MOLECULAR STUDY OF BROAD HOST RANGE BACTERIOPHAGE AGAINST COLISTIN RESISTANT *Salmonella Typhi* FOR POTENTIAL USE IN PHAGE THERAPY

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DEDICATED TO

MY

BELOVED PARENTS

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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:	Anti-Microbial Resistance
AST:	Antibiotic Sensitivity Test
ATCC:	American Type Culture Collection
BIM/s:	Bacteriophage Induced Mutant/s
BLASTN:	Basic Local Alignment Search Tool – Nucleotide
bp:	base pairs
BT:	Bacteriophage Therapy
CDBT:	Central Department of Biotechnology
CDC:	Centers for Disease Control and Prevention
CDS:	Coding DNA Sequence
cfu:	Colony Forming Unit
CRE:	Carbapenem Resistant Enterobacteriaceae
DLAA:	Double Layer Agar Assay
DNA HT library:	Deoxyribonucleic Acid High-Throughput library
DNA:	Deoxyribonucleic Acid
dsDNA:	double stranded Deoxyribonucleic Acid
dsRNA:	double stranded Ribonucleic Acid
EOP:	Efficiency of Plating
GB:	Giga Bytes
GC or G+C content:	Guanine – Cytosine content
gDNA:	genomic Deoxyribonucleic Acid
GPS:	Global Positioning Service
GRAS:	Generally Recognized As Safe
ICTV:	International Committee for Taxonomy of Viruses

JNU-AIRF:	Jawaharlal Nehru University – Advanced Instrumentation Research Facility
Kbp:	Kilobase pairs
MDR:	Multidrug Resistant
MHR:	Multiple Host Range
mRNA:	messenger RNA
MRSA:	Methicillin Resistant *Staphylococcus aureus*
NA:	Nutrient Agar
NCBI:	National Center for Biotechnology Information
NDM-1:	New Delhi Metallo-beta-lactamase-1
ng:	nanogram [one billionth (1×10-9) of a gram]
NGS:	Next Generation Sequencing
nm:	nanometer [one billionth (1×10-9) of a meter]
OD:	Optical Density
ORF:	Open Reading Frame
PCR:	Polymerase Chain Reaction
PDR:	Pan Drug Resistant
PFU:	Plaque Forming Unit
PHASTER:	PHAge Search Tool Enhanced Release
PT:	Phage Therapy
rpm:	revolutions per minute
RNA:	Ribonucleic Acid
rRNA:	ribosomal Ribonucleic Acid
SM buffer:	Sodium Magnesium buffer
SDS-PAGE:	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SPL:	Staphylococcal Phage Lysate
ss DNA:	single stranded Deoxyribonucleic Acid
ss RNA:	single stranded Ribonucleic Acid

TEM:	Transmission Electron Microscopy
tRNA:	transfer Ribonucleic Acid
TSA:	Tryptic Soya Agar
TSB:	Tryptic Soy Broth
TU:	Tribhuvan University
UF:	Ultra Filtration
US-FDA:	United States – Food and Drug Administration
US-NIAID:	United States - National Institute of Allergy and Infectious Diseases
UV:	Ultra Violet
VRE:	Vancomycin Resistant Enterococci
VRSA:	Vancomycin Resistant *Staphylococcus aureus*
WGS:	Whole Genome Sequencing
WHO:	World Health Organization
W/V:	Weight by Volume
XDR:	Extensively Drug Resistant
ZOI:	Zone of Inhibition

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ABSTRACT

Introduction: Emergence of antibiotic resistance worldwide brings a major threat to treat the bacterial infections. In present context, resistance is seen almost all antibiotics even in colistin which is last line drugs. The common pathogens of colistin resistant are *Salmonella spp* and *Escherichia coli* mostly associated with animal infection. So, now it becomes an urgent need to investigate alternative treatment options. The use of Bacteriophages is an important alternative approach in the current era of drug-resistant pathogens. In this study, we aim to isolate lytic phages against colistin resistant *Salmonella spp* and characterize genetically for potential use in phage therapy.

Methodology: Host bacterial strains were identified by biochemical test and 16S rRNA sequencing and antibiotic susceptibility test was performed by disc diffusion method. Bacteriophage isolation was done by Double Layer Agar Assay method. Burst size and latent period of phage was determined by one step growth curve experiment. Phage stability was also checked against pH and temperature. Multi host range property was also determined by spot assay and efficiency of plating method. Most potent phage was confirmed by Transmission Electron Microscopy and protein profiling was done by SDS PAGE. Whole genome sequence was done by Illumina platform to rule out the presence or absence of any virulence gene of bacterial origin.

Results: Colistin resistant S*almonella Typhi* was determined by antibiotic susceptibility test and host was identified from 16S rRNA sequencing and gene sequence submitted to NCBI GenBank. Nine different bacteriophages were isolated against three different strains of *Salmonella Typhi.* Isolated phages showed broad lytic ability against its host and other genus. Burst size of most potent phages, phage TU_sal2T and phage TU_sal5K were found to be 50 and 45 virion per bacterium with latent period of 20min at multiplicity of infection (MOI) 10. Optimum temperature and pH were determined as 37˚C and 8 respectively for both phages. Transmission electron micrograph revealed that the phage TU_sal2T belong to order Caudovirales and family *Myoviridae*. Whole genome sequencing revealed that phage TU_sal2T did not possess any virulent gene of bacterial origin, integrase gene and other toxic gene within their genome.

Conclusion: Our finding showed phages were not extremely host specific, they evolved to achieve broader host range. Phage TU_sal2T could be a potential candidate for the therapeutics and biocontrol of multidrug resistant pathogens specifically for S*almonella spp.*

Keywords: Bacteriophage, Colistin resistant, Host range, MDR, Phage Therapy

Chapter I
INTRODUCTION

1.1 Background

Innumerable life of peoples was saved in the golden age of antibiotic discovery. But these highly potent "miracle" drugs are no longer as effective. They were only lasted a half a century ago. We are now entering an era in which bacteria become resistant to the every antibiotics developed to treat and cure the infections they cause (Golkar *et al.*, 2014).

Increased antibiotic resistance is now becomes a global issue. The CDC has termed the present time the "post-antibiotic" era, because resistance abounds to almost every available antibiotic, and multidrug resistant (MDR) infections are increasingly more common. This problem stems from a variety of factors including widespread agricultural use of antibiotics, inappropriate prescription of antibiotics, a decrease in the number of new antibiotics entering the market, and the increased positive selection of multidrug resistance when gained through the natural prokaryotic exchange of genetic material (Schooley *et al.*, 2017)

The history of antimicrobial drug discovery includes more than 15 classes of compounds, penicillin was first antibiotic discovered by Alexander Fleming in 1928 (CDC, 2018). Penicillin was successful in controlling bacterial infections among World War II soldiers. However, shortly thereafter, bacteria became resistance by penicillin a substantial clinical problem, so that, by the 1950s, many of the advances of the prior decade were threatened. In response, the development and discovery of new antibiotic such as beta-lactum was increasing which restored confidence for treatment. But in 1962, the first case of methicillin-resistant *Staphylococcus aureus* (MRSA) was identified in the United Kingdom during that same decade similar case was observed in the United States in 1968 (Ventola, 2015).

For the control and management of microbial infection, antibiotic became a cornerstone, and have one of the most successful forms of therapy in clinical medicine in 20th century. But due to the widespread overuse and misuse of these compounds in clinical and veterinary medicine and agriculture, this success is compromised by the emergence and dissemination of antimicrobial resistance. The daily increased in morbidity and mortality rates, as well as elevated healthcare costs, it has been brought to the public's attention by several national and international health protection agencies including the Centers for Disease Control and Prevention (CDC), World Health Organization (WHO), Infectious

Disease Society of America (IDSA) and the European Medicines Agency (EMA).(Expert round table on acceptance and re-implementation of bacteriophage therapy *et al*., 2018).

The global spread of drug resistance has been seen among common pathogens like carbapenem resistant *Klebsiella spp, Acenetobacter spp, Pseudomonas spp,* floroquinolones resistant *Escherichia spp* (ESBL producers), methicillin resistant *Staphylococcus spp,* vancomycin resistance *Enterococii.* In present context, resistance is seen not only in 4th generation antibiotics that is carbapenem, there is found to be resistant even in colistin antibiotics which is last line drugs. Recently, the major pathogens that are associated with colistin resistance are *Salmonella, E coli* and *Acinetobacter.*

1.1.1 Salmonella as a pathogen

Salmonella is a genus of non-spore-forming, rod shaped with cell diameter about 0.7-1.5 µm and length ranging from 2 to 5 µm. *Salmonella* consists a facultative genus of rod shape gram negative motile enterobacteria having peritrichous flagella. It belongs to the family *Enterobacteriacea*. The genus *Salmonella* consists of two species, *Salmonella enterica* and *Salmonella bongori*. *S. enterica* is further divided into six subspecies : *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp*. diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), and *S. enterica* subsp. *indica* (VI) (Fabrega and Vila, 2013) and contained > 2600 serotypes (Mahmoud *et al.*, 2018). The serotypes (also serovars) of *Salmonella* defined on the basis of the somatic O (lipopolysaccharide) and flagellar H antigens (the Kauffman– White classification). The full name of a serotype is given as, for example, *Salmonella enterica* subsp. *enterica* serotype *Typhimurium*, but can be abbreviated to *Salmonella Typhimurium* (Brenner *et al*., 2000).

The symptomatic infection caused by the *Salmonella* is Salmonellosis, most common symptoms are diarrhea, fever, abdominal cramps, and vomiting. Infection is usually spread via contaminated meat, eggs, milk or fruits and vegetables. *Salmonella* infections in humans can range from self-limiting gastroenteritis typically associated with nontyphoidal *Salmonella* (NTS) to typhoidal fever, which can be life-threatening. Salmonellosis causes considerable morbidity and mortality in both humans and animals, and has a significant socioeconomic impact worldwide. Infections caused by *Salmonella* have been categorised into four clinical types: gastroenteritis, bacteraemia or septicaemia, enteric fever and convalescent lifetime carrier state (Smith *et al.*, 2016).

In developed countries, nontyphoidal serotypes present mostly as gastrointestinal disease but in sub-Saharan Africa, these serotypes can create a major problem in bloodstream infections. Most cases of invasive nontyphoidal *salmonella* infection (iNTS) are caused by *S. Typhimurium* or *S. enteritidis.* (Feasey *et al*., 2012). Typhoid fever caused by *Salmonella* serotypes including *Salmonella* Typhi, ParaTyphi A, ParaTyphi B and ParaTyphi C are

strictly adapted to humans or higher primates. In the systemic form of the disease, salmonellae pass through the lymphatic system of the intestine into the blood of the patients (typhoid form) and are carried to various organs (liver, spleen, kidneys) to form secondary foci (septic form).

Salmonella spp. cause an estimated 93.8 million illnesses and 155,000 deaths globally each year and it considered the second most common causal agents for foodborne diseases worldwide after *Campylobacter spp* (Sadekuzzaman *et al.*, 2018). In 2015, 90,300 deaths occurred from non-typhoidal and 178,000 deaths from typhoidal salmonellosis (Whang *et al.* 2016*).* In the United States about 1.2 million cases and 450 deaths occur from non–typhoidal salmonellosis a year (CDC, 2015).

The emergence of multidrug resistance *Salmonella* cause major concern among medical and veterinary health professionals. Epidemiological studies showed that the most common source of MDR *Salmonella* in human is via consumption of contaminated food. The evidence of transfer of colistin resistance in bacteria from animals to human, now make it urgent need to investigate alternative treatment options.

Use of bacteriophage as an antimicrobial is an important alternative to antibiotics in the current era of drug resistance pathogens. Owing from the long history of bacteriophage, the use of phages for the treatment of bacterial infections has been extensively studied therapeutic strategy. Bacteriophage therapy involves the use of phage and their products as bioagent for the prophylaxis and control of pathogens. There are many evidences has been accumulated since 1915s from the discovery of phage, in support of the effectiveness of the phage therapy against bacterial infections. Different animal model studied indicated that appropriate administration of phage can be used to treat lethal infection caused by gram-negative bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Vibrio vulnificus*, and *Salmonella* spp., and gram-positive bacteria, such as *Enterococcus faecium* and *Staphylococcus aureus* (Matsuzaki *et al.*, 2005). In western countries different phage therapy centers also conducted and under patenting.

1.1.2 Bacteriophage

Bacteriophages are the group of viruses displaying the ability to infect or kill bacteria while they do not affect cell lines from other eukaryotic organisms. They are the incomplete organism that can only replicate in a live cell and also known as bacterial parasites because they lack the cell structure and enzyme systems necessary for food uptake, protein synthesis or construction of new particles (Wernicki *et al.,* 2017). Frederick Twort in 1915 discovered the bacteriophage as unidentified molecules that inhibit bacterial growth, but in 1917 Felix D'Herelle was the first to isolate and characterize phages (McKinley, 1929). He also proposed that this phenomenon was caused by a virus capable

of parasitizing bacteria and named these viruses 'bacteriophages', a word that is derived from the fusion of 'bacteria' and 'phagein' (to eat in Greek). Alexander Sulakvelidze defined bacteriophages as "the most ubiquitous organisms on Earth, playing a significant role in maintaining microbial balance on this planet" in the occasion of, introduction of the new *Bacteriophage* journal, launched in early 2011. Bacteriophages are widely distributed in nature whose life cycle is strictly associated with the bacterial cell. It has been established that the population number of phages in aquatic systems lies within the range of 10^4 to 10^8 virions per milliliter (ml) and about 10^9 virions per gram(g) in the soil, with an estimated total number of 10^{32} bacteriophages on the planet (Wittebole *et al.,* 2014).

1.1.2.1 General structure of Bacteriophage

Like all viruses, phages are simple organisms that consist of a core of genetic material (nucleic acid) surrounded by a protein capsid. The nucleic acid may be either DNA or RNA and may be double-stranded or single-stranded.

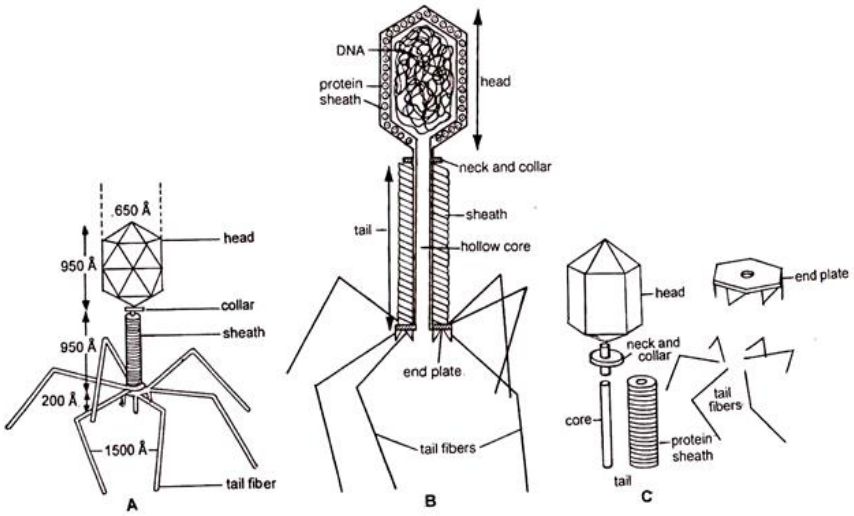


Figure 1.1 Structure of Bacteriophage. A; external structure, B; lateral section of bacteriophage and C; various parts of bacteriophage showing head, tail, base plate, neck and collar region, tail fiber, hollow core etc. *Source: http://www.biologydiscussion.com*

There are three basic structural forms of phage: an icosahedral (20-sided) head with a tail, an icosahedral head without a tail, and a filamentous form. In 1943, H. Ruska was the first to apply electron microscopy for viral taxonomic studies (Ruska, 1943). At present more than 5000 bacteriophages were examined under microscope (Ackermann, 2006). This concept was based on the type of nucleic acid, capsid form, presence or lack of lipid envelope and number of capsomeres. This scheme in 1965 was formally accepted by

Provisional Committee on Nomenclature of Viruses (PCNV) later to become the International Committee on Taxonomy of Viruses (ICTV). The ICTV is the only international body concerned with virus taxonomy.

1.1.2.2 Bacteriophage Classification

Bacteriophage classification was started by Bardley in earnest 1967, this classification scheme was adopted by ICTV (Bardley, 1967). He proposed six basic morphological types, corresponding respectively to tailed phages (with contractile tails, long and noncontractile (rigid) tails, and short tails), small isometric ssDNA viruses with fibrous or spiky surfaces, filamentous phages and small ssRNA phages. The name of order, family and genera of bacteriophage are typically derived from the Latin and Greek root, and end in *-virales, -viridae, -virus* respectively. Based on genomics and proteomics method of ICTV reports, phages were classified into 1 order, 13 families and 31 genera, (Ackermann, 2005) and the vast majority of bacteriophages belongs to the family *Myoviridae, Podoviridae* and *Siphoviridae* under the order *Caudovirales* in the 2015 taxonomy release (Wernicki *et al*., 2017).

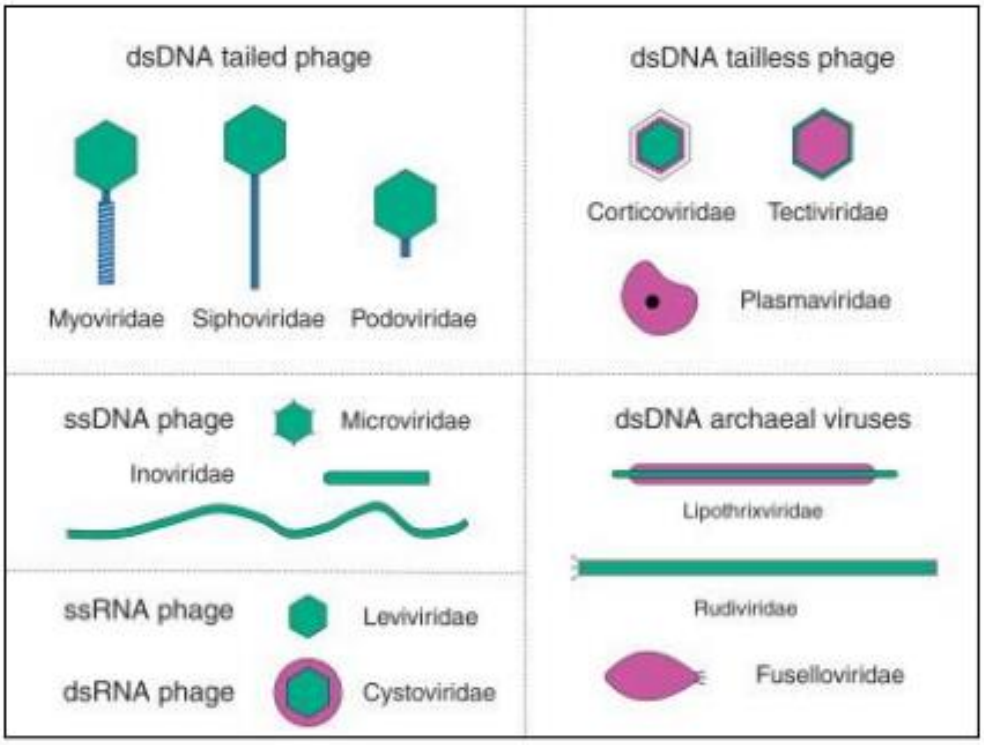


Figure 1.2 Schematic representation of major group of bacteriophages. Double stranded DNA tailed phages belongs to the family *Myoviridae, Siphoviridae, Podoviridae. Myoviridae* family has long contractile tail phage and *Podoviridae* family phage has short tail.

The order *Caudovirales* consists of tailed bacteriophages (*cauda* meaning tail in Latin) represent the most numerous and widely distributed group of bacterial viruses. Phage

capsid is composed of protein coat without envelope and linear double-stranded DNA as a core material. In this order, phages are distinguished by binary symmetry, i.e. cube symmetry for phage head and spiral symmetry for tail. The order is differentiated into 3 families (*Myoviridae, Siphoviridae* and *Podoviridae*) based on the tail peculiarities (Novik *et al*., n.d.). Family *Myoviridae* consists of nearly 25% of bacterial viruses with a long contracting tail composed of inner hollow tube and sheath. Similarly, 62% in the family *Siphoviridae* with non-contractile tail and 14% in the family *Podoviridae* consisting short rigid tail.("Caudovirales -dsDNA Viruses (2011)," n.d.).

Bacteriophages also uses Single stranded DNA (ssDNA) as a genetic material, these phages generally belong to the family *Inoviridae, Microviridae.* These phages are filamentous phages, and replication occurs via rolling circle mechanism. By contrast, according to the latest (2014) report of the ICTV, two families of RNA bacteriophages are also recognized. The single stranded RNA (ss RNA) bacteriophage; family *Leviviridae* and double-stranded RNA (dsRNA) family; *Cystoviridae*. Enterobacteria phage Qβ, Enterobacteria phage F1, Enterobacteria phage MS2, and Enterobacteria phage GA are the examples of *Leviviridae* family and Pseudomonas phage φ6 is only recognize species of *Cystoviridae* (Krishnamurthy *et al.,* 2016).

1.1.2.3 Lytic and lysogenic mechanism of bacteriophage

The bactericidal activity of bacteriophages has been used to treat bacterial infections as an alternative or a complement to antibiotic therapy**.** Bacteriophage undergo five general steps such as adsorption or attachment, penetration, biosynthesis, assembly and release for completely lysis of host cells as eukaryotic viruses. The major processes of host infection by phages include phage attachment via a host receptor, control of the host lytic-lysogenic cycle, and the host cell lysis mechanism. Several host receptors have been experimentally determined and characterized for phage infection such as different flagellar and capsular antigen, lipopolysaccharide (LPS), peptidoglycan(PG), teichoic acid, sex pilus and host outer membrane proteins (OmpC , BtuB, TolC (Ricci and Piddock, 2010), and FhuA (Heller and Braun, 1982; Casjens *et al.*,2005). These receptors play a role in the determination of phage host specificity, suggesting that host receptor study would be able to provide novel insights into the mechanisms of phage infection with host cell (Shin, *et al.*, 2014).

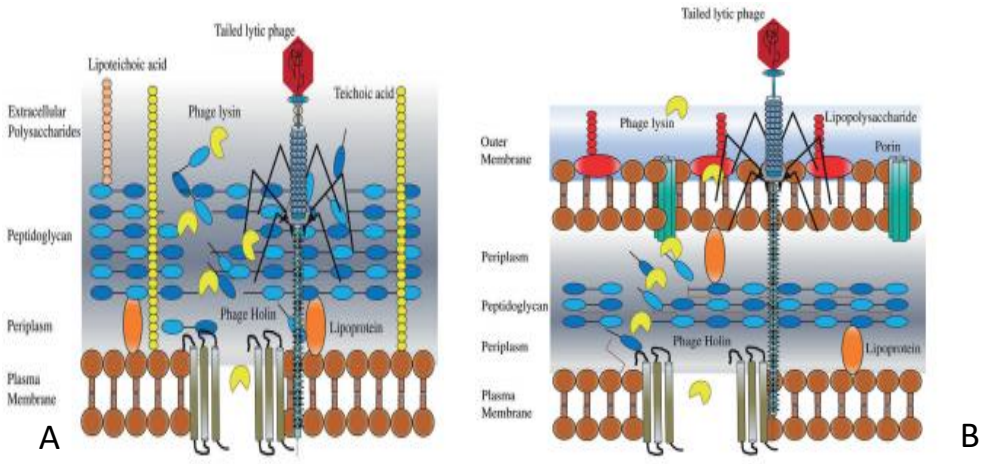


Figure 1.3 Interaction of phage with cell wall of bacteria. Fig. A; Gram positive bacteria and B; Gram negative bacteria (Jiang *et al.*, 2017).

The receptors also help to determine the narrow or broad range of activity of bacteriophages against bacteria. Penetration of nucleic acid takes place after irreversible adsorption of phage, mechanism of this process is specific for each phage or group. Enzymatic splitting of cell wall components, electrochemical membrane potential, availability of ATP play vital role for penetration of phage genetic materials inside the bacterial cell (Rakhuba, *et al.*, 2010). Generally, bacteriophages have two types of activity against bacterial cells during their life cycle; lytic activity and lysogenic activity.

Lysogenic cycle involves the insertion of phage genome into the host bacterial genetic materials. Replications occurs as a part of bacterial DNA resulting in the appearance of prophages. Lysogenic phages generally contain a lysogeny control region consisting of cro, cI, cII, cIII, N, and Q and constitutive bacteriophage promoters, PL and PR. During early gene expression, CII, CIII, and Q proteins are produced. Among these proteins, the CII-CIII complex activates PRE and PI promoters, resulting in the lysogenic cycle by the production of integrase and CI (lytic cycle repressor protein) protein, which are related to phage genome integration and blocking of all phage gene expression (Shin *et al.*, 2014). The lysogenic state can be interrupted spontaneously or by the induction of sunlight, UV radiation, alkylating agent such as methyl methane sulphonate, an antibiotics mitomycin C. Campoy et al., in 2006 also showed that the infection of *Salmonella enterica* with P22 or SE1 bacteriophages triggers the lytic development in *Salmonella enterica* through the induction of SOS response (Campoy *et al.*, 2006). Example of bacteriophages with a lysogenic cycle include lambda (λ) Escherichia coli; Mu, with activity against *E. coli, Salmonella, Citrobacter* and *Erwinia*; MM1, *S. pneumoniae*; and ϕ11 *S. aureus.*

In the lytic cycle, bacteriophages use different biosynthetic materials of bacteria for replication of nucleic acid and proteins constituting the structural part of the capsid somewhere tail and tail fiber whereas replication of bacterial DNA is inhibited or in limiting

stage. This step is also called eclipse period of bacteriophage growth curve. This is followed by the assembly of phage components and maturation of bacteriophage. Lysis of bacterial cells to release phage progeny uses two different ways by different phages. The release of daughter phages have capability to infecting other host cell. Filamentous phages are continuously extruded from bacterial cells without killing them, whereas non filamentous phages induce lytic of the host cell.

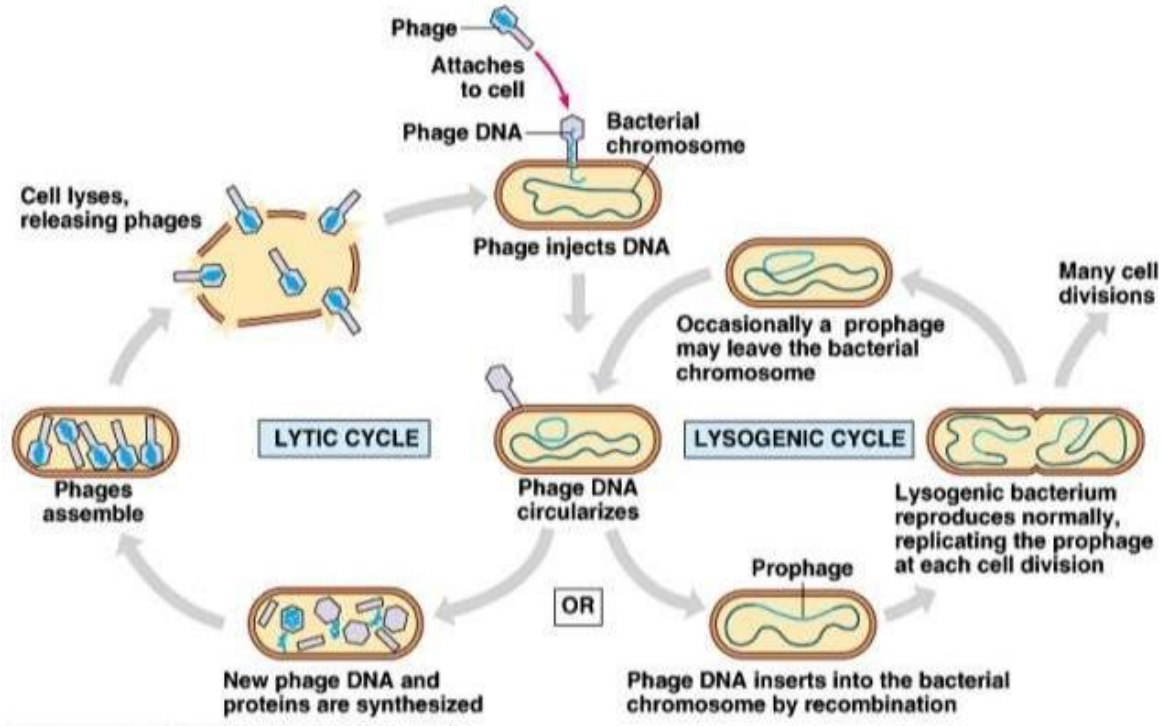


Figure 1.4 Lytic and lysogenic life cycle of bacteriophage. Lytic cycle goes through the synthesis of phage particles to produce phage progeny after the attachment oh host. Lysogenic phages insert its DNA to the host DNA and produce prophages with division of host cell. *Source: https://www.slideshare.net*

Lysis also cause abrupt damage to the cell wall of bacteria and it occurs by two mechanism which are inhibition of synthesis of cell wall components by different protein and enzymatic cleavage of PG or other components by lysin(s) or holin lysin system (Wang *et al.*, 2000). Lysins are the integral components of virion tail that locally digest the cell wall to facilitate the injection of the phage genome into the host cell. These are produced during the late phage of phage life cycle. Some lysins that are exported to the periplasma by the host *sec* system contain N-terminal signal sequences that function as a type II signal anchor (designated as SAR; signal arrest release) or uncleaved signal peptide (Hermoso, *et al.*, 2007).The lysins usually do not have a signal peptide to translocate them to the

periplasmic space to access the PG and phages encode small hydrophobic proteins termed holin, which export lysins across the bacterial inner membrane . Lysin usually possesses only one type of hydrolytic activity but other enzymes such as endopeptidase and lysozyme from phages B30 and NCTC 11261 and endopeptidase and NAM amidase from *Staphylococcus* phage harboring two in depended lytic activities has also been reported. (Rigden, *et al*., 2003; Loessner, 2005)**.** Examples of bacteriophages which undergo lytic life cycle are T_4, T_1.

1.1.2.4 Advantages of Bacteriophage over antibiotics

Bacteriophages were first used successfully to treat bacterial infections a decade before penicillin was discovered. However, the excitement towards phage therapy was short-lived, due to the lack of understanding basic phage biology. Now, the global increase in problem of bacterial resistance to antibiotic, a number of scientists and clinicians are returning again at bacteriophages as a therapeutic option in the treatment of bacterial infections. Phage therapy is a promising and challenging strategy for the development of new antimicrobial treatment approaches. Favor for bacteriophages uses in a therapeutic agent, appear to offer a number of advantages over the use of chemical antibiotics. Some of them are discussed below: (Hanlon, 2007; Ryan *et al.*, 2011; Loc-Carrillo and Abedon, 2011; Golkar *et al.*, 2014)

- Activity: The use of lytic bacteriophage has capability to completely lyse bacteria (bactericidal activity) so that the bacteria are unable to regain their viability. But certain antibiotic such as tetracycline have bacteriostatic activity which may permit bacterial resistivity.
- Dosing: Bacteriophage multiply within susceptible bacterial host exponentially with the single or initial dose and subsequently release outside from host cell. So, there is no need for repeating doses (also called auto dosing). But in case of antibiotic treatment there is needs of repeated dosing.
- Low chances of toxicity: Generally, bacteriophages consist of nucleic acid and capsid proteins, they are inherently nontoxic (Skurnik *et al.,* 2007; Kutter *et al.,* 2010). However, there should be concern about harmful immune responses during treatment. Side effects of antibiotics can range from mild allergic reactions to severe and adverse events and also vary from person to person. Use of unprescribed dosing of antibiotics still cause life-threatening adverse reactions in host body mostly due to defective drug (Yılmaz and Özcengiz, 2017).
- Production cost: Isolation and formulation of new bacteriophage against bacteria take short time and low cost as compared to the development of new antibiotics. Because bacteriophage generally isolated from sewage, water resources and other

waste material which contain high concentration of bacteriophages and bacterial host.

- Specificity: Most bacteriophages are very specific to bacteria, owing to this property, phage only target at the site of infection where its host are concentrated. This makes the minimal impact on health associated normal flora. Antibiotics have broad spectrum activity and easily travel throughout the body which can kill normal flora as well as beneficial organism of the body.
- Resistivity: Due to the use of antibiotic in indiscriminative way, antibiotic resistivity is in increasing. Development of phage resistance to bacteria is lower as compared to antibiotic because the mechanism of action of bacteriophage is completely different from antibiotic. Although, bacteriophages represent last line of antimicrobials for the multiple antibiotic resistance bacteria.

1.2 Current studies

The emergence of multiple drug-resistant bacteria has prompted interest in the use of bacteriophages as antimicrobial agents. Nowadays, using bacteriophage in different purposes; phages as a therapeutic agent, as biocontrol's, phage display, phage typing, Phages as indicators, as food preservatives and decontaminants, transducer considered as tremendous investigate to make facility for human being. Phage endolysin is also a most extensively studied field in recent years specifically for the treatment of drug resistance Gram positive bacterial infection. Therapeutic (lytic or virulent) bacteriophages have been one of promising alternative agents which can be utilized for medicinal and biological control purposes in clinical, veterinary, agriculture and related fields. The idea to treat bacterial infections with phages came out of the pioneering work of Félix d'Hérelle but this was overshadowed by the success of antibiotics.

1.3 Research Hypothesis

This research aimed to assess the efficacy of lytic bacteriophage that have broad host among different drug resistant bacterial pathogens.

Null hypothesis: Lytic *Salmonella* bacteriophage does not show the interspecies host range.

Alternative Hypothesis: Lytic *Salmonella* bacteriophage show the interspecies host range.

1.4 Objectives

1.4.1General Objectives

Isolation and characterization of bacteriophage against colistin resistance *Salmonella Typhi* from different places of Kathmandu valley.

1.4.2 Specific objectives

- Confirmation of host bacteria by 16S r RNA sequencing and submission to NCBI database.
- Isolate the lytic bacteriophage against colistin resistant *Salmonella Typhi.*
- Comparative analysis of physiochemical property of most potent bacteriophages.
- Evaluate intraspecies and interspecies multiple host range of isolated phage.
- Protein profiling of bacteriophage by SDS PAGE
- Morphological identification of the phage using Transmission Electron Microscopy.
- Bioinformatics analysis of whole genome of sequenced phage.

1.5 Rationale

Emergence of drug resistance infection worldwide has become major concern of public health sector. Antibiotic resistant *Salmonella* spp in human are mostly associated with food and animal products. The increasing rate antibiotics resistant pathogens in animal directly leads to the incidence of foodborne illness so that substantial morbidity and mortality has been caused. That's why, there is urgent need of novel approach to fight against such multi-drug resistant pathogen.

Among various approaches, phages have been efficient, specific and possibly cheaper alternatives than other. From the long history of its uses, phage therapy which uses lytic bacteriophage, become one of the key solutions. The detection and production of phage in large scale is a difficult and revealing task. However, with the advancement of molecular techniques like genome sequencing and high-resolution microscopic evaluation, phage diversity can be easily studied now.

The main purpose of this study is to explore and analyze the potential application of lytic bacteriophages against multidrug resistance pathogens. In Nepal, to my best knowledge, isolation and characterization of bacteriophage against colistin resistant bacteria has not been performed yet. So, the primary purpose of this study is to screen, isolate and characterize most potent lytic phage that infect specific as well as interspecific host bacteria. Thus, this study provides basis for an evaluation of phage for potential therapeutic application to control pathogens.

For the successful isolation of specific phage against target bacteria, there should be need to understand the microbial ecology of phage. The simplicity by which phage can be isolated from the natural environment have been attributed to the fact that specific phage has capable of lysing bacteria and phages are there where bacteria are abundantly present. As we know the river water of Kathmandu valley are polluted with fecal materials, untreated sewage and domestic waste, we selected the rivers water as a phage source. Additionally, *Salmonella* generally associated with the animal husbandry, so we also preferred the poultry waste and meat shop waste as phage sources.

Chapter II

LITERATURE REVIEW

2.1 Development of Antibiotic Resistance

The infections caused by bacteria that have become resistant to commonly used antibiotics make serious and major global health problem in the 21st century (Desai, *et al*., 2015) The diagnosis and treatment of this cases are require longer, more complex and also significantly more expensive process. Antibiotic resistance, initially associated with problem of an increased number of hospital-acquired infections usually in critically ill and immunosuppressed patients. Now it has extended into the community causing severe infections which become difficult to diagnose and treat. Bacteria have developed resistance to all different classes of antibiotics discovered to date.

Box 1: CDC assesment of Antibiotic resistance threats (Ventola,2015)

Urgent Threats
- *Clostridium difficile*
- Carbapenem-resistant Enterobacteriaceae (CRE)
- Drug-resistant *Neisseria gonorrhoeae*

Serious Threats
- Multidrug-resistant *Acinetobacter*
- Drug-resistant *Campylobacter*
- Fluconazole-resistant *Candida* (a fungus)
- Extended spectrum beta-lactamase-producing Enterobacteriaceae (ESBLs)
- Vancomycin-resistant Enterococci (VRE)
- Multidrug-resistant *Pseudomonas aeruginosa*
- Drug-resistant nontyphoidal *Salmonella*
- Drug-resistant *Salmonella* Typhimurium
- Drug-resistant *Shigella*
- Methicillin-resistant *Staphylococcus aureus* (MRSA)
- Drug-resistant *Streptococcus pneumoniae*
- Drug-resistant tuberculosis

Concerning Threats
- Vancomycin-resistant *Staphylococcus aureus* (VRSA)
- Erythromycin-resistant Group A *Streptococcus*
- Clindamycin-resistant Group B *Streptococcus*

Antimicrobial resistance (AMR or AR) is the ability of a microbe to resist the effects of medication that once could successfully treat the microbe and the term antibiotic resistance (AR or ABR) is only to bacteria becoming resistant to antibiotics. Microbes resistant to at least one drug in three or more categories of antimicrobials are called multidrug resistant (MDR) (Magiorakos *et al*., 2012). Those microbes which are resistant to at least one agent in all categories, considered as extensively drug resistant (XDR) or totally drug resistant (TDR) are sometimes called "superbugs; concentrating antimicrobial resistance genes in the same cell" (Lerminiaux and Cameron, 2019). There are two (genetic and mechanistic) basis of mechanisms by which bacteria become resistant to antibiotics.

2.1.1 Genetic basis of antimicrobial resistance

Bacteria generally use two major genetic strategies one is mutations in gene(s) often associated with the mechanism of action of the compound another is acquisition of foreign DNA coding for resistance determinants through horizontal gene transfer (HGT) to adapt to the antibiotic "attack" (Munita and Arias, 2016). In general, mutations resulting in antimicrobial resistance alter the antibiotic action via one of the following mechanisms, modifications of the antimicrobial target (decreasing the affinity for the drug,), a decrease in the drug uptake, activation of efflux mechanisms to extrude the harmful molecule, or modification of drug target sites and global changes in important metabolic pathways that bypasses the action of drugs (Tenover, 2006).

Three main strategies that bacteria acquire external genetic material are transformation (incorporation of naked DNA), transduction (phage mediated) and conjugation (Lerminiaux and Cameron, 2019). Similarly, one of the most efficient mechanisms for accumulating antimicrobial resistance genes is represented by transposons and integrons, (Hoek *et al*., 2011) which provide an efficient and simple mechanism for the addition of new genes into bacterial chromosomes, these are site-specific recombination systems capable of recruiting open reading frames in the form of mobile gene cassettes.

2.1.2 Mechanistic basis of antimicrobial resistance

There are several mechanisms that help bacteria become resistance to antibiotics. Most of them are enzymatic basis and some are due to the activity of membrane potentials (intrinsic factors).

2.1.2.1 Chemical alterations of the antibiotic

A well-known mechanism of acquired antibiotic resistance in both gram-negative and gram-positive bacteria is the production of enzymes that have capable of changing chemical structure of the antimicrobial molecule. Different types of modifying enzymes such as aminoglycosides acetyltransferase (ACC), adenyltransferase (ANT) or phosphotransferase (APH) which catalyzes the most frequent biochemical reactions including acetylation (aminoglycosides, chloramphenicol, streptogramins), phosphorylation (aminoglycosides, chloramphenicol), and adenylation (aminoglycosides, lincosamides) (Ramirez and Tolmasky, 2010).

2.1.2.2 Destruction of the antibiotic molecule

Destruction of these compounds is due to the action of different enzymes. β-lactamase is an example of these enzymes which destroy the amide bond of the β-lactam ring,

rendering the antimicrobial ineffective. β-lactamases provide multi-resistance to β-lactam antibiotics such as penicillin, cephalosphorin, cephamycin, carbapenem. Genes encoding for β-lactamases are generally termed *bla* (Lee *et al*., 2015) followed by the name of the specific enzyme (e.g. blaKPC; first identified *klebsella pneumonia*). To date more than 1,000 different β-lactamases have been described and many more are likely to continue to be reported. The Bush-Jacoby classification divides β-lactamases into 4 categories (each with several subgroups) according to their biochemical function and mainly based on substrate specificity but in first, the Ambler classification separates β-lactamases into 4 groups (A, B, C, and D) according to amino acid sequence identity (Bush, 2013)

Carbapenemases is a diverse group of β-lactamases with the ability to hydrolyze carbapenems. These enzymes can be divided into serine based hydrolytic carbapenemases (Ambler class A or D) and metallo-carbapenemases that contain zinc in active sites (Ambler class B enzymes) (Queenan and Bush, 2007). These enzymes are predominantly found in *Klebsiella* spp. (therefore its name KPC, ***K****lebsiella* ***p****neumonia* **c**arbapenemase), they have been reported in several other gram-negatives, including *Enterobacter spp., E. coli, Proteus mirabilis*, and *Salmonella spp.* A total of 22 variants of the blaKPC gene have been described to date, most of them located in plasmids harboring transposable elements (e.g. Tn4401) or in association with insertion sequences like ISKpn6 and ISKpn7 (Nordmann *et al.,* 2009).

More recently (2008), a new carbapenemase was identified in a *K. pneumoniae* and *Eascherichia coli* isolates from Sweden in an indian patient who had been previously admitted to a hospital in New Delhi, India. The enzyme was designated NDM-1, in reference to its origin (**N**ew **D**elhi **M**etallo β-lactamase) (Kumarasamy *et al.,* 2010). The *blaNDM* gene has been found in several types of plasmids readily transferable among different species of gram-negatives, and it has also been associated with the presence of insertion sequences such as the ISAba125. In contrast to other genes encoding metallo-enzymes, blaNDM is not usually related to integron-like structures (Nordmann, 2011).

2.1.2.3 Decreased Antibiotic Penetration and Efflux

Bacteria have developed mechanisms to prevent the antibiotic from reaching its intracellular or periplasmic target by decreasing the uptake of the antimicrobial molecule. Hydrophilic molecules such as β-lactams, tetracyclines and some fluoroquinolones are particularly affected by changes in permeability of the outer membrane since they often use water-filled diffusion channels known as porins to cross this barrier (Pages *et al.,* 2008). Among the best-characterized porins, the three major proteins produced by E. coli (known as OmpF, OmpC and PhoE) and the *P. aeruginosa* OprD (also known as protein

D2)(Fernández and Hancock, 2012) are classical examples of porin-mediated antibiotic resistance.

Many classes of efflux pumps have been characterized these systems may be substrate-specific (for a particular antibiotic such as *tet* determinants for tetracycline and *mef* genes for macrolides in pneumococci) or with broad substrate specificity, which are usually found in MDR bacteria (Poole, 2005). This mechanism of resistance affects a wide range of antimicrobial classes including protein synthesis inhibitors, fluoroquinolone, β-lactams, carbapenems and polymyxins.

2.1.2.4 Changes in target site

Protection and modifications of target site are the two-common strategy for bacteria to develop antimicrobial resistance by avoiding binding and interacting antibiotics with their target site. One of the classic and best-studied examples of the target protection mechanism is the tetracycline resistance determinants *Tet(M)* and *Tet(O)* which were initially described in *Streptococcus spp*. and *Campylobacter jejuni* respectively. But they are now both widely distributed among different bacterial species, likely because they have been found in several plasmids and in broad-range conjugative transposons (Connell *et al.,* 2003).

TetO and *TetM* interact with the ribosome and dislodge the tetracycline from its binding site in a GTP-dependent manner. *TetM* directly dislodges and releases tetracycline from the ribosome by an interaction between the domain IV of the 16SrRNA and the tetracycline binding site. Furthermore, this interaction alters the ribosomal conformation, preventing rebinding of the antibiotic (Donhofer *et al.,* 2012). Modifications of the target site is another most common mechanisms of antibiotic resistance in bacterial pathogens affecting almost all families of antimicrobial compounds. These target changes may consist of point mutations in the genes encoding the target site, enzymatic alterations of the binding site (e.g. addition of methyl groups), and/or replacement or bypass of the original target.

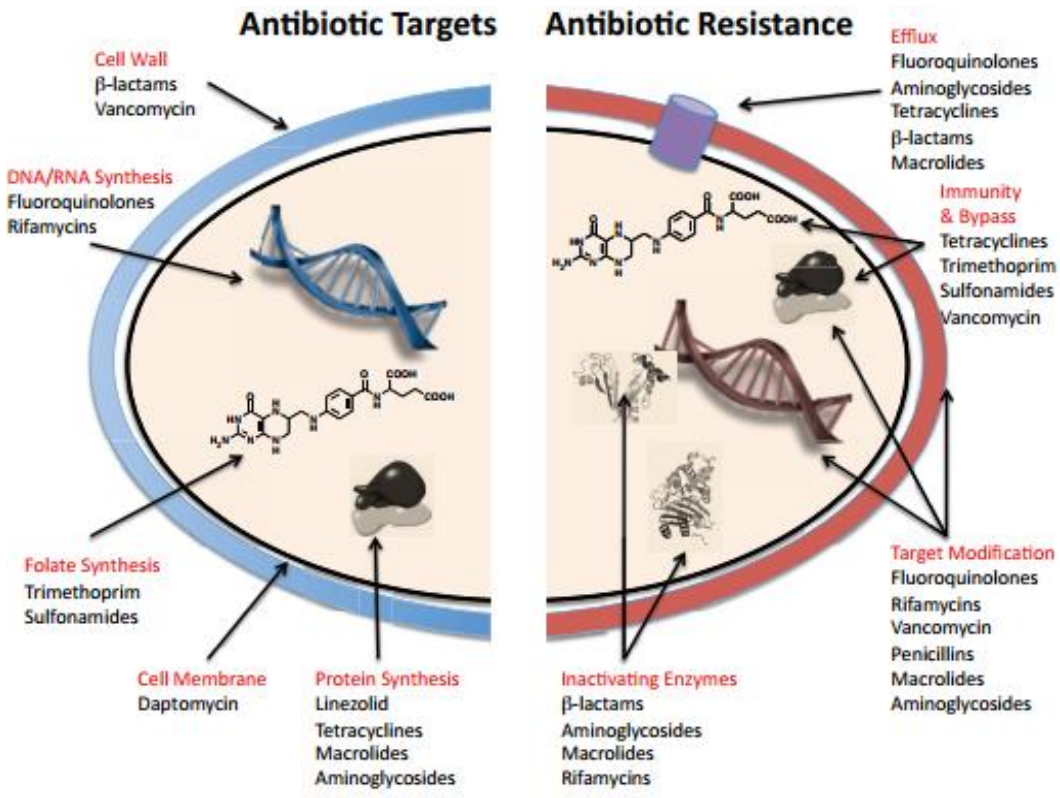


Figure 2.1 Target of antibiotics and mechanism of antibiotic resistance (Wright, 2010). Left part denotes the targets of major antibiotics to the different parts of bacterial cell and right parts is the development of antibiotics due to efflux change, target modification, inactivating enzymes etc.

2.2 Multidrug resistance in Salmonella

The decreased susceptibility of *Salmonella* to antibiotics is mainly due to the widespread use of antimicrobial agents in food-animal production such as chicken, cows, pigs and other livestock. An estimated 23×10^6 kg of antibiotics are used annually in the United States; about half of these are provided to people, and rest are manufactured for animal and agricultural use. Livestock are fed antibiotics not only because they are infected, but also for preventive purposes and growth promoters. These antibiotics are transmitted to humans through food of animal origin (Golkar *et al*., 2014).

The integrin gene cassette system present in *Salmonella* is one of the main reasons for the rapid development of multidrug resistance in clinical strains through horizontal gene transfer. The existence and flexible transmission of integron was proven suitable for the spread of drug resistant genes and the acceleration of multidrug resistance (Lu *et al*., 2014).

Colistin resistance in Salmonella

Colistin usage in human has been limited because of its toxicity when given systematically. However, the increasing number of MDR in healthcare-associated infections has meant that colistin has been re-introduced as a last-resort treatment option in human medicine. It was first authorized in the UK as a veterinary medicine in 2004 and is currently authorized for treatment of gastrointestinal infections caused by *Escherichia coli* in cattle, sheep, pigs and poultry.

Colistin is a mixture of the cyclic polypeptides colistin A and B and it belongs to the class polymixins. The colistin is produced by certain strains of the bacteria *Paenibacillus polymyxa* so it also names as polymixin. Two forms of colistin are available commercially: colistin sulfate (cationic) and colistimethate sodium (anionic). Polymyxin interacts with the lipopolysachharides (LPS) of the outer membrane of Gram-negative bacteria and competitively displaces divalent cations (Ca^{2+} and Mg^{2+}) from the negatively-charged phosphate groups of the lipid A of LPS. Both the positive charged amine groups and the hydrophobic fatty acyl chain of polymyxin B play important roles in the interaction with bacterial LPS (Nation and Li, 2009).

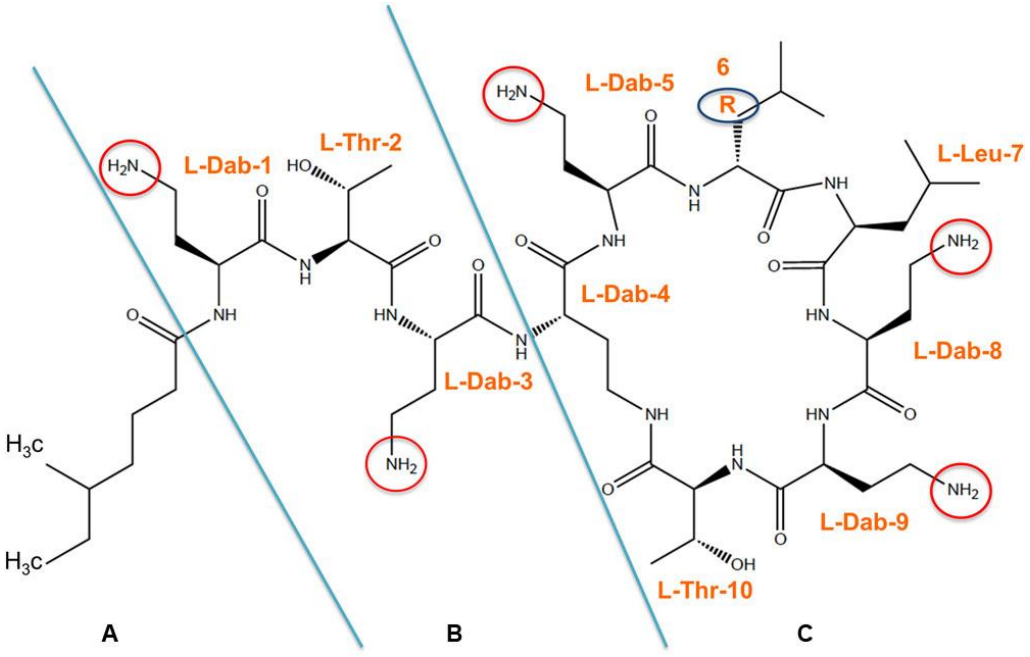


Figure 2.2 Chemical structure of colistin showing three main parts; (A) hydrophobic acyl tail, (B) linear tripeptide segment, (C) hydrophilic heptapeptide ring (Rhouma *et al.*, 2016). Numerical indicate the amino acids present in the structure and encircled amino groups are the reactive amino groups.

Resistance to polymyxins in bacteria is commonly due to modification of lipid A, which anchors the lipopolysaccharide molecule to the outer membrane, resulting in reduction in the affinity to polymyxin. Until recently, all reported polymyxin resistance mechanisms have been chromosomally mediated, involving, for example, SNPs in genes encoding two component regulatory systems (e.g. pmrAB, phoPQ), resulting in modification of lipid A. The PhoPQ system autoregulates the *oprH-phoP-phoQ* operon under limited concentration of divalent cations (Mg^{++}), (Ly *et al.*, 2012) PhoPQ activates the transcription of *pmrAB* gene. Earlier studies have suggested that PmrAB resistance in *Salmonella Typhimurirum* is regulated by the PhoP-PhoQ depended manner (Gunn and Miller, 1996) and in *Acinetobactor baumanii* (Beceiro *et al.*, 2011) and *Peudomonas auregenosa* (Moskowitz *et al*., 2004) independent manner.

Liu *et al.,* reported the first case of a plasmid-mediated colistin resistance mechanism, designated mcr-1, in E. coli and *Klebsiella pneumonia* (Liu *et al.*, 2016). Occurrence of the mcr-1 gene also has been reported in E. coli, Salmonella and *Klebsiella* from Laos, Thailand, Tunisia, Nigeria, South America and several European countries from people, pigs, poultry and foodstuffs, with the list growing rapidly. Furthermore, the first colistin-resistance gene in plasmid which can be transferred between bacterial strains was found in China as part of the human intestinal commensal flora since 2011 (Anjum *et al.*, 2016). More recently, resistance to colistin has been reported among *A. baumannii* clinical strains. Indeed, a surveillance study of US hospitals revealed that 5.3% of all *Acinetobacter* strains were resistant to colistin. Carbapenems have been considered to be appropriate agents for the treatment of MDR *A. baumannii* strains, but colistin methansulfonate (CMS) is required whenever there is carbapenem-resistant *A. baumannii* infection is emergeing (Qureshi *et al.*, 2015)

Now it constitutes a serious public risk for humans, since this resistance trait has been co-transferred along with other antimicrobial resistant genes, such as ESBLs, metallo-β-lactamases (MBLs), and KPCs, a development through the assistance of conjugative plasmids (Wang *et al.*, 2017)

The rapid global spread of bacterial resistance is seen almost all antibiotics even in carbapenem (4^{th} generation antibiotics) and colistin (last resort drug). Now it makes pressing stage to investigate alternative treatment options. Bacteriophages is an important alternative perspective to antibiotics. Bacteriophage therapy could serve as a biocontrol and therapeutic treatment against MDR strains. Bacteriophage seems quite superior to antibiotics, since they are persisting, inactive and non-lytic outside their bacterial hosts. Phage therapy is already evolved and developed concept before the development of antibiotics, but there is need to regenerate and make applicable this concept to fight against the antibiotic resistant bacterial infection.

2.3 History of bacteriophage discovery to bacteriophage therapy

The history of phage therapy starts with discovery of bacteriophage which has been a matter of extensive debates and controversies over decades for priority claims. Prior to the discovery and widespread use of antibiotics, it was suggested that bacterial infections could be prevented and treated with bacteriophages. In 1896, Ernest Hanbury Hankin, a British bacteriologist working as the Chemical Examiner and Bacteriologist to the Government of the United Provinces and of the Central Provinces of India, first reported that the waters from the Indian rivers Ganga and Yamuna contained an antibacterial activity that destroyed cultures of cholera-inducing bacteria (Sulakvelidze *et al.,* 2001a). And he also suggested that these small heat liable substances are passes through fine porcelain filter and were responsible for limiting spread of cholera epidemics. After two years later, Nikolay Fyodorovich Gamaleya observed a similar phenomenon while working with *Bacillus subtilis* (Hermoso *et al.*, 2007).

Frederick Twort, a British microbiologist in 1915, noted that "pure" cultures of bacteria may be associated with a filter-passing transparent material which may break down bacteria of a culture into granules. This transparent material, which was found to be unable to grow in the absence of bacteria, was described by Twort as a ferment secreted by the microorganism for some purpose. But it was not well clear at that time (Duckworth, 1976; Twort,1993). After two year later Félix d'Herelle in 1917, while studying patients suffering or recovering from bacillary dysentery; described a similar experimental finding (McKinley, n.d.-b). Through a number of trials and field experiments, Felix d'Herelle introduced the use of bacteriophages as an antimicrobial agent in clinical medicine (Fruciano and Bourne, 2007). He developed the idea of phage therapy, a therapeutic and prophylactic treatment and published many non-randomized trials from experience all over the world. He even introduced treatment with intravenous phage for invasive infections, and he summarized all these findings and observations in 1931.

Several contributions from other investigators did converge to support d'Herelle's idea that phages were living particles or viruses when replicating in their host cells. The fact that bacteriophages may infect bacteria discovered in 1925 by Bordet and Bail, confirmed the idea that the capacity of reproducing phages within bacteria need the insertion of phage-encoded material into the hereditary units of the host microbe also called as lysogenecity (Wittebole *et al.,* 2014). Frank Macfarlane Burnet, an Australian scientist awarded the Nobel Prize in 1960 for his work on lysogeny, immunity, viral nature of phage and interaction of phage with its bacterial hosts in 1960 (Sankaran, 2010). Schlesinger confrmed the nucleoprotein and biochemical nature of bacteriophages (Pennazio, 2006).

Finally, with the invention of electron microscope (EM), a German doctor, Helmut Ruska in 1940 first observed round particle from phage suspension adhering to a bacterial

membrane(Ackermann and Ackermann, 2011). One year later, Luria and Anderson, in Camden, make a micrograph of T4 phage with a nonhomogeneous round head, thinner tail under EM, giving sperm like appearance (Hendrix, 1998). They also described the different stages of bacteriophage during lysis such as adsorption, bacterial damaging stage and release of newly formed bacteriophage.

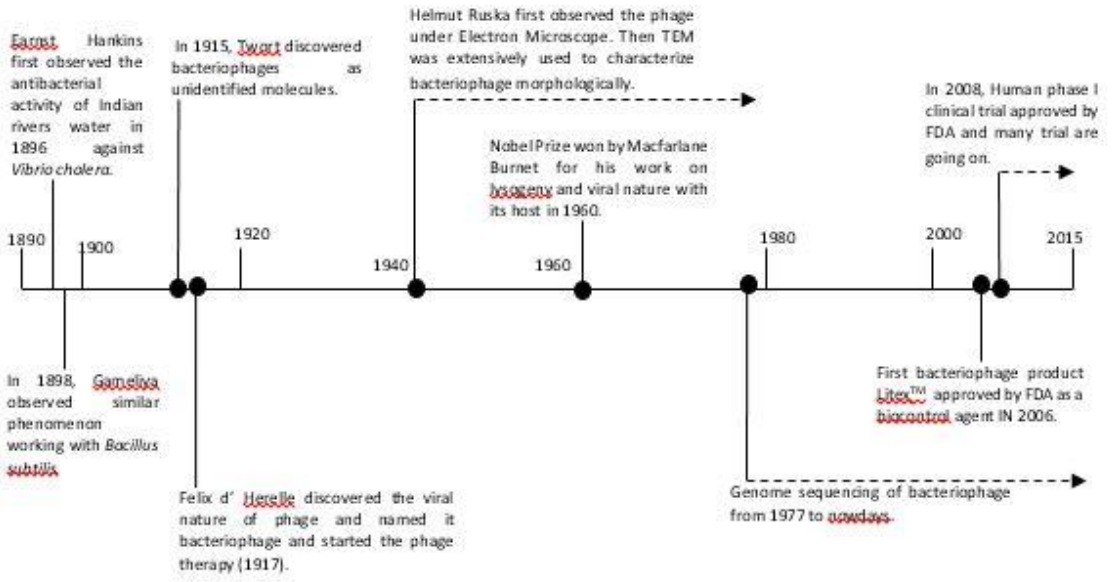


Figure 2.3 Timeline of major events in phage discovery to phage application. Twort (1915) and d'Herelle (1917) independently discovered phage and d'Herelle started the phage therapy. Since the discovery, phages were used therapeutically but much of its use halted after discovery and ease use of antibiotics. After the discovery of its electron microscopic structure, phages have been extensively used in molecular biology to present days. In 2006, FDA approved use of phage cocktail (Litex™) to prevent contamination of *Listeria monocytogenes* in processed meat.

The preclinical phage therapy in animal model was started from the beginning of 1980s. Between the time 1950s to the 1980s there was little published paper on the subject of utility of phage therapy in animal models. For example, in separate work, phages were shown to be effective in rescuing rats from fatal systemic infections (Smith and Huggins, 1982) both induced with *E. coli.* In another animal model, phage therapy was applied in rescuing chicks from fatal diarrhea (induced with *S. Typhimurium*) (Berchieri *et al.,* 1991). Similarly, in mid of 1880s, a series of paper reported on 550 cases of suppurative bacterial infections (empyemas, peritonitis, osteomyelitis, etc.) in humans. Most of the cases were chronic and resistant to all available antibiotics. The targeted bacterial pathogens included *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *E. coli* (Carlton, 1999). Investigators reported approximately 90% of cases have cured by the

use of bacteriophages. Lang et al. reported the use of bacteriophage in seven patients with chronic orthopedic infections caused by resistant organisms. They were able to cure two cases of prostheses, one case of tibial osteomyelitis due to Proteus, *Staphylococcus aureus* and *Klebsiella*; and another case of septic arthritis of the knee due to *Enterobacter* and *Staphylococcus aureus* (Lang *et al*., 1979; Abedon *et al*., 2011). The commercialization of therapeutic phage preparations to treat bacterial infection in humans was started in France by D'Herelle and in the United States in the 1940s by the pharmaceutical company Eli Lilly. However, because of disputed results and the increase in antibiotics uses as a treatment option in the 1940s, the commercial pursuit of therapeutic phages in the West was ceased, although in Eastern Europe phage therapy is going on (Inal, 2003).

2.4 Bacteriophage Therapy

In spite of problem associated with multidrug resistance in microbials, very few antimicrobials with new mechanism of action are under development, bacteriophage therapy is the one alternative approach that could offer to alleviate this challenge. In last two decades, data have been accumulated to show that phage therapy became important alternative to antibiotics in the treatment of bacterial infections. Mostly virulent (lytic) bacteriophages are used in phage therapy. Many cases with successful results have been obtained in combating infections in humans and animals.

Felix d'Herelle in 1917 began testing of phage in human patients. He demonstrated safety and efficacy of phage by administrating to 12 year old boy with severe dysentery under the clinical supervision of Professor Victor-Henri Hutinel at the Hospital desEnfants-Malades in Paris. The patients recovered after single treatment (Setlow *et al.,* 1992). Another successful report of phage therapy came from Bruynoghe and Maisin, (1921) who used phage to treat staphylococcal skin infections (Sulakvelidze, *et al*., 2001b). Soothill (1992) saw protection of mice against systemic infections with both *Pseudomonas* and *Acinetobacter* after use of appropriate phage. He also showed prevention of skin graft in guinea pig by prior treatment with bacteriophages against *Pseudomonas aeruginosa* (Soothill, 1994).

Phage therapy was tried extensively and many successful results were reported for a variety of diseases, including dysentery, typhoid and paratyphoid fevers, cholera and pyogenic (pus-producing) and urinary tract infections. During treatment, phages were poured into lesions, given orally or applied as aerosols or enemas. They were also given as injections-intradermal, intravascular, intramuscular, intraduodenal and intraperitoneal, even into the lung, carotid artery and pericardium.

The production and usage of phages for therapy and prophylaxis is limited to small scale up to now. Several companies have small scale productions of phage for various purposes. The Pasteur Institute in France produced phage preparations against various pathogens (*Pseudomonas, Staphylococcus, Escherichia coli,* and *Serratia*) until 1974 after the successful treatment of dysentery at the Hospital des Enfants Malades in Paris (Sulakvelidze *et al.*, 2001b) These phages were used mainly against skin infections, septicemia, osteomyelitis, wound infections, urinary tract infections, and middle ear and sinus infections. In the United State phages have been used mainly for preparation of animal and human vaccines. *Staphyloccocal* Phage Lysate (SPL) was an example used for animal protection and it was also licensed for human therapeutic usage (Salmon and Symonds, 1963).

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. Unfortunately, owing to regulatory pressure, production of SPL for human therapy was suspended in the 1990s, and the preparation is currently approved and marketed only for veterinary application (Slopek *et al*., 1985). Georgia and Poland are the two European countries where phage therapy is routinely available. Russia also uses phage therapy but information is not so much available. Recently, phage therapy is started in the World Care Center in Lubbock, Texas (Golkar *et al.,* 2013).

Bacteriophage cocktail for therapeutic aspects

Specifity is one of the most interesting property of bacteriophages. The host range of phages can range from narrow (having capacity to infect only single strain) to quite broad (host ranges spanning from different species to multiple bacterial genera). Owing to the benefits of narrow spectrum bacteriophage in therapeutic aspects (minimal chance to harm normal flora), use of single or narrow host range phage must be weighed against the costs associated with identifying the phage susceptibility of pathogens prior to initiation of treatment. By which this issue can be addressed through the combination of individual phage isolates having broad or narrow host range

The simultaneous use of more than one phage type is term as polyphage or phage cocktail therapy. The use of phage cocktails widen the spectrum activity of phage and to prevent the development of phage resistance bacterial mutant (Chan *et al.*, 2013) Formulation of phage cocktails also superior for a presumptive test prior to identification of pathogens. However, having many phages in a cocktail could result detrimental impact on nontarget normal bacteria. Although in most cases this impact is still less than that expected of typical commercial antibiotics. Several studies were published that supports principle activity of phage cocktails to improve the lysis of host. Tanji *et al*. showed that the repeated oral administration of three phages, SP15, SP21, and SP22 was effective for rapid evacuation of *E. coli*O157:H7 from the feces and gastrointestinal tract of mice (Tanji *et al.*, 2005). Yu *et al.* (2018) done comparative study of single phage with phage cocktail. Their phage cocktail had not only wider host range and rapid lysis but also decreased the generation and mutation frequency of phage-resistant strains in vitro than single phage (Yu *et al*., 2018). In another study, use of *Klebsiella* phage cocktail showed significant reduction of 6 log cycles ($p < 0.01$) in burn wound infection BALB/c mice by *K. pneumoniae* B5055 as compared to that of untreated control animals (peak of 8.81 log CFU/ml) (Chadha *et al.*, 2016).

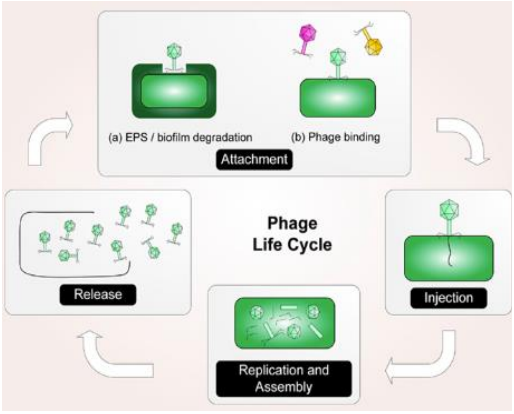


Figure 2.4 Activity of phage cocktail. Receptors recognition by bacteriophage which is specific for target pathogen and lysis of host after phage cocktail administration (Semler *et al.*, 2012).

2.5 Other applications of bacteriophages

2.5.1 Phage as biocontrol agents in Food and Agriculture

Phages can be used to control the growth of bacteria in both food and food contact surfaces. In most studies, the use of high efficacy phages against several major foodborne pathogens resulted in significant reductions of bacterial counts in foods; such reductions are known to substantially decrease a risk of foodborne infections. Main factors that determine the efficacy of bacteriophages used as biocontrol agents include phage particles density in/on a food and agricultural product, level of bacterial contamination, the development of bacterial resistance to phages, and phage stability in different food. Due to their specificity, bacteriophages are attractive for sanitization of ready-to-eat foods (RTE) such as milk, vegetables and meat products.

The status of generally recognized as safe (GRAS) was recently given to the *L. monocytogenes* specific phage Listex P100, which was approved as a food preservative by the U.S. Food and Drug Administration. This has motivated many researchers to investigate the development of phages for biocontrol (Golkar *et al.*, 2013). Goode *et al.* (2003) applied lytic bacteriophages to experimental chicken skin contaminated with *Salmonella enteritica* serovar *enteritidis* and *Campylobactor jejuni* at a multiplicity of infection 1, 100 and 1000, recuced the number of pathogen by 2 $\log_{10}$ over 48 hour (Goode *et al*., 2003). The US Department of Agriculture (USDA) in 2007, approved bacteriophage product designated as spray sanitizers to disinfect cattle hides targeting *Salmonella* spp and *E. coli* (Goodrige and Abedon, 2008).

Simillarly, black spot disease caused by *Xanthomonas Campestris* and *Pseudomonas syringae* on tomato and pepper plant was control by the commercial product of bacteriophage; Agriphage (Monk *et al.*,2010). Recent clearance by FDA of bacteriophage preparations for food applications shows that bacteriophages are gradually gaining acceptance as a means of prevention of foodborne infections.

2.5.2 Bacteriophage for the control of biofilm production

Bacterial cell generally exists with two types of mode; planktonic cell and sessile aggregate which is known as the biofilm. Biofilm is an extracellular polymeric substances (EPS) including enzymes, proteins, polysaccharides, DNA, RNA, in addition water (up to 97%) are major components (Sutherland *et al.*, 2004). Beyond the discovery of microbial biofilm by other scientists, Costerton in 1978, coined the term biofilm and alert the world about the importance of biofilm (Costerton *et al.,* 1999). When the bacterial cell exposed to adaptation under diverse nutritional and environmental condition, the microorganism undergo certain changes after adhering to a surface. During biofilm formation four general steps are followed by microorganism; (a) attachment to a surface (b) formation of micro-colony (exopolysaccharides components) (c) formation of three dimensional structure biofilm (d) biofilm maturation and detachment (dispersal). Nearly, (99.9%) of micro-organisms have the ability to form biofilm on biological and inert surfaces (Jamal *et al.*,2015) *P. aeruginosa, S. epidermidis, E. coli spp, S. aureus, E. cloacae, K. pneumoniae* are mostly reported biofilm producer bacteria.

Increasing rate of biofilm producing organism leads to the emergence of antibiotic and biocide resistance. Mechanism of development of antibiotic resistivity due to the biofilm production includes, direct inactivation of active molecule, altering body's sensitivity to target of action, alteration of efflux pump and membrane permeability, reduction of the drug diffusion and concentration before reaching to the target site.

Bacteriophages have seemed to a greater ability to target this common form of bacterial growth. Bacteriophage act differently on biofilm producing bacteria than antibiotic do.

Bacteriophages have some of properties that make biofilms susceptible to their action. Bacteriophage can induce and express the depolymerizing enzymes that degrade the EPS within host cell. Bacteriophages can progressively remove the biofilm and reduce the potential for regeneration when bacteriophage replicate within cell. Bacteriophage also able to infect persister cells, remaining dormant within them, but when they become metabolically active bacteriophage can also be active (Harper *et al.*, 2014)

Leiman *et al*. (2004) demonstrated that the bacteriophage T4 of *Escherichia coli* have enzymes present on the tail of the virus particle, while they could theoretically play a role in degrading the biofilm matrix and also aid the penetration of bacterial cell wall (Leiman *et al.,* 2004). Alves *et al.* in 2014 applied the combine use of bacteriophage K and novel phage DRA88, which showed significant reduction of biofilm produced by *S. aureus* biomass over 48 h of treatment (Alves *et al*., 2014). The pretreatment of catheters with *Pseudomonas* phage cocktail reduced the 48-h mean biofilm cell density by 99.9% (from 7.13 to 4.13 log10 CFU cm^2; $P < 0.001$) (Fu *et al.*, 2010). These are some examples and experimental works done to supports the applications of phage for biofilm reduction. These results show considerable promise in the control of biofilm by applying phages, especially phage cocktails. However, such applications are still evolving, and large-scale uses are still under development. If effective approaches have been developed and approved, best practices for such uses will, of course, emerge.

2.5.3 Phage display

Phage display technology is one of the most effective molecular diversity technique and valuable tool in biomedical sciences which offers rapid, efficient and relatively inexpensive methods for investigating protein-protein interactions, receptor binding sites, identifying epitopes, mimotopes, generating monoclonal antibodies, improving or modifying the affinity of proteins for their binding partner. Due to the high flexibility of this techniques, phage display has been applied to transfusion medicine, neurological disorders, mapping vascular addresses, tissue homing of peptides, and furthermore it has proved useful in cancer studies and for delivery of vaccines.

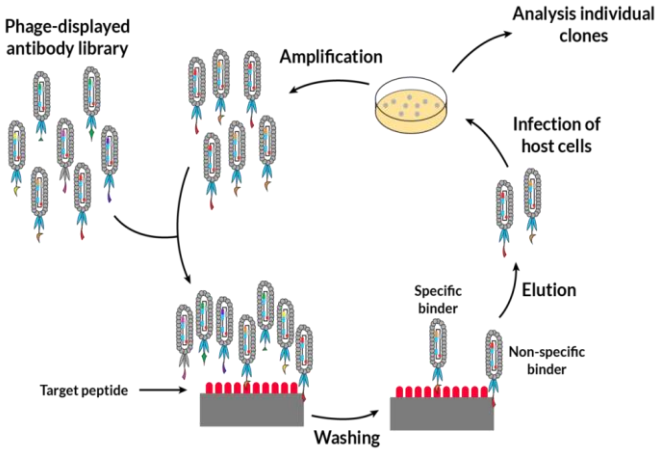


Figure 2.5 Phage display cycle showing different steps in reference to antibody production. Target gene of interest is inserted to the phage genome, phage express protein to the outside and specific binder proteins are selected, amplify and prepared phage displayed antibody library for further use.

Phage display is the linkage between phage genotype and its encapsulated phenotype. In this technique, a gene encoding a protein of interest is inserted into a phage coat and protein gene, causing the phage to "display" the protein on its outside while containing the gene for the protein on its inside. M13 (Smith and Petrenko, 1997), f1 and fd filamentous phages (Kehoe and Kay, 2005) are most common and extensively used bacteriophages in this technique, although T_4, T_7, and λ phage have also been used. George P. Smith was the first who describe display of peptides on filamentous phage by creating fusion of the virus's capsid protein to a library of peptide sequences in 1985 (Smith, 1985).The loss of coat protein functionality was the major limitation of the phage display technology, however this problem was overcome by hybrid phages and coat protein modifications.

The significance of phage display in hematological applications is growing. Anti-ABO, anti-Rh and anti-Kell antibodies were the first obtained antibodies against red blood antigens used for hemagglutination assay (Marks *et al*., 1993). Autoimmune thrombocytopenia (AITP) is one of the most commonly reported autoimmune disorder which has been investigated by phage display. During the random Screening, heptapeptide phage-displayed library was allowed to obtain a phage clones specific to autoantibodies from AITP individuals (Gevorkian *et al.*, 1998). It was also reported that humanized-camel phage display library was utilized in elaborating novel immunotherapeutic strategy for botulism by using a cell penetrating, humanized-single domain antibody that inhibits the botulinum neurotoxin (Thanongsaksrikul and Chaicumpa, 2011). Molecular imaging agents that will be able to visualize pathogenic processes in vivo and tumor targeting peptides obtained by phage display has been one of the main objects of interest in recent years. The scFv (MFE-23) molecule specific for CEA was the first phage displayed recombinant antibody used for tumor targeting (Kelly and Jones, 2003).

2.5.4 Phage Typing

The limited host range of many phages (having capability to infect only a single strain of bacteria) make them useful for distinguishing different strains within the same species. Phage typing is a method used to identify the different strains of pathogens within a community by growing the culture of strain inoculating with different bacteriophages. At first phage-typing was used mainly in short-term investigations of limited outbreaks of sepsis or enterotoxic food-poisoning. Later, the workers were concerned with developing the standardized method and realized the importance of typing procedure (Parker, 1972). Bacteriophage typing is widely accepted as a convenient and highly discriminatory method of identifying epidemic strains of pathogens. (Turbadkar *et al*., 2007). The great advantages of phage typing of bacteria is that it will detect differences between the strain that are identical by serological and other immune-molecular tests. Phage typing is generally more useful for epidemiological purposes. *Salmonella* infection, notably typhoid fever and paratyphoid and infection associated with *Staphylococcus* are the two most successfully typing bacteria by phage typing.

In 1961, Blair and William used phage typing to track outbreak, epidemic and community strains *S. aureus* effectively (Blair and Williams, 1961). Sakamoto *et al.* (1975) had studied phage typing method for *P. aeruginosa* by using phage groups instead of the individual phages with the more clear and reproducible result. They concluded that this system was specific for *P. aeruginosa* or its closely related species (Sakamoto *et al.,* 1975). In the initial phage, bacteriophage typing was the standard method for typing of bacteria. In the past decades (Pulsed Field Gel Electrophoresis) PFGE has replaced bacteriphage typing as the more standard and easy method for typing *S. aureus* isolates. (Zakoks *et al*., 2002; Gustafson *et al*., 2003) It is because PFGE is known to be more discriminatory than phage typing.

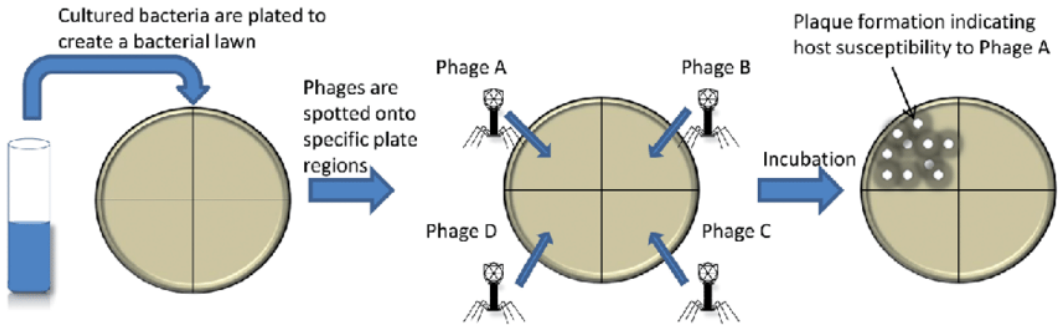


Figure 2.6 Simplified process of phage typing. The bacterial lawn is prepared for bacteria to be typed. Different phages are spotted at their respective labelled plate region and plaques formation is indicative for the host susceptibility to specific bacteriophage (van der Merwe *et al.*, 2014)

2.5.5 Phages as indicators

Detection of human viruses is still a highly skilled, complicated and costly process. Because of the morphological similarity of bacteriophages with human viruses and by means of easy detection, bacteriophages have been studied worldwide as good potential for pollution indicators. Guelin (1948) was the first who recognize the potential of bacteriophage as an indicator and, numerous reports have indicated the potential of bacteriophage as indicator of water pollution (Hilton and Stotzky, 1973; Grabow *et al.*, 1987). Coliphages are the group of bacteriophages that specifically infect coliform bacteria. Somatic coliphages, male specific RNA coliphages or FRNA coliphage and Bacteriods phage are the mostly used indicators phage (Leclerc *et al.,* 2001). Most known heterogeneous group of somatic coliphages found in municipal waste belong to the *Myoviridae, Siphoviridae*, *Podoviridae*, and *Microviridae* families. The F-specific RNA bacteriophage group, also known as sexual coliphages or male-specific bacteriophages, infect bacteria through sex pili belongs to the family *Leviviridae*.

In addition, coliphages have been suggested to be better indicators of enteroviruses as they have been found to be removed at comparable rates with enteroviruses during treatment processes. Somatic coliphages are detected in raw municipal wastewater throughout the world in 100% of the samples tested and values range from 5×10^6–10^7 PFU per 100 mL, with those of F-specific phages ranging from 10^4 to 10^6 PFUs per 100 ml. Kott *et al.* (1974) showed that concentration of coliphages to human enteric viruses in flood waters ranging from as low as 1:1 to as high as 10^3:1. But at various season the concentration was variable; in wastewater the ratio was 10^5:1; trickling filter effluents in winter was 10^4:1; in spring 10^5:1, in summer and rainfall 10^4:1, in oxidation pond effluents in winter the ratio of coliphage to virus was reported as 10^3:1; in spring 10^4:1; and in summer and fall it was 10^3:1 (Kott *et al.*, 1974).

In addition, Sharon *et al*. (2006) reported that, Male-specific (F^+) coliphages have been proposed as a candidate indicator of fecal contamination and of virus reduction in waste treatment. Hence, monitoring of different coliphage groups can indicate the presence of major sources of microbial inputs to surface waters. However, different reports also showed that the limitation of coliphage due to the consistency, reproducibility, complexity for the enumeration of F^+ bacteriophage (Leclerc *et al.*, 2001), low concentration of bacteriophage supports the inclusion of bacteriophages as additional indicators of the efficiency of water quality.

2.5.6 Phages as transducer

Transduction is one of the main classes of Horizontal Gene Transfer (HGT). It is the process by which foreign DNA is transferred from one bacterial cell to another bacterial cell through bacteriophage. Transduction was first discovered in *Salmonella* by Norton

Zinder and Joshua Lederberg at the University of Wisconsin–Madison (Zinder and Lederberg, 1952). There are two types of transduction process, 1.Generalized transducing phage such as the P1 (*E. coli* phage) and P22 (*S. Typhimurium* specific phage package host DNA fragments randomly during lytic growth. 2. Specialized transducing phages such as λ which are formed due to the inexact excision of prophage and induction of different physiochemical and molecular activation of protein (Campbell, 1976).

The process of bacterial transduction is very essential mostly in the molecular biology. At present, the phenomenon of antibiotic resistance is becoming widespread. Transduction is specifically important as it can explain the mechanism by which antibiotics become ineffective because of the transfer of the genes among bacteria. The process of transduction has other importance, mapping of bacterial gene, increase in genetic diversity and it have been also used extensively in genetic manipulations, including bacterial strain engineering, transposon mutagenesis, and plasmid transfer (Matilla and Salmond, 2014).

2.6 Challenges of bacteriophage therapy

The successful launching of a profitable therapeutic product is the most challenging task. The measurements of product identity, quality, purity, and potency are very important considerations of investigational drugs and biologics. Intellectual property rights, safety, reproducibility, stability and robustness of the product are also other major issue in the drug industries. As other drugs and medicines, successful and marketable bacteriophage products also would be a highly purified, containing one or several fully characterized phages, properly controlled efficacy and safety studies. And also have optimized dose, route and method for administrations (Skurnik *et al.*, 2007).

Concerning about the uniqueness of phage as pharmaceuticals, some disadvantages of phage make the therapeutic aspects of phages more challenging. In general existence of phage resistance, narrow host range of phages, lack of understanding of lytic mechanisms, problem association with the use of temperate bacteriophages which can encode the bacterial virulence genes such as endotoxin (Krylov, 2001; Skurnik *et al.,* 2007;Merabishvili *et al*., 2009), difficulties for the fully characterization of phage. In addition, Complexity and sensitivity during product manufacturing, presence of contaminants in phage preparations as well as poor stability, ethical issues and ultimately failure to establish strong scientific evidence are the problem faced by research on phage therapy in human bacterial infection in applicable way (Barbu *et al.*, 2016).

Despite an enormous number of researches and reports that supports the phage have been used successfully for infections, obstructions appear for the expansion of phage therapy is due to the lack of patentability of most phages, which most likely discourage

investors. Addressing these issues related to bacteriophage product manufacturing, designing early clinical trials that have reliable product safety are prerequisites for advancing and to demonstrate effectiveness and assess trails of bacteriophage product in clinical benefit. Regardless of these challenges, phage therapy is an exciting field that have potential to overcome serious conditions (specially for antibiotic resistance conditions) where treatment option are limited (Henein, 2013).

2.7 Recent Advancement on Phage Research

Bacteriophage therapy is an extensive and controversial history in the field of antimicrobial treatment. Recently, increasing incidence of antibiotic-resistant and virulent bacterial pathogens has stimulated interest into these bacteria-specific viruses. In spite of these efforts, certain challenges such as limited host range, bacterial resistance to phages, comprehensive study of every isolated phages, regulation and manufacturing and delivery make bacteriophage therapy remains an underutilized option specially in western medicine. Recent advances in biotechnology, disease diagnosis, macromolecule delivery and synthetic biology may help to overcome these problems. In order to successful advancement of phage therapy in clinical setting, the research efforts must be collaborative with practical approaches at academic, commercial and regulatory level (Lu and Koeris, 2011).

One of the areas that has been looked in recent phage research is the isolation, whole genome characterization and optimization of broad host range bacteriophage. Specifically, for the application, it is better to finish the complete genome sequencing of bacteriophage. Whole genome sequencing is the process of determining complete genome sequence of an organisms at a single time. Sequencing of nearly an entire human genome was first accomplished in 2000 partly through the use of Shotgun Sequencing technology. Since 2005 capillary sequencing has been progressively displaced by high throughput (formerly "next-generation") sequencing technologies such as Illumina dye sequencing, pyrosequeencing and SMRT sequencing. The Whole genome sequencing of bacteriophage is a new and reliable method to better understand phage encoded proteins and biomolecules especially phage lytic enzymes (holing, lysin) which are involved in the bacterial cell lysis and death. Melo *et al.* identified and characterized LM12, a broad host range bacteriophage against *Staphylococcus aureus* having genome size 143,625bp (ds DNA), with endolysin activity against stationary phage cell and biofilm cell reduction (Melo et al., 2018).

Bioinformatics analysis also confirmed the absence of any gene encoding virulence factors, toxins, or antibiotic resistance determinants (Melo *et al*., 2018). Confirmation of their life cycle by identification of gene encoding lysogenic control region, integrase enzyme and even for the presence of any toxic gene in the phage genome could also

analysed by sequencing of genetic material (Klumpp *et al.*, 2012). The information contained in phage genome vary among different phage hosts. To the same extend the sequenced genome is also used to taxonomically classify novel and new phages in orders, families, subfamilies, genus and species (Tulio *et al.*, 2017). For example, Park *et al*. in 2012, isolated and characterized SFP10 novel bacteriophages simultaneously inhibit both *Salmonella enterica* and *Escherichia coli* O157:H7, major food borne pathogen. The SFP10 phage belongs to the family *Myoviridae* having genome size 157,950 bp which contain complete genes required for replication, other nucleotide mechanism and for phage structural protein and the absence of lysogenicity in *Salmonella* (Park *et al*., 2012)

Endolysin, phage encoded enzymes have been nowdays used successfully to control antibiotic resistance pathogens in animal model. Endolysin are murein hydrolases produces in phage infected bacterial cell at the end stage of life cycle of bacteriophage. Endolysin produced at periplasmic space then it is transported to cell wall (specifically target peptidoglycan) of bacteria through membrane pores/lesion formed by holing thereby inducing lysis of bacterial cell and enable the release of phage progeny. As purified recombinant protein, endolysin have rapid degradation of peptidoglycan (PG) and lysis of bacterial cell wall when applied externally. Endolysin can be classified based on their catalytic activity as, N-acetylmuramidases (lysozymes or muramidases), lytic transglycosylases endo-b-N-acetylglucosaminidases (glucosaminidases); all three act upon the sugar moiety glycosides in the cell wall. N acetylmuramoyl-L-alanine amidases (NAM-amidases) hydrolyzes the amide bridge connecting the sugar and peptide constitutes of peptidoglycan and lastly endopeptidases, cleave the peptide bond (Hermoso *et al.*, 2007; Roach and Donovan, 2015).

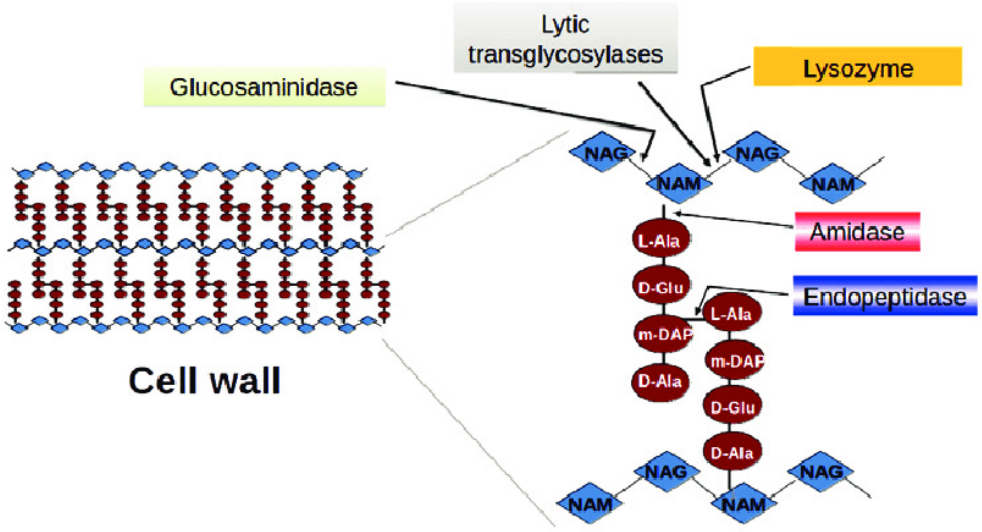


Figure 2.7 Schematic representation of endolysin cleavage sites within the peptidoglycan (Ajuebor *et al*., 2016).

Phage endolysin have generally two functional domain one is cell wall binding domain(CBD) and another is enzymatically active domain(EAD) (Schmelcher *et al.*, 2012). But the structure or domain of endolysin differ between those enzymes targeting Gram-positive and Gram-negative bacteria. EAD confers the cleavage of specific bonds within the bacterial PG (catalytic mechanism of the enzyme) and CBD target the protein to its substrate. It tightly to the cell wall debris after cell lysis, thereby preventing diffusion and also help to destruction of surrounding intact host cell (Loessner *et al*., 2002)

Several advantages of endolysin make them attractive antimicrobials against pathogens. These unique characteristics of endolysin includes, rapid hydrolysis of bacterial cell was both invitro and invivo, species specific strong receptor binding affinity, low probability of developing resistance, safety as compared to whole phage application, can be modified by genetic engineering (Fischetti *et al.*, 2006). There are several reports that supports structure and characterization and the hypothesis towards activity of phage endolysin as an antibacterial. Plotka et al. in 2015 characterized and validated Ts2631 endolysin isolated from extremophilic bacterium *Thermus scotoductus* MAT2631. The enzyme was thermodynamically stable (T_m = 99.82°C, $\Delta H_{cal} = 4.58 \times 10^4$ cal mol^{-1}) and retained 64.8% of its initial activity after 2 hr incubation at 95°C. The endolysin Ts2631 not only have thermostable property but also have broad substrate spectrum that means it can recognize cell wall of other different Gram-negative pathogens such as *Escherichia coli*, *Salmonella panama*, *Pseudomonas fluorescens* and *Serratia marcescens* which make it good for use as an antimicrobial (Plotka *et al.,* 2015). Similarly, Daniel et al. demonstrated that the novel chimeric lysine (ClyS) obtained by fusing the N-terminal catalytic domain of the *S. aureus* Twort phage lysin with the C-terminal cell wall-targeting domain from another *S. aureus* phage lysin (phiNM3) which was expressed in *Escherichia coli* showed synergistic effect with oxacilin invitro to protect against methicillin resistance *S. aureus* (MRSA) (Daniel *et al.,* 2010). This was supposed to be first study to demonstrate the potential of a phage lysin as a therapeutic agent in mucosal and systemic infection caused by methicillin-resistant *Staphylococcus aureus*.

With the pharmacokinetics and pharmacodynamics, clinical trials in animal model and in human are the two most concerning area of phage therapy. Pharmacokinetics is the dose determining steps in the phage therapy development. The clinical information on bacteriophage concentrations, site of phage injection, bodily distribution and subsequent elimination/clearance are crucial in designing the dose because the dose for one application (*e.g.*, skin) may not be suitable for another (*e.g.*, gastro-intestinal diseases) (Parracho *et al.*, 2012).

Recently, trails in animal model are extensively working area that already discussed in different context of literature review part. Under the European Medicines Agency (EMA)

or United States FDA jurisdictions, very few human clinical trials have been conducted up to date in modern standard. Small number of phase I study have been conducted and published (Bruttin and Brüssow, 2005; Merabishvili *et al.,* 2009).

In 2009, an evaluating bacteriophage cocktail targeting *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* in venous ulcers was reported. Which was approved by US Food and Drug Administration (FDA) as bacteriophage phase I clinical trial. But no adverse events were attributed to the study product, efficacy preparation will need to be evaluated in phase clinical trial II efficacy study (Rhoads *et al.*, 2009). In parallel, randomized phase I/II clinical trial targeting chronic otitis caused by antibiotic-resistant *P. aeruginosa* was conducted by Wright *et al*. which was approved by UK medicines and Health products Regulatory Agency (MHRA). This was the first and only regulatory efficacy trial of bacteriophage therapy (Wright *et al.*, 2009).

Regardless of the modern knowledge on genetics and physiology of bacteriophage, there is still lack of formal, well controlled and large-scale clinical studies on their safety and efficacy as therapeutic agents. These regulatory challenges and also patentability of phage halt the movement of phage as a biological therapeutic agent. But industry and regulatory bodies (FDA and EMA) and other agencies are actively and continuously working towards this goal, using the Biological Medicinal Products guidelines for European trials and the Division of Vaccines and Related Product Applications in the USA, as a starting point (Parracho *et al.*, 2012).

2.8 Concluding Remarks

The diversity of phage and its interactive biology with bacteria is reviewed by above study. However, it is premature to confirm exact phage host mechanism for all type of strain without solid result. But we can confidently hypothesize the naturally occurring bacterial viruses (bacteriophages) can infect and kill the drug resistance pathogens of human/animal/plant origin. On the other hand, exploiting the ability of bacteriophage to infect bacteria, and customize them to kill MDR pathogen effectively, ultimately leads to revitalization of using phage therapeutically.

Chapter III

METERIALS AND METHODOLOGY

This study was performed in Central Department of Biotechnology (CDBT), Institute of Science and Technology, Tribhuvan University, Nepal. Major of the work on this study were wet lab based and some of the study were dry lab based experimental study. All of the general requirement for this study such as Media, reagents, glass-wares, plastic-wares, syringe filters etcetera (etc) were purchased from regular supplier and the required equipment such as incubator, centrifuge, hot air oven, vortex, PCR machine etc were made available by the department.

3.1 Bacterial strains collection and preservation

Bacterial strains used in this study were collected from AHRD NARC (glycerol stock) which were already confirmed colistin resistant *Salmonella enterica* subspecies *enterica* serovar *Typhi* at molecular level. Collected bacterial strains were immediately transport to microbiology laboratory at Central Department of Biotechnology, TU Kirtipur at cold box maintaining 2-8˚C aseptically. Bacterial strains were renamed giving unique code.

Table 3.1 List of collected bacterial samples and their codes

SN	Bacterial strains	Bacterial code	Change code
1	*Salmonella Typhi*	1A	Sal 1
2	*Salmonella Typhi*	2B	Sal2
3	*Salmonella Typhi*	5B	Sal3
4	*Salmonella Typhi*	7A	Sal4
5	*Salmonella Typhi*	8A	Sal5
6	*Salmonella Typhi*	MC *Salmonella*	Sal6
7	*Escherichia coli*	*E. coli*	Ec1

Glycerol stock of all collected samples were prepared for preservation and future use. For glycerol stock preparation, a pure colony was cultured in LB broth and incubated at 37°C for 24 hrs. One milliliter of overnight culture was taken in cryovial and centrifuged at 12000rpm for 5min. The supernatant was discarded and pellet was resuspended with

300ul fresh LB broth. Then 700ul 50% autoclaved glycerol was added and incubated at 37˚C for 3 hrs. After incubation, tubes were cooled at 4°C for overnight, then -20°C up to next 24 hour and finally transferred to -80°C for long terms storage.

3.2 Identification by biochemical test

The glycerol stock of *Salmonella* strains collected from NARC were immediately subculture in Luria Bertani (LB) broth media followed by Salmonella Shigella (SS) agar medium which are selective and differential media for *Salmonella spp.* In parallel, biochemical tests were also performed. Urease, MRVP (methyl red Voges Proskuar, citrate and SIM (Sulphur indole motility) test were done for biochemical characterization. For these test, respective media were prepared in test tube and bacteria were cultured on these media. After overnight incubation at 37˚C, colour change were observed for urease, citrate and MRVP test and H_2S production and motility was observed for SIM test.

3.3 Antibiotic Susceptibility Test (AST)

The antibiotic susceptibility test of the *salmonella* species was done to reproduce the result. Kirby-Bauer disc diffusion technique was used for testing the antibiotic susceptibility pattern of bacteria. All antibiotics used for this study were available at the laboratory and classified under sensitive (S), intermediate (I) and resistant (R) according to the Clinics and Laboratory Standard Institute (CLSI) guidelines.

Log phage bacterial culture were lawn on Muller Hinton Agar using sterile cotton swab and it left for air dried. After air drying, antibiotic discs were placed on the lawn culture and incubated at 37°C for 24 hrs. The 'zone of inhibition – ZOI' of antibiotics against confluent lawn of bacterial growth was observed and measured using calibrated ruler.

Table 3.2 Antibiotics and their code used for AST

SN	Antibiotics	Code	Concn of disc(mcg)
1	Colistin	CL	10
2	Piperacilin	PI	100
3	Ertapenem	ETP	10
4	Ceftriaxone	CTR	30
5	Ipimenem	IPM	10
6	Meropenem	MRP	10

3.4 Genomic DNA extraction and 16s rRNA amplification of *Salmonella* strains

3.4.1 Genomic DNA extraction

Genomic DNA extraction of six *Salmonella* strain were performed by CTAB method. For this method, 1 ml of bacterial overnight culture was taken in eppendorf tube then cells were harvested at 13000 rpm for 5min. 567µl of TE buffer was added to the pelleted cells and mixed by gently vertexing or by repeated pipetting for resuspension. 30 µl of 10% SDS followed by 3 µl proteinase K was added to the solution with proper mixing after that it was incubated at 37°C for 1 hour. After incubation, 100 µl of 5M NaCl followed by 80 µl CTAB/ NaCl (0.7m NaCl, 10%CTAB) was added with well mixed. Then the solution was incubated at 65°C for 10min. After incubation, equal volume of Chloroform: Isoamyl alcohol (24:1) was added to the solution and it was well mixed with up and down the tubes then the mixture was centrifuged at 13000rpm for 5min. After centrifugation, upper aqueous layer was transferred to the new tube and 600 µl isopropanol was added to the solution then it was mixed gently until the DNA was precipitated and the solution was centrifuged at 13000rpm for 5min. Remove the isopropanol after centrifugation, 1ml of 70% ethanol was added to wash DNA and centrifuged for 5min. Ethanol was discarded after centrifugation and it was left for air dried. DNA was resuspended with 50 µl TE buffer after air dry and the DNA was stored at 4°C.

3.4.2 Amplification of 16S rRNA gene of Salmonella strains

For the molecular conformation of whether the collected samples were bacteria or not 16S rRNA gene amplification was done. For this amplification, already optimized PCR conditions and PCR reactions volume were used.

Table 3.3 Forward and reverse primer sequences used for PCR

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

PCR reaction mixture was prepared in PCR tubes as mentioned in table 3.4 and were subjected for PCR amplification as table 3.5. After the completion of PCR amplification, 5µl of the amplified product was analyzed by 1 % agarose gel electrophoresis on Tris Acetate EDTA (TAE) buffer containing 0.5 µg/ml Ethidium Bromide (EtBr) as a DNA staining solution. Gel was viewed on a UV transilluminator and image captured with the help of Gel Docsystem (company name).

The PCR product was sent to Xceleris Genomics India for the sequencing of 16S rRNA gene. Sequence obtained from the lab were analyzed by using different bioinformatics tools. Chromas was used for analysis of chromatogram file and to know the sequence similarity between our sequence and nucleotide database sequences, direct BLAST search was done. Similarly, for the evolutionary relationship between other sequences, phylogenetic tree was drawn by MEGA 10 software.

Table 3.4 PCR components, their concentration and volume used for PCR reaction

SN	Components	Concentration	Volume
1	Master mix	1X	12.5µl
2	Forward primer	10pmol/µl	2.5µl
3	Reverse primer	10pmol/µl	2.5µl
4	Template DNA	100ng/µl	2.5µl
5	Nuclease free water		5µl
	Final volume		25µl

Table 3.5 Thermocycling condition for 16S r RNA amplification

SN	Steps	Temperature	Time	Cycle
1	Step 1: Initial Denaturation	95°C	5min	1
2	Step 2: Denaturation	95°C	30sec	
	Annealing	50°C	45sec	34
	Extension	72°C	1min	
3	Step 3: Final extension	72°C	7min	1
4	Step 4: Final hold	4°C	∞	

3.4.3 Detection of bla NDM gene

For *bla NDM* gene amplification, PCR reaction mixture was set up as similar as 16s rRNA amplification which was already mentioned above. But PCR condition was different, so it was run according to condition as mentioned in Table 3.7. PCR product was run in 1% agarose gel electrophoresis. Bands were analyzed after observing the gel in Gel Doc by comparing bands size with ladder.

Table 3.6 Primer sequence and melting temperature (Tm) used for *bla NDM* gene PCR

Primer		**Sequence**	**Tm**	**Product length(bp)**
Forward		AATGCTGAATAAAAGGAAAACT	47.6°C	869
Reverse		GGCAGATTGGGGGTGA	51.8°C	

Table 3.7 Thermocycling condition for PCR amplification of *bla NDM* gene

SN	Steps	Temperature	Time	Cycle
1	Step 1: Initial Denaturation	95°C	5min	1
2	Step 2: Denaturation	95°C	30sec	
	Annealing	55°C	30sec, – 0.5°C PC	15
	Extension	72°C	1:30sec	
3	Step 3: Denaturation	95°C	1min	
	Annealing	49.7°C	30sec	20
	Extension	72°C	1:30sec	
4	Step 4: Final extension	72°C	7min	1
5	Step 5: Final hold	4°C	∞	

3.5 Bacteriophage Isolation, manipulation and processing

3.5.1 Water sample collection and processing

Water and waste sample were collected from different rivers and different places of Kathmandu valley. Water samples were collected from Balkhu river (Balkhu 1, Balkhu 2, and Balkhu3; Bayodha hospital near), Teku river (Teku I and Teku II), Mahadev khola, kirtipur pond and waste samples were collected from Manandhar Excyst Private Limited (poultry farm) and Kirtipur meat shop waste.

Figure 3.1 Water Sample collection sites. Teku river and Bagmati river (behind the Vayodha hospital and Balkhu Tarkari Bazar) in fig. A and B respectively. Water samples were specifically collected from the place where immobile water was there. Water was collected in 50ml falcon tubes.

The samples were collected in 50ml sterile falcon tubes and transported to the laboratory within 1-2 hrs. After transporting sample to the laboratory, 5 gram of solid waste sample was mixed with the 50ml of distilled water and it was vortex thoroughly to detached phage particles from solid substances. Then, both water samples and solid samples were centrifuged at 4000rpm for 30min in order to remove unwanted particles or cell debris. The supernatant was transferred to another falcon tubes without disturbing the cell debris and filtered through 0.20 µm syringe filter (PES Filter Media, Whatman™) and collected in a sterile falcon tube. The prepared sample was ready to use as phage source in our experiment and it was considered to be free from any particulate debris, bacterial or other contamination which has larger than 0.20 µm pore size.

3.5.2 Bacteriophage isolation

Double Layer Agar Assay (DLAA) method was used for the isolation of bacteriophage from different samples. Double Layer Agar Assay (DLAA) is the standard method for isolation of phages from environmental sources like water sample, sewage, dairy waste, animal waste, soil etc. For this assay, a hard agar plate of Tryptic soya agar (1 or 1.5% TSA) which form a base layer, that support the growth of host bacteria and soft agar of Tryptic Soya Broth (TSB, 0.5%) which form top layer were used. 1 ml of syringe filtered water sample was taken is sterile falcon or test tube and 100µl of log phase (OD 0.25) bacterial culture was added on it with mixing and it was allowed to attachment for 5 minutes without any disturbance.

Box 2: Naming of phage

For naming conventional approach has been followed. ICTV also recommend to use genus name followed by virus. For example, a virus is as word "phage" or symbol Ø and followed by representing University such Tribhuvan University (TU) where research is carried out and bacterial code (two uppercase letters if genus and species are known and three sentence lowercase letter code if only genus is known) along with numerical serial code and finally ending with an uppercase letter code that represents sample collection site. Example is given below.

Phage TU_sal2 T

- Sample collection site code
- Numerical code
- Bacterial sample code
- Institution code
- Representative organism

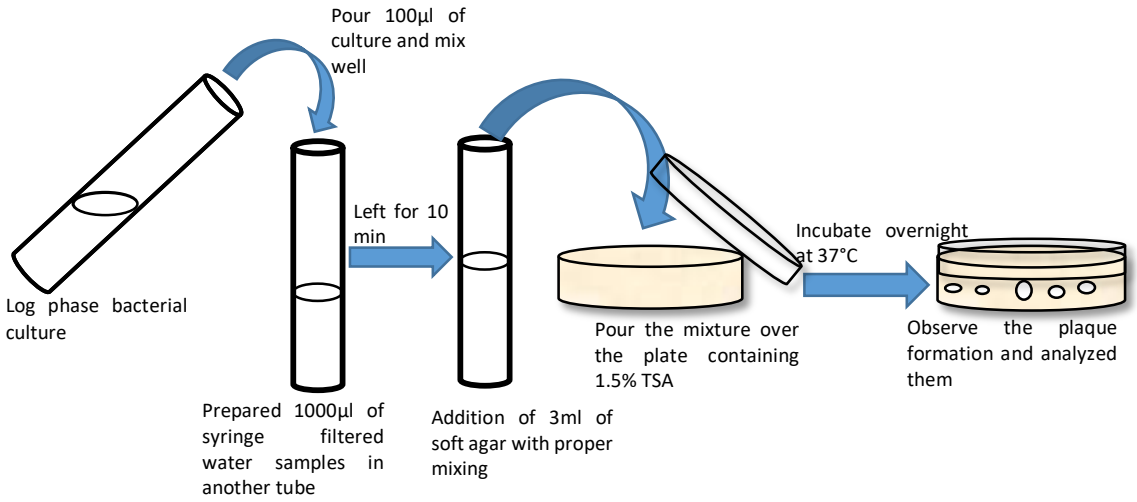


Figure 3. 2 Overall steps of Double Layer Agar Assay (DLAA). After the processing of sewage or water sample, DLAA was performed and plaques were analyzed on the basis of its size, shape and plaque opacity.

After attachment, 3ml of soft agar (Tryptic soya broth with 0.5% agar at 50˚c) was added to tubes, gently shaking the mixture with avoiding bubbles formation and poured over the already prepared hard agar (tryptic soya agar plate with1.5%agar). Plates were incubated in the incubator at 37˚c for 24 hours after solidification. For the negative control, only the bacterial culture was added to semisolid media and poured over TSA plate and incubated at 37°C for 24 hrs.

After 24 hrs incubation, plates were observed for the presence or absence of plaques and examined for clear or turbid lysis, number of plaques count, shape and size of plaque against bacterial host. Plates with positive result having clear lysis was selected for further processing.

The DLAA assay described above was repeated for all of the 6 different bacterial strains (Table 3.1) and all collected water and waste samples separately.

3.5.3 Bacteriophage purification

Bacteriophage purification was done by using the continuous streaking method. First the clear and separate plaque was selected and labelled in the Petri dish. Touched the center of the putative phage with the help of sterile inoculating loop and gently streaked continuously over another TSA (1.5%) plate. The starting line and ending line of streaking was marked. After streaking, 3ml of semisolid TSB (0.5%) was taken in sterile test tubes and 100µl of active log phase host bacteria was added and mixed well. The mixture was carefully poured onto TSA plate slowly from the most dilute (marked end point) of the streaked plate. The mixture was allowed to spread across the plate from the most dilute point to the more concentrated area (marked start point) by gently tilting or tapping the plate. Now, the plates were allowed to harden and incubated at 37˚C for 24 hrs. Next day, the clear lysis and plaques were observed on the streaked line. Three rounds of streaking were done from single plaque in order to obtain pure isolated single plaque morphology.

3.5.4 Bacteriophage stock preparation

Phage stocks were prepared by extracting phage from the plates where clear lysis and plaques were formed after three rounds of streaking. For the extraction of phage, 5ml of sodium magnesium, SM buffer was poured on the plate where the pure plaques were formed. SM buffer help to absorb and detached the phage particles from the media. The upper layer of top agar where plaque remains were collected in falcon tubes by scarping the agar with the help of sterile buds. Then the agar mixture was mixed with vigorous vertexing and then centrifugation was done for 20 to 30 minutes at 4000rpm. The supernatant collected after centrifugation was syringe filtered through 0.22µm pore size to avoid any bacterial cell debris in the solution. Finally, the pure phage stock was prepared and it was stored at 4°C for further use and characterization. For more stock

preparation, streaking was done in several plates from main stock and incubated overnight. Plaques were observed and extraction was done by repeating above mentioned procedure. Phage stock concentration and Phage titer was determined by serial dilution of phage and DLLA method.

3.5.5 Phage titer assay: spot assay

Phage titer determination and spot assay was done from the phage stock solution. This is the basis for determining the concentration of phage particles in a pure phage stock solution. For the spot assay, at first bacterial lawn was prepared by overlaying the mixture of 3ml of semisolid and 100 µl of active log phase host bacteria to the well labelled TSA plate. Then, the plate was allowed for complete dry. Meanwhile, phage stock solution was serially diluted up to 10^{-1} to 10^{-15} dilutions. For this 100 µl of phage stock solution was mixed with 900 µl of sterile SM buffer taken in a 1.5ml Eppendorf tube. Then 100 µl of diluent was withdraw from the first eppendorf tube and it was mixed in second tube containing 900 µl of SM buffer. The dilution was done in subsequent tubes up to 10^{-15} with changing the tips in every dilution. After complete dilution, 5 µl of all respective prepared phage dilutions were transferred aseptically onto the corresponding blocks which was labelled on the bottom of the plate. The droplets were allowed to soak into the agar and incubated at 37°C incubator for 24 hrs. Clear spot or plaques were observed after overnight incubation.

3.5.6 Determination of phage stock concentration

For the determination of phage stock concentration, DLAA was done from 10^{-1} to 10^{-15} dilution. For DLAA, 1ml of serially diluted phage solution and 100µl of log phase host bacteria was mixed and left for attachment for 5min. Then 3ml soft agar was added and overlaid onto the TSA plate. Plates were incubated overnight after solidification. After overnight incubation, the dilution (plate) from which countable plaques were formed with an individually distinguishable plaques were selected for phage titer determination. The total number of plaques on each plate were counted and calculated the number of plaque-forming units per milliliter of original phage solution by using the following formula (vlab.amrita.edu, 2011): which gives the phage stock concentration.

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

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

3.6 Storage of phage solution

As bacterial culture glycerol stock was prepared for long term storage, bacteriophage also should have storage for long term preservation and use. The working solutions of phage were stored in 4˚c after preparation of pure phage stock and works for about 1 year or more without any fluctuation in concentration of phage. But for long term, the storage of phage in 4°C there might be problem in reviving of phage and host both. So here, our seniors designed the storage protocol for both host bacteria and phage simultaneously. For this, first host bacteria were grown in log phase in fresh LB media then 100 µl bacteria and 1 ml of high titer phage solution was mixed and incubated at 37˚C up to the eclipsed period of bacteriophage. At this period, bacteriophage adsorb at the host surface prior to the bacterial generation time and to stop the infection process after the biosynthesis of its all body by using bacterial biosynthesis machinery. Generally, we use 20min incubation for *salmonella* in order to trap the phage particle but it might be different for different host. After incubation, the host and phage mixture were centrifuged at 12000rpm for 5 minutes. The supernatant was discarded and pellet was dissolved in 1ml SM buffer and serial dilution and DLLA was done for checking the infection of phage with host. For storage, 1ml of 50% autoclaved glycerol were mixed with1 ml dissolved pellet and stored in 4˚c for first 24 hour and then transferred to -20˚C.

3.7 Host Range Analysis

3.7.1 Intra host range analysis of phage

Two different isolated *Salmonella* phages (phage TU_sal2T and phage TU_sal5K) and its cocktail were selected for intra host range analysis. Only four available strains of *Salmonella* were used as a host for intra host range which were collected from NARC during the period of isolation and which were also detected as colistin resistance. Host-range is the ability of a specific phage to infect and lyse closely related other bacterial strain. Standard spot assay and DLAA method (Kwon *et al*., 2008) with minor modification was performed to determine multiple host-range of purified phages against other strains within same genus. For spot assay, all the bacteria were grown aseptically to the active log phase. Three milliliters of 0.5 % warm (50°C) soft agar was mixed with 100µl of each bacterial culture in different sterile test-tube and poured on properly labelled separate fresh Tryptic soya agar plate (TSA). Petri plates were swirled so as to distribute top agar evenly and left to solidify. Two different phages sal2, sal5 and one of its cocktails were used for host range and grid were marked in plate for each phage. SM buffer is used as a negative control. After solidification of the top agar layer, 5 µl of the high titer phage stock (10^8-10^{10}) was applied to spots on the corresponding marked line and left for 15-20mins

to dry. The plates were incubated for 24 hours at 37˚C and checked for the presence or absence of bacterial lysis and clear zone. Double layer agar assay was done for those host strain which shows the intra host range during spot assay and number of plaques were counted after DLAA to calculate the efficiency of plating (EOP).

3.7.2 Interspecies host range analysis

Different strain of *Klebsiella*, *Pseudomonas, Acinetobacter* and *Escherichia* spp were collected and revive for interspecies multi host range analysis. These species were already available in the CDBT Laboratory. All the single phages and mixed cocktail of *Salmonella* phages were tested first, spot and then DLAA for those which showed host range, on each bacterium as same in above mention method.

3.8 Characterization and comparative analysis of two different phages

The isolated bacteriophages which possess consistent bacterial cell lysis capability were chosen for further characterization. The characterization of bacteriophage was done by the adsorption assay, growth curve analysis, stability of phage against different temperature and pH and protein profiling by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE). Here, two phages; one isolated from Teku I sample against sal2 host (phage TU_sal2T) and another from Kirtipur meat shop waste against sal5 host (phage TU_sal5K) were used for physiochemical characterization.

3.8.1 One Step growth curve experiment

Bacteriophage follow the 5 steps for the completion of its life cycle and make a curve of its growth from the infection of host cell to the releasing of new phage progeny after the lysis of host cell. Bacteriophage generally take 1 hour or more until the completion of its life cycle. For the phage growth curve experiment the protocol of (Adams and Wassermann, 1956) was adopted with some modifications. First Seven different sterile Eppendorf tubes were taken and labelled as 5min, 10min, 20min, 30min, 40min, 50min and 60min. 1000µl of high titer phage stock 10^8 pfu/ml was transferred on each tube and 100 µl of log phage host salmonella (OD 0.25) was mixed on each seven different tubes then incubated in the 37°C incubator. After 5min incubation, the tube (in which 5min incubation was labelled) was taken out and centrifuged at 12000rpm for 5min. Supernatant was discarded in order to remove the un adsorbed phage, pellet was resuspended with 100 µl SM buffer then 3ml soft agar was mixed to the dissolving solution and overlaid on the TSA plate (DLAA method). Similarly, same work flow was repeated for other 6 different tubes in their subsequent time as mentioned earlier and the plates were left for 10 min. Another set of all requirement was prepared for Sal5 phage and work flow

was going on in similar above-mentioned process. Plates were incubated at 37°C incubator for 24 hour and the plaques were counted (expressed in PFU/ml) after overnight incubation. Graph was plotted PFU/ml versus time of incubation.

3.8.2 Stability of phage against temperature

The determination of Stability of phage against different temperatures was determined by exposing the phage to different temperature and time. The stability against temperature and pH was done by taking a reference from (D'Andrea *et al*., 2017) First, the phage stock was diluted to different dilution in SM buffer and 10^{-8} diluted solution was taken at which countable plaques of phage were found. 1ml of 10^{-8} diluted phage were kept in different sterile Eppendorf tubes and these were exposed to 37°C, 40°C, 50°C, 60°C, 70˚C and 80˚C temperature for different time 10min, 20min, 30min, 40min,50min and 60min. All requirements were prepared in two set for two phages. During incubation all temperature is maintained in dry heat such as incubator, heating mantle and hot air oven. After incubation in different time and temperature, the tubes were withdrawn from the temperature at their respective time and immediately 100 µl of log phage host *Salmonella* at OD 0.25 was mixed with the heat-treated phage solution and DLAA was done without delaying the time. After overnight incubation, the number of surviving plaques were counted and expressed as PFU/ml then graph was plotted PFU/ ml against temperature values.

3.8.3 Stability of phage against pH

The pH stability test of phage was done by treating the phage with different pH. A pH ranges from pH 2 to pH 12 was prepared by adjusting the pH of fresh 10ml Luria Bertani broth. 1M HCl and 1M NaOH was added drop by drop to achieve desired acidic and basic pH. After preparing the LB broth with different pH in different tubes, the tubes were autoclaved. Meanwhile 10^{-8} diluted phage suspension was prepared. 700µl of different pH adjusted media was aliquoted in respective well labelled Eppendorf tubes after autoclaving and 300µl of phage suspension was mixed in each tube and then incubated for 1 hour at room temperature. After incubation, 100µl log phase host *Salmonella* at OD 0.25 was mixed with pH treated phage solution and DLAA was done followed by incubation. The viable phage particles were counted after overnight incubation, the results were expressed as PFU/ ml and graph was plotted against pH values.

3.8.4 Protein profiling of phage by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS- PAGE)

SDS-PAGE of phage was done by Acetone precipitation method and direct heating method.

Acetone Precipitation method

Acetone precipitation of phage helps to concentrate the different proteins of phage particles that's why bands can be easily visualized in gel. For acetone precipitation, 500µl of purified phage solution was precipitated with 4 volumes of ice-cold acetone for 1hr 30 min and supernatant was decanted. Pellet was air dried and it was resuspended in100µl PBS buffer (8 g l-1 NaCl, 0.2 g l-1 KCl, 0.2 g l-1 KH2PO4, 1.44 g l-1 Na2HPO4 × 2H2O, pH7.5).

Sample preparation

Two different salmonella phages (acetone precipitated and not precipitated phages) solution were collected and 15 µl of each phage solution was mixed with 20 µl protein loading dye (62.5 Mm Tris HCl, 2% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.001% (w/v) bromophenol blue). The mixture was heated in 95°C for 10min. Then the samples were ready for gel run.

SDS-PAGE

After preparing samples, discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was carried out as described by Laemmli (Gallagher, 2012). For SDS-PAGE, in short, 12% acrylamide bisacrylamide (29:1) resolving gel and 5% acrylamide bisacrylamide (29:1) stacking gel were used. Different components and volume used for the preparation of these gels are described in Media and Compositions portions. The gel was set in glass plate cascade and properly fitted with the electrophoretic tank. 1X Tris-glycine running buffer (39mM Tris, 48Mm glycine and 0.1% SDS) was used for electrophoresis. 10 µl prepared samples and 5 µl protein marker were loaded on the well and run the gel at 120 volt for 2 hour. Then after electrophoresis, the gels were stained with Bio-Safe Coomassie Blue Stain (company name) kept for overnight with shaking. The gel was destained with Destaining solution (75% Glacial acetic acid, 5% methanol and double distilled water) after staining with commassie blue. Gel was visualized for the presence of different phage protein bands and the marker of Genii is used as standard protein to measure the size of proteins.

3.9 Morphological analysis by Transmission Electron Microscopy (TEM)

Phage TU_sal2T, which was characterized physiochemically was selected for Transmission Electron Microscopy (TEM). The phage titers 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. 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 for overnight. Next day, the fixed phages were

subjected to high-speed centrifugation (35,000g) for 3 hours. Pellet 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.

3.10 Whole Genome Sequencing (WGS) of phage TU_ sal2T

The phage sample was exported to Xceleris Genomics (Ahmedabad, India) in dry ice for Sequencing. Whole genome sequencing of phage was performed on Illumina HiSeq 2000/2500 platform. The sample project ID is XGC-956-A3560.

3.10.1 Isolation, Qualitative and Quantitative analysis of samples

The isolation of gDNA from the culture samples were carried out using Norgen Bacteriophage Kit. Quality of genomic DNA was checked on 0.8% agarose gel (loaded 3µl) for the single intact band. The gel was run at 110 V for 30 mins. 1µl of each sample was used for determining the concentration using Qubit® 2.0 Fluorometer.

3.10.2 Preparation of library

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

3.10.3 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.

3.10.4 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.

3.10.5 Genomic data analysis / Bioinformatics

The whole genome sequence data was received in FASTA file format from the Xcelleris Genomics. The genome file was processed using an online tool PHASTER (PHAge Search Tool Enhance Release). The genome was also visualized by SnapGene viewer. The PHASTER help to genome visualization, ORF prediction, and annotated putative gene and hypothetical proteins. Circular and linearized genome was constructed in PHASTER and genes were predicted using NCBI genome database as a reference. Also BLAST was used for species distributions and to search sequence similarity.

Chapter IV

RESULT AND DISCUSSION

4.1 Identification of host Bacteria

4.1.1 Biochemical test of host bacterial strains

Six collected *salmonella Typhi* strains were revive on Salmonella Shigella (SS) agar. Figure 4.1 showed the formation of black isolated colonies on the streaking line after the overnight incubation.

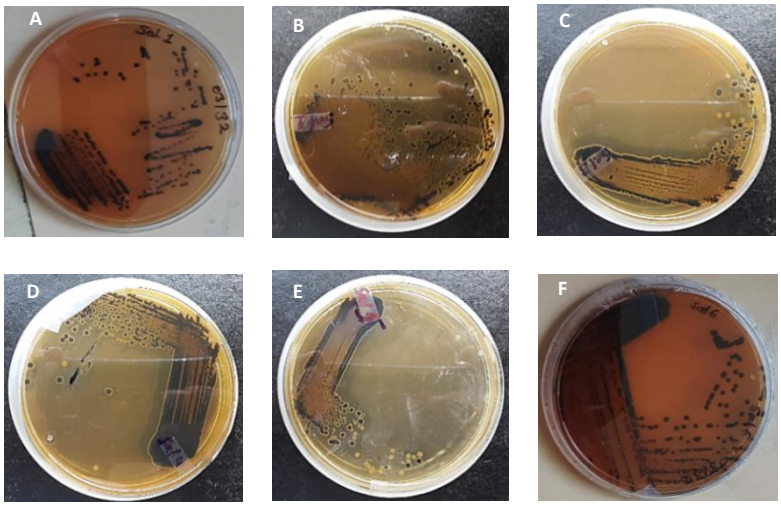


Figure 4.1 Growth of *Salmonella Typhi* on SS agar. Black colonies formation after streaking in fig. A: Sal1, B: Sal2, C: Sal3, D: Sal4, E: Sal5, F: Sal6 respectively.

SS Agar is a moderately selective and differential medium originally developed for the isolation of *Salmonella* and *Shigella* from clinical specimens, suspected foods, and other such samples. The differentiation of bacteria on SS Agar depends on the absorption of neutral red which turns red in the presence of acidic pH thus showing fermentation of lactose has occurred. The presence of bile salts, sodium citrate, and brilliant green in the media serve to inhibit gram-positive and coliform organisms. Sodium thiosulfate in the medium permit as a hydrogen sulfide source, and ferric citrate is added as an indicator for hydrogen sulfide production which is detected by the production of colonies with black centers. *Salmonella* forms with or without black centered colonies, *Shigella* and other non-lactose-fermenting organisms appear as transparent or translucent colorless colonies on SS Agar.

Similarly, biochemical test such as urease, MRVP (methyl red Voges Proskuar, citrate and SIM (sulphur indole motility) test of two host *Salmonella spp* (Sal2 and Sal5) was performed. These bacteria showed that the urease negative, MR positive, VP ngative,

citrate negative and SIM positive. These test help to reconformed the collected samples were *Salmonella Typhi.*

Table 4.1 Biochemical test of *Salmonella*

S. N	Test performed	Results
1.	Urease test	-ve
2.	Methyl Red test	+ve
3.	Vogues proskuar test	-ve
5.	Citrate test	-ve
6.	Sulphur Indole Motility test	Sulphur +ve, motile

4.1.2 Antibiotic Susceptibity Test of salmonella

The collected salmonella was molecularly identified colistin resistant with the amplification of mcr 1 gene. But it also reconfirmed by antibiotic susceptibility test using colistin antibiotic as well as other available antibiotic disc in the laboratory.

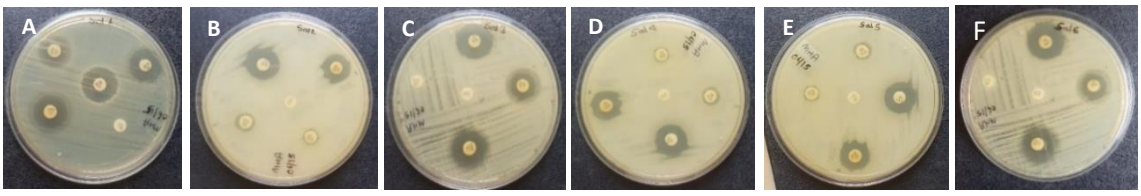


Figure 4.2 Antibiotic Susceptibility Test of A: Sal1, B: Sal2, C: Sal3, D: Sal4, E: Sal5, F: Sal6 respectively on Muller Hinton Agar (MHA) using different antibiotic discs.

The clear zone around the antibiotic disc indicates zone of inhibition (figure 4.2). The diameter of zone of inhibition (ZOI) was measured and expressed in millimeter (mm). The measured value was compared with guidelines provided by CLSI and interpreted as resistance, sensitive and intermediate. All bacteria found to be resistant (Table4.2) to all antibiotics that used for test (CL-Colistin, CTR-Ceftrixone, ETP-Ertapenem, IPM-Ipimenem, MRP- Meropenem, PI- piperacilin).

Table 4.2: Antibiotic susceptibility pattern of host bacterial strain

S.N	Antibiotics	Measured zone of inhibiton (mm)						Std. ZOI for resistivity (mm)	MDR status
		Sal1	Sal 2	Sal3	Sal4	Sal5	Sal6		
1	CL10	0	0	0	0	0	0	≤10	R
2	CTR30	9	4.5	0	0	4.5	0	≤19	R
3	ETP10	8.5	8	9.5	9	7.5	8.5	≤18	R
4	IPM10	8	3	8.5	8.5	3	7.5	≤19	R
5	MRP10	8	7.5	9	8.5	7.5	9	≤19	R
6	PI100	NT	9	NT	NT	8	NT	≤17	R
7	CL25	0	0	0	0	0	0	≤13	R

4.1.3 Amplification of bla NDM gene

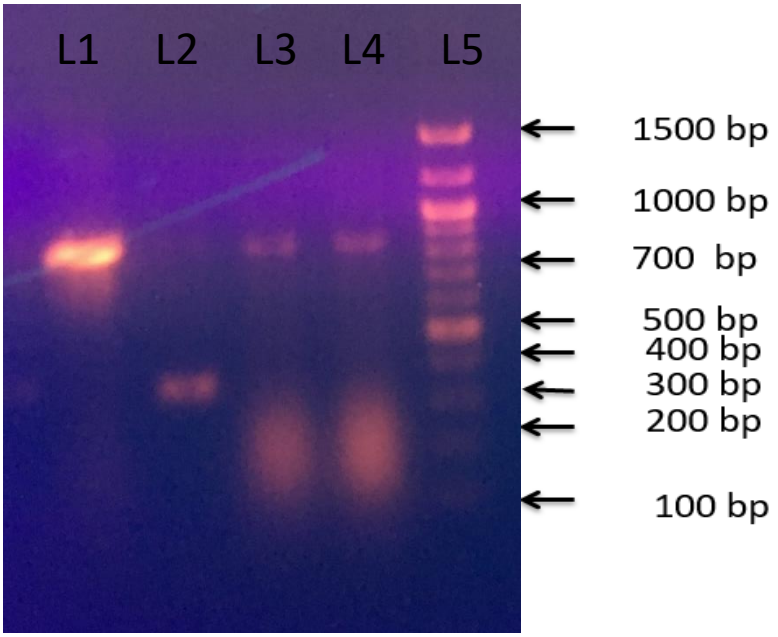


Figure 4.3 PCR amplification of *bla NDM* gene with the size of 865bp (lane1; sal4, lane3; sal2, lane4; sal5). Lane 5 is the 100bp ZR DNA marker. Lane 2 is sal3 where no PCR product was amplified.

The chromosomally mediated *bla NDM* gene was amplified with the size of 865bp only in three *Salmonella* samples sal2, sal4 and sal5). Nonspecific amplification of gene in sal3 might be the low concentration of DNA. The size of amplicon depends on what type of primer we used for detection.

Gene encoding for β-lactamases are generally termed *bla.* Beta lactamases are the enzymes produced by bacteria that provide resistance to beta-lactam antibiotics such as penicillin, cephalosporin, carbapenem etc. by breaking the structure of antibiotics. Carbapenamases are diverse group of β-lactamases that are active against carbapenems and other antibiotics. New Delhi metallo- β-lactamase (NDM) is a class B group of carbapenemase. The gene encoding for NDM-1 is the *bla NDM-1.* It was first detected in a *Klebsiella pneumonia* isolate from a Swedish patient of Indian origin in 2008. The *bla NDM* gene has been identified in bacterial chromosomes and plasmids (Shenoy *et al.,* 2014). The *bla NDM-1* gene spreading in *Enterobacteriaceae* is an alarming risk because these novel multidrug-resistant bacteria could disseminate worldwide very quickly.

Antibiotic resistance is a process at which bacteria become untreatable by antibiotics. Nowdays, bacteria become resistance nearly with all available antibiotics. WHO has priotrized Carbapenem Resistant Enterobactericiae (CRE) as critical group. Resistant developed even in colistin (major pathogens are *Salmonella, E. coli, Acinetobacter*) has causes serious threat to treatment of infections. Due to the improper way of antibiotics use, in Nepal, multidrug resistance bacteria are increasing and create a major public health problem. There had been reported decreased susceptibility to fluoroquinolones with gyrA gene mutation in *Salmonella enterica* serovar *Typhi* and *paraTyphi* (Shirakawa *et al*., 2006). As the *Salmonella* spp contained many pathogenic island regions, emergence of class I integron-associated multidrug resistance in S. *Typhi* was also reported (Tamang *et al*. 2007). So, there should be much attention for urgent need of effective, affordable and reliable alternative to antibiotics.

4.1.4 Molecular identification of host bacterial strain

Genomic DNA extraction and 16s rRNA gene amplification was done for all collected host bacterial samples. All samples were amplified with the size of 1500bp. The amplififed band was compared with ready to use ZR 100bp DNA ladder.

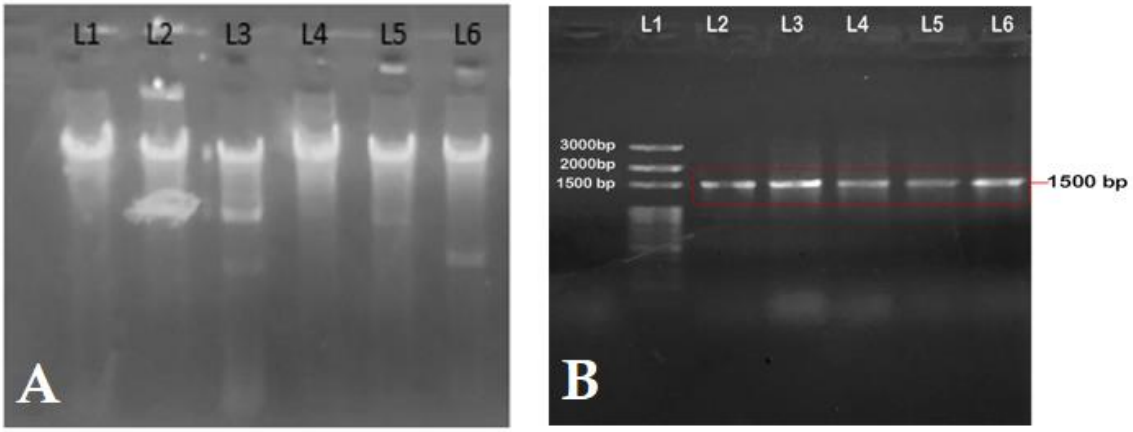


Figure 4.4 DNA and PCR amplification of Salmonella. Figure A is the extracted DNA in lane 1;sal1, lane 2;sal2, lane 3;sal3,lane 4;sal4, lane 5;sal5 and lane 6;sal6 and figure B showed that the amplified 16s rRNA gene of sal1, sal2, sal3, sal4, sal5 and sal6 in lane 1, 2, 3, 4,5,6 respectively. Lane 7 is the 16s rRNA amplicon of EC1 and lane 8 is the ZR100 bp DNA marker.

Bacterial ribosomal RNA has two main subunits; 50S (large subunit) and 30S (small subunit). 16S rRNA is the component of small subunit having the size of 1542 base pairs. The gene in coding 16S rRNA are specially used for the confirmation of organism that are bacteria or not and for working out evolutionary relationships among bacteria. In our sample all bacteria were amplified by 16S rRNA universal primer. For the confirmation of selected bacteria *Salmonella* or not, these samples were sent for 16S rRNA gene sequencing in Xceleris Genomics Company Limited India.

4.1.5 Sequencing of 16S rRNA gene

The strain of *Salmonella Typhi* (Sal2) against which phage was isolated and characterized genomically was send for sequencing to confirm the host that was *Salmonella* or not. Sequence of this gene was obtained in chromatogram file and this was open with Chromas application. The chromatogram file was then search with BLAST tool (nucleotide BLAST) to see the sequence similarity with the NCBI database sequences. The maximum match was seen 91.49% sequence similarity with the *Salmonella Typhi* strain B17 (Accession number KX232359.1) 16S rRNA partial sequence (Refer to Appendix) during direct BLAST hits. Chromatogram also showed the relatively good quality sequences i.e. without the presence of overlapping and flattened peaks. Base call appeared in the top line also help to analyzed the quality of sequences. Full colour in the boxes denotes the less noise in the sequences. Noise in sequences might because of the contamination and degradation of PCR product.

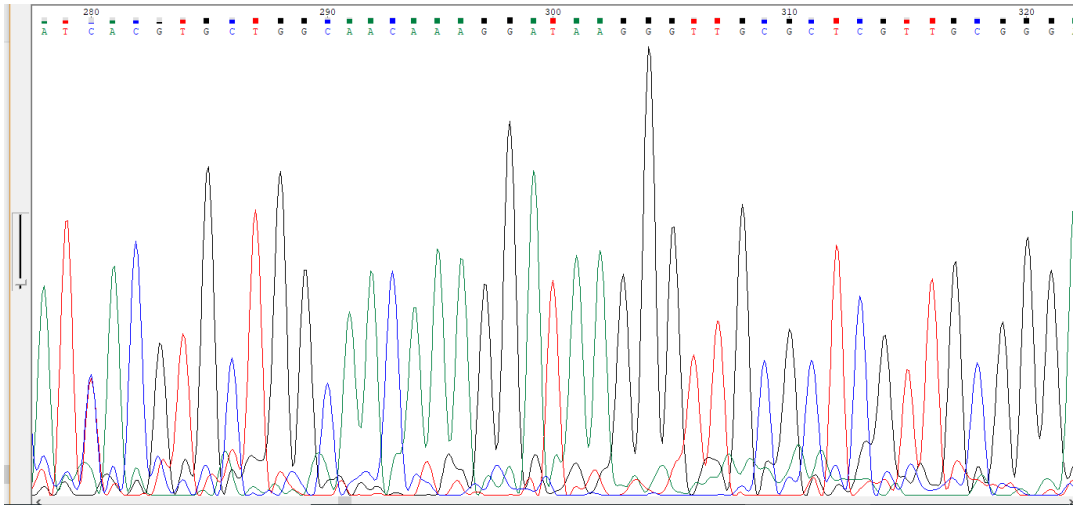


Figure 4.5 Chromatogram of 16S r RNA sequence of Sal2. Different peaks with different colour shows different nucleotide sequences (i.e. ATGC sequences).

To know the evolutionary relationship of this isolated sequences with the reference Salmonella sequences present in the NCBI database, we construct the phylogenetic tree using MEGA X (Molecular Evolutionary Genetic Analysis). Phylogenetic tree or phylogeny is the line of origin of different organism, species or genes from common ancestors. Phylogenies are useful for organizing knowledge of biological diversity, for structuring classifications, and for providing insight into events that occurred during evolution. The branches of the tree represent the time which was required for the mutation of one species into another, new, one (Stamatakis, 2005). After constructing phylogenetic tree, the sequence was submitted to NCBI and NCBI Accession number was obtained as SUB5035396 seq 1 MK481052.

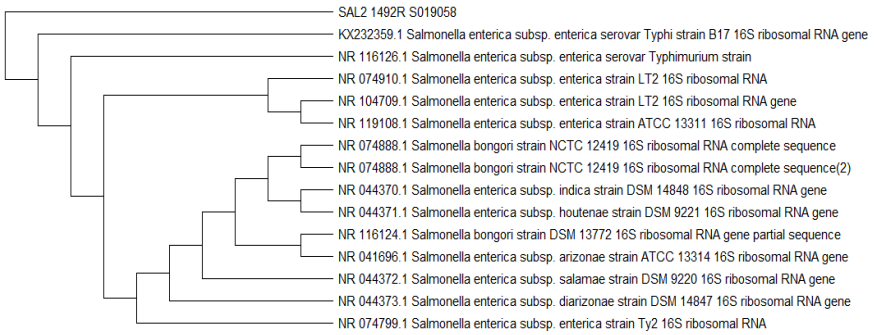


Figure 4.6 Phylogenetic tree of *Salmonella Typhi* (Sal2). Host Sal2 was closely matched with Salmonella Typhi strain B17. Tree was constructed by using MEGA X software.

The phylogenetic tree of Sal2 was constructed by using Neibour Joining (NJ) method. The closely matched strain was found as *Salmonella enterica* subspecies *enterica* serovar *Typhi* strain B17. However, there are other different method to generate phylogeny such as maximum parsimony method, minimum evolution method, distance Wagner (DW) etc. In

this method, a pair of neighbors is a pair of (operational taxonomic unit) OTUs connected through a single interior node in an unrooted tree (Saitou and Nei, 1987). Based on the principle of minimum evolution, NJ method construct a tree by clustering neighboring sequences in a stepwise manner. In each step of sequence clustering, it minimizes the sum of branch lengths (2) and thus examines multiple topologies (Tamura *et al*., 2004).

4.2 Bacteriophage isolation and manipulation

4.2.1 Bacteriophage isolation

From the total eight water and waste collected samples, ten different phages were isolated against four different host. These phages were isolated from four samples Teku I, Teku II, Bayodha hospital near Balkhu water and Kirtipur meat shop waste. Isolated phages have different plaque morphology, different number of plaque counts, turbidity and clarity.

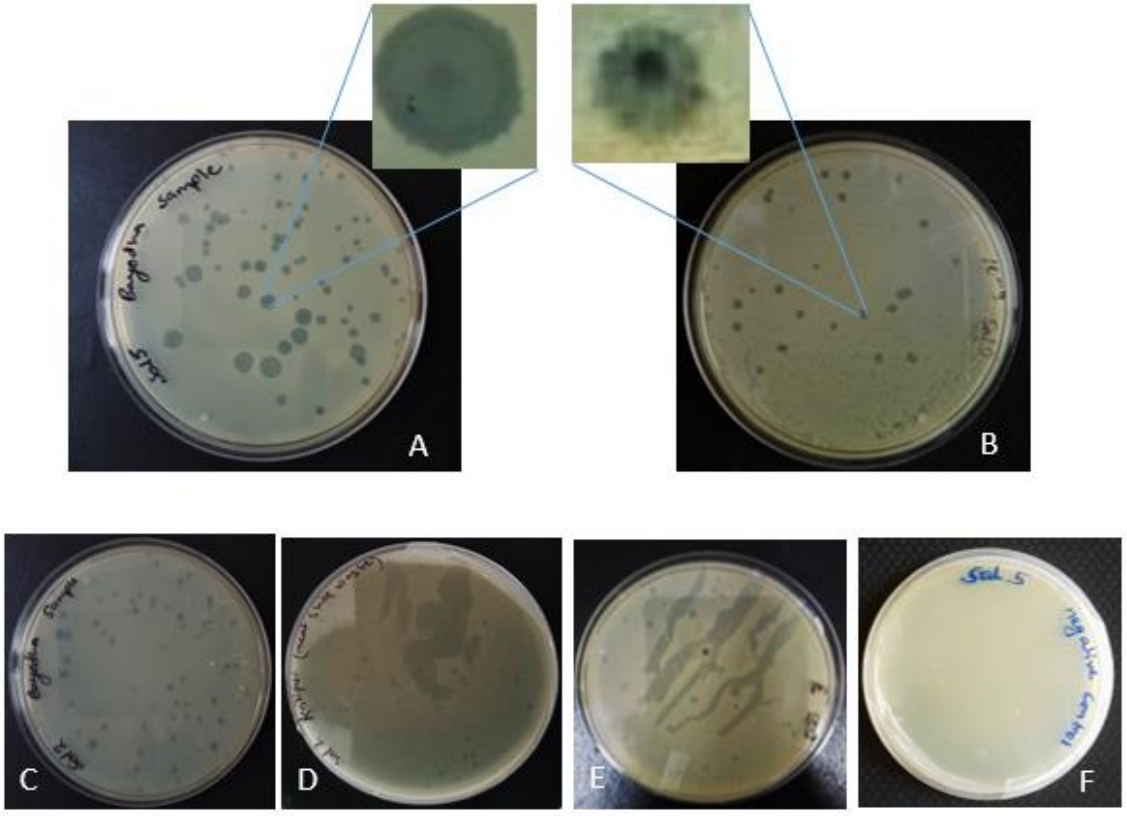


Figure 4.7 Initial isolation of bacteriophage showing different plaques morphology and lysis activity from different samples. Fig A shows that the isolated phage against Sal 5 host from Bayodha hospital near Balkhu river sample. Fig B is the isolated phage against Sal2 host from Teku I sample. C and D are the phage against sal2 from Bayodha sample and Kirtipur meat shop waste respectively. Similarly, fig E is the isolated phage from Teku I sample against *E. coli* (Ec1) and negative control where no lysis was observed is labelled as F.

Bacteriohage isolated from Teku I against sal2 host had small bull eye and clear plaque morphology having plaque diameter 0.2 to 0.5mm. Other isolated phage had similar pinhead morphology and plaque diameter ranging from 0.1 to 0.3mm except phage isolated from Bayodha near balkhu water samples which had large clear morphology as plaque diameter ranging from 0.2 to 0.5mm (table4.3).

Table 4.3 Initial screening of bacteriophage

S.N	Sample source	Host bacteria	Name of phage	Initial no.of plaques	Diameter of plaque (mm)	Plaque opacity	Plaque morphology
1	Teku river I	Sal2	Phage TU_sal2T	31	0.2-0.5	Clear	Small bull eye
		Sal 5	Phage TU_sal5T	18	0.2-0.3	Turbid	Pin head
		Ec1	Phage TU_Ec1T	21	0.2-0.3	Turbid, Clear	pin head
		Sal 3	Phage TU_sal3T	19	0.1-0.2	Clear	pin head
2	Teku river II	Sal2	Phage TU_sal2t	17	<0.2	Clear	Pin head
		Sal5	Phage TU_sal5t	38	<0.2	Clear	Pin head
3	Bayodha near balkhu river	Sal2	Phage TU_sal2B	82	0.2-0.3	Clear	small, clear
		Sal5	Phage TU_sal5B	72	0.2-0.5	Clear	large, clear
4	Kirtipur meat shop waste	Sal2	Phage TU_sal2K	39	0.1-0.3	Clear, Turbid	Pin head
		Sal5	Phage TU_sal5K	21	0.1-0.3	Clear, Turbid	Pin head

The higher number of phages were isolated from Teku river water against four different host than other samples. During the initial screening, phage isolated host were three *Salmonella* (Sal2, Sal3 and Sal5) and one *Escherichia coli* (Ec1). In overall Sal2 and Sal5 were seems to be more susceptible to bacteriophage than another host. Teku river water contained more phage than other samples this suggested that the Teku river was more contaminated than other. The abundancy of Phages is more that places where the bacteria are also adequately present. Teku river was highly contaminated with Human, animal excreta, household, industrial and hospital waste so there might be more bacteria that's why more phages were isolated.

The bacteriophage plaque morphology is the initial point of detection for the presence or absence of bacteriophage in samples. Plaque morphology is one of the foremost criteria for the bacteriophage characterization. In our result also there were formation of plaques with different number, size, clarity and plaque opacity. The major plaque morphology observed were pin head, small and large clear zone and small bull eyed with the formation of halo zones (Table 4.3 and Figure 4.7). Variation in plaque morphology may correspond to the difference in phage strains, addition of cations, bacterial density, and also of gel strength in agar plate (Shende *et al*., 2017). Other factors that affect plaques morphology include virions morphology and diffusibility, various rate constant for phage-host attachment, phage latent period and burst size (Abedon and Yin, 2009). In the study done by Gallet *et al.* in mathematical model, they demonstrated that the phage with higher diffusibility have large plaque size, and too high and too low adsorption rate generally results in smaller plaque size. They also predicted that the plaque size is negatively correlated with latent period and larger burst size would result in the larger plaque size (Gallet *et al*., 2011).

Tryptone Soya Agar (TSA) is mostly adopted media for the isolation of bacteriophage using Double Layer Agar (DLA) assay. TSA contained Calcium and Magnesium (Ca^{++} and Mg^{++}) as a divalent ion. These cofactors increase the enzymatic activity of bacteriophage lytic enzymes. During infection of bacteriophage with host bacteria, bacteria multiply to form confluent lawn of bacterial growth over the surface of plate. Infected bacterium bursts after a short time and releases progeny phages that infect adjacent bacteria, which in turn are lysed. This 'chain' reaction spreads in a circular motion until brought to a halt by a decline in bacterial metabolism and formed zones of lysis on lawns of bacterial cells. This circular zone of bacterial lysis caused by phage action is called plaques (Mullan, 2001).

4.2.2 Bacteriophage Purification

Bacteriophage purification was done by the two to three round streaking of single plaque. Bacteriophage which had clear lytic and bull eyed plaque morphology were selected for purifications. Only three different phages that were isolated from Teku, Bayodha near

Balkhu river and Kirtipur meat shop waste were selected for purification because their plaque morphology were more clear than other. Single plaques were selected for streaking and clear lysis were seen over the streaking line. Phage purification is one of the difficult tasks during phage propagation. Initial phage stocks and unpurified phage contained variability in titer, salts, and bacterial contaminants. Efficient preparation of high titer homogenous bacteriophage (phage) stocks eliminate the variability between phage propagations and improve the molecular characterizations of phage (Bonilla *et al.*, 2016).

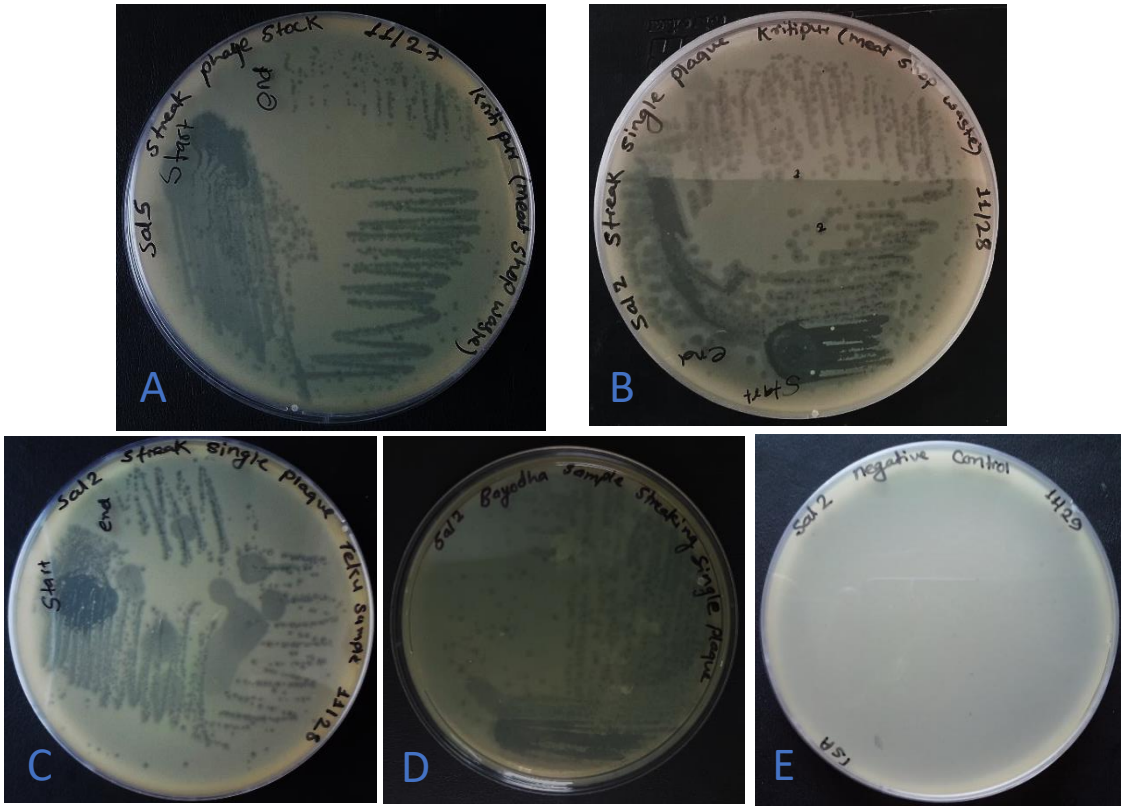


Figure 4.8 Purification of bacteriophage. Streaking of single plague from different sample in different plates showing the lysis of host in streaking line. Fig A and B are streaking of Sal5 and Sal2 phage (namely Phage TU_Sal5K and Phage TU_sal2K) isolated from kirtipur meat shop waste. Fig. C and D are sal2 and Sal5 phage (Phage TU_sal2T and Phage TU_sal5B) from Teku I and Bayodha near Balkhu river sample and E is the negative control where no lysis was observed that because of only bacteria was overlaid without streaking of phage.

After the three four rounds of streaking, the purified selected phage had prepared phage stock by filtering phage suspension through 0.22µm pore sized syringe-filter. Sometimes Chloroform extraction method also used for single plaque purification. Chloroform treatment is generally used to kill any bacteria present in the phage lysate. Chloroform may inactivate some phages and should be used with caution with proper concentration and controls (Mullan, 2001).

4.2.3 Bacteriophage amplification and concentration determination

The bacteriophage against sal2 and sal5 was amplified by streaking and DLAA method from initial pure phage stock in several plate. Spot was observed up to 10^{-10} dilution. It helps to know that up to which dilution phage could lysed host. The concentration of phage stock solution was determined by following formula.

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

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

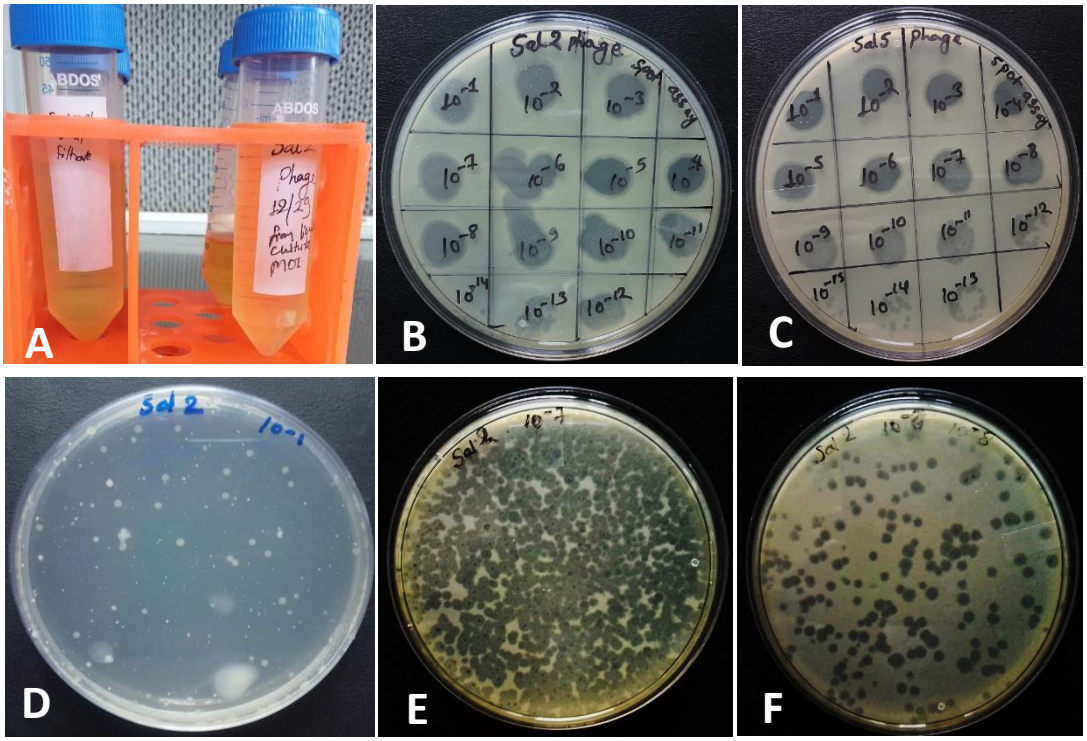


Figure 4.8 Amplification of bacteriophage. In fig.A bacteriophage stock preparation; B and C are spot formation by two phages (Phage TU_sal2T and Phage TU_sal5K). Fig. D is the clear lysis observed at 10^{-1} dilution, E is too much to count plaques formation at 10^{-7} dilution and figure F is countable plaque formation at 10^{-8}.

Since with the limited resources and time, it was very tedious and difficult to amplify and purify all isolated phages simultaneously. Only two phages; Phage TU_sal2T and Phage TU_sal5K were selected for amplification and titer determination and also for further characterization. These phages were selected on the basis of plaques morphology and best lytic capability than other.

The concentration of phage (phage titer) was calculated by using above mentioned formula. Twenty three number of plagues were formed by Phage TU_sal2T against sal2

host at 10^{-9} dilution and 18 number of plaques were formed by Phage TU_sal5K against sal5 host at 10^{-8} dilution (Figure 4.9). The titer of Phage TU_sal2T was found to be 2.3×10^{10} PFU/ml and 1.8×10^{9}PFU/ml of Phage TU_sal5K (Table4.4). This showed that the concentration of Phage TU_sal2T is slightly higher than Phage TU_sal5K.

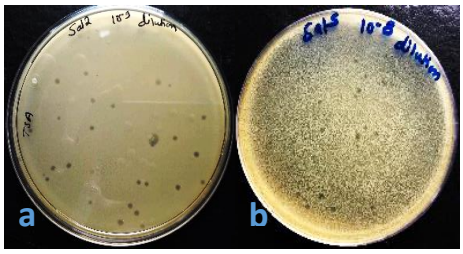


Figure 4.9 plaque formation unit per mililitre (PFU/ml) of Phage TU_sal2T and Phage TU_sal5K at 10^{-9} and 10^{-8} dilution in fig. a and b respectively.

Table 4.4 Titer of phage at different dilution

SN	Phage	Dilution	Phage Titer (PFU/ml)
1.	Phage TU_sal2T	10^{-9}	2.3×10^{10}
2.	Phage TU_sal5K	10^{-8}	1.8×10^{9}

The bacteriophage titer is a quantitative measurement of the biological activity of virus and is expressed as plaque forming units (pfu) per ml. Determination of phage titer in a sample is a key step in the study of the phage involved. It is very important to select suitable dosage in the phage therapy for bacterial infection. As a common method of detection, a plating assay (plaque) is widely used method to detect phage number present in the test samples at various diluted points.

4.3 Multiple Host Range (MHR) analysis of bacteriophage

Multiple host range is a property of phage in which phage can infect or lyse not only for specific host but also for other interspecific or intraspecific host. It is important and highly desirable property in phage therapy because broader the host range broader will be the infectivity of given phage. For MHR analysis, first spot assay of *Salmonella* phages was performed against available strains of bacteria in lab. Then 3

Box 3 Strains used for host range with their abbreviation.

Salmonella spp: Sal1, Sal2, Sal3, Sal4, Sal5, Sal6.

Acinetobacter baumanii: A4, A5, A8, A11, A12, A13, A14, A15, A17, A21, A22, A23, A24, A25, A32, A35, A38, A39, A50, A53, A56, A58, A59 and A70.

Klebsella pneumonia: K12, K23, K42, K70 and NK.

Pseudomonas spp: P19, P42 and P53.

to 4 round of confirmatory test was performed after that DLAA was done to compare the efficiency of phage for lytic capability in nonspecific host than in specific host.

Altogether 38 samples of different host bacteria such as *Acinetobacter baumanii* (24 starins) *Klebsella pneumoniae* (5 strain), *Pseudomonas* spp (3 starins) and *Salmonella spp* (6 strains) were selected for interspecific and intraspecific host range. In spot assay, *Salmonella* phage cocktail showed lytic capability against 15 host (Table 4.5). Among them some of the host were molecularly confirmed by 16s rRNA sequencing. During spot assay first we tested only by cocktail and then individual phages.

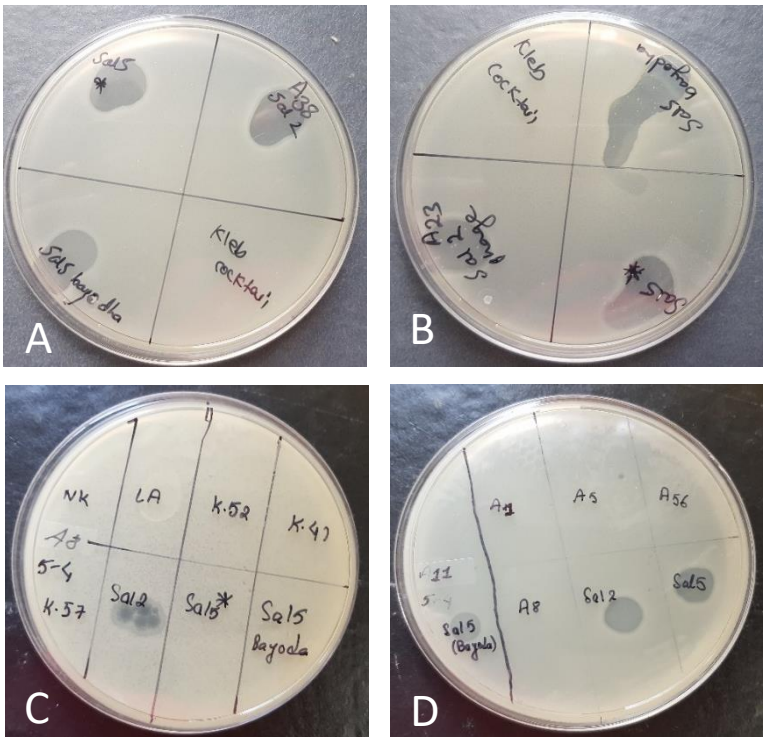


Figure 4.10 Spot assay of phage TU_sal2T and phage TU_sal5K and its cocktail for different nonspecific hosts. Figure A, B and C are the *Acinetobactor baumanii* strains and C is the *Klebsella pneumoniae* strain

After that phages which showed strong lytic capability against host were subjected to perform whole plate (DLA) assay to calculate the Efficiency of Plating (EOP) value. The efficiency of plating (EOP) differs from the spot test in this respect as it measures the titre of particular phage infecting a bacterial strain(Mirzaei and Nilsson, 2015). The positive (+) sign represent the lysis by phage during spot assay. Three and four (+) sign represent the lysis when done repeatedly or by three four times and this was considered as a strong lysis. In parallel, negative (-) sign appointed the absence of lysis (Table 4.5).

Table 4.5 Multihost range of Phage TU_sal2T and phage TU_sal5K and its cocktail.

Host Bacteria	Phage TU_sal2T	Phage TU_sal5K	Cocktail
Sal1	- -	- -	- -
Sal2		+ + + +	+ + +
Sal3	- -	- -	- -
Sal4	- -	- -	- -
Sal5	+ + + +		+ + +
Sal6	- -	- -	- -
A4	+ -	+ -	+ -
A5	+ -	+ -	+ -
A8	+ + + +	+ + +	+ + +
A11	+ + + +	+ + +	+ + +
A12			-
A13			-
A14			-
A15			-
A17	+ + + +	+ + +	+ + +
A21	+ + + +	+ + +	+ + +
A22			-
A23	+ + + +	+ + +	+ + +
A24			-
A25			-
A32			-
A35			-
A38	+ + + +	+ + +	+ + +
A39			-
A50	+ -	+ -	+ -
A53	+ -	+ -	+ -
A56	+ -	+ -	+ -
A58	+ -	+ -	+ -
A59			-
A70	+ + + +	+ + +	+ + + +
K12			-
K13			-
K42			-
K70	-		-
NK	+ -		+ -
P19			-
P42			-
P43			-

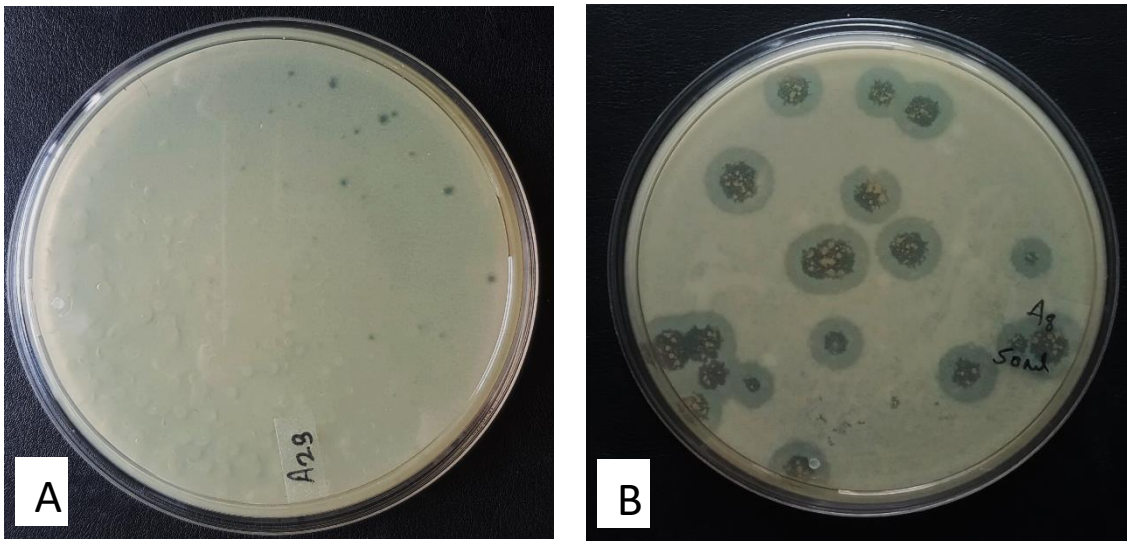


Figure 4.11 Plaques formation of phage TU_sal2T at 10^{-6} dilution against nonspecific host *Acinetobacter baumanii* strains A; A23 and B; A8. The plaque morphology is different in A8 strain.

The efficiency of lysis pattern of single phages and phage cocktail showed no differnces between hosts so phage TU_sal2T was selected as a reference for the determination of EOP value. The similarity between host ranges indicate that these two phages may recognize similar host receptors (Fong et al., 2017). Out of 15 samples, only 6 host were strongly susceptible to *Salmonella* phages which was proven by the four round of spot assay. The nonreproduction of test for all positive sample might be due to the lack of exact spot in the respective labelled place since the test of all host were done at the same time. Mutation in host and contamination in host also other factors for non-reproducible result because we use old stock culture for host range which were characterized by our senior .

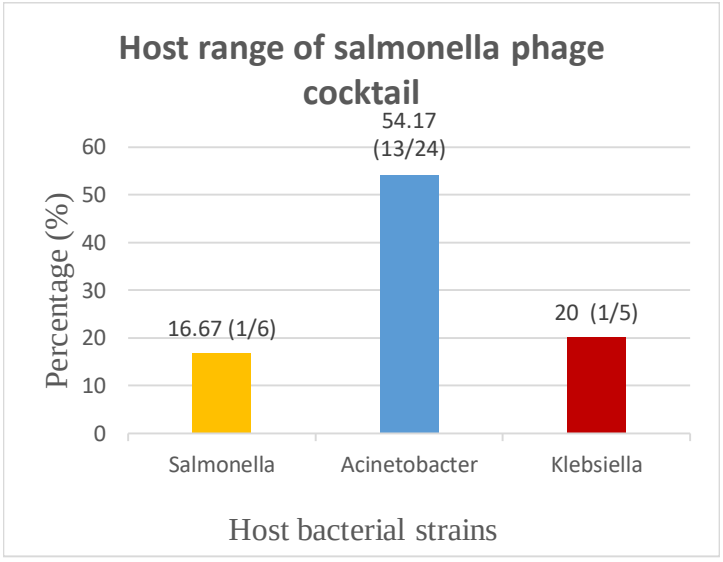


Figure 4.12 Bar graph showing the percentage of host range of Salmonella phage cocktail. Host range of Salmonella phage cocktail showed the highest 54.17 % of activity against *Acinetobacter* out of total *Acinetobacter* strains used.

Table 4.6 EOP value of phage TU_sal2T with different plaque morphology for different host

S N	Bacterial Host	Plaque morphology	Efficiency of phage (EOP) in PFU/ml
1.	*Salmonella spp* (Sal5)	Small clear plaque	2.7×10^{-8}
2.	*Acinetobactor baumanii* (A8)	Large clear plaque with halo zone	1.7×10^{-7}
	Acinetobactor baumanii(A11)	Clear pin head	1.8×10^{-7}
4.	*Acinetobactor baumanii* (A21)	Turbid pin head	9.6×10^{-6}
5.	*Acinetobactor baumanii* (A23)	Clear pin head	1.9×10^{-7}
6.	*Acinetobactor baumanii* (A17)	Turbid pin head	4.8×10^{-6}
7.	*Acinetobactor baumanii* (A38)	Turbid pin head	1.6×10^{-7}
8.	*Klebsella pneumoniae (NK)*	-	-

Efficiency of Plating (EOP) of phage TU_sal2T was determined according to the reference protocol provided by (Kutter, 2009). The EOP means the ability of plaques production on susceptible nonspecific bacterial host. Host in which higher number of plaques were produced was considered as the higher efficiency of plating (EOP). In *Salmonella spps* (sal5) strain, higher number of plaques were formed than other since Sal5 is the intra species host of phage TU_sal2T. In interspecific host range, *Acinetobacter baumanii* (A23) showed the higher efficiency of plating that is 1.9×10^{-7} than other strains with the clear pin head plaque morphology (Table 4.6). But in overall, plaques formation in nonspecific host are less than that of specific host (*Salmonella spp*; Sal2 which have maximum concentration up to 2.3×10^{10}).

Similar type of work was done by Rahaman et al. for *Salmonella* specific phage SAL-PG in which the number of plaques produced on susceptible bacteria was varied from one isolate to another (Rahaman *et al.* 2014). Also plaques morphology formed by phage against *Acinetobacter baumanii* (A8) was quite different than other. It was large clear with halo zone (Figure 4.11). The difference in plaques morphology was affected by different factors such as both phage and host growth characteristics. Previous research showed that the formation of halo zones is an indicator for the presence of phage tail-associated exo-polysaccharide (EPS) depolymerases. When the bacterial growth cycle enters the stationary phase during infection, phage replication also stops or slows down substantially but their tail spikes still can depolymerize bacterial exo-polysaccharide. Which then results the appearance of an increased transparent zone known as the halo zone(Tabassum *et al.*, 2018).

4.4 Characterization and comparative study of two different Bacteriophages

4.4.1 Bacteriophage growth curve analysis

One-step growth curve was performed to identify the different phases of a phage such as infection to lysis of host cell and release of phage progeny. From infection of phage with host cells, latent period, rise period and burst size were determined from the dynamical change of the number of adsorbed phages in different time.

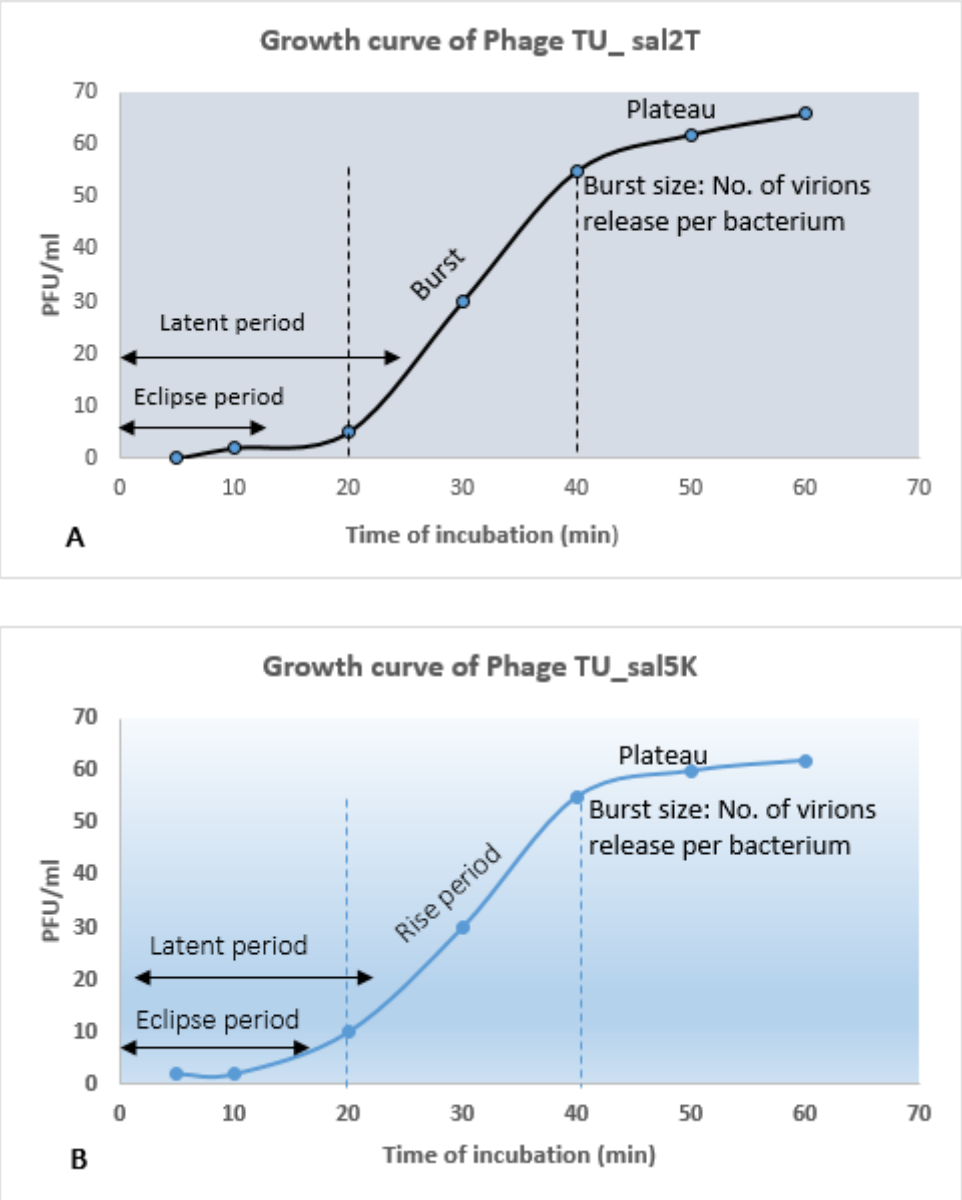


Figure 4.13 Growth curve of Phage TU_sal2T (graph A) and Phage TU_sal5K (graph B). Burst size of Phage TU_sal2T was found to be 50 phages release per bacterium and Phage TU_sal5K was 45 phages release per bacterium. Similarly, latent period was found to 20min for both phages. The phages were expressed in Plaque Forming Unit per bacterium (PFU/ml).

The rise in number of phage progeny was demonstrated during the replication cycle of phage. Following the initial steady state, the drastic rise in phage titer was observed after 20min of incubation in case of both phages. Therefore, the latent period for both phages were identified as 20min. This was similar to that of the phage PSPu-95 and PSPu-4-116 isolated and characterized against *Salmonella* by (Bao *et al.,* 2011). Latent period is the time taken by bacteriophage from the infection or attachment of bacteriophage to the host cell to maturation and release of bacteriophage. There is quite difference between latent and eclipse period of growth. Eclipse is the time period of bacteriophage growth curve in which phage start to attach host cell surface to phage maturation by using the biosynthetic materials of host cell. It took 5 to 10min in accordance with the bacteria, its specific phage and external environment phage host interaction. There may or may not be the plaque formation during this stage. In our study also, there was no plaques formation up to 5 min and very few (2 plaques) formation up to 10 min adsorption (Figure 4.14).

In similar way, burst size and time to require for burst were also determined. Burst size is the no of virions release per bacterium. Burst is also like as exponential phase of bacterial growth cure. It is also called as rise period. In this phase, phages replicate exponentially and releases its progeny outside after the lysis of cell wall. The burst size of Phage TU_sal2T (50 pfu/ml) was found slightly more than Phage TU_sal5K (45 pfu/ml) with the burst time 20min for both phages (Figure 4.13). Burst size is slightly less than that of phage PSPu-95 and PSPu-4-116(77.5 and 86 pfu/cell) (Bao *et al.,* 2011) but much smaller than that of *Acinetobacter baumannii* bacteriophage ΦAB2 (200 pfu/cell; Lin *et al.*, 2010). Burst size was calculated by subtracting the number of pfu/ml of post rise period to the no. of pfu/ml or pre

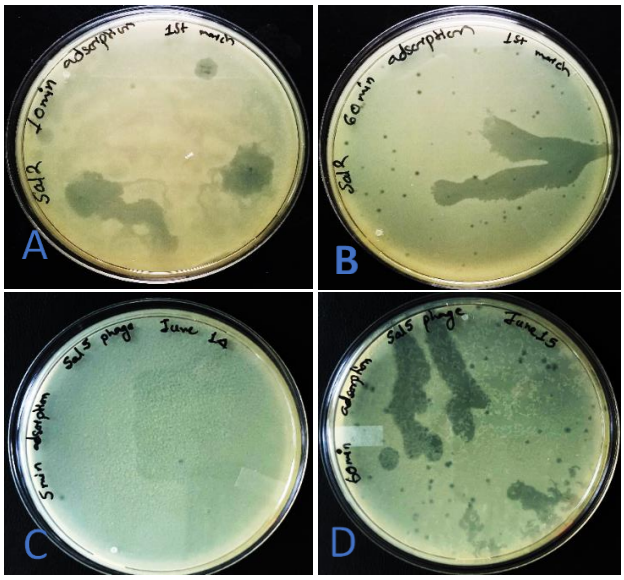


Figure 4.14 Plaques formation by bacteriophage during phage growth experiments. Figure A and C are plaques formation by Phage TU_sal2T and Phage TU_sal5K at 10min and 5min adsorption where a smaller number of plaques were formed. Figure B and D are adsorption of bacteriophage up to 60min where higher number of plaques were formed. This indicated that the high number of viral progenies are released after infection with the host cell with the time increasing.

rise period of growth. Generally, the burst size of bacteriophage (specifically for DNA phages) ranging from 10-100 or more. Small RNA phages have burst size approximately 20,000 virions per bacterium. Small plaques morphology showing phage generally have also small burst size (Gallet *et al.,* 2011). Burst size also depends on the host density, attachment time, specific growth rate, temperature of incubation, medium used for DLAA etc.

4.4.2 Stability of phage at different pH range.

Thermal and pH test of sal2 and sal5 phage was done to check the stability of phage at extreme condition. The PFU/ml of phage was counted by exposing phage at different pH to determine up to which condition phage can survive. The result was compared between two phages.

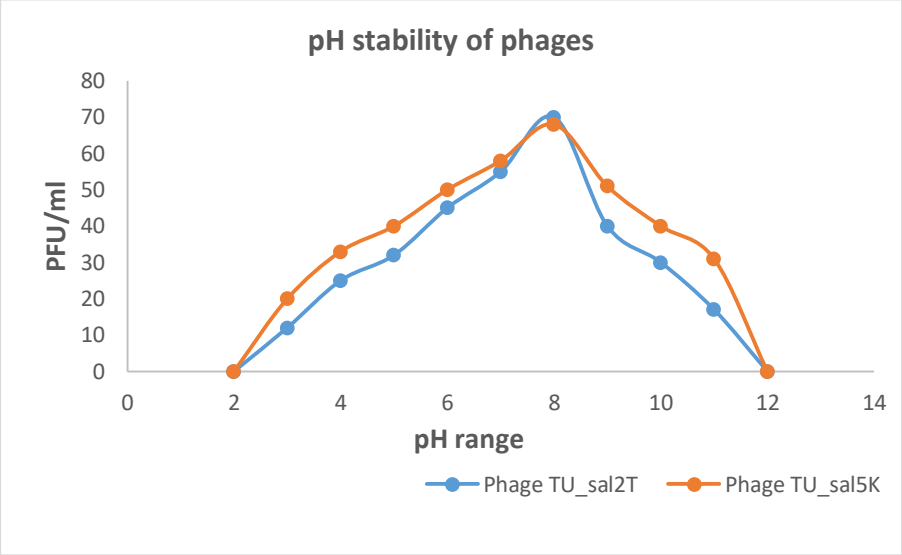


Figure 4.15 pH stability curve of two phages. Red line indicates stability of Phage TU_sal5K and blue line indicates the Phage TU_sal2T against pH from 2 to 12. The higest stability of both phages was observed at pH 8. And the no of plaques survival was higher in alkaline condition than in acidic condition (pH less than 7)

Total number of PFU/ml were counted and graph was plot against pH. Both phage were stable at pH from 3 to 11. The highest no. of survival was found in pH 8 for both phage (figure 4.15). The no. of plaques survival in pH 8 was slightly more in phage TU_sal2T phage then phage TU_sal5K phage but in other pH, no. of plaques was higher in sal5 phage than in sal2 phages. Rahaman *et al.* also isolated and characterized the SAL-PG phage against *Salmonella* which has also wide range of pH(2-9) stability (Rahaman *et al.*, 2014). The survivability of phage at low pH would have allowed them to survive at low pH during the digestive process (Bao *et al.,* 2011).

From above result, we can summarize that the both phages have good stability towards the high and low pH and there was no significant difference between the effect of pH in both phages.

4.4.3 Thermal Stability of Phage

Thermal stability test of phage was done by exposing phage to different temperature for different time. Plaque forming unit per milliliter (PFU/ml) versus time was plotted to detect the survivability of phage at different temperature.

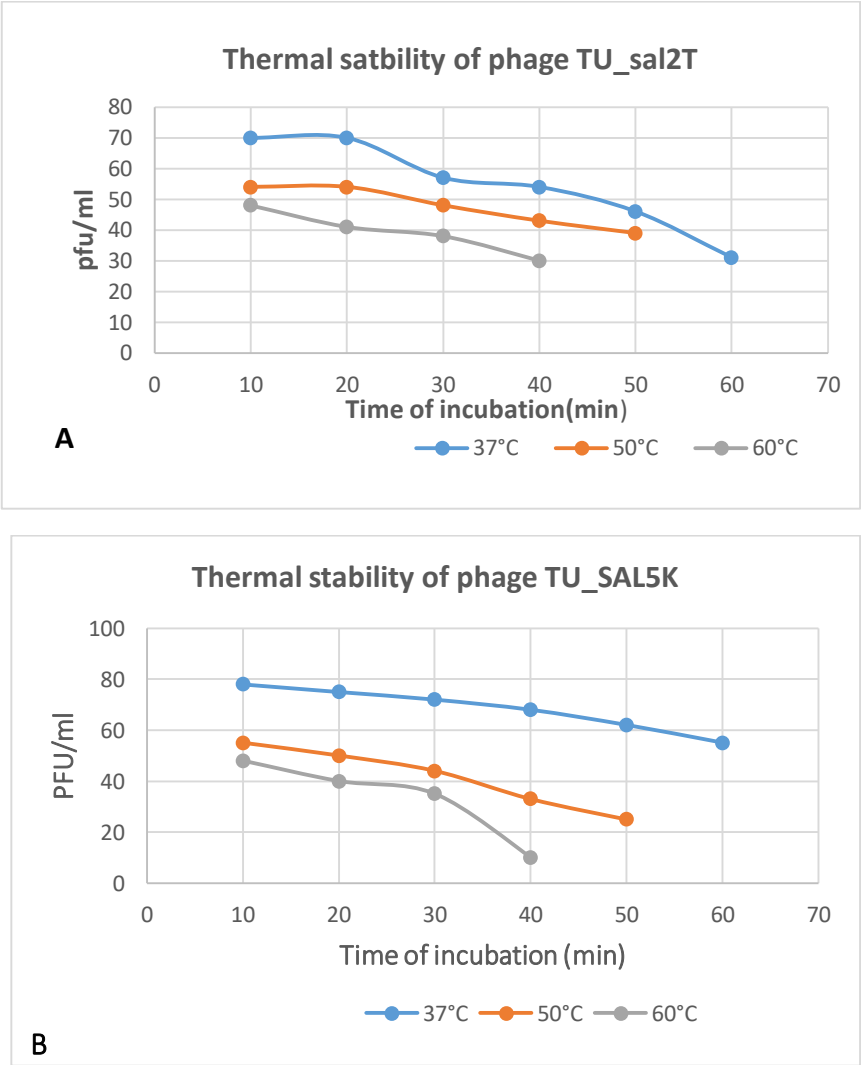


Figure 4.16 Temperature stability of phages. Graph A showing the temperature stability of phage TU_sal2T and graph B shows temperature stability of phage TU_sal5K. Both phages have temperature survilability up to 60°C.

Phage TU_sal5K and phage TU_sal2T were stable up to 60°C for 40min (Figure 4.16). Because of formation of high number of plaques in phage TU_sal2T, Phage TU_sal2T was

seems to be more stable in high temperature than in phage TU_sal5K. The PFU/ml was decreases with the time of incubation increases this showed that the Stability of phage was decreases with time and temperature increases. Both phages were not stable at the temperature of 70°C or above (refer to appendix). This was similar result with that of *Salmonella* phage SAL-PG determined by the Rahaman *et al.* But higher stability to temperature than *Salmonella enterica* serovar Gallinarum biovar Gallinarum bacteriophage SG-JL2 (only stable up to 55°C for 1 hour) (Kwon et al., 2008). There was no gradual decline in pfu at the temperature of 37°C which is indicated this phage could have potential for the phage therapy.

4.4.4 Protein profiling of phage by SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)

Different protein bands of Phage TU_sal2T and phage TU_sal5K were analyzed by using Gel Analyzer software. Two bands of phage TU_sal2T and phage TU_sal5K were analyzed with the reference of protein marker.

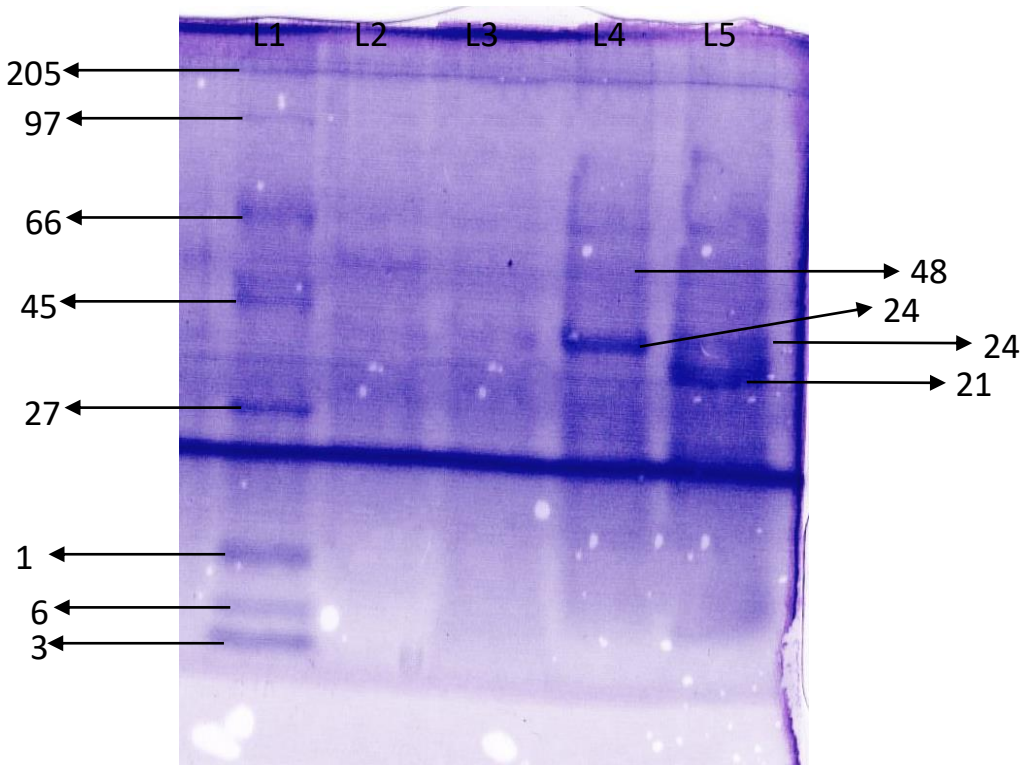


Figure 4.17 Separation of different protein bands of bacteriophage by SDS PAGE. Lane 1 is the protein marker (different bands having molecular weight expressed in Kilo Dalton; KDa unit). Lane 4 is the phage TU_sal2T having bands size 48kDa and 24kDa. And lane 5 is phage TU_sal5K having bands size 24 and 21 KDa. Bands were measure by Gel Analyzer software.

Phage TU_sal2T and phage TU_sal5K have also separate other protein which could be assigned as the internal virion proteins and other minor structural proteins. The bacteriophage Phage TU_sal2T have protein appeared with faint band size of 48 KDa and another protein with distinct band was observed having the size of 24 kDa. By comparing the molecular weight obtained from the sequence of Capsid protein and tail proteins (refer to Genome sequencing portion). These proteins could be assigned as a major capsid protein and tail protein respectively. In the case of phage TU_sal5K two different proteins with band size 24 and 21 KDa were measured. These are also supposed to be the band of major proteins head and tail (Fig. 4.17). The appearance of faint bands might be the error during the SDS PAGE experiment such as improper amount of dye mixing, heating or degradation of protein due to overheating. Also, improper mixing of different gel component during gel preparation, short time running of gel are other factors which affect the bands separation because SDS PAGE is the very sensitive experiment than other. Acetone precipitation method helps to precipitate the protein during SDS PAGE to separate more distinct bands of protein. But here we did not include the result of precipitated bands of phage because these bands were seeming overcrowded.

4.4.5 Transmission Electron Microscope (TEM) of Sal2 phage

On the basis of clear plaque morphology, broad host range, higher number of burst sizes, high thermal and pH activity as compared to Sal5 phage, phage TU_Sal2T was selected for Transmission Electron Microscopy (TEM). The transmission electron micrograph of Sal2 phage was analyzed by Image j viewer software.

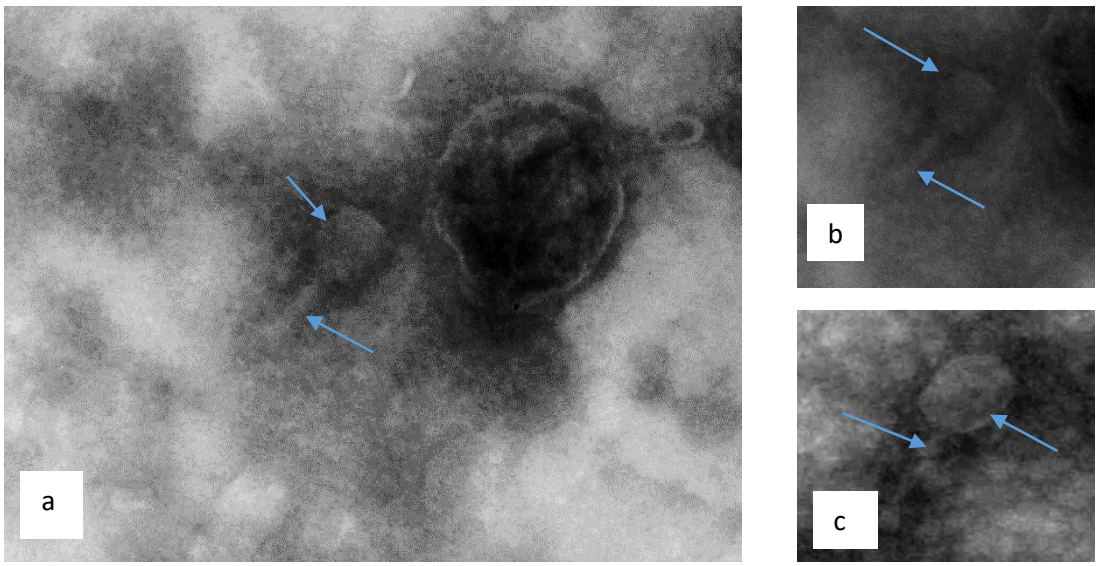


Figure 4.18: Electron micrograph of phage TU_ sal2T, fig. a is the original micrograph of phage TU_sal2 T. Figure b and c are the zoom view of micrograph.

Table 4.7 Morphological Characterization of phage obtained from TEM

Phage Name	Capsid Diameter(nm)	Tail length (nm)	Tail width(nm)	Shape	Putative order/Family
Phage TU_sal2T	66	84	15	Icosahedral	Caudovirales *Myoviridae*

From the above table and micrograph analysis, we can predict that the Phage TU_sal2T is belongs to the putative order Caudivirales and family *Myoviridae.* Only head tail Length cannot consider for the classification of phage. The shape of capsid also taken as a factor for classifies the bacteriophage. The Phage TU_sal2T have icosahedral capsid and contractile tail without envelope. Phage capsid diameter was found as 66nm with the tail length 84nm and width 15nm. Bao *et al.* isolated and morphologically characterized a members of the family *Myoviridae*; *Salmonella* bacteriophage PSPu-4-116 possessed an icosahedral head (diameter, 74.3 nm) and a contractile tail (length, 114.2 nm (Bao et al., 2011).

Generally, bacteriophage is classified according to the guideline provided by International Committee on the Taxonomy of Viruses (ICTV). According to the 7th report of ICTV, three family of bacteriophage *Myoviridae*, *Podoviridae*, and *Siphoviridae* reported the 16 genera of bacteriophages. But in 8th report 18 genera spread over three family (Adriaenssens and Brister, 2017). The *Myoviridae* family phage have general characteristics with nonenveloped double stranded DNA, with head–tail geometries. Tails are contractile, more or less rigid, long and relatively thick (80–455×16–20 nm) (ICTV 9th report, 2011. During contraction, sheath subunits slide over each other and the sheath becomes shorter and thicker, which brings the tail core in contact with the bacterial plasma membrane. By comparing the characteristics obtained from TEM of Phage TU_sal2T with ICTV guideline for *Myoviridae* family phage and also results from the whole genome sequencing, Phage TU_sal2T belongs to the *Myoviridae* family.

4.5 Whole Genome Sequencing of phage TU_Sal2T

4.5.1 Quality check and quantification of phage DNA

The concentration and purity of DNA was checked by Nano Drop reading. The concentration of phage TU_sal2T was found to be 66.6 ng/µl and total yield was 2.6µg (Table 4.8).

Table 4.8 Quantification and purity check of phage DNA.

Xcelris ID	Sample ID	Concentration (ng/µl)	Yield(µg)	$A_{260/280}$
XGC-956-A3560	Phage TU_sal2T	66.6	2.6	1.85

Genomic DNA extraction of phage showed a clear band without smearing under 0.8% agarose gel electrophoresis (Figure 4.19). Comparing DNA band with marker, extracted band size was higher than 23 Kb. The ratio of $A_{260/280}$ is ratio of OD value at 260 and 280 nm which is used to check the purity of DNA whether there is contamination of RNA and protein or not. If this value is less than 1.8 that means there should be presence of RNA contamination and if this value is higher than 2.0 that denotes the presence of protein the DNA product.

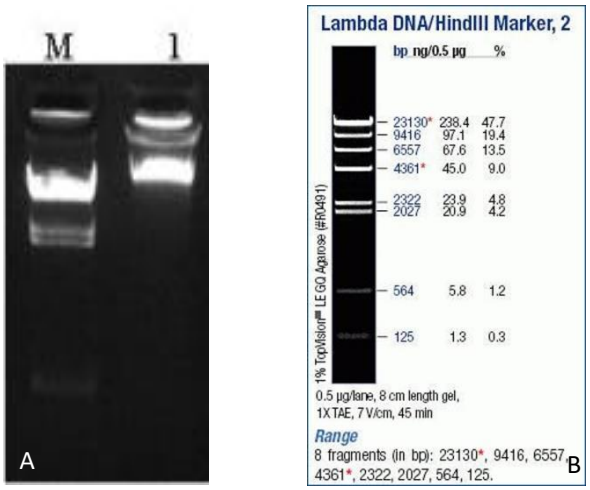


Figure.4.19 Phage DNA on 0.8% agarose gel. Lane M representing the Hind III DNA marker, and lane 1 is the DNA of phage sal2 in figure A and different fragment of marker in bp unit and their concentration are shown as a reference in figure B.

4.5.2 Library preparation of phage.

The library was prepared from the sample S2 phage by *Truseq Nano DNA Library preparation kit*. The average size of library is 309 bp. The library will be sequenced on Illumina platform (2 x 150 bp chemistry) to generate ~2 GB data.

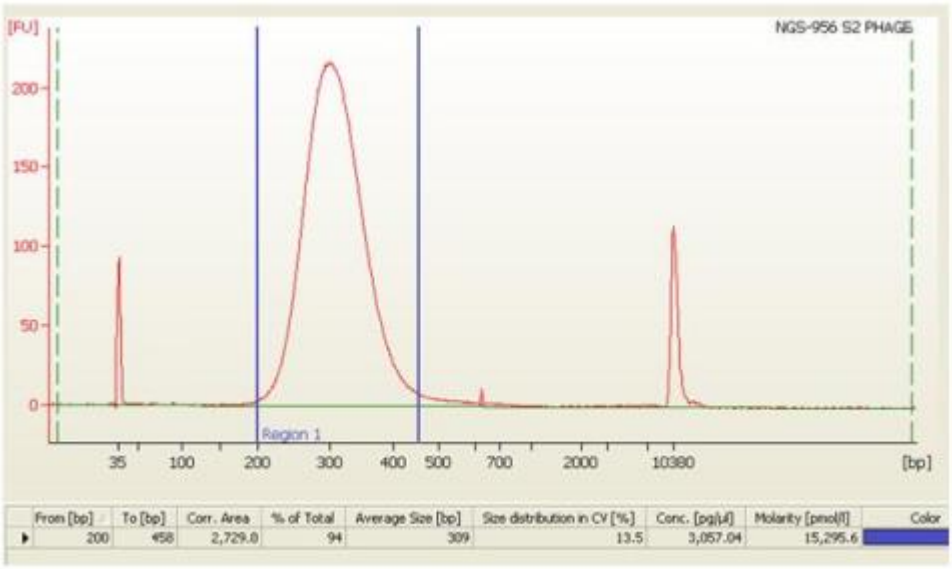


Fig 4.20 Bioanalyzer profile of phage TU_sal2 loaded in Agilent DNA HS chip. The average size of library was 309 bp.

4.5.3 Bioinformatics analysis

4.5.3.1 Data generation and de novo assembly sequence

Paired end data was generated on Illumina platform (2 X 150 bp chemistry). Statistics of high-quality data is provided in the table below.

Table 4.9 Read data statistics for the sample

No. of scaffold	1
No. of contigs	11
Total genome length including gap	164,674
Total genome length without gap	164,595
Average scaffold size	164,674

Final de novo assembly were calculated using in-house PERL script and the details are provided in the below table. High quality reads were assembled using CLC genomics workbench v6.0 and the contigs generated were searched against NCBI NT database. Hence, sequence of the contig_1 obtained from de novo assembly of high-quality reads using CLC genomics workbench was considered for further downstream analysis.

4.5.3.2 Gene ontology analysis

The Gene Ontology project provides controlled vocabularies of defined terms representing gene product properties. These cover three domains: Cellular Component, the parts of a cell or its extracellular environment; Molecular Function, the elemental activities of a gene product at the molecular level, such as binding or catalysis; and Biological Process, operations or sets of molecular events with a defined beginning and end, pertinent to the functioning of integrated living units: cells, tissues, organs, and organisms. Gene Ontology analysis was performed by using Blast2GO command line v1.4.1. Bar chart representation of all the three GO domains viz. Biological Process, Cellular Component and Molecular Function was plotted using Blast2GO command line v1.4.1 at level 2. GO analyzes 45 genes were to Biological Process, 53 to Molecular Function and 35 to Cellular Component.

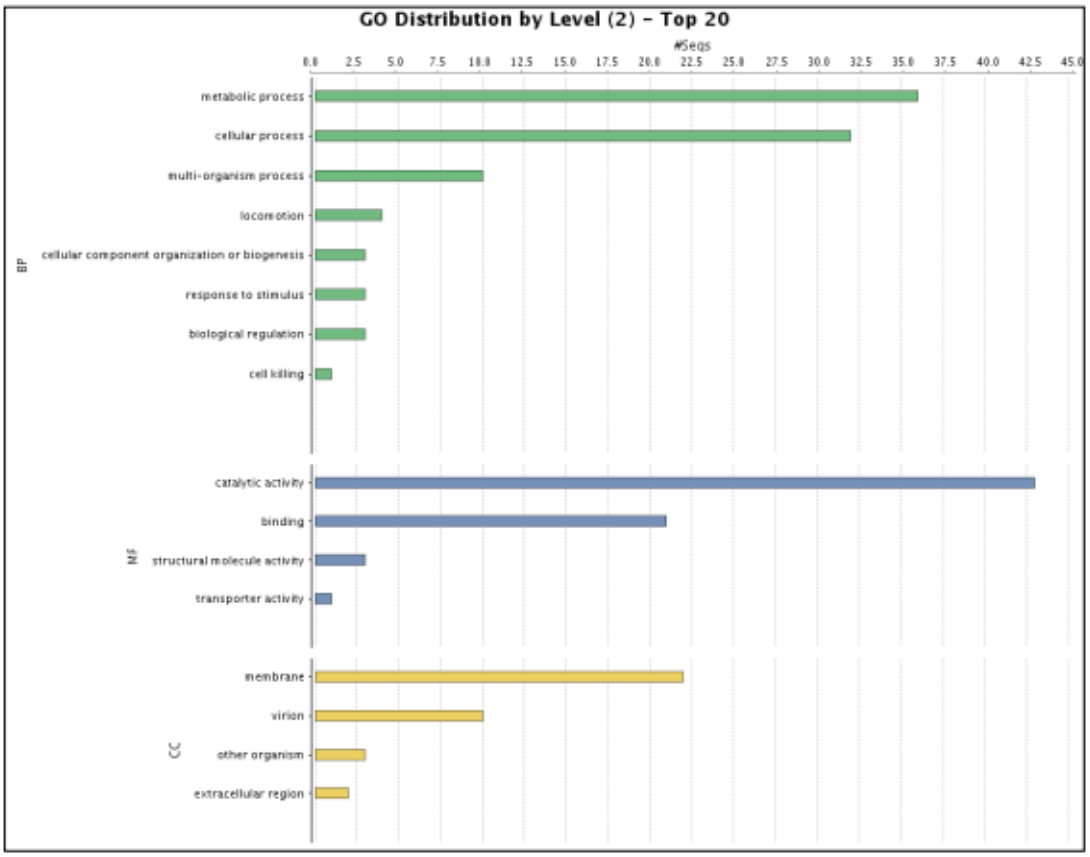


Figure 4.21 GO domain distribution.

4.5.3.3 Gene prediction and functional annotation of gene

Putative genes were identified with Prodigal tool v.2.6.3, which is a highly accurate gene finding program. Total number of predicted genes were 271 with the average gene size 572bp and maximum gene size 3,741bp. Total size of all genes were 154,992bp. Length distribution of the predicted gene was also carried out (see in appendix).

All predicted genes were annotated evaluating the homology by blastx search against NR database (Non-Redundant database). Based on similarity searches with known proteins, we annotated genes with cut-off E-values of 10-6. The statistics of annotated gene sequences with blast hits were found to be 269 and sequences without blast hits were 2 among total genes of 271.

4.5.3.4 Genome analysis of phage

PHASTER (PHAge Search Tool Enhanced Release) was used for the phage genome analysis. PHASTER generated the circular genome of sequenced phage with the genome size, CDS region, GC content and the predicted location. The genome is intact and no significant breaks was reported throughout the region. The total length of the phage was 164.2 Kb. The number of ORF (open reading frame) in the sequence were 267 with the start and end position 247- 164507. Similarly the percentage of GC nucleotide was 40.58% which is shown given below.

Region	Region Length	Completeness	Score	# Total Proteins	Region Position	Most Common Phage	GC %
1	164.2Kb	intact	150	267	247-164507	PHAGE_Entero_GEC_3S_NC_025425(260)	40.58%

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

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

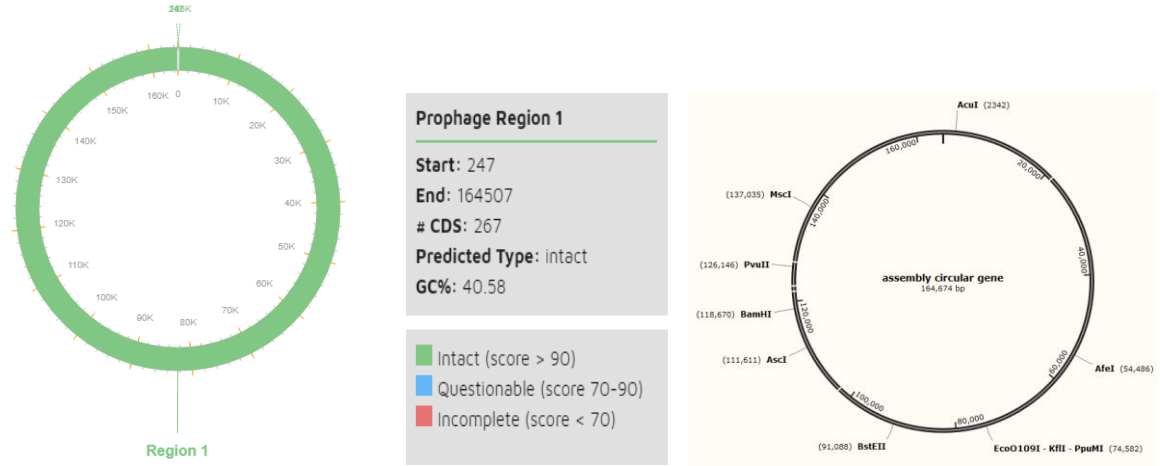


Figure 4. 22 Circular genome of Phage TU_sal2T from PHASTER (region 1) and SnapGene viewer (showing different rectriction enzyme site) respectively.

Genome mapping by SnapGene® however predicted a different number of ORF (usually greater than PHASTER). This is because PHASTER relies only on 'phage database' for

alignment and prediction, whereas SnapGene® relies on hypothetical parameters. The study has comparatively relied more on the prediction of PHASTER rather than SnapGene®. However, due to insufficient database of phage on NCBI, the hypothetical prediction by SnapGene® cannot be completely ruled out as well.

Total gene/protein of 271 were successfully hit and identified by PHASTER annotation (figure 4.21). Genes encoding major capsid protein, head sheath, coat and core protein, neck, tail sheath, tail tube, baseplate, tail fiber protein, holing and lysis protein were suceessfully hits and identified (see in appendix). This verifying the phage was lytic and belongs to the family *Myoviridae.* Further no integrage gene and any virulence or bacterial gene from host within phage genome were predicted. This showed that the phage have most potential candidate for therapeutic purposes.

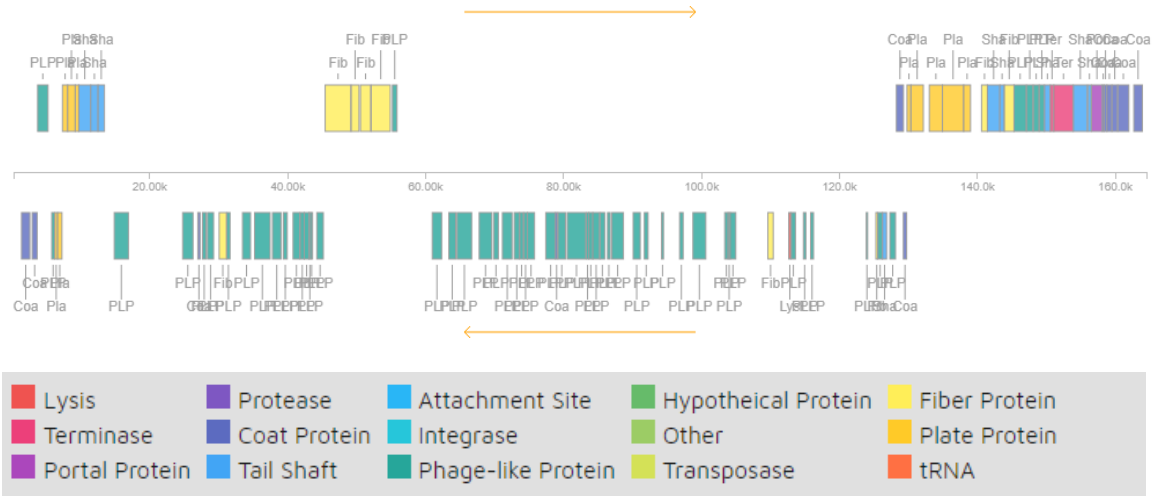


Figure 4.23 Linearized genome annotation of Phage TU_Sal2T. The upper arrow represents forward direction of ORF whereas lower arrow represents the backward direction/strands of ORF. The region position value predicts the location of prophage genome on host genome.

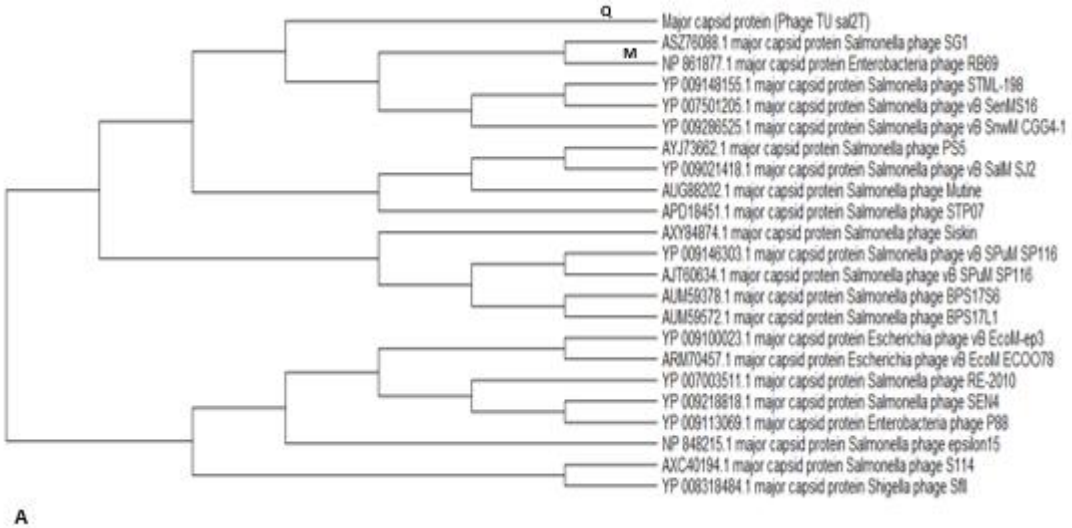
The sequence of Phage TU_sal2T was best match with the *Enterobacteria* group of phage (HE978309.1). Since the specific host (*Salmonella Typhi*) of this phage also characterized molecularly by 16S rRNA sequencing (see on sequencing of host portion) and also *Salmonella* is the genus of Enterobacteria group. That's why we can concluded that the phage TU_sal2T was the *Salmonella* specific phage. Out of the total 271 genes, 269 gene sequences were obtained with hits to NCBI NR database using blastx functional annotation. Majority of the hits corresponds to *Enterobacteria* phage and belongs to the family *Myoviridae.*

In overall functional protein prediction, some of the proteins were identified as hypothetical phage proteins (refer to Appendix). 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.* From the genome annotation it was also revealed that the genome was free of bacterial genes, virulent genes from bacterial origin and other toxic genes. Absence of integrase gene also rules out the transfer of phage genome to bacterial genome (prophage production). In similar way, absence of lytic cycle repressor protein i.e. cI group of protein, make impossible to the production of lysogenicity in phage. Hence, from these above genome characteristics, good lytic capability and with addition to the multihost range property, this phage could be the good candidate for the therapeutic purposes.

4.5.3.5 Phylogenetic tree of Major Capsid Protein and Tail Tube protein of Phage TU_sal2T

The construction of phylogenetic tree of whole phage genome was difficult due to the sophisticated electronic instrumentation (desktop and laptop). Because the data of whole genome was more than 1.5GB this large data could not support by our laptop. So, we constructed the two major protein of phage to know evolutionary relationship of phage proteins with the other reference phage protein available in the NCBI database. Tree was constructed by using the MEGA X, a Bioinformatics tool for phylogeny and its importance was already discussed above in 16S rRNA sequencing portion.



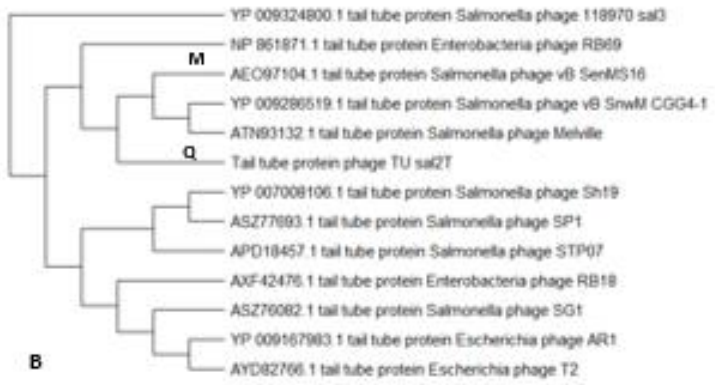


Figure 4.24 Phylogenetic tree of two protein of phage TU_sal2T by using MEGA X software**.** Tree was built by NJ method. Figure A is the major capsid protein and figure B is the tail tube protein. Major capsid protein was best matches with the capsid protein of Salmonella phage SG1 and Enterobacteria phage Rb69 with an equal branch. Tail protein was best matches Salmonella phage Vb SenMS16. Q denotes the query sequence or sample sequence and M refer to the closely match sequence.

The protein molecular weight (MW) of both major capsid protein and tail tube protein was calculated by using ExPASy; a bioinformatics tool. Molecular weight of capsid protein was found to be 56.7KDa with isoelectric point (pI value) of 5.41. Similarly, protein molecular weight of tail tube and tail sheath protein were determined as 18.3(pI; 4.65) and 3KDa respectively. These values were used to compare the different protein bands observed in SDS PAGE.

Chapter V

SUMMARY

Host bacterial strain was identified by 16S rRNA sequencing and colistin resistant also reconfirmed by antibiotic susceptibility pattern. Nine bacteriophages were isolated against the colistin resistant *Salmonella Typhi* by performing standard double layer agar assay method. Almost all phages showed clear lytic effect against host. Phage TU_sal2T and phage TU_sal5K showed the intraspecies and interspecies (against *Acinetobacter* and *Klebsiella strains*) host range showing broad lytic capability.

The physiochemical stability of both phages showed optimum pH with 8 and temperature at 37°C. But they were stable at the range of pH 3 to 11 and temperature up to 60°C for 40min. This was significant characteristics for the use of phage as therapeutics. Burst size and latent period of phage were also an important property in the life cycle of bacteriophage. Burst size of phage TU_sal2T and phage TU_sal5K were determined as 50 and 45 phages per bacterium with latent period of 20min at MOI 10. Phages with large plaque size have high burst size which in turn reduces the latent period of bacteriophage. Burst size, latent period and MOI values are basic factors for the dose determination. In overall phage TU_sal2T showed slightly better effectiveness over the phage TU_sal5K.

Electron micrograph of Phage TU_sal2T revealed it belonged to the order Caudovirales and family *Myoviridae.* The presence of tail head morphology and measurement of its size help to classify phage. Further genome analysis of Phage TU_sal2T showed the phage has genome size 164.5 Kbp with 267 CDS (Coding DNA Sequences). Also, gene prediction suggested that phage genome was free from bacterial genes, virulence genes and any other toxic genes. Absence of integrase and lytic cycle repressor gene also strengthens our argument that 'lytic' phages can be used therapeutically to kill bacterial strains and they [lytic phage] are very unlikely to integrate their genome for the acquisition of a 'prophage' state. Phylogenetic tree construction of capsid and tail protein helps to know the evolutionary relationship of these protein with the protein of closely related species. Comparison of molecular weight of head tail protein with bands of SDS PAGE also suggested that the large MW bands/subunits might be the capsid protein and smaller size protein might be the tail protein.

For the use of bacteriophage as antimicrobial agent; high virulence, effective lysis at lower concentration, broad host range are the mostly prioritized consequences for efficacy and effectiveness of phage as a bio-therapeutics.

Chapter VI

CONCLUSION

Nine phages were isolated from the different places of Kathmandu valley against multidrug resistance *Salmonella Typhi*. These phages could infect and lysed interspecies group of bacteria. Thus, we reject null hypothesis and accept alternative hypothesis which states lytic *salmonella* phage shows interspecies host range. Mixing of sewage and domestic waste directly in rivers water of the Kathmandu valley, there is possibility of heavily contamination of multidrug resistance pathogens, thus it is favorable habitat for bacterial viruses.

Physiological characterization of two *Salmonella* phages (phage TU_sal2T and phage TU_sal5K) showed comparatively less difference activity against host. Stability of phage against pH (optimum 8) and temperature (optimum 37˚C) were almost same. Burst size of phage TU_sal2T (50 virus per bacterium) was slightly higher than that of phage TU_sal5K (45 virus per bacterium) with same latent period of 20 mins.

Transmission electron micrograph and whole genome sequencing revealed that of phage TU_sal2T belongs to the order Caudovirales and family *Myoviridae*. Whole genome analysis also confirmed the absence of any virulent gene of bacterial origin and integrase gene which rule out the possibility of transfer of gene from phage to bacterial strains. Additionally, holin and lysin gene within phage genome were also predicted.

This research builds the framework and successfully proves that the *Salmonella* phages can efficiently kill multidrug resistant *Salmonella* strains as well as other drug resistant pathogens making it good candidate for a phage therapy and other applications.

Limitation of the study

- Small number of sample size is one of the distinct limitations of this study. We do not have satisfactory result of intraspecific host range analysis.
- Sophisticated instrumentation also limited our study. The research required electron microscopy, ultracentrifugation, sequencer, so as to we had exported sample out of country. Further, we did not perform the electron microscopy of another phage because it was costly to export sample and cannot repeat electron microscopy when poor quality of image was observed.
- Whole genome sequence analysis is a stiff task, due to limited knowledge, lack of expertise and skilled manpower in bioinformatics, comprehensive genome annotation could not be performed.

Future Recommendation

- Cell line study: Cell cytotoxicity and cell line analysis after phage infection is potential study that would significantly improve the impact of phage research in invitro model.
- Comprehensive whole genome mapping and annotation: Extensive genome analysis and complete genome submission to genebank is another aspects of study which is yet to be done. A collaborative study with bioinformatics specialist would help to yield better results and conclusion.
- Endolysin gene cloning: Endolysin is an important enzyme play a major role in host lysis mechanism. If extensive research is done in endolysin gene cloning, lysin therapy could be improved as in phage therapy.
- Animal model experiment: Animal model experiment would provide reliable and significant data to improve the acceptance of claim that we made for phage.

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APPENDIX

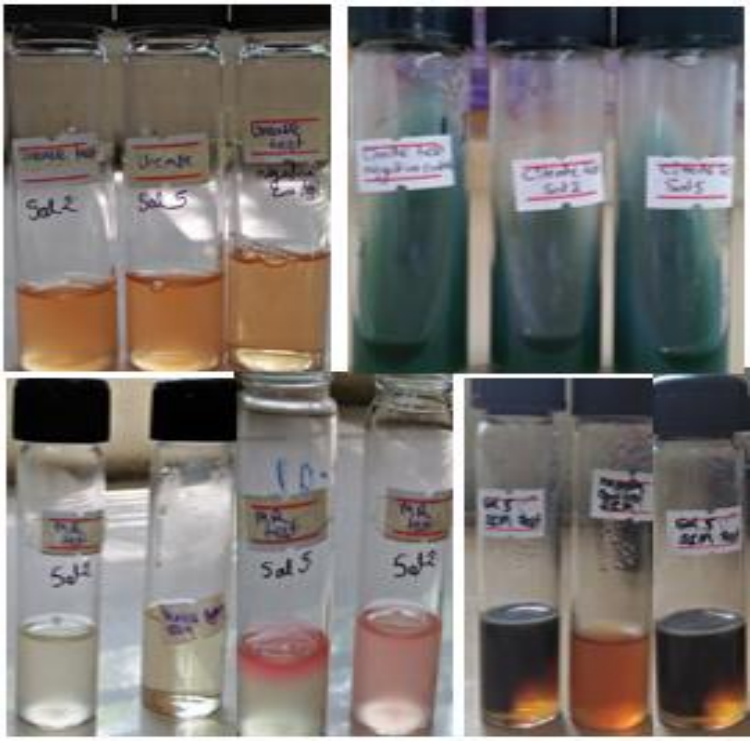


Figure A1: Biochemical test of *Salmonella Typhi*.

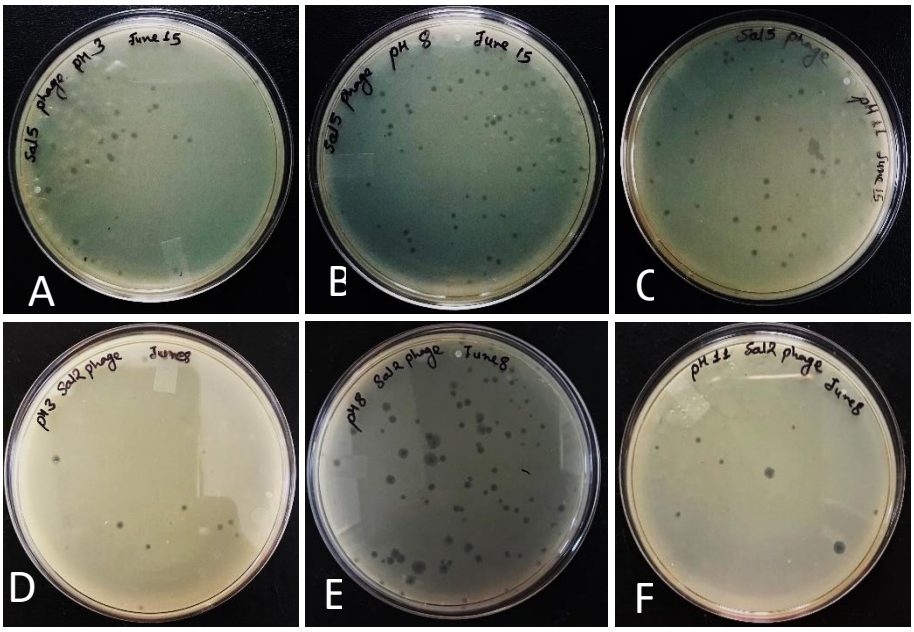


Figure A2: Different no. of plaques survival in p H 3, 8 and 11.

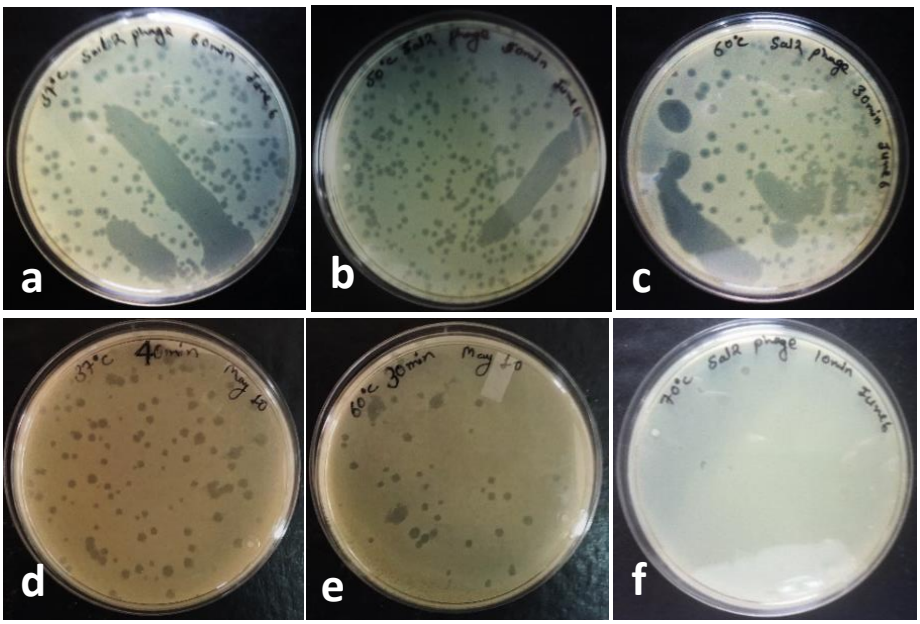
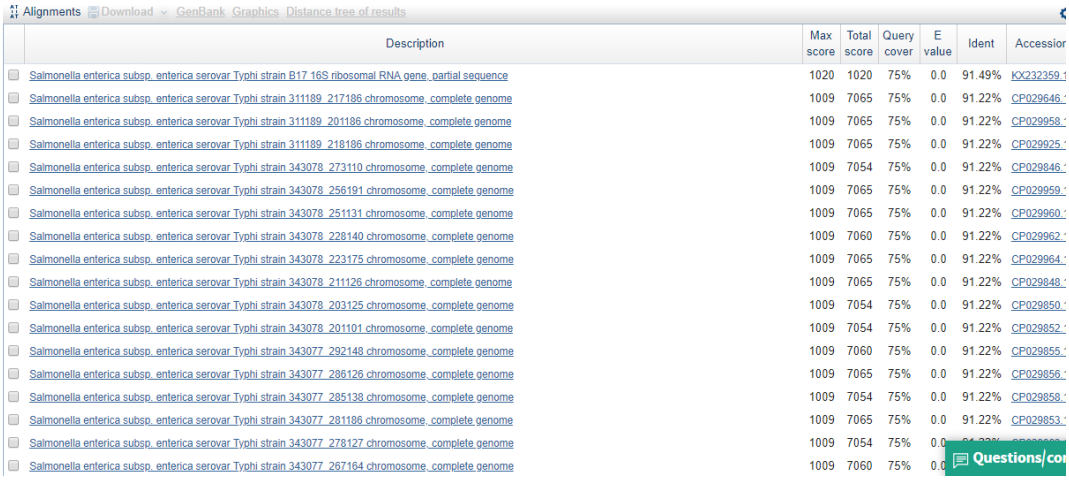


Figure A3: Stability of phage in different temperature.



Alignments Download GenBank Graphics Distance tree of results

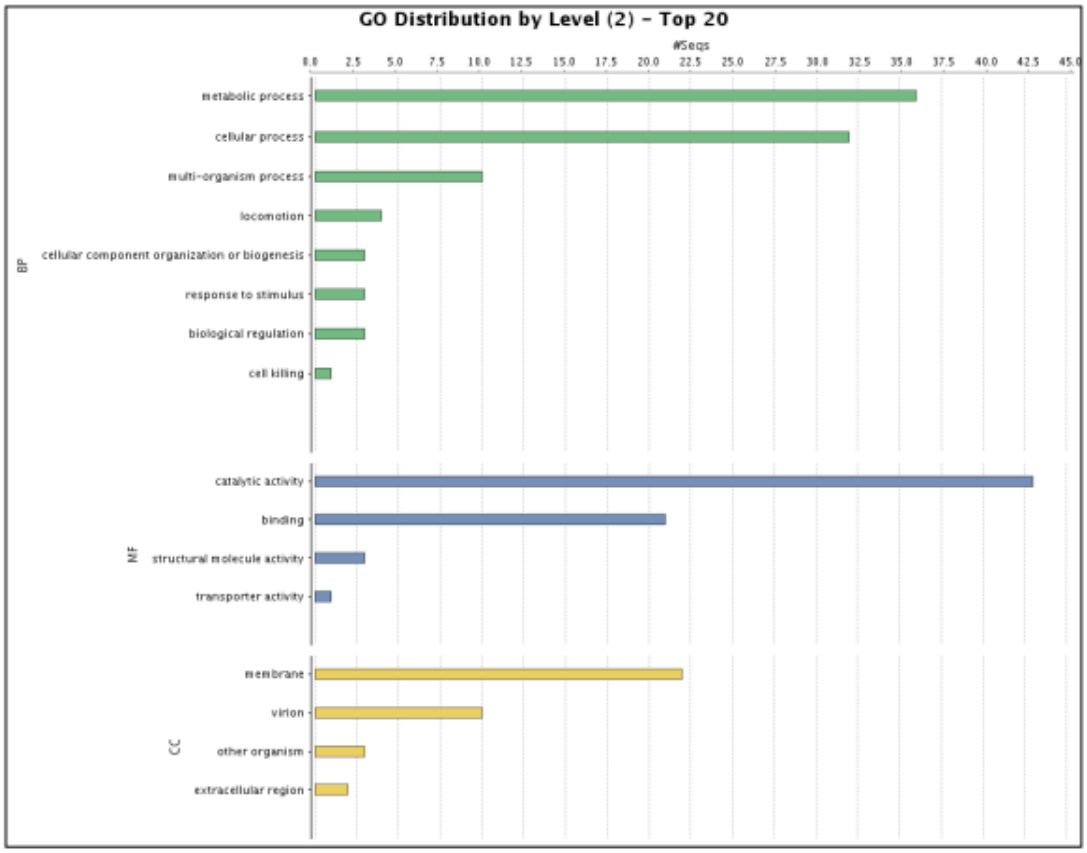
Description	Max score	Total score	Query cover	E value	Ident	Accession
Salmonella enterica subsp. enterica serovar Typhi strain B17 16S ribosomal RNA gene, partial sequence	1020	1020	75%	0.0	91.49%	KX232359.1
Salmonella enterica subsp. enterica serovar Typhi strain 311189_217186 chromosome, complete genome	1009	7065	75%	0.0	91.22%	CP029646.1
Salmonella enterica subsp. enterica serovar Typhi strain 311189_201186 chromosome, complete genome	1009	7065	75%	0.0	91.22%	CP029958.1
Salmonella enterica subsp. enterica serovar Typhi strain 311189_218186 chromosome, complete genome	1009	7065	75%	0.0	91.22%	CP029925.1
Salmonella enterica subsp. enterica serovar Typhi strain 343078_273110 chromosome, complete genome	1009	7054	75%	0.0	91.22%	CP029846.1
Salmonella enterica subsp. enterica serovar Typhi strain 343078_256191 chromosome, complete genome	1009	7065	75%	0.0	91.22%	CP029959.1
Salmonella enterica subsp. enterica serovar Typhi strain 343078_251131 chromosome, complete genome	1009	7065	75%	0.0	91.22%	CP029960.1
Salmonella enterica subsp. enterica serovar Typhi strain 343078_228140 chromosome, complete genome	1009	7060	75%	0.0	91.22%	CP029962.1
Salmonella enterica subsp. enterica serovar Typhi strain 343078_223175 chromosome, complete genome	1009	7065	75%	0.0	91.22%	CP029964.1
Salmonella enterica subsp. enterica serovar Typhi strain 343078_211126 chromosome, complete genome	1009	7065	75%	0.0	91.22%	CP029848.1
Salmonella enterica subsp. enterica serovar Typhi strain 343078_203125 chromosome, complete genome	1009	7054	75%	0.0	91.22%	CP029850.1
Salmonella enterica subsp. enterica serovar Typhi strain 343078_201101 chromosome, complete genome	1009	7054	75%	0.0	91.22%	CP029852.1
Salmonella enterica subsp. enterica serovar Typhi strain 343077_292148 chromosome, complete genome	1009	7060	75%	0.0	91.22%	CP029855.1
Salmonella enterica subsp. enterica serovar Typhi strain 343077_286126 chromosome, complete genome	1009	7065	75%	0.0	91.22%	CP029856.1
Salmonella enterica subsp. enterica serovar Typhi strain 343077_285138 chromosome, complete genome	1009	7054	75%	0.0	91.22%	CP029858.1
Salmonella enterica subsp. enterica serovar Typhi strain 343077_281186 chromosome, complete genome	1009	7065	75%	0.0	91.22%	CP029853.1
Salmonella enterica subsp. enterica serovar Typhi strain 343077_278127 chromosome, complete genome	1009	7054	75%	0.0		
Salmonella enterica subsp. enterica serovar Typhi strain 343077_267164 chromosome, complete genome	1009	7060	75%	0.0		

Questions/co

Figure A4: blast hit of 16S r RNA sequence of *Salmonella Typhi*

Table A1: Read data statistics of phage TU_sal2T DNA

File name	Total reads (R1+R2)	Total bases (R1+R2)	Data in GB
Phage TU_sal2T_R1.fq.gz	14,789,042	2,078,413,621	2.07
Phage TU_sal2T_R2.fq.gz			



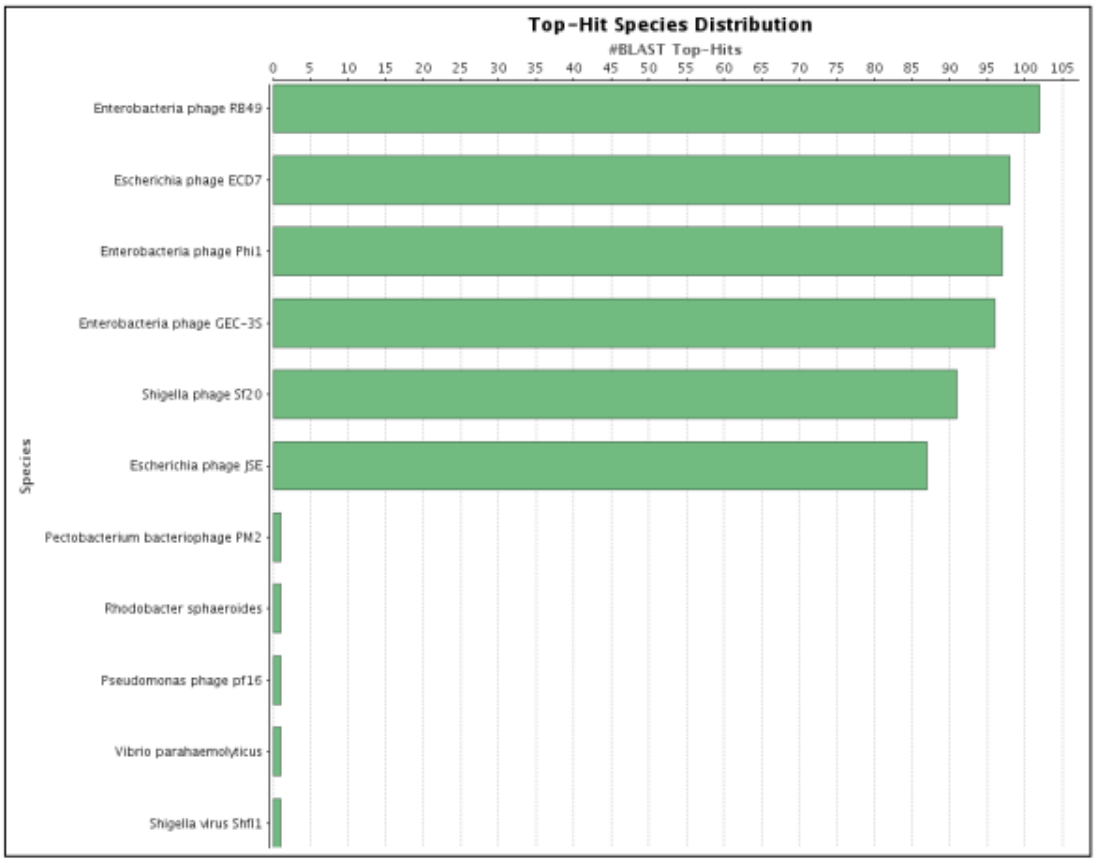


Figure A5: GO distribution and blast hit of phage TU_sal2 T

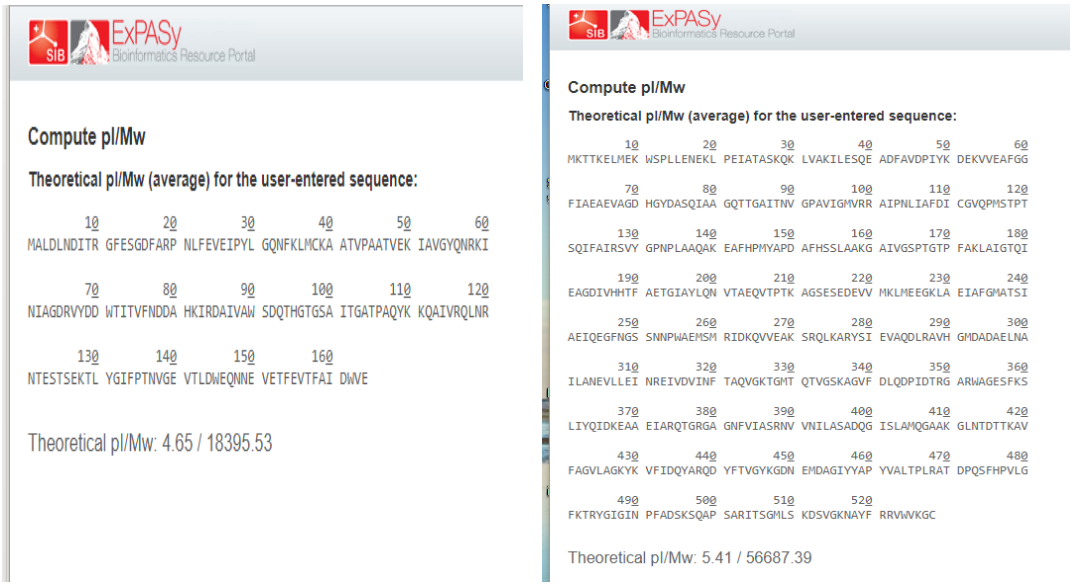


Figure A6: Protein molecular weight determined by ExPAsy

Functional annotation of phage genes using PHASTER:

```
>contig_1


CDS_POSITION                                                                BLAST_HIT
EVALUE              prophage_PRO_SEQ
-------------------------------------------------------------------------------
-------------------------------------------------------------------------------
----------------------------------------


#### region 1 ####

complement(247..684)                        PHAGE_Entero_GEC_3S_NC_025425:
hypothetical protein; PP_00001; phage(gi754380765)          2.32e-104
MKFRKNHVAMKKAMFEAFKAVDQGGVTLIEQINVDVWQSPAITCSRLATAGLLKVHSSTAYDEALQVIKAFA
GDVTSKWVYENKGEIAVAHVGNNIQMEIKIKEGSKRNQMLVRFQPYKTNVVPDDLIISKSNVTIRRNQIVIN
K

complement(731..1006)                       PHAGE_Entero_GEC_3S_NC_025425:
hypothetical protein; PP_00002; phage(gi754380766)          2.17e-60
MDKKTALMVYMLREDHGLNFVDIAKKIGGISGQDAAKAWLMVKDAREAYASREKVVYRKHHINKKFKNKVQK
RVDKPNNTVIMDAFKRAMS

complement(1006..1203)                      PHAGE_Entero_GEC_3S_NC_025425:
hypothetical protein; PP_00003; phage(gi754380767)          8.15e-33
MNEIVRLIKETPIEMYFLALVATMVYQLVACHVWKSENAFIRFIGTVMIQFPVLITILLIVNEAK

complement(1404..2606)          PHAGE_Entero_GEC_3S_NC_025425: Putative
head outer capsid protein; Hoc; PP_00004; phage(gi754380769)      0.0
MAFTVSIQSNKRCFLAGDGFTLTATVAGDEPLPSNLTYTWTKDDQPHENSTATLTVADATSENAGSYKVTVQ
DTDTMESVESEVFLMEEAELIVNITEPQQFYVSSQTNVELHATVKFSGGKTPADNYELHYSWSKGEEVIDTT
```

QDITIQKFTADKNGVYTVKVWGESEDSADSASTKIMLATMNVEQDVVESKTVALGNEISLNYVVSEDIVGDS
SGMPTLTIKHNWYLQREGQLSPTLIGSEVGEALEGFSIMPDGHLFKESATYDDTAKFWCVAKLYQQIEDETV
EVATSTSRKCSMEVVKMFRYVHPIPWRKTSFIYIGWWVFDEIVKFNEAGLEWRNREVYSTSKYAKDLETIAA
AEEKYSDCTCMESRNGFMYHSKELHKLDRETLERVLRIRD

complement(2945..3670) PHAGE_Entero_GEC_3S_NC_025425: Putative inhibitor of prohead protease; PP_00005; phage(gi754380771) 2.28e-176
MFDKEYFEELRAIAASDDKEAQKTAKTELADYAKQFDIKVKKTLSLENMIEFVETELTKLASEMEEETPAEG
TSINDLILAADTADDKVVFDEVDPSLVEAMKEPEIVIEETKEESPAVEPVVEEVKSDVEEAPEVKVESPVAN
IFSDEAEHRSPAHDTDSFCDLTGFRPTICLIGGGRGYYSCPWWIFDWIVNTPDWKKYPERFPHAYVHDTLKS
LIYYIKRDGSVKVRETKHSQFHTLK

3741..5243 PHAGE_Entero_GEC_3S_NC_025425: Putative RNA-DNA and DNA-DNA helicase ATPase; PP_00006; phage(gi754380772) 0.0
MQDIKIHFLNYSHVQIECSDSILLEMRDYFSFEVEGARFQKRFKYGGWDGRIRLLDYNGKLPIGLTKTVGIF
AKNMGYSVWVDPRLFEKEQVTEEEITKWCNDLELYSGANRITPHWYQSKAVFEGIHHKRRMLVLPTSAGKSA
IACMLSRWYLENYEGKVLIIVPTTSLVLQMRDDFVDYRQFPYEAIHTIMSGTSKHVGDRLIVVSTWQSACKQ
PREWFQQFGMVIVDECHKATAKNLLNIVTDMDHCQFKIGMTGSPRDGKANMMQYVGLFGDISKIVSIDRLME
EGQVTKLKINCLFLRYTDEECSAVKGREYAEEIKYITSNPRRNKFACNLALKLAKKGENVFLMFRNTKHGKL
MYDALQKVHDKVYYIDGGVKTEERDEFKKMAEGDTGIICVASYGVFSTGVSIKNLHHVIFGHPVKESTIVRQ
SIGRALRKHGSKDIATVWDLIDHLAVKTKSKNAKKQFSHLNYALKHALLRIQLYNTDKFEYATKTIEI

5287..5523 PHAGE_Entero_GEC_3S_NC_025425: hypothetical protein; PP_00007; phage(gi754380773) 1.86e-49
MISFKRFLVEAAIDGFMSKIYSCNTIEGLNELEAYYEKRKKETELKPADDISIRDAIAGRRKQLEADNEPEE
EPEEDF

complement(5587..5763) PHAGE_Entero_GEC_3S_NC_025425: hypothetical protein; PP_00008; phage(gi754380774) 4.25e-35
MKICICCKQPVEDALAVQTENGVVHTGYCLQLVTERSISEDANGEEQEQLVEQTMLLM

complement(5784..6191) PHAGE_Entero_GEC_3S_NC_025425: Putative recombination protein; UvsY; PP_00009; phage(gi754380775) 4.28e-94
MDLNDLKEQLEADMKIDATKLQWEALNNPVVYSKWLRIYSEAKRETIALEAKKKKAMKNRLDFYTNRSDDWC
RAEYEKSELKVVMAADDEILPLDTKIAYYQMVMDFAGRALDIVKSRGFAIKNAIELRMLESGR

complement(6634..7263) PHAGE_Entero_GEC_3S_NC_025425: Putative baseplate hub subunit; PP_00011; phage(gi754380777) 1.85e-150
MQYFFKIQIGEKLIHARAFTFGEFQKLVAAKETGKLSDVVIEIIYECTGINALELEKCEAEYIFLLLWCHSL
NKMKIDATWVCGVCNKEKVYSLDVSRTQIPEVEPYILDLGQVKIKFRQPKFTEDVDIMQMVIACIEYIVIGD
QQFNIDDLNAQDFDRVLDMLTTDKVEEVIDELTRNQITLAVPIKCECGESGVYSLSGLSSFLKIL

7334..8104 PHAGE_Entero_GEC_3S_NC_025425: Putative baseplate hub assembly catalyst; PP_00012; phage(gi754380778) 0.0
MNIIRIKLPEGVQRFKPFTVKDYRDFLLVSTEIKMNPEEEQTILDELLEEIYPDVDPAFREYIFLNVFTSSI
GKTKIPLCFTCPTCNKERKMLLNLQVEALKPIVLEAAGLKITFRYVKPSTDYAKTFLDAIERVSDGVNDYLW
TELPEEVRDQVIDSISFAEFEEVVKQMHTIKIEQKISCCESHDLKYIGLLPLFKLLLNPDELFIFYRINHLL
AKSNYSISDLMDMLPVERNIALTLVEKDVKEANNAKNGVS

8082..9191 PHAGE_Entero_GEC_3S_NC_025425: Putative baseplate hub subunit; PP_00013; phage(gi754380779) 0.0
MQRTGFPNVSIKLYQDYEAFLNHRFIELGATFVTMTLRDSIRGVNEGLLQFYDNKAMHTKLNGREIIQISLS
TANTDEVFNRIYGISHSNVTIDQKSDNILTFQLKSYHETLNLKFSRALTNNAVNNVTSMIDAIYKEVPLWKP

AIKGDNVVIPRCPWVSTINDYMDFVRAYGQSVETESFVYCWEDFDGITIAGHDTILKNEPIPAIICEPRLMG
QFIDMSPNLMVFNFEWQTKSNAKVKNSFNNVTYTTTIDFVTKQYNKIVVGNGDNIAHFAHGGAYGELTYKNAF
LEGSKLATIEQYDSYATAVCHGNFSLRPGMVIRFFDEKQQARNDFIVDEVVHEISREQSLTHLYMIGNSEEL
QEVKFEGKI

9191..9709 PHAGE_Entero_GEC_3S_NC_025425: Putative baseplate hub distal subunit; PP_00014; phage(gi754380780) 5.83e-122
MTETKPMLKFVVPVKTLEINGKHIKIPKMGIKQHRLLKDVRSCDETLKILLDSICPGLNAAESELVMLNLCA
FNGKCLEEKDGLKLSDVYICTETEFDLNGKTFKFKNPMYTSDPIIDDADFLSHHYHDPSVDFHDFPAFIMDW
ATGLRKTIALDTPEGTIYGGINILDRLS

9706..11439 PHAGE_Entero_GEC_3S_NC_025425: Putative tail length regulator; PP_00015; phage(gi754380781) 0.0
MNDALTSFRGMRKEAEENPIDKLNKLDKLDSIDNLQAATELVAETVEQKSNEVVGAVEDNTAANELTAENTQ
STAGNTQKTYEELQKLNNFSSQMNEKLRGFGVMMERRFGVVSKMASGIGAIEEALKKPEQPQTMPSPQPVLP
TVPEQPNNDNYQGLPKKKPDVDDRKKKNATDKRNADSMENLIKVVRGGFKETIGISNKVLGMLFKITLTAMA
EAAKWGAILMGIVFVIDTLMVHFRYWSDLFETKFDDFMDKAGEWAGPISDILTTIRQVRDYWSKGEYGELIK
SLVMGIGDAFYKTFIQLDRIITTGIAKILRMIPGMGDYADKLEYGALKSAVAQGYTPNERELELMDKVESEH
EKDKYGERTGWTGKARDIGEAIGDSIKDKVNEGLVSLGWRDQKDVDAEKRQEELKRGEYESVSAEQRSASRK
LRIKSEGAINNINEVMENLSGDYDKERMGELKKDIDVYREKVQDPTLVESDRSQLERLIEKFDEMYADKTKG
VVQTQPVPATETETAKQAERTEQMQKQAAIQQQTTNQTSNVNNTQIVTNNRTIKQGAPTTRIDAPGTINMGN
F

11439..12497 PHAGE_Entero_GEC_3S_NC_025425: Putative baseplate tail tube cap; PP_00016; phage(gi754380782) 0.0
MKISVINDAVDSFKAGVKTSAGFTSKNKGKTLTAQFPAERASGNDASGYYINDLYNNGLLFTAYDYTSRTTG
SLRDFRKKKNVASGFGGSVNIAGFDLNLGGRNAAFDREAIANILLPRSQSDVDAASHKFNDVGESVISRGGG
TLGGALSNMASTAVFGGIESITGGYLADHGEQIYNTARSMYAGADARTKNYVWHLTPRSIEDLRNILIIYET
FLELSYGSSGISSTAKELKAEVDAWYKNTLLRKSTPEEAKRNDTLFEGITDFLSNVITVSNPTIWMISNFGK
RTSFEGRSDAFGPAQISSVRLDKSPDGKFNGLAISPNLPSTFVLEVSFREILTLSRGTIFGSAA

12497..13429 PHAGE_Entero_GEC_3S_NC_025425: Putative baseplate tail tube initiator; PP_00017; phage(gi754380783) 0.0
MFDLNDFNEQAANLDFQRSNLFSVAFATTPSNKTQAILESMGGAVYDIIPNALNDYFGITRGDYTDALTNLA
VQGVRRAVDSSGVKKYLLGAMSSRVVQSLLGQFDVGTYALDWFNMAYKTSGLLVYAVKVPENRLNYEIDRNH
NAPNIRITGRDFDPLVLSFRMDSSASNYRAMQDWVNSVEDPVTGLRALPVDVEADIQVNLHNRMGVPHTIMM
FNGCVPVGVSAPELTYENNNEITTFDVTFAYRTMQTGAVGEQAAREWIEDKAINAITNTFGNNLLDSGLSAA
GNALSRLNGVGGRVVNTVTNWF

complement(13463..13948) PHAGE_Entero_GEC_3S_NC_025425: hypothetical protein; PP_00018; phage(gi754380784) 1.89e-109
MKMIKLIGLAAAFMLTGCTNMEAMMNAKSDPVFLLRECKAAYASDDQIESCFISKMERRDVDRVKVGLAPQT
QMFKDAIKEAREEKAREEKKHQEELRKQQEESIQLEKMKARDRCIIMSDMQIEQEARKAALNGDYDRYEELN
SAEFQTKALKYCNDLAK

large tail fiber proximal subunit; PP_00079; phage(gi754380846) 0.0
MADFSQHFKATFGFDAGNEKVVNVALADKNTSTDAVNVEFFNYHNGITQYDTTRGYDQYGTTIYQNRPYYAK
EDITKPAGDFDPTKWQALRTDPRWDYIAATSGDTPLKSGDYIAADGQFANLTFSMPTKPAEGDTITVKDIGG
KCGVNELSFLSSGHEFYHNGNVYNRKWYCTTPYAMNYFIFVRNRWHVYQTGTEPRGVYAQPAIDAIQMQNGD
QVFRRSSLGNITLVLPKFANNGDMIQTTDLDGLTATNHVTVKVHEHAEEQSIGTSGLKEIIGKRSGHGVFIF
DKTENLWRLCDGDQSVRLKPIIDDTRLQPNSYVAVFGDRDVTLTLPTNVEPGDRIQVSMQYMHGNQNCRIIT
ATEDTKILMSKNMVQFPKRSEYPFNGTDTWKEVSELSFNAASDYVPYLEFSYSEGDDGKKHWLVAHSHPIVE
RVDPTRKDRVGVIALATQTETNKNHEENPSDEVAVTPKMLANKTANETRRGIARIATQAETHQDTGSSFLDD

VIVTPKKLNDCVATEARRGVMEIATQHETNEGLDDTRAITPKKLEERRASEDLAGIAEIVQVGGRAAARRGE
AGTGIYNINDHAKIVTPQNINEVKATETSRGVGYLATDAEVQGATESTPQDALLITTRTLTKRTATESRTGI
AEIATQEETNLGQSDNHIITPKKLHTRRATETLHGLAEIATQPEFDAGLDDARISTPLKIKTFFENAQRLKV
DPTQGLNLTGDLWQGIMISGLDATEDTKGVAKVATTQLTDAGQDDTTIITPKKLQGKKATEGKEGIIRVGTQ
AETVAGTLANVAISPKNFKYVVQTEDTWKATEARRGFLKVATQENCFVGDNLQGSTQELGNYQHDGIAVTPK
GLNYALANFLPKMATAQNSLKLGNVEAAKWARRDIDQTIESNYTFNKNVNVKGDLECLKSGSFETLYVTKNS
TDDPSNGHLVLGERGVDGHTGITLHGTTASEGMKNSWSIIVGGTGTAQAVNSGAIAFGQINDGGVVEHYAFA
MEHNGDATAYRDFIAGRNLYAKQGGLYIVDQSNPAMTRSPDGMLNIGHGGSVNIKAGSQTLTTEIAGQKYQI
VHAGNADEVLNRRFVKNAGDTMVGKLTMDNAPIVSVKHEASAATAPAIGNIGFWNMRVTTQNIKETYPEKKN
GTLMQWGTDADGLTQLWSPDGTHKHYIRSGTGGQWTAWGEIYTKQNKPTAQEIGAVVAEGGLMNSMTVRDWI
KVGNVKIIANNLTRTVDFIWED

49202..50341 PHAGE_Entero_GEC_3S_NC_025425: Putative hinge long tail fiber proximal connector; PP_00080; phage(gi754380847) 0.0

MTQDIELRNTGIRFAHFDEDSATFTSISENNSVMYKLEVRGSNADSTKPARIRFNGVEIVGTGYENGLNFKV
LTPTGQLHEEKVFYGRGAVLAMRDYLSLLKGDYIIAMATHGELFADPISDVVFSKMGSVSFPNHILLQQMPR
VSYAAIYSTKMGKIVCEGMQATHGEGQDSSIQIEKVYDTIDDLAITGTPQRFLDYPVEYVSEDAEHFELIQW
PHDEISAPLEDFNIKAGDKLSISFELFRDSAAAAANVTARFYHNYFTDGQYKTGVRYNASKKDQWEKFEAVY
TVPEGVDSVVTGCIRYPTNSNEGIVKIRNILITPISGVVKTTGPTSFGVNGVRTTHIQDNGDFTNPVMSLLK
LPRDNKHITSNNFKEFDVD

50544..52034 PHAGE_Entero_GEC_3S_NC_025425: Putative hinge long tail fiber distal connector; PP_00081; phage(gi754380848) 0.0

MADLKYGSTVGGSPIWTQGNLTIQPAGDQLFYKGHKIYTAFDRPSATDFDAVSASEGGTFQKQVHFEEGLSV
GSASAGETKKNGIFKGQSDAANFDGVSWGFHSWKSIGFVNARDGVIMAYIDTTTGEFKSKGTIEGTTIKDTG
QRVYSPVNKPTNNDLDLVSRRGDTLTGTYNLNTVTVKLSVDSKLIARERDLINFDATKIYYGNVLDTLVFRS
KDEPTIFVNGKEGRFYHTNNKPTKADVGLGNVTNDAQVKRAGDIMEGNLQAPRMLATNDPGGPNELVRLSYF
ERKSMVANPTIIGNVDWNTLINRGIYRVENAGSGTNKPSDSYYNGVLMVYRPEDAVGTRRIVQVYYPESIDH
PMCWRSCSNESWTAWNYVDHRKLADIRYVNVTGDTMTGPLTVPAANGVRTARGAYNGDNYYAGLNSADGTAM
IHRISDIKGTEKLGITHDSKVVFFRSRNNTQTVDRYQLYHEGNKPTPADVGAVPLNAVIDFGTF

52105..54873 PHAGE_Entero_GEC_3S_NC_025425: Putative large distal tail fiber subunit protein; PP_00082; phage(gi754380849) 0.0

MADLSRIQFKRTSTKGRRPDAGTMNPGELAINLADQYLLTKNDSGAIINLSCPPVYDRDVTMAGKVKGNNYI
LSKTANYLEDQTARDLNYFGAFRTNSLDGLMDLTLNVPHSSGKAHGRGFTFRYGTGGSRVETYGFDVQGQKA
FSYKMYHEGDKPTPSELNVYSKQEVDRMFVKTVKLATVPVDIVDGYFKLATALIPQNGRSVFFRIHGGNGYN
VTAYDQVDIVEIVIRSGNNRPKGVNVIAYRRNTNKAFDVLAVNTSGDNYDIYVKYQRYTDNVIVEFGKSVDV
DLVVHDVPDFVVDRPVGDNVIGGRAVTLFNTENKRGVLSFDDNTQNSYDIVHLSNDKGTGRKYIRKFRSNYN
EMIWHETVQGSTYRLATGSTDAQEIITIESSSSIAGTHKGNIISGRMLLNGGSNAITLRRPAGQSNHIAFQD
NRTGDITRQGWLGYANADTDVFEWYSDVGGSSIRQHIDGQIEFQTGNMKRVYTNGQFISLYADGFRTVYGNY
GSWWRNDGSNVYLMSTKSGDTMGPWNTFRPFIYSLANGNVTLGGSDAGNHLMRLNNENRQVEINATTHLGSR
LYWEGSEGSASRFFIKNWGDGTSRAQVWELADETSYHLYSQRDSNGSIQFRVAGSLETGGRAQINGDLVVTN
VLRTNNQIQIYRDNNKEIWFKDADGTNRGVIWGGADGKMRLRNYSSGEYDHVFEAGMIRLERGYANGQGAGL
IRGEVQGGAWSDWRTRAAGLLVDCPNAQTSAYNVWKATKWGLDHIAAMGVHIPSGVVGNSIARLHVGGTNFD
FSATGDFTAGRNGSFNDVYIRSDARLKINKEEYKENATDKVNRLTVYTYDKVKSLTDRTVIAHEVGIIAQDL
EKELPEAVTTSKVGDPDKPEEILTISNSAVNALLIKAFQEMSEELKAVKAELAELKKN

54914..55153 PHAGE_Entero_GEC_3S_NC_025425: hypothetical protein; PP_00083; phage(gi754380850) 1.57e-51

MVYNIVIGALSNFGMKLLSKLISEKMLTKIFFYCARRLAAYTNTPIDDRFVEELYKEFNKDQSEDSNEQDKP
SDSQLPK

55187..55843 PHAGE_Entero_GEC_3S_NC_025425: Putative holin; PP_00084; phage(gi754380851) 3.23e-161
MQVSEKGKDFAISNVLRAIFTTKSTELLVLRVFAAVVLSILAFVVYSKNELFALYKETRYETYAHILQVEKD
RNFDNAAQEQLQIVHVSADADFSAVFSFRPKNLNYFVDLVAYEGKLPHTIDEKNLGGFPINKTSEEYRRHLL
GKSYFTDKDFQYIPSREKKLENIDIGFMYSCPIFNLDNVYSGSIAISWKNKPDIDIENLDTLCNQSARILGR
IR

153924..155924 PHAGE_Entero_GEC_3S_NC_025425: Putative tail sheath protein; PP_00256; phage(gi754380752) 0.0
MTLLSPGFETKETTLSTTIVQSATGRAALVGKFQWGPAFQIIQVTNEVELVNKFGQPDNNTADYFMSGANFL
QYGNDLRVVRVLNKEKAKNATALAGNIEFEITNEGSNYEVGDTIKIKHNRQDIETAGKVTKVDGDGKVKGVF
IPTGKIIAHAKAIGVYPELDGGWTAEFTSSSGNGSAALSVTKIVTDSGLLLTDLETSRANITNQTFLTKLQK
YDMPAVSAIYAGEIGNSLEVEILARSAFKNTAPDLTMYPYGGERTAARNLIPYAPQNDNQYAFIVRRDGVVV
ESYVLSTLKGDKDVYGNSIYMDDFFARGSSQYIYATAQGWVDGFSGIISLAGGVSANEATTGGVGADPFIGA
MMQGWDLFAERESIHVNLLIAGACAGEGDAFSTVQKHAVSIGDERQDCLVMVSPPRSTVVNIPVTTAIDNLI
AWREGSGNYNENNMNINTTYAVIDGNYKYQYDKYNDVNRWVPLAADIAGLCARTDAVSQPWMSPAGYNRGQI
MNVVKLAIEPRKAHRDRLYQAAINPVIGAGGEGFILMGDKTATTVPSPFDRINVRRLFNMLKKNIGDSSKYK
LFENNDNFTRASFRMEVSQYLSTIRSLGGIYDFRVQCDTTNNTPDVIDRNEFVASMFIKPAKSINYIMLNFT
AVATGSDFDEIIGPANQA

155998..156492 PHAGE_Entero_GEC_3S_NC_025425: Putative tail tube protein; PP_00257; phage(gi754380753) 1.74e-119
MALDLNDITRGFESGDFARPNLFEVEIPYLGQNFKLMCKAATVPAATVEKIAVGYQNRKINIAGDRVYDDWT
ITVFNDDAHKIRDAIVAWSDQTHGTGSAITGATPAQYKKQAIVRQLNRNTESTSEKTLYGIFPTNVGEVTLD
WEQNNEVETFEVTFAIDWVE

156551..158116 PHAGE_Entero_GEC_3S_NC_025425: Putative portal vertex protein; PP_00258; phage(gi754380754) 0.0
MFSRLKMLARWADFDNDKYEEQIKDKAESIAAPKNNDGATEVEINDNSPASSWNSLTQQFYSTDQKISTTKQ
LVNTYRGLMNNHEVENAVQNIVNDAIVFEEGHEVVSLNLEATGFSESVKERIHEEFKDLLNTIQFDRRGQDM
FRRWYVDSRIFFHKIIGKNPKDGIIELRQLDPRNLEYVREIITEDTPEGKIYKATKEYFIYTVGNSSYCAGG
QVFSPNSRVKIPRSAITYAHSGLMDCDDKYIIGYLHRAVKPANQLKLLEDAMVVYRITRAPERRVFFIDTGN
MNNRKAAQHMNSVAQSFKNRVVYDASTGKLKNQQANLSMTEDYWLQRRDGKAITDVTTLPGASGMSDIDDIR
YFNRKLYEALRVPLSRSNLSDANMVIGGDGSEITRDELEFSKFIRTLQSQFSEVLRDPLKYNLILKNVITED
DWDREINNIKVVFHRDSYYTEVKDAEILERRIGLIERITPYIGKYFSNQTVMRDILKYTDDQMDTEKKQIEE
EANDPRFKQTPDEIEDF

158117..158356 PHAGE_Entero_GEC_3S_NC_025425: Putative prohead core protein I; PP_00259; phage(gi754380755) 7.62e-42
MKQFIEAIKTRDLTLIEKEFDKIMEAKKAQIIESQRVEIASSIMIEGEEEKDRDDEEDMEDEDDEDEDRKDD
KKDKEDE

158369..158776 PHAGE_Entero_GEC_3S_NC_025425: Putative prohead core protein II; PP_00260; phage(gi754380756) 5.06e-91
MIKDFTPKADLETFLPEAQEKLDMLAMFESEELLKIVEGIAEDEPELAVAMLSIIDGITVNEAMVKHVNARG
EVARKKDRKTRERNAYMTTGLSKAKRRQIARRAAKTKRANPSIERHAERKRKKAMRKRKALGL

158788..159483 PHAGE_Entero_GEC_3S_NC_025425: Putative prohead core scaffold protein and protease; PP_00261; phage(gi754380757) 4.10e-168
MEKNELLIEQWGVPCARGSSSLLESFDDKATGNLYIQGIFLQAETVNRNKRWYPRSVLENAVTKYIHEQVET

HQALGELNHPARAMPDPSNACIIIEKLWWEGNNVMGKARVIEGDKGAGDKLAALIRAGWIPGVSSRGLGRLK
DSGRGYNIVQEGFVLTVGVDVVWGPSAPDAWVTPVVESTDIDSSASDAPESDKTEKVTNPTAPQQNIVELNN
SAFMALAEALSKRYK

159515..160309 PHAGE_Entero_GEC_3S_NC_025425: Putative prohead core scaffold protein; PP_00262; phage(gi754380758) 0.0
MLKEMLLEEAKQITVDVALDDLFESVELSPEVKENFGTIYAQAVKANAVALAESHIEKIAAKADELVESKVE
EARTEIETKLYEDADKFLNHLGAKWLAENKEAVTRNIKADLCESLIGSLKDVFVTHNVVVPEESVDVVAELD
EALKEEKEKTSELFDAKLQLESEIRGMKREQAINESTRDLSDTQKEKVTALIEGLEYSETFDKKLTAIVEMV
AKKEDKPAKIEESLNTDADKLNVIAEAVEESEKKATVDSGVSRYLNFI

160369..161955 PHAGE_Entero_GEC_3S_NC_025425: Putative major capsid protein; PP_00263; phage(gi754380759) 0.0
MKTTKELMEKWSPLLENEKLPEIATASKQKLVAKILESQEADFAVDPIYKDEKVVEAFGGFIAEAEVAGDHG
YDASQIAAGQTTGAITNVGPAVIGMVRRAIPNLIAFDICGVQPMSTPTSQIFAIRSVYGPNPLAAQAKEAFH
PMYAPDAFHSSLAAKGAIVGSPTGTPFAKLAIGTQIEAGDIVHHTFAETGIAYLQNVTAEQVTPTKAGSESE
DEVVMKLMEEGKLAEIAFGMATSIAEIQEGFNGSSNNPWAEMSMRIDKQVVEAKSRQLKARYSIEVAQDLRA
VHGMDADAELNAILANEVLLEINREIVDVINFTAQVGKTGMTQTVGSKAGVFDLQDPIDTRGARWAGESFKS
LIYQIDKEAAEIARQTGRGAGNFVIASRNVVNILASADQGISLAMQGAAKGLNTDTTKAVFAGVLAGKYKVF
IDQYARQDYFTVGYKGDNEMDAGIYYAPYVALTPLRATDPQSFHPVLGFKTRYGIGINPFADSKSQAPSARI
TSGMLSKDSVGKNAYFRRVWVKGC

hypothetical protein; PP_00264; phage(gi754380761) 1.60e-23
MLDLLNSTNFRSNCMAKGPMNEMSVHVDIVLDRFGRYTVLVYADLVMTFQRDASGNFWFWYAVP

162442..162594 PHAGE_Entero_GEC_3S_NC_025425: hypothetical protein; PP_00265; phage(gi754380762) 2.92e-28
MSKKENSYVMTRKEYNELMLALAYAIIRSDHAINLVNKVNRSVDNVSRKK

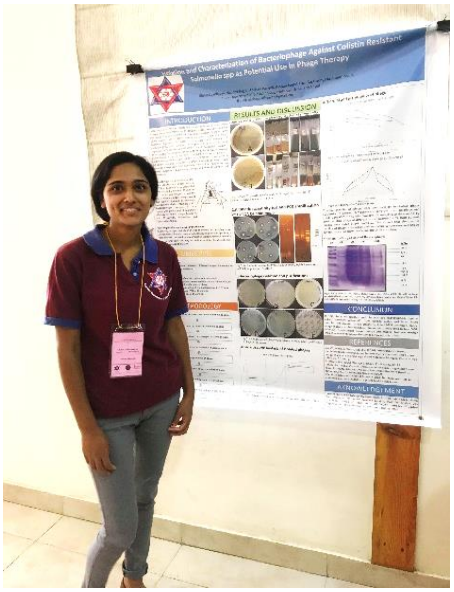
162678..163919 PHAGE_Entero_GEC_3S_NC_025425: Putative head vertex protein; PP_00266; phage(gi754380763) 0.0
MTHIFTELLRESTSSVANQTARPQLLSLTRAVNNLIFSDLVAIQPTDQPVSALYGLRYLNRDGQMTFRTAAT
YGGAVGDRTEIEEFSKEKSYDEGALFKSGDVVYEVVTAGTVGEDAATPEAAIFKGVMTNKIRFYSDCASVEY
FEDKNTEIASTSMQFDKWQVNVGSRKLKTSFTTELMQDLEASQINSENSVIDLLATVASEEINKDIIQKLIT
VSSRYKIKGITPDGVLSLVSVQDAPTQARELYRYACEMSNQMLRTSSFAGTYVLASSRVVGLLQSSGWMEET
DNALSEGRLRCGLEVYADTTTPFDYMLVGCKHMIGDMESVGSLFYSPYTEADGAGAYKSVIDPNSFQHHVAI
MNRYSLSVNPYTSKVDQEEHQVIKGDDWGKMAGRSEMSYILGIELPPLEIDNA

ACTIVITY AND ACHIVEMENTS

Some clicks during lab work

Prof. Dr. Rajani Malla awarded by NAST

Group photo with all 7th batch colleagues



Best Poster presentation award on ISIABA 2018

Batch for the completion of 3 month online course entitled on Research writing in the sciences organized by AUTHORAID; an online institution.

Attending seminar on ICBB 2018 held at KU; above and world DNA 2018 held at NAST; below


Attending SHREEMAT BHAGWAT GYAN MAHAYAGYA

Thesis presentation with external, internal examiners and Supervisor.

ANNUAL DAY and extra activities of the department.

With my beloved FAMILY