_0|}$$ (Jiang et al., 1998)

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, Be is the bacterial number at the end of the experiment, and B_0 is the bacterial number at the beginning of the experiment.

The graph was plotted with PFU/ml against time. The latent period was identified as time in between after phage absorption and the initial rise in plaque number. The burst size was identified by dividing the average of pfu/infected-cell in the post-rise period of growth curve by the average of pfu/infected-cell in the pre-rise period of the growth curve (Ellis & Delbrück, 1939).

3.29. Phage genomics / Whole genome sequencing (WGS)

PhageTU-Kleb27 sample was exported to Xcelris Genomics (Ahmedabad, India) in dry ice for Next Generation Sequencing. Whole genome sequencing of phage was performed on Illumina HiSeq 2000/2500 platform under Project ID: NGS-913.

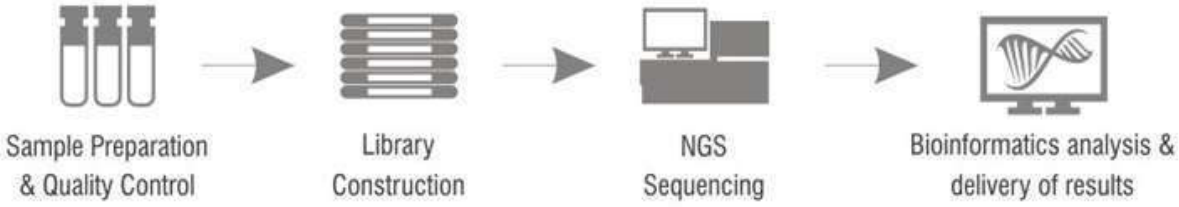


Figure 3. 2: Work flow of Whole Genome Sequencing (WGS) workflow at Xcelris Genomics, Ahmedabad, India.Source: http://www.xcelrisgenomics.com

3.30. Isolation, Qualitative and quantitative analysis of gDNA

Genomic DNA was isolated from PhageTU-Kleb27 sample using Norgen Phage DNA Isolation kit. 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. 1 µl of each sample was used for determining concentration using Qubit® 2.0 Fluorometer.

3.31. Preparation of library

The paired-end sequencing library was prepared using Truseq Nano DNA Library preparation kit. The library preparation process was initiated with 200 ng g-DNA. The g-DNA was mechanically sheared into smaller fragments by covaris 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.

3.32. 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.

3.33. Cluster Generation and Sequencing:

After obtaining the Qubit concentration for the library and the mean peak size from Bioanalyser profile, library will be 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 will bind to complementary adapter oligos on paired-end flow cell. The adapters are designed to allow selective cleavage of the forward strands after re-synthesis of the reverse strand during sequencing. The copied reverse strand is then used to sequence from the opposite end of the fragment.

3.34. Genomic data analysis / Bioinformatics

The whole genome sequence (WGS) data was curated and delivered to us by Xcelris Genomics in fasta format (.fa files). The genome files were primarily processed using an online tool PHASTER – PHAge Search Tool - Enhanced Release, http://phaster.ca – (Arndt et al., 2016; Zhou et al., 2011) Throughout the project, NCBI database was used as the sole source for reference genome for bacterial and phage genomes. Also Blast+ was used for species distribution. PHASTER tool was also used for prediction of putative genes and RAST was used for gene annotation.

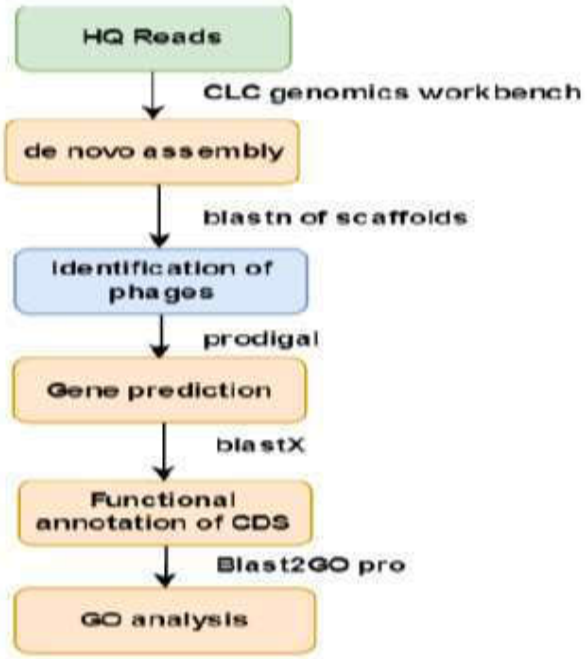


Figure 3. 3: Bioinformatics analysis workflow

3.35. Phylogenetic tree construction

The phylogenetic analysis of PhageTU-Kleb27 capsid protein and tail fiber protein was done with closely related species of Klebsiela phages present in NCBI data base through Phylogeny.fr an online server.

3.36. Applications of Bacteriophage as antimicrobial agent

3.37. Determination of Biofilm producing bacteria- Tissue culture plate method

This quantitative test described by Christensen et al. is considered the gold-standard method for biofilm detection (O'Toole, 2011). Different strains of Klebsiella broths were incubated at 37˚C for 24 h. The cultures were then diluted 1:100 with fresh medium to meet upto optical density 0.5 to 0.8.Individual wells of sterile 96 well-flat bottom polystyrene tissue culture treated plates (Tarson, India) were filled with 200 µL of the diluted cultures. The control organisms were also incubated, diluted and added to tissue culture plate. Negative control wells contained inoculated sterile broth. The plates were incubated at 37ºC for 24 h. After incubation, contents of each well were removed by gentle tapping. The wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) four times. This removed free floating bacteria. Biofilm formed by bacteria adherent to the wells were fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying at last 95% ethanol was addedin each well and Optical density (OD) of stained adherent biofilm was obtained by using ELISA reader at wavelength 620nm. The experiment was performed in triplicate and repeated three times. The interpretation of biofilm production was done according to the criteria of Stepanovic et al (Stepanovic et al., 2007). Optical density cut-off (ODc) was determined. It is defined as average OD of negative control + 3× Standard deviation (SD) of negative control.

Table 3. 6: Classification of biofilm formation abilities by Micro-titre Plate method

	Biofilm formation abilities
OD > 4×ODc	Strong
2×ODc < OD ≤4×ODc	Moderate
ODc< OD ≤ 2×ODc	Weak

3.38. Biofilm Disruption by use of bacteriophage

Phage treatment to biofilm disruption was done according to Forti et al with slight modifications (Forti et al., 2018).For biofilm evaluation by crystal violet staining, an overnight culture of eight strong biofilm producing organism (K27, K57,NK,K3, K6, P25, P6 and P53) were diluted 1:100 and 200µl of each bacteria (0D600 standard with 0.5 macfarland) inoculated into 96-well polystyrene microtiter plates. The plates were incubated at 37°C for 24 h to allow biofilm formation. Broths containing planktonic cells were gently removed, and the wells were washed with 200µl of LB broth for two times. Then, 200µl of phage lysate at 105 PFU/ml was added, and incubation was continued for

4 h.The bacteria and bacteriophages were selected on the basis of biofilm formation of bacteria and host range of phage to lyse those bacteria. After incubation, the wells were carefully emptied and gently washed with PBS for two times. The bacteria adhering to the walls of the plate were it was fixed with 200µl sodium acetate for 30minutes at 60˚c and the content was again discarded. Finally the adhered cells were stained with 200 µl of 0.1% crystal violet solution for 10 min .Excess stain were removed by using deionized water and plates were kept for drying and at last 95% ethanol was added in each well and adherent biofilm was quantified by measuring the optical density at 620 nm. Each treatment was repeated in 5 wells, and the median value and standard deviation (SD) were calculated. PAO1 Strain was used as Positive control and only Lb broth was used as negative control.

Table 3. 7: Biofilm producer bacteria and Bacteriophage used for disruption of biofilm

SN	Bacteria strain code name	Phage used
1	K3	PhageTU-Kleb57
2	K6	PhageTU-Kleb57
3	NK	PhageTU-KlebNK
4	K27	PhageTU-Kleb27
5	K57	PhageTU-Kleb57
6	P6	PhageTU-P19
7	P25	PhageTU-P19
8	P53	PhageTU-P53

CHAPTER FOUR

RESULTS

4.1. Antibiotic Sensitivity Test (AST)/ Antibiogram Assay

From the antibiotic susceptibility test (AST) using Kirby Bauer Disk diffusion method, both the bacteria K27 and P53 showed resistance to more than 3 antibiotics thus they were found to be multi drug resistant and both the bacteria even showed resistance to imipenem and meropenem drugs which confers the carbapenem resistant.

Table 4. 1: Antibiotic susceptibility pattern of K27 and P53 bacteria

Antibiotics	**Bacteria kleb 27**	**Bacteria P53**
Amoxicilline (Amx)	Resistant	-
Ceftibuten(CB)	-	Resistant
Ceftriaxone(CTR)	Resistant	Resistant
Cefotaxime(CTX)	-	Resistant
Gentamycin(GN)	-	Resistant
Levofloxacin(LE)	-	Sensitive
Nitrofurantoin(NIT)	Sensitive	-
Norfloxacin(NX)	Resistant	-
Oflaxacin(OF)	Resistant	Resistant
Cefixe(CFM)	Resistant	Resistant
Imipenem	Resistant	Resistant
Meropenem	Resistant	Resistant

4.2. Molecular identification of bacteria by 16srRNA gene amplification and sequencing

The genomic DNA was extracted from CTAB (Cetyl Trimethyl Ammonium Bromide) method. Distinct band of DNA was observed of 5 different putative strain of Klebsiella pneumoniae. Similarly, Intense and distinct bands were observed in case of 3 different putative strain of Pseudomonas aeruginosa. The concentration of genomic DNA were found to be approximately~100ng by nanometer reader and were used as template for PCR(polymerase chain reaction) .Then PCR products showed visible distinct band of size 1500bp in uv transilluminator and then products were subjected to Sanger sequencing. The different strain of host bacteria was characterized by 16S rRNA gene sequence analysis.

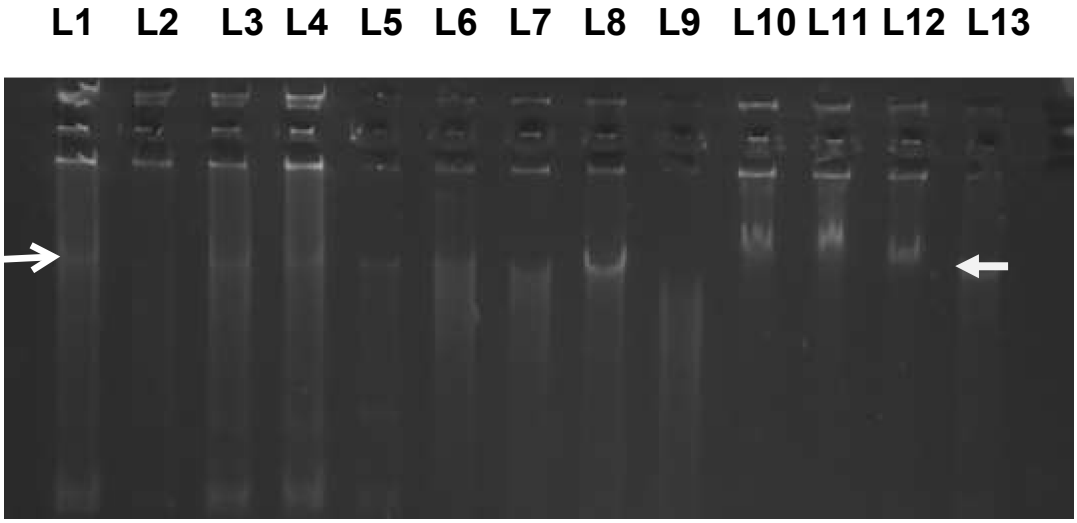


Figure 4. 1: Gel electrophoresis in 0.8% agar of Bacterial genomic DNA extraction using CTAB method

L1: K27, L3:K41, L4:K52, L6:K57, L7: NK, L8: P19, L12:P43, L13:P53

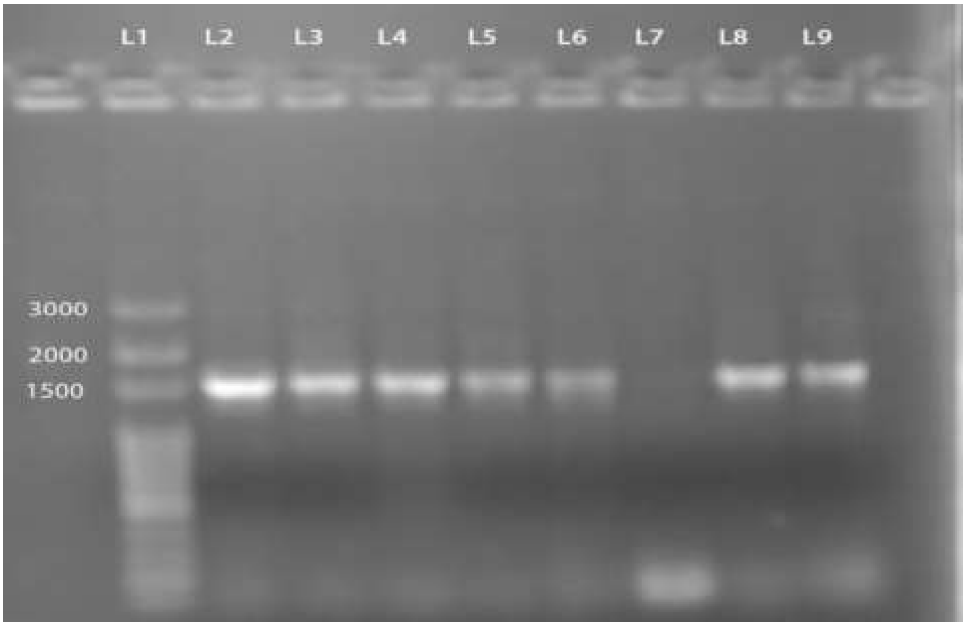


Figure 4. 2: Gel electrophoresis of Molecular amplification of 16srRNA gene of bacterial DNA using universal primer(1500bp) in 1% agarose gel. L1: Ladder (1kb) solid biodyne L2: K27, L3:K41, L4:K52, L5:K57, L6:P19, L8:P43 and L9:P53

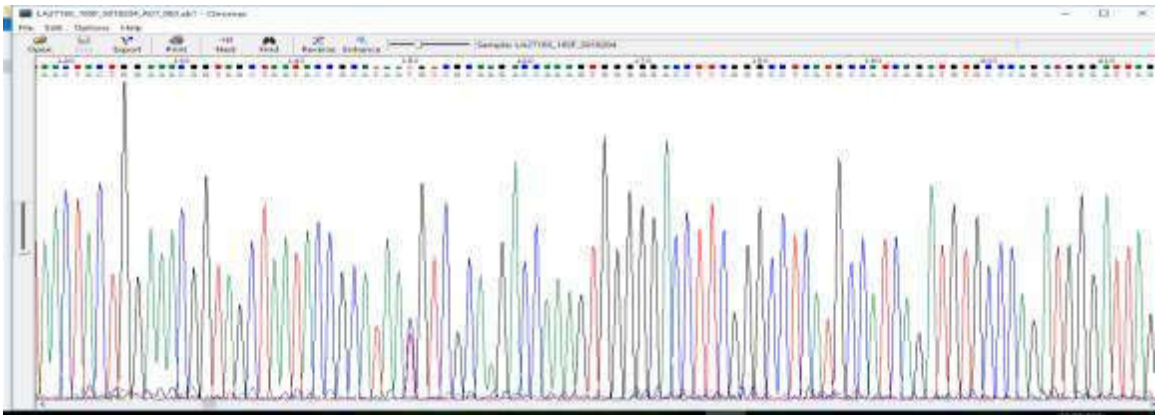


Figure 4. 3: Chromatogram file of Bacteria kleb27of 16srRNA gene using chromas software.

The evenly spaced peaks, each with only one colour determines the quality of clean sequence.

The sequences were submitted to NCBI and are available for public under following accession number.

k27: SUB4157635 Kleb27 MH482940

K52: SUB4811522 Kleb52 MK192090

Remaining bacteria samples (K41, K57, NK, P43, P53 and P19) were under processing to get accession number.The chromatogram files of all remaining strains of *Klebsiella pneumoniae* were given in Appendix 3.

4.3. Molecular amplification of carbapenem resistance gene (blandm, blakpc etc)

Klebsiella pneumoniae strain Viz: K41, K52 and K57 were already confirmed BLaNDM gene positive strain from the previous thesis work whereasK27 strain was further processed for detection of Carbapoenem resistance gene. The full length metallo-betalactamase gene, bla (NDM-1) and KPC gene were amplified in k27.As, shown in figure the desired product size was 890 of NDM and 540 of internal Blakpc. Similarly, among *Pseudomona aeruginosa* strain, P43 is already confirmed as bla NDM positive in previous thesis work. Carbapenem resistant gene blaNDM was found to be positive n both strain *P.aeruginosa* Viz: P19 and P53.

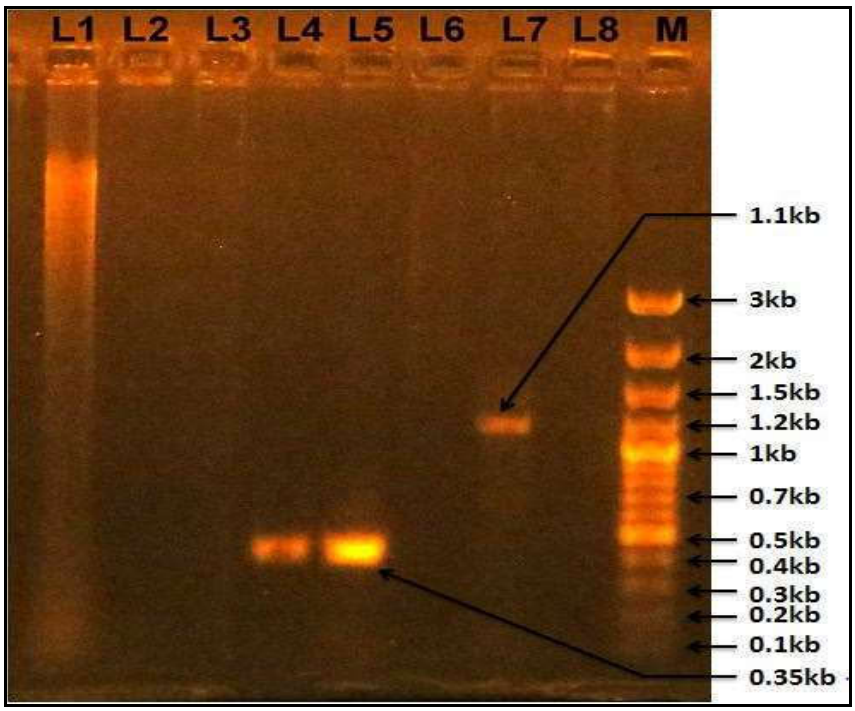


Figure 4. 4: Gel electrophoresis in 1% agarose gel showing kpc positive, blandm positive and 16SrRNA positive result in kleb 27

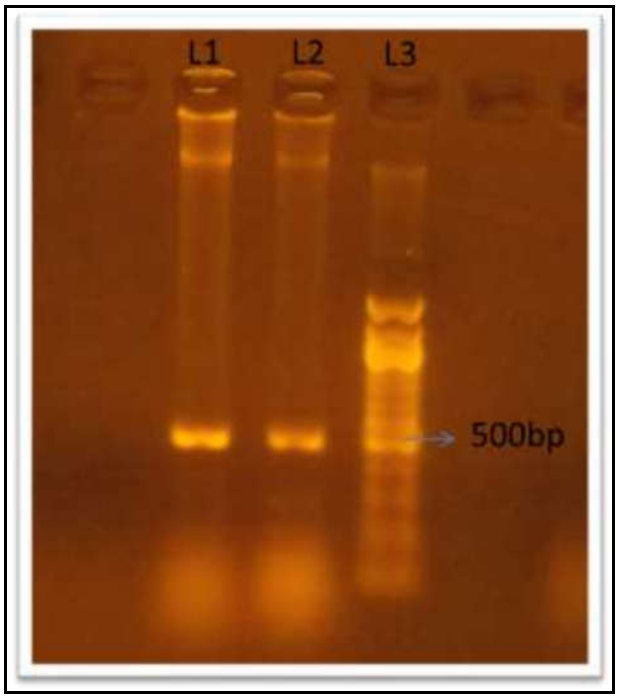


Figure 4. 5: Gel electrophoresis in 1% agar showing molecular amplification of blaNDM internal gene (500bp)

L1:P19 L2:P53 and L3: ladder 100bp (ZR, zymo research ladder)

4.4. Isolation of bacterophage by DLAA method

Alltogether 21 bacteriophages isolated from water samples of nine different sizes were found to lyse carbapenem resistant 5 strains of *Klebsiella pneumoniae* and 3 strains of Pseudomonas aeruginosa .The result showed that all the water samples contained huge number of bacteriophage populations. Morphology of phages observed from small pin headed clear plaque to large clear plaque, some are halos forming and the most dominant one is Bull's eyes plaque(Figure 4.7 and 4.8). The details of phage number in different water samples along with it's morphology are listed in below table (Table 4.2). The high abundance of phage number was found in Teku sewage sample near slaughter house.

Table 4. 2: Bacteriophage Isolation Table

Sn	**Location of sample collection**	**Host Bacteria**								
		E. coli	K27	K41	K52	K57	NK	P53	P43	P19
1	Balkhu 1	+	NT	-	-	-	NT	NT	-	-
2	Salinadi sample	+	NT	-	+	-	NT	NT	-	-
3	Balkhu 2	-	NT	-	-	-	NT	NT	+	-
4	Bishnumati sample	-	NT	-	+	+	NT	+	-	-
5	Mahedevi khola	+	-	+	-	-	NT	NT	-	+
6	Teku1	-	-	+	+	+	-	-	-	-
7	Teku2	+	NT	NT	NT	NT	+	NT	NT	NT
8	Basundhara	-	+	+	+		+	-	+	
9	Kirtipur	NT	NT	NT	NT	NT	NT	NT	NT	+

Note: '+' denotes the presence of lytic phages, '-'denotes the absence of lytic phages and NT denotes not tested in those sample.

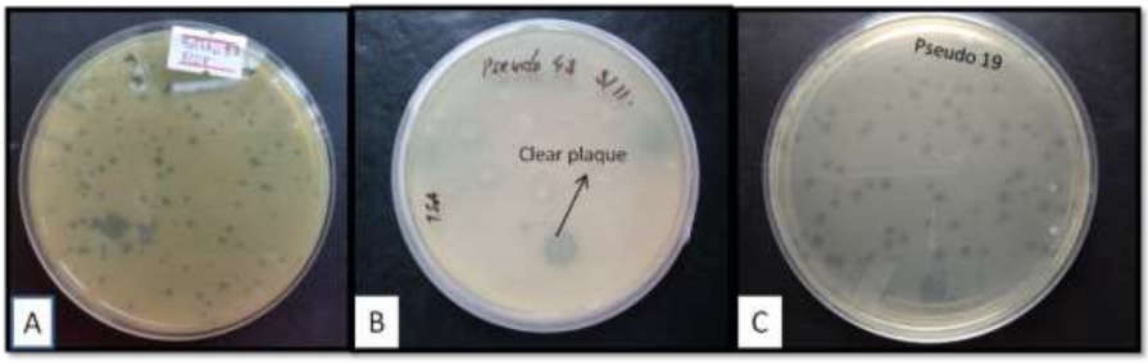


Figure 4. 6: Initial Bacterophage isolaton in *Pseudomonas* strain By DLAA methodFig A: clear lysis plaque formed in P53 bacteria sample in Balkhu sample and Fig B: clear plaque formed in Teku sample in P43 bacteria. Fig C: Clear plaque formed in P19 bacteria

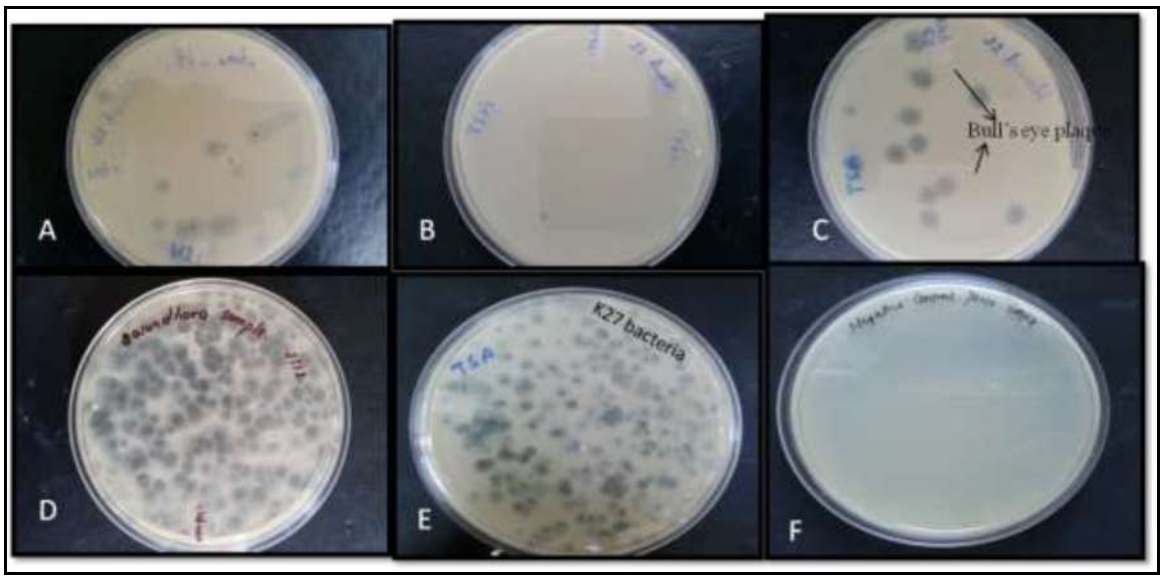


Figure 4. 7: Initial Bacterophage isolaton in *Klebsiella* strain By DLAA method

Bull's eye plaque formed in Teku sewage sample against bacteria K41, K52 and k57 respectively in fig A,B and C. Fig C:numerous small and large size Bull's eyes. Plaque formed in New kleb bacteria from Basundhara sample. FigD: Bull's eyes plaque formed in Basundhara sample in bactera K27.Fig E.negative control where only bacteria were incubated**.**

Table 4. 3: Isolation of bacteriophage and their morphology

SN	**Host Bacteria**	**Sample site**	**Initial number of plaques**	**Plaque morphology**	**Lysis pattern**	**Diameter of plaque**
1	K27	Basundhara	32	Bulls eye	Clear center (halo) surrounded by turbid ring.	0.5cm
2	K41	Teku	12	Bulls eye	Clear center (halo) surrounded by turbid ring.	0.65cm
3	K52	Teku	4	Small Bulls eye	Clear center (halo) surrounded by turbid ring.	0.29cm
4	K57	Teku	12	Large Bulls eye	Clear center (halo) surrounded by turbid ring.	0.84cm
5	NK	Basundhara	TMTC	Bulls eye	Clear center (halo) surrounded by turbid ring.	Large:0.6cm Small:0.2cm
6	P19	Kirtipur	18	Clear plaque	Complete lysis	0.3cm
7	P43	Balkhu	10	Clear plaque	Complete lysis	0.42cm
8	P53	Balkhu	TMTC	Clear plaque	Complete lysis	0.36cm

4.5. Purification of Bacteriophages

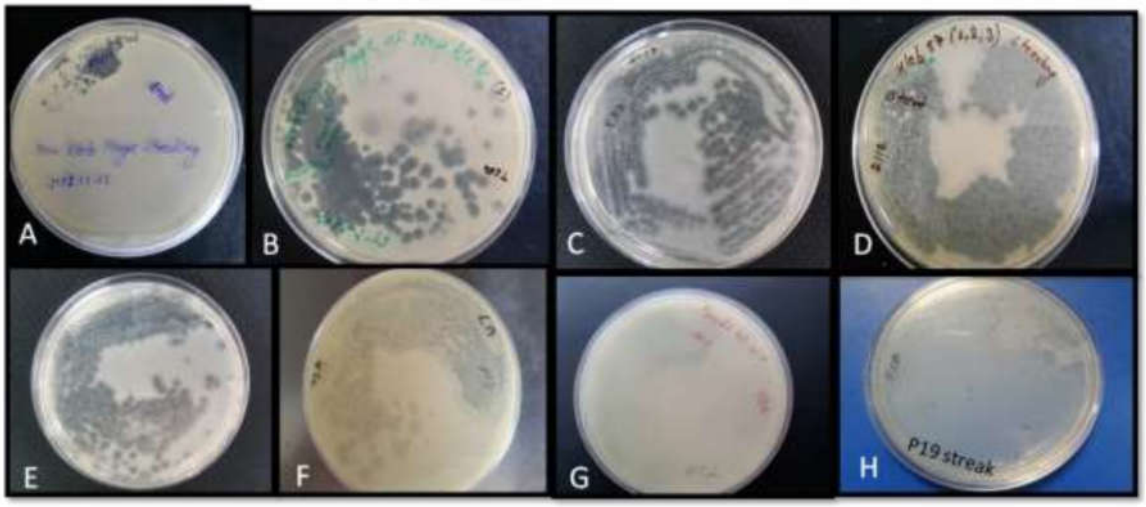


Figure 4. 8: Purification of phage by continuous streaking method

Bull's eye plaque formed in Teku sewage sample against bacteria K41,K52 and k57 respectively in fig A,B and C. Fig C:numerous small and large size Bull's eyes. Plaque formed in New kleb bacteria from Basundhara sample. FigD: Bull's eyes plaque formed in Basundhara sample in bactera K27.Fig E.negative control where only bacteria were incubated.

Phage streak results the uniform plaque and as in above figure (Figure4.9).After 3 round of streaking from sigle isolated plate, it gives the pure plaque of uniform size.The plaques were formed in high number in starting line and decreased in ending line.

4.6. Spot assay of phage and titer determination

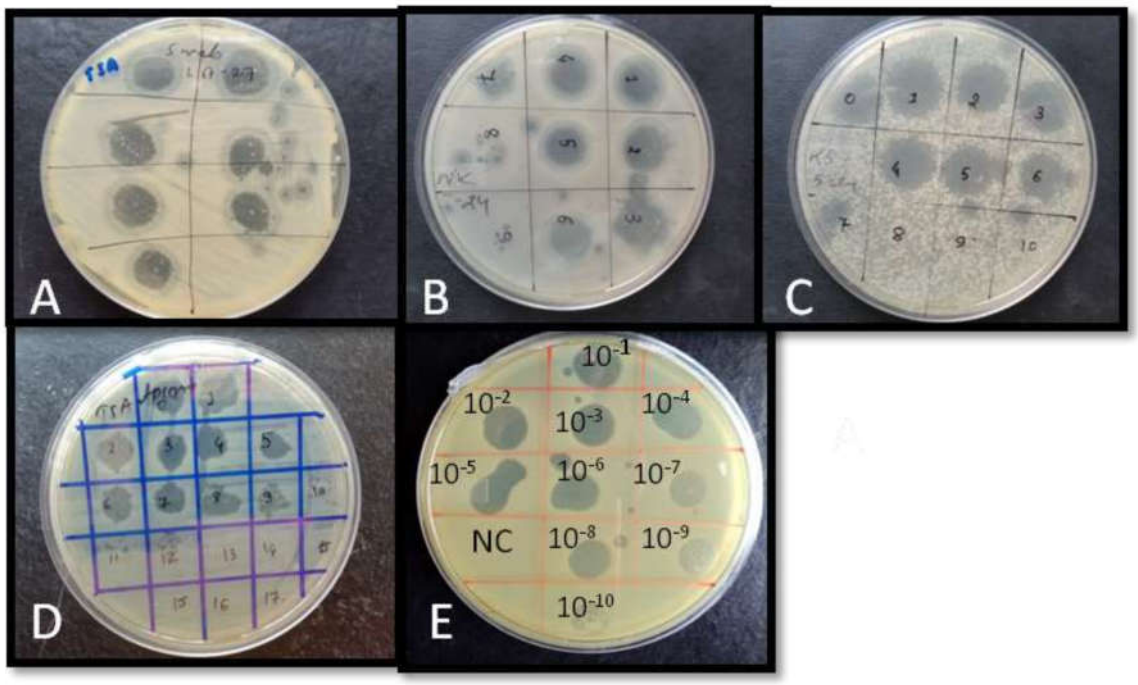


Figure 4. 9: Spot assay of bacteriophage stock to determine the phage titre. Each block in plate has different dilutions. Clear lysis was observed over bacterial lawn in each dilution.

Fig A: spot assay of PhageTU-Kleb27 showing lysis upto 10^{7} Fig B: spot assay of NK phage showing lysis upto 10^{-9} dilutions. Fig C: spot assay of K57 phage showing lysis upto 10^{-7} dlutions. Fig D: spot assay of P43 phage showing lysis upto 10^{-13} dilutions. Fig E: spot assay of P53 phage showing lysis upto 10^{-10} dilutions. NC denotes negative control where only SM buffer was used.

Figure 4. 10: Titre determination of Phages.

DLAA plates showing countable plaques in higher titre value. Fig A: plaques formed in maximum dilution of 10^{9} in PhageTU-Kleb27 Fig B and Fig C: Plaques formed in dilution of 10^{4} and 10^{10} in PhageTU-Kleb52 respectively. Fig D: Plaques formed in dilution 10^{8} in PhageTU-P43.FigE: Plaques formed in dilution of 10^{19} in PhageTU-P53. PhageTU- P53 has higher phage concentration.

Table 4. 4: Plaque count upon serial dilutions and Phage titre determination (pfu/ml)

Phage	10	10^{1}	10^{2}	10^{3}	10^{4}	10^{5}	10^{6}	10^{7}	10^{8}	10^{9}	10^{10}	10^{11}	10^{12}
K27	Clear	clear	TMTC	TMTC	60	32	14	9	10	8	2	1	
K52	Clear	clear	Clear	clear	TMTC	TMTC	180	75	39	16	16	5	2
K57	Clear	clear	TMTC	TMTC	160	75	5	no	no	no	no		
Nk	Clear	clear	TMTC	TMTC	89	42	12						
P43	Clear	clear	Clear	clear	clear	Clear	TMTC	83	71	49			
P53	Clear	clear	Clear	clear	clear	Clear	TMTC	TMTC	TMTC	96	88	19	6
P19	Clear	clear	Clear	TMTC	60	23	5						

4.7. Intrahost range analysis of Klebsiella phage and Pseudomonas phage

The ability to infect and produce lytic zone by the PhageTU-kleb27, PhageTU-Kleb57,PhageTU-Kleb52, PhageTU-NKphage and PhageTU-Kleb41 phage against 20 different bacteria were tested by spot test. Among them PhageTU-Kleb57 was able to infect 6 strain of *K. pneumoniae*.Similarly, Phagetu-Kleb52 showed lytic activity in 5 strains of *K.pneumoniae*, PhageTU-kleb41 showed lytic activity in 3 strains, PhageTU-NK Kleb showed lytic activity in 2 strains while PhageTU-Kleb27 showed no any lytic activity to other strains of *K. pneumoniae*.

Similarly, host range activity in Pseudomonas phage was done among 18 different strains of *Pseudomonas aeruginosa* and PhageTU-P19 showed lytic ability in 9 strain of Pseudomonas, PhageTU-P43 showed lytic ability in 3 strains of *Pseudomonas* and PhageTU-P53 showed lytic ability in 6 strain of Pseudomonas and the mixture of phage cocktail has significant efficiency to lyse 12 strains. The result of host range activity of 5 different types of klebsiella phages and 3 different types of Pseudomonas phages were listed below in pie chart (4.12 and 4.13) and the detailed result of host range were showed in appendix.

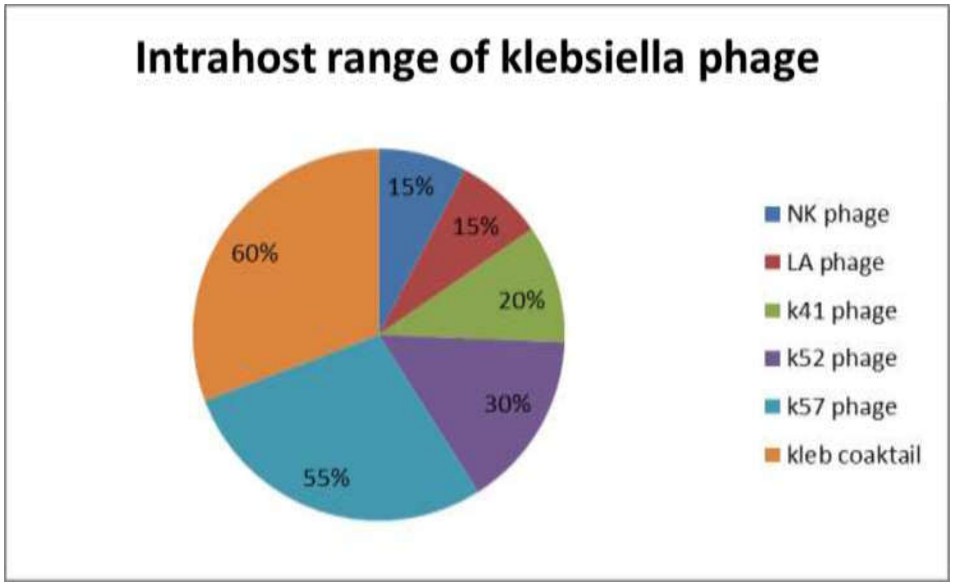


Figure 4. 11: Host range activity of Klebsiella phage among 20 Klebsiella strain.

Among individual, PhageTU- Kleb57 showed wide host range activity.

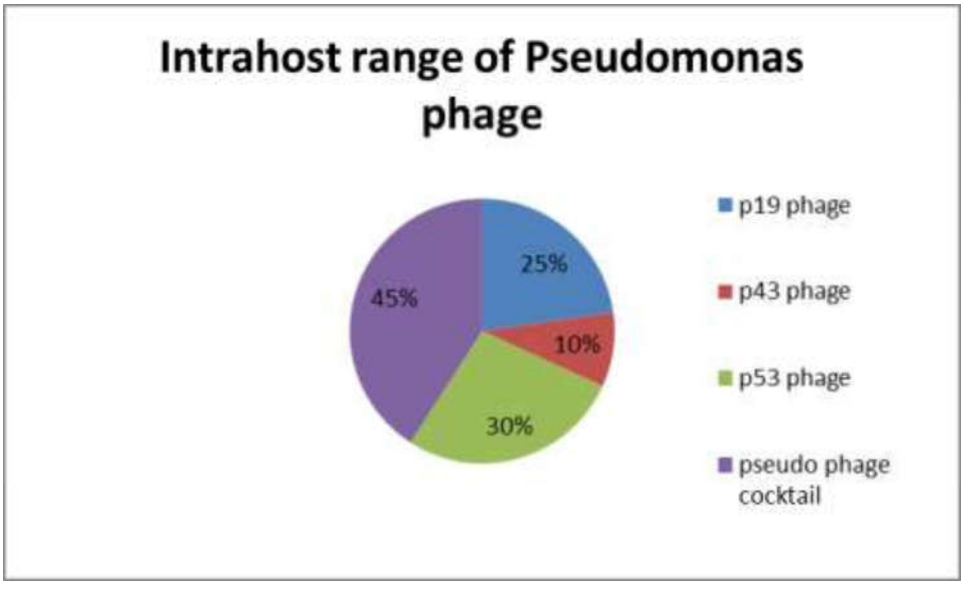


Figure 4. 12: Graphical representation of host range of Pseudomonas phage in 18 different types of *Pseudomonas* strain.PhageTU- P53 showed higher lytic capability

4.8. Interspecies or Multihost range analysis

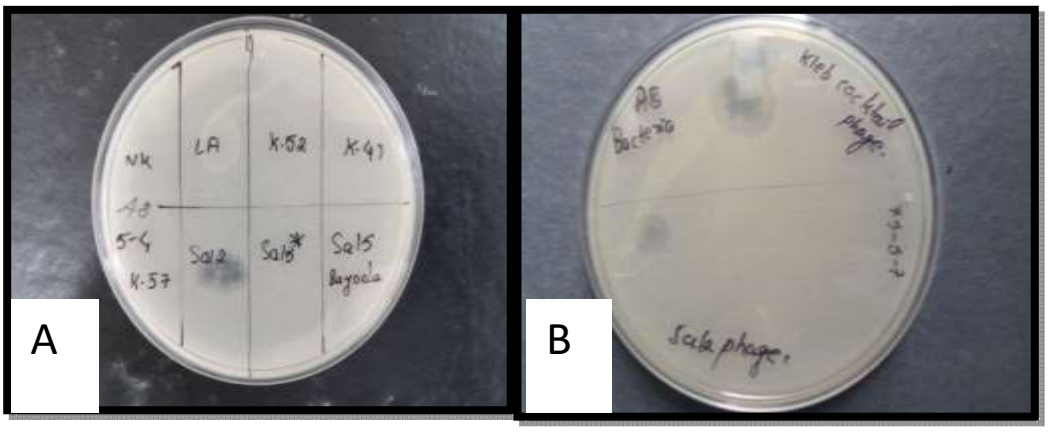


Figure 4. 13: Multihost range analysis of klebsiella phage

Fig A: individual phage of klbsiella (K27, k41, k52, k57 and Nk) did not lyse the A8 bacteria Fig B: cocktail of klebsiella phage (K27, k41, k52, k57 and Nk) lyse the A8 strain of *Acinetobacter baumannii.*

Table 4. 5: Positive result of multihost range analysis

Bacteria	Kleb cocktail(mixture of phage k27, k41, k52, k57 and NK)	Pseudo phage cocktail(mixture of P19,P43 and P53 phage)
A8	+++	--
A17	+++	--
A70	+++	--

Note: '+' denotes the positive result and '+++' denotes that it gives positive result on 3 times repeated test.'-' result showed that negative result for host range and '---' indicates negative result in 3 time repetition.

The phages were tested against several other gram-negative microorganisms such as carbapenem resistant Pseudomonas aeruginosa, Klebsiella pneumoniae, Acinetobacter baumanni, and colistin resistant Salmonella spp. Eighty eight different multidrug resistant bacteria were used as host .Based on spot testing results, Pseudomonas phages did not show any inter host range property but Klebsiella phage and its cocktail showed unique property of host range .Among them, only positive results are shown in above table(Table 4.5). Those phages were able to infect various bacterial hosts that can easily encounter a susceptible prey and replicate in it. Here the most interesting finding was that only the cocktail of Klebsiella phage lysed three carbapenem resistant Acinetobacter baumanni strains (A8, A17 and A70) however individual phage of klebsiella did not lyse those bacteria as shown in figure (Fig.4.14).

4.9. Storage of phage solution: Infection of host with phage

Table 4. 6: Storage efficacy of Phage : Phage titer enumerations after six months intervals when stored inside the host bacterial cells at −80 °C. PFU= Plaque Forming Unit

Phage name	**Phage Titer**				
	Initial phage titer	**Phage titer after 6moth**	**Phage titer in 12 month**	**Log_{10}phage titer reduction in 1st 6 month**	**Log_{10} phage titer reduction in 12 th month**
PhageTU-Kleb27	8.9×10^{7}pfu/ml	6.2×10^{7}pfu/ml	2.1×10^{7}pfu/ml	0.157	0.6217
PhageTU-Kleb52	1.78×10^{8}pfu/ml	1×10^{8} pfu/ml	8.6×10^{7}pfu/ml	0.2504	0.3159
PhageTU-Kleb57	8.33×10^{4}pfu/ml	5.62×10^{4}pfu/ml	1.1×10^{4}pfu/ml	0.1709	0.879
PhageTU-KlebNk	1.33×10^{5}pfu/ml	1×10^{5}pfu/ml	6.4×10^{5}pfu/ml	0.12138	0.3177
PhageTU-P53	2.11×10^{10}pfu/ml	1.1×10^{10}pfu/ml	8.9×10pfu/ml	0.2829	0.375

Phage preservation is very much crucial steps in phage research. The titer decreased steady state over a year.The higher titer reduction was seen in PhageTU-Kleb57 after 12 month among all phages.from the above table it was found that the reductions were always below 1 log unit after 12 months for all the phages. This phage-host storage method is advantageous due to recovery of both host bacteria and phage .The Phage titer of bacteriophages did not change significantly after 12 months of storage when stored inside the host bacterial cells at −80 °C.While recovery of phage from storage condition after 6month and 12 months, titre value was found to be less than initial titre.The statistical significance of the changes in phage titer was assessed by Student's t test at ($p<0.05$).So storage of phage infected with its host could be the best method for preservation for phage and host itself.

4.10. Characterization of phage

4.11. SDS

The protein profiling of bacteriophage was analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). Multiple bands were observed in both phage proteins. PhageTU-P53 and showed two bands of proteins with apparent size of 80KDa and 45KDa and PhageTU-Kleb27 was found to be major band of 83KDa and minor band of 44KDa which was compared with 205 KDa ladder (3.5 KDa -205 KDa). The proteins which were precipitated by acetone showed intense band than by direct heating method.

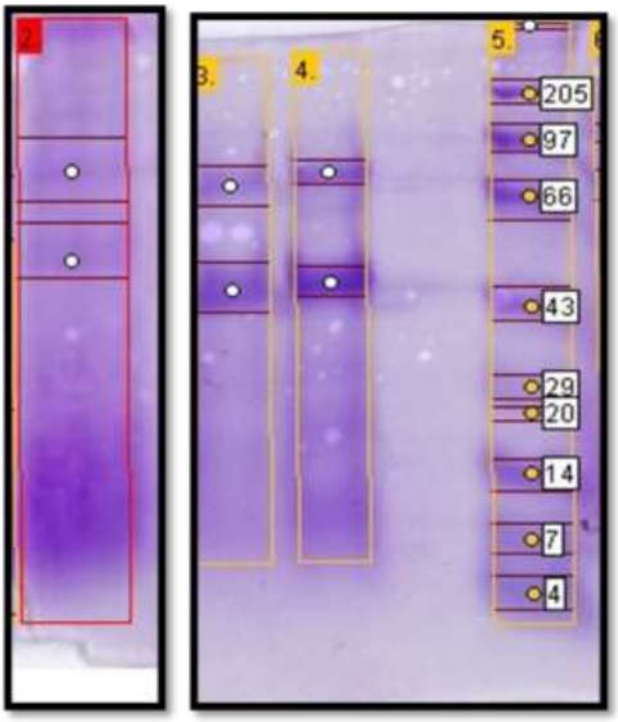


Figure 4. 14: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis of Bacteriophage Protein.

4.12. Transmission electron microscopy

The TEM micrograph of phage is very much essential for characterization of phages as it gives the basis for classification of phage. We had done TEM of two phages one of Pseudomonas phage PhageTU-P53 and another from Klebsiella phage PhageTU-Kleb27.

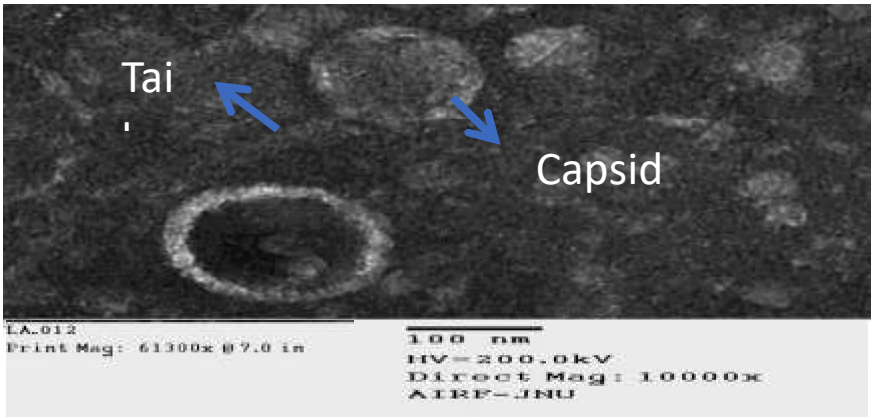


Figure 4. 15: Transmission Electron Micrograph image of PhageTU-Kleb27 negatively stained with 1%uranyl acetate.

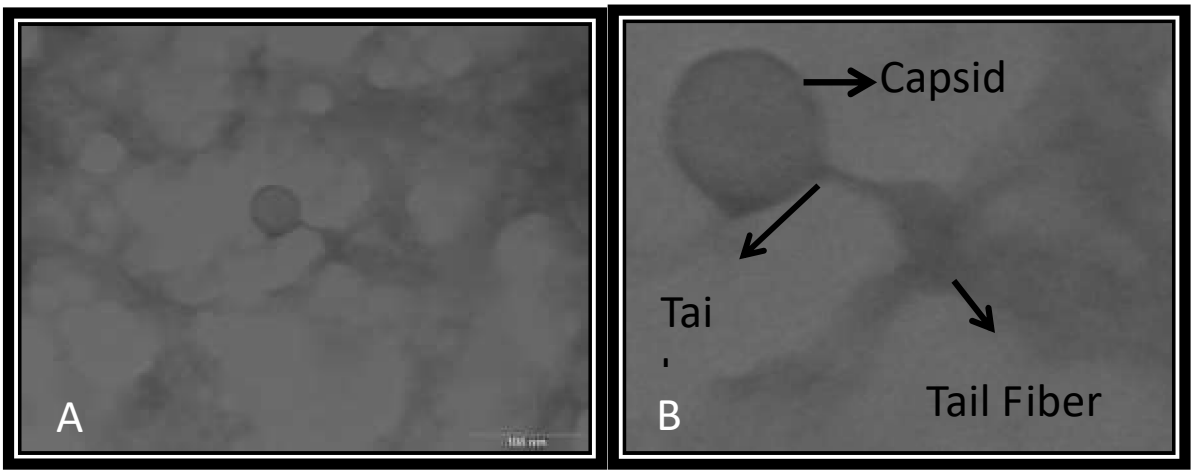


Figure 4. 16: Transmission Electron micrograph image of PhageTU-P53 negatively stained with 1% uranyl acetate.

Fig A: Original image of PhageTU-P53. Scale bar in image denotes the distance. FigB: magnifying image of FigA. The phage was found to be caudovirales.

Table 4. 7: Classification of phages according to ICTV guidelines (9th report) based on transmission electron micrograph.

Phage Name	**Capsid (in nm*)**	**Tail**		**Shape**	**Order**	**Putative family**
		Width	Length			
PhageTU-Kleb27	135	15	48	icosahedral	Cauodovirals	Podoviridae
PhageTU-P53	85	13	56	icosahedral	Caudovirales	Siphoviridae

*nm= Nanometer

The micrograph revealed that the PhageTU-Kleb27 has icosahedral capsid with an extremely short tail which resembled with the phages belonging to the Podoviridae family, Caudovirales order (Fig 4.15).Similarly, incase of PhageTU-P53 it has an icosahedral capsid with long non-contractile tail which are typical morphological features of Siphoviridae family, caudovirales order (Fig 4.16).This classification was done based on the ICTV guidelines 9th report which was shown in appendix (Appendix).

4.13. Effect of temperature on phage viability

The thermal stability was carried out to measure the heat resistance of phage. In this study, two phages PhageTU-Kleb27 and PhageTU-P53 were selected for thermal tolerance. Temperature stability of Phages was determined at different temperature in different time of incubations. Phages were found to be stable even at 70˚c upto 20mins. This result showed that viability of phage decreases as the temperature increases and as the temperature increases plaque morphology also changed to small size.

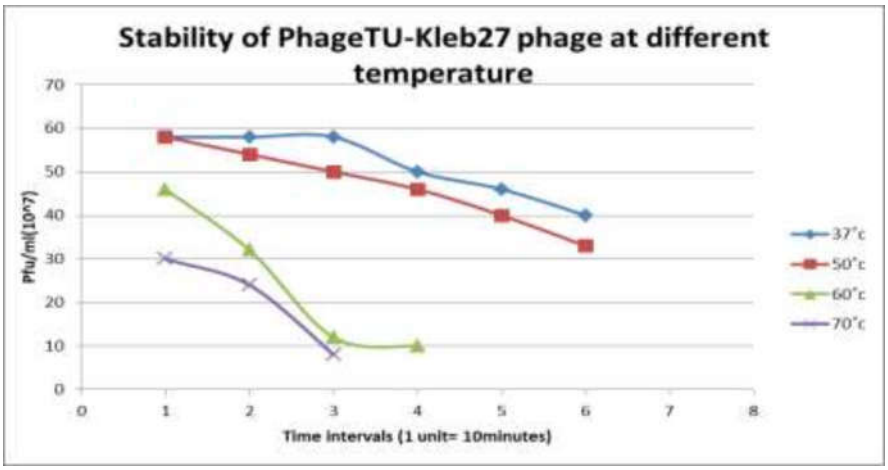


Figure 4. 17: Graphical representation of stability of PhageTu-Kleb27 at different temperature.

PhageTU-Kleb27 showed stable upto 70˚c.This graph showed that phage PhageTU-Kleb27 is heat stable upto 70˚c.The phage titre decreased after 30 minutes incubation at 60˚c and significant decrease in 70˚c.

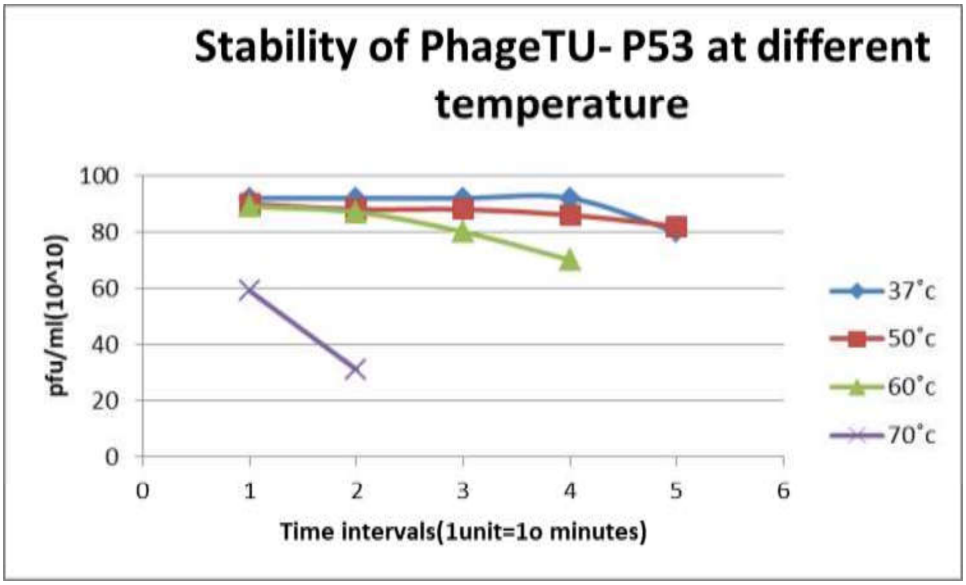


Figure 4.18: Graphical representation of stability of phage P53 at different temperature .It showed stability upto 70˚c.

4.14. Effect of pH on phage viability

pH stability test was carried out in order to determine the optimal pH of kleb27 phage and P53 phage at different pH for 1 hour at room temperature. The titers of phage dropped at lower pH and lost its activity at pH 2and 3 and then the phage titer continued to increase dramatically in higher ph also showing its effective lysis upto pH 12. As shown in graph, phage Kleb27 showed its lytic activity even at pH 12 where it was inactivated at pH 2 and 3 and its optimal pH was in range of 6 to 10. The PhageTU-P53 phage showed lytic activity even in acidic condition at pH2 and it completely lost its activity at pH 12 and optimal pH for this is 6 to 10 where no any significant decrease in phage titre.

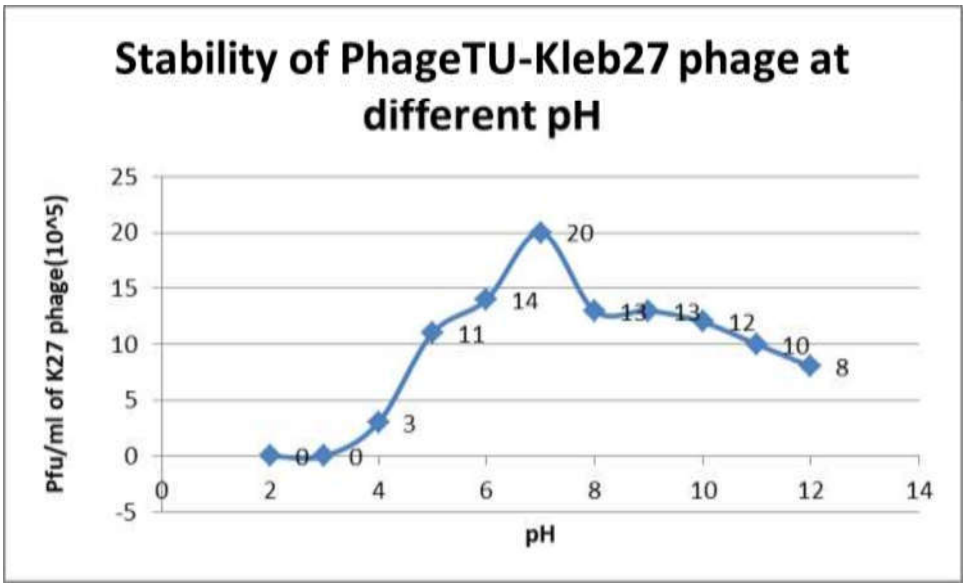


Figure 4. 19: Graphical representation of pH versus Pfu/ml of PhageTU- Kleb27 to check stability in different pH.

Phage was incubated under different pH values for 60 min in pH adjusted LB broth at room temperature (25˚c). It showed wide range of pH activity, and number in each point indicates the total number of plaques formed in each plate after DLLA.

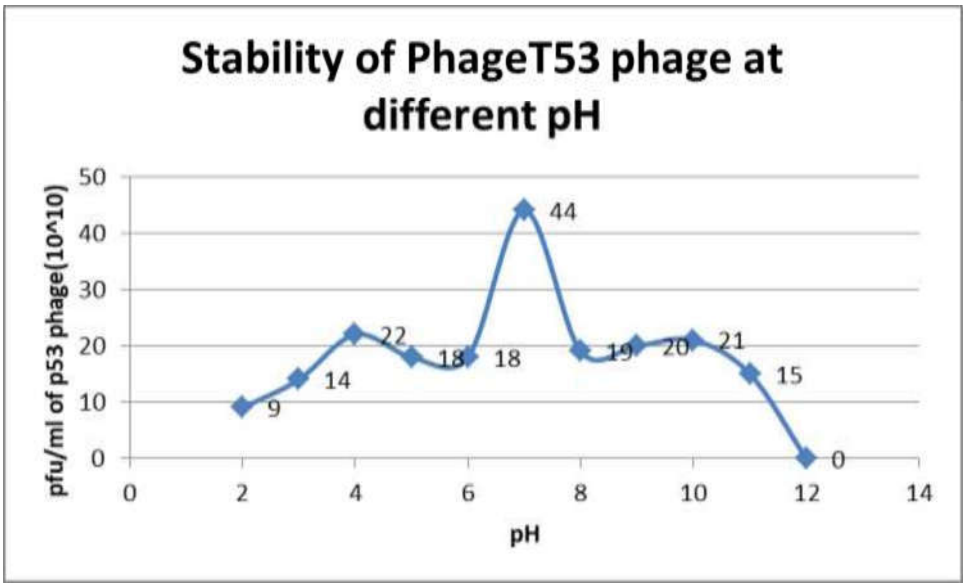


Figure 4. 20: Graphical representation of pH versus Pfu/ml of PhageTU-P53 to check stability in different pH.

Phage was incubated under different pH values for 60 min in pH adjusted LB broth at room temperature (25˚c).It showed wide range of pH activity and it shows lytic activity even in lowest pH 2 and number in each point indicates the total number of plaques formed in each plate after DLLA.

4.15. One Step growth curve experiment

One step growth curve was performed to determine Latent period and burst size of the phage infection. The change in the number of phages during one replicative cycle was calculated.

Following the initial steady period, the rise in phage titer was observed after 20 minutes. Therefore, the latent period for both phage K27 and P53 phage was identified as 20 minutes. P53 phage was found to be the latent period of 20min, burst time was found to be 10min and burst size was computed to be 160 phage particles per infected cell. Similarly, phage K27 was found to be the latent period of 20min, burst time was found to be 10min and burst size was computed to be 20 phage particles per infected cells. The latent period and burst size were determined according to Hyman and Abedon, (2009). The latent period was identified according to time in between after phage absorption and the initial rise in plaque number. The burst size was identified by dividing the average of pfu/infected-cell in the post-rise period of growth curve by the average of pfu/infected-cell in the pre-rise period of the growth curve.

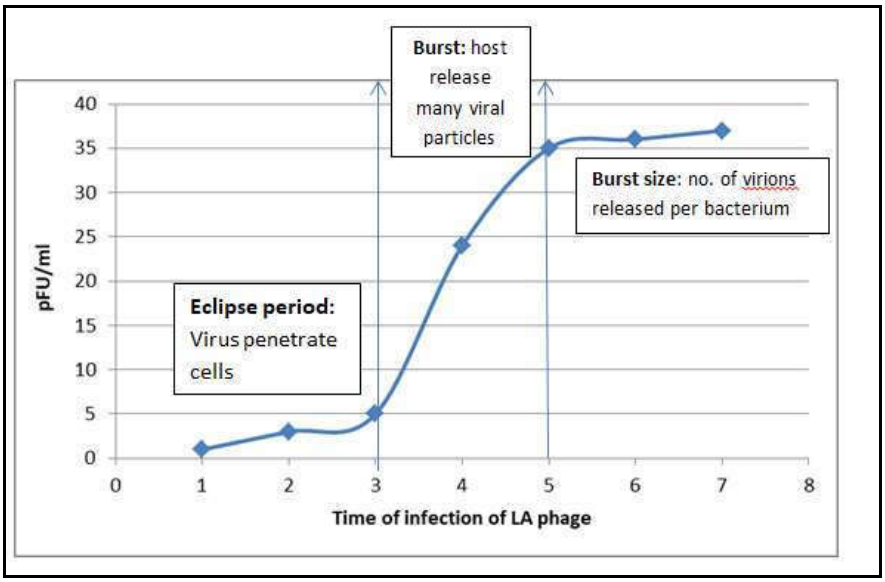


Figure 4. 21: One step growth curve of PhageTU-Kleb27

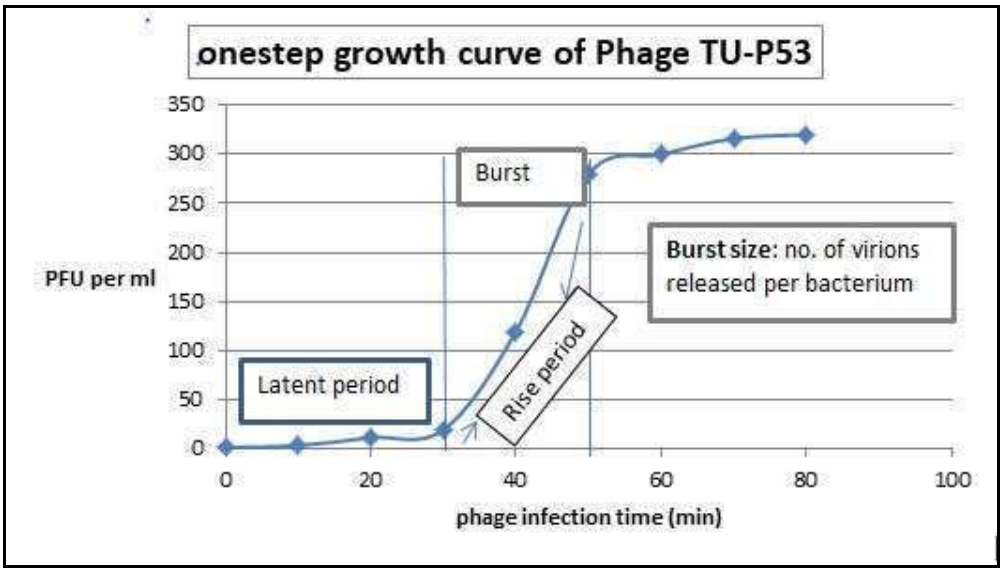


Figure 4. 22: One step growth curve of Phage TU-P53

4.16. Phage genomic study

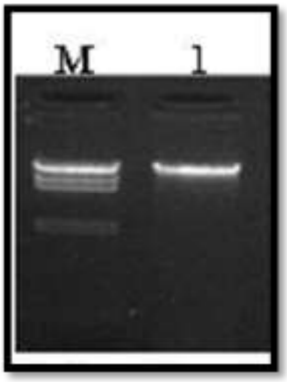


Figure 4. 23: Gel electrophoresis of phage DNA on 1% agarose gel Lane M: Hind III marker Lane 1: Phage TU-Kleb27

A common prerequisite to all DNA sequencing technologies is the necessity for high-quality nucleic acid preparations, free from contaminating RNA, proteins or solvents. Genomic DNA was extracted by cracking the phage capsid with heat and proteinase K by using Norgen Phage DNA Isolation kit and purified. The DNA of Phage TU-Kleb27 showed A280/260 values of ~1.85 and formed a clear, sharp band on agarose gel electrophoresis. After gel electrophoresis, compared with ladder the genomic DNA of the phage was found to be 23130bp.

Table 4. 8: Quantification using Qubit Fluorometer

SN	Phage name	Concentration (ng)	Yield (µg)	A260/280
1	K27phage	102	4.0	1.85

4.17. Bioanalyzer profiles of library loaded in Agilent DNA HS chip:

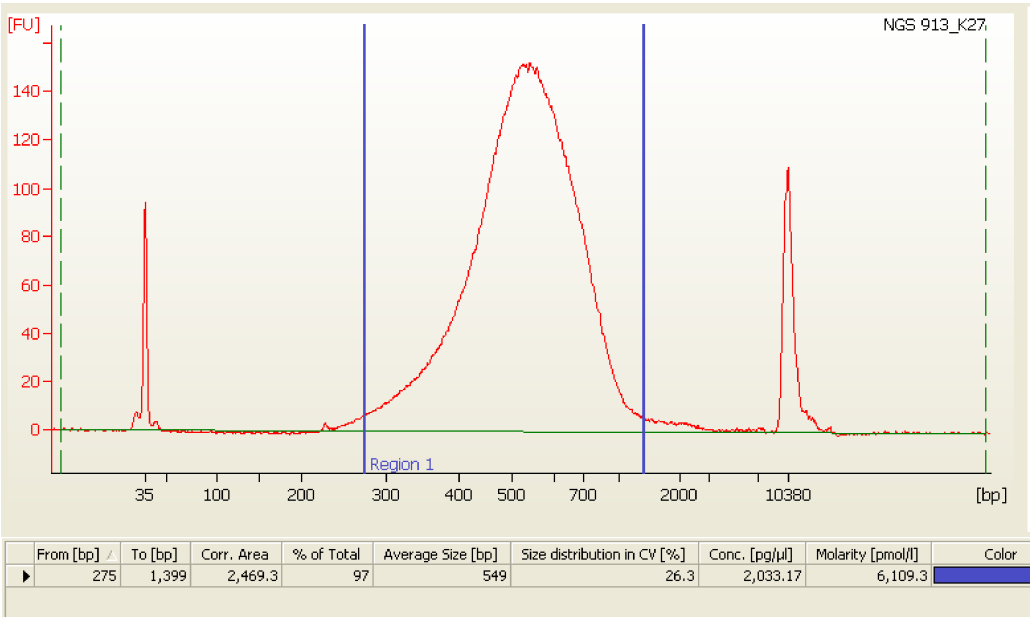


Figure 4. 24: Bioanalyzer profile of PhageTU- Kleb27

The library was prepared from the samples Phage TU-Kleb27 by Truseq Nano DNA Library preparation kit. The average size of library of this phage is 549 bp. The library of Phage TU-Kleb27 was then sequenced on Illumina platform (2 x 150 bp chemistry) to generate ~1.5 GB data.

4.18. Data generation

The paired end data was generated on Illumina platform (2 X 150 chemistry)

Table 4. 9: Data generation on illumine platform

File Name	Total Reads	Total Bases	Data in GB
K27_Phage_Stock_R1.fastq.gz	23266482	3483316312	3.48
K27_Phage_Stock_R2.fastq.gz			

4.19. Denovo Assembly

Assembly is the process of merging reads together to reconstruct original sequences. The most popular de novo assemblers are based on the De Bruijn graph [28] to merge overlapping reads into contigs. 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). A scaffold is a portion of the genome sequence reconstructed from end-sequenced whole-genome shotgun clones. Scaffolds are composed of contigs and gaps. Denovo assembly found 7480 scafolds platforms in PhageTU-Kleb27. Most of the phage scaffolds were 30-50 kbp long in size. Except some scaffold (4, 6, 23) were quite large (400-500bp).It may be due to host genome contamination.

Table 4. 10: Statistical elements of genome assembly

Description	Phage TU-Kleb27
No. of scaffolds	7480
No. of contigs	8327
Total genome length including gaps (in bps	8575500
Total genome length without gaps (in bps	8521860
Average scaffold size (in bps)	1146
Scaffold N50	93957
Maximum scaffold size	506656

4.20. Genome Annotation

Blastn analysis was carried out taking the scaffold sequences as query sequences. The assembled scaffold sequences were searched for similarity against NCBI's NT database.The E value of 10-6 was used as filter to retain significant blast hits and remove short ambiguousalignments. Scaffold sequences homologous to phage scaffolds were identified based on the blastN hit description.Further Blastx was done for functional annotation. In PhageTU- Kleb27, 46 scaffolds were found to share homology with phage. It was found that majority of the hits were correspond to Pseudomonas phage followed by Klebsiella phage in PhageTU-Kleb27 as shown in

below figure of species distribution of phage. The Top hit species distribution was plotted based on count of hits obtained from blastx functional annotations in which It showed that the top most hit was found to homologous with pseudomonas phage PAE1 with 40 BLAST top hits.Blast was done in scaffold representing different phages and other organisms also. From, Bastn of scaffolds it was found that it was similar with Pseudomonas phage and Klebsiella phage. As species distribution did not precisely predict the relatedness of phage genome to their respective phages, we have relied on the results of PHASTER which is a curated phage database. For the identification of phage further annotation and gene prediction was done.

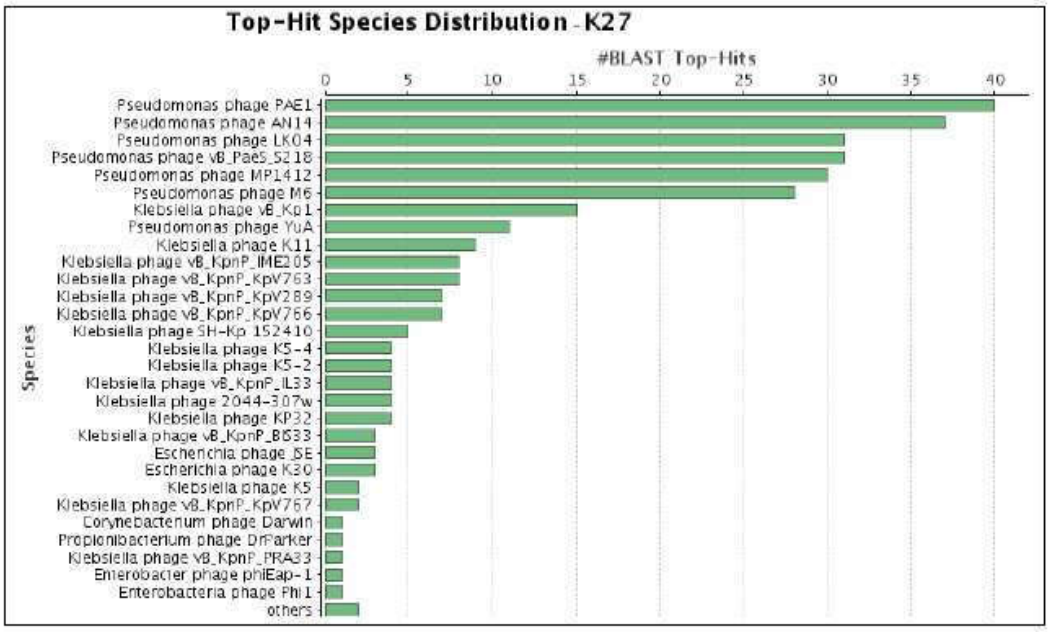


Figure 4. 25: Top hit species distribution for PhageTU- Kleb27

The X-axis represents the count of hits obtained from blastx functional annotations.

The putative genes that is the scaffold homologus to phage were identified with Prodigal tool v.2.6.3 which is highly accurate gene finding programme.In total 170 genes were predicted in Phage TU-Kleb27 with a average gene size of 592bp and maximum size was found to be 3966bp.All the predicted genes were annotated evaluating the homology by blastx search against NR database (Non-Redundant database).The snapshots of the blastx analysis of phage was given in table.Scafold 35 of PhageTU-Kleb27 98% similar with klebsiella phage (KP1 and Kp11) and the gene of DNA polymerase, exonuclease,head-to-tail joining, capsid scaffolding,major capsid and tail fiber are present in this scaffold. It can be said that our phage is tailed phage containing tail fiber protein.

Sequence name	Sequence description	Sequence length	Hit description	Hit accession	E-Value	Similarity	Alignment length
gene_3_scaffold_35	DNA polymerase	2118	gi|338826830|ref|YP_004678744.1|DNA polymerase [Escherichia phage K30] gi|335335293|gb|AEH41037.1| DNA polymerase [Escherichia phage K30]	YP_004678744, AEH41037	0	99.29	705
gene_7_scaffold_35	exonuclease	906	gi|966200899|ref|YP_009190981.1|exonuclease [Klebsiella phage vB_Kp1] gi|940325618|gb|ALJ98084.1| exonuclease [Klebsiella phage vB_Kp1]	YP_009190981, ALJ98084	0	99.66	301
gene_12_scaffold_35	head-to-tail joining	1608	gi|1093424764|gb|AOZ65559.1|putative head-to-tail joining protein [Klebsiella phage vB_KpnP_KpV766]	AOZ65559	0	99.43	535
gene_13_scaffold_35	capsid scaffolidng	957	gi|194100449|ref|YP_002003822.1|gp9 [Klebsiella phage K11] gi|193201388|gb|ACF15866.1| gp9 [Klebsiella phage K11]	YP_002003822, ACF15866	0	98.74	318
gene_14_scaffold_35	major capsid	1032	gi|194100450|ref|YP_002003823.1|gp10A [Klebsiella phage K11] gi|193201389|gb|ACF15867.1| gp10A [Klebsiella phage K11]	YP_002003823, ACF15867	0	99.70	343
gene_17_scaffold_35	tail fiber	2376	gi|194100452|ref|YP_002003825.1|gp12 [Klebsiella phage K11] gi|193201391|gb|ACF15869.1| gp12 [Klebsiella phage K11]	YP_002003825, ACF15869	0	98.8622	791

Figure 4. 26: Snapshot of blastx functional annotation for sample K27.It showed that genes present at scaffold-35 represent different functions DNA polymerase, exonuclease, and Head-to tail joining, capsid and tail fiber protein.

4.21. Genome analysis of Phage: PHASTER annotation

PHASTER (PHAge Search Tool Enhanced Release) is an upgrade web server for the rapid identification and annotation of the phage sequence. It is faster, better, easier to use and robust technique for phage genome analysis. PHASTER generated circular genome of sequenced phages which gives the information about the genome size, number of CDS region, GC content and predicted the location (start and end) of the prophage genome on the host genome.Circular genome map of PhageTU-Kleb27 was created by phaster with approximate genome size is of 8521.86 kbp. It showed that total 14 prophages in which 7 regions were intact, 6 regions are incomplete and 1 region are questionable. 8 prophages regions have identified from scaffold 4, 15, 22,24 ,32,34 ,35 and 144.From the phaster , it is found that some of the scaffolds have very good homology with some known phages as given in below snapshot.

Further, we analysed two scaffolds 24 and 35 which showed Klebsiella phage from phaster. The scaffold 24 seems to be a plasmid and scaffold 35 seems be a phage from BLAST output which was shown in Appendix.

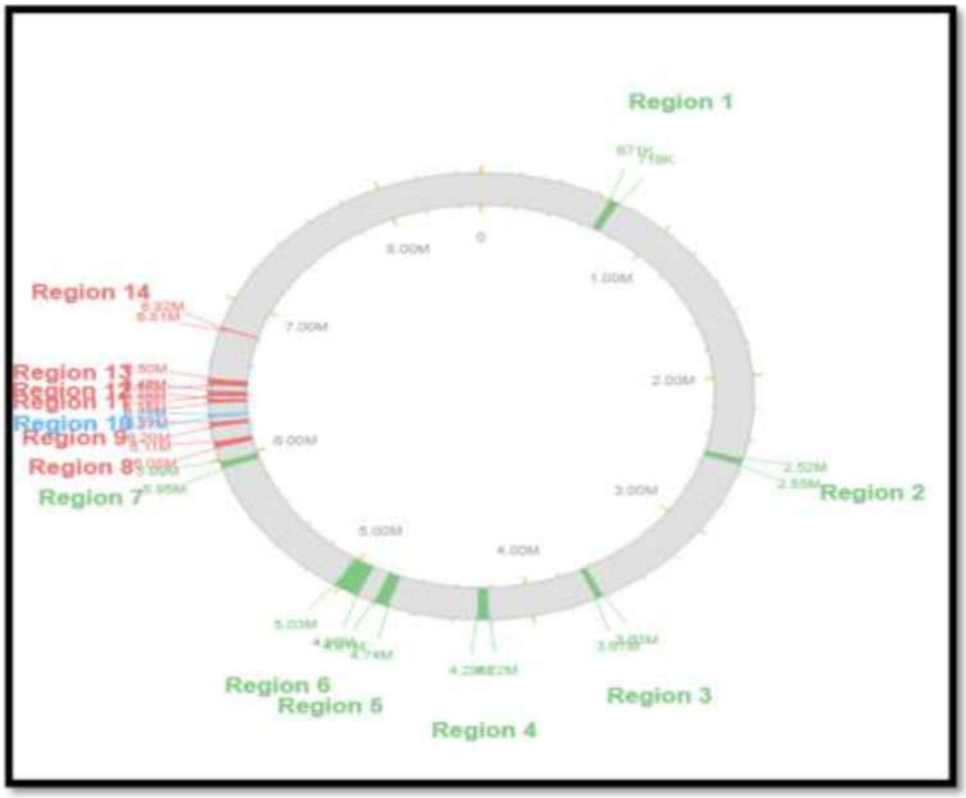


Figure 4. 27: Circular genome map of Phage TU- Kleb27 generated by Phastrer .It showed 7 intact regions along with 6 were incomplete and 1 is found questionable.

Length: 8521860 bps

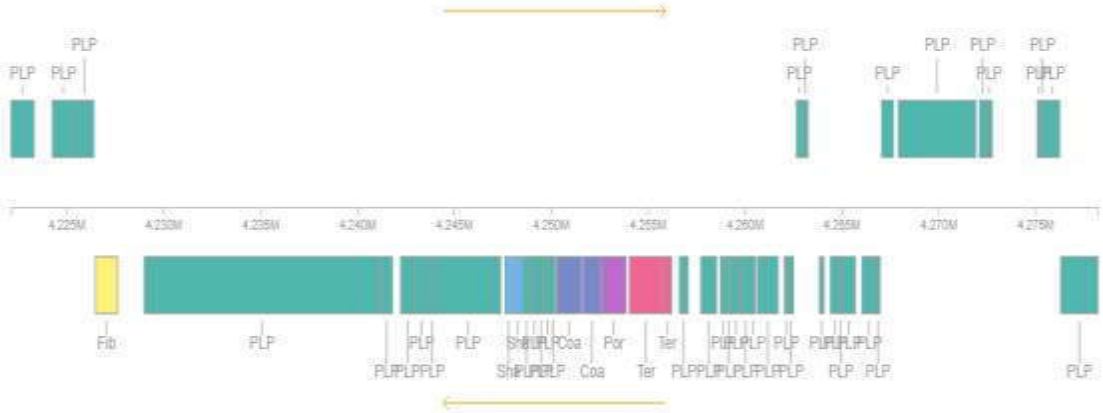


Figure 4. 28: Linearized genome annotation of Phage TU_Kleb27 (region 4) in which GC% was found to be 53.90%.

The upper arrow represents forward direction of ORF whereas lower arrow represents the backward direction/strands of ORF. The region position value predicts the location of prophage genome on host genome.

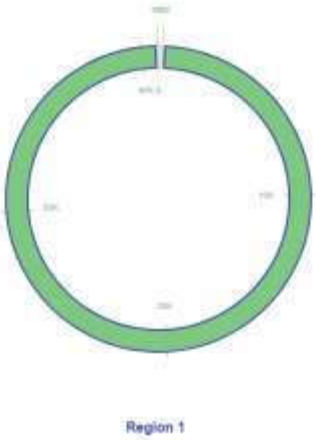


Figure 4. 29: Circular genome of Scafold 35 prophage region 1 which contains 53.01% GC% and 45 CDS region.

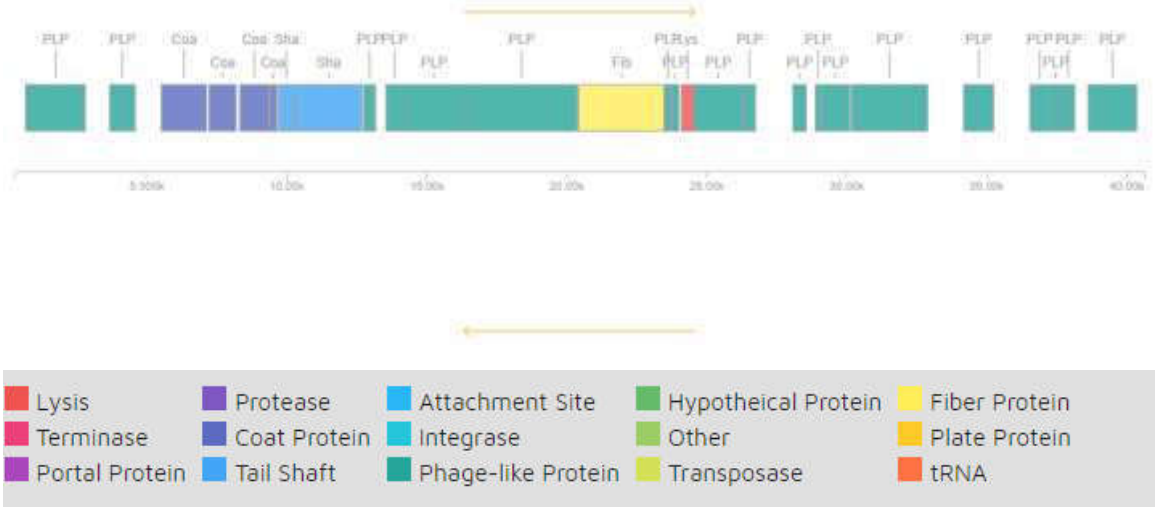


Figure 4. 30: Linearized genome annotation of Scafold 35 of PhageTu-Kleb27 which resembles with klebsiella phage.

4.22. Genome analysis :RAST Annotation

RAST (Rapid Annotation using Subsystem Technology) is a fully-automated service for annotating complete or nearly complete bacterial and archaeal genomes. It provides high quality genome annotations for these genomes across the whole phylogenetic tree. The RAST annotation showed that the total GC % of the PhageTU-kleb27 was found to be 59.8%. The sequence size uploaded for annotation was 110829 and after soplitting into scaffold it was of 109986bp size.Typical features that can be found in a phage genome include protein-encoding genes, noncoding RNA genes, insertion elements and transposons, direct and indirect repeats, origins of replication, and attachment or integration sites.The RAST annotated the genes and gives

information about Gnee identification, t RNA identification, r RNA identification ,functional annotation and subsystem assignment.During subsystem assignment, only 13% are in subsystem distribution with total 21 proteins as shown in above figure.The subsystems cover all modules of cellular machinery and not just the metabolic pathways.The important features was found to be there are no any transposable elements, virulence and toxic genes and there are no any genes that are transferable from host to phage.This is desired features of the phages to used as phage therapy.Similarly ,phage proteins were annotated with different features, tail proteins, capsid protein, assembly ,lysis and holing gene etc. Subsystems-based technologies were developed in the SEED with the view that the interpretation of one genome can be made more efficient and consistent if hundreds of genomes are simultaneously annotated in one subsystem at a time".

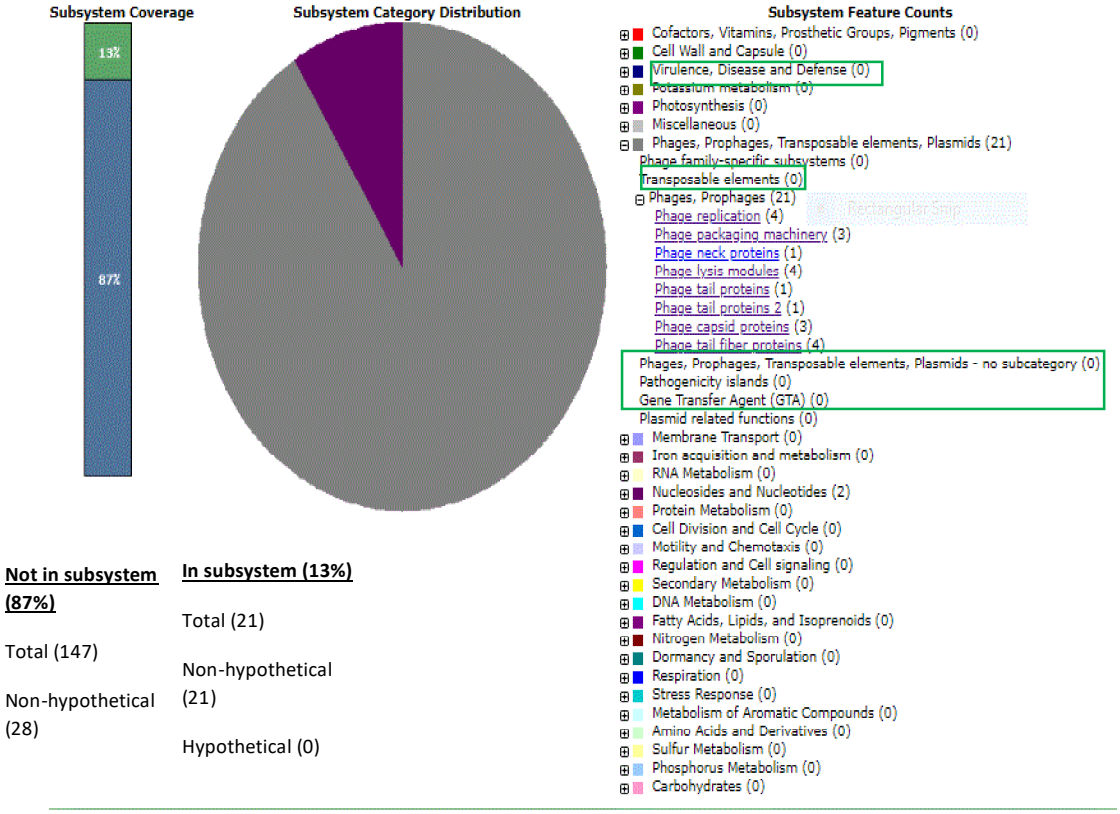


Figure 4. 31: RAST annotation of PhageTU-Kleb27 in subsystem statistics.

Genes connected to subsystems and their distribution in different categories.The entire cellular metabolism is given in figure. The highlighted green box showed that there is no any virulence gene and transposons and gene transfer elements.Blue colour letter showed that there is presence of phage tail protein, lysis and holin gene.

4.23. Phylogenetic tree construction

Phylogenetic analysis of Phage TU-Kleb27 was also carried out to elucidate the evolutionary history of the phage.As our phage genome was not completely anotated so we only analysed tail protein and capsid protein for phylogeny.To reveal the relationship between PhageTU-Kleb27 and other member classified, the phylogenetic analysis of major capsid protein of PhageTU-Kleb27 and tail fiber protein of same klebsiella phage with various related species were analysed separately by using phylogeny .fr with character based approach of phylogenetic analysis (Maximum likelihood method).It showed that major capsid protein of PhageTU-Kleb27 is closely

related with klebsiella virus Kp32isolate 192 and klebsiella phage VBkpnpkpv763 which are all the clades with most common ancestor .While,other different klbsiella phages are closely related but are not the clades in which Enterobacter phagephiEap-1 is also closely related apart from klebsiella species. However, Enterobacter phageTS, BacteriophagephiYe03-12 Escherichia PhageECA2, klebsiella phage Sci are distantly related with PhageTU-Kleb27 however, the distantly related species are clades of each other further, Psedomonas phage UNOSLW4 is distantly related to PhageTU-Kleb27 capsid protein as displayed in the tree with TreeDyn.

From the tree diagram of tail protein of PhageTU-Kleb27 with different species it showed that it is closely related with eight different klesiella phages as well as salmonella phage SG-162. However, it is distantly related with Enterobacter phage E-3.

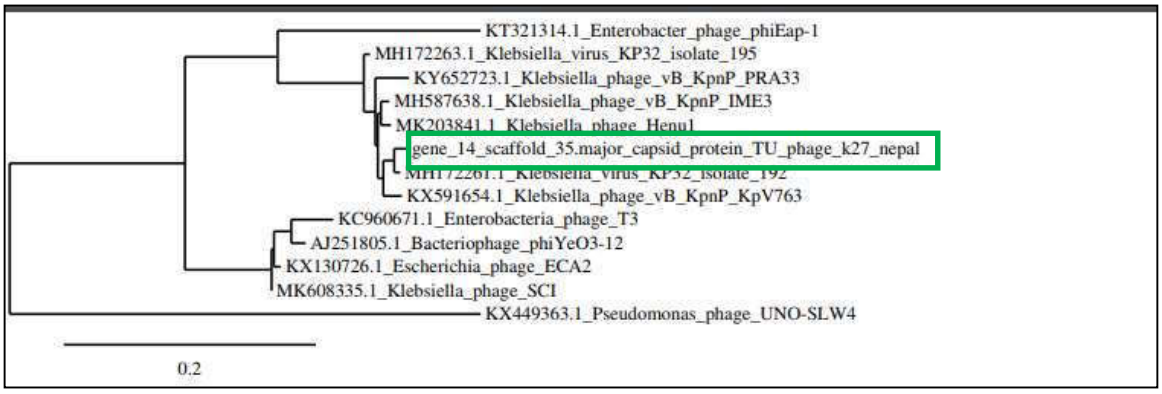


Figure 4. 32: Phylogenetic tree construction of PhageTu-kleb27 major capsid protein by phylogeny FR.online server

Capsid protein of gene 14 scafold 35 PhageTU-Kleb27 .The GenBank accession numbers are also provided before phage name.This tree showed the relationship of Capsid of PhageTU-Kleb27 with closely and distant bacteriophage.

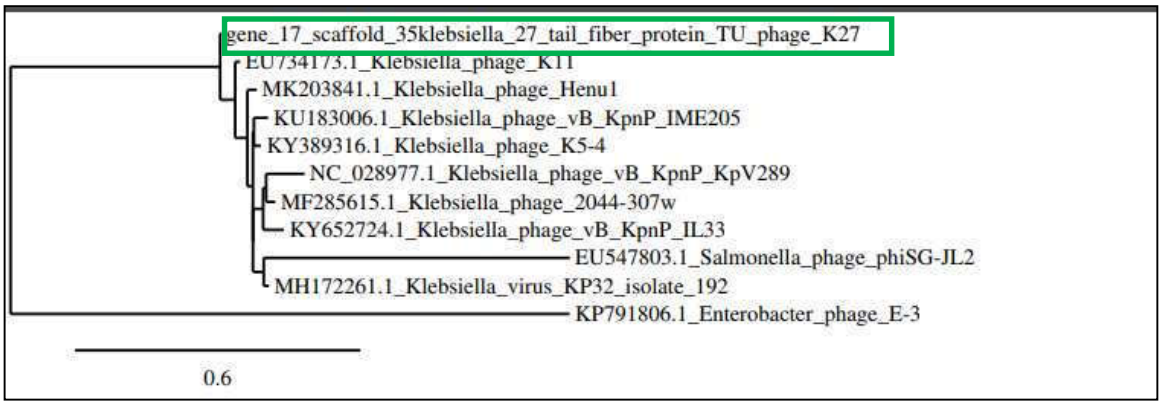


Figure 4. 33: Phylogenetic tree construction of PhageTu-kleb27 tail protein by phylogeny fr online server.

Tail fiber protein of gene 17 scafold 35 PhageTU-Kleb27 .The GenBank accession numbers are also provided before phage name.This tree showed the relationship of Capsid of PhageTU-Kleb27 with closely and distant bacteriophage.

4.24. Biofilm detection in bacteria

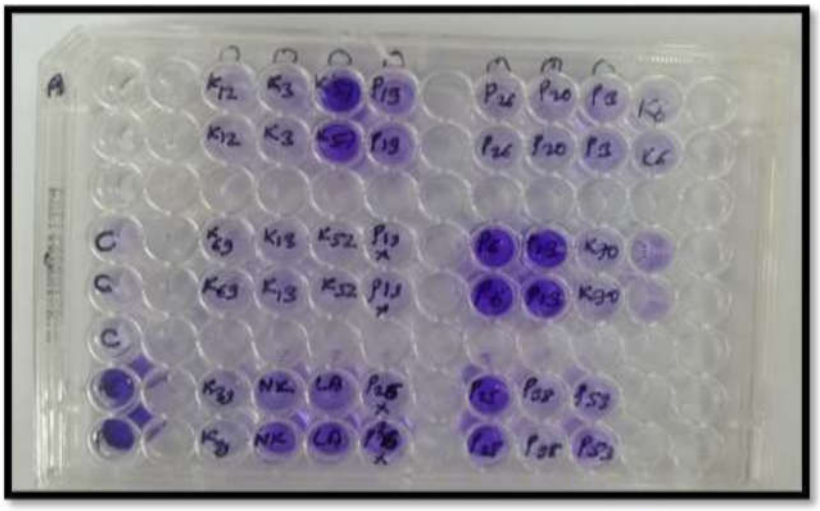


Figure 4. 34: Biofilm detection in ELISA plate reader using 1% crystal violet.Only LB broth is used as control and Positive control is used as PAO1. Intense blue colour indicates the strong biofilm producer group. K57 and P25 bacterium were found to be strong biofilm producer.

Biofilm detection is done by tissue culture method using crystal violet (0.1%) stain.All the strains were categorized into the following categories: non-adherent (0), weakly (+), moderately (++), or strongly (+++) adherent, based upon the ODs of bacterial films. We defined the cut-off OD (ODc) for the microtiter-plate test as three standard deviations above the mean OD of the negative control. Strains were classified as shown in table. All tests were carried out three times and the test results were averaged.

Table 4. 11: Calculation of cutoff value Table

Mean OD of NC	0.1035
SD of NC	0.006795
3× SD of NC	0.020385
Cut off value	0.123885

Table 4. 12: Reference value calculated

Od value.	**Biofilm Production**
≤ 0.123885	Negative
0.123885 – 0.24777	Weak
0.24777-0.49554	Moderate
> 0.49554	Strong

Table 4. 13: Classification of biofilm producer based on optical density

SN	Bacteria	Mean OD at 600nm	Inference
1	K12	0.152	Weak
2	K3	0.14275	Weak
3	K57	1.3435	Strong
4	P19	0.967	Strong
5	P26	0.12925	None
6	P20	0.118	None
7	P3	0.1755	Weak
8	K6	0.12175	None
9	K69	0.1065	None
10	K13	0.132	Weak
11	K52	0.104	None
12	P6	1.235	Strong
13	P13	1.149	Strong
14	K70	0.1225	None
15	K39	0.1265	Weak
16	NK	0.3625	Moderate
17	LA	0.46825	Moderate
18	P26*	0.29925	Moderate
19	P25	0.90625	Strong
20	P38	0.1345	Weak
21	P53	0.24425	Weak

The Tissue culture plate assay is an important tool for the study of the early stages in biofilm formation and has been applied primarily for the study of bacterial biofilms. The biofilm mode of bacterial survival and growth is now being seen as a serious threat to public health and awareness about such cases draws importance among the scientific as well as social communities. The bacteria residing in biofilms have enhanced extent of virulence pathogenicity as well as antibiotic resistance.

4.25. Application of Bacteriophage: Biofilm Disruption by Bacteriophages

The biofilm formation in 8 different clinical isolates and phage treatment in biofilm disruption were tested using the microtitre dish biofilm assay.Absorbance readings from the crystal violet biofilm assays of al isolates at 48h after phage treatments were displayed in Figure.In this study, measurement of biofilm density made using crystal violet/OD620 over 24h showed a clear reduction following phage inoculation compared with non-treated controls. Thus, it showed promising result in disruption of bacterial

biofilm. Decrease in OD after addition of phage was assumed to be distrupted of bacterial growth by phage. The biofilm reduction by the PhageTU-Kleb57 was 64.95% for K3 bacteria, 72.07% for K6 bacteria and 58.17% for K57 bacteria for its own host.Similarly, PhageTU-Kleb27 showed 57.46% reduction for K27 bacteria and PhageTU-KlebNK showed 45.49% reduction for its own host NK bacteria. Incase of Pseudomonas phage, the biofilm reduction by PhageTU- P19 is 68.85% for P6 bacteria and 62.14% for P26 bacteria and reduction by PhageTU-P53 was found to be 55.87% for P53 bacteria. Statistically significant reduction in biofilm biomass were seen in all phages at ($p<0.05$) was assessed by Student's t test.Phages P19, P53 and K57 showed good result even in control of biofilm produced by other host strain. From above graph, it is found that there is significant decrease in reducing the biofilm produced by bacteria .In this study, phages showed significant reduction in other host bacteria compared to it sown host.It showed that phages are capable to clear the biofilm of other host showing wider host range.PhageTU-P19 phage is capable of disruption of biofilm produced by P25 and P6 bacteria and PhageTU-P53 phage disrupts the biofilm of P53 bacteria. Similarly, PhageTU-Kleb27, PhageTU-kleb57, PhageTU-KlebNK phage along with its host, PhageTU-Kleb57 disrupts the biofilm produced by K6 and K3 bacteria.

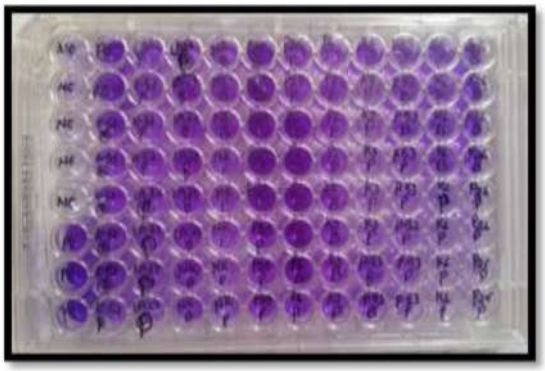


Figure 4. 35: Microtittre plate showing formation of biofilm as well as disruption of biofilm by bacteriophage.

NC denotes negative control where only LB broth was used. PC control indicates positive control in which PAO1 strain was used.Each sample was tested in 4 well, B indicates bacteria and P denotes the bacteria infected with phage.

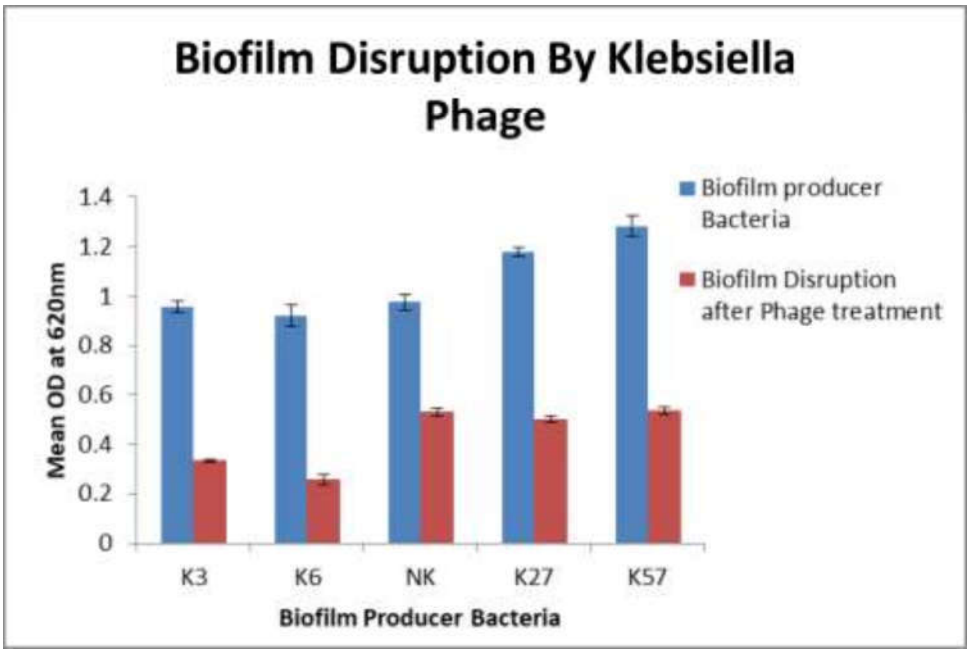


Figure 4. 36: Graphical representation of biofilm disruption by klebsiella phage.

Twenty four hour biofilms of the indicated 5 different strains of *Klebsiella pneumoniae* were exposed for 4 h to the different klebsiella phages.The reduction of the biofilm biomass following treatment with the phage was compared with that following no treatment by measuring the OD620 after crystal violet staining.The blue colour indicates that biofilm producing bacteria not treated with phage and red colour showed the treatment with phage which indicates disruption of biofilm. It showed that significant decrease in biofilm after addition of bacteriophage. The biofilm reduction by the PhageTU-Kleb57 was 64.95% for K3 bacteria, 72.07% for K6 bacteria and 58.17% for K57 bacteria for its own host.Similarly, PhageTU-Kleb27 showed 57.46% reduction for K27 bacteria and PhageTU-KlebNK showed 45.49% reduction for its own host NK bacteria. The errorbars indicate standard error, and the statistical significance of the biofilm reduction (at $P < 0.05$) was assessed by Student's t test.

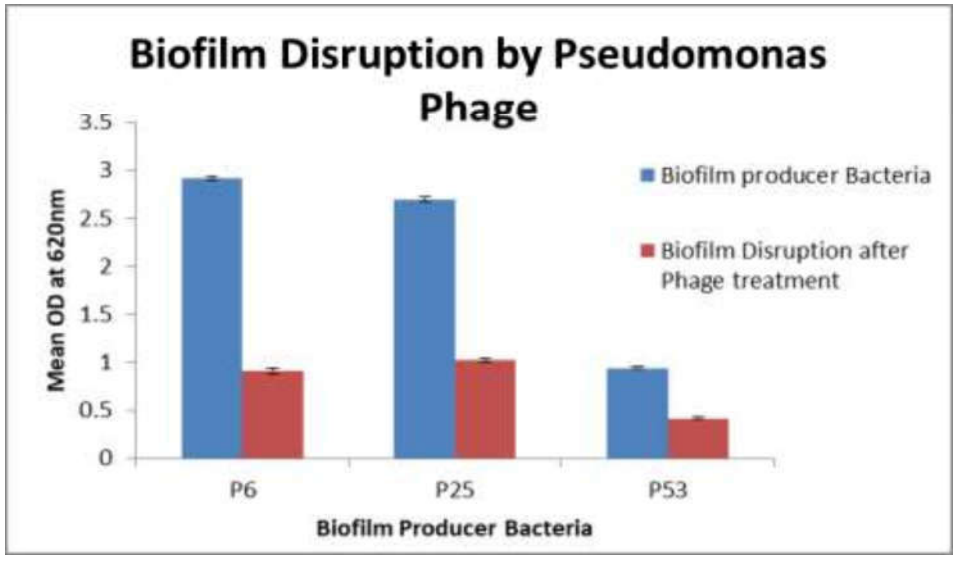


Figure 4. 37: Graphical representation of biofilm disruption by Pseudomonas phage.

Twenty four hour biofilms of the indicated 3 different strains of *Pseudomoas aeruginosa* were exposed for 4 h to the different klebsiella phages.The reduction of the biofilm biomass following treatment with the phage was compared with that following no treatment by measuring the OD620 after crystal violet staining.The blue colour indicates that biofilm producing bacteria not treated with phage and red colour showed the treatment with phage which indicates disruption of biofilm. It showed that significant decrease in biofilm after addition of bacteriophage. The biofilm reduction by PhageTU-P19 is 68.85% for P6 bacteria and 62.14% for P26 bacteria and reduction by PhageTU-P53 was found to be 55.87% for P53 bacteria. The errorbars indicate standard error, and the statistical significance of the biofilm reduction (at $P < 0.005$) was assessed by Student's t test.

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CHAPTER FIVE

DISCUSSION

Multidrug resistance bacteria are emerging across the globe and this resistance is being a critical problem even in carbapenem drugs. As, WHO has priotrized carbapenem resistant enterobactericiae as crtical group, resistance developed even in last resort drug has become serious threat causing various infections which is increasing the number of morbidity and mortality of patients. Along with several intrinsic and extrinsic mechanisms, misuse and overuse of antibiotics with inadequate and improper use results the resistance to antibiotics. In, Nepal increasing carbapenem resistant gram negative bacteria seems to create the major public health problem. Discovery of potential alternative therapeutic agent has regained much attention.

The prospects of lytic phages as potential antimicrobial agents gainst pathogenic multidrug resistant bacteria are being reconsidered worldwide (Sulakvelidze & Alavidze, 2001). Recent review also hints the potential of phages as alternatives to antibiotics and for phage therapy.Phages are natural viral pathogens of bacteria. They are the most abundant entities in the biosphere, with total estimated numbers ranging from 10^{30} to 10^{32} (Kutter et al., 2004).Advantages of bacteriophages and their components include activity against antibiotic resistant bacteria and targeting of a very narrow-spectrum of infecting pathogens, with minimal impact on the resident microbiota.

The emergence and dissemination of carbapenem resistance among Enterobacteriaceae, especially *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* constitute a serious threat to public health, since carbapenems are the agents of last resort in the treatment of life-threatening infections caused by drug-resistant Enterobacteriaceae. One particular group of transmissible plasmid-encoded carbapenemase enzymes, designated *K. Pneumoniae* carbapenemase (KPC), confers carbapenem resistance to enterobacterioceae is rapidly spreading worldwide (Tabassum et al., 2018).

KPC is the most common class A carbapenemase and is encoded by the blaKPC gene. Along with its variants KPC-2 to KPC-13, which differ only in terms of amino acid mutations, it has spread globally. The blaKPC gene is plasmid-mediated and is transported in a Tn3-based transposon, Tn4401, which makes it readily transferable between bacterial isolates. Indeed, although most often found in *K. pneumoniae*, blaKPC genes have also spread to other enterobacteria and to *Pseudomonas aeruginosa* (Naas et al., 2008).

NDM-1-producing *K. pneumoniae* are an emerging group of highly resistant bacteria that have spread from the Indian subcontinent across the globe. Encoded by the blaNDM-1 gene, NDM-1 is encoded on a readily transferable plasmid. NDM-producing bacteria are resistant to many groups of antibiotics, including fluoroquinolones, aminoglycosides and β-lactams (especially carbapenems). Many NDM-1 producers remain susceptible only to

tigecycline, colistin and, to a lesser extent, fosfomycin .NDM-1 is mostly associated with travel to the Indian subcontinent, where it appears to be endemic. However, it has also been reported in other countries, including China, Australia, the United States, Canada and many parts of Europe, most recently the Balkan region (Dortet et al., 2014).

In this study, we screen, isolate and characterize the lytic bacteriophage that is potential to use as biocontrol and therapeutic agent against Carbapenem-resistant *K.pneumoniae* (CRKP) and *Pseudomonas aeruginosa* both can cause several types of healthcare-associated infections, including pneumonia, bloodstream infections, wound or surgical site infections, urinary tract infections and meningitis, which are associated with high mortality rates.

Total 5 strains of *Klebsiella pneumoniae* and 3 strains of *P. aeruginosa* were used as host in the isolation of lytic phages from river water of Kathmandu valley. All of the bacteria except one strain viz: New kleb were recovered from clinical specimens.New Kleb strain was isolated from the environment (water). These bacteria were confirmed by 16SrRNA sequence analysis as *Klebsiella pneumoniae*. The use of 16S rRNA gene sequencing is one of the easiest methods for identifying bacteria (Janda & Abbott, 2007).

Among all Klebsiella strains, Kp41, Kp52, Kp57 were already identified as carbapenem resistant by amplification and sequencing of bla NDM gene. *K pneumoniae* K27 ,*Pseudomonas aeruginosa* P19 and P53 were confirmed as carbapenem resistant by amplifying bla NDM gene and further Kpc gene was found to be positive in K27 bacteria.Resistance to carbapenems in some species is intrinsic or in some case acquired by mutational events or gene acquisition via horizontal gene transfer and certain species are able to prevent carbapenems reaching their PBPs by diminishing the permeability of their outer membrane. OprD for example, is an outer membrane porin of *Pseudomonas aeruginosa* through which carbapenems enter its periplasmic space where PBPs are located. All beta-lactamases are categorized into four molecular classes according to the Ambler classification (Ambler, 1980). Among them, the class A enzymes KPC,GES/IBC , IMI/NMC-A , SFC-1, the class B MBLs IMP,VIM, NDM ,SPM , GIM, SIM, AIM , DIM, FIM, POM, and several class D (OXA-type) enzymes, possess the ability to hydrolyze at least partially a carbapenem antibiotic (Bush & Jacoby, 2010). The most effective carbapenemases, in terms of carbapenem hydrolysis and geographical spread are KPC, VIM, IMP, NDM and OXA-48 types. KPCs inactivate all beta-lactam antibiotics and are only partially inhibited by beta-lactamase inhibitors like clavulanic acid, tazobactam and boronic acid.

Isolation of Bacteriophage

In this study, total of 21 phages were isolated from 8 different water samples, against carbapenem resistant bacteria. Phages were isolated employing double agar overlay method of Adams,(1959). It helps to form greater uniformity of plaque and formed greater size plaque due to greater rates of phage diffusion in soft agar. Thus, soft agar

permits diffusion of phage to nearby infected cells but does not permit new phages to move to remote parts of plate. For the phage extraction/storage, we used SM (sodiumchloride and magnesium sulphate) buffer. The gelatin used 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 higher titre without enrichment. It shows that the rivers of Kathmandu valley are heavily polluted with human/animal excreta as well as waste produced by the nearby hospitals, industries and household without treatment and another major concern is that there is presence of pathogenic bacteria which may results serious outbreak of disease .The high abundance of phages in kathmandu valley river water is also supports by (Bhetwal et al., 2017) from Nepal, in which total 67 phages were isolated from 8 samples.Similarly work done by our Senior Roshan Nepal also found huge abundance with divergence of phages ie:34 lytic phages against all MDR clinical isolates (16 phages of *E. coli*, 13 phages of *Salmonella*, 2 phages of *Shigella*, 2 phages of *Klebsiella,* 1 phage of *Citrobacter*) from water samples of 5 holy rivers of Kathmandu valley (Nepal R, 2016).

In our study, the high phage titre was found to be in PhageTU-P53 from Balkhu and in NK from Basundhara sample at initial isolation.The difference in abundance rate of bacetriophages is due to difference in abundance of host bacteria in sample collection sites as bacteriophages grow only where its host grow.If the host bacteria and phage infection and consecutively phage proliferation may not occur as for most phage a successful multiplication requires at least 10^3 -10^4 cfu/ml bacteria.

We faced lots of problem in initial days during isolation of the bacteriophages, we tried in various samples but it was hard to find the phage. That might be due to either there is absence of phage in those particular host or there might be some error in our work. We keep on trying on different new sewage sample until we isolate the new bacteriophage. Finally, we isolated the phage on *Escherichia coli* bacteria which found easily in river water, which gave an idea to work in rest of the bacteria.It gave the clear plaques as shown in Appendix. Various factor determines the plaque formation; host culture, attachment time, sewage sample etc. In some water sample, there was absence of lytic phages that might be due to absence of host or error in water sample collection, which is collecting water without sediment. As phage particle tends to attach to sediment and over time these phage attached sediment aggregate in the bottom of the water body and with gentle shaking the ground floor phages are released into the water so it can be said that phages are generally more abundant in the sediment than overlying water.

Surprisingly we even isolated bacteriophages against extensively drug resistant bacteria from a holy river of fresh water source like that of Sali Nadi, Bhaktapur, a well known holy river for Hindu people and their impact was found to be significant as well. The phages were isolated against 2 bacteria from Sali nadi sample but unfortunately it could

not be amplified and later phages were isolated against these bacteria from different water sample. Similarly, various phages isolated were not amplified so that it took long time to isolate phage and further processing. Alltogether 21 different putative phages were isolated, only the lytic clear phage and which can be amplified easily were selected for further processing.

Plaque morphology, Purification of phages and titer determination

Various types of plaques were observed from small pin headed clear plaque to large clear plaque and the most dominant one is Bull's eyes plaque. Only the clear lytic plaques were selected for further processing. In one plate there were different size plaques which denoted the presence of more than one phage type 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 (Gallet et al., 2011). 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 plaques. And another fact is that the 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 pseudomonas phage (P19, P43 and P53). Bull's eye plaque form predominantly because phage-induced bacterial lysis is less efficient or complete later on 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 (www.phage.org).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 other bacteria's CPS (Adams M H, 1959).The Bull's eyes plaque morphology of klebsiella phages were similar with the phage produced by Kumari et al against *K. pneumoniae* B5055 where plaques were surrounded by a large halo, indicating the production of large amounts of depolymerase enzyme (Kumari et al., 2010) .Similarly, same type of plaque morphology was seen in bacteriophage ZCKP1 against *K. pneumoniae* (Taha et al., 2018).The formation of

halozones is an indicator for the presence of phage tail-associated exopolysaccharide (EPS) depolymerase (Cornelissen et al., 2011). Thus from halo forming morphology, it can be assumed that the isolated halo phages are of tailed phage.

The concentration and purification of virus particles are essentials for structural and functional characterization of phages .All phages were purified and concentrated and titer was determined before further characterization. Three round of streaking method is applied to obtain single pure plaques and the phage solution was further filtered through syringe filter.In this study we did not use PEG precipitation. For the characterization of phage and TEM and sequencing higher titer value is preferred so we made the phage stock of higher titer.During titer determination, in serial dilutions plate web lysis pattern were observed from initial lower titer plates (web lysis pattern in Appendix).The threadlike pattern on the surface of an agar plate were observed called web pattern or web lysis due to complete lysis of bacterial lawn by phage .

Host range analysis

Host range is the key characteristics of bacteriophage for use in therapeutics. Spot assay is a quick way to check whether a phage sample can infect a bacterium by placing a small drop or “spot” of phage 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, we found that, each phage had a distinct host range, with no individual phage being able to lyse all strains. PhageTU-Kleb 57 and PhageTU-P53 were found to be effective with broad host range in lysis of different bacteria.PhageTu-Kleb 27 was found to be of naarow host range.The PhageTU-Kleb27 being narrow host range represents the specificity of bacteriophage which lyse only the targeted bacterial populatios.From the result it was found that the phage cocktail was able to lyse a range of bacterial strains broader than the range that any of the individual phages that made up the cocktail could lyse. As expected, the cocktail phage cocktail had a broader host spectrum for all MDR strains and cocktails of phage were found to be typically more effective. After triplicate spot test, of positive result it was confirmed that lysis was done by phage rather than any bacterial toxins. Spot test is the most common way of testing host range, in which a small volume of phage is placed on a growing lawn of bacteria. This is simple and rapid, this technique can sometimes cause false positives because of lysis of bacterial cells without phage infection.Further it canbeconfirmed by killing assay which is not done in our study.To explore the host range intercations and lysing capability we can analyze through killing assay.Wider host range may be due to production of Depolymerase enzyme.López-Cuevas et al. (2011) hypothesised that the differences of host range might be due to environmental origin of bacteria tested in which a loss of bacteriophage receptors may have happened as a result of antagonistic co-evolution between bacterium and bacteriophage (López-cuevas et al., 2011). Similarly, it could be associated with the prevention of adsorption by bacterial receptor

mutations or with degradation due to restriction or modification of the resistance bacterial system (Abedon et al., 2010).

Interestingly, the cocktail of klebsiella phage lysed 3 carbapenem resistant *Acinetobacter baumannii* strains (A8, A17 and A70) but when tested with single Klebsiella phage, they did not show any lytic effect. This means there is synergistic effect between the phages during infection process. Research conducted by Schmerer et al. (2014), observed that the size of plaques formed by two phages J8-65 and T7 is larger than the plaque formed by single one (Schmerer et al., 2014). The result of our study is different than others. Only cocktail was able to lyse the bacteria not by individual phage alone. By this result it can be predicted that such synergy effect might be due to the one phage facilitate the infection to the same bacterium by providing certain mechanism so that another phage easily cause infection. This might be also due to that pahages having the different types of receptors.Synergy offers a potential tool for improving phage therapy however process of 'synergy' between phages is relatively unexplored. In latest research, the synergistic action of phage cocktail was seen in lowering the burden of the wound and preventing the spread of infections in case of *Acinetobacter baumannii* infections in mouse model experimentSimilar with our result, in this study also the cocktail is composed of four phages that do not kill the parent strain of the infection (Regeimbal et al., 2016).Further killing efficacy of phage cocktail invivo and invitro experiment was remaining to study this synergistic action of klebsieela cocktail phage. While often the killing potential of a cocktail is greater than the combined killing potential of the single phage – referred to as synergy, it is important to note that phages in a cocktail may also interfere with each other and this was observed in food trials using E. coli phages CEV1 and CEV2, whereby CEV1 prevented CEV2 reproduction in *E. coli* cells. In this regard, it is critical that phages within a cocktail are compatible. Indeed, Gill and Hyman advise using phages which adsorb to independent bacterial receptors (López-cuevas et al., 2011).Thus, phage cocktail represents the new class of therapeutics, which is effective than antibiotics on the basis of mechanism of action and production .The preparation of phage library of diverse phages of well characterized with sequenced phages could give the rapid action of phage cocktail in therapeutics.

Storage of phage solution: Infection of host with phage

Low-temperature preservation techniques have been widely used to maintain phage stocksduring long-term period.We stored working phage at 4 °C in SM buffer which showed minimal reduction in titer for short-term storage.It is important to determine the optimalstorage conditions to ensure long-term phage stability. It is said thatbacteriophage susceptibility to storage and processing conditions differs among phages. Therefore, a one universal method of virion preservation does not exist. There were various method applied for phage preservation and storage ie: storage of lysates at 4°C, freezing and storage at -80°C or in liquidnitrogen, and storage of dried or lyophilized phages, are used most commonly.in this study ,phage storage with infection

with host was done where titer was decreased at a steady state .the resukt were different from Golec et all where it was sshowed that there is no any titer reduction in 12 month in E coli phage and staphylocuus phage while stored with infecting the host(Gonzalez-Menendez et al., 2018).The result may be different in this case due to different phages and due to the phage storage condition and buffer in which phage stored .The cryopreservation of bacteria in appropriate growth media supplemented with glycerol is a standard storagemethod in the majority of strain collections .It can be said that the storage of phage infected with host will be the best method for preservation as it would be a convenient technique for preservation of master stocks for industrial productionof phages.

Protein profiling of Bacteriophage

Analysis of phage proteins was done by SDS-PAGE through entire phage particles. Both major structural proteins and minor proteins were found to be in both isolated phages. Similar types of distict bands were observed in both types of phages, thus. It can be said that the major bands were capsid protein and minor bands were of tail protein which resembles with the size of capsid and tail protein of other phage. Further, it was confirmed by TEM analysis.Thus this is the presumptive test for the structura protein analysis of phage from crude whole phage solution. It was said that even though the phage belongs to the same family, the structural proteins are unique to each phage and depends on their morphotypes (Yoon et al., 2002).

Transmission Electron Microscopy

The TEM of bacteriopahge contributes for the characterization and classification of phage.TEM anlaysis is relatively faster than genome analysis and according to shape and size of phage it can be classified in a very short time.In this study klebsiella phage was found to be of podoviridae family having short tail and pseudomonas phage has long non-contractile tail which belongs to siphoviridae family. Phages with contractile tail like ϕP100 present a higher genetic complexity and different mechanisms of DNA injection during infection when compared with phages having non-contractile tails (e.g. ϕApr-1 and ϕIZSAM-1). Among the tailed phages of the genus Pseudomonas, there are about 36% from the family Myoviridae, the same percentage from the family Siphoviridae and 28% belonging Podoviridae.Thus, TEM was a major tool used in analysis of phage morphology and initiated a process of classification of viruses.

Effect of pH on phage viability

Acidity or alkalinity of the environment is another crucial factor in phage survivability. pH stability showed that no reduction of phage.The viability of PhageTU-Kleb 27 and Phage TU-P53 were observed when incubation at pH 5, 6, 7, 8, 9 and 10. Therefore, both of the phages were stable over a wide pH range (5 to 10). Our PhageTU-Kleb27 has higher stability even in higher alkaline condition compared to φBO1E in which phage activity is completely reduced at pH 12 (D'Andrea et al., 2017b). Similarly, our PhageTU-

P53 shows stability even at pH3 contradictory with result shown by phage ϕPA-HF17 stability from pH 4 to 10 (Han et al., 2014). 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) (Langlet et al., 2007). 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 (Jończyk et al., 2011). At the higher and lower pH, the plaque morphology was changed and there is difficulty in set of agar at lower pH as agar hydrolysis occurs so it also affects the plaque morphology.

Effect of temperature on phage viability of PhageTU-Kleb27 and PhageTU-P53

Response of phages on exposure to varying temperatures is considered as a key model for understanding the ability of the organism under question to adapt to 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 influence of temperature variation on PhageTU-Kleb27 and PhageTU-P53 viability revealed that this phage was able to withstand exposure to temperature upto 70˚C, without affecting phage viability. In this experiment we used dry heat.From the study and from our experiment it is found that the phages were stable more in moist heat compared to dry heat. However its activity lost at 80˚c.PhageTU- P53 results similar with the result of phage ϕPA-HF17 which shows stability upto 70˚c for 20 minutes (Han et al., 2014) .PhageTU- kleb27 is more stable to temperature than phage φBO1E in which it stable upto 60˚c only (D'Andrea et al., 2017b). And another interesting result is that there is no significant loss of phage number at 37˚c even after 24 hours and 48 hours.The phage plates after 24 hour incubation at 37˚c was shown in figure (appendix). So the stability of phages at human body temperature can be considered as therapeutic agent. We found that as the temperature increases, the plaque morphology also changes.

One step growth curve experiment

One step growth experiment allows one to identify the effect of changes in the yield of virus per infected host cell and in chemical and physical properties on the period of an infectious cycle (Adams, 1959). The growth curve for phage is similar like of bacteria except in the last stage. The phage growth curve starts with a latent or eclipse period (similar to the bacterial lag phase). The next phase is called the maturation or release stage (similar to the log phase in bacteria) when new phage particles are assembled and released. The cycle then start over with the infection of new cells.The curve seemed to be of stepwise process and thus it is called as "one-step phage growth curve". Through one-step growth experiment, latent period and burst size were measured for PhageTU-Kleb27 and PhageTU-P53 phage. These two parameters are influenced by host culture,

the temperature of incubation, medium on which the experiment was done, and specific growth rate (Keogh & Shimmin, 1974). In this study, PhageTU-P53 showed a short latent period of 20 minutes and a high burst size of around 160 pfu/infected cells. This short latent period and high burst size are of an obvious advantage since its results in the production of more infective phages in a short period of time which in turn increases the probability of host bacteria to interact and consecutively lysis of host organism.And small burst size depends on the host culture density, and attachment time. Burst size value varies in accordance with the specific virus and may range from 10 to 100 for the DNA transducing phages to approximately 20,000 pfu for the RNA virus.We can see in the graph (Figure 4.20 and 4.21) at the start of the experiment, the plaque count is relatively constant over a time period because each infected bacterium will yield only one plaque. A rise in plaque forming units (pfu) to a plateau level occurs as bacteria are lysed and the newly synthesized phages are released into the medium.

Phage genome analysis

Whole genome sequence of bacteriophage is necessitous to understand the phage biology and its characterstics.There is no universal marker for phages in the same way the 16SrRNA gene can be used to reliably place the phylogenetic affinity of all bacteria. This is because there are no any genes that were conserved within all phages, or in any taxonomic group.So, for the characterization, classification and analysis of toxic gene in phages whole genome sequence is necessary.

In this study, PhageTU-Kleb27 was sequenced through illumina sequencing in which low-quality sequencing data were first trimmed depending on qualityscores using PHRED with the minimum quality score of 20 and reads with less than 150 bases in length were discarded. The cleaned-up sequencing data were processed by merging the paired-end sequence reads using fast length adjustment of short reads to obtain complete sequence. Denovo assembly found 7480 scaffolds in phageTU-Kleb27 .In phaster annotation, 7 intact prophages were found which were integrated in host genome. In overall functional protein prediction, some of the proteins were identified as hypothetical phage protein. Some of them were Hoc head outer capsid, inhibitor of prohead protease, RNA-DNA and DNA-DNA helicase ATPase, DNA-primase-helicase subunit, Rec-A like recombination protein, translational repressor protein, thioredoxin, tRNAs, head completion protein, terminase, DNA packaging enzyme large subunit, gp13 neck, short tail fibers, tail sheath protein, tail tube protein, gp48 baseplate tail tube cap, tail length regulator, hinge connector of long tail fiber distal connector, large distal tail fiber subunit, gp27 baseplate hub subunit, portal vortex of head, major head protein, major capsid, gp22 prohead core scaffold, DNA primase helicase subunit, lysis inhibitory accessory proteins, head assembly chaperone protein, endonuclease II, DNA topoisomerase subunit I etcetera.

In phaster annotation we observed various hypothetical proteins.Many of phage, predicted proteins represents “Hypothetical proteins” with homology among phages but

with none described function.This is due to the very few study concerning genome sequencing.

Due to unique features of phage genome, ORF calling, genome annotation, noncoding RNA (ncRNA) identification, and the identification of transposons and insertions are all complicated in phage genome analysis.The whole genome analysis and annotation is a crucial, complicated and time consuming with expertise in bioinformatics skill ,so the genome annotation is still going on. Till the date, only 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.

To date, 28 genomes of dsDNA phages specific to Klebsiella have been deposited into the NCBI database.These have been classified within three famiies: podoviridae (14), Myoviridae (9) and Siphoviridae (5) (Maciejewska et al., 2017).A major difficulty in the functional annotation of protein encoding genes on phage genomes by homology searches is the fact that most proteins have no close homologs in the reference databases. Especially for novel phages, the majority of encoded ORFs havie not no annotated function, or a hypothetical function at best. For the understanding of complete phage genome annotation, identification of the host bacteria is very important as phages are dependent on its host cell. In this study, the host bacteria was identified by 16SrRNA sequencing and confirmed as *Klebsiella pneumoniae*.

With the annotation with RAST, it combines multiple methods and criteria to produce an annotation for a genome. It gives various data and information of phage geome however we could not anlayse all the data.The subsystem distribution provised the genes connected to subsystems and their distribution in different categories by comparing with other known genome sequneces.It gives all the information of metabolic and cellular machinery. Along with this, from RAST annotation we have found that PhageTU-Kleb27 has no any virulence gene, transferable gene and trasposons gene which may be harmful to asses the phage as therapeutics. The subsystem is populated by connecting these functional roles to specific genes in specific genomes, producing a subsystem spreadsheet, where each row represents one genome and each column corresponds to one functional role as shown in Appendix. The principle concept of functioning is illustrated by having genes for all the functional roles that compose a variant of a subsystem. It showed that there is presence of holin and lysine gene which are responsible for lyse the bacterial cels being lytic phage and similarly group of tail protein capsid gene which showed that the phage is tailed phage. Similarly GC% was found to be of 59.8%. There is correlation between G+C content between organism size.Unlike in bacteria and plasmid genome, lower the G+C content in bacteriophage the size of the phage increased.It is said that intracellular pathogens have a G+C content lower than their host organism (Almpanis et al., 2018).

The phylogenetic analysis of respective phage proteins with closely related species were performed through Phylogeny.fr an online server which analyse the molecular sequences in four different steps .Firstly, multiple sequence alignment of obtained DNA sequences with closely related sequences is done by MUSCLE(MUltiple Sequence Comparison by Log- Expectation) ,a bioinformatics tool which is quite faster and have better average accuracy than that of clustalW2 and curation of the alignment is done by Gblocks that eliminates poorly aligned position and divergent region of DNA for much better phylogetic analysis .Further the phylogenetic tree is constructed by using PhyML, a web source for maximum likelihood based phylogenetic analysis .Finally phylogenetic tree is visualized through Tree Dyn which uses annotation and dynamic graphical method for editing and analyzing multiple trees.

Starting from plaque morphology , being halo assumed to have phage tail-associated exopolysaccharide, and further from TEM it is found to be of caudovirales group and finally from genome anlaysis and phylogenetic analysis its resembles with klebsiella phage of caudovirales group.Thus, it can be said that Phage TU-Kleb27 was found to be of tailed phage of caudovirales with short tail and there is no any toxic gene and virulence gene and no any resistance gene of host bacteria integrate in it (confirmed from RAST and Phaster annotation).However, being a wild phage isolated from natural environment it might be contaminated with lysogens also but the lytic activity of the therapeutics is not hindered as we can see effective lysis and no any presence of such gene is confired by annotation of genome sequence.Further it showed homology with pseudomonas phage while blasthit this means they may have share the similar receptor, which indicates the symbol the broader host range with in other species too.As, complete geneome mapping is still remaining, for now we can only classified this phage

Detection of bIofilm producing Bacteria

The microtiter dish assay is an important tool for the study of the early stages in biofilm formation, and has been applied primarily for the study of bacterial biofilms. It does not allow for the formation of the mature biofilms typically associated with flow cell systems. This simple microtiter dish assay allows for the formation of a biofilm on the wall and/or bottom of a microtiter dish. Motile microbes typically adhere to the walls and/or bottoms of the wells, while non-motile microbes typically adhere to the bottom of the wells. The optimal conditions for biofilm formation (i.e., growth medium, temperature, time of incubation) must be determined empirically for each microbe. The extent of biofilm formation is measured using the dye crystal violet (CV).CV gives the both viable and dead cells count and it is often preferred due to its simplicity, reliability, and quick throughput. The CV nonspecifically stains all biomass, both living and dead, as well as the matrix composed of extracellular polymeric substances. This stain makes the assay useful to assess the overall biofilm response of an isolate. Through this method, an isolate can be classified as high, moderate, or non-biofilm producer. In this study after

calculating the cut off value, The bacteria strains k57, P19 ,P6, P13 and P25 were found to be strongly biofilm producer.

Disruption of Bacteria using bacteriophage

The presence of biofilms in bacterial infections can increase the pathogenicity of the bacteria and protects the bacteria from being destroyed by external treatment. Biofilm formation is an ancient mode of survival for bacteria in hostile environments. Biofilms protect the cells from assaults like UV radiation, pH stress, chemical exposure, phagocytosis, dehydration and antibiotics. The biofilm mode of bacterial survival and growth is now being seen as a serious threat to public health and awareness about such cases draws importance among the scientific as well as social communities. The bacteria residing in biofilms have enhanced extent of virulence and pathogenicity. For example, *P. aeruginosa* can form biofilms in a wide variety of environmental conditions which can lead to chronic persisting infections (Bjarnsholt, 2013). So to control the formation and development of biofilm becomes mandatory.

Biofilm are microbial community and shows ineffective against antibiotic treatment.In this study phages showed promising result in disruption of bacteria. The bacteria were selected based on the host range result to check biofilm production and PhageTU-P19 phage was found to control biofilm not in only its host but in other host P25 and P6 bacteria. It showed lytic effect in multistrains of same species. Similarly, PhageTU-Kleb57 was found to be effective in controlling of other bacteria. Similar result was observed in case of phge ZCKP1 phage which removed 50% of biomass (Taha, et al., 2018). The biofilm nearly disappeare after incubation with phages. Phages are able to enter the biofilm destroying the biomass and reaching the bacteria embedded inside. Literature showed that different phages have been used to infect a variety of bacterial biofilms and in general, all these phage-biofilm interaction studies reveal that phages are capable of decreasing the bacterial populations (Abedon, 2011; Gutiérrez et al., 2016).

CHAPTER SIX

SUMMARY

The pathogenic MDR samples were collected from NPHL and from AST result, they are found to be carbapenem resistant and it was further found to be bla-NDM positive in 3 strains. The host of klebsiella which were previously characterized in the lab, were included in this study as host bacteria and the remaining host bacteria were identified by 16SrRNA gene anlaysis and the gene segment were submitted to NCBI.

Altogether 21 lytic phages were isolated by double layer agar method which lyses carbapenem resistant bacteria without enrichment. The Bull's eyes plaques are found in Klebsiella phage and in clear lytic plaques in case of Pseudomonas phage. The phages were purified by repeated continuous streaking method and then titre was determined from phage stocks. PhageTU-Kleb27 was found to be of host specific and contrasting to the specificity of the bacteriophage property, most of the phages showed multiple host range property. Among, them PhageTU-Kleb57 showed broader host range lysing 6 strains out of 20. Similarly, PhageTU-P19 showed lysis in 9 strains out of 18 strain showing broader host range capability. Wide host range within the genus is considered to be a desirable quality of phage as a biocontrol agent. Similary the effectiveness of phage cocktails and synergistic action of phage cocktail was observed in present study which are the impotant aspects of phage therapy. The phages were stored by infecting the host bacteria which showed no any significant reduction in titre value after 6 month and 12month.Among al the phages, only two phages were selected for further characterization due to lack of time and resources.

One of the Phage, PhageTU-Kleb27 against kpc producing *Klebsiella pneumoniae* and PhageTU-P53 against *Pseudomonas aeruginosa* showing clear lytic property were selected for further study. The protein profiling showed the distinct band of 76KDa and 27KDa, which may be of major capsid protein and minor tail protein. The TEM morphology showed that PhageTU-Kleb27 was found to be of podoviridae with short tail and PhageTU-P53 was found to be of siphoviridae with long tail.

From the construction of the one step growth curve of PhageTU-Kleb27, the latent period was calculated to be approximately 20 minutes and the rise period to be 20 minutes, while the burst size was 20 phages per bacterial cell and burst time was found to be 10min. The one step growth curve of PhageTU-kleb27 was indicated that the latent period and rise period was 20 minutes each and the burst size was 160 phages per bacterium and burst time was found to be 10min. Similarly, physiochemical characterization is important for the application of phage which was studied in ph and temperature. Exposure to high temperatures ranging from 50°C to 70°C drastically reduced phages viability, although both of the phages were able to withstand exposure to temperatures as high as 70°C being the optimal temperature at 37˚C. Similarly, optimal pH was found to be 7 for both of the phages, where PhageTU-Kleb27 showed its

viability even in alkaline pH of 12 contrasting to this, PhageTU-P53 showed lytic ability even in acidic condition of pH2. The stability of phages in different temperature and pH is the indicative characteristic of being potential candidate for phage therapy.

Further in the phage genomics, from Phaster analysis PhageTU-Kleb27 was found to be of size 852.1860 kbp which contains 8 intact proteins, and 6 incomplete regions. The phaster annotation showed that there is no any virulence gene present in it thus making likely candidate for phage therapy and functional gene predictions showed that there is presence of lysis gene, capsid protein, tail fiber protein, which confirms the presence of tailed phage. The phylogenetic analysis of phage capsid proteins and phage tail proteins with closely related species showed that it belongs to klebsiella phage of caudovirales group. Similarly from RAST annotation GC % was found to be of 59.8% and no any presence of virulence gene.

Finally the evaluation of phages as biocontrol agent was done for the disruption of biofilm. The phages showed significant reduction in biofilm formation after phage treatment (p-value < 0.05). The 5 different phages were used for 8 different strong biofilm producer clinical isolates.

Our result showed that phages showed multiple host range as well as effectively killed multi-drug resistant bacteria and even it clear the biofilm very successfully of strong biofilm producer which can possibly be used in therapeutics and as professed in scientific world. Now it can be said that with increasing level of antibiotic resistance, in near future phage might move from our last resort against antibiotics-resistant bacteria to our first line drug.

CHAPTER SEVEN

CONCLUSION

With the ever increasing incidence of antibiotic resistance, the scientificworld is therefore looking for effective alternatives to counteract this imminent crisis. The bacteriophages are nowadays found to be potent alternative to tackle this antibiotic crisis. Carbapenem resistant *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are critical pathogens priotrized by WHO causing serious nosocomial infections.This work aimed to isolate and characterize carbapenem resistant strain of *Klebsiella pneumonia* and *Pseudomonas aeruginosa* specific lytic bacteriophage from different environmental water samples in an attempt to isolate a therapeutically potential phage. Alltogether 21 lytic phages were isolated that could infect and lyze carbapenem resistant bacteria (clinical isolates) of human origin. Thus, we reject the null hypothesis and accept the alternative hypothesis which states – There are no bacteriophages in the water of Kathamnadu valley that can lyse the carbapenem resistant human pathogenic bacteria and cannot used in the control of biofilm production. Among 8 phages, PhageTU-Kleb 57 phage and PhageTU-P53 were found to be of multi host range capacity. The phage may produce depolymerase enzyme and indicates a broad host range confined within genus *Klebsiella* and *Pseudomonas*. The PhageTU-Kleb27 was found to be of podoviridae and PhageTU-P53 was found to be of siphoviridae group. The phage was then characterized for stability under the different adverse condition, and a couple of important parameter of its growth dynamics. The result of those experiments demonstrated that PhageTU-Kleb27 and PhageTU-P53 phage is pretty stable under pH and temperature. The stability of phages under different conditions along with phage-associated depolymerase production makes it a worthy candidate to use as a potential alternative in phage therapy to treat the infections. And another important thing is to the best of our knowledge; this is the first study demonstrating such synergistic effect of bacteriophages lytic infection to the bacteria other than its primary host genus. Synergy between phages during infection and lysis of host bacteria is a somewhat unexplored field to some extent. The effectiveness of phage cocktails in conferring better protection was evident from the present study. However, in the present study, we demonstrated synergistic effect phenotypically only. More detailed studies are needed to understand the mechanisms at molecular level of phage host interacton. From whole genome sequencing of Phage it is further confirmed that of tailed phage of Klebsiella and did not contain any virulence gene. Another promising result from our study is the ability of the phages to clear the biofilms formed by multidrug resistant bacteria which is serious problem in hospitals. Based on this result, we can conclude that this promising effect of phages has raised the possibility of expansion of host range and utilization of these phages in phage therapy as well as for biocontrol of pathogenic bacteria and clearance of biofilm. Still, more works are left to be done in molecular characterization of some phages, however it has already provided a good platform for further advance research

with understanding of phage biology and phage characterizationin in control of todays burning issue of antibiotic resistant nosocomial infections Thus, in near future phages being our friend it may help to control various antibiotic resistant infections by using the phages of Nepal.

LIMITATIONS OF THE STUDY

1. Small sample size: Altogether 21 Bacteriophage were isolated but characterization was done only in two samples 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.Due to unavailabilitry of TEM facility we could not study about phage-host infection process where fresh smaple is needed.
3. Lack of experience and expertise: Although Whole genome sequencing is not available in Nepal, we have done it in India. But we faced lots of trouble in genome analysis of that huge data which is crucial steps for characterization of phage for approval in phage application. Due to limited bioinformatics skill and due to lack of powerful computer for data processing we were unable to annotate the phage sequence.
4. Cell line study /mouse model: Due to lack of time and and unavailability of animal house facility we could not performed mouse model experiment.

Challenges to fulfill the objective of the Research and possible sollutions

Owing to specificity of bacteriophages, it will be somehow difficult to target each strain thus phage cocktail strategy will definetly help to lyse bacterial infections being broad spectrum.Narrow host range bacteriophage can be used as personalized medicine.Besides this, safety regulations of phage are another challenges for the application of bacteriopahges. In Nepal, antibiotic resistant case is increasing day by day and very few works related to phages has been done.So awareness on the misue of antibiotics and its potential alternative should be given.Still, there is no sufficient knowledge on bacteriophage and phage therapy as antimicrobial agent in hospitals environmentand clinical persons so collaboration with hospitals and researcher should be made to avoid this gap and the last one is, if we are ready to apply the phage as phage therapy there are several ethicial issues to be obeyed and people are afraid the name of virus itself being pathogen.It is necessary to explore the phage therapy among clinicians and stake holders as bacteriopahges are not our enemy it’s our friend which destroys our enemy (pathogenic bacteria).

RECOMMENDATIONS

In this study the phages have been characterized morphologically, physiochemically as well as in a molecular basis. Further, it has been found to have role in clearance of biofilm formation. Also, the synergistic action of phage cocktail has been observed .This research work is highly encouraged to be studied in further phage therapy. Following are the highly anticipated work that is recommended on the basis of the results that have been obtained from this important research work.

1) Pharmacokinetics phenomenon of phage is needed to be studied, when live viruses are used therapeutically.Similarly, the phage impact on host is recommended to study through cytokine profiling.
2) Study of Host-pathogen interaction and receptor gene analysisis to be done to explore the synergistic action of cocktail phage and to enhance the efficacy of cocktail.
3) Cell line study: Cytotoxic effect of phage in cell line should be analysed for quality assurance, safety and efficacy of phage in phage therapeutics.
4) Cloning of endolysin gene and engineering of phage endolysin-based proteins for phage therapy is the potential area of phage research.
5) Genome annotation: Genome annotation of phage is crucial for depth understanding of phage biology. As, in this study the annotation has not been completed so It is recommended that the collaboration with expert of bioinformatics to deal with huge data of NGS. The collaboration will for sure help to improve phage genome annotation and exploring of phage genomics in future.
6) Preparation of phage library with specific hosts: Preservation of phage and phage host is very important and for collection of phage, phage bank is established in our laboratory. So in future more characterized phages and phage hosts should be preserved in large scale, which can be used in phage therapy when needed in future days.
7) Phage used as disinfectant: The morphological and physiochemical characterization of phages was completed so now it could be used as disinfectant to minimize the rate of nosocomial infections.

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APPENDIX

Appendix 1: Media compositions and Reagent preparations

Nutrient Broth/Agar (NB/NA) – HiMedia

Ingredients	**Grams / Liter**
Peptone	10.000
Beef extract	10.000
Sodium chloride	5.000
pH (after sterilization)	7.3±0.1

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

Ingredients	**Grams / Liter**
Pancreatic digest of casein	17.000
Papaic digest of soyabean meal	3.000
Sodium chloride	5.000
Dextrose	2.500
Dibasic potassium phosphate	2.500
Final pH (at 25°C)	7.3±0.2

Sodium Magnesium Buffer (SM Buffer)

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

Mueller Hinton Agar (MHA) – HiMedia

Ingredients	**Grams / Liter**
Meat, infusion solids from	2.000

300g

Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.000
Final pH (at 25°C)	7.3±0.1

Luria Bertani Broth-HiMedia

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

Phosphate Buffered Saline (PBS)

NaCl - 8.0 g

KCl - 0.2 g

Na2HPO4 - 1.44 g

KH2PO4 - 0.24 g

Appendix 2: Reagents for Sodium dodecyl sulphate electrophoresis

Solution components	**Resolving gel (12%)** **Solution final volume: 10ml**	**Stacking gel (5%)** **Solution final volume: 2ml**
TDW	3.3 ml	1.4ml
30% Acrylamide	4 ml	0.33ml
1.5% Tris (pH 8.8)	2.5ml	0.25ml
10% SDS	0.1ml	0.02ml
10% (NH4)2S2O8	0.1ml	0.02ml
TEMED	0.004ml	0.002ml

30% Acrylamaide solution

S.No.	**Constituents**	**Weight/Volume**
1	Acrylamide; C3H5NO: MW=71.08	29g
2	Bis Acrylamide(N,N Methylene Bisacrylamide); C7H10N2O2: MW=154.17	1g
3	TDW	Maintain upto 100ml

Tris Buffer

	Lower Tris buffer For 100ml (pH 8.8)				**Upper Tris buffer For 50ml (pH 6.8)**	
SN.	Constituents	Amount		SN.	Constituents	Amount
1	Tris(Tris base)	1.5M /18.17g		1	Tris(Tris base)	0.5M/3.03g
2	TDW	Maintain 100ml		2	TDW	Maintain 50ml

Loading sample buffer: pH 6.8 for 10ml

S.No.	**Constituents**	**Amount**
I.	Upper Tris pH 6.8	1.25ml
II.	10% SDS	3.0ml
III.	Glycerol	4.75ml
IV.	Beta-mercaptoethanol	0.5ml
V.	0.1%Bromophenol Blue	0.5ml

Staining solution: Comassie Brilliant Blue G-250 (CBB G-250) for 500ml

S.No.	**Constituents**	**Amount**
i.	Comassie Brilliant Blue G-250	500mg
ii.	Glacial acetic acid	25ml
iii.	Methanol	250ml
iv.	TDW	225ml

Destain solution preparation: For 500ml

S.No.	**Constituents**	**Amounts(ml)**
i.	7.5% Glacial acetic acid	37.5
ii.	5% Methanol	25
iii.	TDW	437.5

Running buffer/Electrolysis buffer preparation: For 1000ml: pH8.4

S.No.	**Constituents**	**Amount**
i.	39mM Tris	4.724g
Ii.	48mM Glycine	3.603g
iii.	0.1% SDS	0.37g

Appendix 3: Chromatogram file of 16SrRNA sequencing

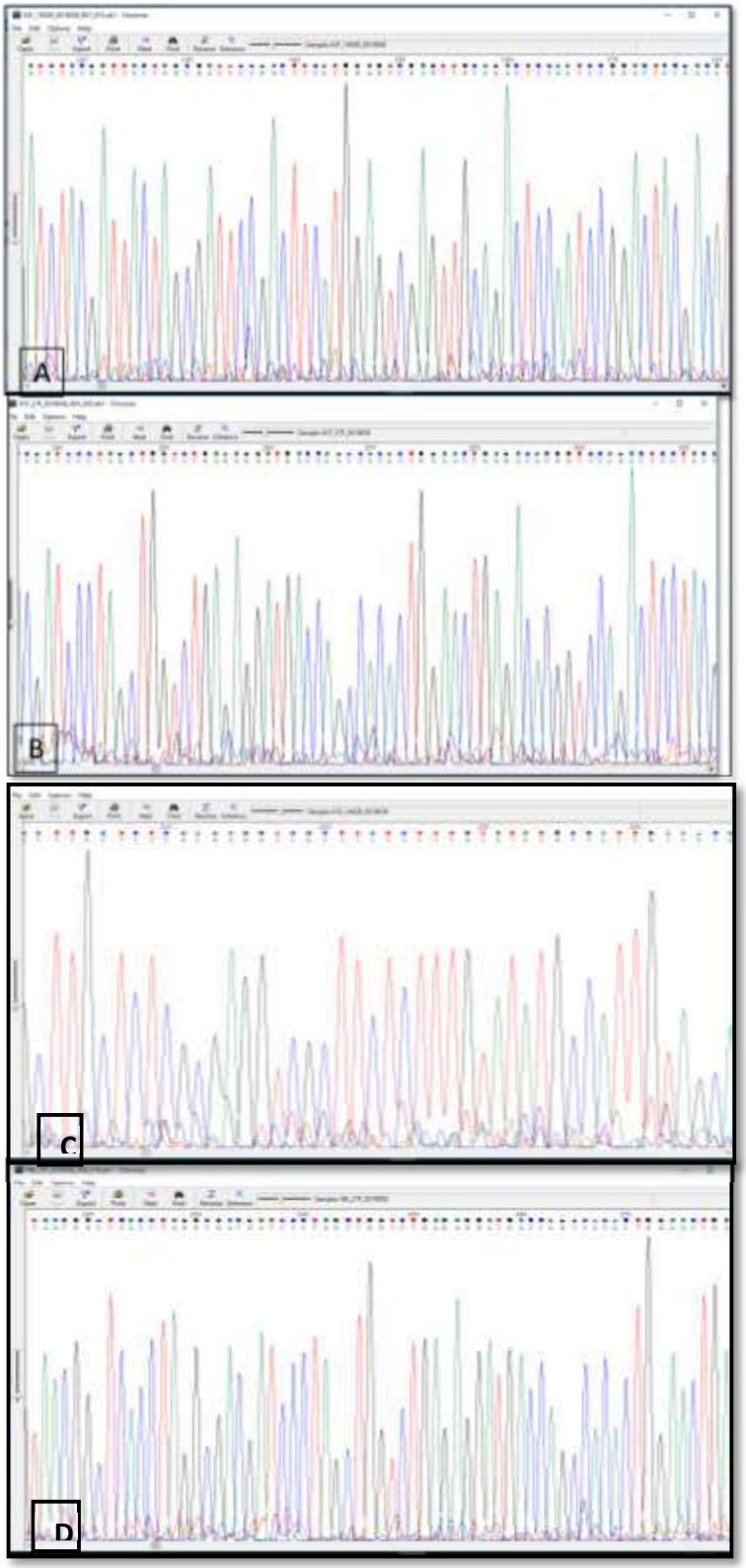


Figure : fig A,B,C and D Chromatogram file of Bacteria K41, K57, K52 and NK of 16SrRNA gene usingchromas software respectively.

Appendix 4: Phage plates

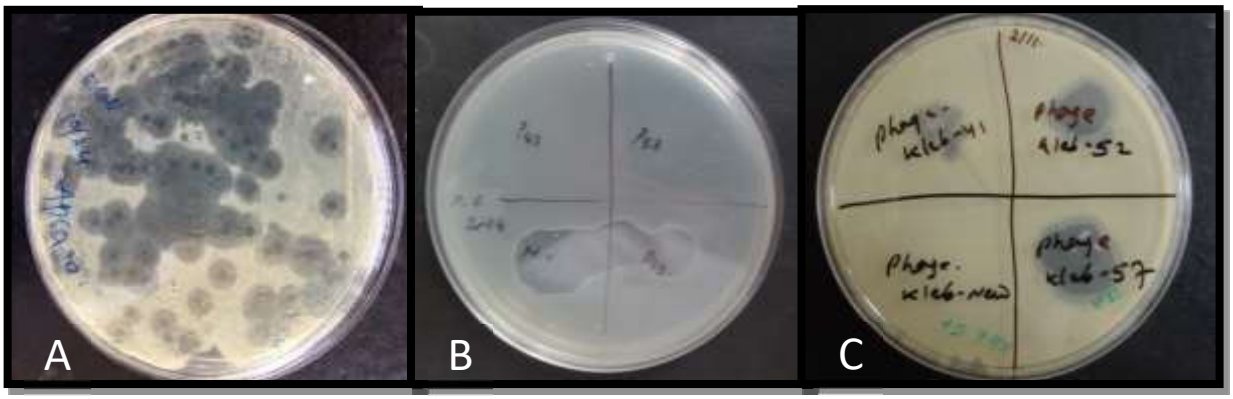


Figure A: Plaques isolated against *E. coli* host for the first time Fig B: host range of Pseudomonas phage showing lysis by PhageTU-P19 in P6 bacteria. FigC:Host range of Klebsiella phage showing lysis by PhageTU-Kleb 41, PhageTU-Kleb 52 and PhageTU-Kleb 57 in Klesiella bacterial strain K57.

Appendix 3.2: Representative appearance of plates from serial dilution pf phage lysates of PhageTU-Kleb52 Fig A : No lysis so no plaquesin 10^{5} dilutions. Fig B: Discrete plaques, PFU can be counted in 10^{4} dilutions FigC and FigD : "Web Pattern plate". Fig E: complete lysis of bacteria in original stock .

Appendix 5: Host range Table

Table: Host range spectrum of 5 different klebsiella phage against 20 different Kelbsiella strain

Bacteria	K57 phage	K52 phage	NK phage	LA phage	K41phage
K3	+	-	-	-	-
K13	+	+	-	-	-
K44	-	-	-	-	-
K27	-	-	-	-	-
K1	-	-	-	-	-
K12	+	-	-	-	-
K23	-	-	-	-	-
K8	-	-	-	-	-
K69	-	+	-	-	-
K6	+	-	-	-	-
K4	-	-	-	-	-
K70	-	+	-	+	-
K15	-	-	-	-	-
K39	-	-	-	-	+
K57	+	-	+	-	+
K52	+	+	-	-	-
K41	-				-
NK	-	+	+	-	+
LA					-
K72	-	+	-	-	-

Table: Host range of Pseudomonas phages in Different *Pseudomonas* strains

Bacteria	p19phage	p43phage	p53phage	Phage cocktail
p3	_	+	-	+
p26	+	-	+	+
p38	+	+	+	+
p6	+	-	-	+
p56	+	-	-	+
p19	+	-	-	+
p25	+	-	-	+
p54	-	-	-	-
p52	-	-	-	-
p26	+	-	-	+
p32	-	-	-	-
p20	+	-	+	+
p13	-	-	+	+
p33	-	-	-	-
p11	-	-	-	-
p57	-	-	-	-
P43	-	+	+	+
P53	-	-	+	+

Appendix 6:WHO priority pathogens list of 12 families of bacteria that pose the greatest threat to human health

WHO priority pathogens list for R&D of new antibiotics

Priority 1: CRITICAL

1. *Acinetobacter baumannii*, carbapenem-resistant
2. *Pseudomonas aeruginosa*, carbapenem-resistant
3. *Enterobacteriaceae*, carbapenem-resistant, ESBL-producing

Priority 2: HIGH

1. *Enterococcus faecium*, vancomycin-resistant
2. *Staphylococcus aureus*, methicillin-resistant, vancomycin-intermediate and resistant
3. *Helicobacter pylori*, clarithromycin-resistant
4. *Campylobacter* spp., fluoroquinolone-resistant
5. *Salmonellae*, fluoroquinolone-resistant
6. *Neisseria gonorrhoeae*, cephalosporin-resistant, fluoroquinolone-resistant

Priority 3: MEDIUM

1. *Streptococcus pneumoniae*, penicillin-non-susceptible
2. *Haemophilus influenzae*, ampicillin-resistant
3. *Shigella* spp., fluoroquinolone-resistant

Source: https://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed

Appendix 7 Classification and Nomenclature of Viruses based on Ninth Report of the International Committee on Taxonomy of Viruses

Table: Families and Genera of Viruses Listed According to the Nature of the Genome

Order	Family or unassigned genus	Nature of the genome	Presence of an envelope	Morphology	Virion size	Genome configuration	Genome size (kbp or kb)	Host
Caudovirales	Myoviridae	dsDNA	-	icosahedral head with tail	icosahedral heads: 60–145 nm; elongated heads: 80 × 110 nm; tail: 16–20 × 80–455 nm	1 linear segment	31–317	B, Ar
Caudovirales	Podoviridae	dsDNA	-	icosahedral head with short tail	head: 60–70 nm tail: 10–20 nm	1 linear segmen	t 16–78	B
Caudovirales	Siphoviridae	dsDNA	-l	icosahedral head with tail	head: 40–80 nm; tail: 5–10 nm ×100–210 nm	1 linear segment	21–134	B Ar

Source : (King et al., 2012)

Appendix 8 : Fasta sequence used for phylogenetic tree analysis

Capsid protein of PhageTU- Kleb27, Nepal

>gene_14_scaffold_35

ATGGCTAACATGCAAGGTGGACAGCAGCTCGGTACTAACCAAGGCAAAGGTCAATCCGCAGCAGACAAGCTGGCGCTATTCCTGA
AAGTATTCGGCGGTGAAGTCCTGACCGCATTCGCTCGTACCTCTGTGACCACCAACCGTCACATGCAGCGTCAAATCAGCTCCGGT
AAGTCCGCACAGTTCCCTGTGATTGGCCGCACCAAGGCTGCTTACCTGCAACCGGGCGAGTCTCTGGATGACAAACGTAAAGACA
TCAAGCACACCGAGAAGACCATTAACATTGATGGCCTGCTGACCGCTGACGTGCTGATTTACGACATCGAAGACGCGATGAACCA
CTATGACGTTCGCTCCGAGTACACCTCTCAGATTGGCGAATCTCTGGCGATGGCAGCCGATGGCGCGGTTCTGGCTGAGCTGGCT
GGTCTGGTTAACCTCGCTGATTCCGTCAACGAGAACATCGCTGGTCTGGGCAAACCGTCCCTGCTGGAAGTTGGTGCTAAGGCTG
ACCTGACTGACCCGGTCAAACTGGGCCAAGCGGTTATCGCGCAGCTGACCATTGCTCGTGCGGCTCTGACCAAGAACTACGTCCC
GGCTAACGACCGTACGTTCTACACCACCCCGGACGTGTACTCTGCGATTCTGGCAGCTCTGATGCCTAACGCTGCGAACTATGCGG
CTCTGATTGACCCTGAGCGTGGTTCTATCCGTAACGTGATGGGCTTCGAAGTCGTTGAGGTTCCGCACCTGACCGCTGGTGGTGCT
GGTGATGACCGCCCGGACGAAGATGCAGAAGCGACCAACCAGAAGCACGCCTTCCCGGCAACTGGTGGTAAAGTAAACAAAGAG
AACGTTGTGGGCCTGTTCCAGCACCGTTCCGCTGTCGGTACCGTCAAGCTGAAAGATCTGGCTCTGGAGCGTGCTCGTCGTACCGA
GTATCAGGCTGACCAGATTGTTGCTAAGTACGCGATGGGTCACGGTGGTCTGCGTCCAGAATCTGCTGGTGCACTGGTTTTCACAG
CAGCCTAA

Tail fiber protein of PhageTU- Kleb27 ,Nepal

>gene_17_scaffold_35

ATGGCTCTCGTATCACAATCAATCAAGAACCTCAAGGGAGGCATTAGCCAGCAGCCTGAAATCCTACGGTACCCAGAGCAGGGTACACTTCAGGTC
AACGGTTGGTCCTCCGAGACTGAGGGTCTCCAGAAGAGGCCACCTATGGTGTTCATCAAGTCCTTGGGAGCGCGTGGATACCTCGGGGAAGACCC
GTACATTCACCTCATCAACCGAGATGAATACGAGCAGTATTACGCAGTGTTCACTGGGAACGACGTTAGGGTATTCGACCTGTCCGGCTATGAGTAC
CAAGTCAGAGGTGACCGCTCGTATATCTCCGTAGTCAACCCTAAGGATAACTTGCGGATGATAACCGTGGCCGACTACACGTTCATCGTTAACCGTA
CCCGACAGGTCCTCGAGAACCAGAACGTGACCAACGGTGGTACCTTCAGGGACAACGTGGACGGTATTGTCAACGTCCGTGGTGGTCAGTATGGT
CGTAAGCTCGAAGTGAACATTAATGGTGTATGGGTCAGCCACCAGCTGCCTCCGGGTGACAACGCTAAGGAAGACCCGCCCAAGGTTGACGCACA
GGCCATTGCGACCGCACTAGCTGACCTACTCCGTGTGGCCCACCCAACGTGGACATTCAACGTGGGGACTGGTTATATCCACTGTATCGCCCCGGCT
GGTGTTACTCTCGACGAGTTCCAGACGAGGGATGGCTACGCGGACCAGCTGATTAACCCGGTGACCCACTACGTCCAGAGCTTCTCTAAGTTGCCA
CTTAACGCGCCTGACGGGTACATGGTGAAGATTGTCGGGGACACGTCCAAGACTGCTGACCAGTATTACGTGAAGTATGACGCTTCTCAGAAGGTC
TGGAAGGAAACCGTGGGCTGGAACATCTCGGCCGGCCTTGAGTATCACACGATGCCTTGGACTCTGGTACGTGCGGCTGATGGTAACTTTGACCTA
GGGTATCGCGAGTGGAGGGACCGCCGTGCTGGTGATGACGACACTAACCCTCAGCCGTCCTTTGTTAACTCGACGATAACCGATGTGTTCTTCTTCA
GGAACCGCTTAGGGTTCATCTCTGGGGAGAACATCGTACTGTCCCGCACCAGTAAATACTTTGAGTTCTACCCGCCGTCAGTGGCCAACTATACGGA
TGATGACCCGCTGGATGTTGCTGTGAGTCATAACCGAGTGTCGGTCCTGAAGTACGCTGTGAGCTTCGCTGAGGAGCTGCTGCTATGGTCTGACGA
GGCGCAGTTCGTTCTGTCGGCCAACGGTGTGTTATCCACTAAGACTGCACAGCTGGACCTGACCACTCAGTTCGATGTGTCAGACCGTGCGCGTCCT
TATGGTATCGGTCGAAACATCTACTACGCGTCTCCTCGCAGCTCCTTTACGTCCATCATGCGTTACTACGCGGTACAGGATGTAAGCTCTGTGAAGA
ACTCAGAGGACATGACGGCCCACGTCCCGAACTACATCCCGAACGGTGTGTACAGCATCAACGGGTCCGGTACTGAGAACTTCGCGTGTGTGCTGA
CCAAGGGTGCTCCCAGCAAGGTGTTCATTTACAAGTTCCTCTACATGAATGAGGACATCCGTCAGCAGTCGTGGTCACACTGGGACTTCGGAGACG
GTGTGGAGGTGATGGCGGCAAACTGTATCAACTCAACGATGTACCTGCTGATGCGGAACGCCTACAACGTGTGGATAGCTGCTGTGGACTTTAAGA
AGGAGTCGACTGACTTCCCGTTCGAGCCTTACCGATTCCACGTGGACGCCAAGCGGTCGTATCACATCTCAGAGACTGCGTATGACATCGAGACCA
ACCAGACGGTAGTTAACGTCAAGGACATCTACAGTGCGTCGTTCTCTAAGGGTACCGTGGCAATCTGCGAGAGTGACGGTAAAATCACTACGTATG
AGCCGATGGGTGACTCTTGGGATTCCACGCCGGACATCCGCATTAGTGGTGACATCTCTGGTAAGGATATCGTCATTGGGTTCCTGTACGACTTCCG
CTATGTGTTCAGTCGGTTCCTCATCAAGCAGGAGCAGAACGATGGCACAACGTCCACCGAGGACTCTGGTCGTCTACAGCTGCGTAGAGCGTGGGT
GAACTATCAGGACACTGGTGCGTTCACTGTGAGCGTCGAGAATGGCAACCGTGAGTTCAACTATCTGGTCAACGCCAGAGTGGGTTCTACGGGTCT
ACGTCTGGGCCAGAAGGCCACAACTACTGGTCAGTATCGTTTCCCGGTGACAGGCAACGCATTGTACCAGAAGGTGTCCCTGAGTTCCTTCAATGCT
TCCCCGGTGTCAATCATTGGGTGCGGCTGGGAGGGTAACTACAGCAGACGCGCCAACGGTATTTAA

Appendix 9: BLAST output of scaffold 35:

Job title: gb|MK134560.1| (40539 letters)

RID	2ETDUA8J11N (Expires on 12-30 19:16 pm)		
Query ID	MK134560.1	**Subject ID**	lcl|Query_144151
Description	Klebsiella phage kpssk3, complete genome	**Description**	scaffold_35
			See details
Molecule type	nucleic acid	**Molecule type**	nucleic acid
Query Length	40539	**Subject Length**	40740
		Program	BLASTN 2.8.1+

Graphic Summary

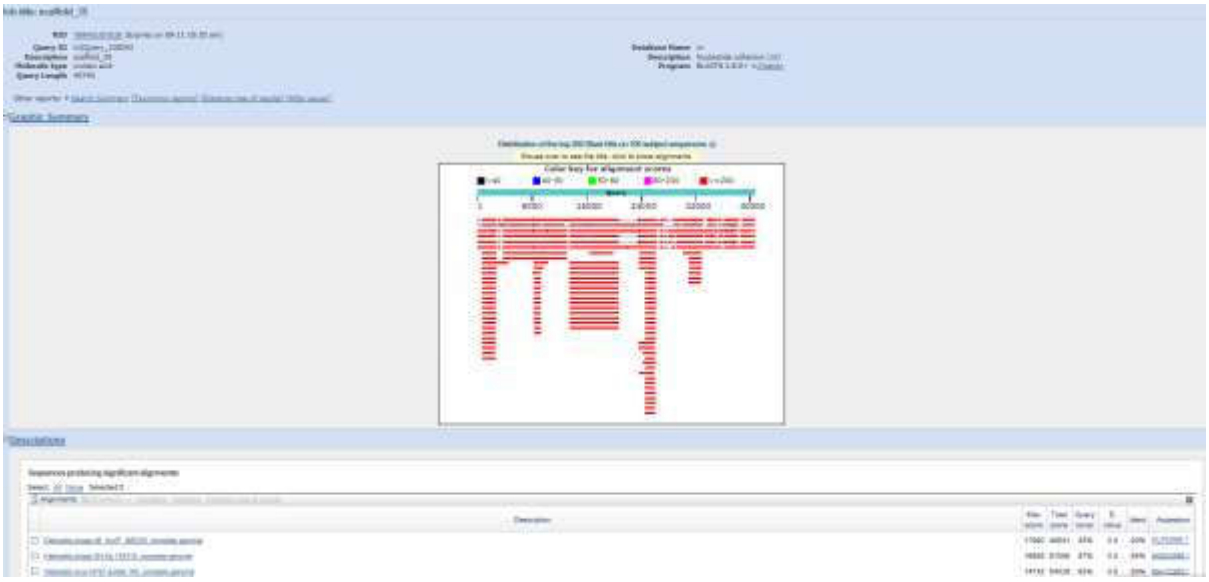


Figure: The BLAST hit of scaffold 35 showed that 93% identity in Klebsiella phage.

BLAST output of Scafold 24

BLAST® » blastn suite-2sequences » RID-2ETUSJR1114

BLAST Results

Questions/comments

Blast 2 sequences

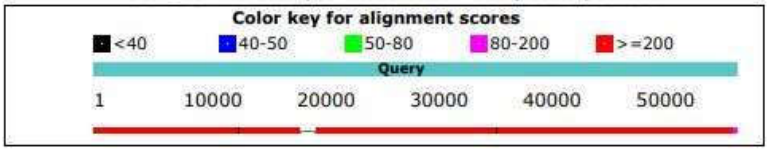
Job title: gb|CP027046.1| (55018 letters)

RID 2ETUSJR1114 (Expires on 12-30 19:23 pm)

Query ID	CP027046.1	**Subject ID**	ld|Query_194751
Description	Klebsiella pneumoniae strain 1_GR_13 plasmid unnamed, complete sequence	**Description**	scaffold_24 See details
Molecule type	nucleic acid	**Molecule type**	nucleic acid
Query Length	55018	**Subject Length**	54679
		Program	BLASTN 2.8.1+

Graphic Summary

Distribution of the top 5 Blast Hits on 1 subject sequences



Dot Matrix View

Appendix 10: RAST Annotation Results

Table :Subsystem :Subsystem of spreadsheet of Phage tail fiber proteins

Diagram | Functional Roles | Subsystem Spreadsheet

Group Alias	Abbrev.	Functional Role	Reactions	Scenario Reactions	GO	Literature
	SaTFP	Tail fiber protein [SA bacteriophages 11, Mu50B]	-	-	GO:0005737, GO:0006817	none
	TFP	Phage tail fiber protein	-	-	-	none
	TFP1	Phage tail fiber protein 1	-	-	-	none
	TFAP	Phage tail fiber assembly protein	-	-	-	none
	TFP2	Phage tail fibers	-	-	-	none
	LTFPS	Phage long tail fiber proximal subunit	-	-	-	none
	LTFPC	Phage long tail fiber proximal connector	-	-	-	none
	TFC	Phage tail fibers chaperone	-	-	-	none

Table :Subsystem Spreadsheet of Phage tail proteins

Diagram | Functional Roles | Subsystem Spreadsheet | Additional Notes

Group Alias (all)	Abbrev.	Functional Role	Reactions	Scenario Reactions	GO	Literature
	TP	Phage tail protein	-	-	-	none
	Maj	Phage major tail protein	-	-	-	none
	Min	Phage minor tail protein	-	-	-	none
*Measure	TMP'	Phage tape measure protein	-	-	-	none
*Measure	TMP	Phage tail length tape-measure protein	-	-	-	none
*Measure	TMP1	Phage tail length tape-measure protein 1	-	-	-	none
*Measure	TMP2	Phage tail length tape-measure protein 2	-	-	-	none
*Shaft	SHA	Phage tail shaft protein	-	-	-	none
*Shaft	MTSP	Phage major tail shaft protein	-	-	-	none
*Tube	TubM	Phage tail tube monomer	-	-	-	none
*Tube	TubP	Phage tail tube protein	-	-	-	none
*Tube	MTub	Phage major tail tube protein	-	-	-	none
*Sheath	TSM	Phage tail sheath monomer	-	-	-	none
*Sheath	TSP	Phage tail sheath protein	-	-	-	none
*Sheath	MShe	Phage major tail sheath protein	-	-	-	none
	Spi	Phage tail spike protein	-	-	-	none
	TCP	Phage tail connector protein	-	-	-	none
	TComp	Phage tail completion protein	-	-	-	none
*Assembly	TAC	Phage tail assembly chaperone	-	-	-	none
*Assembly	TAP	Phage tail assembly protein	-	-	-	none
*Assembly	TAP1	Phage tail assembly protein I	-	-	-	none
*Assembly	TAP2	Phage tail assembly	-	-	-	none
	HRP	Phage tail protein involved in host-recognition	-	-	-	none
	Circ	Phage tail/DNA circulation protein	-	-	-	none

Table : Subsystem of phage lysis modules

Diagram | Functional Roles | Subsystem Spreadsheet | Description | Additional Notes

Group Alias (all)	Abbrev.	Functional Role	Reactions	Scenario Reactions	GO	Literature
*genLysins	Lys	Phage lysin (EC 3.2.1.17)	-	-	-	none
*genLysins	Endolys	Phage endolysin	-	-	-	none
*genLysins	PGH	Phage peptidoglycan hydrolase	-	-	-	none
*Lysins	Lys1	Phage lysin, 1,4-beta-N-acetylmuramidase (EC 3.2.1.17) or lysozyme	-	-	-	none
*Lysins	Lys2	Phage lysin, N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)	-	-	-	none
*Lysins	Lys3	Phage lysin, endo-beta-N-acetylglucosaminidase (EC 3.2.1.96)	-	-	-	none
*Lysins	Lys3'	Phage lysin, glycosyl hydrolase	-	-	-	none
*Lysins	Lys4	Phage peptidoglycan endopeptidase	-	-	-	none
*Lysins	Lys4'	Phage lysin, peptidoglycan endopeptidase (EC 3.4.99.17)	-	-	-	none
*Lysins	Lys5	Phage lysin, L-alanyl-D-glutamate peptidase (EC 3.4.-.-)	-	-	-	none
*Lysins	Lys6	Phage {Gamma}-D-glutaminyl-L-lysine endopeptidase	-	-	-	none
*Lysins	Lys7	CHAP domain-containing phage lysin	-	-	-	none
*Lysins	TransI	Phage lysin, phospho-MurNAc-pentapeptide translocase inhibitor	-	-	-	none
*Myco	mLysB	Mycobacteriophage lysis protein, LysB	-	-	-	none
*Holin	Hol	Phage holin	-	-	-	none
*Holin	Hol1	Phage holin, class I	-	-	-	none
*Holin	Hol2	Phage holin, class II	-	-	-	none
*Rz/Rz1	Rz	Phage spanin Rz	-	-	-	none
*Rz/Rz1	Rz''	Phage outer membrane lytic protein Rz	-	-	-	none
*Rz/Rz1	Endo	Phage endopeptidase (EC 3.4.-.-) Rz	-	-	-	none
*Rz/Rz1	Rz1	Phage outer membrane lipoprotein Rz1	-	-	-	none
*Rz/Rz1	LysA	Phage lysis regulatory protein, LysA	-	-	-	none
*Rz/Rz1	LysB	Phage lysis regulatory protein, LysB	-	-	-	none
*truncLysins	tAmid	Truncated phage lysin, N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)	-	-	-	none
*Lysins	Lys8	Phage lysin, cell wall-binding domain	-	-	-	none

Appendix 11 Participation in Extra activities

1) Organizer of WORLD DNA DAY 2017(April-24-25) held in NAST
2) Participated in Fifth International Conference SCIENCE AND SCIENTIST-2017, 18th and 19th August,2017 Nepal Pragya Pratisthan, Kamaladi-kathmandu,Nepal
3) Volunteer in conducting the International workshop on Applications OF Flow Cytometry in Biotechnology-2017 held at CDBT, Kathmandu, Nepal (September 14-17,2017)
4) **Tutor** at CDBT-TU WINTER SCHOOL-2018 in applied molecular biology. (January 7-10 2018)
5) **Poster presentation** in 2nd INTERNATIONAL CONFERENCE ON BIOSCIENCE & BIOTECHNOLOGY (ICBB-2018) Held in Dhulikhel, Nepal February 17-20,2018 organized by RIBB and FIMM ,Finland
6) **Oral presentation** in WORLD DNA DAY 2018 (April 24-25, 2018) with different events organized by BSN.
7) **Tutor** on 5-day workshop on "Applied Microbiology, Molecular Biology and Bioinformatics" organized by Central for Health and Disease Science on 9-13 July 2018
8) Completion of 8 weeks online course 'Research Writing in the Sciences' of the AuthorAid Programme at INASP from 4 September to 2 October
9) Participation in the workshop of speed-dating event of Swiss-Nepal Technology Transfer Workshop 2019, where the idea of "Phage spray as disinfectant" was selected.
10) Involved in the research," Pharmacokinetics study and cytokine profiling of bacteriophages in mouse model."

Publications:

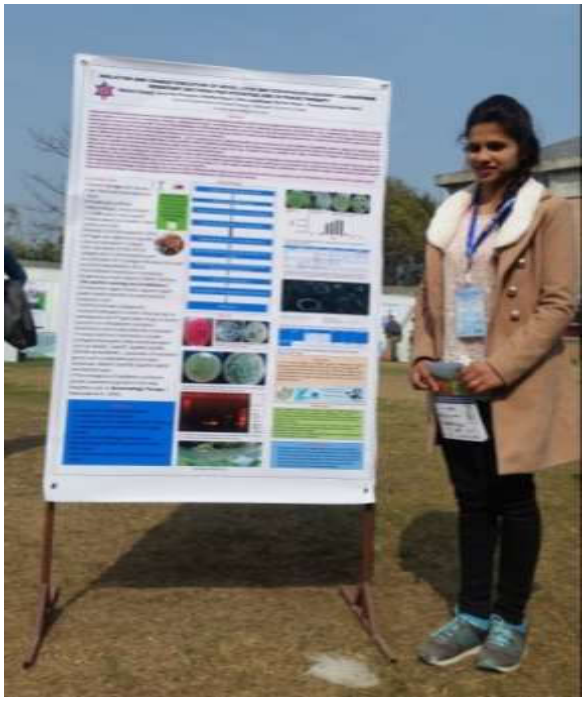
The manuscript is submitted entitled 'Enhanced phage host range by the synergistic effect of newly isolated phage cocktails from the rivers of Kathmandu, Nepal.' This is on peer review process.

Appendix 12: Some photographs

Working on laminar hood

Detection of OD value of Biofilm producer in ELISA Reader

Phage team late night work



Poster Presentation: (ICBB-2018) Held in Dhulikhel, Nepal February 17-20, 2018

Prof. Dr.Rajani Malla awarded with **Nabil Science and Technology Award** – NAST june10 2018

Oral Presentation: in WORLD DNA DAY 2018 (April 24-25, 2018)

Participating in ICET-2016 December 9-11, 2016

Participation at SABC-2017 3rd INTERNATIONAL SOUTH ASIAN BIOTECHNOLOGY CONFERENCE (March 16-18, 2017) in Kathmandu Nepal

Tutor at CDBT-TU WINTER SCHOOL-2018 in applied molecular biology. (January 7-10)

CDBT teams at αφ̂ϕ μρ†ª≠ ¡ ρ± ∂øρ∑¡ - ûπº ∑ρ∏ρ July (8-14) 2018

Teacher day at CDBT : 2017 July 9

Farewell of our 6th batch: 27 June 2018

Participation at SABC-2017 3rd INTERNATIONAL SOUTH ASIAN BIOTECHNOLOGY CONFERENCE (March 16-18, 2017) in Kathmandu Nepal

Thesis defense: 2018-December 31st

CDBT 6th batch: Seminar at Nagarkot 18th July 2016