MORPHOLOGICAL CHARACTERIZATION AND WHOLE GENOME ANALYSIS OF 'NOVEL' LYTIC PHAGES AGAINST DRUG RESISTANT HUMAN PATHOGENS – AN ALTERNATIVE APPROACH TO ANTIBIOTIC THERAPY

M.Sc. Thesis

2016

Submitted to:

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Institute of Science & Technology, Tribhuvan University

Kirtipur, Kathmandu, Nepal

Roshan Nepal

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Submitted by:
Roshan Nepal

Supervisor:
Prof. Dr. Rajani Malla

T.U. Registration No: 5-2-366-31-2007

TRIBHUVAN UNIVERSITY
CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Ref. No.: .....

Date: Dec 4, 2016

RECOMMENDATION

This is to certify that **Mr. Roshan Nepal** has successfully completed his dissertation work entitled **"Morphological characterization and whole genome analysis of 'novel' phages against drug resistant human pathogens - an alternative approach to antibiotic therapy"** under my supervision.

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

Rajani Malla

Prof. Rajani Malla, PhD
(Supervisor)
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

Central Department of Biotechnology, Kirtipur, Kathmandu, Nepal. Tel.: 977-1-4336221
E-mail: biotechtu.edu.np@gmail.com
Website: www.biotechtu.edu.np

TRIBHUVAN UNIVERSITY

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CERTIFICATE OF EVALUATION

This is to certify that this thesis entitled "**Morphological characterization and whole genome analysis of 'novel' phages against drug resistant human pathogens - an alternative approach to antibiotic therapy**" presented to evaluation committee by **Mr. Roshan Nepal** is found satisfactory for the partial fulfillment of Master of Science in Biotechnology.

.....

Prof. Rajani Malla, PhD
(Supervisor)
Head of Department
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

.....

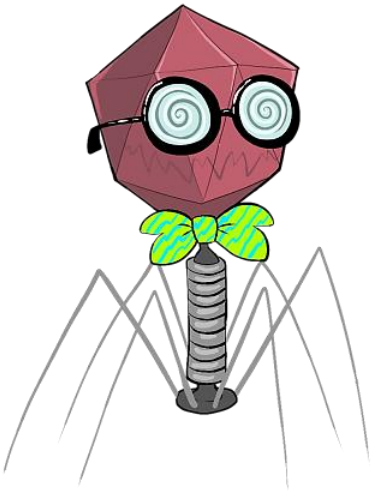
Dr. Sameer Mani Acharya Dixit
(External Examiner)
Center for Monicular Dynamic Nepal
Kathmandu, Nepal

.....

Prof. Krishna Das Manandhar, PhD
(Internal Examiner)
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

Central Department of Biotechnology, Kirtipur, Kathmandu, Nepal. Tel.: 977-1-4336221
E-mail: biotechtu.edu.np@gmail.com
Website: www.biotechtu.edu.np

DEDICATION



With love, to all the phage hunters reading this page right now, who believe in bacteriophage/phage therapy with all their heart and soul.

Peace.

Happy PHAGE HUNTING!

ACKNOWLEDGEMENTS

With heart full of gratitude and soul full of compassion, I'd like to seize this moment to thank my beloved ones whose continuous support and unyielding confidence in me have brought me this far. Foremost, I'd like to thank my supervisor Prof. Dr. Rajani Malla who believed in me with her dream project on 'Bacteriophage' and steered the research in best possible way with her keen supervision and observation. I would also like to remember my co-supervisor Mr. Buddha Basnet for his guidance in initial days. My sincere gratitude to Sneh Panwar, Gajendra Saini and all the lab members at CIF-JNU, New Delhi, India. I would also like to acknowledge Sisir sir, Prof. Dr. Mohan Kharel, Prof. Dr. Tribikram Bhattarai, Prof. Dr. Krishna Das Manandhar, Bal Hari Poudel, Jarina Joshi, Smita Shrestha including all the faculty members of Central Department of Biotechnology, Prof. Dr. Chirik Shova Tamrakar (Dean, Institute of Science and Technology, Tribhuvan University) and all the faculties of IOST-TU for their support and constructive criticisms.

Special thanks to my brother Dipen, parents, elders, and all family members. Thanks also to friends Sujindra, Mukesh, Santosh, Sandipty, Bimala, Gauri, Nirmal×2, Rajindra, Mitesh, Nirman dai, Narayan dai, Hari sir, Elen dee, Binu dee, Mohan dai, Radheshyam sir, Bhupendra sir, Ujjwal dai, Shuva didi, Sumit Rastogi, Amreesh dai, Shabnam Pratap Sircaik, Shivani Ror for their support and encouragements.

I would also like to bestow my sincere appreciations to Kathmandu Center for Education and Research, CAS & TU for the 'Thesis Grant for M.Sc. students – 2015' for financial assistance. This research would not have come this far without financial support from Chinese Academy of Sciences (CAS).

Last but not least, I would also like to thank all my well-wishers who directly and indirectly motivated me to keep going, dream bigger and not lose hope even in the face of adversity.

Thank you all.

ACRONYMS

µg	: microgram [one billionth (1×10^{-9}) of a kilogram]
µl	: microliter [one millionth (1×10^{-6}) of a liter]
ABR / AMR	: Anti-Bacterial Resistance / Anti-Microbial Resistance
AST	: Antibiotic Sensitivity Test
ATCC	: American Type Culture Collection
BALB/c	: Bagg ALBino/genotype 'c'
BIM/s	: Bacteriophage Induced Mutant/s
BLASTN	: Basic Local Alignment Search Tool - Nucleotide
BPT	: Bacteriophage Therapy
CDBT	: Central Department of Biotechnology
CDC	: Centers for Disease Control and Prevention
CDI	: *Clostridium difficile* Infection
CDS	: Coding DNA Sequence
cfu	: Colony Forming Unit
CRE	: Carbapenem Resistant Enterobacteriaceae
CRISPR	: Clustered Regularly Interspaced Short Palindromic Repeats
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
MDR	: Multidrug Resistant
MHR	: Multiple Host Range
mRNA	: messenger RNA
MRSA	: Methicillin Resistant *Staphylococcus aureus*
MSSA	: Methicillin Sensitive *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]
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
RNA	: Ribonucleic Acid
rRNA	: ribosomal Ribonucleic Acid
SM buffer	: Sodium Magnesium buffer
SPL	: Staphylococcal Phage Lysate
ssDNA	: single stranded Deoxyribonucleic Acid
ssRNA	: single stranded Ribonucleic Acid
TEM	: Transmission Electron Microscopy
tRNA	: transfer Ribonucleic Acid
TSB	: Tryptic Soy Broth
TUTH	: Tribhuvan University Teaching Hospital
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

Morphological characterization and whole genome analysis of 'novel' lytic phages against drug resistant human pathogens – an alternative approach to antibiotic therapy

Introduction: Antimicrobial resistance – a condition when microbes are no longer sensitive to the drugs used to kill them – is a global concern and emergence of superbugs have threatened advances of modern medicine pushing us closer to post antibiotic era. Once easily treatable infections are now killing and alternatives to antibiotics are being explored. Among all alternatives, phage therapy – though used longest in clinics – is largely ignored by western world. Bacteriophages are viruses that infect bacteria but leave animal and plant cells unscathed. As MDR becomes a threat, interest in phage therapy is revitalized and now even US-NIAID lists 'phage' as research priority to address antibiotic crisis. Here, we explore availability of phages against MDR bacteria in our environment and assess their efficacy in-vivo followed by morphological identification.

Methodology: Forty different drug resistant human pathogens representing 12 genera were collected from hospital after biochemical identification and antibiotic susceptibility test. Water sample from 5 holy rivers was screened for presence of lytic phages by double layer agar assay. Phages were purified by successively sub-culturing single plaque thrice & standard spot assay was employed to determine multiple host range. Most potent phages were confirmed by TEM. Whole Genome Sequence of three phages were then analyzed for presence of lytic proteins / enzymes than may be potential antibiotic candidate.

Results: Thirty-four lytic phages - 16 *E. coli*, 13 *Salmonella*, 2 *Shigella*, 2 *Klebsiella*, 1 *Citrobacter* - were isolated. Except 2 *Klebsiella* phages all 32 phages showed multiple host range (lysed more than 1 strains). All *Salmonella* phages showed interspecific (*S. typhi* and *S. paratyphi*) lytic ability. Bacteriophage induced mutants were only observed against *Klebsiella* phages. All except *Klebsiella* phage completely lysed bacterial host up to five consecutive cycles. Whole genome sequencing of three phages revealed that they do not possess any 'virulent' bacterial genes within their genome.

Conclusion: Our findings showed 'phages against MDR bacteria' that could be used therapeutically exists in real world and they are not extremely host specific as professed in scientific world. They've evolved to achieve broader host range and even possess interspecific lytic capability. Conclusively – we are not out of weapons to kill drug resistant pathogens yet, and co-evolving phages may be the 'once and for all' alternative to antibiotics.

Keywords: AMR, ABR, Alternative medicine, Bacteriophage, Holy rivers

CHAPTER – ONE
INTRODUCTION

1.1 Background

Let's face the truth: Antimicrobial resistance (AMR) is urgent and real, not figments of imagination. The golden age of antibiotics is over and we are definitely approaching a post-antibiotic era, much quicker than predicted if nothing is done to control, contain and cure current 'global threat' of antibiotic resistance. Bacterial resistance to antibiotics already poses a serious health threat and the rise of antimicrobial resistance undeniably is the biggest global health crisis of this century (Henein, 2013 & Chan, 2015). Humans have always been in an endless war against bacteria, indeed it can kill us and for decades, antibiotics have been our only real weapon against them. Discovery of penicillin and the discoveries of other antibiotics henceforth revolutionized medicine in the 20th century, and together with vaccination have led to the near eradication of many diseases in the developed world and in developing world. But, despite their amazing success, drug resistant superbugs like methicillin resistant *Staphylococcus aureus* (MRSA), Carbapenem-resistant Enterobacteriaceae (CRE) and vancomycin-resistant Enterococcus (VRE) are slowly winning. Every year more and more people die from drug resistant infections as we inch closer back to the pre-penicillin days. Their effectiveness and easy access led to overuse, especially in live-stock raising, prompting bacteria to develop resistance sooner than anyone expected.

Since research into new antibiotics is not progressing at the same rate as the development of bacterial resistance, widespread calls for alternatives to antibiotics have been made (Chan, 2015). Antimicrobial resistance (AMR) – also specifically called Antibiotic resistance (ABR) – is the ability of a microorganism (like bacteria, viruses, and some parasites) to stop an antimicrobial (such as antibiotics, antivirals and anti-malarials) from working against it rendering them ineffective for treatment (who.int/antimicrobial-resistance). As a result, standard treatments become unsuccessful, infections persist and may spread to others. AMR is no longer a prediction but a very real possibility for 21st century. It's happening right now, in every region – developed, developing and poor – of the world and has the potential to affect anyone, any age, and in any country. Antimicrobial resistant bacteria cause more than 2 million infections and 23,000 deaths each year in the United States only (cdc.gov/drugresistance). WHO's first global and most comprehensive report on antibiotic resistance – Antimicrobial Resistance: global report on surveillance 2014 – which included data from 114 countries reveals serious worldwide emergency from antimicrobial resistant infections (WHO, 2014). 'Without urgent, coordinated action by

many stakeholders, the world is headed towards post-antibiotic era, in which common infections and minor injuries which have been treatable for decades can once again kill' warns Dr. Keiji Fukuda, WHO's Assistant Director General for Health Security (WHO, 2014). According to the same report, carbapenem resistant *Klebsiella pneumonia*, floroquinolones (most widely used) resistant *Escherichia coli*, cephalosporins (last resort, 3rd generation) resistant *Neisseria gonorrhea*, methicillin resistant *Staphylococcus aureus* (MRSA) has been reported from every part of the world. This clearly implies that achievement of modern science in healthcare is already threatened and magnitude of AMR is disturbingly widespread and expanding in alarming rate.

No one denies that AMR is burgeoning problem in South East Asia Region too. Report shows high level of *E. coli* resistant to 3rd generation cephalosporins and flouroquinolones. Resistance to 3rd generation cephalosporins in *K. pneumonia* is also alarmingly widespread and in some parts of region more than one quarter of *S. aureus* infections are reported to be MRSA (WHO, 2016). Looking into South-East Asia, AMR is far greater problem because of non/un regulated antibiotics use (WHO, 2014). There is a dire need of an effective alternative therapy to antibiotic therapy as news of in-effectiveness of last line lifesaving drugs have been frequently reported. Multi drug-resistant (MDR), extensively drug-resistant (XDR), pan drug-resistant (PDR) and even 'superbugs' are now spreading rapidly and taking lives of people in the region. Major factors that speed up the process of antibiotic resistance are: use of antibiotics in agriculture, evolution of bacterial itself and over/non-regulated use of antibiotics. To be honest, very less is being done by concerned stakeholders to minimize the effects of these factors and no 'concrete plans/alternatives' are within sight that could solve the issue immediately.

Concerns with cost and benefit have always become the crucial aspect of decision making. As drug resistance is a dynamic process, and investors need to invest again-and-again when resistance develops, the benefit of drug development is outweighed by the cost involved in bringing a drug in market. As such, pharmaceuticals are more concerned with research on 'non communicable diseases' like obesity, genetic disorders, diabetes, heart diseases et cetera which is related to 'lifestyle' rather than communicable infections. But, economic arguments convince that antimicrobial-drug resistance is also a serious issue (McGowan, 2001). In the US, Institute of Medicine estimates the cost of infections caused by antibiotic resistant bacteria to be $4-$5 million (Gandra *et al.*, 2014). Cost of treating resistant infections place a substantial burden on society and also impacts the in-hospital cost of managing possible resistant infections outbreaks which in turn will impact the overall economic and public health status of a country – eventually world.

Lack of attention means that funding to solve the problem is unlikely to be found. A change in perception and action is needed to give the 'drug resistance' a status of important economic impact and the priority it deserves. This led the World Health Organization last year to classify antimicrobial resistance as a serious threat [that] is no longer a prediction for the future and also urged world leaders to take significant steps in 'antibiotic resistant' crisis management. Further, the United Nations held a meeting to discuss about the 'impact' of AMR giving AMR the attention it deserved.

Although significant recommendations are made to control the AMR crisis – such as: **a)** controlling the choice of antimicrobial agents by individual prescribers, **b)** limit use of inappropriate agents by removing specific drugs from the list of available agents in over-the-counter pharmacies, **c)** restricting some misused agents to certain specialist only, **d)** banning use of antibiotics in animal farming, **e)** awareness campaign about effective use of antibiotics to name few – we believe it is not enough given the magnitude of the problem and reckon an alternative approach must be considered to tackle the ongoing AMR crisis. In addition, the alternatives should be as effective, efficient, cheap and [if possible] available locally to have a greater impact.

The hunt for ways to kill 'nasty' drug resistant pathogens has already begun and non-antimicrobial approaches are gaining traction even in the West and Europe. Various alternatives – herbal remedies, cannibalistic/predatory bacteria (*Bdellovibrio bacteriovorus*), phages, antibiotic peptides, gene editing enzymes (clustered regularly interspaced short palindromic repeats - CRISPR), metals (silver, copper, gold) – are available that may be our savior in our fight against drug resistant pathogens (Reardon, 2015). Among various approaches/alternatives to antibiotics, phages have been used longest in clinics and thus phage therapy which uses 'good viruses that cure' may represent one of the key alternatives (Ryan *et al.*, 2011; Sulakvelidze, 2011; Kuchment, 2012). Bacterial infections can be treated with specific viruses that have the ability to infect and lyse/kill bacteria, culminating in their destruction. After listing 'phages' as one of the research priority against antibiotic crisis by United States - National Institute of Allergy and Infectious Diseases (US-NIAID) in 2015, scientists worldwide are once again turning to the 'natural predator' of bacteria – bacteriophage. The research is of more importance because no any effective alternative/antibiotics have been discovered/developed recently. If the situation remains same for few more decades, we will soon lose all the advancements in medicine and a simple 'thorn prick' aided with infection by drug resistant bug would suffice to make any healthy being sick.

Keeping in mind the urgent need of an alternative to antibiotics so as to mitigate the current antimicrobial crisis, this study aims to perform a preliminary study on assessing

availability of lytic phages in natural environments like river waters that are capable of infecting and killing various drug resistant clinical pathogens (bacterial) of human origin.

1.1.1 Bacteriophage

Bacteriophage/s – also referred as phage/s and occasionally as bacterial virus – are any group of viruses that infect bacteria or more generally 'viruses of prokaryotes'. In other word, they are obligate intracellular parasites of bacteria. It was discovered independently by Frederick Twort – a bacteriologist from England in 1915, and Félix d'Hérelle – a French-Canadian microbiologist – two years later in 1917 (Duckworth, 1976). d'Hérelle coined the term 'bacteriophage' meaning 'bacteria eater', from the word 'bacteria' and the Greek word 'phagein' which means 'to eat or to devour'. The size of most phages range from 22 nm – 200 nm in length. The largest bacteriophage known as T4 is about 200 nm long and about 80 – 100 nm wide. Phages are made up of proteins that coat an inner core of nucleic acid, either DNA (deoxyribonucleic acid) or RNA (ribonucleic acid) but never both and [but not necessarily] a tail (Figure 1.1). The tail region is associated with other characteristic structures called the tail fibers, tail pins, and base plate. They are specific to one or a limited number of bacterial host strains; thus, they are generally named after the bacteria group, strain, or species they infect. For example, the phage that infect the bacterium *Escherichia coli* are called coliphage in general.

Phages are everywhere where their bacterial host is present; 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 and about 10^9 virions per gram in the soil, with an estimated total number of 10^{32} bacteriophages on the planet (Wittebole *et al.*, 2013).

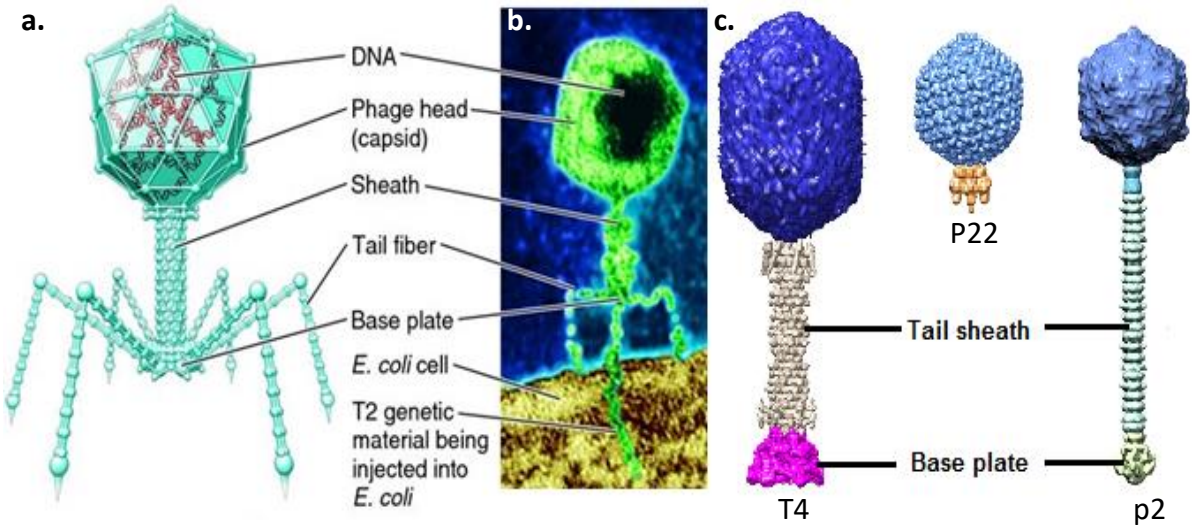


FIGURE 1.1 | Typical Bacteriophage. a) Schematic drawing of T2 bacteriophage. **b)** An electron micrograph of T2 bacteriophage infecting an *Escherichia coli*. **c)** From left to right. Three families – Myoviridae (T4), Podoviridae (P22) and Siphoviridae (p2) – of Caudovirales order.

1.1.1.1 Classification of phages

Phages are classified by the International Committee on Taxonomy of Viruses (ICTV) according to morphology and nucleic acid (Figure 1.2 & Figure AF2). Phages are altogether classified into 10 different families according to ICTV guidelines (19 families that infect bacteria and archaea). Among them, nine families infect bacteria only, and one (*Tectiviridae*) infects both bacteria and archaea (King, 2012 & Figure AF2). Of these, only three (Myoviridae, Podoviridae and Siphoviridae) belong to order Caudovirales – meaning tailed virus – and all other are tailless and are not assigned any order till date (ictvonline.org). Although new approaches for classification of tailed phages based on their neck organization are under development (Lopes *et al.*, 2014), ICTV approach – though not comprehensive and easy – is most widely used till date.

BOX 1 - Etymology of Viral

Caudo	: from Latin *cauda*, 'tail'.
Myo	: from Greek *myos*, 'muscle', referring to contractile tail.
Sipho	: from Greek *siphon*, 'tube', referring to long tail.
Podo	: from Greek *pous*, *podos*, 'feet', referring to short tail.

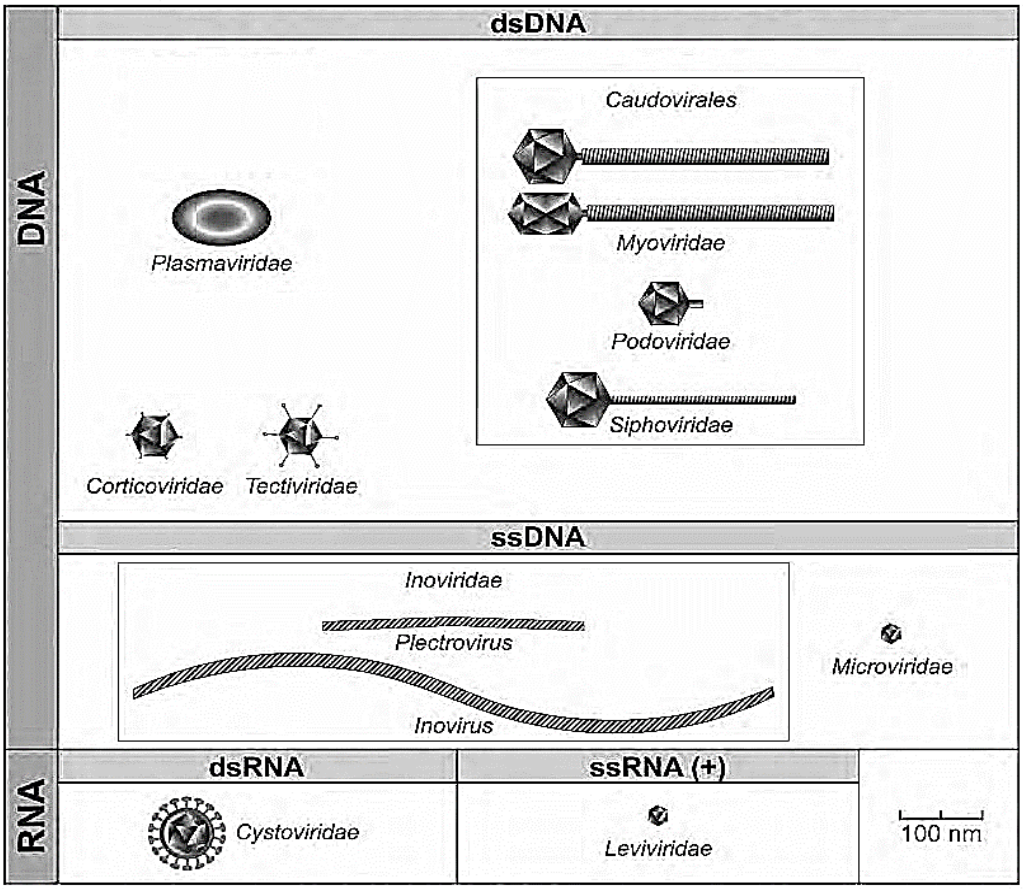
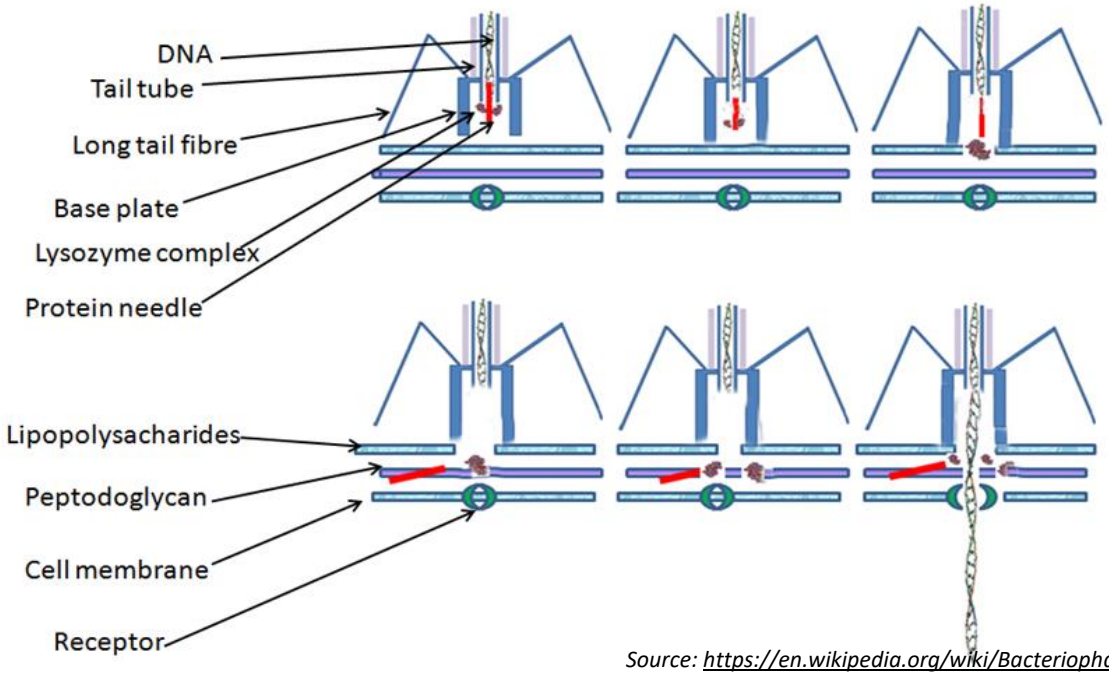


FIGURE 1.2 | Virus taxa infecting only bacteria according to 'Ninth Report of International Committee on Taxonomy of Viruses' released in 2012. Bacterial virus can be divided into altogether 10 families according to ICTV. Among them the most abundant/studied are tailed virus from order Caudovirales. Figure source: (King, 2012. pp: 16)

1.1.1.2 How bacteriophages work

In order to infect a specific host cell, bacteriophage recognizes and adheres itself to the bacterial surface, specifically on a receptor found on the bacteria's surface. This process is called adsorption and cannot occur randomly**.** To successfully adhere, molecules on the phage tail and/or tail fibers must match specific molecules on the bacterial surface that serve as receptors. A bacterium lacking these molecules is resistant to infection and this causes specificity in phages. Once it becomes permanently bound to the cell, the bacterial virus injects its genetic material (its nucleic acid) into the bacterium in a step called penetration. This involves the contraction of the helical sheath, which forces the hollow tube into the cell cytoplasm, much like a microscopic syringe. In the process, the viral DNA is released into the cell's interior. The viral capsid does not enter the cell. It remains as an empty shell, attached to the cell exterior. Depending on the type of phage, one of two cycles will follow thereafter – **the lytic** or **the lysogenic cycle**.

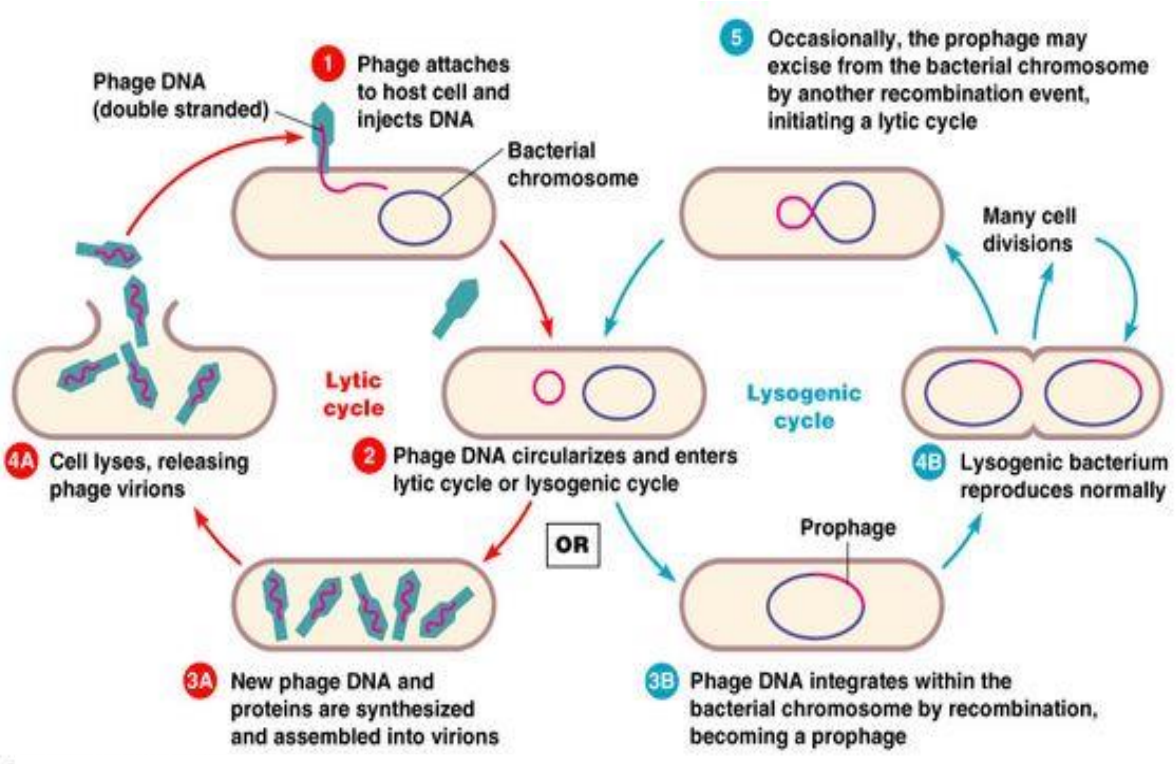
During a lytic cycle, the phage will make use of the host cell's chemical energy as well as its biosynthetic machinery to produce phage nucleic acids (phage DNA and phage mRNA) and phage proteins. During this, the metabolic activity of the cell is blocked and the cell begins producing proteins coded for by the viral DNA. These include proteins that block normal host-cell activity, enzymes required for viral replication, and structural proteins needed to construct new capsids. The viral DNA is also repeatedly copied. All energy



Source: https://en.wikipedia.org/wiki/Bacteriophage

FIGURE 1.3 | Overview of phage-host reorganization, interaction and phage genome injection into host. First, receptors in long tail fibers recognize the receptors in host's surface. This temporary recognition is further strengthened by permanent recognition and attachment with the base-plate. Lysozyme complex (primarily holin and lysin) lyze the host's membrane thereby creating unrestricted 'tube like' path for phage genome (DNA or RNA) injection.

required for these processes is provided by the host cell. Once the production phase is finished, the phage nucleic acids and structural proteins are then spontaneously assembled. After a while, certain proteins – collectively called lysins – produced within the cell will cause the cell wall to lyse, allowing the assembled phages within to be released and to infect other susceptible bacterial cells. Example: *E. coli* bacteriophage T4.



Source: Pearson Education Inc., 2004 (publishing as Benjamin Cummings)

FIGURE 1.4 | Lytic and Lysogenic life cycle of bacteriophage. Phages depending upon environmental condition follows either lytic cycle or lysogenic cycle. During lytic cycle, phages infect and lyse the host cell, whereas during lysogenic cycle, phages infect and integrates their genome in host's genome until favorable environment. It is lytic phages that are being explored for therapeutic usage because of their ability to kill host cell.

However, not all phages undergo a lytic cycle. Viral reproduction can also occur through the lysogenic cycle where host cells are not necessarily lysed/killed. Once the host cell is infected and genetic material (DNA/RNA) is released into a susceptible bacterium, lysogenic phage does not immediately reproduce. Rather it enters an inactive period called the prophage state (Figure 1.4-3B). In the prophage state, the viral genome is actually incorporated into the bacterial genome. If the bacterium divides, the viral genome will also replicate along with it, but otherwise, the infected cell remains largely unaffected. This state of viral inactivity inside an infected cell is called lysogeny (Figure 1.4). Thus, the daughter cells also contain the prophage which carries the potential of producing phages. The lysogenic cycle can continue indefinitely (daughter cells with prophage present within continuing to replicate) unless exposed to adverse conditions

which can trigger the termination of the lysogenic state and cause the expression of the phage genome and the start of the lytic cycle. These adverse conditions include exposure to UV or mutagenic chemicals and desiccation. Example: *E. coli* with phage lambda.

1.1.1.3 Applications of bacteriophages

Phage Therapy (PT) – if the target host of a phage therapy treatment is not an animal, the term 'biocontrol' as in phage-mediated biocontrol of bacteria is usually employed, rather than 'phage therapy' – is practicable both in veterinary science and in agricultural and food industry. No doubt there is immense application of bacteriophage in health care, food preservation, agriculture and environmental sanitation to name few. In some countries such as Russia, Georgia and other Eastern European nations, phages are used therapeutically for treatment of pathogenic bacterial infections that are resistant to antibiotics. Popularly known as phage therapy, this method involves use of a phage to destroy the infective bacteria such as *E. coli* or *Salmonella*. Phages are also used in identifying pathogenic bacteria (phage typing) in diagnostic laboratories. One other use of phage is for killing specific bacteria found in food. For example, as sterilizing agent ListShield™, LISTEX™ by Micreos is made up of phages that can kill the *L. monocytogenes* bacteria in cheese and have been regarded as Generally Recognized as Safe (GRAS) by United States - Food and Drug Administration (US-FDA). Another approved product treats plant pathogens such as *Xanthomonas campestris, Pseudomonas syringae* pv tomato. Further products are in development against pathogen including *E. coli* strain (0157:H7) and *Salmonella enterica.* Microphage Inc. recently received US-FDA approval for blood culture test that used phage infection to detect MRSA (phages.org).

Fighting and destroying bacterial infections (both in humans and animals) are the primary applications of phage therapy, but it can also be employed for other uses like environmental sanitation, agriculture, food-preservatives *et cetera.* It can be the key to fighting the NDM-1 (New Delhi Metallo-beta-lactamase-1 – a gene that can be included in the DNA of bacteria) enabling them to resist antibiotics.

Waste water from sewage systems are not really considered waste because it is a rich source of phage strains for various kinds of bacteria that lead to the most up-to-date medicines. Skin grafting for extensive wounds, trauma, burns, and skin cancer can also be improved by using phage therapy to lessen the *Pseudomonas aeruginosa* infection. Some experiments for cells in tissue culture have also discovered antitumor agents in phages (Wu *et al.*, 2002). Bacteria cause food to spoil faster, and phages have been studied for their potential to increase the freshness of food and decrease the incidents of food spoilage (Sillankorva *et al.*, 2012).

1.1.2 Phage therapy (PT)

Phage therapy or viral phage therapy is the therapeutic use of bacteriophages to treat pathogenic bacterial infections. Phage therapy has many potential applications in human medicine as well as dentistry, veterinary science, and agriculture (wikipedia.org/wiki/Phage_therapy). Immediately after discovery of phages, the thought of using phages to fight bacterial infections was already apparent. d'Hérelle began testing the therapeutic effects that phages may have on chickens and cows first, and the tests were successful. Eventually, human tests were conducted and the development of phage therapy became more extensive especially with the foundation of the Eliava Institute in 1923 (Wittebole *et al.*, 2013). The pharmaceutical company Eli Lilly began commercialization of phage therapy in the US during 1940s. During World War II, phages were used to treat bacterial diseases among soldiers of the Soviet Union, particularly gangrene and dysentery (phages.org). Spotty results, however, combined with the development of powerful antibiotics (in 1950s) dampened interest in 'phage therapy' as the use of antibiotics became more favorable. However, in recent years, with the development of antibiotic resistance by many bacteria, some researchers are taking a fresh look at this approach and renewed their interest in the development and employment of phage therapy (Sulakvelidze, 2015). Countries like France, Germany, the UK and the US itself (Eli Lilly, Swan-Mayers, Squibb, AmpliPhi Biosciences) have re-focused their priority on phage therapy. Earlier in 2014, phage therapy was highlighted as one of seven approaches to 'achieving a coordinated and nimble approach to addressing antibacterial resistance threats' in a 2014 status report from the National Institute of Allergy and Infectious Diseases (NIAID) (Madhusoodanan, 2014; Reardon, 2015).

Although US-FDA has approved a handful of phages for treating food products, it is still a long way to get approval for therapeutic usage. The efforts to harness the benefits in medicine is still hindered by instability and extreme specificity nature of phages. However, a new technique that allows changing/swapping of 'few genes' on tail fibers – as genes in tail region actually dictates what it targets – with appropriate ones can generate phage that can target and inject its lytic genes in broad range of bacteria. A team of scientists from Massachusetts Institute of Technology (MIT) have identified a code for key tail fiber gp17 that plays crucial role in phage infection (Ando *et al.*, 2015). 'As new resistant bacteria appear, the same engineering strategy can be applied over and over' reckons Prof. Lu – one of the researcher from same study.

FIGURE 1.5 | A history of fighting infection. In 1896 Hanin reported some agents capable of lytic action against certain pathogens in Ganges and Jamuna/Yamuna river of Indian (now). Bacteriophages were co-discovered independently by Twort and de'Herelle in 1915 and 1917 respectively. Althought phages were used to treat infections in early days of their discovery, advent of penicillin in 1928 pushed back phage research. After 25 years of discovery, electron micrograph of phage was published by brothers Ernst and Helmut. Figure source: (Madhusoodanan, 2016)

1.1.3 Advantages of phage-therapy over antibiotic-therapy

Phages are target specific, meaning only one or very few bacterial strains are targeted by a phage for destruction. So each phage attacks only one type of bacterium, so treatment leave harmless (beneficial) bacteria unscathed. The host specificity is dependent on evolution of recognition system of virus based on 'lock and key' theory. Also phages are abundant in nature, so researchers have ready replacements for any therapeutic strain that bacteria evolve to resist. As bacteria and phages co-evolve with time, it is easier to develop new phages than new antibiotics. A time period of only a few days or weeks is needed to acquire new phages for resistant strains of bacteria, whereas it can take years to obtain new antibiotics. When resisting bacteria evolve, the assigned phages also evolve, so when super bacterium appears, an equivalent super phage fights it as long as the phage is derived from the same environment.

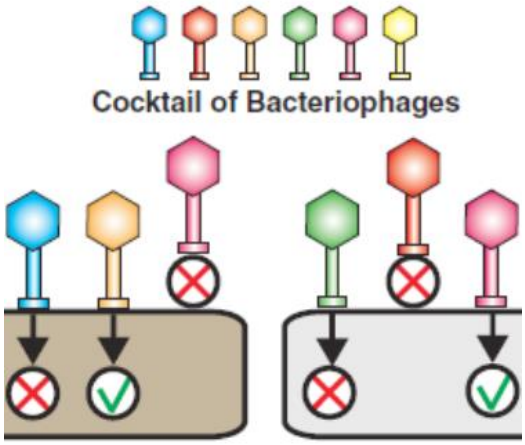


FIGURE 1.6 | Phage cocktail. This illustrates the benefit of using a cocktail of bacteriophages by broadening the host-range to confer greater infectivity against multiple bacterial strains. Figure source: (Liu, 2014)

Compared to antibiotics, phages go deeper into the infected area. Antibiotics, on the other hand, have concentration properties that quickly decrease as they go below the surface of the infection. The replication of phages is concentrated on the infected area where they are needed the most, while antibiotics are metabolized and removed from the body. In addition, secondary resistance does not happen among phages, but happens quite often among antibiotics. Secondary resistance is acquired and occurs when there aren't enough blood drug levels.

Certain infections in people and experimentally infected animals have been proven to be more effectively treated with phage therapy than using antibiotics. Since 1966, the average success rate of studies that used phages in various ways (systematically, topically, intravenously, or orally) is from 80 to 95%, with minimal or no allergic and/or gastrointestinal side effects. The infections studied are from *E. coli, Acenatobacter, Psuedomonas*, and *Staphylococcus aureus* (phages.org). Comparison in cost of phage therapy and antibiotic therapy revealed that treatment with phages was approximately 50% cheaper than treatment with antibiotics like *vancomycin, linezolide, teicoplanin and chinupristin+dalfopristin* (Miedzybrodzki *et al.*, 2007). This clearly means wider application of phage therapy could lead to substantial saving in healthcare costs and make antibacterial therapy accessible to those who otherwise cannot afford treatment.

Further, because of its simplicity, phages can be easily manipulated and modified for treatment if bacteria acquire resistance against them whereas discovery of new effective antibiotics requires several years. Bacteriophages being very specific in phage-host range, can only infect specific bacteria, so this minimizes the chance of secondary infections or sometimes super-infections that can be caused by use of antibiotics. This is certainly an advantage in the age of targeted medicine. Antibiotics may be considered as nukes that kills any bacteria – good and bad, whereas phages can be regarded as snipers that precisely targets its host thereby killing them. Also, bacteriophages replicate at the site of infection where host is present causing its lysis, but antibiotics travel throughout the body irrespective of the site of infection. Further antibiotic resistance is fueled by misuse and overuse of antibiotics which is so common in developing countries that people do not even require prescriptions to buy antibiotics from pharmacist. So treating with antibiotic is indirectly creating more resistant bacterial strains and more diseases. Lastly, bacteriophages are environment friendly and are based on natural selection.

Moreover, although bacteria can become resistant to phages, phage-resistance is not nearly as hazardous as drug resistance. Just like bacteria mutate, phages too can mutate and evolve to counter phage-resistant bacteria naturally (Matsuzaki *et al.*, 2005). Furthermore, development of phage-resistance can be forestalled altogether if phages are used in cocktails and/or in conjugation with antibiotics which is reported to have synergistic effect (Kutateladze & Adamia, 2010).

TABLE 1.1 | Advantage of bacteriophage therapy: Comparison of the prophylactic and/or therapeutic use of phages and antibiotics

SN	Bacteriophages	Antibiotics	Remarks
1.	Very specific (i.e., usually affect only the targeted bacterial species); therefore, dysbiosis and chances of developing secondary infections are avoided.	Antibiotics target both pathogenic microorganisms and normal microflora. This affects the microbial balance in the patient, which may lead to serious secondary infections.	High specificity may be considered to be a disadvantage of phages because the disease-causing bacterium must be identified before phage therapy can be successfully initiated. Antibiotics have a higher probability of being effective than phages when the identity of the etiologic agent has not been determined.
2.	Replicate at the site of infection and are thus available where they are most needed.	They are metabolized and eliminated from the body and do not necessarily concentrate at the site of infection	The 'exponential growth' of phages at the site of infection may require less frequent phage administration in order to achieve the optimal therapeutic effect.
3.	No serious side effects have been described.	Multiple side effects, including intestinal disorders, allergies, and secondary infections (e.g., yeast infections) have been reported.	A few minor side effects reported for therapeutic phages may have been due to the liberation of endotoxins from bacteria lysed in vivo by the phages. Such effects also may be observed when antibiotics are used.
4.	Phage-resistant bacteria remain susceptible to other phages having a similar target range.	Resistance to antibiotics is often class-wide. Multiple antibiotics with similar mechanism of action will become ineffective once resistance develops.	Because of their more broad-spectrum activity, antibiotics select for many resistant bacterial species, not just for resistant mutants of the targeted bacteria.
5.	Selecting new phages (e.g., against phage-resistant bacteria) is a relatively rapid process that can frequently be accomplished in days or weeks.	Developing a new antibiotic (e.g., against antibiotic-resistant bacteria) is a time-consuming process and may take several years.	Evolutionary arguments support the idea that active phages can be selected against every antibiotic-resistant or phage-resistant bacterium by the ever-ongoing process of natural selection.

Source: Phage International, phages.org, and BTER Foundation

1.2 Statement of problem

The problem with today's treatment strategy is: we are solving a dynamic problem (evolving bacteria) with a static solution (antimicrobial). This is clearly a temporary solution and predestined to fail someday. To everyone's surprise, the day has already arrived – too early than predicted – with emergence of extremely powerful 'superbugs'. Today we have resistant bacteria to almost every antibiotic in use and/or we have some bacteria that are resistant to all the antibiotics in use – infamously called 'superbugs'. Emergence of 'superbugs' not only threatens the achievement of modern medicine but are already taking lives. Diseases that were easily treatable few years back are now untreatable, even in developed countries like the US and the UK – for example MRSA and *E. coli* outbreak in Germany few years back. Reports of 'superbugs' from India, its spread to the USA and China has already alarmed the global health community. MRSA only kills more Americans than Emphysema, HIV/AIDS, Parkinson's disease and homicide combined (Golkar *et al.*, 2014). Reports of plasmid mediated colistin resistant gene (mcr-1) in *E. coli* from China, Europe, Canada and now the US confirms the spread of pan-drug-resistance (cdc.gov[1]). This implies that bacteria are evolving more rapidly than human beings and for this they have two advantages. First, co-evolution occurs in nature and almost all our antibiotics are natural in origin. So its cent percent sure that those bacteria are going to find out a way to tolerate these antibiotics somehow someday and be more powerful! Second, they have great advantage over numbers. That is, they replicate faster (have small generation time) and thus there is higher chance of co-evolving a resistant variety in a population.

We need to treat these rapidly evolving antibiotic resistant bacterial infections or else human may suffer from an outbreak of 'superbug' that, if not controlled, contained and cured, are highly likely to be a pandemic and cause a human catastrophe.

1.3 Objectives

1.3.1 General objectives

1. Isolation and characterization of lytic phages against drug resistant bacterial human pathogens from river waters of Kathmandu valley and assessment of their potential therapeutic application.
2. Next Generation Sequencing of potential phages and comparative analysis of their genome.

1.3.2 Specific objectives

1. Isolation of lytic phages against drug resistant human pathogens from sewage/water samples of Kathmandu valley.
2. Study plaque morphology, lytic capability and lytic efficiency of isolated phages.
3. Evaluate multiple host-range of isolated phages against bacterial species of different origin.
4. Perform transmission electron microscopy (TEM) and characterize selected phages morphologically.
5. Whole genome sequencing of selected phages [probably] with high therapeutic application.
6. Bioinformatics analysis and prediction of putative genes that beholds greater therapeutic and diagnostic value.

1.4 Hypothesis

This research broadly aims to assess the availability and efficacy of lytic phages in nature that can successfully lyse drug resistant bacterial pathogens of human origin.

H_0: There is no significant amount of lytic phages in our river waters/sewages that can effectively kill drug resistant bacterial pathogens of human origin that has significant therapeutic value.

H_1: Lytic phages that can effectively kill drug resistant bacterial pathogens of human origin are abundantly present in river waters of Kathmandu valley as river waters of valley is heavily contaminated with drug resistant human pathogens, and thus is favorable habitat for bacterial viruses of its kind.

1.5 Research questions

Steering our research in the direction of set hypothesis, with this dissertation and extensive review on phage research, we seek answers to the following questions.

1. Are there therapeutically potential lytic phages in our nature (river waters) that can effectively lyse drug resistant bacterial pathogens of human origin?
2. Do phages have broad host range like antibiotic or are extremely host specific as professed in today's scientific world?
3. How frequent is induction/introduction of bacteriophage induced mutants (BIMs)?
4. Are phages only 'active' lytically during log phase of host's growth cycle or are equally effective during stationary phase as well?
5. Are there any 'virulent' genes of bacterial origins in phages?
6. Identify phage lysin (endolysin) genes.
7. Can bacteriophage therapy be as effective as it is claimed to be?
8. Do we have enough data/publications to claim about the efficacy and efficiency of phages therapeutically?
9. What makes phages excellent candidate for 'alternative to antibiotics'? What are the setbacks we have already faced in the past and what challenge lies ahead?
10. Characterizing the isolated phages, this study also aims to assess the possibility of phage typing for rapid diagnosis of diseases on both humans and other animals.

1.6 Rationale

The impetus for this research on phages associated with drug resistant bacteria can be attributed to the increasing cases of drug resistant infections worldwide and possible therapeutic use of phages as an effective biological control agents and/or curing agents.

No doubt, antibiotic resistance has been catastrophic and requires alternative immediately. A novel approach to treat drug resistant infections and strategies to control spread of resistant genes undoubtedly is the need of an hour. In addition to being highly targeted and efficient towards such nasty bugs, preferably cheap, locally available alternative would be boon to medical sciences.

Among various approaches, phages have been used longest in clinics, are specific, and possibly cheaper alternatives. Thus, phage therapy which uses 'good viruses that cure' may represent one of the key alternatives. Viruses (particularly phages) influence virtually all of the biogeochemical processes occurring on our planet, but remain enigmatic because it has proved difficult to detect, isolate and classify them in large-scale studies. However, recently, advancement of molecular techniques like genome sequencing and high resolution microscopy, phage diversity can be easily studied now. Failure of early clinical studies – when current sophisticated research tools were not available – should never impede future investigations that may hold immense possibilities.

This study therefore aims to explore, analyze and expose the potential application of lytic bacteriophage – a forgotten cure – against antibiotic resistant bacterial infection. In Nepal, to my best knowledge, study on lytic phages against drug resistant pathogenic bacterial strains of human origin has not been performed yet. So, primary purpose of this study is to screen, isolate and characterize (morphologically) number of lytic phages that infect various drug resistant bacteria and investigate their lytic properties under controlled laboratory conditions. The study thus provides the basis for an evaluation of potential phages to control various diseases caused by drug resistant pathogenic bacteria and consequently assesses their potential therapeutic application

The need to understand the microbial ecology of phages is an inevitable prerequisite to successful isolation of phages specific against target bacteria (drug resistant). The relative simplicity by which phages can be isolated from the environment can be attributed to the fact that the natural environment of the bacteria of interest is likely to contain the specific phages capable of infecting and lysing the microorganisms (Gill & Hyman, 2010). As we know our rivers are polluted with animal/human fecal materials, untreated sewages from hospitals, we deliberately selected river waters of Kathmandu valley as phage source.

CHAPTER – TWO

LITERATURE REVIEW

2.1 Historical review

Bacteria and their viruses have a shared evolutionary history stretching for billions of years. Although phages were discovered only in 1915/17, Earnest Hanbury Hankin had reported antibacterial activity against *Vibrio cholera* in waters of Ganges and Yamuna in India in 1896 (Abedon *et al.*, 2011; Hankin, 2011; Chanishvili, 2012). Felix d'Herelle had also proposed the use of bacteriophages for the therapy of human and animal bacterial infections at the beginning of the 20th century. Not long after his discovery, d'Herelle used phages to treat dysentery, which was probably the first attempt to use bacteriophages therapeutically. The studies were conducted at the Hospital des Enfants-Malades in Paris in 1919 under the clinical supervision of Professor Victor-Henri Hutinel, the hospital's chief of pediatric (Summers, 2001, 2012). The phage preparation was ingested by d'Herelle, Hutinel himself, and several hospital interns in order to confirm its safety before administering it the next day to a 12-year-old boy with severe dysentery. The patient's symptoms ceased after a single administration of d'Herelle's anti-dysentery phages, and the boy fully recovered within few days. The efficacy of the phage preparation was 'confirmed' shortly afterward when three additional patients having bacterial dysentery and treated with one dose of the preparation started to recover within 24 hour of treatment. However, results of these studies were not published immediately and, therefore, the first reported phase-based clinical trial to treat infectious diseases of humans came later from Richard Bruynoghe and Joseph Maisin (1921), who used bacteriophages to treat staphylococcal skin disease (Lavigne & Robben, 2012; Wittebole *et al.*, 2013). The bacteriophages were injected into and around surgically opened lesions, and the authors reported regression of the infections within 24 to 48 hour (Wittebole *et al.*, 2013). Over the next ten years, thousands of people were treated with a variety of phage preparations for other infections, including cholera and/or bubonic plague in India and elsewhere.

There were also several hundred studies on phage therapy that followed, mostly in Eastern Europe and the former Soviet Union. Unfortunately, many of the older studies are of poor quality (compared to our current research standards) and/or they are not available in English (bterfoundation.org/phage). Human phage therapy was also practiced in France since 1919 and since then Pasteur Institute in France had been producing phage

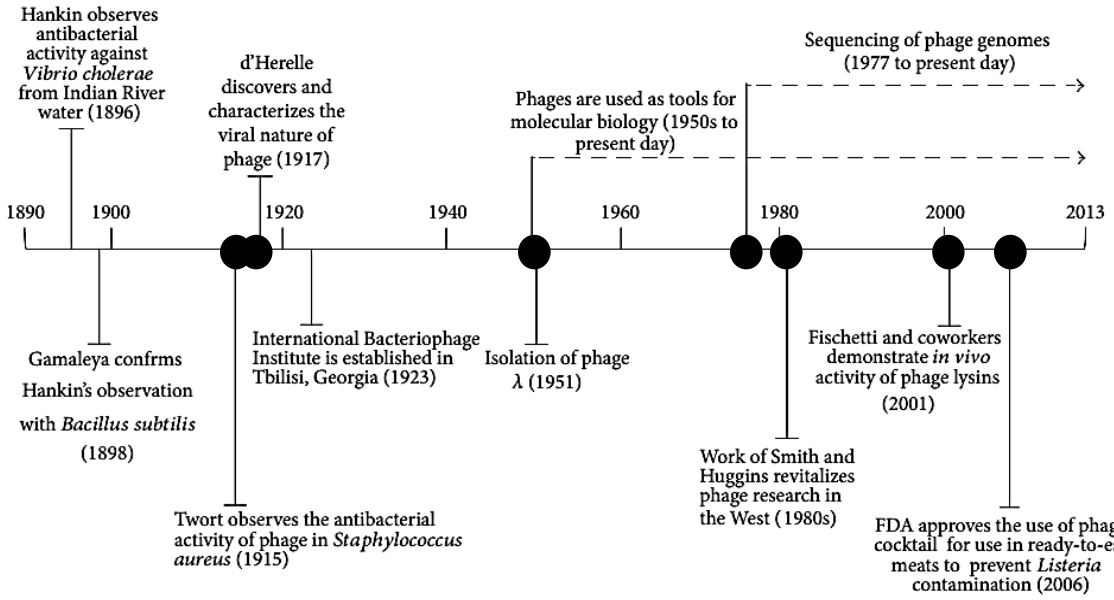


FIGURE 2.1 | Timeline of major milestones in phage discovery and phage therapy. Phages were discovered independently by Twort (1915) and d'Herelle (1917). Since the discovery, phages were used therapeutically but much of its use halted after advent of antibiotics. However, since 1950s, phages have been used in molecular biology to present day. Works of Smith and Huggins revitalized phage research in West (1980) and Fischetti demonstrated effectiveness of 'phage lysin' in 2001. In 2006, FDA approved use of phage based cocktail to prevent contamination of Listeria ion processed meat. Since then, phage research has been encouraging in recent days. Figure Source**:** (Elbreki *et al.*, 2014, pp: 6)

preparations against various pathogens like *Pseudomonas*, *Staphylococcus*, *E. coli*, and *Serritia* until 1979. This approach, however, was not widely accepted in the West. Even after the advent of antibiotics in therapy, phage therapy was widely practiced in the Soviet Union due to collaboration of d'Herelle with his Georgian colleagues. In the United States, Eli Lilly Company produced seven therapeutic phage products for human use. However, the efficacy of phage preparations was controversial and is controversial. Clinical studies were not vigorously pursued in the United States and Western Europe, and what little research there was all but ceased in the 1940s with the availability of penicillin and other commercial antibiotics. d'Herelle's laboratory ceased production of bacteriophage, although the laboratory still operates today, under the name L'Oreal (Golkar *et al.*, 2014).

The majority of the articles dedicated to phage research dates from the 1930s' and 1940s'. The old Soviet literature indicates that phage therapy was used extensively to treat wide range of bacterial infections in the areas of dermatology, ophthalmology, urology, stomatology, pediatrics, otolaryngology, and surgery (Chanishvili, 2012). While West and Europe halted the phage research Soviet Union 1940s, phage research was diverted to a more fundamental level. At the same time, phage continued development in 1920s' and former Soviet Union countries like Russia, Georgia and other Eastern European nations continually used [even today] phages therapeutically for the treatment of pathogenic

bacterial infections that are resistant to antibiotics (Reardon, 2015). This method involves use of a phage to destroy infective bacteria such as *E. coli* or *Salmonella* (Kuchment, 2012).

Bacteriophage is also used in identifying pathogenic bacteria (also called phage typing) in diagnostic laboratories. One other use for bacteriophages for killing specific bacteria found in food. For example, LISTEX by Micreos is made up of bacteriophages that can kill the *L. monocytogenes* bacteria in cheese (phages.org).

2.2 Recent advancements in phage therapy

Experimental phage therapy tested in mice had reported efficacy against up to 95% of all tested *Staphylococcus aureus* (Golkar *et al.*, 2014). In Poland, thousands of people have been treated with phages. With safety trails completed in 1959, Staphylococcal phage lysate (SPL) was licensed for human therapeutic usage and was successfully administered using different routes (intranasal by aerosol, topically, orally, subcutaneously and also intravenously) (Golkar *et al.*, 2014). Researchers from Italy have also identified a bacteriophage active against *S. aureus* including MRSA in mice and possibly humans (sciencedaily.com, 2007). Studies have also reported treatment of MRSA using phages which can be accomplished by local application for local infection or, if necessary, and with substantially more caution, more systemic dosing, including intra-peritoneally for systemic infections (Straub, 1933). Since bacteriophages can be applied as spray and MRSA resides on skin and nasal epithelial linings, phage-therapy may work in the most efficient way without harming gut commensals. Also in hospital as MRSA are transmitted by use of contaminated equipment, phage-spray may be handy and effective method to curb MRSA wherever it is most prevalent.

Most serious criticisms leveled at bacteriophage therapy are:

i. Phage induce neutralizing antibodies,
ii. Phages are active only when administered shortly after bacterial infection,
iii. Phage resistant bacteria – called BIMs – emerge rapidly in course of therapy,
iv. Phages have too narrow host so act against only one types/strains.

But a study on *Salmonella enterica* showed that phages induced non-neutralizing antibodies and were active 2 weeks after experimental infection of mice. Phage resistant bacteria were a-virulent and short lived in in-vivo. More importantly phage resistant bacteria were excellent vaccines, protecting against lethal dosage of heterologous *S. enterica* serovars (Capparelli *et al.*, 2010). Also in 2008, the US Food Safety and Inspection

Service approved *Salmonella*-specific phage preparation to reduce contamination level in live poultry before processing (Capparelli *et al.*, 2010).

In a different study, Tiwari *et al.*, reports an efficient bacteriolytic effect of *Salmonella* Enteritidis specific lytic bacteriophage (phage SE2) with biofilm dispersing ability. The phage SE2 could maintain its virulence even at extreme pH and temperature (Tiwari *et al.*, 2013). As *Salmonella enterica* serovar *enteritidis* is one of the major food borne pathogens, utilizing lytic bacteriophages can be a potential biotherapeutic approach for the prevention of food-contamination and food-borne infection against *Salmonella enteritidis*.

Publications on phage pharmacokinetics suggests that phages get into bloodstream of laboratory animals (after single oral dose) within 2-4 hours and are found in internal organs (liver, spleen, kidney, et cetera) within approximately 10 hours. Also, data concerning the persistence of administered phages indicate that phages can remain in human body for relatively prolonged periods of time - up to several days (Rashel *et al.*, 2007). This property clearly suggests that phages can also be used to treat systemic infections. Another study used four types of phages to treat 72 strains of MRSA and reported that injections of 8×10^8 bacteria intra-peritoneally caused bacteremia and eventual death in mice, but when administered simultaneously with purified phage ØMR11 (MOI ≥ 0.1) they suppressed *S. aureus* induced lethality (Matsuzaki *et al.*, 2003). Further high doses of phage ØMR11 used on uninfected mice showed no adverse effects. Similar effects of anti-staphylococcal phage were demonstrated while dose related phage-treatment was studied (Capparelli *et al.*, 2007). Phages were administered intravenously and reported that minimal effective dose was 10^9 pfu/ml per mouse and also showed that lower doses were ineffective. Phages were active against systemic as well as local infections and within 4 day bacteria in bloodstream were completely eradicated (97% of mice survived). Most importantly, according to Capparelli *et al.*, the phages also successfully lysed MRSA. A study in BALB/c mice suggests combination therapy (mupirocin+phage) represents more attractive option in decolonizing MRSA from nasal cells (Capparelli *et al.*, 2007; Chhibber *et al.*, 2014).

Characterization of 22 *F. psychrophilum* phages from Danish rainbow trout farms revealed phage genome size into three major size classes (8.5 to 12 kb, 48 kb, and 90 kb). The phage host ranges comprised from 5 to 23 of the 28 tested *F. psychrophilum* strains, and 18 of the phage isolates showed unique host ranges. Each bacterial strain had a unique pattern of susceptibility to the 22 phages, and individual strains also showed large variations (up to 10^7 fold differences) in susceptibility to specific phages. The discovery and characterization of broad-host-range phages with strong lytic potential against numerous

pathogenic *F. psychrophilum* host strains thus provided the foundation for future exploration of the potential of phages in the treatment (Wittmann *et al.*, 2014).

No doubt that whole phages can destroy bacterial cells, but these viruses/phages also produce specific enzymes called endolysins which are involved in rapid degradation of cell wall and can destroy bacterial cell wall as even isolated agents (Brzozowska *et al.*, 2011). Bacteria developing resistance to activity of endolysin is very low because of the fact that endolysin targets unique and highly conserved peptidoglycan bonds (Nelson *et al.*, 2012). Prevalence of MRSA generated substantial interest in highly active staphylococcal endolysins, thus a number of staphylococcal endolysins have been characterized including those from following phages: phi11, Twort, P68, phiWMY and phageK (Kaźmierczak *et al.*, 2014). The most extensively and best described endolysin isolated from staphylococcal phage is MV-L which was able to lyse all tested strains, even MRSA and VISA strains (Rashel *et al.*, 2007). In another study, Gu, *et al.* used phage endolysin (LysGH15) as a prophylactic to protect mice against MRSA infections (Gu *et al.*, 2011). Their results demonstrated that 50 µg of LysGH15 was sufficient to protect mice against injections at double the minimum lethal dose of MRSA when administered 1 hour prior to the bacterial challenge. Chhibber *et al.* in their study on BALB/c mice reported that phages were able to significantly reduce in-vitro adherence, invasion and cytotoxicity of MRSA 433000 as well as other clinical MRSA strains on murine nasal epithelial cells as compared to untreated controls (Chhibber *et al.*, 2014). The same study also reported frequency of emergence of spontaneous mutants decreased to negligible levels when phage MR-10 and mupirocin were used together i.e.: combination-therapy. This study proves that MR-10 and mupirocin had additive/synergistic effect and the combined therapy was able to effectively eradicate the colonizing MRSA from nares of mice by 5^{th} day. This same approach may be used to other drug resistant strains as well and may be as effective as on MRSA.

Targeted gene deletion has produced phages with the capacity to bind to their target receptors and inject their DNA, but not replicate or lyse bacteria. This potentially results in inhibition of toxin production and in bacterial killing. Exposure of MRSA to such construction resulted in ≥ 99.9 % kill rate in 5 minutes with an inoculum of 1.0×10^5 organisms and a ≥ 99.9 % kill rate in 10 minutes with an inoculum of 1.0×10^7 organisms (Golkar *et al.*, 2014).

Scientists from Tel Aviv University recently have been able to re-programme temperate and lytic bacteriophage to sensitize and selectively kill antibiotic-resistant bacteria using CRISPR-Cas system. The system can restore antibiotic sensitivity to drug-resistant bacteria. In this experiment, researchers used phages for delivering a programmable DNA nuclease, CRISPR-Cas to reverse antibiotic resistance and eliminate the transfer of

resistance between strains. This approach combined CRISPR-Cas delivery with lytic phage selection of antibiotic-sensitized bacteria. The strategy may reduce the prevalence of antibiotic-resistant bacteria in treated surfaces and on skin of medical personnel, as it uses phages in a unique way that overcomes many of the hurdles encountered by phage-therapy (Yosef *et. al*, 2015).

In another study at University at Leicester, using biofilm and waxworms as models, *Clostridium* phages (against *Clostridium difficile*) reduced *C. difficile* bacterial counts when administered as a preventative measure. Furthermore, combinations of phages and vancomycin led to a marked decrease in *C. difficile* colonization in the waxworms. The study successfully demonstrated that *C. difficile* phages were particularly effective when used to prevent infection, but they are also good at targeting harmful bacterial infections once biofilms have formed. University of Leicester scientists have previously identified the potential of using a bacteriophage cocktail to eradicate *Clostridium difficile* infection (CDI) using an insect model, and now they show that their prophylactic use can prevent infection forming in the first place (Nale *et al.*, 2016). 'The results suggest that it may be possible to reduce the threat of *C. difficile*, and potentially other bacterial infections, through the use of phages both prophylactically to prevent infection, and as therapy once an infection is established. Phage therapy targets specific pathogenic bacterial populations while sparing patients' beneficial microbiome.' says Professor Clokie – one of the researcher.

It can now be said with near certainty that bacteriophage therapy (BPT or PT) can be used successfully with minimal or no side-effect/risks and is cheaper than traditional antibiotic therapy. Its efficacy has already been studied and found to be more than satisfactory. Personally, I believe with advent of new biotechnological tools and genetic engineering, phages are going to be more effective and beneficial than they are in their natural state. Keeping these facts in mind, we can easily and efficiently use phages in hospitals and even in communities to defeat various drug resistant/antibiotic resistant bacterial infections. Thus, rapid emergence of such antibiotic resistant strains calls for alternative option and phage therapy has been showing promising results. Mostly serious skin lesions, carbuncles, furuncles may be treated with aerosol spray or subcutaneous application of purified phage preparations that are caused by *S. aureus* (Kang, 2013; Jensen *et al.*, 2015). Further more serious systemic infections could be easily treated with intravenous injections and/or intra-peritoneal injections. Since animal model trials have already proved intravascular application to be safe, there is none or minimal chance of its failure in human body. Further, phages have shown no residual of phages in blood once infecting bacteria is cleared off and no harmful effect in blood have been reported yet. Phages

colonize specifically only in those places where host are present, multiplying inside them and finally killing them which may be extremely beneficial to clear-off systemic infections while no affecting other body parts.

Despite all the scaremongering of using 'live virus' for treatment, on a different note, advancement of genetic engineering in recent years has strengthened the natural prowess of phages. In recent study, researchers successfully used bacteriophages to deliver a specific CRISPR/Cas system into antibiotic-resistant bacteria (β-lactam antibiotics) to reverse the resistance and thereby sensitize the microbes to the drugs (Yosef *et al.*, 2015). Unlike classic phage therapy, which uses one or more types of phages to infect and lyse specific bacterial strains, the crux of this new approach is using these specialized viruses to supply CRISPR/Cas to rid bacteria of antibiotic-resistance plasmids in the environment before the microbes are able to infect a host.

2.3 Other applications of phage

Beside pure therapeutic application (including veterinary), there are numerous fields where importance of phages is yet to be explored. Some of the potential fields are:

1. **Microbiome research:** As reports link Chron's disease to presence of specific strain of *E. coli* (Rhodes, 2007; Chassaing *et al.*, 2011; Strober, 2011; Martinez-Medina & Garcia-Gil, 2014) application of specific phage (as antibiotic kill numerous strains in the microbiome) has been gaining traction (Wagner *et al.*, 2013; Babickova & Gardlik, 2015; Norman *et al.*, 2015). It would be fascinating to look at the specific function of specific bacteria in [gut] microbiome. Scientists only recently started recognizing the role of the microbiome – the bacteria in and on the body, and the bacteria's genes – in illness. For example, changes in the gut microbiome have been linked to obesity, diabetes, metabolic syndrome and inflammatory bowel diseases. Possibility that viruses may have unrecognized roles in obesity and diabetes and the two most common inflammatory bowel diseases, Crohn's disease and ulcerative colitis may suggest that scientists should be studying the virome as closely as the microbiome.
2. **Phage display:** Phage display is another fascinating field that uses phages waiting for myriad of possibilities. Phage display technology is an in vitro screening technique for identifying ligands for proteins and other macromolecules. It is one of the most effective molecular diversity techniques available today (Brzozowska *et al.*, 2011; Bazan *et al.*, 2012). This technology is based on a direct linkage between phage phenotype and its encapsulated genotype, which leads to presentation of molecule libraries on the phage surface. Phage display is utilized in studying protein-ligand interactions, receptor binding sites and in improving or modifying the affinity of

proteins for their binding partners. Techniques originating from phage display have been applied to transfusion medicine, neurological disorders, mapping vascular addresses and tissue homing of peptides. Phages have been applicable to immunization therapies, which may lead to development of new tools used for treating autoimmune and cancer diseases (Hoogenboom *et al.*, 1998; Bazan *et al.*, 2012). The past innovations and those to come promise a bright future for this field (Pande *et al.*, 2010).

3. **Phage typing:** In today's world, where precise diagnosis and strain identification of primary source of outbreak is equally important as treatment and control of the infections, phages may be a handy tool in identification/diagnosis (Ridley *et al.*, 1998). When a bacterial isolate is exposed to a panel (collection of phages that infect similar bacteria) of phages, a profile is generated – a listing of which phages are capable of infecting and lysing the bacteria. The phage profile may be used to type/identify bacterial strains within a given species (Tang *et al.*, 1997). As such, scientists are exploring the capacity of phages to identify the source strain while in outbreaks – a method called 'phage typing'. Phage typing is rapid, economical, reliable and reproducible technique, requiring no specialized equipment for fingerprinting disease-causing agents for epidemiological investigation and surveillance and thus has greater potential in disease diagnosis as well (Clokie & Kropinski, 2009).
4. **Environmental biocontrol agent:** Phages are highly abundant in aquatic environments, ranging from 10^4 to 10^8 pfu/mL (Bergh *et al.*, 1989). These viruses – for sure – have a role in [bio] controlling the diversity and population of bacterial communities/species. It has been suggested that phages are functionally important components of activated sludge systems (Ewert & Paynter, 1980; Hantula *et al.*, 1991; Khan *et al.*, 2002; Thomas *et al.*, 2002). Phage lysis of such systems has potential to influence treatment performance by controlling any unwanted bacterial communities. On the other hand, lytic control of bacterial pathogen while sludge treatment is direct control of pathogens from contaminating agricultural land ant ultimately preventing transmission to humans through food. Thus phages have immense potential as an 'natural biocontrol agent' that would drastically reduce/control the use of chemicals for waste/sludge treatments.
5. **Food preservative/biocontrol:** Phages have wide range of potential application in non-clinical settings where regulations for their use as biocontrol agents in foods may not be as stringent. In fact, phages preparations as food preservative has already been given a GRAS status and phage preparations against *L. monocytogenes* (ListShield™ and LISTEX™ marketed by US company Intralytix Inc. and Micreos respectively) has already been approved by US-FDA for control of *L. monocytogenes* in refrigerated foods (intralytix.com, listex.eu/products). Micreos also has US-FDA approved food

safety product SALMONELEX™ against *Salmonella* spp. (micreos.com). Thus, there lies a technically 'unexplored and potential' field for application of phage beside its biomedical application. Additionally, phages have also been used to treat plant diseases like bacterial spots on tomatoes and blight on geraniums caused by *Xanthomonas campestris* pv. *Vesicatoria* (Flaherty *et al.*, 2000; Flaherty *et al.*, 2001).

6. **Agriculture and crop protection:** The concept of using phages as biocontrol agents in pest management had emerged shortly after their discovery, but as in healthcare, was halted with advent of more powerful antibiotics. In recent years, phages have been found to be effective for control of several phytobacteria such as *Erwinia* spp., which cause bacterial soft rot and fire blight on apple and pear, *Xanthomonas* spp., which cause bacterial spot of tomato, peach, geranium, citrus, walnut blight, leaf blight of onion and citrus canker, *Ralstonia solanacearum* which causes bacterial wilt of tobacco, *Pseudomonas* spp., which causes bacterial blotch of mushrooms, and *Streptomyces scabies*, which causes potato scab (Flaherty *et al.*, 2000; Frampton *et al.*, 2014; Frampton *et al.*, 2012; Jones *et al.*, 2007; Nagy *et al.*, 2011). In spite of this increasing research success, so far only one phage based biopesticide (AgriPhage™) is commercially available for controlling plant pathogenic bacteria (http://www.omnilytics.com/products/agriphage) (Nagy *et al.*, 2011). It would thus be exciting to look into the prospects of phage based biocontrol in farming as well.

2.4 Concluding remarks: The future of phage research

Though it would be premature to confirm without concrete 'clinical and or field trails', we can confidently hypothesize that naturally occurring bacterial virus can infect and kill drug resistant pathogens of human/animal/plant origins thereby revitalizing the possibility of using phage therapeutically. On the other hand, exploiting the natural ability of bacterial virus – phages – to infect bacteria and tailoring them to kill the MDR pathogens more effectively and efficiently may be the next big discovery after 'antibiotics' given the hypothesis that phages possess anti-CRISPR capability by which it escapes the CRISPR/Cas attack by its host.

With advancement of genetic engineering tools in recent days, it excites me to look into the possibilities this miniscule entity beholds in balancing nature, healthcare, food safety, environmental cleansing, agriculture and so on.

CHAPTER – THREE

MATERIALS AND METHODS

This study is entirely a 'lab based experimental study' that aims to uncover the possible usage of lytic phages therapeutically to kill – and thus to cure – infections caused by drug resistant bacterial pathogens. All of the experiments – otherwise mentioned – were performed in Central Department of Biotechnology (CDBT), Institute of Science and Technology, Tribhuvan University, Nepal. Media, reagents, glass-wares, plastic-wares, incubators, syringe filters, bench-top centrifuge et cetera and all other required equipment were either purchased from regular supplier or made available by the university.

The study method is largely 'analytical and quantitative' and results are primarily interpreted based on 'visual analysis' (plaques formation, lysis spots, electron micrograph) and compared with available databases and/or published research for verification. Statistical tools were rarely used for data interpretation as the study was purely experimental/lab based and also our sample size was not large enough for comprehensive statistical analysis.

3.1 Bacterial strains, manipulations and growth media

3.1.1 Bacterial strain collection and transport

All of the bacterial stains used in this study (except ATCC as reference strains) are multi-drug resistant human pathogens collected from Tribhuvan University Teaching Hospital (TUTH), Maharajgung (Table 3.1, 23 [1-23] strains generously provided by Prof. Dr. Basista Rijal, Mr. Hari Kattel and Mr. Narayan Parajuli) and Manamohan Hospital and Research Center (kindly provided by Mr. Santosh Khanal, Sorhakhutte, 3 strains -

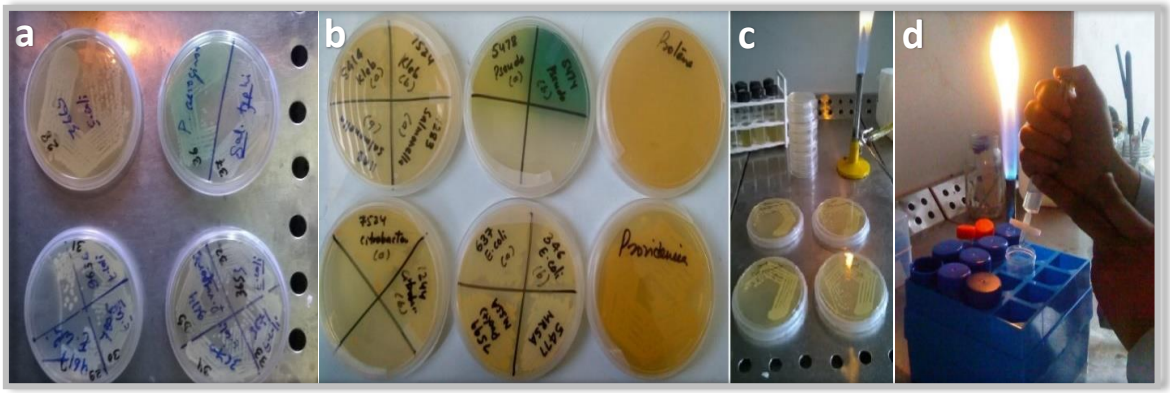


FIGURE 3.1 | Bacterial host strains collection and processing of water sample. a) and b) bacterial strains collected from TUTH hospital, **c)** host bacteria reay for sub-culture and **d)** syringe filtration of the sewage/river water in lab.

*Acenatobacter*_22, *Pseudomonas*_23 and *Salmonella paratyphi*_24) during the month of December-January, 2014/2015.

Additional batch of bacterial samples (Table 3.1 [24-41]) were collected from TUTH during the month of June, 2015 for wider multiple-host range determination as the 'Gorkha Earthquake – 25th April, 2015' destroyed some of the stored cultures in the lab. Bacterial cultures collected from different hospitals in nutrient agar (in petri dish) were immediately transported to microbiology laboratory at Central Department of Biotechnology, Kirtipur in chilled condition following standard protocol as instructed by the hospital staffs as all of the strains collected were pre-confirmed to be multidrug resistant (MDR) by respective hospital staffs following their own standardized protocol.

TABLE 3.1 | Bacterial strains (MDR) collected from TUTH and Manamohan Hospital and Research Center, Dec-Jan 2014/2015 and June 2015.

SN	Bacterial strains	Bacterial code
1	*Proteus spp._1*	Pro1
2	*Providencia spp._2*	Prv2
3	*Citrobacter spp._3*	Cit3
4	*Citrobacter spp._4*	Cit4
5	Methicillin resistant *S. aureus_5* (MRSA)	SA5
6	Methicillin sensitive *S. aureus_6* (MSSA)	SA6
7	*Escherichia coli_7*	EC7
8	*Escherichia coli_8*	EC8
9	*Klebsiella spp._9*	Kle9
10	*Klebsiella spp._10*	Kle10
11	*Salmonella typhi_11*	ST11
12	*Salmonella spp._12*	Sal12
13	*Pseudomonas spp._13*	Pse13
14	*Pseudomonas spp._14*	Pse14
15	*Escherichia coli_15*	EC15
16	*Enterobacter aerogens_16*	EA16
17	*Escherichia coli_17*	EC17
18	*Escherichia coli_18*	EC18
19	*Shigella spp._19*	Shi19
20	*Burkholderia cepacia* complex_20	BC20
21	*Escherichia coli_21*	EC21
22	*Acenatobacter spp._22* (Carbapenemase R)	Ace22
23	*Pseudomonas spp._23* (OF/Lev R)	Pse23
24	*Salmonella paratyphi_24* (NARS)	SP24
25	*Escherichia coli* ATTC 25922_25	EC25
26	*Staphylococcus aureus* ATTC 25923_26	SA26
27	*Pseudomonas aeruginosa*_27	PA27
28	*Escherichia coli*_28	EC28
29	*Escherichia coli*_29	EC29
30	*Escherichia coli*_30	EC30
31	*Escherichia coli*_31	EC31
32	*Escherichia coli*_32	EC32
33	*Escherichia coli*_33	EC33
34	*Escherichia coli*_34	EC34
35	*Pseudomonas vulgaris*_35	PV35
36	*Pseudomonas aerogenosa*_36	PA36
37	*Salmonella typhi*_37	ST37
38	*Escherichia coli*_38	EC38
39	*Klebsiella pneumoniae*_39	KP39
40	*Klebsiella pneumoniae*_40	KP40
41	*Klebsiella pneumoniae*_41	KP41

3.1.2 Bacteria sub-culturing, storage and growth media

Bacterial cultures collected from hospitals were immediately sub-cultured into freshly prepared nutrient agar (HiMedia, India) plates after being transported to laboratory at Central Department of Biotechnology. Glycerol stocks (in triplicates) were prepared and stored for future use. For glycerol stocks, bacteria were initially cultured in nutrient broth (HiMedia, India) at 37°C for 24 hours and 0.5 ml of overnight broth culture was added to 0.5 ml of autoclaved 50% glycerol

(HiMedia, India) in 1.5 ml cryovial tubes. The tubes were then cooled at 4°C for an hour, chilled at -20°C for an hour and finally transferred to -80°C for long time storage.

Tryptic soy broth - TSB (HiMedia, India) was used for phage isolation, purification and amplification. Agar was separately added to TSB in appropriate concentration whenever required.

3.2 Antibiotic sensitivity test (AST) / Anti-biogram assay

Although antibiotic sensitivity test was already performed in respective hospital, the sensitivity assay was repeated in Central Department of Biotechnology - CDBT laboratory for data verification and confirmation. Widely accepted Kirby-Bauer disc diffusion technique – as per the modification by Jan Hudzicki – (Hudzicki, 2013) was used to test the antibiotic sensitivity of all collected bacterial strains against 10+1 (Methicillin used only for MRSA and MSSA) different antibiotics (HiMedia, India; list of antibiotics in Table 3.2) available at the laboratory and classified under sensitive (S), intermediate (I) and resistant (R) according to the company's guidelines. The numerical value in the 'Code' section of Table 3.2 represents respective concentration (in µg) of the 6.0 mm disc used in this study.

TABLE 3.2 | Antibiotics used in CDBT laboratory for MDR verification.

SN	Antibiotic	Code
1	Gentamycin	GEN 10
2	Meropenem	MRP 10
3	Piperacillin / Tazobactam	PTI 100/10
4	Vancomycin	VA 30
5	Nalidixic Acid	NA 30
6	Ampicillin	AMP 10
7	Amikacin	AK 30
8	Ofloxacin	OF 5
9	Piperacillin	PI 100
10	Cefotaxime / Cephotaxime	CTX 30
11	Methicillin	MET 5

Briefly, drug resistant bacterial pathogens (from overnight cultured broth, compared to 0.5 McFarland standard) were lawn cultured on Mueller Hinton Agar using sterile cotton swab. After air-drying, appropriate antibiotic disc/s were placed on the lawn culture and incubated at 37°C for 24 hours. The 'zone of inhibition – ZOI' against confluent lawn of bacterial growth was recorded with the unaided eyes from back of the petri dish using calibrated ruler against black, non-reflecting surface illuminated with reflected light. Bacterial strains/cultures were then classified (as sensitive, intermediate, resistant) according to the company's parameters/guidelines.

3.3 Water/sewage sample collection and processing

Five sewage mixed water samples (100 mL each, one sample per day) – also referred as water samples – were collected in sterile culture bottles from different rivers of Kathmandu valley (Table 3.3) and transported to CDBT laboratory. Global positioning service (GPS) co-ordinates were recorded at the point of sample collection using Google maps. Upon arrival, 50 mL water sample was transferred to sterile falcon tube and centrifuged at 4,000 rpm for 1 hour to clear-off larger debris and sand particles. The supernatant was filtered through 0.20 µm syringe filter (Sartorius, Germany) and collected in a separate sterile falcon tube, thereby removing any microscopic contaminants larger than 0.20 µm pore size. This filtered water sample was then used as the phage source in our study and was considered to be free from any particulate debris, bacterial and/or diatomic contamination.

FIGURE 3.2 | Location of water samples. Sewage / Water sample collection points around Kathmandu Valley as determined by the GPS co-ordinates (represented by star & pop-ups with sample code for clarity) and saved in Google Maps for future references as phage population significantly varies from place to place.

TABLE 3.3 | Geographic mapping of Sewage/Water Sample collection points/area.

SN	Location / Code	Volume	Date and Time	GPS co-ordinates	Remarks
1	Gongabu / G	100 mL	February 25, 2015 1035 hours	27°44'06.2"N 85°18'25.5"E	Bishnumati river, near New Bus Park bridge
2	Kalanki / K	100 mL	March 19, 2015 1148 hours	27°41'23.3"N 85°17'01.6"E	Balkhu-khola, Khasi Bazaar, under the bridge
3	Balkhu / B	100 mL	March 25, 2015 1124 hours	27°40'27.9"N 85°17'34.3"E	Bagmati river, downstream to Vayodha Hospital
4	Chabahill / O	100 mL	June 02, 2015 0912 hours	27°43'19.0"N 85°20'43.8"E	Dhobi-khola, near Om Hospital & Research Center
5	Pulchowk / P	100 mL	June 19, 2015 1219 hours	27°41'20.4"N 85°18'59.8"E	Bagmati river, under Bagmati bridge

** Romanized Nepali words (khola, khasi-bazaar) used without translation as they appear on google maps for reproducibility.*

3.4 Phage manipulations

3.4.1 Phage screening

Phage screening was performed by standard Double Layer Agar Assay (DLAA) and plates with clear, round plaques against bacterial lawn culture (visual observation) were considered positive and stored for further processing.

One milliliter of processed water sample was pipetted into sterile 25.0 mL culture tube and approximately 100 µL (1-2 drops) of overnight broth culture (cultured previous day in Nutrient broth) of host bacteria was added to it. The mixture was left still for 5 minutes for proper, irreversible attachment of phage (if present) to the host bacteria. Afterwards, 3.0 mL soft agar (Tryptic Soy Broth with 0.5% agar, stored at 50°C) was added to the mixture, gently vortexed rotating the tubes, and poured over previously readied hard agar (Tryptic Soy Broth with 1.0% agar) petri plates. The plates were left to solidify and finally after solidification incubated at 37°C for 24 hours in upright position.

Next day, the plates were visually examined for presence/absence of plaques (uniform circular, clear zones against even bacterial lawn). As our research was completely based on isolation and characterization of potentially therapeutic lytic phages, plates with clear visible plaques were regarded as positive and stored for further analysis, whereas plates that did not have significantly clear plaques were considered as negative and discarded, though they may harbor lysogenic phages. Plaques were analyzed visually for their morphological characteristics (shape, size, opacity, presence/absence of halo).

The DLAA assay described above was repeated for all of the 26 different bacterial strains (collected in first batch, Table 3.1 [1-26]) and all collected 5 water samples separately.

3.4.2 Phage isolation, purification, amplification and storage

Only one, completely isolated plaque per plate was selected for further study on the basis of their visual characteristics like shape (round), size (largest was selected, pinhead plaques were discarded), opacity (low), uniqueness (dissimilar from plaques obtained within same plate and also other plates) and efficiency to lyse the host bacteria. Selected plaque was carefully cut-out using pipette tip (previously cut to match the size of plaque) and immediately dissolved in 1.0 mL of SM buffer. After gentle vortexing for few seconds, SM buffer was filtered through 0.20 µm syringe filter (Sartorius, Germany). The filtrate was serially diluted up to 10^{-10} and DLAA was again performed for all dilutions using same bacteria as host from which phage was initially isolated.

After proper incubation, the process of sub-culture (plaque selection, cutting, dissolving in SM buffer, filtering, serial dilution and finally DLAA) was repeated again from appropriate plate. This arbitrarily confirms the purity of phage and assumes that the phage stock obtained after three round of sub-culture is pure and mono-culture of a single phage that formed plaque in screening assay.

Plaque/phage sub-culture was performed separately for all phages, isolated and selected from screening assay to obtain nearly pure/mono-culture of single phage. The purity of purified phage strain was checked by DLAA culture method. A serially diluted pure culture (up to 10^{-10}) was cultured with host culture and plaque morphology was observed next day after proper incubation.

BOX 2 - Double Layer Agar Assay (DLAA)

Double Layer Agar Assay (DLAA) is the standard method for isolation of phages from environmental sources like sewage water, dairy waste, animal waste, soil et cetera.

PROCEDURE: *A thin layered hard agar (1% agar, any media that supports growth of host bacteria, here we have used Tryptic Soy Broth) is prepared and left for solidification. At the same time soft agar (same media used to prepare hard agar but, half the agar percentage, here 0.5 %) is prepared and stored at 50°C. In a separate sterile culture tube, 1.0 mL of phage stock (any sample that is believed to have phage particles and filtered through 0.20 µm syringe filter) is added. Few drops (around 100 µL) log phase bacterial culture is added to the phage tube, mixed well and allowed to stay still for 2 minutes. This will ensure permanent attachment of phages (if present) with the host bacteria. 3.0 mL of soft agar is added to the tube, mixed well and poured into hard agar aseptically. The top layer of soft agars is spread evenly rotating the plate and left for solidification. Finally, after solidification, the plates are incubated at optimal temperature (as per bacterial hosts' requirements, here 37°C as all our experimental strains are human pathogens and grow well at 37°C) in upright position.*

After successfully selecting phages for further analysis, phages were amplified so that the lysate contained enough number of pure phages. For amplification, 0.5 mL purified phage stock was added to 10 mL of log phase host culture (cultured 3-4 hours at 37°C in TSB, growth of bacteria observed visually) and incubated overnight at 37°C. Next day, the broth culture which now contains high number of phage particles, was centrifuged at 4,000 rpm for 30 minutes to separate the bacterial debris and filtered through 0.20 µm syringe filter (Sartorius, Germany) to remove any smaller bacterial contaminants. The filtrate was now used as phage stock for phage amplification in plate.

DLAA was again performed in 3 plates per each phage using filtered phage stock and their respective host and incubated overnight at 37°C. Next day, the plates were flooded with 10 mL SM buffer per plate and kept in rotating shaker for 20 minutes at 80 rpm. The SM buffer from all three plates (now contains phage particles) were collected in a 50.0 mL falcon tube, centrifuged at 4,000 rpm for 30 minutes and finally filtered through 0.20 µm syringe filter. The filtrate thus obtained was used for all other processes like multiple host range analysis, TEM and efficacy testing in lag, log and stationary phase of bacterial life cycle.

3.4.3 Multiple Host-Range (MHR) spectrum analysis

Multiple host-range (also rarely called broad host-range) is the ability of a specific phage to infect and lyse closely related bacterial strains other than the host strain. Standard spot-assay with minor modification was performed to determine multiple host-range of purified phages against other strains within same genus (Garbe *et al.*, 2010; Karumidze *et al.*, 2013). For spot assay, few drops (approx. 100 µl) of log phase bacterial culture was mixed with 3.0 mL of soft agar in a sterile culture tube in aseptic condition. The mixture was gently mixed and poured in hard agar (bottom agar) in a petri dish (previously prepared). Petri plates were swirled so as to distribute top agar evenly

BOX 3 - Phage purification protocol

The process of three round sub-culturing for purification is entirely based on hypothesis that three-round of sub-culture of single isolated plaque gives pure progeny of single phage that formed plaque in the first round of assay (screening assay) because we assume, a plaque results from infection of a single phage.

BOX 4 - Naming of Phages

Although International Committee on Taxonomy of Viruses (ICTV), in 2015, recommends to use genus name followed by virus (for example Escherichia virus for phage that infects Escherichia coli), conventional naming approach has been followed (unless mentioned) for greater clarity. Throughout this dissertation (unless mentioned) a phage is named such that a word 'phage or a symbol Ø wherever deemed appropriate' is followed by TU (representing Tribhuvan University - the university where the research was carried out), bacterial code (two uppercase letters if genus and species are known and three sentence case letters code if only genus is known) along with numerical serial code assigned to each strains and finally ending with an uppercase letter code like K, P, O, B, G that represents water/sewage sample collection site.

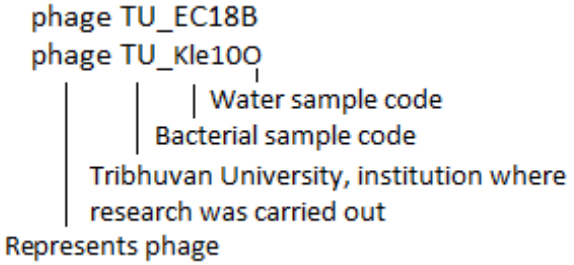


FIGURE 3.3 | Representative example of phage name. The first word phage represents 'phage'; TU represents 'Tribhuvan University'; the 2-3 letter code with numerical afterwards represents 'bacterial strain' and finally last alphabet represents 'sampling site'.

and left to solidify. After solidification plates were divided into required parts and spots were marked for spot testing. Ten microliters of different pure phage lysate were applied to different spots (previously marked) and again left for about 10 minutes to dry. The plates were then incubated overnight at optimum temperature for bacterial growth. Next day, presence/absence of clear zones (lysis of bacteria) were observed at the spots where phage was applied.

Spot assay of all 34 purified phages were performed and result was interpreted on the basis of clearing zones observed visually. A heat killed (1 hour in boiling water) phage lysate was used separately as a control.

Mean value of host range - whenever deemed necessary - was also calculated using following formulae.

$$\text{Mean (MHR)} = \frac{\Sigma \text{ Host Range within a genus}}{\text{Total number of phages isolated per genus}}$$

3.4.4 Multi-generation infection efficiency of a phage and host bacteria

As often times, efficiency and efficacy of phages are challenged by researchers claiming that bacteria are wise enough to mutate and escape phage infection giving rise to phage induced mutants infamously called Bacteriophage Induced Mutants (BIMs), we assessed the infectivity and efficacy of a single pure phage in 5 subsequent generations of same host bacterium. For this, all 34 phages (from stock) were subjected to DLAA using freshly sub-cultured bacterial host in duplicates. The process was again repeated after a week with same phage stock, but a freshly sub-cultured bacterial host. This process was repeated for 5 generations of a bacterial host. Result interpretation was made based on visual analysis, that is emergence/absence of BIMs and compared with the plates from first generation.

The goal of this test was only to report BIM (if emerged), so only plates that had significant BIM in them were marked positive and chosen for further analysis. Plates that were completely clear or that showed the similar lysis pattern as in first generation signifying similar lysis were marked negative, meaning there were no induction of BIM.

3.4.5 Efficacy analysis of selected potent phages on lag, log and stationary phase of bacterial life cycle

Efficacy of selected 7 phages (only those selected for electron microscopy) were also analyzed for their efficacy on lag, log and stationary phase of host bacteria's life cycle. For this, liquid broth culture was used and results were analyzed by subsequent plating onto fresh medium. One milliliter of phage (phage titer >10^{10} pfu/ml) was added to 10 mL of lag (broth culture of host bacteria cultured for 5 minutes), log (broth culture of host bacteria cultured for 2 hours) and stationary (broth culture of host bacteria cultured overnight) phase of host culture and incubated overnight at 37°C. Next day, 100 µl from each tube/broth was cultured on nutrient agar (spread plate) and incubated at optimum temperature. After 24 hours, plates were observed for presence/absence of bacterial colonies.

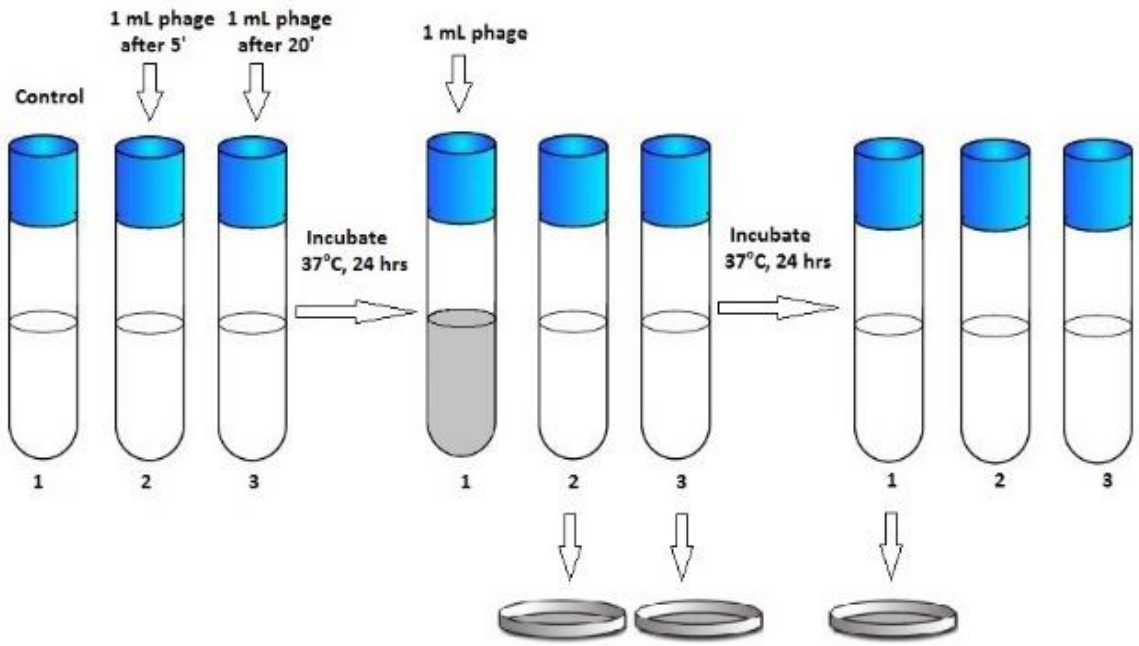


FIGURE 3.4 | Workflow – efficacy analysis of phages on lag, log and stationary phase of bacterial life cycle. The representation presents a positive result where bacteria are completely killed/lysed by phages in all of their lag, log and stationary phase.

3.5 Transmission Electron Microscopy (TEM)

Seven most potent phages were selected for TEM analysis on the basis of its multiple host range, lytic efficiency on the host bacterial strain and diversity. Serial dilution was performed to enumerate the phage concentration and ensured that the phage titrate met the requirement for TEM analysis (higher than 10^7 pfu/ml). Selected phage lysates were transported to Advanced Instrumentation Research Facility – Jawaharlal Nehru University (AIRF-JNU), New Delhi, India in cold chain. Upon arrival, the phage lysates were fixed with

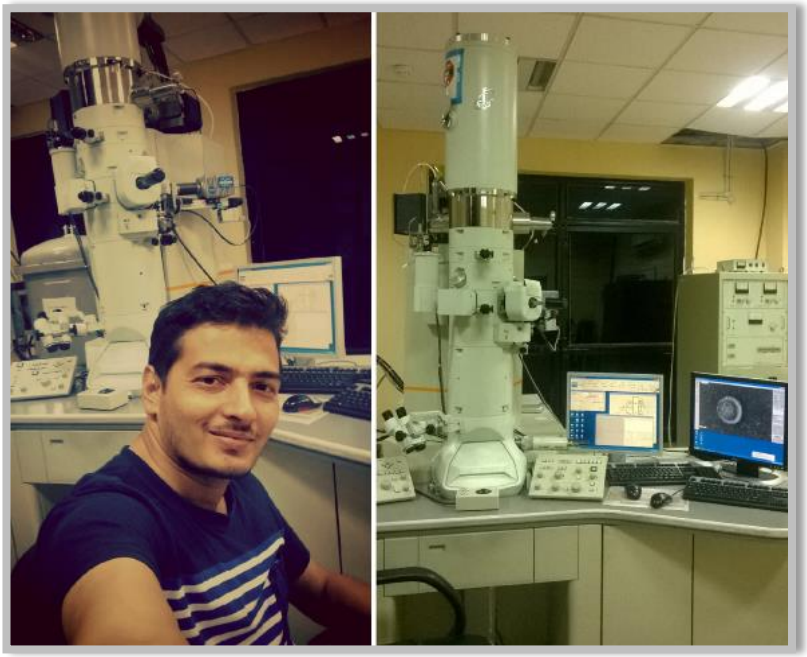


FIGURE 3.5 | Transmission Electron Microscope. Performing Transmission Electron Microscopy (TEM) at JNU-AIRF, New Delhi, India.

fixative (2.5% glutaraldehyde and 2% paraformaldehyde prepared in 0.1 M sodium phosphate buffer (pH 7.2)). For fixation, equal volume of phage lysate and fixative were added, mixed and left overnight. Next day, the fixed phages were subjected to high-speed centrifugation (35,000×g) for 3 hours. Contrary to our expectation, pellets were not visually observed, so, lower portion of phage lysate were processed for TEM viewing 10.0 µL fixed phage lysate (per sample) was deposited on separate 300 mesh carbon-coated copper grid and after 2 minutes' excess phage lysate was soaked-off with a blotting paper. The copper grid was then flooded with 2% (w/v) uranyl acetate (pH 4.5) and after 2 minutes, excess stain was soaked-off with blotting paper. The copper grid was dried and finally examined in JEM-2100F Transmission Electron Microscope (JEOL, USA, 200 KV) under different magnifications.

TEM micrographs were processed using ImageJ 1.50i (https://imagej.nih.gov/ij) for determining tail size (width and length) and size of phage capsid / head. Three readings of head and tail (width and length) were recorded and mean value was calculated to minimize the human error.

3.6 Phage genomics / Whole genome sequencing (WGS)

Three phage samples (phage TU_Kle10O, phage TU_EC18O and phage TU_SP24B) were exported to Xcelris Genomics (Ahmedabad, India) in dry ice for Next Generation Sequencing. Whole genome sequencing of phages were performed on Illumina HiSeq 2000/2500 platform under project ID: 625.

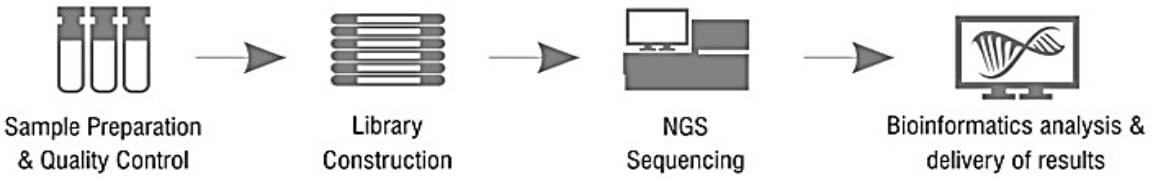


FIGURE 3.6 | Next Generation Sequencing. Whole Genome Sequencing (WGS) workflow at Xcelris Genomics, Ahmedabad, India. Source: http://www.xcelrisgenomics.com

3.6.1 gDNA isolation

The genomic DNA (gDNA) of three phages were isolated using Phage DNA Isolation Kit (Cat. #46800, Norgen Biotek Corp., Canada). Qualitative and quantitative checks were performed using conventional electrophoresis and Qubit® 2.0 Fluorometer respectively. Five microliter DNA of each sample was loaded on 1% agarose gel and run for 30 minutes at 110 Volt. One microlitre of each sample was loaded in Nanodrop 8000 for determining A260/280 ratio and 1.0 µl sample was again loaded in Qubit® 2.0 Fluorometer for determining concentration of DNA.

3.6.2 Library preparation

The paired-end sequencing library was prepared using Illumina TruSeq Nano DNA HT Library Preparation Kit. Two hundred nanograms of gDNA was fragmented by Covaris shearing that generated dsDNA fragments with 3' or 5' overhangs. The fragments were then subjected to end-repair. This process converts the overhangs resulting from fragmentation into blunt ends using End Repair Mix. The 3' to 5' exonuclease activity of this mix removes the 3' overhangs and the 5' to 3' polymerase activity fills in the 5' overhangs. A single 'A' nucleotide is added to the 3' ends of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction. A corresponding single 'T' nucleotide on the 3' end of the adapter provides a complementary overhang for ligating the adapter to the fragment. This strategy ensures a low rate of chimera (concatenated template) formation. Indexing adapters ligates to the ends of the DNA fragments, preparing them for hybridization onto a flow cell. The ligated products were purified using SP beads supplied in the kit. The size-selected product was PCR amplified as described in the kit protocol.

3.6.3 Quantity and quality check (QC) of library on Bio-analyzer

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

3.6.4 Cluster generation and sequencing

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

3.6.5 Genomic data analysis / Bioinformatics

The whole genome sequence (WGS) data was curated and delivered to us by Xcelris Genomics in fasta format (.fa files). The genome files were primarily processed using an online tool PHASTER – PHAge Search Tool - Enhanced Release, http://phaster.ca – (Arndt *et al.*, 2016; Zhou *et al.*, 2011) and genome visualization, ORF prediction and annotation was further enhanced by SnapGene® 3.2.1 tool. Throughout the project, NCBI database was used as the sole source for reference genome for bacterial and phage genomes. Also Blast+ was used for species distribution.

PHASTER tool was also used for prediction of putative genes. A linearized genome was constructed in PHASTER and genes were predicted using NCBI genome database as reference. Hypothetical proteins were removed from annotation to avoid cluttering while mapping of genes/proteins.

A comprehensive genome analysis and extensive gene annotation is ongoing as part of the project. Also, phylogenetic analysis will be presented by co-researcher of the project in the coming days. We are at the final stage of submitting the whole genome sequence to GenBank® (https://www.ncbi.nlm.nih.gov/genbank/) and finally make an Genome Announcement (http://genomea.asm.org/) as well.

CHAPTER – FOUR

RESULTS AND DISCUSSION

4.1 Antibiotic susceptibility test (AST)

Antibiotic susceptibility testing (AST) - also called antibiogram assay - of 41 bacterial strains (Table 4.1) collected from Tribhuvan University Teaching Hospital and Manamohan Hospital and Research Center in two batches revealed that all but 7 (*Salmonella typhi*_11, *Salmonella paratyphi*_24, *Pseudomonas* spp._13, *Pseudomonas* spp._23, *Pseudomonas aeruginosa*_27, *Pseudomonas vulgaris*_35, *Pseudomonas aeruginosa*_36) were multi-drug resistant. Here, a strain was regarded as 'multi-drug resistant (MDR)' if it was resistant to more than one antibiotic tested in the CDBT lab irrespective of the group they belong. This assumption was made because, all of the collected strains were pre-confirmed by hospital staff as MDR strains (resistant to at least one antibiotic from 3 different groups of antibiotic), but limited resources in our laboratory and a different priority confined our antibiotic testing to only 10 available antibiotics.

The discrepancy on the result may be because of the difference in antibiotics used in hospital lab and university lab which is justified, given the limited number of available antibiotics used in university lab. Also, as these bacterial pathogens were intentionally selected from hospital cases, all strains being MDR does not necessarily imply that all of the hospital cases are multi-drug resistant. However, we cannot deny the worldwide crisis of AMR and the urgent need of effective, affordable and reliable alternative to antibiotics cannot be overlooked in present scenario.

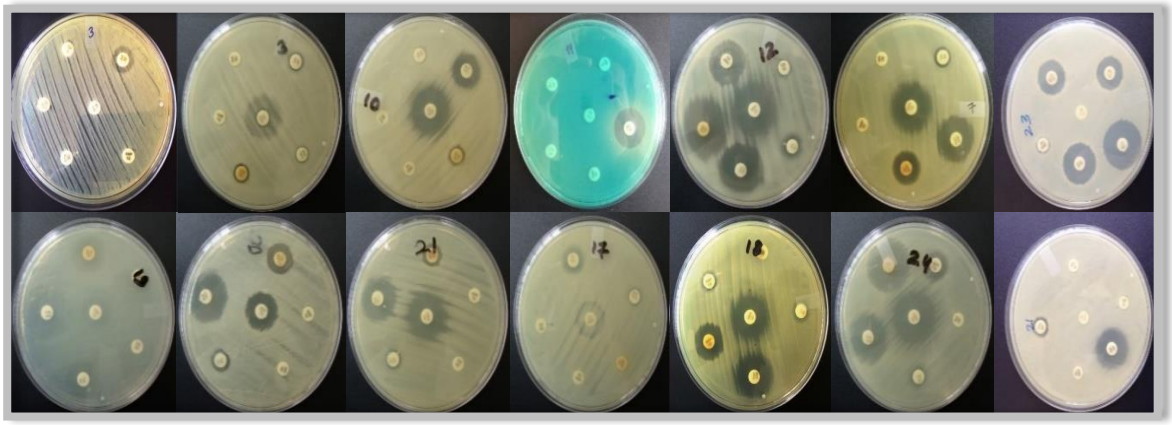


FIGURE 4.1 | Antibiotic susceptibility testing (AST) / Anti-biogram assay of collected human pathogens. All of the bacterial strains (pathogens of human origin) are resistant to more than one antibiotics used. The clear zones around the antibiotic discs are zone of lysis where bacteria are killed by drug, whereas no lysis zone around the disc signifies that the bacterium is drug resistant.

TABLE 4.1 | Antibiotic Susceptibility Testing (AST) / Anti-biogram assay of bacterial host strains.

SN	Bacteria strains / Antibiotics	GEN 10	MRP 10	PIT 100/10	VA 30	NA30	AMP 10	AK 30	OF 5	PI100	CTX 30	MET 5	MDR Status
1	*Escherichia coli*_7	22	20	20	NA	0	0	23	13	14	0	NA	+
2	*Escherichia coli*_8	17	14	24	NA	0	0	21	9	0	0	NA	+
3	*Escherichia coli*_15	17	0	7.5	NA	0	0	15	0	0	0	NA	+
4	*Escherichia coli*_17	16	0	18	NA	0	0	9	0	0	0	NA	+
5	*Escherichia coli*_18	24	15	22	NA	0	19	21	14	21	16	NA	+
6	*Escherichia coli*_21	20	10	18	NA	0	0	19	0	0	0	NA	+
7	*Escherichia coli*_25 (ATTC 25922)	22	27	22	NA	8	11	22	22	20	26	NA	+
8	*Escherichia coli*_28	10	11	0	NA	NA	18	25p	25p	20p	0	NA	+
9	*Escherichia coli*_29	18	13	20	NA	NA	0	20	10	12	20	NA	+
10	*Escherichia coli*_30	20	32	20	NA	NA	0	22	30	0	0	NA	+
11	*Escherichia coli*_31	18	22	18	NA	15	0	18	18	0	0	NA	+
12	*Escherichia coli*_32	11	11	16	NA	0	14	17	25p	22p	25p	NA	+
13	*Escherichia coli*_33	8	10	0	NA	0	0	20p	0	0	0	NA	+
14	*Escherichia coli*_34	10	11	0	NA	0	0	15p	8	0	0	NA	+
15	*Escherichia coli*_38	0	33	0	NA	21	0	0	23	0	0	NA	+
16	*Klebsiella* spp._9	17	0	9	NA	0	0	11	0	0	0	NA	+
17	*Klebsiella* spp._10	21	10	15	NA	0	0	20	0	0	0	NA	+
18	*Klebsiella pneumoniae*_39	9	15	0	NA	0	0	13	10	0	0	NA	+
19	*Klebsiella pneumoniae*_40	0	16	0	NA	0	0	0	12	0	0	NA	+
20	*Klebsiella pneumoniae*_41	0	9	0	NA	NA	0	0	0	0	0	NA	+
21	*Salmonella typhi*_11 (NARS)	21	25	22	NA	0	24	24	24	22	24	NA	-
22	*Salmonella* spp._12 (NARS)	25	29	24	NA	7	22	26	23	23	22	NA	+
23	*Salmonella paratyphi*_24 (NARS)	22	25	23	NA	0	16	24	18	19	27	NA	-
24	*Salmonella typhi*_37 (NARS)	22	24	22	NA	10	9	22	22	15	25	NA	+
25	*Citrobacter* spp._3	20	9	7	NA	0	0	12	0	0	0	NA	+
26	*Citrobacter* spp._4	18	0	11	NA	0	0	15	0	0	0	NA	+
27	*Proteus* spp._1	19	11	21	NA	0	0	15	0	16	16	NA	+
28	*Enterobacter aerogens*_16	18	7	8	NA	0	0	7	0	0	0	NA	+
29	*Shigella* spp._19	15	15	22	NA	0	0	21	13	0	0	NA	+
30	*Pseudomonas* spp._13	24	30	22	10	0	0	22	22	17	0	NA	-
31	*Pseudomonas* spp._14	21	0	0	0	0	0	18	0	0	0	NA	+
32	*Pseudomonas* spp._23	27	24	20	15	21	8	19	24	15	15	NA	-
33	*Pseudomonas aeruginosa*_27	28	29	24	12	9	0	23	20/29	19	20	NA	-
34	*Pseudomonas vulgaris*_35	17	26	20	0	21	0	18	26	14	16	NA	-
35	*Pseudomonas aeruginosa*_36	23	28	21	0	0	0	26	24	19	9	NA	-
36	Methicillin resistant *S. aureus*_5 (MRSA)	20	19	9	18	0	0	20	10	0	0	0	+
37	Methicillin sensitive *S. aureus*_6 (MSSA)	18	18	20	17	8	10	22	15	10	10	16	+
38	*Staphylococcus aureus*_26 (ATTC 25923)	24	31	34	17	10	30	20	25	26	28	NA	+
39	*Providencia* spp._2	0	11	0	0	0	0	0	0	0	0	NA	+
40	*Burkholderia cepacia* complex_20	0	0	0	0	0	0	0	0	0	0	NA	+
41	*Acenatobacter* spp._22	27	27	23	17	22	0	26	22	13	0	NA	+

Color codes Resistant, Intermediate, Sensitive, Not tested / Reference data not available. Numerical value inside differently colored boxes represent bacterial lysis zone in 'mm', NA represents 'Not tested' and or reference data 'not available', 'p' after numerical value represents partial lysis and a fractional number represents inner and outer lysis zone respectively.

4.2 Phage manipulations

4.2.1 Phage screening

Initial screening of sewage/river water from different rivers of Kathmandu valley (Bagmati river, Bishnumati river, Dhobi-khola, Balkhu-khola) revealed that, all the river water harbor phages against different genus of MDR bacteria (human pathogens) especially against family Enterobacteriaceae like *Escherichia* spp., *Klebsiella* spp., *Salmonella* spp., *Shigella* spp. and *Citrobacter* spp. (Table 4.2 and Figure 4.2).

Altogether 34 plates were positive in initial screening which were then further processed. This clearly proves that waters from rivers of Kathmandu are heavily contaminated with bacterial pathogens of human origin, as phages are 'strictly specific' to bacterial host strains and a phage that infects and replicates inside an environmental strain is extremely unlikely to infect a human pathogen which may have entirely different receptors. Additionally, numerous plaques with different morphology and lytic ability were also observed in a single plate (i.e. a single water sample had more than one type of phages that could infect and lyse a single bacterial host strain), clearly implying that numerous phages per host were present in the water sample.

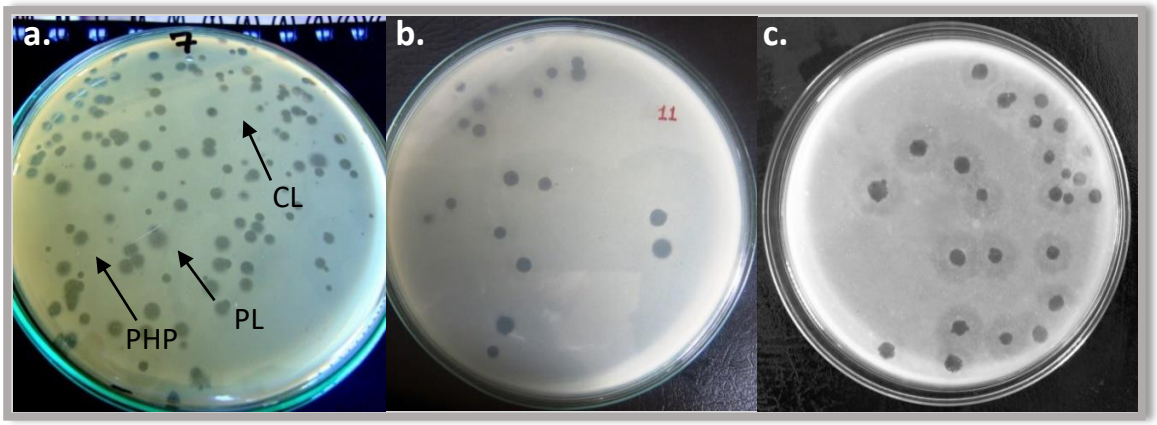


FIGURE 4.2 | Bacteriophage screening. Three representative plates showing different types of plaques – formed by lytic activity of phage – against three different bacterial hosts. **a)** More than one types (complete lysis - CL, partial lysis - PL, pin-head plaques - PHP et cetera) of plaques are isolated on same host, **b)** Similar plaque on a plate but without prominent halo zone and **c)** A single type of plaque with noticeable halo zone.

The results thus suggest presence of 'lytic phages' in astoundingly high numbers in our sewage mixed river waters that could kill drug resistant pathogens of human origin. As this study does not consider presence of 'lysogenic phages' that do not produce visible plaques, the number may even be higher. Presence of phages that can kill drug resistant bacterial strains in such voluminous amount implies that drug resistant strains spilled in the environment are extensively controlled by their natural predators - bacterial virus - that could have impacted the spread of disease outbreaks in Kathmandu valley.

4.2.2 Phage isolation, purification, amplification and storage

As stated earlier, this research was entirely focused on isolation and characterization of potentially therapeutic phages, so only lytic phages that successfully formed clear plaques (round clear zones in bacterial lawn formed by phage infection, lysis and propagation) were selected for purification. Relying to the above agenda, 34 phages were selected for further study (only one from each plate, however some plates contained phages with different plaque morphology) based on plaque morphology, purified and amplified. As the isolation was entirely based on visual analysis of lytic zones (plaques) against confluent bacterial lawn culture, we were able to isolate only lytic phages that formed clearly visible plaques and all the lysogenic phages (may present) that do not form visible plaques were unintentionally omitted from the study. This further justifies our objective of isolating phages of potential therapeutic usage, because it is lytic phages that can infect and lyse the bacterial host at the end of its life cycle.

Visually similar plaques (not identical, as plaque size may vary on other factors like thickness of agar and uneven distribution of host culture) were observed in plates cultured to check the purity of isolated phages. This indirectly confirmed that the phages were of same origin and thus rectified the process of purification.

A total of 34 phages were isolated from 5 water samples using 26 different bacterial strains representing 12 genera as hosts. Among them, 16 were *Escherichia* phage, 13 were *Salmonella* phage, 2 were *Shigella* phage and *Klebsiella* phage each and only one was *Citrobacter* phage. Contrary to our expectation, phages were not isolated against other bacterial strain such as *Pseudomonas* spp., *S. aureus*, *Enterobacter* spp., *Proteus* spp., *Providencia* spp., *Burkholderia* spp., and *Acenatobacter* spp. although various study have reported presence of phages against these bacterial group in sewage water. Phages were isolated from all water samples against 3 bacterial strains namely *Salmonella typhi*_11, *E. coli*_18 and *Salmonella paratyphi*_24. This implies that all river waters are heavily contaminated with these 3 bacterial strains. Further, 4 water sample contained phages against *E. coli*_7 and *E. coli*_21 rectifying the fact that our river water contains more coliforms. Also 2 phages against *Shigella* spp._19 and *Klebsiella* spp._10 each were isolated which indirectly proved presence of those bacteria in river water. A single phage against *Citrobacter* spp._4 was also obtained. Presence of phages against 5 different genera (out of 12 genera) of bacteria (*Escherichia, Salmonella, Shigella, Klebsiella* and *Citrobacter*) indicated abundancy of phages in river water (Table 4.2 & Table 4.3) representing these genus of bacteria.

TABLE 4.2 | Phage Screening against all 26 different bacterial strains using 5 sewage/water samples collected from rivers of Kathmandu valley.

SN	Bacterial strains	Bacterial code	Sewage/water sample					Total phages / strain
			G	K	B	O	P	
1	*Proteus spp._1*	Pro1						**0**
2	*Providencia* spp._2	Prv2						**0**
3	*Citrobacter* spp. _3	Cit3						**0**
4	*Citrobacter* spp. _4	Cit4						**1**
5	MRSA_5	SA5						**0**
6	MSSA_6	SA6						**0**
7	*Escherichia coli_7*	EC7						**5**
8	*Escherichia coli_8*	EC8						**1**
9	*Klebsiella* spp_9	Kle9						**0**
10	*Klebsiella* spp._10	Kle10						**2**
11	*Salmonella typhi_11*	ST11						**5**
12	*Salmonella* spp._12	Sal12						**3**
13	*Pseudomonas* spp._13	Pse13						**0**
14	*Pseudomonas* spp._14	Pse14						**0**
15	*Escherichia coli*_15	EC15						**0**
16	*Enterobacter aerogens_16*	EA16						**0**
17	*Escherichia coli_17*	EC17						**1**
18	*Escherichia coli_18*	EC18						**5**
19	*Shigella* spp._19	Shi19						**2**
20	*Burkholderia cepacia* complex_20	BC20						**0**
21	*Escherichia coli_21*	EC21						**4**
22	*Acenatobacter* spp._22	Ace22						**0**
23	*Pseudomonas* spp._23	Pse23						**0**
24	*Salmonella paratyphi_24*	SP24						**5**
25	*Escherichia coli* ATTC 25922_25	EC25						**0**
26	*Staphylococcus aureus* ATCC 25923_26	SA26						**0**
	Total phages isolated / water sample		**6**	**6**	**8**	**8**	**6**	**34**

Thirty-four plates were positive (16 against *Escherichia* spp., 13 against *Salmonella* spp., 2 against *Shigella* spp., 2 against *Klebsiella* spp. and 1 against *Citrobacter* spp.) in initial phage screening. Only one phage per plates was selected for further study, so this table only accounts one phage/plate/water sample although this may not be technically true, because more than one type of phage was observed in a single plate as shown in Figure 4.2.

Only one phage per plate / host were selected for further processing. Most common phages were against *Escherichia* spp. and *Salmonella* spp., implying our river waters are heavily contaminated with these two bacterial strains.

Although not studied, presence of phages against drug resistant human pathogens in such great numbers could have direct impact on controlling the number of such pathogens in environment and this may be the reason why, despite poor sanitary practices, Kathmanduities have not suffered a major disease outbreak.

TABLE 4.3 | Plaque record of all positive plates from initial screening.

SN	Water Sample	Host Bacteria	Total plaques	Types / how many of each?	Diameter of plaque (in mm ±0.4)	Plaque Opacity
1	**Gongabu / G Bishnumati river**	*Escherichia coli_7*	9	2 / 7, 2	pinhead – 3.0	all clear
2		*Salmonella typhi_11*	22	2 / 21, 1	pinhead – 4.0	all clear
3		*Salmonella* spp._12	15	3 / 10, 3, 2	pinhead – 4.0	12 - clear 3 - turbid
4		*Escherichia coli_18*	84	2 / 81, 3	pinhead – 9.0	28 - clear 56 - turbid
5		*Escherichia coli_21*	TMTC	1 / TMTC	pinhead – 2.0	all clear
6		*Salmonella paratyphi_24*	TMTC	1 / TMTC	not isolated	all clear
7	**Kalanki / K Balkhu-khola**	*Escherichia coli_7*	168	2 / 139, 29	pinhead – 4.0	all clear
8		*Salmonella typhi_11*	25	1 / 25	2.0 – 5.0	all clear
9		*Salmonella* spp._12	23	1 / 23	2.0 – 5.0	all clear
10		*Escherichia coli_18*	TMTC	3 / TMTC	pinhead – 3.0	clear + turbid
11		*Escherichia coli_21*	22	2 / 21, 1	pinhead – 3.0	1 - clear 21 - turbid
12		*Salmonella paratyphi_24*	13	1 / 13	5.0 – 12.0	all turbid
13	**Balkhu / B Bagmati river**	*Citrobacter* spp. _4	102	2 / 94, 8	pinhead – 4.0	8 - big, clear 94 - turbid
14		*Escherichia coli_7*	26	2 / 16, 10	pinhead – 5.0	10 - clear 16 - turbid
15		*Escherichia coli_8*	TMTC	1 / TMTC	pinhead – 3.0	all turbid
16		*Klebsiella* spp._10	TMTC	2 / TMTC, 5	pinhead – 2.0	5 - clear
17		*Salmonella typhi_11*	19	1 / 19	2.0 – 4.0	all clear
18		*Escherichia coli_18*	29	2 / 17, 2	pinhead – 3.0	all clear
19		*Shigella* spp._19	112	2 / 95, 17	pinhead – 3.0	17 - clear 95 - turbid
20		*Salmonella paratyphi_24*	137	1 / 137	1.0 – 5.0	all turbid
21	**Chabahill / O Dhobi-khola**	*Escherichia coli_8*	37	1 / 37	pinhead – 3.0	all clear
22		*Klebsiella* spp._10	TMTC	1 / TMTC	isolated plaques not visible	plate was nearly clear
23		*Salmonella typhi_11*	78	2 / 62, 16	pinhead – 5.0	16 - clear 62 - turbid
24		*Escherichia coli_17*	22	1 / 22	pinhead – 3.0	all turbid
25		*Escherichia coli_18*	TMTC	1 / TMTC	TMTC	all clear
26		*Shigella* spp._19	TMTC	2 / TMTC	pinhead – 4.0	clear + turbid
27		*Escherichia coli_21*	156	2 / 136, 20	pinhead – 3.0	clear + turbid
28		*Salmonella paratyphi_24*	141	1 / 141	pinhead – 1.0	all turbid
29	**Pulchowk / P Bagmati river**	*Escherichia coli_7*	32	2 / 22, 10	pinhead – 3.0	10 - clear 22 - turbid
30		*Salmonella typhi_11*	4	1 / 4	pinhead – 3.0	all clear
31		*Salmonella* spp._12	103	1 / 103	pinhead – 5.0	all turbid
32		*Escherichia coli_18*	TMTC	1 / TMTC	pinhead – 1.0	all turbid
33		*Escherichia coli_21*	14	1 / 14	pinhead – 2.0	all turbid
34		*Salmonella paratyphi_24*	26	2 / 19, 7	pinhead – 4.0	all clear

Different characteristics of plaques suggest that our river water harbors variety of phages that are capable of lysing drug resistant human pathogens. The word 'pinhead' implies that the plaques were too small to be measured with available scaling technique and TMTC refers to 'too many to count'.

***** *As it was strenuous to determine size (diameter) of each plaque, record of smallest and largest have only been recorded/shown.*

TABLE 4.4 | List of phages isolated from different water samples.

SN	Water Sample	Bacterial host strains	Phage name/code	Total phages per water sample
1	**Gongabu / G Bishnumati river**	*Escherichia coli_7*	phage TU_EC7G	**6**
2		*Salmonella typhi_11*	phage TU_ST11G	
3		*Salmonella* spp._12	phage TU_Sal12G	
4		*Escherichia coli_18*	phage TU_EC18G	
5		*Escherichia coli_21*	phage TU_EC21G	
6		*Salmonella paratyphi_24*	phage TU_SP24G	
7	**Kalanki / K Balkhu-khola**	*Escherichia coli_7*	phage TU_EC7K	**6**
8		*Salmonella typhi_11*	phage TU_ST11K	
9		*Salmonella* spp._12	phage TU_Sal12K	
10		*Escherichia coli_18*	phage TU_EC18K	
11		*Escherichia coli_21*	phage TU_EC21K	
12		*Salmonella paratyphi_24*	phage TU_SP24K	
13	**Balkhu / B Bagmati river**	*Citrobacter* spp. _4	phage TU_Cit4B	**8**
14		*Escherichia coli_7*	phage TU_EC7B	
15		*Escherichia coli_8*	phage TU_EC8B	
16		*Klebsiella* spp._10	phage TU_Kle10B	
17		*Salmonella typhi_11*	phage TU_ST11B	
18		*Escherichia coli_18*	phage TU_EC18B	
19		*Shigella* spp._19	phage TU_Shi19B	
20		*Salmonella paratyphi_24*	phage TU_SP24B	
21	**Chabahill / O Dhobi-khola**	*Escherichia coli_8*	phage TU_EC8O	**8**
22		*Klebsiella* spp._10	phage TU_Kle10O	
23		*Salmonella typhi_11*	phage TU_ST11O	
24		*Escherichia coli_17*	phage TU_EC17O	
25		*Escherichia coli_18*	phage TU_EC18O	
26		*Shigella* spp._19	phage TU_Shi19O	
27		*Escherichia coli_21*	phage TU_EC21O	
28		*Salmonella paratyphi_24*	phage TU_SP24O	
29	**Pulchowk / P Bagmati river**	*Escherichia coli_7*	phage TU_EC7P	**6**
30		*Salmonella typhi_11*	phage TU_ST11P	
31		*Salmonella* spp._12	phage TU_Sal12P	
32		*Escherichia coli_18*	phage TU_EC18P	
33		*Escherichia coli_21*	phage TU_EC18P	
34		*Salmonella paratyphi_24*	phage TU_SP24P	
		Total phages isolated from 5 water samples		***34***

Thirty-four phages were isolated in total from 5 different water samples collected from different rivers (holy) within Kathmandu valley using 26 bacterial strain (from 12 genus) as host.

All of phages isolated were against bacterial species of Enterobacteriaceae family like *Escherichia* spp., *Salmonella* spp., *Klebsiella* spp., *Shigella* spp. and *Citrobacter* spp. Presence of phages against fecal coliforms (more accepted as 'thermotolerant coliform') like *E. coli* and *Salmonella* is an indirect indicator that the water in rivers are contaminated with feces/fecal coliform of human origin.

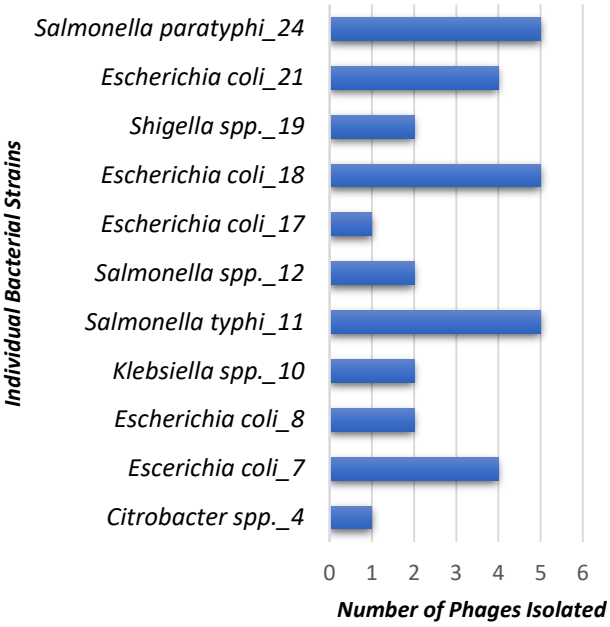


FIGURE 4.3 | Total phages isolated per individual host strain. Five phages were isolated against *Salmonella typhi*_11, *Escherichia coli*_18 and *Salmonella paratyphi*_24 each. 4 phages were isolated against *Escherichia coli*_7 and *Escherichia coli*_21 each. 2 phages were isolated against *Escherichia coli*_8, *Klebsiella* spp._10, *Salmonella* spp._12, *Shigella* spp._19 each. Similarly, a single phage was isolated against *Citrobacter* spp._4 and *Escherichia coli*_17 each.

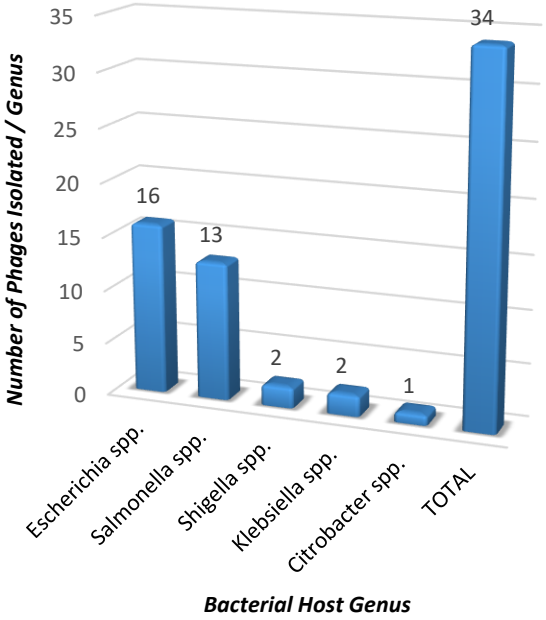


FIGURE 4.4 | Total phages isolated per individual host genus. Sixteen phages were isolated against *Escherichia* spp., 13 against *Salmonella* spp., 2 against *Shigella* spp., and *Klebsiella* spp. each and a single phage was isolated against *Citrobacter* spp. Contrary to our expectations, no any phages were found against *Pseudomonas* spp. and *Staphylococcus* spp.

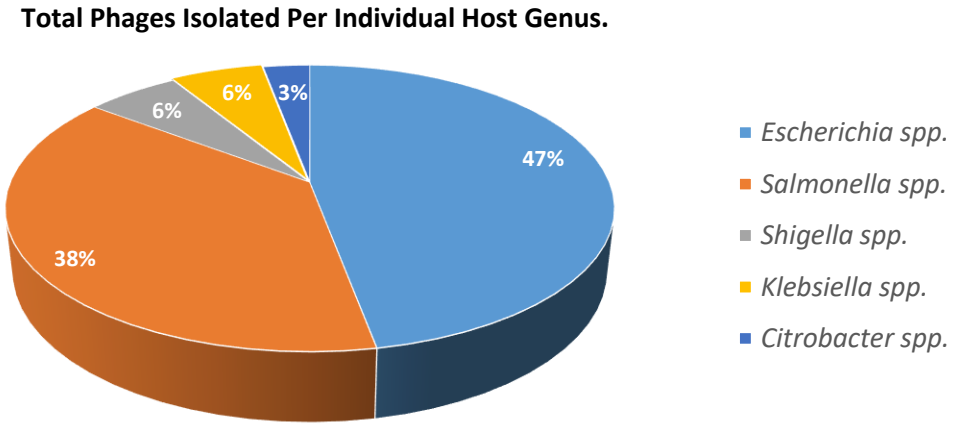


FIGURE 4.5 | Percentage of phages isolated per host genus. Highest 47% of phages were isolated against *Escherichia* spp., 38% of phages were against *Salmonella* spp. 6% of isolated phages were against *Shigella* spp. and *Klebsiella* spp. each and finally 3% of phage was isolated against *Citrobacter* spp.

4.2.3 Multiple Host-Range (MHR) spectrum / Spot assay

Spot assay of all 34 phages on different bacterial cultures (within same genus) revealed that all but two *Klebsiella* phages had multiple host range. Here, we have arbitrarily assumed strains from different patients to be different which may not be necessarily true. The assumption is rationalized by difference in antibiotic resistance pattern and different plaque characteristics formed by same phage. However, more reliable approaches like 16S rRNA PCR/sequencing, restriction digestion analysis of genomic/plasmid DNA are more appropriate and thus recommended for differentiating the bacterial strains whenever possible.

4.2.3.1 MHR spectrum of *Escherichia* phages

Spot assay of *Escherichia* phages was performed against all available (15) strains of *Escherichia* genus. All sixteen *Escherichia* phages (100 %) possessed multiple host-range (were able to lyse multiple strains) with a mean value of 7.0 (actual mean = 6.875) bacterial strains/phage out of 15 tested strains. The most potent phages were able to lyse 8 out of 15 (53.33 %) different strains of bacteria (Table 4.5). Multiple host range is highly desirable property in phage therapy because broader the host range broader will be the infectivity of given phage.

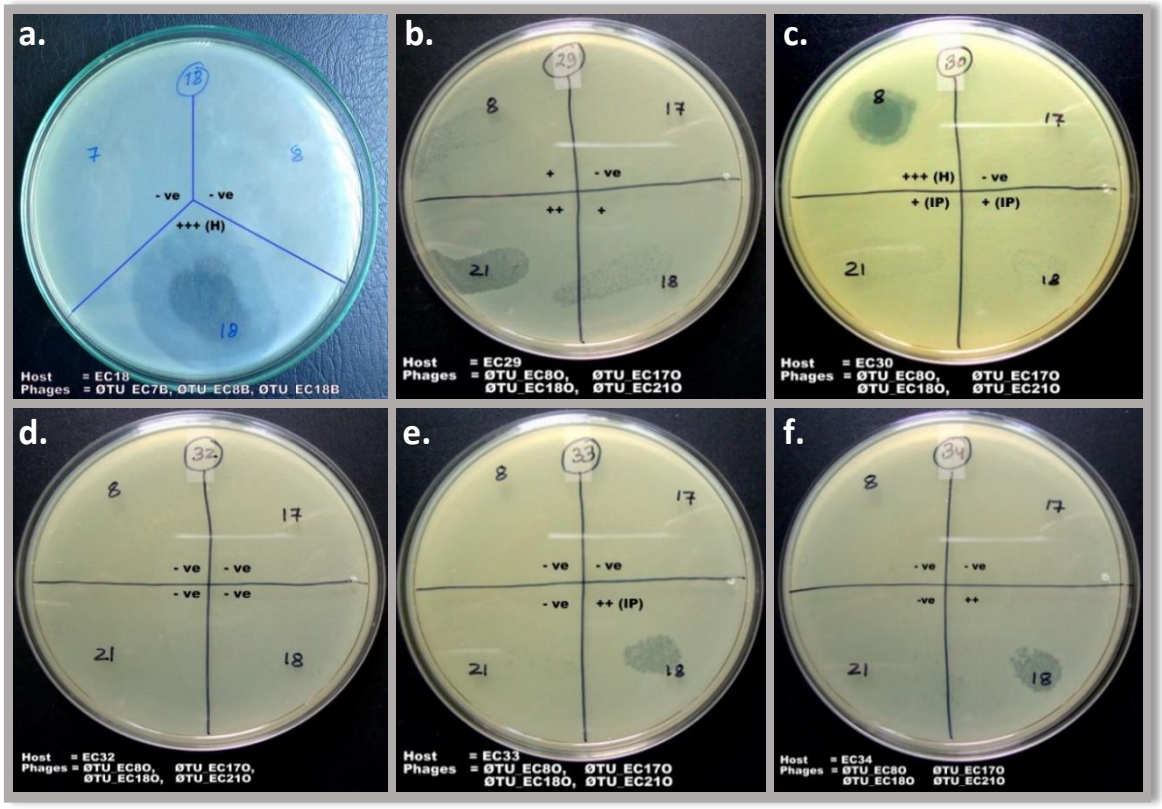


FIGURE 4.6 | Spot testing of *Escherichia* phages showing various lysis efficiency. Multiple host-range assessment of purified *Escherichia* phages revealed broad host range of Escherichia phages with varying efficiency. Figures above shows the varying lytic efficiency of different phages during spot assay.

TABLE 4.5 | Multiple host-range (MHR) analysis of all (16) isolated *Escherichia* phages against all (15) available strains of same genus.

SN	Ø STRAINS	BACTERIAL STRAINS (15 available strains of same genus)														Host Range (HR)
		EC 7	EC 8	EC 15	EC 17	EC 18	EC 21	EC 25	EC 28	EC 29	EC 30	EC 31	EC 32	EC 33	EC 34	EC 38	
1	ØTU_EC7G	+++	-	-	+	-	-	++	+	-	++	-	-	+ (S)	++	-	7
2	ØTU_EC18G	+	-	+	+	+++ (H)	-	+	++	-	-	-	-	+	+	-	8
3	ØTU_EC21G	-	-	++	-	-	+++	-	-	++	+++ (H)	++	-	+++	-	+	7
4	ØTU_EC7K	+++	++	-	-	++	-	-	++	-	++	+	-	+	+	-	8
5	ØTU_EC18K	+	++	-	-	+++	+++	+	++	-	++	-	-	-	-	-	7
6	ØTU_EC21K	-	-	-	-	-	+++	+	+++	+	-	-	-	+	-	+	6
7	ØTU_EC7B	+++ (H)	+++	-	-	-	-	-	-	-	++	-	-	-	-	-	3
8	ØTU_EC8B	+	+++ (H)	-	-	-	++	++	-	++	+++	+	-	-	-	-	7
9	ØTU_EC18B	+++	-	-	-	+++ (H)	++	+	-	++	+	+	-	-	-	-	7
10	ØTU_EC8O	+++	+++ (H)	-	-	++	++	-	-	+	+++ (H)	-	-	-	-	-	6
11	ØTU_EC17O	+	-	+	+++	+	-	+++	-	-	-	-	-	-	-	+++	6
12	ØTU_EC18O	-	-	+++	-	+++ (H)	+++	-	++	+	+ (IP)	-	-	++ (IP)	++	-	8
13	ØTU_EC21O	+++ (H)	+++	-	-	+++	+++	-	-	++	+ (IP)	-	-	-	+	-	7
14	ØTU_EC7P	+++	-	-	+	++	++	+	+	++	-	-	-	+	-	-	8
15	ØTU_EC18P	-	-	-	+	+++	-	-	++	-	+++	+	-	++	+	-	7
16	ØTU_EC21P	+	-	++	+	-	+++	-	++	-	+	++	-	-	-	+	8
															Mean		**7.0**

All isolated *Escherichia* phages showed multiple host-range (i.e. were able to lyse other strains of bacteria than their initial host) with a mean value of 7 different bacterial strains per individual phage. **Symbol interpretation:** +++ = complete lysis, ++ = partial lysis, + = faint lysis, (H) = presence of halo zone around lysis zone, (IP) = small individual plaques inside lysis area.

Presence of *Escherichia* phage against pathogens of human origin in such abundancy suggests that our river waters are extensively polluted with human pathogens, probably through sewages and/or hospital effluents that are directly mixed into rivers without appropriate sterilization. Time and again, coliforms are reported from river waters as well as drinking waters sources of Kathmandu valley by news media (Wash-Media South Asia, August 10, 2012; Republica, August 21, 2015) and Prasai *et al.* (2007) which indirectly justifies the presence of phages against them in such water resources. However, we are astonished to find that the phages can act upon drug resistant clinical strains, implying that their host in water resources also may be drug resistant strains as phages are extremely specific in host recognition and infection.

Although not proven experimentally in this experimentation, we can infer from Table 4.5 that a cocktail of 'three or more *Escherichia* phages' can theoretically lyse 15 out of 16 (93.75 %) drug resistant *E. coli.* Only one strain of *E. coli* (EC32) was resistant to all the phages isolated. Thus, we can confidently say – phages against drug resistant strains are extremely common and efficient.

However, without large scale studies and extensively controlled trails, it would be unjustifiable to conclude about the 'effectiveness' on clinical application of phages.

4.2.3.2 MHR spectrum of *Salmonella* phages

Spot assay of *Salmonella* phages was performed against all available (4) strains of *Salmonella* genus to determine its multiple host-range. Spot assay of *Salmonella* phages showed that all 13 *Salmonella* phages possessed extremely efficient multiple host-range (was able to lyse multiple strains) with a mean value of 4.0 (actual mean = 3.92) bacterial strains/phage out of 4 tested strains. The most potent phages were able to lyse all 4 different strains (out of 4) of bacteria.

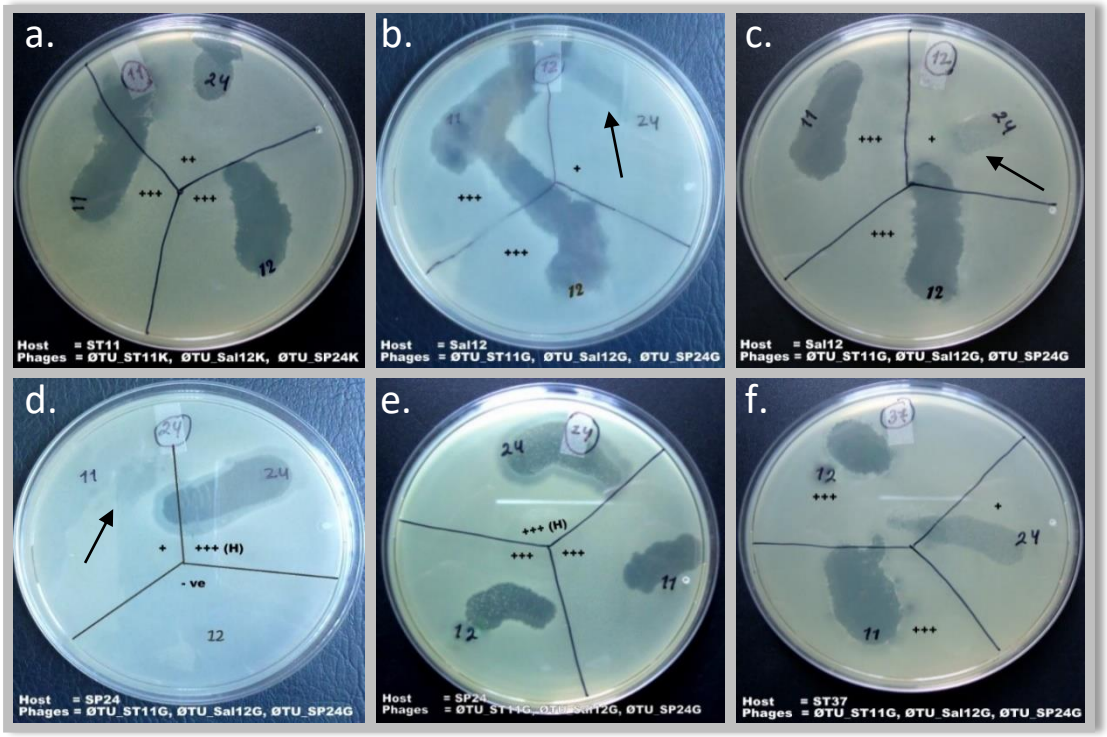


FIGURE 4.7 | Spot testing of *Salmonella* phages showing various lysis efficiency. Multiple host-range assessment of purified *Salmonella* phages reveals that phages of this group have multiple host range of varying efficiency. The arrows in the figure shows 'faint' lysis spots. The most significant result from the above figure is (e) where a host strain, *Salmonella typhi*_24 (NARS - Nalidixic acid resistant *Salmonella*) is lysed/killed by 3 different phages (phage TU_ST11G, phage TU_Sal12G and phage TU_SP24G).

TABLE 4.6 | Multiple host-range (MHR) analysis of all (13) isolated *Salmonella* phages against all (4) available strains of same genus.

SN	Ø STRAINS	BACTERIAL STRAINS (4 available strains of same genus)				Host Range
		ST11	Sal12	SP24	ST37	
1	ØTU_ST11G	+++ (H)	+++	+	+++	4
2	ØTU_Sal12G	++	+++	-	+++	3
3	ØTU_SP24G	+++	+	+++ (H)	++	4
4	ØTU_ST11K	+++	+++	++	+	4
5	ØTU_Sal12K	+++	+++	+++	+++	4
6	ØTU_SP24K	++	+	+++	+	4
7	ØTU_ST11B	+++	+++	++	+++ (H)	4
8	ØTU_SP24B	+	+	+++ (H)	++	4
9	ØTU_ST11O	+++	+++	+++	+++	4
10	ØTU_SP24O	+++	+	+++ (H)	++	4
11	ØTU_ST11P	+++	+++	+++	+	4
12	ØTU_Sal12P	+++	+++	+	+++ (H)	4
13	ØTU_SP24P	+	+	+++	+++	4
					Mean	**4.0**

All isolated *Salmonella* phages showed multiple host-range (i.e. were able to lyse other strains of bacteria than their initial host) with a mean value of 4 different bacterial strains per individual phage. **Symbol interpretation:** +++ = complete lysis, ++ = partial lysis, + = faint lysis, (H) = presence of halo zone around lysis spot.

The results from Salmonella phages are astonishing because of their wider host range and efficient lytic property. Although, available strains of Salmonella were few, all 13 (100 %) Salmonella phages showed extremely broad host range. Twelve out of thirteen (92.3 %) *Salmonella* phages were able to form lysis spot on all 4 different Salmonella strains implying that 'only' one phage could kill every *Salmonella* strains irrespective of their different serovar. A single phage could infect and lyse two different strains/serovars (*typhi* and *paratyphi*) implying phages can have broader host spectrum as antibiotics.

4.2.3.3 MHR spectrum of *Klebsiella* phages

Spot assay of *Klebsiella* phages was performed against all available (5) strains of *Klebsiella* genus. In contrast to our expectation, spot assay of *Klebsiella* phages revealed that both *Klebsiella* phages (phage TU_Kle10B and phage TU_Kle10O) were unable to lyse other strains beside their own primary host and thus did not possess multiple host-range (was unable to lyse multiple strains). Published reference data was not available for *Klebsiella* phage and thus we could not compare the result, but the result was in contrast to what we had observed in other phages in our own study, and suggested phages are 'extremely' host specific as professed in scientific arena. This property can be either 'boon or curse' depending upon how we perceive. However, personally I regard this as an 'undesirable'

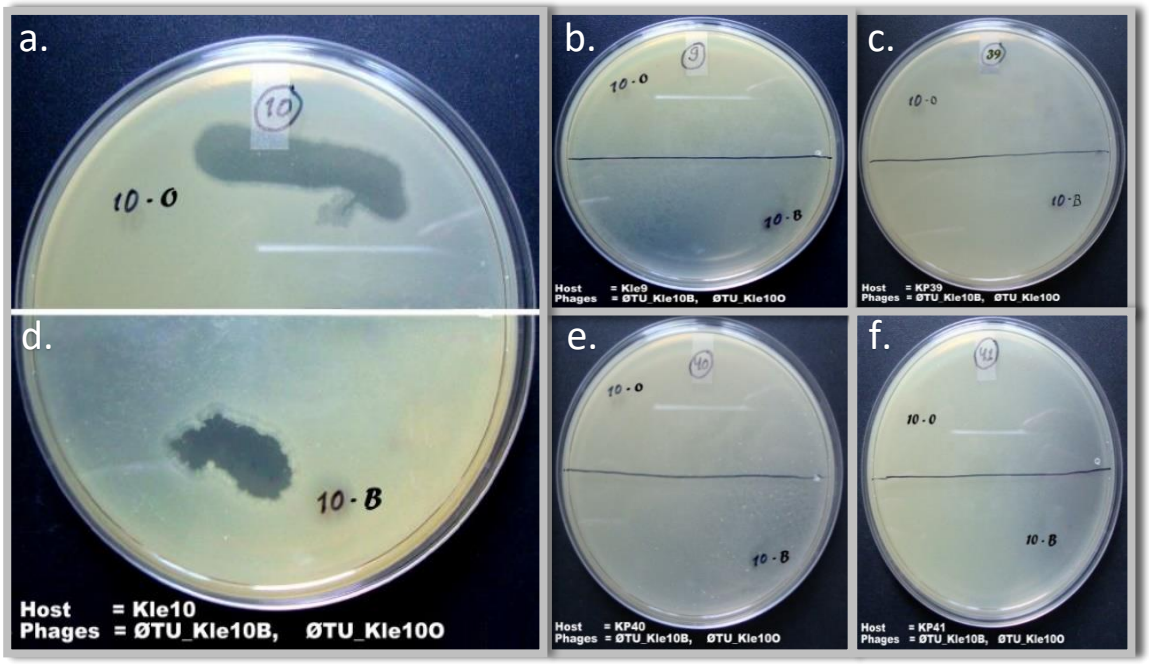


FIGURE 4.8 | Spot testing of *Klebsiella* phages showing extreme specificity. Multiple host-range assessment of purified *Klebsiella* phages reveals that phages of this group did NOT show multiple host range. In other words, Klebsiella phages (ØTU_Kle10B, ØTU_Kle10O) are extremely host specific and are not able to lyse strains other than their primary host from which they were isolated.

TABLE 4.7 | Multiple host-range (MHR) analysis of all (2) isolated *Klebsiella* phages against all (5) available strains of same genus.

SN	Ø STRAINS	BACTERIAL STRAINS (5 available strains of same genus)					Host Range
		Kle9	Kle10	KP39	KP40	KP41	
1	ØTU_Kle10B	-	+++	-	-	-	1
2	ØTU_Kle10O	-	+++	-	-	-	1

All isolated *Klebsiella* phages showed absence of multiple host-range (i.e. did not lyse other strains of bacteria than their initial host). **Symbol interpretation:** '+++' represents complete lysis, and '-' represents absence of lysis spot.

as our aim was to assess the therapeutic value of phages. Form therapeutic point of view, such specificity is not desirable, because when phages show higher specificity, every person should receive different phage that can kill the pathogen. This resembles a 'personalized medicine' specifically tailored to kill the specific pathogen which may be desirable but, this process requires an enormous phage library consisting specific phage for every specific strains. Library preparation of such diversity would itself be very daunting and further, finding an appropriate phage that can kill a specific phage would be 'finding needle in a haystack' when there is such high degree of specificity.

4.2.3.4 MHR spectrum of *Citrobacter* phages

Spot assay of a Citrobacter phage (phage TU_Cit4B) was performed against all available (2) strains of Citrobacter genus. Spot assay indicated that phageTU_Cit4B was able to partially lyse both (100 %) of the available strains and thus exhibited multiple host-range (Figure 4.9 and Table 4.8). However, the lytic property of phage TU_Cit4B was not satisfactory [therapeutically] as lysis zones were faint indicating inefficient lytic efficiency. But, a single phage was able to lyse both of the available drug resistant strains which is encouraging. No any reference data was available for comparison of multiple host-range for *Citrobacter* phage till summing up this manuscript. As only two strains were available for spot assay, statistical analysis was not deemed necessary.

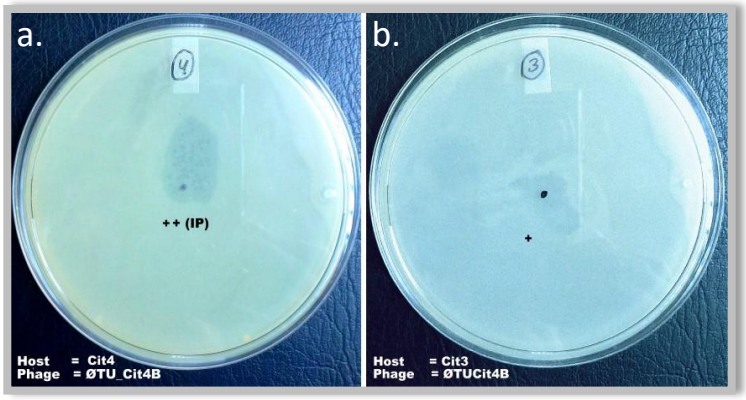


FIGURE 4.9 | Spot testing of only isolated *Citrobacter* phage. Multiple host-range assessment of purified *Citrobacter* phage (ØTU_Cit4B) against two available bacterial host of same genus *Citrobacter* spp._3 and *Citrobacter* spp._4. The phage ØTU_Cit4B was able to lyse both available *Citrobacter* strains (Cit3 and Cit4). However, the lysis spot is too faint suggesting that the phage may not be completely lytic.

TABLE 4.8 | Multiple host-range (MHR) analysis of all (1) isolated *Citrobacter* phage against all (2) available strains of same genus.

SN	Ø STRAIN	BACTERIAL STRAIN (2 available strains of same genus)		Host Range
		Cit3	Cit4	
1	**ØTU_Cit4B**	+	++ (IP)	**2**

Only isolated Citrobacter phage showed multiple host-range (i.e. was able to lyse other strains of bacteria than their initial host within same genus). **Symbol interpretation:** ++ = partial lysis, + = faint lysis and (IP) = small individual plaques inside lysis area.

The genus *Citrobacter* is distinct group of aerobic, gram negative bacilli from the Enterobacteriaceae family, widely distributed in water, soil, food and intestinal tract of man and animals. Urinary tract infections (UTIs) caused by *Citrobacter* species have been described in 5 to 12% of bacterial urine isolates in adults (Metri, 2013). As we can easily find phages against *Citrobacter*, phage therapy also has possibility in addressing infections caused by *Citrobacter* species including UTIs.

Two phages (ØTU_Shi19B and ØTU_Shi19O) were also isolated against *Shigella* spp., but since we only had one strain of *Shigella* spp., we could not perform MHR of *Shigella* phage. But, a spot testing on the host strain showed a complete lysis (+++). The presence of *Shigella* phage in river waters against MDR pathogen of human origin clearly implies that our river waters are also contaminated with *Shigella* spp. (of human origin) which is one of the major cause of Shigellosis – an infectious disease caused by a group of bacteria called *Shigella*. Most who are infected with *Shigella* develop diarrhea, fever, and stomach cramps starting a day or two after they are exposed to the pathogenic bacteria. (https://www.cdc.gov/shigella/)

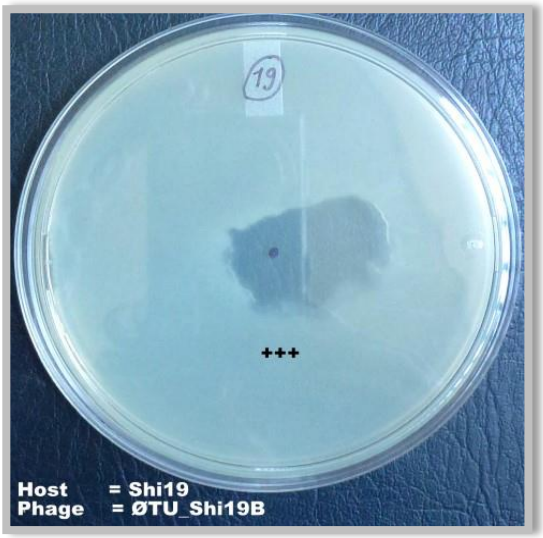


FIGURE 4.10 | Spot testing of isolated *Shigella* phage on host bacterium. The host Shi19 – a MDR Shigella (human pathogen) is completely lysed by a phage ØTU_Shi19B on spot testing.

Although, spot assay seems straightforward to perform in resource limited settings and convincible as well, a recent study has challenged the results from spot assay only and suggested to perform an additional Efficiency of Plating (EOP) test to confirm the host-range analysis (Mirzaei & Nilsson, 2015). It reports that spot assay overestimates the host range of phage and the results are also not in co-ordination with EOP analysis. The study further clarifies that no single study (spot assay or EOP) is sufficient enough to make concluding remarks on multi-host spectrum and thus also suggest to explore other definitive alternatives. As this study was already completed at the time of publication of the new finding, we could not adopt the suggestion in our study, but we highly recommend to verify the data obtained from spot assay by performing EOP test as well in future studies that includes spot assays.

4.2.4 Multi-generation infection efficiency of a phage and host bacteria / BIM analysis

Citrobacter phage (phage TU_Cit4B) was also not analyzed for multi-generation infection as it did not produce completely clear plates in its first cycle of infection and as such were unable to differentiate between partial lysis and newly emerged mutants. Multi-generation (up to 5 cycles) infection analysis of all other 33 phages revealed that all but two Klebsiella phages (phage TU_Kle10B and phage TU_Kle10O) were able to effectively lyse their host bacterium with same efficiency (as in the first generation) and did not give

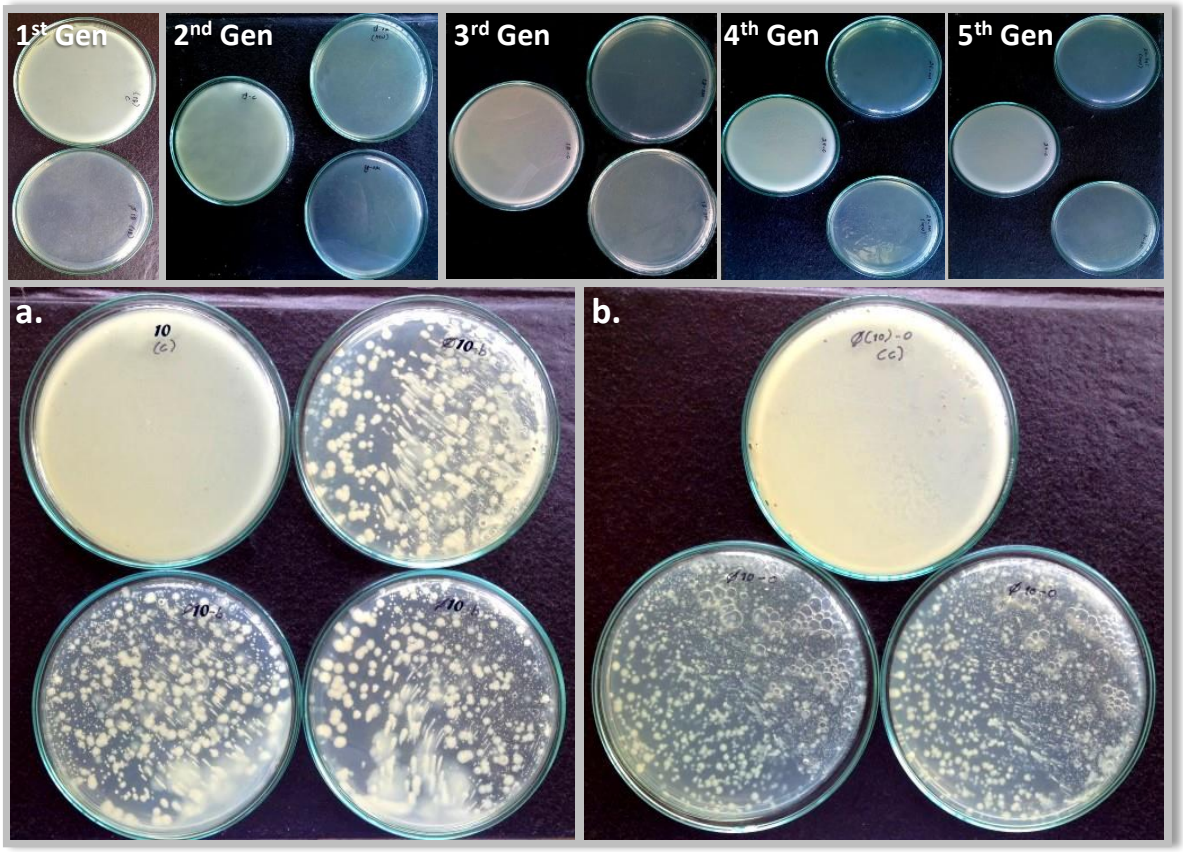


FIGURE 4.11 | Multi-generation infection analysis to report bacteriophage induced mutants. Upper row: A representative plate showing completely clear (lysed) plates indication the efficient infection and lysis of given phage (phage TU_EC18O) up to 5th generation. **Lower row: a)** Host bacteria Kle10 showing BIM against phage TU_Kle10B in its first generation and **b)** Host bacteria Kle10 showing BIM against phage TU_Kle10O in its first generation.
*The results for all other 31 phages showed clear plates up to 5th cycle (figures not shown) without induction of any mutants.

any BIM up to five subsequent cycles. Both *Klebsiella* phages did not produce completely lysed clear plates even in first generation suggesting that the bacteria had efficient mechanism to escape phage infection very quickly. Thus we can say that BIM were induced within first generation in case of both *Klebsiella* phages, which makes it extremely non-efficient for therapeutic purpose – our primary concern. However, hypothetically this problem could be minimized using 'phage cocktail' and/or a mix therapy (antibiotics + phage/phage cocktail) and thus may be a savior when a single phage is in-effective.

However, all other thirty-one phages showed similar infective and lytic ability up to five consecutive cycles (Table 4.9). As our primary concern was to report BIM (if any), all other negative pictures/results are not shown here.

TABLE 4.9 | Multi-generation infection efficiency of a phage and host bacteria/BIM analysis.

SN	Bacterial host	Phage	Emergence of BIM (up to 5th cycle)
1	*Citrobacter* spp. _4	phage TU_Cit4B	Not completely lytic
2	*Escherichia coli_7*	phage TU_EC7G	No
3		phage TU_EC7K	No
4		phage TU_EC7B	No
5		phage TU_EC7O	No
6		phage TU_EC7P	No
7	*Escherichia coli_8*	phage TU_EC8B	No
8	***Klebsiella* spp._10**	**phage TU_Kle10B**	**Yes**
9		**phage TU_Kle10O**	**Yes**
10	*Salmonella typhi_11*	phage TU_ST11G	No
11		phage TU_ST11K	No
12		phage TU_ST11B	No
13		phage TU_ST11O	No
14		phage TU_ST11P	No
15	*Salmonella* spp._12	phage TU_Sal12G	No
16		phage TU_Sal12K	No
17		phage TU_Sal12P	No
18	*Escherichia coli_17*	phage TU_EC17O	No
19	*Escherichia coli_18*	phage TU_EC18G	No
20		phage TU_EC18K	No
21		phage TU_EC18B	No
22		phage TU_EC18O	No
23		phage TU_EC18P	No
24	*Shigella* spp._19	phage TU_Shi19B	No
25		phage TU_Shi19O	No
26	*Escherichia coli_21*	phage TU_EC21G	No
27		phage TU_EC21K	No
28		phage TU_EC21O	No
29		phage TU_EC21P	No
30	*Salmonella paratyphi_24*	phage TU_SP24G	No
31		phage TU_SP24K	No
32		phage TU_SP24B	No
33		phage TU_SP24O	No
34		phage TU_SP24P	No

Lysogenic (prophage) and or BIM (as we cannot determine from our experiments) were only observed in case of one *Citrobacter* phage and two *Klebsiella* phages. All other phages produced completely lytic plates up to 5th cycle of host (subculture). This clearly indicated that phages are efficiently 'lytic' and thus holds possibility of being used in therapeutics.

4.2.5 Efficacy of selected lytic phages on lag, log and stationary phase of bacterial life cycle

Efficacy – infectivity and lytic ability of phage – testing of selected 7 phages on lag, log and stationary phase of bacterial life cycle revealed that except two of them (phage TU_Cit4B and phage TU_Kle10O) all other (phage TU_EC8O, phage TU_EC18O, phage TU_Shi19O, phage TU_SP24B) lysed the host bacteria in lag, log and stationary phase of their life cycle

efficiently. No any bacterial colonies were reported after plating the phage infected broth culture (24 hours at 37°C) in lag and log phase. However, during stationary phase, few mL extra media had to be added, as phages only infect actively multiplying host cells. The idea behind this test was to analyze the efficacy of phage on already chronic infections that may have developed a biofilm and/or older less metabolically active cultures. But, as we lacked the appropriate techniques, this test is not considered extremely reliable scientifically. This was why only 7 phages that were identified morphologically by TEM analyzed in this test. Although, small amount of media had to be added in case of stationary phase tubes, older cultures were also completely lysed by the phages (Figure 4.12 & Table 4.10). Clear plates in subsequent plating of phage infected stationary phase culture provides a preliminary information that, yes, phages can infect and lyse a revived stationary/inactive phase host cells. This can be of therapeutic value to control the chronic/old infection and keep the bacterial growth in check when infection has already spread.

Assessment of ability of phage to cross biofilm is of immense importance in phage therapy as some papers have reported phages being more efficient than antibiotic in treatment of pathogens forming biofilm like *Salmonella* spp., *Pseudomonas* spp. (specifically in cystic fibrosis infection), *E. coli*, *Staphylococcus epidermidis*., *Acenatobacter baumannii* to name few and as such, suggested to look into the applicability of phage therapy against biofilm forming pathogens (Azeredo & Sutherland, 2008; Ahiwale *et al.*, 2011; Thawal *et al.*, 2012; Tiwari *et al.*, 2013; Parasion *et al.*, 2014; Motlagh *et al.*, 2016). Noting the preliminary results of this study and the possibility phage holds in treatment of 'difficult-to-treat' biofilms, we recommend a more sophisticated quantitative experimental study to analyze the effects of phage on biofilms and also old, metabolically less active cultures.

Nonetheless, phage mediated clearance of older biofilms and metabolically inactive cultures can be challenging, though not necessarily impossible given the extended periods of treatment and/or repeated phage dosing/application. Alternatively, a study by Goldman *et al.* suggests on potential use of specific lytic phages to prevent ultrafiltration (UF) membrane biofouling using three bacterial model organisms (*Pseudomonas aeruginosa, Acinetobacter johnsonii* and *Bacillus subtilis* – separately and also combined) (Goldman *et al.*, 2009). Application of specific bacteriophages concept in other membrane processes such as nano-filtration and reverse osmosis, that encounter less bacterial species diversity, can be successful.

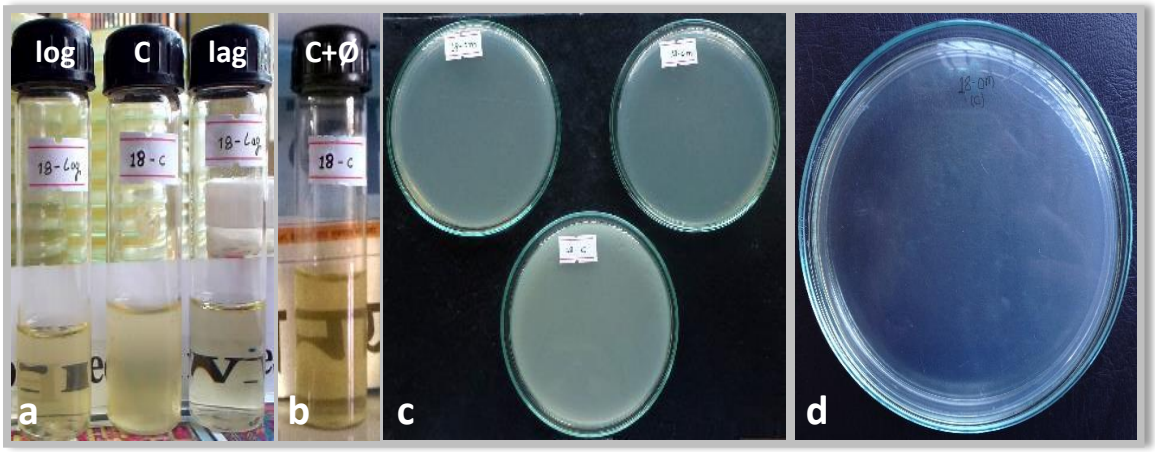


FIGURE 4.12 | Assessment of phage infection and lytic efficiency on lag, log and stationary phase of host's life cycle. a) Broth analysis showing lytic efficiency of phage TU_EC18O on lag and log phase of host's life cycle compared with a control in the middle tube. Host bacteria on both tubes on either side of control are completely lysed by phage TU_EC18O. **b)** The control tube from previous figure after inoculation of phage TU_EC18O and 24 hours' incubation at 37°C. As compared to control in fig. a, the tube is relatively clearer meaning bacterial lysis has occurred. **c)** Plates showing no bacterial growth from lag and log phase tubes (upper two plates). The lower one is control plate. **d)** Plates showing no bacterial growth from control tube after inoculation of phage.

FIGURE 4.13 | Assessment of phage infection and lytic efficiency on lag and log phase of host's life cycle. From left to right: control, lag and log phase of host's life cycle. **a)** phage TU_Cit4B **b)** phage TU_Kle10O. Both phages did not show complete lytic efficiency but partial lytic activity. The leftmost tube in both pictures (control) is more opaque than the rest of the tubes [two] right to it. This clearly shows that the lytic efficiency of those two phages were extremely low even in cases of actively growing phase of host's lifecycle. As this was undesirable, the tubes were not further processed. However, we cannot confidently say if it was because of partial lysis or induction of BIM (and its growth) that caused the turbidity.

TABLE 4.10 | Phage efficacy assessment on lag, log & stationary phase of host's life cycle.

SN	Phage	Host	Bacterial Growth after Phage Inoculation					
			In Broth			On Plates		
			Lag	Log	Stationary	Lag	Log	Stationary
1	phage TU_Cit4B	*Citrobacter* spp._4	+ ve	+ ve	NT	NT	NT	NT
2	phage TU_EC8O	*Escherichia* coli_8	- ve	- ve	- ve	- ve	- ve	- ve
3	phage TU_Kle10O	*Klebsiella* spp._10	+ ve	+ ve	NT	NT	NT	NT
4	phage TU_ST11B	*Salmonella typhi*_11	- ve	- ve	- ve	- ve	- ve	- ve
5	phage TU_EC18O	*Escherichia* coli_18	- ve	- ve	- ve	- ve	- ve	- ve
6	phage TU_Shi19O	*Shigella* spp._19	- ve	- ve	- ve	- ve	- ve	- ve
7	phage TU_SP24B	*Salmonella paratyphi*_24	- ve	- ve	- ve	- ve	- ve	- ve

**NT refers to 'not tested'. When growth occurred on lag and log phase of bacterial life cycle after inoculation of phage, the plates were not cultured on plates, as that was deemed un-necessary.*

Although we aimed to test the efficiency of lytic phage infection and its lytic capability on inactive bacterial culture and also biofilm, we were not satisfied with the approaches we used and because of insufficient amount of quantifiable data, we are not in a position draw a definitive conclusion about the efficiency of phage in inactive strains of bacteria. However, we recommend to apply better approaches with this test, as reports have claimed 'phage penetration' to be more effective in biofilm than the antibiotics.

4.3 Transmission Electron Microscopy (TEM)

Seven phages were selected for transmission electron microscopy (TEM) on the basis of multiple host-range and diversity. Transmission electron micrograph of selected phages revealed that all phages belonged to order Caudovirales – meaning tailed phages. The phages were classified according to the ICTV guidelines (Table 4.11 & Appendix – Figure AF2) (King *et al.*, 2012) and cross-checked for verification in online ICTV database (ictvonline.org/virusTaxonomy.asp). Online database was used instead of print release to incorporate the latest findings as recent print-release was made on 2012, but recent releases were made on 2015. Among seven Caudovirales phages, 3 belonged to Myoviridae, 2 belonged to Siphoviridae and 2 belonged to Podoviridae family (Figure 4.15). As most of the published literatures report 'tailed virus' to be abundant in nature, our result is coherent to the scientific data we have currently. However, this does not rule out the presence and abundancy of other 'tail-less' phages in the nature.

Although we could not perform electron microscopy of all 34 isolated phages, we can observe that out of 7 phages two belonged to Podoviridae family and both of them were not efficiently lytic in their activity during multi-generation infective analysis and lag, log and stationary phase infection analysis. To add to this, *Klebsiella* phages (one Podoviridae, next 'not analyzed') did not show any multiple host range and Citrobacter phage (only one, Podoviridae) too did not show satisfactory multiple host range. What we can suggest – but not confirm – from this analysis is that phages with better lytic capability tend to have longer tail implying that evolution of phages from 'tail-less to tailed' considering evolution increases the adaptability/infectivity of a virus to broader host range. From what we have observed, we can

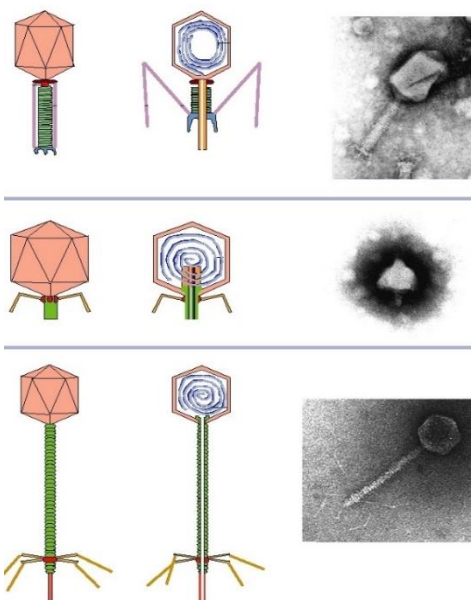


FIGURE 4.14 | Three [representative] tailed virus family from Order: Caudovirales. From up to down: Myoviridae, Podoviridae and Siphoviridae. From left to right: Outline structure, tentative cross section and electron micrograph.

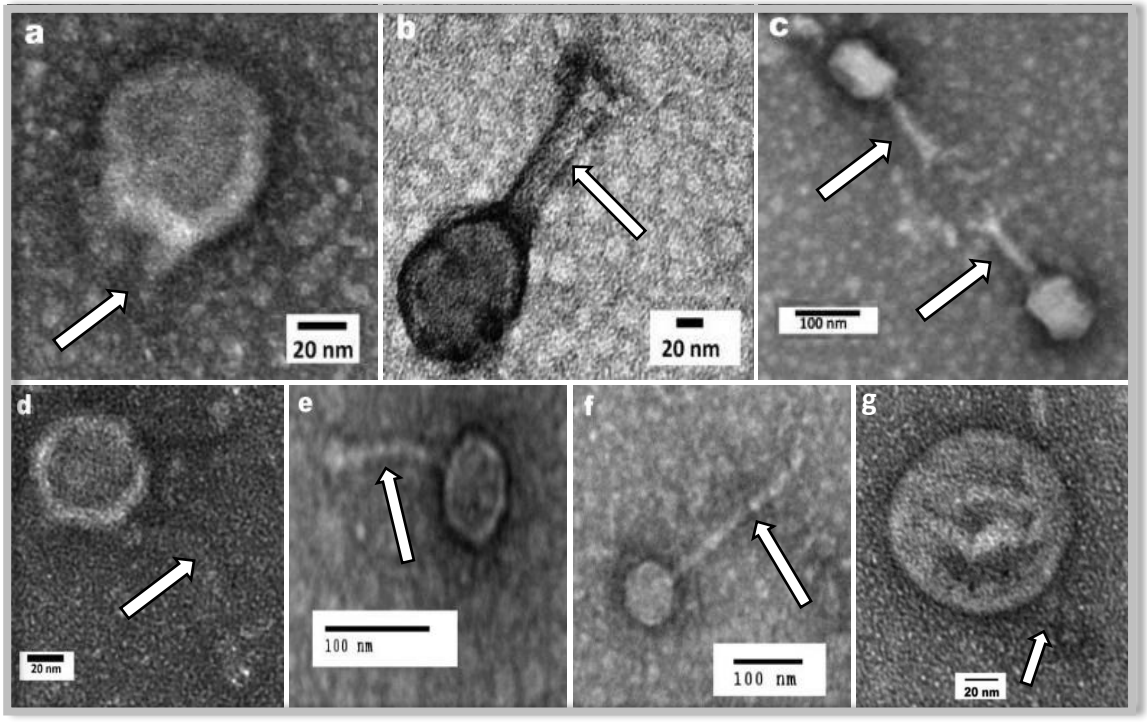


FIGURE 4.15 | Transmission Electron Microscopy (TEM) of selected phages (negatively stained). The micrograph reveals that all of the phages selected for TEM were tailed virus (Order: Caudovirales) **a)** phage TU_Cit4B, **b)** phage TU_EC8O, **c)** phage TU_EC18O, **d)** phage TU_Shi19O, **e)** phage TU_SP24B, **f)** phage TU_ST11B **g)** phage Kle10B. White arrow represents position of tail in each figure and the white rectangular box mentions the scale of individual figure.

comfortably say – for the first time – that 'Myoviridae and Siphoviridae' may be better in 'lytic' capability than 'Podoviridae'. However, we cannot confirm this as the study sample is not enough, and no reference data is available till finalizing this draft.

TABLE 4.11 | Classification of phages according to ICTV guidelines (9th report) based on transmission electron micrograph.

SN	Phage Name / Phage genus	Capsid (in nm*)	Tail (in nm*)		Shape	Order / Putative Family
			Width	Length		
1	phage TU_Cit4B / *Citrobacter* phage	73	11	13	icosahedral	Caudovirales / Podoviridae
2	phage TU_EC8O / *Escherichia* phage	82×99	25	109	elongated	Caudovirales / Myoviridae
3	phage TU_EC18O / *Escherichia* phage	82×108	19	111	elongated	Caudovirales / Myoviridae
4	phage TU_Shi19O / *Shigella* phage	60	8	124	icosahedral	Caudovirales / Siphoviridae
5	phage TU_SP24B / *Salmonella* phage	63	9	106	icosahedral	Caudovirales / Siphoviridae
6	phage TU_ST11B / *Salmonella* phage	73	16	236	icosahedral	Caudovirales / Myoviridae
7	phage TU_Kle10O / *Klebsiella* phage	99	18	34	icosahedral	Caudovirales / Podoviridae

* nm = nanometer

From above table (Table 4.11), what we can predict is that, although tail length is considered to be primary while phage classification, 'tail length' only cannot be the sole criteria for characterization of phages. Other factors such as 'capsid size' and 'capsid shape' also should be taken into consideration. The largest phage reported in this study was phage TU_EC18O (capsid = 82×108) which was elongated in shape with 111 nm tail attached to it and belonged to Myoviridae family. Similarly, phage with longest tail was phage TU_ST11B that had 236 nm long tail attached to 73 nm icosahedral head which also belonged to Myoviridae family. Among 7 phages, two phages had 'elongated' capsid while 5 phages were with 'icosahedral' capsid. As all three families (Myoviridae, Podoviridae and Siphoviridae) from Caudovirales order are dsDNA phages, all 7 phages in this study - which belonged to Caudovirales - is also considered to be dsDNA phages/bacterial virus. None of the phages had any envelope outside their capsid and according to ICTV guideline, all of these 7 phages must have a 'single linear genomic configuration' enclosed in a capsid/head bearing no outer envelope as all of these belonged to tailed virus - Order: Caudovirales.

According to our findings, phage TU_Cit4B (*Citrobacter* phage) belongs to Podoviridae family of Caudovirales order, but the online database of ICTV reports only one *Citrobacter* phage (with keyword #Citrobacter) that belongs to Siphoviridae family (Figure 4.16). So, the result reports an entirely different member of *Citrobacter* phage that belongs to Podoviridae family which opens a new avenue in the field of *Citrobacter* phage research adding/growing the database. Although ICTV database (as of November 18, 2016) does not report *Citrobacter* phage that belongs to Podoviridae family, a recent study by Hamdi *et al.* (2016) reports existence of various *Citrobacter* phages belonging to Podoviridae family. If taken appropriate steps to report and submit the findings, this could be an opportune moment for researchers coming from universities of developing country like Nepal for global recognition.

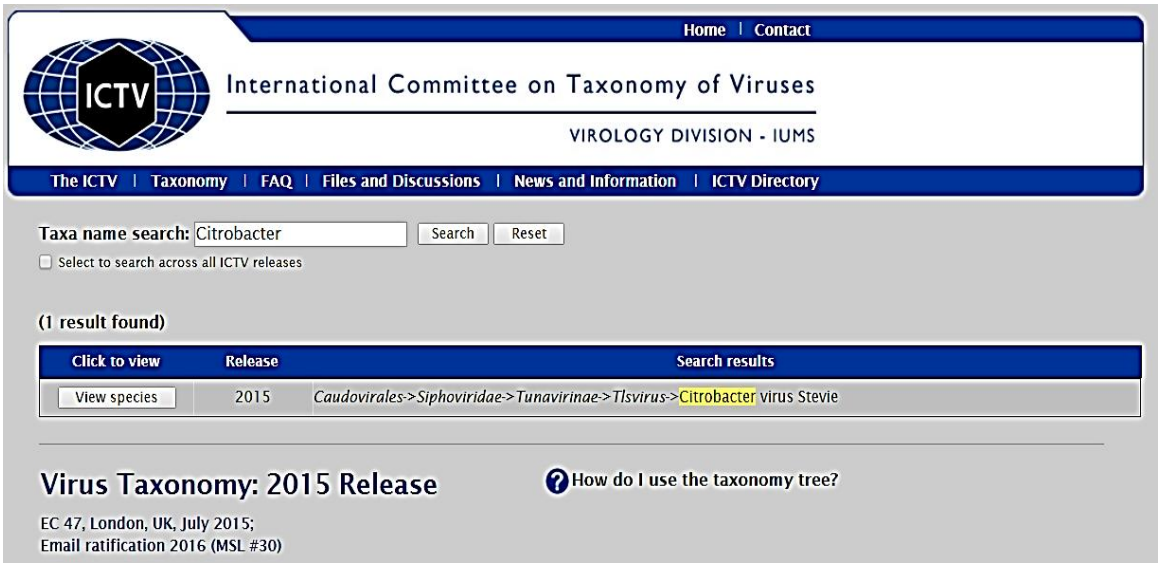


FIGURE 4.16 | ICTV online database search result for Citrobacter phage/virus. Only one *Citrobacter* phage was found/deposited in ICTV database that belongs to Siphoviridae family of order Caudovirales.

All other identified phages perfectly matched with the ICTV online database (Figure 4.17 to Figure 4.20) that have been described separately in the following paragraphs.

A similar database search for *Escherichia* phage with keyword #Escherichia resulted 133 matched output with all three types of phages – Podoviridae, Myoviridae and Siphoviridae – belonging to Caudovirales order along with many more belonging to unassigned order as well (Figure 4.17). Two *Escherichia* phages (phage TU_EC8O, phage TU_EC18O) in this study belonged to Myoviridae family of Caudovirales order, which has already been reported by ICTV with various entry. This justifies the findings of this study.

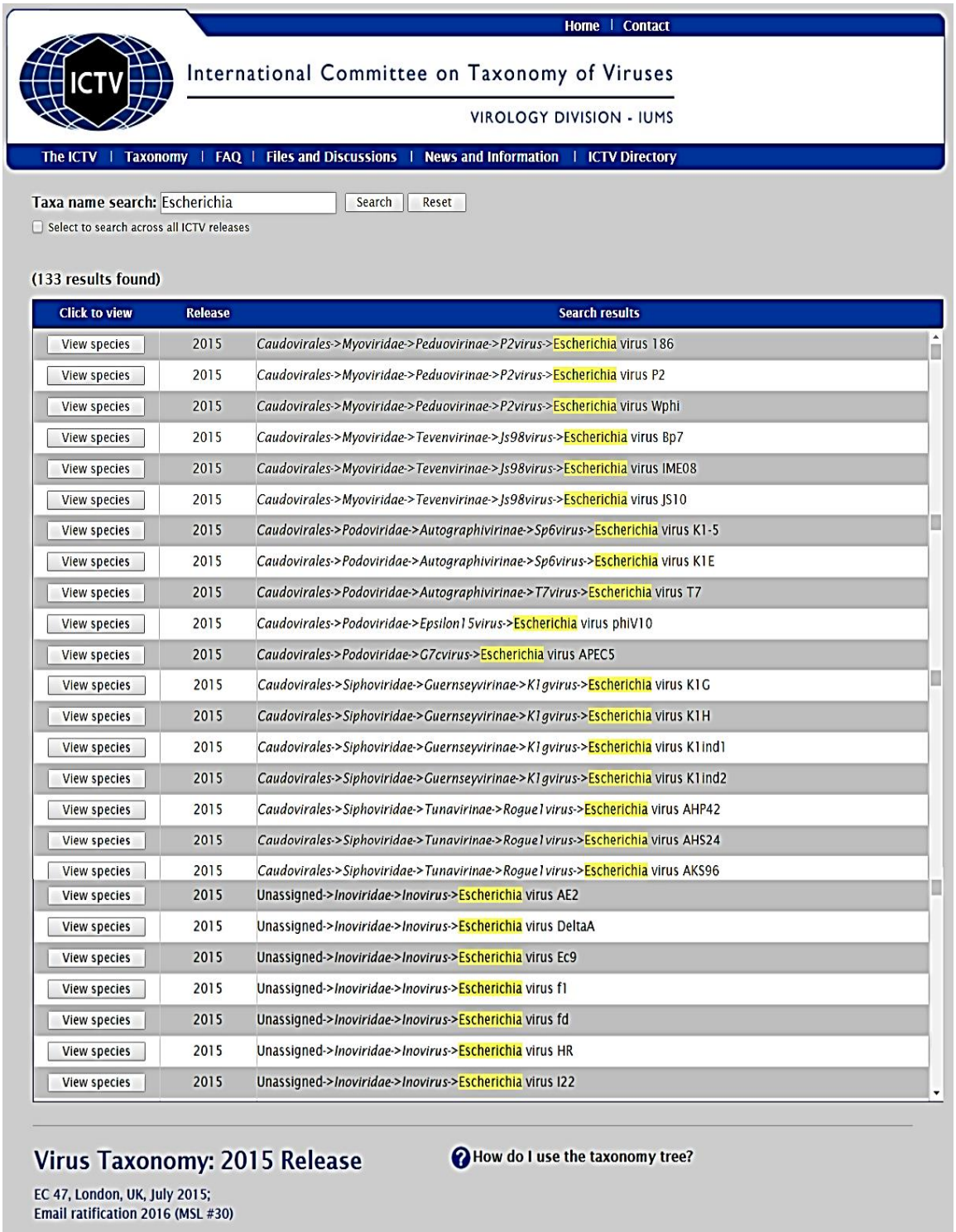


FIGURE 4.17 | ICTV online search results for Escherichia phage/virus. One hundred thirty-three *Escherichia* phages were found/deposited in ICTV database that belongs to all three Myoviridae, Podoviridae and Siphoviridae family of order Caudovirales. Also many phages are unassigned to any order, but still deposited in the database which may be tailless.
***Note:** All 133 results are NOT shown here for brevity.*

A similar search result for *Shigella* phage with keyword #Shigella in ICTV online database resulted in 10 matches with all three types of phages – Podoviridae, Myoviridae and Siphoviridae – belonging to Caudovirales order. According to the electron micrograph, the *Shigella* phage (phage TU_Shi19O) from our study belongs to Siphoviridae family (having long tube like tail) which has already been reported by ICTV. Hence, we can conclude with certainty that the bacterial virus that infected drug resistant *Shigella* belongs to Siphoviridae family.

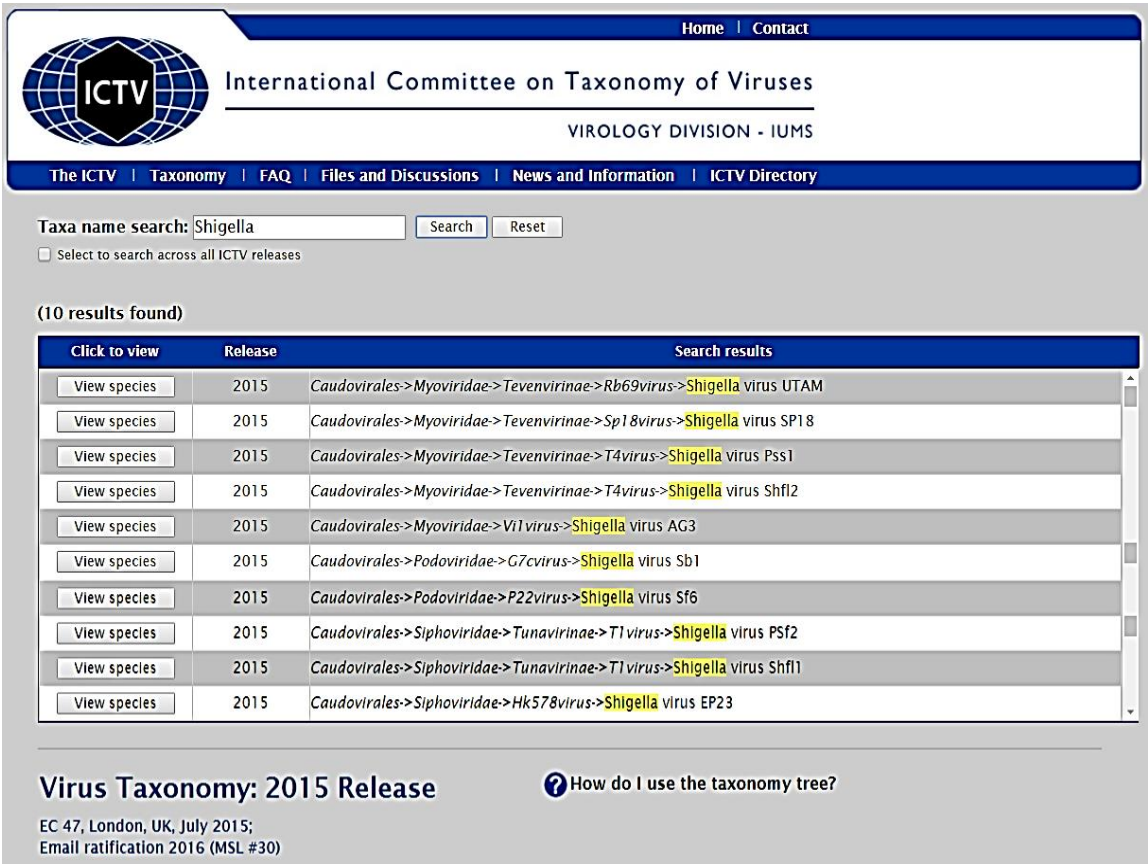


FIGURE 4.18 | ICTV online search results for Shigella phage/virus. Ten *Shigella* phages were found/deposited in ICTV database that belongs to all three Myoviridae, Podoviridae and Siphoviridae family of order Caudovirales.

Similarly, ICTV database search for *Salmonella* phage with keyword #Salmonella gave 52 matched output with all three types of phages – Podoviridae, Myoviridae and Siphoviridae – belonging to Caudovirales order along with many more belonging to unassigned order as well. This signifies the expansive diversity of *Salmonella* phages in nature. Two micrographs of *Salmonella* phage from this study suggested that one belonged to Myoviridae (phage TU_ST11B) and the other to Siphoviridae (phage TU_SP24B) family. As both types of *Salmonella* have been reported and recorded in ICTV database already, these may be the most commonly found *Salmonella* phages in nature.

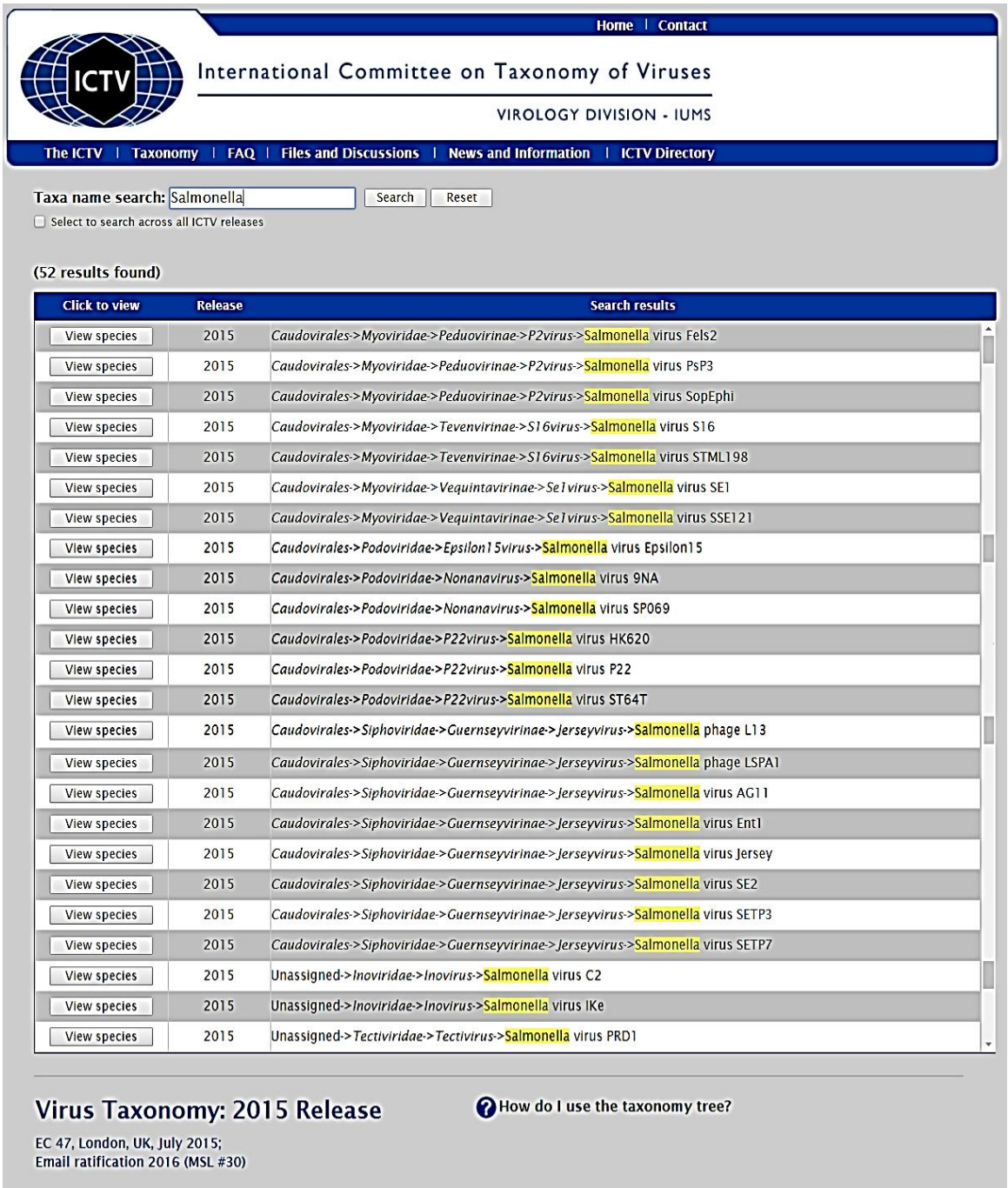


FIGURE 4.19 | ICTV online search results for *Salmonella* phage/virus. Fifty-two *Shigella* phages were found/deposited in ICTV database that belongs to all three Myoviridae, Podoviridae and Siphoviridae family of order Caudovirales. Also many phages are unassigned to any order, but still deposited in the database which may be tailless. ***Note**: All 52 results are NOT shown here for brevity.*

A similar search for *Klebsiella* phage with keyword #Klebsiella delivered only 7 matched output with only two types of phages – Podoviridae and Siphoviridae – belonging to Caudovirales order. The database does not report any phages from Myoviridae family as of today (November 18, 2016). This clearly signifies that research on *Klebsiella* phages is

FIGURE 4.20 | ICTV online search results for Klebsiella phage/virus. Seven *Klebsiella* phages were found/deposited in ICTV database that belongs to Podoviridae and Siphoviridae family of order Caudovirales. No any Klebsiella phage belonging to Myoviridae was found on ICTV database.

not as satisfactory as on *Escherichia* phages. However, independent studies have reported *Klebsiella* phage belonging to Myoviridae that can infect multidrug resistant *Klebsiella* spp**.** (Kęsik-Szeloch *et al.*, 2013) and as drug resistant *Klebsiella* are one of the most troubling pathogens, *Klebsiella* phage hunting may be one of the most attractive field in phage research. *Klebsiella* phage (phage TU_Kle10O) from our study belongs to Podoviridae family which has already been reported by ICTV and thus is cross verified.

4.4 Phage genomics

Rather than using a conventional phage names that is used throughout this dissertation to this point for simplification, a ratified names of phages according to ICTV guidelines (as of 2015 modification) are used hereafter for greater acceptance/referencing while submitting genome to GenBank and other genome databases. 'Virus Taxonomy: 2015 Release' (ictvonline.org, 2015) suggests to drop the use of 'phage' and replace it with 'virus' and only use non-italicized genus name of host bacteria instead of genus + species name while naming a phage (for example change '*Escherichia coli* phage' to 'Escherichia virus'). A personalized code can be added to identify the specific phage strain. As such, sequenced phages are re-named accordingly and used henceforth (unless specified).

TABLE 4.12 | Re-naming of sequenced phages according to ICTV guidelines (as of 2015 modification).

SN	Host bacteria	Conventional phage name	Ratified phage name
1	*Klebsiella* spp._10	phage TU_Kle10O / Ø TU_Kle10O	Klebsiella virus TU_Kle10O
2	*Escherichia coli*_18	phage TU_EC18O / Ø TU_EC18O	Escherichia virus TU_EC18O
3	*Salmonella paratyphi*_24	phage TU_SP24B / Ø TU_SP24B	Salmonella virus TU_SP24B

4.4.1 Genomic DNA extraction and quality check (QC of gDNA)

Agarose gel electrophoresis of extracted genomic DNA showed a clear single band (Figure 4.21) suggesting a high quality DNA extraction from the 'Phage DNA Isolation Kit' (Norgen Biotek, Canada). Readings from NanoDrop 8000 further ruled out the protein and/or RNA contamination in the extracted gDNA.

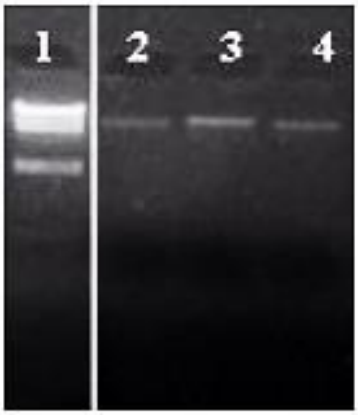


FIGURE 4.21 | Quality control on agarose gel. Lane 1: Hind III marker**, Lane 2:** Klebsiella virus TU_Kle10O, **Lane 3:** Escherichia virus TU_EC18O, **Lane 4:** Salmonella virus TU_SP24B

TABLE 4.13 | Quantification using Qubit® fluorometer.

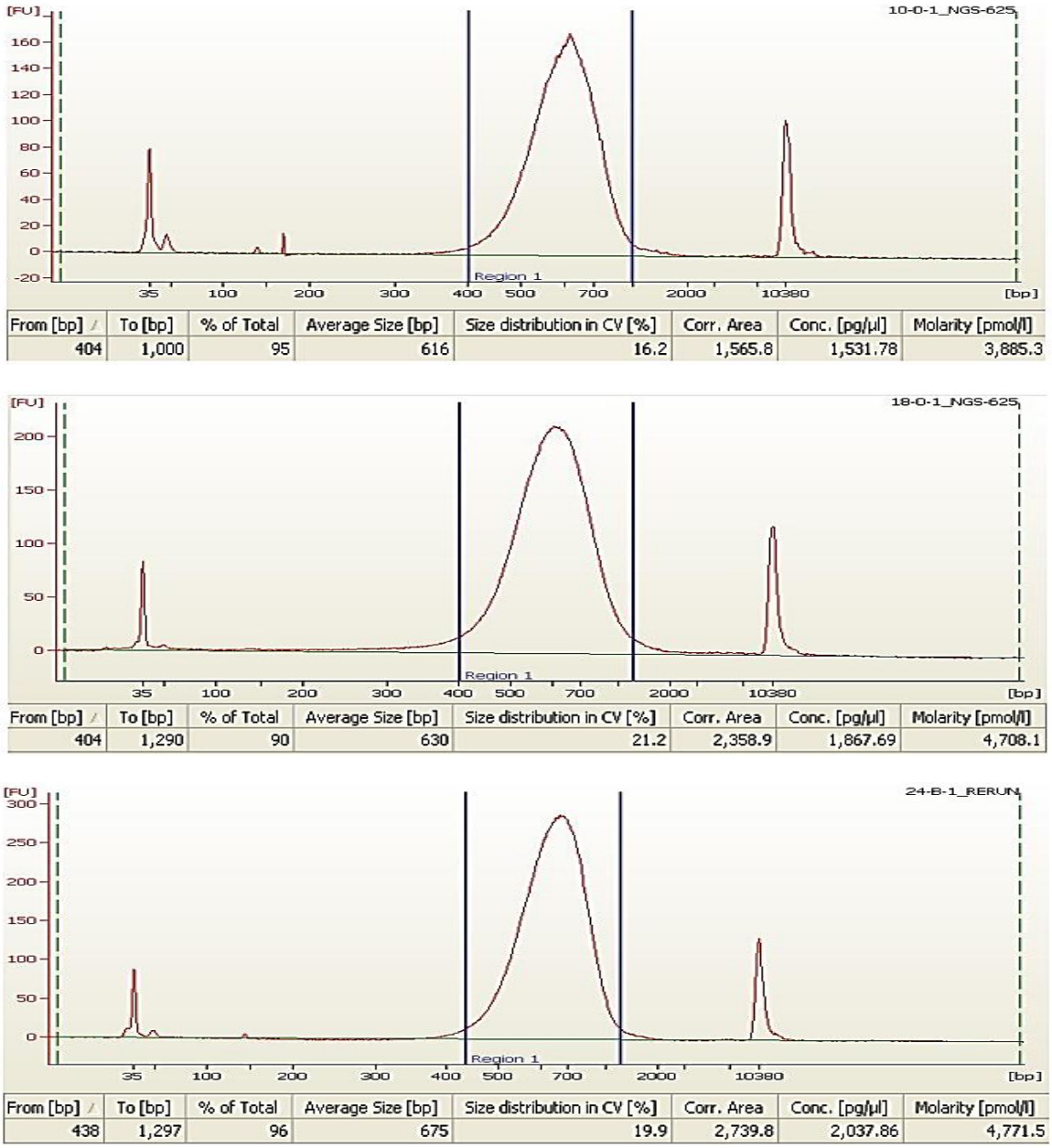
SN	Phage	Concentration (ng/µl)	Volume (µl)	Yield (µg)
1	Klebsiella virus TU_Kle10O	4.94	25	0.1
2	Escherichia virus TU_EC18O	9.36	25	0.2
3	Salmonella virus TU_SP24B	4.88	25	0.1

* ng = nano gram; µl = micro liter; µg = micro gram

As recorded by Qubit® fluorometer, the concentration of extracted gDNA was found to be 4.94 ng/µl, 9.36 ng/µl, 4.88 ng/µl and the total yield of gDNA was 0.1 µg, 0.2 µg and 0.1 µg for Klebsiella virus TU_Kle10O, Escherichia virus TU_EC18O and Salmonella virus TU_SP24B respectively. The final yield being satisfactory for genome sequencing, the samples were further processed accordingly.

4.4.2 Library preparation and quality check (QC of library)

The libraries were prepared from gDNA samples of Klebsiella virus TU_Kle10O, Escherichia virus TU_EC18O and Salmonella virus TU_SP24B using Illumina TruSeq Nano DNA HT Library Sample Preparation Kit. The mean sizes of the fragmented libraries were 616 bp, 630 bp and 675 bp respectively (Figure 4.22). The libraries were then sequenced (2x150 bp chemistry) to generate around 2 GB data per sample.



From [bp]	To [bp]	% of Total	Average Size [bp]	Size distribution in CV [%]	Corr. Area	Conc. [pg/µl]	Molarity [pmol/l]
404	1,000	95	616	16.2	1,565.8	1,531.78	3,885.3

From [bp]	To [bp]	% of Total	Average Size [bp]	Size distribution in CV [%]	Corr. Area	Conc. [pg/µl]	Molarity [pmol/l]
404	1,290	90	630	21.2	2,358.9	1,867.69	4,708.1

From [bp]	To [bp]	% of Total	Average Size [bp]	Size distribution in CV [%]	Corr. Area	Conc. [pg/µl]	Molarity [pmol/l]
438	1,297	96	675	19.9	2,739.8	2,037.86	4,771.5

FIGURE 4.22 | Bio-analyzer profiles of library loaded in Agilent DNA HS chip. From top to bottom: Klebsiella virus TU_Kle10O (coded as 10-O-1), Escherichia virus TU_EC18O (coded as 18-O-1) and Salmonella virus TU_SP24B (coded as 24-B-1). The mean sizes of the fragmented libraries (gDNA) of Klebsiella virus TU_Kle10O, Escherichia virus TU_EC18O and Salmonella virus TU_SP24B were 616 bp, 630 bp and 675 bp respectively.

4.4.3 Bioinformatics Analysis

4.4.3.1 Data generation

The next generation sequencing of the samples were generated on Illumina platform. The raw read statistics are as follows:

TABLE 4.14 | Read data statistics for the samples phage Kle_TU10O, phage TU_EC18O and phage TU_SP24B

SN	Sample	Reads	Total reads (Individual)	Total reads (R1+R2)	Bases (Individual)	Total bases (R1+R2)
1	Phage Kle_TU10O	R1.fq	5793266	11586532	868989900	1737979800
		R2.fq	5793266		868989900	
2	Phage TU_EC18O	R1.fq	3601618	7203236	540242700	1080485400
		R2.fq	3601618		540242700	
3	Phage TU_SP24B	R1.fq	5098098	10196196	764714700	1529429400
		R2.fq	5098098		764714700	

4.4.3.2 De novo assembly

De novo assemblies of high quality PE reads (paired-end reads) were accomplished using CLC Genomics Workbench 6.0 at default parameters (Minimum contig length: 200, Automatic word size: Yes, Perform scaffolding: Yes, Mismatch cost: 2, Insertion cost: 3, Deletion cost: 3, Length fraction: 0.5, Similarity fraction: 0.8). The statistical elements of the assemblies were calculated by in house perl scripts and are given below (Table 4.15).

TABLE 4.15 | Statistics of assembled reads for phage TU_Kle10O, phage TU_EC18O, & phage TU_SP24B

Description	Phage TU_Kle10O	Phage TU_EC18O	Phage TU_SP24B
Num_scaffolds	1662	519	150
Num_contigs	2105	663	202
Total genome length incl. gaps	1872856	962289	492375
Total genome length w/o gaps	1857113	944961	487329
Avg_scaffold_size incl. gaps	1126.86883	1854.12139	3282.5
Avg_scaffold_size w/o gaps	1117.39651	1820.7341	3248.86
Avg_contig_size	882.238955	1425.28054	2412.5198
Contig N50	916	2331	50124
Scaffold N50	1046	3111	59124
Max scaffold size	166976	86083	111266
Min scaffold size	500	502	503

4.4.3.2 BLASTN annotations

The assembled scaffolds were blasted against 'nucleotide' database using BLAST+ 2.2.30 (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/LATEST). Below figures (Figure 4.23) show the species distribution of all three samples. (refer to Appendix – AF3 to AF5 for supplement data)

For two samples (phage TU_Kle10O and phage TU_EC18O) the results were filtered to show species which have hits more than or equal to 10. Rest all were clubbed into 'others' category. It was observed from BLASTN that sample 'phage TU_Kle10O' has much of the genome similarity with bacterial species *Klebsiella pneumonia* (428 hits) (Figure 4.23(a)). Similarly, it was observed that genome of 'phage TU_EC18O' was most closely related to *Triticum aestivum* (64 hits). Further the genome was also closely related to *Shigella* phage (54 and 34 hits), *Enterobacter* phage (42 and 36 hits) and *Escherichia* phage (19 hits) (Figure 4.23(b)). This abnormal result was also consistent with PHASTER analysis (Table 4.16) where the genome 'phage TU_EC18O' has been found to be closely related with phages of other species like *Enterobacter* and *Shigella* on the basis of 'total hit gene count'. (PHAGE_Escher_vB_EcoM_JS09_NC_024124(**266**), PHAGE_Escher_vB_EcoM_PhAPEC2_NC_024794(**229**), PHAGE_Entero_RB69_NC_004928(**229**), PHAGE_Shigel_Shf125875_NC_025437(**226**)).

For phage TU_SP24B, the results were filtered to show species which have hits more than or equal to 2 and rest were clubbed into 'others' category. It was observed from BLASTN that sample 'phage TU_SP24B' has much of the genome similarity with bacterial species *Salmonella enterica* Serovar *paratyphi* (41 hits) (Figure 4.23(c)).

As species distribution did not precisely predict the relatedness of phage genome to their respective phages, we have relied on the results of PHASTER which is a curated phage database that is used extensively for phage characterization and genes prediction. Although species distribution showed close relatedness of phage genome to their respective host bacteria, the results were not in co-ordination with the PHASTER results and PHASTER precisely predicted absence of any complete genes of bacterial origin within phage genome.

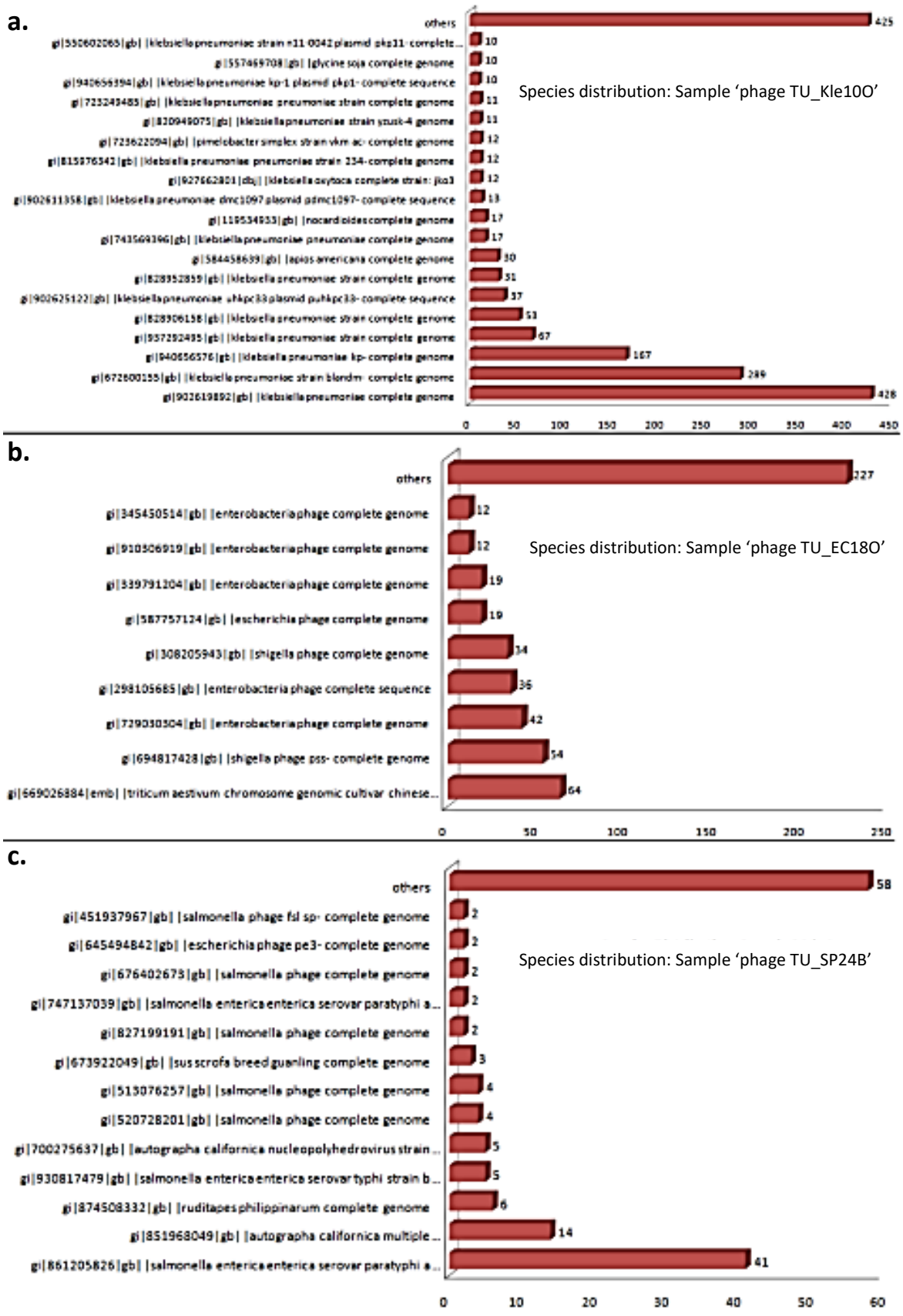


FIGURE 4.23 | Species distribution. a) *Klebsiella* phage (phage TU_Kle10O) **b)** *Escherichia* phage (phage TU_EC18O) **c)** *Salmonella* phage (phage TU_SP24B)

* please refer to APPENDIX (AF3, AF4 and AF5) for supplementary data.

4.4.4 Comparative genome analysis of sequenced phages.

PHASTER generated circular genome of sequenced phages (Klebsiella virus 11TU_Kle10O, Escherichia virus TU_EC18O and Salmonella virus TU_SP24B) revealed the genome size, number of CDS region, GC content and predicted the location (start and end) of the prophage genome on the host genome. All of the genomes are 'intact' and no significant breaks were reported throughout the genome.

The tool predicts 286 CDS regions for Klebsiella virus TU_Kle10O, 274 CDS regions for Escherichia virus TU_EC18O and 54 CDS regions for Salmonella virus TU_SP24B (Figure 4.23).

A comprehensive and customized 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 hypothetical parameters used for ORF prediction in SnapGene® was: minimum length of amino acid = 75, required start codon = ATG, genetic code for ORFs and new features = Bacterial, Archaeal and Plant Plastid. In this scenario, the study has comparatively relied more on

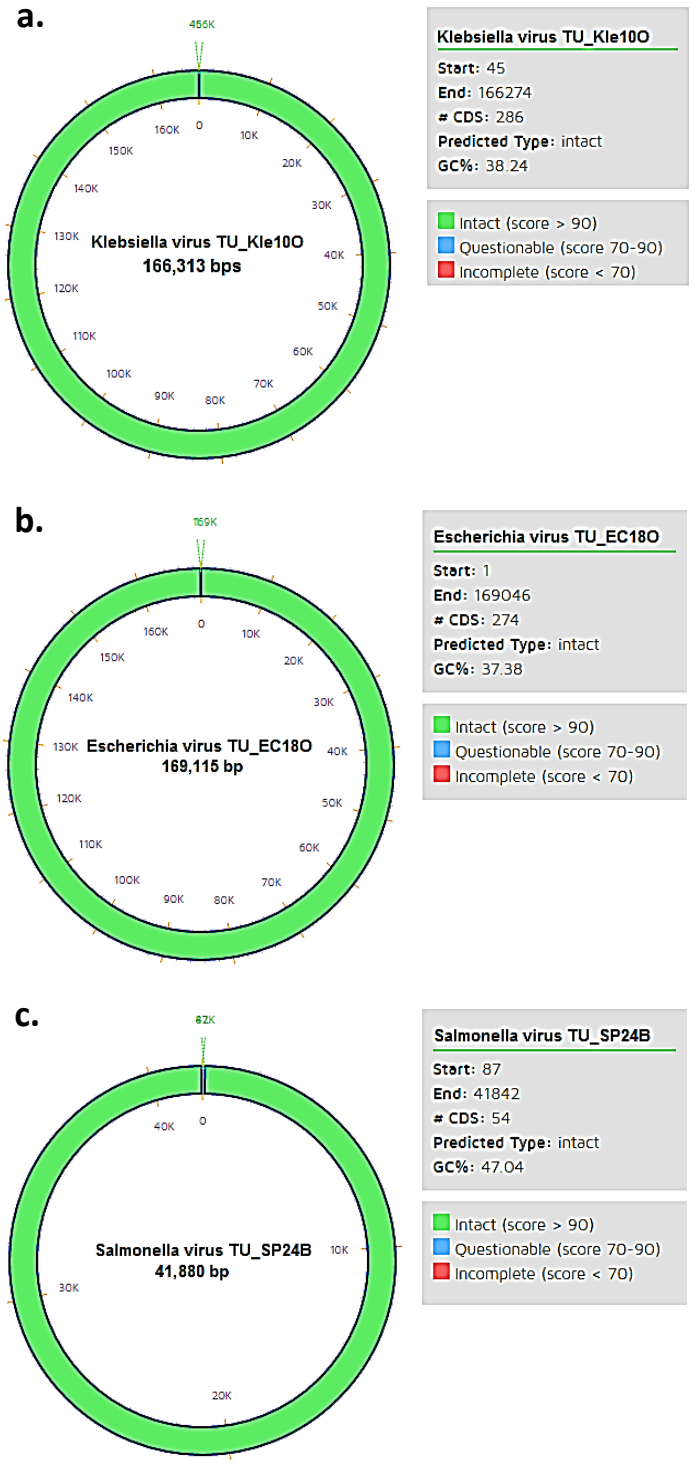


FIGURE 4.24 | Comparison of circular genome of all three sequenced phages. PHASTER generated circular genome of **a)** Klebsiella virus TU_Kle10O, **b)** Escherichia virus TU_EC18O and **c)** Salmonella virus TU_SP24B.

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.

Genes/proteins like head, terminase, tail, portal, lysis, plate, capsid, protease and lysis were successfully hit/identified. All three phages had lysis/lysis gene responsible for lysis of bacteria verifying that the phages were lytic. Further no 'integrase' genes were hit suggesting that the phages are lytic.

Bacterial proteins were not predicted by the PHASTER tool within the genome of sequenced phages (Table 4.16) clearly proving that the phage genome do not carry any virulent/bacterial genes within their genome by itself. This makes them an excellent candidate for therapeutic purpose and debunks the idea that phages may transfer their virulent genes to bacterial hosts making them more virulent. However, this does not rule out the possibility of 'gene transfer' from one host to another host by transduction.

TABLE 4.16 | Comparative analysis of sequenced phages (Klebsiella virus TU_Kle10O , Escherichia virus TU_EC18O and Salmonella virus TU_SP24B) as generated by PHASTER tool.

Phage Parameters	Klebsiella virus TU_Kle10O	Escherichia virus TU_EC18O	Salmonella virus TU_SP24B
REGION LENGTH	**166.2 Kb**	**169 Kb**	**41.7 Kb**
COMPLETENESS (SCORE)	intact (140)	intact (150)	intact (129)
SPECIFIC KEYWORDS	**head, terminase, tail, portal, lysis**	**lysis, tail, head, plate, capsid, portal, terminase, protease**	**terminase, tail, lysin**
REGION POSITION	45-166274	1-169046	87-41842
# TRNA	16	0	0
# TOTAL PROTEINS	**270**	**274**	**54**
# PHAGE HIT PROTEINS	**263**	**274**	**51**
# HYPOTHETICAL PROTEINS	**7**	**0**	**3**
PHAGE + HYPOTHETICAL PROTEIN %	**100 %**	**100 %**	**100 %**
# BACTERIAL PROTEINS	**0**	**0**	**0**
ATTACHMENT SITE	no	no	no
# PHAGE SPECIES	4	7	2
MOST COMMON PHAGE NAME (HIT GENES COUNT)	**PHAGE_Klebsi_JD18_NC_028686(260)** PHAGE_Entero_CC31_NC_014662(94) PHAGE_Salmon_S16_NC_020416(90) PHAGE_Entero_PG7_NC_023561(89) PHAGE_Citrob_Moon_NC_027331(81) PHAGE_Salmon_STML_198_NC_027344(77)	**PHAGE_Escher_vB_EcoM_JS09_NC_024124(266)** **PHAGE_Escher_vB_EcoM_PhAPEC2_NC_024794(229)** **PHAGE_Entero_RB69_NC_004928(229)** **PHAGE_Shigel_Shf125875_NC_025437(226)** PHAGE_Entero_HX01_NC_018855(54) PHAGE_Escher_Av_05_NC_025830(22)	**PHAGE_Salmon_LSPA1_NC_026017(50)** PHAGE_Salmon_Ent1_NC_019539(18) PHAGE_Salmon_Jersey_NC_021777(18) PHAGE_Salmon_vB_SenS_Ent2_NC_023608(17) PHAGE_Salmon_SS3e_NC_006940(15) PHAGE_Salmon_SE2_NC_016763(14)

	PHAGE_Salmon_STP4_a_NC_026607(66) PHAGE_Edward_PEi20_NC_028683(37) PHAGE_Yersin_phiR1_RT_NC_019909(20) PHAGE_Pectob_PM2_NC_028940(20) PHAGE_Entero_QL01_NC_028847(19) PHAGE_Yersin_vB_YenM_TG1_NC_028820(18) PHAGE_Entero_JS98_NC_010105(18) PHAGE_Entero_JS10_NC_012741(16) PHAGE_Shigel_SP18_NC_014595(13) PHAGE_Entero_vB_EcoM_VR5_NC_028881(11) PHAGE_Escher_vB_EcoM_JS09_NC_024124(11) PHAGE_Entero_Bp7_NC_019500(11) PHAGE_Entero_vB_EcoM_VR7_NC_014792(10) PHAGE_Entero_vB_EcoM_VR25_NC_028925(10) PHAGE_Entero_vB_EcoM_VR26_NC_028957(10) PHAGE_Entero_RB51_NC_012635(8) PHAGE_Entero_vB_EcoM_VR20_NC_028894(8) PHAGE_Entero_RB69_NC_004928(8) PHAGE_Entero_IME08_NC_014260(8) PHAGE_Serrat_PS2_NC_024121(7) PHAGE_Escher_wV7_NC_019505(7) PHAGE_Shigel_Shf125875_NC_025437(6) PHAGE_Escher_slur14_NC_028448(6) PHAGE_Escher_vB_EcoM_PhAPEC2_NC_024794(6) PHAGE_Klebsi_KP27_NC_020080(6) PHAGE_Yersin_PST_NC_027404(5) PHAGE_Klebsi_KP15_NC_014036(5) PHAGE_Entero_RB68_NC_027979(4) PHAGE_Entero_RB27_NC_025448(4) PHAGE_Entero_RB3_NC_025419(4) PHAGE_Escher_slur02_NC_028927(4)	PHAGE_Escher_slur14_NC_028448(19) PHAGE_Escher_wV7_NC_019505(16) PHAGE_Yersin_phiD1_NC_027353(16) PHAGE_Shigel_pSs_1_NC_025829(15) PHAGE_Edward_PEi20_NC_028683(13) PHAGE_Escher_HY01_NC_027349(13) PHAGE_Entero_RB51_NC_012635(12) PHAGE_Entero_JS98_NC_010105(12) PHAGE_Yersin_PST_NC_027404(11) PHAGE_Entero_vB_EcoM_VR5_NC_028881(11) PHAGE_Entero_IME08_NC_014260(11) PHAGE_Entero_ime09_NC_019503(11) PHAGE_Shigel_Shfl2_NC_015457(10) PHAGE_Entero_vB_EcoM_VR7_NC_014792(10) PHAGE_Entero_RB32_NC_008515(10) PHAGE_Entero_RB3_NC_025419(10) PHAGE_Escher_slur02_NC_028927(10) PHAGE_Entero_vB_EcoM_VR25_NC_028925(8) PHAGE_Shigel_SP18_NC_014595(8) PHAGE_Entero_RB27_NC_025448(8) PHAGE_Entero_RB14_NC_012638(7) PHAGE_Entero_AR1_NC_027983(7) PHAGE_Entero_T4_NC_000866(6) PHAGE_Escher_e11/2_NC_024125(6) PHAGE_Entero_JS10_NC_012741(6) PHAGE_Entero_QL01_NC_028847(5) PHAGE_Escher_ECML_134_NC_025449(5) PHAGE_Entero_vB_EcoM_VR20_NC_028894(4) PHAGE_Escher_slur07_NC_028780(4) PHAGE_Entero_vB_EcoM_ACG_C40_NC_019399(4) PHAGE_Citrob_Moon_NC_027331(3)	PHAGE_Salmon_SETP7_NC_022754(14) PHAGE_Salmon_SETP13_NC_022752(14) PHAGE_Salmon_vB_SenS_Ent3_NC_024204(13) PHAGE_Salmon_SETP3_NC_009232(12) PHAGE_Escher_K1_dep(1)_NC_027994(11) PHAGE_Escher_K1_dep(4)_NC_027993(10) PHAGE_Salmon_f18SE_NC_028698(9) PHAGE_Salmon_L13_NC_021317(8) PHAGE_Entero_phiEap_2_NC_028695(2) PHAGE_Entero_JSE_NC_012740(1) PHAGE_Klebsi_JD001_NC_020204(1) PHAGE_Vibrio_KVP40_NC_005083(1) PHAGE_Yersin_phiR1_RT_NC_019909(1) PHAGE_Sodali_SO1_NC_013600(1) PHAGE_Salmon_vB_SalM_SJ2_NC_023856(1) PHAGE_Vibrio_ValKK3_NC_028829(1) PHAGE_Yersin_vB_YenM_TG1_NC_028820(1) PHAGE_Aeromo_phiAS4_NC_014635(1) PHAGE_Salmon_SFP10_NC_016073(1) PHAGE_Shigel_EP23_NC_016566(1) PHAGE_Cronob_vB_CsaM_GAP31_NC_019400(1) PHAGE_Entero_4MG_NC_022968(1) PHAGE_Salmon_Marshall_NC_022772(1) PHAGE_Salmon_SSE_121_NC_027351(1) PHAGE_Salmon_PhiSH19_NC_019530(1) PHAGE_Entero_EK99P_1_NC_024783(1) PHAGE_Edward_eiAU_183_NC_023555(1)

	PHAGE_Cronob_S13_NC_028773(4) PHAGE_Entero_RB32_NC_008515(4) PHAGE_Entero_ime09_NC_019503(4) PHAGE_Escher_Lw1_NC_021344(3) PHAGE_Shigel_pSs_1_NC_025829(3) PHAGE_Escher_HY01_NC_027349(3) PHAGE_Shigel_Shfl2_NC_015457(3) PHAGE_Entero_T4_NC_000866(3) PHAGE_Entero_RB14_NC_012638(3) : :	PHAGE_Entero_RB68_NC_027979(3) PHAGE_Salmon_S16_NC_020416(2) PHAGE_Entero_vB_EcoM_VR26_NC_028957(2) PHAGE_Entero_PG7_NC_023561(2) PHAGE_Acinet_Acj61_NC_014661(1) PHAGE_Pectob_PM2_NC_028940(1) PHAGE_Yersin_phiR1_RT_NC_019909(1) PHAGE_Entero_CC31_NC_014662(1) PHAGE_Phage_Gifsy_1_NC_010392(1) : :	
FIRST MOST COMMON PHAGE #	260	266	50
FIRST MOST COMMON PHAGE %	96.29%	97.08%	92.59%
GC %	38.24%	37.38%	47.04%

Region Length : The length of the sequence of that region (in bp).

Completeness : A prediction of whether the region contains an intact or incomplete prophage based on the above criteria.

Specific Keyword : The specific phage-related keyword(s) found in protein name(s) in the region.

Region Position : The start and end positions of the region on the bacterial chromosome.

tRNA : The number of tRNA genes present in the region.

Total Proteins : The number of ORFs present in the region.

Phage Hit Proteins : The number of proteins in the region with matches in the phage protein database.

Hypothetical Proteins : The number of hypothetical proteins in the region without a match in the database.

Phage + Hypothetical Protein % : The combined percentage of phage proteins and hypothetical proteins in the phage region.

Bacterial Proteins : The number of proteins in the region with matches in the nrfilt database.

Attachment Site : The putative phage attachment site.

Phage Species : The number of different phages that have similar proteins to those in the region.

Most Common Phage : The phage(s) with the highest number of proteins most similar to those in the region.

First Most Common Phage # : The highest number of proteins in a phage most similar to those in the region.

First Most Common Phage % : The percentage of proteins in # Phage Hit Proteins that are most similar to the Most Common Phage proteins.

GC % : The percentage of GC nucleotides of the region.

4.4.5 Klebsiella virus TU_Kle10O

The complete genome of Klebsiella virus TU_Kle10O is comprised of 166,313 bps (166.2 Kb) and has G+C content 38.24%. A total of 270 proteins (including homology to hypothetical proteins) were predicted within the genome map. Based on genomics and 'hit genes count', Klebsiella virus TU_Kle10O was most common to PHAGE_Klebsi_JD18 [NCBI Reference Sequence: NC_028686] (Table 4.16 & Figure 4.25). Genome of PHAGE_Klebsi_JD18 is also 166,313 bps (~166.3), is also a dsDNA virus with no RNA stage and also belonged to Caudovirales.

Region Length	Completeness	Score	# Total Proteins	Region Position	Most Common Phage	GC %
166.2Kb	intact	140	270	45-166274	PHAGE_Klebsi_JD18_NC_028686(260)	38.24%

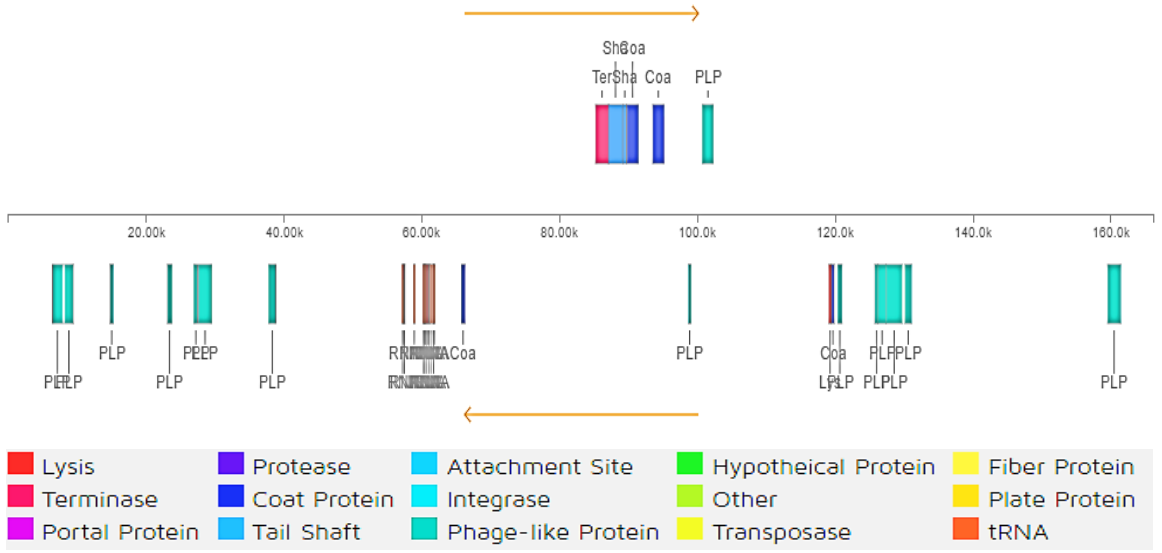


FIGURE 4.25 | Linearized genome annotation of Klebsiella virus TU_Kle10O. The upper arrow represents forward direction/strand of ORF whereas lower arrow represents backward direction/strand of the ORF. The region position value predicts the location of prophage genome on host genome. Source: http://phaster.ca/submissions/ZZ_84200931a3

Out of 270 hit/predicted genes, 260 matched to this phage, signifying high degree of similarity between these two phages. But, despite such similarity, Klebsiella virus TU_Kle10 belonged to Podoviridae family whereas PHAGE_Klebsi_JD18 is reported to be Myoviridae – an entirely different family. As the work is unpublished (ncbi.nlm.nih.gov/nuccore/NC_028686) we could not analyze the micrograph and genome sequences comparatively.

Out of 270 predicted proteins, a total of 41 genes were identified (hypothetical hits omitted) from Klebsiella virus TU_Kle10O. (Refer to Appendix for complete list. Table AT1) Some of them were DNA-primase-helicase subunit, Rec-A like recombination protein, translational repressor protein, thioredoxin, tRNAs, head completion protein, terminase

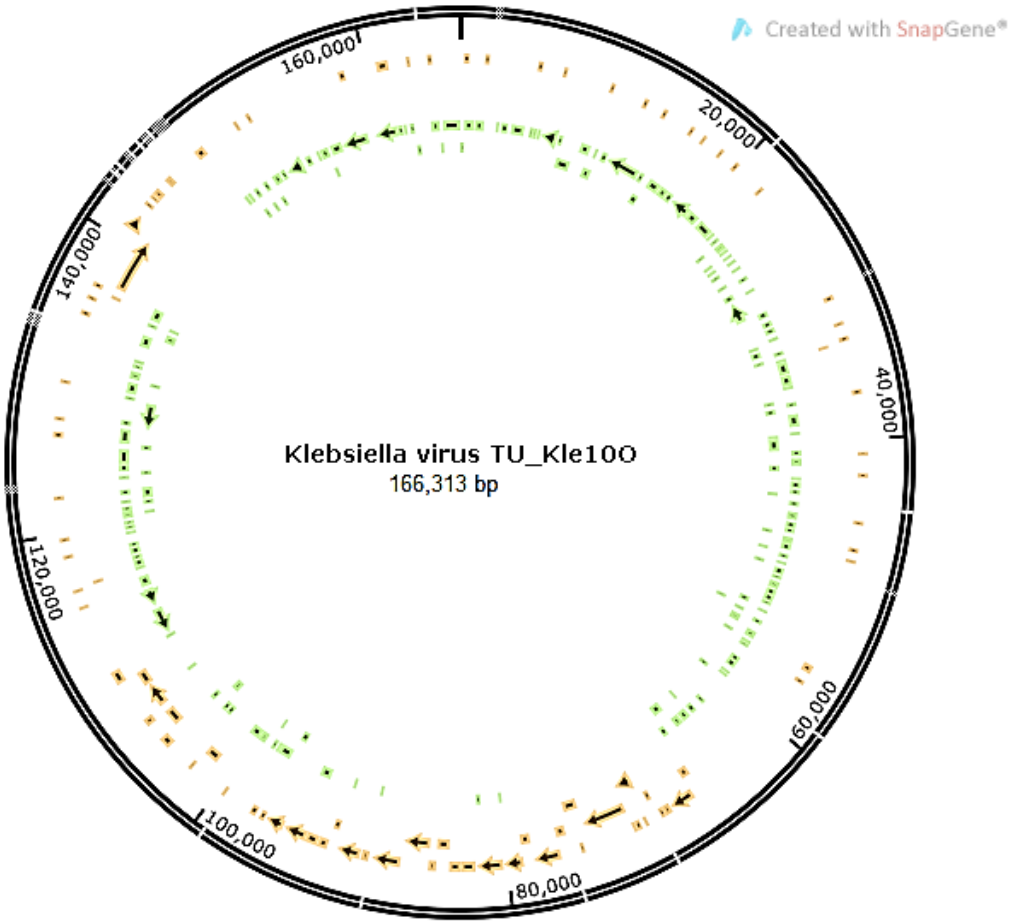


FIGURE 4.26 | Circular genome mapping / annotation of Klebsiella virus TU_Kle10O created with SnapGene®. The genome size of given phage is 166,313 base pairs. The position of all predicted ORF are shown with 'arrow' in six concentric inner circles. The parameters for ORF prediction was: minimum length of amino acid = 75, required start codon = ATG, genetic code for ORFs and new features = Bacterial, Archaeal and Plant Plastid.

DNA packaging enzyme large subunit, tail sheath protein, tail tube protein, portal vortex of head, major head protein, DNA primase helicase subunit, lysis inhibitory accessory proteins, head assembly chaperone protein, endonuclease II, DNA topoisomerase subunit I *et cetera*. Absence of genes of bacterial origin within the genome of Klebsiella virus TU_Kle10O rules out the possibility of transfer of 'virulent genes' from phage genome to bacterial genome. This makes Klebsiella virus TU_Kle10O an excellent candidate for therapeutic purpose. However, on the other hand poor lytic efficiency, absence of multiple host range and induction of BIM makes this phage a poor candidate for therapeutic application.

4.4.6 Escherichia virus TU_EC18O

The complete genome of Escherichia virus TU_EC18O comprised of 169,115 bps (169 Kb) and has G+C content 37.38%. A total of 274 proteins were hit/predicted within the genome map. Based on genomics and 'hit genes count', Escherichia virus TU_EC18O was most common to PHAGE_Escher_vB_EcoM_JS09 [NCBI Reference Sequence: NC_024124] (Table 4.16 & Figure 4.27). Genome of PHAGE_Escher_vB_EcoM_JS09 is 169,148 bps (~169.1 Kb), is a dsDNA virus with no RNA stage and belongs to Myoviridae family from order Caudovirales. Electron micrograph also identifies Escherichia virus TU_EC18O as a Myoviridae.

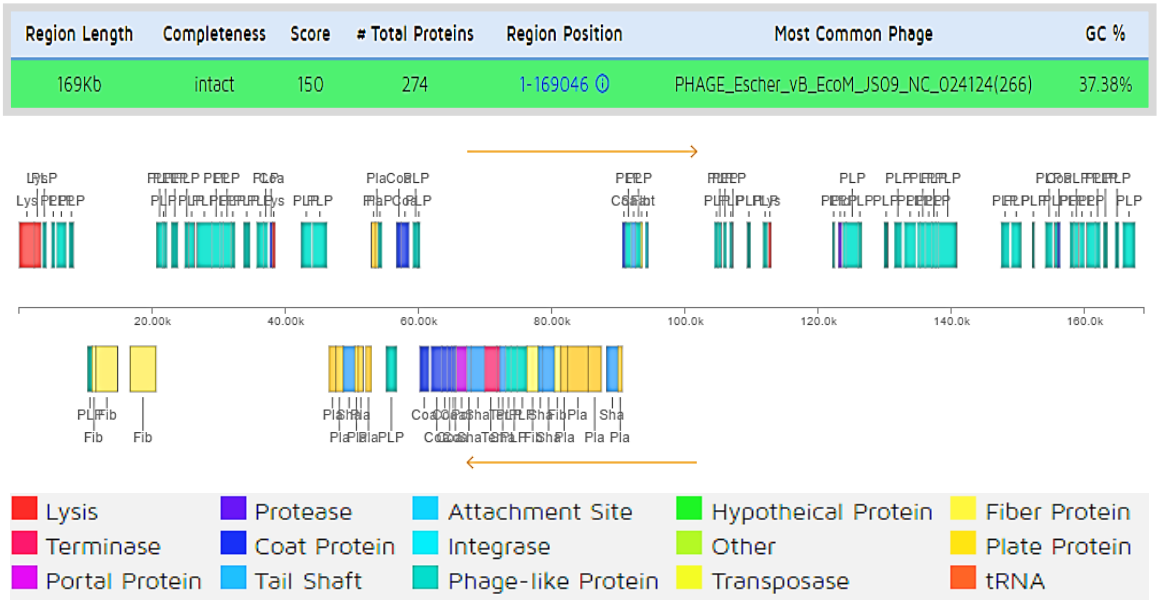


FIGURE 4.27 | Linearized genome annotation of Escherichia virus TU_EC18O. The upper arrow represents forward direction/strand of ORF whereas lower arrow represents backward direction/strand of the ORF. The region position value predicts the location of prophage genome on host genome. Source: http://phaster.ca/submissions/ZZ_6df63c8de8

Out of 274 hit/predicted genes, 266 matched to this phage, suggesting similarity between two phages. Further, three more phages in the PHASTER database (PHAGE_Escher_vB_EcoM_PhAPEC2_NC_024794(229), PHAGE_Entero_RB69_NC_004928(229), PHAGE_Shigel_Shf125875_NC_025437(226) were closely related to Escherichia virus TU_EC18O based on matched genes (229/274, 229/274 and 226/274 respectively). As third and fourth most common phages were *Enterobacter* phage and *Shigella* phage respectively, with significant amount of similarity based on hit genes, we can arbitrarily predict that phages from this group (coliphage) have higher degree of similarity between them. This may explain (although not studied in this research) why some specific phages show expansive host spectrum. This property thus holds immense applicability if further study is performed to determine the mechanism of phage infection and identify the factors proteins/enzymes that determine phage specificity.

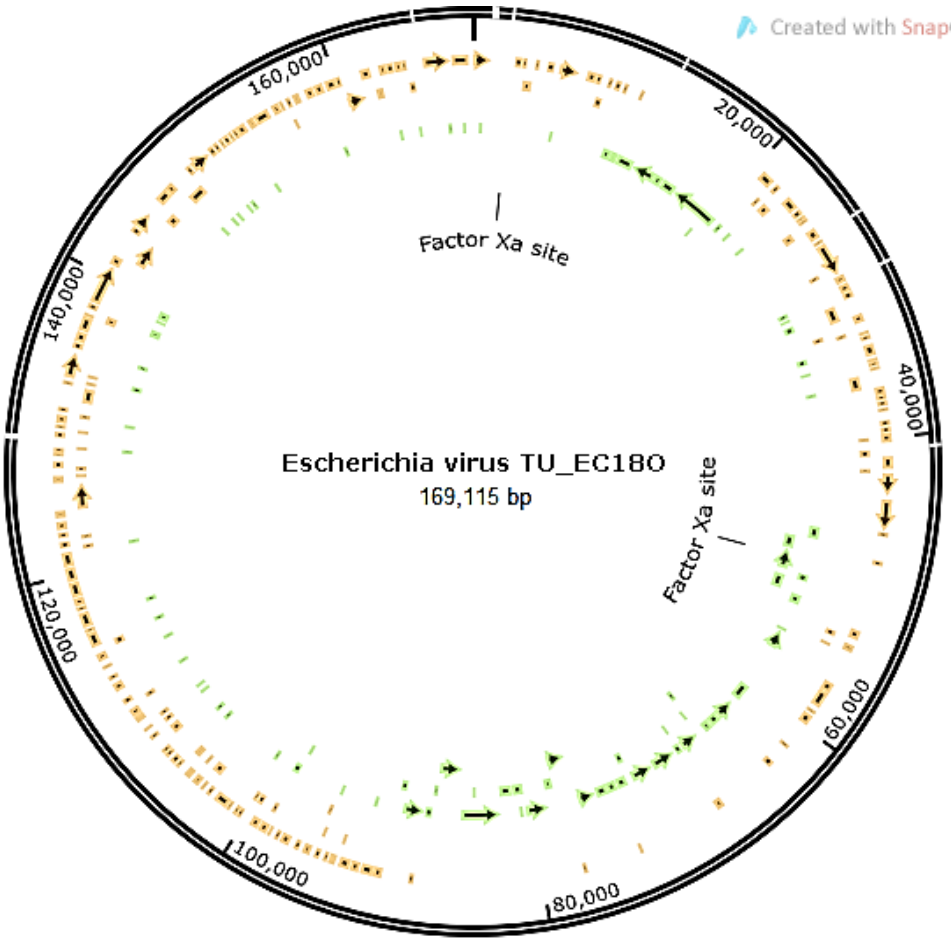


FIGURE 4.28 | Circular genome mapping/annotation of Escherichia virus TU_EC18O created with SnapGene®. The genome size of given phage is 169,115 base pairs. The position of all predicted ORF are shown with 'arrow' in six concentric inner circles. The parameters for ORF prediction was: minimum length of amino acid = 75, required start codon = ATG, genetic code for ORFs and new features = Bacterial, Archaeal and Plant Plastid.

Out of 274 predicted proteins, 110 were identified (Refer to Appendix for complete list, Table AT2) from Escherichia virus TU_EC18O. Some of the identified proteins were rII A/B protector protein (from prophage induced lysis), nucleotide disruption protein, phage holin, short and long tail fibers, endonucleases, head assembly chaperone with GroEL, baseplate subunits (clustered together), tail tube, tail sheath, terminase proteins, neck proteins, fibritins, baseplate wedge subunit and tail pins, head completion proteins, DNA end protector proteins, tail completion and sheath stabilizers, thioredoxins, endonucleases, sliding clamps, clamp loaders, Rec-A like recombinase immunity to superinfection, membrane protein and other outer capsid proteins *et cetera.*

4.4.7 Salmonella virus TU_SP24B

The complete genome of Salmonella virus TU_SP24B comprised of 41,880 bps (41.7 Kb) and has G+C content 47.04%. A total of 54 proteins were predicted within the genome map. Based on genomics and 'hit genes count', Salmonella virus TU_SP24B was most common to PHAGE_Salmon_LSPA1 [NCBI Reference Sequence: NC_026017]. (Table 4.16 & Figure 4.29) Genome of PHAGE_Salmon_LSPA1 is also 41,880 bps (~41.8 Kb), is a dsDNA virus with no RNA stage and belongs to Siphoviridae family from order Caudovirales just like Salmonella virus TU_SP24B from this research.

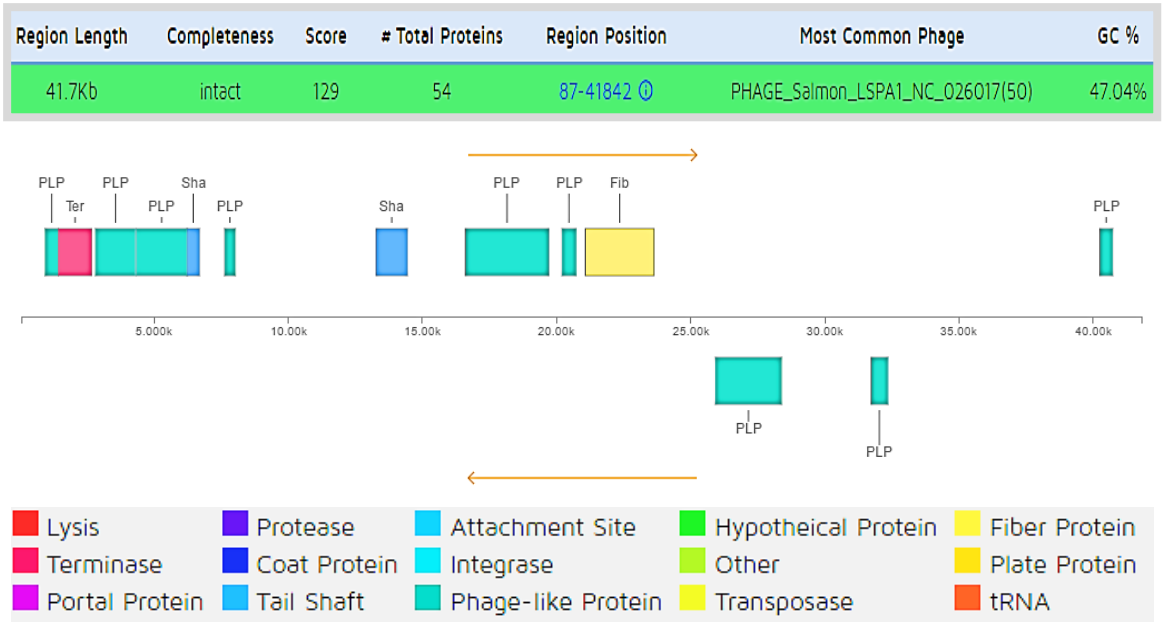


FIGURE 4.29 | Linearized genome annotation of Salmonella virus TU_SP24B. The upper arrow represents forward direction/strand of ORF whereas lower arrow represents backward direction/strand of the ORF. The region position value predicts the location of prophage genome on host genome. Source: http://phaster.ca/submissions/ZZ_02080bfb54

As out of 54 hit/predicted genes, 51 matched to this phage, had similar genome size, and both infected *Salmonella enterica* Serovar *Paratyphi* suggesting strong possibility of both phages being identical. Further, Switt *et al.* have reported 'four' *Salmonella* phage with similar genome size (belonging to Siphoviridae family) in their comprehensive study of 22 *Salmonella* phages (Switt *et al.*, 2013) and Tiwari *et al.* also have reported *Salmonella* phage having 43,221 bps and G+C content of 49.6% (Tiwari *et al.*, 2012). The results are thus aligned.

However, this does not conclude about the range of genome size of *Salmonella* phages being around 41 Kb, as *Salmonella* phages with varying genomic size has also been reported (Switt *et al.*, 2013; Zhang *et al.*, 2014) that supports with significant diversity of phages in nature.

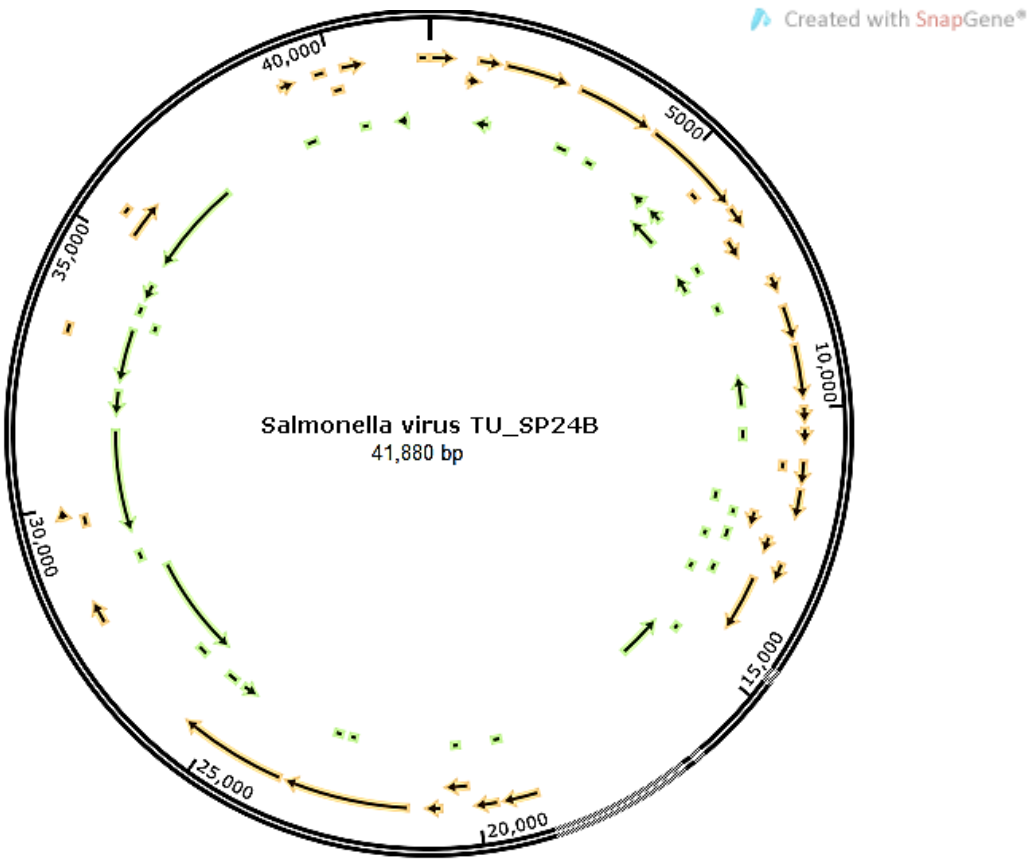


FIGURE 4.30 | Circular genome mapping/annotation of Salmonella virus TU_EC24B created with SnapGene®. The genome size of given phage is 41,880 base pairs. The position of all predicted ORF are shown with 'arrow' in four concentric inner circles. The parameters for ORF prediction was: minimum length of amino acid = 75, required start codon = ATG, genetic code for ORFs and new features = Bacterial, Archaeal and Plant Plastid.

Out of 54 predicted proteins, only 12 proteins were identified against the database available while all other hypothetical proteins have been omitted (Refer to Appendix for complete list. Table AT3). Some of the identified proteins were terminase, structural proteins, putative tail proteins, putative spanins, putative major tail protein, putative tape measure proteins, enolase like proteins, putative tail fiber protein, putative helicase, putative DNA polymerase and putative endolysin. The minimal number of protein may be contributed to small genome size and inadequate reference database for rigorous alignment unlike the case of Escherichia virus (the most studied one). However, it completely rules out the possibility of any virulent genes of 'bacterial origin; within the genome of Salmonella virus TU_SP24B which is the most desirable.

Finally, comprehensive phage genome analysis will definitely allow easy identification of phages lacking integration machinery, which will facilitate safe and effective use of phages as biocontrol agents, although long-term studies on the emergence of resistant mutants and consumer safety should be performed for any phage to be applied commercially (Mahony *et al.*, 2011). While in the United States and Western Europe, phage therapy is still considered investigational, a systemically controlled intensive study on lytic phages against drug resistant infections might be groundbreaking for developing countries like us and we – scientist from developing world – should be looking out for locally available therapies instead of commercially developed drugs as far as possible.

4.5 Possible impacts on 'healthcare' as a possible 'alternative' to antibiotic therapy

Knowing how phages affect humans and/or human microbiome will allow us to determine whether they are to be prevented, treated, ignored or even encouraged. In recent years' giant leaps in genomic sciences have allowed researchers to detect viruses (including phages) in and on the human body that resulted in an entirely new school of thought collectively called 'human variome'. Now, with metagenomics analysis scientists can identify virus present by comparing the 'raw genes' floating around with the vast amount of data available at databases like NCBI, European Nucleotide Archive (ENA) et cetera. Studies even have reported phages that adhere to mucus of human body (primarily gut) are able to induce non-host derived immunity (Barr *et al.*, 2013) while other lytic phages directly kill the pathogens in any environment. Thus, the perception that every virus causes disease therefore no longer holds true, and scientists predict a much more complex biological phenomenon between human-virus microenvironment. In fact, some of our residential viruses, like phages have been proved to be beneficial. So the possibility of using phages as therapeutics – either as alternative to antibiotics or along the antibiotics – is real that holds immense possibilities.

Although, the approach seems straightforward, the concern of gene swapping still remains and as some bacteria (*Vibrio cholera*) have even acquired their virulence thought genes of 'phage' origin, before commercial investment and or therapeutic use, the disease causing abilities of phages must be stringently vetted.

4.6 Possible impacts on 'environmental cleansing'

The importance of phages in environmental ecology is indisputable and has been clearly established in past decades. Phages control the bacterial population in the environment as 'natural bio-control agent'. Phages may also control the composition of bacterial communities and as they can infect bacteria and integrate their genes, may also influence the whole bacterial genetic diversity (enviphage.eu/en). Thus it would be fascinating to look into the roles played by phages in environmental cleansing including municipality sewages/sludge. As numerous phages have been isolated in this study against pathogenic bacteria, it may also be possible that phages play role in controlling number of pathogens in their natural environment. Phages may have been indirectly protecting us killing or acting as bio-controlling agents in the environment. This may explain why despite strong predictions of outbreak of diseases such as typhoid, cholera and diarrhea, we Kathmanduities have not seen one regardless of our poor sanitary practices. Many a times, coliforms have been reported in the river and drinking waters of Kathmandu valley, but rarely has an outbreak occurred! Presence of phages against such coliforms (*Escherichia, Salmonella, Klebsiella, Shigella* and *Citrobacter*) may explain why we are protected from such outbreaks.

4.7 Concluding remarks

It is indisputable from the observations that 'yes' phages can efficiently kill/lyse drug resistant bacterial pathogens of human origin in laboratory setting and phages that infect and kill such pathogens can easily be found around us in our natural environment. However, this research cannot and does not guarantee about the efficiency of its use in human and or animal model without more comprehensive data, but guarantees about the probability of finding phages against drug resistant bacterial pathogens of human origin in the environment that could possibly be used therapeutically. Although research institutes and scientists from many countries claim to be using 'lytic phages' therapeutically to cure bacterial infections (including drug resistant like MRSA), none of them are approved by FDA for human and or animal therapeutic use. Although phages do not possess 'virulent genes' by themselves, there is always chance of virulent genes getting swapped between different strains of bacteria by 'bacteriophage transduction'. The speculation that bacteriophage transduction plays a role in horizontal transfer of genes (including antibiotic resistant genes and other mobile elements' is one of the main concern and hindrance for success of phage therapy, which in my view is genuine and thus cannot and should not be overlooked.

4.8 'Paradigm shift' for success of phage therapy

Phage therapy and application of phage in diverse field seems genuinely promising, but we are not interested and/or fully benefiting from this 'natural gift' and was almost forgotten if it wasn't for antibiotic resistance which rekindled the possibility and interest in phage therapy once again. The present limitations on phage therapy depends mainly in two factors: Firstly, large natural variation in infecting phage and bacterial host, and secondly on regulatory framework of clinical application (Mirzaei & Nilsson, 2015). As phages are live organisms and have entirely different pharmacokinetics, killing mechanism and host specificity, a detailed and comprehensive study is must for advancement of phage therapy. Proper dosing, interval of administration, duration of treatment, monitoring adverse effects have to be taken into consideration before 'getting too excited' and claiming the effectiveness of phage therapy. As each family of phage have different genome organization and life cycle, it is strenuous to engineer the phage and thus poses challenges for regulatory approval for clinical use. However, a different approach must be developed and applied for recognition and approval of phage for human use and clinical trials. The stringent FDA rules for antibiotics – that is not practically applicable in case of phages – is one of the hurdle that has been hindering phage research as FDA requires each component of drug (phage in this case) to be characterized before going into trail. But as phage is dynamic, practically it is almost impossible to follow same rules for phages that are being followed for antibiotics. Thus, there needs to be a 'paradigm shift' and phages should be considered as a different entity and thus be allowed to follow a different approach like 'flu vaccines'.

CHAPTER – FIVE
SUMMARY

Thirty-four bacterial viruses – more popularly called phages – were isolated against different drug resistant pathogenic bacteria of human origin, specifically from Enterobacteriaceae family. All but two Klebsiella virus showed multiple host range – meaning were able to lyse bacterial strains other than their primary host. Salmonella virus even showed inter-species host range (in between *typhi* and *paratyphi* serovars) which undermines the established hypothesis about phages being extremely host specific. Electron micrograph of seven phages revealed that all selected phages belong to order Caudovirales (tailed virus).

Bacteriophage Induced Mutants (BIM) were only seen against both Klebsiella virus in multi-generation infection cycle and Citrobacter virus did not show completely lytic property. Both – Klebsiella virus and Citrobacter virus – belonged to Podoviridae family, while all other belonged to Myoviridae or Siphoviridae. Further, virus from Podoviridae also did not lyze their own host bacteria completely in lag and log phase during multi-generation lytic analysis. This indisputably is a setback to our primary hypothesis, but a startling discovery may be looming behind this characteristic feature. The multiple host range and lytic efficiency may be closely related to 'tail' of phages, as during our observation we saw that Klebsiella virus (a Podoviridae) did not show any multiple host range efficiency. Citrobacter virus (also a Podoviridae) also did not show efficient multiple host range. To add to this, they also did not show complete lytic ability in lag and log phase of host's life cycle. This trend encourages us to propose a new hypothesis that 'longer the tail of phage, they are more adaptable and efficient'. We can also predict phages with longer tails are more evolved ones, as evolution certainly makes organism more adaptable to broader host spectrum.

Further genome analysis of 3 phages (Klebsiella virus TU_Kle10O, Escherichia virus TU_EC18O and Salmonella virus TU_SP24B) revealed the genome size of phages to be 166.2 Kb, 169 Kb and 41.7 Kb respectively. The tool also predicted 286 CDS regions for Klebsiella virus TU_Kle10O, 274 CDS regions for Escherichia virus TU_EC18O and 54 CDS regions for Salmonella virus TU_SP24B. Fortunately no any 'bacterial genes' were predicted within the phage genome, which ruled out the concern for transfer of 'virulent' genes from phage to bacterial strains. In addition, no 'integrase' genes were identified, which 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 and acquire a 'prophage' state. Also 'lysin' genes were identified in all three phages which

confirmed the presence of very enzyme in all strains. As ‘lysis therapy’ is gaining traction because of its ‘natural availability’, this could be a milestone in phage research in coming days. And genetically engineered ‘lysin’ enzyme may be the new antibiotic that could kill the bacterial bugs from outside, just like antibiotics.

High virulence and effective lysis are the key trait for microbial biocontrol agents, broad infectivity (broad host range) and efficient lethality at lower concentration (robust lysin enzyme) will have the most dramatic consequences for efficacy and for the cost-effectiveness of the bio-therapeutics like phages. As lytic phages have shown complete lysis of host bacterial strains for multiple generations, therapeutics use of phage have immense possibilities. Additionally, phage replicate whenever host is present, so we can hypothesize that ‘lower’ concentration of phage may suffice for efficient killing of drug resistant bacterial strains as they tend to replicate as long as host strain is present.

To address the concern of BIMs, application of ‘phage cocktails’ have shown greater promise because given bacterial strain is very unlikely to acquire multiple mutations at any given timeframe. Further, as phages are ‘host specific’, they would not exert selective pressure to other commensals like antibiotics. This approach (if proven) would result in dramatic improvements in treatment of infections without harming ‘beneficial’ commensals and equip us with efficient arm that can ‘kill’ drug resistant bacterial strains as soon as they emerge. Genetic engineering (tweaking phage receptors) would further enhance the efficiency of phage and ultimately help us resolve a ‘dynamic problem with a dynamic solution’.

CHAPTER – SIX
CONCLUSION

Thirty-four lytic phages were isolated that could infect and lyze drug resistant bacteria [clinical isolates] of human origin. Thus, we reject the null hypothesis and accept the alternative hypothesis which states - Lytic phages that can effectively kill drug resistant bacterial pathogens of human origin are abundantly present in river waters of Kathmandu valley as river waters of valley is heavily contaminated with drug resistant human pathogens, and thus is favorable habitat for bacterial viruses of its kind. Among them, 32 (except 2 *Klebsiella* phages) showed multiple host range within own genus and also did not induce any BIM up to 5th cycle of host's life cycle. However, lytic ability of *Citrobacter* phage (phage TU_Cit4B) was extremely low after purification and BIM were readily induced in case of *Klebsiella* phages (phage TU_Kle10O and phage TU_Kle10B). Transmission electron micrograph of deliberately selected 7 phages revealed that all of them were Caudovirales, of which two phages (phage TU_Cit4B and phage TU_Kle10O) were from Podoviridae family and did not show complete lytic property in lag and log phase of host's life cycle whereas other phages belonged to Siphoviridae and Myoviridae family that showed complete and efficient lytic ability in lag, log and also stationary phase of host's life cycle. This sufficiently proves that phages - natural predators of bacteria - can efficiently kill drug resistant bacteria. Exploiting lytic potential of such phages may lead the way for discovery of efficient antibacterial agent/enzyme that holds immense potential in therapeutics.

Genome analysis of three different phages confirmed absence of any potential 'virulent' genes of bacterial origin within phage genome which ruled out possibility of virulent gene transfer from phage to bacterial strain making it an excellent candidate for therapeutic use. Additionally, lysin proteins are predicted in all three strains, which may be separately used as an antibiotic in lysin therapy.

It is clear that without much additional work, it is unjustifiable to conclude about the possibility, efficiency and efficacy of phages in biomedical application. However, this research undoubtedly builds the framework and successfully proves that we are not out of weapons in fight against drug resistant bacteria yet. Phages – natural predator of bacteria – which have been functioning as biocontrol in the environmental microbiome since ages, can also efficiently kill drug resistant bugs. With availability of advanced techniques in molecular biology, a 'genetically customized phage' with rectified infective ability may be the 'next big thing' in the history of medicine anytime soon that could solve current antibiotic resistance crisis 'once-and-for-all'.

Limitations of the study

1. Small sample size: Limited sample size (bacterial) one of the distinct limitation of this study. We do not have satisfactory results of MHR spectrum analysis because of sample restriction.
2. Sophisticated instrumentation: As the research required electron microscopy, and ultracentrifugation, we had to export the sample where the facility was available. This limited our study and we could only observe 7 phages under electron microscopy, although we had 34 purified phages. Further, we also did not have privilege to repeat the electron microscopy for Klebsiella virus TU_Kle10O when it turned out to be entirely 'different' than its closest relative.
3. Assumption made in case of bacterial strains: Regarding bacterial strains to be different based on AST and plaque morphology in them (when infected by phage) is one of the main limitation of this study.
4. Lack of experience and expertise: Whole genome analysis is an arduous task – for sure. Due to limited knowledge and skilled bioinformatician, this study lags behind in various aspects of genome analysis like comprehensive genome annotation and phylogenetic analysis.

Future prospects / Recommendations

- Phylogenetic analysis: A detailed phylogenetic study is underway that would let us know about the similarity of our sequenced phages with the available database.
- Comprehensive Genome Mapping/Annotation: This dissertation only reports preliminary genome annotation. A comprehensive genome mapping, submission to GenBank and Announcement of Genome is another aspect of the study which is yet to be done. As I have encountered extreme lack of knowledge while working with 'whole genome' and its 'comprehensive annotation', I personally recommend a collaborative study (with bioinformatics specialist) would yield better publishable results/conclusions.
- Cell line study: Cell line analysis (after phage infection) is another potential study that would significantly improve the impact of phage research.
- Mouse/insect trail: Mouse trail in controlled animal house would provide us reliable data and significantly improve the acceptance of the claim we made. As pharmacokinetics of potential 'drug' differs in 'plates' and in 'live organisms', it is highly recommended to perform mouse trail (if possible) and/or insect trail in future studies.

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APPENDIX

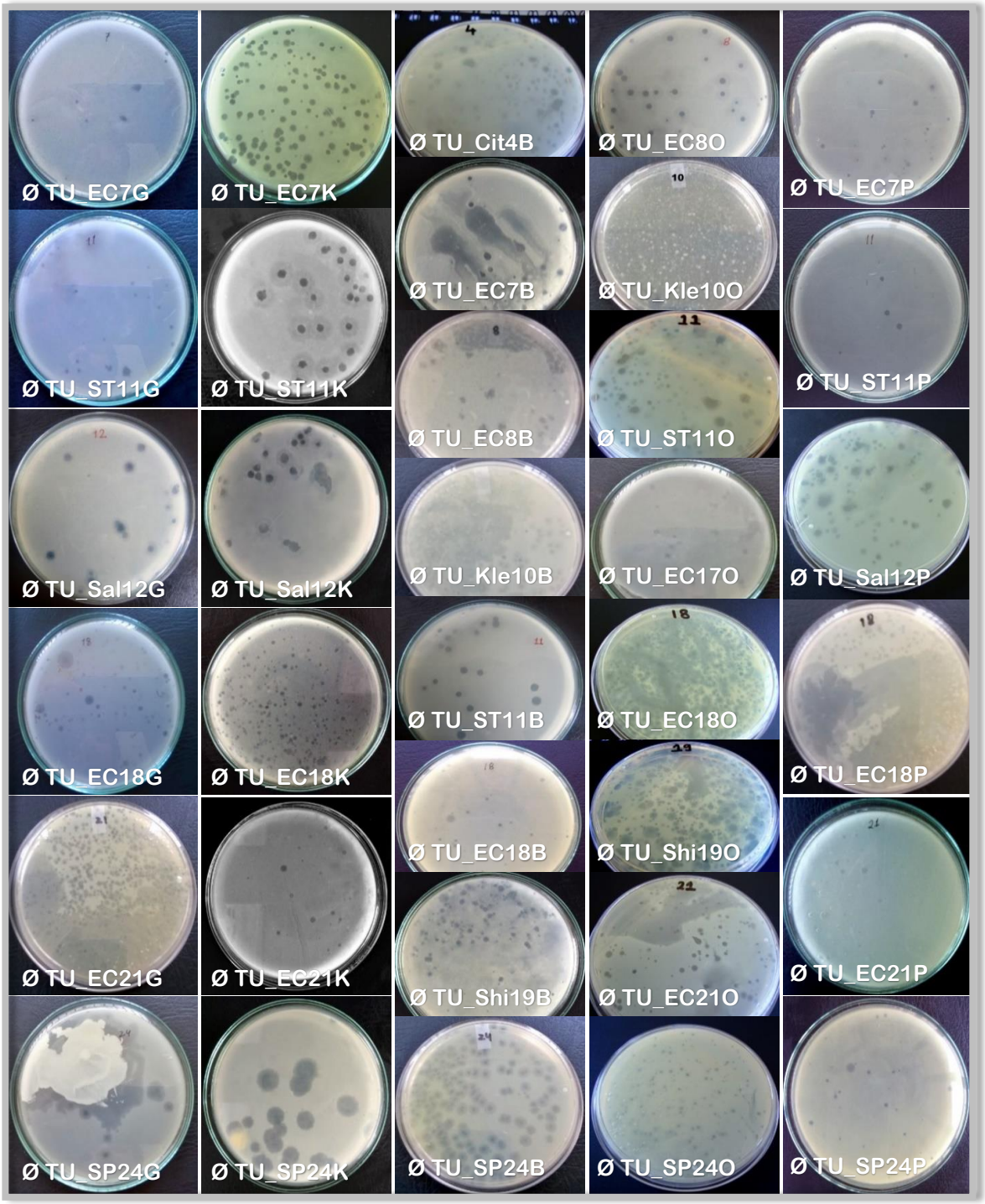

FIGURE AF1 | Phage screening. All 34 isolated phage from initial screening of 5 water samples. Conventionlal naming approach has been followed for simplicity and the word 'phage' is replaced by symbol 'Ø'. A single plaque was selected from each plate for isolation of pure culture. The selection was made based on visual analysis and a single clearest, round and biggest isolated plaque was selected from each plate for purification.

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

Order	Family or unassigned genus	Nature of the genome	Presence of an envelope	Morphology	Virion size	Genome configuration	Genome size (kbp or kb)	Host
Caudovirales	*Myoviridae*	dsDNA	−	icosahedral head with tail	icosahedral heads: 60–145 nm; elongated heads: 80 × 110 nm; tail: 16–20 × 80–455 nm	1 linear segment	31–317	B, Ar
Caudovirales	*Podoviridae*	dsDNA	−	icosahedral head with short tail	head: 60–70 nm tail: 10–20 nm	1 linear segment	16–78	B
Caudovirales	*Siphoviridae*	dsDNA	−	icosahedral head with tail	head: 40–80 nm; tail: 5–10 nm × 100–210 nm	1 linear segment	21–134	B, Ar
Unassigned family	*Corticoviridae*	dsDNA	−	icosahedral	60 nm	circular supercoiled	10	B
Unassigned family	*Plasmaviridae*	dsDNA	+	quasi-spherical, pleomorphic	50–125 nm	circular supercoiled genome	12	B
Unassigned family	*Tectiviridae*	dsDNA	−	icosahedral	66 nm	1 linear segment	15	B
Unassigned family	*Inoviridae*	ssDNA (+)	−	inoviruses: filamentous; plectroviruses: rod-shaped	inoviruses: 7 nm × 700–3500 nm; plectroviruses: 15 nm × 200–400 nm	circular genome	inoviruses: 5.8–12.4; plectroviruses: 4.5–8.2	B
Unassigned family	*Microviridae*	ssDNA (+)	−	icosahedral	25–27 nm	circular genome	4.4–6.1	B
Unassigned family	*Cystoviridae*	dsRNA	+	spherical	85 nm	3 linear segments	6.4–7.1, 3.6–4.7, 2.6–3.2	B
Unassigned family	*Leviviridae*	ssRNA (+)	−	icosahedral	26 nm	1 linear segment	3.5–4.3	B

FIGURE AF2 | ICTV Guidelines (according to 9th Report) for Classification of tailed and tail-less bacterial virus. (King *et al.*, 2012. pp 10-13)

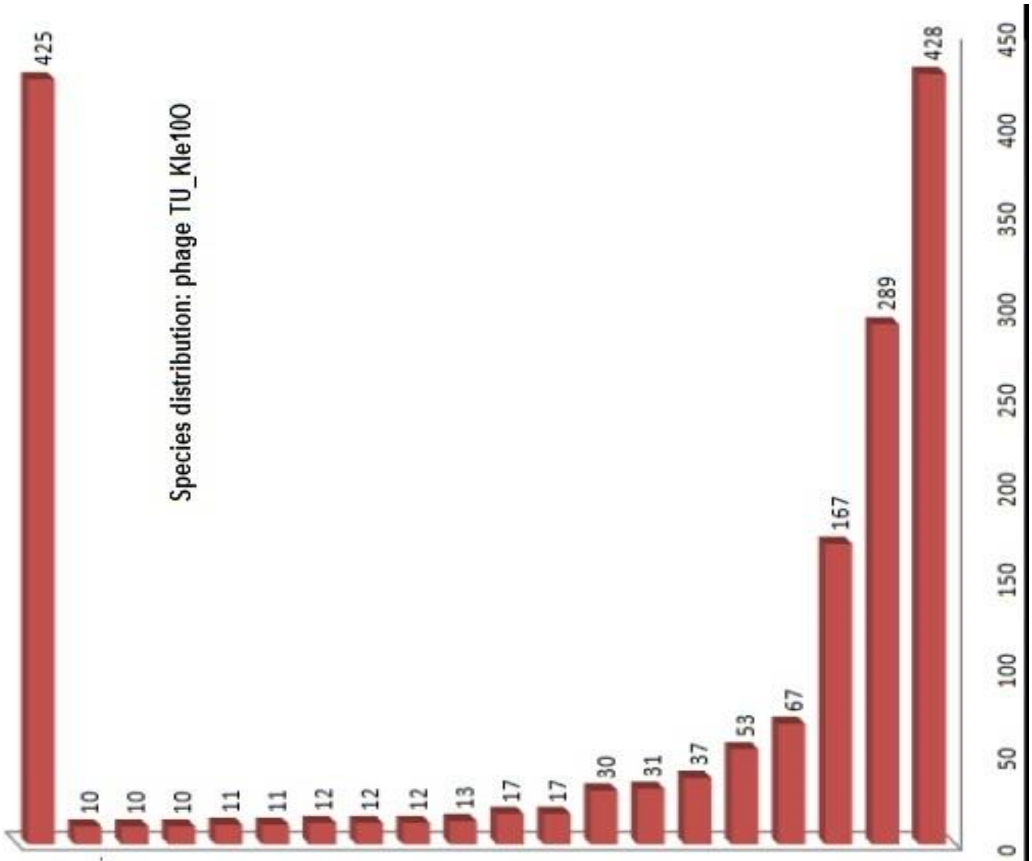

FIGURE AF3 | Species distribution for 'phage TU_Kle10O' (Klebsiella phage)

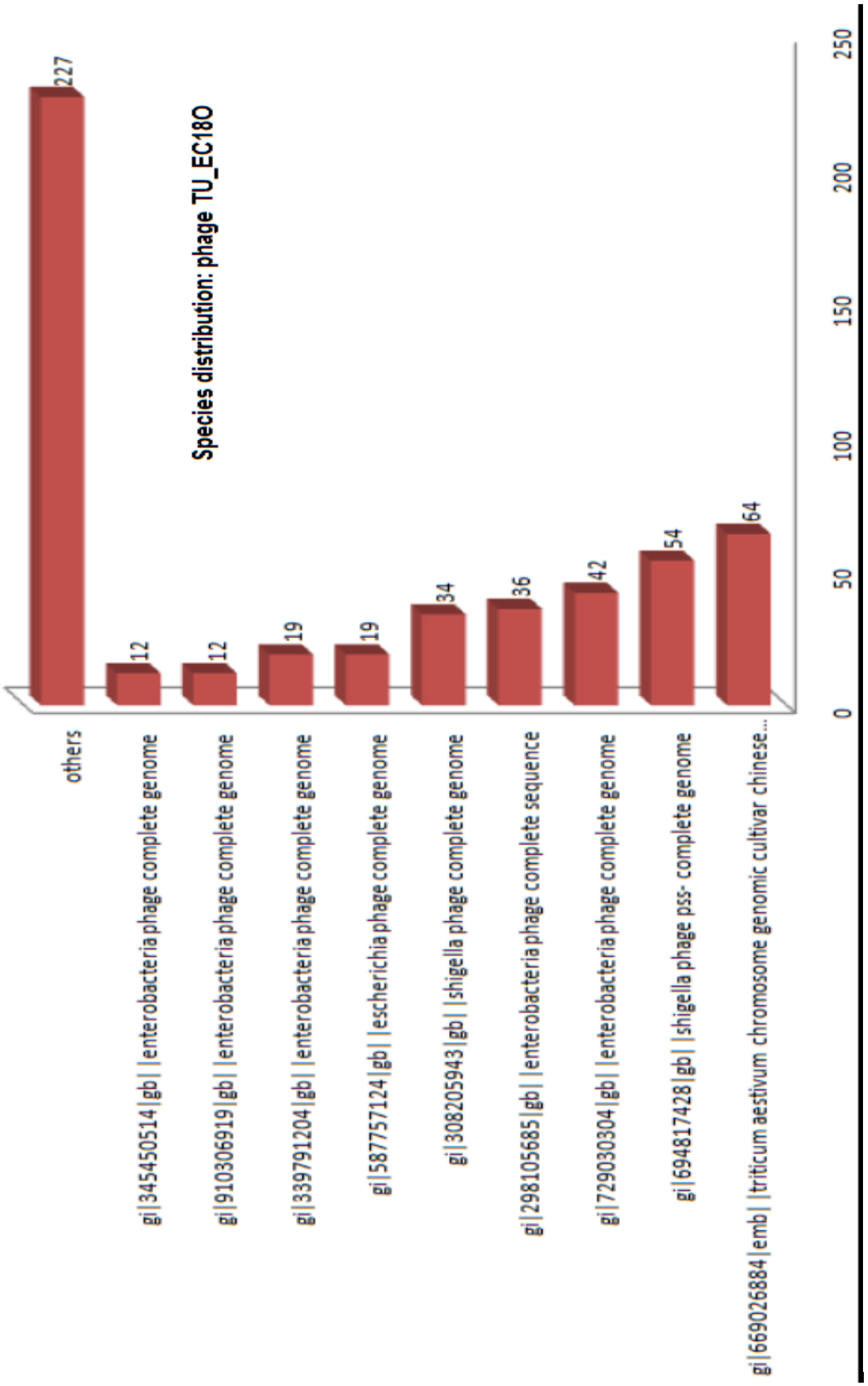

FIGURE AF4 | Species distribution for 'phage TU_EC18O' (Escherichia phage)

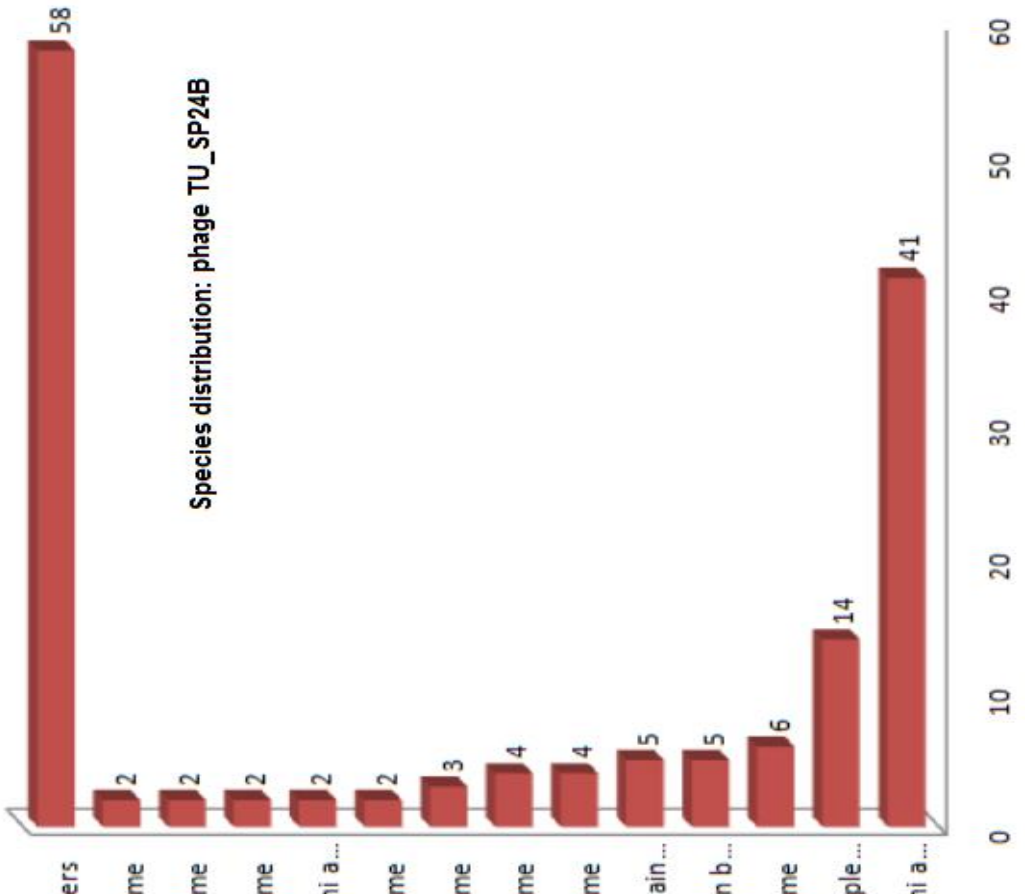

FIGURE AF5 | Species distribution for 'phage TU_SP24B' (Salmonella phage)

MEDIA / CHEMICAL COMPOSITIONS

1. Sodium Magnesium Buffer (SM Buffer)

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

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

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

3. Nutrient Broth/Agar (NB/NA) – HiMedia

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

* Note: 0.5 % and/or 1.0 % pure agar was added for semi-/solidification of media whenever required.

4. Mueller Hinton Agar (MHA) – HiMedia

Ingredients	Grams / Liter
Meat, infusion solids from 300g	2.000
Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.000
Final pH (at 25°C)	7.3±0.1

GENE/S IDENTIFICATION (CDS position, Blast hit and Protein sequences)

TABLE AT1 | Total genes identified from Klebsiella virus TU_Kle10O, their position on genome and complete protein sequences as identified by PHASTER tool.

SN	CDS POSITION	BLAST HIT	prophage_PRO_SEQ
1	complement (6406..7845)	PHAGE_Klebsi_JD18_NC_028686: DNA primase-helicase subunit; PP_00016; phage(gi966200514)	VVETILANLIYNQAFFTKVWPYMDKEYFEQGPAQTVFNIIKKHVNEYT AIPSKTALCVALDNSSITETEHEGAKKLIDKLSDAPEDLNWLVKETEKY VQEKAMYNATSRIIEIQANAQLEPNKRDKRLPDXGAIPDIMREALSVS FDSYIGHDWMEDYEARWLSYQNKARKVPFKLSILNKITKGGAETGTL NVLMAGVNVGKSLGLCSLAADYLQMGHNVLYISMEMAEEVCAKRI DANLLDVSLDDIDDGCVSYAEYKGKMEKWRSSSTLGRLIIKQYPTGG ANANTFRALLNELKLKKNFKPTVIIIDYLGICASCRIRQYTENSYTLVKAI AEELRALAVESETVLWTAAQVGRSAWDASDMDMSDIAESAGLPAT ADFMLAVIETPELAQMKQQLIKQIKSRYGDKNINNKFFMGVHKANQ RWVEIEKQNDPTKPNPTNTVREGAGAQNRVAESNRQERVSRSKLD ALAEELKF
2	complement (8195..9370)	PHAGE_Klebsi_JD18_NC_028686: RecA-like recombination protein; PP_00018; phage(gi966200516)	MSDLKSRLIKASTSKMTAELTKSKFFNEKDVVRTKIPMLNIAISGALDG GMQSGLTIFAGPSKHFKSNMSLTMVSAYLNKYPDAVCLFYDSEFGIT PAYLKSMGVDPDRVIHTPVQSVEQLKIDMVNQLEAIERGEKVIVFIDS IGNMASKKETEDALNEKSVADMTRAKSLKSLFRIVTPYFSIKNIPCVAV NHTIETIEMFSKTVMTGGTGPMYSADTVFIIGKRQIKDGTELEGYQFV LNAEKSRTVKEKSKFFIDVKFDGGIDPYSGLLDMALDIGFVVKPKNG WYAREFLDVETGEMIREEKSWRAKDTSSTEFWGPLFKHEPFRDAIKA RYQLGAIDSNAAVDEAVAEMINSKVSTKVDGVKLPESGSVSAAEVED ELENFMNED
3	complement (14806..15177)	PHAGE_Klebsi_JD18_NC_028686: translational repressor protein; PP_00025; phage(gi966200523)	MVKMIEITLKKPEDFLKVKETLTRMGIANNKDKVLYQSCHILQKQGRY YIVHFKEMLKLDGRPVTIDLEDEIRRDSIAQLLADWGLLSINRGQTLA QMQNNFRVITFKQKHEWTLKSKYTIGA
4	complement (23125..23673)	PHAGE_Klebsi_JD18_NC_028686: sigma factor for late transcription; PP_00039; phage(gi966200537)	MSNYVNNKELYKSICAWKEKCRESEAAGGPRVVKQNDTIGLAIMLIA EGLSKRFNFSGYTQSWKQEMISDGIEAAIKGLINFDETKYDNPHAYIT QACFNAFVQRIKKERKEMAKKYSYFVHNVYDSRDDDMVALADETFI QDIYDKMTQYESTAYKAPGSAKKSEPTSDGPNLEFLYEAED
5	complement(26973..27443)	PHAGE_Klebsi_JD18_NC_028686: anaerobic NTP reductase small subunit; PP_00052; phage(gi966200550)	MNFDRIYPSDFVNGPGCRVVLFVTGCLHKCEGCYNKSTWNARNGQ LFTMNTVKEIASHLSKSYTQGLTLTGGDPLYPQNREEISNLVSWVKAR FPEKDIWMWTGYKFEDIKDLDLLQHIDVIIDGKYEKSLPTTKNWRGS DNQRLWVRNGSTWTHD
6	complement (27620..29458)	PHAGE_Klebsi_JD18_NC_028686: anaerobic ribonucleotide reductase; PP_00054; phage(gi966200552)	MNIELEIQGLINKTNKDLLNENANKDSRVFPTQRDLMAGIVSKHIAN QVIPFSVMEAHKEGVIHFHDMDYSPALPFTNCCLVDLKGMLQNGFK LGNAQIETPKSIGVATAIMAQITAQVASHQYGGTTFANVDLVLAPYV EKTFAKHVRDARKYQVALVKDYAISKTEKDVFDAFQAYEYEVNTLFSS NGQTPFVTITFGMGTSWEEKLIQRAILDNRIRGLGRDGITPIFPKLVM FVEEGINLRKEDPNYDIKQLALECAAKRMYPDIISARNNRAITGSETPV SPMGCRSFLGAWRDSSGKPVLDGRNNLGVVTLNLPRIALDANYKSS DDSNKLFKLLDERLDICKEALLTRIKSLEGVTASVAPILYQEGAFGVRM KPDDEILELFKNGRSSISLGYIGIHEFDMLTFKGSGKLVLKYINAKLNKW TEETGYAFSLYSTPAESLCYRFCKIDQAKFGDVKGVTDKGWYTNSFHV SVEENLSPFEKIDREAPYHSIAKGGHISYVELPDMKRNLEGLEAVWDY AIEKLDYFGVNMPVDKCLSCGSTHEMTPTENGFTCSICGETDPKKMN TIRRTCGYLGNPSERGFNLGKNKEIMHRVKHVRETNEAI
7	complement (37826..38803)	PHAGE_Klebsi_JD18_NC_028686: thioredoxin; PP_00077; phage(gi966200577)	MSTITIKKGIYFGKEISGTYELLGEWFPDSLSAEDSRQGDGKVFVELNG KKRGVWVFKDDITIDGVAAKIEVVESVDEMKERIKKRFNVMGLMTN GLVHGNIRSLIISGAAGIGKTYSLDKALQHAHDTNAIDYKSVNGKISGI GLYCRLWESREANSVLLIDDVDVFSDMDILNLLKAALDSGEKRKVCW STASSFLEDKGIPNEFEFEGTVVFITNVDIDRELERGSKLAPHLQALVSR

			SVYLDLGVHTNEEIMVRVQDVIMTTSMLQNRGLRNSEVIEVLEFMK DNVNRLRNVSLRTALYIGDFVATDRKNWREIAEVTMLK
8	complement (57172..57247)	tRNA	GCCGATTTAGCTCAGCTGGTAGAGCGCTTCACTTGTAATGAAGAT GTCGCGGGTTCGACTCCTGCAATCGGCACCA
9	complement (57348..57432)	tRNA	GCATCGATGGTGGAACTGGTATACACAcctcACTTAAAATgaggCGC CGCAAGGATTGAGGGTTCGAATCCCTCTCGGTGCACCA
10	complement (57437..57513)	tRNA	CGGGGCATAGCTCAGTTGGATAGAGCAGCGGACTTCTAATCCGCA GGTCGAAGGTTCGAATCCTTCTGCCTCGACCA
11	complement (58860..58936)	tRNA	GGCCCTGTAGCTCAATTGGTAGAGCGTTCCCCTCATAAGGGATTG GTTGCATGTTCGAGTCTTTGCCAGGGTCACCA
12	complement (60204..60280)	tRNA	CTCCGTATAGCTCAGTCTGGTAGAGCGCTCCATTTGGGATGGAGA GGTCGAATGTTCGAGTCATTCTATGGAGACCA
13	complement (60286..60360)	tRNA	GCATCCATCGTATAGCGGATATTATGTCTGGCTTCCACCCAGAAG ATGGGAGTTCGATTCTCCCTGGATGCTCCA
14	complement (60366..60440)	tRNA	AGGTTCTTAGTATAACGGCTATTATGCTGGGCTCCAAACCCAGTG ATGAGGGTTCGATTCCTTCAGGGCCTGCCA
15	complement (60447..60522)	tRNA	GGGAGTATAGCTCATTTGGTAGAGCTCTCGACCGATAATCGAGCG GTGACTGGTTCGAGTCCAGTTACTCCCACCA
16	complement (60589..60673)	tRNA	GGAGAGTAGCGCTAGTGGTAGCAAACCGGACTTGAAATCCGGGC CATCGGAAACGGTGAGGGTTCAACTCCTTTACTCTCCGCCA
17	complement (60770..60845)	tRNA	GTGGCCGTAGTTCAGTTGGTAGAACTCGAGATTGTGATTCTCGTA GTCATGGGTTCAACTCCCATCGGTCACCCCA
18	complement (60942..61016)	tRNA	TGGACTATAGACAAGCGGTTAAGTCACCGGCCTTTGACTCCGGTA TCTCTGGTTCGAATCCAGATAGTCCAGCCA
19	complement(61023..61099)	tRNA	TGCGGGGTAACTTCAGTTGGTAGAATGTTGGGCTCATATCCCGAC ACGCGCAGGTTCGAGTCCTGCCTCCGCCTCCA
20	complement (61261..61336)	tRNA	GGACCTATAGTTTCAGCGGTTAAAATACTCGCCTGTCACGCGAGA GTCACGGATTCGAATTCCGTTAGGTCCGCCA
21	complement (61590..61674)	tRNA	GGGTCGTTGGCTGAGAGGGTAAGCGACGGACTGTTAATCCGTGT CAGAAATGACTAGGCAGGTTCGATACCTGCACGGCCCGCCA
22	complement (61680..61756)	tRNA	GGGATACTAGCTCAGTTGGTTAGAGCACCGGACTTTTAATCCGGG TGTCCGAAGTTCGAGTCTTCGGTGTCCCACCA
23	complement (61763..61849)	tRNA	GGGGAGTTAGACCGTAGGGGTAGCGGGACAGACTGTAAATCTGT TGCTCAAAAGGCTCGAGTGGTTCGACTCCATTACTCCCCACCA
24	complement (65764..66213)	PHAGE_Klebsi_JD18_NC_028686: head completion protein; PP_00137; phage(gi966200639)	MAYSGKFMPQNLHKYKGDFRKITYRSTWEQYMMRWLDNHPDVV QWNSEEVVIPYFSNADGKKRRYFMDFWAKFSNGQQFFFEVKPKKET RPPVKPTKLTTSAKKRYIDEIYTWSVNVDKWKAAQATASKMGIEFRLI TEDSLKKLGWKG
25	85231..87069	PHAGE_Klebsi_JD18_NC_028686: terminase DNA packaging enzyme large subunit; PP_00154; phage(gi966200656)	MEQTQPFNVLSDAHPLNDGKQVVIRHPSEMETKIENGVRFFKSQW DDKWYPEKFEDYLKLHGIVKIRLQGEDPAHFQTFKDKNNKRTRYMG LPNLKRANIKMQLTREIVAEWKKCRDDIVYFAETYCAITHIDYGTIKVQ LRDYQRDMLEIMAAKRMTCCNLSRQLGKTTVVAIFLAHFVCFNKDK AVGILAHKGSMSAEVLDRTKQAIELLPDFLQPGIVEWNKGSIELDNGS SIGAYASSPDAVRGNSFAMIYIDECAFIPNFIDAWLAIQPVISSGRRSKI IITTTPSGLNHFYDIWTAAVEGKSGFTPYTAIWNSVKERLYNDEDMFD DGWQWSLQTISASSLEQFKQEHCAEFHGTSGTLISGMKLANMDWI EVTPDSHGFYKFKEAEADHKYIATLDSAEGRGQDYHALNIIDVTTSEW EQVGVLHSNTISHLILPDIVIKYLMEYNEAPIYIELNSTGVSVAKSLYMD LEYENVICDSIVDLGMKQTKRSKAVGCSALKDLIEKDKLIIHHRATVQE FRTFSEKGVSWAAEDGYHDDLIMSLVIFAWLTTQQKFADFVDKDEM RLASEVFKRELEDMNDDYAPVVFVDAVNSAEYAPQEHGLSFV
26	87103..89076	PHAGE_Klebsi_JD18_NC_028686: tail sheath protein; PP_00155; phage(gi966200657)	MPLVSPGIELKETSVQSTVVLNATGRAAIVGKFQWGPAYQVTQITNE VELVDMFGGPNNQTADYFMSAMNFLQYGNDLRTVRVVNREAAKN ASPLVDNIEWTITTAGSNYEVGDKITVKYADQTVDDTGSVTEVDSDG KIKSVFIPTSKIIAYAKSINQYPDLGSSWTTTITSQSSGVSAVITLGKIISES

			TVLLTEHETAHEEMTKTEFQTALAQYKMPGIVAAYPGELGNQLEIEIV SKAAFDKGEQLTIYPGGGQRASTAKAVFGYGPQTDTQYAIIVRRDGA VVESAVLSTSRQDKDIYGNNIFMDDYFSKGSSRYVFATAQGWPEGFS GVIRLGGGVSANESVTAGDLIQGWDLFGDREALRVNLLIAGACAGET DEIASTVQKHVSSIADERQDCLALISPPRSTIVNIPLTRAVDNLIDWRQ GEGSYDSANMNINTTYAAIDGNYKYQYDKYNDVNRWVPLAADIAG LCARTDDVAQPWMSPAGYRRGQILNCIKFAIEPRQAHRDRMYQAGI NPVTGQGTGDGFILFGDKTATTVPTPFDRINVRRLFNMLKNNIGDSS KWQLFELNDNFTRSSFRMETSQYLAGIKALGGVYDFRVVCDTTNNT PAVIDRNEFVASFYIKPXXXXXXXXXXXXXXXXXDFDELIGPQ
27	89204..89695	PHAGE_Klebsi_JD18_NC_028686: tail tube protein; PP_00156; phage(gi966200658)	MELTDITRAFESGDFARPNLFEVEIPYLGRNFSFKCKAAPMPAGIVEK VPVGYMNRKINVAGDRTYDDWTVTIYNDDKHEVRKAIIAWQAQAH AQGNDISGMTPADYKKVATVRQFSRDGKTITNEHTITGLWPTNVGE VQMDWDSNNEVETFETTFAIDWWE
28	89800..91368	PHAGE_Klebsi_JD18_NC_028686: portal vertex of the head; PP_00157; phage(gi966200659)	MAFHILDLFAPWEKRDEAEYKQQINNDLESITAPKFDDGAREVESNE NEIQYNSFNQMMFGSNEPGMKTTADLINTYRSLMNNYEVDNAVEE IVSDAVVYEDGHPVVSLDLDSTDFSQAIKDRILEEFNTVLTCLNFERKG ADHFRRWYVDSRIFFHKIVNTKKMKDGIQELRRLDPRNLQFIREIVTA DDAGTKIVKGYKEYFIYDTGKESYYADGRLYSAGTKIKIPRDAIVYAHS GLVDCSGQNIIGYLHRAVKPANQLKLLEDALVIYRITRAPDRRVFYIDT GNMPSRKAAAHMQHIMNTMKNRVVYDASTGKIKNQQHNMSMT EDYWLQRRDGKAVTEVDTLPGMSGMSDMDDVRYFRTALYMALRV PLSRMPDANNQGGVQFDAGTSITRDELDFAKFIRRLQHKFEEIMLDP LRTNLILKKVLSKDEWEDEINNIKIVFHKDSYFTELKDAEVMERRINML TMAEPFIGKYISHKTAMKDFLQMSDEEIEQEAKQIELESKEARFQDQE NEEDF
29	93530..95089	PHAGE_Klebsi_JD18_NC_028686: major head protein; PP_00162; phage(gi966200664)	MKKNKLIEKWQPLLENEALPEIVGASKKALIAKIFENQEADINQAPEY RDEKIAEAFGSFLSEAEIGGDHGYDAQNIAAGQTSGAVTQIGPAVM GMVRRAIPNLIAFDICGVQPMSNPTGQVFALRAVYGKDPLAAGAKE AFHPMYAPDAMFSGQGATEKFEAVKAGDALTVGDIVVHDFAQTGR AHLQVVEDFTVDAGATDAAKLDAAVTAALEAGKVVEIAEGMATSVA ELQEAFNGSKDNPWNEMGFRIDKQTIEAKSRQLKAQYSIELAQDLRA VHGMDADAELSSILATEIMLEINREVVDWINYSAQVGKSGMTQTVG SKAGVFDFQDPIDIRGARWAGESFKALLFQIDKESAEIARQTGRGEG NFIIASRNVVNVLAAVDTNVSPAAQGLGRGYNTDTTKAVFAGVLGG RYRVYIDQYARQDYFTIGYKGANEMDAGIYYAPYVALTPLRGSDPKN FQPVMGFKTRYGIGINPFADSAAQQPKGRITSGMPSIVNSVGKNAYF RRVWVKGI
30	complement (98745..98972)	PHAGE_Klebsi_JD18_NC_028686: DNA primase-helicase subunit; PP_00167; phage(gi966200670)	MKALQAHLMHESGKDFQEIARALDITPAEAAKLWVSVEKAHERFKQ KEKVVYRKRLTNVGIKSRHKKLVKHMRTL
31	100756..102258	PHAGE_Klebsi_JD18_NC_028686: RNA-DNA and DNA-DNA helicase, ATPase; PP_00170; phage(gi966200673)	VHDIQVKFKDFSHVHIECDDSIFYELRDYFSFEADGYRFNPKYRYGHW DGRIRLLDYNRLLPFGLVGQIRKFADQFGYKVYFDPAIFEQETLSREDF DTWLASKEIYSGLTKIEPHWYQKDAVYEGLVNRRRILNLPTSAGKSLI QALLARYYVENYEGKILIIVPTTALVDQMIDDFCDYRLFPRNAMLGIR GGTARDSNALVYVSTWQTAVKQPKEWFSQFGMMMNDECHLATG KSISTIIAGLTNCMFKYGLSGSLKDGKANIMQYVGMFGEIFRPVSTSKL MEDGQVTELKINTIFLRYPDAAANALKGKTYQEEIKFITNVKKRNRW VANLASKLASRDENAFVMFKHVAHGKELFEMIKATGHEQVYYVSGE VNTETRNALKAMAENGKGIIIVASYGVFSTGISVKNLHHVILAHPVKS KIIVLQTIGRVLRKHKDKSLATVWDIIDDLGVKPKSANAKKKYTHLNYC LKHALERIQRYADEKFNYVMKTVNL
32	complement (119111..119359)	PHAGE_Klebsi_JD18_NC_028686: lysis inhibition accessory protein; PP_00196; phage(gi966200700)	MNKQLTKALELQRNAWNSGHENYGASIDIYAEALEVLKGFKHLNPA QAEFRDTLEAMDELKYAKHLGSAARKAVRHFVVTLK

33	complement (119478..119801)	PHAGE_Klebsi_JD18_NC_028686: head assembly chaperone protein; PP_00197; phage(gi966200701)	MELPIKALGEYVILVSEPAQQGDEIVSPSGIILGKEEQGQLPDMCEIYSIGDDVPKGFVEVGDLTPLPVGNIRNVPHPLVAAGVKKPKEIRQKFVTCHYKSLACVYK
34	complement (120364..120933)	PHAGE_Klebsi_JD18_NC_028686: deoxycytidylate deaminase; PP_00200; phage(gi966200704)	MKASTVLQIAYLVSQESKCCSWKVGAVIEKNGRIISTGYNGSPSGGVNCCDHAAEQGWIGEIPYKSTGLRQDGFQVKKVGLLKEHRAAHSAWSKVNEIHAELNAILFAARNGSSIDGATMYVTLSPCPDCAKAIAQSGIKKLVYCETYDKNEPGWDDILRSAGIEVFNVPKRNLDKLNWYNIKEFCGIE
35	complement (125754..126170)	PHAGE_Klebsi_JD18_NC_028686: endonuclease II; PP_00211; phage(gi966200715)	MNDIANEFSFIKYVQLELEPDFTIKPILIANKLNVVYAIAVDDELVYIGKTKNLRKRINYYRTAINRKDQTSDSAKSAKIFEALMAGKKVEFYARQCFNLLINNELGEMSISTMDLEEPMFIKKFNPPWNTQHKVKKC
36	complement (126198..127340)	PHAGE_Klebsi_JD18_NC_028686: ribonucleotide reductase of class Ia (Aerobic) beta subunit; PP_00212; phage(gi966200716)	MSTVFNTKQVDIMTEPMFFGSGLGIARYDIQRHKVFEELIEKQLSFFWRPEEVNVMMDRGQFEKLPEHQRNIFTDNLKYQSLLDSIQGRAPAAVLSALISDPSLDTWNQTWTFSETIHSRSYTHIMRNLYVDPAKIFDEIVLDEAIMKRAESIGVYYDDVIAKTRAWENAKNRCFNQDNIEIKEAKRDLMKSLYLCLHVINALEAIRFYVSFACTFNFHKNMEIMEGNAKIMKFIARDEQLHLKGTQYILRQLQTGTDGEEWVEIAKECEQEAIKIFMEVNRQEKDWAIHLFRNGGLPGLNVKILHDFIDYLTVSRMRSCGLPCPITDAPTRHPIPWIREYLNSDAVQSAPQEVEISSYLVAQIDNDVTDDVLIGFKKYL
37	complement (127397..129643)	PHAGE_Klebsi_JD18_NC_028686: ribonucleoside-diphosphate reductase; PP_00213; phage(gi966200717)	MQVIKSSGVSQEFDMQKIIKVLEWACEGTKVDPYELYEIIKSHLRDGMSTADIQKTIVKVAANSISIDEPDYQYVASNAAMFEIRKRVYGQFEPPAFIDHISRCVNANKYDKEILSKWSAEEITLLDSYIKHERDFTMTYAGTMQLIEKYLVKDRHTGELYETPQFAFMLIGMCLHQDDGENRLANVIRFYDAVSTKKISLPTPIMSGVRTPTRQFSSCVVIEGGDSLNSINEAAASITKYISKRAGIGINAGMIRAEGSKIGFGEVKHTGVIPFWKHFQTAVKSCSQGGVRGGAATLYYPIWHLEVENLLVLKNNKGVDENRIRHLDYGVQINNLMIERLIKNDYITLFSPDVCLGTLYTEYFRDAQAFRTLYEELEKNPDIRKKRIKARELFELFLTERAGTARIYPYMVDNVGEYGPFIRDVATVKQSNLCLEIALPTSDVGQEDGEIALCTLAAFVLDNFNWQDQEEVNEIAEVMVRALDNLLDYQDYPVDKALKAKDRRALGVGITNYAAWLASNFASYADANDITHEMMERIQYALIKASVKLASEKGPCTLYKETRYGRGELPIDWYNERIDQLAAPNYVCDWELLREDLKRYGIRNSTLSALMPCESSSQVSNSTNGIEPPRGPVSVKESKEGSFNQVVPNVEHNASLYDYAWQLAKQGNKPYLNQVLIMQKFVDQSISANTYYDPANFPKGKVEMSVMMDDLLYFWYFGGKTLYYHNTRDGSGNDDMIQDSADCAACKL
38	complement (130139..131014)	PHAGE_Klebsi_JD18_NC_028686: thymidylate synthase; PP_00216; phage(gi966200720)	MREYQELIKDIFENGYETDDRTGTGTIAKFGTQLRFDLQKGFPAVTTKRLAWKACIAELIWFLQGSTNVHELRLIQHGSLLEGKTVWDDNYENQAKDMGYSGGELGPVYGKQWRDFMGVDQLKLVIDRIKQLPTDRRQIVTAWNPIDLDKMALPPCHLLYQFNVRNGYLDLQWYQRSVDVFLGLPFNIASYAALVHIIAKMTNLKPGHLVFTGGNTHIYLNHIEQCKEILRREPKELCELEINYWPPVKDKEFHELATEEQLAWVTGMMKPSDFVLKGYESHPTIKGKMAV
39	complement (159536..161374)	PHAGE_Klebsi_JD18_NC_028686: DNA topoisomerase subunit; PP_00258; phage(gi966200764)	MIKNEIKILSDREHIIKRSGMYIGSSAFEAHDRFLFGKFQSVKYVPGIIKLIDEIIDNSVDEAIRTNFKHANKISVDIKGNKIIVTDNGRGLPQAPVVTPEGETIPGPVAAWTRPRAGGNFGDDAERKTGGMNGVGSALTNIFSVTFVGATCDGKNEIVVRCSNGAENVSWEEHPAKDKEFIKDKTGTVVSFIPDFTHFESDGLTDVDQSIIHDRLMTLAVVYPDIEFKFMGKRVQGKFKAYAHMYDENAVVQDSDTCAIAIGRSDDGFRQLSYVNNIHTKNGGTHVDLVLDELSNELIPALKRKYKLEVNKARIKECLTVIMFIRDMSNMRFDSQTKERLTSPWGEIRSHIDIDYKKLANAIMKSEDIHMPIIEAMLARKLAAEKAAETKAAKKAQKAKVAKHIKANKYGKDADTTLFLTEGDSAIGYLLTTRDRELHGGYPLRGKFMNTWGMSAADAMKNKEVFDICAITGLTIGEPAENTNYRNIAIMTDADVDGVGSIFPSLLAFXSNWPELFEQGRIRFVKTPVIILTKGKEQRWFYSLGEYEDHKDDFKGWKLRYIKGLGSLEEDEYERVIQDPVYDVVTLPENWKELFELIMGNDAAPRKTWMSE

TABLE AT2 | Total genes identified from Escherichia virus TU_Kle10O, their position on genome and complete protein sequences as identified by PHASTER tool.

SN	CDS POSITION	BLAST HIT	prophage_PRO_SEQ
1	1..2214	PHAGE_Escher_vB_EcoM_JS09_NC_024124: rIIA protector from prophage-induced early lysis; PP_00001; phage(gi642905971)	MIIETAKETIIXSGGKSTAFTIQGNSKVYKILSNXLYTNKELACVRELITNCI DXQILNGCTDKFIVQAPGRLDPRFVVRDFGPGMSDFTIRGNDEEPGIYN SYFASTKTSSNDFIGGFGLGSKAPLAYTDTFNLTSYHXXEVRGYVIYQDDS GPQIKPTFVDKMGPDDRTGVEVVVPVNPEDFEKFAXEIAYVMRPLGXIA EVRGVKDIKYFPEFDDVYLAKEAPWGERGNIMAVYGGIXYPIGSVIKEQT WMMTXCTTAYIKFPMGELDVAPSREALSFDKRTVANIHKRVAEIDAKLF AEDSKKWIDCEXPRHVFREIDSLGYTARKYMEKAGSNIEXLKYSKEQLTYS ELYKRFKMGPEWCNLGVVYDIIEDPRLRRIRESGSSSAXISINSLIGXXXXXI DIVIDXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXSVLFVDPEDEDA MKLLERLKXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXPRPKSXSAHRF FKNENDVWVSEDLFIPASEADEIQGYAIVRNRSNAECLDADKGWLNYD SNFLTRIADLSGIKEFTVVRPQIAKKVRKLGEVECLFEKSIXDXIXLIDKVDY DEYVSPSRRAQPYLNHITRNEEXNFLSKYFSSKNKEISKDFAKLSTVNNR WGWNRFSGATNXXLNKKLELCAKIFDKLKDNAYDNDDKMVIEFETNYH IVSEYMGRRGTLSKEQVAQIVKFMKAVEAAK
2	2224..3171	PHAGE_Escher_vB_EcoM_JS09_NC_024124: rIIB protector from prophage-induced early lysis; PP_00002; phage(gi642905972)	MYNIKCLSQEEQLEAYDLXKSGKWTRKEIADYFDISTDTLRKIVKNFKAEX XXXXXXXXXXXXEEVPENDEPIIGGHRPEIVWNASSKFISIIEGRVAXNAT PSSHANFEEIKANLVAGNLSKAVELINIKKAITKFVXGNVTIEGGSLFYQGI EIRSGLVNRIIDXMEKGEDFKFYLPFLENLLENPXEKAVQRLFDFLVANDI EITEDGHFYAWKVVRKDYLDCYSGTFDNSPGKVVSMPRTRVNDDDTQT CXXGXXVCAKSYIRYFGSSSDKVVKVKVHPRDVVSIPVDYGDAKMRTCR YEVISDVTELFAE
3	3518..3994	PHAGE_Escher_vB_EcoM_JS09_NC_024124: DenB; PP_00004; phage(gi642905974)	MKVKIADTARYSLSQNPDQNKEXVVRRCKVAVFAEYVVANWXDGYVN KGIEDVEDPYTYAWDVLAHPKYCGIXVEVKTXQIDSKWISVTTGYSGDYP GGNGINIXPFLNHRVADCIIILDVKETSPDVFSYSIKFVGDHEDLKKIVRKS NYNGWYLQL
4	4854..5300	PHAGE_Escher_vB_EcoM_JS09_NC_024124: nucleoid disruption protein; PP_00007; phage(gi642905978)	MSKYLTRKDLLAVXGEVVAVVRNGEYGXEVSKEFRSREGFYFFVKGSSD WRQVAARFFVGRQRSKQGLDAILSHIRQGRSQLARTMGTNNIEYDVIF VAAKNMKPLTTGYGKGQLALAFTRNHTSEYQTLTEMNRLLADNFKFILQ SY
5	5644..6969	PHAGE_Escher_vB_EcoM_JS09_NC_024124: DNA topoisomerase II medium subunit; PP_00009; phage(gi642905980)	MQLNSRNLKSIIDNEALAYAMYTVENRAIPNMIDGFKPVQRFVVHRAL DLARGNKEKFHKLASIAGGVADLGYHHGESSAQDAGALMANTWNNN YPLLDGQGNFGSXTVQKAAASRYIFARVSKNFYNVYKDTEYAPAHEDKE HIPPKFYLPIIPTVLLNGVSGIATGYATNILPHSFKSVKKAVLQALQGKNVT KPKVEFPEFRGEXHEVDGRYEIHGTYKFTSRTQMQITEIPYKFDRETYVSK VLDPLEDKGLISWEDDCGEHGFGFKVKFRKEYSLPDDEELRHEKIMKDFS LIERRSQNITVINEKGKLAVYDNVVDLIKDFVEVRKTYVQKRIDNKILESEK AFKLAFAKAHFIKKVXTGEIVIQGKTRKALTEELAQIEMYKEHVDKLVGLN IFHITSDEARKLAEEAKAKKEENEYWKSTDVVTEYTKDLEAL
6	7476..8108	PHAGE_Escher_vB_EcoM_JS09_NC_024124: activator of middle period transcription; PP_00011; phage(gi642905982)	MSKVTYIIKASENALNEKTAAIMVYIIKNNFTTAANVREALEAEYNASVV NSNIGVLIKKGLVEKSGDGLIATGEAMDIIQKAADLFAQENAPELLQKRN TRKARGVTPEMHELANFVFENIKDKVEVKEIGENRSNLEVRFAKRVLGIR QIEIRRDGALRIFAYNMTETESKLFTSLENDVKIKIGGKYTYIDFPNVSKEII TLVTNVL
7	complement (10258..10917)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: phage holin; PP_00020; phage(gi642905798)	MATPKVSFSPSDILFGLLDRIFKDNATGKVLMSRVAVIILLFLMALIWYKG SILLDYYVRAKYDTYTEIIQKERALRFESAALEQLQIVHISSGADFSAVYSFR PKNLNYFVDLIAYEGKLPSTISEKSLGGFPVDKTADEYIIHLSGRHFSSKSEF AFLPTKSKTAELSYMYSCPYFNLDNIYAGTVTMYWYKGNNILEEDRLAAI CNQAVRILGRAK
8	complement (10927..11478)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: tail fibers; PP_00021; phage(gi642905799)	MKLYHYYFDTKEFYKEENYKPLKGVGFPAHSTPKKPLDPKEGYAVIFDER EQEWVYVEDHRGKTVWTYDKQKIVIDTPGALQNVTTEEPGEFDIWTG DGWKEDEAYKRVVQRDKKIHELYKLFSVLSSLIDAEVANREEKRKFKELK KFFALLEKHEHLGGEFPKFPDLENKSIVKAIINIFK

9	complement (11509..14751)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: long tail fiber distal subunit; PP_00022; phage(gi642905800)	MATLKQIQFKRSKVAGVNPTAAQLAEGELAINLKDRTLFTKDDLGNIISL GFAKGGDINGNVTQNGDYLQNGSYNLNGNQFVYAGKYIEFLPKTAGN GAWANQHLNKAPIFTDLSSTTSTSEYHPLIKQRYKDGTFSVGTLVSEGSF RVHYIDSTAPDNSKHWVFNRNGNFIVDSGNIEVRTGNISASGNINSANG IVSAPQVNTKTIVLDTKAFGQYDSQSLVQYVYPGTGEENGVNYLRKVRA KSGGTIYHEIASAQTGKNDEISWWTGNTLTTKLMGLRNDGAMVLRRSL AIGTITTDENTNNYGSPTPMGERYIALGDAATGLKYIKQGVYDLVGNWN SVASITPDSFRSTRKALFGRSEDQGTXWIMPGTNXAFLSVQTQADENNX XXGXXXIGYNXGGKMNHYFRXKGKTNINTQQGMEINPGIXKLVTGSDN VQFYADXTISXIQXVKLDXELFLNXXNNXAGLKXXAPSKVDGSRTIQWN AGTRAGQNKSYLTMKAWGNAFDPTAGDRETVFELYDGQGYHFYXQRL APXGSETVGTLQFRISGALRVGGGIISAGXIVTESSLVXNNGXSVXXQAKX XGXADXLRIWNAEYGAIFRRXETALHIIPTLKDQGENGEIGNLRXXXXXX NXGMXQMXHXVXLGDDXXGXNMVTVXNDSKLVXITSHSXISPNYRMX LXQSAYIDAECTDTARPAGAGXFASXNXEXVRAPFYMNIDRXDTSTYVPI VKQRYVQNNSCYSIGTLINGGNFRXXYXEGGDGVSTGAVIKDLGWEFN KNGDFYSPGKLGAGNVRIGTDGNITGGSGNFANLNTTINNLKTDIVSSYP IGAPIPWPTDTPPEGYAIMEGQTFDAGLYPKLAAVYPSGALPDMRGQTI KGKPSGRAVLSTEADGVKSHSHSASASSTDLGTXTXSSFDYGSKTTSSFD YGTKTSNTTGNHNHTVSGNTSSAGAHQHARSGPQXQGGIPTSVFYDG YNSAGPNGNAKXTATVSGATASSNMAKTSSDGAHTHSWSGTTSATGN HAHTVGIGAHTHTVGIGAHSHTVAIGSHGHTITVNSTGNTENTVKNVAF NYIVRLA
10	complement (16624..20499)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: putative long tail fiber proximal subunit; PP_00025; phage(gi642905803)	MADIKRKFRAEDGLDAGGDKIVNVALADRTVGTDGVNVDFLVQENTV QVYDSTRGYNKGFVVLYDNRLYQAINDIVSPSGAFNLLQWRAVRTDAQ WITVASGTYQLSSGESISVNTGAGNDMVFTLPNTPVDGDTVVLADIGG RTGTVKVQINAPVQSILNFRGEQVRTVLMTRPRSQLLFVFSNRLWQMYI TDYGKESITVTPAAPYQAQANDFIXRRFTSAAPINITLPRNANNGDIISLV DLDXLNPLYHTIVKTFDDTTSIGEVGTHIAEGRNDSDAFFVFDAASSLWRI WEGDQKSRLRIIRADSNIRPNEEVLIFGTNNATAGTINLTLPSGILSGDTV KISMNYMRKGQTVKIKAAEGDTIASSVALLQFPKRSEYPPDAQWVSVTE LEFNGTTSYVPVLELAYIEDTVAXXRYWVVQQNVPTVERVDAGSDXXXA RVGVIALATQAQANVDLENTPAKEVAITPETLANRTATESRRGIAKIATT AQVNQNSTASFVDDTIVTPKKLNERTATETRRGLAEIATQTETDAGLDD TTIITPKKLQARQXXETLSGIVKYVSTTSATPAETRGAAGTNVYNKDTXXL TISPKALDQYKATYAQQGAVILAVDSEVIAGQSQAGYSHAVVTPETLHKK TSTDGRIGLIEIATQAETNAGTDYTRAVTPKTLNDRRATEGLSGIAEIATQ VEFDTGTDDTRISTPLKIKTHFDSSDRTSVNSDSGLIEEGTLWNHYTLDIS KANETQRGTLRVATQAESNAGTLDDVLITPKKLLGTKSTETSEGVIKVAT QAETVTGTSANTAVSPKNLKWIVQNEPTWAATTAXXGFVKTSSGSITFV GNDTVGSTQPLESXEKNSYXVSPYELNRVXANXLPLKAKAADTNLLDGL DSSQFIRRDIAQTVNGSLTLTQQTNLRAPLVSSSTATFGGSVSANSTLTIS NTGXATRLIFEKGPQTGTNPAQTMTIXXWXXXXXGGXDTTRSTVFEVG DETSNHFYSQRNKDGNIAFSINGTVMPININASGLMNVNGXATFGRSV TANGEFISKSANAFRAINGDYGFFIRNDAANTYFMLTASGDQTGGFNGL RPLAINNASGQVTIGESLIIAKGATINSGGLTVNSRIRSQGTKTSDLXTXAP TSDTVGFWSIDINDSATYNXXPGYFKMVEKTNEVTGLPYLERGEEVKSP GTLTQFGNTLDSLYQDWITYPTTPEARTTRWTRTWQKTKNSWSSFVQV FDGGNPPQPSDIGALPSDNATIGNLTIRDFLRIGNVRIIPDPVNKSVKFE WIE
11	20603..21520	PHAGE_Escher_vB_EcoM_JS09_NC_024124: RnaseH; PP_00026; phage(gi642905991)	MDLEMMLDEDYKEGIALADFSXIALAAALNNFEDGDKITVPMVRHVILN SIRKNVVMFRKQGYTKFVLCMDNATSGYWRRDFAYYYKKNRKTDREAS KWDWEGYFTALRQVVDEIKKYMPYVVMDIDKYEADDHIGVLTKYLSLA GHKVCIVASDGDFTQLHKYPNVKQWSPPQKKWVKIKNGSAEIDCMTKI LKGDRKDGVASVRVRGDFWFTRVDGERTPSMKTSIIEAIANDRSQAEVL LSAEEYKRYQENLVLIDFDYIPDNIASTIIEYYNSYKPQPKGKIYSYFVKSGL SKLTSVINEF
12	21528..21797	PHAGE_Escher_vB_EcoM_JS09_NC_024124: double-stranded DNA binding protein;	MAKKEAVEFNQDVHGEELAKLVKEASDNKLKISGYNELIKDIRTRAKEEL GVDGKMFNRLLALYHKDARDQFEAENEEVVELYDTVFTK

		PP_00027; phage(gi642905992)	
13	21775..22113	PHAGE_Escher_vB_EcoM_JS09_NC_024124: late promoter transcription accessory protein; PP_00028; phage(gi642905993)	MTQFSLSDIKPVDEAGLSEQELAVKHDKDDIAKLLDRQENGFIIESMVEQFGMSYLEATTAFLEENSIPETQFAKFIPTGIVEKITSEAIDENMLRPSVARGEKTNTLDFLL
14	22886..23785	PHAGE_Escher_vB_EcoM_JS09_NC_024124: single-stranded DNA binding protein; PP_00030; phage(gi642905995)	MFKRKSTADLAAQMAKLNGNKGFSSEDKGEWKLKLDASGNGQAVIRFLPAKTDDALPFAILVNHGFKKNGKWYIENCSSTHGDYDSCPVCQYISKNDLYNTNKAEYSQLKRKTSYWANILVVKDPQAPDNEGKVFKYRFGKKIWDKINAMIAVDTEMGETPVDVTCPFEGANFVLKAKQVSGFSNYDESKFLGQSEIPRINDEAFQKELYDQMVDLTTLTAKDQFKSFEKLNESFAKVLGTAALGGAAAAAASVADKVASDLDDFDKDMEAFSSAKTEDDFMSTSSSDDGDLDDLLXGL
15	24860..25447	PHAGE_Escher_vB_EcoM_JS09_NC_024124: dihydrofolate reductase; PP_00034; phage(gi642905999)	MLKLVFACAPSKTVDDKLEYAFGLGDGLPWKHIKQDMSNFVARTKNTVMVMGAKTFASLPRLLPDRTHVVVTDMARKLPRTKTNELAHFYITQAEFITLVMGSEINLFSPSTDVPFKLSTEHVDVSVIGGPALLKQALPYADEIVMTKIIKKCRVNSDVQLDKDFVQDIMLQRSMVESHYYRIDELTEITESVYK
16	25444..26304	PHAGE_Escher_vB_EcoM_JS09_NC_024124: dTMP thymidylate synthase; PP_00035; phage(gi642906000)	MKQYQYLIKDILENGYETDDRTGTGTIALFGTKLRWDLTKGFPAVTTKKLAWKACIAELLWFMSGSTNVNDLRIRTHGSLIQGKTIWDDNYENQAKDLGYHSGELGPIYGKQWRDFGGVDQLVETIDRIKKLPTDRRQIVSAWNPAEINQMALPPCHMFYQFNVRNGHLDLQWYQXSVDVFLGLPFNIASYAALTHIVAKMCNLIPGDLVFSGGNTHIYSNHVEQCKEVLRREPKELCEXXXXXXXXXXXXXXXXXXXXXXXXXXXDFILKNYESHPAIKAKMAV
17	26648..28903	PHAGE_Escher_vB_EcoM_JS09_NC_024124: ribonucleoside-diphosphate reductase subunit alpha; PP_00037; phage(gi642906002)	MQVTKSSGVSQNFDAQKIIQVLTWQCEGTQIDPYELYEEIKTHLVDGMSTKDIQKICIKVAANSISVEEPDYQYVAAKGLMFALRKDVYGQFEPPAFIDHISYCVNEGKYDPELLSRYSAEEIIYLDSQIKHERDFDLTYAGAMQLKEKYLVKDKTTGKIYETPQFAIMAIGMALHQDETHDRLKHIIRFYDAVSTHQVSLPTPIMAGARTPTRQFSSCVVIEAGDSLKSINKTSASIIEYISKRAGIGINVGMLRAEGSKIGMGEVRHTGVIPFWKHFQTAVKSCSQGGIRGGAATAYYPMWHLEVENLLVLKNNKGVDENRIRHMDYGIQINDLMMERLGKNDYITLFSPHEMSGELYYSFFEDQDKFRELYEKAEKDPTIRKKRIKALELFELFMTERSGTARIYPQFVDNTNNYTPFIREKAPIRQSNLCCEIAIPTNDVNGPDAEIGLCTLSAFVLDKFDWQDQDKINELAEVQVRALDNLLDYQDYPVPEALKAKKRRNLGVGVTNYAAWLASNFASYEDANDLTHELFERLQYGLIKASIKLAKEKGPCEYYSDTKWSRGELPIDWYNKKIDQIAAPNYVCDWEELRAELKEHGIRNSTLSALMPCESSSQVSNSTNGIEPPRGPVSIKESKEGNFRQVVPNIEHNMSLYDYAWKLAKKGNKPYLTQVAIMLKWVCQSASANTYYDPAVFEKGKVPMSVMLDDLLYFWYFGGKNLYYHNTRDGSGTDDYELETPKAEDCSACKL
18	28956..30134	PHAGE_Escher_vB_EcoM_JS09_NC_024124: NrdB; PP_00038; phage(gi642906003)	MSTVFNTQQVDVLNEPMFFGSGLGIARYDIQRHKVFEDLTEKQLSFFWRPEEVNLMLDSAQFNKLPQFQQDIFTNNLKYQSLLDSIQGRAPSAVLMALISDPSLDTWVATWTFSETIHXRSYTHIMRNLYTDPSKVFDEIVLDEAIMKRAESIGKYYDDVXXKTRXWENXXXXXXXXXXXXXXXXXXXXXXXXXXXXXAKRDLMKSLYLCLHVINALEAIRFYVSFACTFNFHKNMEIMEGNAKIMKFIARDEQLHLKGTQYIIRQLQSGTDGDEWVQIAKEXEQEAVDIFMEVNRQEKEWAAHLFKDGTCPGINTQSMCAFVDYLXVSRMKQCGLPCPITDAPTKHPYPWIREYLNSDLVQAAPQEVEISSYLVAQIDNDVDQNVINSYKKYF
19	30161..30571	PHAGE_Escher_vB_EcoM_JS09_NC_024124: endonuclease II; PP_00039; phage(gi642906004)	MKDIAAEYSFIKYTELELLEDATIKTVDVPNKKNVIYAIAIDDELVYIGKTKNLKKRINYYRTAINRKDQTSDSRKSLMILDALMQGKKVEFWARQCFDLSVTNELGSMTIATMDLEEPLFIKKFNPPWNIQHKKK
20	30626..31750	PHAGE_Escher_vB_EcoM_JS09_NC_024124: RNA ligase A; PP_00040; phage(gi642906005)	MEKLYYNLLSLCKSSSDRKFFYSDDVSPIGKKYRIFSYNFASYSDWLLPDALECRGIMFEMDGETPVRIASRPMEKFFNLNENPFTLSIDLDDVKYLMTKEDGSLVSTYLDGGTVRFKSKGSIKSDQAVSATSILLDIDHKNLADRLLELCNDGFTANFEYVAPTNKIVLTYPEKRLILLNIRDNNTGEYIEYDDIYLDPVFRKYLVDRFEVPEGDWASDVKSSTNIEGYVAVMKDGSHFKLKTDWYVALHTTRDSISSPEKLFLAIVNGASDDLKAMYADDEFSFKKVELFEKAYLDFLDRS

			FYICLDTYDKHKGKDRKTYAIEAQAVCKGAQTPWLFGIIMNLYQGGSKE QMMTALESVFIKNHKNFIPEGY
21	31813..32313	PHAGE_Escher_vB_EcoM_JS09_NC_024124: inhibitor of host transcription; PP_00041; phage(gi642906006)	MNMQLITNDMVVAAFGDSTDGIXVFKKGRPVGYLTDLRVTLAKNXKKK LXQKEYSTRYAEEKREAMPEAVNAMVEFLQNNLTKYDANVFVNISQPN VHIAGIKFYIICDPLTDKFNRLGISSPYHTAEELCVLFESYKIQCDGXKTVLI NGLSRDEIIEIIKTCLK
22	33704..34603	PHAGE_Escher_vB_EcoM_JS09_NC_024124: polynucleotide kinase; PP_00047; phage(gi642906012)	VKKIILTVGCPGSGKSTWAREFIAKNPGFFNINRDDYRQSIMGHEERDEY KYTKKKESIVTYMQHDAAHMILCQDGTKGVIISDTNLNPERRLVWEEYA KQWGHEVVYQVFDVPWTELVKRNAKRGTKAVPIDVLRSMYSRMHEYK GLPVYKGTPGKPKAVIFDLDGTLALHVARGPYELDKLSTDEPNPMVVEY VKMLHQAGYTIITVSGRESGTKEDSMCYYEATKKWMDTFSIPWEMHIQ RNQGDTRKDDVVKEELFWNCIAPYYDVKLAVDDRTQVVEMWRRIGLE CWQVASGDF
23	35700..36692	PHAGE_Escher_vB_EcoM_JS09_NC_024124: phospho-2-dehydro-3-deoxyheptonate aldolase; PP_00053; phage(gi642906018)	MLPVVGKLISPKELAEIIPLSEAYASQVASHREQVNSIMNGEDPRKLIVVG PCSIHDPIAAVEYGKRLAELQTRLPNVLLVMRVYFEKPRTTVGWKGLVN DPYLDGSFDMNHGLIVARTLCRKLLRMGLPLATEVLDPFTIKYLSGIFSW VAIGARTTESQTHREIASGLPMCVGFKNATNGSIKVATDAMYSAAWPH RYMGMDVDGTVGIVEAEGNQNTHIVLRGGTNGPNYHSSDIQEASNKA RAVGLNHYVMVDCSHANADGHYSNQIGIGKNLAANDLVKGIMIESFLH EGNQKISDNMSYGVSITDACISWEQTKELLTYINKVQ
24	36692..37273	PHAGE_Escher_vB_EcoM_JS09_NC_024124: deoxycytidylate deaminase; PP_00054; phage(gi642906019)	MKASTYLQIAYLISQESKCCSWKVGAVIEKDGRIISTGYNGSPAGGVNCC EHASEXGWLKNKPSPVIVAGHKEGTTAFGRVDNFVLAKEHRAAHSAWS ANNEIHAELNAILFAARKGNSIEGATLYTTLSPCPDCTKAITQSGIKKVVYA ELYXRSPENWADILKQAGIEVIQYSRNNLRSLNWEQIRNFCGE
25	37629..37961	PHAGE_Escher_vB_EcoM_JS09_NC_024124: head assembly cochaperone with GroEL; PP_00056; phage(gi642906021)	MSEVQLPIRAVGEYVILVSEPAQAGDEEVTESGLVIGKRVQGEVPELCVV HSVGPDVPEGFVEVGDLTPLPVGQIRNVPHPFVALGLKQPKEIKQKFVT CHYKAIPCLYK
26	38086..38334	PHAGE_Escher_vB_EcoM_JS09_NC_024124: rIII lysis inhibition accessory protein; PP_00057; phage(gi642906022)	MTKQLQHALELQRXAWNNGHXXXGASIDVEAEALEIXRYFKHLNXAQA TLAAELEXKDELKYAKPLASAARKAVRHFVITLK
27	42358..43851	PHAGE_Escher_vB_EcoM_JS09_NC_024124: DNA ligase; PP_00067; phage(gi642906032)	MILDILNQIAAIGSTKTKQEILKKNKDNKLLERVYRLTYARGIQYYIKKWPG PGERSQAYGLIELDDMLDFIEFTLATRKLTGNAAIKELMGYXXDGKPDDV EVLRRVMMRDLEVGASVSIANKVWPGLIQLQPQMLASAYDEKLITKNIK WPAFAQLKADGARCFAEVRDDGVQFFSRAGNEYHGLTLLADELMEMT KEARERHPNGVLIDGELVYHSFDIKKVVSSGNDLSFLFGDNEESEEVQVA DRSTSNGLANKSLQGTISPKEAEGMVLQAWDYVPLDEVYSDGKIKGQK YDVRFAALENMAEGFKRIEPIENQLVRNLDEAKVVYKKYVDQGLEGIILK NRDSYWENKRSKNLIKFKEVIDIALEVVGYYEHSKDPNKLGGVELVSRCR RITTDCGSGFKDTTHKTVDGVKVLIPLDERHDLDRERLMTEAREGKLIGRI ADCECNGWVHSKGREGTVGIFLPIIKGFRFDKTEADSFEDVFGPWSQTG L
28	44095..46182	PHAGE_Escher_vB_EcoM_JS09_NC_024124: ADP-ribosyltransferase; PP_00069; phage(gi642906034)	MSEQLNEVFESEGSLPVVNLNPKAKVPQVWKIXDVDTNIVVRLFSYLSE GDAVKQVKLGXKXAHVVIMSLSEKGNLAELKNGLGPAPIDAINTIFNTVY EQVKALRMDAVLFRFPTKKLKGRGQXLQTLLARLVSTKTGGRFKVLSAM YQFTGKHTYVMMVRKNANIEDIKGIPNINTELXTKVDSDVGEVYXSKKT GEKVTKETAIAGSIAAVEEKRKDKPVIAXTKISRRAIAASQSLEADRQEGEL FQKYENSAKEVSGPATAELLPEAYEIVLAQASSTAKGTLVADIENKIYNRI DESFKFADEVSYGSVIKPTLEKFAKKIKTEKTTSVKALAAFVEAANEIADSI KDEWFEDFRRDNFQLPDDVLAEVSERTWKQRKSAFLSNVMYTYXXESA RGTFNITMNRDPKQYSVAEKRAIREYASSAYTDINNMLLGRYKPDFYDV ADEDEVKRAIDGLDSAFLNGDRLPEGLTLYRAQSIRMPIYEAMVKNKVF YFRNYVSTSLAPIIFGGFKENVALGLAPEEVRKELNIDNNDEGVTISPSQV

			RTAMHAPEQIRVNVGWAIDGAHKVNVIYPGQLSNHPNEQEIILPRGILL QINKITDASSDTGAGLESNLKFIQAEVMSSDQLDEAVIYDGDVLMETGE VVAMTGEIESDEPVSFSSFVEKTSAPKGLKLLASLMDLESVPFKFVQG
29	complement (46567..47529)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: baseplate subunit; PP_00071; phage(gi642905804)	MYSLEEFNNQAINADFQRNNMFSCVFATTPSTKSSSLISSIGSFAYNNLG LDSDWLGLTQGDINQGVTTLITAGTQKLIRKSGXSKYLIGAMSQRTVQSL LGEFTVGAYLIDFFNMAYNNTGLMIYSVKMPENRLSYETDFNYNSPNIRI TGREMDPLVISFRMDSEASNFRAMQDWVNSVQDPVTGLRALPQDVE ADIQVNLHARNGLPHTAVMFTGCIPVSVSSPELTYDGDNQITVFDVTFA YRVMQSGAVNRQAALEWLESGLISSVSGMFGNNQNDSGLGSAVSRLS RLGGTAGGVSNINTLTGVVNSASRVLGL
30	complement (47529..48638)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: baseplate subunit; PP_00072; phage(gi642905805)	MISVKEIVVDAKDLNKAIPVSESAGQSTKTETTTKTYVAQFPTGRAAGND STGDFQVTDLYKNGLLFTAYNMSARDSGSLRNLRPAYAGTSSNGIISDLT DNVKDAVTKFSNGLLPAGANKPTINKTPVANILLPRSKSDVDTTSHRFND IXDSLITKGGGTATGVLSNIASTAVFGALDSITQGLMADNNEQIYTTSRS MYGGAENRTKVFTWDLTXRSTEDLMAIINIYQYFNYFSYGETGKSQYAQ EIKSYLDEWYRSTFIEPMTPDDAVKNKTLFEKITASLTNVLVVSNPTIWM VKNFGYTSKFDGLTDVFGPCQIQSVRFDKTPNGQFNGLAVAPNLPSTFX LEITMREIITLNRSSLYAGTF
31	complement (48647..50419)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: baseplate hub subunit, tail length determinator; PP_00073; phage(gi642905806)	MKPEEMKSMRRNKVIADNKPQKVAATAATDSLEALNNISSKLDDVQA ASELTSQSVEDKGNGIIESIGDLKNSSDNTAEGTELIAEVIEKQTEVTKSIN EVSSAISSKLDRLATLLEQKLQTSTAIQNTGGTSLEVIENAIPVKVVENETS DELFKALPTPEKIDNKPDEDFFPAPVQESANSTSDSKGGISFKLSDKIAML TKTVQTGFNKSISISDRIAGMLFKYTITAAIEAAKMAALILGIVIGIDLLIVH FKYWTDKFTSAWDLFDENFTKFSDEAKEWGKFLSDIFTSIDSIKQLWEA GDWGGLTVAIVKGVGTALMNLGELIQLGMAKLSASILRAIGFGDTADEI EGRALEGFQETTGNKLKKEDQEKVAKYQMKRDDGELGTVSKGLDMLQ RGKTFVTNWVRGNDNKEEFSTSDERSAESAKLKELPEEERKEAYIKANET RAALVRFEDYIDKIDMTNPENAKNVEKSYADLSKLIKDPELNKTPVVKKEL DARFEKLNNKMAEAKKAQTTVKPESSTKSPEAKQVQSIEKGRASESKQQ QPVAAISNTNNVVKKNTVVQNMTPVTSTTAPGIFHATGVN
32	complement (50416..50886)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: baseplate hub distal subunit; PP_00074; phage(gi642905807)	MIPKLGLKHYNLLKDVKGPDENMKILADSICKNMSPADFDFACLHILEFN NKLKSEVEKDGFTYKLDDVYVCQRTEFQFQGNTFYFRPPGKFEQFATISE MLSNCLIKVNDEEKEISFLEMPAFVIKWAEDLSTTIAIPGPNGPIKGIAEII GLLE
33	complement (50897..51667)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: baseplate hub subunit; PP_00075; phage(gi642905808)	MLGVIYKDRTLITPPINTINAYVPDIPWTSTFDDYLAYVRELGLATASDKF VFVWQDILGVNMIDYDTLIGQEGIKMIVGEPNTVGQYIQELEYPLVWDF TWMTKANQFTRDPIKNATIFAHSFLDTSIPVIVTGDGDNAILVSRSGGYS EMTYRNGFEEASRLQTMAQYDGYAKCTTTGNFNITPATKIIFVDQKNQF KSEFYVDEVIHELSNNNSQTHLYMFTNSMVLEPVNPVKVKNELKSDSTS KENNSTTV
34	complement (52066..52818)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: baseplate hub assembly protein; PP_00076; phage(gi642905809)	MANIIRCKLPDGVHRFKPFTVADYRDFLLVRNDIEHRSPQEQKEIIADLID DYFGEYPKTWQPFIFLQVFAGSIGKTKVPVIFTCPKCSKEKTAPFEIYQKEL VEPELDVAGIKIRFSFPEKFYDNKALMISENIKEIYYNDEWYPWNDLTEE NQIQVIEAIDIDSLEKVIASMNPINLTLRLGCCERHVKTYTDILEVFKLLVN PDEIFTFYQINHSLVKSQYTLDSIMQMIPVERGIALTLVEKDHKK
35	52866..53492	PHAGE_Escher_vB_EcoM_JS09_NC_024124: baseplate hub subunit; PP_00077; phage(gi642906036)	MYKYTFTVRLGDKEVNCRAFTLKEYLGLIGARATGTIESAVNNIITNCSNA KNLTKQEAELLLVNLWAHSLGEVNQEHTWNCSCGHSFQVYMNLLHTQ LDEQADPWYSFSGIKIKFRQPKLFDDKNIALMIASCIEAVFVNGESIPVED LTEAEINDLYGLITEDDMINIKNLLVSPSIYLATPIKCPKCGASHVHTIRGLK EFFELL
36	53492..53890	PHAGE_Escher_vB_EcoM_JS09_NC_024124: baseplate wedge subunit; PP_00078; phage(gi642906037)	MSNINKLYSDIDPEMRMDWNKDVARSVGLRSIKNSLLGIVTTRKGSRPF DPEFGCDLVDELFENMTPLTADTIERNIQAAVRNYEPRIDKLSVSVTPVY DDYTVIVEIRFSVVDNPDDIEQIKLQLASSNRV
37	53914..54384	PHAGE_Escher_vB_EcoM_JS09_NC_024124: recombination, repair and ssDNA binding	MVLGPSDTAGRLLVKRENIMKLEDLQEELDADLAIDMTKLQYETANNV KLYSKWLRKHSFIRKEMLRIETQKKTALKARLDYYSGRGDGDEFSMDRYE KSEMKTVLAADKDVLKIETTLQYWGILLEFCSGALDAVKSRSFALKHIQD MREFEAGQ

		protein; PP_00079; phage(gi642906038)	
38	complement (55124..56638)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: helicase; PP_00083; phage(gi642905811)	MTDIKVHFYDFSHVRIECDESTFYELRDFFSFEADGYKFNPKYRYGQWD GRIRLLDYNRLLPYGLVGQIKKFCNNMSYSVWIDPKIFETEDLTREDFDA WLSKQEIYSGNSKIEPHWYQKDAVYEGLVNRRRILNLPTSAGKSLIQALL ARYYLENYEGKILIIVPTTALTTQMANDFVDYRLFSHSMIKKIGGGADKA DRAKNDAPIIVGTWQTVVKQPKEWFSQFGMMMNDECHLATGKSISSII SGLNNCMFKFGLSGSLRDGKANVMQYVGMFGEIFRPVSTSKLMEDGQ VTELKINSIFLRYPDEFTVKLKGKTYQEEVKIITGLKRRTKWIAQLSVKLAKK DENAFVMFKHVTHGKEIFEAIKELGYEKVYYVSGEVDTETRNALKVMAE NGKGIIIVASYGVFSTGISVKNLHHVIFAHGVKSKIIVLQTVGRVLRKHGSK AVATVWDLIDDAGVKPKSLNTKKKYTHLNYLLKHGIDRIQRYADEKFNY VMKTINL
39	56689..57357	PHAGE_Escher_vB_EcoM_JS09_NC_024124: inhibitor of prohead protease; PP_00084; phage(gi642906041)	MIDKDYIEELRALSDKKEAKAKLFEYAAQFGISVKKTKSFDNIVIDIEEALN ALADEPLPETDGLSITDLITAADDVDGVNFTNEEVKEEAILLFDSPTEQVE VLEVVEQEKEFDHDKFEEAITQVVESEKEPETEVNKEVKFVLPENFSPTLI KLGKGPGYVTVPWWIYQWIAETPDWKSRPTSFVHASAHQTLFSLIYYIN RDGSVLIRETRNSSFVTLK
40	57367..58497	PHAGE_Escher_vB_EcoM_JS09_NC_024124: large head outer capsid protein; PP_00085; phage(gi642906042)	MAFTVDITPKTPTGVIDETKQFTATPSGETGGGTITYAWSVDDVPQDG AEATFSYVXKGPAGQKTIKVVATNXXPXSEAETAEAXTTITVQNKTQTTT LAVTPNSPSAGVIGTPVQFTAALASQPVXASATYQWYVDXXQVXGETN STFSYTPTTXGVKRXKCVAQVTATDYDAKEVTSNEVSLTVNKKTMNPQV TLTPPSINVQQDASATFTANVTGXPXXAQITYSWKKDSSPVEGSTNVYT VDTSSIGSQTIEVTATITATDYDSKTITAEGQVQVTDKVAPEPEGELPYVH PLPHRTSAYIWCGWWVMDEIQKMAEEGKDWKTEDPDSKYYLHRYTL QKMMKDYPEVDVQESRNGYIIHKTALETGIIYTYP
41	58599..58793	PHAGE_Escher_vB_EcoM_JS09_NC_024124: hypothetical protein; PP_00086; phage(gi642906043)	MRAEVEVYTLHEAGFSFVEIAQKIGLQPKEVGLLWTKAETARSKFKAKEK VVYRKRLINKKVKK
42	59161..59880	PHAGE_Escher_vB_EcoM_JS09_NC_024124: RNA ligase 2; PP_00088; phage(gi642906045)	MFKKYSSLENHYNSKFIEKLYTNGLTTGVWVAREKIHGTNFSLIIERDNVT CAKRTGPILPAEDFYGYEIVLKKYDKAIKAVQEFMYTARAVSYQVFGEFA GGGIQKGVDYGEKDFYVFDILINTESGDNTYLTDYEMQDFCNEFGFKM APMLGRGTFDALIMIPNDLDSVLAAYNATASEDLVEANNCVFDANVIG DNTAEGYVLKPCFPKWLPNGTRVAIKCKNSKFSEKKKSDKAY
43	59849..60160	PHAGE_Shigel_Shf125875_NC_025437: RNA ligase 2; PP_00089; phage(gi725949987)	VKRKSLIKPIKTQVPLTEIDKNLLDVLACYVTLNRINNVISKIGTVTPKDFGK VMGLTVQDILEETSREGIVLTSSDNPNLVKKELVRMVQDVLRPAWIELV S
44	complement (60191..61474)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: capsid vertex protein; PP_00090; phage(gi642905812)	MSKIQKLLRESTTSTSNSVGRPNLVALTRATTKLIYSDIVATQRTNQPVAA FYGIKYLNPDNEFTFKTGATYAGEVGYVDREKITELTEESKVTLNKGDFFK YNNIVYKVLEDTPFADIQESDLELALQIAVVHLKVRLFSDAAVTSKFESSD SEISDARFQINKWQTSVKSRKLKTGLTVELAQDLEANGFDAPNFLEDLLA TEMADEINKDILQSLITVSKRYKVTGITDTGFIDLSYASAPEAGRSLYRMV CEMVSHIQKETTYTATFCVASARAAAILAASGWLKHKPEDDKYLSQNAY GFLANGLPLYCDTNSPLDYVIVGVVENIGEKEIVGSIFYAPYTEGVDLDDP EHVGAFKVVVDPESLQPSIGLLVRYALSANPYTVAKDEKEARIIDGGDM DKMAGRSDLSVLLGVXXXKIIIDE
45	complement (61898..63466)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: major capsid protein; PP_00092; phage(gi642905813)	MTTIKTKAQLVDKWKELLEGEGLPEIANSKQAIIAKIFENQEKDFEVSPEY KDEKIAQAFGSFLTEAEIGGDHGYNAQNIAAGQTSGAVTQIGPAVMG MVRRAIPNLIAFDICGVQPMNSPTGQVFALRAVYGKDPIAAGAKEAFHP MYAPDAMFSGQGAAKKFPALAADTTTVVGDIYTHFFQETGTVYLQASA VVTLDSGATDAAKLDAEVKKQMEAGALVEIAEGMATSIAELQEGFNGS TDNPWNEMGFRIDKQVIEAKSRQLKAAYSIELAQDLRAVHGMDADAEL SGILATEIMLEINREVVDWINYSAQVGKSGMTLTPGSKAGVFDFQDPIDI RGARWAGESFKSLLFQIDKEAVEIARQTGRGEGNFIIASRNVVNVLASVD TGISYAAQGLASGFNTDTTKSVFAGVLGGKYRVYIDQYAKQDYFTVGYK GANEMDAGIYYAPYVALTPLRGSDPKNFQPVMGFKTRYGIGVNPFAES SLQAPGARIQSGMPSILNSLGKNAYFRRVYVKGI

46	complement (63484..64296)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: prohead core protein; PP_00093; phage(gi642905814)	MIKEQLLKEAQNIETSVALNSVFESVELSPDVKANFSTVFEATVKQEAVK LAESHIKAIAEKAEEEVEKAKEDAEEKADKKLAEQASKFLDHLAKEWLAE NQIAVDKGIKADLFESMLGGLKELFVEHNVVVPEESVDVVAEMEEELAE HKEETARLFEEVTKRDAYINYVQRETAINESVKDLTESQKEKVIGLVEGM DYSDAFGTKLTAIVEMVKGSTKEEAAITESINTVDNDAAGLNFVAEAVDT TTTQVEQNSNVSLYAKVASRF
47	complement (64330..64971)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: prohead core scaffolding protein and protease; PP_00094; phage(gi642905815)	MNKPELLIETWGQPGEIIDGVPMLESHDGKDSGLKPGLYIEGIFLQAEVV NRNKRLYPKRVLEKAVSDYIKEQVETKQALGELNHPPRANVDPMQAAIII EDMWWKGNDVYGRARIIEGDHGPGDKLAANIRAGWIPGVSSRGLGSL TDTNKGYRIVNEGFKLTVGVDAVWGPSAPDAWVTPKQISESENSVEITK NSADEAFKALAESLKAL
48	complement (64971..65396)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: prohead core protein; PP_00095; phage(gi639163158)	MYLIPESYELVLENVEALIPEAQGRIDALSSALDIDDINTIIENMLETETDLA VAMASIINEEQLNEFIVKHVSSRGEVTRTKDRKTRERNAFQTTGLSKAKR RQIARKVVKAKKANPSGQVKGLRKRKKALKRRKALGL
49	complement (65396..65632)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: prohead core protein; PP_00096; phage(gi639163159)	MDDLIQAIKSNDLVATRKFFESAMAEKTVRLIEARKAEIASQFLIEGEEPE EEKKAKASEDDADEGDDDEDEDDEDDE
50	complement (65632..67203)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: portal vertex protein; PP_00097; phage(gi639163160)	MKFNILSLFAPWAKMDERDYKDQEKENLESITXPKLDDGAKEYEVSENE AQQTYNAMFQRMFGSQEPGLKSTRELIDTYRNLMTNYEVDNAVSEIVS DAIVYEDDTEVVSINLDNTKFSPNIKSMMLDEFNEVLNHLSFQRKGSDH FRRWYVDSRIFFHKIIDPKRPKEGIKELRRLDPRQVQYVREVITTTEAGVKI VKGYKEYFIYDTSHESYACDGRIYEAGTKIKIPKAAIVYAHSGLVDCCGKNI IGYLHRAIKPANQLKLLEDAVVIYRITRAPDRRVWYVDTGNMPSRKAAE HMQHVMNTMKNRIAYDATTGKIKNQQHIMSMTEDYWLQRRDGKAV TEVDTLPGADNTGNMEDVRWFRNALYMALRIPITRIPSDQGGIQFDAG TSITRDELSFGKFIRELQHKFEEIFLDPLKTNLILKGIITEDEWNDEINNIKIK FHRDSYFSELKDAEILERRINMLQMAEPFIGKYISHRTAMKDILQMSDEEI EQEAKQIEEESKEARFQDPDQEQEDF
51	complement (67288..67779)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: tail tube protein; PP_00098; phage(gi639163161)	MFVDDVTRAFESGDFARPNLFEVEISYLGQNFSFQCRATALPAAIVEKVP VSYMNRKINVAGDRTFDDWTITVMNDDAHSIRQKFVDWQGIAAGQG NEITGGKPAEYKKTAIVRQFARDAKTVTKEVEIVGLWPTNVGEVSLDWD SNNEIETFEVTLALDWWE
52	complement (67892..69874)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: tail sheath protein; PP_00099; phage(gi639163162)	MALLSPGVELKETTVQSTVVNNSTGTAALAGKFQWGPAFQIKQITDEV ALVDMFGTPNTDTADYFMSAMNFLQYGNDLRVVRAVDRDTAKNSSP VAGNIEFTISAAGSNYAVGDKIIVKYLTETVEADGYVTSVDVDGKILNIYIP TGKIIARAKEINEYPALGSNWTAEVASSSSGLSGVITIGSIVTDSGILLTEVE TSEEAITSLTFQESIKKYGVPGVVALYPGELGDQLEIEIVSKADYDKGASA QLKIYPDGGTRASTAKAIFGYGPQTDDQYAIIVRRNDAVVQSVVLSTKRG ERDIYGSNIFIDDFFAKGASNYIFATAQGWPKGFSGVIKLGGGLSSNETV DAGDLMEAWDLFADRESVNAQLFIAGSCAGESLEVASTVQKHVVAIGD SRQDCLVLCSPPRATIVGIPVNRAVDNLVDWRTASGTYTDNNFNVSSTY AAIDGNYKYQYDKYNDVNRWVPLAADIAGLCARTDNISQPWMSPAGY NRGQILNVIKLAIETRQPQRDRLYQEAINPVTGTGGDGYVLYGDKTATSV PSPFDRINVRRLFNMVKTNIGSASKYRLFELNNAFTRSSFRTETSQYLQGI KALGGVYNFKVVCDTTNNTPAVIDRNEFVATFYLQPXXSINYITLNFVAT ATGADFDELIGAVGG
53	complement (69905..71740)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: large terminase protein; PP_00100; phage(gi639163163)	MEQPINTLNDNHPLNEGDKVVILPPHLAERKEEDGIHWIKSQWDGKW YPEKFSDYLRINKIVKIPNNSDKPELFQTXKDKNNKRTRYMGLPNLKRANI KTQWTYEMVAEWKKCRDDIVYFAETYCAITHIDYGTIKVQLRDYQRDM LKIMSSKRMTVCNLSRQLGKTTVVAIFLAHFVCFNKDKAVGILAHKGSM SAEVLDRTKQAIELLPDFLQPGIVEWNKGSIQLDNGSSIGAYASSPDAVR GNSFAMIYIDECAFIPNFIDSWLAIQPVISSGRRSKIIITTTPNGLNHFYDI WTAAVEGKSGFEPYTAIWNSVKERLYNDEDIFDDGWQWSKQTISASSL SQFRQEHTAAFEGTSGTLISGMKLAILDYIEVTPDSHGFHRFKKPEEGHK YIATLDCSEGRGQDYHAMHIIDVTTDKWEQVGVLHSNTISHLILPDIVFK

			YLMEYNECPIYIELNSTGVSVAKSLYMDLEYENVICDSMNDLGMKQSRR TKPVGCSTLKDLIEKDKLKINHRATIQEFRTFSEKGVSWAAEEGYHDDLV MGLVIFGWLTTQQKFADYADKDDMRLASEVFSRELQDMNDDYAPVIF VDCASNSAEYNPSAHGLSMV
54	complement (71724..72218)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: small terminase protein; PP_00101; phage(gi639163164)	MEGLDINKLLDISDLPGISGEEVEVYAPLQLVEVQSNPQNRTPDLEDDYS VVRKNMHFQQQMLMDAAKIFLETAKNADSPRHMEVFATLMGQMTT TNKEILKLHKDMKEITSEQVGTKSTAPSSQMNIQNATVFMGSPTELMEE VGDAYEAQEAREKVINGTAN
55	complement (72228..73004)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: tail sheath stabilizer and completion protein; PP_00102; phage(gi639163165)	MMGDLFSNIQVNRQLSTGNKLIRVPITYASKEHFMMKLNKWTSINSQE DVAKVETILPRINLQMVDFVYNPTFKTNILNNSLLSKSTKDIVDQYNPSPI KMIFELSIFTRYEDDMFQIVEQIIPYFQPHFNTTMIEQYGQDIPFERDIKV VWMAAAMDEQIDGDNLSRRRLEWSLTFEVNGWMYPPVGAAEGLIKT TYLDFHANERDLQTAASVFESVDTEIKPRDVEAQDWNGEVEQTYTHDIP IPTPPTPPGPRKQ
56	complement (73102..73866)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: neck protein; PP_00103; phage(gi639163166)	MATYDKNLFAKLENRGGYSQTNETEILNPFVNFNNYENSQTLADVLVAE SIQMRGIECFYVPREYVAVDLIFGEDLKNKFTKAWKFAAYLNSFEGYEGA KSFFSNFGMQVQDEVTLSINPGLFKHQVNNQEPKEGDLIYFPMDNSLFE INWVEPYDPFYQVGKNAIRKITAGKFIYSGEEINPVLQKNEGINIPEFSDLE LNPVRNLDGIHDINIDEYSEVEQINSEASEYVEPYVVVNNRGRQNSPFDD GFMN
57	complement (73868..74794)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: neck protein; PP_00104; phage(gi642905784)	MSGYNAQNPKELKDVILRRLGAPIVNVELTTDQIYDCISRALELYGEYHF DGLNKGFHVFFVDDEEKYRHGVFDMRGSNVFAVTRIIRTNVGSITSMD GNATYPWFTDFLLGMAGVSGGMGSNCGKFYGPNAFGADLGYYSQLV SYMGMLQDMIAPLPDFWFNSANEQLKVMGNFRQKDVIIVESYVRSYIE THKMVGNTVGYGQVGPRDSWSISERYDNPDHNLVGXRVGEDPATKQ GAYNNRWVKDYATALAKELNGQILAKHQGMMLPXXVTVDGVRLIEEA RLEKEALREELYLLDPPAGILVG
58	complement (74827..76275)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: fibritin; PP_00105; phage(gi897635951)	MTDITLVDLPYVDGPPDAGQSRIEWIKNDEEITGADTKYGSEGVMNRPI VEVQRNVEALDVNIKTIASSLNTANEDIKVIQGILDVSGDVDALAQIGQN TQDIDVIKVTVQTHASELNDLNEXIDANTDDIGVYNQETDSVYRTVRND XLWIKNELGQYTGQDINGVPTPGNDSTGMKRRIMTNSXVIVDHGVRLT DLETKFADXDVGSLTIEVEHXREEMXPRTSFTTPVFSRLSGIDLTLSRQNA DLDGIKQSIGYPNTTSIITTTNANSSAIXDINLELNQSXGIKPRLTXVETAIG XDDLPTTIQGRIKLNTDAIGALNTVVGSDSSSGLRFNVSWLNNVVGVDA SXGKXEPDGSLLYRTRXLEGTISGMSNDIQNIQTDVGTNTTGIKGSVNTL NTLIXGTNPNGSTVEERGILPTVKSHDTTINGLTSRIATLETDLAAAEAEIQ TLKDAGYIEDAPSDGKFYVRKDXAWVELPTA
59	complement (76275..77948)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: putative short tail fiber protein; PP_00106; phage(gi897635952)	MSNNNTYQHVSNESIYVEFDPTGSNFPDTVTNVQAALAAISPSGVNGV PEATTTTPGIIQIATPQEVIDGLDNSKAVTPASLNARLQYPNATETQYGLT RYATEDEAVTGAIDDASITPLKLNQKIDNVFQTKVSTEATNGVIKISTSAA ALAGSDDTTAMTPLKTQQLAIKLISQIAPNNSDATESITGVVRLATVAQA QQGTLREGYAISPYTFMNSVASETNKGVVKLGTQTEVNSXNSSVVVTG ATLNGRGATTSLRGVVRLTTQAGSQSGGDGSSALAWNADVIHQRGGQ TINGTLRINNTLTVGSGGANITGNVNMTGGYIQGNRIVTQNELDRILPV GAITMWAADSLPSSDWRFCHGGTVSASECPVYASRIGTRYXGSSSNPG LPDMRGLFVRGSXRGNHLTNPNVNGNDQFGKPRLAVGCTGGYVGEV QKQQMSYHKHAXGFGEHDDSGAFGNTRRSNFVGTRKGLDWWDNRS YFTNDGYEIDPASXRNSKYTLNRPEDNRSYFTNDGYEIDPASXRNSKYTL NRPELIGNETRPWNISLNYIIKVK
60	complement (77945..78604)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: baseplate wedge subunit and tail pin; PP_00107; phage(gi642905787)	MTVLNTKAGVISRHADFLNYRKNPAQIDVLNNQAVGSVTISQLARGFYD ANVESAINDVHNMARADIGTVVTNTSGVSPEGAAQVDYWTFSGDVLD DTLTTGTPVVINVFGLAVKATIGMTDNELTAQVRIALQEAIANQIAISSYK DHPTEGNKLQVTYLDNQRHVLPTYASFGVTISQEILSEAKSGYGTWNLL GAQTVTLDNHNTPTVFYHFVRAS
61	complement (78604..80409)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: baseplate wedge subunit and tail pin;	MKQNIKIGNVVDDGQGDYLRRGGEKINENFDEIYYELGDGEVPYAAGA WKTYDAADGLNLKAAWGKSYAINTTSGRVSVNLPKGTIADYNKVIRAR DVFATWNINPVTLIPANGDTIKGSSSPVEINVQFSDLELVYCAPGRWEYI KNKQIDKIVSSDISNVARREYLVETQGQTDFMDVFNGTSYNVNNIRVKH

		PP_00108; phage(gi642905788)	RGNELYYGDVFSDNSDFGSPGANPGELVALDGFNIRLRQPCNVGDTVQ IETFLDGVSQWRSSYTRRQVRILDSKLTAKKSIDGSIYVTDLSTFRSIPFTAF GINPTEPPNPNSLEVRFNGILQELAGTVGTPIFRCEGVDADSEESCANLG GTWTQSYTDYAIEYTDNIPSAILFDRQFEDQDIITITWFNNDLGTLLEME EILDVADEKYVSQGSNIEITGDVALTDFDKIGWPNVEPVPKYEREFSTISAI FDTIYPVGTIYENAVNPNNPATYLGFGSWKIWGQGKVLAGWNDDASD PNFALNNNDLDVNGNPTHTAGGTVGVTTITLENADLPATQTDEKVLISD DNGTVIIGGCQYDPDEEGPIYTKYREDHAKTNGSHVPARSITNIQPSITVY RWVRIA
62	complement (80409..81281)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: baseplate wedge tail fiber connector; PP_00109; phage(gi642905789)	MAFFKQTPKQLIDTGEIGNASTGDILFDGGNKINDDINAIYNAFGDQRK MAVADGTGPTGQTIHATGYYQKGKAVDYQTPIKPGTMHDIDASDGGV IVIIDRPELGDSVEFINSNGSISVNNPLSIQVTDGSIKGVLNNLVISTPYTHV VLRCIELNGPTSVWNYSTDSMFGQKESPVDGTWSLTGNTVSIPLFYRTD YNMAKLLVTCQSADGRKIKTCEINILIDTINSEVLSTEFAVMRVGNVDET DEIANISFSIINNFATMTITSDIIGLRSAVKIISTQKIGVAQ
63	complement (81354..82358)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: baseplate wedge subunit; PP_00110; phage(gi642905790)	MNDSSVIYRAIVTSKFRTEKMLNFYNSVGDGDDKNSIYITFGRSEPWAS NENEVGFAPPYPTDSVLGVTDMWTHMMGTVKVLPSMLDSVIPRRDW GDVRYPDPYTFRINDIVACNTAPYNATEVGAGWLIYRCLDVPDVGLCSIE SLDNKDECLKLGGKWTPSVRSMSPPEGRGDANGVIETGDGYIWEYLYEI PADVSINRCTNEYLVVPWPEEIKEDPTRWGYENNLTWQQDDYGLIYRV KANTMRFKAYFDSVYFPEAALPGNKGFRQISIIANPLEAKKNPNDPNVK AEKDYYDPRDLQRHSGEMIYMENRPPVIMAMDQTEEINILFMF
64	complement (82351..85449)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: baseplate wedge subunit; PP_00111; phage(gi642905791)	MTVKAPAVTSLRISKLSANQVQIRWDDVGANFYYFVEIAETKTADGXIIP PASYRWINLGYTPNSNWFESSLDPLTSYIIRVSTAAEGFERSDWVYTEIFE SFQTNAYTFQHMIEMQLANTFIREKFTNNNMAYVDFNNDTIMAALM NESFQFSPAYADISSIKNFIIGENEYHEIQGSIQQVCHDINRVYLMESEGIL YLFERYQPVVKVSNDKGQTWKSVKLFNDRVGYPVSRTVYYQTDYTTYVL GYDKLFYGRKSTDVRWSADDVRFSSQDITFAKIGDQLNLGFDVEIFGNY ASLPANVSRIAEAIVCNDDYVYVAARDKVRFIKTSNAPIDSDPLSPTYSER LFEQETFNITGNPKAVCYKMDSVGGKIFALIIGEVVNLNDDPRTTPIVDSV DKGIYVLDHDAGTFKRVFGNTEEERRRIEPGYTSMSTDGVELSISSSNFKF LESDIVDDPKTQVKYGLIGAVKYEYPREWLADKHYHMMAFISTEDSQW ESFTPQPMEYYAEPFFSYSRKSGTRCWINNSDKAVVIYSDLLYTKVIENHP LSSSDREVHEYWKDGDCTIVMPNVDFTGFKKYASGMIFYKASGEIISYYD FSYRVRDNVSIIWKPTNIFLTASLQNQEKDTTWTPVEENGIADPDLRPLL TTMMPESYLLDNTNFEAFCEAYIQYLSDGYGTHYNNLLNLIKNQYPREE HAWEYLWSEIYKRNIYLNAEKRDLVSRFFESRSYDFYSTKGTEASYKFLFK VLYNEDVEIEIESSSGTEYDIVIESDSLTEDLVGQTIYTATGRCNVTYIERSY SKGKLQWTVTIHNLLGRLIQGQEVKAERLPGFEGEIVRGIKGKELSQNNI DYINRGRAYYVMKIKSKLPSSRWKSDVLRFVHPVGFGFIAITLLTMFINTG LTLKHVETIINKYKNYKWDSGLPTKYADRVAKLDPQGNVEFDPITGKAIY EIGPMAGQDYPLPEDYNAENNNSIFQGQLPSERRKLMSPLFDASGTTFS RFRDLVEQRLIDNVGNPRDPVNPPQVKLDE
65	complement (85446..87419)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: baseplate wedge subunit; PP_00112; phage(gi642905792)	MANTPVNYQLTRTANAIPEIFIGGTFAEIKQNIIEWLNGQNEFLDYDFEG SRLNVXCDLLAYNTLYIQQFSNSAVYESFMRTANLRSSVVQAAQDNGYL PSSKSAAQTEIMLTCTDALNRNYITIPRGTRFLAYAKDTSVNPYNFVSTED VIVVKDKNNQYFPRLRLAQGRIVRTELTFDRLKPIIIRDKNIDRNLVKLYVD GAEWINWTRKSMVHAGSTSTIYYMRETVDGNTEFYFGEGEISINTSEGA LTSNYIGGLKPVQGSKIVIEYISTNGADANGAVGFSYADTLANITVIDINE NPSNNPDFVGADGGGDPEDIERIRELGTIKRETQQRCVTASDYDTFVSE RFGSIIQAVQTFTDSSKPGYAYIAAKPKSGLYLTSVQRDDIKNYLNDYNLG TITPVVISPDYLFIKMNIKVTYALNKLQESEQYLEGQIINKIDXXYIEDVEIF NSSFAKSKMLTYVDDADISIIGSSATIQMVREVQNFYKTPEAGIKYNNQI KDRSMESNIFSFDSHRPNPENANETIKYGVRIVSSDRNDRGIGQVIIGPF ADGDVVENENIQPYTGNDFNKLTVTDGRNKYYSIGEVNYPADSIYWNIA KIDLTSDRFEVQTIELYSDPADDIIFTKDGSLIVFENDLRPQYLTIDLEPISQ
66	complement (88243..89922)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: baseplate hub subunit and tail lysozyme; PP_00115; phage(gi642905795)	MDPLKQGRVRVRVVGLHPAQRAQGDVQGIPTEKLPWMTVIQPITSAS MSGIGGSVTGPVEGTRVYGHFLDKWKTNGIVLGTYGGIVREKPNKLEGF SDPTGQYPRRLGNDTNVLNQGGEAGYYSNSNVIQDNNLDYGINPDDT DLANIPEDDDPNFTITEMLRRDEGLRDKVYWDHLGYPTVGIGHLIVMEK TRDMSRINKLLSDQVGREVTGNPGTITLEEATALFEKDLAKMQKDIRSNS KVGPVYAKMNRSRQMALENMCFQMGVGGVAKFNTMLKAMAAGD

			WKTAYKSGRDSLWFQQTKGXASRVTTIILTGNMESYGVPVKTPPSPGV GADLVTRNTDPEDPAGPPVPLDSRILFKEPESSYRGEYPYVHAMETESG HIQEFDDTPGNERYRLVHPTGSYEEVSPSGRRTRKTVEDLFDITNGDGNF LISGDKLVNVGANEIYYNMADRLHQIDGNDTIFIRGNQTKTVEGDGTLY VKGNIKIVVDGNADILVKGDAKTQVEGNHDYTVNGNVKWTVNGNVD MTVAGDWAETMSTMSSVASGQYTVDGSRIDIG
67	complement (89976..90551)	PHAGE_Escher_vB_EcoM_JS09_NC_024124: baseplate wedge subunit; PP_00116; phage(gi642905796)	LLFTFFDPIGYSAKTVDKXAPTIPMTDIFRNYKEYFKRVAANYRLRSYYIKG SPRPEELANIIYGNPQLYWVLLMCNDNYDPYYGWITSQEAAYQASIQKY ANAGGDQVLYHINENREKFYNLVSYPDEPLVWYDKGDEARKYPQYKGP LAAVDTYEAAVLDNEKLRKIKIVAKEDINSFITDLIREMEIA
68	90613..91062	PHAGE_Escher_vB_EcoM_JS09_NC_024124: head completion protein; PP_00117; phage(gi642905816)	MAYSGKWVPKNLKKYRGDHTKITYRSNWEKFFFEWLDKNPEIIAWGSE TAVIPYFCNAEGKKRRYFMDIWMKDASGQEFFVEIKPKKETQPPIKPVN LTXAAKKRYMNEIYTWSVNCDKWKAASAVAEKRNIKFRVLTEDGLRKL GYKG
68	91065..91886	PHAGE_Escher_vB_EcoM_JS09_NC_024124: DNA end protector protein; PP_00118; phage(gi642905817)	MSIFQIISEGVQAPKIAQSMNERKWIEIGLEYKKAKEKGITAKAFAESKGI KYSSFTSAMSRYASSIKTAQKIETLESKPKNKLNKQERQLLMINSFRSSIRE KIRNEGAAVNNKSSKWFVETIKKSVKGHKVVKPTPGKIYTYVYDAKHKD TLPYWDRYPLIIYLGLGKHNLMYGLNLHYIPPKARQQFLEELLKQYASTTT ITNNTKLKIDWSKVKGFQGADKMIKAYLPGHIRGTITEIAPKDWANIIML PTQQFMSKGKRFSANTVWK
69	91989..92573	PHAGE_Escher_vB_EcoM_JS09_NC_024124: tail completion and sheath stabilizer protein; PP_00119; phage(gi642905818)	MTRPARQIFNQTNITNFVVDIPDSTRTSGFVLNAQTAPLPGVRIPVVETV TGTMGLGRAMRAGTTFEYDPFVVRFIVDEDMTSWMNMYKWMLSTN NYITGHNTAHSDGPEFVTLHILNNNKTEVVLTANFYKPWISDLSEIEFSYT EDSDPAVTCVATIHYAYMTIEKDGEVIVSQQPRQAVESKSISRHPSLR
70	92627..93361	PHAGE_Escher_vB_EcoM_JS09_NC_024124: deoxynucleoside monophosphate kinase; PP_00120; phage(gi642905819)	MELIFLSGIKRSGKDTTADYINSNFKSVKYQLAYPIKDALAIAWGRRHAE NPDVFTELKYEYFEGVGYDRETPLNINKLDVIELLEEALIYLQSQYLPINNV KVLSSLEGGYSYLDIKPYEALREAINNINDAWSIRRLMQALGTDVVVNLF DRMYWVKLFALNYMDYIGSDFDYYVVTDTRQVHEMETARAMGATVI HVVRSGTESTDKHITEAGLPIEEGDLVITNDGSLEELYSKIETILR
71	93366..93596	PHAGE_Escher_vB_EcoM_JS09_NC_024124: chaperone for tail fiber formation; PP_00121; phage(gi642905820)	MSTETIEKLQSEIVTLKSRILDTQDQAAAIQQESQQLGSALAKIANLVGIT GESVKIDDLVEAVRELVKAETTEEE
72	94129..94416	PHAGE_Escher_vB_EcoM_JS09_NC_024124: internal head protein; PP_00123; phage(gi642905822)	MKTYKEFISPDANVSTLTEATLTSEVIKANKGREGKPMISLVDGEEVKGT VYLGDGWSAKKDGATIVISPAEETALFKAKHISVAQLKIIAKTLL
73	complement (..95924)	tRNA	NA
74	complement (..96002)	tRNA	NA
75	104497..104955	PHAGE_Escher_vB_EcoM_JS09_NC_024124: nudix hydrolase; PP_00147; phage(gi642905846)	MMSKKNKVKELSAGIIFLTEDKELFMGRVTGSRPKGSLAHRWDIPKGRV EPGESPIEAAIRECEEETGFTQYDPAFLKDLGEHHYSDNKNIHLFLYTIPVE HEQFRNSVCNSYHTFPDGRQIPEFDAFALIKPSQWEYVMGKSLYKVLTT LL
76	104990..105478	PHAGE_Escher_vB_EcoM_JS09_NC_024124: e lysozyme murein hydrolase; PP_00148; phage(gi642905847)	MNIFEMLRNDEGLRLTLYKDTEGFWTIGIGHLVTKNPSLAVAKAELDRM IGRKCNGTITLDEAEKLFNEDVDKAVRGILGNAKLKPVYDSLDAVRRCAL VNMVFQMGVTGVAGFTNSLRMLQQKRWDEAAVNLAQSKWYRQTP NRAKRVISTFKTGTWKAYI

77	105815..106228	PHAGE_Escher_vB_EcoM_JS09_NC_024124: endonuclease V N-glycosylase UV repair enzyme; PP_00150; phage(gi642905849)	MTRINLTLVSELTDQHLMAEYRELPRVFGAVRKHVQNGKRVKDFKISPTFILGTGHVTFFYDKLEFLRLRQIELIAECLKRGFKIKDTTVQDISDIPAEFRNNYVPSEASIAISQARLDEKIAQRPTWYKYYGKPIY
78	106756..107058	PHAGE_Escher_vB_EcoM_JS09_NC_024124: ipII protein; PP_00153; phage(gi642905852)	MKXYXEFXTEARVTAGKLEAAINKKAHSFHDLSDKDRKKLVSLYIDKERILALPGANEGKQAKPLNAVEKKIDNFASKFGMSMDDLQQAAIEAAKVIKGK
79	107116..107244	PHAGE_Escher_vB_EcoM_JS09_NC_024124: endonuclease V N-glysidase; PP_00154; phage(gi642905853)	MTYINLTLVSELADQHLIAEYREXXRVFGIVRKHVAKIFTFK
80	109358..109816	PHAGE_Escher_vB_EcoM_JS09_NC_024124: site-specific RNA endonuclease; PP_00161; phage(gi642905860)	MTINSDVFIRRNKLRRIFETEFRKINAGIKDAAKSLGLPGFHIKYSQHLLDRAIQREIDENYVFELFRKVKNHVKEVAEFLSMPARPDVDEDFVEGVEYRPGRLEITDGNLWLGLTVCRENPAFKMKTLQCRMAIINSKRLPGKASKAVIKI
81	111772..112353	PHAGE_Escher_vB_EcoM_JS09_NC_024124: thymidine kinase; PP_00167; phage(gi642905866)	MASLIFTYAAMNAGKSASLLTAAHNYKERGMGVLVLKPAIDTRDSKGEIVSRIGIRQDANIITKDMDIFEFYKWAAAQKDIHCVFVDEAQFLTTEKVYQLSRIVDVYNVPVMAYGLRTDFKGNLFEGSQALMAIADKLVELKGVCHCGKKATMVARVTEDGLPITDGSQIEIGDTDRYVSLCRKHWNDLTGLL
82	112606..112908	PHAGE_Escher_vB_EcoM_JS09_NC_024124: rI lysis inhibition regulator, membrane protein; PP_00169; phage(gi642905868)	MALRAIAVMAMLGFFAATTPNVGTAYTDPYFDNFMESGIKNVYTLFEIQNVENSEKFYKYMAKHYKNSPCDDAFECHEQGIKTARQFAEFMKIKLEPTSI
83	122196..122459	PHAGE_Escher_vB_EcoM_JS09_NC_024124: thioredoxin; PP_00189; phage(gi642905887)	MFKVYGYDSTIHKCIYCDNAKRLLTVKKQPFEFINVMPEKGVFDDEKIAELLVKLGRESQVGLTMPQVFAPDGXSIGGFDQLREYFK
84	123113..123526	PHAGE_Escher_vB_EcoM_JS09_NC_024124: protease inhibitor; PP_00193; phage(gi642905891)	MNISETRGKWFKIVKEDEELQXKFPELKKXTIVKVIGTTTEDGYGDHGIIEVMLNTGEKLCIYDRDTSLWCFWESHSIDELEEIDQVVCDXGLGEFEGERISYALAKLAAQENNDGYEGNLMQAAAEYIEYLERRLS
85	123754..124227	PHAGE_Escher_vB_EcoM_JS09_NC_024124: recombination endonuclease VII; PP_00195; phage(gi642905893)	MLLSGKLXKEEKQKFYDAQNGKCPICHRELDPDVQGNHLDHDHELNGPKAGRVRGLLCNLCNAAEGQMKHKFNRSGLKGRDVDYLEWLEALLAYLKQDYSDNNIHPNFVPDKTKEFSRLGKEEMMAEMLQRGFEYNESDTKTQLIASFKKQLRKSLK
86	124224..126041	PHAGE_Shigel_Shfl2_NC_015457: putative NrdD anaerobic NTP reductase large subunit; PP_00196; phage(gi330858602)	MKIEKEIEGLIHKTNKDLLNENANKDSRVFPTQRDLMAGIVSKHIAKNMVPSFIMKAHESGIIHFHDIDYSPALPFTNCCLVDLKXMLENGFXXGNAQIETPKSIGVATAIMAQITAQVASHQYGGTTFANVDKVLSPYVKRTYAKHIEDAEKWQIADALNYAQSKTEKDVYDAFQAYEYEVNTLFSSNGQTPFVTITFGTGTDWTERMIQKAILKNRIKGLGRDGITPIFPKLVMFVEEGVNLYKDDPNYDIKQLALECASKRMYPDIISVKNNKAITGSSIPVSPMGCRSFLSVWKDSTGNEILDGRNNLGVVTLNLPRIALDSYIGTQFNEQKFXXXXNERMDLCFEALMCRISSLKGVKATVAPILYQEGAFGVRLKPDDDIIELFKNGRSSVSLGYIGIHELNILVGRDIGQEILTKMNARLKQWTERTGFAFSLYSTPAENXXYRFCKLDTEKYGSVXDVTDKGWYTNSFHVSVEENITPFEKISREAPYHFIATGXHISYVELPDMKNNLKGLEAVWDYAAQHLDYFGVNMPVDKCFTCGSTHEMTPTENGFVCSICGETDPKKMNTIRRTCGYXGNPNERGFNLGKNKEIMHRVKHQ

87	126038..126508	PHAGE_Escher_vB_EcoM_JS09_NC_024124: anaerobic NTP reductase small subunit; PP_00197; phage(gi642905895)	MNYDRFYPCDFVNGPGCRVVLFVTGCLHKCEGCYNKSTWNARNGIPFTGETLEQLIECLNNDYIEGLTITGGDPLYPDNRDVVHCIAQTVKNLYXNKSIWLWTGYKFEDIKQLEMLKYVDVIIDGKYEKNLPTKKLWRGSDNQRLWSNTDGVWKHD
88	129941..130498	PHAGE_Escher_vB_EcoM_JS09_NC_024124: RNA polymerase sigma factor; PP_00211; phage(gi642905908)	MTEQKPKNNYVNNKELLAAIIEWKKELLNNKDPNKIIRQNDTIGLAIMLIAEGLSKRFNFSGYTQSWKQEMIADGIEASIKGLHNFDETKYNNPHAYITRACFNAFVQRIKKERKEVAKKYSYFVHNVYDSHDDDMVALVDETFIQDIYDKMTHYEESAYKAPGAEKKEVVSDSPSLDFLYEDDN
89	131486..132505	PHAGE_Escher_vB_EcoM_JS09_NC_024124: recombination endonuclease subunit; PP_00216; phage(gi642905913)	MKILNLGDWHLGVKADDEWVQSIQLDGIKQAIEYSKKXGITTWIQYGDIFDVRKAIXHKTMXFXREIVQMLDDAGITLHTXVGNHDMHFKNTLTPNASTELLAKYPNVKVYDKXTXVDFDGCLIDLIPWMCEENTGEILEHIKTSSAAYCVGHWELNGFYFYKGMKSHGLEPDFLKTYKQVWSGHFHTISEAANVKYIGTPWTLTAGDENDPRGFWVFDTDVERMEFIPNETTWHRRITYPFKGKINYSDYTNLAVRVIVSEIDSELTKFESELEKVVHSLRMVSKVDNSVESDDDQEIEIKSLQDIIKEYIDAIPDISDEDREALVKYSNELYIEATQ
90	132982..134670	PHAGE_Escher_vB_EcoM_JS09_NC_024124: endonuclease subunit; PP_00219; phage(gi642905916)	MKSFKLNRVRYQNIMSVGGNPIDIQLDKVQKTLITGKNGGGKSTMLEAITFGLFGKPFRDVKKGQIINSTNKKELLVELWMEFDGKKYFIKRGQKPNIFEISVDGVRLDESASSRDFQEEFERSIGMSYASFKQIVVLGTAGYTPFMALSTPARRKLVEDLLEVGTLAEMDKINKSQVRELNSQGQVLDAKKDGVIQQIKIYNENIERQKKLSGDNVARLQNMYDDLAKEARSLKAEIEEANERLLNIVLDEDPTEAFNKIGQEALLIKSKIDSYNKVIKMYHDGGTCPTCASQLHQGDPIVSKITNKLHECNHSFEQLTCHRDNLSVLVDEYRANVKTKQDLASDIRTKKQAMIATIDKAKKVKAAIEQASAEFIDHADEIALLQKELDKIIKTKSDIVLEKYHRGIITDMLKDSGIKGAIIKKYVPLFNKQINHYLKIMEADYVFSIDEEFNESIKSRGREDFSYASFSQGEKARIDIALLFTWRDIAEKVSGVKINTLILDEVFDGAVDSEAVKAIDTILGSLQNTNVFIISHRDHDPQRYGQHLQMSKVGRFTVMTVS
91	134926..135342	PHAGE_Escher_vB_EcoM_JS09_NC_024124: RNA polymerase binding protein; PP_00221; phage(gi642905918)	MFTTAKGFTAADLKVTSIRTDANPHNHNRVRKAWVLHCDDASAKKLQSLPQETRFMIYGFIDNDVSDMWIHLMRKHYKDSIEAGGKIVLDKDGSERLEDLYCVDADEQLIAAGEIVASKIPEYIESLPEAIKKQMVAA
92	135385..136071	PHAGE_Escher_vB_EcoM_JS09_NC_024124: sliding clamp; PP_00222; phage(gi642905919)	MKLSKDTIAILKNFASINSGILLSQGKFIMTRAVNGTTYAEANISDEIDFDVALYDLNSFLSILSLVSDDAEISMHTDGNIKIADTRSTVYWPAADKSTIVFPNKPIQFPVASVITEIKAEDLQQLLRVSRGLQIDTIAITNKDGKIVINGYNKVEDSGLTRPKYSLTLTDYDGSNNFNFVINMANMKIQPGNYKVMLWGAGDKVAAKFESSQVSYVIAMEADSTHDF
93	136147..137109	PHAGE_Escher_vB_EcoM_JS09_NC_024124: clamp loader small subunit; PP_00223; phage(gi642905920)	MITINSKEHILEQKYRPSSIDECILPAYDHETFKSLVSKGKLPHIILHSPSPGTGKTTVAKALCNDINAEMMFVNGSDCKIDFVRGPLTAFARSVSMEGKPKVIVIDEFDRSGLAESQRHLRTFMEEFSSNCSIVITANNIDGIIEPLRSRCRVIEFGRPTEEDKISMMKKMIHRMVEICKNENIEIADMKVVAALVKKNFPDFRRTIGQLDQYSSKGVLDAGILSIVTNDRGTVSDVIEAMKNKDIKQLRALAPKYAADYSWFIDKLVSECYDQVAPGKSIISLYEIAGENNKFHGLASNIELHVMYMLLQLTCELTWK
94	137111..137674	PHAGE_Escher_vB_EcoM_JS09_NC_024124: clamp loader small subunit; PP_00224; phage(gi642905921)	MNLFDDDVRLNEHQIAWKSNDADAIQKCADMFKEKPENEFFKIINAINEKKSMSIAQVDYSKFMVENSLSQFPECMPAVYMMNLVGSELSDEAHFNYMMAAIPRGRRFSKWAKLVEDTSELLVIKLLMKRYTINMNDATEYKRLLEKNNKLPIVLKELKAMVTDEFLKEVTKNVKEQKQFKKLALEW
95	137677..138045	PHAGE_Escher_vB_EcoM_JS09_NC_024124: translation repressor protein; PP_00225; phage(gi642905922)	MIEIKLKNPEDFLKVKETLTRMGIANNKDKVLYQSCHILQKQGKYYIVHFKEMLRMDGRQVDIDGEDYQRRDSIAQLLEDWGLIVIEDSAREDLFGLTNNFRVISFKQKDDWTLKAKYTIGN
96	138127..140838	PHAGE_Escher_vB_EcoM_JS09_NC_024124: DNA polymerase;	MKEFYLTVEQIGDSIFERYIDSNGRERTREVEYKPSLFAHCPESQATKYFDIYGKPCTRKLFANMRDASQWIKRMEDIGLEALGMDDFKLAYLSDTYNYEIKYDHTKIRVANFDIEVTSPDGFPEPSQAKHPIDAITHYDSIDDRFYVFDLLNSPYGNVEEWSIEIAAKLQEQGGDEVPSEIIDKIIYMPFDNEKELLMEYLN

		PP_00226; phage(gi642905923)	FWQQKTPVILTGWNVESFDIPYVYNRIKNIFGESTAKRLSPHRKTRVKVIE NMYGSREIITLFGISVLDYIDLYKKFSFTNQPSYSLDYISEFELNVGKLKYDG PISKLRESNHQRYISYNIIDVYRVLQIDGKRQFINLSLDMGYYAKIQIQSVF SPIKTWDAIIFNSLKEQNKVIPQGRSHPVQPYPGAFVKEPIPNRYKYVMS FDLTSLYPSIIRQVNISPETIAGTFKVAPLHDYINAVAERPSDVYSCSPNG MMYYKDRDGVVPTEITKVFNQRKEHKGXMLAAQRNGEIIKEALHNPNL SVDEPLDVDYRFDFSDEIKEKIKKLSAKSLNEMLFRAQRTEVAGMTAQIN RKLLINXLYGALGNXWFRYXDLRNATAITTFGQMALQWIERKVNEYLNE VCGTEGEAFVLYGDTDSIYVSXDKIIDKVGESKFRDTNHWVDFLDKFARE RMEPAIDRGFREMCEYMNNKQHLMFMDREAIAGPPLGSKGIGGFWT GKKRYALNVWDMEGTRYAEPKLKIMGLETQKSSTPKAVQKALKECIRR MLQEGEESLQEYFKEFEKEFRQLNYISIASVSSANNIAKYDVGGFPGPKCP FHIRGILTYNRAIKGNIDAPQVVEGEKVYVLPLREGNPFGDKCIAWPSGT EITDLIKDDVLHWMDYTVLLEKTFIKPLEGFTSAAKLDYEKKASLFDMFDF
97	147528..148700	PHAGE_Escher_vB_EcoM_JS09_NC_024124: RecA-like recombination protein; PP_00235; phage(gi642905932)	MSDLKSRLIKASTSKMTADLTKSKLFNNRDEVPTRIPMLNIALGGALNAG LQSGLTIFAAPSKHFKTLFGLTMVAAYMKKYKDAICLFYDSEFGASESYFR SMGVDLDRVVHTPIQSVEQLKVDMTNQLDAIERGDKVIIFIDSIGNTASK KETEDALNEKVVGDMSRAKALKSLFRIVTPYLTIKDIPCVAINHTAMEIGG LYPKEIMGGGTGILYSANTVFFISKRQVKEGTELTXXXFTLKAEKSRTVKEK STFPITVNFDGGIDPFSGLLEMATEIGFVVKPKAGWYAREFLDEETGEMI REEKSWRAKATDCVEFWGPLFKHKPFRDAIETKYKLGAISSIKEVDDAVN DLINCKATTKVPVKTSDAPSAADIENDLDEMEDFDE
98	149044..150486	PHAGE_Escher_vB_EcoM_JS09_NC_024124: helicase; PP_00237; phage(gi642905934)	VVEIILSHLVYDQAYFSKVWPYMDSEYFERGPAKNVFKIIKSHVNEYNA MPSINALKVALDNSSLTEAEYKGTSDLIEKLADTPEDHEWLVKETEKYVQ QKAMYNATSKIIEIQSNAELPPEQRNKKMPDVGAIPDIMRQALSISFDSY VGHDWMEDYEARWLSYLNKARKVPFKLNILNKITKGGAETGTLNVLMA GVNVGKSLGLCSLAADYLQTGHNVLYISMEMAEEVCAKRIDANMLDVS LDDIDDGNVSYAEYKAKMEKWRSKTTLGRLVVKQYPTGGANANTFRAL LNELKLKKNFVPSVIMVDYLGICGSCRIRVYTENSYTLVKAIAEELRALAVE SETVLWTAAQVGRAAWDASDMNMSDIAESAGLPATADFMLAVIETEE LAQAEQQLIKQIKSRYGDKNKWNKFLMGVRKGNQKWIEIEQEGMNTP NTVNENAGAQMRQAEVNRTERVGKAKTTRADLDSLANELKF
99	152215..152463	PHAGE_Escher_vB_EcoM_JS09_NC_024124: immunity to superinfection membrane protein; PP_00243; phage(gi642905940)	MELFVASSVAVLIAGIGSIIIYMLPWVIALIRGTKSTTAIFFVSLLFNWTMV GWIGTLIWSIVAEKKSAQQPQQVIIIREKE
100	154198..155220	PHAGE_Escher_vB_EcoM_JS09_NC_024124: DNA primase; PP_00248; phage(gi642905945)	MAEWIDNEFAYRAFSHLPRFRQINNSSTFKLTFRCPVCGDSQTDAMKA RGWYYGGTPGNVHCYNCQYHNTISGYLKEYDEELYREYLMEVRKEKAR MEPKIEKVPEHKPEPEKKTINSLPSCSRLDKLPEEHPIVKYVKTRCIPKESW NRLWFTLEWPKLVNKIQPGTYKKEIPEPRLVIPIFNKDGKAESFQGRALR KDAPQKYITIKAFESATKIYGVERVKEGDVWVMEGPIDSLFIPNAIAITGG SIDLDVVPFKERRVWVMDNEPRHPDTIARMKRLVDAGERVMFWDRA PWRSKDVNDMVMKENATPQEILEYMKQNISSGLHAKMRLSRYSKI
101	155503..156024	PHAGE_Escher_vB_EcoM_JS09_NC_024124: dCTP pyrophosphatase; PP_00250; phage(gi642905946)	MAHFNECSQLISGVDKAEEAYFNALIHEDKDPLQVMLDMQKSLQVRLA NDKPEHNKHPDELATAGDVVDWLRNQKDYIDDEFRELLTSLGGMSNG EKAASSVWKPWKAQHAEYRNRRIDELSPEDQLEIKFEMIDXLHFVLNMF QGLGLSAEEIFKLYYLKNQHNFERQDNGY
102	156070..156306	PHAGE_Escher_vB_EcoM_JS09_NC_024124: small outer capsid protein; PP_00251; phage(gi642905947)	MGGYVNIKTFTHPAGEGKEVKGMEVSVPFEIYSNEHRIADSHYQIFPSEX AAYSXVVSDAXTWKTKNAAMFTPTXXGG
103	157878..158459	PHAGE_Escher_vB_EcoM_JS09_NC_024124: ADP-ribosylase; PP_00258; phage(gi642905954)	MTCQSELQEREYLTNLIDSKFTRQEQNILWACTDSKEDPDFHYELDHLV RKHMTSTVPVELYRGVTPEEVERLSLLSVGCHWSPGRVTSFTTDFSTAR QFSGRWEYQTYTILSLRNAPFIFDYYQNMVNIVLAGKNPKHVMEETRX DVLDMIESEQEYMVSGISRFEIVEIEDLEYDPLSRLYKIIHLKMLNF

104	158517..159125	PHAGE_Escher_vB_EcoM_JS09_NC_024124: RNA polymerase ADP-ribosylase; PP_00259; phage(gi642905955)	MFMPYNECEIEEMVRRXQNPEYQDVLQYEXDSQFSDIEQSVLWQCMENKPDSIQQDLSAIVRRNWXSXVPETMYRGISKKTMATLDDKGXGSIIKFDRVMSFSPVFGVARNFASYNFYGTFNMFCIKDAPFAFNFREHMLNMILAAPSCEFNGAFPEATRRSNARLISDECEFMLPIGTTLRVDSIIQDGRYTIWNLSIVSY
105	159278..160024	PHAGE_Escher_vB_EcoM_JS09_NC_024124: putative anti-sigma factor; PP_00260; phage(gi642905956)	MLSNNKINKINRRLDHTRASAKRRSKDFNLDFNYLKNILDQKVCAYSGESFNNSVEGEKLSLERFNNDIGYIKGNVIPVKKKYNTARSDLTLEELIEKRDXIARRIANPSARKVEKLNLDEKKWAQIKKVYGTILKIRAKRENRVKHMANMMKNQPLSNESKLRIVALKARINGSHQAEGHELTKLXVLLKGSDWKTKTKLTDAESLFDTYDKVIQGLQRFEKIGFXGKLKLKRGLPLSAXLFQLIKG
106	160335..161648	PHAGE_Escher_vB_EcoM_JS09_NC_024124: DNA helicase; PP_00262; phage(gi642905958)	MTFDDLTEGQKNAFNVVMXAIKEKKHHVTINGPAGTGKTTLTRFIVEALISSGESGIILAAPTHAAKKVLSKLAGQEASTIHSILKINPTTYEENVLFEQKKVPDLALCRVLLFDEASMCDRSLFKIMLASIPKWCTVIGIGDRCQIRPVDPGESTAYLSPFFTHKDFVQCNLTEVKRSNAPIIDVATDIRNGKWIYDKVVDGHGVHGYTDLKSYMMNYFNVVKTPEDLFENRMMAFTNKSVDKLNSIIRRKLLETEKPFIKDEVIVLQEPFTKTYKLDGKNMTEMLFNNGQFVRIRDAVETSTFVKAXGVSGEYMIRYWNLTVETYGDDEEYIREDIKVISSEEELYKFNLFLAKTAETYKFWNKGGKAPWSEFWDAKRMFTKVKXLPVSTFHKAQGMSVDTAFVYTPCIHXADSELAKQLLYVGTTRGRFDVHFI
107	161658..162335	PHAGE_Escher_vB_EcoM_JS09_NC_024124: exonuclease; PP_00263; phage(gi642905959)	MSTDVIIDFETFGNTSKAAVIDLAVIAYNSDPEVVESFEELXQRGKRIKFNLASQKGKRVFAKSTMKWWKEQXAEXRKNLAPSEDDVTTLEGIKIFLDYCRANKVDQWKSQMWXRGMSFDFPILVDLIRDLYRDEGVLEPEIDTDKLEPVKFWAQRDIRTAIEAYSLTRGLSMCPLPMGTLKGFVAHDSIHDCAKDILMLKYAQRYALGLEDVPENPDPLSVKQR
108	162917..163414	PHAGE_Escher_vB_EcoM_JS09_NC_024124: modifier of transcription; PP_00265; phage(gi642905961)	MEYNEFNLNKERKIMITIGEIKRVSTXXXSKXAGRLVEVVGLXXXGMGRXKEVKVRIIPSFGRQDXQXAYVSPKFLETPVXAPFXSTEPNAQXKWAXCKGVEFQTDKEFDYIDEYXYPSKTDIMCGFISDQWVEDGEKLYNIVFLGDFRVVKESEITRYVSPRKA
109	164712..165122	PHAGE_Escher_vB_EcoM_JS09_NC_024124: mRNA metabolism modulator; PP_00269; phage(gi642905965)	MANKFRVNSWYQFIDKRAQEEFIKDHTDXGIYARRLGMEPFKILDVDHLXRPTKILTSTGTVGYATGGDILDENFIWLSTNEAEFFDEVENPYQASEEQDSDELDELSEFPVMTITIENNDQAWSLYQMLKAHFKE
110	165793..167610	PHAGE_Escher_vB_EcoM_JS09_NC_024124: DNA topoisomerase II large subunit; PP_00272; phage(gi642905968)	MIKNEIKVLSDVEHIKKRSGMYIGSSAKEXXERFLFGKYQQVEYVPGLVKLIDEIIDNSVDEAIRTSFKFANKIDVQIKNNQVSVEDNGRGIPQGLVTDQTGEQIPGPVAAWTIPKAGGNFGDDSERKTGGMXGVGSSLTNIFSTLFTGITXDGENEITVNCSNGMENKXWSSKKSKGKGTKVIFTPDFSXFEEHNLSQIYLDITLDRLQTLAVVYPDIKFTFNGKKVDGNFKRFAKQFGEDNIIQENDKVSIXFTTSPXGFRHLTYVNNIHTKNXGHHVECVMDDICEHLLPXIKKKYKGIEVTKARVKECLTMLMFIRDMSNMRFDSQTKERLTSTYGDIRNHIQLDAKKIAQALLKXEALIMPIVEAALARKLXAEKAAETKAAKKATKAKVHKHIKANQXGKXADTTLFLTEGDSAIGYLIDVRDRELHGGFPXRGKVMNSWGMSYADMMKNKELFDICAITGLILGEKAENTNYRNIAIMTDADHDGLGSIYPALLAFFSNWPELFEQGRIRFVKTPVIIAQIGKTQKWFYTVAEYEEAKDTLPKHSIRYIKGLGSLEKSEYREMIQNPVYDVVKLPENWKELFEMLLGXDSELRKEWMS

TABLE AT3 | Total genes identified from Salmonella virus TU_SP24B, their position on genome and complete protein sequences as identified by PHASTER tool.

SN	CDS POSITION	BLAST HIT	prophage_PRO_SEQ
1	1436..2662	PHAGE_Salmon_LSPA1_NC_026017: terminase; PP_00003; phage(gi744692837)	MLLLPHQYELLADTTTKIIGLCSGFGGGKTYSAARKAVHLALLNPGVDG IITEPTFPLLVQVMFPELMAALDFFGVKYKFNKVESIFYCDINGQTTRIIC GSMENYQRLIGINAAWCVCDEFDTAKQETAYAAYMKLLGRLRRGNV RQLVIVSTPEGFRAMYQIFVTEADASKRLIRAKTTDNYHLPADYVESMR SQYPEQLINAYLNGEFVNLTSGVVYYGYQRGKCESNETIKPGEPLYIGQ DFNVGKMASCVYVKRGTDFHAVAELVDLFDTPDVVRVIKERWAGHHI IMYPDASGRNRKSNSASTSDIAILEQAGFEIRAKSKNPPVKDRVAASNA AFEKGRVFINSKACPATARGLEQQAYDKNGEPEKNGVIDHMVDAATY LIAYEMPVAKPVINIPVTFAL
2	2812..4296	PHAGE_Salmon_LSPA1_NC_026017: structural protein; PP_00004; phage(gi744692838)	MLNMNGQDQGVKTKHREWSHHFNKWQKVRHALAGDLIRYLRNVG KNEPDPTYAAQRQEEYENGAICYNFTKRTLAGMVGSVMRKEAEINIPK ELEYLLKNADGSGVGLIQHAQDTLMEIDAVGRGGLLVDAPETGAATA AEQNAGLLNPTIAFYTTENIVNWRLARVGSVNRVTMVVLRETWEYYE PENEFETKYGEQYRVLDIDTDGNYRQRLFRFDAEGGAQEGVVEIYPDL GESLRGVIPFTFIGATNNDATIDDAPLLPLXXXNIGHMRNSADVEEASFI TGQPTLFIAPGENMNMEQWKEANPHGVRMGSRSGHNIGYGGNAFL LQAVETNLARQNMLDKEQQAIQIGAQLITPTQQITAESARIQRGADTS VMATXXRNVSQAYTDALRWVAVMLGKPEDTEVEFRLNMDFFLEPM TAQERAAWMADINAGLLPATAYYAALRRAGVTDWTDEDILNAMED APLPLGGVTQVXXEIPQAAQQQDTTQQ
3	4333..6225	PHAGE_Salmon_LSPA1_NC_026017: structural protein; PP_00005; phage(gi744692839)	MLLTLAPQGLSMSLLTSLISHQIWLQRTASGEVKDLTPFIKEMRDEIKR QVLLFGDDGRSTARLNKLLRDLEEALAGLTGDWQTKLTEDLKGLAAYE AEWNVKTLTTNVNAEFVTPTAEQVWAAAEFQPLSLSDKPVDFTKLM AGWGETEVARLVTGVKMGFVQGQTTRQIVKNVVGAGGLADISERNA ATVIRTALSHVSNEARNETYRQNDDIIEKYEWVSTLDSRTSTICRARDG MTWEIGKGPMPPAHPNCLLGDTVVSTGSPVSNIFKRAYKGVIVYVSTK SGRTLSITPNHQVLTTSGWVASGLLNVGDKLVCAKDSALSLKHKEHNV VAKFSDLFSAANVAVNPAAVSTSPTSPEDFHGDGTDGEVEIVLVDRLS WDKVKSGLNKQIIDKELPVTAGVDDSLPGFGSAKQLGMVGLSPSDSF MRRGGESEAILGGSFSHSDKHSVATSANGNAVLSENAYDWATRNAD NLSDFNWSDAVGVELDDVVDLVFSEADFCGHVYNLENEQNWYLANG IIAHNCRSTTAPVISPEFDFLDKGAKRAARGADGGQQVSANTTYYEFLK QQPAWFQDEALGPVRGKIFRNSGVTPEEFRVISVDGFGRPLSLKEMAE LDKRVADYLKEE
4	6228..6686	PHAGE_Salmon_LSPA1_NC_026017: putative tail protein; PP_00006; phage(gi744692840)	MGFFKVKDVPSRRVVQYSRVSGSSENVVFIEDESVLGTPVDDMPFAD KTGIALPVAGMLYEIPYLADAGGVYFSVQPQDVELADGSATITVEVKA GKAPYVLTWYKDGKEVVNVPEEALSLTVNAVGEYFVKVTDADGVEAV SKAAKVTKPE
5	7628..8014	PHAGE_Salmon_LSPA1_NC_026017: putative spanin; PP_00008; phage(gi744692842)	MNLKAAIITTACSAFLVFAYGKYNYLQGWNEGRANLVSQQQQKAQA ELAKKTQRQQQDESKAAAADNEGKTKSEVITREVIKYITRPGRTVCEFP PERVSIKRRAAENANSISGYDVNAASVQNGAAK
6	13271..14437	PHAGE_Salmon_LSPA1_NC_026017: putative major tail protein; PP_00020; phage(gi744692854)	MAQPYKGAMTAQFYVPETTPGVTPDSPMWQPLRNTGGIPAVTRDT LVSNELDGSRETSSIRTGNRQVTGEYAIELSAASQDELLAGAMTSSWV AGSTAQAISITVDPVVKTFTRTTGSFVADGVDVGDLVQFDGLSGNNDK AFLVTAVTATVVTGAGIQHTLTAESDVQADLRIADKLETGNLCKTYSILT WLKGKCGNPDSYIITRGVEFTGFTIEQAVNAMVTGSFPFIGLNQEILEE LPSGSNFTTNFSARPFASVDVSVYDGTAPLKLIDTFTITSDNSASAQFEL GNNSVAFVERGRAANTFSLAGKLYDMTLLNKFLNETQMEVSSVLDGP DGAMSFTLKRASLTSATPEIGGPESVTLSLEGQATGNQFQSSIVIQRVK YA
7	16598..19705	PHAGE_Salmon_LSPA1_NC_026017: putative tape measure protein; PP_00023; phage(gi744692861)	MRLSDFYXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

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8	20204..20719	PHAGE_Salmon_LSPA1 _NC_026017: enolase-like protein; PP_00025; phage(gi744692863)	MSQESVEAAYRRKLASNPDGEMDFITLEIYHPLLSKRWLLVRGVNDLT ATLETGEVVTFEGTPMEVKNAANNNDMDQTASFSLPDVLNILDEEM DRIPFDNKELPKFIFRRYVGTDLSYPCDGPVVYELQTLTQEKGVFTAETG TPMLNQRATGILMTPEEIPLLRGILTS
9	21072..23630	PHAGE_Salmon_LSPA1 _NC_026017: putative tail fiber protein; PP_00027; phage(gi744692865)	LALVIHYTRNEDGTFDVKHYRDNPINFAVTHVPDGVPFRVFIDEIGEDN DVTEDFEALKENATFHIVESAGGGAIKGVMKIFSVILKPLAKLLSPSVKG ASSNLANSQADSPNNSLTDRNNKARPYERSYDICGTVQTIPNNLMTTY KVFNAAGKIVEYGYYDAGRGYLDIHPEGITDGDTRVSDITGTSVAVYAP YTSPNNTSTPQVMVGDPIEQGLYITVESNEVDGVVLKAPNGLGISFSY MSGYPSLSGNIGTIYDPTGGSDFSGVLVPDDTFSLVAAWTNTDVDLSG GGYQVVSVSEGTVTFIVPGGLIGRWQEIRPGSFFRGDGEASLQPDSTY EKTLTDWVSINRTEVERIVANIAAANGMYKDNGKSKTLASVTAEIQYQ LLDENSTPYGPIYTAQGTVSGRTPDYNGVTIYADLPVVSRVRVRARRVT DLDFNFEGSVVDEITYVNLYGQTRDNTPHYGNRTTVHSMRKQTPRAA EVKQPQLRMIATEMVYKYLGNGVFEDTMTPNTQAVQSLIRLARDPDV GGLNLTVRNMDKLLAVQNEVEAYFGDKQAGEFCYTFDDYKTTMQDI VSAIADAIFCTPYRRGADILLDFERPRMGPEMVFTHRSKAGTSEKWTR TFNDAQVFDSLKFSYIDPKTNVKETITIPETGGLKTETYDSKGIRNYKQA FWAANRRHQKNILKKISVSFTATEEGIFALPNRAVSVVKGSRMATYDG YVTAVNGLTVELSQPVKFTSGDDHSLILKLRDGGVQSVNVVPGAHDR QVIMTSVPQEAIYTGNSALKTEFSFGNEARHNAQMILVSTVDPGDDRT VKITGFNYDKDFYKFDNVPPFGRAFSNGFDNGFN
10	complement (25916..28381)	PHAGE_Salmon_LSPA1 _NC_026017: putative helicase; PP_00030; phage(gi744692868)	MSKFTRRPYQKLMTSFMLWHPRCNIWASMGSGKCLKRGTEVIMFD GTTKKVEDVIVGDVLMGPDSTPRNVLSLGRGREMMYEVKPRKGESYT VNESHILSLRTTTGIAKGSWPDNTVFDISVRDWLKLPKYVTGPNGCLK GWRVPVDFPRKEQDEALLPPYLMGLWLGDGTSSSGAITSGENEKEIR AYLESYAARNRMQIRKEGLTWSISHGNTGHKKHGFTHALKSAGVLNN KHIPHNYKCGDRRQRLELLAGLLDSDGYRDLSKAGFDWISVSERLADD FCYLCRSLGFAAYKKKTRKRCTNTDVWGDYFRVSVSGDFSEVPFVRGR HQNLPKRNINKNVLNVGIESITPVGVDDYFGFTIDGDSRFLLGDFTVTH NTSSTLWVLNRLFRNGQLTEDDRVLILAPLRVASGTWPAEQARWNFP CLRVVDATGNEKRRVAALESDANVVCTNYEVIEWLINYYGKDDWPFT VIVADESTKLKSFRSRSGGSKRARALSKVAFGKVKRFINLTGTPSPNGLK DLWGQNWFIDAGERLGSSYTSFTDRWFNSVQKGKSAMAREYHARP GADNEIHQKMKDVSLTIDAAEWFGCEAPIIVPVEIDLPKKARQAYIDM EEKLFAELESGEVEAANAAAKTSKCLQIASGAVYVSGPDGEATKDWEK VHDTKLDALESIVEELQGAPLLVAYQFKHELERILKRFPQAQAFAKGSK GNKQMEAWNRGEIEILCVHPASAGHGLNLQDGGHHLAFISQGWNLE HYLQVVERIGPVRQKQAGHERPVFLYHIVAKDTLDEVVATRTDEKKSV QEELLNYMKRRGKK
11	complement (31723..32346)	PHAGE_Salmon_LSPA1 _NC_026017: putative DNA polymerase; PP_00036; phage(gi744692874)	MGIKLNLRKVNTAWVNVFEREKDRENDDGSITKGQYSATIILPSDHAQ IDALYDTVYAVVEEALGAAAAEKWMKSNYGEGKHMDKCAIKDIAERD NPFEDFPEGFYFKAKAQKQPLIVTSKKGETQVEQDFNVDGEQIEGEQV YSGCVANVSVEIWFSQKYKVLGVNLLAIKYVGEGKAFGGSKVAASVDD LEDDEEDEAPRRGRRR
12	40245..40730	PHAGE_Salmon_LSPA1 _NC_026017: putative endolysin; PP_00049; phage(gi744692887)	VSRGISDNGLKFTAAWEDFRGTAYRATPSEKYLTIGYGSYGPHVYEGQ KITKGQGLLLLNRDMAKAVAAVAAVAHHSLTQAQFDAVCDLVYNAG AGVISATTGTGKALRAGDIATLRAKLALFINQNGKPLLGLRRRTAGRLA LFDGKPWQEAEAIGRAAK

EXTRA THESIS ACTIVITIES & OTHER ACHIEVEMENTS

Awards / Achievements:

1. ASM Student and Postdoctoral Travel Award for ASM Microbe 2016, held from 16-20 June, 2016 at Boston, Massachusetts, USA.
2. ASM-ICIDN Registration Grants for SAARC Microbiologists for 2nd International Conference on Infectious Disease and Nanomedicine – 2015, held from December 15-18, 2015 at Kathmandu, Nepal
3. Kathmandu Center for Education and Research, CAS & TU Thesis Grant for M.Sc. students - 2015 grant.
4. Runner-Up award in an event International Young Microbiologist Competition-2014 (an international scientific article writing competition) on topic *"Defeating MRSA from the hospitals using efficient techniques."*

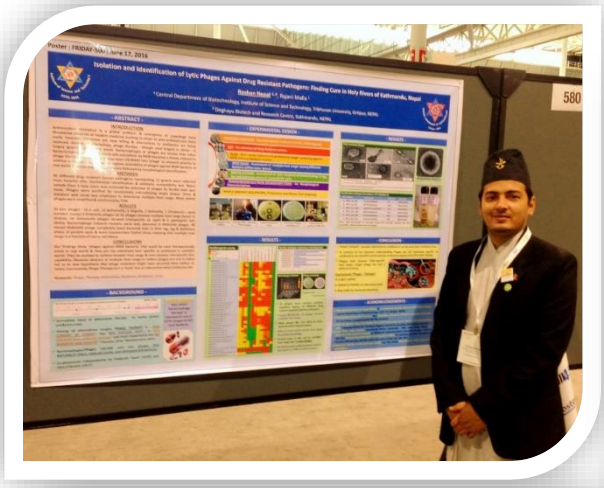
Seminar / Conference Presentations / Attendances:

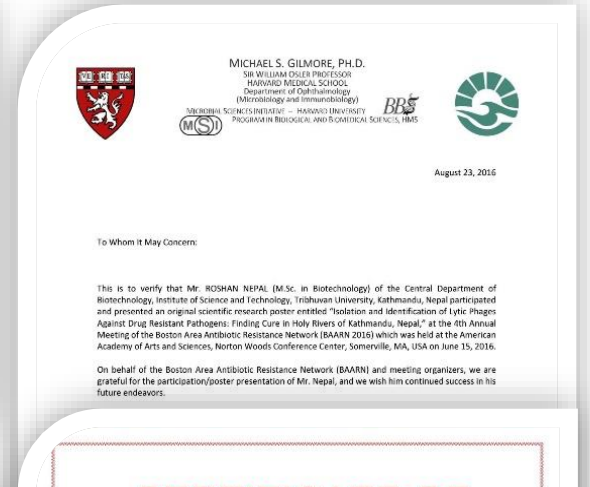
1. **Poster Presentation** – American Society for Microbiology (ASM Microbe 2016), June 16-20, 2016. Boston, USA.
2. **Poster Presentation** – Boston Area Antimicrobial Research Network (BAARN 2016), June 15, 2016. Massachusetts, USA.
3. **Oral Presentation** – Second National Summit of Health and Population Scientists in Nepal, April 11-12, 2016. Kathmandu, Nepal.
4. **Oral Presentation** – 7th National Conference on Science and Technology. March 29-31, 2016. Kathmandu, Nepal.
5. **Poster Presentation** – 2nd International Conference on Infectious Disease and Nanomedicine. December 15-18, 2016. Kathmandu, Nepal.

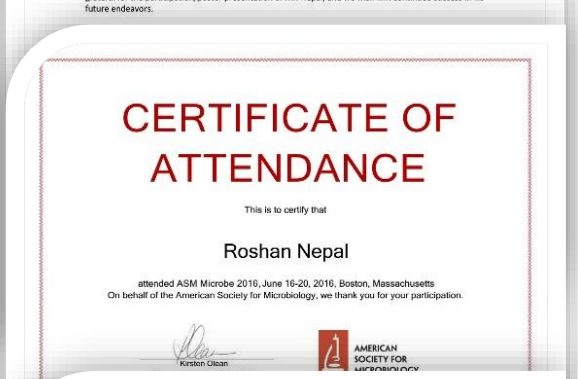
Publications:

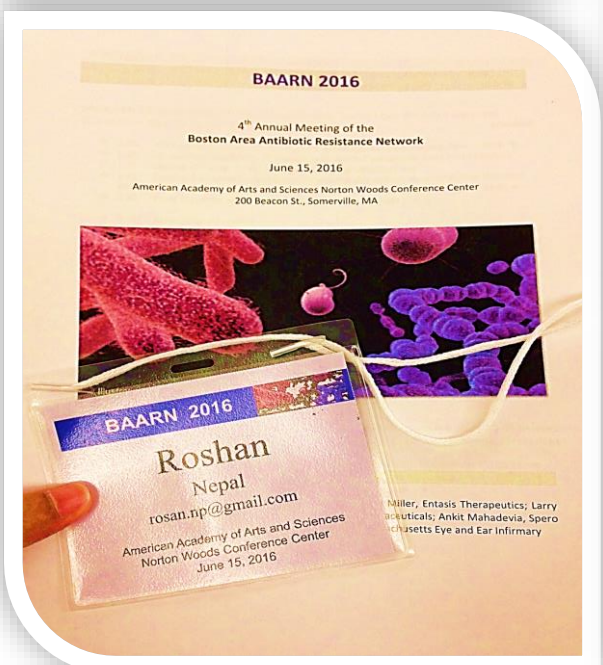
1. **Nepal, R.** (2016). Defeating MRSA with Phage Therapy – a Possible Natural Alternative Amidst Antibiotic Resistance Frustrations. *The Transcript.* Vol. 2(1). ISSN 2505-1083. Nepalese Society of Biotechnology. pp. 133-138
2. Maharjan, A**.**, **Nepal, R**., Shrestha, M., Malla, R. (2016). Bacteriophages – an alternative to antibiotics: Challenges and its future possibility. *The Transcript.* Vol. 2(1). ISSN 2505-1083. Nepalese Society of Biotechnology. pp. 50-53
3. Malla, R., **Nepal, R**., Maharjan, A. (2016) Bacteriophages – solution to a global crisis lies in rivers of Kathmandu valley. *RevoScience*. Vol. 06(03). June 2016. pp. 33

PHOTOGRAPHS / CERTIFICATES





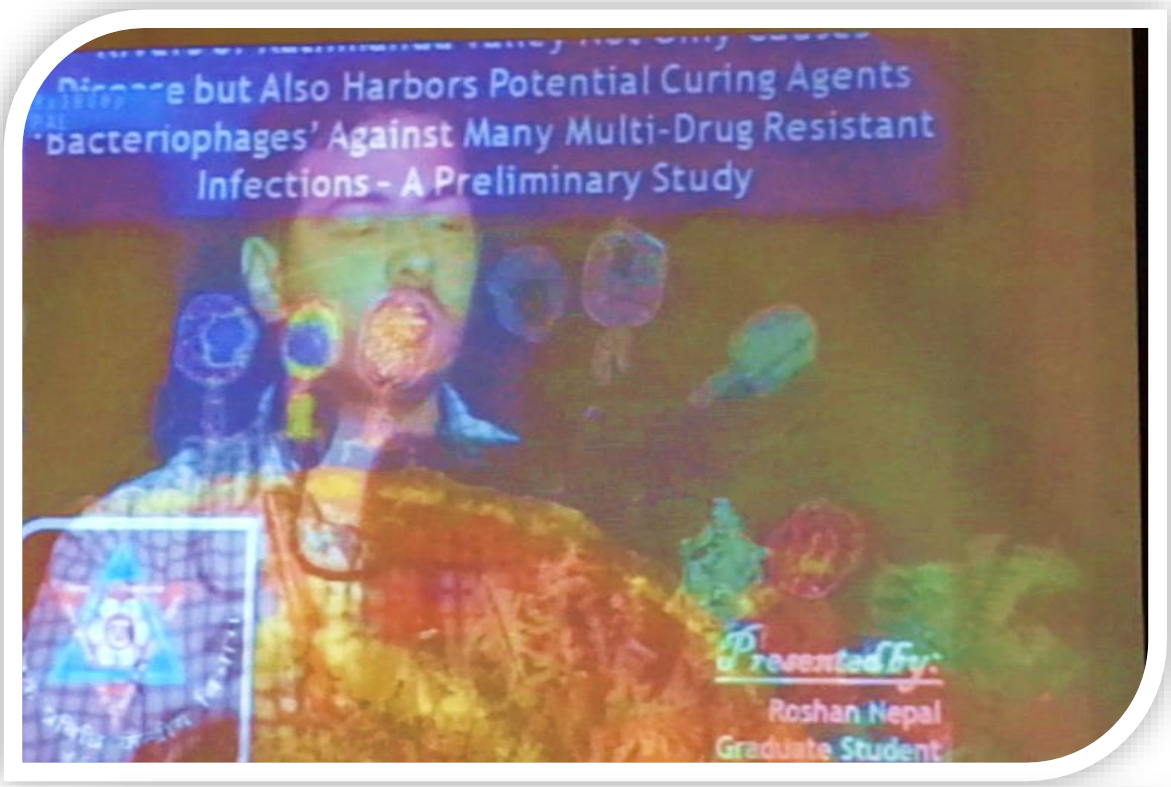




ASM Microbe 2106 & BAARN-2016
June 16-20, 2016
Boston, MA, USA

International Workshop on microRNAs: Novel Tools for Diagnostics
October 15-16, 2016
South Asian University, New Delhi, India

Advanced Instrumentation Research Facility (AIRF)
Jawaharlal Nehru University (JNU)
New Delhi, India

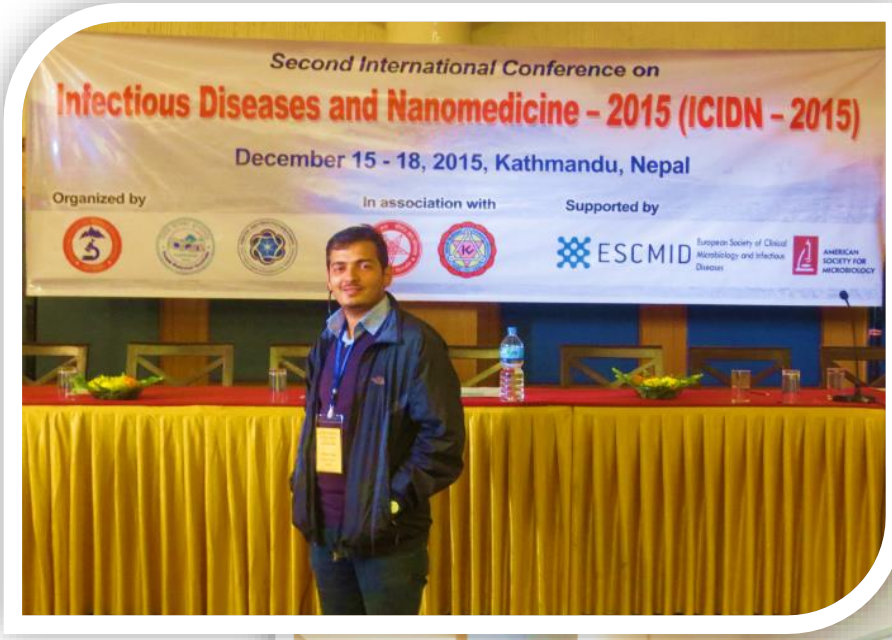


2nd National Summit of Health and Population Scientists in Nepal
April 11-12, 2016
Kathmandu, Nepal

7th National Conference on Science and Technology

March 29-31, 2016

Kathmandu, Nepal



2nd International Conference on Infectious Disease and Nanomedicine – 2015 (ICIDN-2015)

December 15-18, 2015

Kathmandu, Nepal