APPLICATION OF BACTERIOPHAGE IN NOSOCOMIAL INFECTION CONTROL, A NEW CONCEPT TO DISINFECT HOSPITAL ENVIRONMENT

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GLOSSARY ACRONYMS:

µg: Microgram [one billion (1×10^{-9}) of Kilogram]

µl: Microliter [one millionth (1×10^{-6}) of a liter

ABR: Anti-bacterial Resistance

AMR: Antimicrobial Resistance

AST: Antibiotic Sensitivity Test

BLAST: Basic Local Alignment Search Tool

BLASTN: Basic Local Alignment Search Tool- nucleotide

CDBT: Central Department of Biotechnology

CDC: Centre for disease control and prevention

CDS: Coding DNA Sequence

CF: Cystis Fibrosis

CFU: Colony Forming Unit

CNS: Central Nervous System

CPS: Capsular Polysaccrides

CFU: Colony Forming Unit

CRE: Carbapenem Resistant Enterobacteriaceae

DLAA: Double Layer Agar Assay

DNA HT library: Deoxyribonucleic Acid High-Throughput library

DNA: Deoxyribonucleic Acid

dsDNA: double stranded Deoxyribonucleic Acid

dsRNA: double stranded Ribonucleic Acid

EOP: Efficiency of Plating

FDA: Food and Drug Administration

GB: Giga Bytes

GC or G+C content: Guanine – Cytosine content

gDNA: genomic Deoxyribonucleic Acid

GPS: Global Positioning Service

GRAS: Generally Recognized as Safe

HCAI: Health Care associated infection

Ip: Intraperitonial

ICTV: International Committee for Taxonomy of Viruses

IOM: Institute of Medicine

Kbp: Kilobase pairs

LB: Luria Bertani

Log: Logarithm

LPS: Lipopolysaccharide

MDR: Multidrug Resistant

MHR: Multiple Host Range

MOI: Multiplicity of Infection

mRNA: messenger RNA

MRSA: Methicillin Resistant *Staphylococcus aureus*

MTB: *Mycobacterium Tuberculosis*

NA: Nutrient Agar

NCBI: National Center for Biotechnology Information

NDM-1: New Delhi Metallo-beta-lactamase-1

ng: nanogram [one billionth (1×10^{-9}) of a gram]

NGS: Next Generation Sequencing

NIH: National Institute of Health Science USA

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

NPHL: National Public Health Laboratory

OD: Optical Density

ORF: Open Reading Frame

PCR: Polymerase Chain Reaction

PDR: Pan Drug Resistant

PFU: Plaque Forming Unit

PHASTER: PHAGE Search Tool Enhanced Release

PT: Phage Therapy

RBP: Receptor Binding Protein

rpm: revolutions per minute

RNA: Ribonucleic Acid

rRNA: ribosomal Ribonucleic Acid

SM buffer: Sodium Magnesium buffer

SDS-PAGE: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SPL: Staphylococcal Phage Lysate

ss DNA: single stranded Deoxyribonucleic Acid

ss RNA: single stranded Ribonucleic Acid

TAE: Tris-Acetate EDTA Buffer

TE: Tris- Chloride EDTA Buffer

TEM: Transmission Electron Microscopy

tRNA: transfer Ribonucleic Acid

TSA: Tryptic Soya Agar

TSB: Tryptic Soy Broth

TU: Tribhuvan University

UF: Ultra Filtration

US-FDA: United States – Food and Drug Administration

US-NIAID: United States - National Institute of Allergy and Infectious Diseases

UV: Ultra Violet

VRE: Vancomycin Resistant Enterococci

VRSA: Vancomycin Resistant *Staphylococcus aureus*

WGS: Whole Genome Sequencing

WHO: World Health Organization

W/V: Weight by Volume

XDR: Extensively Drug Resistant

ZOI: Zone of Inhibition

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ABSTRACT

Introduction: Health care associated infections (HAIs) are one of the important public health problems which might result in significant rise in mortality and morbidity, predominantly in immune compromised patients of Intensive Care Unit (ICU). A study showed that in the Asian region, risks of HAIs have been estimated to be 2-20 times higher than in developed countries up to 25% of hospitalized patients having acquired infection (Ling et al., 2015) and also a report shows that in United States, roughly 9.2 out of every 100 patients acquire a nosocomial infection, according to Healthline and among them also some HAIs is quite serious and potentially life-threatening. The common bacteria causing nosocomial infections include *Pseudomonas aeruginosa, klebsiella pneumonia, Acinetobacter baumanii, Enterococci etc.* (Khan, Baig, & Mehboob, 2017). Therefore, disinfectants like alcohols, hypochlorite, hydrogen peroxide, amyl phenol, glutaraldehyde etc. are widely used in hospital to kill these organisms which cause NAs but sometimes these chemical disinfectants might be hazardous, irritants and toxic to us and environment. Among many alternatives, bacteriophage mediated bio-control of the pathogenic bacteria is considering as one of the best options. Our objective of the study is to isolate, identify the pathogenic bacteria from the hospital environment and evaluate the efficacy of newly isolated lytic bacteriophage to minimize the bacterial load on hospital fomites.

Methodology: The study time period of this research was 6 months and, in this study, we isolated phage against *Pseudomonas aeruginosa* bacteria collected from ICU of Teku hospital. Then most potent phage was characterized morphologically and Physiochemically. Burst size was obtained from one step growth curve. Intraspecific and interspecific host range was assessed by spot assay. During this research work, we used sterile fomite cloth pieces and marble tile as they are most potent sources were bacteria reside. Firstly, we contaminate the fomites and tiles with *Pseudomonas aeruginosa* bacteria then after we used *Pseudomonas aeruginosa* phage to decontaminate those cloths and tile.

Result: Altogether 16 bacterial strain, 4 *Pseudomonas aeruginosa* and 12 *Staphylococcus aureus* were confirmed by Gram staining and Biochemical test and among them 2 *Pseudomonas aeruginosa* strains and 5 *Staphylococcus aureus* strains were found to be Carbapenem resistance and Multi Drug Resistant (MDR) respectively. Total six bacteriophages against *Pseudomonas aeruginosa* were isolated from different sewage samples. And one of the most potent phage P4 was characterized morphologically and physiochemically. Burst size of the phage was found to be 28 virions per bacterium. Protein profiling was done by SDS-PAGE where protein band between 20-250Kda were found and Phage P4 belongs to order Caudovirales and family Siphoviridae. Similarly, Phage was found to tolerate temperature of 70°C for 20 minutes, pH 3-12, exhibiting multiple host range as well. Decontamination assay was done on the sterile fabric cloth which showed that the P4 phage having MOI value 1 showed higher rate of decontamination with log reduction of 1 and p-value (0.002) i.e., significant. Further Comparison was done between P4 phage and normal disinfectant Phenol where we found that single phage has more disinfectant rate than phenol.

Similarly, time period up to which bacteriophage can show their effectivity as disinfectant was also done and it showed highest level of effectivity up to 6 hours at MOI 1 with the CFU/ml log reduction of 1.

Conclusion: The result from the present thesis reveals several characteristics of the bacteriophage (P4), for instance, effective lytic capability, multiple host range, and stability in wide range of pH and temperature. Higher rate of decontamination with the log reduction of 1 CFU/ml and p-value (0.002) was showed by P4 phage having MOI value of 1. While doing comparison single phage have more disinfectant rate than normal disinfectant & P4 phage can show its highest level of effectivity up to 6 hrs at MOI 1.

Keywords: Nosocomial Infection, disinfectant, *Pseudomonas aeruginosa*, bacteriophage, decontamination

1. INTRODUCTION

1.1Background:

Persistent contamination of hospital environments by pathogenic microbes is one of the major causes of healthcare-associated infections (HAI) (Allegranzi *et al.,* 2011), which represent a main concern in all western hospitals and most common bacteria responsible for hospital acquired nosocomial infection are *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli, Staphylococcus aureus,* Clostridium *difficile, Bacteroides fragilis* etc. (Khan, Baig, & Mehboob, 2017). Among them Multi drug resistant (MDR) *Pseudomonas aeruginosa* is a causative pathogen in healthcare associated infection and is also considered as the most common Gram-negative pathogen causing nosocomial pneumonia in the United States, which is frequently implicated in hospital-acquired urinary tract, lungs, sepsis and bloodstream infections (Nathwani, Raman, Sulham, Gavaghan, & Menon, 2014) and they are directly responsible for about 37,000 deaths each year, and, economic loss of approximately € 7 billion, (ECDC, 2013). The control of these pathogen contaminations has been approached so far by conventional sanitation which is based on the use of chemical-based sanitizers and disinfectants, including chlorine-derivatives, triclosan, chlorhexidine and others. All these approaches are unable to decrease microbial contamination in a stable way and it is not capable of preventing recontamination that occurs continuously due to the presence of inpatients, medical staff, visiting persons, etc. And also, chemical sanitizers kill microbes indiscriminately; thus, both the pathogenic and the potentially beneficial normal microbiota are targeted equally (Fiessinger et al., 1981)

The solution behind all these problems could be bacteriophage. Lytic bacteriophages are safe for humans, being able to only infect bacteria and unable to transduce them. And also their action is rapid and they can be applied successfully on surfaces, as reported for treatment of food or food-processing surfaces (Abuladze *et al*., 2008; Tomat *et al*., 2014) and against AMR bacteria (Sulakvelidze, 2005; Jensen *et al*., 2015). Phages are indicated as an interesting safe and green technology for bacterial decontamination and they have specific characteristic that are specific to single host bacteria only (Jensen *et al*., 2015), and in aqueous solution (Abuladze *et al*., 2008), showed that they can be successfully used to rapidly decrease the amount of pathogens commonly associated with HAIs on different types of surfaces, including AMR strains (D'Accolti *et al*., 2018). Here in this study we aimed to analyze the feasibility and the effectiveness of routine phage decontamination.

1.1.1 Bacteriophage

Bacteriophages are the group of viruses displaying the ability to infect or kill bacteria while they do not affect cell lines from other eukaryotic organisms. They are the incomplete organism that can only replicate in a live cell and also known as bacterial parasites because they lack the cell structure and enzyme systems necessary for food uptake, protein synthesis or construction of new particles (Wernicki et al., 2017). Bacteriophage or “phage” is viruses of prokaryotes (Abendon, 2009) which was discovered independently by Frederick Twort- a bacteriologist from England in 1915 and

Felix d' Herelle- a French Canadian microbiologist- two year later in 1917 (Duckworth, 1976). Felix d' Herelle coined the term 'Bacteriophages', a word that is derived from the fusion of 'bacteria' and 'phagein' (to eat in Greek), size of the most bacteriophage range from 22nm-200nm in length. The largest Bacteriophage T4 is 200nm long and about 80-100nm wide, they are found everywhere where bacterial host is present. It has been established that the population number of phages in aquatic systems lies within the range of 10^4 to 10^6 virions per milliliter (ml) and about 10^9 virions per gram(g) in the soil, with an estimated total number of 10^{32} of bacteriophages on the planet (Wittebole et al. 2014).

Generally, phage consists of nucleic acid molecule also called genome surrounded by coat protein known as capsid or head. The genetic material of phage consists of single stranded or double stranded RNA or DNA. The head or capsid is made up of morphological subunits called promoters. Many but not all phages have tails; length of tail varies among family of phages. Phage have contractile sheath which surrounds tail and contract during infection of the bacterium in some phages. Some phages also have a base plate and one or more tail fibers are attached to the tail end. Phages are specific to one or a limited number of bacterial host strains (Yuan & Gao, 2017). Most of the phages are tailed bacteriophage (Order: Caudovirales), accounting for 96% of all phages present on earth and are the oldest known virus group (Ackermann, 2009).

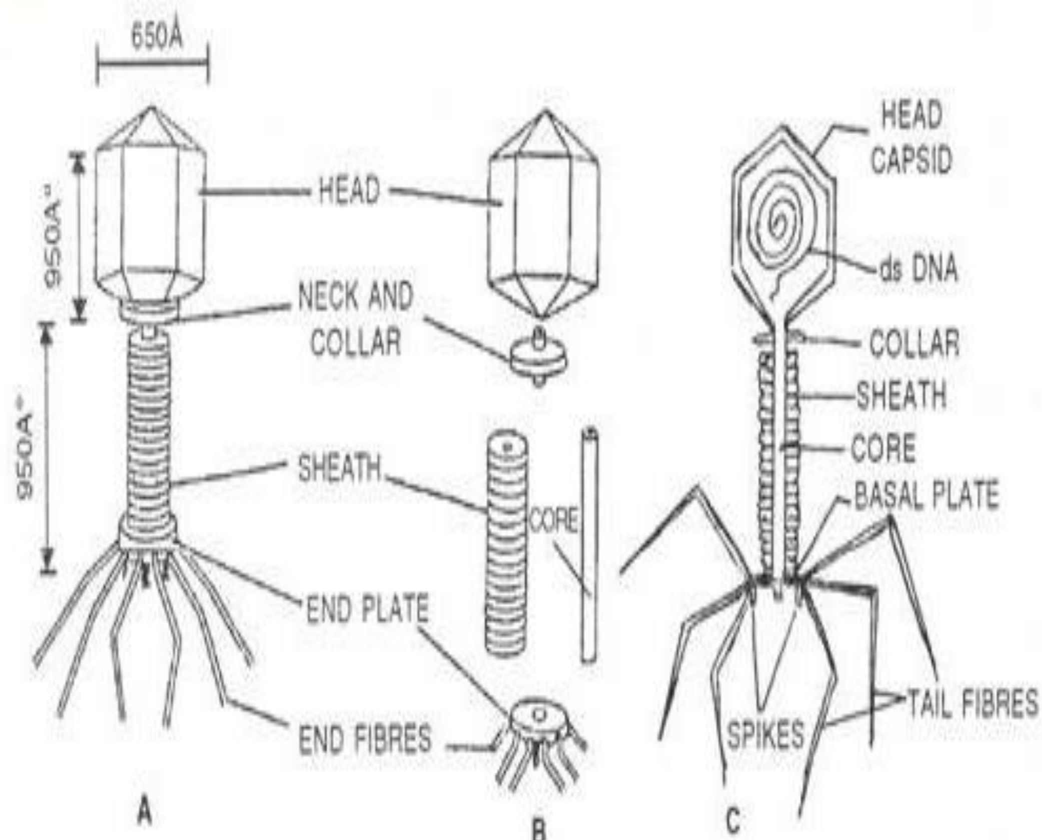


Figure1.1: Typical Bacteriophage. A) Schematic drawing of T4 bacteriophage, B) External Structure of Bacteriophage and C) LS of Bacteriophage. Figure Source: Biology discussion .com

International Committee on Taxonomy of Viruses (ICTV) classified phages on the basis of morphology and nucleic acid. They are classified into 13 orders based on: Tailed

bacteriophage (Order Caudovirales), RNA- containing bacteriophage with polyhedral capsids, DNA- containing bacteriophage with polyhedral capsids, Non tailed phages, Pleomorphic bacteriophage, Filamentous bacteriophage. The Caudovirales are classified into three different families: Podoviridae (Short non-contractile tail, eg: Enterobacteria T7), Myoviridae (Long contractile tail, eg: Enterobacteria phage T4) and Siphoviridae (Long non-contractile tail, eg: HK97) (Fokine & Rossmann, 2014).

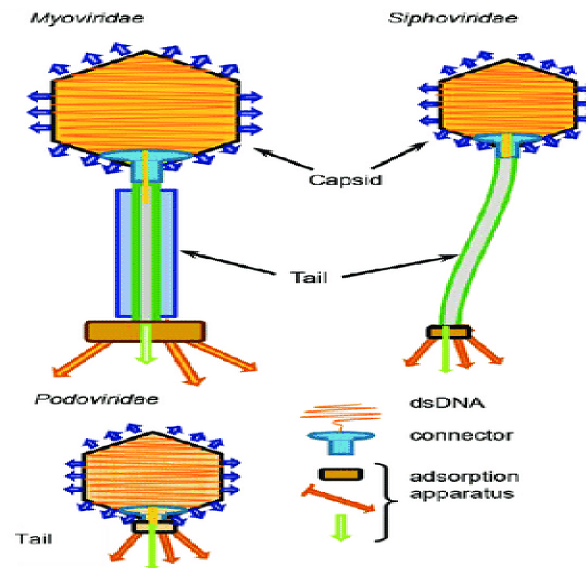


Figure1. 2: Member of the Caudovirales Family. Figure source: (White & Orlova, 2020)

There are seven other families of filamentous, pleomorphic phages which comes under unassigned group as per the recent 9th report (Karthik, 2014). List of phageswith details about shape and structure is given below:

FAMILY	PROPERTIES	SHAPE
Myoviridae	Contractile tail	
Siphoviridae	Noncontractile long tail,	
Podoviridae	Short tail	
Microviridae	ssDNA (C), 27 nm, 12 knoblike capsomers	
Corticoviridae	dsDNA (C), complex capsid, lipids, 63 nm	
Tectiviridae	dsDNA (L), inner lipid vesicle, pseudo-tail, 60 nm	
Leviviridae	ssRNA (L), 23 nm, like poliovirus	
Cystoviridae	dsRNA (L), segmented, lipidic envelope, 70–80 nm	
Inoviridae	ssDNA (C), filaments or rods, 85–1950 x 7 nm	
Plasmaviridae	dsDNA (C), lipidic envelope, no capsid, 80 nm	

Figure1. 3: Different family of Bacteriophage. Fig source (Kartik, 2014)

1.1.2 Bacteriophage biology

Phages are the obligatory parasite thus; require host cellular machinery for the replication of their genetic material. Based on the mode of replication, the phage has two types of life cycle: Lytic and lysogenic cycle where they undergo five general steps such as adsorption or attachment, penetration, biosynthesis, assembly and release for completely lysis of host cell as eukaryotic viruses. In order to infect a specific host cell, bacteriophage recognizes and adheres itself to the bacterial surface, specifically on a receptor found on the bacterial's surface. This process is called adsorption and cannot occur randomly. To successfully adhere, molecules on the phage tail or tail fiber must match specific molecules on the bacterial surface that serves as receptors. A bacterium lacking these molecules is resistant to infection and this causes specificity in phages. Once it becomes permanently bound to the cell, the bacterial virus injects its genetic material into the bacterium in a step called Penetration. This involves the contraction of helical sheath, which forces the hollow tube into the cell cytoplasm, much like the microscopic syringe. In this process, the viral DNA is released into the cell's interior. The viral capsid does not enter the cell. It remains as an empty shell, attached to the cell exterior. Depending on the type of phage, one of two cycles will follow- lytic or lysogenic cycle (Orzechowska & amp; Mohammed, 2019).

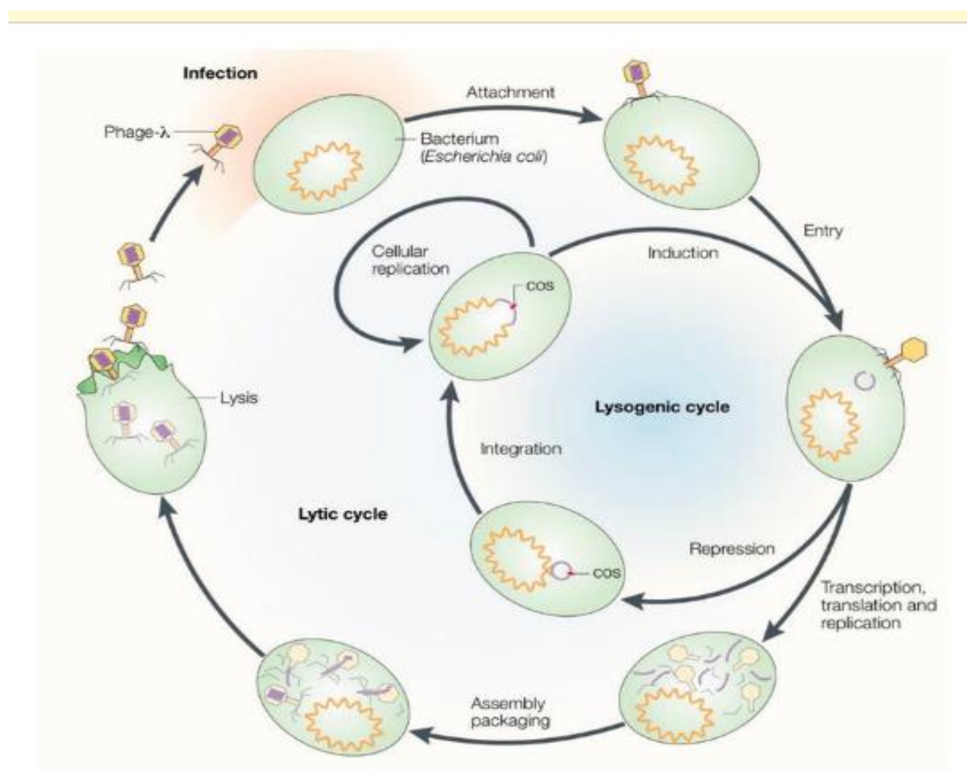


Figure1. 4: Mechanism of Bacteriophage

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

phase is finished, the phage nucleic acids and structural proteins are then spontaneously assembled. After a while, certain protein collectively known as lysin are produced within the cell which causes the cell wall to lyse, allowing the assembled phages within to be released and to infect other susceptible bacterial cells (Ofir & sorek, 2018). All the phages do not under goes lytic cycle. Viral reproduction can also occur through the lysogenic cycle where host cells are not necessarily killed or lysed.

In lysogenic cycle, after the injection of phage genome into a host cell, it gets incorporated into the host genome and rest as a prophage until its lysogenic conversion and the state is called lysogeny. Usually, the lysogenic conversion occurs when the host comes under stress (UV or mutagenic chemicals and desiccation). The phage genome multiply along with the host genome and the daughter host cells are also capable of producing phage upon lysogenic conversion. Lysis of bacterial cell after phage maturation occur several methods depending on the type of phage involved. Double stranded DNA containing phages produce endolysin an enzyme which digest peptidoglycan in the cell wall; it reaches the cell wall by the action of a second protein called Holin which permeabilizes the cell membrane. Some phages also contain genes which code for two alternative lysis proteins, and are supposed to interfere with the cell membrane or cell wall. Single NA containing phages possess only one lysis protein which is presumed to interrupt with peptidoglycan synthesis. Filamentous phages can emerge from the infected bacterial cells through phage encoded channels in the bacterial cell wall. The Lysogenic cycle, although do not cause lysis and is not lethal to bacteria, but has a negative impact on the bacterial growth (Wang, Smith *et al.,* 2000).

For the isolation of phage in laboratory, DLAA (Double Layer Agar Assay) method is widely used. In this method, a hard layer serves as a base layer and the mixture of phage particles, host cell and soft agar forms the upper overlay in the petri plate. When the plate is incubated, host bacterium form a confluent growth and phages attached with the host lyses the bacterium releasing new phage particles which then infect neighboring bacterium. The spread of virus to the whole plate is limited by gel. The area in which phage kills the bacterium is seen as a clear plaque and hence this method is also called "plaque assay". The individual plaque in the plate is supposed to result from a single plaque forming unit and hence the plaque count gives the number of plaque forming units (PFU) in the original phage culture.

1.1.3 Host bacteria

1.1.3.1 *Staphylococcus aureus*

Staphylococcus aureus is a Gram- positive, round-shaped bacteria frequently found in the upper respiratory tract and on the skin (Masalha et al., 2001), they are both a commensal bacterium and a human pathogen. Approximately 30% of the human population is colonized with *S. aureus* and it is a leading cause of bacteremia and infective endocarditis (IE) as well as osteoarticular, skin and soft tissue, pleuropulmonary, and device-related infections (Wertheim et al., 2015). According to the WHO *S. aureus* is one of the leading bacteria that is resistant to fourth generation of antibiotic, now superbug needs to be developed to cure infection caused by *S. aureus.* Each year, around 500,000 patients in hospitals of the United States contract a Staphylococcal infection, chiefly by *S. aureus* (Tracey, 2018).

1.1.3.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is gram negative, rod shaped opportunistic pathogen of animals, ubiquitous in nature (Palumbo, 1972). According to United States National Nosocomial Infection Surveillance System, it accounts for 16.1% of all nosocomial infections and ranked second among Gram negative pathogens (Ranjan et al, 2010). It is the third leading cause of 12 % of hospital-acquired urinary tract infections, upper and lower respiratory tract infections like cystic fibrosis that is associated with high mortality rate in immune-compromised patients (Todar, 2011) and also gender-wise prevalence showed 61.78% male and 38.22% females were infected by *P. aeruginosa* (Khan et al, 2008). Souli and colleagues (2008) published data from 23 countries on the European Antimicrobial Resistance Surveillance System and it was shown that 18% of all isolates were multidrug resistant *P. aeruginosa* strains (Souli et al, 2008). Aman Ullah and their colleagues (2012) carried out a study in Islamabad and showed that *P. aeruginosa* is 94% resistant to Chloramphenicol, 88% to Colistin /sulphate, 73% to Tetracycline and 3% to Imipenem (Ullah et al, 2012).

1.1.4 Nosocomial infection

The term "Nosocomial" comes from two Greek words: "Nosus" meaning "disease" + "komeion" meaning "to take care of." Hence, "Nosocomial" should apply to any disease contracted by a patient while under medical care. However, common usage of the term "Nosocomial" is now synonymous with hospital-acquired. Nosocomial infections are infections that are found in a hospital and are potentially caused by organisms that are resistant to antibiotics (Rosenthal et al., 2012). The Centers for Disease Control and Prevention (CDC) defines nosocomial infection (NI) as an infectious event that is diagnosed >48 hours after admission without evidence that the pathogen was already in the incubation phase (Yangco, 1989

In the United States, roughly 9.2 out of every 100 patients acquire a nosocomial infection, according to *Healthline and among them also* some HAIs can be quite serious and potentially life-threatening (Chalik, 2014). We can find different factors that can influence the development of HAIs, contaminated instruments, objects, and substances, poor use or maintenance of catheters and ventilators, patient/health care worker contact, contaminated air conditioning systems, understaffing, congested hospitals (beds in close proximity to one other), improper sterilization and disinfection practices, reusing syringes and needles. Although there are significant recommendations to control hospital acquired infections such as by controlling antimicrobial agents by individual prescribers, by making the hospital rooms well ventilated, proper cleaning and decontamination of surgical clothes, surgical instruments and floor of the hospitals, by using gloves during surgery, by not allow anyone to walk bare foot. Despite of all these control measures, it is unable to control the hospital acquired nosocomial infection due to the resistant mechanism of bacteria against the antibiotic (Reardon, 2015).

1.2 Rationale/ Statement of Problem

Nosocomial infections (NIs) are an important public health concern due to increasing numbers of immune compromised hospitalized patients. The World Health Organization (WHO) has reported that 1.4 million people around the world are suffering from NIs at any one time. Mostly nosocomial infections in hospitals nowadays are caused by

antibiotic resistant microorganisms and improper sterilization and disinfection practices (WHO, 2017). Disinfectants by chemicals are hazardous, harmful and irritant when used in excess amount and also, they equally target normal microflora of our body (Fiessinger et al., 1981). Therefore, most useful and effective replacement is natural agents (Virus) that kill or inhibit bacteria, which is Bacteriophage. Recent findings also support the potential of phage to be an alternative for decontaminants. Bacteriophage against Acinetobacter baumanii when used in hospital environment showed significant decrease in Acinetobacter-associated HAIs (Ho *et al*., 2016).

This study aims to focus on the study of phage activity as a disinfectant to decontaminate bacteria on fomite clothes and marble tile. Study of phage activity as disinfectant help us to study the efficacy of phage to minimize the bacterial load in fomites and marble tiles, responsible for nosocomial infection in the hospital.

1.3 Objectives

1.3.1 General Objectives

1. To evaluate the efficacy of bacteriophage to minimize the bacterial load responsible for nosocomial infection.

1.3.2 Specific Objectives

1. To isolate and identify bacteria from ICU ward of Teku hospital that are responsible for nosocomial infection
2. To perform antibiotic susceptibility test and molecular identification of isolated bacterial pathogen.
3. To isolate the lytic bacteriophage against the bacteria isolated from ICU ward.
4. To perform multi host range of the isolated phage.
5. To characterize the most potent phage morphologically and physiochemically.
6. To evaluate the reduction of bacterial load with bacteriophage lysate on hospital fomites.

1.4 Hypothesis

This research aims to answer “Can phages be used effectively as potential agent to minimize the nosocomial infection?”

1.4.1 Null hypothesis

H0: The bacteriophage does not reduce the load of the bacteria responsible for nosocomial infection on fomite.

1.4.2 Alternative hypothesis

H1: The bacteriophage significantly reduces the load of the bacteria responsible for nosocomial infection on fomite.

2. LITERATURE REVIEW

2.1 Nosocomial infection and its prevalence

Nosocomial infections are infections that occur while receiving health care that are developed in a hospital or other health care facility that appears in first 48 hours or more than 48 hours after hospital admission, or within 30 days after having received health care. There are multiple studies that indicate the common types of adverse events affecting hospitalized patients are adverse drug events and surgical complications. The US Center for Disease Control and Prevention studied that nearly 1.7 million hospitalized patients annually acquire NIs while being treated for other health issues and that more than 98,000 patients, which causes death of one among seventeen (Haque, M. 2018) and is a recognized public health problem world-wide with a prevalence rate of 3.0-20.7%, an incidence rate of 5-10%. It has become increasingly obvious that infections acquired in the hospital have led to increased morbidity and mortality which causes economic burden as well (Samuel, S et al., 2010).

Other studies conducted in high-income countries found that 5%–15% of the hospitalized patients acquire HCAIs which can affect from 9% to 37% of those admitted to intensive care units (ICUs) (Vincient, J-L. 2008). In the United States, it has been estimated that as many as one hospital patient in ten acquires a nosocomial infection, or 2 million patients a year. Estimate of the annual cost ranges from $4.5 billion to $11 billion and up. Nosocomial infections contributed to 88,000 deaths in the US in 1995. In France, prevalence was 6.87% in 2001 and 7.5% in 2006, some patients were infected twice. In Italy, in the 2000s, about 6.7% of hospitalized patients were infected, i.e, between 450,000 and 700,000 patients, which caused between 4,500 and 7,000 deaths. In Switzerland, extrapolations assume about 70,000 hospitalized patients are affected by nosocomial infections, between 2 and 14% of hospitalized patients. In India, 30 to 35 percent of persons admitted to hospitals develop HAIs. Among hospital-acquired infections 30 to 40% are urinary tract infections, 15 to 20% surgical wound infections, 15 to 20% lower respiratory tract infections and 5 to 15% blood stream infections. (The +Hindu article, 2008). A survey (2002-2007) study at Sir H.N. Hospital & Research Center Mumbai, found the highest incidence of *P. aeruginosa* (28.35%) was found in ICU, which was followed by IMCU (26.86%), NICU (16.41%), IPCU (14.92%), and ICCU (13.43%) (Balikaran, R. 2010).

2.2 Prevalence of nosocomial infection in Nepal

Nosocomial infection/Hospital acquired infections (HAI) are one of the most common complications of hospital care, leading to high morbidity and mortality. Hospital acquired infection includes Ventilator Associated Pneumonia (VAP), Central Line Associated Bloodstream Infection (CLABSI), Catheter Acquired Urinary Tract Infection (CAUTI), and Surgical Site Infection (SSI) (Koirala, A. 2018). There are different studies done in Nepal about prevalence of nosocomial infection in hospitals, a study conducted in the Grande International Hospital to review the profile of hospital at the time interval of one year where 664 patients were taken where majority of infections (21.08%) were seen among the age group of 65- 78 years and most of the HAIs in ICU were caused by MDR Gram Negative Organism & *Acinetobacter baumanni* was most common and

overall mortality due to HAIs was 12.61% (Acharya, D.S. 2018). Hospital acquired pneumonia at Tribhuvan University Teaching hospital was the most common HAI (49.61%) and Ventilator-associated pneumonia (VAP) had the highest incidence rate. *Escherichia coli* was the most frequently isolated microorganism (24.41%), among them 38% of the pathogens isolated were MDR (Shrestha, S. K., & Shrestha, P. K. 2018), among 491 patients of intensive care unit of Tribhuvan University Teaching Hospital, Kathmandu, Nepal patients suspected as hospital-acquired pneumonia (16%), bloodstream infections (5.7%), surgical site infections (4.7%), and urinary tract infections (3.9%) (Parajuli, N. P. et al., 2017).

Prevalence of Hospital Acquired Infection and its Preventive Practices among Health Workers in a Tertiary Care Hospital Prevalence of Hospital Acquired Infection and its Preventive Practices among Health Workers in a Tertiary Care Hospital Prevalence of Hospital Acquired Infection and its Preventive Practices among Health Workers in a Tertiary Care Hospital Prevalence of Hospital Acquired Infection and its Preventive Practices among Health Workers in a Tertiary Care Hospital Prevalence of Hospital Acquired Infection and its Preventive Practices among Health Workers in a Tertiary Care Hospital Another study done at National Trauma Center showed that prevalence of bacteria causing Hospital Acquired Infections was 11.83% in critical units (Shrestha, P., Rai, S., & Gaihre, S. 2017). Similary, Nine hundred clinical specimens were used for study at Tribhuvan University Teaching Hospital (TUTH), Kathmandu, Nepal during a period of March 2011 to February 2012, where prevalence of bacteria causing nosocomial infection were *Escherichia coli* followed by *Acinetobacter* species, *Klebsiella pneumonia* and *Staphylococcus aureus* (Sah, MK. *et.al*., 2014).

2.3 Bacteriophage to treat *P. aeruginosa*

Bacteriophages specific for the *Pseudomonas* genus were first described in the middle of the 20th century, and there are currently 137 completely *Pseudomonas* phage genomes found in public databases which was reported in January 27, 2015. Since *Pseudomonas aeruginosa* is one of the most problematic opportunistic pathogens involved in hospital-acquired infections, large fractions of the phage application study and genome sequencing projects have been focused on this bacterium (Alves, et al., 2014).

P. aeruginosa is one of the major opportunistic pathogens that causes nosocomial infections (Breidenstein et al., 2011) that causes chronic lung infections in cystic fibrosis but can be treated by using both phage lysins and live phage from back more than 50 years (Soothill, 2013). A cocktail of six phages was observed to successfully treat respiratory *P. aeruginosa* in mice and *Galleria mellonella* models (Forti et al., 2018). Phages were stable in spray dried formulations with less than 1-log10 titre reduction (Chang et al., 2017, 2018). A phage cocktail therapy done to treat acute *Pseudomonas aeruginosa* lung infection showed 95% efficacy in *invitro* when phage was administrated through pulmonary route and 90% survival at 48hours when aerosolized with 4 different nebulizers (Bodier-Montagutelli et al., 2018).

The efficacy of phage therapy was observed up to 7 days post infection in a biofilm-associated murine model where *P. aeruginosa* establishes a natural long-term chronic lung infection and also in an artificial sputum medium biofilm model, phage can effectively kill *P. aeruginosa* (Waters, E. M., 2017). Pires et al. 2011 used a broad-host-range phage for *P. aeruginosa* biofilm control and the effect of lytic phages in the prevention of *P. aeruginosa* biofilm formation in hydrogel-coated catheters (Fu et al., 2010) resulted in a 3-log reduction of biofilm cell populations after 48 h, compared with untreated catheters.

Overall, phage therapy has significantly contributed to control and even prevention of *P. aeruginosa* infections *in vivo*. Regarding the clinical trials in mouse models, most of the reported cases showed survival rates that ranged between 80% and 100% after phage treatment, and intraperitoneal or intranasal administration seemed to be the most efficient route of administration. Phages were also able to increase the life span of both *P. aeruginosa*-infected wax moth larvae and *P. aeruginosa*-infected *Drosophila melanogaster* (Pires, D. P., 2015). Finally, phage lysin research is also on the increase: Guo et al. described a novel endolysin with *in vitro* activity against *P. aeruginosa* and other Gram-negative bacteria on the critical priority pathogens list (Guo et al., 2017).

2.4 Bacteriophage to treat nosocomial infection

Due to the increasing resistance of bacteria against various antibiotics Bacteriophages have been considered as a potential antibiotic alternative for treating bacterial infections (Cao, F. et al., 2015). Morello, et al., 2011 showed that intranasal administration of Phage 1513 can also effectively treat pneumonia that is caused by *K. pneumoniae*. Another clinical trial was done by Aleshkin and his team; evaluate therapeutic effectiveness of the cocktail in an intensive care unit, caused by multi-resistant strains of *Acinetobacter baumannii, Klebsiella pneumonia, Pseudomonas aeruginosa* and *Staphylococcus aureus* where 79% of the initial samples from 14 patients' endotracheal aspirate, blood and urine were contaminated. Twenty-four hours after the 3-day phage therapy, contamination level dropped to 21% (Aleshkin, et al., 2016). LM33_P1 was infected with 025b *E. coli* strains which show 70% coverage on the two major antibiotic resistant pandemic clonal complexes ST13 1-025b:H4 and ST69-025 and the in vivo activity of bacteriophage LM33_P1 using three different extra intestinal virulence murine models showed that it infects bacteria in several organs. Prazak. et al., 2019 compared the efficacy of intravenous phage cocktail therapy ($2–3 \times 10^9$ pfu/ml, n = 12) with teicoplanin (3 mg/kg, n = 12), and combination of both (n = 11) showed that treatment with either phages or teicoplanin increased survival from 0% to 58% and 50%, respectively ($P < 0.005$).

Application of Commercial bacteriophage cocktail targeting *K*. *pneumoniae* which was administered orally over 5 days to patients hospitalized in the neonatal intensive care unit, where the rate of nosocomial *K. pneumoniae* infections decreased to zero and remained at this level for more than a month of surveillance in the ICU (Aslanov. et al., 2018). The bacterial pathogens *Klebsiella pneumonia* causes urinary tract infections in immune compromised patients of local hospital in Shanghai, China used Phage 117 as a biocontrol agent against *K. pneumoniae* isolates with carbapenem resistance (Tan, et al., 2019).

2.5. Bacteriophage as a disinfectant

Recently, phages have been widely used for the decontamination of food, but limited data are available in which phages are used for decontamination of hospital environment. Some research papers are found in which bacteriophages are used as disinfectant. A two-phase prospective intervention study was performed by Ho.etal.,2016 at a 945 bed public teaching hospital Taiwan where 264 Carbapenem-resistant *A. baumannii* (CRAB) were isolated and mainly responsible for healthcare-associated infection in intensive care units (ICUs) and to control this, the bacteriophage decontamination was conducted where 8 selected phages of 10^7 PFU (plaque forming unit) stock was passed singly through ultrasonic humidifier for 5 min which convert it in the form of cool fog and spread equally in the room and then the patients were sifted to the aerosolized ICU rooms. The result showed that CRAB in the intensive care units decreased from 8.57 per 1000 patient-days in the pre-intervention period to 5.11 per 1000 patient-days in the intervention period ($p = 0.0029$) where the mean percentage of resistant isolates CRAB decreased from 87.76% to 46.07% in the intensive care units ($p = 0.001$).

A comparative study was done by Accoti, et al., 2018, where the decontamination ability of phage and phage plus probiotic-based sanitation system (Probiotic Cleaning Hygiene System, PCHS) was tested in vitro (plastic, glass, and ceramic surfaces) and in situ (ceramic sink of a bathroom), against drug-susceptible or resistant *Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa* strains, and result showed that the combined use of phages and PCHS not only resulted in a rapid reduction (up to >90%) of the targeted pathogens, but also, due to the stabilizing effect of probiotics, the pathogens were maintained at low levels (>99%) at later times too whereas a significant reduction, compared to controls. In 2019 Accoti, et al., analyze the feasibility and effectiveness of phage addition to PCHS sanitation, which showed that a daily application of phage by nebulization induced a rapid and significant decrease in *Staphylococcus* spp. load on treated surfaces, up to 97% more than PCHS alone ($P < 0.001$). Treatments with phage cocktails produced complete inactivation (ca. 5–6 log CFU/ml) of EPEC920 and O157:H7 STEC464 on glass coverslips, and of EPEC920, non-O157 STEC ARG4827 and O157:H7 STEC464 on stainless steel coupons, at both temperatures (4 °C and 37 °C) at multiplicity of infection ca. 103 and 107 respectively (Tomat et al., 2014). Decontamination of fabric, glass slide and coverslip inoculated with MRSA strain treated with singular phage yield a 1-1.5, 1.5 and 0.5 log reduction in CFU/ml compared with mock treatment with a p-value ≤ 0.01, ≤ 0.005 and ≤ 0.02 respectively whereas cocktails of phage were typically more effective at decontaminating MRSA than single phage (Jensen, et al., 2015).

2.6: Application of bacteriophage

A part from bacteriophage as a disinfectant, there are many applications of bacteriophage:

2.6.1. Phage therapy

Phage therapy has many advantages over antibiotics for therapy, but there are also concerns. This approach has been used in animal, plants, and human beings with varied degrees of success. One of the advantages of using phage is their specificity to the target

bacteria and lack of interference with the host normal flora. After phage administration, they spread quickly through the human body reaching every organ. However, the immune response generates antibodies that clear the systemic phage and that is one of the major concerns about using phage therapy. One way to circumvent this is to use the phage's lytic enzymes such as endolysins and holins for therapy instead of using the whole viron particle (Haq et al., 2012).

2.6.2 Phage display

In the phage display technique, DNA, which encodes the desired polypeptide, is fused within the coat protein genes for the phage. Then the desired protein is produced and expressed on the surface of the phage (Smith, 1985). Phage display can be used to generate antibody fragments libraries using the filamentous phage such as M13 phage. These display libraries have been involved in many applications such as in the treatment of cocaine addiction. First, the phages are administrated nasally until it reaches the central nervous system where the displayed antibody binds to the molecules of cocaine and prevent its effect on the brain (Dickerson et al., 2005).

2.6.3 Phage typing

The use of phages as a diagnostic tool or for phage typing depends upon the sensitivity patterns of bacteria to certain phages. There are various methods that can be used to detect pathogenic bacteria. For example, phages can be used to deliver reporter genes, which can be detected post infection. Phage adsorption can be detected by using phage that has fluorescent dye attached to their coats (Goodridge et al., 1999). Detection of released bacterial proteins following bacterial lysis due to phage infection, such as adenylate kinase, can be detected by using antibodies produced by phage display that will bind specifically to these complexes (Petrenko and Vodyanoy, 2003). The phage amplification assay is the most technique that have been used to detect bacteria such as Pseudomonas, E.coli, Mycobacterium tuberculosis, Salmonella, Campylobacter and Listeria species (Barry et al., 1996).

2.6.4 Bioprocessing and bio control

Bacteriophages are used in bioprocessing to decrease the bacterial load in foods. Especially, foods that are minimally processed to avoid cooking associated texture or flavor (Garcia et al., 2010). Phage bioprocessing has been employed to reduce the growth of many food pathogens such as *Salmonella enteritidis* in cheese, *Campylobacter* and *Salmonella* on chicken skin, and *Listeria monocytogenes* on meat. In addition, this approach can be used to extend the shelf life of animal products (Dykes and Moorhead, 2002).

Phages can be used as well as predators of bacteria that associated with fungi, plants or their products. Phage biocontrol of plants pathogens has been a successfully used against *Xanthomonas pruni* on peaches, peppers, and cabbage plants. These methods also worked against *Ralstonia solanacearum* on tobacco and *Xanthomona campestris* on tomatoes.

3. MATERIALS AND METHODS

3.1 Preparation of media

Nutrient Agar (NA), Maconkey Agar (MA), and Luria Bertain (LB) broth were used to culture and subculture bacteria. To confirm Multi Drug Resistant (MDR) bacteria, Muller Hinton Agar (MHA) was used. Similarly, Tryptic Soya Broth and Sodium chloride and Magnesium sulfate (SM) Buffer were used for Bacteriophage isolation, purification and amplification. Cetrimide agar (CA) was used for growth of bacteria after decontamination assay by phage on apron pieces and tiles.

3.2 Bacteria collection, isolation and identification

Bacterial samples were collected from ICU ward of Teku Hospital by plate exposure and swabbing method. Swabbing was done from different utensils and lab coat of the nurse of ICU ward. Similarly, NA (nutrient agar), MSA (Mannitol Salt Agar) and cetrimide agar plates were exposed for 30 minutes in the ICU ward. The plates were safely transported to the Central Department of biotechnology (CDBT). The plates were then incubated at 37°c for 24 hours. After incubation, next day individual yellow mucoid colony from MSA plate and greenish colony from cetrimide plate were subculture on the NA plate for further biochemical test and Gram staining. Biochemical tests performed were Indole test, Methyl Red (MR) test, Voges Proskauer (VP) test, Citrate utilization test, Triple Sugar Iron Agar (TSIA) test, Oxidase test and Urease test.

Table3. 1: Bacteria used and their code name

SN	Bacteria	Code name
1	*Pseudomonas aeruginosa*	P_1
2	*Pseudomonas aeruginosa*	P_2
3	*Pseudomonas aeruginosa*	P_3
4	*Pseudomonas aeruginosa*	P_4
5	*Staphylococcus aureus*	Sa_1
6	*Staphylococcus aureus*	Sa_2
7	*Staphylococcus aureus*	Sa_3
8	*Staphylococcus aureus*	Sa_4
9	*Staphylococcus aureus*	Sa_5
10	*Staphylococcus aureus*	Sa_6
11	*Staphylococcus aureus*	Sa_7
12	*Staphylococcus aureus*	Sa_8

13	*Staphylococcus aureus*	Sa_9
14	*Staphylococcus aureus*	Sa_{10}
15	*Staphylococcus aureus*	Sa_{11}
16	*Staphylococcus aureus*	Sa_{12}

3.3 Antibiotic susceptibility test

All the isolated bacteria were tested for antibiotic susceptibility test by Kirby- Baure disc diffusion method. Different classes of antibiotics were used like, Meropenem, Imipenem, Ciprofloxacin, Methicillin, Vancomycin, Ampicillin. For AST, a small quantity of bacterial culture was inoculated in LB broth and incubated at 37 degree Celsius. A 0.5 McFarland was prepared by adding 9.95ml of 1% H2SO4 and 0.05ml of 1% Barium chloride. Bacterial density in the culture was compared with 0.5 McFarland and when similar turbidity was achieved, the bacterial culture was taken for AST.

For AST testing, 100 microliters of above culture was spread plated in MHA (Muller Hinton Agar) plate and antibiotic discs were aseptically put firmly in the plates. The plates were then cultured at 37 degree Celsius for 24 hours. After incubation the clear zone around antibiotics (zone of inhibition) was measured in millimeters using ruler. The size of zone of inhibition was compared with provided standard data to determine if the organism was sensitive, intermediate or resistant with that antibiotic.

3.4 Molecular detection of *Pseudomonas aeruginosa* bacteria

Isolated *Pseudomonas aeruginosa* was further confirmed by sequencing the 16S rRNA gene Xceleris Genomics India.

3.4.1 Genomic DNA extraction of bacterial strain

Genomic DNA extraction was done by using bacterial DNA isolation Kit. Quality of gDNA was checked on 0.8% agarose gel for the single intact band. Genomic DNA samples were loaded into the wells of 0.8% agarose gel and run gel at 110V for 30 mins. The gel was then observed in UV transilluminator for single intact band.

3.4.2 PCR amplification of 16SrRNA gene

Molecular identification of the host bacteria was done by PCR using primer16SrRNA. PCR amplification was done. For PCR amplification already optimized PCR condition and PCR reaction volume was used shown in table 3.4 and 3.3 respectively.

Table3. 2: Forward and Reverse primer sequence used for PCR

Primer name	Tm	Sequence	Amplicon size
27F	56.4	AGAGTTTGATCMTGGCTCAG	1500
1492R	56.4	CGGTTACCTTGTTACGACTT	1500

PCR reaction mixture was prepared in PCR tubes as mentioned in table and subjected for PCR amplification. After the completion of PCR amplification, 5µl of the amplified

product was analyzed by 1 % agarose gel electrophoresis on Tris Acetate EDTA (TAE) buffer containing 0.2µg/ml Ethidium Bromide (EtBr) as a DNA staining solution. Gel was viewed on a UV transilluminator and image captured with the help of Gel Doc.

Table3. 3: PCR component, their concentration and volume used for PCR reaction

SN	Component	Concentration	Volume
1	Master mix	1X	12.5
2	Forward primer	10pmol/µl	1
3	Reverse primer	10pmol/µl	1
4	Template	100ng/µl	1
5	NFW		9.5
	Total		25µl

PCR product was then sent to Xceleris Laboratory, India for sequencing of 16s rRNA. Sequence obtained from the lab was analyzed by using different bioinformatics tools. Chromas was used for analysis of chromatogram file and to know the sequence similarity between our sequence and nucleotide database sequences, direct BLAST search was done.

3.4.3 PCR Condition of 16srRNA gene amplification

Table3. 4: Thermo cycling condition for 16srRNA amplification

SN	Steps	Temperature(ºC)	Time	Cycle
1.	Enzyme Activation	95	5min	
2.	Initial Denaturation	95	30sec	
3.	Annealing	55	30sec	35
4.	Extension	75	2.5min	
5.	Final extension	72	5min	
6.	Final hold	4	∞	

3.5 Sample collection for bacteriophage isolation

Water samples were collected from different river of Kathmandu valley. Sample was taken from Balkhu River, Teku River and Basundhara River, Sali Nadi River. While collecting the sample stacked water was preferred rather than the running water. 30 ml water was collected in sterile falcon tubes, carried to the Central Department of Biotechnology (CDBT) lab and it was then centrifuged to 4000 rpm for 30 minutes in order to remove unwanted contaminants and other bacterial cell debris. Now, with the help of membrane filter of size 0.22µm (PES Filter Media,Whatman™) syringe filter was

done to remove bacterial contamination and other unwanted materials .The filtrate was collected in sterile falcon tubes and stored at 4°C for further use.

Figure 3. 1: Representative figure of water sample collection

3.6 Bacteriophage isolation

Bacteriophage isolation was done by standard technique of Double Layer Agar Assay (DLAA) method using Kropinski et al., 2009 method. Briefly, the processed (syringe filtered) 1ml water sample was taken in a sterile tube and log phase bacterial growth (100µl) was added on it and allowed to remain for attachment. Then, 3ml semisolid Tryptone soya broth (0.5% agar) was added to tubes and poured into the already prepared Tryptone soya agar plate (1% agar). After solidification of the media then plates were incubated in the incubator at 37°C for 24 hours (Chase & Bradely, 2011). After incubation presence/absence of plaque was observed. Two types of plaques were observed on the plate, round bull's eye and small pin head. Small pin head plaques were selected for further processing. Plaques were characterized by size, shape and turbidity.

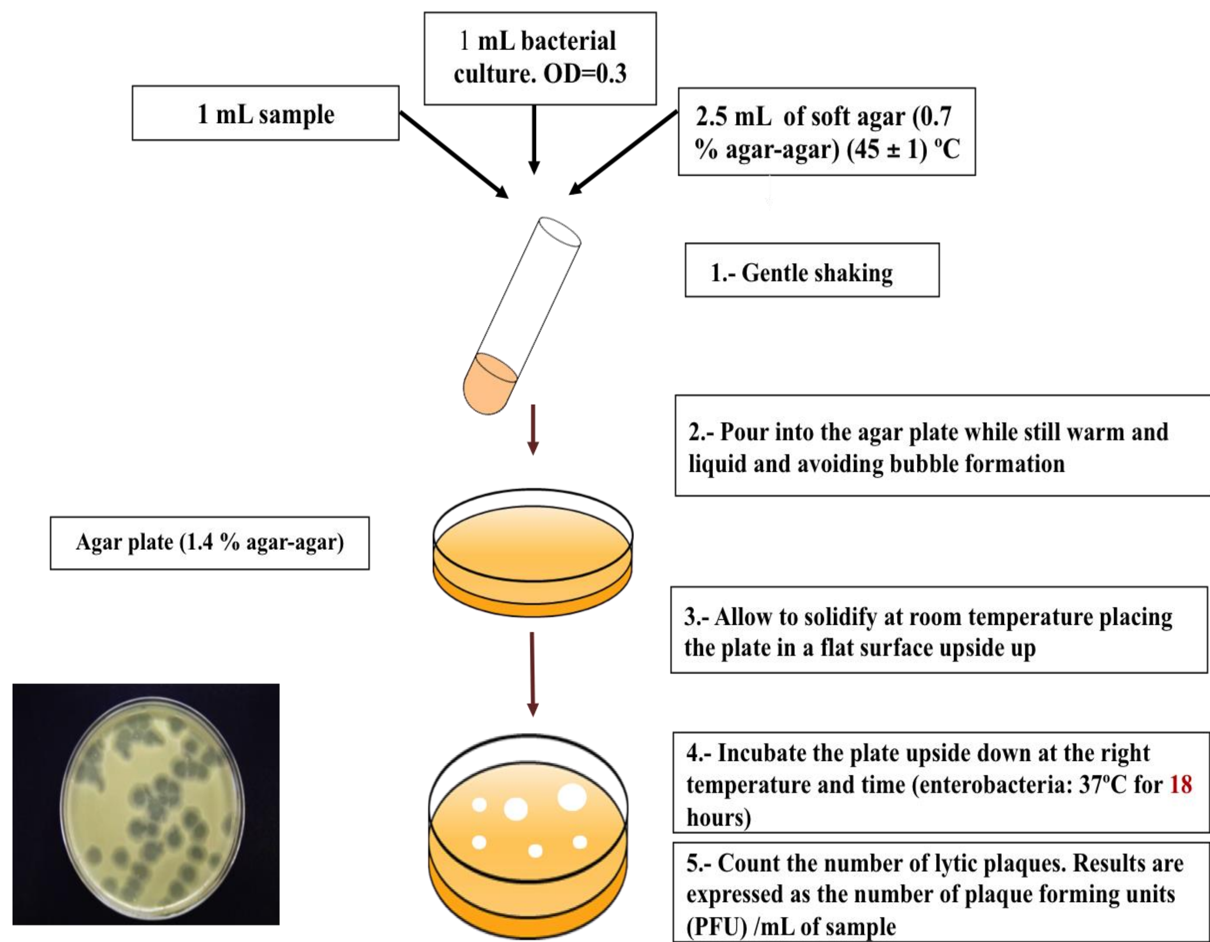


Figure 3. 2: DLAA technique for phage isolation and culture

3.7. Bacteriophage purification: Phage streak protocol

The initially formed plaques may be of different types, so for further processing and characterization of phage it should be obtained in pure form. Analogous to bacteria, each phage plaque is presumed to be clonal, having originated from a single virion. The picking and sub-culturing of plaques ensures that a phage population is descended from a single virion and is therefore clonal, or "pure". Streaking the phage on agar plate and overlaying with bacterial lawns is an efficient way of isolating single plaques from sample putatively heterogeneous populations. The technique is very similar to streaking for single bacterial colony. First the single isolated phage was touched at the center with a sterile wooden toothpick and streaked on 1.5% TSA plate making primary, secondary and tertiary inoculums. Then 100 µl of host bacterial culture was mixed with 3ml of soft agar in a sterile falcon tube and poured in streaked petri plate starting from the end point of streaking then incubated overnight at 37˚C after solidification of agar. Three subsequent rounds of streaking were performed to obtain pure phage plaques (Clokie & Kropinski, 2009).

3.8. Phage lysate/stock preparation

Phage stock solution was prepared by amplifying phages in petri dishes. Eight TSA plates were streaked with a single plaque by continuous streaking method and allowed to dry. Then after 100 ul of bacterial culture in LB was mixed with 3ml of soft agar and overlaid on streaked plates. The petri plates were then incubated at 37˚C overnight after

solidification. Next day 3 ml of SM buffer was poured on each plate containing plaques and allow agitating in shaker for 5 hours at 100rpm. SM buffer help to absorb and detach the phage particles from the media. Then the upper layer of soft agar along with SM buffer was scraped with sterile cotton buds and transferred to falcon tube. The mixture was then centrifuged at 4000 rpm for 30 minutes to settle down the debris. Transferring the filtrate to another falcon tube centrifugation was repeated for next 15 minutes. Then the filtrate was filtered through 0.22µm pore size syringe filter in a sterile falcon tube and thus the stock solution of phage was ready. The stock was stored at 4°C for further use and characterization (Fortier & Moineau, 2009).

3.9 Determination of phage titer

For the titer determination of phage, the phage stock was diluted into different dilution ranging from 10^{-1} to 10^{-14}. The stock culture is the 10^{0} dilution. To dilute stock, 100ul of stock was added to 900ul SM buffer and labeled 10^{-1} dilution and from each upper dilution 100ul was added to 900ul SM buffer to produce 10-fold lower dilutions.

Spot assay was utilized for titer determination. In this, grids were drawn on the bottom of TSA plate for spot test of each dilution. Then the bacterial lawn was prepared by pouring the mixture of 100 µl active log phase bacteria (P3) in 3ml soft agar into the labeled TSA plates. After allowing drying, 5 µl of respectively prepared phage dilutions were spotted aseptically onto corresponding grids as labeled. Only SM buffer was used as negative control. The droplets were allowed to soak into the agar and plates were incubated at 37°C for 24 hours in inverted position. Next day, the plates were observed for the clear zone of bacterial lysis/plaques on spots (Anderson et al., 2011).

For determining the concentration of phage in stock solution, the last three dilutions which showed clear lysis on spot assay were used. DLAA was performed taking individual dilutions and host bacteria (P3) and incubated overnight after solidification. After overnight incubation, the plates were observed for plaque formation and the distinguishable plaques were counted. The plaque forming unit per ml was calculated by using the following formula:

$$\text{pfu/ml} = \frac{\text{number of plaques observed}}{\text{dilution} \times \text{volume of sample}}$$

3.10 Determination of Optimum Multiplicity of Infection (MOI)

MOI is defined as the ratio of the number of phages infecting a number of bacterial host cells. To determine the optimal MOI of phage, different dilution of bacteria and phage are mixed with each other so that the ratio between the numbers of phages to number of bacteria gives MOI (Abedon, 2016).

$$\text{MOI} = \frac{\text{number of phage}}{\text{number of bacteria}}$$

Different MOI of 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 were prepared and added to 10 ml of sterile LB broth and allowed to incubate in shaking incubator at 37°C for 24 hours. The solution was observed for clarity as turbidity refers to growth of bacteria. The

lowest MOI at which clear solution without bacterial growth was observed was taken as optimal MOI.

3.11 Characterization of phage

Newly isolated *Pseudomonas aeruginosa* phage was further characterized:

3.11.1. Host Range Analysis

Both intra specific and inter genus host range analysis was done by spot assay with slight modification of Verma et al., 2009. For intra specific host range, all the Pseudomonas strains were grown aseptically to the active log phase. Three milliliters of 0.5 % warm (50°C) soft agar was mixed with 100µl of each bacterial culture in different sterile test-tube and poured on properly labeled separate fresh Tryptic soya agar plate (TSA). Petri plates were swirled so as to distribute top agar evenly and left to solidify. Isolated *Pseudomonas* phage was used for host range and grid were marked in plate for each phage. SM buffer was used as a negative control. After solidification of the top agar layer, 5µl of the phage stock was applied to spots on the corresponding marked line and left for 15-20mins to dry. The plates were incubated for 24 hours at 37˚C and checked for the presence or absence of bacterial lysis and clear zone. Double layer agar assay was done for those host strain which shows the intra host range during spot assay and number of plaques were counted after DLAA. For interspecies host range, different strain of *Klebsiella*, *Pseudomonas, Acinetobacter* and *Salmonella* were collected and revived these species were already available in the CDBT Laboratory. Single phage of Pseudomonas, cocktail of *Pseudomonas, Klebsiella, Acinetobacter,* and *Salmonella* were tested. At first, lawn culture of all 28 bacteria was prepared by mixing 100µl active log phase bacteria with 3ml soft agar (Vermal et al., 2009). The mixture was then poured into TSA plates and allowed to set for few minutes. Like the spot assay performed above, blocks were already made for different phages to be spotted. 5µl of each phage was pipetted on corresponding blocks and allowed to soak for about 15 minutes. Then the plates were incubated overnight at 37˚C. Presence of clear spot after incubation shows lysis of bacteria by corresponding phage.

3.11.2. One step growth curve analysis and burst size determination

For one-step growth curve, 0.01 MOI of bacteria and phage were prepared and added to 30 ml sterile LB broth (Jin *et al,* 2012). The LB broth was then incubated in shaking incubator at 37°C for 1.5 hour and during this time; samples were taken at 5 mins, 10 mins, 20 mins, 30 mins, 40 mins, 50 mins, 60 mins, 70 mins and 80 mins. The samples were then centrifuged at 12000 rpm for 2 mins to settle bacterial debris. The supernatant was taken and mixed with 3ml soft agar, 100ul bacterial culture and plated on TSA plate. During plating after 30 mins, the supernatant was diluted 10 times and for both diluted and original supernatant, DLAA was performed. The plates were incubated at 37°C for 24 hours and numbers of plaques were observed.

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

3.11.3. Protein profiling by Sodium Dodecyl Sulfate Polyacrylamide Electrophoresis (SDS-PHAGE)

SDS-PAGE of phage protein was done, in which, sample was prepared by direct heating method and acetone precipitation method.

3.11.3.1 Sample preparation

a) Direct heating method: In this method, 25µl of purified phage was mixed with equal volume of 2X sample buffer and heated in heating mantle at 95°C for 10 minutes to denature protein (kumari, Harjal *et al*, 2009.

b) Acetone precipitation method: For acetone precipitation method, purified phage solution and ice-cold acetone were mixed in the ratio of 1:4 (200µl:800µl) and vortexed (Sillankorva, 2009). Then, the vortexed sample was incubated for 60 minutes at -20°C for phage precipitation (Urban-Chmiel, et al., 2018). After incubation, the sample was centrifuged at 13000rpm for 10 minutes. The supernatant was decanted and pellet was air dried and was resuspended in 50µl PBS buffer (8 g l-1 NaCl, 0.2 g l-1 KCl, 0.2 g l-1 KH2PO4, 1.44 g l-1 Na2HPO4 × 2H2O, pH7.5). Acetone precipitation of phage helps to concentrate the different proteins of phage particles so that proteins can be easily visualized in the gel. Denaturation of protein was performed as in direct heating method.

3.11.3.2 SDS-PAGE

SDS-PAGE was performed as described by Laemmli (1970) with slight modification. Separation was carried out in 12% resolving gel (Tris–HCl buffer with pH 8.8), and 4% polyacrylamide in Tris–HCl buffer (pH 6.8) was used as a stacking gel. Electrophoresis was carried out in standard Tris–glycine chamber buffer at a constant current of 400 mA till the tracking dye reached the bottom of the gel. A molecular weight standard (Protein Ladder, (Genei) with a molecular weight range from 7 to 240 kDa was used as protein marker (Urban-Chmiel, et al., 2018). After electrophoretic separation, the gels were stained with Bio-Safe Coomassie brilliant blue solution for certain hours in a shaker and then suitably destained with destaining solution for best visibility of protein bands. The gel was scanned in scanner and photograph of separated protein bands was taken (Sangha, et al., 2014).

3.11.4. Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) the potent lytic phages were selected for Transmission Electron Microscopy. The phage titer was maintained at a range of 10^{-8} - 10^{-10} pfu/ml. Phage lysates were transported to Advanced Instrumentation Research Facility – Jawaharlal Nehru University (AIRF-JNU), New Delhi, India in cold chain. TEM of bacteriophages was performed using the method described by Ackermann (Ackermann, 2009). Phages lysates were fixed with fixative (2.5% glutaraldehyde and 2% paraformaldehyde, prepared in 0.7M sodium phosphate buffer (pH 7.2). For fixation, equal volume of phage lysate and fixative were added, mixed and left overnight. Next day, the fixed phages were subjected to high-speed centrifugation (35,000g) for 3 hours. Pellet of phages was deposited on separate 300 mesh carbon coated copper grid and then flooded with 2%(w/v) uranyl acetate (pH 4.5) and after 2 min excess stain was soaked-off with blotting paper. The copper grid was dried and examined in Transmission

electron microscope under various magnifications. Phage morphology was observed from the micrographs. The TEM micrographs were analyzed through ImageJ 1.52a (https://imagej.nih.gov/ij) for determining tail size (width and length) and size of phage capsid/ head. Three readings of head and tail, width and length, were recorded and mean value was taken. The bacteriophage isolate was assigned to a respective family in accordance with the recommended guidelines of the International Committee on Taxonomy of Viruses (ICTV), based upon examination of virion particle morphology.

3.11.5. Stability of phage against temperature

The determination of Stability of phage against different temperatures was determined by exposing the phage to different temperature and time. The stability against temperature and pH was done by taking a reference from (D'Andrea *et al*., 2017) first, the phage stock was diluted to different dilution with SM buffer and 10^8 diluted solutions was taken at which countable plaques of phage were found. 1ml of 10^8 diluted phages were kept in different sterile eppendorf tubes and these were kept to 37°C, 40°C, 50˚C, 60˚C, 70˚C and 80˚C temperature for different time period 10min, 20min, 30min, 40min,50min and 60min. Incubation was done in dry heat such as incubator, heating mantle and hot air oven. After incubation in different time and temperature, the tubes were withdrawn from the temperature at their respective time and immediately 100µl of log phase host *Pseudomonas aeruginosa* at OD 0.1(10^8PFU/ml) was mixed with the heat-treated phage solution and DLAA was done. After overnight incubation, the number of surviving plaques were counted and expressed as PFU/ml then graph was plotted PFU/ ml against temperature.

3.11.6. Stability of phage against pH

pH stability was done according to (Han *et al.,* 2014) with slight modification. pH ranging from 1-13 was prepared by adjusting the pH of fresh LB broth. 1M HCL was added drop by drop to achieve desired acidic pH and 1M NaOH was used to achieve desired basic pH. 900µl of pH adjusted LB broth was taken in thirteen different eppendorf tube (pH from 1-13) and autoclaved. 100µl of phage suspension of high titer (10^8PFU/ml) was mixed to make a final solution 1ml and incubated for 1hours at 37°C. Phage suspension having pH7 was used as control. After the incubation period, phage titer was determined by double layer agar method against host bacteria *Pseudomonas aeruginosa*. The counts of surviving phage were expressed as PFU/ml and plotted against pH values.

3.12 Application of phage as disinfectant

3.12.1. Use of bacteriophage on contaminated cloth

Cloth decontamination assay was performed according to (Jensen et al., 2015) with slight modification. Laboratory coat materials are composed of 35% cotton and 65% polyester. 1.5×1.5cm pieces were prepared and autoclaved to achieve sterility. *Pseudomonas aeruginosa* bacteria were cultured to logarithmic growth, and then diluted to obtain 10^5 CFU/ml. 100µl of 10^5 CFU/ml bacterial culture was added to the pieces of lab coat (pieces of lab coat were kept on glass slide and covered with Petridish) and allowed to remain for 30min at 37°C. After that 100µl of phage titer was then added and incubated at 37°C for 1hr. Phage titers added to the cloth ranged from 10^5PFU/ml to 10^9PFU/ml for a range of MOI of 1 to 10,000. For control, sterile phage buffer with bacteria was added in one lab coat piece. After one-hour incubation bacteria were

removed by placing the cloth into 500µl LB broth and vortexes at high speed for 10second. Cetrimide agar plate were prepared and labeled as control, MOI 1, MOI 10, MOI 100, MOI 1000, MOI 10,000. 100µl broth was taken and put on the cetrimide agar plate, spreading was done and incubated at 37°C overnight. Next day colonies were counted on the plates and compared with the colonies on the control plate.

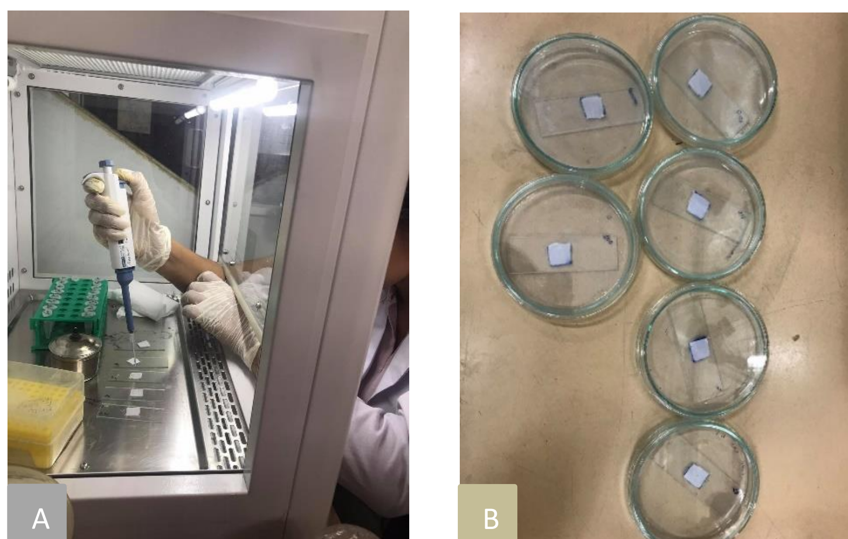


Figure 3. 3: Decontamination assay on fabric cloth.

Figure A is the addition of 100µl of bacterial culture added to the piece of lab coat in slides. Figure B is the pieces of lab coat kept on glass slide, covered with Petridis and ready to be incubated at 37°C.

3.12.2. Comparison between *Pseudomonas aeruginosa* phage and phenol

Comparison between *Pseudomonas aeruginosa* phage and phenol was done using above procedure. This experiment was done to compare the effectivity of phage and phenol on the cloth contaminated with bacteria. Concentration of phenol was kept ranging from 10^{-5} dilution to 10^{-9} dilution and concentration of bacteria was kept 10^{5}CFU/ml.

3.12.3 Decontamination by bacteriophage on marble tiles

Decontamination by bacteriophage on marbles was done by following (Accolti., 2018) protocol with slight modification. Host bacteria was grown on LB broth reaching the logarithmic growth phase 0.1 at OD600nm, then diluted with LB broth to obtain a final concentration of 10^{6} CFU/ml. 8pieces of 5×5cm surface marbles tiles were taken and labeled as control after 1hr, MOI 1 after1hour, MOI 1 after 3 hours, MOI 1 after 6hrs, MOI 1 after 24hrs, MOI 10 after 1hr, MOI 10 after 3hrs, MOI 10 after 6hrs. 10µl of bacterial suspension was spread on the surface of tile obtaining final concentration of 10^{6}CFU/ml and allowed to dry at room temperature for 15minutes. After that 100µl of phage lysate of titer 10^{6}PFU/ml and 10^{7}PFU/ml was added to the surface to obtain the MOI 1 and MOI 10 respectively and incubated at 37°C. After 1hr, 3hrs, 6hrs and 24hrs, surface of tiles were directly touched on the surface of the Cetrimide agar plate and spreading was done, incubated at 37°C. Next day colonies on the plates were counted and compared with control.

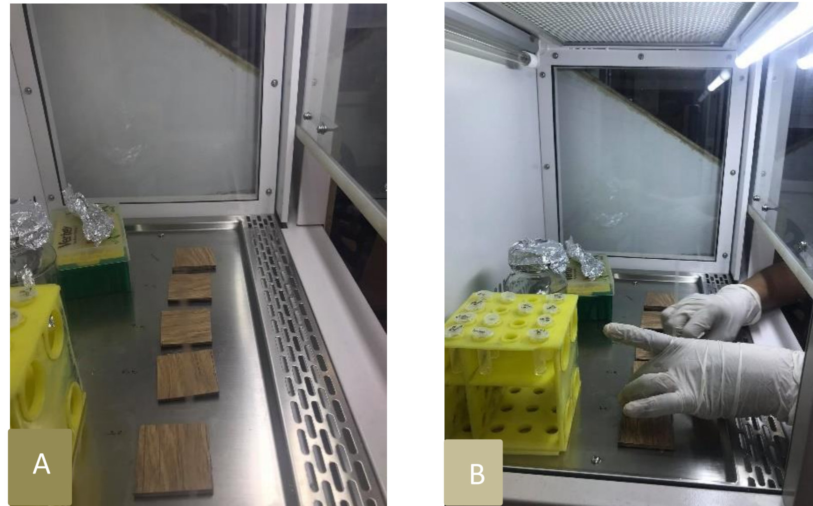


Figure3. 4: Decontamination assay on marble tiles

Fig A: Sterile marble tiles ready for decontamination assay. Fig B: Bacterial sample was added by swabbing in the tile.

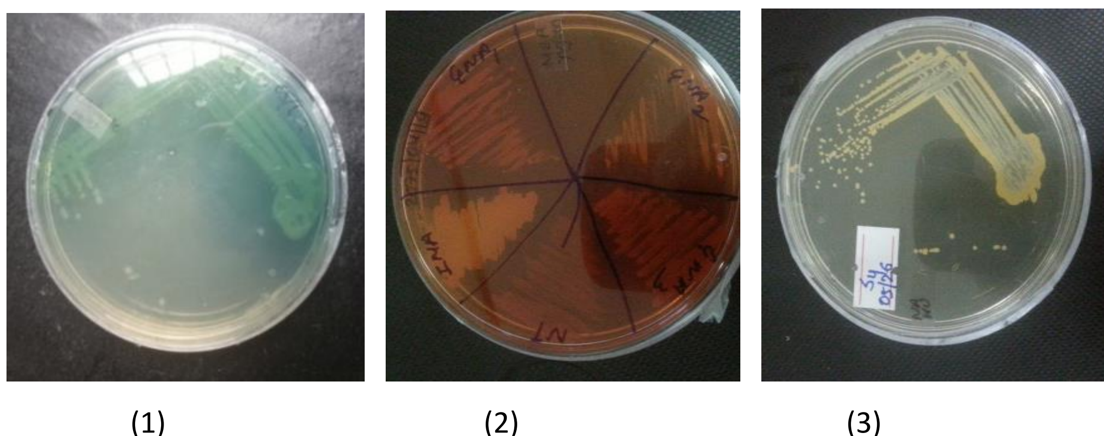
3.13 Statistical analysis

Statistical analysis was done by using Statistical Package for social science (SPSS). The data were evaluated using paired t- test.

4. RESULTS

4.1. Isolation and identification of bacteria

Sixteen samples of *Pseudomonas aeruginosa* (4) and *Staphylococcus aureus* (12) were isolated from ICU ward of Teku hospital by plate exposure method. The colony with green pigment on cetrimide agar was presumed to be *Pseudomonas aeruginosa* and colony with yellowish pigment on Mannitol Salt Agar (MSA) was presumed to be *Staphylococcus aureus* which was then picked and sub cultured in NA agar for Gram staining and biochemical identification. Cultures were named as Sa1, Sa2 and so on up to Sa12 for *Staphylococcus* species and P1, P2 and so on up to P4 for *Pseudomonas* species.



(1) (2) (3)

Figure4. 1: Subculture on NA from cetrimide agar plate in (1) and subculture in NA plate from MSA plate in NA plate in (2 & 3).

4.1.1: Gram staining

The colony from NA plates were Gram stained and organisms were found to be: Both Gram positive, dioplococci in figure (A) and Gram Negative, Rod Shaped in figure (B)

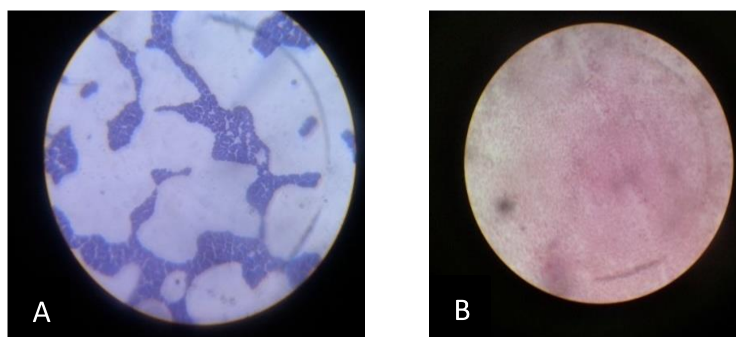
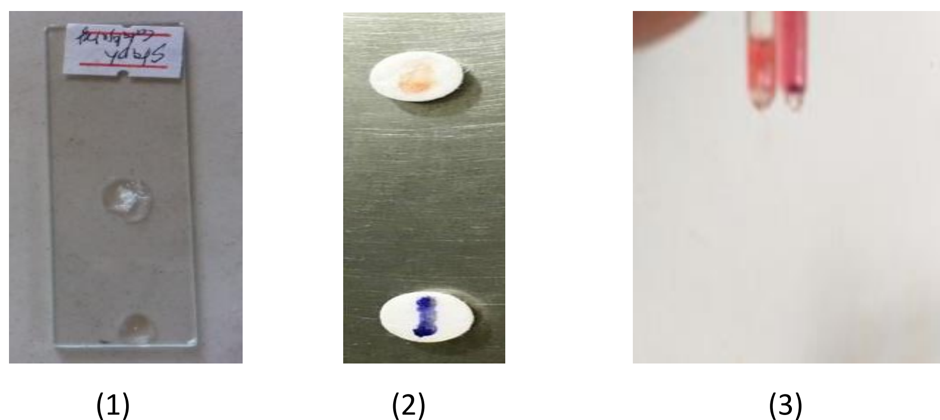


Figure4. 2: Gram staining

4.1.2: Biochemical tests

Biochemical test was performed for both Gram positive and Gram-negative bacteria. For Gram Positive bacteria, Catalase, Coagulase and Oxidase tests were done and for Gram negative bacteria, Indole, Methyl Red, Voges Proskauer, Citrate (IMViC) tests were done.

For Gram positive bacteria



(1) (2) (3)

Figure4. 3: Biochemical test for Gram positive bacteria

In figure (1), Gram positive sample on the top showing catalase positive and sample on the bottom is control, in figure (2), Gram positive sample on the top showing oxidase negative and sample on the bottom is control showing oxidase positive of Gram-negative bacteria and in figure (3), Gram positive sample on right side showing coagulase test and sample on the left is control.

Form these above tests for Gram positive bacteria; we can confirm that the bacteria are *Staphylococcus aureus*.

For Gram negative bacteria

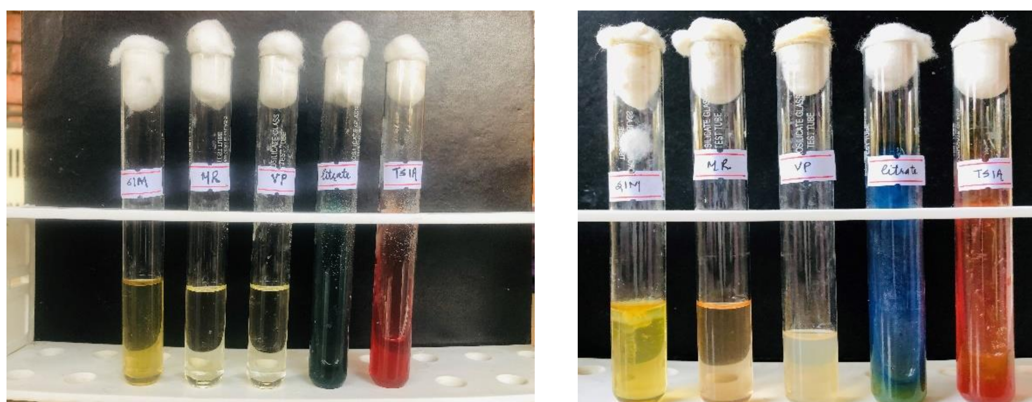


Figure4. 4: IMViC and TSIA test result (Left: Control, right: with test organism) Media are SIM (Simons Indole Motility), MR, VP, Citrate and TSIA from left to right in each individual figure.

The biochemical tests of all the isolates of Gram negative showed following result:

Indole: Negative

Methyl Red (MR): Negative

Voges Proskauer (VP): Negative

Citrate: Positive

Triple Sugar Iron Agar (**TSIA) test:** No H_2S producer

Form these above tests for Gram negative bacteria, we can confirm that the bacteria are *Pseudomonas aeruginosa.*

4.2 Antibiotic susceptibility test

Antibiotic Susceptibility Test (AST) of 16 bacterial strains (4 strain of *Pseudomonas aeruginosa* and 12 strains of *Staphylococcus aureus)* was done. Among them 2 strains of

Pseudomonas aeruginosa (P3 & P4) and 5 strains of *Staphylococcus aureus* (Sa3, Sa5, Sa12, Sa15 and Sa11) were found to be Multidrug Resistant (MDR), Sa3 was found to be Vancomycin Resistant *Staphylococcus aureus* (VRSA). Both the bacterial strain of *Pseudomonas aeruginosa* showed resistance to imipenem and meropenem drugs which confers the carbapenem resistant.

Table4. 1 Antibiotic Susceptibility pattern of host bacterial strain

Antibiotics	Reference Zone (mm)			Zone of Inhibition Diameter (mm)			
	S	**I**	**R**	**Sa1**	**Sa3**	**P3**	**P4**
Ampicillin	>17	14-16	<=13	12 (R)	0 (R)	NT	NT
Ciprofloxacin	>35	28-35	<=27	29 (I)	0 (R)	0 (R)	0 (R)
Methicillin	>21	18-20	<=17	22 (S)	8(R)	0 (R)	0 (R)
Vancomycin	>21	17-21	<=16	26(S)	12	0 (R)	0 (R)
Meropenem	>31	22-30	<=21	NT	NT	0 (R)	0 (R)
Imipenem	>32	24-31	<=23	NT	NT	0 (R)	0 (R)
Ofloxacin	>22	17-21	<=16	NT	NT	0 (R)	0 (R)

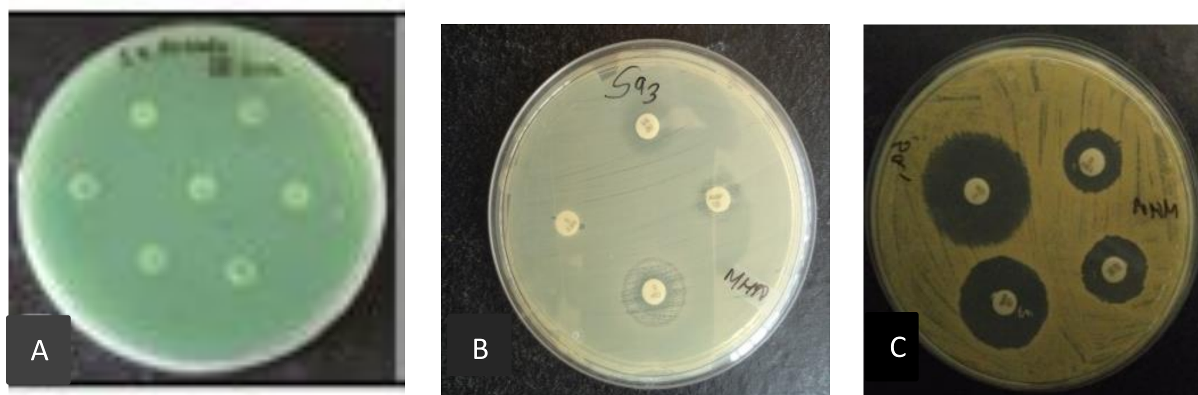


Figure4. 5: AST of *Pseudomonas aeruginosa* and *Staphylococcus aureus* bacteria

4.3 Bacterial genomic DNA extraction

Bacterial DNA extraction was done by Kit method, distinct bands of DNA was observed for 2 different strains of *Pseudomonas aeruginosa* analyzed in 0.8% agarose electrophoresis which showed smiley bands when observed in UV transilluminator. Smiley bands represent low concentration of genomic DNA in the sample as shown below.

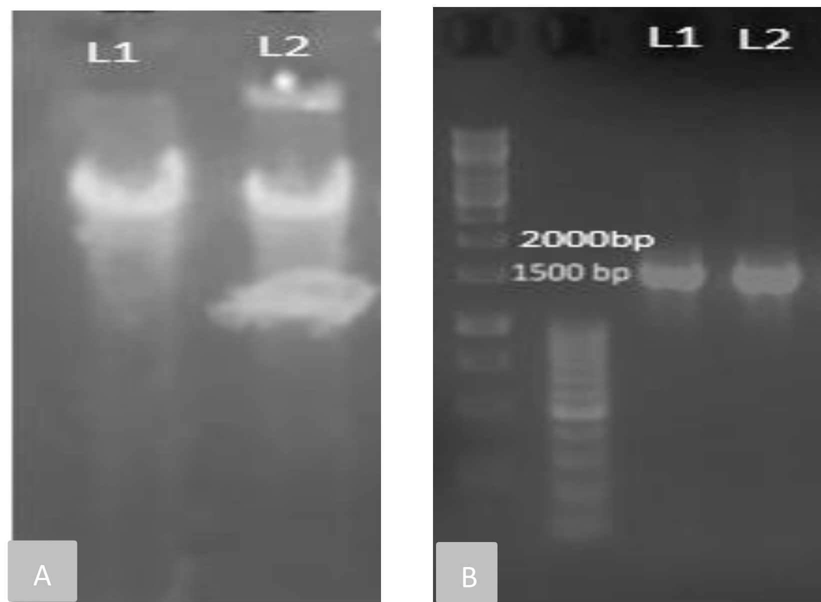


Figure4. 6: A) Gel electrophoresis in 0.8% agar, B) Gel electrophoresis in 1% agar

Fig A: bacterial genomic DNA visualized with UV transilluminator. Lane L1: P3 and Lane L2: P4 and fig. B: Amplification of 16srRNA gene of bacterial DNA using Universal primer (Expected size of band = 1500bp) in 1% agarose gel. Lane L1: P3 and lane L2: P4

The 16srRNA sequence of bacterial strain *Pseudomonas aeruginosa* was received. The sequence was then analyzed using chromas software showed chromatogram as follows:

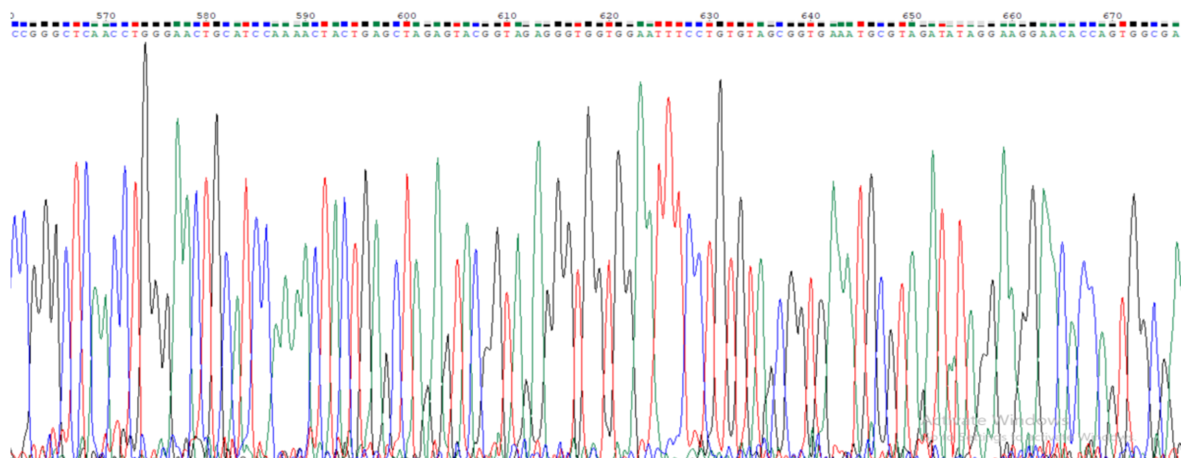


Figure 4. 7 Chromatogram file of bacteria P3 of 16srRNA gene using chromas

The evenly spaced peaks, each with only one color determines the quality of clean sequence and BLAST of obtained chromatogram samples showed maximum (96-99%) similarity with *P. aeruginosa* from which the biochemically unidentified samples were identified as *P. aeruginosa* strains.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
☐	Pseudomonas aeruginosa strain KRF 102 16S ribosomal RNA gene, partial sequence	1689	1689	99%	0.0	98.49%	gi|353025318|JN604532.1
☐	Pseudomonas aeruginosa strain Hualan_196_1 16S ribosomal RNA gene, partial sequence	1686	1686	98%	0.0	98.58%	gi|1723195100|MN314684.1
☐	Pseudomonas aeruginosa strain H25883 chromosome, complete genome	1686	6732	98%	0.0	98.58%	gi|1518045211|CP033686.1
☐	Pseudomonas aeruginosa strain TLPA 1 16S ribosomal RNA gene, partial sequence	1684	1684	98%	0.0	98.58%	gi|1631898696|MH998020.1
☐	Pseudomonas aeruginosa strain Xuyi_330_1 16S ribosomal RNA gene, partial sequence	1684	1684	98%	0.0	98.58%	gi|1723195334|MN314803.1
☐	Pseudomonas aeruginosa strain SZH16 16S ribosomal RNA gene, partial sequence	1684	1684	98%	0.0	98.58%	gi|288189621|GU384267.1
☐	Pseudomonas aeruginosa strain R8-589 16S ribosomal RNA gene, partial sequence	1683	1683	98%	0.0	98.48%	gi|381217606|JQ659967.1
☐	Pseudomonas aeruginosa strain GQ-2 16S ribosomal RNA gene, partial sequence	1682	1682	98%	0.0	98.68%	gi|528794346|KF453952.1
☐	Pseudomonas aeruginosa strain AP-CMST 11 16S ribosomal RNA gene, partial sequence	1682	1682	98%	0.0	98.38%	gi|407750815|JX465662.1
☐	Pseudomonas aeruginosa strain OE5 16S ribosomal RNA gene, partial sequence	1682	1682	98%	0.0	98.48%	gi|1735137052|MN416143.1
☐	Pseudomonas aeruginosa strain GIMC5002:PAT-169 chromosome	1682	6728	98%	0.0	98.48%	gi|1736694455|CP043549.1
☐	Pseudomonas aeruginosa strain GIMC5001:PAT-23 chromosome	1682	6728	98%	0.0	98.48%	gi|1735629040|CP043483.1
☐	Pseudomonas aeruginosa isolate ID40 genome assembly, chromosome: ID40_omosome	1682	6728	98%	0.0	98.48%	gi|1735417931|LR700248.1
☐	Pseudomonas aeruginosa strain CCUG 51971 chromosome, complete genome	1682	6728	98%	0.0	98.48%	gi|1733063483|CP043328.1
☐	Pseudomonas aeruginosa strain DD.SS.SJ_03 16S ribosomal RNA gene, partial sequence	1682	1682	98%	0.0	98.48%	gi|1730123917|MN371810.1

Figure 4. 8 NCBI BLAST of obtained sequence

4.4. Isolation of bacteriophage

All together 6 bacteriophages were isolated against two strain of bacteria P3 and P4. Water samples were collected from different rivers of Kathmandu valley Sali nadi, Basundhara river and Balku river, followed with isolation of Bacteriophage. The initial plate contain variation in lysis and morphology of plaque which differ from small pin head clear plaque to hallow forming Bull’s eye but most dominant was small pin head plaque. Among numerous plaques single clear plaque was selected for further processing.

Table 4. 2 Table showing phage and their site of collection

S.N	Sample site	Host Bacteria				
		P3	**P4**	**Sa3**	**Sa2**	**Sa5**
1	Teku	_	NT	_	_	_
2	Balku	+	+	_	NT	_
3	Basundhara	+	+	_	NT	_
4	Sali nadi	+	+	_	NT	_
5	Om Hospital side river	NT	_	_	_	_
6	Chabel	_	NT	_	_	_
7	Pashupati	NT	_	_	_	_
8	KMC Hospital side river	_	NT	_	_	NT

Note: ‘+’ denotes presence of phage, ‘-‘denotes absence of p9hage and NT denotes not tested

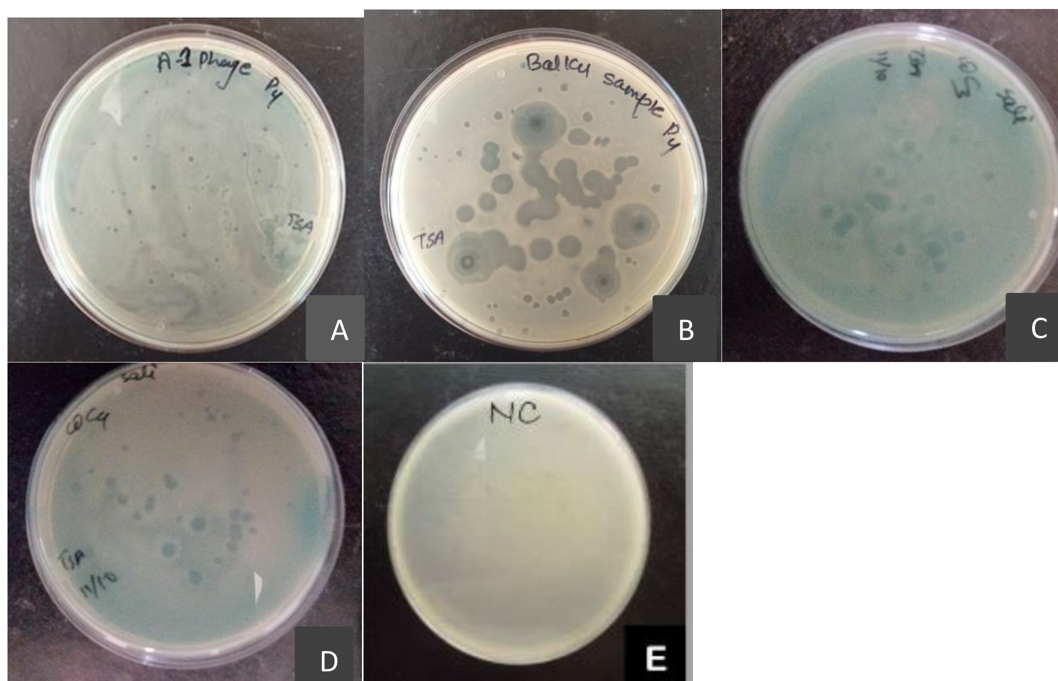


Figure 4. 9 Initial isolation of Bacteriophage against *Pseudomonas aeruginosa* strain P3

In figure A, B and C mixed type of plaques are seen (bull's eye and pin head) from Balkhu, Sali nadi and Sali nadi water sample of bacterial strain P4 and P3 respectively, in fig D pin head plaques are seen from Basundhara water sample against P4 bacterial strain.

Table 4. 3 Initial Screening of Bacteriophage

S.N	Host Bacteria	Sample site	Phage Morphology	Lysis pattern
1	P3	Sali nadi	Bull's eye and pin head	Clear center surrounded by turbid ring and complete lysis
		Basundhra	Clear Plaque	Complete lysis
		Balkhu	Clear Plaque	Complete lysis
2	P4	Sali nadi	Bull's eye and pin head	Clear center surrounded by turbid ring and complete lysis
		Basundhara	Clear Plaque	Complete lysis
		Balkhu	Bull's eye and pin head	Clear center surrounded by turbid ring and complete lysis

4.5. Purification of Bacteriophage

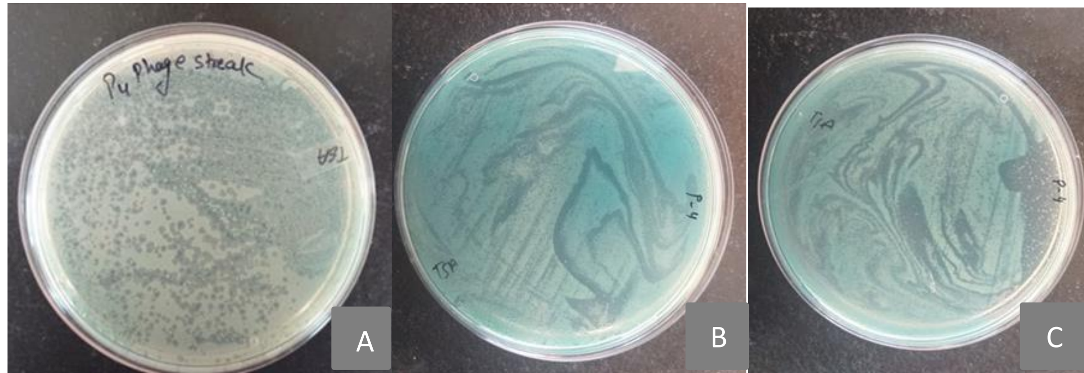


Figure 4. 10 Purification of phage by streaking method

Fig A, B and C is the three rounds of purification of phage P4 by continuous streaking method.

Bacteriophage purification was done by three round streaking of single plaque. Bacteriophage which had clear lytic plaque morphology was selected for purification. Among different phages that were isolated from Balkhu River, Basundhara River and Sali nadi, Single plaque of bacteriophage from Basundhara River was selected for purification by streaking because their plaque morphology was clearer than other and clear lysis was seen over the streaking line.

4.6. Phage titer determination

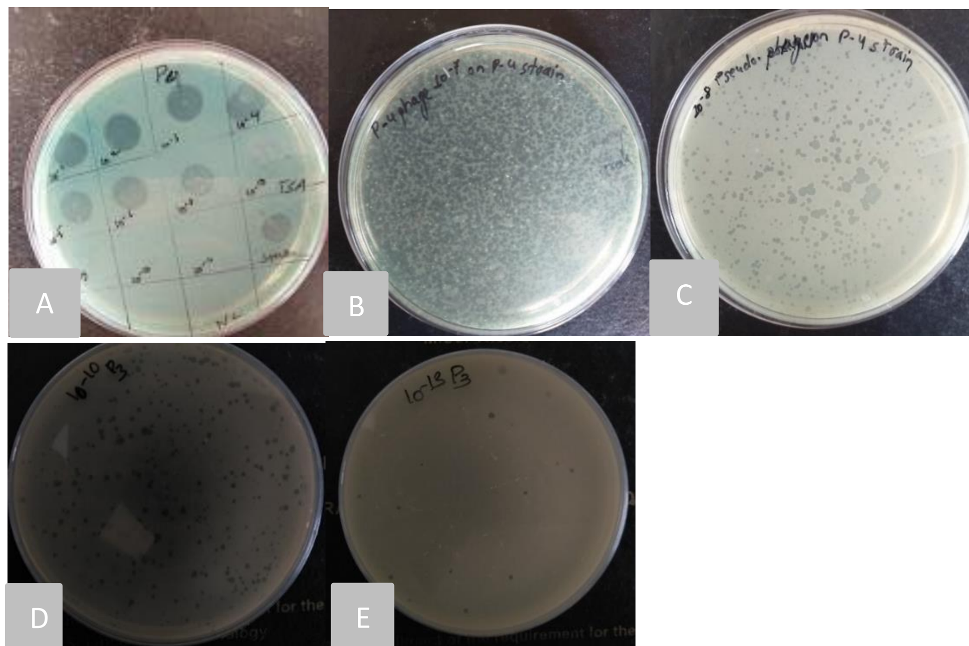


Figure4. 11 A) Spot assay of P4 showing lysis up to 10^8 dilutions, Fig B, C, D and E are plaques formed in the dilution 10^{-7}, 10^{-8}, 10^{-11} and 10^{-13} respectively of P4 phage

The spot assay was used to determine the titre/concentration of phages in the original stock solution. The figure above shows plaques of phages at different dilutions up to dilution 10^{-13}. But clear lysis above 10^{-8} is not observed in first figure of 4.11 that might be due to less amount of phage dilution (5µl) pipetted in respective areas.

For further confirming the presence of phage as shown by spot assay, individual dilutions were taken and plated on individual. After incubation the plaques on each plate was counted and total concentration of phage was calculated as below:

Table 4. 4 Plate count upon serial dilutions and phage titre determination (PFU/ml)

Phage	10^{-1}	10^{-5}	10^{-6}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	10^{-13}
P4	Clear	TMTC	TMTC	TMTC	TMTC	366	106	38	16

The concentration of phage stock solution was determined by following formula.

PFU/ml= Number of plaques/ Volume of phage× dilution

$= 16/1\times10^{-13}$

$= 1.6\times10^{14}$

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

4.7: Determination of Multiplicity of infection (MOI)

MOI or in vitro lysis bacteriophage was calculated by comparing the optical density at 600nm in calorimetry, increase in bacterial density in the culture medium where as other graphs contain bacteria with phage at different MOI. The optimum lysis was seen at 1 MOI.

Figure4. 12 Invitro Lysis of bacteria by phage

Culture of bacteria P3 and phage P4 at different MOI. Tubes are bacteria only (no phage), blank (no bacteria or phage), bacteria and phage at MOI 100, MOI 10, MOI 1, MOI 0.1, MOI 0.01 and MOI 0.001 from left to right

The tubes incubated for 24 hours with different MOI showed some turbidity in 0.001 and 0.01 MOI, though it was not as turbid as the bacterial culture. This shows

incomplete but high degree of bacterial lysis even in lower MOI. Clear solution, like blank (no bacteria and phage, only sterile LB broth) were seen in the tubes with MOI 1 and above. This indicates the optimal MOI for the complete lysis of bacteria is 1 MOI. The result indicates that although there is high degree of bacterial lysis even at MOI 0.001, these MOI cannot completely lyse the bacteria and some bacterial population still remains in solution which can further grow and multiply, after the activity of phage is decreased. But at MOI 1 and above, complete bacterial lysis can be obtained by the phage particles, showing the ratio of 1:1 for phage: bacteria is enough to completely get rid of bacteria in-vitro in the LB broth.

4.8. Characterization of phage P4

4.8.1. Multiple Host Range Analysis by Spot Assay

Inter host range analysis and intra host range analysis was done by spot assay. While doing intra host range analysis only 8 strains of *Pseudomonas aeruginosa* were used. The strain used were P4, P3, P19, P53, P6, P10, P9 and P20 and the P4 phage showed intra host range for strains P4, P3, P9, P19 and P20. Inter host range analysis was done by using different strains of *Acinetobacter baumanii, Salmonella* spps, *Klebsiella pneumonae.* Altogether 28 strains were used for inter host range analysis by spot assay. Along with the P4 Phage, phage cocktail of *Pseudomonas aeruginosa* was used to observe synergistic mechanism and other phages of *Pseudomonas aeruginosa* were provided by Senior from Phage lab of Central Department of Biotechnology, T.U.

Table4. 5 Intra host analysis by spot assay

S.N	Phage	Host Bacteria							
		P3	P4	P6	P9	P10	P19	P20	P53
1	P4 Phage	+++	+++	×	++	×	+++	++	×

+ sign indicate the presence of lysis by P4 phage to the host bacteria and × sign indicate

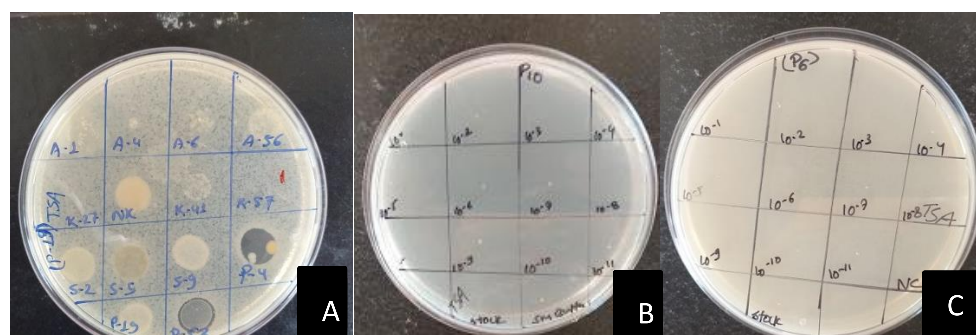


Figure4.13. Intra host range analysis

Fig A: P19 is the bacterial strain lawn in the TSA plate where P4 phage showed lysis in the spotted region, Fig B: Dilution of P4 Phage did not show lysis in the P10 bacterial strain lawn in the TSA plate similarly in Fig C: Dilution of P4 phage did not showed lysis in the P6 bacterial strain.

Table4. 6 Inter host range of phages by spot assay

Bacterial Genus	Code Name	Lysis of phage			
		Phage P4	Phage P19	Phage P53	Pseudomonas phage cocktail
***Acinetobacter* Strains**	A1	+	+	+	+
	A4	-	+	+	+
	A5	-	+	+	++
	A6	+	+	+	+
	A7	+	+	+	-
	A8	-	-	-	-
	A11	+	+	+	-
	A17	+	+	-	+
	A21	+	+	+	+
	A23	+	-	+	-
	A38	+	+	+	+
	A47	-	-	-	-
	A50	-	-	-	-
	A53	-	-	-	-
	A56	-	-	-	+++
	A70	-	-	-	+
***Klebsiella* Strains**	NK	+++	-	-	++
	K27	-	-	-	+
	K41	-	-	-	-
	K57	-	-	-	+
	K52	-	-	-	-
***Salmonella* Strains**	S2	-	-	-	+++
	S3	-	-	-	-
	S4	-	-	-	-
	S5	-	-	-	+++
	S6	-	-	-	-

[Note: 'x' indicates no lysis of bacteria, '+' indicates very slight lysis with turbidity on lysis zone, '++' indicates better lysis with slightly larger lysis zone but irregular with turbid areas, and '+++' indicates clear lysis zone with uniformity in lysis (best lysis).]

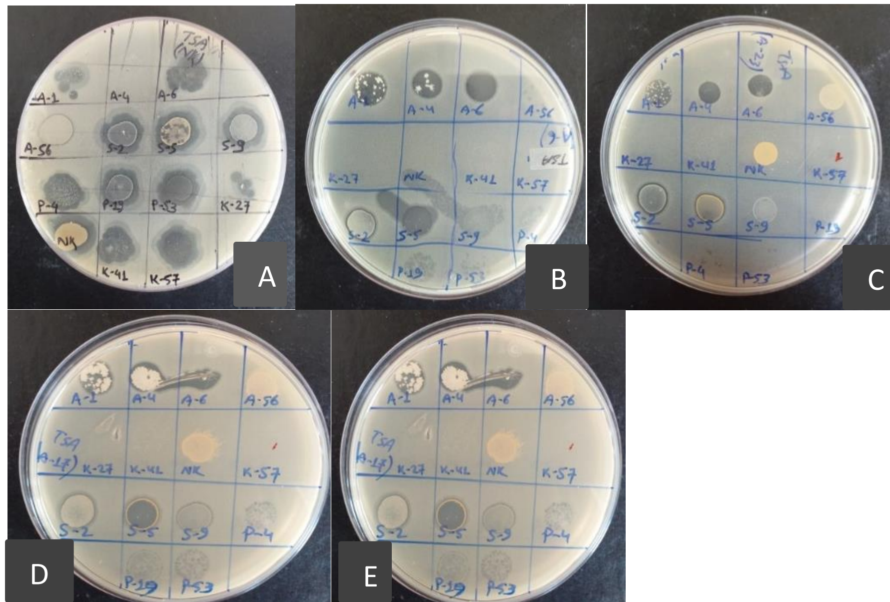


Figure4. 14: Inter host range of phage P4. In figure A, B, C, D and E phage P4 showed lysis against bacterial strain A6, A23, A15, NK and A27 respectively. Lawn of Bacterial strain was made on TSA plate and Host range analysis was done by spot assay method.

During the multi-host range analysis of the bacteriophage, out of 28 bacterial samples taken, *Salmonella* and *Klebsiella* were not lysed by any of the 4 phages taken for study except NK strain of Klebsiella by P4 phage. The largest host range was shown by phage cocktail, as phage cocktail lyse bacterial strain which were not lysed by all the three phages, which is known as synergistic mechanism. Pseudomonas phages (P4, P53, P19) showed wide host range to Acinetobacter spps. Even though all the phages of Pseudomonas showed wide host range, we took phage P4 for further study.

While doing Host range analysis, we made lawn of bacterial strain and phages were tested by spot assay. In figure 4.14. A, we can see that there is contamination (bacterial growth) in spot S5 and NK phages. The contamination was due to the contamination in stored NK and S5 phage. During the study me and my friend both were doing research on bacteriophage, we took single plate of bacterial for host range analysis and my thesis work is mainly focused on Phage P4 not on Phage NK and S5, in the figure 4.14. we can clearly observe that there is no contamination or bacterial growth on spot P4 which defines that our Phage P4 stock was not contaminated during storage.

4.8.2. SDS-PAGE

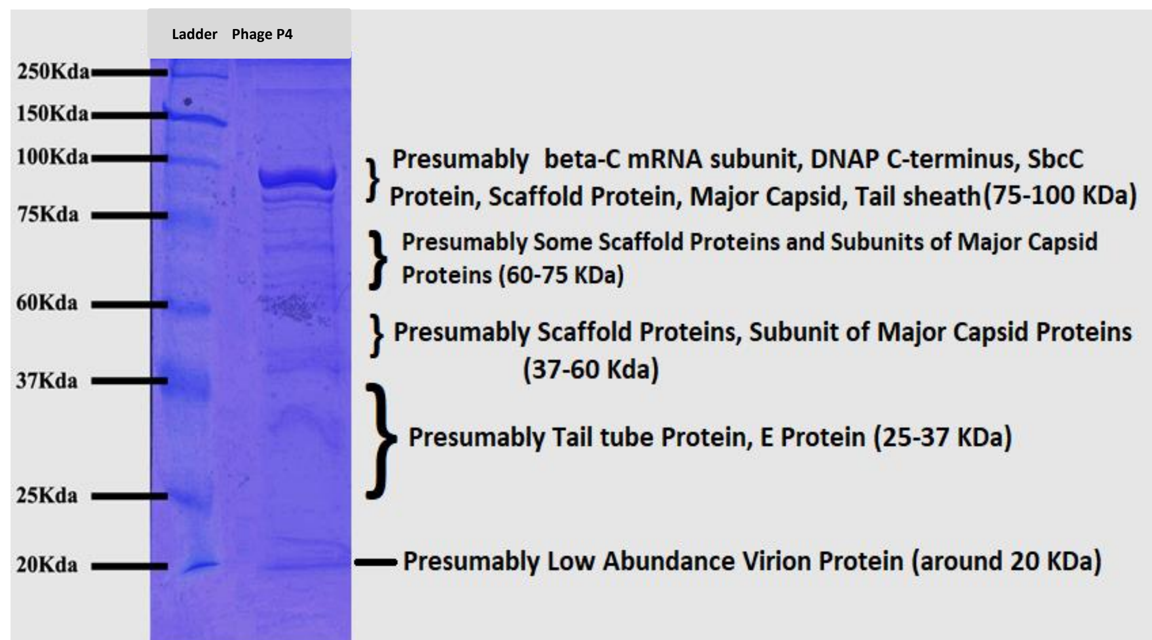


Figure4. 15: SDS PAGE and protein bands of phage P4.

Protein profiling was done by SDS-PAGE and the band size obtained was compared with standard protein marker (20-250 KDa protein ladder, Genei). Clear bands were seen below 100 KDa which is the largest protein and might be capsid protein present in phage. Multiple bands between 20 and 100 Kdal were observed.

4.8.3. Transmission electron microscopy

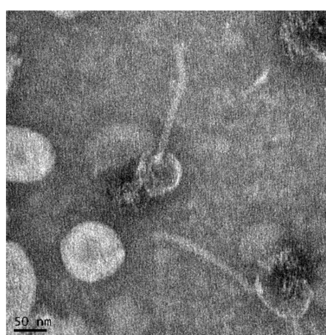


Figure4. 16: Negative staining images of phages P4 by TEM

TEM micrograph of phages was obtained and the result was analyzed by using ImageJ i1.52a software and interpreted according to according to ICTV guidelines. The criteria for classification of phages according to ICTV guideline is capsid size, capsid symmetry, absence or presence of tail, tail length and genome size. Depending on these criteria, phages were classified as given in table 4.7.

Table4. 7: TEM image analysis and classification of phages based on ICTV guidelines.

Phage name	Capsid 'nm'	Tail 'nm'		Shape	Order	Putative Family
		Width	Length			
Phage P4	60.77	11.62	179.67	Isohedral	Caudovirales	Siphoviridae

4.8.4: Effect of temperature on phage stability

Temperature stability of phage was determined at different temperature from 30°C to 80°C at different time of incubation. The result we obtained was that the phage was found to be stable even at 70°C for 20 min. The viability decreased with the increase in temperature and vice versa.

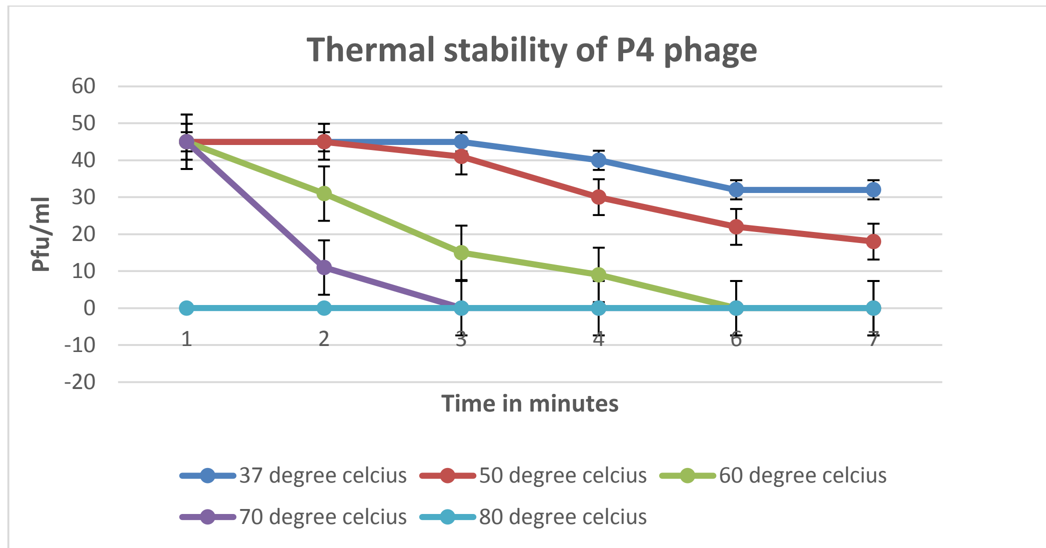


Figure4. 17 Thermal Stability of Phage P4

Temperature stability of phage was determined at different temperature in different time of incubations. Phage was found to be stable even at 70˚C up to 20 minutes. The phage titer was decreased after 40min incubation at 60°C. This result showed that infectivity of phage decreases at the temperature increases.

4.8.5: Effect of pH on phage P4 viability

pH stability test showed that phage P4 is stable in pH3 to pH12. Phage showed most efficient lytic activity at pH6 and pH7 and lost its lytic activity below pH3 and above pH12.

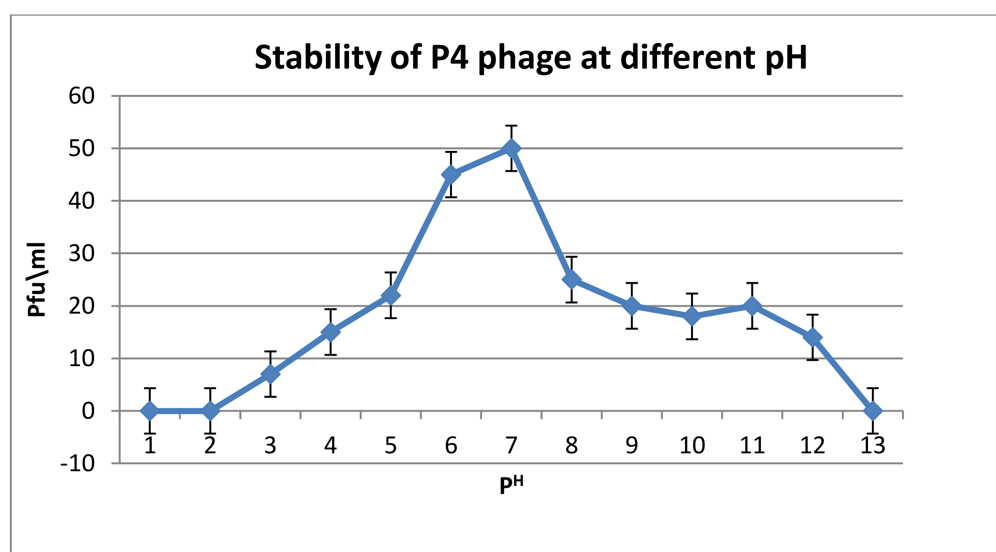


Figure4. 18 Stability of Phage P4 at different pH

Phage P4 was found to be stable from the pH ranging from 3-12 and it could not survive below 3.

4.8.6: One step growth curve determination

One step growth curve was done to determine the latent period, rise period and burst size of our phage. The result showed that the latent period was found to be 30min and burst size was found to be 28 virions per bacterium. The phage P4 has short latent period meaning that it can lyse bacterial cell more rapidly.

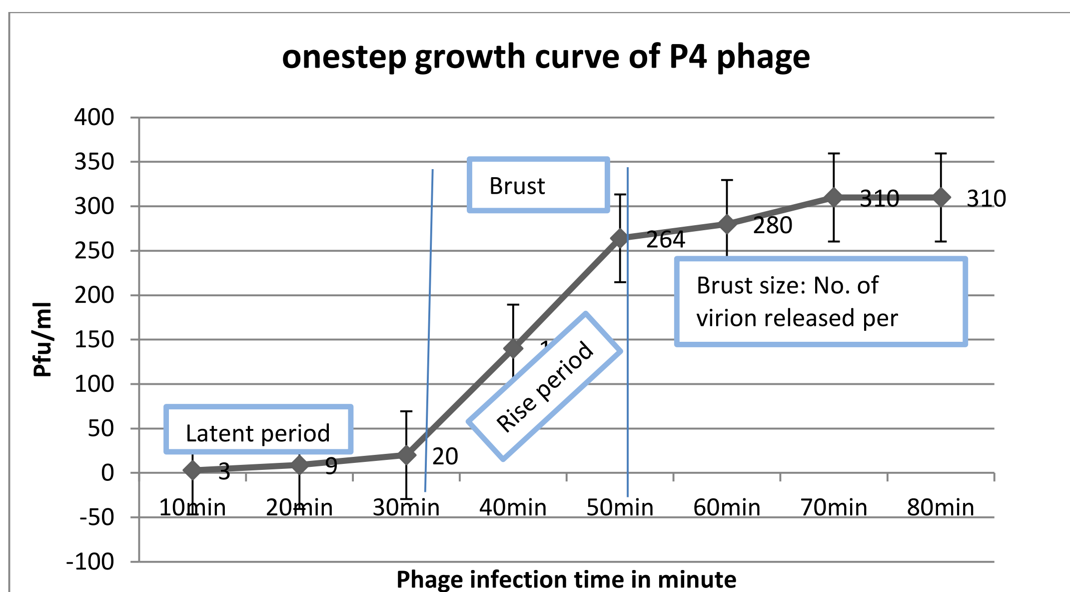


Figure4. 19 One step growth curve of P4 Phage

The one-step growth curve of phage P4 showed a latency period of 30 minutes after which rapid infection takes place and phage virions are released afterwards. The latency period means the time for adsorption and infection by phage particles which happened in about 30 minutes during our experiment and the burst size was found to be 28 virions per bacterium.

The explosion of plaque forming units at 60 mins is due to bursting and release of phage virions after infecting the host. From the experiment, the burst size was calculated to be 280/10 i.e. 28 phage particles per infected bacterial cell (28 PFU/CFU).

4.9: Decontamination assay of bacteriophage

During decontamination assay comparative study was done. Along with the phage P4, phenol was also used as decontaminant. Decontamination was done in marble tile pieces and apron cloth pieces. Firstly, decontamination assay was done on cloth using bacteriophage P4 of different MOI values to determination the MOI value at which phage P4 can show more lysis of bacteria on cloth. Phenol (which is regularly used in hospitals to decontaminate floors) of different MOI were also used from the comparison between the effectively of lysis of phage P4 and phenol.

4.9.1: Decontamination assay Using phage P4

*Pseudomonas aeruginosa (*P3) bacterial strain was inoculated on to sterile fabric cloth (from a lab coat similar to one worn by clinicians) and then added a single phage P4 and bacterial load was determined in the form of CFU. Phage P4 of different MOI (1, 10, 100,

1,000 and 10,000) and control which contain only bacteria was prepared. The result showed that the bacteriophage with MOI value 1 showed higher rate of decontamination i.e. Phage P4 applied on cloth piece was able to lyse more bacteria in cloth as, we observed less no, of colonies on cetrimide agar plate. The result obtained showed that phage P4 have higher decontamination (less number of bacterial colonies on Cetrimide agar plate) at MOI 1 with the CFU/ml log reduction of 1 and p-value was 0.002 i.e., statistically significant by using paired T- test. The decontamination rate

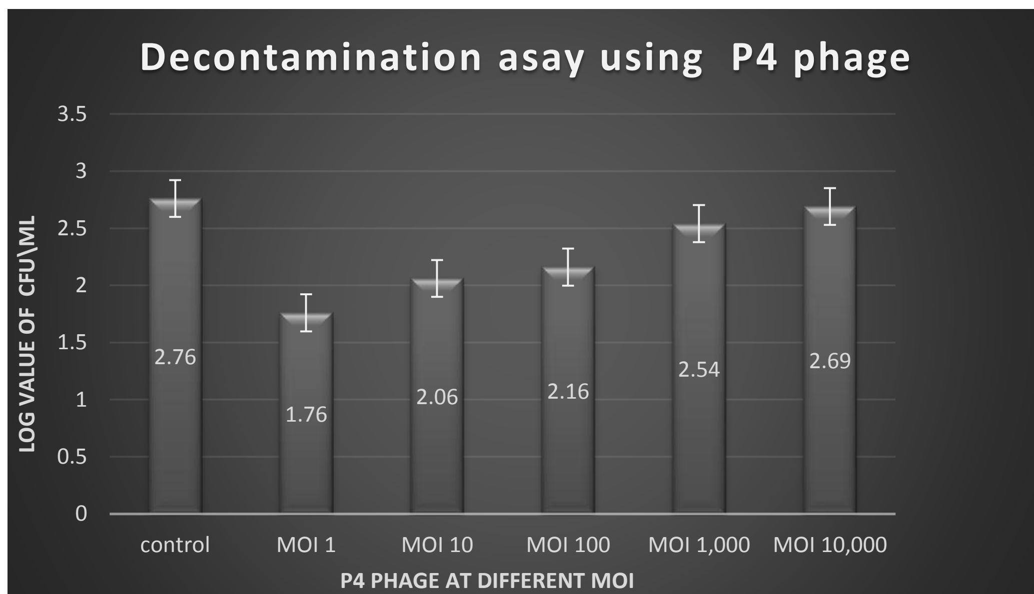


Figure4. 20 Decontamination assay of phage P4 at different MOI values

decreases with increase in the MOI values.

Phage P4 showed higher rate of decontamination at MOI value of 1 which means that one phage P4 can lyse one bacteria at a time

4.9.2: Comparison between phage P4 and phenol as disinfectant

A Pseudomonas aeruginosa (P3) bacteria *was* inoculated on to sterile cloth and phage P4 and phenol was added separately in different cloth pieces. Then, bacterial load was determined in the form of CFU. Comparison was done to determine the effectivity of phage P4 and phenol. Comparison was done between the MOI values 1 and 10. The result obtained showed that single phage has higher decontamination rate at MOI 1 with the CFU/ml log reduction of 1 and p value was 0.002 whereas MOI 10 showed less decontamination rate than that of MOI 1 with the CFU/ml log reduction of 0.692 and p value was 0.004. Likewise, Phenol did not show significant decontamination at MOI value 1 and 10 than that of single phage. While comparing, phage P4 with MOI value 1 was found to be more effective than phenol.

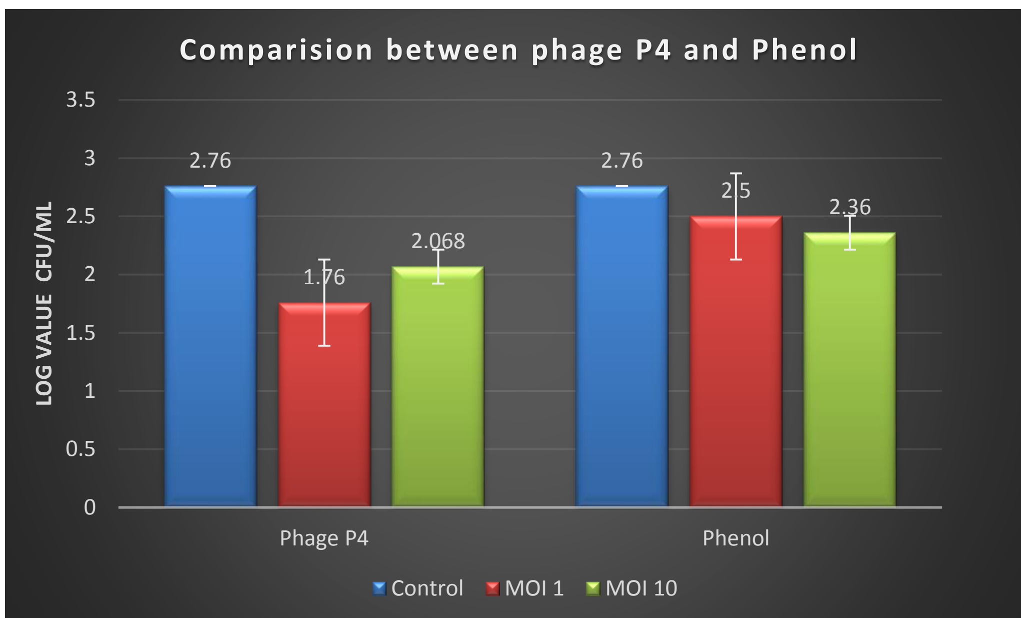


Figure4. 21 Comparison between Phage P4 and phenol as disinfectant

P4 Phage showed higher rate of decontamination at MOI value of 1 which means only 1 phage is required to lyse 1 bacteria at a time, Phenol did not show significant decontamination at MOI value of 1 and 10.

4.9.3 Decontamination by bacteriophage on marble tiles

Decontamination assay by bacteriophage on marbles was done to determine the time period up to which bacteriophage can show their effectivity on marble. The result showed that bacteriophage has highest level of effectivity up to 6 hours at MOI 1 with the CFU/ml log reduction of 1 and p value was 0.002 and the effectivity level goes on decreasing. But bacteriophage showed its effectiveness for more than 24 hours with the log reduction of 0.47 and p value 0.098**,** it was also checked on MOI 10 and was effective up to 6 hours with the pfu/ml log reduction of 0.62 and p value was 0.009.

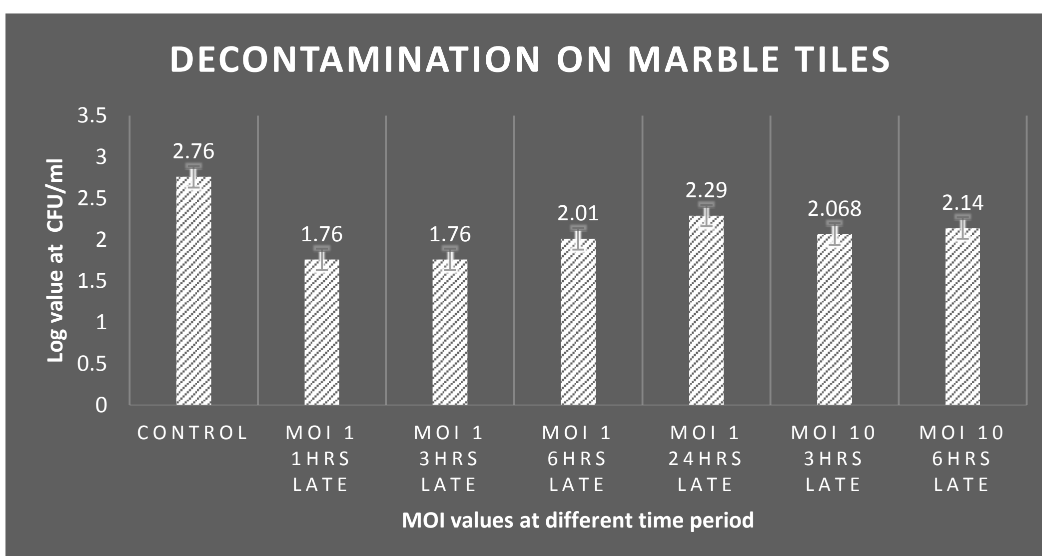


Figure4. 22 Decontamination by Phage P4 at different time period on marble tiles

Phage can show its effectivity up to 24 hrs at MOI1 but its effectivity goes on decreasing from 1 hour to 24 hour and MOI 10 also showed its effectivity after 6 hrs on marble tiles.

5. DISCUSSION

In this study, we screen, isolate, characterize and also check their potent ability of the lytic of bacteriophage against *Pseudomonas aeruginosa* that can be used to decontaminate the hospital floor which can help some how to control the hospital acquired nosocomial infections because *Pseudomonas aeruginosa* is one of the most frequent and severe causes of acute nosocomial infection, mainly affecting immune compromised patients or those who are admitted to the intensive care unit (ICU) (Lila, et al., 2018).

Among 4 strain of *Pseudomonas aeruginosa* and 12 strains of *Staphylococcus aureus,* only 2 strains of *P. aeruginosa* and 5 strains of *Staphylococcus aureus* were found to Carbepenem resistance and methicillin resistance respectively, also one strain of Staphylococcus was found to be VRSA. *P. aeruginosa* strains were found to be Carbapenem resistant. We performed only disc method to check the susceptibility of the bacteria towards antibiotics. Therefore altogether 3 strains of *Staphylococcus aureus* (Sa_2, Sa_3 and Sa_5) and 2 strains of *Pseudomonas aeruginosa* (P3 and P4) were used to isolate the lytic bacteriophages. The P3 bacteria which was used for further processing against lytic bacteriophage was confirmed by 16SrRNA sequence analysis as *Pseudomonas aeruginosa*. The use of 16SrRNA gene sequencing is one of the easiest methods for identifying bacteria (Janda & Abbot, 2007).

We faced lots of problems during isolation of the bacteriophage against *Staphylococcus aureus*, we tried in various samples but it was hard to find the phage. That might be due to either there is absence of phage in those particular host or there might be some error in our work (attachment time period between phage and bacteria, improper collection of sewage samples). Up to 1 and half month we keep on trying on different new sewage sample but we were unable to isolate the new bacteriophage. In some water sample, there might not be presence of lytic phages that might be due to absence of host or error in water sample collection, which is collecting water without sedimentation. As phage particles are attached to sediment and with over time these phages attached sediment aggregate in the bottom of the water body and with gently shaking the ground floor phages are released into water so it can be said that phages are generally more abundant in the sediment than overlying water. A study done by Mattila, Ruotsalainen, & Jalasvuori, 2015 also showed that isolation of new phages against methicillin resistant Staphylococcus aureus strains was found to be very difficult. They were able to isolate phages for only a single S. aureus strain, SA10, from 117 enrichment attempts (Mattila, Ruotsalainen, & Jalasvuori, 2015)

We were only able to isolate 6 phages against *Pseudomonas* strain from 3 different water samples. This result would suggest water of Kathmandu valley might not contain the lytic phages against that MDR Staphylococcus aureus and as phages are strictly specific to their host it is difficult to isolate phage against these bacteria. Phages were isolated by using double layer agar assay method of Adam, (1959). It helps to form greater uniformity of plaque and formed greater size plaque due to greater rates of phage diffusion in soft agar. Thus, soft agar permits diffusion of phage to nearby infected cells but does not permits new phages to move to remote parts of plate. We were only able to isolate bacteriophage against P3 and P4 bacteria from 3 rivers (Sali nadi, Basundhara river and Balkhu river) of Kathmandu valley.

Different types of plaques were isolated from these three rivers. The morphology of plaques were small pin head to the large bull's eye, among them pin head plaques were dominant so, we choose pin head plaques for further processing. There was a different sized and different morphological plaque which indicates there is more than one type of phage in the water sample. The size of the plaque is proportional to the efficiency of adsorption, the length of the latent period and the burst size of the plaque. A diversity of plaque sizes can result, if the phage can infect cells at different time during the bacterial growth phase ie., the phage that infect early makes larger plaques than that of which infect later and also the another factor is that the physical size of the bacteriophage also plays important role in the overall size of the plaque morphology, smaller phage has less physical size which can diffuse more quickly and easily through the semi-solid media in plaque assay plate in order to produced zone of lysis whereas larger plaque have difficulty in diffusion and form small size plaques that is a slowly proliferating phage, one which yield low number of progeny phage will more likely to produce a smaller plaque compared to quickly proliferating phage.

A clear plaque indicates that the host is completely susceptible to the phage which is formed in *Pseudomonas aeruginosa* (P3 ad P4). The Pin head morphology of *Pseudomonas* phage was similar with the phage produced by Tang and his team against *P. aeruginosa* TC6 (Tang et al., 2018). Similar type of plaque morphology was seen in *Pseudomonas* phage BrSP1 against *Pseudomonas aeruginosa* (Melo et al., 2019). Pin head plaques are dominant during the phage isolation which indicates phage induced bacterial lysis is more efficient or complete early during the lysis. Decreasing lytic efficiency can be a consequence of the bacterial lawn, associated increases in the size of the micro colonies making of the bacterial lawn, or because of the less general phenomenon such as lysis inhibition phenotype seen in the T-even phages (www.phage.org). Bull's eye plaque indicates bacterial lysis is less efficient or complete later on during plaque development than it is early on during plaque development. The halo around the plaque indicate the decapsulation of the bacterial host cell by phage produced soluble enzyme depolymerase and the hazy ring indicate phage produced a depolymerase enzyme that defused through the agar layer and degraded the bacterial capsular polysaccharides (CPS) into different oligosaccharide components. Early studies showed that certain *Klebsiella pnuemoniae* bacteriophages produced depolymerase during phage proliferation and released the enzyme from infected bacteria that targeted other bacteria's CPS (Admans M H, 1959)

After isolation of bacteriophage, purification, amplification and titer determination was done. Single phage colony was taken with the help of sterile loop and streak on the TSA plate and the soft agar with bacteria was lawn over the plate, this helps in the purification of the single colony of bacteriophage and amplification of a single selected colony. Stock preparation of bacteriophage was done on the SM (sodium, chloride, magnesium) buffer. The magnesium salt contained in SM buffer help to absorb the phage but not bacteria, gelatin helps to maintain the stability of the phage particles during storage and chloroform maintain the sterility of the phage particles by hindering the bacterial growth. For the characterization of phage higher titer value is preferred so we made the phage stock of higher titer. The bacteriophage titer is a quantitative measurement of the biological activity of virus and is expressed as plaque forming units (PFU) per ml. Determination of phage titer in a sample is a key step in the study of the

phage involved. It is very important to select suitable dosage in the phage therapy for bacterial infection. As a common method of detection, a plating assay (plaque) is widely used method to detect phage number present in the test samples at various diluted points.

The multiplicity of infection is the ratio of phage to the bacteria. When referring to the bacterial culture inoculated with virus particles, Multiplicity of infection or MOI is the ratio of number of virus particles to the number of bacteria present in a definite space. Lysis of *Pseudomonas aeruginosa* was determined by comparing the optimal density of bacterial culture with bacteriophage at different MOI with the growth of bacterial culture free of bacteriophage. P4 phage showed efficient reduction of bacterial cell in liquid culture medium. The optimum lysis was observed at MOI 1. Here the optimum MOI 1 means that the optimum lysis occurs at the ratio of 1 phage: 1 bacteria. A study done by Scarascia showed, a MOI value of 10 was the optimal for bacteriophage infection (Scarascia, Yap, Kaksonen, & Hong, 2018). It is likely that this MOI value maximized encounter rates between bacteriophage and bacterial cell, hence ensuring that nearly all host cells are infected by at least one bacteriophage particle**.**

Multiple host range is a property of phage in which phage can infect or lyse not only for specific host but also for other interspecific or intraspecific host. It is important and highly desirable property in phage therapy because broader the host range broader will be the infectivity of given phage. 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. In this study we used three phages of *Pseudomonas aeruginosa* (P4, P19 and P53). Phage P19 and P53 was provided by our Senior Apshara Parajuli. Intra and inter host analysis was done. From this study P4 phage showed narrow range of lysis against *Pseudomonas strain* and at the same time we used only 8 strains of *Pseudomonas.* But P4, P19 and P53 phages showed broader range of lysis against strain of *Acinetobacter baumanii* and it also showed lysis against some strains of *Klebsiella* and *Salmonella.* Lysis pattern was also checked by making cocktail of those three phages. As expected, phage cocktail had broader host range for the MDR strain and cocktails were found to be more effective. Spot assay is the most common way of testing host range, in which a small volume of phage is placed on a growing lawn of bacteria. As it is simple and rapid, this technique can sometimes cause false positive because of lysis of bacterial cells without phage infection. It can also be confirmed by killing assay but it was not done in this study. Wider host range may be due to production of depolymerase enzyme. Lopez-Cuevas et al. 2011 hypothesized that the difference of host range might be due to the environmental origin of bacteria tested in which a loss of bacteriophage receptors may had happened as a result of antagonistic co evolution between bacterium and bacteriophage. At the same time, it could be associated with the prevention of adsorption by bacterial receptor mutations or with degradation due to restriction or modification of the resistance bacterial system (Abedon et al., 2010).

In this study we found interesting result, the cocktail of *Pseudomonas* phage lysed 2 Carbapenem resistant *Acinetobacter baumanii* (A70 and A56)*,* 2 colistin resistant *Salmonella typii* (S2 and S5) and 2 Carbanepem resistant *Klebsiella pneumonae* (K27 and K57) but when tested with each of the single phage of *Pseudomonas,* they did not show any lytic effect, which means there is synergistic effect between the phages during

infection process. From this result we can hypothesized that such synergistic effect might be due to the one phage that facilitates the infection to the same bacterium by providing certain mechanism so that another phage easily cause infection. This might be also due to that phage having different types of receptors. Synergy provides a potential tool for improving phage therapy however process of synergy between phages is relatively unexplored. Similar result was suggested by Regeimbal et al., 2016 in which phage cocktail was seen lowering the burden of the wound and preventing the spread of infections in case of *Acientobacter baumanii* infections in mouse model experiment. In this study also, the cocktail is composed of four phages that do not kill the parent strain of the infection. Another result showed that combination of T7 and J8-65 phage have 100-fold greater killing efficiency than T7 and J8-65 phages alone have their killing efficiency (Schmerer, Molineux, & Bull, 2014). Thus, phage cocktail represents new way of therapeutics, which is more effective than single phage and antibiotics on the basis of mechanism of action.

Protein profiling done by SDS- PHAGE showed clear protein band ranging from 20Kda to 250Kda. Comparing our protein bands with the data of protein mass published on ASM (2018), Journal of Virology, we can make various presumptions about the proteins present in our phage P4. The presumed proteins along with their respective size are labeled in the figure 4.15. Protein bands obtained in SDS show a wide variety of proteins present in our phage ranging from large capsid, scaffold, tail proteins to smaller tail tube proteins and low abundance virion proteins. Similarly, TEM analysis is relatively faster than genome analysis and according to shape and size phages can be classified into respective families in a very short time. Our Phage P4 belongs to Caudovirales order and Siphoviridae family with tail width 11.62nm and length 179.77nm.

pH plays crucial role in phage survivability. It should be noticed that at higher and lower pH, the plaque morphology was changed as there is difficulty in setting agar. Our phage P4 was able to tolerate over wide range of pH (3-12). A study done to obtain pH stability of *Pseudomonas aeruginosa* phages vB_PaeM_SCUT-S1 (S1) and vB_PaeM_SCUT-S2 (S2) showed phages were stable at pH range of 4-10 (Guo, Chen, Lin, & Wafng, 2019), which suggest our phage P4 has higher stability in higher acidic and alkaline condition which indicate that they could be maintained in human physiological condition that our phage is highly stable at wide range pH. It was shown that hydrogen ion concentration influences phage aggregation. For example, MS2 phages showed significant ability to aggregate when pH was less than or equal to the phage isoelectric point (PI =3.9) (Jończyk, Kłak, Międzybrodzki, & Górski, 2011). Their aggregate could be up to 6μm in diameter. This may cause decline in phage count and easier elimination of aggregates through their adsorption on membrane than single virion.

Temperature plays a fundamental role in attachment, penetration, multiplication and latent period of phage. Attachment is dependent on capsid proteins of bacteriophages and surface proteins on bacterial hosts, both of which are sensitive to high temperatures (Scarascia, Yap, Kaksonen, & Hong, 2018). Stability of phage on exposure to varying temperature is considered as a key model for understanding the ability of to adapt into novel environment. It determines the occurrence, viability and storage of bacteriophage. The result showed that phage P4 was able to tolerate up to 70ºC for 20min. The phage was not stable above 80ºC when exposed for 10min and nor phage was able to tolerate at 70ºC when exposed for 30min. Here in this study, we used dry heat but from different

studies we found that phages were more stable at moist heat. Our phage showed less stability to the temperature as compared to Pa1 and Pa2 (up to 90°C) (Marei, 2020) but our phage P4 showed similar result with phage OMKO1 (up to 70°C) (Blazanin, Lam, Chan, & Turner, 2019). The effect of different temperature showed that phage P4 was able to withstand exposure to temperature up to 70°C for 20minute.

One-step growth curve was performed to determine lysis through phage multiplication, abortive infection or lysis pattern. From this study we determined different stages of virus growth curve; latent period, raise period and burst size. These parameters are influenced by the temperature of incubation, medium on which the experiment was done and specific growth rate. In our study phage P4 showed short latent of 30minute and high burst size of around 28 virions per bacterium. In a study (Gadagkar & Gopinathan, 1980) found that the burst size of the phage is inversely correlated with the multiplicity of infection and our finding supports this finding as our phage has a large burst size of 28 virions per bacterium and optimum MOI 1. The high adsorption rates, burst sizes, and short latent periods agree with the lytic activity, which makes them potentially good bio control agents (Magin, Garrec, & Andrés, 2019)**.**

Latent period of a phage is a time period in which phage synthesized early protein needed for replication, coat protein and other structure protein and in the raise period the phage assembles all the protein to make a mature virion. And after the assembly, phage is released by lysing the host bacterial cell. Burst size of a bacteriophage is the number of virions released after the lysis of an infected bacterium, and burst size depends upon the various factors such as nutritional availability in a bacterial culture and functions of phage coated proteins such as polymerase and other regulatory proteins essential for replication; inherently low efficiency of proteins may not allow the phage to make full use of bacterial machinery and consequently the burst size may be limited (Gadagkar & Gopinathan, 1980). Burst size value varies in accordance with the specific virus and may range from 10 to 100 for the DNA transducing phages to approximately 20,000 PFU for the RNA virus.

Nosocomial transmission is a major problem, especially when the immune compromised and those with underlying health issues become infected. We sought to determine if our phages could effectively decontaminate fomites associated with nosocomial transmission. We used the results of the spot tests to design combinations where the phages were predicted to have a host range that would include the bacterial targets. Decontamination of fabric was also analyzed as a more likely source of nosocomial transmission. We used pieces of marble tiles to represent decontamination of solid surfaces. Firstly, we used fabric cloth pieces to determine the MOI at which our phage could show the effective lytic activity against bacteria spread on the fabric cloth and it was found to be MOI 1. This result completely agrees with the result which we did in this research previous but that time the MOI was determined in the liquid culture medium.

The result on the fabric cloth was high significant with 1 log reduction in CFU_S of bacteria with P value was found to be 0.002 and log reduction in CFU_S goes on increasing with increase in MOI values. The attachment time for bacteria and phage on fabric cloth was kept 30min as we knew that the latent period of our phage was 20 min, which we got from one step growth curve result. After determination of MOI, comparative analysis was done with phenol (which is used as daily decontaminating agent).

The effective time period up to which Phage could show significant lytic activity was determined which was done on the marble tiles, which represent decontamination of solid surfaces. At this time, we took single phage P4, as we came to know that single phage has high rate of lytic activity than phenol. But we took phage of 2 different MOI (MOI 1 and MOI 10). After 1, 3, 6, 18 and 24 hours, surfaces were directly sampled by contact Rodac plates on TSA, to collect residual viable bacteria. Each plate, containing samples taken at the different time points, was incubated for 24 hours at 37°C and bacterial load was evaluated by enumerating plate CFU. The result showed that phage can show its lytic activity for more than 24 hours and it can suppress the recontamination of bacteria even though bacteria get recontamination after 1 hour. A significant reduction, compared to controls, was detected at 1 hour post treatment at 10 MOI (−40±15%, $p<0.05$) (D'accolti et al., 2018).

6. SUMMARY

The pathogenic MDR *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacteria were collected from ICU ward of Teku Hospital and from AST result, 2 strain of *Pseudomonas aeruginosa* and 5 strains of *Staphylococcus aureus* were found to be Carbapenem and methicillin resistant. *A pseudomonas aeruginosa* (P3) *bacterium was* identified by 16srRNA gene analysis and the gene was submitted to NCBI. P3 bacteria showed 98% similarity with *Pseudomonas aeruginosa* when performed BLAST in NCBI database Altogether 6 lytic phages were isolated by double layer agar assay method which lyses carbapenem resistant bacteria without enrichment. Mixed plaques (Bull's eye and pin head) were found in Pseudomonas aeruginosa phage, we choose phage P4 having pin head plaques for further study. The phage was purified by repeated continuous streaking method and then titer was determined from phage stock. Phage P4 showed titer value above 10^8 concentrations.

Physiochemical Characterization is important for the application of phage which was studied in host range, SDS-PAGE, TEM, pH and temperature. Phage P4 showed wider intra and inter host range lysis. The invitro lysis of host bacteria by phage showed optimum lysis of MOI 1 and burst size 28 virions per bacterium.

Protein profiling of phages was done by SDS-PAGE using acetone precipitation method (to obtain concentrated protein). Proteins of different band size between 20-250 Kda were obtained. From Transmission electron microscopy, the phages were found to be of order Caudovirales and siphoviridae family. Exposure to high temperature ranging from 50°C to 70°C drastically reduces the phage viability, although phage P4 was able to tolerate exposure to temperature as high as 70°C for 20 minutes. Similarly, optimum pH is 7 but our phage was also able to tolerate even in alkaline pH3 and acidic pH12.

Decontamination assay was done in cotton fabric cloth and marble tiles. Firstly, we used fabric cloth pieces to determine the MOI at which our phage could show the effective lytic activity against bacteria spread on the fabric cloth and it was found to be MOI 1. The result on the fabric cloth was highly significant with 1 log reduction in CFU_s of bacteria with p value was found to be 0.002. Similarly, comparative analysis was done with phenol (which is used as daily decontaminating agent). Our phage P4 was found to be more effective and showed significant lytic activity against bacteria than phenol. Our P4 phage showed 1 log reduction of CFU_s with p-value of 0.002 at MOI 1 but phenol showed 0.2 log reductions at MOI 1. We also determined the effective time period up to which Phage could show significant lytic activity which was done on the marble tiles, as it represents decontamination of solid surfaces. Phage P4 can show its lytic activity for more than 24 hours and it can suppress the recontamination of bacteria even though bacteria get recontamination after 1 hours.

7. CONCLUSION

- Pathogenic MDR *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacteria were identified and were found to be methicillin and Carbapenem resistant respectively.
- Total Six phages against two multidrug resistant *Pseudomonas aeruginosa* were isolated which were lytic in nature; plaques were pinhead and Bull's eye.
- Phages were tested for multi host susceptibility and most phages were found to be susceptible to *Acinetobacter baumanii*.
- Phage P4 was found to tolerate wide range of pH and temperature from pH 3-12 and temperature up to 70°C.
- Burst size of phage was found to be 28 virions per bacterium which was calculated using one-step growth curve.
- Our phage P4 was found to be more effective and showed significant lytic activity against bacteria than phenol. Our P4 phage showed 1 log reduction of CFU_s with p-value of 0.002 at MOI 1 but phenol showed 0.2 log reductions at MOI 1.
- The effective time period up to which Phage showed significant lytic activity for MOI 1 hrs. Late but can show its lytic activity for more than 24 hours and it can suppress the recontamination of bacteria even though bacteria get recontamination after 1 hours.

8. LIMITATIONS OF THE STUDY

As our study was conducted in research lab of Central Department of Biotechnology, all the requirements to conduct our study was not available in our lab

- For bacterial identification by 16s rRNA sequencing, bacterial samples were sent to India and it took time for identifying the biochemically unidentified bacteria.
- We were unable to get the whole genome sequencing of our phages and study the mechanism of synergy.
- Due to lack of sophisticated instruments like TEM we had to export our sample to abroad, and could not get TEM result clear as expected.
- We were not able to study in more than one host MDR bacteria due to time limitation.

9. RECOMMENDATION

As our study was the preliminary work on studying the use of bacteriophage as a disinfectant to cure hospital acquired infection by using fomite cloth and tiles, following would be the recommendations for further extension of research.

- First and foremost, whole genome sequencing and complete genome annotation of phage need to be done to understand the phage biology and genetics.
- Aerosol should be made and applied in hospital
- Study should be done in more than one host MDR bacteria.
- Study should be done on the synergistic mechanism of bacteriophage and normal micro flora inside our body.
- Further experiment should be done in hospital, before that it might be done inside CDBT laboratory.

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APPENDICES

Media composition/Reagent preparation

A) Luria Bertani (LB) Broth

Ingredients	**Grams/litre**
Casein enzymic hydrolysate	10.00
Yeast extract	5.00
Sodium chloride	10.00
Final pH (at 25°C)	7.5 ± 0.2

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

Ingredients	**Grams / Litre**
Pancreatic digest of casein	17.00
Papaic digest of soyabean meal	3.00
Sodium Chloride	5.00
Dextrose	2.50
Dibasic Potassium Phosphate	2.50
Final pH (at 25°C)	7.3 ± 0.2

C) Salt of Magnesium (SM) buffer

Ingredients	**Grams / Litre**
Sodium chloride	100mM
Magnesium sulphate	10mM
Tris-HCL	50mM
Gelatin	0.01% (w/v)

Reagents for SDS-PAGE

A) 30% acrylamide solution: (For 100ml)

Constituent's	**weight/volume**
Acrylamide; C_3H_5NO	29g
Bis Acrylamide; $C_7H_{10}N_2O_2$	1g
TDW	Maintain upto 100ml

B) Casting Constituents

Solution components	**Resolving gel (12%) :10ml**	**Stacking gel (5%) :3ml**
TDW	3.3	2.1
30% Acrylamide	4	0.5
1.5% Tris (pH 8.8)	2.5	_
1.5% Tris (pH 6.8)	_	0.38
10% SDS	0.1	0.3
10% $(NH4)2S2O8$	0.1	0.3
TEMED	0.004	0.003

C) Tris buffer

Lower tris pH 8.8: for 100ml				**Upper tris pH 6.8: for 50ml**		
S.N.	Constituents	Amount		S.N.	Constituents	Amount
1	Tris (Tris base)	1.5M/18.17 g		1	Tris (Tris base)	0.5M/3.03g
2	TDW	Maintain 100ml		2	TDW	Maintain 50ml

D) Loading (Sample) buffer (pH 6.8): For 10ml

S.N.	Constituents	Amount (ml)
1	Upper Tris pH 6.8	1.25
2	10% SDS	3.0
3	Glycerol	4.75
4	Beta-mercaptoethanol	0.5
5	0.1% bromothymol blue	0.5

E) Staining solution CBB G-250: 500ml

S.N.	Constituents	Amount
1	CBB G-250	500mg
2	Glacial acetic acid	25ml
3	Methanol	250ml
4	TDW	225ml

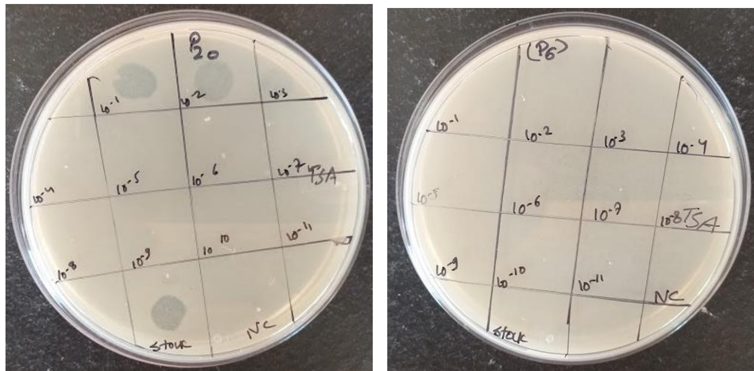
F) Destain solution: 500ml

S.N.	Constituents	Amount (ml)
1	7% glacial acetic acid	37.5
2	5% methnol	25
3	TDW	437.5

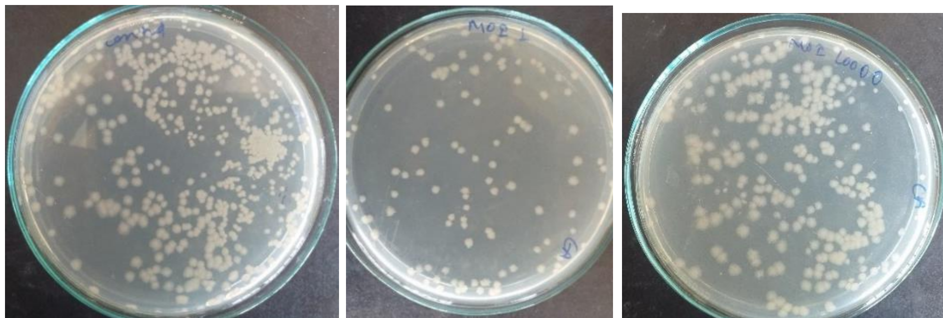
G) Running buffer/ Electrolysis buffer (pH 8.4): 1000ml

S.N.	Constituents	Amount
1	39mM tris	4.724g
2	48mM glycine	3.603g
3	0.1% SDS	0.37g

Intra Host Range analysis



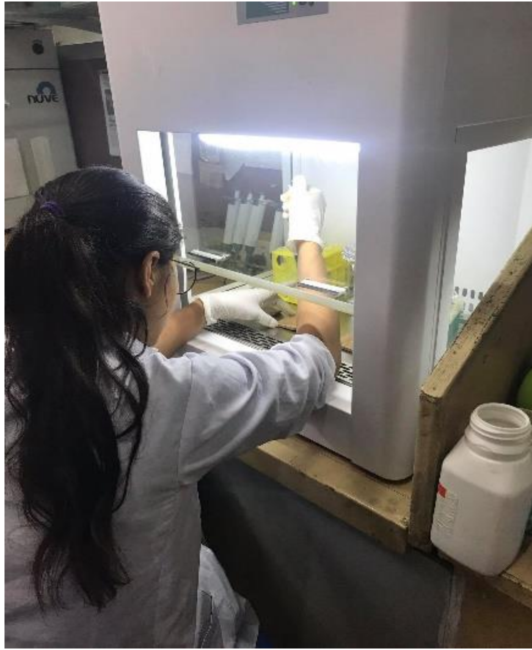
Phage P4 as disinfectant by using bacteria on Fomite cloth



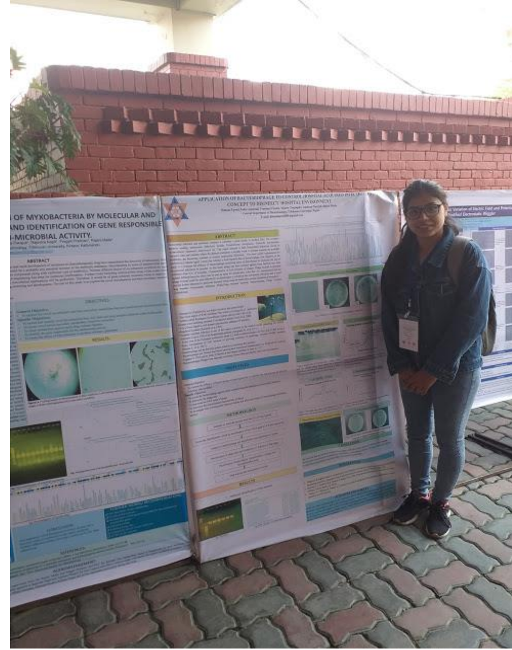
Some pictures during CDBT Thesis Journey

Preparing SDS_PAGE apparatus

Working at laminar hood



Loading bacterial sample on marble tiles



Poster Presentation in International Youth Conference organized by NAST, October 2019

Thesis defense day with my Supervisors and HOD Sir

Holi program at CDBT

CDBT's Stall at NAST on World DNA Day by we third semester students