Isolation and characterization of bacteriophages derived from environmental waste water samples with specificity towards *Klebsiella pneumoniae* and *Salmonella paratyphi*

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of Bachelor of Science in Biotechnology

Department of Mathematics and Natural Sciences

BRAC UNIVERSITY

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Declaration

It is hereby declared that-

1. The thesis submitted is our own original work while completing a degree at BRAC UNIVERSITY.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. We have acknowledged all main sources of help.

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Ethics Statement

This study does not involve any human or animal trials and is devoid of any unethical incidents.

Dedicated to our beloved parents and our faculty members for always believing in us.

Abstract:

Multiple drug resistance of *Klebsiella pneumoniae* and *Salmonella* variants are constantly evolving and posing a significant threat to global healthcare. Furthermore, the global rise in multidrug-resistant strains of *Klebsiella pneumoniae* has made therapy more challenging, the prevalence of multidrug-resistant *K. pneumonia* is responsible for around 80% of nosocomial infections (Cao, 2015). On the other hand, *Salmonella paratyphi* is the most common foodborne pathogen responsible for over 135,000 deaths annually worldwide (Hoffman & Luby, 2024). The therapeutic use of certain phage particles that target bacterial pathogens is one potential non-antibiotic treatment because it provides advantages such as efficient, non-toxic, ubiquitous, easy to prepare and can result in targeted lysis of the host. To gain extraordinary insight between phage and host interaction in the aquatic environment, samples from Buri-Ganga River, Turag River and Mirpur wastewater were collected and phage *K1, K2, K3 and S* were isolated and their pathogenic host systems were examined. Initially, these phages were evaluated for thermal stability, and the results showed that they were resistant at moderate temperatures but completely inactivated at higher ones. Phages *K1, K2, K3, and S* showed varying reactions to pH changes, but were remarkably stable in the pH range of 4.0 to 10.0. They were also assessed for their susceptibility to the organic solvents' ethanol and chloroform, which revealed resistance to ethanol but total deactivation in the presence of chloroform. Furthermore, the study also looked at the separated phages' host range selectivity against 29 different clinically relevant bacterial strains, which showed promise for their therapeutic efficacy against a range of strains. Phages *K1, K2, K3, and S* demonstrated lytic efficacy against a variety of bacterial strains, including those having clinical significance, indicating their possible application in phage therapy.

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Atiar Md. Suja Sami

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List of Acronyms

Acronyms	*Full Form*
TNF	*Tumor Necrosis Factor*
LPS	*Lipopolysaccharide*
MDR	*Multi drug resistant*
PSC	*Primary Sclerosing Cholangitis*
KP	*Klebsiella pneumoniae*
DNA	*Deoxyribonucleic acid*
ICTV	*International Committee on Taxonomy of Virus*
IBD	*Inflammatory bowel disease*
FDA	*Food and Drug Administration*
RNA	*Ribonucleic acid*
EPEC	*Enteropathogenic Escherichia coli*
PDT	*Phage Display Technology*
VAL	*Virosome associated lysozyme*
BPs	*Bacteriophages*
hvKP	*Hyper virulent Klebsiella pneumoniae*
cKP	*Classical Klebsiella pneumoniae*
KC	*Kupffer cells*
RTE	*Ready to eat*
ALP	*Alkaline Phosphatase*
PJI	*Prosthetic joint infection*
PFU	*Plaque forming unit*

List of Acronyms

Acronyms	*Full Form*
LB	*Luria-Bertani broth*
LBA	*Luria-Bertani agar*
DLA	*Double layer assay*
MCT	*Microcentrifuge tube*
CFU	*Colony forming unit*

Chapter 01

Introduction

1.1 Objective :

The principal objective of the thesis project is to isolate a bacteriophage that selectively targets clinical strain *Salmonella paratyphi* and *Klebsiella pneumonia*. The procedure includes isolation of phage samples from water and identifying the bacteriophages onto different physiological and molecular characteristics. The term “physiological traits" describes the bacteriophage's functional characteristics, such as its capacity for infection and replication. Conversely, molecular attributes are related to the bacteriophage's genetic and molecular characteristics.

This investigation looked at the effects of variables like temperature, pH, salinity, and host range of the phage sample. The goal of our work was to measure the degree of variation in the phage samples' performance against various phage strains and species, as well as characterizing the ***K1, K2, K3,*** *and* ***S*** according to the parameters**.**

1.2. Intended Goal of the Research:

The goal of the study is not just to characterize the isolated bacteriophage. It implies that the results and the chosen phage might find use in biotechnology, medical research, or other domains where managing *Salmonella paratyph*i and *Klebsiella pneumonia* is important. Ultimately, the goal of the study is to better understand bacteriophages and their potential uses in a variety of scientific fields. The initial target of the research is to use the isolated and characterized bacteriophage, as This suggests that the chosen bacteriophage is seen to be quite effective and appropriate for further research.

Research on phage therapy with an emphasis on *Salmonella paratyphi* and *Klebsiella pneumoniae* aims to investigate the possibility of bacteriophages as a targeted treatment for illnesses caused by these organisms. Research has demonstrated that phage therapy is effective in treating *Klebsiella pneumoniae* infections, particularly when the strains are resistant to drugs (Torabi et al., 2021). Studies have indicated that in order to improve the efficacy of phage therapy, it is crucial to identify and isolate bacteriophages unique to *Klebsiella pneumoniae*.

Furthermore, phage cocktails have been found to be an essential tactic for maximizing the effect of phages on Klebsiella populations, which in turn increases the effectiveness of phage therapy.

In general, the goal of phage therapy research on *Salmonella paratyphi* and *Klebsiella pneumoniae* is to use the effectiveness and selectivity of bacteriophages to fight antibiotic-resistant strains of these diseases and offer alternate forms of treatment. Researchers are investigating novel strategies to tackle the increasing problem of antibiotic resistance by isolating, characterizing, and employing bacteriophages designed for these particular bacterial species.(Torabi et al., 2021)

Chapter 02

Literature Review

2.1 Bacteriophage

Bacteriophages, also known as phages, are viruses that only infect bacterial cells and exhibit extraordinary variation in size, appearance, and genetic structure. Despite differences, all phages have the same basic structure: a nucleic acid genome enclosed in phage-encoded capsid proteins. While phages appear to have distinct “heads,” “legs,” and “tails”. Brownian motion, rather than motility, is used to reach their bacterial prey. Phages are very particular to their hosts, infecting only a single bacterial species or strains within a species. They use either lytic or lysogenic replication methods. During the lytic cycle, phages reproduce quickly within the host, causing cell lysis and the release of additional phages. The lysogenic cycle entails incorporating the phage genome into the bacterial chromosome, allowing replication without cell death. Despite their inability to infect human cells, phages play an important function in the human microbiome by allowing genetic exchange between pathogenic and non-pathogenic bacteria. Bacterial genes are transferred either at random (generalized transduction) or through the excision of bacterial DNA during lysogenic phage replication (specialized transduction). While phages have been studied for their potential to treat bacterial illnesses, obstacles remain. Bacterial resistance, a wide range of strains, and the immunogenic nature of phages all pose difficulties. Phage therapy, the clinical use of lytic bacteriophages, faces challenges such as limited efficiency against various bacterial strains and quick clearance from the bloodstream. Some argue that phage enzymes could be used as an alternate method, although thorough human trials are lacking (Maciejewska et al., 2018).

2.1.1 Early Historical Perspectives on Bacteriophages

In 1896, Ernest Hanbury Hankin, a British bacteriologist working in India, discovered that the Ganga and Yamuna rivers had a biological substance capable of killing cholera-causing bacteria. This material was able to pass through millipore filters, which generally trap larger microbes. Hankin published his findings in the Pasteur Institute Annals(Wittebole et al., 2014). In 1915, while studying the growth of vaccinia virus on agar media devoid of cells, British microbiologist Frederick Twort discovered a filter-passing translucent substance linked with "pure" bacteria. Twort called this substance a "filterable agent," citing its ability to break down bacteria in a

culture into granules (Twort, 1915). In his tests with vaccinia-isolated micrococci, Twort discovered that certain colonies' material, which could not be sub-cultured, might infect new micrococcal growth for several generations. This transparent material, described as a ferment released by the microbe, lacked the ability to develop independently and served no apparent purpose at the time (Wittebole et al., 2014).

Two years after this discovery, Félix d'Herelle separately reported a similar experimental finding while researching patients with or recovering from bacillary dysentery. D'Herelle recovered a material he referred to as an "anti-Shiga microbe" from the stools of shigellosis survivors. He achieved this by filtering stools that had been cultured for 18 hours. When the resultant filtrate was added to a Shiga bacilli culture or emulsion, it was able to stop the culture, promote bacterial mortality, and eventually cause bacilli lysis. D'Herelle described his **discovery as a** true microbe with immunity and an obligatory bacteriophage. He also demonstrated the efficacy of this anti-Shiga microbe by giving it to experimental animals as a treatment for shigellosis. This supported the clinical relevance of his findings and aligned with some of Koch's postulates (Wittebole et al., 2014).

In 1938, John Northrop maintained his idea that bacteriophages were created by live hosts through the production of an inert protein, which was then changed into active phages by an auto-catalytic mechanism (Northrop, 1938). However, data from many investigators, including Wollman in 1928 and Bordet and Bail's discovery of lysogeny in 1925, supported d'Herelle's theory that phages were live particles or viruses while replicating in host cells. The invention of the electron microscope enabled Helmut Ruska to describe phage particles, while Luria and Anderson visualized several phage kinds, revealing their shared structure (Wittebole et al., 2014). Despite continuous research in the former Soviet Union and nations such as Poland, interest in phage therapy revived in the English literature in the 1980s, with human trials beginning in the 2000s. The Phage Summit in 2004 was a large worldwide conference that sparked a fresh interest in phage biology. Bacteriophages are one of the most studied bacteria in the world, as evidenced by the large phage literature, which includes thousands of papers

During World War II, regions in the Soviet Union and Eastern Europe used phage therapy because they had limited access to antibiotics. This procedure, which began over 80 years ago, is still common in Russia and Eastern European countries, particularly at the Eliava Institute in Tbilisi, Georgia. In 1952, the Hirszfeld Institute launched a programme to treat suppurative illnesses with phage therapy. In the 1980s, controlled investigations in English scientific literature concentrated on animals. Recently, certain Western European countries legalized therapeutic use. In 2016, Paul Turner and colleagues identified a phage that can restore antibiotic susceptibility in multidrug-resistant P. aeruginosa. This phage successfully healed a patient with a prolonged aortic graft infection that was resistant to surgery and rigorous antibiotic therapy with a single phage application (McCallin et al., 2019).

Following his discovery, d'Herelle pioneered early attempts at phage treatment, including employing phage to treat dysentery. The trials were undertaken at the Hôpital des Enfants-Malades in Paris in 1919, supervised clinically by Professor Victor-Henri Hutinel. To ensure safety, d'Herelle, Hutinel, and hospital interns took the phage preparation before giving it to a 12-year-old dysentery patient. The patient's symptoms disappeared after a single phage dosage, and he recovered completely within days. While the findings were not immediately published, Richard Bruynoghe and Joseph Maisin reported the first known use of phages to treat human infectious illnesses in 1921. They treated staphylococcal skin disease using bacteriophages injected into surgically opened lesions, and the infection resolved within 24 to 48 hours. Subsequent positive experiments, especially those conducted by d'Herelle in India against cholera and/or bubonic plague, cleared the door for active commercial phage production against a variety of bacterial diseases (Summers, 1999).

2.1.2 Taxonomic Classification of Bacteriophages

Phage classification has developed over time. Burnet and Ruska set the groundwork in 1937 for comprehending phage size and morphological variety. In 1948, Holmes developed a taxonomy based on host range and symptoms, which is now considered historical. Lwoff, Horne, and Tournier proposed classifying viruses based on virion characteristics and nucleic acid in 1962, and the International Committee on Taxonomy of Viruses (ICTV) established the foundation for phage taxonomy in 1971 by identifying six phage “genera”. The ICTV, the sole international

viral taxonomy body, publishes reports using a variety of criteria. Phage classification began in earnest in 1967, when Bradley proposed six basic morphological groups, which were later adopted by the ICTV. Currently, the ICTV classifies virions based on nucleic acid, shape, and physicochemical features.

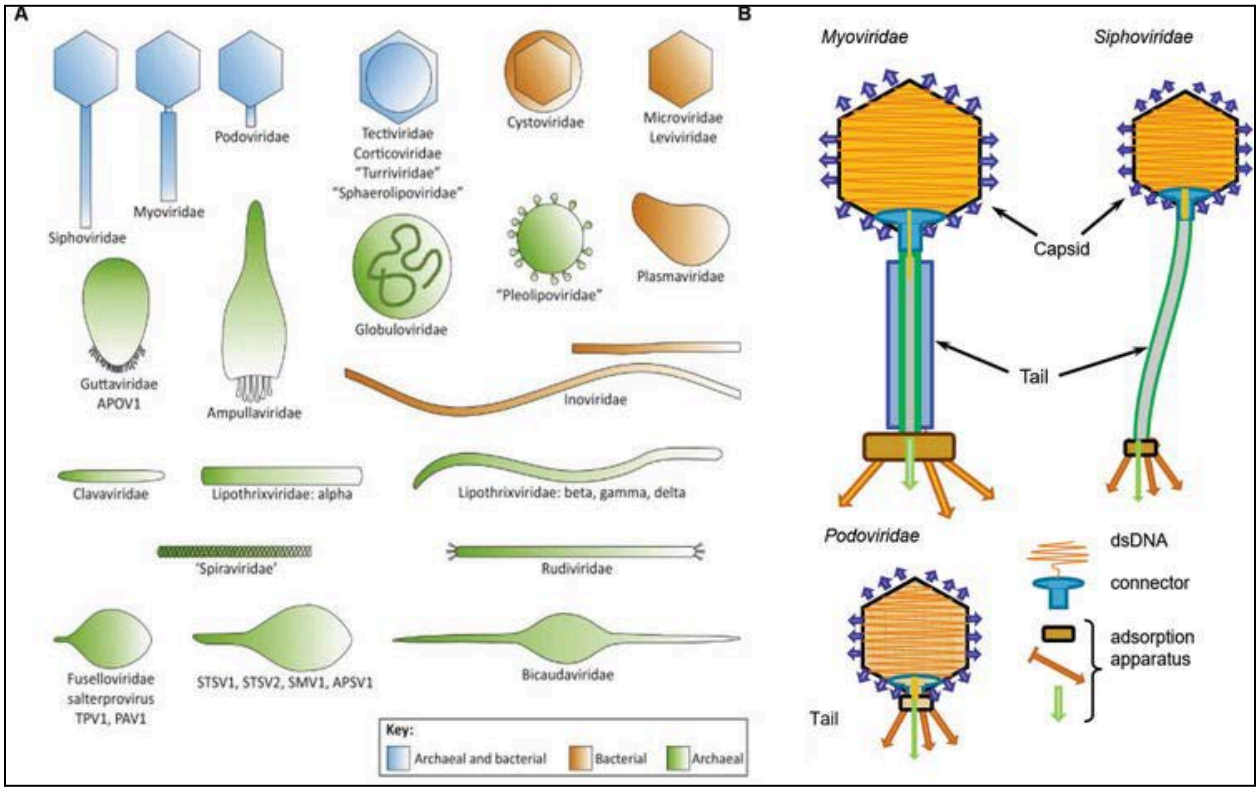

Figure 2.1: Structure of a bacteriophage *(Adapted from White et al., 2018).*

Phages include a variety of properties, including double-stranded or single-stranded DNA or RNA, and particle morphologies that range from tailed or polyhedral to filamentous or pleomorphic. The appearance, physicochemical features, and physiological characteristics of phage families have been widely studied. Over 96% of phages are tailed phages, which are classified into three families: Myoviridae (contractile tails), Siphoviridae (long noncontractile tails), and Podoviridae (short tails). These phages usually feature icosahedral or nearly comparable heads. Tailed phages frequently cause complications in phage classification due to their overwhelming quantity and huge, but occasionally low-quality, data. The eighth International Committee on Taxonomy of Viruses (ICTV) Report includes 17 genera of tailed phage.(Fauquet & Fargette, 2005) In addition, seven groups of polyhedral, filamentous, and

pleomorphic phages can be distinguished by considerable changes in nucleic acid composition and structure. These families, which are distinguished by distinctive characteristics such as lipid-containing virions and lipoprotein envelopes, are generally tiny, with some having only one member, offering less taxonomic issues. The ICTV now uses the "polythetical species definition," which defines a viral species as a polythetic group of individuals that form a replicating lineage and share a certain biotic habitat(Fauquet & Fargette, 2005).

2.1.3 Environmental Abundance of Bacteriophages

Bacteriophages, at times known as phages, have received a lot of interest since they are the most abundant biological entities in the world. Their impact on bacterial community composition is a topic of great interest. This review investigates the existence of phages in natural and manmade habitats, as well as their effects on communities. Phages are abundant in natural habitats such as the human body, soils, and the marine environment, indicating that they may play a role in maintaining bacterial community balance. Bacteria in artificial environments, such as wastewater treatment facilities, industrial processes, and pharmaceutical formulations, attract phages. In these conditions, phages can either inhibit the bacteria required for efficient processes or, conversely, contribute favorably by eliminating resilient organisms (Batinovic et al., 2019).

Phages infect hosts via a variety of surface receptors, including carbohydrates, lipopolysaccharides, and proteins, resulting in host range specificity (Hyman & Abedon, 2010). The lytic and temperate phage life cycles determine their effect on microbial populations. After injecting their DNA into the host, lytic phages use host metabolic processes to create viral progenies, thus commencing an infection cycle. Temperate phages, on the other hand, can go through a lysogenic lifetime by integrating into the host genome or generating plasmids, with the possibility of subsequently switching to the lytic cycle (Howard-Varona et al., 2017). Because of their potential to modify microbial populations, lytic phages are thought to be excellent candidates for phage treatment or biocontrol. Their effects can be both positive and negative, impacting microbial community functioning. This review focuses on the prevalence and roles of lytic phages in both natural and artificial environments, emphasizing the significance of

understanding phage communities as important components of microbial ecology(Batinovic et al., 2019).

The human body contains over 10^{12} bacteria, predominantly in the stomach, which influence host-microbiota interactions (Shkoporov & Hill, 2019). Bacteriophages (phages) are abundant on body surfaces and have control on bacterial ecosystems. Phages, estimated at 31 billion per day, cross intestinal epithelial cells and influence host cell signaling. Mucosal surfaces, critical interaction zones, symbiotic microorganisms, and phages. Mucus helps lysogenic phages create symbiotic connections with bacteria. Phages elicit immunological responses; TLR9 detects viral DNA during phage transcytosis. The consequences of phage-mediated immunity are multifaceted, with the potential to either contribute to or mitigate inflammatory reactions(Janeway & Medzhitov, 2002). Alterations in phage populations are associated with disorders like as inflammatory bowel disease (IBD) and type 1 diabetes. Phages may be used as biomarkers to identify disease. Immune-modulating phage characteristics may alter host-bacterial interactions and therapeutic outcomes. Understanding phage populations in the human microbiome has potential for disease detection and treatment(Batinovic et al., 2019).

Oceans, which occupy more than 70% of the Earth's surface, are essential for biodiversity and oxygen generation. Microbes, particularly bacteria, dominate marine ecosystems, accounting for 90% of ocean biomass. Viruses, especially phages, are abundant in ocean waters, estimated at 4×10^{30}, outnumbering bacteria and archaea by more than 15 times. Despite their frequency, roughly 60% of marine phages lack taxonomic data, indicating gaps in our understanding of their variety Siphoviridae is the most widespread, occurring not only in maritime environments but also in a variety of habitats. Deep-sea settings, characterized by harsh conditions, support different phage populations, with Caudovirales dominating. Temperate phages, unlike surface layers, are abundant in deep-sea ecosystems and may have an impact on bacterial populations (Batinovic et al., 2019).

Compared to marine habitats, soil virome is understudied but critical for biogeochemical processes. Soil, a primary biome, has a varied range of microbial communities that influence soil quality and plant growth. Bacteriophages are believed to play an important role in bacterial population regulation and horizontal gene transfer. Virus extraction challenges in soil have

hampered study, however studies estimate virus-like particle counts in the range of 10^9 per gramme of dry soil. The ratio of viruses to bacteria varies by soil type and is regulated by moisture, pH, and temperature (Batinovic et al., 2019). Tailed viruses predominate, with their abundance indicating soil variety. Soil phages influence bacterial growth rates, nitrogen cycling, and symbiotic relationships in the rhizosphere. Soil phages have been effectively used in biocontrol applications to battle plant diseases.

The activated sludge wastewater treatment technology uses a bacterial colony to filter water. Phages, particularly those of the Siphoviridae family, are prevalent in activated sludge, and new metagenomic investigations have revealed their variety. While the impact of phages on the activated sludge community is uncertain, studies indicate that they may have a deleterious impact on bacterial composition and plant performance. Understanding host-phage connections in activated sludge is difficult because of the presence of CRISPR-Cas sequences in bacterial genomes. Phages may operate as environmental controls for harmful bacteria in activated sludge, potentially resolving problems such as bulking and foaming caused by filamentous bacteria.

2.1.4 Life Cycle Dynamics of Bacteriophages

Bacteriophages have lytic and lysogenic life cycles and play an important role in bacterial infections. During the lytic cycle, phages connect to bacterial surfaces using receptor-binding proteins before entering the host and commencing viral DNA injection. This procedure involves enzymatic cleavage of the bacterial cell wall, with certain phages using a sheath to insert a tube into the bacteria. Replication occurs when phage enzymes inhibit bacterial DNA, RNA, and protein production, allowing the phage to duplicate its genome through host metabolism. The maturation step entails assembling phage components around the genome, and a gene disintegrates bacterial cell wall peptidoglycans, resulting in bacterial lysis. The final stage, reinfection, generates multiple phages from each infected bacterium, effectively killing the host in the lytic cycle (Alsobhi, 2021).

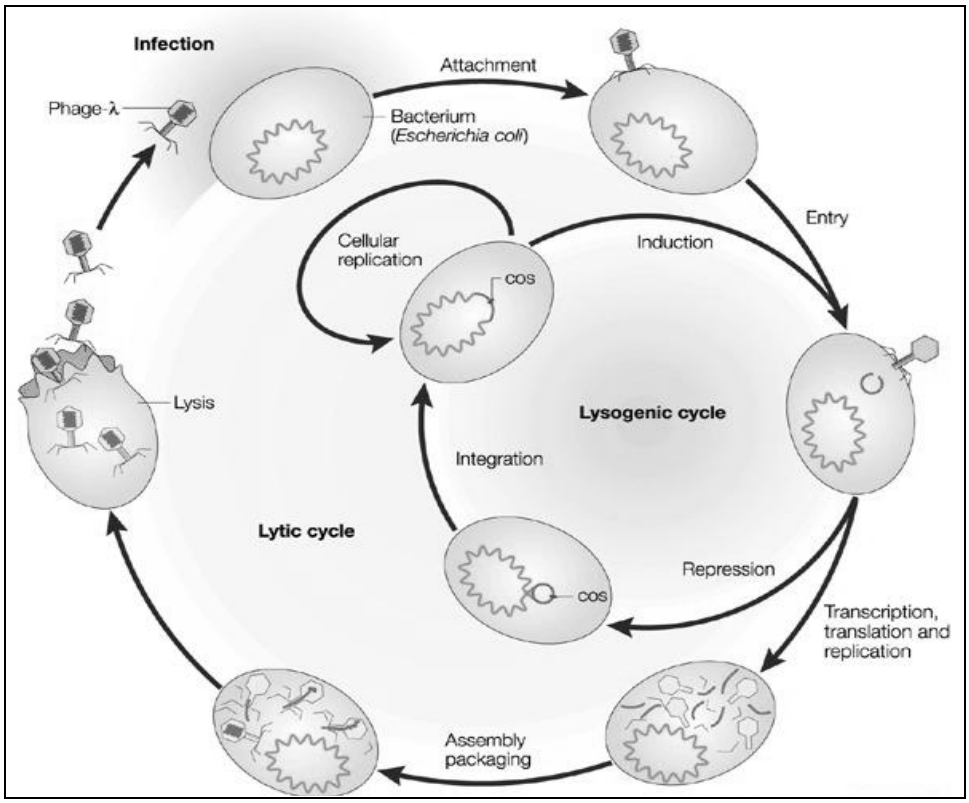

Figure 2.2: Stages of the bacteriophage lytic and lysogenic cycle *(Adapted from Jamal et al., 2018).*

Temperate phages, also known as non-virulent phages, have lysogenic properties and use the host to stay inactive (Batinovic et al., 2019). During the lysogenic cycle, the phage's injected genome integrates into the bacterial genome to become a prophage. When bacteria are exposed to difficult conditions such as ultraviolet light or antibiotic treatment, temperate phages can be driven to enter the lytic cycle. This switch causes bacterial lysis and the release of viral progeny. The complicated dynamics of these life cycles demonstrate bacteriophages' resilience and different methods in their interactions with bacterial hosts (Howard-Varona et al., 2017).

2.1.5 Influence of Phage on Bacterial Populations and Host Fitness

While the precise impact of natural phages on microbial communities is unknown, they are likely to shape these communities by favoring certain bacterial species while eliminating others. This impact is mediated by mechanisms such as host selectivity, horizontal gene transfer, bacterial

evolution, and competition among bacterial communities. The mutual selective pressure between phages and bacteria appears to have a considerable impact on bacterial diversity, pathogenicity, and evolutionary history (M. R. Clokie et al., 2011).

The lytic life cycle is crucial for eliminating bacterial species, extensively studied for medical applications. However, the lysogenic, pseudo-lysogenic, and chronic cycles are also vital in phage-bacterium interactions. The lysogenic cycle aids bacterial survival against lytic phages by integrating genomic sections and transferring resistance genes, providing colonies with a selection advantage. Chronic phages reduce bacterial development, resulting in a drop in their abundance relative to uninfected bacteria in an environment, demonstrating phages' power to shape microbial communities (Naureen et al., 2020).

Phages have evolved a variety of techniques to combat bacterial defense, including the alteration of attachment proteins and anti-CRISPR proteins. To coexist with bacteria and survive, phages use a variety of methods, including nucleic acid degrading systems, point mutations, and anti-CRISPR mechanisms. The Acr gene, identified in temperate *Pseudomonas* phages, inhibits the CRISPR-Cas evolutionary process of bacteria, demonstrating the complexities of phage-bacteria interactions (Wittebole et al., 2014).

Phages act as predators, influencing bacterial populations in a variety of ways. They can have an impact on bacterial species' population and diversity, as well as alter their physiology, competitive power, and pathogenicity. Phages can carry genes that are similar to bacterial metabolic genes, known as auxiliary metabolic genes, which may influence bacterial metabolism in favor of the infecting phages (M. R. Clokie et al., 2011). Cyanophages, for example, may have photosynthesis-related genes that allow them to create energy when the host's photosynthetic protein synthesis stops, allowing phage replication to continue.

Phages can influence bacterial virulence by adding virulence genes, increasing bacterial survival, perhaps expanding the phage host range, and promoting continuous replication. This mechanism, at times, causes enhanced host pathogenicity, which contributes considerably to bacterial infections in people and animals. The techniques include transferring toxin-encoding genes, replicating virulence factors, and altering regulatory sequences that govern bacterial virulence genes. Furthermore, phages can promote biofilm development, antimicrobial resistance,

immunological resistance, and increased pathogenicity in bacterial hosts, as seen in Vibrio cholerae, Pseudomonas aeruginosa, and Shigella dysenteriae (Naureen et al., 2020).

The specificity of phage-bacteria interactions affects the relative fitness of bacterial species in a given setting. Phages that infect two hosts can vary in adsorption rate, time to cell lysis, and burst size, providing one bacterial species an advantage in the presence of the phage. If both hosts develop resistance to the phages at different costs, phage-mediated selection may modify their competition even when the phage is not present. This competition intensifies when one host is less sensitive and the other is more vulnerable to the same phage, resulting in the tolerant species dominating a certain niche (Naureen et al., 2020).

2.1.6 Applications of Bacteriophages

Bacteriophages, which outnumber bacteria in quantity, have important roles in global ecology, biogeochemical cycles, and health (Batinovic et al., 2019). Strathdee and colleagues present a thorough overview of phage biology, including excellent properties for therapy, engineering, and contemporary uses. Aside from therapy, bacteriophages are versatile in domains such as agriculture, pest control, microbiome regulation, and disinfection.

1. Food products, such as salads and greens, are prone to bacterial contamination, resulting in outbreaks. Pathogens like Bacillus cereus, Escherichia coli, and Salmonella spp. Are dangerous. Phage use after harvest or before packaging, using FDA-approved products like as Intralytix and Phagelux, can reduce infection. Phages can help prevent Cronobacter sakazakii in baby formula and Clostridium botulinum in honey (Liu et al., 2022).

2. Phages are a viable technique for rebalancing microbiomes associated with illnesses. They can target specific pathogens, such as Helicobacter pylori for gastric ulcers, Clostridium difficile for chronic diarrhea, and germs that cause acne and vaginosis. Furthermore, in complicated dysbiosis such as periodontal disease, lytic phages have shown promise against important bacteria. Phages can improve prebiotic and probiotic

compositions, giving a focused strategy to treat health-damaging disorders like obesity(García-Cruz et al., 2023).

3. Bacteriophages have shown potential in fighting degenerative disorders such as Parkinson's and Alzheimer's by focusing on misfolded protein aggregates. The M13 phage capsid protein, which interacts with the amyloid fold, has the ability to reverse plaque formation.(Krishnan et al., 2014) Phages provide immunomodulation and gene transfer in viral infections, making them useful as supplementary therapy for viruses such as SARS-CoV-2. Engineered phage vectors demonstrate efficacy in circumstances such as glioblastoma multiforme. This strategy is also applicable to viral treatments, which use phage-based vectors to target genome editing in infected cells. Despite manufacturing obstacles, phage-based vectors' specificity and safety make them potential for clinical use in non-bacterial illnesses.(García-Cruz et al., 2023)

4. Bacteriophages are becoming increasingly used in agriculture as an antibiotic option for combating bacterial infections. In addition to targeting disease-causing bacteria, phages have the ability to weaken or eliminate insect pests that rely on symbiotic bacteria. Recent research found that phages drastically lower P. aeruginosa in housefly stomachs, altering gut microbiome makeup and influencing fly development. In mosquitos, utilizing phages to control larval microbiomes shows potential for changing life-history features. Introducing phages that target certain bacteria in Anopheles larvae lowered survival and larval development, demonstrating phages' potential for altering insect microbiota and influencing their biology.

Despite the tremendous potential of phage therapy, significant research gaps remain. Priority areas include increasing the repertory of lytic phages for specific diseases such as H. pylori, C. difficile, and G. vaginalis, which necessitates the identification of appropriate phages and the conversion of lysogenic ones to lytic forms. Diseases with complicated dysbiosis, such as periodontal diseases, necessitate a comprehensive strategy that takes into account the spatiotemporal interactions of multiple bacterial species. Concerns are raised about phage-induced bacterial lysis, which releases toxic chemicals that may injure hosts and increase inflammation.

2.1.7 Current States of Phage Therapy in Human Health

While certain applications show potential, further research into phage biology and ecological interactions with bacteria is required. Approximately 70% of phage genes with uncertain functions require judicious selection. Unintended consequences, such as potentially selecting more hazardous bacteria during dysbiosis treatment, must be considered. Scaling up phage preparations and overcoming financial and technical barriers to commercial availability present problems. Learning from past failures to avoid a repeat of the antibiotic crisis is critical for appropriate phage use(Xu et al., 2022).

Phage treatment has proven effective against common gastrointestinal illnesses such as bacillary dysentery and cholera. Fecal sterile filtrate with phages efficiently cures refractory Clostridium difficile infections. While the therapeutic efficacy of Escherichia coli-T4 phage in acute bacterial infectious diarrhea is debatable, phage therapy in mice shows promise against Enteropathogenic *Escherichia coli* (EPEC). In a rabbit model infected with Vibrio cholerae, a phage "cocktail" delivered within 6 hours greatly lowers *Vibrio cholerae* growth. In comparison to antibiotics, phage therapy is efficient in reducing Clostridium difficile colonization in hamsters while having minimal influence on gut flora(Xu et al., 2022).

Phage treatment effectively treats respiratory infections. Nasal inhalation of the *Klebsiella pneumoniae* phage protects mice from deadly pneumonia induced by drug-resistant strains(Cao et al., 2015.) Phage treatment is useful in treating recurrent urinary tract infections, especially in cases with highly drug-resistant *Klebsiella pneumoniae*. Combining phages with antibiotics produces effective results. Phages prevent bacterial biofilm formation during long-term catheter use, minimizing the risk of infection. Clinical investigations, such as intravesical Pyo-phage, reveal that phage therapy outperforms antibiotics in postprostatectomy infections, with a 66.67% efficacy rate and no adverse effects. Renal transplant patients with refractory urinary tract infections caused by *Klebsiella pneumoniae* respond well to phage treatment(Kuipers et al., 2019).

Phages, recognized for their immunogenicity, stability, and low cost, are being investigated for vaccine development. Staquicini et al. created a new coronavirus-targeted vaccination with phages that induces specific humoral protection in mice (Staquicini et al., 2021). Li et al.

discovered phage Ab8, which had a high affinity for antibody V and neutralized SARS-CoV-2 in animal models, suggesting both preventative and therapeutic efficacy(W. Li et al., 2020).

Type 2 diabetes patients had a higher abundance of intestinal phages, with seven phages connected to illness onset. Phage changes coincide with changes in the relative quantity and variety of host microorganisms. In type 1 diabetes, viral genome modifications occur before illness onset, and decreased intestinal phage diversity precedes changes in serological markers. The interaction of phages and gut microbiota may lead to the emergence of autoimmune disorders in type 1 diabetic patients (Ma et al., 2018).

Colorectal cancer, a highly malignant tumor with significant morbidity and death, sometimes exhibits no early signs, resulting in late-stage diagnoses. According to studies, colorectal cancer patient's microbiota are significantly altered, with an increase in intestinal phage diversity. Dysregulation of intestinal viromes is associated with cancer progression, with over 20 different virus genera differing between patients and healthy persons. Colorectal cancer patients have significantly altered Siphoviridae and Myoviridae phages, which helps us comprehend the disease's virome.

Phage Display Technology (PDT) is a molecular biology method that uses phages as carriers to display foreign proteins on their surfaces. Its clinical applications include the quick manufacture of high-specificity monoclonal antibodies, as seen with adalimumab for rheumatoid arthritis.(Frenzel et al., 2016) PDT is critical to the development of vaccines against infectious diseases such as COVID-19, Ebola, and influenza (Xu et al., 2022). It also assists illness diagnosis and treatment by targeting tumors and organs, which has the potential to advance early cancer screening and therapy. Specific polypeptides, when combined with medicines, allow for specific targeting of tumor cells. In essence, PDT appears to be a flexible technology with applications in antibody generation, vaccine formulation, and targeted disease therapies(He et al., 2019).

2.1.8 Current Landscape of Commercial Phage-Based Products

Phages have been used to treat human ailments since 1921, when they were successfully injected to cure dysentery in France. (Luong et al., 2020) Initial applications included L'Oréal, Antipiol, Enterofagos, and EliLily's "Staphylofel." During WWII, the Soviet and German military employed phages to treat wound infections. However, interest in phage therapy decreased after antibiotics were discovered. There are currently five phage therapy institutions across the world, the first of which is Eliava Phage Therapy Centre (Kakasis & Panitsa, 2019). Although phage products are effective against a variety of bacteria, none are licenced for human use in the EU or the United States. The FDA has provided a regulatory pathway for emergency usage. Preclinical research focuses on multidrug-resistant microorganisms (MDRs). Phage mixtures for a variety of illnesses, including bone and joint infections, diabetic foot ulcers, and more, are being developed. The vast majority of preclinical phage products are liquid mixtures. The routes of administration affect efficacy, with oral administration being the most common. Endolysins and virosome-associated lysozyme (VAL), which are produced from phages, show potential for digesting peptidoglycans. Commercial endolysin products, such Staphefekt SA. 100 and Artilysin®, have been created. Rephasin®SAL200 and Exebacase CF301 are undergoing phase II clinical studies. The translation of preclinical analysis from small molecule antibiotics to phage lytic enzymes allows for speedier clinical evaluation (Huang et al., 2022).

Table 2.1 : Overview of Phage Products in Human Health

Phage Product	Origin Country	Application	Form/ State
Antipol	France	Not specified	Not specified
Enterofagos	Germany	Not specified	Not specified
Staphylofel	USA (Indianapolis)	Not specified	Not specified

Phage Product	Origin Country	Application	Form/ State
Polyfagin	Germany	Dysentery treatment	Not specified
Phage Cocktail	Not specified	Investigated for wound treatment	Not specified
Phagoburn Program	Multinational (France, Belgium, Switzerland)	Burn wound treatment	Not specified
Staphefekt SA.100	Netherlands (Micreros)	Chronic *S.aureus* associated diseases	Not specified
Artilysin product Line	Gremany (Lysando AG)	Effective against resistant *P.aeruginosa* and *A.baumannii*	Various forms (spray, nebulizer, solution, lyophilization, gel coating)
Rephasin SAL200	Korea (Intron Biotechnoology)	Phase II of human clinical trials	Not specified
Exebacase CF301	USA (ContraFect)	Effective against *S.aureus* including MARS	Not specified

The US Environmental Protection Agency (USEPA) has licenced a number of phage products for plant health. Commercial solutions like OmniLytics' AgriPhage™ for bacterial infections and Erwiphage PLUS for fire blight demonstrate phages' potential in plant health. Biolyse®BP and agriPHIX™ are solutions that improve crop storage and prevent soft rot. Overall, phage-based biopesticides offer promising treatments to plant diseases (Huang et al., 2022).

The widespread use of antibiotics in animals has resulted in antibiotic resistance (AMR), requiring regulatory limits. Phage therapy has been used in veterinary medicine since 1919, and it is gaining popularity again due to AMR concerns. Applications include preventing E. coli and Salmonella infections in poultry, S. aureus-induced mastitis in cattle, and fish mortality in aquaculture.. Companies like Intralytix concentrate on pet food safety, PhagePharm on environmental enhancement, and Fixed-Phage on phage cocktails. While most gel formulations are taken orally, some, such as Staphage Lysate (SPL)®, treat cutaneous infections. More study is needed on companion animals, particularly in bacte rial dermatitis(Huang et al., 2022).

2.1.9 Advantages of Employing Phage Therapy

Bacteriophages(BPs) are viruses that selectively infect and destroy bacteria while causing no harm to human or animal cells, have been used to treat bacterial infections for over 100 years. BP therapy, pioneered by Felix d'Herelle, was widely utilized in the Soviet Union and produced good results, nevertheless it is not well known in the Western world. The image of BPs has shifted over the last three decades as a result of the rise of multidrug-resistant bacteria and a fall in innovative antibiotic research. Recent research in the United States and Europe, as well as European Commission-funded programmes, demonstrate a renewed interest in BPs for a variety of applications, including clinical trials. However, obstacles remain, and no BP preparation has been approved for human use by regulatory agencies (Domingo-Calap & Delgado-Martínez, 2018).

- **Broad Bacterial Lysis:** BPs can theoretically lyse any bacteria, making them more effective than antibiotics, which do not have a broad range against all bacterial species.

This adaptability increases their ability to treat a wide spectrum of bacterial illnesses(Principi et al., 2019).

- **Specificity of Action:** BPs are highly specific, preferentially targeting and destroying just the recognised pathogen while excluding non-pathogenic bacteria. This precision reduces collateral damage to the host microbiome(Domingo-Calap & Delgado-Martínez, 2018).
- **Narrow Spectrum:** Unlike antibiotics, BPs have a narrow range of activity, preventing microbiome disturbances and associated concerns such as secondary pathogen overgrowth and the emergence of resistance bacteria. This tailored method minimizes the ecological impact of helpful bacteria(Domingo-Calap & Delgado-Martínez, 2018).
- **Safety and Tolerance:** BPs are regarded as safer and more tolerable than antibiotics since they multiply exclusively in target bacteria and do not infect mammalian cells. Various investigations have shown the absence of severe adverse effects with BP treatment, emphasizing the safety profile(Kakasis & Panitsa, 2019).
- **Localized Effect:** The impact of BPs is restricted to the site of infection, making them effective even in bodily organs or systems where antimicrobials are difficult to penetrate. This tailored action increases medicinal efficacy while reducing systemic side effects(Principi et al., 2019).
- **Biofilm Disruption:** Engineered BPs can dissolve biofilms, hence improving the eradication of illnesses that are difficult to treat with conventional antibiotic therapy. This dual-action technique targets both bacterial cells and the protective biofilm matrix(Lu & Collins, 2007).
- **Potential Cost Savings:** BPs may be cost-effective, particularly in the treatment of multidrug-resistant infections, helping to cut healthcare expenditures. This economic advantage makes BPs an attractive option for resource-efficient healthcare plans(Principi et al., 2019).

2.1.10 Limitations Associated with Phage Therapy

There is limited and often inconsistent data on the use of Bacteriophages (BPs) to treat bacterial infections in humans. Trials are frequently non-randomized with no placebo controls. The difficulty of preparing BPs for clinical application persist, and not all questions concerning BP biology have been answered(Principi et al., 2019).

- **Narrow Cleavage Spectrum:** Bacteriophages have a narrow spectrum, functioning only on specific genera or species of bacteria, making it difficult to target all pathogenic strains of a single bacterial species (Hyman & Abedon, 2010). Specific bacteriophages may struggle to produce the intended therapeutic effects in complicated, multi-bacterial illnesses(Gill & Hyman, 2010).
- **The lysogenic phenomenon and the transmission of toxins:** Lysogenic phages can integrate with host bacteria without lysing them, limiting the lytic action of other phages and perhaps spreading toxins and antibiotic resistance genes (Carascal et al., 2022). Bacteriophages in the lysogenic condition can help disseminate poisons and antibiotic-resistant genes.
- **Complex Composition and Quality Evaluation:** Unlike protein medications, evaluating the quality and curative effects of phage treatment preparations is difficult due to their complex mix of proteins and nucleic acids (Tsonos et al., 2014).
- **Lack of Relevant Policies:** The lack of clear policies and regulations for clinical phage treatment applications creates obstacles and highlights the importance of a specific regulatory framework (Fauconnier, 2019). Standardization challenges in phage isolation and purification contribute to variations in the efficacy of isolated phage preparations.
- **Bacterial resistance to bacteriophages:** Prolonged usage of a single phage may result in the evolution of phage-resistant bacterial strains via natural selection mechanisms. Bacteria use a variety of anti-bacteriophage methods, including adsorption inhibition, CRISPR-Cas systems, and other mechanisms (Hyman & Abedon, 2010).
- **Absence of Phage Pharmacokinetic Data:** Clinical applications are hampered by the difficulty of standardizing phage treatment preparations, ambiguous dosage definitions, and defining administration strategies. Limited pharmacokinetic data make it difficult to

determine effective dosages and comprehend the destiny of bacteriophages in the human or animal body(Lin et al., 2022).

- **Interaction With the Body:** Bacteriophages producing bacterial toxins during lysis can exacerbate bacterial infections and, in some situations, cause septic illnesses. Despite the fact that phages are generally benign, the possibility of immunological reactions in response to foreign proteins carried by them raises concerns (Górski et al., 2007).

2.2 Klebsiella pneumoniae

Carl Friedlander described *Klebsiella pneumoniae* for the first time in 1882. It is a gram-negative, encased, non-motile bacterium that belongs to the Enterobacteriaceae family. *Klebsiella pneumoniae* is a member of the ESKAPE pathogens (Enterococcus faecium, Staphylococcus aureus, *Klebsiella pneumoniae*, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species), which are the most common cause of nosocomial infections worldwide. The majority of them are multidrug resistant isolates, which pose one of the most difficult issues in clinical practice (Santajit, 2016). *Klebsiella pneumoniae*, an important opportunistic pathogen in the Enterobacteriaceae family, causes a wide range of serious illnesses, with drug resistance on the rise.

According to Shiri et al., it contributes to one-third of Gram-negative infections, resulting in high mortality, protracted hospitalization, and significant economic costs. The rise of multidrug-resistant bacteria heightens the importance of comprehending and tackling this clinical dilemma.Treatment of K. pneumoniae infections is difficult due to -lactamase resistance, which reduces beta-lactam efficacy. Carbapenems and fluoroquinolones, which are commonly used to treat ESBL-producing strains, are being challenged by carbapenem-resistant K. pneumoniae (CR-Kp), raising global illness risk.

Furthermore, MDR-Kp strains may emerge after ESBL-producing and CR-Kp pathogens establish resistance to second-line antibiotics(Yang., 2023). Antibiotic resistance is acquired by K. pneumoniae via factors such as plasmids and mutations in different proteins. The growth of biofilms increases resistance. Combination therapy is effective against resistant bacteria,

highlighting the critical importance of administering antibiotics in hospitals in preventing resistance emergence and transmission (Karami., 2023).

2.2.1 Clinical significance of Klebsiella pneumoniae

When compared to classical *Klebsiella pneumoniae* (cKp), hypervirulent *Klebsiella pneumoniae* (hvKp) is a more virulent pathotype. hvKp infects mostly healthy people in the population and has a global presence, with a larger incidence in the Asian Pacific Rim. It is prone to multi-site infections and metastatic spread, requiring source control.

Notably, hvKp has a greater proclivity to produce central nervous system infections and endophthalmitis, necessitating prompt diagnosis and site-specific treatment. Genetic components discovered on a virulence plasmid, maybe with integrated conjugal elements, produce the hypervirulent phenotype. Increased capsule and aerobactin synthesis are important virulence factors. However, hvKp strains, like cKp, are gradually acquiring antimicrobial resistance via mobile elements, which might lead to nosocomial infections when highly drug-resistant cKp strains integrate hvKp-specific virulence factors (Russo, 2019).

Table 2.2: Demographic and clinical factors that can help differentiate infection caused by hypervirulent and classical K. pneumoniae strains.

Parameter	Searching for pathotypes	
	hvKp	**cKp**
Location of infection development	Typically, the community	A health care setting is more typically used
Host	All ages; frequently otherwise healthy	Older, but with some kind of compromise
Ethnic origins	Frequently Asian, Pacific Islander, or Hispanic	There is no ethnic preference
Hepatic abscess	Usually occurs when there is no biliary illness	This is more common in the presence of biliary illness
The number of infection sites	Frequently multiple	Typically single
Unusual K. pneumoniae infectious syndromes.	Endophthalmitis, meningitis, brain abscess, necrotizing fasciitis, splenic abscess, and spinal abscess are all possibilities.	None
Copathogens at the infection site	Monomicrobial organisms are uncommon	It is not rare, particularly in cases of abdominal, soft tissue, or urinary catheter infection

K. pneumoniae colonizes the colons of 5-35% of healthy people in Western countries and the nasopharynx of 1-5% in Asian countries, with rates ranging from 18.8% to 87.7%. Nasopharyngeal colonization rises with age and is influenced by environmental factors such as sanitation, food contamination, age, smoking, alcohol consumption, and rural life. The prevalence of K. pneumoniae contamination in Malaysian street food is 32%. Notably, it was the most prevalent agent in community-acquired pneumonia in Indonesia, highlighting the probable relationship between colonization and infection prevalence (Farida et al., 2020).

2.2.2 Virulence Determinants in Klebsiella pneumoniae

Capsule production: *Klebsiella pneumoniae* capsule is an important virulence component that protects against the host immune system. The Wzx/Wzy machinery (polysaccharide polymerization) produces over 80 different capsule types, which impedes phagocytic clearance, conceals cell architecture, and increases bacterial survival. Despite its relevance, the capsule's precise role in K. pneumoniae pathogenesis is unknown. The capsule, which has been identified as an appealing vaccine antigen and possible therapeutic target, is critical in countering invasive Klebsiella infections (Kobayashi et al., 2018).

The liver is a significant organ for capturing microorganisms in the bloodstream. Kupffer cells (KCs), liver-resident macrophages in sinusoids, account for over 90% of all resident macrophages in the body. KCs are critical in hepatic anti-infection capabilities because they use receptor-mediated pathogen recognition pathways to capture blood-borne germs. CRIg, a KC-specific complement receptor, is required for complement-mediated bacterial clearance, and Von Willebrand factor(vWF) recruits platelets to encapsulate bacteria attached to KCs. Although KCs have been shown to be indispensable in the management of *Klebsiella pneumoniae* infection, the particular interaction between KCs and the *K. pneumoniae* capsule is unknown (Wanford et al., 2021).

Aerobactin production: *Klebsiella pneumoniae* virulence factors include the capsule, lipopolysaccharide, fimbriae, and siderophores. Aerobactin, enterobactin, salmonocin, and yersinomycin are siderophores that aid in the acquisition of iron, the promotion of bacterial

growth and metabolism, and the exacerbation of infections. Aerobactin, the most common siderophore, is connected to invasive infections and accounts for more than 90% of active siderophores. Aerobactin is encoded by the gene iucABCDiutA, with iucB being the most important component.(Russo et al., 2014) Aerobactin has been shown in studies to enhance iron uptake from host tissue cells, even in iron-depleted situations, making it a key and crucial virulence factor in *K. pneumoniae* siderophores (Li et al., 2019). Aerobactin plays a significant impact in increasing *K. pneumoniae* pathogenicity both in vitro and in vivo, according to experimental findings (Russo et al., 2015).

Biofilm formation: *Klebsiella pneumoniae* forms biofilms on abiotic surfaces such as medical devices and catheters, as well as host tissues such as the respiratory, urinary, and gastrointestinal tracts. Biofilm production is influenced by a variety of variables, including the polysaccharide capsule, fimbriae, and pili. Iron metabolism and interactions with several bacterial species also contribute to K. pneumoniae biofilm development.

Klebsiella pneumoniae's polysaccharide capsule protects the bacterium by blocking complement deposition and preventing bacterial opsonization and phagocytosis. Beyond their direct role in biofilm production, *K. pneumoniae* capsular polysaccharides have anti-biofilm characteristics against other bacteria, offering a competitive advantage in mixed bacterial environments (Goncalves et al., 2014).

Lipopolysaccharide (LPS): LPS, a critical component of the outer membrane in *Klebsiella pneumoniae*, leads to biofilm formation. Balestrino et al. found that LPS is important in the initial attachment of *K. pneumoniae* to abiotic surfaces, impacting the early phases of biofilm development. Mutant strains lacking LPS production or transport genes have delayed biofilm formation, implying that LPS charge is essential in the proper folding of Type 1 pili, as seen in their tests. Vuotto et al.'s recent findings emphasize the overexpression of LPS-associated genes (wbbM and wzm) in biofilm-grown *K. pneumoniae*, emphasizing their function in biofilm generation(Guerra et al., 2022).

K. pneumoniae expresses two types of fimbriae, type I and type III, which act as adhesins to both biological and abiotic surfaces. There are at least ten gene clusters in the genome that encode chaperones, ushers, and adhesin proteins for fimbria assembly, including the fim, mrk, ecp, and

kpa to kpg gene clusters. Notably, fim, mrk, ecp, and kpf, which encode type I and type III fimbriae, common pilus, and type I-like fimbriae, respectively, are experimentally well-characterized. These fimbriae are critical in increasing surface binding, aiding tissue invasion, and contributing to biofilm formation, especially on medical devices (Paczosa & Mecsas, 2016).

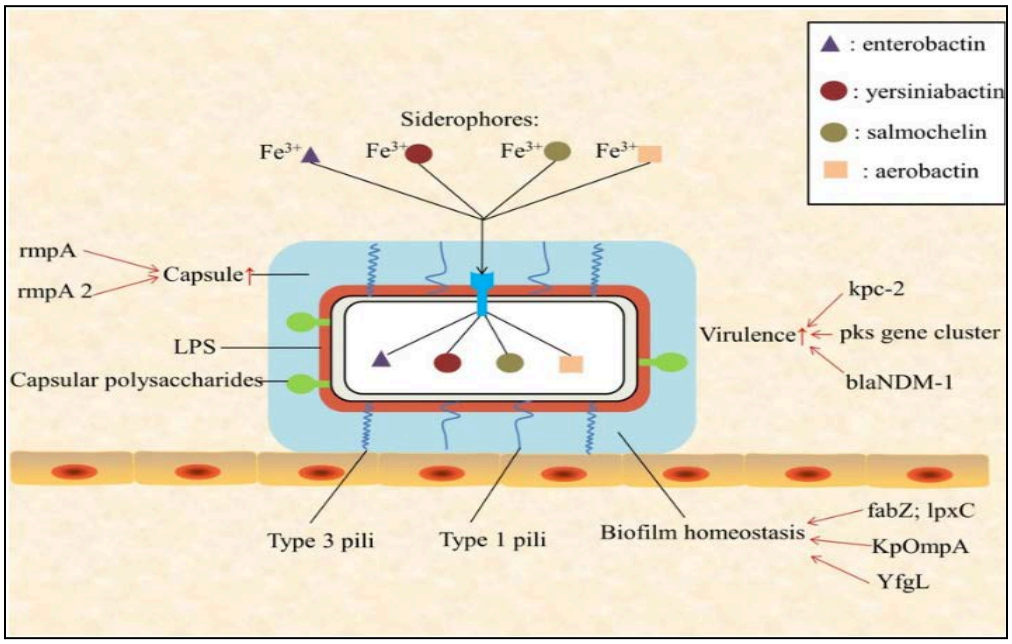

Figure 2.3: Schematic presentation of *Klebsiella pneumoniae* virulence factors, and biofilm homeostasis. *(Adapted from Xhao et.al 2020).*

2.2.3 Widespread Dissemination of Antibiotic-Resistant Klebsiella pneumoniae

Sharma et al. investigated 82 instances of *Klebsiella pneumonia*, including 40 isolates from January to June 2018 and 42 from January to June 2022. Antimicrobial resistance was substantial, particularly in the 2018 group, with significant resistance to amoxicillin and decreased sensitivity to meropenem and colistin. Tigecycline demonstrated consistent sensitivity. Overall, resistant *Klebsiella pneumonia* increased from 7.5% in 2018 to 21.4% in 2022, whereas

XDR-Klebsiella pneumonia rose from 62.5% to 71%. MDR-*Klebsiella pneumonia* decreased non-significantly from 17.5% in 2018 to 7% in 2022(Sharma et al., 2023).

Lin et al. investigated variations in *Klebsiella pneumoniae (K. pneumoniae)* detection rates among hospital wards between 2008 and 2020. Increases were seen in the ICU and AIDS ward, particularly between 2015 and 2017. Sputum and urine were the most common sources of isolation (53.77% and 14.70%, respectively). Resistance to six antibiotics, most notably imipenem and ciprofloxacin, has grown over time. Cefazolin consistently demonstrated the strongest resistance across many specimens. Carbapenem-resistant *K. pneumoniae* (CRKP) was more commonly detected in older patients, with substantial differences observed across specimen types and hospital wards, particularly in the ICU(Z. Lin et al., 2022, pp. 2006–2020).

Between June 1, 2017, and January 31, 2018, Pham et al. conducted a prospective observational cohort study with 74 ICU-admitted patients, resulting in 69 individuals and 34 environmental samples. The sequencing of 357 *Klebsiella pneumoniae* isolates revealed 95 antimicrobial resistance profiles, with 64% of them containing multiple ESBL and carbapenemase genes. The most common sequence types were ST15 (37%), and ST16 (19%). Despite slight changes in resistance patterns, no substantial hospital-associated signals were detected in patient transmission. ST15 demonstrated chronic carriage, within-hospital transmission, and multihospital or community dissemination. Notably, the carbapenem resistance gene blaKPC-2 was found globally within ST15, with a separate clade in Hanoi restricted to the 2017/18 isolates. The plasmid pMHP-KPC2, which carries blaKPC-2, was found in many isolates, emphasizing its significance in carbapenem resistance (Pham et al., 2023).

Between June 1, 2017, and January 31, 2018, Hartantyo et al. discovered that 21% of raw and ready-to-eat (RTE) food samples in Singapore tested positive for *Klebsiella pneumoniae*, with raw food items testing higher (45%) than RTE samples (15%). *K. pneumoniae* was identified in 58% of raw veggies, as well as 27% of raw chicken and pork liver. Of the foodborne isolates, 8% were potentially pathogenic and carried virulence factors, while 10% were multidrug-resistant. Antibiotic resistance rates were observed, with ampicillin showing the highest resistance rate at 98%. The investigation identified various antimicrobial resistance phenotypes and related resistance patterns in foodborne *K. pneumoniae* isolates(Hartantyo et al., 2020).

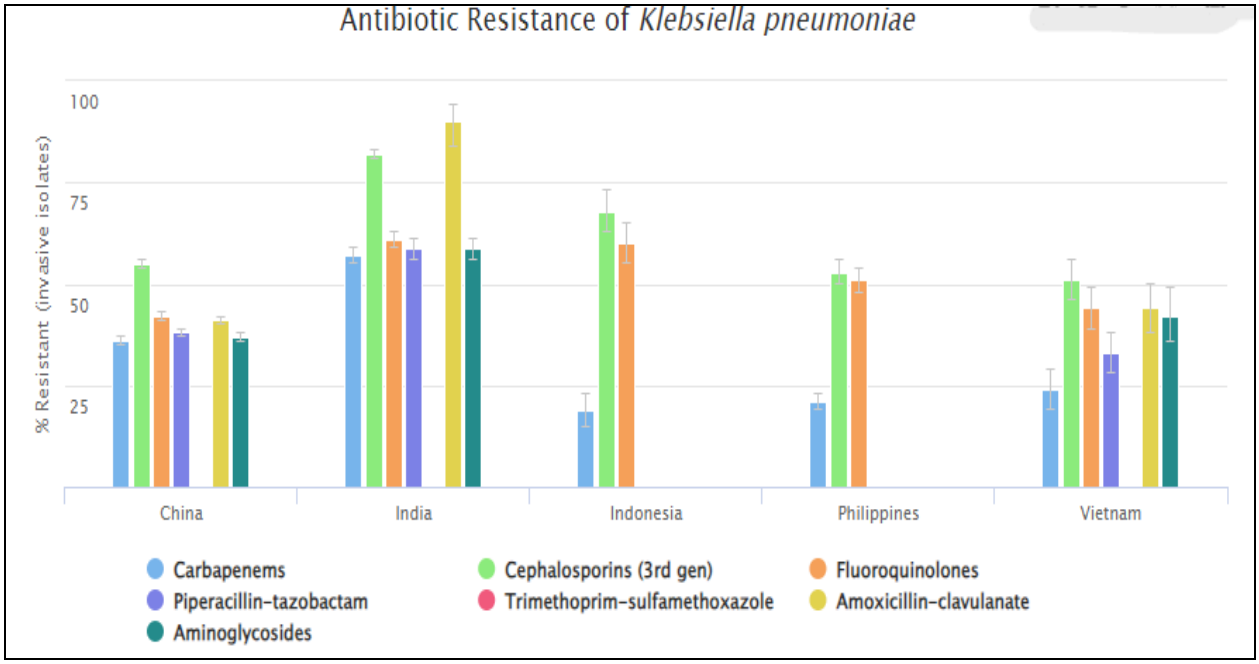

Figure 2.4: Graphical representation of antibiotic resistant Klebsiella pneumoniae in Asian countries. (Data used and created from Center for Diseases Dynamics, Economics & Policy, CDDEP. ***Retreived from*:** *https://resistancemap.onehealthtrust.org/AntibioticResistance.php*)

2.2.4 Bacteriophage Intervention for Klebsiella pneumoniae infection

A substantial link was found between the presence of *Klebsiella pneumoniae* (Kp) and Enterococcus gallinarum (Eg) and the progression of Primary Sclerosing Cholangitis (PSC) in a study performed by Ichikawa et al.(2023) The study found that these bacteria are linked to PSC regardless of the existence of colonic inflammation. Notably, combining Kp and Eg was connected to higher serum alkaline phosphatase (ALP) levels and worse clinical outcomes. The researchers proved the efficiency of a bacteriophage cocktail particularly tailored to target Kp in a mouse model of hepatobiliary injury in the study, which proposes Kp as a possible therapeutic target. Phage treatment, which is thought to be a safer and more precise alternative to antibiotics, demonstrated promise in lowering Kp levels and minimizing hepatobiliary damage. The findings point to certain microorganisms, such as Kp and Eg, as possible indicators and therapeutic targets in PSC. However, the researchers emphasize the importance of additional large-scale,

long-term investigations to corroborate these findings. By detecting particular bacterial associations and providing focused therapy approaches, this study provides new insights into understanding and perhaps treating PSC (Ichikawa et al., 2023).

The study, led by Gan et al.(2022), looks at the efficacy of phage therapy against pneumonia caused by multidrug-resistant *Klebsiella pneumoniae* (MDR K. pneumoniae) strains, notably ST11 and ST383. Through screening, the researchers discovered two lytic phages, pKp11 and pKp383, which showed high activity against a wide spectrum of MDR K. pneumoniae isolates. These phages destroyed bacterial cells efficiently in vitro and remained stable at human-body temperatures, recognising lipopolysaccharide (LPS) as receptors with specificity to ST11 and ST383 host strains. Both phages rapidly reduced bacterial loads, cytokine concentrations, and lung tissue damage in an experimental mouse model, showing their efficacy in pneumonia treatment. Phage treatment, which targets proinflammatory cytokines such as IL-1, IL-6, and TNF-, has the ability to modulate inflammatory responses. The study also discovered no rise in proinflammatory cytokines after phage therapy, verifying its safety. Phage cocktails outperformed single-phage solutions in terms of efficacy, implying that they may have broader applications. While the results show that phage therapy is effective and safe against ST11 and ST383 infections, the researchers emphasize the need for additional research to better understand phage-bacterium interactions, the evolution of phage resistance in vivo, and the translation of these findings into human therapeutics (Gan et al., 2022).

In a study led by Anand et al.(2020), the temperature-tolerant phage VTCCBPA43 displayed optimal activity at pH 5 with a burst size of 172 PFU/mL and a restricted host range similar to KP36. Shotgun proteomics found its features. A single intranasal dose of VTCCBPA43 (2109 PFU/mouse) resulted in the presence of active phage in vivo in a murine model challenged with virulent *K. pneumoniae*. Notably, there was a significant reduction in lung bacterial load across all time points, which was associated by a decrease in lesion severity, demonstrating that VTCCBPA43 phage therapy had positive consequences in the pneumonic mouse model (Anand et al., 2020).

According to Taha et al. (2018) a single application of phage ZCKP1 to formed biofilms of K. pneumoniae KP/01 isolated from diabetic foot patients resulted in a considerable reduction in

crystal violet stainable biofilm content and the proportion of live cells seen by MTT staining after 4 h. The most successful treatment had the greatest MOI (50 PFU/CFU). Despite this initial disturbance, there was a subsequent recovery in biofilm estimations as well as a resurgence in cell viability. Notably, several treatments of phage ZCKP1 at 4 h intervals on formed K. pneumoniae KP/01 biofilms resulted in sustained decreases in biofilm content and inhibited cell viability recovery throughout the 24 h trial.

Phage therapy resulted in the eradication of local symptoms and signs of infection, as well as functional recovery. In a case study by Cano et al. (2020) involving a 62-year-old patient with prosthetic joint infection (PJI). The patient was still getting minocycline 34 weeks after finishing treatment and remained asymptomatic. There was a tendency in biofilm biomass reduction 22 hours following exposure to KpJH462 (P =.063). Overall, the addition of phage therapy yielded a satisfactory result in the treatment of intractable biofilm-associated prosthetic knee infection. The study reveals that phage therapy has the potential to cure device-associated infections, but more research is needed to determine its efficacy and safety(Cano et al., 2021).

Rahimi et al. (2023) investigated the phage PSKP16, a member of the Drexlerviridae family with a genomic size of 46,712 bp and 67 predicted ORFs. The researchers investigated the effectiveness of rapid phage treatment in reducing bacterial load and boosting survival rates in a BhvKp-induced pneumonia model. The results demonstrated that rapid phage therapy achieved these results faster than a synergistic model with delayed treatment. BhvKp strain distribution in the lung linked with histopathological findings and serum tumor necrosis factor- (TNF-) levels. The use of phages reduced severe lesions, alveolar edema, and inflammatory cell infiltration. In comparison to gentamicin, phage treatment did not cause over-inflammation and resulted in a faster repair of blood cell count abnormalities. Furthermore, the phage effectively removed biofilms in vitro. Overall, the study demonstrates that PSKP16 phage therapy has the ability to address bacterial load, survival rates, and biofilm clearance in pneumonia models.(Rahimi et al., 2023)

2.3 Salmonella paratyphi

Salmonella paratyphi is a bacterial strain that has become a significant public health concern. Discovered in 1880 by Karl Eberth, it is a gram-negative, mobile bacterium that belongs to the Enterobacteriaceae family. Along with *Salmonella Typhi*, it is a leading cause of enteric fever, also known as paratyphoid fever, which can cause a range of discomforting symptoms, including fever, abdominal discomfort, and gastrointestinal issues.

Salmonella paratyphi spreads through contaminated food or water, and it is prevalent in regions where sanitation and hygiene practices are inadequate. Unfortunately, the emergence of multidrug-resistant strains of *Salmonella paratyphi* has made treatment challenging, similar to its counterpart, *Salmonella typhi*. The increasing incidence of antimicrobial resistance among *Salmonella paratyphi* isolates highlights the importance of comprehending its clinical significance and virulence mechanisms.

2.3.1 Clinical significance of Salmonella paratyphi

Salmonella paratyphi is one of the major causative agent of paratyphoid fever, which is a serious global health issue. This illness is characterized by fever, abdominal discomfort, headache, and gastrointestinal symptoms. Unlike *Salmonella typhi*, which causes a more severe form of typhoid fever, Paratyphi infections are typically milder but still pose a significant burden on public health systems, particularly in endemic regions. Salmonella typhi and Salmonella Paratyphi A are pathogens that are limited to humans as their reservoir.

Table 2.3: Demographic and clinical factors distinguishing infections caused by *Salmonella paratyphi* and *Salmonella typh*i adapted from data presented by Crump and Mintz (2010)

Parameter	*Salmonella paratyphi*	*Salmonella typhi*
Location of infection development	Community-acquired infections	More commonly associated with travel to endemic regions
Host	All age groups; otherwise healthy individuals	All age groups; higher incidence in younger populations
Ethnic origins	Varied; not specific to any ethnicity	Varied; predominant in regions with poor sanitation
Hepatic abscess	Less commonly associated with biliary illness	Often associated with biliary illness
Number of infection sites	Typically single	Typically single or multiple
Unusual infectious syndromes	Less commonly associated with severe extraintestinal manifestations	Can lead to severe extraintestinal complications such as peritonitis and intestinal perforation

Unlike other Salmonella strains that cause inflammation and diarrhea in the intestines, *Salmonella typhi* and *Salmonella paratyphi* A, B, and C enter the bloodstream from the gastrointestinal tract, persist in macrophages, and may lead to long-term presence in 1-4% of cases. These types of *Salmonella* cause more than 135,000 deaths annually. Typhoid fever is more common in densely populated metropolitan areas where human fecal matter is present in the drinking water supply. The increasing prevalence of antibiotic-resistant strains is a potential threat to the future, as it may lead to more severe illness and higher case fatality ratios. Studies have shown a stronger link between chronic carriage and gallbladder cancer over the past decade.

2.3.2 Virulence Determinants in Salmonella paratyphi

Salmonella paratyphi has several virulence factors that help it evade host immune responses. The factors are as follows:

- **Capsule Production:**

  Salmonella paratyphi produces a polysaccharide capsule that protects the bacterium from being engulfed by phagocytes and enhances its survival within the host. The capsule is a major antigenic determinant and is essential for systemic dissemination.

- **Type III Secretion System (T3SS):**

  Salmonella paratyphi uses a T3SS to inject effector proteins into host cells. This facilitates invasion and intracellular survival, allowing the bacterium to manipulate host cell processes and evade immune detection.

- **Adhesion and Invasion Factors:**

  Salmonella paratyphi expresses adhesins and invasins that promote attachment to intestinal epithelial cells, followed by invasion. These factors include fimbriae, type 1 pili, and outer membrane proteins involved in host cell recognition.

- **Resistance Mechanisms:**

  Antimicrobial resistance in *Salmonella paratyphi* is mediated by various mechanisms, including acquiring resistance genes via mobile genetic elements such as plasmids and integrons. The emergence of multidrug-resistant strains limits treatment options and underscores the need for alternative therapeutic approaches.

2.3.3 Global Dissemination of Antibiotic-Resistant *S. paratyphi*

According to a study conducted by Biwas et al.(2022), multidrug-resistant *Salmonella* spp., particularly *Typhi*, are a developing issue in South-East Asia, as they are resistant to traditional anti-typhoidal medications such as ampicillin, cotrimoxazole, and chloramphenicol. Because of this resistance, fluoroquinolones have become the primary therapeutic option. However, in recent years, there has been a significant increase in fluoroquinolone resistance, with 82.2% of *Salmonella* isolates demonstrating resistance in the current study. This shift in resistance patterns is consistent with reports of growing fluoroquinolone resistance across the Indian subcontinent. Indiscriminate antibiotic use and the re-emergence of chloramphenicol susceptibility are two factors contributing to resistance (Chitnis et al., 1999). Understanding the molecular mechanisms of resistance is critical to future study. Alternative medicines such as cephalosporins and azithromycin, as well as immunization programmes, have shown potential in combating antimicrobial resistance and typhoid fever. The rise of extensively drug-resistant (XDR) *Salmonella Typhi*, particularly in neighboring countries such as Pakistan, presents a considerable concern (Klemm et al., 2018). Resistance to third-generation cephalosporins remains low in India, but ongoing monitoring and surveillance are critical to preventing future rise. Furthermore, a single isolate of carbapenem-resistant *Salmonella paratyphi* A highlight the importance of continuing surveillance and research into new treatment options (Biswas et al., 2022).

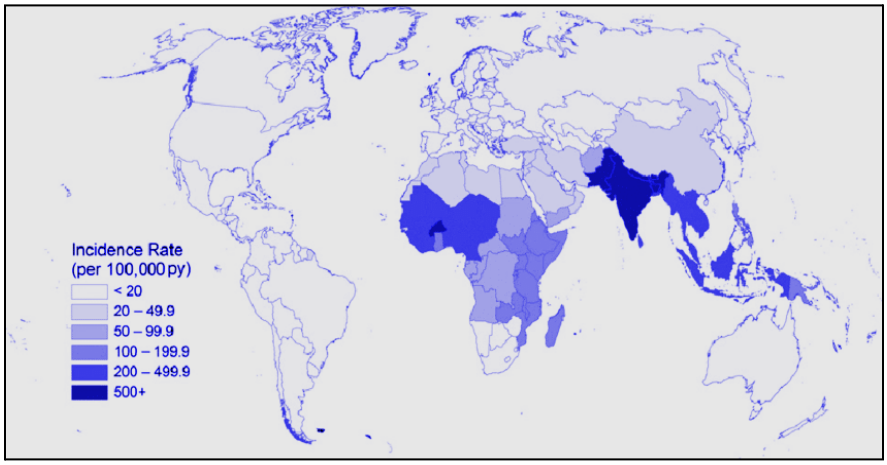

Figure 2.5: Schematic representation of Estimated occurrence of typhoid and paratyphoid fevers per 100,000 population by country in 2015. *Adapted from a study by Radhakrishnan et al. (2018).*

Browne and colleagues gathered a large dataset from 601 sources and analyzed 184,225 *Salmonella Typhi* and *Salmonella paratyphi A* isolates from 45 countries during a 30-year period. This thorough dataset yielded 3008 location-years of data for six pathogen-drug combos. The data distribution reflected the burden of enteric fever, with the majority coming from South Asia, followed by other regions. MDR *Salmonella Typhi* prevalence varies across time and space, dropping in South Asia while increasing in Sub-Saharan Africa. FQNS *Salmonella Typhi* developed and spread rapidly across all regions, particularly in Pakistan, Bangladesh, and India. MDR *Salmonella paratyphi.* The incidence remained modest, but FQNS varied greatly by country, with high rates in Pakistan and Nepal and lower levels in Papua New Guinea. Except for Pakistan, there was little third-generation cephalosporin resistance. The findings of Browne and colleagues' study show the complicated dynamics of antibiotic resistance in *Salmonella Typhi* and *Paratyphi A*, emphasizing the importance of targeted therapies and continued surveillance(Browne et al., 2024).

Pokharel and colleagues conducted a study that found rising antibiotic resistance in *S. enterica*, which is especially alarming in underdeveloped nations where typhoid and paratyphoid fevers are prevalent. They discovered a higher prevalence of serotype *Paratyphi A* than *Typhi*, as well as increased rates of multidrug resistance and ESBL synthesis in *Paratyphi A*. Chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole, which were once the major treatments for enteric fever, demonstrated decreased efficacy, reflecting global patterns of resistance. While third-generation cephalosporins and carbapenems remained effective against MDR S. enterica, fluoroquinolones had low performance, possibly due to their extensive usage and overuse Furthermore, the study discovered ESBL production by *S. enterica* in Nepal for the first time. Alternative antibiotic testing yielded good findings with gatifloxacin and ertapenem, indicating potential replacements for tackling resistance(Gales et al., 2002). The study emphasizes the importance of ongoing antibiotic resistance surveillance, routine ESBL screening, and investigation into the efficacy of novel antibiotics against *S. enterica* (Pokharel et al., 2006).

2.3.4 Phage Therapy for Treating S. paratyphi Infections

Paratyphoid fever, caused by *Salmonella paratyphi*, poses a substantial health risk in countries where it is prevalent. The growing antimicrobial resistance poses significant obstacles to conventional antibiotic therapies. Phage treatment presents an alternate approach by selectively targeting particular bacterial strains while minimizing damage to the surrounding commensal flora.

Combating *Salmonella paratyphi* infections through Phage therapy is difficult due to fewer infections and limited phage options compared to *Klebsiella pneumoniae* infections. Nonetheless, bacteriophages are a viable answer to *Salmonella* contamination in the poultry sector. They serve two purposes: reducing economic losses by battling bacterial infections in animals and improving food safety for people. Phage selection, concentration, application method, and treatment time all have an impact on their efficacy (Wernicki et al., 2017). Phage mixtures can be given orally, sprayed on, or added directly to items to target Salmonella transmission horizontally and vertically. Notable phages such as P22 and Felix-O1 exhibit extensive activity against *Salmonella* strains (Carvalho et al., 2017). While preliminary research shows promise, large-scale trials are required for safety evaluation. One such research, including more than 34,000 broiler chickens, proved the safety and efficacy of phage treatment in a commercial setting, highlighting its potential for wider application (Clavijo et al., 2022).

Phage therapy for *Salmonella paratyphi* in humans remains limited due to several factors. Primarily, there has been relatively less research conducted on phage therapy specifically targeting *Salmonella paratyphi* compared to Salmonella Typhi. The specificity of phages poses a challenge, as finding phages that effectively target Paratyphi strains may be difficult due to strain diversity and varying susceptibility. Additionally, developing phage therapy for human use necessitates rigorous clinical trials to demonstrate safety and efficacy, which may not have been extensively pursued for Paratyphi. Antibiotic treatment availability further diminishes the urgency for alternative treatments like phage therapy. While phage therapy holds promise as a potential treatment for bacterial infections, including those caused by Salmonella, its development and adoption for specific pathogens like *Salmonella paratyph*i may lag behind due to research priorities, regulatory hurdles, and existing treatment modalities.

Chapter 03

Methodology

3.1 Location of Research:

Research was carried out in the Biotechnology and Microbiology lab of BRAC University's department of Mathematics and Natural Science in Dhaka, Bangladesh.

3.2 Adherence to Standard Laboratory Practices

The laboratory glassware, such as test tubes, conical flasks, and beakers, was washed first with tap water and then with distilled water. Before use, the culture media, which includes agar-based and broth solutions, as well as pipette tips, centrifuge tubes, and empty test tubes for the double-layer agar method, were autoclaved at 121 °C and 15 psi for 15 minutes. The aseptic conditions at 4 °C were maintained for the autoclaved equipment and stored culture media. During experimental lab safety protocol was maintained following by using a clean lab coat and gloves were worn, and the experiments were conducted within vertical laminar flow cabinets that had been pre-cleaned with 70% ethanol to prevent contamination.

3.3 Preparation of Culture Media, Reagents, and Solutions

Luria-Bertani Broth (LB):

In our project, we primarily used Luria-Bertani broth (LB) to enhance the bacteriophages and create a young culture of bacteria for the Spot test & DLA test. The ingredients of LB broth are a combination of yeast extract, sodium chloride, and tryptone. Ten grams of tryptone, five grams of yeast extract, and ten grams of NaCl were mixed with one liter of distilled water to make this broth. To guarantee optimal mixing, the mixture was heated to 100 °C. It was then autoclaved for 25 minutes at 120 °C. If a product is available for immediate use that has been pre-mixed with powder, please refer to the product instructions to find out how much powder is needed to make one liter of Luria-Bertani broth.

Luria-Bertani Agar (LA) / Top Agar:

Every testing technique we utilized Luria-Bertani agar as the medium. This agar medium, called **LB**, has an additional agar mixture in addition to the same ingredients as Luria-Bertani broth in addition to Agar as a solidifying medium. This medium includes 10 grams of tryptone, 5 grams of yeast extract, 10 grams of NaCl, and 15 grams of agar were combined into one liter of Luria-Bertani agar. The resultant mixture was autoclaved at 120 °C for 25 minutes. The precise amount of powder needed to make one liter of Luria-Bertani agar can be found in the package instructions, if there is a commercially accessible pre-mixed powder product.

Preparation of 0.6 % top LB Agar:

In order to make the top layer or top agar, a different batch of 0.6% LB agar powder was mixed in order to perform the double-layer agar experiment. Initially, 500 milliliters of distilled water were mixed with 13 grams of measured nutritional broth powder. Then, to make the final volume of 1000 ml, 6 grams of agar powder were added, along with more deionized water. Using a Bunsen burner, the mixture was heated until it was clear and homogenous, and began to boil. After that, the solution of the Agar mixture was placed at 121 °C for 15 minutes. Following autoclaving, the Falcon tubes holding the agar media were kept in storage at 4 °C for further use.

Normal Saline:

The mixture consisted of sodium chloride (NaCl) salt and distilled water. 0.9g of NaCl was added to dissolve in 100ml of water for standard saline recipe and that was autoclaved for 25 minutes at 121 °C. In order to perform salinity experiments, saline solutions of 0.2%, 0.4%,0.9%, 1%, 1.2%, and 1.5% were made accordingly.

SM buffer:

In order to make SM Buffer solution Sodium chloride (NaCl), magnesium sulfate ($MgSO_4$), gelatin, and Tris-HCl make up the SM buffer, which is used for normal phage suspension storage , characterization. 15.76g of Tris-HCl, 0.20g of $MgSO_4$, 0.58g of NaCl, 0.1g of gelatin, and 100 ml of distilled water were combined and heated to 100 °C to ensure a thorough blending. After

that, any bacteria that might have been in the solution were removed from the medium by autoclaving it for 25 minutes at 121 °C**.**

3.4 Selection of Host Bacterium

The screening of the phages was conducted using 2 bacterial strains that are *Klebsiella pneumoniae* reference strain from **NIDCH** and *Salmonella paratyphi* reference clinical strain from **ICDDR,B.**

All the bacteria were cultured in LB medium by following laboratory standard procedure outlined at the BRAC UNIVERSITY laboratory.

3.5 Bacteriophage Isolation

3.5.1 Collection of Environmental Water Samples

Four water samples in total were collected from different parts of Dhaka city for this investigation in order to isolate *Klebsiella pneumoniae* (K1, K2 and K3) and *Salmonella paratyphi* for host specific Bacteriophage. sample K1 was collected from Turag River where the water was transparent and clear, sample K2 was isolated from Buri-ganga River mixed with soil and wastage, and K3 and S1 was isolated from Mirpur waste water which contain bad odor and the water color was black/dark in normal visualization.

3.5.2 Bacteriophage Enrichment and the Detection of Phages

- ***Isolation of Bacteriophage from environment water sample and enrichment:***

1. The water was collected and poured into a bottle for further process.

2. Water samples were centrifuged 3–4 times at 13500 rpm for around 10 mins and then filtered with paper.

3. Then the water samples were again filtered via 0.22- or 0.20-micron filter and stored into a falcon tube

4. For further processing and assuring the phage detection, one single colony of Pneumonia and S. Paratyphi was mixed with 8 ml of LB broth and put it into incubator for 1.5-2h to make young culture.

5. 2ml of that filtered liquid solution was added to the young culture and again incubated overnight in a shaker incubator overnight for phage enrichment.

6. Next day, the enriched solution was centrifuged at 13500 rpm for 10 to 15 min and filtered

using a 0.22- or 0.2-micron filter and kept in the fridge at 40 °C.

7. Initial Spot test done for assurance of bacteriophage from the enriched incubated solution.

8. Lysis diameter of plaques determined the presence of phage in the media.

3.5.3 Phage Purification Using Double-Layer Agar Assay

1. 100µL of freshly isolated phage solution was mixed to 900µL LB broth in an MCT and diluted as per needed.

2. 8 ml of liquid soft agar (6%), 100µL of bacterial young culture and 20µL of diluted phage was vortexed and poured into an LA plate then cooled for 30 mins.

3. The plates are incubated less than 16 hr and after that, results are observed.

4. Small lysis plaques obtained from the experiment were visualized.

3.5.4 Preparation of Phage Stocks via serial dilution and spot test /DLA

1.all the bacterial strains were mixed with LB broth for 2hr to make bacterial young culture.

2. 5ml of (6%) soft agar was kept at 50-to-55-degree Celsius temp and 100µL the young bacterial culture was taken into a test tube.

3. After that, the young bacterial cultures were mixed to molten soft agar were poured and dried onto an LA plate.

4. Following the cooling of LA plates, 5µL of freshly enriched phage solution was given as droplets and dried for 15–20 mins.

5. The plates were further incubated for 10-12h and the results were obtained.

6. After the incubation, the presence of plaque on the dropped zone of the plate, would be determined as the presence of phage via the Spot Test

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3.5.5 Determination of Phage Titer

1. After conducting the DLA experiment, the obtained number of plaques present on the plates were counted as per factor of the dilution.

2. The number of plaques formed in each dilution was recorded.

3. By using the below formula, phage titer or PFU/ml of a phage sample were calculated

The PFU formula:

The PFU count for each plate is $\frac{Plaque\ count}{Dilution\ Factor \times Volume\ Plated}$.

3.6 Phage Characterization

3.6.1 Assessment of Thermal Stability

The isolated bacteriophage's stability was assessed through temperature testing. Several sterile Eppendorf tubes were filled with 1ml of phage solution. The tubes were immersed in a water bath at temperatures of 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C for 3hr. Following the water heat treatment, the double layer assay method was used to determine the titer of bacteriophage.

3.6.2 Evaluation of pH Stability

This procedure involved the collection of Eight different pH values (pH 4, pH 6, pH 7, pH 8, pH 9, pH 10) values. To begin with, LB broth was created following the usual procedure. In addition to that, Glacial Acetic acid droplets were introduced to acidify the buffer, while NaOH was used to make it basic. Following the preparation of the eight buffer solutions, 200µL of buffer was combined with 100µL phage stock in an MCT for each pH. Additionally, the MCT was left to incubate for two hours. After that, each buffer-mixed phage was diluted to the desired concentration, and a DLA test was performed, and the obtained plaques were reviewed and the quantity of plaques in each pH buffer was counted.

3.6.3 Determination of Stability under Saline Conditions

Six distinct salinity levels of saline water were used in this experiment: 0.2%, 0.4%,0.9%, 1%, 1.2%, and 1.5%. First, distilled water was used to create saline solutions by replacing 0.9% NaCl with 0.2%, 0.4%,0.9%, 1%, 1.2%, and 1.5% NaCl. 200µL of the saline solution and 100µL of phage stock were mixed together in a Microfuge Tube (MCT) and incubated for two hours after each salinity was prepared. Following a suitable dilution of the phage combined with saline, a

DLA test was carried out for every salinity, documenting the quantity of plaques generated under each concentrated saline condition

3.6.4 Sensitivity Test to Organic Solvents :

The stability of bacteriophages in organic solvents was tested in chloroform and ethanol. A newly enriched phage suspension and an equal volume of organic solvent were mixed separately and incubated at room temperature for 1 hour with intermittent hand shaking. Following incubation, centrifugation was performed at 10000 rpm for 10 minutes to separate the mixtures, and the phage titer in the aqueous phase was determined using the double layer agar method. To determine the relative inactivation of phage by organic solvents, phage suspended in LB broth and held in 4ºC was used as the control for comparison.

3.6.5 Determination of Host Range:

Host range refers to the variety of host species that a pathogen can infect. Understanding a pathogen's host range is vital for studying its epidemiology and pathogenicity. It influences how pathogens are transmitted and survive and helps predict evolutionary processes. A pathogen's host range can reveal insights into its ability to diversify, incorporate new host species, switch between hosts in response to environmental changes, and persist in the environment between outbreaks.

To determine the host range, the method described by Verma et al. (2009) was followed. WA double-layer assay by mixing 6mL of 0.6% warm (45-50ºC) top agar with 150µl of each specific bacterial culture in separate sterile vials. This mixture was then poured onto fresh LA plates. Once the top layer of the agar solidified, we spotted 5µl of high titer phage stock dropwise, along with 5 µl of sterilized saline as a control. The plates were then incubated at 37ºC for 24 hours, and bacterial lysis was observed.

A total of 29 bacterial cultures (listed in Table 3.1) were used to assess the host range of the isolated bacteriophage, where 12 of those bacterial strains had clinical significance. The spot test results are also depicted within the table.

Table 3.1 : Determination of Host Range for bacteriophage sample of K1,K2,K2 & S against various bacterial cultures

Serial no.	Bacterial Strain	Spot Test Results				Source
		K1	***K2***	***K3***	***S***	
1.	*Klebsiella pneumoniae* *(Reference strain)*	+	+	+	-	NIDCH[(1)]
2.	*Salmonella paratyphi** (Reference strain)	-	-	-	+	ICDDR,B[(2)]
3.	*Klebsiella pneumoniae* Isolate 1	-	-	+	-	SHISHU[(3)]
4.	*Klebsiella pneumoniae* Isolate 6	+	-	+	-	SHISHU
5.	*Klebsiella pneumoniae* Isolate 11	+	-	+	-	SHISHU
6.	*Klebsiella pneumoniae* Isolate 22*	-	-	+	-	SHISHU
7.	*Klebsiella pneumoniae* Isolate 26*	+	-	-	-	SHISHU
8.	*Klebsiella pneumoniae* Isolate 28*	-	-	-	-	SHISHU
9.	*Klebsiella pneumoniae**	-	-	-	-	ICDDR,B
10.	*Salmonella paratyphi*	-	-	-	+	NIDCH

Serial no.	Bacterial Strain	Spot Test Results				Source
		K1	***K2***	***K3***	***S***	
12.	*Klebsiella spp*	+	-	+	-	ICDDR,B
13.	*Shigella flexneri*	-	-	-	-	ICDDR,B
14.	*Shigella dysenteriae**	-	-	-	-	ICDDR,B
15.	*Salmonella typhi**	-	-	-	+	ICDDR,B
16.	*Bacillus cereus*	-	-	-	-	ICDDR,B
17.	*E. coli* 0157:H7	-	-	-	-	ICDDR,B
18.	*Escherichia coli*/ATCC: 25922	-	-	-	-	SHISHU
19.	*Enterotoxigenic Escherichia coli**(ETEC)	-	-	-	-	ICDDR,B
20.	*Enteropathogenic Escherichia coli**(ETEC)	-	-	-	-	ICDDR,B
21.	*Streptococcus pneumoniae**	-	-	-	-	SHISHU
22.	*Pseudomonas aeruginosa**	-	-	+	-	ICDDR,B
23.	*Staphylococcus aureus*/ATCC:25923	-	-	-	-	SHISHU

Serial no.	Bacterial Strain	Spot Test Results				Source
		K1	***K2***	***K3***	***S***	
25.	*Proteus vulgaris*	-	-	-	-	BUL[(4)]
26.	*Hafnia alvei** (Reference Strain)	-	-	+	+	ICDDR,B
27.	*Vibrio cholerae**	-	-	-	-	ICDDR,B
28.	*Vibrio parahaemolyticus**	-	-	-	-	ICDDR,B
29.	*Kocuria rhizophila*/ATCC: 9341	-	-	-	-	SHISHU

(1) The National Institute of Chest Diseases and Hospital (NIDCH)

(2) The International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B)

(3) Dhaka Shishu Hospital (SHISHU)

(4) BRAC UNIVERSITY lab stock (BUL)

(+)Produced a distinct lytic zone

(–)Failed to generate a lytic zone

(*)Strains having clinical significance

Chapter 04

Results

Results

4.1 Isolation and Purification of the Isolated Bacteriophages

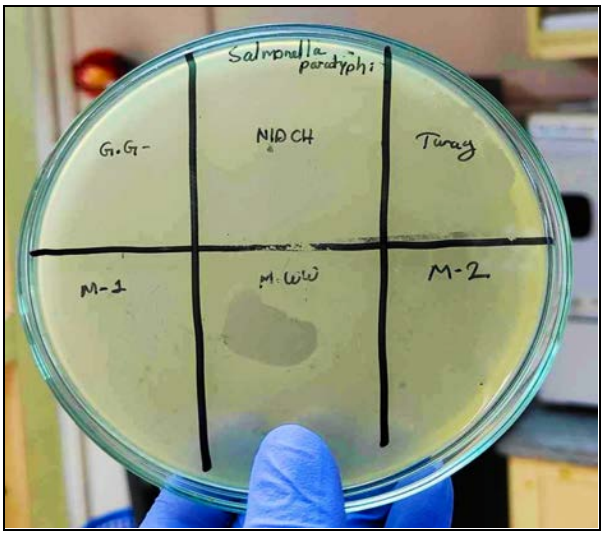
4.1.1 Spot test

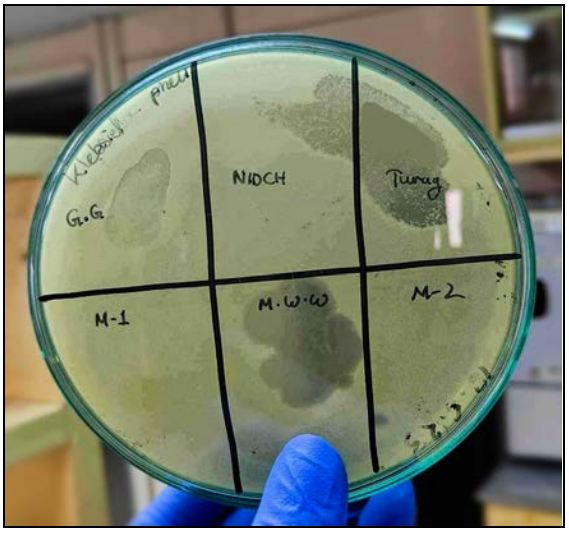
Water samples from 4 different species were collected and enriched to isolate *Klebsiella pneumoniae* and *Salmonella paratyphi* specific bacteriophages. The screening of the phages was conducted using 2 bacterial strains that are ***Klebsiella pneumoniae*** reference strain from **NIDCH** and ***Salmonella paratyphi*** reference clinical strain from **ICDDR,B.** In the process of isolation, 3 ***Klebsiella pneumoniae*** specific bacteriophages (**Phage *K1, K2 and K3***) were obtained alongside 1 *Salmonella paratyphi* specific bacteriophage (**Phage S**). Among the isolated results, all four of them exhibited clear lytic zones in the spot test conducted against their own host strains. The enriched sample water was analyzed through a double layer assay, which confirmed the presence of individual plaques at different dilutions. The isolated plaques showing the clearest and individual bacteriophages were selected for characterization, identification, and further study. Sampling was conducted in the month of July 2023.

Table 4.1: Water sample collection for Spot test from different areas for phage *K1, K2, K3 & S.*

Isolated Phages	Locations	Date of collection	Host	Remarks
K1	Turag River	24-07-2023	***Klebsiella pneumoniae*** *Reference strain (Environmental source) from NIDCH*	Water was blackened due to sewage disposal. There was no rain 5 days prior to collection.

Isolated Phages	Locations	Date of collection	Host	Remarks
K2	Buriganga River	24-07-2023	***Klebsiella pneumoniae*** *Reference strain (Environmental source) from NIDCH*	Water was heavily polluted and had a black hue. There was no rain 5 days prior to collection.
K3	Mirpur Sewage Waste Water	24-07-2023	***Klebsiella pneumoniae*** *Reference strain (Environmental source) from NIDCH*	Water was filthy with solid particles and debris from human waste. There was no rain prior to 5 days of collection
S	Mirpur Sewage Waste Water	24-07-2023	***Salmonella paratyphi*** *Reference strain (Clinically significant) from ICDDR,B*	Water was filthy with solid particles and debris from human waste. There was no rain prior to 5 days of collection





(a) **(b)**

Figure 4.1: Initial Spot test results obtained from collected water samples. (a) Initial spot for *Salmonella paratyphi* for page sample S**. (b)** Initial spot for *Klebsiella pneumonia* for phage sample K1, K2 & K3 .

4.1.2 Phage morphology

Individual plaques were produced by the isolated phage against the host bacteria on the double-layer agar plate. The plaques formed by the phages were amorphous and oversized in nature, and hence the plaque size couldn't be measured accurately.

4.1.3 Determination of phage titer

Following the first purification process, the phages ***K1, K2, K3 and S*** which were isolated were subjected to enrichment, and subsequently, the phage titer was assessed via the DLA technique. The phages ***K1, K2, K3 and S*** exhibited phage titers of $\mathbf{10^5}$, $\mathbf{10^2}$, $\mathbf{10^6}$ **and** $\mathbf{10^4}$ PFU/ml, respectively.

4.2 Characterization of the Isolated Bacteriophages

4.2.1 Assessment of Thermal Stability:

The study examined the thermal stability of phage isolated from environmental water samples *K1, K2, K3 and S*. The phage infectivity was measured in the study after it was freshly enriched and exposed to different temperatures for almost 2 hours. To examine their effects on phage thermal stability, additional assessments were carried out at temperatures of 30,40,50, 60, 70, and 80 °C. Upon incubated on water bath at different temperature, all the phages showed resistance at 30,40 and 50 °C. However, they inhibited their activity with temperature rise. At 60 °C, all the four-phage sample titers decrease by 2 log and completely lose their activity at 70 °C and 80 °C.

Table 4.2: Phage titer for *K1, K2, K3 and S* at different temperatures

Temp in °C	Assessed Bacteriophages			
	K1	***K2***	***K3***	***S***
30	1.75E+07 PFU/ml	2.35E+07 PFU/ml	4.25E+07 PFU/ml	2.30E+07 PFU/ml
40	7.50E+06 PFU/ml	1.80E+07 PFU/ml	4.00E+07 PFU/ml	2.10E+07 PFU/ml
50	4.00E+06 PFU/ml	1.00E+07 PFU/ml	3.50E+07 PFU/ml	2.00E+07 PFU/ml
60	2.50E+06 PFU/ml	5.00E+06 PFU/ml	2.25E+07 PFU/ml	1.00E+07 PFU/ml
70	0 PFU/ml	0 PFU/ml	0 PFU/ml	0 PFU/ml
80	0 PFU/ml	0 PFU/ml	0 PFU/ml	0 PFU/ml

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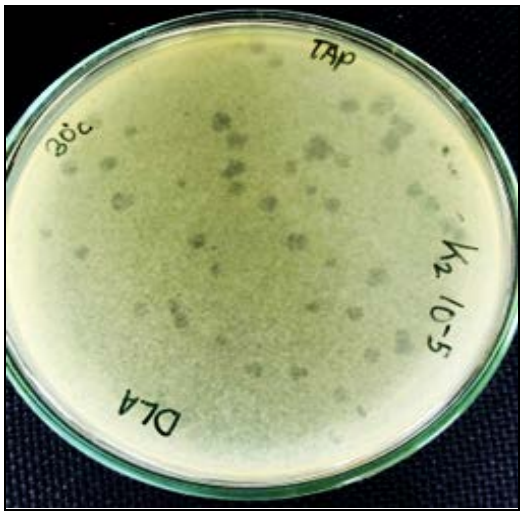

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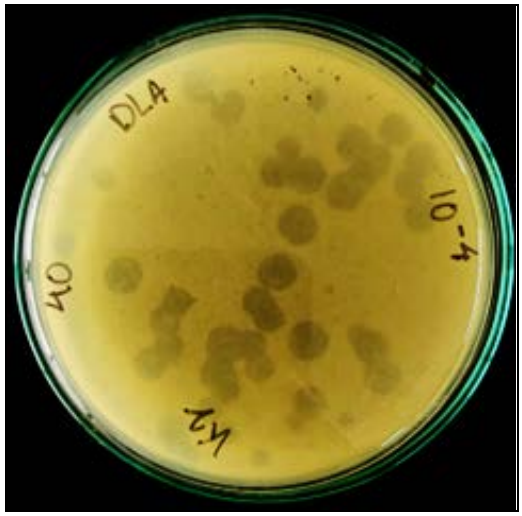

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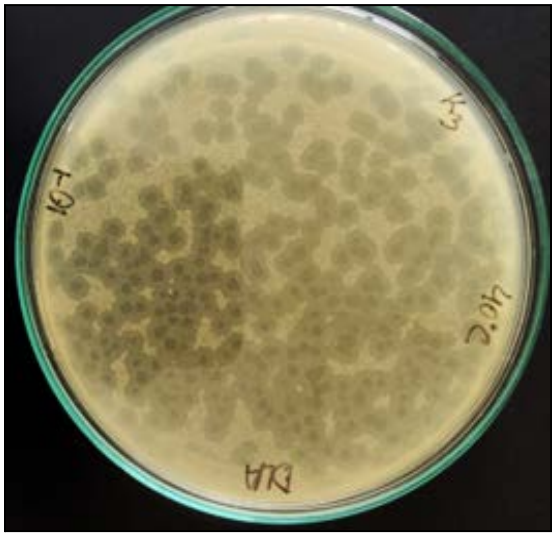

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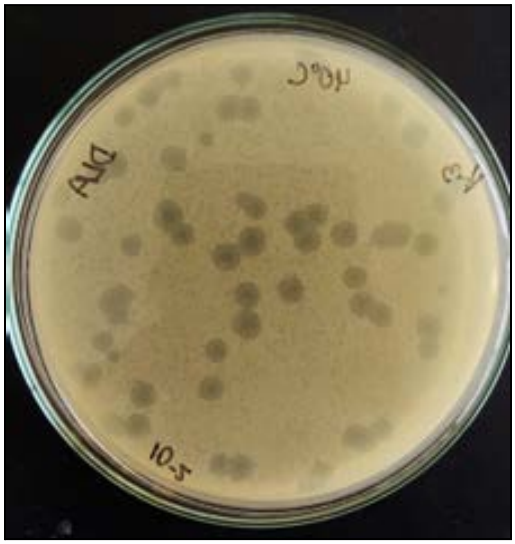

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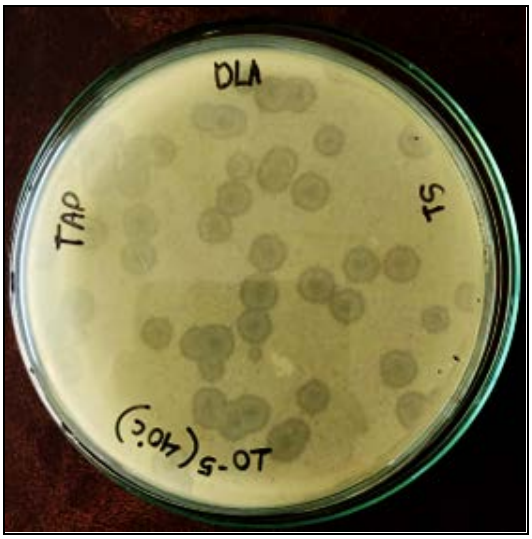

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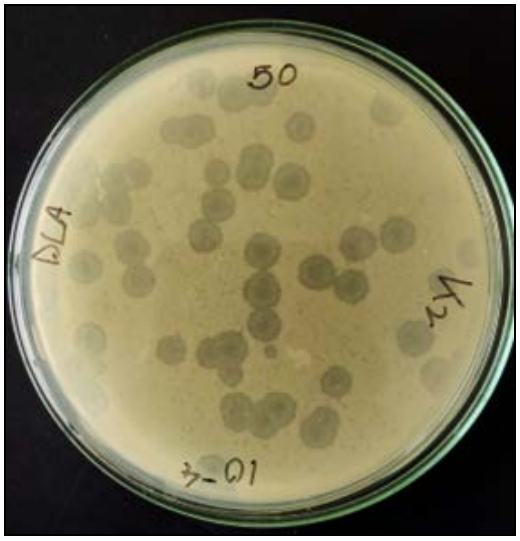

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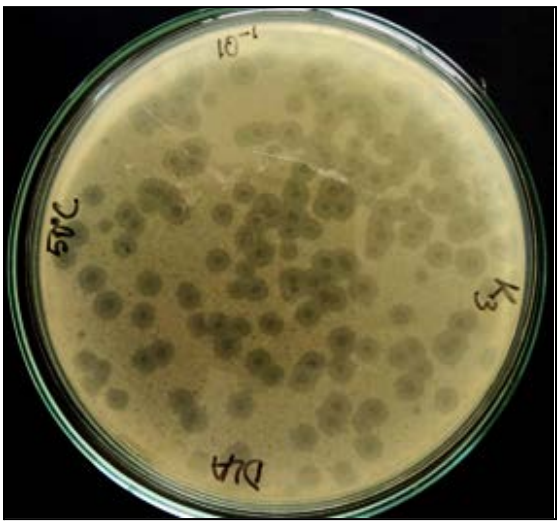

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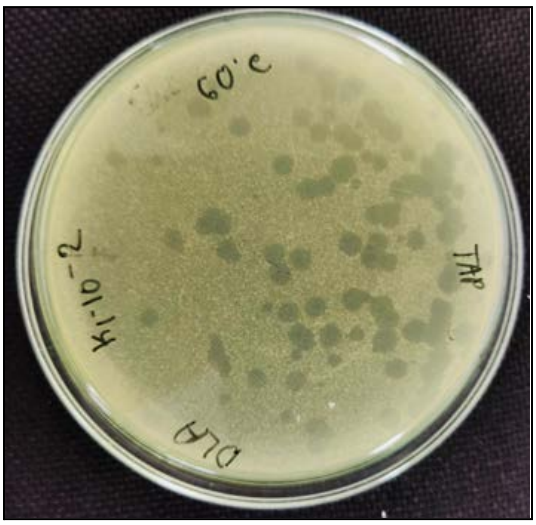

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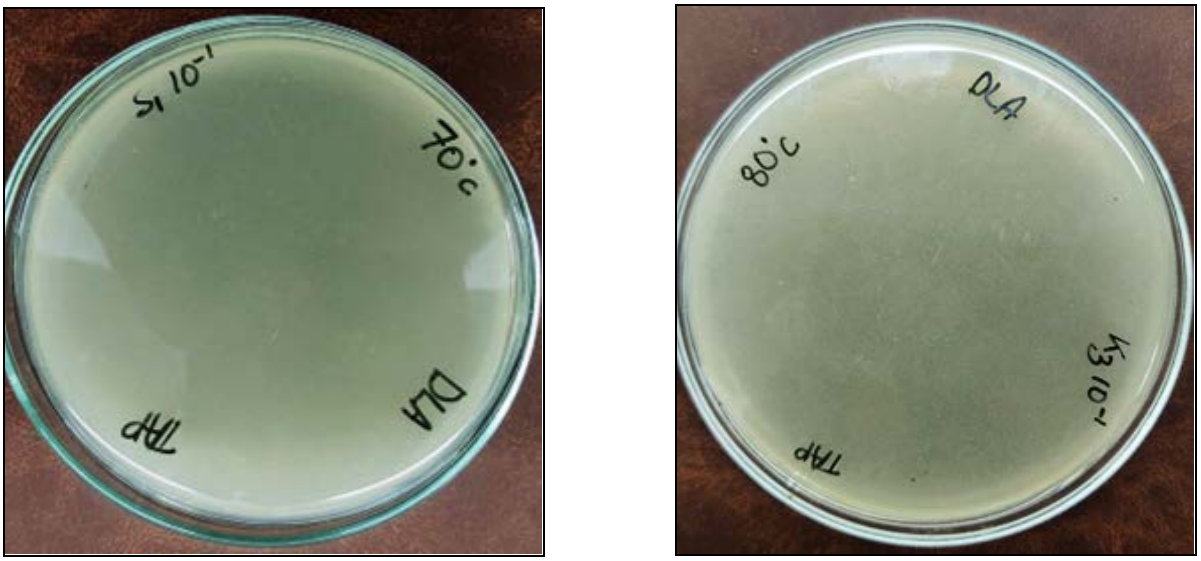


(i) **(j)**

Figure 4.2: Showing the visible Plaques done by DLA of Phage sample ***K1, K2, K3 & S*** at various temperatures. **(a)** DLA test results for K2 at 30 °C. **(b)** DLA test results for K2 at 40 °C. **(c)** DLA test results for K3 at 40 °C. **(d)** DLA test results for K3 at 40 °C at different dilution levels. **(e)** DLA test results for S at 40 °C. **(f)** DLA test results for K2 at 50 °C. **(g)** DLA test results for K3 at 50 °C.(h) DLA test results for K1 at 60 °C.**(i)** DLA test results for S at 70 °C.**(j)** DLA test results for K3 at 80 °C

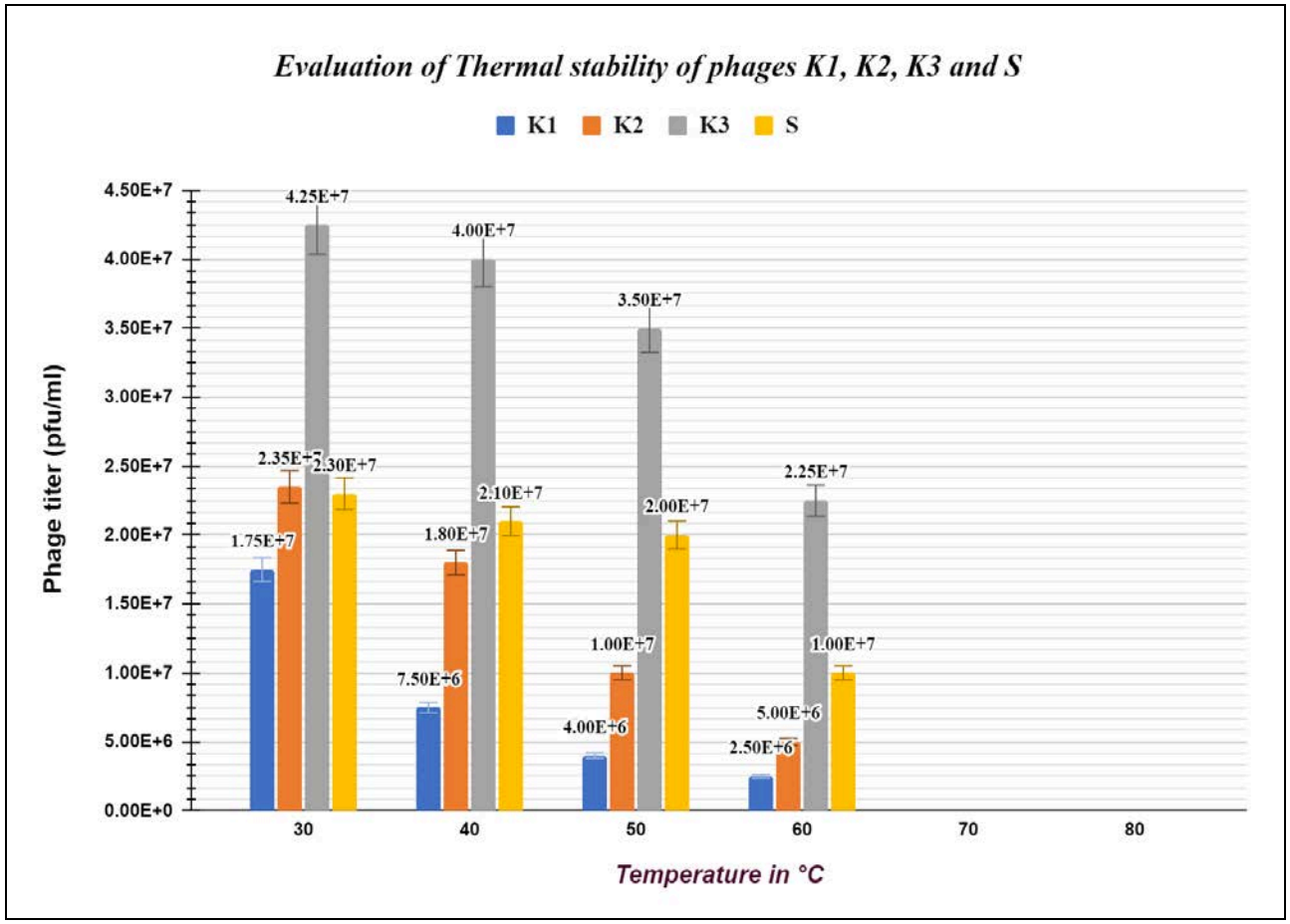


Figure 4.3: Illustration of the Assessment of Thermal Stability of the isolated bacteriophages at different temperatures.

4.2.2 Evaluation of pH Stability

To evaluate the impact of pH on the phage's stability, the phage stock was separately incubated at various pH values. The findings showed that an incubation period at pH 4.0 and pH 6.0 resulted in countable plaques while pH 7.0 had the most number of plaques which started to slowly decline at pH 8.0, 9.0 and 10.

Table 4.3: Phage titer for phages K1, K2, K3 and S for a variety of pH values.

pH	Assessed Bacteriophages			
	K1	*K2*	*K3*	*S*
2	0 PFU/ml	0 PFU/ml	0 PFU/ml	1.50E+06 PFU/ml
4	3.15E+07 PFU/ml	1.90E+07 PFU/ml	4.05E+07 PFU/ml	5.65E+07 PFU/ml
6	5.45E+07 PFU/ml	2.05E+07 PFU/ml	3.00E+07 PFU/ml	6.95E+07 PFU/ml
7	1.64E+08 PFU/ml	8.15E+07 PFU/ml	1.90E+08 PFU/ml	1.47E+08 PFU/ml
8	7.32E+07 PFU/ml	1.55E+07 PFU/ml	6.00E+07 PFU/ml	4.50E+07 PFU/ml
10	4.35E+07 PFU/ ml	1.70E+07 PFU/ml	4.65E+07 PFU/ml	4.25E+07 PFU/ml

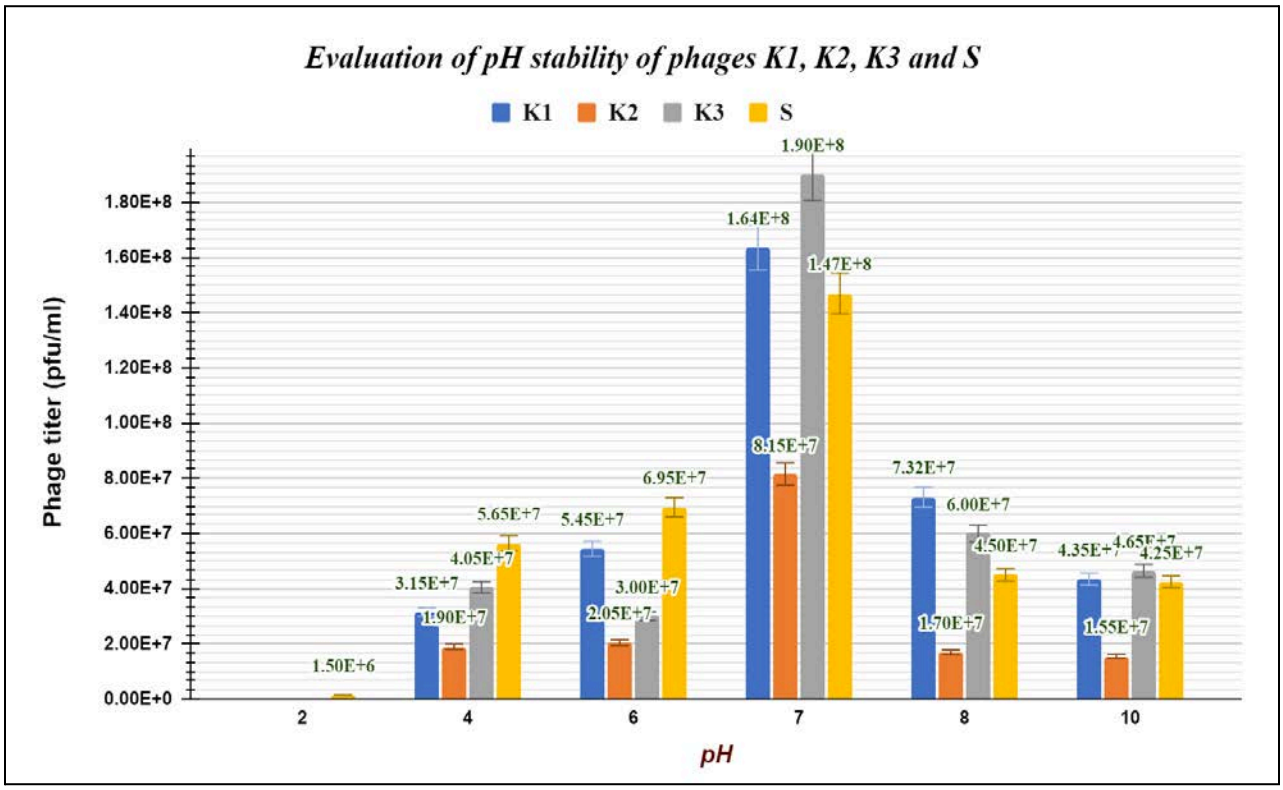
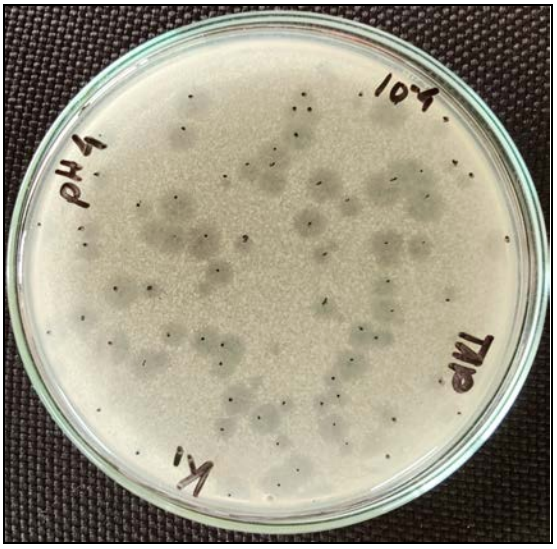
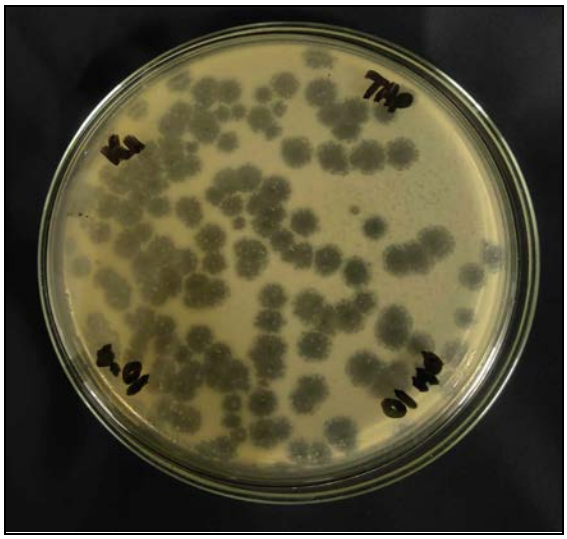


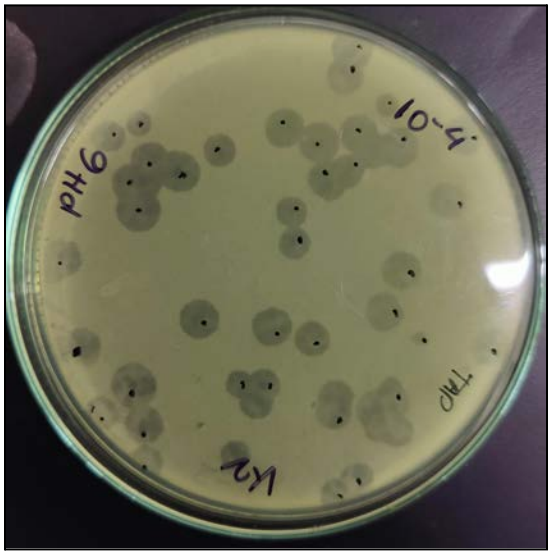
Figure 4.4 : Illustration of the Assessment of bacteriophage stability at different pH values.



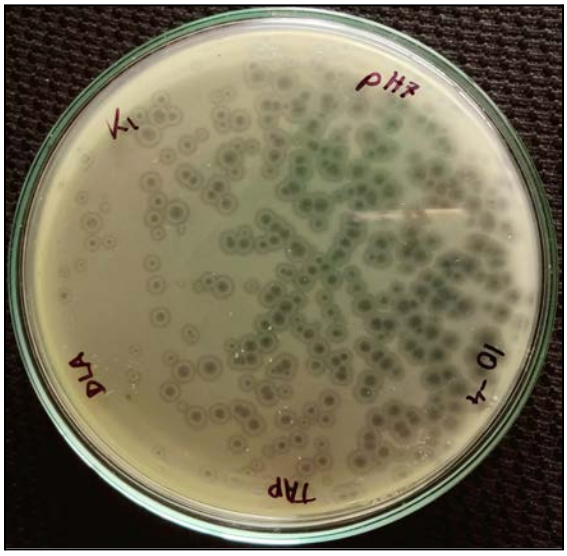
(a)



(b)



(c)



(d)

Figure 4.5 : Showing the visible Plaques done by DLA of Phage sample *K1, K2, K3 & S* at various PH values . **(a)** DLA test results for K1 at pH 4. (b) DLA test results for K2 at pH 10.**(c)** DLA test results for K2 at pH 6. **(d)** DLA test results for K1 at pH 7

4.2.3 Determination of Stability under Saline Conditions

To evaluate the impact of salinity on the phage's stability, the phage stock was separately incubated at various salinity concentrations. The findings showed that an incubation period at every saline concentration resulted in countable plaques while at saline concentration of 0.9 and 1.0 resulted in the most number of plaques, and *Salmonella paratyphi* provided the highest tolerance amongst the four phages in every concentration tested.

Table 4.4: Phage titer for phages K1, K2, K3 and S for a variety of saline concentrations.

Salinity	Assessed Bacteriophages			
	K1	K2	K3	S
0.2	3.60E+07 PFU/ml	3.80E+07 PFU/ml	4.40E+07 PFU/ml	6.95E+07 PFU/ml
0.4	3.80E+07 PFU/ml	4.15E+07 PFU/ml	4.55E+07 PFU/ml	8.20E+07 PFU/ml
0.9	4.60E+07 PFU/ml	5.20E+07 PFU/ml	5.50E+07 PFU/ml	8.45E+07 PFU/ml
1	4.20E+07 PFU/ml	5.35E+07 PFU/ml	5.85E+07 PFU/ml	8.70E+07 PFU/ml
1.2	4.15E+07 PFU/ml	4.80E+07 PFU/ml	5.30E+07 PFU/ml	7.30E+07 PFU/ml
1.5	3.65E+07 PFU/ml	4.00E+07 PFU/ml	4.75E+07 PFU/ml	6.00E+07 PFU/ml

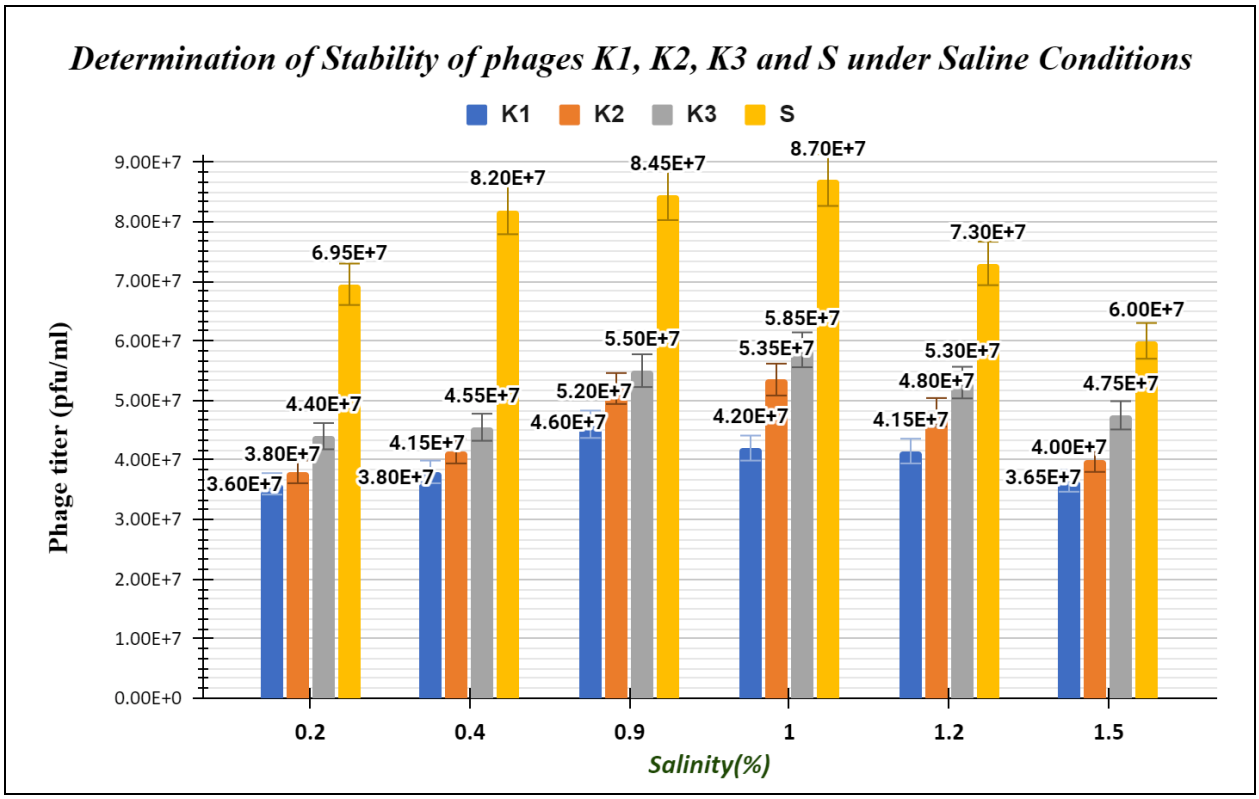
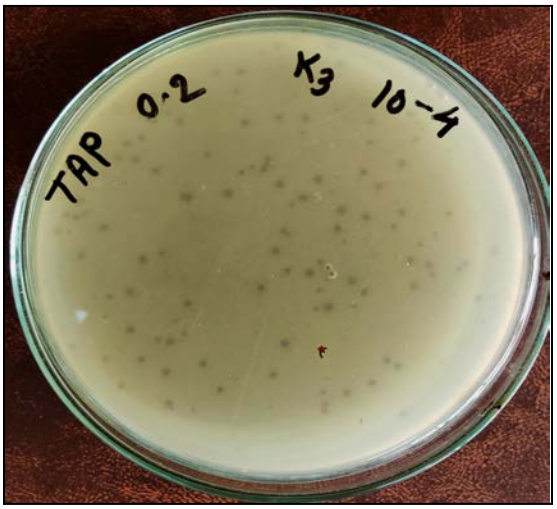
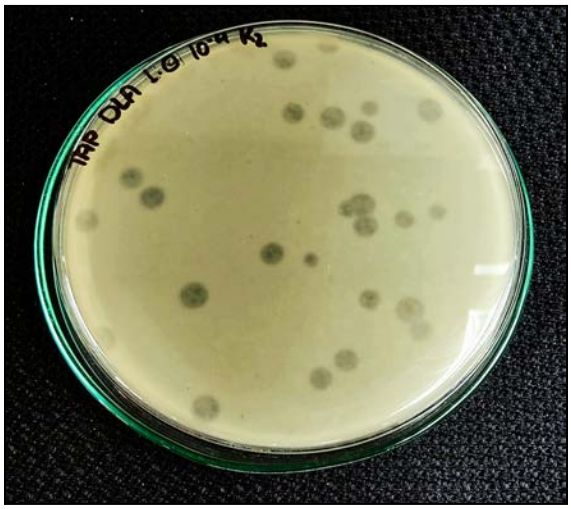


Figure 4.6: Assessment of bacteriophages stability under different saline concentrations





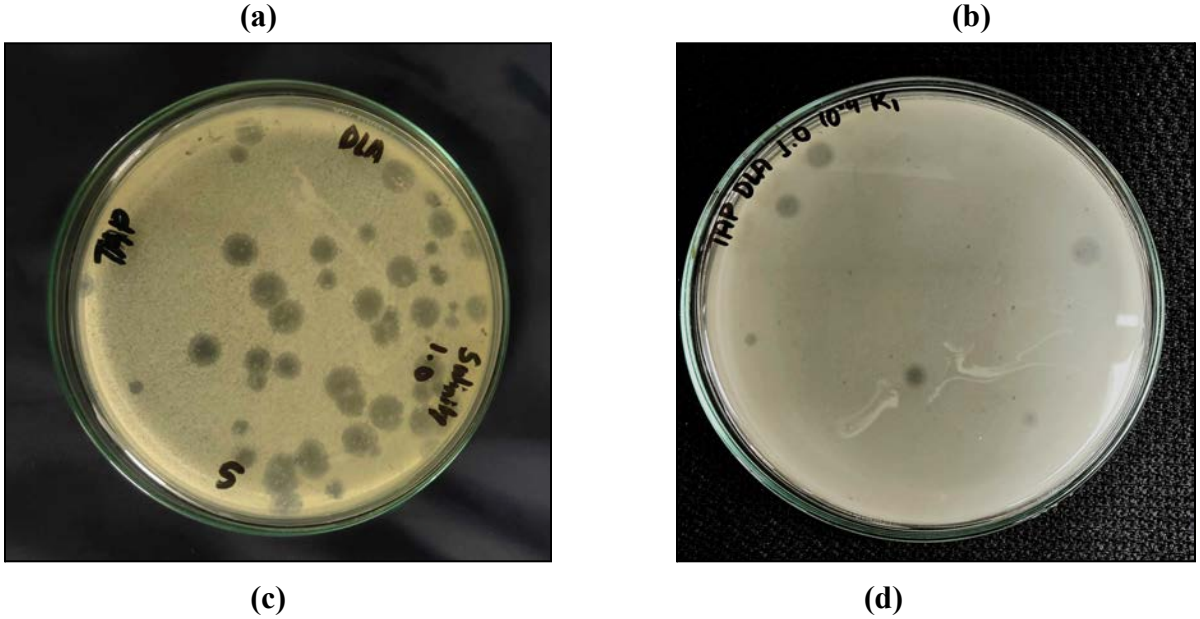


Figure 4.7: Showing the visible Plaques done by DLA of Phage sample *K1, K2, K3 & S* at various saline concentrations. (a) DLA test results for K3 at 0.2 saline concentrations. **(b)** DLA test results for K2 at 0.4 saline concentrations. **(c)** DLA test results for S at 3.0 saline concentrations**. (d)** DLA test results for K1 at 1.0 saline concentrations

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4.2.4 Organic Solvent Susceptibility Test

The susceptibility of the isolated bacteriophages *K1, K2, K3,* and *S*, towards organic solvents chloroform and ethanol was evaluated. After a 1 hour (60-minute) incubation period, the results indicated that there was no observable disparity in the titer levels between the phages subjected to chloroform compared to the control group. On the other hand, when the phages were exposed to ethanol for the equal amount of time, they were completely rendered inactive. These obtained results clearly illustrate the susceptibility of the isolated phages towards organic solvents, wherein chloroform exerts a minimal influence on their activity, and ethanol fully deactivates them upon incubation.

Table 4.5: titer of the isolated phage against organic solvents :

Solvent Type	Assessed Bacteriophages			
	K1	K2	K3	S
Control	1.45E+09	1.60E+09	1.35E+09	2.50E+08
Chloroform	1.30E+09	1.40E+09	1.10E+09	1.00E+08
Ethanol	0	0	0	0

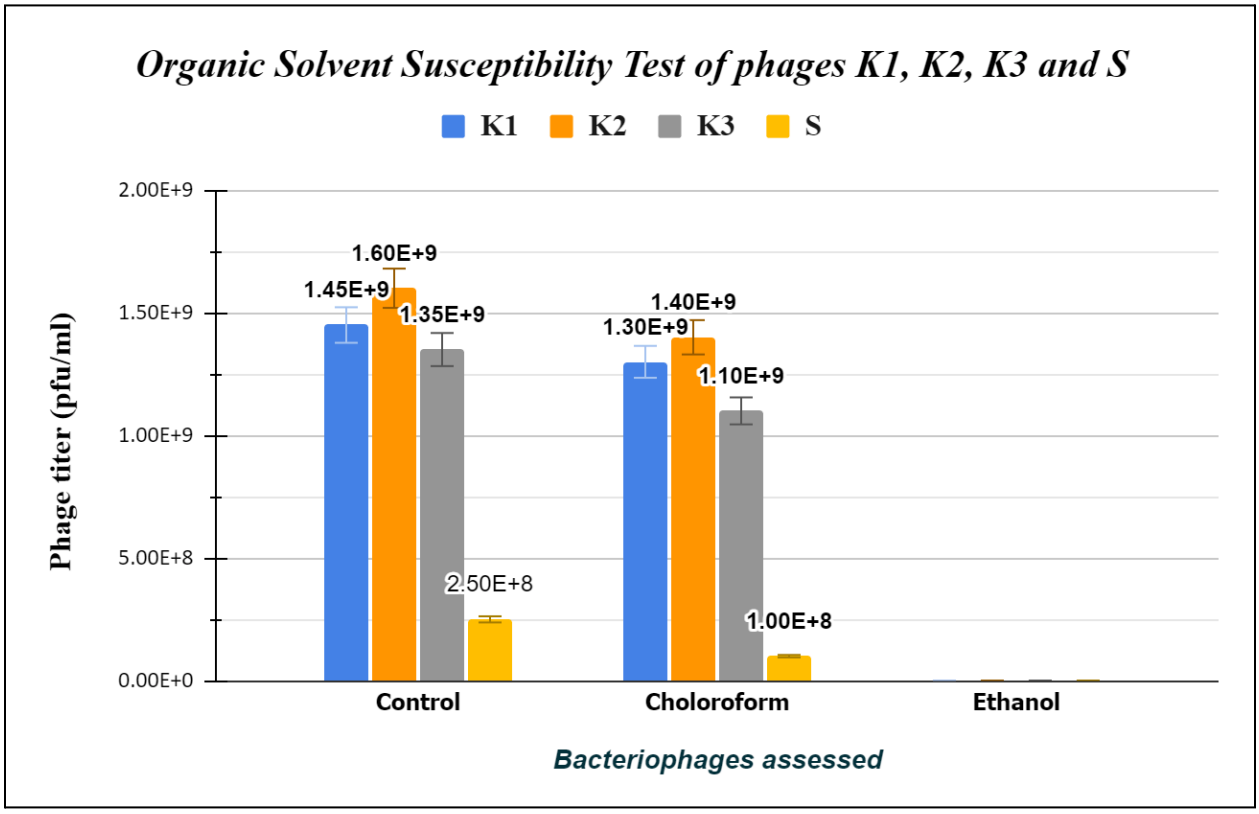
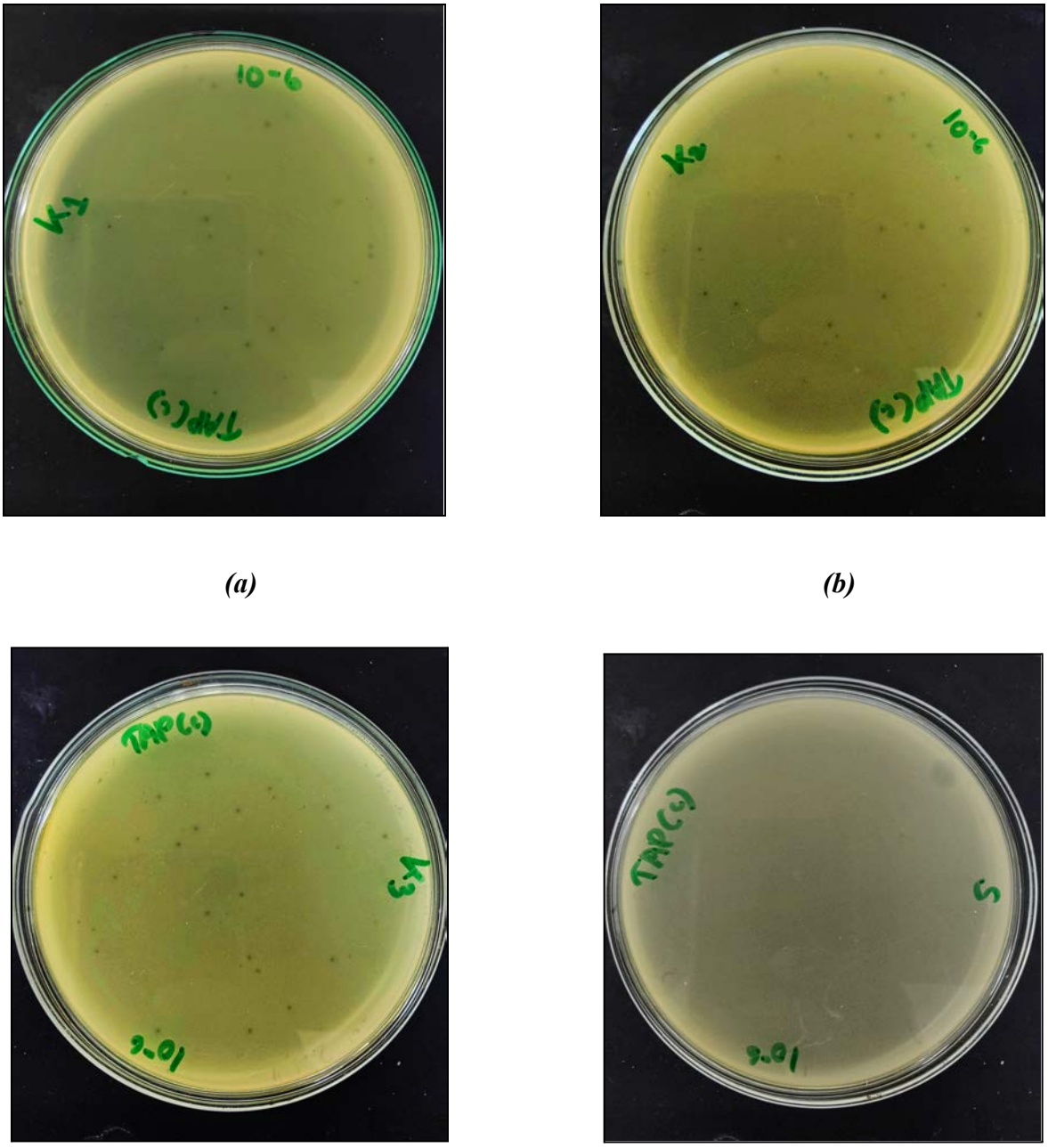


Figure 4.8: Stability of bacteriophages *K1, K2, K3, and S* in organic solvents chloroform and ethanol.



(a) *(b)*

(c) *(d)*

Figure 4.9: Showing the visible Plaques done by DLA of Phage sample K1,K2,K3 & S in organic solvents chloroform and ethanol. ***(a)*** *DLA test results for K1 in organic solvents chloroform and ethanol.* ***(b)*** *DLA test results for K2 in organic solvents chloroform and ethanol.* ***(c)*** *DLA test results for k3 in organic solvents chloroform and ethanol.* ***(d)*** *DLA test results for S in organic solvents chloroform and ethanol.*

After analyzing the obtained results, no substantial changes in titer of the phages incubated with chloroform were seen when compared to the control group. However, incubating the phages with ethanol for the same period of time resulted in the complete deactivation of all the phages.

4.2.5 Determination of Host Range Susceptibility

The isolated bacteriophage's host range was assessed using 29 bacterial cultures, as listed in Table 3.8.1. Among these cultures, 12 strains had high clinical significance. The spot test results are also depicted within the same table since the ability to infect and produce lytic zones by the isolated *K1, K2, K3, and S* bacteriophages against different bacterial strains were tested through spot test. The results are demonstrated below as follows:

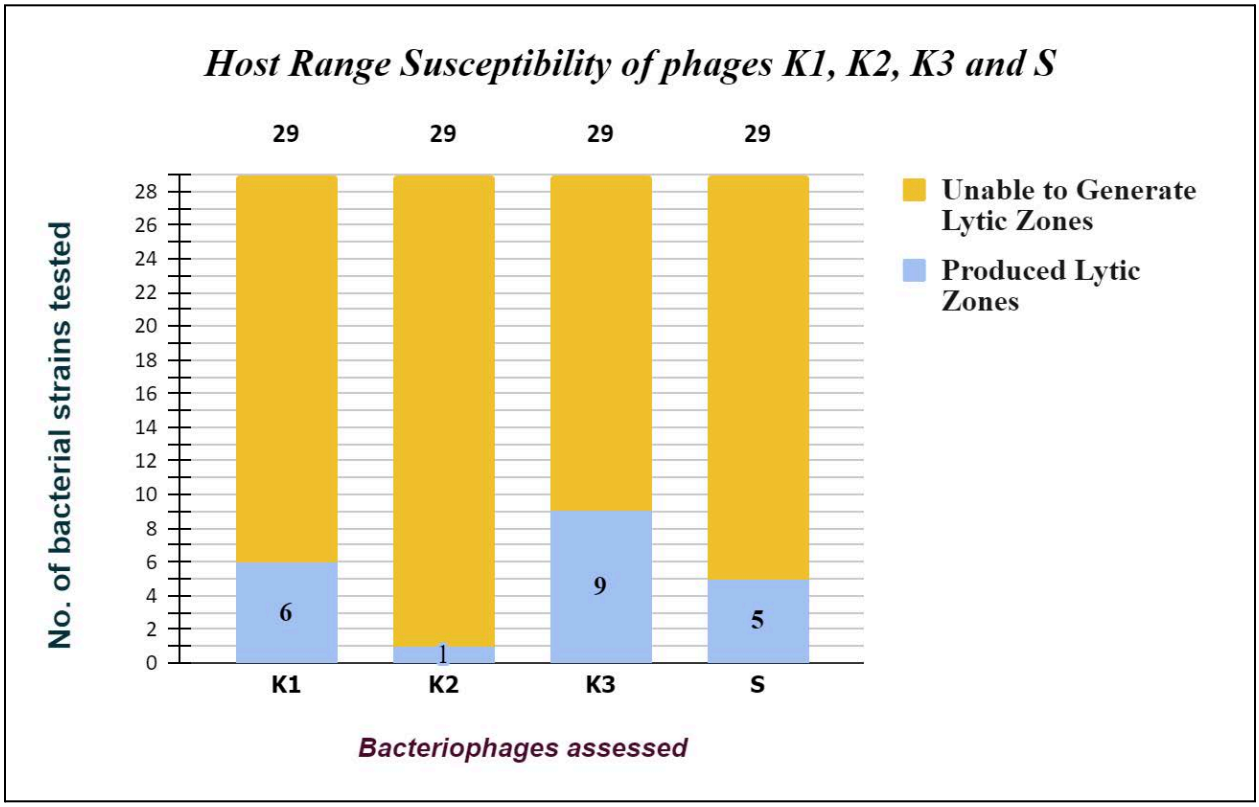


Figure 4.10: Host range Susceptibility of phages *K1, K2. K3 & S.*

The results of the spot test turned out very promising. Out of the 29 bacterial cultures tested, the isolated phages were able to produce lytic zones with 12 of the strains (excluding their host strains), among which:

- **Phage *K1*** produced lytic zone in **6 strains** out of the 29 bacterial strains tested through spot test. Of those 6 strains, the notable ones were the ***Klebsiella pneumoniae* Isolate 26** and ***Klebsiella variicola*** *as both of these strains had clinical significance.( )*
- **Phage *K2*** was unable to produce any lytic zone in any of the 29 bacterial strains tested besides its host strain.
- **Phage *K3*** produced lytic zone within **9 strains** out of the 29 bacterial strains tested through spot test. Of those 9 strains, the notable ones were the ***Klebsiella pneumoniae* Isolate 22, *Klebsiella variicola, Pseudomonas aeruginosa and Hafnia alvei*** *as all of these strains were clinically significant.*
- **Phage *S*** produced lytic zone within **5 strains** out of the 29 bacterial strains tested through spot test. Out of those 5 strains, the notable ones were the ***Salmonella typhi*, *Salmonella paratyphi, Enterococcus faecalis and Hafnia alvei*** *as all of these strains were clinically significant.*

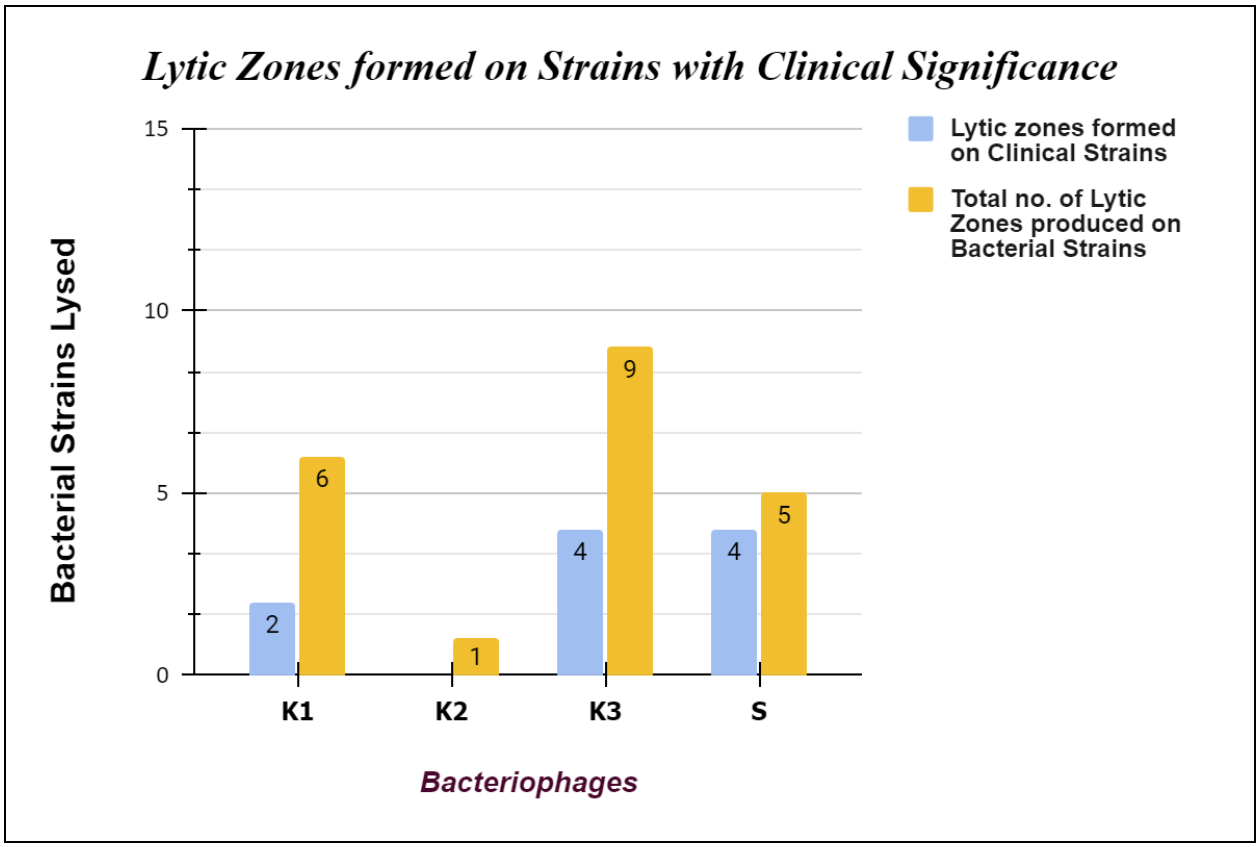


Figure 4.11 : Lytic Zones formed on strains having clinical significance.

Chapter 05

Discussion

Discussion

5.1 Bacteriophage Isolation and Purification:

The water samples, stored at 4ºC and processed within 14 days, were analyzed to isolate and purify bacteriophages ***K1, K2, K3,*** *and* ***S.*** This timeframe adheres to the recommendation by Townsend et al. (2021), as storing phages at -20ºC is discouraged due to the potential destruction caused by the ice crystal structure, as demonstrated by Warren and Hatch (1969). The absence of phages in three water samples could be attributed to the collection method, potentially omitting sediment. Phage particles often adhere to sediment, aggregating over time at the bottom of water bodies, with shaking releasing ground-floor phages into the water (De Flora et al., 1975). Phages are typically more abundant in sediment than overlying water (Drake et al., 1998). The two lakes sampled had placid waters, with sediment deposited at the bottom, reducing available phage-attached sediment at the surface. In contrast, the sewage water sample contained black particle-like beads, to which phages might have attached and been collected. Before initial phage enrichment, water samples were shaken, likely detaching phages from particles and thoroughly mixing them with the sample.

Various factors in aquatic environments can render phages non-infective. Heterotrophic bacteria and protists can degrade viruses, while heavy metals and high-energy photons can bind with and disrupt phages (Weinbauer, 2004; Fujioka et al., 1980; Bitton, 1980; Murray & Jackson, 1993). However, sunlight, particularly ultraviolet (UV) light, is the primary factor causing viral decay. UV-B in sunlight, accounting for 50-90% of virus inactivation, damages viral genomic material irreparably (Kirchman, 2012; Caldwell, 1971; Setlow, 1974; Suttle & Chen, 1992). In open water bodies like those where K1 and K2 were sampled, UV light likely played a significant role, especially during the summer when sunlight intensity is highest. Conversely, little to no sunlight interacts with the waters in continuously flowing water bodies, such as where K3 and S were sampled.

During isolation, only clear plaques were selected for further study. This selection was not arbitrary, but based on the understanding that clear plaques indicate complete lysis of bacterial hosts, a characteristic of lytic phages. Lytic phages release many progeny phages upon infecting

host cells, leading to the expansion of the lysis zone. The size of the clear plaques varied, suggesting the presence of multiple phage types in the water sample. Factors such as the proliferation rate and physical size of phages influence plaque size, slowly proliferating or smaller phages producing smaller plaques (Irving et al., 1990).

Water samples from different locations were collected and enriched to isolate *Klebsiella pneumoniae* and *Salmonella paratyphi*-specific bacteriophages for isolation and purification. Screening was performed using reference strains of *Klebsiella pneumoniae* from NIDCH and *Salmonella paratyphi* from ICDDR, B. Three *Klebsiella pneumoniae*-specific phages (K1, K2, and K3) and one *Salmonella paratyphi*-specific phage (S) were isolated. All four phages exhibited clear lytic zones against their respective host strains. The isolated plaques were selected for further characterization and identification.

The morphology of individual phage plaques formed on double-layer agar plates was amorphous and oversized, making accurate size measurement challenging. Following purification, the phages K1, K2, K3, and S were enriched, and their titers were determined using the DLA technique. The phage titers were 10^5, 10^2, 10^6, and 10^4 plaque-forming units (pfu)/ml, respectively.

5.2 Analysis of Thermal Stability :

Thermal performance curves are used to forecast how Bacteriophage interactions would alter under projected temperature change, and consequently how range shifts, disease dynamics, and community composition will change along with phage infectivity. The effects of temperature change on host-phage interactions are a frequently disregarded issue. Given that temperature affects the physiology, ecology, and evolution of both hosts and bacteriophages, its impact on species interactions is probably extensive (Padfield, 2020). Here, we study the effects of temperature-dependent alterations on a microbial host-phage connection and how this relationship influences the bacteriophage's ecological and evolutionary processes. Assessment of Thermal Stability of bacteriophage offers important information regarding their growing

requirements, host interactions, stability, enzyme activity, and applicability in many contexts—including research and possible therapeutic applications.

The investigated phages' heat stability and reconstitution showed notable results. Overall, all the samples responded in a similar pattern to high temperatures. Initially K1 started at 1.75E +07 pfu/ml at 30 °C and was stable until it reaches 60 °C which dropped to 2.50E +06 pfu/ml. Here K1 activity dropped by at least 10 logs pfu/ml upon exposure to 70 °C and 80 °C, the activity dropped below the limit of detection pfu/ml.

Following that, in terms of sample K2 and K3, after 2 hours of exposure heat, both K2 and K3 showed a similar infective pattern. This overall decreases from 2.35E+07 pfu/ml to 5.00E+06 pfu/ml for K2 and 4.25E+07 pfu/ml to 2.25E+07 pfu/ml for K3 following the temperature at 30 °C. 60 °C. However, both the phages' activity was completely eliminated at 70 and 80 °C.

Furthermore, after treatment at 30 °C to 60 °C, phage *S* remained stable between titer 2.00E+07 to 1.00E+07 pfu/ml, but shows overall declination along with increasing temperature (70°C and 80 °C) by dropping the titer nearly at 0 PFU m showing complete deactivation of the samples

5.3 Examination of pH Stability:

The survival of phages *K1, K2, K3, and S* was tested in relation to the acidity or alkalinity of the environment . After 3 hours of incubation, these phages displayed resilience to pH levels ranging from 4.0 to 10.0. Notably, phages *K1, K2, and K3* were highly stable within the optimum pH range of 4.0 to 10.0. However, phage S demonstrated extraordinary resistance at pH 2.0, making it the only phage capable of withstanding this acidic state, whilst the other three phages were severely corroded. This unusual behavior shows that, whereas *K1, K2, and K3* are well-suited for a variety of uses, including possible therapeutic formulations for treating *Klebsiella pneumoniae* and *Salmonella paratyphi* infections, phage S may have a particular advantage in pH settings as low as 2.0. Nonetheless, phage S's overall stability throughout a wide pH range makes it a viable therapeutic agent, particularly for treating urinary tract infections or inhibiting biofilm in urinary catheters, which is consistent with the applications described accordingly.

5.4 Determination of Stability under Saline Conditions:

The stability of phages, including *K1, K2, K3, and S*, was examined under different saline concentrations (0.2, 0.4, 0.9, 1, 1.2, and 1.5). Across these salinity ranges, the phages were most resistant at concentrations of 0.9 and 1. Resistance decreased slightly at both lower (0.2 and 0.4) and higher (1.2 and 1.5) saline contents, as opposed to the substantial stability found at 0.9 and 1. This finding suggests that the ideal saline concentrations for the phages are between 0.9 and 1, implying that they may be successful in moderate salinity situations.

The observed decrease in resistance or pfu/ml at higher and lower saline concentrations (1.2 and 1.5, and 0.2 and 0.4, respectively) could be due to osmotic shock, a process in which sudden changes in osmotic pressure affect the stability and functionality of phage. While the phages showed the greatest resistance at concentrations of 0.9 and 1, indicating optimal performance under moderate saline conditions, a slight decrease in resistance at a higher saline percentage (1.2 and 1.5) and lower saline percentage (0.2 and 0.4) indicates a potential sensitivity to osmotic variations.

It is worth noting that the negative effects of saline conditions, notably the amount of sodium chloride, on the phages were minimal, demonstrating a degree of resilience. This report emphasizes the importance of understanding how osmotic circumstances affect phage stability, providing insights into their behavior in various saline environments. Such insights are critical for assessing the phages' practical uses in a variety of situations with varying salt levels.

5.5 Assessing the Susceptibility of the Isolated Bacteriophages towards Organic Solvents

The susceptibility of isolated bacteriophages *K1, K2, K3, and S* to organic solvents chloroform and ethanol was assessed. This assessment is crucial for considering the potential use of bacteriophages in therapeutic or biocontrol preparations involving various organic solvents. The stability of bacteriophages in different organic solvents primarily hinges on the stability of their proteins, which is altered by the solvent's ability to either reinforce or disrupt specific inter and intramolecular hydrophobic and electrostatic interactions (Olofsson et al., 1998). There exists a competition between stabilization and destabilization of these interactions upon exposure to organic solvents (Olofsson et al., 1998).

All four bacteriophages *K1, K2, K3, and S* exhibited resistance to chloroform in a chloroform-aqueous environment. This finding suggests that storing the phages in chloroform can prevent bacterial contamination, as chloroform possesses antimicrobial properties and has been commonly used in phage stock preparation (Cotton and Lockingen, 1963). Conversely, all isolated bacteriophages were sensitive to absolute ethanol, aligning with similar findings reported by Verma et al. (2009). A previous study by Olofsson et al. (1998) demonstrated a significant decrease in phage viability with ethanol concentrations higher than 40%. Therefore, the use of 70% ethanol, commonly employed in laboratories to maintain aseptic conditions, is recommended for preserving a sterile environment where the presence of the isolated phages is deemed unacceptable. This principle of organic solvent sensitivity of bacteriophages underscores their potential utility in various therapeutic or biocontrol applications involving organic solvents.

5.6 Identification of Host Range Specificity of the Isolated Bacteriophages

Promising insights have been obtained from the spot test outcomes undertaken to assess the host range specificity of bacteriophages *K1, K2, K3, and S*. Among the 29 bacterial cultures that were assessed, the phages that were recovered shown the capacity to generate lytic zones in 12 strains. The phages that were recovered showed the capacity to generate plaques within **12 strains**, while excluding the strains of their respective hosts. This result has important consequences for the possible use of phage treatment in fighting bacterial infections.

The lytic activity of **phage K1** was seen against **6** out of the 29 bacterial strains that were tested. Significantly, some bacteria, including ***Klebsiella pneumoniae*** **Isolate 26** and ***Klebsiella variicola***, were shown to be impacted. The clinical significance of these strains lies in their ability to induce severe infections, such as pneumonia and bloodstream infections. These findings indicate that phage K1 has the potential to be employed as a treatment for illnesses caused by these viruses.

In contrast, the lytic activity of **phage K2** was shown to be absent against all bacterial strains that were examined, except its host strain. Although its value in phage treatment may be limited, conducting additional research on its mode of action might yield significant insights into the interactions between phages and their hosts.

Phage K3 demonstrated lytic activity against 9 out of the 29 bacterial strains, including ***Klebsiella pneumoniae*** **Isolate 22**, ***Klebsiella variicola, Pseudomonas aeruginosa, and Hafnia alvei***, all of which are clinically significant. This broad host range suggests that phage K3 may be effective against a diverse range of bacterial pathogens besides its host strain.

In a similar vein, **phage S** demonstrated lytic efficacy against five bacteria, namely ***Salmonella typhi, Salmonella paratyphi (NIDCH strain), Enterococcus faecalis, and Hafnia alvei***, all of which had therapeutic relevance. In addition, the findings propose the potential existence of strain-level selectivity, wherein certain phages may only target closely related strains within a given species.

The significance of comprehending the host range specificity of bacteriophages in phage treatment is emphasized by these findings. Researchers can enhance treatment tactics and customize phage cocktails to efficiently target certain bacterial infections by finding phages that have either broad or limited host ranges. Furthermore, a comprehensive investigation of the processes that govern phage-host interactions might significantly augment our comprehension of phage treatment and its prospective use in addressing antibiotic-resistant diseases.

5.7 Identification of Limitations and Suggestions for Future Studies

Identification of Limitations of the Research :

The Double Layer Agar (DLA) method, which is extensively used to determine phage titers, has various drawbacks that may hinder the accuracy and repeatability of the results. One key problem is the subjective interpretation required during plaque counting, which necessitates careful observation and distinction of individual plaques, especially when they are densely packed or overlap on the agar surface. Subjectivity among observers can add variability and bias, potentially leading to inconsistencies across replicates or among laboratories.

Furthermore, the creation of satellite plaques poses a constraint, as these smaller and less distinguishable plaques may be ignored or miscounted, particularly in samples with high phage concentrations, confounding result interpretation. Variations in plaque size and morphology, caused by variances in host bacteria sensitivity to phage infection, complicate reliable plaque counting and titration with the DLA method.

Moreover, the method's dynamic range may be limited, especially for samples with exceptionally high or low phage concentrations, potentially leading to underestimating or overestimation of

titers. The DLA method's time-consuming nature, taking hours or days for plaque production and enumeration, may not be suitable for circumstances that need rapid or high-throughput titer determination. Finally, the danger of cross-contamination between plaques during counting, particularly with manual approaches, raises concerns about assay integrity and results accuracy. These limitations emphasize the significance of rigorously validating experimental techniques when using the DLA approach for phage titer measurement, as well as the need for continued attempts to solve these issues in phage research methodologies.

Suggestions for Future Studies:

- ➔ The results obtained might be unique to the bacteriophage sample, like *Salmonella Paratyphi* and *Klebsiella pneumoniae*. By characterizing environmental phages in greater detail, this methodology can be expanded and used to create a framework of reference for additional research.
- ➔ Isolating the phage DNA and sequencing it for further bioinformatic research could be an additional characterization step.
- ➔ In vivo studies might be used to assess the potential of bacteriophages to combat bacterial infections in human, animal, and aquaculture.
- ➔ One way to evaluate isolated phages' capacity to break down biofilms is to use them.
- ➔ Building a phylogenetic tree with the isolated phages can help comprehend their evolutionary history.
- ➔ Moreover, a build-by-design strategy based on synthetic genomics might be used to modify the genome by creating phages according to known phage biology principles, possibly for use in phage therapy applications.
- ➔ In the future, phages might be designed to deliver payloads that are intended to change host reactions or reduce the chance of infection
- ➔ One example that will revolutionize the future aspect of phage therapy includes a therapeutically useful phage that is administered by aerosolization to treat lung infections. It is imperative to take into account the stability of every phage in the particular nebulizer that is being utilized for administration.

Conclusion

Given the increasing prevalence of antibiotic resistance, phage therapy appears to have a bright future in the fight against Salmonella and *Klebsiella pneumoniae* infections. It provides a focused and possibly efficient substitute for traditional medicines. To fully realize the therapeutic promise of phage treatment and address the growing danger posed by these resilient bacterial pathogens, more research and innovation in phage isolation, characterization, formulation, and clinical translation are needed. We might be able to change the way infectious illness management is approached by taking advantage of the bacteriophages' inherent specificity and effectiveness. Moreover, given that the bulk of antibiotics are used in agriculture and animals, it is doubtful that phages will ever completely replace antibiotics; however, phage-based techniques have the potential to greatly enhance antibiotic stewardship from the One Health perspective.

To sum up, the evaluation of the saline conditions, pH stability, thermal stability, and susceptibility to organic solvents provide important information about the behavior and possible uses of isolated bacteriophages, especially those that target *Klebsiella pneumoniae* and *Salmonella Paratyphi*. The results emphasize how crucial it is to take temperature swings, pH values, saline concentrations, and solvent exposure into account when developing and putting into practice phage-based methods. For example, the temperature-dependent changes that were found show how significant it is to properly maintain phage viability in handling and storage settings. Analogously, the evaluation of pH stability indicates fluctuations in phage resistance at various acidity levels, indicating customized methods for certain uses.Studying saline circumstances provides information on the range that is ideal for phage stability and how well the organisms function in different salinity environments. Furthermore, in order to preserve phage integrity, appropriate handling and storage procedures are crucial due to the vulnerability to organic solvents.

To successfully use phages in research and therapeutic interventions, as well as other contexts, it is imperative to comprehend how these environmental conditions affect phage stability and activity.

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Appendix-I

Media Composition

During our research, the following media were utilized. Here, all the components were autoclaved at 121ºC and 15 psi for about 15 minutes, unless otherwise noted.

- **Luria-Bertani Agar**

Component	Quantity(g/L)
Agar	15.0
Peptone	10.0
NaCl	10.0
Yeast extract	5.0
Final pH	7.0 ± 0.2

- **Luria-Bertani Broth**

Component	Quantity(g/L)
Peptone	10.0
NaCl	10.0
Yeast extract	5.0
Final pH	7.0 ± 0.2

Appendix-II

Reagents Utilized

The following reagents were used consistently throughout our research. The reagents are:

- **Luria-Bertani Broth**

Component	Quantity(g/L)
Peptone	10.0
NaCl	10.0
Yeast extract	5.0
Final pH	7.0 ± 0.2

- **SM Buffer**

Component	Quantity(g/L)
NaCl	5.8
$MgSO_4.7H_2O$	2.0
Tris HCl	50 ml
Gelatin	5.0
Final pH	7.5 ± 0.2