



Enhanced Host Range and Reduction of Biofilm by Synergistic Effect of Phage Cocktails Isolated from Rivers of Kathmandu, Nepal

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ACRONYMS

BCE	: Bacterial Cell Envelope
BLAST	: Basic Local Alignment Search Tool
CBB	: Coomassie Brilliant Blue
CDBT	: Central Department of Biotechnology
cfu	: colony forming unit
DLAA	: Double Layer Agar Assay
DNA	: Deoxyribonucleic Acid
dsDNA	: double-stranded DNA
EDTA	: Ethylene Diamine Tetra-Acetic Acid
ELISA	: Enzyme Linked Immunosorbent Assay
EMA	: European Medicines Agency
EOP	: Efficiency of Plating
EPS	: Extracellular Polysaccharide
FDA	: Food and Drug Administration
gDNA	: genomic Deoxyribonucleic Acid
ICTV	: International Committee on Taxonomy of Viruses
kDa	: KiloDalton
LB	: Luria Bertani
LF	: Lactose Fermenter
LPS	: LipoPolySaccharide
MDR	: Multi Drug Resistance
MHR	: Multi Host Range
ml	: microliter
NCBI	: National Center for Biotechnology Information
NIH	: National Institute of Health
NLF	: Non-Lactose Fermenter
nm	: nanometer
OD	: Optical Density

OMP	: Outer Membrane Porin/s
PBS	: Phosphate Buffer Saline
PCR	: Polymerase Chain Reaction
pfu	: plaque forming unit
PG	: Peptidoglycan
RBPs	: Receptor Binding Proteins
RNA	: Ribonucleic Acid
rpm	: revolution per minute
SM	: Salt of Magnesium
TAE	: Tris Aetate EDTA
TEM	: Transmission Electron Microscopy
TSA	: Tryptic Soy Agar
TSP	: Tail Spike Protein/s
TTC	: Triphenyl Tetrazolium Chloride
UPEC	: Uropathogenic <i>Escherichia coli</i>
UTI	: Urinary Tract Infection
UV	: Ultraviolet
VALs	: Virion Associated Lysins
VF	: Virulence Factor
WHO	: World Health Organization

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ABSTRACT

Introduction: The world is running out of antibiotics and search for new therapeutic tools to tackle this problem is a major concern worldwide. Using phages in therapeutics has great potential to fight antimicrobial resistance, which can be a solution to this global threat. Bacteriophages are viruses that specifically infect the bacterial host and lyse the host bacteria during their lytic cycle. Phages in therapeutics has been investigated from over a century, now it has been developed as a revitalized therapy. Narrow host range of phages possess both benefits and limitations; highly specific phages may not harm beneficial microbes while such phages cannot be effective to treat multi-bacterial infections. Combination of different phages with complementary features are often used to mitigate such issues. Concept of phage cocktails in treatment of infectious diseases is the most highlighted topic in phage therapy. The use of different phages combined in cocktails allows for the treatment of multiple pathogens, broadening the phages' action spectrum. In this study, we aimed to test the efficacy of phage cocktails to enhance host range along with effective biofilm reduction.

Methodology: Phages were isolated from the river water using different clinical strains of bacteria and were combined to make cocktails. Intraspecific and interspecific host range with both individual phages and cocktails phages was assessed by spot assay and liquid lysis assay/killing assay. Reduction of biofilm by individual phage and phage cocktails was determined by crystal violet assay.

Results: From this research work, we found that *Klebsiella* phage cocktails were effective against *E. coli* pathogenic strains. On multi host range analysis with individual and cocktail *Klebsiella* phages, host range was enhanced by cocktails, which was confirmed by efficiency of plating assay. Morphological identification of phages by TEM showed all the *Klebsiella* phages were of Order Caudovirales and family Podoviridae. The maximum bacterial growth inhibition was at 4 hours of infection, with phage cocktails, showing the bacterial inhibition upto 50%. The bacteria used in our experiment were found to be moderate biofilm producers and there was substantial reduction in biofilm with phage cocktails as compared to individual phages.

Conclusion: The results indicate that the application of the phages in the form of a cocktail have their potential to be used presumptively to control multi-bacterial infection. Phage cocktails of *Klebsiella pneumoniae* can effectively lyse *E. coli* bacterial strains, though different genus, than individual phages, along with effective biofilm reduction. With the extensive research, such phage therapy can treat infection in-vitro; as an application to treat multi-bacterial infections as well as such phage cocktails can be used as disinfectant to decontaminate hospital indwelling devices.

Keywords: Antibiotic resistance, Bacteriophage, Biofilms, Host range, Phage cocktails

CHAPTER 1 INTRODUCTION

1.1 Background

Antibiotic resistance is one of the most pressing public health issue modern world is facing. Antimicrobial resistance (AMR) has possessed a greatest challenge for effective treatment of infections globally, which is why, it has become a growing concern to human, animal, and environment health. Excessive use of antibiotics, antibiotics sold over-the-counter, and increased release of non-metabolized antibiotics or their residues into the environment through manure/feces are the major causes of AMR. In developing countries, another cause of antimicrobial resistance is non-human use of antimicrobials such as to prevent and treat disease in animals, used as growth promoters in animal breeding and as additives in plant agriculture. Multidrug-resistant (MDR) bacteria or “superbugs” are evolving in such a rate that the speed of production of novel antibiotics cannot keep up with the rate at which bacteria are developing and spreading resistance. WHO in its latest report of 2017 has clearly declared that the world is running out of antibiotics and therefore, the search for an alternative is a matter of urgency (Aslam *et al.*, 2018).

On searching alternative tools to treat infections, scientists from different parts of world came up with different alternative tools such as, bacteriophage therapy, predatory bacteria, bacteriocins, and competitive exclusion of pathogens all of which are highly specific in their action than antibiotics (Allen, 2017). Among all alternative options, bacteriophage therapy is among the most heavily researched and have the longest history. Phages in therapeutics has been investigated from over a century, now it has been developed as a revitalized therapy. Many researches regarding the use of phages in therapeutics are ongoing which has given a hope that phages can be a potent solution to battle the war of antibiotic resistance. For the phages to be commercialized as therapeutics, more insights into phages and their mechanism of infecting bacteria is to be studied. Using phages as cocktails to make the treatment more effective is one of the highlighted topics in phage therapy.

1.1.1 Bacteriophage: Phage Biology Basics and Brief History of Phage

Bacteriophage, or simply phages, are the viruses which attack and infect bacteria. Phages are obligatory parasites of bacteria that carry all the genetic information required to undertake their reproduction in an appropriate host. Phages are the most abundant genetic agents on Earth, with estimations of total phage numbers ranging to in excess of 10^{30} virions (Chibani-Chennoufi *et al.*, 2004). Most phages are infectious only to the

bacteria that carry their complementary receptor, thus are highly specific. Host specificity varies among phages, many of which are strain-specific, whereas others can infect across a range of bacterial strains and even genera (Lin *et al.*, 2017). Phages are extremely diverse in size, morphology, and genomic organization. The size of most phage ranges from 22nm – 200nm in length. The largest bacteriophage known is T4 phage which is about 200 nm long and about 80 – 100 nm wide. The genome size of phages varies from a few thousand base pairs up to 498 kilobase pairs in phage G,

which is the largest phage sequenced to date (Romero-Calle *et al.*, 2019). However, structurally all phages consist of a nucleic acid genome encased in a shell of phage-encoded capsid proteins, which protect the genetic material and mediate its delivery into the next host cell. Phage virions could be effectively visualized with electron microscopy; specifically, Transmission Electron Microscopy (TEM). TEM has allowed the detailed visualization of hundreds of phage types, many of which appear to have heads, legs, and tails. The head or capsid is made up of morphological subunits called protomers. Most of the phages are tailed phages representing 96% of known phages (Ackermann, 2009). Tailed phages additionally consist of structures such as; collar, sheath, base plate, spikes and tail fibers.

Discovery of phages dates back over a century. A British bacteriologist, Ernest Hankin, in 1896 reported something in the waters of the Ganges and Jumna in India, which had unexpected antibacterial activity against cholera and limiting the spread of cholera epidemics. That unidentified substance could pass through fine porcelain filters and was heat labile. But he didn't explore his findings. In 1915, the British bacteriologist Frederick Twort discovered a small agent that killed colonies of bacteria in growing cultures and published his results. But it was Felix d'Herelle, a French-Canadian microbiologist, who in 1917, identified the source of this phenomenon and coined the term "bacteriophage" (literally "bacteria eater") (Orlova, 2012). d'Herelle observed that cultures of the dysentery bacteria disappear with the addition of a bacteria-free filtrate obtained from sewage. Many scientists accepted the independent discovery of bacteriophages and simply referred to it as the "Twort-d'Herelle phenomenon" and, later, the "bacteriophage phenomenon" (Sulakvelidze, *et al.*, 2001).

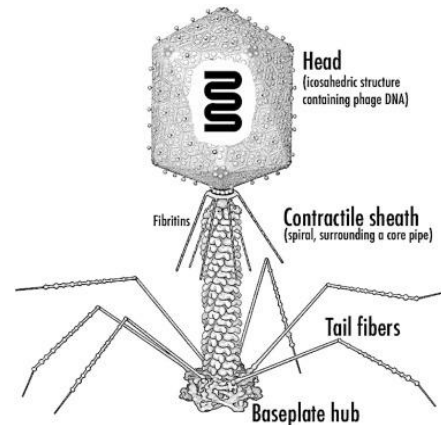
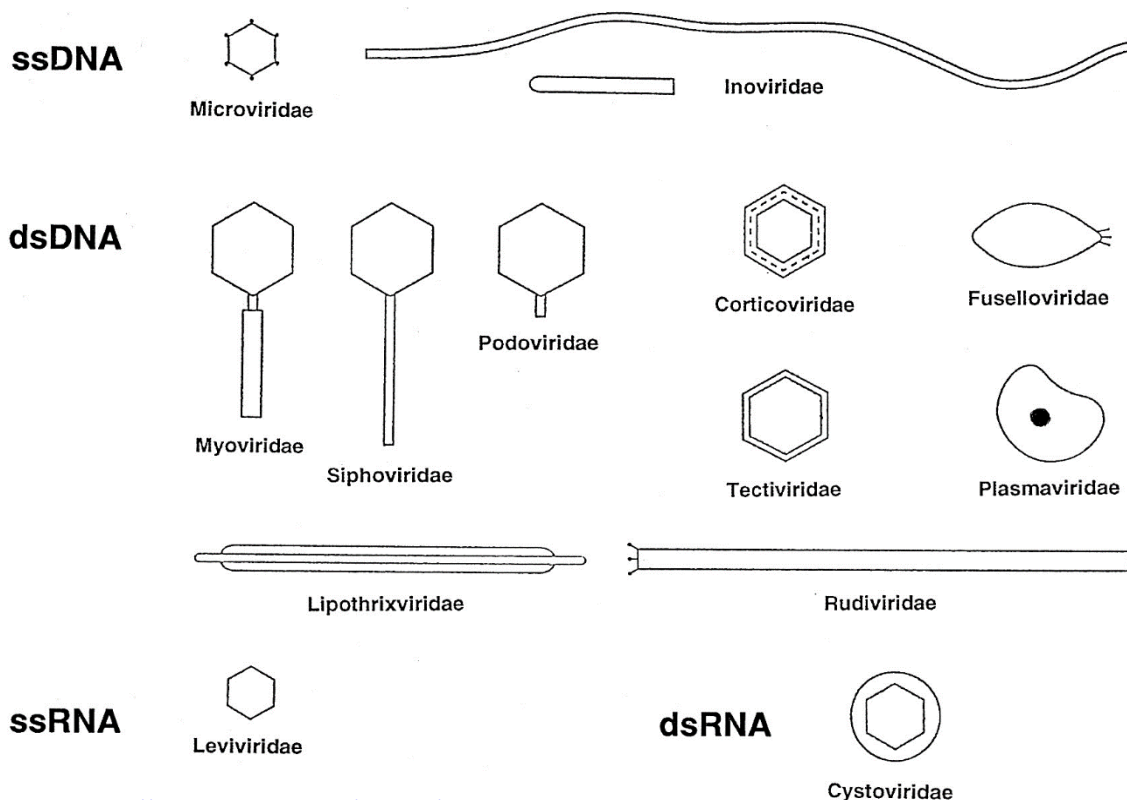


Figure 1.1 Schematic representation of prototypical Bacteriophage

Image source: Harada, *et al.*, 2018

1.1.2 Classification of bacteriophages

Virus classification is based on characteristics such as morphology, type of nucleic acid, replication mode, host organism and type of disease. Over 40 criteria are engaged for phage differentiation into genera and species. Phages can be classified to a first approximation in terms of their genome type and virion morphology, with genome size representing an additional interesting means of distinguishing among phages (Hyman & Abedon, 2012). The **International Committee on Taxonomy of Viruses (ICTV)**, latest scheme of classifying viruses, has produced an ordered system for classifying viruses. The ICTV taxonomic system requires visualization of the phage structure using electron microscopy. Nearly 5500 bacterial viruses have been characterized by electron microscopy. As of ICTV 2019 update, there are 22 families of phage that infect bacteria and archaea (Stone, *et al.*, 2019). Phages are found in a variety of morphologies: tailed phages, polyhedral phages, filamentous phages, phages with a lipid-containing envelope and phages with lipids in the particle shell. They have a genome, either DNA or RNA, which can be single or double stranded.



Source: http://thebacteriophages.net/chapters/0020_figure_001.htm

Figure 1.2 Schematic representation of major phage groups

The genome contains information on the proteins that constitute the particles, additional proteins that are responsible for switching cell molecular metabolism in favor of viruses. The genome can be one or multipartite and is located inside the phage capsid. The shape of viruses is closely related to their genome, and a large genome indicates a large capsid

and therefore a more complex organization (White & Orlova, 2019). The most studied group of phages are the tailed phages of order *Caudovirales*.

1.1.3 Bacteriophage life-cycle- Crucial differences

Like any other virus, phages are also obligatory parasites requiring the help of specific host bacteria to reproduce. There are generally two types of life cycle in tailed phages (*Caudovirales*): lytic and lysogenic. However, sometimes pseudo-lysogeny is also seen. The typical phage cycle begins with the adhesion of viral particle to the surface of bacterial cell (**adsorption**). Right after adhesion, phage activates various molecular mechanisms leading to the injection of viral DNA into the host cell (**penetration**). The following process differs in lytic and lysogenic phages (Ofir & Sorek, 2018).

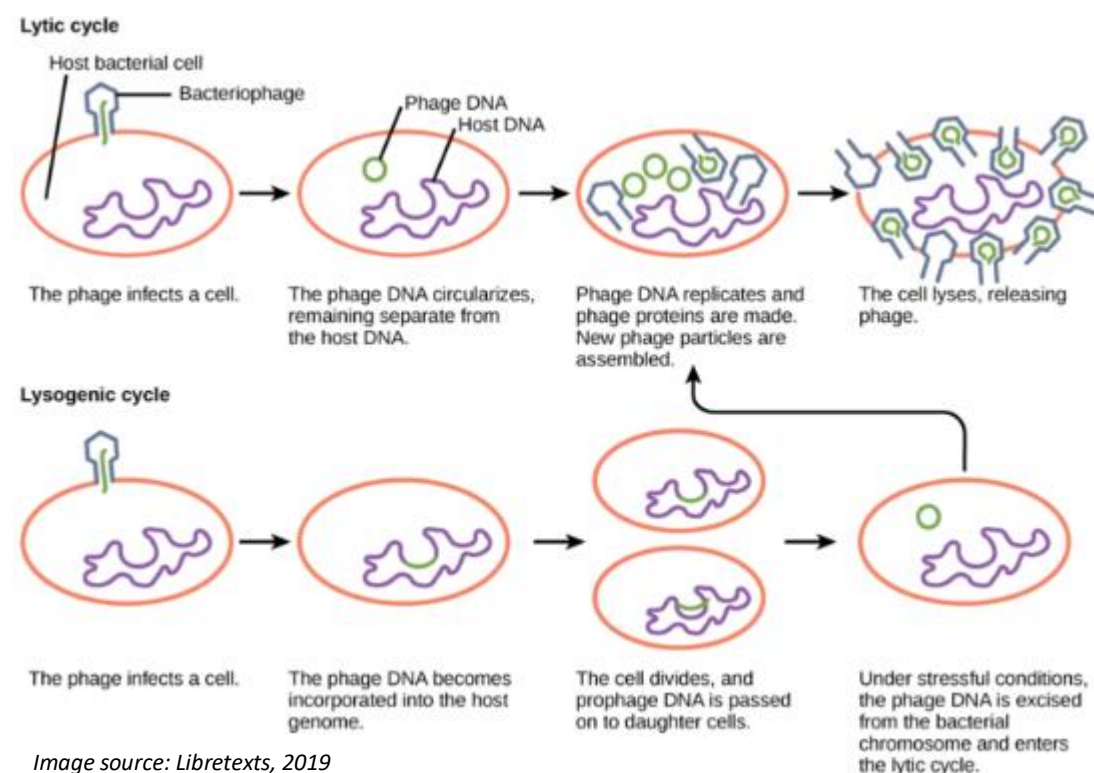


Figure 1.3 Lytic versus lysogenic cycle

In the lytic cycle, the phage replicates and lyses the host cell. In the lysogenic cycle, phage DNA is incorporated into the host genome, where it is passed on to subsequent generations. Environmental stressors such as starvation or exposure to toxic chemicals may cause the prophage to excise and enter the lytic cycle.

In lytic cycle, immediately following injection into the host cell, the phage genome synthesizes early proteins that break down the host DNA, allowing the phage to take control of the cellular machinery. Then phage uses the host cell to synthesize the remaining proteins required to build new phage particles. Consequently, phage capsids are assembled and packed with genetic material. During this process, the host cells gradually become weakened by phage enzymes, lysin, and eventually burst, releasing on average 100-200 new phage progeny into the surrounding environment. That means the

phages cause the complete lysis of a host cell, and the cell subsequently dies after phage replication (Steward, 2018).

Lysogenic phages, can propagate in two different ways; either in the lytic strategy or by simultaneous propagation with the cell host as a prophage (lysogeny). It is governed by several phage-encoded repressors and regulators, as well as, specific phage enzymes such as integrases and excisionases. If environmental condition is favorable, the repressor maintains the phage in lysogenic state. Following the injection of the phage genome into the host cell, it integrates itself into the host genome, with the help of phage-encoded integrases, where it is then termed a prophage. Thus, bacterium continues to live and reproduce normally, but the phage genome is transmitted to progeny cells at each subsequent cell division. Under stressed condition, bacterial cells may mobilize the SOS response system (especially RecA co-protease) and inactivate the phage repressor, which triggers the expression of lytic cycle genes causing the lysis of host cells (Kim & Ryu, 2013).

The third and rare type of phage existence is **pseudo-lysogeny** when the viral DNA is present within a host cell as an independent episome (plasmid-like form). The host is thus only a phage carrier and the episome segregates asymmetrically during cell division. This phenomenon occurs because of unfavorable growth conditions such as starvation occurring to the host cell and is often terminated with the instigation of either true lysogen or lytic growth where the growth conditions are restored (Miller & Ripp, 2002).

For medical treatments we need to use only lytic phages, since phages have to replicate in sufficient number and lyse the bacterial cell. Prophages/lysogenic phages are not preferred in phage therapy as such phage population insert their genome into the host chromosome and can lay dormant or alter the phenotype of the host. It could lead to the transfer of genes that increase the bacterial host's virulence through general or specialized transduction mechanisms (Sabouri & Mahommadi, 2012).

1.1.4 Enterobacteriaceae

Enterobacteriaceae are diverse group of facultative Gram-negative rods, common pathogens of healthcare and community-associated infections worldwide. Among the major public health concerns of MDR pathogens worldwide, MDR Enterobacteriaceae is also on a top list. Within the family Enterobacteriaceae, *E. coli*, and *Klebsiella* spp. ranked highest in the WHO critical priority list of antibiotic-resistant bacteria (Tacconelli, *et al.*, 2018). Both the bacteria can be highly isolated from clinical settings.

Klebsiella pneumoniae is a Gram-negative, facultatively anaerobic rod-shaped bacteria of genus *Klebsiella* and family Enterobacteriaceae. *Klebsiella* spp. are frequent nosocomial and community-acquired pathogens recognized for their MDR status. The pathogenicity of *K. pneumoniae* is multifactorial including capsular serotype, lipopolysaccharide, iron-scavenging systems, and fimbrial and nonfimbrial adhesions urease, outer-membrane

proteins and biofilms. Because of the collective threat of several virulence factors, *K. pneumoniae* has attained superbug status and is one of the most common antibiotic-resistant bacteria (Wu & Li, 2015).

E. coli is the most common pathogen causing diarrhoea, neonatal septicemia, UTI, bacteremia and urosepsis, though many strains are commensal enteric. It is responsible for 80% of community-acquired UTIs and 30% of nosocomial infections. Extraintestinal *E. coli* is one of the leading causes of bloodstream infections and comprises 17–37% of all bacteria isolated from patients with bloodstream infections. Such strains have also been associated with ventilator-associated pneumonia (VAP), a common life-threatening hospital-acquired infection (Messika, et al., 2012). Bloodstream infections with extraintestinal *E. coli* are frequently associated with patients who have undergone major surgeries; who were admitted to hospitals for long durations; or who had a peripheral or urinary catheter (Johnson & Russo, 2002). Virulence is multifactorial, and on the basis of functional groups, the *E. coli* VFs can be categorized as adhesins, toxins, iron uptake, protectins, biofilms and others, such as pathogenicity-associated islands (Nagarjuna, et al., 2015).

Being from same family and associated with some common type of nosocomial infections, researches regarding the use of bacteriophages to treat such infections in common is important.

1.2 Current studies

With the rapid emergence of MDR pathogens globally, the Western world has developed prompt interest in phages as alternative to antibiotics. Though bacteriophages can be applicable in various fields, current studies on bacteriophages focus on clinical application of phages as therapeutic tools to treat bacterial infections. Properties of phages to be used as therapeutic tools, molecular mechanism of phage- host interaction and how phages can actually kill the pathogens are the most focused areas of bacteriophage research. Several clinical trials have been conducted in recent times and clinical trials for the treatment of venous leg ulcers and chronic otitis are among the first controlled clinical trials in humans conducted in the western world. More recently, a number of clinical trials have been registered; chronic otitis, infected burn wounds, diarrhea, UTIs, gastrointestinal disorders and others (Furfaro, et al., 2018). Most recently, the first US clinical trial of intravenously administered bacteriophage therapy has received FDA approval, for the treatment of participants with ventricular assist devices (VADs) infected by resistant *Staphylococcus aureus* (Technology Networks, 2019).

In context of Nepal, AMR is at an emerging rate. Researches regarding the isolation, purification and host range analysis of phages from sewage samples of different parts of Nepal have published in different journals. Researches regarding the application of phages

in mice models had also conducted, in Central Department of Biotechnology, Nepal, though the publication process is not completed yet. With the growing interest of using phage cocktails as effective treatment option worldwide, and as suggested by many literatures, my research here is focused on the *in-vitro* study of phages and formulated phage cocktails isolated from rivers of Kathmandu and study the phage cocktails effect on enhancing the host range of broad host range phage in different genus of bacteria.

1.3 Research hypothesis

1.3.1 Alternative hypothesis, H₁:

Phage cocktails enhance the host range and effectively reduce the biofilm formed by different bacterial strains.

1.3.2 Null hypothesis, H₀:

Phage cocktails do not enhance the host range and do not effectively reduce the bacterial biofilm.

1.4 Objectives

1.4.1 General objective

- To determine the efficiency of phage cocktails in enhancing the host range and reducing bacterial biofilm.

1.4.2 Specific objectives

- Biochemical and/or molecular identification of bacterial host
- Characterization of phages morphologically and their protein profiling
- Determination of host range of individual and phage cocktails using different strains of MDR bacteria
- Determination of bacterial growth inhibition rate by individual and phage cocktail
- Determination of phage cocktails role in bacterial biofilm reduction

1.5 Rationale of the study

Phages are an 'intelligent' drug which multiplies at the site of the infection until there are no more bacteria. In past, use of phages as antimicrobial agent was limited due to lack of knowledge and sophisticated instruments to study about bacteriophage. But now, with the advancement in technology and vigorous research on phage therapy more doubts are clear. With ongoing vigorous researches, many advantages of using phages in therapy have studied. Using single phages to treat specific infection has a chance to develop phage resistance so, concept of phage cocktails in therapy has been recently developed. Cocktail phages can also broaden the host range which is important factor to be considered to use phages in therapy. And there are various success stories of phage cocktails as therapeutic agent to treat a patient near death from a multidrug-resistant bacterium. Unlike other researches our research is focused on using the prepared phage cocktails of one bacterial genus to lyse the bacterial of other bacterial genus of same family; Enterobacteriaceae and determine whether the phages as cocktails can broaden host range or not. Also, we will be determining the biofilm reduction by phage cocktails.

CHAPTER 2 LITERATURE REVIEW

2.1 Phage-Host interaction mechanisms

Phages are the viruses which attack and infect bacteria. Like other viruses, phages are obligatory parasites, that is, depend on their host for the production and release of new viral particles. Understanding the structural and molecular mechanisms of phage–host interactions is crucial for the application of phages in medicine and biotechnology. In addition, understanding this mechanism also provide deeper mechanistic insight into phage ecology and evolution, as well as their role in shaping the human microbiome and microbial ecology in general. Phage-host interactions require both a host-encoded receptor(s) and a phage-encoded receptor binding protein (RBP). All tailed phages use the tail and associated RBPs to interact with the host and to create a channel through which the DNA enters the cell. Despite this common strategy, the protein machinery responsible for host adsorption has specific features depending on phage taxa. Such features can influence the phage host range.

2.1.1 Receptor Binding Proteins

RBPs are major determinants of phages' host-range, have a high genetic plasticity, which enables the phages to adapt to and evolve to infect new hosts. The RBPs of tailed phages, can localize in different substructures of the tail, namely fibers, spikes or baseplates. Tail spikes have largely conserved structures but vary in their receptor-binding domains and the host specificities that they confer. Furthermore, tail spikes often carry enzymes that degrade the cell wall and facilitate DNA ejection. In contrast to tail spikes, tail fibres usually have no enzymatic activity although regions encoding depolymerases have been found in tail fibre genes. As with tail spike, tail fibres have highly variable receptor-binding domains and are involved in reversible and/or irreversible adsorption. Tail fibres are involved in triggering conformational changes that lead to DNA ejection from the phage capsid. Other are the membrane-penetrating proteins which are structurally and functionally distinct from tail fibres and tail spikes. Their main function is breaching of the cell boundary, although virtually nothing is known about penetration of the inner membrane. Also, the phages harbor many different types of peptidoglycan-degrading enzymes, including endopeptidases, N-acetylmuramyl-l-alanine amidases, N-acetyl-glycosaminidase, lysozymes and lytic trans-glycosylases which locally degrade or rearrange peptidoglycan. Phages can also have enzymes that target other bacterial structures (Nobrega, *et al.*, 2018).

2.1.2 Host receptors

Like phage RBPs, the type and structure of the host receptors have a crucial role in determining the specificity of phage–host interactions. Receptor localization, amount and

density on the cell surface also influence specificity. Gram-positive and Gram-negative bacteria differ substantially in the thickness, uniformity and lipid and lipoprotein content of the cell wall and in the receptors available for phage adsorption. The receptor presented on the cell surface may be a carbohydrate, protein or teichoic acid moiety, or a combination of these (Casey, *et al.*, 2018).

The main receptor in Gram-negative bacteria is lipopolysaccharide (LPS). In Gram-negative bacteria, the peptidoglycan layer is relatively thin and is located inward of the outer membrane. The outer membrane is a sophisticated structure composed of a lipid bilayer ornamented with proteins, and lipopolysaccharides (LPS). Generally, LPSs are polysaccharide (O-antigen). Cells that contain all three components of the LPS are smooth (S) type and those that lack the O-polysaccharide portion are distinguished as rough (R) type. Furthermore, O-antigens differ between different host bacteria.

Phages recognize the O-antigen with their tail fibres or tail spike proteins, and they can hydrolyze the O-antigen to enable penetration of the tail. For example: Podophage T7 and siphophage SSU5 use the gp17 and gp22 proteins of tail fibres, respectively, to bind rough LPS in *Shigella* spp., *Escherichia* spp. and *Salmonella* species. Notably, the same phage can bind to different structures of the LPS core when interacting with different bacterial species and genera. Some phages bind outer membrane porins, such as OmpC and OmpF. For example, myophage T4 can use either OmpC or LPS for adsorption. Other transport proteins have also been used as receptors, including vitamin B12 uptake protein BtuB, maltoporin LamB, efflux pump TolC, ferrichrome transport protein FhuA and TonB.

In Gram-positive bacteria, the main component of the cell wall is peptidoglycan, which is a heteropolymer of N-acetylglucosamine and N-acetylmuramic acid, and is often involved in phage adsorption. Another main component of the cell wall of Gram-positive bacteria that can be involved in phage adsorption is teichoic acid—polysaccharides composed of glycerol phosphate or ribitol phosphate and amino acids. They are bonded to the muramic acid of peptidoglycans. When teichoic acids are bonded to the lipids of the plasma membrane, they are called lipoteichoic acids (LTA). Phages can also bind the backbone of the teichoic acid. Phages can also bind proteins of Gram-positive bacteria, for example, the cell wall protein GamR in *Bacillus anthracis*, the phage infection protein (Pip) in the membrane of *Lactococcus* species. Fewer phage receptors have been found in Gram-positive bacteria than in Gram-negative bacteria. It is expected that recent technological advances, such as single particle cryo-electron microscopy, will improve the identification of phage receptors in Gram-positive bacteria.

Structures other than cell wall components such as, flagella, pili and capsules have also proven to act as phage receptors. **Flagella** are long thin helical structures that confer motility to cells. They are composed of a basal body, a flagellar hook and a flagellar

filament composed of subunits of flagellin proteins. Phages that use flagella as receptor, such as iEPS5, SPN2T (both infecting *Salmonella* spp.), typically bind flagellin proteins, such as FliK and FliC at the distal tip of the flagellum using their tail fibres. The phages are

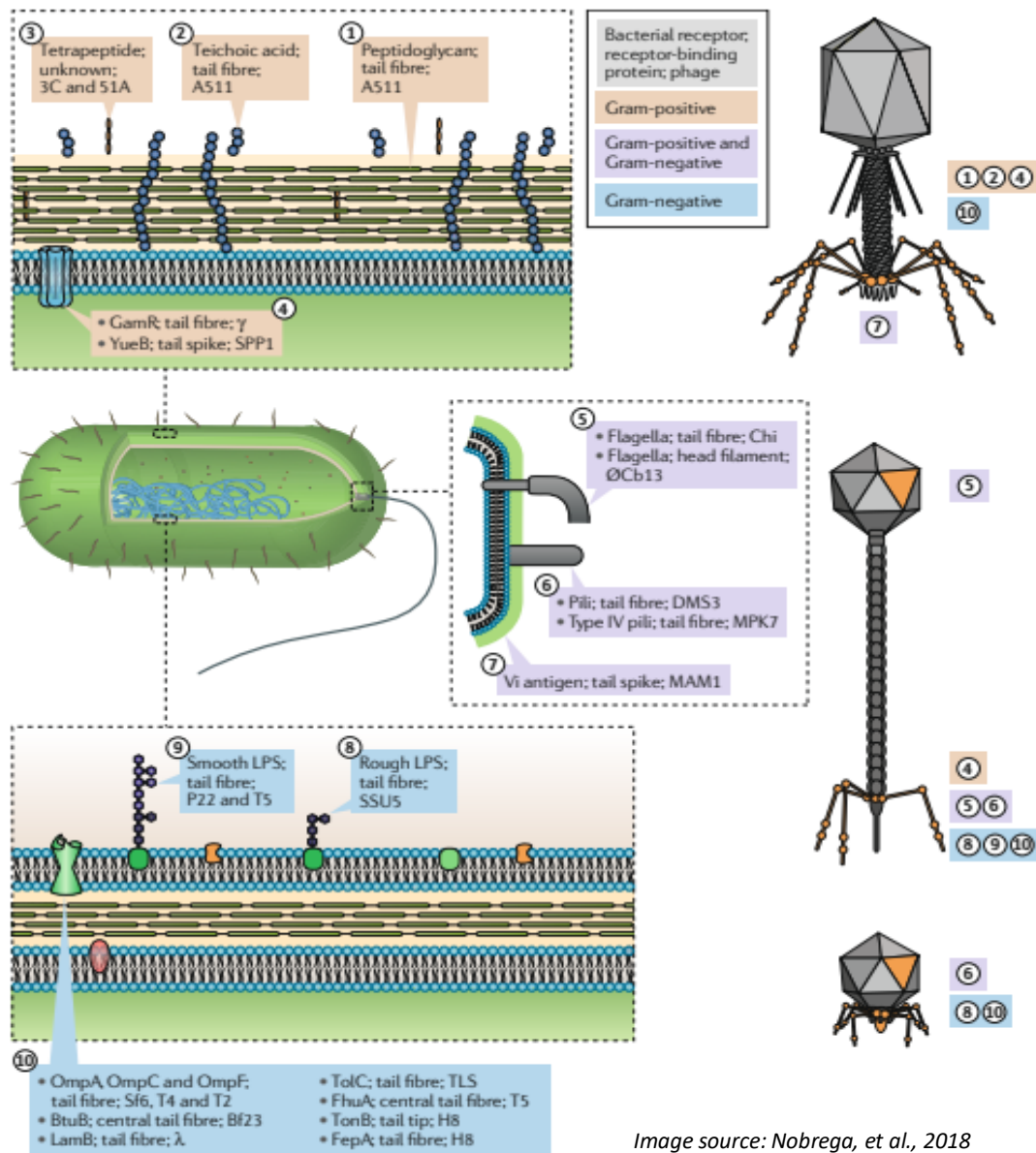


Image source: Nobrega, et al., 2018

Figure 2.1 Gram-negative and gram-positive bacterial receptors for the attachment of tailed phages

The legend for both bacterial receptors is given in the middle panel. The Gram-positive and Gram-negative cell walls and thus the structures available for phage binding differ substantially. Some of the main phage receptors in Gram-positive bacteria are peptidoglycan and teichoic acids. In Gram-negative bacteria, tailed phages bind LPS. Both Gram-negative and Gram-positive bacteria can have flagella, pili and Vi antigen, for example, those found in the capsule of *Salmonella* spp., represent potential phage receptors. Notably, phages often bind these latter bacterial structures with their head or the side of the tail fibre or spike to leave room for the other receptor-binding proteins to engage the ultimate bacterial receptors on the bacterial cell surface. Numbering on the left indicates the bacterial structure bound by the phage receptor-binding protein identified on the right with the same numbering. BtuB, vitamin B12 transporter BtuB; FepA, ferrienterobactin receptor; FhuA, ferrichrome outer membrane transporter/phage receptor; GamR, cell wall protein GamR; LamB, maltoporin; TolC, outer membrane protein TolC

then transported by flagellum motility along the filament to their ultimate receptors. **Pili** are rod-shaped filamentous appendages used for bacterial conjugation. Tailed phages can also attach to pili, a common mechanism of *Pseudomonas* spp. phages, such as phiKMV and MPK, which specifically bind to type IV pili. Some bacteria produce a polysaccharide **capsule** and/or a slime layer; flexible cementing substances with sticky nature that extend radially from the cell wall. Adsorption of phages to capsules or slime layers is mediated by enzymatic cleavage of the EPS that compose the layers. The hydrolysis of the layer is a reversible step, whereas irreversible binding is achieved through bonding of the phage with receptors on the cell wall. Examples include the phages of *S. Typhimurium* which uses Vi capsular antigen as receptor, K11 phages of *Klebsiella* species.

(Rakhuba, *et al.*, 2010; Silva, *et al.*, 2016; Nobrega, *et al.*, 2018)

2.1.3 Enzymes involved to cross the bacterial cell barriers

Tailed phages of both Gram-negative and Gram-positive bacteria commonly harbor degrading enzymes, that target extracellular polysaccharides (EPS), structural polysaccharides like the LPS or the PG glycan stands or other components of the bacterial surface. These enzymatic activities may be important for the phage to go through protective substances that block the access to cell receptors, to mediate the actual process of receptor binding, or to overcome barriers of the Bacterial cell envelope (BCE). Most of these enzymes belong to a broad group of polysaccharide-degrading proteins, also known as polysaccharide depolymerases, which degrade the EPS layer composing capsules or biofilm matrices. LPS-targeting enzymes are mostly referred to as TSPs. In accordance to their mechanism of action, phage depolymerases can either be hydrolases or lyases, each of which causes the breakdown of polysaccharides into soluble oligosaccharides. Since these enzymes target and compromise structures important for cell survival and virulence they may find applications in the control of bacterial pathogens. Phages that infect bacteria with a polysaccharide capsule often encode endoglycosidases, endosialidases and enzymes with antibiofilm activity. Peptidoglycan-degrading enzymes, include endopeptidases, N-acetylmuramyl-L-alanine amidases, N-acetylglucosaminidase, lysozymes and lytic transglycosylases. These enzymes locally degrade or rearrange peptidoglycan, and although they are not always required for infection, they provide advantages under particular conditions.

Upon irreversible binding, most phages use their tail dynamics to penetrate the different layers of the BCE and to deliver the viral DNA into the bacterial cytoplasm. Insertion of the tail substructures across the CW can be assisted by virion-associated lysins (VALs) that promote a local degradation of the PG network. VALs are actually virion-associated depolymerases and are named so because they are capable of provoking host cell lysis. As

components of the virion structure, VALs may play key roles in the morphogenesis, stability and infectivity of phage virus particles. (Fernandes & São-José, 2018; Yan, *et al.*, 2013).

2.1.4 Crossing the Bacterial Cell Envelope and LPS to Get Outside

By highly specific irreversible phage-receptor binding, several enzymatic activity and conformational changes, phage insert their genetic material into host cell. And in doing so, cell viability is not compromised. Quite oppositely, the mechanisms for virion progeny release from infected bacteria typically involve considerable destruction of the BCE, with consequent host cell burst (lysis). For most phages, active and timed lysis involves three steps corresponding to disruption of each of the three layers of the cell envelope: inner membrane, peptidoglycan and outer membrane. Tailed phages accomplish bacterial cell lysis through the concerted action of at least two phage-encoded functions, the holin and the endolysin. Additional proteins called spanins are involved in the lysis process in Gram-negative hosts. **Holins** are hydrophobic proteins which oligomerize in the cytoplasmic membrane at mid or late stages of phage replication. Holins create small pores in the membrane and allow the endolysins to reach their substrates after reaching a genetically defined threshold concentration. **Endolysins** are PG-degrading enzymes synthesized during the late phase of gene expression in the lytic cycle. The most commonly found catalytic domains in these enzymes have muramidase or amidase activity. Depending on the absence or presence of a signal sequence, endolysins are classified as canonical endolysins (no signal sequence) and signal-anchor-release endolysins (Signal Arrest Release endolysins, N-terminal type II signal anchor). Canonical endolysins require holing to reach their substrate, and are released at a precise timing through the large holes generated by the aggregation of holin molecules. While with non-canonical endolysins, their release occurs through membrane depolarization performed by pinholins (membrane depolarizers), which make small holes in the host cell membrane. In Gram negative bacteria lysis additionally requires the disruption of the outer membrane. **Spanins** are essential for the lysis of such bacteria with both internal and outer membranes (Fernandes & São-José, 2018; Young, 2014).

2.2 Bacteriophages and their potential application

Phages, the viruses that infect and lyse host bacteria using host cell machinery, are considered as great potential tools due to their multiple benefits. Since their discovery, phages have been used as models to understand fundamental genetic processes and as great tools in molecular biology. Phages can be used for pathogen detection, as biocontrol agent in food, agriculture, disinfectant in hospitals, as vaccine carrier as well as therapeutic tool in veterinary and medicals (Harada, *et al.*, 2018). In spite of the rapid success of antibiotics, the emergence of multi-drug resistant bacteria is a general concern

which has led to the need for new treatments. Our emphasis is on the potential application of phages against pathogenic bacteria, phage cocktails as host range enhancers and phages as biocontrol agents. Different approaches using phages are proposed, and some of the most relevant ones in the fight against bacterial resistance are reviewed here.

2.2.1 Phage types used in therapeutics

Not all type of phages can be used as a therapeutic tool. Only the lytic phages which can clearly lyse the bacterial host are preferred. Virulent tailed phages, of Order *Caudovirales* have been the best described for phage therapeutic applications (Romero-Calle, *et al.*, 2019). The order consists of the three families of tailed bacterial viruses infecting Bacteria and Archaea. The virion has no envelope and consists of two parts, the head and the tail. The head is a protein shell and contains a single linear dsDNA molecule, and the tail is a protein tube whose distal end binds the surface receptors on susceptible bacterial cells. DNA travels through the tail tube during delivery into the cell being infected. Heads have icosahedral symmetry or elongated derivatives thereof (with known triangulation numbers of T=4, 7, 13, 16 and 52). Isometric heads are typically 45–170 nm in diameter. Elongated heads derive from icosahedra by addition of equatorial belts of capsomers and can be up to 230 nm long. Tails usually have base plates, spikes, or terminal fibers at the distal end. Some viruses have collars at the head–tail junction, head or collar appendages, transverse tail disks, or other attachments. Tailed phages can be found everywhere and represent 96% of known phages and are separated into three main phylogenetically related families.

- *Myoviridae*: Phages in *Myoviridae* family have a complex contractile tail (only tailed phages with a contractile tail) more or less rigid, long and relatively thick (80–455×16–20 nm). They consist of a central core built of stacked rings of six subunits and surrounded by a helical contractile sheath, which is separated from the head by a neck. Contractile tails are the most complex structures used for the delivery of phage genomes. Example: T4 and Mu phages that infect enterobacteria.
- *Podoviridae*: Podoviridae phages, such as coliphage T7 and *Salmonella* spp. phage P22, have short, non-contractile tails about 20×8 nm that consist of an upper tail adaptor protein that connects the tail to the capsid and a lower nozzle that is surrounded by six or twelve tail fibres or tail spikes, but they have no baseplate.

- *Siphoviridae*: Phages of *Siphoviridae* family, such as the enterobacterial phages λ and T5, have long, flexible, non-contractile, thin tails (65–570×7–10 nm) without a sheath. Siphophages that infect Gram-negative bacteria generally have both a central, straight fibre and side fibres, but they lack baseplate. While some siphophages infecting Gram-positive bacteria have baseplates less complex than of myophages (Nobrega, *et al.*, 2018).

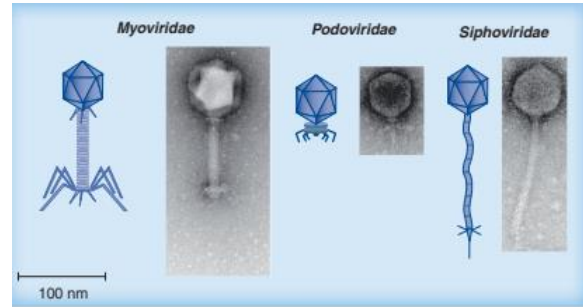


Figure 2.2 Morphology of the tailed bacteriophages (Caudovirales)

Image source: Harper, *et al.*, 2011

Host range of phages is one of the important characteristics to be understood for using phages in therapeutics. The host range of a bacteriophage is the taxonomic diversity of hosts it can successfully infect. Host range is determined by a range of molecular interactions between phage and host throughout the infection cycle. Mostly phages have a narrow host range and infect specific bacteria or its strain and is considered advantageous as such phages cannot infect the normal flora. However, phage–host coevolution studies have shown that host specificity can be altered through mutations in the RBP gene as well as in other genes. Most mutations do not lead to a change in host range due to the more constrained nature of gain-of-function mutations in the phage versus loss-of-function mutations in the host. Some phages possess molecular mechanisms that allow for targeted genetic diversification of RBP, thereby allowing controlled host switching. Polyvalent broad-host-range phages encode mechanisms for binding multiple different receptors (Jonge, *et al.*, 2019). High specificity of phages is not always an advantage as such phages cannot be effective to treat multi-bacterial infections. Concept of using phage cocktails can better solve the problem with narrow host range as well as development of resistance. Phages as cocktails can increase the chance of receptor binding to phage by providing different receptor binding proteins to bind the host cells.

2.2.2 Phage Therapy

Phage therapy is based on the therapeutic use of phages to treat bacterial infections. One of the basic principles to select phages for therapy is excluding temperate phages because the bactericidal effect is only guaranteed for lytic phages. The main application of phage therapy is its use as a therapeutic agent to eliminate pathogenic bacteria involved in disease or infection as well as those that form biofilm (Domingo-Calap & Delgado-Martínez, 2018).

2.2.2.1 Early phage Research to Revitalization

After the discovery and naming of bacteriophage by himself, d'Herelle immediately continued on his findings and used phages to treat dysentery, and it was probably the first attempt to use bacteriophages therapeutically. The first reported application of phages to treat infectious diseases of humans came in 1921 from Richard Bruynoghe and Joseph Maisin, who used bacteriophages to treat staphylococcal skin disease. The bacteriophages were injected into and around surgically opened lesions, and the authors reported regression of the infections within 24 to 48 hours. With such several promising and encouraging results, d'Herelle and others continued studies of the therapeutic use of phages (e.g., d'Herelle used various phage preparations to treat thousands of people having cholera and/or bubonic plague in India (Summers, 1999). In addition, several companies began active commercial production of phages against various bacterial pathogens. In 1923, the Eliava Institute was opened in Tbilisi, Georgia, to study bacteriophages and to develop phage therapy. Since then many scientists have been involved in developing techniques to study phages and using them for various purposes. The prestigious Nobel Prize in Physiology and Medicine (1969) was awarded to Max Delbrück, Alfred Hershey and Salvador Luria for their discoveries of the replication of viruses and their genetic structure (Sulakvelidze, *et al.*, 2001; Orlova, 2012).

The discovery of penicillin in 1928 began the golden age of antibiotics, which pushed aside the interest in the development of phage as clinically-used antibacterial agents, especially in the West (in the East, phage therapy quietly pressed onward). However, in recent years antibiotic resistance is widespread, and new antibiotics to fill the gaps have not emerged at a pace sufficient to eliminate concern. Thus, in the last 15 years or so, development of phages as therapeutic drugs has gained momentum. Various trials including randomized ones, have been described since 2000 in Western countries while in Eastern world it was an ongoing process since discovery. Such well-controlled trials and studies have addressed common nosocomial infections that have the potential to cause bacteremia, including the culprits *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus*, as well as infections of the urinary, respiratory, and gastrointestinal tract (Romero-Calle, *et al.*, 2019).

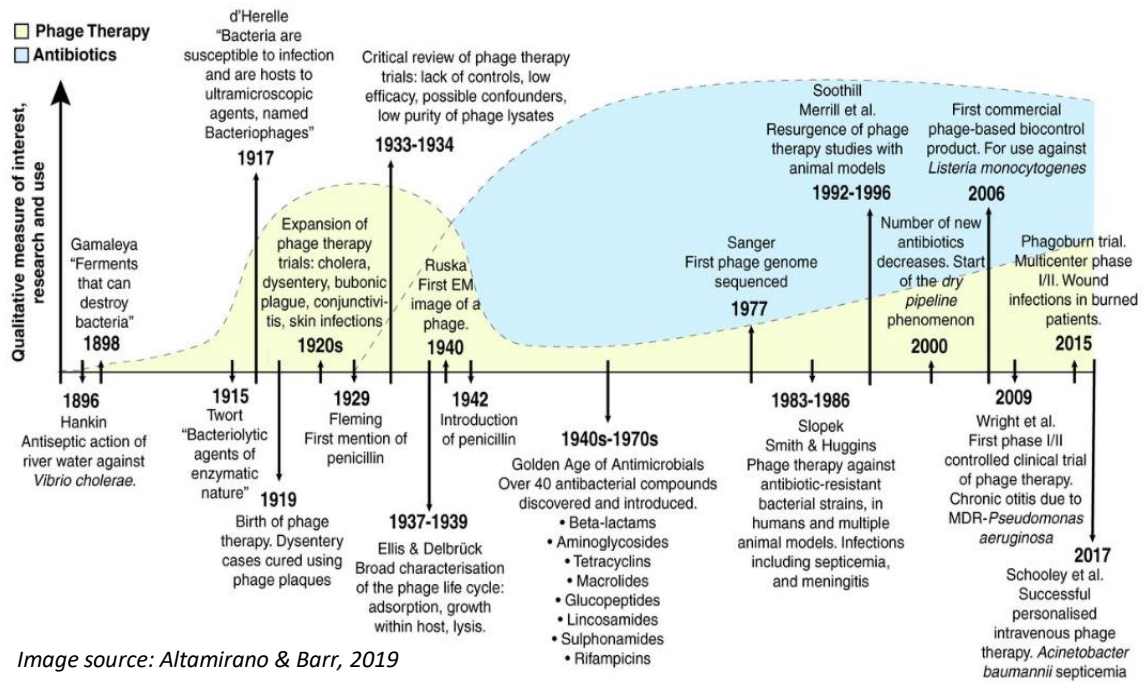


Image source: Altamirano & Barr, 2019

Figure 2.3 Timeline of major events in history of phages, phage therapy, and antibiotics

Background curves represent a qualitative measure of the overall interest, research, and use of phage therapy (yellow) and antibiotics (blue), showing how the introduction of antibiotics and the critical review of the early phage therapy studies coincided to bring phage therapy research and development to an almost complete standstill around the 1940s.

The intensity of investigation using controlled models is increasing, and phages are demonstrated to be effective at reducing bacterial burdens and improving the health of test subjects. This has led even more adventurous attempts to use phage in the treatment of human infections in the West. The recent first-in-human systemic and successful administration of phages in the United States to clear an *Acinetobacter baumannii* infection, Tom Patterson case, is also evidence of the growing willingness to move this technology forward. There are several successful studies of phage therapy till date, (Green, *et al.*, 2018), most of the cases being treated with phage cocktails. Phage cocktails has been designed and used so as to broaden/ enhance broad host range as well as to reduce the chance of phage resistance mutants.

2.2.2.2 Polyphage therapy

Polyphage therapy is the application of a phage cocktail, that is, therapy involving the simultaneous use of more than one phage type. Despite the effectiveness of a single type of bacteriophage against a bacterial strain, because most infections in the body are polymicrobial, either polyvalence phage or phage cocktails can be an interesting strategy to develop a broader host spectrum and solve issues relating to resistance. (Domingo-Calap & Delgado-Martínez, 2018). Thus, it is imperative to employ effective and efficient phage cocktails, curated from a combination of multiple selected phages. There have been several notable clinical trials of phage therapy within recent time most of which uses

phage cocktails for effective result. In Patterson's case, patient with necrotizing pancreatitis and infection with multi-drug resistant *Acinetobacter baumannii*, nine different bacteriophages that could lyse *A. baumannii* were administered intravenously and intracavitary, which save the patient from near death (<https://www.infectioncontroltoday.com>). Phage cocktail of genetically engineered lytic phages were administered intravenously to treat a young patient with cystic fibrosis and bilateral lung transplantation who had developed a *Mycobacterium abscessus* infection (LeMieux, 2019). Also, phage cocktails can play a decisive role in biofilms by allowing for the phages' effects to last longer by delaying the emergence of resistance to all the phages that are part of the cocktail.

2.2.3 Advantages and Disadvantages of Phage therapy

2.2.3.1 Key Advantages

- ❖ Specificity: Most phages are very specific to bacteria, targeting the site where host is concentrated, avoiding the killing of beneficial microbes.
- ❖ Lack of reported side effects: To date, phages have also not shown any significant side effects or risks of toxicity on mammalian cells.
- ❖ Self-reproducing, allowing dose-independent administration: able to produce therapeutic dose only at infected sites from a very small input dose.
- ❖ Able to counter the development of resistance: bacteriophages undergo adaptation *in vivo*, including antagonistic co-evolution with their bacterial targets, countering the development of resistance. Although resistance to phage has been reported, it is claimed that it can be avoided by using phage cocktails.
- ❖ Simple and inexpensive to manufacture: the process of isolation and selection of new phages is less expensive, in terms of time and costs, than the development process required for antibiotics: bacteriophages grow to high titers with simple protocol.
- ❖ Biofilm clearance: phages have been demonstrated to lyse and penetrate through some biofilms that have shown resistance to antibiotics. This is partially attributed to the presence of depolymerases and lysins that can chew through the biofilm extracellular polymeric matrix.

2.2.3.2 Key challenges

Regardless of the numerous known advantages of bacteriophages, there remain outstanding challenges and un-addressed limitations to this approach that must be addressed and further investigated. These are predominantly due to gaps in knowledge and regulations, which may be resolved in the future.

- ❖ Need to know nature of infection: As phages can multiply only at the infection site, we need to know the nature of infection; for determining administration route.
- ❖ Need to avoid lysogenic bacteriophages and confirm lack of transduction

- ❖ Need to assemble multiple agents into a single therapeutic (but this can be simple to do and helps to counter resistance).
- ❖ Phage inactivation: Phage inactivation by human serum can pose a limitation in phage therapy.
- ❖ Regulatory issues: Even though phage therapy was employed in Western medicine before antibiotics took over, it currently does not have the approval from the Food and Drug Administration (FDA) or the European Medicines Agency (EMA) for human administration. Phage is allowed only on a compassionate care basis for human therapeutic use.

(Romero-Calle, *et al.*, 2019; Harper, *et al.*, 2011)

2.2.4 Phages in biofilm control

2.2.4.1 Bacterial Biofilms

Biofilm is an organized aggregate of microorganisms in which cells are frequently embedded in a self-produced matrix of EPS that are adherent to each other and/or a surface (Jamal, *et al.*, 2018). Most of the biomass of biofilm comprises hydrated EPS rather than microbial cells. Different types of components are present in extracellular polymeric substances. The main component of the matrix is water (up to 97%), which contains the structural and functional components of the matrix: soluble, gel-forming polysaccharides, proteins and extracellular DNA, as well as insoluble components such as amyloids, cellulose, fimbriae and pili (Flemming, *et al.*, 2016). Bacterial biofilms appear to be the predominant or even “most successful” bacteria state and nearly all (99.9%) of microorganisms have the ability to form biofilm on a wide range of surfaces i.e. biological and inert surface. Common biofilm producing bacteria are *P. aeruginosa*, *S. epidermidis*, *E. coli*, *S. aureus*, *E. cloacae*, *K. pneumoniae*. According to NIH, about 65% of all microbial infections, and 80% of all chronic infections are associated with biofilms (Lewis, 2001; Jamal, *et al.*, 2015). Biofilms are the defensive systems of bacteria, which have a vital role in bacterial pathogenesis, and are associated with an emergence of antibiotic resistant bacteria. Biofilms are thought to underlie much of the reported resistance to antibiotics in clinical use. The bacteria in a biofilm are 1,000- fold more resistant to antibiotic treatment than the same organism that are grown planktonically (Chadha, 2014).

2.2.4.2 Phages in biofilm disruption

While searching for alternative tools to tackle the problem of antibiotic resistance, it is also important to focus on biofilm disruption of bacteria. Bacteriophages have been considered as a promising strategy against bacterial biofilms. Phages act differently on bacteria contained within biofilms than do antibiotics. Several studies have shown that phages can effectively infect and lyse cells present in single and polymicrobial species biofilms (Pires, *et al.*, 2017). Bacteriophages replicate within their host cells, resulting in

localized increase in bacteriophage numbers, which releases increasing numbers of infectious progeny bacteriophages into the biofilm. By spreading through the biofilm, phages eliminate the bacteria producing the EPS material, remove the biofilm and reduce the potential for regeneration. Natural bacteriophages can and often do express enzymes capable of disrupting biofilms. Phages can produce and/or induce depolymerizing enzymes i.e. depolymerases that are able to degrade the exopolymeric matrix facilitating the migration of the phage through the biofilm, while it has been recorded that all biofilm diffusing phages need not produce depolymerases. However, with phages which do not produce depolymerases, not all studies have shown efficient infection within biofilms. Persister cells can be infected by bacteriophages; although bacteriophage cannot replicate within and destroy inactive cells, they can remain within these bacteria until they reactivate and then commence a productive infection, which then destroys the cells (Harper, *et al.*, 2014). Interaction of phages and biofilm cells depends on phage-host characteristics and biofilm structure. Biofilm features such as, biofilm matrix, reduced metabolic activity of biofilm cells, resistant variants hamper phage infection. Phage biofilm killing can be enhanced by overcoming biofilm barriers that offer protection to cells (Azeredo & Sutherland, 2008).

In order to improve the performance for an efficient biofilm control, different approaches, such as synergistic combinations with other phages or antimicrobials, mechanical debridement of biofilms, and genetic engineering of phage genomes have been addressed. Multiple phages with different host ranges and targeting different receptors can be combined forming phage cocktails to expand their spectrum of activity and prevent the development of phage-resistant bacterial variants (Pires, *et al.*, 2017). Several studies have reported the use of this strategy to treat bacterial biofilms and have shown successful results. The use of phage cocktails could be potentially applied, especially, in indwelling medical devices to prevent bacterial colonization and biofilm formation, which might inhibit the development of bacterial infections. Studies regarding the pretreatment with phages as well as biofilm disruption after its formation have shown promising results. In an experiment conducted by Fu *et al.*, pretreatment of catheters with phage

cocktail resulted in a reduction of 3 orders-of-magnitude in the number of biofilm cells after 48 h, compared with untreated catheters (Fu, *et al.*, 2009). Phage cocktails have also been successfully used to target *S. aureus* biofilms. In a biofilm prevention study, crystal violet staining assays revealed a complete inhibition of *S. aureus* biofilm formation over a

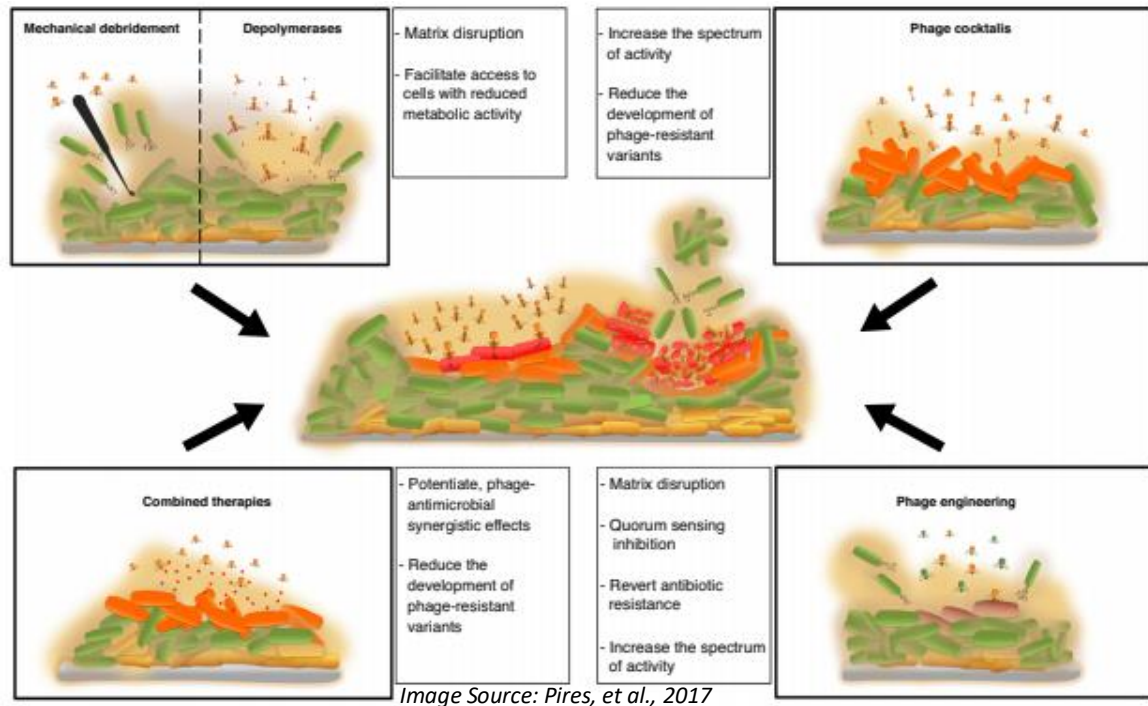


Figure 2.4 Schematic representation of strategies to overcome biofilm barriers using phages and combined therapies

The biofilm is represented by cells under different metabolic conditions and susceptibility to phages (red cells: phage infected cells; green cells: metabolically active cells; orange cells: phage-resistant variants; yellow cells: low metabolic activity)

period of 48 hours (Alves, *et al.*, 2014). Phage cocktails have also been studied to eradicate *E. coli* biofilms. Chibeu *et al.* (2012) recently assessed the ability of three phages to eradicate UPEC biofilms and Sybesma *et al.* (2016) described the lytic activity of commercial bacteriophage cocktails on *Escherichia coli* and *Klebsiella pneumoniae* strains isolated from UTI patients. Galtier *et al.* (2016) also isolated three virulent phages from wastewater that target an antibiotic-resistant strain of UPEC. Phage efficacy was characterized both *in vitro* and *in vivo*, and it was found that a single dose of the three-phage cocktail was able to dramatically reduce gut carriage of UPEC. These studies are among many that hold promise for the potential of bacteriophage therapy for the treatment of *E. coli* acquired UTIs (Bolocan, *et al.*, 2016). The study of Cao *et al.* (2015) supported the efficacy of bacteriophage treatment of pneumonia caused by biofilm producing multidrug-resistant *K. pneumoniae*.

CHAPTER 3 MATERIALS AND METHODS

3.1 Bacterial (Host) sample collection and culture

All the bacterial strains used for this research were obtained from the research lab of Central Department of Biotechnology, as glycerol stock, which were previously collected from Institute of Medicine (IOM), Maharajgunj, and Nepal Public Health Laboratory (NPHL), Teku. Most of the bacterial samples used were already identified by 16srRNA sequencing. Bacterial samples were revived on Luria Bertani (LB) broth, then streaked on nutrient agar and then on Mac-Conkey agar. Bacterial strains were confirmed as respective strains by Gram staining and few confirmatory tests.

Putative Bacteria used and their code name:

Bacterial strains	Code name
<i>Klebsiella pneumoniae</i>	K27, NK, K57(Host strains), K23, K41, K52
<i>Escherichia coli</i>	A1, A4, A6(Host strains), A5, A7, A17, A21, A23
<i>Salmonella</i>	S2, S3, S4, S5, S6
<i>Pseudomonas aeruginosa</i>	P3, P4, P9, P19, P53
<i>Acinetbacter baumannii</i>	A38, A47, A70

3.2 Molecular identification of Bacteria by 16srRNA Gene Amplification and sequencing

Before performing the Multi host range analysis assay, the bacteria to be used for this assay were identified biochemically and few strains, which were not confirmed by biochemical tests, were identified by 16srRNA sequencing. Total 23 bacterial strains were revived for intra and inter species host range analysis.

3.2.1 Bacterial Genomic DNA extraction

Genomic DNA of 5 biochemically unidentified bacterial strains was isolated using bacterial DNA Isolation kit. Quality of gDNA was checked on 1% agarose gel for the single intact band. Genomic DNA samples were loaded into the wells of 1% agarose gel and run gel at 110V for 30 mins. The gel was then observed in UV transilluminator for single intact band.

3.2.2 PCR amplification of 16s rRNA gene

For the amplification of 16srRNA gene, universal primers were used which amplify a segment (1.5kb) of 16srRNA gene. PCR reaction mixture was prepared in PCR tubes as mentioned in table 3.1 and subjected to amplification in thermocycler.

Table 3.1 PCR components, their concentration and volume used for PCR reaction

S.N.	Components	Concentration	Volume
1.	Master mix	2X	12.5 μ l
2.	Forward primer	10pmol/ μ l	2 μ l
3.	Reverse primer	10pmol/ μ l	2 μ l
4.	Template	100ng/ μ l	1 μ l
5.	NFW	-	6 μ l

PCR program was set up as mentioned in table 3.3. After the completion of PCR amplification, aliquots (3 μ l) of the amplified products were analyzed by electrophoresis on 1 % agarose gel in Tris Acetate EDTA (TAE) buffer. Ethidium bromide (0.5 μ g/ml) was used as a DNA staining solution. The gel was run at 60V for 60 minutes in electrophoresis chamber and viewed on a UV transilluminator and image captured with the help of Gel Doc system.

The PCR product was sent to Xcelaris Genomics India for the sequencing of 16s rRNA gene. Sequence obtained for each bacterial strain were analyzed by using different bioinformatics tools. Chromas was used for analysis of chromatogram file. The bacterial identity was determined by comparing the sequences obtained with the gene sequences available in the Genbank database using Basic Local Alignment Search Tool (BLAST) software at NCBI site. The sequence was deposited in the Genbank database to get the accession number. Similarly, for the evolutionary relationship between other sequences, phylogenetic tree was drawn by MEGA6 software. Details of primers used and PCR conditions are given in table 3.2 below.

Table 3.2 Forward and reverse primer sequences used for PCR

Primer	Melting temperature	Sequence	Amplicon length
27F	56.4	AGAGTTTGATCMTGGCTCAG	1500bp
1492R	56.4	CGGTTACCTTGTTACGACTT	

Table 3.3 Thermocycling condition for 16srRNA amplification

S.N.	Steps	Temperature (°C)	Time
1.	Enzyme activation	95	5 minutes
2.	Initial denaturation	95	30 seconds
3.	Annealing	55	30 seconds
4.	Extension	72	2.5 minutes
5.	Final extension	72	5 minutes
6.	Hold	4	∞

3.3 Revival of phages against specific host strains

Phages against the respective host strains were revived from the phage stock stored in phage bank of Bacteriophage laboratory in Central Department of Biotechnology. Phages were revived against *Klebsiella pneumoniae* by Double layer agar assay method while new phages were isolated against *E. coli* strains from sewage samples.

3.4 Sewage samples collection and processing

Sewage samples were collected from Balkhu River and Teku River of Kathmandu valley as a site mostly polluted with clinical wastes. Sewage water was collected in 50 ml falcon tubes and transported to Central Department of Biotechnology. The samples were then processed by centrifugation at 4100 rpm for 30 minutes for the removal of unwanted contaminants and cell debris. The centrifuged sewage sample was then filtered through 0.22µm (PES Filter Media, Whatman™) syringe filter and filtrate was collected in sterile falcon tubes. This processed filtrate was used as phage source in experiment.

3.5 Bacteriophage isolation

Bacteriophage isolation was done using Adams Double Layer Agar Assay (DLA) method, a standard method for the isolation of phages from environmental sources, with small modifications (Pallavali, *et al.*, 2017).

For this assay, a thin layer hard agar plate of Tryptic soya agar (1.5% TSA) and semi-solid/soft agar *i.e.* 0.6% TSA were prepared. The hard agar serves as a base layer (to form gel), and soft agar with a mixture of few phage particles (from sewage sample) and large number of host cells was used as top layer. 1 ml of syringe filtered water sample was taken in a sterile falcon tube and 100µl of log phase (OD 0.3) bacterial culture was added on it with mixing, and incubated for 5 minutes for proper adsorption/attachment without any disturbance.

After attachment, 3ml of soft agar (0.6% TSA at 50°C) was added to the mixture gently shaking the mixture avoiding bubbles formation and immediately overlaid on already prepared hard agar plate and swirled. Plates were then incubated at 37°C for 24 hours after solidification. Phage-free cultures (containing only bacterial host) were used as control. After 24 hours incubation, plates were observed for the presence or absence of plaques and examined for clear or turbid lysis, number of plaques formed, shape and size of plaque against bacterial host. Plates with positive result having clear lysis were selected for further processing.

3.6 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).

3.7 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 µl 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 to agitate in shaker for 5 hours at 100 rpm. 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 4100 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.

For the phages revived from phage bank, new stock was prepared in the same way as above.

3.8 Phage titer determination: Spot assay

This is the basis for determining the concentration of phage particles in a pure phage stock solution. For the determination of phage titer, phage stock solution was serially diluted upto 10^{12} dilutions using sterile tips and changing the tips for each dilution. 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 μ l active log phase bacteria in 3ml soft agar into the labelled TSA plates. After allowing to dry, 5 μ l of respectively prepared phage dilutions were spotted aseptically onto corresponding grids as labelled. 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.

3.9 Determination of phage stock concentration

For determining the concentration of phage in stock solution, the last three dilutions which shows clear lysis on spot assay were used. For DLAA, 1ml of diluted phage solution and 100 μ l of log phase host bacteria were mixed and left for attachment for 5min. Then, the phage-bacteria mixture was poured into sterile falcon tube containing 3 ml of soft agar stored at 50°C, swirled and overlaid onto the TSA plate. Plates were incubated overnight after solidification. After overnight incubation, the plates were observed for plaque formation and the plate with individually distinguishable plaques were selected for phage titer determination. 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 Characterization of phage:

Three phages of *Klebsiella pneumoniae*, which were used as cocktails, and three phages of *Escherichia coli*, which were newly isolated, were used for further characterization.

3.10.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS- PAGE) for phage protein profiling

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

3.10.1.1 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.
- b) Acetone precipitation method: For acetone precipitation method, purified phage solution and ice-cold acetone were mixed in the ratio of 1:4 (200 µl:800 µl) and vortexed. 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.

3.10.1.2 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).

3.10.2 Transmission Electron Microscopy (TEM)

The potent lytic phages were selected for Transmission Electron Microscopy. The phages titre was maintained at a range of 10⁸-10¹⁰ 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%gluteraldehyde and 2%paraformaldehyde, prepared in 0.7M sodium phosphate buffer (pH 7.2). For fixation, equal volume of phage lysate and fixative were added, mixed and left overnight. Next day, the fixed phages were subjected to high-speed centrifugation (35,000g) for 3 hours. 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 Multi-host Range analysis (For individual phages and phage cocktails)

3.11.1 Composition and Preparation of phage cocktail

Phage cocktails were prepared by mixing the selected phages of each host group in equal volume having phage lysate titre 10^7 pfu/ml. That means the individual phages were diluted to reach the concentration of 10^7 pfu/ml, then mixed in equal volume to form cocktails (Manohar, *et al.*, 2019). For *Escherichia coli* phage cocktail, three selected phages (A1, A4 and A6) were used. Similarly, *Klebsiella* phage cocktail was prepared by mixing three different selected phages (K27, NK and K57).

3.11.2 Spot assay

Both intra specific and inter genus host range analysis was done, by spot assay. Individual phage stocks as well as phage cocktails were used for host range analysis. Different strains of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Salmonella* available in the CDBT laboratory were used for spot assay. Total 23 bacterial strains revived (8 *E. coli* strains, 5 *K. pneumoniae*, 5 *Salmonella* strains, and 3 *Pseudomonas aeruginosa* strains) were used for intra and inter species host range analysis. At first, lawn culture of 23 host bacteria were prepared by mixing 100 μ l active log phase bacteria with 3ml soft agar. 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. 5 μ l of each phage was pipetted on corresponding blocks and allow to soak for about 15 minutes. Then the plates were incubated in inverted position overnight at 37°C.

Similarly, for spot assay of phage cocktails, bacterial lawn was prepared and experiment was performed in the same way as for individual phages. Next day, the plates were checked for the presence or absence of bacterial lysis and clear zone. For the reproducibility of result these tests were repeated in triplicate.

To ensure whether the lysis was due to excess phage or actually due to bacterial lysis by phage, the bacterial strains showing positive spot test except that of host strains were again tested with different dilution of phages and phage cocktails.

3.11.3 Efficiency testing of individual *Klebsiella* phage (K57 phage) and *Klebsiella* phage cocktails on *E. coli* strains

K57 phage and *Klebsiella* phage cocktails showing inter-genus host range with *E. coli* strains (A5, A7, A17, A21 and A23) in spot assay were selected and tested for their efficiency to lyse bacteria. The phages were diluted upto certain dilutions and tested for their efficiency to lyse, in the same way as spot assay. After this, DLAA was performed by attachment of active log phage bacteria with phage cocktail (MOI 1), then mixing with 3ml of semi-solid agar and then overlaying on TSA plates. The plates were then incubated at 37°C for 24 hours and plaques were observed next day.

3.12 Bacterial growth inhibition assay/Killing assay

Bacterial growth inhibition assay was performed as described previously by (Mclaughlin 2007; Knezevic, *et al.*, 2011) with little modifications. Individual *Klebsiella* phages and cocktails of three *Klebsiella* phages on *E. coli* bacterial strains were used. Briefly, bacteria were cultured in LB broth and incubated overnight at 37°C. The overnight cultures were diluted (1:100) into LB broth and incubated at 37°C until OD650 reached 0.3 (approximately 1.5×10^8 CFU/ml). Then, 50 µl of culture were inoculated in 50 µl double strength LB broth into 96-well microtiter plate wells flat bottom. Sterile LB broth was used as a negative control. Then, 100 ul of the prepared bacteriophage cocktail was added to each well. After this, 50 ul of 0.1% filter sterilized TTC (LOBA Chemie) was added. The plate was incubated at 37°C in the dark for 18 hours. The absorbance was taken at an interval of 1 hour for upto 8 hours and final absorbance was taken at 18 hours. The absorbance was measured at OD551 by ELISA plate Reader. The experiment was replicated twice with triplicate samples. The percentage inhibition of the bacteriophage cocktail bacterial strains was calculated using the following formula:

$$\% \text{ inhibition} = \frac{\text{absorbance of controls} - \text{absorbance of treated wells} \times 100}{\text{absorbance of controls}}$$

3.13 Phage cocktails in biofilm control

3.13.1 Detection of Biofilm producing bacteria

Among the different methods used for detecting biofilm producers, tissue culture plate method described by Christensen *et al.* is considered the gold-standard method for biofilm detection (Hassan, *et al.*, 2011).

Bacterial strains that showed positive spot test with phage cocktails were incubated in fresh LB broth at 37°C for overnight. The cultures were then diluted 1:100 with fresh medium to reach the OD₆₅₀ of 0.5-0.7. Then, 50 µl of each diluted culture were inoculated, one bacterial broth in 5 wells, in 50 ul double strength LB broth into 96-well flat-bottomed microtiter plate wells. In this study, *Pseudomonas* strain PA01 (strong biofilm producer) served as positive control and sterile LB broth served as negative control.

The microtiter plate was then incubated at 37°C for 24-48 hours to allow biofilm formation. After incubation, contents of each well were removed by gentle tapping. The wells were washed thrice with 200 µl of 1X PBS and left to dry. This removed free floating bacteria. Attached bacterial cells were fixed with 250µl of absolute methanol for 15 minutes. The fixative was removed and the plates were air-dried. Into each well, 200µl of 0.4% crystal violet was added and after 20 minutes stain was removed. The plates were washed by stream of tap water in order to remove excessive amount of the stain and left to dry. Into each well of dried plates, 250µl of 33% acetic acid was added and left for 20 min to allow stain to dissolve. The absorbance was measured at 551 nm using the microtiter plate reader. In this experiment all bacterial strains were inoculated in five wells simultaneously and repeated twice (on different days), and then, OD values were averaged and SD was calculated.

The interpretation of biofilm production was done according to the following criteria (Prakash, *et al.*, 2017). Optical density cut-off (OD_{cut}) value was determined.

$$\text{OD}_{\text{cut}} = \text{OD}_{\text{avg of negative control}} + 3 \times \text{standard deviation (SD) of ODs of negative control}$$

Table 3.4 Interpretation of biofilm production

Average OD value	Biofilm production
$\text{OD} \leq \text{OD}_{\text{cut}}$	Non-biofilm former
$\text{OD}_{\text{cut}} < \text{OD} \leq 2 \times \text{OD}_{\text{cut}}$	Weak biofilm-former
$2 \times \text{OD}_{\text{cut}} < \text{OD} \leq 4 \times \text{OD}_{\text{cut}}$	Moderate biofilm-former
$\text{OD} > 4 \times \text{OD}_{\text{cut}}$	Strong biofilm-former

3.13.2 Biofilm disruption by phage cocktail

In order to determine phage effect on biofilm disruption, the experiment was performed according to Knezevic and Petrovic with slight modifications (similar to bacterial inhibition assay). The bacteria determined as moderate biofilm producer in biofilm assay were used to study the cocktail effect. The microtiter plate was filled with 50µl of double strength LB

broth and diluted bacteria after overnight culture were inoculated into the wells as in previous experiment. The plates were incubated overnight to allow biofilm formation. When biofilm was formed after 24 hours, planktonic bacteria were removed and plate was washed once with PBS thrice (Knezevic & Petrovic, 2008). The plates were left to dry for 10 min and then the wells were filled with 100µl of appropriate phage cocktail (10^7 pfu/ml). The incubation was continued for additional 4 hours. After incubation, the wells were carefully emptied and gently washed twice with PBS. The bacteria adhering to the walls of the plate were fixed with 200µl absolute methanol for 15minutes. Finally, the adhered cells were stained with 200 µl of 0.4% crystal violet solution for 20 minutes. Excess stain was removed by washing the plate with stream of tap water and left to dry. Into each well of dried plates, 250µl of 33% acetic acid was added and left for 20 min to allow stain to dissolve. The absorbance was measured at 551 nm using the microtiter plate reader. Each treatment was repeated in 5 wells, and the median value and standard deviation (SD) were calculated. PA01 strain was used as Positive control and only LB broth was used as negative control. The experiment was repeated twice.

CHAPTER 4 RESULTS AND DISCUSSION

4.1 Revival and identification of host bacteria and bacteria to be used for MHA

Total 23 strains of different bacteria were revived from glycerol stock. These were the bacteria to be used as host for phage isolation and amplification as well as for multi- host range analysis. Identification process was same for all bacterial strains and some are shown below. On Gram staining, all bacterial strains were Gram negative rods (red rod-shaped under microscopic observation). 3 bacterial isolates which were mucoid and lactose fermenting (pink colonies on MA) and 4 isolates which were non- lactose

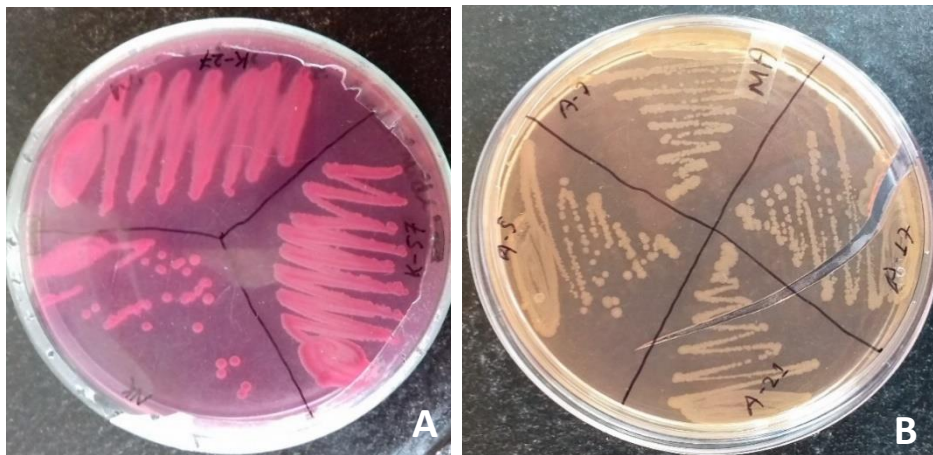


Figure 4.1 Bacterial Streak on MA plate A) LF mucoid pink mucoid colonies of; B) NLF colonies

fermenting on MA are shown in Figure 4.1. Confirmatory biochemical tests identify the bacteria as respective strain.

Table 4.1 Biochemical test of bacteria

S.N.	Tests Performed	Results for	
		Lactose fermenter	Non-lactose fermenter
1.	Catalase	Positive	Positive
2.	Oxidase	Negative	Negative
3.	Indole test	Negative	Positive
4.	Methyl red test	Negative	Positive
5.	Voges Proskauer test	Positive	Negative
6.	Citrate utilization test	Positive	Negative
	Urease test	Positive	Negative
	Nitrate reduction test	Positive	Positive

From biochemical tests, 3 lactose fermenting isolates were identified as *Klebsiella pneumoniae*. NLF were not confirmed as particular bacterial strain. Further the NLF isolates were identified as *E. coli* strains by molecular identification tools. Though most of the *E. coli* strains are lactose fermenters, it has been reported that *E. coli* can be NLF too and have very similar pathogenic potential as of lactose fermenters (Kaczmarek, *et al.*, 2017). *Pseudomonas aeruginosa* strains were identified by growth on Cetrimide agar.

4.2 Molecular identification of bacteria

Bacteria which were not confirmed by biochemical tests were identified by 16srRNA sequencing.

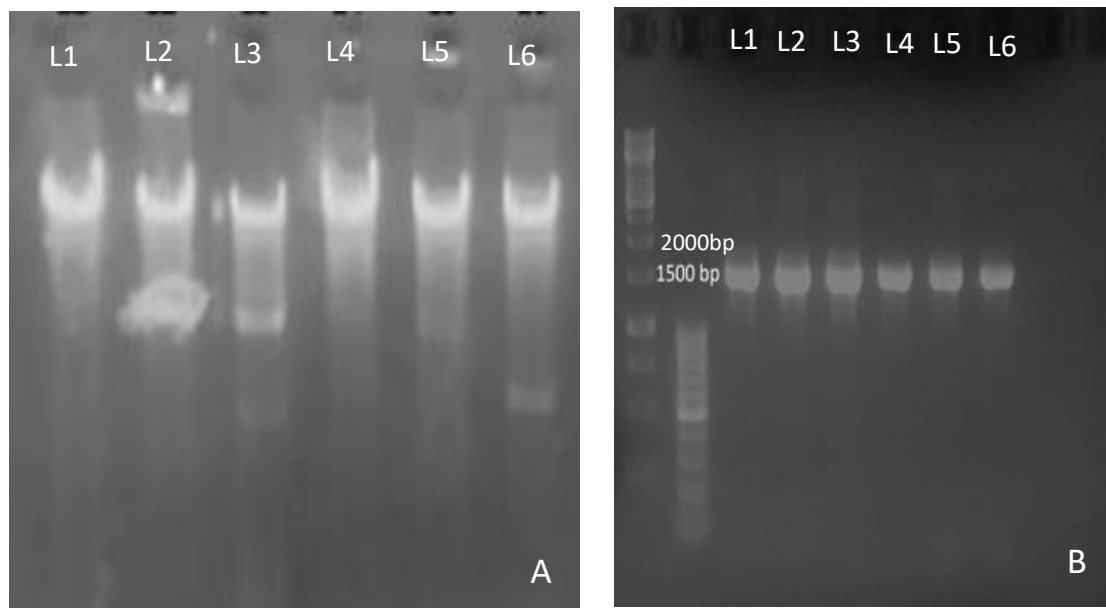


Figure 4.2 Genomic DNA extraction and PCR amplification; A represents the bands of genomic DNA where L1, L2, L3, L4, L5 represents respective strains A5, A7, A17, A21 and A23 of *E. coli*; B represents PCR amplified product on agarose gel where L1, L2, L3, L4 and L5 represents respective strains

Distinct bands were obtained after agarose gel electrophoresis of genomic DNA as shown in figure 4.2A. Nanodrop reading was taken to determine the concentration of genomic DNA from which concentration was near about 100 ng for all samples. PCR amplification was done using 16srRNA universal primer and on visualization of PCR amplified gel run product on gel documentation system bands were seen near 1500 bp which was determined by comparing with 100 bp ladder as in figure 4.2B.

PCR amplified products were sent for sequencing. Sequence of 16srRNA genes were obtained in chromatogram file which was then searched with BLAST tool (nucleotide BLAST) to see the sequence similarity with the NCBI database sequences. The chromatogram and sequence similarity result of each strain was obtained as follows. BLAST of obtained sequencing results identified the biochemically unidentified non-lactose fermenters as *E. coli*. BLAST of all samples showed maximum (96-99%) similarity

with *E. coli* from which the biochemically unidentified samples were identified as *E. coli* strains.

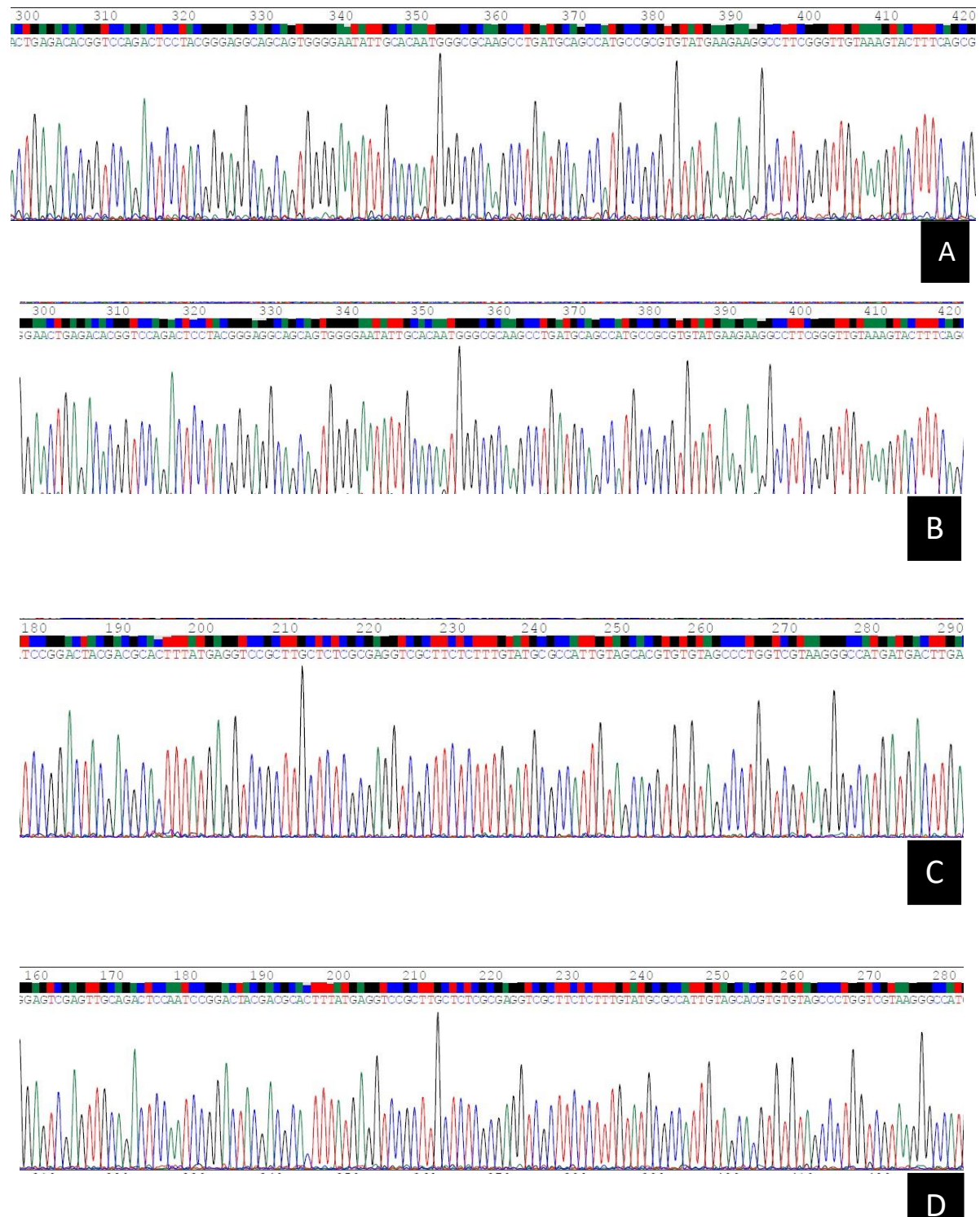


Figure 4.3 Chromatogram file viewed on *Chromas* software. Fig A, B, C and D represent the chromatogram of sample strain A5, A7, A21 and A23 respectively. Different color on the top of peak determine different bases and the quality of bases. . Lesser the noise higher the base call and higher the quality of base and vice-versa

Phylogenetic tree constructed by neighbour joining method showed sample A7, A21 and A23 were closely related with each other while the sample A5 was distantly related to these three samples.

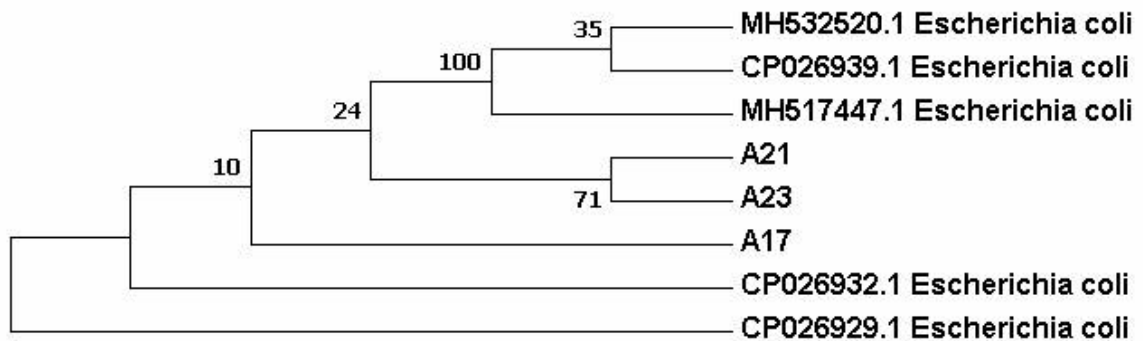


Figure 4.4 Molecular phylogenetic tree constructed using neighbour joining method by MEGA6 software

4.3 Bacteriophage isolation (new phages) and revival (already isolated)

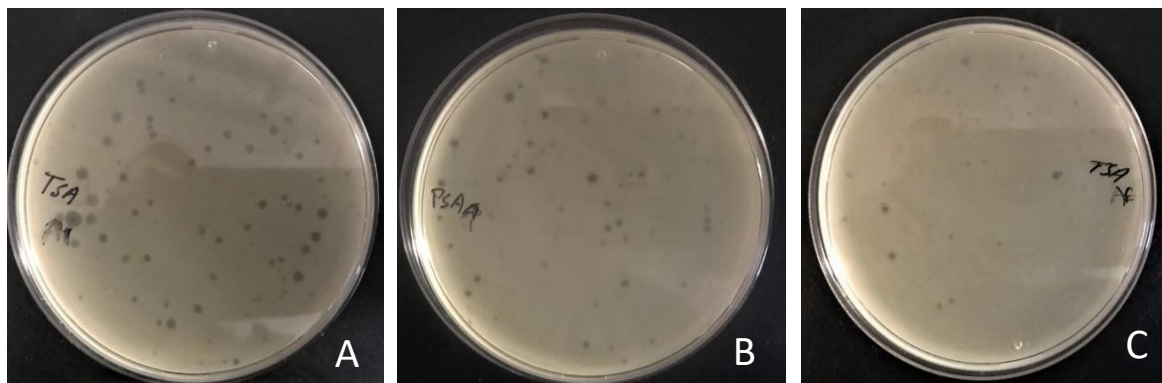


Figure 4.5 Initial phage isolation in *E. coli* strains by DLAA showing different plaque types

A) Phage of strain A1; B) phage of strain A4; C) phage of strain A6, all 3 bacterial strains show mixture of phages with different plaque morphologies

New phages were isolated against 3 different strains of *E. coli* (A1, A4 and A6) by DLAA method. The initial plates contained mixed population of phages showing variation in plaque morphology and lysis. The phages against *E. coli* were all isolated from sewage samples of Balkhu near Vayoda hospital. The phages were of different size and different morphology which indicates the presence of more than one type of phage in the water sample. Most of the plaques formed in all three strains of *E. coli* were pin-headed in isolation step. Already isolated phages (Kp27, NKp and Kp57) of *K. pneumoniae* were revived by streaking on TSA plates. The phages revived against host bacteria, *K. pneumoniae*, were all bull's eye shape as previous.

The size of the plaque is proportional to the efficiency of adsorption, the length of the latent period, and the burst size of the phage (Abedon & Yin, 2009). In general, plaque size increases as the velocity of phage diffusion increases. The diffusion rate is dependent on certain phage properties e.g. phage dimensions and whether the phage

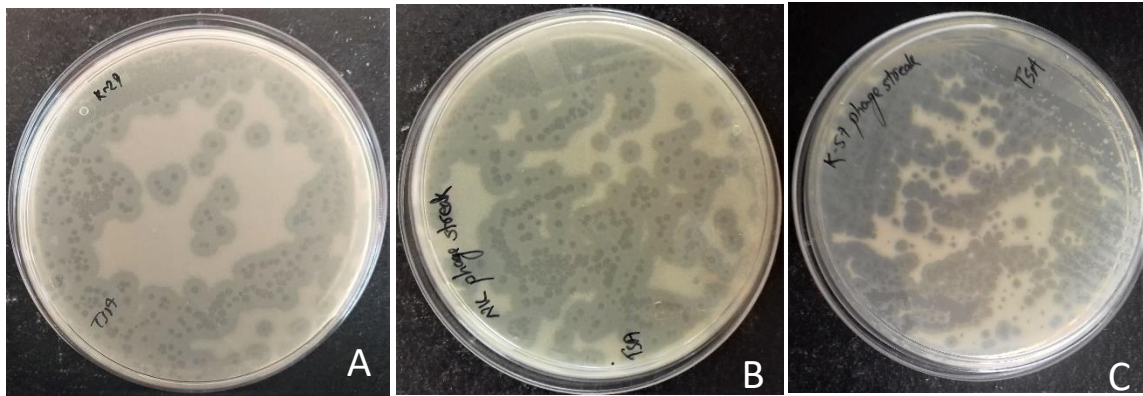


Figure 4.6 Phages of different *K. pneumoniae* strains (K27, NK and K57 from left to right) streaked on TSA plates all of which were bull's eye shape

aggregates. Referring to the shape, a clear circular plaque is formed when the host is completely susceptible to the phage. Formation of bull's eye plaque represents the presence phage tail-associated exopolysaccharide (EPS) depolymerase enzyme. The hazy ring suggests that phage produced a depolymerase enzyme that diffused through the agar layer and degraded the bacterial capsular polysaccharide (CPS) into different oligosaccharide components (Yan, *et al.*, 2013). Early studies have already shown that certain *Klebsiella pneumoniae* bacteriophages produce depolymerase during phage proliferation and release the enzyme from infected bacteria that targeted another bacteria's CPS. Thus, the bull's eye plaques were formed by our phages due to the presence of depolymerase enzyme in them to degrade the capsule.

4.4 Purification and amplification of isolated phages

Among numerous plaques, single clear plaque was selected for further processing. After several round of streaking with single plaque, pure phages (pin-headed) of *E. coli* strains

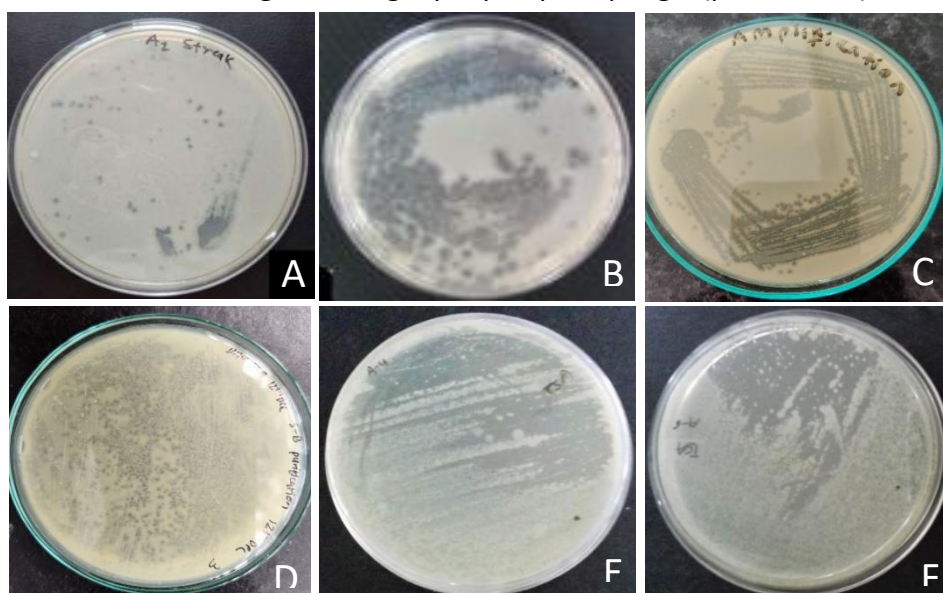


Figure 4.7 Purification and amplification of bacteriophages; A, B and C represents quadrant streaking of A1, A4 and A6 phages respectively for purification while D, E and F represents continuous streaking of respective phages A1, A4 and A6 for amplification

were obtained. Continuous streaking of phages after purification results in uniform phages of same type of morphology.

The plaque formed decreased from starting to the ending line. Pin headed clear plaques were formed due to complete lysis of bacteria by purified phages. Phage stock solution of newly isolated and revived phages was prepared in SM buffer. The gelatin present in SM buffer helps to stabilize the phage particle while storage.

4.5 Phage spot assay and determination of phage titre

All 6 phage stocks were diluted up to 10^{-10} dilution and spot assay was performed. Spot assay result shows that the phages of *Klebsiella* K27, NK and K57 can form plaques even at high dilution of 10^{-10} . Phages of *E. coli* were less concentrated than *Klebsiella* phages, with positive spot test up to 10^{-6} dilution only. Spot assay is important for determining the titre of phages.

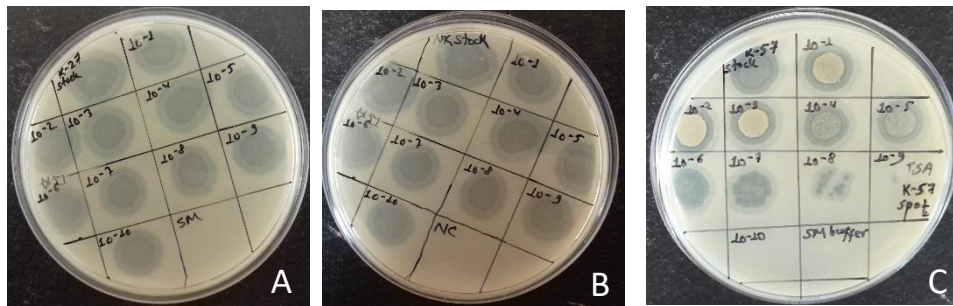


Figure 4.5 Spot assay of phage stock and its different dilution showing lysis of bacterial lawn
A, B and C represents spot assay of *K. pneumoniae* phage stocks K27, NK and K57; Showed lysis up to 10^{-10} , 10^{-10} and 10^{-9} dilutions respectively

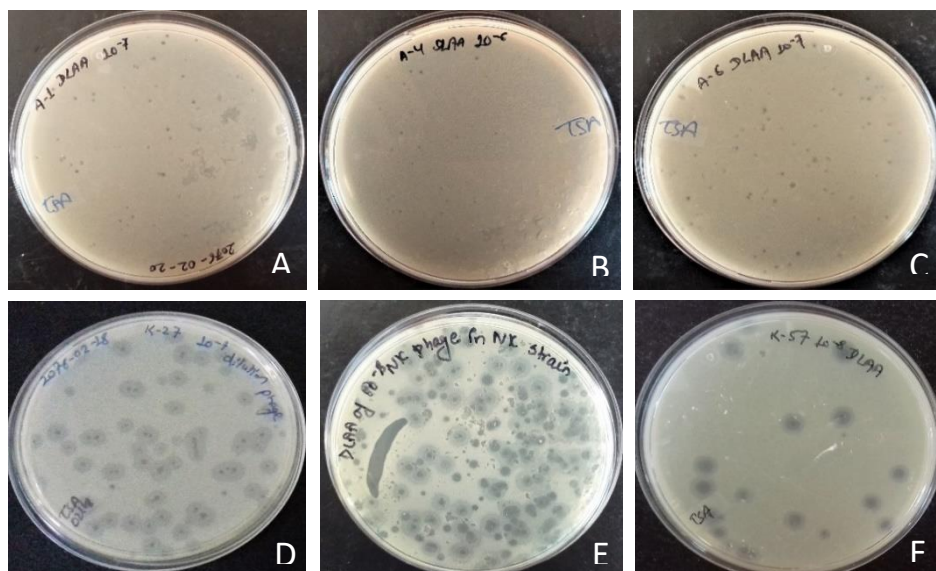


Figure 4.8 DLAA plates showing countable plaques at different dilution of phages

Figure A, B and C represents pin headed plaques of A1, A4 and A6 phages respectively. A1 phage showed 35 countable plaques at 10^{-7} dilution, A4 showed 12 countable plaques at 10^{-6} dilution and A6 phage showed 51 countable plaques at 10^{-7} dilution. Figure D, E and F represents bull's eye plaques of K27, NK and K57 phages respectively. K27 phage showed 55 countable plaques at 10^{-7} dilution, NK phage showed 110 countable plaques at 10^{-8} dilution and K57 phage showed 18 countable plaques at 10^{-8} dilution

Phage titre was calculated and titre value was found different for different phages as shown in table below. Countable plaques of A1, A4 and A6 phages were obtained at 10^{-7} , 10^{-6} , and 10^{-7} and that of K27, NK and K57 phages were obtained at 10^{-7} , 10^{-8} and 10^{-7} dilution respectively. The concentration and purification of virus particles essential for structural and functional characterization of phages. Characterization requires high titre phages of at least 10^7 concentration and all our isolated phages were of that concentration. Also, phage titer is essential to determine the MOI and for phage dosing in phage therapy experiments.

Table 4.2 Determination of phage titre

Phage Name	Plaque counts at different dilutions						Titer in Pfu/ml= plaques no./ (dilution× volume of sample)
	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	
A1	>200	100	35	6	-	-	3.5×10^8
A4	49	12	-	-	-	-	1.2×10^7
A6	TMTC	88	51	4	-	-	5.1×10^8
K27	TMTC	>200	55	10	-	-	5.5×10^8
NK	TMTC	TMTC	TMTC	110	20	1	1.1×10^{10}
K57	TMTC	TMTC	144	18	2	-	1.8×10^9

4.6 Characterization of phage

4.6.1 SDS-PAGE

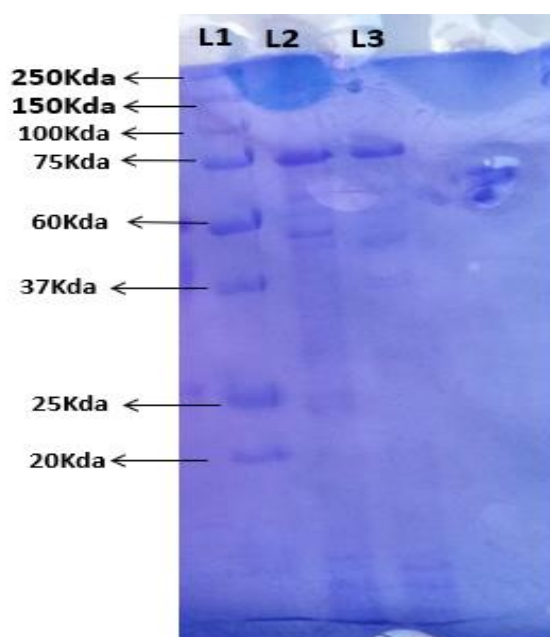


Figure 4.9 SDS-PAGE of phage protein; L1- marker, L2, L3- A4, K57 phage

Protein profiling was done by SDS-PAGE and the band size obtained was compared with standard protein marker. Multiple bands between 50-120 kDal were observed in all phage proteins representing different proteins present in phage tails and capsid. The larger band size can be assigned to major capsid protein while the smaller band size can be assumed of structural proteins such as, head-tail connector protein, internal virion protein.

4.6.2 Transmission electron microscopy

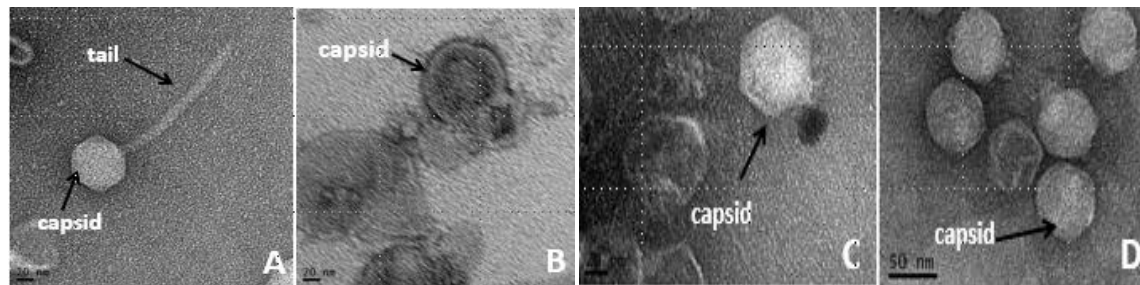


Figure 4.10 Negative staining images of phages by TEM; A, B, C, D represents phages of A4 (*E. coli*) and K27, NK, and K57 (*K. pneumoniae*)

TEM micrograph of phages were obtained and the result was analyzed by using ImageJ 1.52a software and interpreted 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 4.3. 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.

Table 4.3 TEM image analysis and classification of phages based on ICTV guidelines

Phage Name	Capsid (in nm*)	Tail (in nm)		Shape	Order	Putative family
		Width	Length			
TU-A4phage	60.77	11.62	179.67	Icosahedral	Caudovirales	Siphoviridae
TU-K27phage	90.64	10.23	9.6	Icosahedral	Caudovirales	Podoviridae
TU-K57phage	65.88	12.08	11	Icosahedral	Caudovirales	Podoviridae
TU-NKPhage	60.32	17.77	13.97	Icosahedral	Caudovirales	Podoviridae

4.7 Multi- host range analysis

4.7.1 Spot assay

A total of 23 different bacteria were used for testing the host range of 6 different phages. Among the bacterial strains used, 5 were *Klebsiella pneumoniae* strains, 8 *E. coli* strains, 5 *Salmonella* strains and 3 *Pseudomonas aeruginosa* strains. Neither individual phages nor cocktails showed positive spot test on *P. aeruginosa*. All 3 phages isolated against *E. coli* as well as their cocktail showed positive spot test with two *Salmonella* strains. Individual phage K57 showed turbid spot like dot on 5 strains of *E. coli* while with *Klebsiella* phage cocktails the spot was clear. Though phages are highly specific, we observed that phages showed positive spot test with different genus of bacteria too, which is reported only in few research articles. All spot tests were repeated in triplicate to confirm results of phage lysis rather than bacteriocin induced lysis. The *E. coli* strains which showed positive spot test with *Klebsiella* phage cocktails were all non-lactose fermenters, while lactose fermenting strains were not lysed. The table showing the results of MHR analysis is shown in Table 1 and Table 2 of **appendix portion**. Spot assay is simple and rapid, this technique can sometimes cause false positives because of lysis of bacterial cells without phage infection. So, efficiency testing and killing assay were further performed.

4.7.2 Efficiency testing of positive spot result

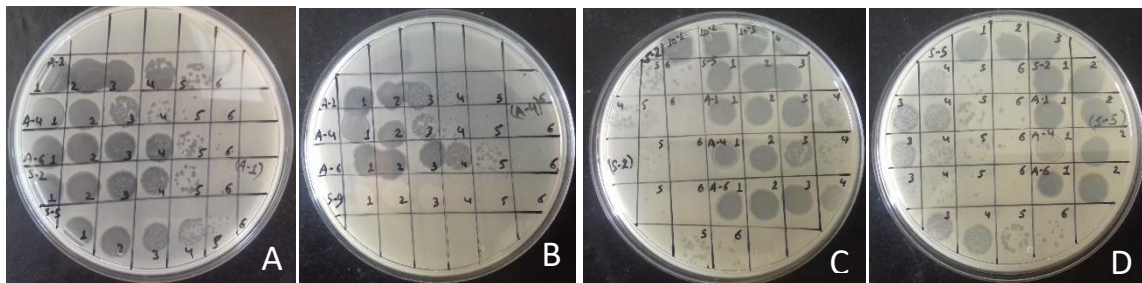


Figure 4.12 Spot test of different dilutions of phages on host bacteria and other bacteria showing positive spot test with stock

Figure A shows the spot assay of A1, A4, S2 and S5 phages up to 10^{-6} dil. on A1 bacteria, figure B shows the spot assay of A1, A4, A6 and S9 phages on A4 bacteria, figure C and figure D shows the spot assay of S2, S5, A1, A4 and A6 phages on S2 and S5 bacteria respectively.

Phages stocks which showed positive spot test on bacterial strains other than their host were diluted upto 10^{-6} dilutions and again tested for efficiency to lyse bacteria by spot assay. Dilution of *E. coli* phages and spot testing on different bacteria, showing positive result with phage stock, gave positive spot up to 10^{-6} dilution in all tested bacteria as shown in figure 4.12.

On testing the efficiency of K57 phage on *E. coli* strains it was found that the dilution of K57 phage did not show positive spot test, which means the lysis was not efficient, while the cocktail phages (10^7 concentration each) of 3 different *K. pneumoniae* strains showed positive spot test even upto 10^{-4} dilution.

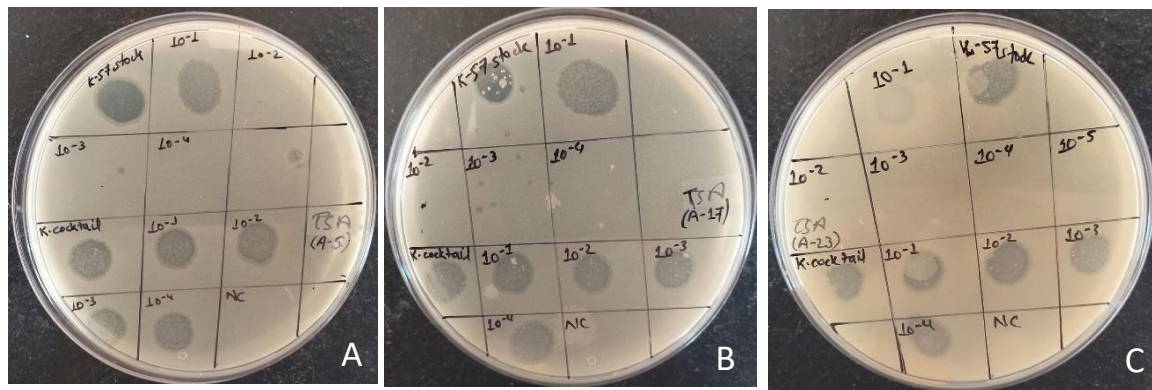


Figure 4.13 Efficiency testing of K57 phage and Klebsiella phage cocktails on different *E. coli* strains

Figure A, B and C represents the spot of dilutions of K57 phage and Kleb. phage cocktails on A5, A7 and A 23 strains of *E. coli* strains respectively. K57 phage did not show spot after dilution while cocktail phage showed spot even after dilution

4.7.3 Double Layer agar assay to determine plating efficiency

From DLAA it was found that the Klebsiella phage cocktails can effectively lyse *E. coli* bacterial strains, which individual phages cannot. DLAA with individual phages didn't show any plaques after incubation for appropriate time.

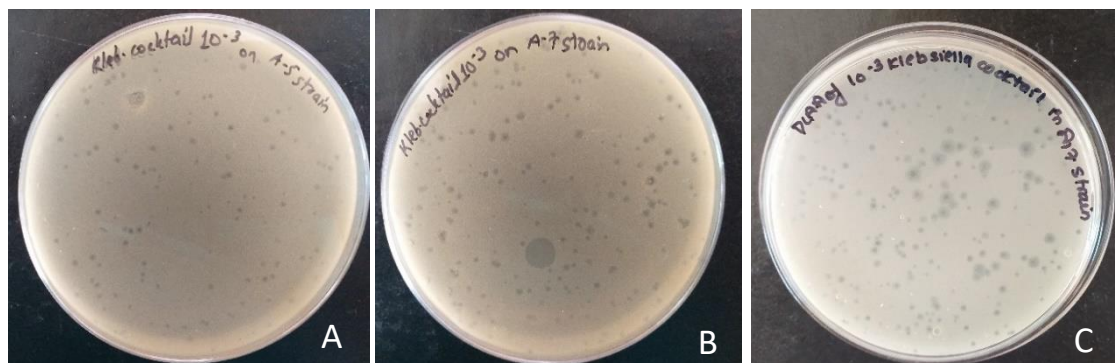


Figure 4.11 DLAA plates of *E. coli* strains with Klebsiella phage cocktails showing plaques

A, B and C shows the plaques formed by Klebsiella phage cocktails on A5, A7 and A17 strains of *E. coli* respectively. All plaque formed are with clear center surrounded by turbid zone (Bull's eye), but with small diameter

Host range is one of the important characteristics for the therapeutic use of bacteriophages. The wider the host range of phages, the more likely they can kill the broad range of MDR bacteria. Broad host range is used to describe a bacteriophage that can infect multiple species, as well as, the same term is used to describe a bacteriophage that can infect multiple strains of the same species of bacteria and sometimes the term polyvalence is also used. While preferring the broad host range phages for therapeutics it should also be taken into account that one of the important characteristics of phages is their specificity. Host specificity is generally observed at a strain level, species level, or, more rarely, at genus level. Some phages can infect multiple bacteria, especially the bacterial members of the same family. For example, bacteriophage Mu is able to infect species of *Escherichia coli*, *Citrobacter freundii*, *Shigella sonnei* all of which are the

members of Enterobacteriaceae. Staphylococcal phage ϕ 812 infects 95% of 782 strains of *Staphylococcus aureus* and 43% of other *Staphylococcus* species tested (Ross, *et al.*, 2016). Such broad host range phages can be used to treat multi-bacterial infections.

In our study, we found that the Klebsiella phage K57 was able to lyse *E. coli* bacterial strains by spot test but the lysis was not efficient. But, when Klebsiella phage cocktails were prepared and used, effective lysis was observed which was confirmed by DLAA. Also, phage cocktails were used to check for the lysis on non-pathogenic *E. coli* strains available in the lab and no lysis was found. The result of our study is different than others. Most of the studies use either the phage cocktails on different strains of same bacterium from which individual phages were isolated or formulate cocktails from phages of different species or genus of bacteria and study the cocktail effect. But in our study, we used the Klebsiella phage cocktails to lyse the *E. coli* strains, to explore the phage cocktails effect on different bacteria of same family, that is, Enterobacteriaceae. As expected, phage cocktails enhanced the host range and found to be effective than individual phages. Enhanced host range by phage cocktails was obtained due to some type of synergy between the formulated cocktails. Studies have suggested that when two or more phages in the cocktail attack the same bacterium, the combination may produce better killing than any single phage (synergy) or the combination may be worse than the best single phage (interference). Synergy is a dynamical phenomenon in which greater bacterial killing is achieved by mixture of phages than by either phage alone. Such synergy effect might be due to the one phage facilitate the infection to the same bacterium by providing certain mechanism so that another phage easily cause infection. The synergy likely stems from a tail spike enzyme produced by one of the phages. For example: one phage produces a depolymerase that strips the bacterial capsule enhancing infection by another phage (Schmerer, *et al.*, 2014).

4.8 Bacterial Growth inhibition assay/Killing assay

Killing assay was performed to determine the bacterial growth inhibition rate by phage cocktails using tetrazolium dye staining. Bacterial growth was significantly inhibited by phage cocktails compared to individual phage, as shown in graph in figure 4.12. Bacteria with only SM buffer was used as a control. The rate of inhibition at different time intervals is shown in as a graph in figure 4.13. *E. coli* growth was highly inhibited by Klebsiella phage cocktails with maximum inhibition, that is 50%, at 4 hours of phage cocktail infection. Killing assay is important to ensure that the *in-vitro* lysis of host was by phage cocktails rather than other mechanisms.

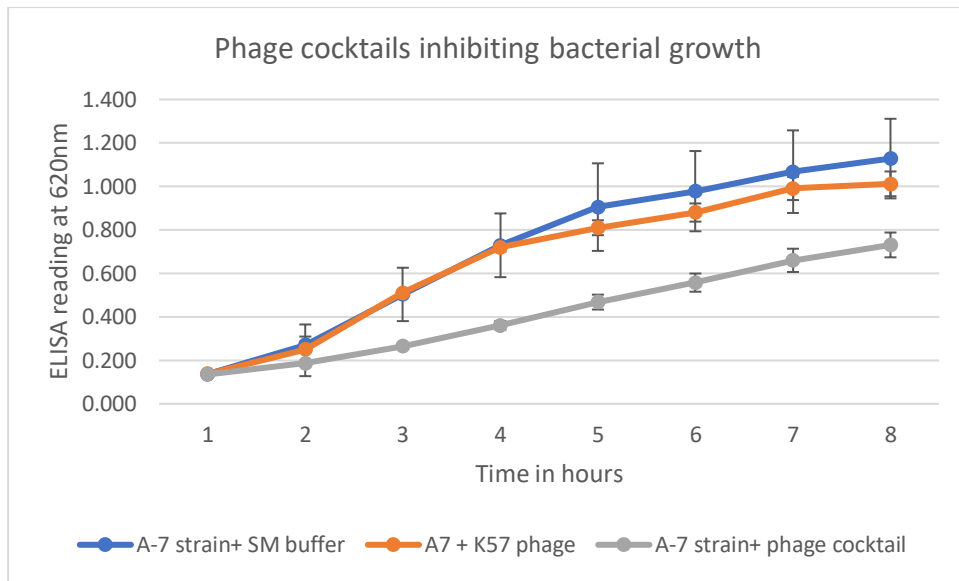


Figure 4.12 Comparison of *E. coli* bacteria growth inhibition (representative strain A7) by individual phage(K57) and Klebsiella phage cocktail

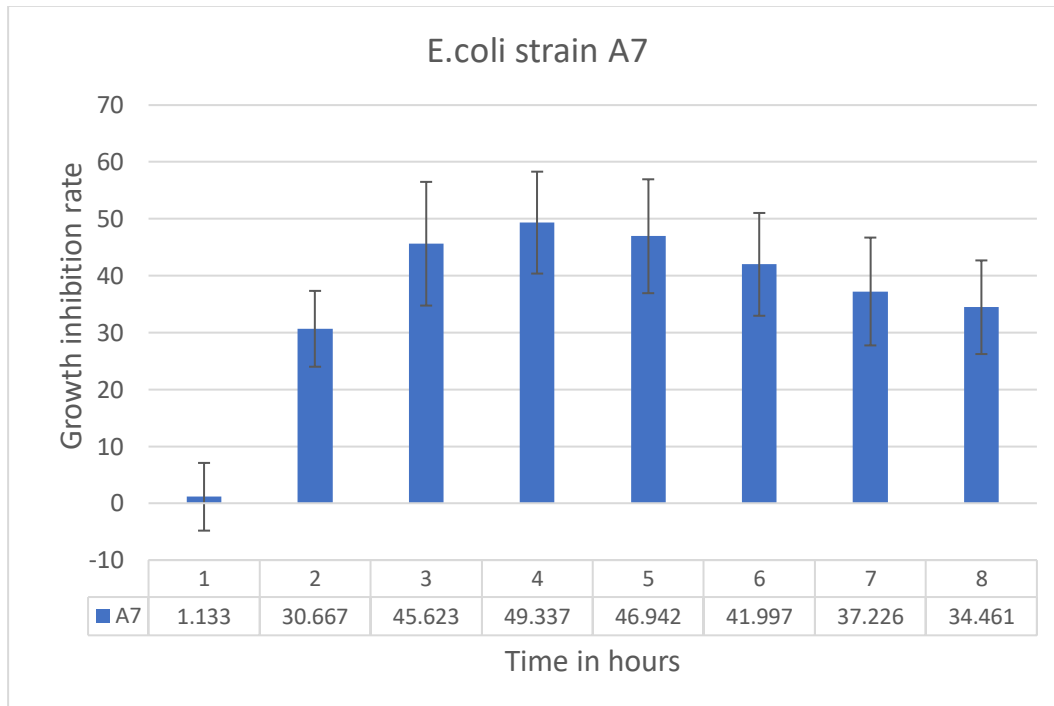


Figure 4.13 Graphical representation of *E. coli* bacteria inhibition by Klebsiella phage cocktails; representative strain A7. The bar graph depicts the percent growth inhibition and represents the mean percentage \pm SD of triplicate experiments

4.9 Biofilm assay of *E. coli* strains and biofilm disruption by *Klebsiella* phage cocktail

Table 4.4 OD551 values obtained from biofilm assay

Bacteria strains	Mean OD value of bacteria tested	Mean OD after phage cocktail
A5	0.1778	0.064
A7	0.1844	0.096
A17	0.134	0.067
A21	0.2102	0.069
A23	0.201	0.085
PA01(positive control)	0.545	0.5102

Table 4.5 Calculation of cut-off value

Penta-plate reading of NC	0.075, 0.076, 0.078, 0.067, 0.069
Average OD of NC	0.073
Standard deviation of NC	0.00474
3 × S.D. of NC	0.0142
Cut-off value	0.0872

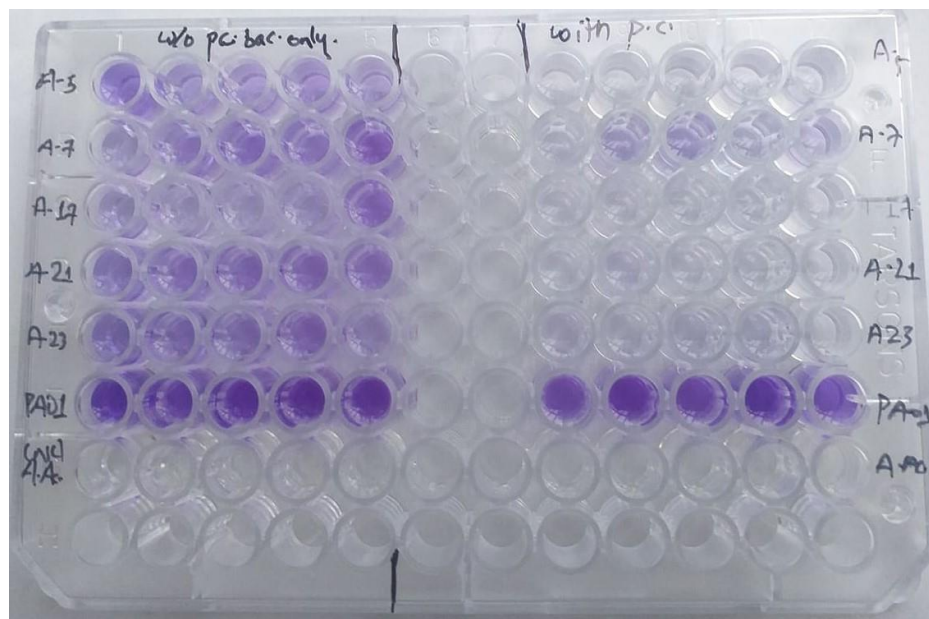


Figure 4.14 Microtiter plate of biofilm assay showing solubilized crystal violet in wells.

NC denotes negative control where only LB broth was used. PA01 is a positive control. A5, A7, A17, A21 and A23 denotes different *E. coli* test strains. Each sample was inoculated in 5 wells. As indicated in figure, w/o pc indicates biofilm assay with bacteria only while with pc indicates phage cocktails treatment of formed biofilms

4.9.1 Interpretation of result

For the interpretation of obtained result, cut-off value is required. As cut off value is calculated as three S.D. above the average of negative control, the value is different for different experiments performed. Cut-off value was calculated (shown in table 4.5) and obtained to be 0.0872 for this study. None of the *E. coli* strains used for biofilm assay were non-biofilm producer; since $OD > OD_{\text{cut-off}}$ for all. From the OD values obtained and interpreting them, it was found that *E. coli* strain A7 is weak biofilm producer and A5, A17, A21 and A23 are moderate biofilm producers. The positive control used was the already known strong biofilm producing *Pseudomonas aeruginosa* strain PA01.

Table 4.6 Categorization of biofilms made by *E. coli* strains

Average OD ₅₅₁ value	Biofilm grade	Bacterial strains
≤ 0.0872	Non-biofilm former (NBF)	-
> 0.08722 but ≤ 0.1744	Weak biofilm producer	A17
> 0.1744 but ≤ 0.3489	Moderate biofilm producer	A5, A7, A21, A23
> 0.3489	Strong biofilm producer	PA01(positive control)

Biofilm disruption of *E. coli* strains by *Klebsiella* phage cocktails:

The *Klebsiella* phage cocktails were used for disrupting biofilms of *E. coli* bacteria which were lysed in spot assay and growth inhibition assay, and found to be effective in disrupting biofilms. While in positive control, there was no significant effect of phage cocktails in biofilm formation (OD values shown in table 4.4).

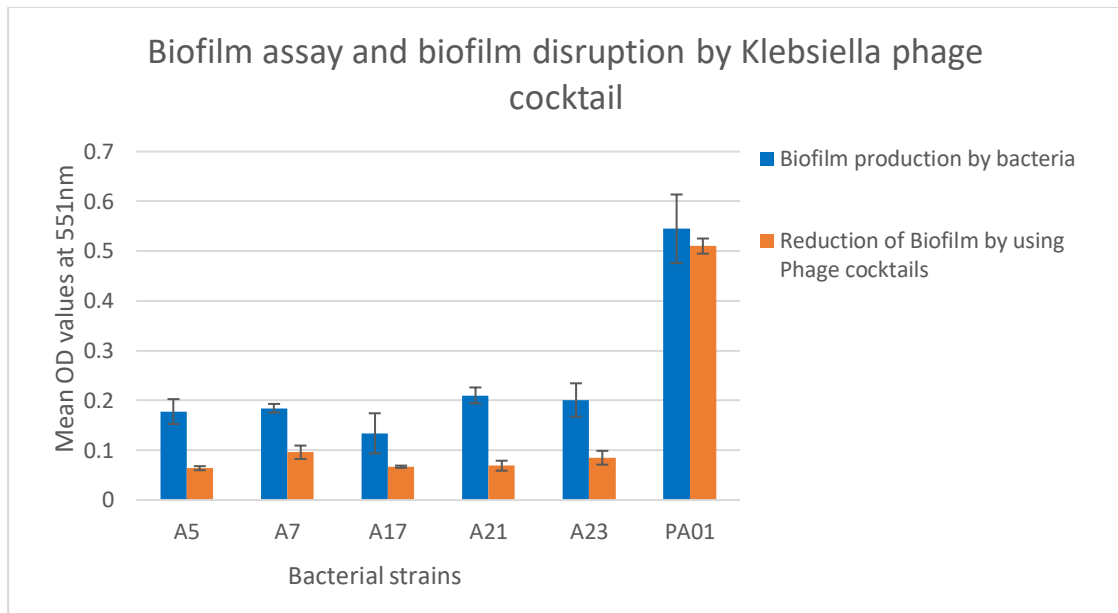


Figure 4.15 Graphical representation of biofilm disruption of *E. coli* by Klebsiella phage cocktails

The OD value of respective strains of *E. coli* after biofilm disruption assay was reduced substantially to the value less than the cut-off value, indicating that the biofilm was disrupted by the phage cocktails.

The biofilm producing bacteria have high pathogenicity compared to non- biofilm producers due to the presence of extra virulence factor of biofilm forming EPS. The biofilm mode of bacterial survival and growth is now being seen as a serious threat to public health and awareness about such cases draws importance among the scientific as well as social communities. So, disrupting the biofilm while killing the pathogen is important for effective therapy. In this study, formulated phage cocktails showed promising result in disruption of bacterial biofilm. The bacteria were selected based on the host range result to check biofilm production and the biofilm nearly disappear after incubation with phages. Phage modification of biofilm architecture also increases susceptibility to antibiotics. Biofilm disruption by phage cocktails might be due to the production of sufficient amount depolymerases enzymes which allow the penetration through the biofilm matrix, like with capsule degradation.

CHAPTER 5 SUMMARY

The pathogenic MDR samples of *K. pneumoniae* and *E. coli* available in the phage research lab, confirmed as MDR by seniors, were collected and revived from glycerol stock. To ensure that no contamination have occurred biochemical tests for respective bacteria was performed. The bacteria to be used for determining the multi host range lysis were also revived and identified as respective strains. Biochemical test and those which were not identified by biochemical test were identified by 16srRNA sequencing. For molecular identification, first bacterial DNA was isolated, PCR was performed with universal primers, 16srRNA sequencing was done, sequence obtained was BLAST and, from sequence similarity obtained bacteria were identified. For obtaining the bacteria accession number gene segment was submitted to NCBI database.

Using the host bacteria, new phages were isolated against three *E. coli* strains while previously isolated phages were re-isolated for *K. pneumoniae* strains. The host bacteria were already characterized in the lab. So, molecular identification was not necessary. Purification and amplification of phages was done. Bull's eyes plaques were formed by *Klebsiella* phages while the plaques of *E. coli* phages were all pin-headed. On determining the phage titer, all phages showed a titer of above 10^7 concentration.

The next step was phage characterization. Protein profiling of phages was done by SDS-PAGE using acetone precipitation method (to obtain concentrated protein). Proteins of different band size between were 50-150 kDa were obtained in all 6 phages. From Transmission electron microscopy, the phages were found to be of order *Caudovirales*. All phages of *K. pneumoniae* were of Podoviridae family and among the *E. coli* phage, phage A4 was of siphoviridae family. Phages were sent for genome sequencing but the result was not obtained due to time limitation and related factors.

MHR analysis was performed by Spot assay with individual phages as well as phage cocktails. Efficiency testing was done by spot assay of different dilution of phages and performing DLAA. The interesting result was obtained in MHR analysis, that is, *Klebsiella* phage cocktails effectively lysing non-lactose fermenting pathogenic *E. coli* due to the synergistic action of phage cocktails used. While with some non-pathogenic strains tested no lysis was found. From killing assay, *E. coli* growth inhibition rate by phage cocktails for different interval of time was obtained. *E. coli* strains were first assayed for biofilm production by crystal violet assay and then detected to determine the role of *Klebsiella* phage cocktails in biofilm disruption of *E. coli*, biofilm disruption assay by phage cocktails was performed. The moderate biofilm formers biofilm was disrupted on application of phage cocktails.

Our result showed that phages as cocktails can enhance the lysis of bacteria by the synergistic action. There must be some type of receptor similarity between *K. pneumoniae*

and *E. coli*, and some similar type of lysis mechanism due to which *E. coli* strains were lysed by Klebsiella phage cocktails. Such phage cocktails can be used to treat some type of multi-bacterial infections. Along with the enhanced lysis capability, Klebsiella phage cocktails were successful in disrupting the formed biofilms. This supports the use of phages to disinfect the medical indwelling devices and other inanimate surfaces.

CHAPTER 6 CONCLUSION

- Phages of *Klebsiella pneumoniae* used as cocktails were all Podoviridae family
- Polyphages can enhance host range compared to individual phages
- Klebsiella phage cocktails effectively lysed pathogenic *E. coli* bacterial strains in- vitro, which can be used as an application to treat multi-bacterial infection
- Also, phages as cocktails were effective in disrupting the biofilm formed by bacteria

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

- i. 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.
- ii. Due to time limitation, we were unable to get the whole genome sequencing of our phages and study the mechanism of synergy through whole genome sequence result analysis.

RECOMMENDATION

As our study was the preliminary work on studying the effect of phage cocktails on different genus of bacteria of same family Enterobacteriaceae, following would be the recommendations for further extension of research.

- i. First and foremost, whole genome sequencing and complete genome annotation of phages used as cocktails need to be done to understand the phage biology and genetics.
- ii. Study of phage-host interaction mechanism is important to understand the mechanism of synergy shown by phage cocktails.
- iii. Exploring the synergy, further the experiment on animal model can be conducted.
- iv. And for biofilm disruption property phage cocktails can be used as disinfectant in hospitals and medical indwelling devices and tested for their efficacy to eliminate pathogens.

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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)

Constituents	weight/volume
Acrylamide; C ₃ H ₅ NO	29g
Bis Acrylamide; C ₇ H ₁₀ N ₂ O 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	0.03
10% (NH ₄) ₂ S ₂ O ₈	0.1	0.03
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/18.17g	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

Multi-host range analysis table

Table 1: Host range analysis of individual phages on different bacterial strain

Bacterial strains	K27 phage	NK phage	K57 phage	A1 phage	A4 phage	A6 phage
K23	×	(+++)	(+++)	×	×	×
K27	(+++)	(++)	×	(+)	×	×
NK	(+++)	(+++)	(+++)	(+++)	×	(+++)
K41	×	×	×	×	×	×
K52	×	×	(+++)	×	×	×
K57	×	(++)	(+++)	×	×	×
S2	×	×	×	(++)	(+++)	(+++)
S3	×	×	×	×	×	×
S4	×	×	×	×	×	×
S5	×	×	×	(+++)	(+++)	(+++)
S6	×	×	×	×	×	×
A1	×	×	×	(+++)	(+++)	(+++)
A4	×	×	×	(+++)	(+++)	(+++)
A6	×	×	×	(+++)	(+++)	(+++)
A5	×	×	(++)	(+++)	(+++)	(+++)
A7	×	×	(++)	(+++)	(+++)	(+++)
A17	×	×	(++)	(+++)	(+++)	×
A21	×	×	(++)	(+++)	(+++)	(+++)
A23	×	×	(++)	(+++)	(+++)	(+++)
P4	×	×	×	×	×	×
P19	×	×	×	×	×	×
P53	×	×	×	×	×	×

'+++' represents clear spot '++' represents clear at side and turbid at center '+' represents small plaques on spot

Table 2: Host range analysis of phage cocktails on different bacterial strains

Bacterial strains	Klebsiella phage cocktail	E. coli phage cocktail
A1	✓	✓✓✓
A4	✓	✓✓✓
A6	✓	✓✓✓
A5	✓✓✓	✓✓✓

A7	✓✓✓	✓✓✓
A17	✓✓✓	✓✓
A21	✓✓✓	✓✓
A23	✓✓✓	✓✓✓
K23	✓✓✓	x
K27	✓✓✓	✓✓
NK	✓✓✓	✓
K41	✓✓✓	x
K52	✓✓	x
K57	✓✓✓	x
S2	✓✓✓	✓✓✓
S3	x	x
S4	x	x
S5	x	✓✓✓
S6	x	x
P4	x	x
P19	x	x
P53	x	x

"✓✓✓" represents clear spot "✓✓" represents clear at sides and turbid at center "✓" represents small plaques on spot