Characterization of Bacteriophages Against Multi-drug Resistant *Pseudomonas aeruginosa* and Phylogenetic Analysis of Multi Host Range Bacteriophages

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ACRONYMS:

µL	: Microlitre
ABR	: Antibiotic Resistant Bacteria
AST	: Antibiotic Susceptibility Test
BLAST	: Basic Local Alignment Search Tool
CDBT	: Central Department of Biotechnology
CFU	: Colony Forming Units
DLAA	: Double Layer Agar Plaque Assay
DNA	: Deoxy Ribonucleic Acid
dsDNA	: Double Standard Deoxy Ribonucleic Acid
dsRNA	: Double Standard Deoxy Ribonucleic Acid
EAE	: Experimental Allergic Encephalomyelitis
EDTA	: Ethylenediaminetetraacetic acid
EPA	: Environmental Protection Agency
FDA	: Food and Drug Administration
gDNA	: genomic Deoxy Ribonucleic Acid
GRAS	: Generally Recognised as Safe
ICTV	: International Committee for Taxonomy of Viruses
KDa	: Kilodalton
L	: Litre
LB	: Luria Bertaini Broth
MDR	: Multi Drug Resistant
MEGA	: Molecular Evolutionary Genetic Analysis
MHR	: Multi Host Range

mm : millimeter

MOI : Multiplicity of Infection

MRSA : Methicillin-Resistant Staphylococcus aureus

NCBI : National Center of Biotechnology Information

ng : nanogram

nm : nanometer

OD : Optical Density

PBS : Phosphate Buffer Saline

PCR : Polymerase Chain Reaction

PFU : Plaque Forming Units

RNA : Ribo Nucleic Acid

Rpm : Revolutions per Minute

SDS-PAGE : Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SM : Sodium Chloride and Magnesium Sulfate

ssDNA : Single Standard Deoxy Ribonucleic Acid

ssRNA : Single Standard Ribonucleic Acid

TBE : Tris-Borate-EDTA

TDW : Triple Distilled Water

TSA : Tryptic Soya Agar

TUTH : Tribhuvan University Teaching Hospital

UPGMA : Unweighted Pair Group Method with Arithmetic mean

USDA : United States Department of Agriculture

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen and frequent cause of healthcare-associated infections. Multi Drug Resistant (MDR) strains of *P. aeruginosa* have been increasingly reported worldwide. Bacteriophages are often considered potential therapeutic candidates in treating infectious diseases. The present study aimed to isolate phages from holy rivers of Kathmandu valley against MDR *P.aeruginosa* and characterize them. Bacteriophages were isolated by Double Layer Agar Assay (DLAA). The 3 isolated bacteriophages were able to lyse *Pseudomonas aeruginosa.* However, among 3 isolates, phage CDBT-PA31 demonstrated lytic activity against *Pseudomonas aeruginosa_31 and Pseudomonas aeruginosa*_11. Three isolated phages were purified and checked for their Multi host range (MHR) spectrum. The most potent phage CDBTPA-31 was selected for further characterization after spot test on selected bacteria panel. phage CDBT-PA31 was stable at pH 3-11 and extreme temperature 50^{o}C. In one-step growth assay, phage CDBT-PA31 showed latent period of 30 minutes, with corresponding burst sizes of 423-525 PFU/cell. The genome of the bacteriophage was extracted by phage DNA isolation kit. Phage proteins were analysed on the basis of protein profiling through Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Four bands of phage CDBT-PA31 and 3 bands of phage CDBT-PA11 was clearly distinguished in the gradient gel ranging from approximately 3.5-29 KDa. Efficacy of phage therapy was performed on mouse model. Superficial wound infection on its back healed within 5 days of phage treatment. Klebsiella virus TU_Kle10O showed 97% homology with *Klebsiella* phage KPV 15, 97% homology with *Klebsiella* phage PKO 111, 76% homology with *Escherichia* phage ECML-134 and 76% homology with *Enteriobacteria* phage RB59. Similarly Salmonella virus TU_SP24B showed 91% homology with *Salmonella* phage SE2, 87% homology with Salmonella phage f2SE, 87% homology with *Salmonella* phage fSE1C and 81% homology with *Escherichia* phage G AB-2017. Escherichia virus TU_EC18O shows 98% homology with *Escherichia* phage vB EcoMJSO9 96% homology with *Escherichia* phage Av-o5, 80% homology with *Escherichia* phage YUEELO1 and 79% homology with Shigella phage SHFML-26 RB59. MDR bacteria are emerging day by day and it is global problem, phages are the alternative sources of antibiotics.

Keywords

Antibiotics, DLAA, SDS-PAGE, Phage therapy, Deoxy Ribonucleic Acid, homology,

Chapter One
INTRODUCTION

1.1 Background

Viruses are microorganisms which are widespread throughout the world, and infecting almost all organisms showing no mercy even to the bacteria. Phages are the special group of viruses which includes the bacteriophages and virophages. Bacteriophages are bacterial viruses that infect and multiply within the bacterial hosts with high specificity. Since their discovery in 1915, they are interesting and researchers are working on their therapeutic potential against various drug resistant bacteria (Dhama et al., 2013; Tiwari et al., 2014). The estimated global phage population size is extremely high. It is estimated that aquatic habitats have total phage numbers above 10^{31} terrestrial ecosystems have revealed 10^{7} viruses per gram of soil and sewage present total phage numbers in the range of 10^{8}-10^{10} per milliliter (Sharp, 2001). They are considered to be the predominant life form in biosphere.

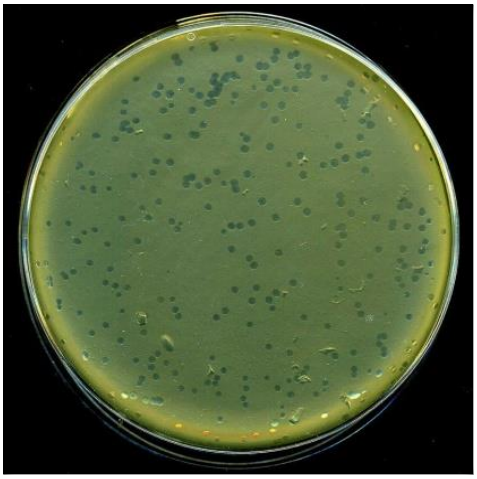

FIG 1.1: A culture of bacteria infected by bacteriophages, the "plaques" are areas where the bacteria have been killed by the virus.

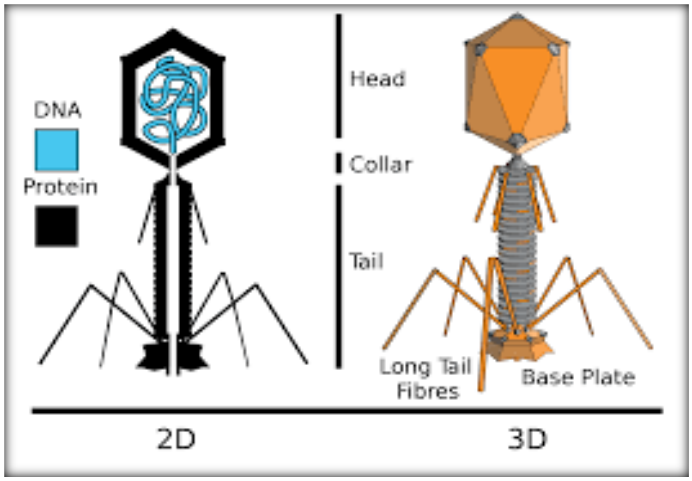

FIG 1.2: 2D and 3D structure of bacteriophage.

Source: en.wikipedia.org

Structurally, they contain a core nucleic acid encapsulated with a protein or lipoprotein capsid which is connected with a tail that interacts with various bacterial surface receptors by the tip of the tail fibers. This interaction shows an affinity that is specific to certain group

of bacteria or even to a particular strain. Phages are extremely diversified group. They are known to be most abundant and self-replicating organisms on earth and they are ten times more prevalent than their bacterial host (Silankorva, 2009). Most of the phages are tailed bacteriophage (Order: Caudovirales), accounting for 96% of all phages present on earth. According to International Committee on Taxonomy of Viruses (ICTV), they are classified into three families: the Myoviridae (long contractile tail), the Siphoviridae (long non-contractile tail) and the Podoviridae (short non-contractile tail) (Tan et al., 2014). Phages are named according to the bacteria from which they were isolated [e.g. phage ϕIBB-PF7 stands for Institute for Biotechnology and Bioengineering (IBB), the host bacterium *Pseudomonas fluorescens* (PF) and sample number 7] (Silankorva, 2009).

1.1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a gram-negative, aerobic rod belonging to the family Pseudomonadaceae. They are common inhabitants of soil and water. It tends to inhabit sites where moisture accumulates, including ventilator tubing. The organism has a characteristic grape-like odor and contains the pigment pyocyanin, which gives it a bluish-green color on culture media (Cavalieri and Stephen, 2005).

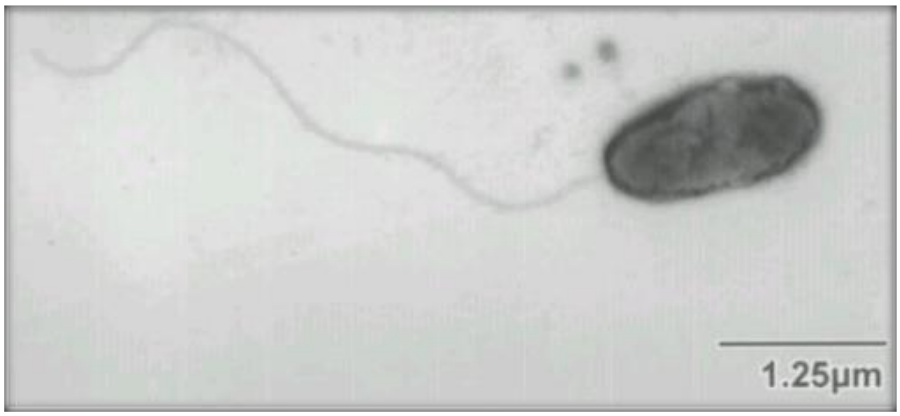

FIG 1.3: Electron micrograph *of Pseudomonas aeruginosa*. The bacterium measures 0.5µm -0.8µ by 1.5-3.0 µm. The single flagellum can be distinguished (Dasgupta et al., 2000).

P.aeruginosa can be further characterized on the basis of motility by means of a single polar flagellum. It can also characterized on the basis of non-spore forming, capsulated "polysaccharide capsule", aerobic, oxidase and catalase positive. Their optimum temperature for growth is 37°C, and it is able to grow at temperatures as high as 42°

Pseudomonas aeruginosa is opportunistic pathogen and associated with a variety of infections including:

- Urinary tract infections (UTI)
- Wound and burn with blue green pus
- Respiratory system infections
- Eye infection that may lead to blindness
- Ear infection (external ear or otitis media)
- A variety of systemic infections

P. aeruginosa is inherently resistant to narrow-spectrum penicillins, first- and second-generation cephalosporins, trimethoprim, and sulfonamides. The anti-pseudomonal agents include extended-spectrum penicillins, such as ticarcillin and piperacillin, extended-spectrum cephalosporins, such as ceftazidime and cefepime, carbapenems, aminoglycosides, and fluoroquinolones (Cavalieri and Stephen, 2005).

1.2 Current Studies

Bacteriophages were used in the treatment of human diseases almost instantly after their discovery due to their remarkable antibacterial potency (Tan et al., 2014). They appeared as the frontline therapeutics against infectious disease before the discovery of the broad spectrum antibiotics and were used in various countries until the Second World War. The therapeutic use of bacteriophage was dampened after the emergence of the age of antibiotic chemotherapy with introduction of sulfa drugs in 1930s and penicillin in 1940s (Ta et al., 2014). Bacteriophages had been extensively used in therapeutic application to prevent and treat bacterial infections in humans (Sulakvelidze and Kutter, 2005). They have raised interest in various agricultural settings to improve the safety of food and to control food borne diseases as well as to reduce the use of antibiotics in livestock. Phage therapy has shown success in treating infections in livestock, plants, aqua-cultured fish and human (Sulakvelidze and Kutter, 2005). Recently, bacteriophages have received much attention as tool for DNA vaccination and as cloning and expression vectors in the field of genetic engineering (Verheust et al., 2010). Besides, bacteriophages provide a more convenient and inexpensive alternative technique for environmental monitoring and epidemiological surveillance by using phage typing method (Faruque et al., 2003).

1.3 Rationale of the Study

The increased incidence of bacterial illness has caused substantial morbidity and mortality worldwide annually, often associated with disease outbreaks. Bacterial antimicrobial

resistance (AMR) has become a public health concern as the increased of antibiotic resistant bacteria poses increasing threat to human health. There is need for development of novel non-antibiotic approach to fight against the increased incidence of multi-drug resistant pathogen due to the shortage of new antibiotics in developmental pipeline. Recently, there has been an increased interest in the application of bacteriophage as an alternative antimicrobial chemotherapy in various fields including human infections, food safety, agriculture, and veterinary applications. Despite the increasing antibiotic resistant strains, people are demanding natural cure of diseases. Recently, it has been recognized that bacteriophages, the natural predators of bacteria can be used efficiently in modern biotechnology. Thus study on phage research need to be highly encouraged.

Our study mainly focused on isolation and characterisation of lytic bacteriophage against *Pseudomonas aeruginosa* that have potential to be used for therapeutic applications.

1.4 OBJECTIVES

1.4.1 General Objectives

- Proteomic and genomic characterization of bacteriophage against multidrug resistant (MDR) *Pseudomonas aeruginosa* and phylogenetic analysis of multi host range bacteriophages.

1.4.2 Specific Objectives

- Isolate lytic bacteriophage from holy rivers of Kathmandu valley against MDR bacteria.
- Perform double-layer-agar assay & spot assay to identify multi host range phage.
- Determine burst size from one step growth curve.
- Determine pH and thermal stability of isolated phages.
- Perform in vitro mediated bacterial lysis by lytic phage.
- DNA extraction and restriction digestion.
- Protein profiling through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).
- Perform mouse-model-therapeutic assay of characterized phage.
- Phylogenetic analysis of multi host range phages after whole genome sequencing.

1.5 RESEARCH HYPOTHESIS

- **Null Hypothesis**

  Bacteriophages do not cure significantly infect/ lyse diseases caused by MDR strains of bacteria

- **Alternative Hypothesis**

  Lytic bacteriophages are present in nature that infect / lyse disease infected with MDR bacteria.

Chapter Two
LITERATURE REVIEW

2.1 Bacteriophage Discovery

Bacteriophage was first predicted by a British Bacteriologist; Ernest Hankin in 1896 (Abedon et al., 2011). He reported the presence of bactericidal activity against *Vibrio cholerae* in the water of the Ganges and Jumna rivers in India. He proposed that a heat labile unknown substance which passed through fine porcelain filters was responsible for preventing the spread of cholera epidemics. After two years, Gamaleya, the Russian bacteriologist observed similar phenomenon while working with *Bacillus subtilis* (www.biologydiscussion.com). Almost 20 years after Hankin's study, Frederick Twort, a medically trained bacteriologist from England reported similar cases and hypothesised that it was most probably caused by virus. However, Twort did not pursue his discovery due to various reasons such as financial difficulties (www.biologydiscussion.com). Two years later of Twort discovery, Felix d' Herelle, a French Canadian microbiologist at the Pasteur institute in Paris reported the same observation. Felix d' Herelle proposed that it was virus that parasitised bacteria based on the appearance of small, clear zones in the lawn of bacterial culture and the lysis in broth culture. He initially named it as taches, then taches (spot) vierges and later plaques. Felix d'Herelle officially named it as "bacteriophage" that formed from "bacteria" and "phagein" which means phages "eat" or "devour" bacteria (Sulakvelidze et al., 2001). He called phages as "exogenous agents of immunity" due to their function as therapeutic and prophylactic agents in eradicating various types of infectious disease (Sulakvelidze et al., 2001; Summers, 2005).

2.2 Bacteriophage

2.2.1 Classification

Bacteriophages are classified into 1 order, 13 families and 31 genera (Ackermann, 2005). The current ICTV report lists 2284 virus and viroid species distributed amongst 349 genera, 19 subfamilies, 87 families and 6 orders (King et al., 2012). The International Committee on the Taxonomy of Viruses (ICTV) classifies phages into 13 families according to the nature of phage nucleic acid and overall virion morphology traits. Most of the characterized phages (95%) are in the order of Caudovirales or tailed dsDNA phages (Guttman et. al., 2005).

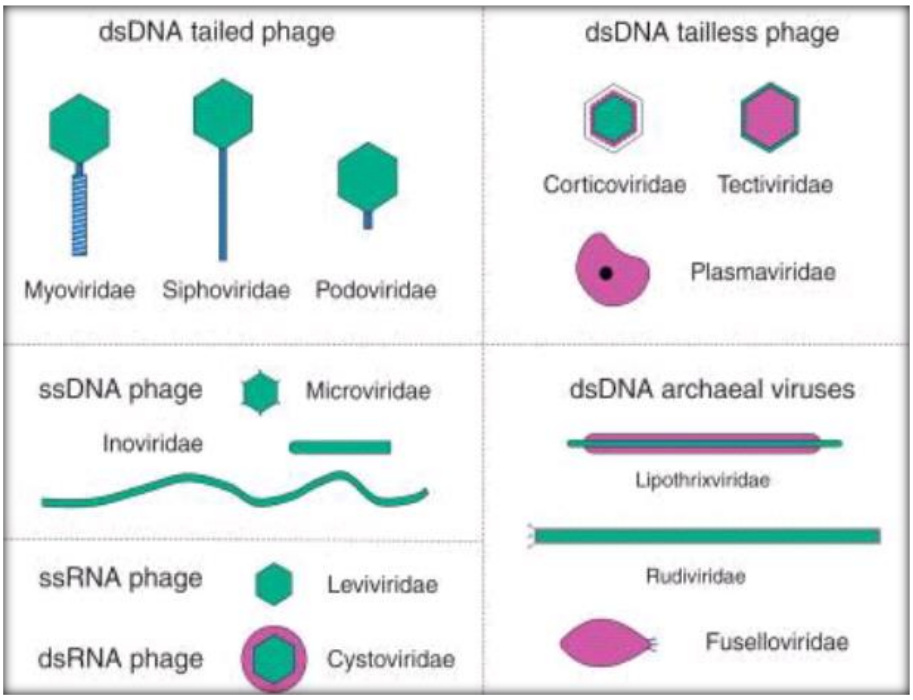

FIG 2.1: Basic morphologies of different families of prokaryote viruses (Hyman and Abedon 2009).

The ICTV classification is based mainly on morphological analysis, nucleic acid type and host organism. However, other properties, like physio-chemical, biological, protein amount and size, lipid content and characteristics, among others standards, are equally used for phage classification. The ICTV taxonomical system requires electron microscopy (EM) visualization however this doesn't allow the classification of the numerous prophage genomes that are found within the sequenced microbial genomes. Recently, a new strategy for phage classification has become popular and relies on sequencing of the phage genomes (Sillankorva, 2009).

2.3 Genome of Bacteriophage

The majority phages contain dsDNA, but there are also small proportion phage groups with ssDNA, ssRNA or dsRNA (Ackermann, 2005). The differentiation of bacteriophages into four genome size categories: very small (single stranded RNA phages), small (single-stranded DNA phages), medium (lipid-containing, double-stranded DNA, tailless phages) and large (double-stranded DNA, tailed phages) (Abedon, 2011). Very small numbers of phages belong to the members of family Leviridae with genome size that range between 3.5 to 4 kb. Small phages have genomes that are slightly larger than the members of family Leviridae, which range from 4.5 kb up to about 9 kb. Medium-sized phages have genomes

that range in size from approximately 9 to 15 kb. Last but not the least, the larger genome tailed phages, members of virus order Caudovirales, have genomes with 16 kb or greater. Phages with larger genome size have higher gene number, greater infectivity and virion sophistication, higher possibility of gene acquisition through horizontal gene transfer as well as additional genetic redundancy both within and between genomes (Abedon, 2011).

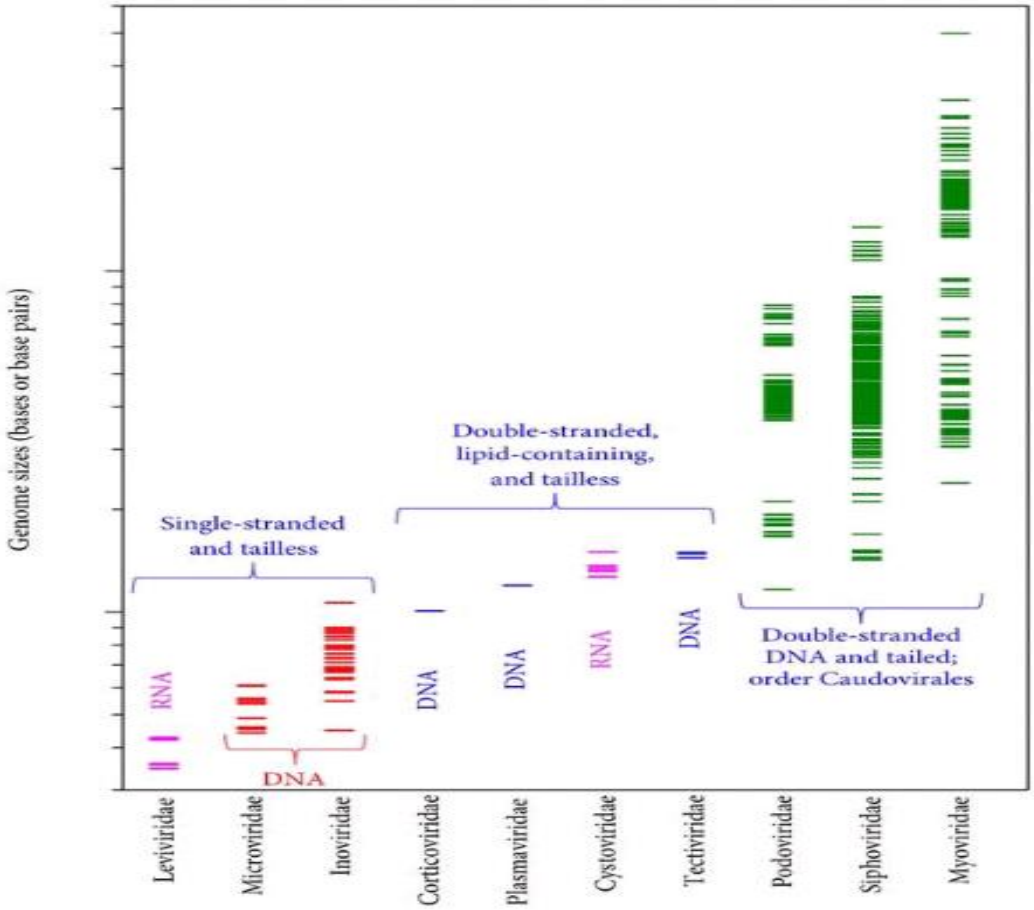

FIG 2.2: Phage morphologies and genome sizes (Hyman and Abedon, 2012).

2.4 Life Cycle of Bacteriophages

Phages carry their genetic information either in the form of DNA or RNA. Phage interaction with a host bacterium occurs via their tail and tips which recognize the necessary receptors (carbohydrate, protein and lipopolysaccharide molecules and flagella) present on the host's surface. Most phages are highly specific for their receptors and there is poor or no interaction with structurally variant receptors. This high specificity is the basis of phage typing methods, widely used for the identification of bacterial species or subspecies

(Silankorva, 2008). Depending upon phage lifecycle, they may be classified as **lytic** or **lysogenic**.

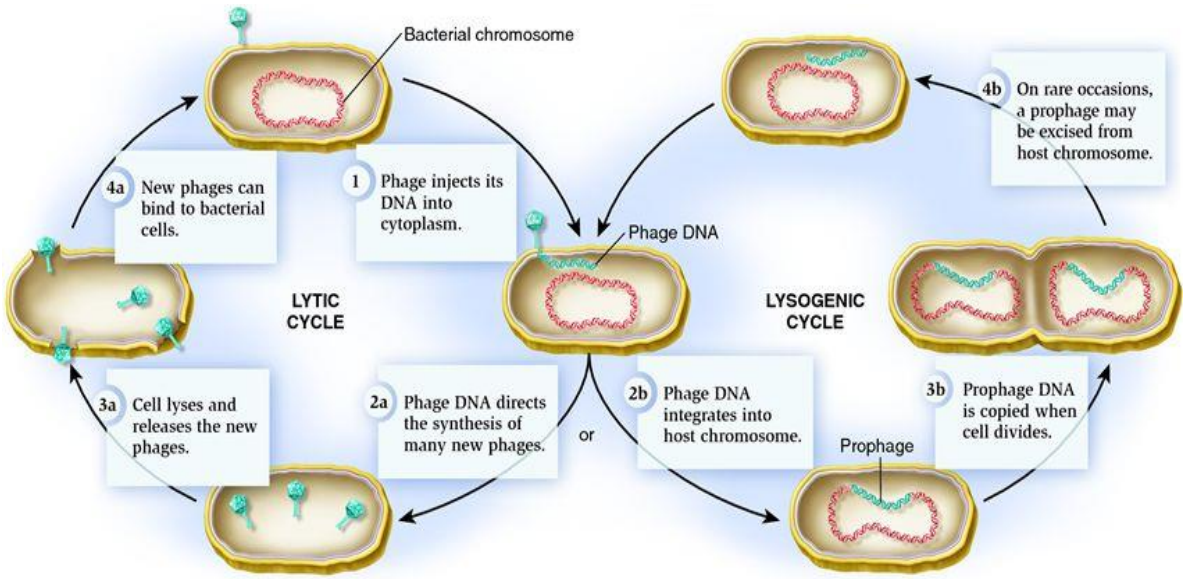

FIG 2.3: Lytic and lysogenic life pathways. 1: Phage injects its DNA into cytoplasm, **2a:** Phage DNA directs the synthesis of many new phages, **2b:** Phage DNA integrates into host chromosome (socratic.org) **3a:** Cell lysis and releases the new phages, **3b:** Prophage DNA is copies when cell divides, **4a:** New phages can bind to bacterial cells, **4b:** Prophage may be excised from host chromosome (socratic.org)

2.5 Advantages of Bacteriophages over Antibiotics

The increase in bacterial antimicrobial resistance has become a burning problem in 21st century as the increase of antibiotic resistant bacteria poses life threatening diseases to human health. Antibiotics are getting failure to treat bacterial infection. They are seeking for natural products of medicine. Thus, there is need for development of novel non-antibiotic approach to fight against the increased incidence of multi-drug resistant pathogen. Therefore phage therapy can be the alternative to treat bacterial infections in various fields including human infections, food safety, agriculture and veterinary applications.

Table 2.1: Advantages of Bacteriophages over antibiotics; phages as an alternative to antibiotics

Bacteriophages	Antibiotics
Replicate at site of infection and thus available where they are most needed	Metabolized and eliminated from the body and do not need necessarily concentrate at site of infection
No serious side effects reported yet.	Multiple side effects, including intestinal disorders, allergies and secondary infections
Phage resistant bacteria remain susceptible to other phage having similar target range	Resistant to antibiotics is not limited to target bacteria
Selecting new phage is relatively rapid process	Developing a new antibiotic is time consuming process and may take several years.

2.6 Application

The emergence of multi drug resistant (MDR) bacteria is a problem in modern medicine. To ease problem of antibiotic resistant bacteria, bacteriophage therapy can be used as efficient and effective tool. The concern of humankind is reentering the "pre antibiotics" era has become very real. Thus phages need to be revitalized. Bacteriophages are considered as novel therapeutic agent and it can be used against MDR bacteria in different way in different sector.

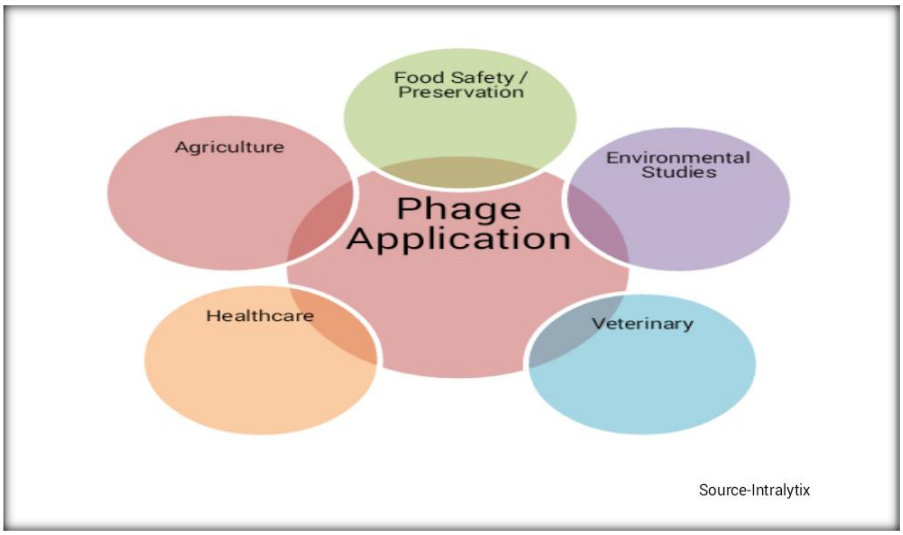

FIG 2.4: Venn diagrammatic representation of application of bacteriophage in different sector. Phages can be used against MDR bacteria in health care, agriculture, veterinary, environmental studies and food safety/preservation.

2.7 Bacteriophages for Biocontrol of Pathogens in Food

Bacteriophage-based bio control have great potential in food industry based on their long history of safe use, relatively easy handling and their high and specific antimicrobial activity. The concept of combating pathogens in food by means of phages can be applied at all stages of production in the classic "farm to fork" approach throughout the entire food chain (Garcia et al., 2008). Bacteriophages are suitable to prevent or reduce colonization and diseases in livestock, to de-contaminate carcasses and other raw products, such as fresh fruit and vegetables, and to disinfect equipment and contact surfaces (phage bio-sanitation and biocontrol) and to extend the shelf life of perishable manufactured foods as natural preservatives (Garcia et al., 2008).

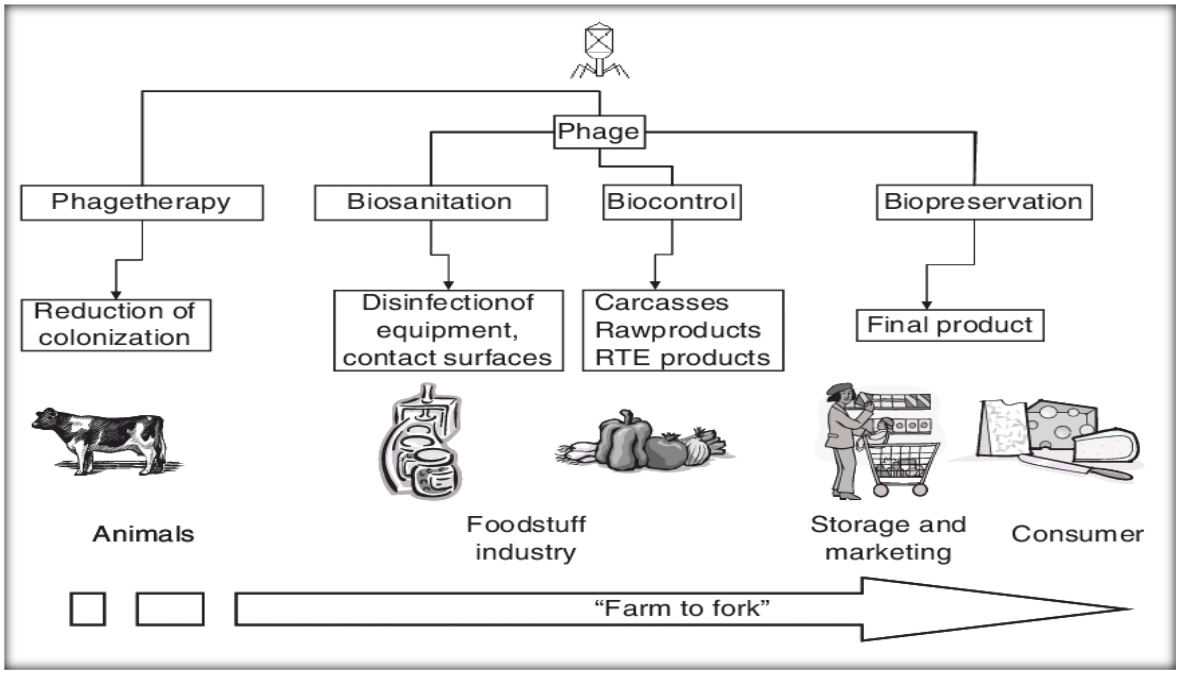

FIG 2.5: Examples of phage application along the food chain. Phages can be applied at all stages of production in the classic 'farm to fork' approach throughout the entire food chain (Garcia et al., 2008)

2.8 Role in Health Care and Medicine

The United States Department of Defense sponsored carefully designed phage therapy experiments for animal models of infectious diseases. Despite the encouraging results obtained in these experiments this approach was almost derelict in the United States following the discovery and development of broad-spectrum antibiotics (Merril et al. 2003, 2006). However, with the advent of antibiotic-resistant pathogenic bacterial strains,

research on the potential of phage therapy has been rejuvenated. In addition, with the expanded knowledge of phage molecular biology and interactions with mammalian immune systems, it is possible to genetically engineer phage that might be more efficacious than the wild types found in nature. This potential was shown in the development of long circulating phage strains that could stay in the circulatory system longer than the laboratory strains from which they were derived. There have been a number of successful demonstrations of the effectiveness of phage in animal models of bacterial infectious diseases (Dubos et al., 1943; Smith and Huggins, 1982; Merril et al. 1996; Biswas et al., 2002; Bull et al., 2002; Gu et al. 2012) including a recent success in the treatment of rats using an animal model system for sepsis and meningitis caused by S242, a fatal neonatal meningitis *E. coli* strain (Pouillot et al., 2012).

2.8.1 Clinical Studies

The evaluation of phage therapy for the treatment of *Escherichia coli* and *Pseudomonas aeruginosa* wound infections was performed in burned patients (PHAGOBURN). The objective of PHAGOBURN is to assess tolerance and efficacy of local bacteriophage treatment of *E. coli* or *P. aeruginosa* wound infections in burned patients. Phase I/II clinical trial randomized, multicentric, open label, standard of care (silver sulfadiazine) controlled aiming at assessing tolerance and efficacy of bacteriophage treatment on wound pherecydes pharma. Anti-*Escherichia coli* and anti-*Pseudomonas aeruginosa* bacteriophages have been developed for treatment of infections caused by these organisms but efficiency of single native phage is reported to be poor. One of the mechanisms of ameliorating the deficiency of the treatment regimen could be use of mixture or cocktail of phages which could be further enhanced through genetic modifications of these phages. This Project is a European Research & Development (R&D) Project Funded by the European Commission Under the 7th Framework Programme for Research and Development Involving 7 Clinical Sites in EU (clinical trial.gov)

Chronic otitis, known less formerly as swimmer's ear, is a *P. aeruginosa* ear infection that in many cases can resist antibiotic treatment. The company, Bio control (recently acquired by targeted genetics of Seattle to form a new joint company, AmpliPhi Biosciences), has been developing anti-*P. aeruginosa* phages targeting this condition (Marza et al., 2006 and Hawkins et al., 2010). In 2009, they published the results of their double-blinded phase 1/2a (safety and small-number efficacy) trial in human patients also suffering from the condition (Wright et al., 2009).

The oral phage therapy was used in carrier patient (nurse) for targeting methicillin resistant *Staphylococcus auerus* (MRSA). This individual was MRSA colonized in her gastrointestinal tract and also had a urinary tract infection. The result of phage therapy was complete elimination of culturable MRSA (Leszczynski et al., 2006)

Ninety two percentage of positive cases for phage treatment of 550 single- and mixed-etiology infections was selected infecting *Staphylococcus aureus*. Efficacy of phage treatment of suppurative staphylococcal infections, was reported with 93% "effective" rate "based on case history and data contained in a special questionnaire (Slopek et al., 1983)

One well-controlled anti-dysentery trial was conducted in Tbilisi on 30,769 children. Neighborhoods were split up with one side of each street treated prophylactically with a phage cocktail and the other a placebo. The result was a 3.8-fold decrease in dysentery incidence with phage treatment. Similar follow up trials were undertaken with 20,000 and 5,000 children respectively as well against *Salmonella*-associated disease (Babalova et al., 1968).

Relatively, early in the development of phage therapy its power was directed towards the treatment of infections by *Vibrio cholerae* as well as treatment of wells in India with anti-cholerae phages, both with some success, as reviewed very extensively by Summers (d'Herelle, 1930 and Summers, 1999).

2.9 Environment Sanitation

Phage use to prevent *Staphylococcus* infections has been both proposed and employed. Use of phages for disinfection has been carried out in Georgia to sanitize operating rooms and medical equipment and prevent nosocomial infections (Abedon et al., 2011). A complementary approach proposed by the company Novoltyics is to use a gel containing a cocktail of phages; to treat nasal carriage of MRSA. The result showed significantly reduced the infection rate (www.novolytics.co.uk/technology.html) and removal of *S. aureus* infection via experimental hand washing with a phage-containing ringer's solution. Approximately 100-fold reductions in bacterial densities were observed after washing with a solution containing 10^8 PFU/ml of phage particles (O'Flaherty et al., 2005). Phage treatment of inanimate hard surfaces contaminated by *E. coli* O157:H7 showed that the bacteria significantly decrease on using phage as a sanitizer (Abuladze et al., 2008).

2.10 Role in Agriculture and Horticulture

Plant pathogens are rarely dangerous for humans and they are one of the main causes of product loss. Plant pathogens such as *Ralstonia solanacearum* and *Pseudomonas syringae* cause bacterial wilt, foliar spots and blights and affect a variety of products (potato, tobacco, tomato, banana, peanut and soybean) (Sillankorva, 2008). Now, phage research is going on protecting plants, vegetables and fruits from decay and spread of bacterial diseases. The main products to which phages have been applied to are: geranium, tobacco, tomato, potato, mushroom, sprouts, peach and apple. Thus in agriculture and horticulture phage therapy can be potential way (Erskine, 1973). Two of these phage products, LISTEX^{TM} 100PM and LMP-2, target *Listeria monocytogenes* present in cheese, meat and fish products and both have been approved by the U.S. Food and Drug Administration (FDA). Additionally, LISTEX^{TM} 100PM has also received the Generally Recognised as Safe (GRAS) status by the FDA and by the United States Department of Agriculture (USDA), to be incorporated in food production processes. Another phage product named BacWash^{TM} has been issued by the USDA, and it targets *Salmonella* and *E. coli* O157:H7. BacWash^{TM} phage was especially created to be used on animals prior to slaughter and it can be applied as a wash, mist, or sprayed directly to the live animal. The Environmental Protection Agency (EPA) approved, in 2005, the product AgriPhage^{TM} for control purposes of *Xanthomonas campestris* pv. vesicatoria and *Pseudomonas syringae* pv. in tomato, and pepper plants among others (Sillankorva, 2008).

2.11 Phage to Control Bio-film

Phages are capable of killing early stage bio films (or adhered cells) (Sillankorva, 2008). McKenna et al in 2001 reported that single cells adhered to glass surfaces of a parallel plate flow chamber during 60 minutes and under laminar flow, were efficiently killed with phage S1. Cell removal was fast (20 minutes) and efficient leading to a biomass reduction of approximately 90%. The surfaces exposed to phages where impossible to be re-colonized by the bacteria (McKenna et al., 2001). Another strategy studied and proven to reduce bio film formation by *Staphylococcus epidermidis* is the pre-treatment of catheter surfaces with phages (Curtin et al., 2006).

The proximity of host cells in bio film communities can be an advantage in bio film treatment using phages, as the released phages stay concentrated in close proximity and therefore can start infecting a neighboring cell much faster than in planktonic cultures where cells are not as accessible (Wiggins et al., 1985).

2.12 Phages as Probiotics

Phage-based probiotics can be used prophylactically rather than therapeutically, if administered early in bacterial infections. Phage-based probiotics aid the Gastro Intestinal-tract balance by targeting specific pathogenic bacteria. They are likely to be most successful for managing pathogens such as *Salmonella* spp., *Clostridium difficile*, diarrheagenic *E. coli* and other bacteria that have an oral portal of entry and require short- or long-term colonization of the GI tract in order to cause disease (Mai et al., 2010). One recently-published paper suggests that the approach has merit, while additional research is currently underway (Sulakvelidze, 2004). Because of their high specificity, phages are likely to be unique tools for manipulating the microflora composition of the GI tract in a much more specific way than has been possible with other probiotics organisms or other antibacterial agents (Abedon et al., 2011). Hence, a phage-based approach is likely to open new and exciting avenues for subsequent research in many areas related to basic and applied probiotics research, the GI tract's microflora and nutrition (Mai et al., 2010).

2.13 Phage Display

The development of phage display enables the synthesis of novel polypeptides with high binding affinity to a particular substrate. In this system, the DNA that encodes the polypeptide is cloned as gene fusions with the phage coat protein genes. The desired protein is then incorporated into the mature phage particle and expressed on the surface of the phage (Rees and Loessner, 2005). Libraries of phage particles can also be used for the screening and identification of clones expressing peptides that are highly specific and possess desired binding characteristics (Rees and Loessner 2005; Haq 2012).

This approach has been extensively used for medical or pharmaceutical applications. Phage display has led to the revolution of phage antibody technology where phages were used to display antibody molecule with specific antigen binding domain that can be used for affinity selection process (Rader and Barbas, 1997). This antibody-derived peptide has been successfully developed as therapeutic agent that serves the function of agonist and antagonist in receptor-ligand interaction. The effectiveness of this method for the treatment of cocaine addiction is by bacteriophage displaying cocaine-sequestering antibodies to block the action of cocaine in the brain (Dickerson et al., 2005).

Furthermore, phage display can be applied for detection assay to detect for the biological threat agents. This assay utilizes phage-borne peptide as diagnostic detector or probe that specifically binds to bacterium, spore, virus and toxin. This technique has proved to be successful in the identification of various types of viruses (Petrenko and Vodyanoy, 2003),

detection of *Bacillus* spores (Zhou et al., 2002) and differentiation of *Candida* species in clinical samples (Bliss et al., 2003).

2.14 Phage Typing

Phage typing is a procedure for characterizing and identifying bacterial strains by their reaction (susceptibility or resistance) to various known strains of phages. It is a relatively rapid, simple and inexpensive method for the typing of bacteria (Hagens and Loessner, 2007). The host specificity of phages is a useful tool for the classification of bacteria and the detection of pathogenic bacteria (Clark and March, 2006). Many phages are highly specific for the receptors on host cell surface and only receptors with similar structure and configuration can interact with the respective phage. Phage typing is easy method and convenient to be performed. It can analyses large number of bacterial isolates can be at the same time. For example, *Escherichia coli* O157:H7 has been successfully subdivided into 66 different phage types (Frost et al., 1993). Phages are not only specific towards species of bacteria, but also strains of bacteria, permitting typing beyond the level of species (Welkos et al., 1974). Phage typing method appears to be more sensitive than colicin (bacteriocin produced by and toxic to some strains of Escherichia coli) typing and antibiograms in differentiating bacterial strains (Pruneda and Farmer, 1977). Susceptibility to infection by a particular phage enables the phenotypic differentiation of strains and identification of the strain that causes an outbreak of disease. This property can also be employed for epidemiological investigations to trace the causative agent responsible for the infection. Isolated phage SF-9 has epidemiological applications in tracing and monitoring the presence of *Shigella dysenteriae* type 1 from environmental waters in Bangladesh (Faruque et al., 2003). This method has proved to be useful in predicting outbreaks and the spread of shigellosis, which occurs as epidemics in many developing countries. Besides, this system provides reliable, sensitive and fast results for epidemiologists in the surveillance of outbreaks (Pruneda and Farmer, 1977).

2.15 Phylogenetic Analysis of Tree after Whole Genome Sequencing Using MEGA 7

Molecular Evolutionary Genetics Analysis (MEGA) computer software was used for conducting statistical analysis, molecular analysis of molecular evolution and for construction of phylogenetic trees. It includes many sophisticated methods and tools for

phylogenomics and phylomedicine. It is licensed as proprietary freeware. The project for developing this software was initiated by the leadership of Masatoshi Nei in his laboratory at the Pennsylvania State University in collaboration with his graduate student Sudhir Kumar and postdoctoral fellow Koichiro Tamura (Kumar et al., 1994). MEGA is intended to be easy-to-use and include solid statistical methods only.

A phylogenetic tree is a tree diagram showing the evolutionary relationships among various biological species or other entities. Their phylogeny is based upon similarities and differences in their physical or genetic characteristics. The taxa joined together in the tree are implied to have descended from a common ancestor. Phylogenetic trees (evolutionary tree) are central to the field of phylogenetics. Phylogenetic analysis of whole genome of phage was performed by bioinformatics tool Molecular Evolutionary Genetic Analysis (MEGA) 7 software.

2.16 Transmission Electron Microscopy (TEM)

Transmission electron micrograph of selected phages revealed that phages belonged to order Caudovirales – meaning tailed phages (Nepal and Malla, 2016). The phages were classified according to the ICTV guidelines. The literatures report 'tailed virus' to be abundant in nature, but this does not rule out the presence and abundance of other 'tail-less' phages in the nature. All these three phages belonged to Caudovirales order. Klebsiella virus TU_Kle10O belonged to Podoviridae, Escherichia virus TU_EC18O belonged to Myoviridae and Salmonella virus TU_SP24B belonged to Siphoviridae.

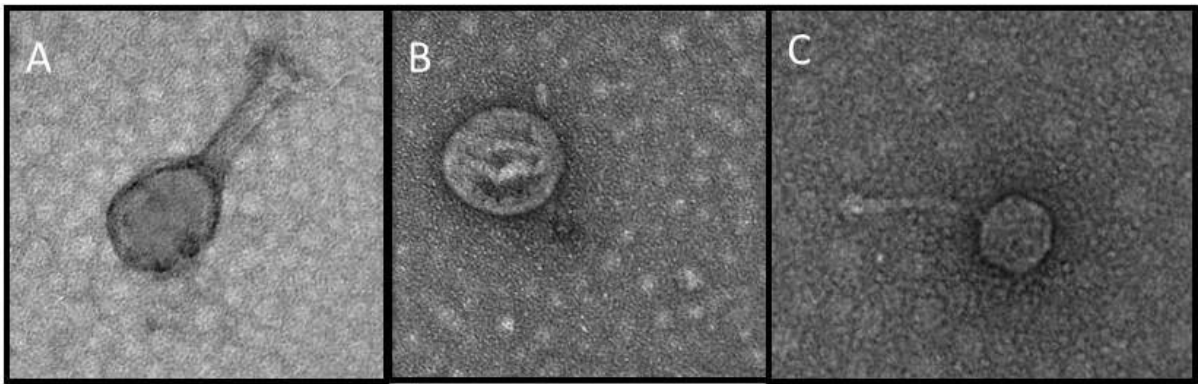

FIG 2.6: Transmission Electron Microscopy of phages (negatively stained) (Nepal and Malla, 2016. **A:** Escherichia virus TU_EC18O, **B:** Klebsiella virus TU_Kle10O, **C:** Salmonella virus TU_SP24B. Escherichia virus TU_EC18O is elongated in shape. Capsid is about 82×108 nm, length of tail is 19 nm and width is 111 nm. Klebsiella virus TU_Kle10O is icosahedra in shape, capsid is about 99 nm, length of tail is 18 nm and width is 34 nm. Salmonella virus TU_SP24B is icosahedral, capsid is 63nm, length of tail is 9nm and width is 106 nm.

The tail length can be considered as primary while phage classification, however '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 nm) which was elongated in shape with 111 nm tail attached to it and belonged to myoviridae family (Nepal and Malla, 2016). phage TU_Kle10O (capsid=99nm) was icosahedral in shape with tail 18nm long and 34nm width. phage TU_SP24B (capsid=63nm) was icosahedral with tail 9 nm long and 106 nm width. phage TU_EC18O belonged to myoviridae, phage TU_Kle10O belonged to podoviridae, phage TU_SP24B belonged to Siphoviridae order. The order belongs to caudovirales and they are considered as dsDNA bacterial virus.

None of the phages had any envelope outside their capsid and according to ICTV guideline, these phages have a 'single linear genomic configuration' enclosed in a capsid/head bearing no outer envelope as all of these belonged to tailed virus caudovirales.

2.17 BALB/CJ Mouse as an Experimental Model

BALB/CJ mice are frequently used for a variety of immunological studies because they demonstrate TH2-biased immune responses. BALB/CJ mice are particularly well known for the production of plasmacytoma on injection with mineral oil, forming the basis for the production of monoclonal antibodies. Mammary tumor incidence is normally low, but infection with mammary tumor virus by fostering to MMTV+ C3H mice dramatically increases tumor number and age of onset. BALB/c mice develop other cancers later in life, including reticular neoplasm, primary lung tumors, and renal tumors. Rare spontaneous myoepitheliomas arising from myoepithelial cells of various exocrine glands have been observed in BALB/CJ mice. JAX® Ready Strain™ BALB/CJ Strain Common Name: BALB C Type: Inbred (https://www.research.uky.edu)

Characteristics:

- Commonly develops ulcerative blepharitis and periorbital abscess
- Exhibits incomplete penetrance of callosal agenesis
- Exhibits spontaneous dystrophic cardiac calcinosis
- Susceptible to pristane induced arthritis
- Exhibits TH-2-lymphocyte driven pulmonary inflammation, a model for asthma
- Susceptible to TMEV-induced demyelinating disease
- Relatively resistant to diet-induced atherosclerosis
- Male mice are resistant to multi-dose streptozotocin (STZ)-induced diabetes

- Resistant to the induction of experimental allergic encephalomyelitis (EAE)
- Useful in vaccine development and studies of infectious disease (https://www.research.uky.edu)

2.18 Pseudomonas aeruginosa

Pseudomonas aeruginosa is an opportunistic pathogen and a frequent cause of healthcare-associated infections. It is a major cause of infections in cystic fibrosis patients. Nowadays it is commonly isolated from cases of nosocomial infections especially from immune compromised hosts, such as patients suffering from respiratory diseases, cancer, children and young adults with cystic fibrosis and burns. According to the National Nosocomial Infections Surveillance System, *P. aeruginosa* is the third most common pathogen associated with all hospital acquired infections, accounting for 10.1% of all nosocomial infections and is associated with a high mortality rate (Moreau-Marquis et al., 2008)

In England, there was an overall 3.9% reduction in the rates of *Pseudomonas* bacteraemia between 2008 and 2015 (from 7.2 to 6.9 reports per 100,000 population); however, there was a year-on-year increase in rates between 2013 and 2015. Comparing 2011 and 2015, the highest increases in rates of *Pseudomonas* bacteraemia observed among Public Health England's (PHE) centers were in South Midlands and Hertfordshire (34.7%; from 4.7 to 6.4 reports per 100,000 population) and Avon Gloucestershire and Wiltshire (27.8%; from 5.0 to 6.4 reports per 100,000 population) In England, Wales, and Northern Ireland between 2011 and 2015, the most frequently identified *Pseudomonas* species causing bacteraemia was *Pseudomonas aeruginosa* (~80% in all years). Similarly, the percentages of the other named *Pseudomonas* species identified within the same period remained relatively stable. In 2015, the highest rates of *Pseudomonas* bacteraemia in England was observed among the elderly (>74 years) at 54.7 and 21.9 reports per 100,000 population for males and females, respectively.

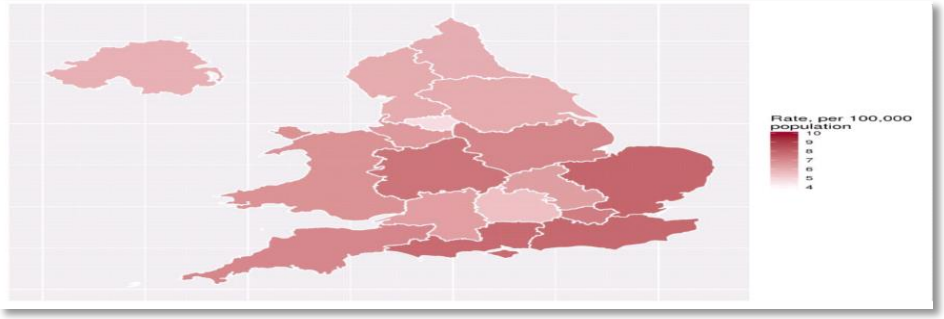

FIG 2.6: Geographical distribution of *Pseudomonas* bacteraemia rates per 100,000 population in England, Wales and Northern Ireland from 2011 A.D to 2015 A.D (Health

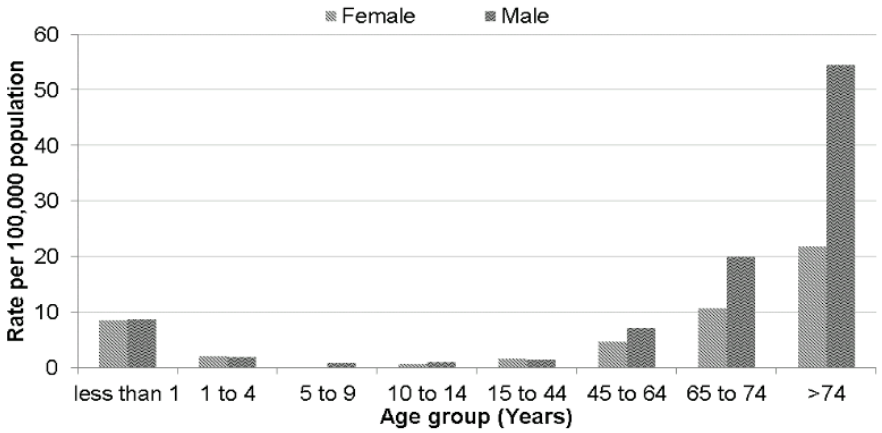

FIG 2.7: *Pseudomonas* bacteraemia rates per 100,000 population by age and sex at England) from 2011 to 2015 (Health protection report, Public Health England, 2016)

The percentage of *Pseudomonas* isolates non-susceptible to the antimicrobials viz gentamicin, ciprofloxacin, ceftazidime, meropenem, imipenem, tobramycin, amikacin, piperacillin/tazobactam reduced between 2011 and 2015 except for piperacillin/tazobactam, which increased from 6.8% to 10.1%. The highest reduction in non-susceptibility was observed for tobramycin, with a decrease from 5.7% to 3.4% within the same period i.e 2011-2015 (Health Protection Report.; Public Health England, 2016).

In 2015, the highest percentages of non-susceptibility to the antimicrobial agents were observed for imipenem (13.3%), piperacillin/tazobactam (10.1%), ciprofloxacin (9.4%) and meropenem (7.8%). These results are not surprising as *P. aeruginosa*, which accounts for the bulk of reported *Pseudomonas* bacteraemia, is often resistant to multiple antibiotics through intrinsic and adaptive mechanisms to these agents (Elena et al., 2011). The relatively higher percentages of non-susceptibility to imipenem, meropenem and ciprofloxacin observed in this report is likely due to the pathogen's ability to develop antibiotic resistance through three main mechanisms - alteration in DNA gyrase by mutations in gyrA or gyrB genes, decreased drug accumulation by decreased permeability of the cell wall, and enhanced efflux (Cambau et al., 1995). *P. aeruginosa* cells possess low outer membrane permeability caused by reduced expression of OprD which will limit the penetration of imipenem into its cell.

Table 2.2: Distribution of *P. aeruginosa* various age groups (Shrestha et al., 2015)

Age	Number	Percentage
Upto 1 yr	2	1.9
>1-20	12	11.8
>20-40	22	21.6
>40-60	30	29.4
>60	36	35.3

In Nepal, bacteremia *P. aeruginosa* was also studied. Shrestha et al in 2015 found that the occurrence of the isolates to be higher in the age group of patients who were more than 60 years of age (35.3%) (Shrestha et al., 2015).

On the other hand, non-susceptibility to meropenem is considered to be as a result of over expression of multi-drug efflux pumps (MexAB–OprM and MexCD–OprJ) which leads to the antibiotic's expulsion from its cells. While the mechanism of the pathogen's resistance to ciprofloxacin involves mutations in gyrA or gyrB genes (gyrase), as well as parC and parE (topoisomerase IV) which reduces fluoroquinolones binding affinity (Elena et al., 2011 and Cambau et al., 1995).

Beta-lactam resistance in *P. aeruginosa* is due to a combination of:

- Beta-lactamases
- Efflux systems
- Changes in outer membrane proteins (permeability barriers)
- Changes in penicillin-binding proteins

P. aeruginosa can become resistant to gentamicin, tobramycin, and amikacin in several ways:

- Low-level aminoglycosides resistance is due to lack of permeability of the outer membrane to these drugs.
- High-level aminoglycosides resistance is due to amino glycoside-modifying enzymes.
- Some isolates are resistant as a result of both impermeability and amino-glycoside-modifying enzymes (Cavaleiri, 2005).

Resistance to only amikacin (but not gentamicin and tobramycin) is highly un-usual. Ciprofloxacin remains the most active of the fluoroquinolones against *P. aeruginosa*. Resistance to fluoroquinolones is due to impermeability, efflux or mutations affecting the DNA gyrase and topoisomerase IV enzymes (Cavaleiri, 2005).

The bacterium's virulence depends on a large number of cell-associated and extracellular factors. The virulence factors play an important pathological role in the colonization, the survival of the bacteria and the invasion of tissues. There are two types of virulence factors:

- Factors involved in the acute infection: The pili allow adherence to the epithelium. The exoenzyme S and other adhesins reinforce the adherence to epithelial cells. The exotoxin A is responsible of tissue necrosis. Phospholipase C is a thermolabile haemolysin. The pathogenic role of exoenzyme S is attributable to the disruption of normal cytoskeletal organization, the destruction of immunoglobulin G and A, leads to depolymerization of actin filaments and contributes to the resistance to macrophages. *P. aeruginosa* produces at least four proteases causing bleeding and tissue necrosis (Cambau et al., 1995).
- Factors involved in the chronic infection: siderophores (pyoverdin and pyochelin), allow the bacteria to multiply in the absence of ferrous ions. The strains isolated from patients with cystic fibrosis have a pseudocapsule of alginate that protects the bacterium from phagocytosis, dehydration and antibiotics. Moreover, it improves adherence to epithelial cells forming a bio film. Two different types of regulation systems control the expression of the majority of these virulence factors:
    - The quorum sensing system
    - Transcriptional regulatory system

These two mechanisms are necessary to the survival and the proliferation of this microorganism in the host.

Recently, at United States of America a woman named called Nevada was killed by a superbug which was resistant to last resort of antibiotics. The most worrisome kind of colistin resistance is caused by single gene called *mcr-1*. The bacteria that killed this woman did not have *mcr-1***;** it's still unclear how they became resistant (www.theatlantic.com, 2017). *mcr-1* contains loop of free floating DNA called plasmid on which bacteria of different species can pass back and forth and there are many plasmids out there with genes that confer resistance to this or that class of antibiotics.

WHO in 2017 published its first ever list of antibiotic-resistant "priority pathogens" – a catalogue of 12 families of bacteria that pose the greatest threat to human health (www.who.int). The list highlights in particular the threat of gram-negative bacteria that are

resistant to multiple antibiotics. These bacteria have built-in abilities to find new ways to resist treatment and can pass along genetic material that allows other bacteria to become drug-resistant as well. The WHO list is divided into three categories according to the urgency of need for new antibiotics: critical, high and medium priority (www.who.int).

The most critical group of all includes multidrug resistant bacteria are *Acinetobacter*, *Pseudomonas* and various Enterobacteriaceae (including *Klebsiella*, *E. coli*, *Serratia*, and *Proteus*). They can cause severe and often deadly infections such as bloodstream infections and pneumonia.

These bacteria have become resistant to a large number of antibiotics, including carbapenems and third generation cephalosporins. The second and third tiers in the list – the high and medium priority categories – contain other increasingly drug-resistant bacteria that cause more common diseases such as gonorrhoea and food poisoning caused by *Salmonella*.

The list was developed in collaboration with the Division of Infectious Diseases at the University of Tübingen, Germany, using a multi-criteria decision analysis technique vetted by a group of international experts to promote research and development (R&D) of new antibiotics.

Chapter Three
MATERIALS AND METHODS

3.1 Preparation of Media and Solutions

The media used for the study were tryptic soya agar (TSA), tryptic soya broth (TSB), Luria Bertaini broth (LB). All these media were prepared as instruction given by their manufactures. Chemicals used during this research includes sodium chloride and magnesium sulfate buffer (SM buffer), phosphate buffer saline (PBS), agarose powder, Tris HCl, sodium hyrdroxide, ammonium per sulphate, glycerol, beta mercaptoethanol, bromophenol blue, acrylamide, bisacrylamide.

3.2 Bacterial Strains

Clinical strains of *Pseudomonas aeruginosa* were collected from Tribhuvan University Teaching hospital (TUTH), Maharajgunj. The bacteria were grown on nutrient agar by streaking and incubating at 37°C for 16-18 h. The plates were stored at 4°C. Exponential growing bacteria culture was used in all the tests in this study. This was done by inoculating single isolated colony of bacteria in Luria Bertaini (LB) broth and incubated at 37°C with constant agitation (200 rpm) until mid-log phase was reached. (1.0 OD corresponded to 2.04×10^8 CFU/ml with well-defined linear relationships of Y (CFU/ml) = 2.04×10^8 X (OD_{600}) (Kim et al., 2012

3.3 Antibiotic Susceptibility Test (AST)

Bacterial samples were collected from TU teaching hospital, Maharajgunj, Nepal. For identification of multidrug resistant (MDR) bacteria, antibiotic susceptibility test was performed by Kirby Bauer disc diffusion method. AST were already performed at hospital, however it was reconfirmed at CDBT lab for confirmation. Different antibiotics were used for AST. Beta lactams, carbapenems, macrolide, fluoroquinolones, aminoglycosides antibiotics were used (http://www.emedexpert.com/lists/antibiotics.shtml).

For AST, single isolate colony was mixed in normal saline, compared to 0.5 Mac Farland standard then lawn cultured into Mueller Hinton Agar (MHA). The plates were left for few minutes to air dry and then incubated at 37°C for 24 hrs. Next day, zone of inhibition were

measured using standard calibrated scale.. Bacterial strains were classified as sensitive, intermediate, resistant according to standard AST chart of Himedia.

Table 3.1: Antibiotics discs used for Antibiotic Susceptibility Test

S.N	Antibiotics	Code
1	Ofloxacin	0F 5
2	Amoxyclav	AMC 30
3	Bacitracin	B1 10
4	Teicoplanin	TEI 30
5	Amikacin	AK 30
6	Cefotaxime	CTX 30
7	Gentamycin	GEN 10
8	Meropenem	MRP 10
9	Penicillin –G	P 10
10	Piperacillin	PI 100
11	Nalidixic acid	NA 10
12	Piperacillin/Tozobactam	PI 100/10
13	Methicillin	MET 5
14	Vancomycin	VA 30
15	Cloxacillin	COX 10
16	Cephoxitin	CX 30
17	Ceftazidime	CA 30
18	Ampicillin	A/S 10
19	Ciprofloxacillin	CIP 5
20	Nitrofurantoin	NIT 300
21	Imipenem	IPM 10
22	Co-Trimoxazole	COT 25
23	Levofloxacin	LE 5

The antibiotics used for the study manufactured by Hi-media. The numbers indicates concentration of drug in µg.

3.4 Sample Collection

Sewage samples were collected from holy rivers of Kathmandu valley (Bagmati river of Balkhu and Kalanki River). Samples were collected in sterile 50 ml conical tubes. The sewage sample was mixed thoroughly and the sediments were collected together with the overlying water from collection site.

FIG 3.1: Sample collection sites: Bagmati river of Balkhu (sample A) and Bishnumati River of Kalanki (sample B)

3.5 Bacteriophage Manipulations

3.5.1 Bacteriophage Isolation

The sewage samples were centrifuged at 4000 rpm for 20 min to remove large particulates and bacteria. Supernatant was decanted into a new sterile falcon tube and passed through syringe filter (0.2 micrometer, Axiva). The filtrate was collected in new sterile falcon tube. Bacteria were cultured overnight in Luria Bertaini broth (LB). Then 2 drops of mid exponential phase culture was taken and then 1 ml of filtrate was mixed in sterile Eppendroff tubes and allowed to incubate at room temperature for 5-10 minutes. The mixture was poured into Tryptic Soya Broth (TSB) culture tubes containing soft agar (0.5%) mixed properly and then poured into TSA petri plates containing hard agar (1%). The process is called as Double Layer Agar Assay (DLAA). The plates were incubated at 37°C for 12-24 hours and inspected for plaques. Plaques were tested for containing only one single type of phage by repeated transfer of the lysates to new plates. Final lysates were stored at 4° C for further use (Sillankorva, 2008).

3.5.2 Preparation and Purification

When purifying a single strain from a heterogeneous stock, a single isolated plaque was picked. The plaque was removed from the plate using a sterile open-ended capillary tube or Pasteur pipette to pierce the agar (both top and bottom layers). The 'plug' was withdrawn by holding the index finger over the open end. One milliliter SM buffer and a drop of chloroform was poured into it; shaked briefly to suspend the phage and DLAA was performed for preparation and purification of phage. Ten milliliter of sodium chloride–magnesium sulfate (SM) buffer (5.8 g/L NaCl, 2 g/L $MgSO_4 \times 7\ H_2O$, 50 ml 1 M Tris, pH 7.5, 2% gelatin) was poured into petri dish containing plaques and 2 drops of chloroform was added into it; shaked at 80 rpm for 30 minutes. The phage suspension was centrifuged at

4000 rpm for 20 mins. Pellet was discarded and supernatant was passed through 0.2µm syringe filter. Phage supernatant was stored at 4°C for further use. Two – three cycles of purification should be carried out to get pure plaques (Sillankorva, 2008).

3.5.3 Amplification and Titration

Hundred microlitre of overnight culture of *Pseudomonas aeruginosa* was poured into LB media and incubated at 37°C for 3 hours. Hundred microlitre of phage suspension was taken from stock and serially diluted from 10^{-1} to 10^{-4} dilutions. Two drops of bacteria infected with 1 ml of 10^{-2}, 10^{-3}, and 10^{-4} phage suspension dilutions was allowed to incubate at room temperature for 10 to 30 minutes and mixed with 3 ml of soft agar and then poured into hard agar petri plates. The plates were allowed to cool until top agar solidified and incubated at 37°C overnight. The next day, many plaques were visible and the number of phages will reflect each plate's respective dilution. Plaques morphology, size, numbers and types were recorded. The phage gets amplified and their concentration was also determined. Three rounds of purification is believed to produce a pure lysates. It is of utmost importance that the final plaque purification contains only a single phage. Different factors such as incubation time, temperature, concentration of phage affects the amplification and purification of phage. Thus, the standard procedure should strictly be followed to ensure successful purification and amplification (Maniatis, 1982).

3.6 Intra Specific Host Range of Isolated Bacteriophage

Host range spectrum of phage was determined by spot test. The method was spotting 10 µL of phage preparation (10^{-8}, 10^{-9} and 10^{-10} PFU/ml) on lawn cultures of the bacterial strains and allowed to evaporate for 20-30 mins (Bao et al., 2015).

In negative control, 10 µl of phage buffer was spotted in an appropriate place and allowed to adsorb. The plates were incubated at 37°C for 16 – 24 hours. The next day, spots were checked for clearing. A positive spot test will appear as complete obliteration of the entire drop area, whereas a negative spot test will result in the bacterial lawn growing normally in the region of the spot (www.pitt.edu).

3.7 pH and Thermal Stability Test

The pH of the LB was adjusted with either 1 M HCl or 0.5 M NaOH to obtain a pH ranging from 1-14. A total of 100 µl of bacteriophage suspension (5×10^8 PFU/ml) was inoculated into 5 ml of pH-adjusted medium (Han et al., 2014). After incubation for 1 hour at 37°C, the surviving phage particles were counted immediately using the double-layer method. Thermal stability of phage at different temperatures (50°C, 60°C, 70°C, 80°C and 90°C) was determined by incubating the phage (10^7 PFU/ml) (Han et al., 2014)at the indicated temperature for 30 mins and 60 mins at pH 7.0 in nutrient broth; the surviving phages were then calculated .

3.8 In Vitro Mediated Phage Lysis

Overnight cultures of *Pseudomonas aeruginosa* with optical density 1.0 i.e (1.0 OD corresponded to 2.04×10^8 CFU/ml) in fresh medium was prepared (Kim et al., 2012). A single phage stock was added to give Multiplicity of Infection (MOI) of 10^2 and then mixtures were incubated at 37°C for 1, 2, 3, 4 and 5 hours respectively with gentle shaking. Phage-free culture (containing only bacteria) and bacteria-free culture (containing only phage) were also included as controls. Bacterial cell densities were determined at 0, 1, 2, 3, 4 and 5 hours by spectrophotometry at 600nm (Bao et al., 2015).

3.9 One Step Growth Assay

Ten milliliters of a mid-exponential-phase culture was harvested by centrifugation (7,000xg, 5 min, 4°C) and resuspended in 5 ml LB medium in order to obtain an OD_{600} of 1.0 i.e approximately (2.04×10^8 CFU/ml using spectrophotometer) (Kim et al., 2012). To this suspension, 5 ml of phage solution were added in order to have a MOI of 0.001 i.e (2.04×10^5 PFU/ml) and phages were allowed to adsorb for 5 min at room temperature. (The concentration of phage stock solution was 8.3×10^{12}). The mixture was then centrifuged as described above and the pellet was resuspended in 10 ml of fresh TSB medium. Samples were taken every 10 min interval over a period of 2 hours. The sample was plated immediately by DLAA method and incubated at 37°C for 16-24 hours. Next day plates were monitored for plaques formation (Sillankorva, 2009; Han et al., 2014).

3.10 Molecular characterization

3.10.1 DNA Extraction

DNA extraction of nucleic acid was conducted according to the Norgen's kit method (Phage DNA Isolation Kit, Norgen Biotek Corporation).

3.10.2 Agarose Gel Electrophoresis

The extracted phage genomic DNA was analysed using agarose gel electrophoresis. Five microlitre of the DNA was mixed with 1 µL loading dye and loaded into wells of a 1.0% agarose gel in 1X Tris-Borate-EDTA (TBE). The gel was subjected to electrophoresis at 50 V for 90 min until the dye front was near to the bottom of the gel. After electrophoresis, the gel was visualized using transilluminator to visualise the presence of DNA bands.

3.10.3 Restriction Digestion

EcoRI restriction enzyme was used to digest phage genomic DNA. The reaction mixture was prepared and mixed gently and allowed for incubation at 37°C for 2 hour. The restriction enzyme was then inactivated by incubating the reaction mixture in water bath. The digested DNA was separated by agarose gel electrophoresis process. The restriction patterns were visualised by transilluminator with UV light after staining with ethidium bromide.

Table 3.2 Restriction enzyme inactivation by heating (Thermo Scientific, 2012; BIORON, 2013).

S.N	Restriction Enzyme	Temperature (°C)	Time (min)
1	EcoRI	65	20

Table 3.3 Restriction enzyme digestion reaction for phage DNA.

S.N	Components	Volume
1	Phage DNA	10 µl
2	Restriction enzyme (EcoRI)	0.5 µl
3	Restriction enzyme buffer (10x)	2µl
4	BSA	1 µl
5	Nuclease free water	5 µl
	Total	20 µl

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

Protein profiling of purified phage was carried out by at CDBT laboratory by 2 methods.

- Acetone Precipitation
- Direct Heating with 2x sample buffer on boiling water bath for 10 minutes.

3.11.2 Acetone Precipitation

Five hundred microliter purified phage solution was precipitated with 4 volumes of ice-cold acetone for 90 minutes. Supernatant was decanted and pellet was air dried, resuspended in 100 µl PBS buffer (8 g/L NaCl, 0.2 g/L KCl, 0.2 g/KH_2PO_4 1.44 g/L $Na_2HPO_4 \times 2H_2O$, pH 7.5). SDS-PAGE was carried out according to Laemmli. Briefly, 25 µl of sample was added to 25 µl of 2 × Laemmli buffer and boiled for 10 mins. Samples were then loaded to 10 % PAGEr precast gels and electrophoresed with tris-glycine buffer. Five µl of protein marker with 1 µl of loading dye was also loaded after boiling for 5-10 mins. After electrophoresis the gels were stained with coomassie brilliant blue R-250 (CBB) for overnight and then bands were visualized after adding destaining solution (Sillankorva, 2008).

3.11.3 Direct Heating with 2x Sample Buffer on Boiling Water Bath for 10 minutes

For Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis (SDS-PAGE) separation, purified 25 µl *Pseudomonas* phages were mixed with equal volume of 2x sample buffer (0.125 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 2%-mercaptoethanol (ME), 0.02% bromophenol blue) heated in a boiling water bath for 3-5 minutes (Sen and Ghosh 2005). Samples were then loaded to 10 % PAGEr precast gels and electrophoresed with tris-glycine buffer. SDS-PAGE was carried out according to Laemmli. After electrophoresis the gels were stained with coomassie brilliant blue R-250 (CBB) for overnight and then bands were visualized after adding destaining solution (Kumari et al., 2009).

3.12 Mouse Model Experiment for Analysis of Phage Therapy

Pathogen free 7 weeks old, 12 BALB/CJ male mice were used for infection experiment. The experiment was performed in triplicates. Multi Drug Resistant (MDR) *P. aeruginosa* culture was grown in LB medium at 37°C. Log phase culture of MDR *P. aeruginosa* to optical density at 600 nm of 1 (approximately 2.04×10^8 CFU/ml), followed by centrifugation at 3000 rpm was used (Golkar et al., 2013). Pellet was washed and resuspended in PBS and was stored at 4°C. This suspension was used as infectious agent on by superficial scratches, mainly to epidermis layer and perhaps and dermis layer at some places. Animals were maintained in strict sterile condition, according to guidelines for the housing of rodent in scientific institutions. Bedding (single type), cage, cage shelter, bars, cage lid, water container were autoclaved and changed every day. Food was stored in a clean, dry, vermin-free, well-ventilated sterile area to reduce any possible contamination. Water delivery system was monitored during study to ensure proper function.

The efficacy of phage therapy was evaluated in four groups of mice, (positive control, negative control, treated with phage, and healthy) using MDR *P. aeruginosa* as pathogen. Two groups of mice were infected with *P.aeruginosa*_31 3×10^4 CFU/ml by superficial scratches. Whereas the negative control, received 3×10^4 PFU of phage without bacterial infection. Each animal in these groups were treated with a two injections of lytic phage CDBTPA-31 of the appropriate dose (9×10^8 PFU), administered intrapertonially 30 mins after bacterial infection. Twenty four hours after the first injection, the animals were administered a second injection of phage and then given a daily dose of phage orally (3×10^8). The positive control group was not treated with phage. Infected animals and controls were observed under sterile condition for one week and the status of the wounds were monitored and recorded by photography (Golkar et al., 2013).

3.13 Multi-host Range Phage Genomics

3.13 Analysis of Phylogenetic Tree after Whole Genome Sequencing Using MEGA 7

Molecular Evolutionary Genetics Analysis (MEGA) computer software was used for conducting statistical analysis, molecular analysis of molecular evolution and for construction of phylogenetic trees. For phylogenetic analysis, the accession numbers of closely related phages were retrieved from NCBI and then pate in the word with respective phage sequences. These sequences are paste in MEGA software in MEGA format and then

only phylogenetic tree was analysed. It includes many sophisticated methods and tools for phylogenomics and phylomedicine.

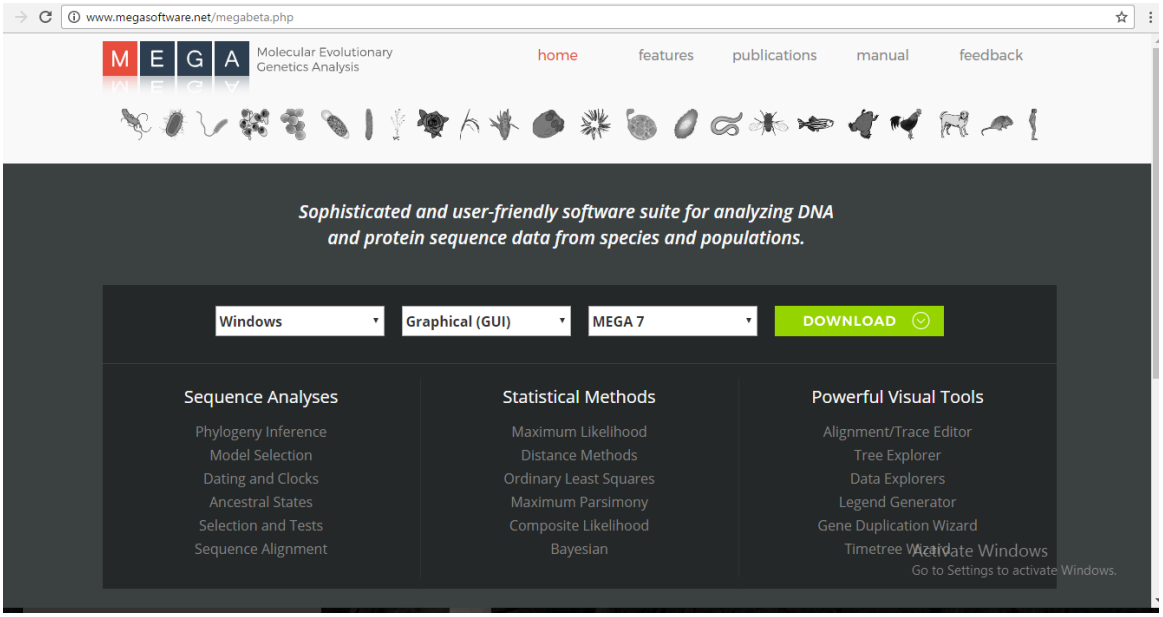

Fig 3.2: Molecular Evolutionary Genetics Analysis (MEGA). MEGA is an integrated tool for automatic and manual sequence alignment, inferring phylogenetic trees, mining web-based databases, estimating rates of molecular evolution, and testing evolutionary hypotheses.

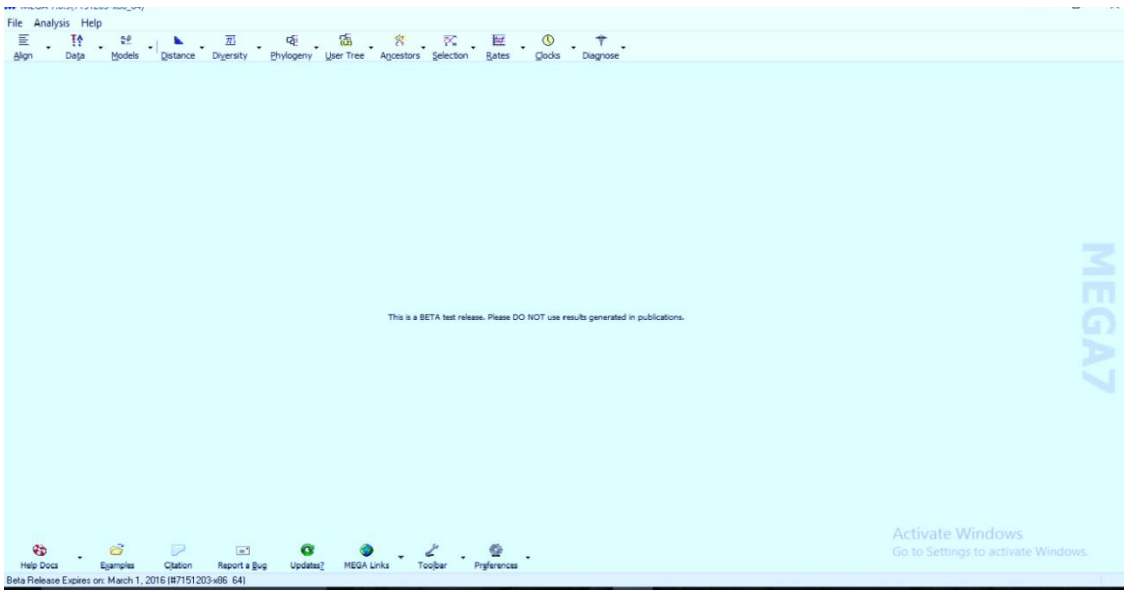

FIG 3.3: Molecular Evolutionary Genetics Analysis (MEGA). MEGA 7 is the latest release of MEGA software for phylogenetic analysis.

Chapter Four
RESULT AND DISCUSSION

4.1 Antibiotic Susceptibility Test (AST) / Antibiogram Assay

Bacterial samples were collected from Tribhuvan University Teaching Hospital (TUTH), Maharajgunj, Nepal. The bacterial strains were found to be resistant to more than 2 antiibiotics thus confirmed as multidrug resistant bacteria. Altogether, 35 MDR bacteria were collected. Out of 35, 7 were *Pseudomonas aeruginosa* and others were *E.coli.* Different antibiotics were use AST which were available in our laboratory.

Table 4.1: Antibiotic susceptibility Test (AST) performed to confirm MDR *Escherichia coli*.

S.N	Bacteria	CIP 5	AMX 10	A/S 10/10	AK 30	CTX 30	PIT 100/10	NIT 300	IPM 10	COT 25	LE 5
1	*E.coli* 1	24	0	11	12	27	11	19	21	18	21
2	*E.coli* 2	15	18	20	20	30	23	20	22	28	16
3	E.coli 3	31	0	17	15	30	21	11	30	21	21
4	*E.coli* 4	30	11	20	12	21	0	22	27	28	18
5	*E.coli* 5	0	0	12	10	25	16	19	17	0	10
6	*E.coli 6*	18	0	10	15	0	23	20	28	0	14
7	*E.coli* 7	7	0	11	11	23	20	13	27	27	10
8	*E.coli* 8	32	0	16	21	28	21	18	18	0	26
9	*E.coli* 9	12	0	18	27	11	23	15	24	29	15
10	*E.coli* 10	13	0	15	15	16	25	31	31	0	12
11	*E.coli* 11	12	0	16	18	19	25	18	20	26	11
12	*E.coli* 12	7	0	11	0	21	20	19	25	26	12
13	*E.coli* 13	14	0	11	12	8	11	19	22	0	11
14	*E.coli* 14	0	0	17	11	9	21	13	20	20	10
15	*E.coli* 15	9	0	12	0	0	0	12	13	0	13
16	*E.coli* 16	0	0	11	0	18	12	12	20	0	8
17	*E.coli* 17	0	0	16	13	19	18	18	25	20	9
18	*E.coli* 18	6	0	16	9	17	18	11	25	2	9
19	*E.coli* 19	10	0	17	11	21	20	19	24	23	11
20	*E.coli* 20	10	0	14	16	23	29	16	25	11	19

The numerical value indicates zone of inhibition measured in millimeter (mm). Red, green, yellow and orange colors in boxes indicate resistant, not available, sensitive, and intermediate respectively.

CIP 5-Ciprofloxacillin, AMX 10-Amoxicillin, A/S 10/10-Ampicillin, AK 30-Amikacin, CTX 30-Cefotaxime, PIT100/10- Piperacillin/Tozobactam, NIT300- Nitrofurantoin, IPM 10-Imipenem, COT25-Co-Trimoxazole, LE 5- Levofloxacin.

Table 4.2: Antibiotic susceptibility test (AST) chart of *Pseudomonas aeruginosa*

S.N	Bacteria	0F 5	AMC30	B 10	TEI30	AK 30	CTX 30	GEN 10	MRP 10	P 10	PI 100	PIT 100/10	MET 5	VA 30	COX 10	CX 30	CAZ 30
1	*Pseudomonas aeruginosa _11*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	*Pseudomonas aeruginosa_31*	33	0	0	0	27	0	21	32	0	23	26	0	11	0	0	20
3	*Pseudomonas aeruginosa_35)*	0	0	0	0	25	0	24	0	16	0	0	0	0	0	0	0
4	*Pseudomonas aeruginosa_37*	0	0	0	0	21	0	28	14	0	0	30	0	0	0	0	0
5	*Pseudomonas aeruginosa_56*	18	0	0	0	20	0	26	24	0	19	25	0	0	0	0	18
6	*Pseudomonas aeruginosa_57*	24	0	0	0	27	0	29	25	0	11	20	0	11	0	0	14
7	*Pseudomonas aeruginosa_58*	0	0	0	0	31	0	23	32	0	21	29	0	14	0	0	0

The numerical value indicates zone of inhibition measured in millimeter (mm). Red, green, yellow and orange colors in boxes indicate resistant, not available, sensitive, and intermediate respectively. Seven strains of *P. aeruginosa* were tested and all were found to be MDR. OF 5- Ofloxacin, AMC 30- Amoxyclav, B 10- Bacitracin, TEI 30- Teicoplanin, AK 30- Amikacin, CTX 30- Cefotaxime, GEN 10- Gentamycin, MRP 10- Meropenem, P 10- Penicillin –G, PI 100/10- Piperacillin/Tozobactam, PI 100- Piperacillin, MET 5- Methicillin, VA 30- Vancomycin, C0X 10- Cloxacillin, CX 30- Cephoxitin, CAZ 30- Ceftazidime.

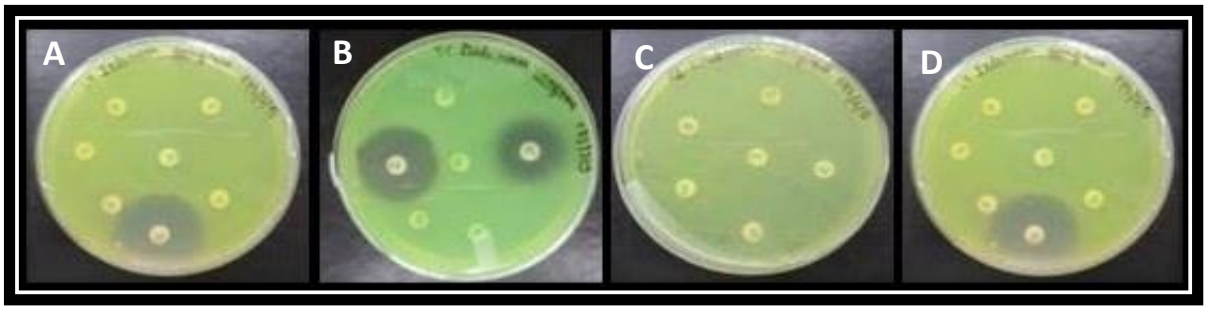

FIG 4.1: Antibiotic susceptibility test by Kirby Bauer disc-diffusion method. A, B, C, D showing AST to confirm MDR strains of *P. aeruginosa*. The bacterial isolates were considered as MDR since they were resistant to more than one antibiotic. The clear zones indicates zone of lysis of particular bacteria.

There are several classification schemes for antibiotics, based on bacterial spectrum (broad versus narrow) or type of activity (bactericidal vs. bacteriostatic), the most useful is based on chemical structure. Antibiotics within a structural class will generally have similar patterns of effectiveness, toxicity, and allergic potential. The main classes of antibiotics are beta-lactams (penicillins, cephalosporins), macrolides, fluoroquinolones, tetracyclines, aminoglycosides (http://www.emedexpert.com).

4.2 Isolation of Bacteriophages

4.2.1 Isolation of Bacteriophages by Double Layer Agar Assay (DLAA)

Altogether 3 phages were isolated against MDR *Pseudomonas aeruginosa* from sewage sample of Bagmati river (sample A), 3 phages against MDR *E.coli* from sewage sample of Bishnumati river (sample B). Pin shaped, clear and transparent plaques were observed against *Pseudomonas aeruginosa_31*. Round clear and transparent plaques were observed against *Pseudomonas aeruginosa_56 and Pseudomonas aeruginosa_58* indicating that the sewage samples collected from different sites contained bacteriophages that were contagious against MDR *P. aeruginosa.* phage CDBTPA-31 produced plaques of 0.1 mm in diameter (pinhead) and absence of halo with well-defined edges in bacterial lawn of *P.aeruginosa*_31. It showed that the isolated phage have lytic effect against *P. aeruginosa_31* whereas phage CDBTPA-56 produce plaques of 0.4 mm in diameter with halo and phage CDBTPA-58 also produce plaques of 0.4mm with bulls eyes. Bull's eyes means phage mediate lyses a bacterial lawn in which the center is clearer than the outer edges (www.archaealviruses.org). Bull's eye form predominantly because phage-induced bacterial lysis is less efficient or complete later on during plaque development than it is early on during plaque development. Decreasing lytic efficiency can be a consequence of aging of the bacterial lawn, associated increases in the size of colonies making up the bacterial lawn, or because of less general phenomena such as the lysis inhibition phenotype seen with T-even phages (www.archaealviruses.org). Different types of bacteriophages were found in each plate based on the formation of plaques with different degree of transparency and sizes. The plaques count on bacterial lawn of *Pseudomonas aeruginosa_31* was 480, 18 on bacterial lawn of *Pseudomonas aeruginosa*_56 and 8 on bacterial lawn of *Pseudomonas aeruginosa_56.*

Table 4.3: Number of Bacteriophages isolated against MDR bacteria

S.N	Microorganism	Sewage sample source	Phage isolated (number)
1	*P. aeruginosa*	Bagmati river	3
2	*E. coli*	Bishnumati river	3

Three bacteriophages against *P.aeruginosa* and three bacteriophages against *E.coli* were isolated from two different holy rivers of Kathmandu valley.

Table 4.4: List of phages isolated from sewage samples

S.N	Phage name	Number of plaques	Plaque diameter (mm)	Turbidity	Halo
1	ø CDBT-PA31	480	0.1	Clear	-
2	ø CDBT-PA56	18	0.4	Clear	+ with bull's eye
3	ø CDBT-PA58	16	0.4	Clear	+
4	ø CDBT –EC24	14	0.2-0.3	Clear	-
5	ø CDBT –EC27	20	0.3-O.4	Clear	+
6	ø CDBT –EC30	19	0.1	More turbid	-

øCDBT-PA31 stands for Central Department of Biotechnology (CDBT), the host bacterium name e.g. *Pseudomonas aeruginosa* (PA) and sample number]. Number of plaques, plaques diameter, turbidity and halo were recorded.

Similarly, round, turbid plaques with 0.2-0.3 mm diameter and pin shape plaques were observed against *E.coli_24*. Clear, round with 0.3-0.4 mm diameter with bull's eyes were observed against *E.coli_27* and round turbid plaques were formed against *E.coli_30* showing that the isolated phages have lytic effect against MDR *E. coli.* The study mainly focuses on characterization of *Pseudomonas aeruginosa* bacteriophage and to analyze efficacy of phage therapy on mouse model. Thus, most of the study was done on *Pseudomonas aeruginosa* bacteriophage.

Three bacteriophages against *Pseudomonas aeruginosa* were isolated, on the basis of lytic and amplification rate. Among three, phage CDBT-PA31 was selected for further work.

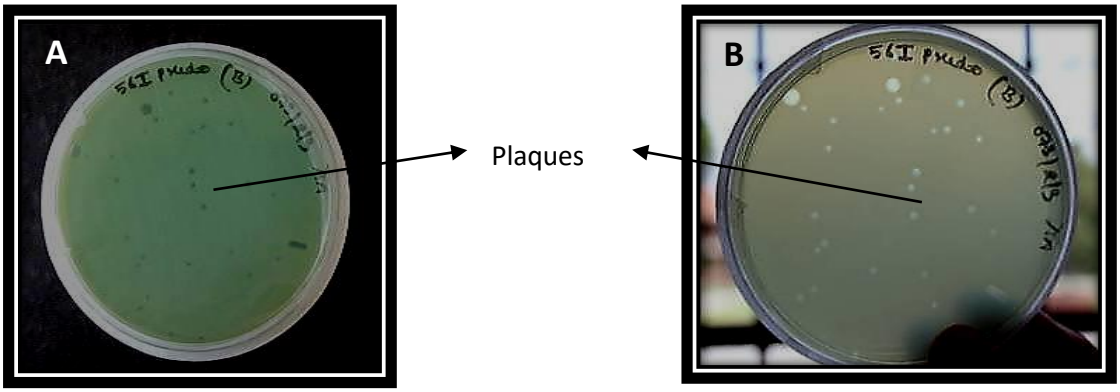

FIG 4.2: Plaques formation on bacterial lawn of MDR *P.aeruginosa_56* from sewage sample A. A and B: Bacteriophages isolated against MDR *P .aeruginosa*_56. Clear plaques were observed of its diameter about 0.4 millimeters. Plaques counts were about 18.

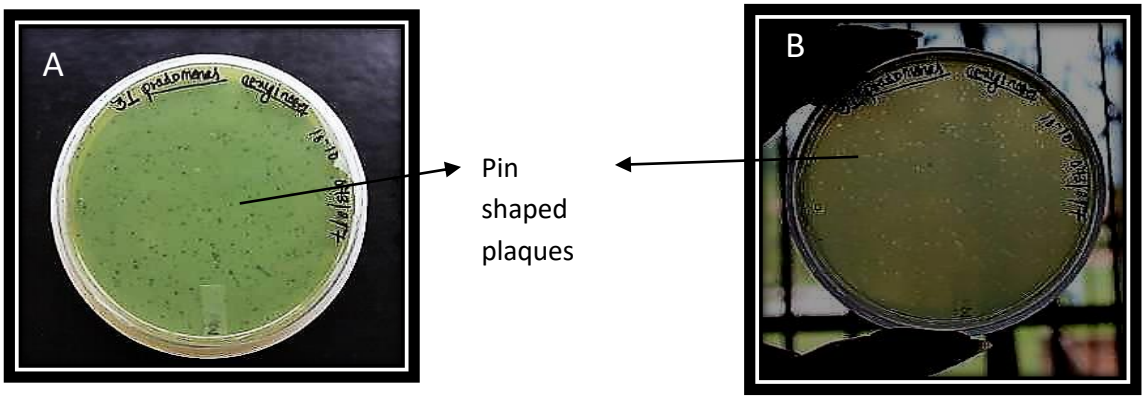

FIG 4.3: Plaques formation on bacterial lawn of MDR *P.aeruginosa_31* from sewage sample A. Bacteriophages isolated against MDR *P.aeruginosa*_31. Clear, pin shaped plaques were observed of its diameter about 0.1 millimeters. Plaques counts were about 480.

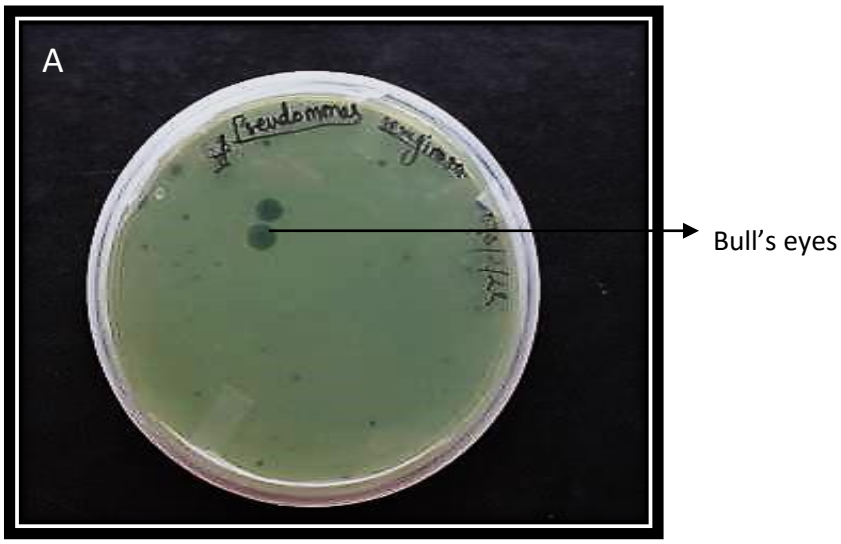

FIG 4.4: Plaques formation on bacterial lawn of MDR *P.aeruginosa_58* from sewage sample A. Bacteriophages isolated against MDR *P.aeruginosa*_58. Clear with halo plaques were observed of its diameter about 0.4 millimeters. Plaques counts were about 16. Among all plaques 2 plaques observed with bull's eyes.

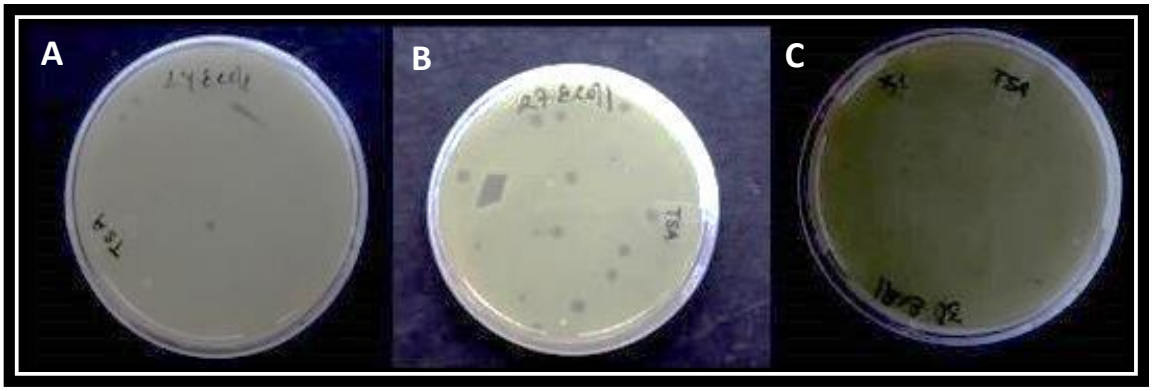

FIG 4.5: Double layer plaque assay for isolation of bacteriophages against MDR *E.coli* from sewage sample B. A: Bacteriophages isolated against MDR E.coli_27. Turbid plaques were observed counted numbers were 14. **B:** Twenty clear plaques were seen of about 0.3 -0.4 mm. **C:** Most turbid plaques were observed. Plaques count were 19 of about 0.1 mm

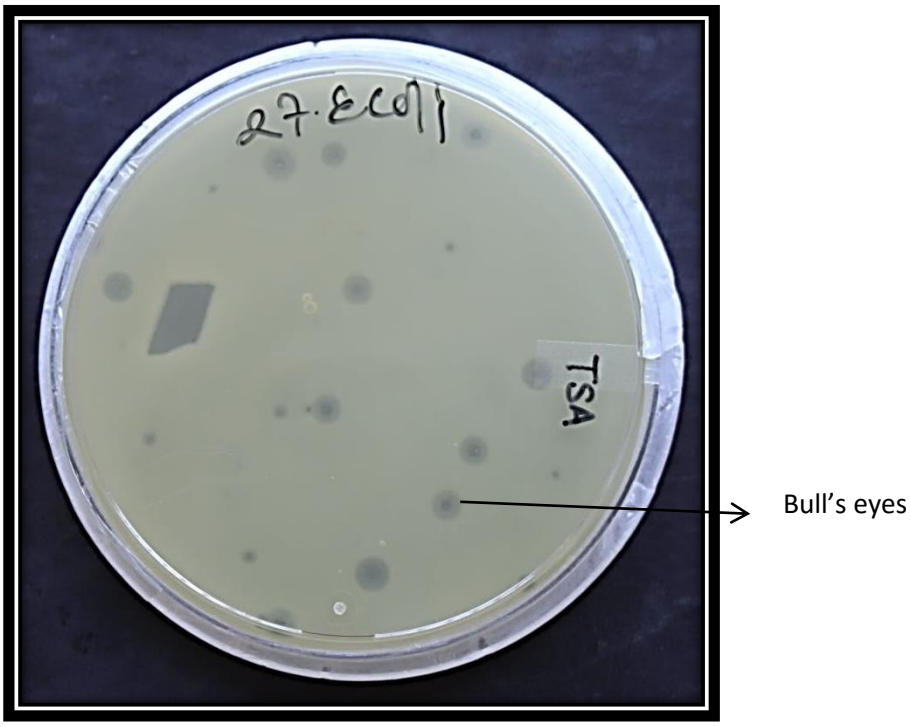

FIG 4.6: Bull's eye formation on lawn of *E. coli*_27. Bull's eyes form predominantly because phage-induced bacterial lysis is less efficient or complete later on during plaque development.

Similarly, some work on previously isolated phage against *Salmonella typhi* and *Salmonella paratyphi* was also carried out during my thesis work. The research work was entitled of "Morphological characterization and whole genome analysis of novel lytic phages against multidrug resistant human pathogens - an alternative approach to antibiotic therapy" (Nepal and Malla, 2016). For continuation, we have done study on application of bacteriophage against food borne pathogens like *Salmonella* spp and *E. coli* but this research was not succeeded because result was not satisfactorily. Thus the study was limited up to bacteriophage amplification, high titer determination of *Salmonella* phage. *In vitro* intra specific phage mediated lysis was also observed.

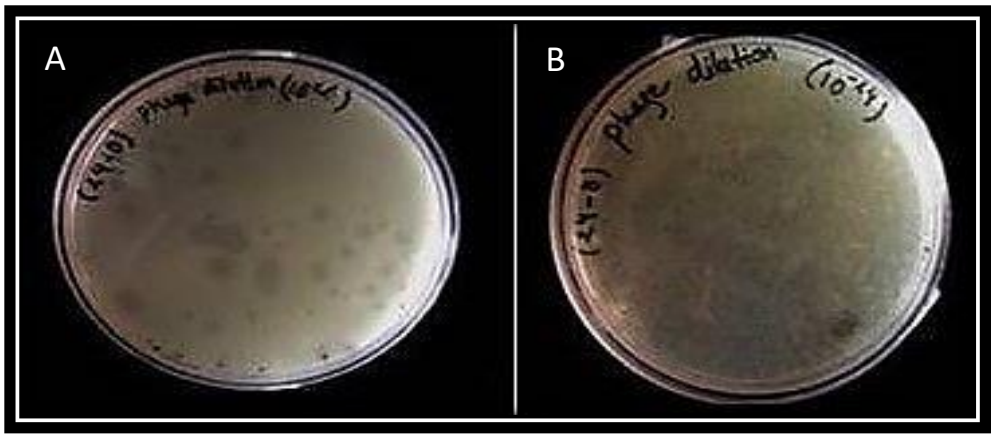

FIG 4.7: Phage amplification and its high titer determination. A: Phage amplification at 10^{-26} dilutions. **B:** Phage amplification at 10^{-24}. Plaques count on 10^{-22} was too many too count (TMTC) and at 10^{-24} were 48.

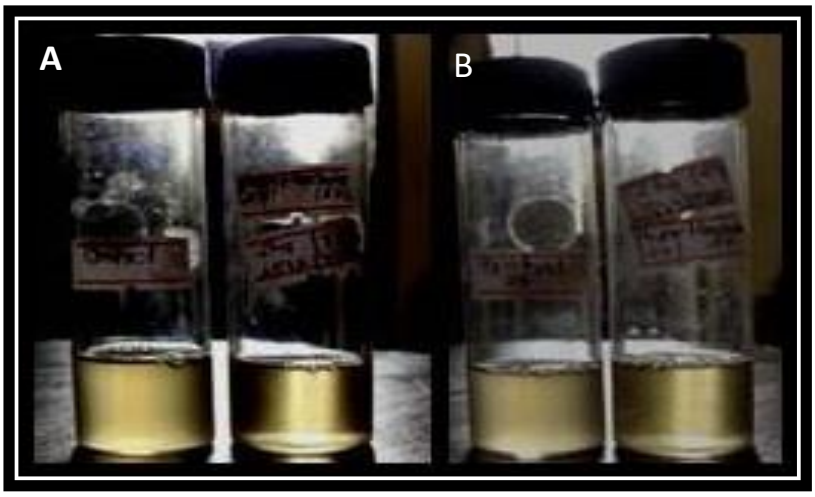

Fig 4.8: *In-vitro* intra specific phage mediated lysis. A: Phage mediated lysis on *Salmonella typhi_12*, after 2 hours and **B:** Phage mediated lysis on *Salmonella paratyphi_24*. Bacterial number analysed through turbidity after incubation at 37°C for 2 hours.

4.3 Amplification and Titration

The isolated bacteriophage against *Pseudomonas aeruginosa_31* (phage code/name) was further amplified and titrated by DLAA method to determine concentration of phage and prepared high titer phage stock. Viral titer was determined by following formula.

$$\boldsymbol{Viral\ Titre} = \frac{\boldsymbol{Number\ of\ plaques}}{\boldsymbol{Dilution}} \times \boldsymbol{Volume\ of\ diluted\ virus\ added\ to\ the\ plate}$$

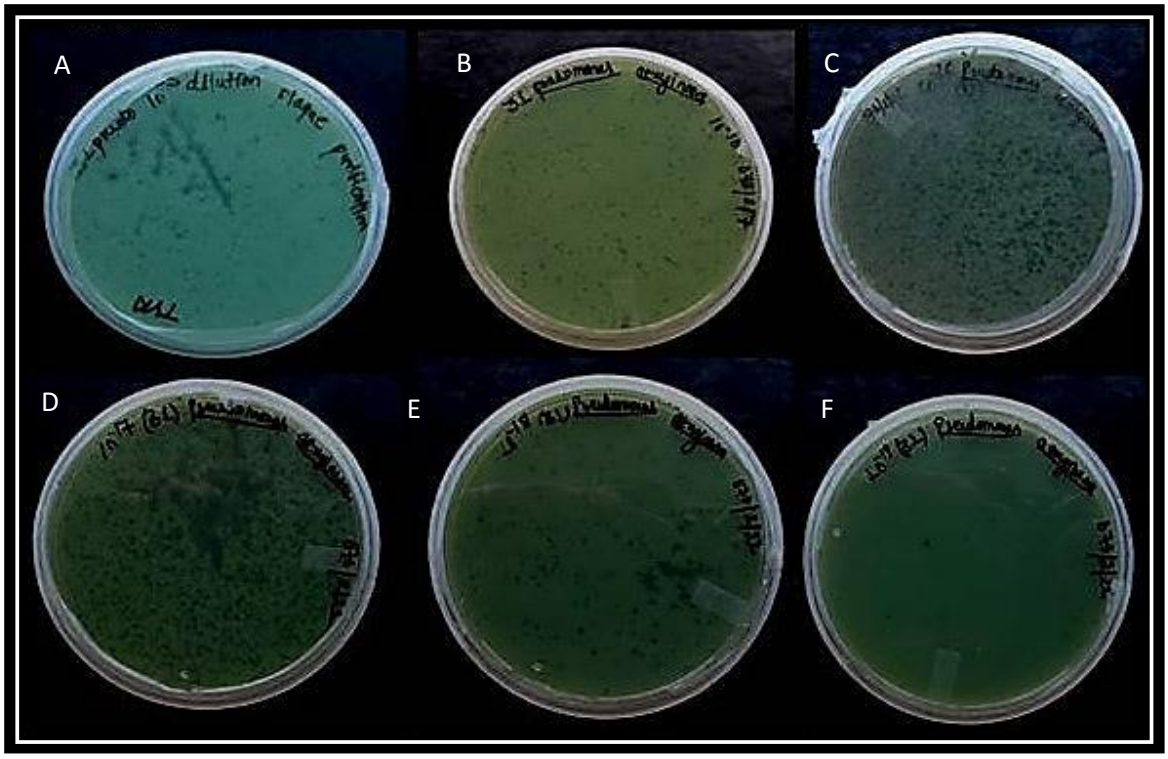

FIG 4.9: Amplification of bacteriophage by DLAA method. **A**: Amplification of bacteriophage at 10^{-3}, **B**: Amplification of bacteriophage at 10^{-10} , **C**: Amplification of bacteriophage at 10^{-16}, **D**: Amplification of bacteriophage at 10^{-17}, **E**: Amplification of bacteriophage dilution at 10^{-18}, **F**: Amplification of bacteriophage at 10^{-19}. Plaques were observed from 10^{-3} to10^{-19} and high titer of phage from 10^{-10} and 10^{-19} were store as a stock. Phage amplification was carried out by DLAA method.

On amplification, we were unable to count at 10^{-3} and for high titer determination, stock was further diluted and phage concentration were determined at 10^{-11} dilution i.e 8.3×10^{12} and so on. Plaques count at 10^{-10} was 170 and at 10^{-11} were 83. After that plaques number started to decrease, so we performed second round of amplification. We found plaques count from 10^{-14} to 10^{-16} was too many too count (TMTC). Plaques count at 10^{-17} was 1160,

10^{-18} was 250, 10^{-19} was 23 and 10^{-20} was 2. Phages were eluted on SM buffer and purified by chloroform. Samples in SM buffer were stored at 4°C until further use.

All the isolated phages were used for further screening assays using the phage spot test on bacterial lawns of the other strains in order is to characterize the host range of each phage and to select a phage with the broadest host range.

4.4 Intra Specific Host Range (Spot Assay)

Spot assay is a quick way to check whether a phage sample can infect a bacterium by placing a small drop or "spot" of phage onto a plate inoculated with the bacterium. This test will determine if the putative plaque will propagate phage (www.phagehuntingprogram.com). A positive spot test appeared as complete obliteration of the entire drop area, on lawn culture of *Pseudomonas aeruginosa_11* whereas a negative spot test resulted in the bacterial lawn growing normally in the region of the spot. Thus, result showed that the isolated bacteriophage phage CDBTPA-31 shows lytic activity against MDR *Pseudomonas aeruginosa_11*. Therefore, phage CDBT-PA31 was selected. After observing spots, the size of spot was bigger on bacterial lawn with high titer of phage i.e spot on 10^{10} greater than spot on 10^{9} greater than spot on 10^{8}. Thus we assumed that higher the titer of bacteriophage efficiency of lytic activity on bacterial strains was more.

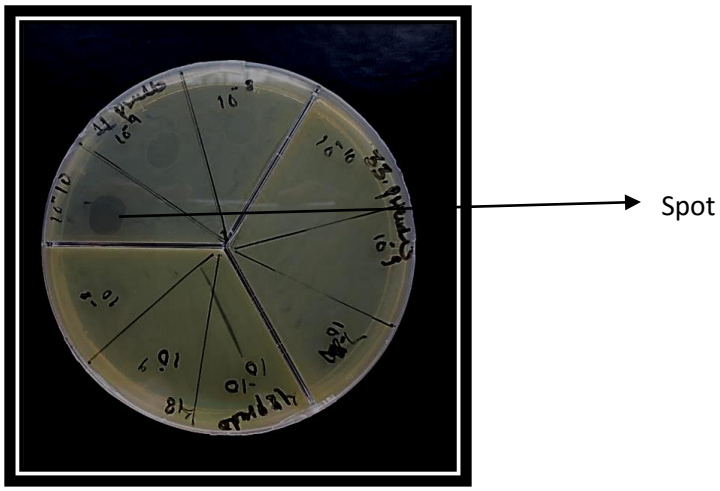

FIG 4.10: phage CDBT–PA31 showed intraspecific host range against *P. aeruginosa* _11 whereas other stains of *P. aeruginosa* spots were not formed. Spot were observed on the bacterial lawn of *P. aeruginosa_11* of different concentration i.e 10^{-8}, 10^{-9} and 10^{-10} after incubation at 37°C for overnight.

Further, the study was confirmed by DLAA method. Double layer agar assay (DLAA) is one of the most important procedures used in virology for measuring the virus titer by using double layer agar i.e soft agar (0.5% agar) and hard agar (1% agar. Renato Dulbecco modified this procedure in 1952 for use in animal virology, and it has since been used for reliable determination of the titers of many different viruses (www.virology.ws).

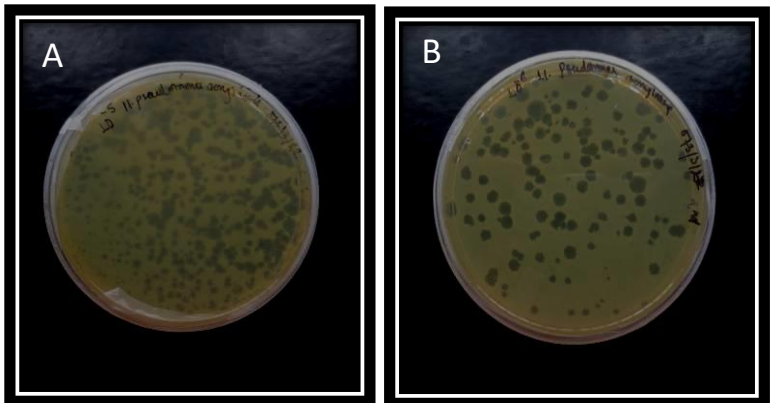

FIG 4.11: Intra specific host range confirmed by DLAA method. **A:** Phage amplification at 10^{-5} on bacterial lawn of *P. aeruginosa*. **B:** Phage amplification at 10^{-6} on bacterial lawn of *P. aeruginosa*. Plaques were observed and further amplified, titrated and store as stock. Plaque size, number, turbidity was analysed. Clear plaques were observed of about 0.2-0.4 mm diameter. Plaques count on 10^{-5} was 168 and plaques count on 10^{-6} was 77.

From plaque assay, it was confirmed that, phage CDBT-PA31 possessed lytic activity against *P. aeruginosa_31* and *P. aeruginosa_11*. Thus phage CDBT-PA31 was selected for further characterization. While phage CDBT-PA11 was also eluted out by DLAA and stored at 4°C.The stock phage were used for analysis of phage proteins analysis and genomic DNA extraction Spot test and plaque assay both showed intraspecific host range. These two methods are quick and reliable method that are feasible in our CDBT lab.

4.5 pH and Thermal Stability

The effect of pH on phage CDBT-PA31 activity was observed after 1 hour of incubation at pH levels ranging from 1 to 12. phage CDBT-PA31 decreased its activity at pH 11. The initial viral titer was 5×10^{8} PFU/ml. However it completely lost its activity at higher pH greater than 11 and lower than viral titer was also determined. When incubation at pH 3 for 1 hour, a titer of 5.8×10^{4} PFU/ml was found and at pH 10, a titer of 5.08×10^{4} PFU/ml of active phage was detected. The maximum stability of phage was observed at a pH 3, 4, 5, 6, 7, 8, 9 and

10. Viral titer was decreased at pH 11 i.e 4.35×10^2 PFU/ml at the end of incubation for 1 hour.

Heat resistant capability of phage CDBT-PA31 at pH 7.0 was also performed. The initial viral titer was 10^7PFU/ml. The results showed that phage CDBT-PA31 heat stable after 30 min and 60 min at 50°C. Viral titer at 50°C at 30 min was 6.15×10^3 PFU/ml and at 60 min was 5.63×10^3 PFU/ml. However, the number of viable phages decreased from 6.15×10^3 PFU/ml to 3×10^2 PFU/ml after 30 min incubating temperature at 60°C and 1×10^2 PFU/ml after 30 min at 70°C. No activity was observed at 80°C and higher.

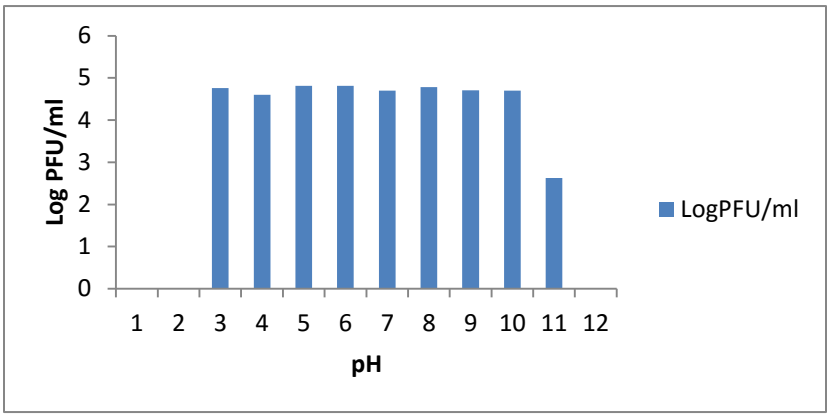

FIG 4.12: pH stability of phageCDBTPA-31. Effect of pH on phage CDBT–PA31 activity was observed after 1 hour of incubation at 37°C pH ranging from 3 to 11. phage CDBTPA-31 was stable from pH ranging from 3-11. It could not survive at lower pH ranging from 1 to 2 and higher pH 12 and above.

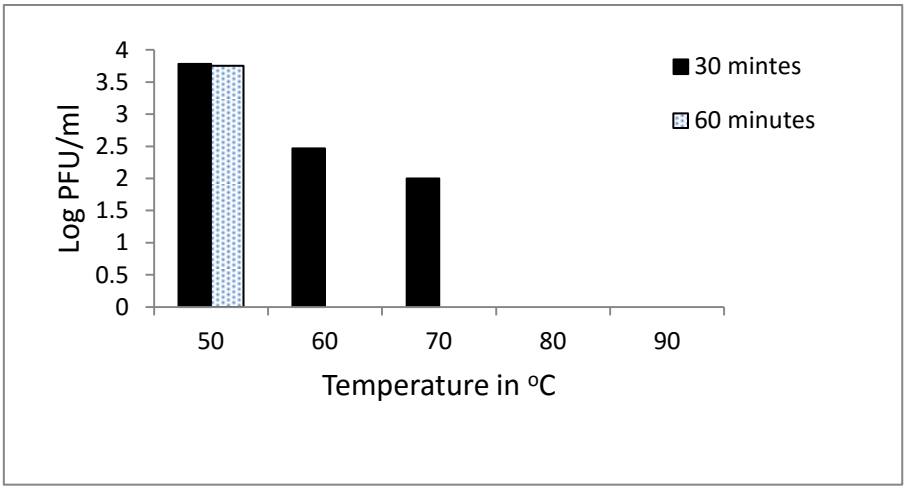

FIG 4.13: Thermal stability of phage CDBT-PA31. phage CDBT-PA31 was stable at 50°C at both time period i.e 30 mins and 60 mins. After incubating phage CDBT-PA31 at 60°C and 70°C stability decreases. At high temperature from 80°C it could not survive.

4.6 In Vitro Phage Mediated Lysis on its Host

The reduction of *Pseudomonas aeruginosa* concentration was compared to phage-free control (Multiplicity of Infection (MOI) = 0). When phages were added at MOI 10^2 (2.04×10^{10}) to host cells initially present at 2.04×10^8 CFU/ml is equal to 8.31 log CFU/ml (Bao et al., 2015), phage CDBT-PA31 achieved a reduction of 1.13, 1.38, 2.05, 2.23 and 2.4 log CFU/ml after 1, 2, 3, 4 and 5 hours respectively. The reduction of bacterial cells in percentage were also calculated and it was found to be 12.29%, 15.01%, 22.30%, 24.26% and 26.11% respectively. From 1 to 5 hour of incubation, the number of viable *P. aeruginosa* was reduced by about 2.4 log i.e 26.11% when treated with phage at MOI of 10^2 compared to the phage-free control after 5 hour. This study was preliminary study, so work was done on low MOI. This study can also be carried out on high MOI.

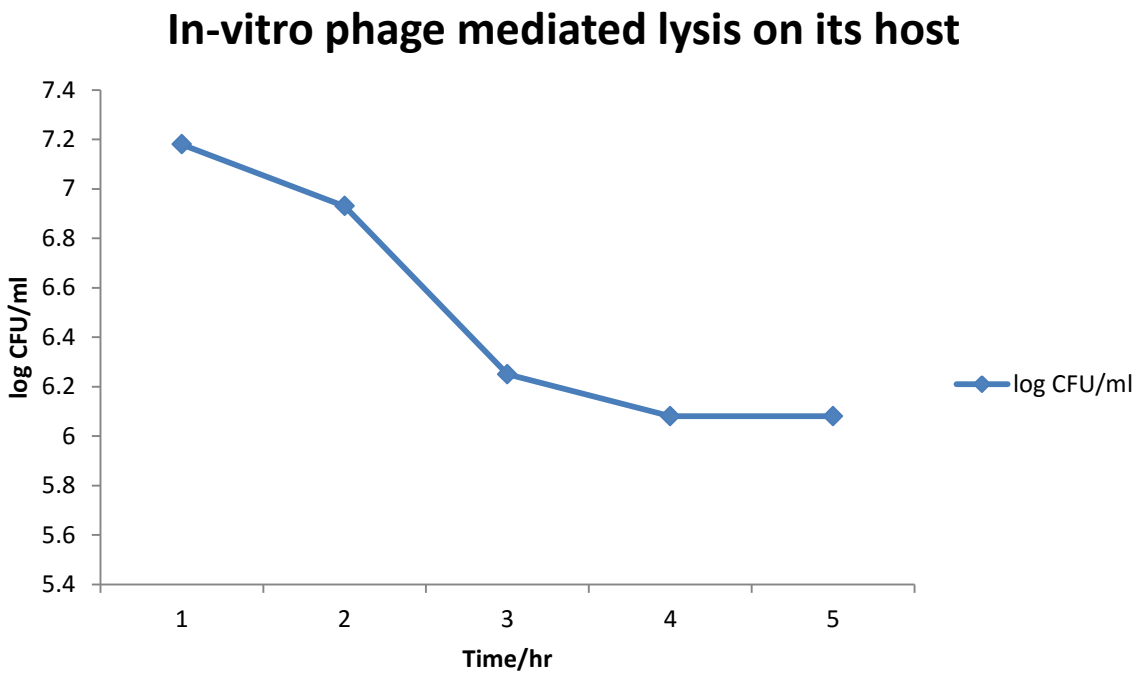

FIG 4.14: Lytic effect of phage CDBT-PA31 on its specified host in vitro. At the end of incubation at 37°C for 5 hours, bacterial cell concentration of *P. aeruginosa* was reduced by about 2.29 log when treated with phage at MOI of 10^2 compared to the phage-free control

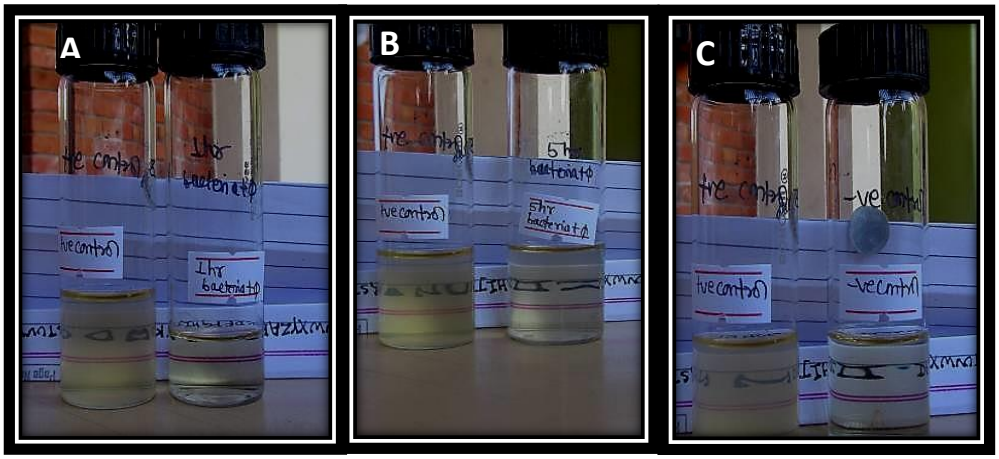

FIG 4.15: *In vitro* phage mediated lysis on its specified host. A: *In vitro* phage mediated lysis on its specified host after 1 hour **B:** *In vitro* phage mediated lysis on its specified host after 5 hour **C:** positive and negative control for experiment.

Table 4.5 : Spectrophotometer reading of bacterial cells at 600nm from 1 to 5 hours

S.N	Time (in hours)	Optical Density	CFU/ml	Log CFU/ml	Log decreased in bacterial density	Decreased in bacterial density (%)
1	1	0.075	1.53×10^{7}	7.18	1.13	12.29
2	2	0.042	8.56×10^{6}	6.93	1.38	15.01
3	3	0.009	1.83×10^{6}	6.26	2.05	22.30
4	4	0.006	1.22×10^{6}	6.08	2.23	24.26
5	5	0.004	8.16×10^{5}	5.91	2.4	26.11

The study on the basis of turbidity showed the growth of bacteria was halted by bacteriophage. The experiment was performed incubating cultures at different period i.e 1 hour to 5 hour. Optical density was determined by spectrophotometer at 600 nm and then CFU/ml was also determined.

4.7 One Step Growth Assay

This study was aimed to characterize phage CDBT-PA31's life cycle. Firstly, one-step growth studies were performed to identify the different phases of a phage infection process. After infection of phage CDBT-PA31 host, phage growth cycle parameters - the latent period, and burst size, were determined from the dynamical change of the number of free and total phages. In this study, latent period of phage CDBT-PA31 was 30 minutes, and yielded a burst size of 423-525 PFU per infected cell.

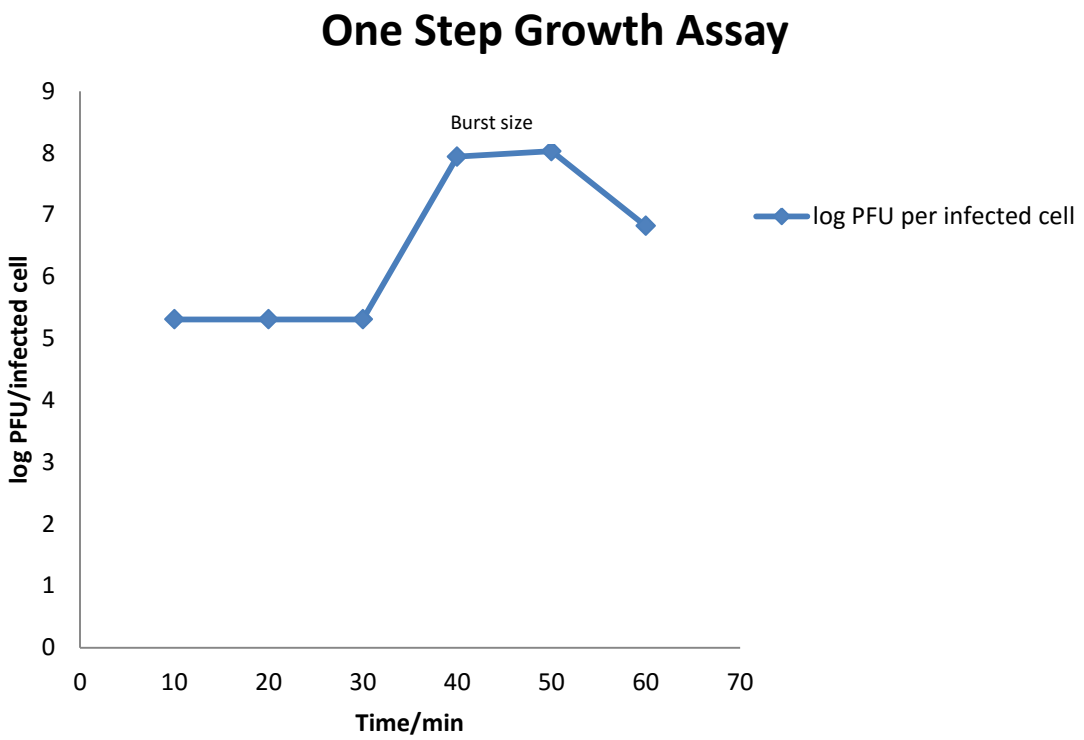

FIG 4.16: One step growth curve of phage CDBT-PA31. Phage infection process was studied. Latent period of phage CDBT-PA31 was 30 minutes, and yielded a burst size of 423 to 525 PFU per infected cell.

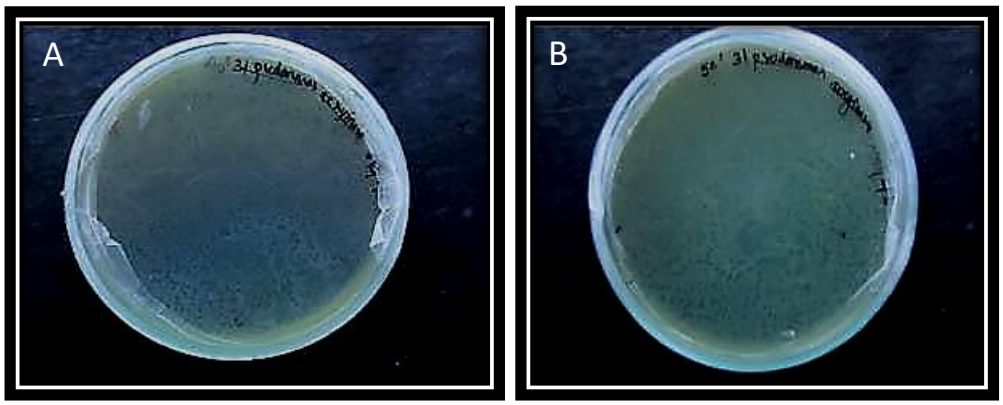

FIG 4.17: Burst size determination of phage CDBT-PA31 by one step growth curve. A: viral progeny cells released after at 40 mins of incubation **B:** viral progeny cells released after at 50 minutes of incubation. Burst size was started from 40 minutes of incubation.

4.8 Molecular Characterisation

4.8.1 DNA Extraction

The phage genomes were successfully extracted using phage DNA isolation kit (Norgen's Biotek Canada). Genomic DNA (gDNA) was visualized on UV transilluminator. The band for Lane 1 was genomic DNA of phage CDBT-PA11, Lane 4 and 5 were genomic DNA of phage CDBT-PA31 from first elution and second elution respectively. The genomic DNA bands were clear. The nucleic acid and protein concentration were also obtained from Nano Drop reading.

FIG 4.18: phage DNA extracted using phage DNA isolation kit (Norgen's Biotek Canada). Lane 1: gDNA of phage CDBT-PA11, Lane 4: gDNA of phage CDBTPA-31 from first elution, Lane5: gDNA of phage CDBTPA-31 from 2nd elution

Table 4.7: Nanodrop reading of phage Nucleic acid concentration, protein concentration at O.D 260/280 and protein concentration at O.D 260/230

S.N	PHAGE NAME	NUCLEIC ACID CONCENTRATION	PROTEIN CONCENTRATION AT 0.D 260/280	PROTEIN CONCENTRATION AT O.D 260/230
1	phageCDBT-PA31	31.6 ng/µl	2.16	1.92
2	phageCDBT-PA11	463.35ng/µl	2.17	2.20

*ng= nanogram, µl=microlitre**,**

According to the Nanodrop reading, nucleic acid concentration of phage CDBT-PA31 and phage CDBT-PA11 was 3.16 ng/µl and 463.3 ng/µl respectively. Protein concentration of phage CDBT-PA31 and phage CDBT-PA11 at0D 260/280 was 2.16 and 2.17 respectively. Similarly, protein concentration at OD 260 /230 was 1.92 and 2.20 respectively.

4.9 Protein Profiling by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

To further characterize phage CDBT-PA31 and phage CDBT-PA11, its protein composition was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Numbers of protein bands were observed. Four bands of phage CDBT-PA31 and 3 bands of phage CDBT-PA11 can be clearly distinguished in the gradient gel ranging from approximately 3.5-29 kDa.

In case of protein profiling of phage CDBT-PA31, the most predominant polypeptide appeared at a size of approximately 29 KDa. It could be assigned to the major capsid protein. Also other three protein bands could be correlated with structural proteins: they could be assumed as head-tail connector protein capsid assembly protein, internal virion protein. Like phage CDBT-PA31, on the protein profiling of phage CDBT-PA11, major structural protein appeared at size of 14.3 KDa and other bands could be assumed as minor structural protein.

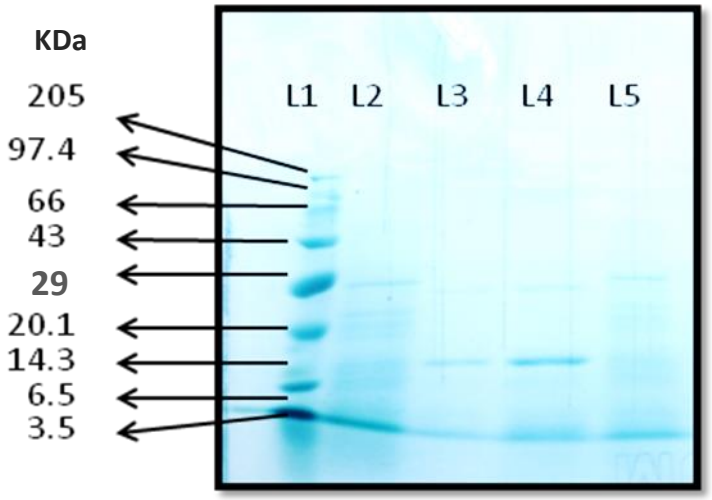

FIG 4.19: Protein profiling of by SDS-PAGE. Lane1 = Marker (Genei), 3.5-205 Kilodalton (KDa) Lane2: phage CDBT-PA31, Lane3: phage CDBT-P11, Lane4: phage CDBT-PA11, Lane5: phage CDBT-PA-31. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to Laemmli. On lane 2 and lane 4 are phage proteins collected from acetone precipitation were loaded whereas on lane 3 and lane 5 phage proteins collected from direct heating were loaded.

On lane 2 and lane 5 phage proteins of phage CDBT-PA31 and on lane 4 phage protein of phage CDBT-P11 were loaded. On analyzing phage proteins bands, clear bands were formed on lane 2 and lane 4. While on lane 3 and 5 bands were not so clear. The clear bands were formed by acetone precipitation method and faint bands were formed from direct heating method. Thus, we can conclude that phage proteins ruptured on precipitation and on heating at 100°C. Both methods can be used for analysis of phage proteins but according to my study acetone precipitation method was better than direct heating method.

4.10 Effectiveness of Phage Therapy on Mouse Model

In our investigation, the efficacy of phage treatment in BALB/C mouse model, after superficial infection with MDR *P. aeruginosa*, showed that administration of lytic phage phage CDBT-PA31 in the infected animal resolved the infection.

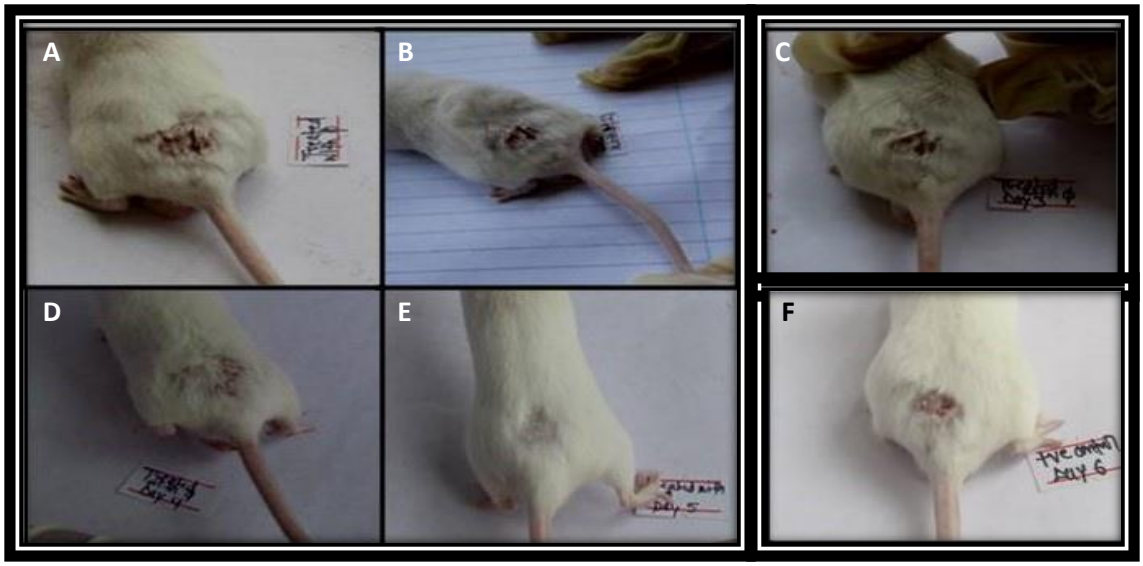

FIG 4.20: MDR *P. aeruginosa* acute infection of mice treated with phage CDBT-PA31 (from day 1 to day 5). Phage application (3×10^4) after 30 minutes on superficial infection on mice infected with MDR *P. aeruginosa* and observed for 5 days. Phage treatment A. Day 1**, B.** Day 2, **C.** Day 3, **D.** Day 4 **E.** Day 5 and wound started to heal from day 4.

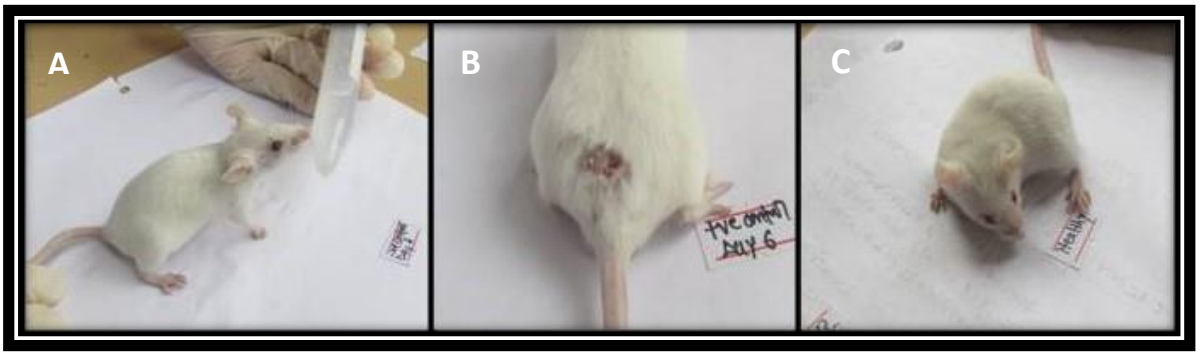

FIG 4.21: Experimental phage therapy on mouse model. A: Negative control for experiment, **B:** Positive control for experiment and **C:** Healthy mouse for experiment.

Concerning the timing of phage treatment, our results showed that administration of phage after 30-45 minutes after the bacterial challenge was successful. The scientific literature shows, simultaneous injection is the easiest method for examination of the antibacterial effects of phage or drug in vivo (Watanabe et al., 2007). Furthermore, the bacteria and the phage immediately transferred into blood, as this is assumed to be the most suitable in vivo situation for the phage–bacterium interaction (Watanabe et al., 2007).

In these animals, the lesion might not be cleared by the immune system of the animals, but healing was apparent by the lytic phages. Recovery of the animals was achieved by administering two doses of phage daily for a week and maintaining a continuous infusion of phage via the drinking water. The effectiveness of lytic phage CDBT-PA31 in treatment of experimental infected mouse model suggested that MDR *P. aeruginosa* is susceptible for both oral and intrapertonially applications of the lytic phage CDBT-PA31.

In our study, superficial lesion healed within 5 days. Same doses of phage (i.e doses given to infected mice) were also administered on negative control mice for same period. No side effects were monitored. On the basis of physical activity, they showed similar activities comparing with healthy mice. Thus we can conclude that phage therapy can be used to control bacterial infections.

4.11 Phylogenetic Analysis Klebsiella virus TU_Kle10O by Molecular Evolutionary Genetics Analysis (MEGA 7)

A phylogenetic tree, also known as a phylogeny, is a diagram that depicts the lines of evolutionary descent of different species, organisms, or genes from a common ancestor. Phylogenies are useful for organizing knowledge of biological diversity, for structuring classifications, and for providing insight into events that occurred during evolution (Baum., 2008). The pattern of branching in a phylogenetic tree reflects how species or other groups evolved from a series of common ancestors. In trees, two species are more related if they have a more recent common ancestor and less related if they have a less recent common ancestor. Phylogenetic trees can be drawn in various equivalent styles. Rotating a tree about its branch points doesn't change the information it carries (www.khanacademy.org)

In this study, phylogenetic tree of whole genome sequences of Klebsiella virus TU_Kle10O, Escherichia virus TU_EC18O and Salmonella virus TU_SP24B were made by using bioinformatics tool i.e MEGA 7. The data was obtained from research work entitled "Morphological characterization and whole genome analysis of novel lytic phages against multidrug resistant human pathogens- An alternative approach to antibiotic therapy" (Nepal and Malla, 2016) for continuity of ongoing research at Central Department of Biotechnology.

Initially accession numbers or bacteriophages names of closely related phages were listed from National Center for Biotechnology Information (NCBI) by Basic Local Alignment Search Tool (Blast) then only phylogenetic analysis were done. National Center for Biotechnology Information is an open access to provide information about biomedical and genetic information. BLAST finds regions of similarity between biological sequences. This program helps to compares nucleotide or protein sequences to sequences databases and calculates the statistical significances. In our study, we use BLASTn for retrieving the nucleotide sequences of closely related phages. Now, a new version of BLAST RNA –SEQ mapping tool is also available.

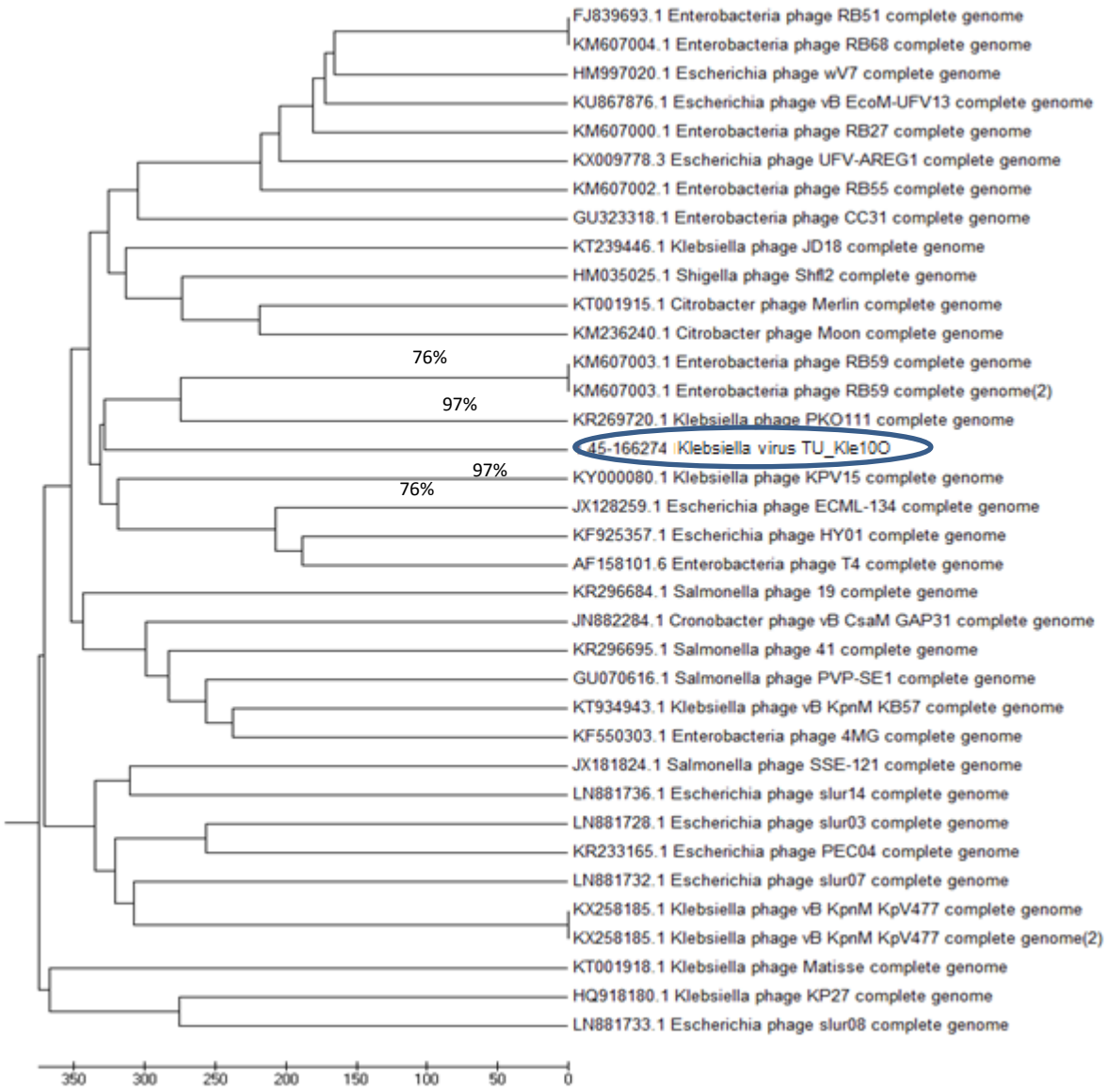

FIG 4.22: Phylogenetic Analysis of *Klebsiella* virus TU_Kle10O by MEGA 7 using command called Unweighted Pair Group Method with Arithmetic mean (UPGMA). *Klebsiella* virus TU_Kle10O shows 97% homology with *Klebsiella* phage KPV 15, 97% homology with *Klebsiella* phage PKO 111, 76% homology with *Escherichia* phage ECML-134 and 76% homology with *Enteriobacteria* phage RB59.

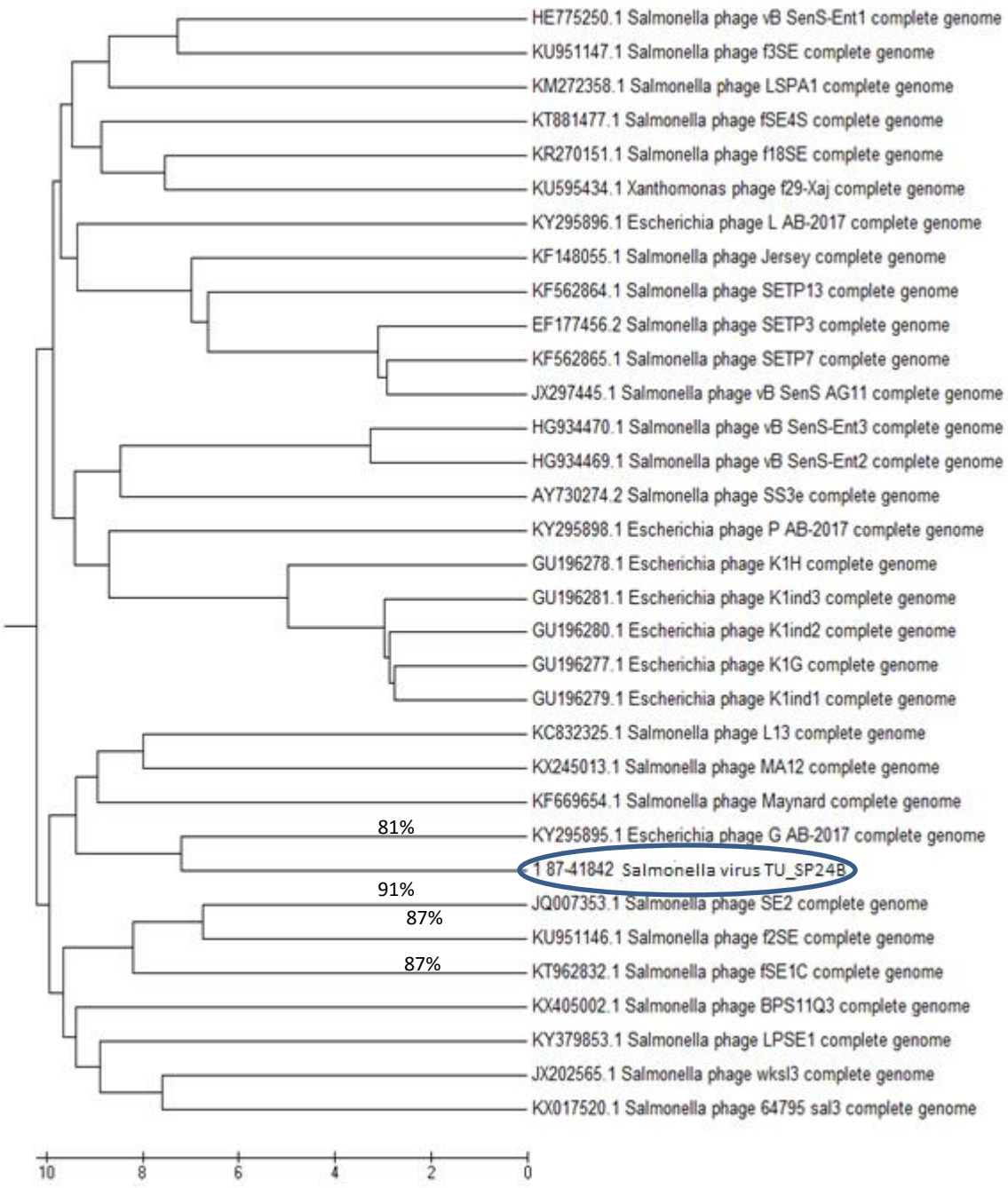

FIG 4.23: Phylogenetic Analysis of Salmonella virus TU_SP24B by MEGA 7 using command called Unweighted Pair Group Method with Arithmetic mean (UPGMA). Salmonella virus TU_SP24B showed 91% homology with *Salmonella* phage SE2, 87% homology with *Salmonella* phage f2SE, 87% homology with *Salmonella* phage fSE1C and 81% homology with *Escherichia* phage G AB-2017.

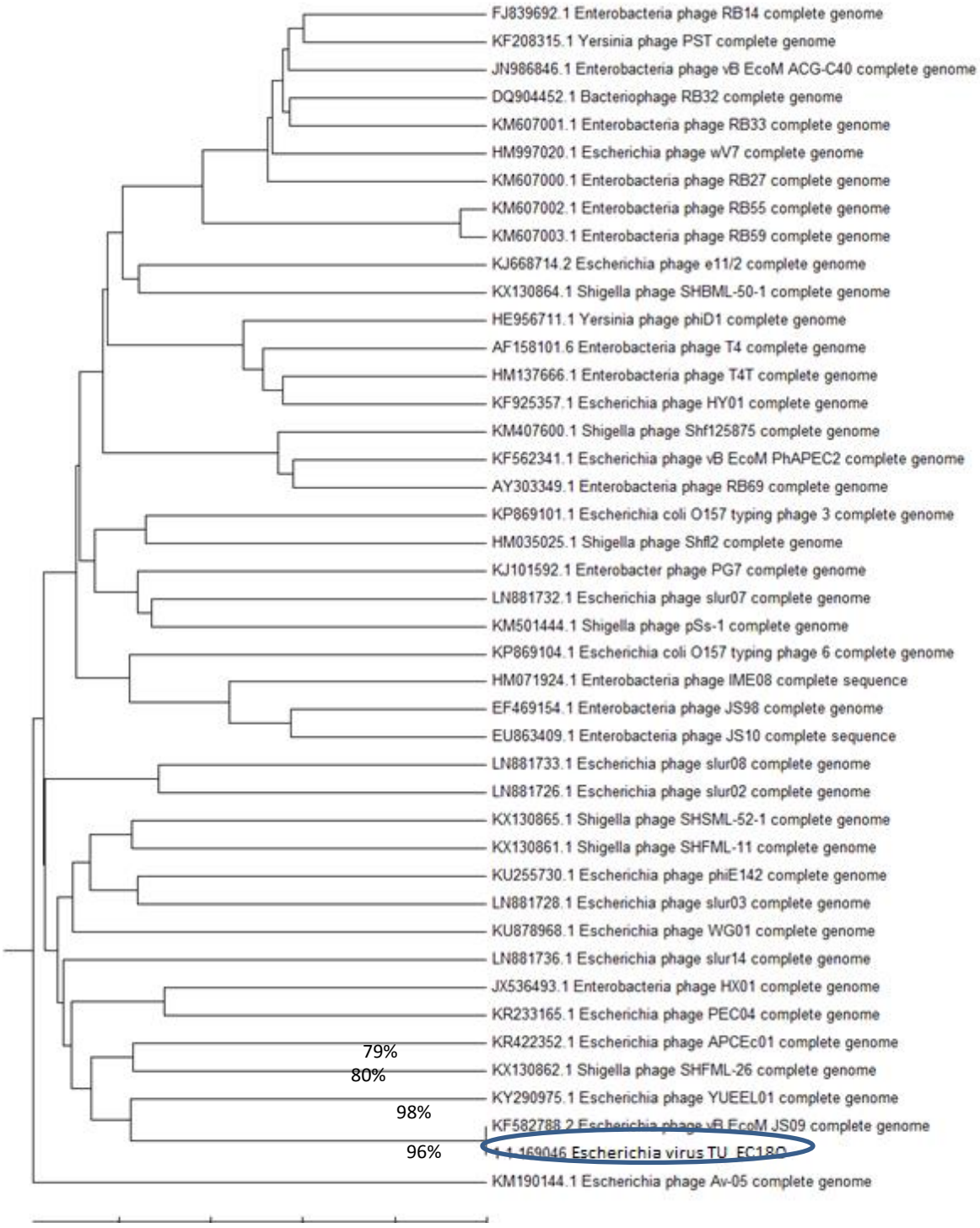

FIG 4.24: Phylogenetic Analysis of Escherichia virus TU_EC18O by MEGA 7 using command called Unweighted Pair Group Method with Arithmetic mean (UPGMA). Escherichia virus TU_EC18O shows 98% homology with *Escherichia* phage vB EcoMJSO9 15, 96% homology with *Escherichia* phage Av-o5, 80% homology with *Escherichia* phage YUEELO1 and 79% homology with *Shigella* phage SHFML-26 RB59.

Phylogenetic tree of was prepared using command called Unweighted Pair Group Method with Arithmetic mean (UPGMA) tree of MEGA 7 software. This tree-making method assumes that the rate of evolution has remained constant throughout the evolutionary history of the included taxa. Therefore, it produces a rooted tree.

The phylogenetic result showed Klebsiella virus TU_Kle10O showed 97% homology with Klebsiella phage KPV 15, 97% homology with *Klebsiellla* phage PKO 111, 76% homology with *Escherichia* phage ECML-134 and 76% homology with *Enteriobacteria* phage RB59. Similarly, Salmonella virus TU_SP24B showed 91% homology with *Salmonella* phage SE2, 87% homology with *Salmonella* phage f2SE, 87% homology with *Salmonella* phage fSE1C and 81% homology with *Escherichia* phage G AB-2017.

Escherichia virus TU_EC18O showed 98% homology with *Escherichia* phage vB EcoMJSO9 15, 96% homology with *Escherichia* phage Av-o5, 80% homology with *Escherichia* phage YUEELO1 and 79% homology with *Shigella* phage SHFML-26 RB59.

Chapter Five
SUMMARY

Pseudomonas aeruginosa is a gram negative rod shape bacteria that cause diseases not only to human but also in animals and plants. Medically, *P. aeruginosa* has become an important cause of gram-negative infection, especially in patients with compromised host defense mechanisms*. P. aeruginosa* is MDR bacteria and it is emerging day by day, antibiotics failed to control. It is the burning problem in medicine. Scientist developed new drugs but bacteria are so clever that they would changes their binding sites for drugs and those drugs work no more. Thus the superbugs are emerging due to the antimicrobial resistance. In my view global concern should focus on phage therapy.

Our study was based on phage therapy. Bacteriophage isolation, characterization was preliminary process and after that sequencing, electron microscopy and analyzing the efficacy of bacteriophage treatment on mice model are secondary process.

Three bacteriophages were isolated against *Pseudomonas aeruginosa* from Bagmati river of Balkhu by Double Layer Agar Assay (DLAA). Plaques size, numbers, type were analysed. Phage CDBT-PA31 showed lytic activity against *Pseudomonas aeruginosa* _11. phage CDBT-PA11 showed intra specific host range analysis against *Pseudomonas aeruginosa* _11 and further confirmed by DLAA. Thus phage CDBT-PA31 was selected to further work on it. Bacteriophages were amplified for high titer determination. High titer of phage of PFU/ml about 10^{12} PFU/ml to10^{17} PFU/ml were store as stock at 4°C for further use.

Phage CDBT-PA31 was stable at pH 3-11 and extreme temperature 50^{o}C. Phages DNA were extracted by phage DNA isolation kit (Norgen's Biotek Canada). Phage proteins were analysed on the basis of protein profiling through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Four bands of phage CDBT-PA31 and three bands of phage CDBT-PA11 can be clearly distinguished in the gradient gel ranging from approximately 3.5-29 kDa. One-step growth study was performed to identify the different phases of a phage infection process. phage CDBT-PA31 showed a latent period of 30 minutes, with corresponding burst sizes of 423-525 PFU/cell.

Efficacy of phage therapy was analysed on performing experimental phage therapy on mice model. Superficial wound infection on its back healed at 6 days of phage treatment.

Similarly, electron microscopy images and phylogenetic analysis of multi host range bacteriophages were also analyzed. Escherichia virus TU_EC18O belonged to myoviridae, Klebsiella virus TU_Kle10O belonged to podoviridae, Salmonella virus TU_SP24B belonged to Siphoviridae.

Klebsiella virus TU_Kle10O showed 97% homology with Klebsiella phage KPV 15, 97% homology with *Klebsiellla* phage PKO 111, 76% homology with *Escherichia* phage ECML-134 and 76% homology with *Enteriobacteria* phage RB59. Similarly, Salmonella virus TU_SP24B shows 91% homology with *Salmonella* phage SE2, 87% homology with *Salmonella* phage f2SE, 87% homology with Salmonella phage fSE1C and 81% homology with *Escherichia* phage G AB-2017. Escherichia virus TU_EC18O shows 98% homology with *Escherichia* phage vB EcoMJSO9 15, 96% homology with *Escherichia* phage Av-o5, 80% homology with *Escherichia* phage YUEELO1 and 79% homology with *Shigella* phage SHFML-26 RB59.

In context of developing country like Nepal, further in vivo trial on animal model and clinical trial need to encourage supporting phage therapy for the commercial production of phage medicine.

Chapter Six
CONCLUSION

Three bacteriophages were isolated against *Pseudomonas aeruginosa* from Bagmati river of Balkhu by Double Layer Agar Assay (DLAA). Isolated bacteriophages showed lytic activity against multi drug resistant *P. aeruginosa.*

On the basis of plaque size, number and plaque clarity, only one bacteriophage, phage CDBT-PA31 was selected for further study. phage CDBT-PA31 was purified, amplified and titrated for further work. phage CDBT-PA31 was molecularly characterized further information. On Invitro and Invivo phage trial, *P. aeruginosa* bacterial densities decreased on using lytic phage CDBT-PA31. Phylogenetic analysis of multi host range phages as performed on MEGA software by UPGMA method.

In context of developing country like Nepal, further Invitro and in vivo trial on mice model and clinical trial need to do to support phage therapy and their commercial production of phage medicine. Study on genetic engineering on phage genes facilitates determination of cloning sites that helps gene cloning, drug designing.

Multi drug resistant (MDR) bacteria are the global problem, more study on bacteriophages and its therapy facilitates to produce phage drugs to overcome or to control MDR bacterial pathogens.

LIMITATIONS OF THE STUDY

- **Number of small sample size:** Small sample size (bacterial) one of the main limitation of this study. We were unable to isolate more numbers bacteriophages. It also effects on determining multi host range analysis.
- **Lack of highly sophisticated instrumentation:** Facility of electron microscopy, whole genome sequencing, and ultracentrifugation are not available in our country. So for further electron micrograph, sequencing analysis samples need to be exported where available. Thus, we were unable to perform these works in our lab.
- **High cost:** The cost for whole genome sequencing and electron microscopy were expensive too.
- **Lack of chemicals:** Phage genome could not extracted as many times due to limitation of chemicals required for phage DNA extraction. Thus DNA was limited and we could not repeat our experiment on Restriction Digestion (RE). We did not get result of RE. Polymerase Chain Reaction (PCR) could not performed due to lack of primers.

FUTURE PROSPECTS / RECOMMENDATIONS

- **Electron Microscopy:** For phenotypic identification, purified phage samples require electron microscopy.
- **Whole genome sequencing:** The study on whole genome sequencing helps in gene analysis, genome annotation; determine cloning sites, to identify novel phages. If we get result on electron microscopy and whole genome sequencing, the result of our work will be best and it's good for publication.
- **Mouse Trial:** Invivo trail in mouse model on aseptic and controlled animal house would provide us reliable data on phage research that helps to achieve new findings for further study.
- **Cell line study:** Cell line analysis is another potential study that would significantly improve the impact of phage research.
- **Clinical trial:** If possible, clinical trial on phage therapy should carry out that will be the landmark in the field of medicine and drug designing but it is very difficult task.

Chapter Seven

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APPENDIX

Media/Chemical composition

Sodium Magnesium Buffer (SM Buffer)

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

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

Nutrient Broth/Agar (NB/NA) – HiMedia

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

Final pH (after sterilization) 7.3±0.1

Mueller Hinton Agar (MHA) – HiMedia

Ingredients	Grams / Liter
Meat, infusion solids from 300g	2.000
Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.500

Final pH (at 25°C) 7.3±0.1

Luria Bertaini broth(LB) – Himedia

Ingredients	Grams/Liter
Tryptone	10
Yeast extract	5
NaCl	10

Phosphate Buffer Saline 10× (PBS)

Ingredients	Grams/Liter
Sodium chloride	8
Potassium chloride	0.2
Disodium hydrogen phosphate	1.44
Potassium dihydrogen phosphate	0.24

Final pH (at 25°C) 7.2-7.4

5 ml stacking gel:

Ingredients	Volume(ml)
H2O	2.975
0.5 M Tris-Hcl, pH 6.8	1.25
10% (w/v) SDS	0.05
Acrylamide/bis-acrylamide	0.67
Ammonium per sulfate (APS)	0.05
TEMED	0.005

10ml separating gel (10%)

Ingredients	Volume
H2O	3.8ml
Acrylamide/bis-acrylamide (30%/0.8% w/v)	3.4ml
1.5M Tris(pH=8.8)	2.6ml
10% (w/v)SDS	0.1ml
ammonium per sulfate (APS)	100µl
TEMED	10µl

Note: APS and TEMED must be added right before each use.

Sample buffer (2x)

Ingredients
0.125MTris-HCl pH6.8
20% glycerol
4%SDS
2% beta mercaptoethanol
0.02%bromphenolblue

1×Running Buffer

Ingredients
25mM Tris Hcl
200mM Glycine
0.1%SDS

Coomassie Brilliant Blue 0.1%

Ingredients	Concentration/volume
Coomassie brilliant blue G-250	500mg
Glacial acetic acid	25ml
Methanol	250ml
Triple distilled water(TDW)	225ml

Destaining solution

Ingredients	Volume
7.5% Glacial acetic acid	37.5ml
5% methanol	25m
TDW	437.5ml