SYNERGISTIC EFFECT OF BACTERIOPHAGE WITH ANTIBIOTIC IN THE ERADICATION OF *VIBRIO CHOLERAE* INFECTION: AN IN-VIVO ANALYSIS IN MICE MODEL

By

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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
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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing 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.
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Approval

The thesis titled **"Synergistic Effect of Bacteriophage with Antibiotic in the Eradication of *Vibrio cholerae* Infection: An In-Vivo Analysis in Mice Model"** submitted by Fariya Akter (22176005) of Spring, 2022 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Master of Science in Biotechnology on 28th August 2024.

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Ethics Statement

This study was approved by the Institutional Ethical Review Committee (IERC) of the Department of Biochemistry and Molecular Biology, University of Dhaka.

Abstract

Cholera remains a significant global health threat, especially in regions like Bangladesh with annual outbreaks. The causative agent, *Vibrio cholerae*, thrives in aquatic environments and forms biofilms, complicating treatment. While antibiotics are effective, their unregulated use has led to rapid resistance, raising concerns of a potential pandemic. Bacteriophages present a promising alternative, yet phage resistance has also emerged. This study explores the synergistic effect of combining reduced doses of antibiotics with a bacteriophage cocktail to treat cholera. Ampicillin was tested at 500 µg/kg and 50 µg/kg, yielding 50% and 0% survival in infected mice, respectively, compared to 100% survival at the standard 50 mg/kg dose. When combined with a cocktail of two bacteriophages (10^9 PFU/ml each), survival rates improved to 88% and 75%, respectively. These results highlight the potential of combined therapy to reduce both antibiotic and bacteriophage resistance, offering a more effective treatment strategy.

Keywords: *V. cholerae*, Cholera, Bacteriophage cocktail, Antibiotic resistance, Phage-antibiotic synergy, JSF2, JSF25.

Dedication

I dedicate this work solely to my family and supervisor Dr. Iftekhar Bin Naser.

Acknowledgement

This dissertation holds a special place in my heart, as I have carefully and diligently chosen this topic of research. It may seem like a small step, but for me, it represents a significant leap towards fulfilling my passion for research. Throughout this journey, I faced numerous challenges and setbacks, and I am immensely grateful for the support and encouragement I received during those difficult times.

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List of Acronyms

WHO	World Health Organization
OCV	Oral Cholera Vaccine
CDC	Centers for Disease Control and Prevention
ORS	Oral rehydration saline
WASH	Water, sanitation, and hygiene
TCP	Toxin-coregulated pilus
TCBS	Thiosulfate, Citrate, Bile salts, Sucrose
LB	Luria-Bertani
PEG	Polyethylene Glycol
OD	Optical Density

Chapter 1

Introduction

Cholera is an acute diarrheal disease caused by *Vibrio cholerae*, giving rise to a huge public health concern worldwide. It has already caused seven distinct pandemics since the onset of the first pandemic in 1817 (Devault et al., 2014). *Vibrio cholera* is a member of the Vibrionaceae family, a gram-negative curved rod and motile microorganism. The distinction within its species is given based on serogroup, cholera enterotoxin production, and the potential to cause an epidemic. Until today, 200 serogroups have been identified and grouping has been done based on the heat-stable O-antigen present on the bacterial surface. Among all the serogroups, O1 and O139 are causing major outbreaks around the globe. Other than these two, a few other serogroups have caused occasional outbreaks but did not impose potential threats for the future (Mandal et al., 2011). Colonization of toxigenic *Vibrio cholera* in the human small intestine influences the production of enterotoxin, cholera toxin (CT) that causes the disease and can kill an individual within hours if proper treatment is not given. Areas including southern Asia, parts of Africa, and Latine America experience seasonal cholera outbreaks due to inadequate access to safe drinking water and sanitation (Faruque, Albert, & Mekalanos, 1998). Moreover, *Vibrio cholerae* has recently become endemic in areas where cholera had not been previously observed. (Almagro-Moreno, Pruss, & Taylor, 2015). According to the report of the World Health Organization, each year there are 1.3 to 4.0 million cholera infection cases have been observed, and 21000 to 143000 deaths have been worldwide (World Health Organization, 2023).

The immediate treatment for cholera involves administering oral or intravenous hydration to all patients to restore electrolyte levels. Moreover, antibiotic therapy is recommended in conjunction with rehydration for patients who are severely or moderately dehydrated or have

experienced significant stool loss. This dual approach aims to effectively manage cholera and prevent complications associated with dehydration (Sharifi-Mood & Metanat, 2014). Having said that, continuous antibiotic treatment contributes to the proliferation of antimicrobial resistance. Therefore, an alternative strategy is necessary for treating primary infections and preventing secondary spread. Phage therapy has emerged as a promising alternative; however, concerns about the development of phage resistance remain prevalent (Bhandare et al., 2018).

To address these challenges, we aimed to investigate the synergistic effects of bacteriophages and antibiotics in mice infected with cholera. Our approach seeks to reduce the development of antibiotic and phage resistance over time by using antibiotics at sub-inhibitory concentrations and employing a bacteriophage cocktail to minimize the risk of phage resistance. We infected P4 - P5 mice with *Vibrio cholerae* and initiated treatment one-hour post-infection, with a bacterial input ranging between 10^8-10^9 CFU/ml. We analyzed infection time, survival time, and the synergistic effect of the bacteriophage cocktail and antibiotic. The overall data indicate a positive correlation between the bacteriophage cocktail and antibiotic in treating cholera in the mouse model. This approach shows potential for further analysis and possible application in human treatment.

Chapter 2

Literature Review

2.1 Cholera

Cholera is an acute diarrheal disease caused by ingesting food or water contaminated with the bacterium *Vibrio cholerae* (Abdulhadi et al., 2018). This pathogen produces a potent enterotoxin that disrupts normal ion transport in the gut, leading to severe watery diarrhea, rapid dehydration, and, if untreated, potential death. Symptoms range from mild diarrhea to severe dehydration and electrolyte imbalance, which can occur within hours of infection (Cohen, 2022). The work of John Snow during the 1854 London outbreak, where he traced the source of infection to a contaminated water pump, was pivotal in identifying the link between water contamination and cholera transmission, laying the groundwork for modern epidemiology and public health measures (Newsom, 2006). Cholera has a storied history, with seven pandemics recorded since the 19th century. The first pandemic began in 1817 in the Ganges Delta and spread across Asia, the Middle East, Africa, and Europe, marking cholera's devastating global impact (Cockburn & Cassanos, 1960). Cholera remains a significant global health challenge today, affecting an estimated 1.3 to 4.0 million people annually, with death tolls reaching up to 143,000. The disease is most prevalent in regions with poor sanitation and limited access to clean drinking water, primarily affecting Africa, South Asia, and parts of the Americas (Ali et al., 2015). Recent outbreaks, such as those in Yemen, Haiti, and several African countries, have underscored the disease's persistent threat. In Yemen, ongoing conflict has exacerbated the cholera crisis, with over 2 million cases reported since 2016 (Federspiel & Ali, 2018). In Haiti, a devastating outbreak followed the 2010 earthquake, resulting in over 800,000 cases and nearly 10,000 deaths. These outbreaks highlight the critical need for robust public health infrastructure and rapid response mechanisms (Piarroux et al., 2022).

The impact of cholera on affected areas is profound. Outbreaks can quickly overwhelm local healthcare systems, leading to high morbidity and mortality rates, significant economic burdens, and social disruption. The mortality rate for untreated cholera can reach 50%, but with prompt and adequate treatment, particularly rehydration therapy, it can be reduced to less than 1%. Infection rates and outcomes vary depending on local infrastructure, the effectiveness of response measures, and the availability of medical supplies. Efforts to combat cholera involve various international organizations. WHO plays a central role in coordinating global response efforts, including deploying OCVs in endemic and epidemic settings (Legros, 2018). UNICEF focuses on improving WASH infrastructure, and promoting practices that reduce the risk of transmission. CDC and other agencies contribute to surveillance, outbreak response, and public health education (UNICEF, 2020). Preventive measures are crucial for controlling cholera. Key strategies include ensuring access to safe drinking water, improving sanitation facilities, promoting hygiene practices such as handwashing, and providing education on the importance of these measures. Rapid rehydration therapy, using ORS or intravenous fluids in severe cases, is essential for treating cholera. Antibiotics may also be administered to shorten the duration of the illness and reduce bacterial shedding. However, their use is generally reserved for severe cases due to the risk of developing antibiotic resistance (Global Task Force on Cholera Control, 2017). Despite these efforts, cholera remains endemic in more than 50 countries, largely due to ongoing challenges such as poverty, conflict, and inadequate infrastructure. Climate change and rapid urbanization further complicate efforts to control the disease, as they can exacerbate the conditions that facilitate the spread of *Vibrio cholerae* (Nelson et al., 2009). Effective cholera control requires a multifaceted approach, including vaccination, improved WASH infrastructure, rapid response to outbreaks, and public education. Continuous investment and coordinated global action are essential to mitigate the impact of cholera and prevent future outbreaks.

2.2 The Cholera Pathogen

Vibrio cholerae is a Gram-negative, comma-shaped bacterium responsible for the disease cholera. This pathogen thrives in aquatic environments such as rivers, estuaries, and coastal waters, often associating with plankton, shellfish, and other marine organisms. There are over 200 known serogroups of *V. cholerae*, but only two serogroups, O1 and O139, have been linked to epidemic and pandemic cholera. The O1 serogroup is further divided into two biotypes: classical and El Tor (Prouty & Klose, 2014). While the classical biotype was responsible for previous pandemics, the El Tor biotype has been the dominant strain in the current seventh pandemic, which began in 1961. The El Tor biotype's ability to cause asymptomatic infections and its enhanced environmental stability have contributed to its persistence and widespread distribution. *Vibrio cholerae* inhabits both freshwater and marine environments and can form biofilms on surfaces such as aquatic plants, zooplankton, and crustaceans (Nelson et al., 2009), (Harris et al., 2012). Biofilm formation enhances the bacterium's survival in adverse conditions and facilitates its transmission. Environmental factors such as temperature, salinity, and nutrient availability influence the abundance and activity of *V. cholerae* in aquatic habitats. Seasonal variations, particularly in tropical regions, often correlate with cholera outbreaks due to changes in water temperature and plankton blooms that provide a conducive environment for bacterial growth (Huq et al., 1983). The bacterium has developed several sophisticated mechanisms to evade the host immune response, ensuring its survival and pathogenicity within the human host. These strategies include structural modifications, the production of protective barriers, the secretion of immunomodulatory molecules, and the manipulation of host signaling pathways.

2.2.1 Pathogenesis of *Vibrio cholerae*

The pathogenesis of *Vibrio cholerae* involves a complex interplay of bacterial factors and host responses, leading to the characteristic severe diarrheal disease known as cholera. Here is an in-depth explanation of the pathogenesis process:

Ingestion and Colonization

- **Ingestion**: *Vibrio cholerae* is typically ingested through contaminated water or food. The infectious dose is relatively high, requiring about 10^6 to 10^{11} bacteria to cause disease in healthy individuals.
- **Survival in the Stomach**: The acidic environment of the stomach poses a significant barrier to the bacteria. However, *V. cholerae* can survive due to its acid tolerance response, which is enhanced when the bacteria are consumed with food, buffering the stomach acid.
- **Colonization of the Small Intestine**: Upon reaching the small intestine, *V. cholerae* must navigate through the viscous mucus layer lining the intestinal walls. The bacterium uses its polar flagellum for motility to penetrate this mucus barrier. *V. cholerae* then attaches to the epithelial cells of the small intestine using pili and other adhesion molecules. A crucial adhesin in this process is the TCP, which is essential for colonization and virulence (Nelson et al., 2009).

Toxin Production and Mechanism of Action

- **Cholera Toxin (CT)**: The primary virulence factor of *V. cholerae* is the cholera toxin, an A-B type exotoxin. The CT consists of one A subunit and five B subunits. The B subunits bind to GM1 ganglioside receptors on the surface of intestinal epithelial cells, facilitating the entry of the A subunit into the cell (Harris et al., 2012).

- **Toxin Internalization and Activation**: Once inside the host cell, the A subunit is cleaved into two parts, A1 and A2. The A1 fragment ADP-ribosylates the Gs alpha subunit of the adenylate cyclase enzyme, leading to the activation of adenylate cyclase and a subsequent increase in cyclic AMP (cAMP) levels within the cell (Harris et al., 2012).
- **Ion Secretion and Water Loss**: Elevated cAMP levels lead to the opening of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels in the cell membrane. This results in the secretion of chloride ions into the intestinal lumen. Sodium ions and water follow the chloride ions osmotically, causing a massive outflow of electrolytes and water into the intestinal lumen. This process leads to the characteristic watery diarrhea of cholera (Harris et al., 2012).

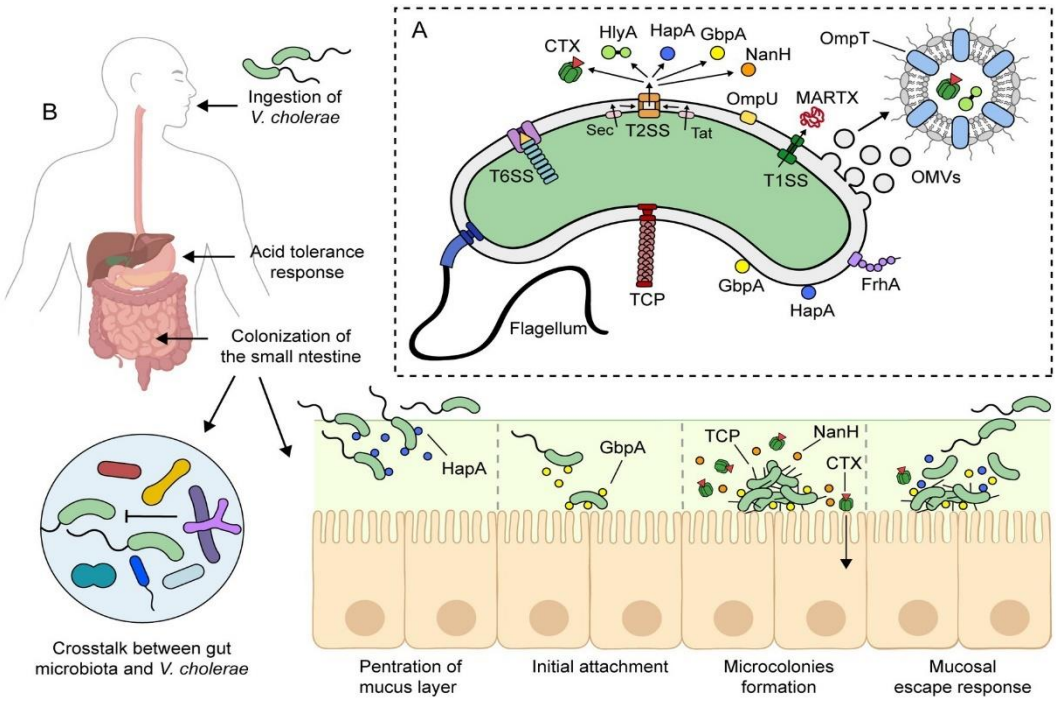

Figure 1: Pathogenesis of V. cholerae (Montero et al., 2023)

2.2.2 Pathological consequences

Following infection, the incubation period of cholera ranges from a few hours to five days, with symptoms typically appearing within two to three days. The severity of the disease varies,

with most infections being asymptomatic or mild. However, in about 5% of cases, the disease progresses to severe dehydration and shock if not promptly treated. This severe diarrhoea is accompanied by vomiting and can lead to rapid dehydration and electrolyte imbalance, particularly if untreated. Patients typically present with intense thirst, dry mucous membranes, decreased skin turgor, and sunken eyes—a reflection of severe fluid loss. The host immune response to *Vibrio cholerae* infection involves a complex interplay of innate and adaptive mechanisms aiming to control and eliminate the pathogen (Nelson et al., 2009). The disease's acute nature and ability to cause widespread fluid loss underscore the importance of timely medical intervention to mitigate its potentially fatal outcomes (Harris et al., 2012). Survivors of cholera infection can become carriers, shedding the bacteria in their stool for up to several weeks, which can contribute to the spread of the disease, especially in areas with poor sanitation (Faruque et al., 2003). Beyond acute symptoms, some individuals may become asymptomatic carriers of *Vibrio cholerae*, contributing to ongoing transmission. Thus, Effective public health measures, including vaccination campaigns, improved sanitation infrastructure, and rapid response to outbreaks, are critical in mitigating the impact of cholera and preventing its resurgence in endemic regions (World Health Organization, 2017).

2.2.3 Management strategies

Cholera management integrates a comprehensive array of clinical treatments and public health strategies aimed at reducing morbidity and mortality while preventing further transmission. Central to clinical management is the swift administration of rehydration therapy, primarily through ORS for mild to moderate cases and intravenous fluids for severe dehydration to restore electrolyte balance and fluid levels (World Health Organization, 2017). Antibiotics like tetracycline, doxycycline, or azithromycin are often prescribed to shorten the duration and severity of symptoms, particularly in severe cases where prompt intervention is crucial to

prevent complications (Ali et al., 2015). Supportive care includes meticulous monitoring of electrolyte levels and nutritional support to aid recovery and prevent secondary complications. Public health interventions focus on early detection and rapid response through surveillance systems to detect outbreaks swiftly and implement control measures. Improving access to safe water through methods such as chlorination and filtration, and promoting adequate sanitation practices, are fundamental to interrupting the fecal-oral transmission cycle of *Vibrio cholerae* (World Health Organization, 2017). Vaccination campaigns with OCVs are pivotal in endemic regions and during outbreaks to reduce transmission and protect vulnerable populations (World Health Organization, 2017; Ali et al., 2015). These vaccines contribute significantly to cholera prevention efforts by enhancing community immunity and reducing the spread of the disease. Community engagement and education are essential components, empowering individuals with knowledge about hygiene practices such as handwashing with soap and safe food handling to minimize cholera transmission. By integrating these multifaceted approaches, effective cholera management aims to mitigate the impact of outbreaks, reduce mortality rates, and improve public health outcomes in endemic regions.

2.3 Bacteriophages: Nature’s Tiny Predators

Bacteriophages, or phages, represent a fascinating group of viruses that have evolved to specifically target and infect bacterial cells. Their discovery dates back to the early 20th century, with the pioneering work of Frederick Twort and Félix d'Hérelle, who independently observed the lytic activity of these viruses against bacterial cultures. Since then, phages have been extensively studied and harnessed for various applications, ranging from bacterial typing and diagnostic tools to therapeutic agents against bacterial infections. These are viruses that specifically infect bacteria. They are ubiquitous and play a crucial role in controlling bacterial populations and facilitating horizontal gene transfer. Bacteriophages comprise of several components including capsid, tail, tail fibers or spikes, baseplate, and accessory proteins that

enable their infectivity and replication within bacterial cells (Krupovic & Bamford, 2008; Ackermann & Prangishvili; 2012, Taylor et al., 2016; Bertozzi Silva et al., 2016; Young, 2014). Understanding the classification of bacteriophages is crucial for unravelling their complex biology and harnessing their potential in various applications, including biotechnology and medicine. Phages exhibit remarkable diversity in morphology, genomic composition, life cycles, and host interactions, leading to their classification into distinct groups based on these fundamental characteristics. Morphologically bacteriophages are classified into myoviridae, siphoviridae, podoviridae, and cystoviridae (Ackermann & Prangishvili, 2012; Young, 2014, Kutter et al., 1995). Based on the genetic material they can be DNA phages or RNA phages (Calendar, 2006; Young, 2014). Depending on the life cycle they follow, they can be lytic phages of lysogenic phages (Calendar, 2006; Young, 2014; Ptashne, 2004). Moreover, in nature, we have some specialized phages. These phages can be categorized into several types based on their specialized functions and characteristics, including transducing phages, filamentous phages, and phages containing the CRISPR-Cas system (Boyd & Brussow, 2002; Smith, 1985; Rakonjac et al., 2011; Hynes et al., 2014)

2.3.1 Bacteriophage Infection Procedure

The infection process of bacteriophages, or phages, is a highly coordinated sequence of events that ensures the successful replication of the phage within the bacterial host. This process can be broadly divided into several key stages: attachment, penetration, biosynthesis, maturation, and release.

- **Attachment (Adsorption):** The infection begins with the phage's attachment to the bacterial cell's surface. Specific interactions mediate this attachment between the phage's tail fibers or spikes and receptors on the bacterial cell surface. These receptors are often specific proteins, lipopolysaccharides, or other molecules present on the

bacterial membrane. The specificity of these interactions determines the host range of the phage, meaning which bacteria can be infected by a particular phage (Hyman & Abedon, 2010). For example, bacteriophage T4 attaches to Escherichia coli using its long tail fibers that recognize and bind to the outer membrane protein C (OmpC).

- **Penetration (Injection):** Following attachment, the phage injects its genetic material into the bacterial cell. For tailed phages, this involves the contraction of the tail sheath (in Myoviridae phages) or other mechanisms that facilitate the passage of the phage DNA or RNA through the bacterial cell wall and membrane. The capsid, or protein coat, of the phage remains outside the bacterial cell during this process (Molineux, 2006). During this stage, the bacteriophage T4 contracts its tail sheath, driving a needle-like tube through the bacterial envelope to deliver its DNA into the host cytoplasm.
- **Biosynthesis:** Once inside the host cell, the phage's genetic material hijacks the bacterial machinery to begin the synthesis of phage components. Early genes are expressed first, often coding for proteins that degrade the host DNA and protect phage DNA from host defenses. This stage includes the replication of phage nucleic acids and the transcription and translation of phage proteins. The host's resources are diverted towards the production of new phage particles, including capsid proteins, tail fibers, and other necessary components (Calendar, 2006). For instance, the bacteriophage T4 utilizes its early gene products to degrade the host's DNA and modify RNA polymerase to preferentially transcribe phage genes.
- **Maturation (Assembly):** During maturation, newly synthesized phage components are assembled into complete virions. This assembly process is highly ordered and involves the formation of the capsid, packaging of the phage genome into the capsid, and the attachment of tail structures. In some phages, the DNA is inserted into preformed capsids through a specialized motor protein (Catalano, 2005). The assembly process

ensures that each new virion is correctly formed and capable of infecting new bacterial cells. The bacteriophage T4, for example, assembles its head and tail separately before combining them into a mature virion.

- **Release:** The final stage of the phage life cycle involves the release of mature phage particles from the bacterial cell. This is achieved through lysis in lytic phages, where enzymes such as endolysins degrade the bacterial cell wall, causing the cell to burst and release progeny phages. In filamentous phages, the release process is non-lytic, with phage particles extruding through the bacterial membrane without killing the host cell immediately (Rakonjac et al., 2011). The bacteriophage T4 uses holins and lysins to break down the bacterial cell wall, leading to cell lysis and release of phage particles.

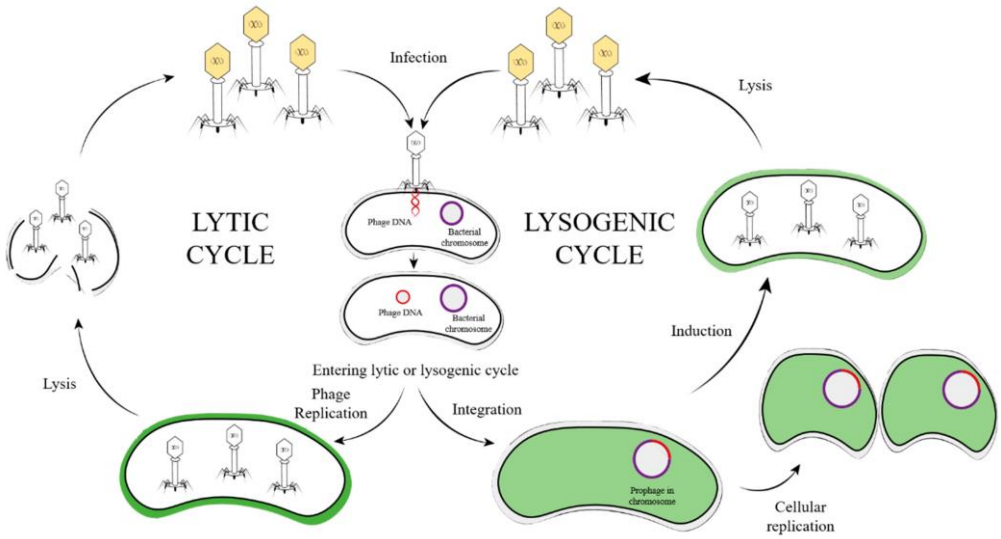

Figure 2: Infection cycle of Bacteriophage (Zhou et al., 2023)

2.3.2 JSF2 and JSF 25

JSF2 and JSF25 are specific bacteriophages that have garnered significant attention for their effectiveness against *Vibrio cholerae* infections. JSF2 belongs to the Myoviridae family, known for its long, contractile tails used to inject genetic material into host cells (Weinbauer, 2004). This phage initiates infection by attaching to specific receptors on the *V. cholerae* cell surface, injecting its DNA through the contraction of its tail sheath, and subsequently hijacking the bacterial machinery for replication and assembly. The process culminates in the lysis of the

bacterial cell, releasing new phage particles (Comeau & Krisch, 2008). On the other hand, JSF25 is classified under the Podoviridae family, characterized by short, non-contractile tails. This phage also binds to specific receptors on *V. cholerae*, injects its genetic material into the host cell, and follows a similar replication and lysis process as JSF2, albeit with a simpler tail structure (Chibani-Chennoufi et al., 2004).

Genomic studies of JSF2 and JSF25 have revealed the genes responsible for their replication, assembly, and lysis mechanisms, providing insights into their infection processes and host specificity (Weinbauer, 2004). Structural analyses through techniques such as electron microscopy have detailed the intricate structures of their tails and capsids, contributing to the understanding of their functionality (Comeau & Krisch, 2008). The specificity and effectiveness of JSF2 and JSF25 make them strong candidates for phage therapy, particularly in addressing antibiotic-resistant *V. cholerae* strains (Górski et al., 2017).

Research suggests that these phages can effectively recognize and bind to *V. cholerae* receptors, leading to the bacterial cell's destruction. Further studies are needed to explore their efficacy and safety in clinical settings, with animal models and clinical trials essential for validating their therapeutic potential (Chibani-Chennoufi et al., 2004). Understanding the interactions between these phages and their bacterial hosts is crucial for optimizing their application in phage therapy. This includes examining how these phages overcome bacterial defenses and their impact on bacterial populations within the gut microbiome (Weinbauer, 2004; Comeau & Krisch, 2008; Górski et al., 2017).

2.4 Antibiotic Therapy for Cholera: Efficacy and Considerations

Antibiotic therapy has been a critical component of cholera treatment, particularly in severe cases, complementing the primary treatment of rehydration therapy. The administration of

antibiotics is required to reduce the duration and severity of symptoms, preventing complications, and limiting the spread of the disease. Tetracycline, doxycycline, ciprofloxacin, and azithromycin are among the antibiotics commonly used for treating cholera infections.

The use of antibiotics in cholera treatment began in the mid-20th century. Early studies demonstrated that antibiotics could reduce the volume and duration of diarrhea and shorten the period of bacterial shedding in stool, thereby reducing transmission. The choice of antibiotic and treatment duration may vary depending on factors such as the severity of the infection, antibiotic susceptibility patterns, and local guidelines. The efficacy of antibiotics in treating cholera is well-documented. Antibiotics can reduce the volume of diarrhea by up to 50%, shorten the duration of diarrhea by 1-2 days, and decrease the period of *V. cholerae* shedding by several days (Nelson et al., 2009). These effects not only benefit the individual patient but also help control outbreaks by reducing the transmission potential.

Tetracycline, doxycycline, ciprofloxacin, and azithromycin were the first antibiotics used extensively in the treatment of cholera, showing significant efficacy in reducing the severity and duration of the illness (Levine et al., 1978; Sack et al., 2004). The success rate of antibiotic therapy in cholera is generally high when used appropriately. However, the emergence of antibiotic-resistant strains of *V. cholerae* poses a significant challenge. For instance, multi-drug-resistant strains have been reported in various parts of the world, including South Asia and Africa, complicating treatment efforts (WHO, 2017). Therefore, judicious antibiotic use, adherence to treatment guidelines, and surveillance of antibiotic resistance patterns are essential for mitigating the risk of resistance development and preserving the efficacy of available antibiotics for cholera treatment.

2.5 Phage Therapy

Phage therapy began with the discovery of bacteriophages by British bacteriologist Frederick Twort in 1915 and independently by French-Canadian microbiologist Félix d'Hérelle in 1917. D'Hérelle's work at the Pasteur Institute led him to coin the term "bacteriophage" (meaning "bacteria eater") and to recognize its therapeutic potential, leading to the first documented cases of phage therapy for treating bacterial infections like dysentery and cholera.

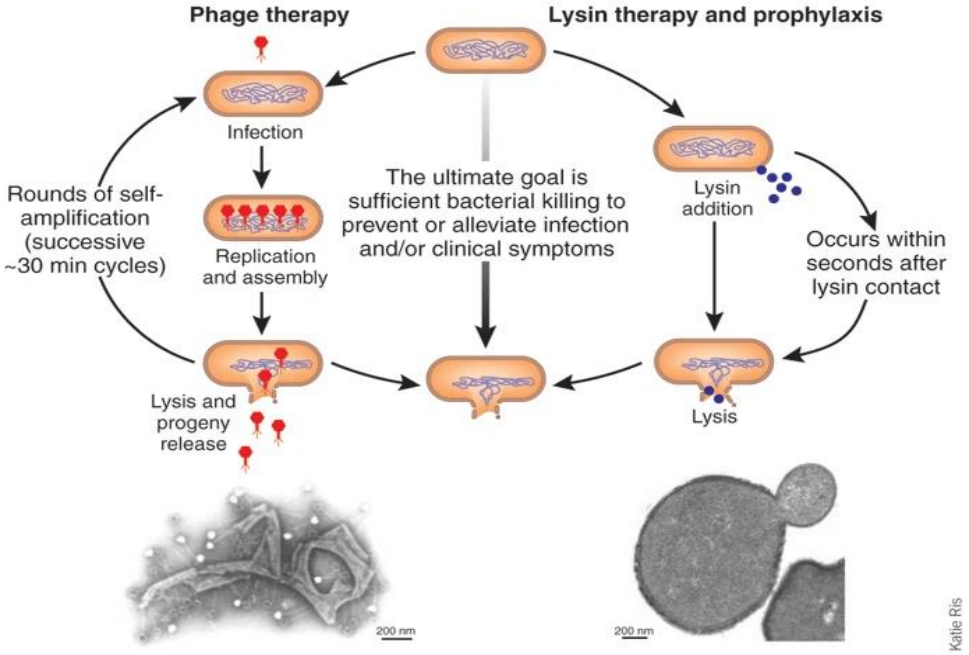

Figure 3: Phage Therapy (Fischetti et al., 2006)

The use of bacteriophages to treat bacterial infections, has gained renewed interest due to the rise of antibiotic-resistant bacteria. Bacteriophages specifically infect and lyse bacterial cells, offering a targeted approach to combating bacterial infections. This method exploits the natural predator-prey relationship between phages and bacteria, where phages attach to specific receptors on the bacterial surface, inject their genetic material, and replicate within the bacterial cell. The subsequent lysis of the bacterium releases new phage particles, which can then infect

other bacterial cells. This process continues until the bacterial population is significantly reduced or eliminated (Kutter et al., 2010).

The research landscape for phage therapy has expanded significantly over the past few decades. Early studies focused on isolating and characterizing bacteriophages from various environments, including soil, water, and clinical samples. These efforts have led to the discovery of numerous phages with potential therapeutic applications. Advances in genomics have played a crucial role in this field, allowing researchers to sequence phage genomes and understand their interactions with bacterial hosts at a molecular level. Bacteriophage T4 is a well-studied lytic phage that infects Escherichia coli. T4 has been extensively researched for its complex structure and efficient infection process, making it a model organism in molecular biology. Its success in laboratory settings has paved the way for understanding phage biology and its potential therapeutic applications (Kutter et al., 2010). Bacteriophage M13 is a filamentous phage that infects E. coli. Unlike lytic phages, M13 does not cause cell lysis but rather extrudes through the bacterial cell membrane, allowing continuous production of phage particles without killing the host. This characteristic makes M13 useful in genetic engineering and biotechnology, particularly in phage display technology (Smith & Petrenko, 1997).

One notable study by Chan, Abedon, and Loc-Carrillo (2013) emphasizes the adaptability of phages in evolving alongside bacteria. This evolutionary advantage allows phages to counteract bacterial resistance mechanisms that typically render antibiotics ineffective. The study discusses the potential of phage cocktails, which combine multiple phages to target a broader range of bacterial strains, thereby reducing the likelihood of resistance development (Chan et al., 2013). Another significant study by Kutter et al. (2010) highlights the efficacy of phage therapy in treating bacterial infections in animal models, providing a foundation for human clinical trials (Kutter et al., 2010). Another case involved a patient with a multidrug-resistant

Acinetobacter baumannii infection. Despite receiving all available antibiotics, the patient's condition continued to deteriorate. As a last resort, doctors administered a cocktail of bacteriophages specifically tailored to target the patient's bacterial strain. The treatment led to a dramatic reduction in bacterial load and a significant improvement in the patient's condition, ultimately resulting in recovery (Schooley et al., 2017). This case underscores the potential of phage therapy to save lives in situations where conventional treatments are ineffective.

2.6 Phage Therapy for Treating Cholera

Phage therapy has been explored as an alternative to traditional antibiotics for treating cholera, given the increasing antibiotic resistance observed in *V. cholerae* strains. Research has demonstrated that phages specific to *V. cholerae*, are effective in lysing the bacterium, thereby reducing bacterial load and mitigating the symptoms of cholera (Khan et al., 2020).

The procedure for phage therapy involves isolating specific phages from the environment or sewage that are active against the target bacterial strain. These phages are then amplified in bacterial cultures and purified for therapeutic use. For cholera, phages are isolated based on their ability to infect and lyse *V. cholerae*. Once a suitable phage preparation is developed, it can be administered orally or rectally, depending on the site of infection. The administration method ensures that phages reach the gut where *V. cholerae* resides (Kutter et al., 2010).

The success of phage therapy in treating cholera has been documented in various studies. For instance, a study by Sarker et al. (2016) demonstrated the efficacy of oral phage therapy in reducing *V. cholerae* counts in a rabbit model. The study highlighted that phage therapy led to a significant decrease in bacterial load and improved survival rates compared to untreated controls. Moreover, phage therapy was well-tolerated, with no adverse effects reported, underscoring its safety and potential as a viable treatment option. In the context of cholera

treatment, phage therapy holds particular promise due to its specificity for *V. cholerae* strains and its ability to target antibiotic-resistant variants. Phages have demonstrated lytic activity against *V. cholerae* in preclinical studies, utilizing mechanisms such as receptor binding, DNA injection, and bacterial lysis to effectively reduce bacterial load and alleviate symptoms of infection. These phages offer a targeted approach to cholera treatment, minimizing collateral damage to the host microbiota and potentially reducing the risk of antibiotic resistance development. For instance, research by Yen et al. (2017) demonstrated the effectiveness of a cocktail of three virulent bacteriophages in preventing *V. cholerae* infection in animal models. The study highlighted the therapeutic potential of phages in mitigating cholera transmission and reducing bacterial burden.

One of the key advantages of phage therapy is its ability to overcome antibiotic resistance, a growing concern in the management of cholera and other infectious diseases. This adaptability allows phages to effectively combat antibiotic-resistant *V. cholerae* strains, offering a valuable therapeutic option for patients with multidrug-resistant infections. Moreover, phage therapy may offer a more sustainable and environmentally friendly approach to cholera treatment compared to antibiotics.

2.7 Enhancing Antibacterial Efficacy: Phage-Antibiotic Synergy

Phage-antibiotic synergy, a novel therapeutic approach, involves the combination of bacteriophages (phages) with antibiotics to enhance the efficacy of bacterial infection treatment. This synergistic interaction between phages and antibiotics has gained significant attention in recent years due to its potential to overcome antibiotic resistance, improve treatment outcomes, and reduce the risk of treatment failure. The rationale behind phage-

antibiotic synergy lies in the complementary mechanisms of action of phages and antibiotics, which together can target bacteria more effectively than either treatment alone. By combining phages with antibiotics, researchers aim to exploit the lytic activity of phages to weaken bacterial cells and enhance the susceptibility of bacteria to antibiotics. Phages can penetrate bacterial biofilms, a protective matrix that shields bacteria from antibiotics, and disrupt bacterial cell walls, allowing antibiotics to penetrate and exert their antimicrobial effects more effectively. Additionally, phages can target antibiotic-resistant bacteria, including those harbouring multidrug resistance mechanisms, by exploiting alternative pathways for bacterial killing. Conversely, sub-lethal concentrations of antibiotics can stress bacteria, making them more susceptible to phage infection. This dual assault can overwhelm bacterial defence mechanisms, leading to more effective bacterial clearance.

Several studies have demonstrated the potential of phage-antibiotic synergy in enhancing the efficacy of antibiotic treatment against bacterial infections. For example, a study by Torres-Barceló et al. (2018) investigated the synergistic interaction between phages and antibiotics against *Pseudomonas aeruginosa* infections in vitro and in vivo. The researchers found that the combination of phages with antibiotics resulted in greater bacterial killing compared to either treatment alone, suggesting a synergistic effect between phages and antibiotics in combating antibiotic-resistant *P. aeruginosa* strains. Another study by Comeau et al. (2007) demonstrated that the combination of phages and antibiotics could prevent the emergence of bacterial resistance. In this study, the use of phages in conjunction with sub-inhibitory concentrations of antibiotics significantly reduced the development of antibiotic-resistant mutants compared to antibiotic treatment alone (Comeau et al., 2007). This finding highlights the potential of PAS to mitigate one of the most pressing challenges in contemporary medicine: antibiotic resistance. Moreover, observational studies and retrospective analyses have provided valuable insights into the real-world application of phage-antibiotic synergy in clinical practice. Sarker et al.

(2016) conducted a retrospective study to assess the efficacy of phage-antibiotic combination therapy in treating bacterial infections in patients with burn wounds. The study reported favorable outcomes, including reduced infection rates and improved wound healing, in patients treated with the combination therapy compared to antibiotics alone.

2.8 Advantages of Phage-Antibiotic Synergy

The advantages of phage-antibiotic synergy (PAS) are numerous and hold significant promise for addressing the burgeoning issue of antibiotic resistance. First and foremost, PAS can significantly reduce the dosage and duration of antibiotic therapy needed to achieve clinical success. By employing a dual approach that leverages both phages and antibiotics, lower doses of antibiotics are often sufficient to clear infections. This reduction in antibiotic use minimizes the side effects and toxicity associated with prolonged antibiotic treatments, making therapy safer for patients. Research has demonstrated that lower antibiotic dosages, when combined with phages, can achieve effective bacterial clearance, thereby reducing adverse effects and the risk of antibiotic-associated complications such as Clostridioides difficile infection (Abedon et al., 2011). Another key advantage of PAS is its ability to restore the effectiveness of older antibiotics that have become less effective due to widespread resistance. Phages can weaken bacterial defenses by disrupting cell walls and membranes, making the bacteria more susceptible to antibiotics. This "revitalization" of antibiotics can extend their useful lifespan and provide additional treatment options against resistant bacteria. For instance, studies have shown that phages can sensitize methicillin-resistant Staphylococcus aureus (MRSA) to beta-lactam antibiotics, thereby enhancing the antibiotics' effectiveness against otherwise resistant strains (Torres-Barceló et al., 2018). PAS also offers a multifaceted attack on bacterial populations, reducing the likelihood of resistance development. Bacteria exposed to both phages and antibiotics face simultaneous lethal stressors from different mechanisms of action,

making it more difficult for them to develop resistance. This combinatory approach exploits the weaknesses of bacteria, with phages targeting specific receptors and antibiotics interfering with essential cellular processes. The result is a synergistic effect that can overwhelm bacterial defenses. Studies have demonstrated that this dual approach significantly reduces the emergence of resistant bacterial mutants compared to monotherapy with either phages or antibiotics alone (Chan et al., 2016). Furthermore, PAS can be particularly effective in treating biofilm-associated infections, which are notoriously resistant to antibiotics. Biofilms protect bacteria from hostile environments and reduce the penetration of antibiotics. However, phages have a natural ability to penetrate and disrupt biofilms, thereby enhancing the efficacy of antibiotics. This combination can lead to the complete eradication of biofilms, which is essential for treating chronic infections in wounds, medical devices, and respiratory conditions. Research has shown that phage-antibiotic combinations can effectively eradicate biofilms formed by Pseudomonas aeruginosa and Staphylococcus aureus, significantly improving clinical outcomes (Alves et al., 2014; Donlan, 2009).

Chapter 3

Materials and Methods

3.1 Bacterial Culture Media

TCBS agar

TCBS is a Selective and differential medium primarily used to isolate and identify Vibrio species, including *Vibrio cholerae*. This media contains citrate and thiosulfate that support *vibrio* growth by generating a high pH environment. Bile Salt inhibit the growth of Gram-positive and most non-enteric Gram-negative bacteria. D-sucrose helps in the fermentation. Thymol And Bromothymol Blue indicate the change in acid production from sucrose fermentation. *Vibrio cholerae* grows as yellow-coloured colonies in TCBS agar.

During this research, TCBS agar has been used as a selective media for growing required *vibrio cholerae* strains including, 1877, and WT346.

LB media

LB media is widely used in various applications related to microbial growth due to its rich nutrient composition, which supports the rapid and robust growth of bacteria. In this research, both LB broth and LB agar media were utilized for bacterial culture as needed. Additionally, soft agar (LB + 0.6% agar) was prepared for DLA and plaque assays of bacteriophages. To evaluate the antibiotic susceptibility of the bacteria used in this research, ampicillin (100ug/ml) and kanamycin (50ug/ml) have been added to LB agar plates.

During In vivo analysis, the mouse intestine was homogenized with, LB media supplemented with 20% glycerol.

3.2 In-Vitro Experiments

3.2.1 **Plaque Assay** or **Spot Assay**

Day 1

- Retrieve the required bacteria *Vibrio cholerae* 1877 for bacteriophage selection.
- Streak *Vibrio cholerae* 1877 on a TCBS agar plate from the bacterial stock.
- Incubate the plate overnight at 37°C.

Day 2

- Select 2-3 single colonies of *Vibrio cholerae* and prepare a young culture.
- After incubation, young culture of *Vibrio cholerae* 1877 was added to soft agar (Luria Bertani broth containing 0.6% Bactoagar, Difco) and overlaid on the freshly prepared LA plates.
- Spot each phage dilution onto the surface of the solidified soft agar layer.
- Allow the spots to absorb into the agar, and incubate the plates at 37 degrees
- Examine the plates for clear zones (plaques) where the phages have lysed the bacteria.

3.2.2 Enrichment of Bacteriophage

Day 1:

- Retrieve the host bacteria (WT 346) for the specific bacteriophages (JSF2, JSF25).
- Streak WT 346 on a TCBS agar plate from the bacterial stock.
- Incubate the plate overnight at 37°C.

Day 2

- Select colonies of WT 346 from the plate and prepare a young culture.
- Once the young culture is prepared, add pure bacteriophage from the stock.

- Incubate the mixture in a shaker incubator at 37°C, centrifuge, and sterilize using a 0.22-micron syringe filter.
- Store the phage stock at 4°C.

3.2.3 Double Layer Agar Assay: JSF2, JSF25

Day 1:

- Retrieve the host bacteria (e.g. WT 346).
- Streak the bacteria onto an LB agar plate from the bacterial stock.
- Incubate the plate overnight at 37°C to obtain isolated colonies.

Day 2:

- Select colonies of the host bacteria from the plate, and prepare young culture.
- Prepare serial dilutions of the bacteriophage stock using SM buffer. Typically, prepare dilutions from 10^{-1} to 10^{-9}.
- Mix each phage dilution with the host bacterial culture.
- Add the mixture to soft agar (0.6% agar).
- Pour, and gently swirl the plate to ensure an even distribution of the mixture.
- Incubate the plates at 37°C.

Day 3

- Examine the plates for clear zones (plaques) where the phages have lysed the bacteria.
- Count the number of plaques at the dilution level where they are countable (usually 20-200 plaques per plate).
- Calculate the phage titer (plaque-forming units per ml, PFU/ml) based on the dilution factor and the volume plated.

3.2.4 Polyethylene Glycol Precipitation of Bacteriophage: JSF2, JSF25

- Combine the freshly enriched phage solution with PEG-NaCl solution.
- Incubate the mixed solution at 4^{o} Celsius.
- Centrifuge, and carefully discard the supernatant without disturbing the supernatant.
- Resuspend the bacteriophage pellet in the desired volume of SM buffer.
- Perform DLA to evaluate the bacteriophage titer.

3.2.5 Bacterial Growth Curve Analysis

- *Vibrio cholerae* was inoculated from a single colony into LB broth and incubated overnight at 37°C with shaking.
- The overnight culture was diluted to an initial OD600 of 0.05 in fresh LB broth
- OD600 of the culture was measured at regular intervals (every hour) using a spectrophotometer.
- The OD600 values were recorded to monitor bacterial growth over time.
- The OD600 values were plotted against time to create a bacterial growth curve.
- The growth phases (lag, exponential, stationary) were analysed based on the plotted curve.

3.2.6 Antibiotic Susceptibility Testing for Selection of Antibiotic

- Four to five 1877 strains have been taken with a sterile inoculation loop and suspended in 2ml of sterile saline (0.9% NaCl). The saline tube was vortexed and the turbidity of the suspension was checked by comparing with 0.5 McFarland standard.
- A sterile swab was dipped into the inoculum tube and inoculated on MH agar plate.

- Selected antibiotic disks had been placed on the MH agar plate with the help of a sterile forceps.
- The plate was incubated in 37^0C incubator for 16 to 18 hours.
- After incubation the diameter of the zone was measured and compared with the given CLSI guideline.

3.3 In-Vivo Experiments

3.3.1 Infection Model Preparation

Bacterial Inoculum Preparation for Cholera Infection Model Preparation

- Inoculate *Vibrio cholerae* 1877 into LB broth and incubate overnight at 37°C with shaking.
- Transfer overnight bacterial culture into a centrifuge tube, centrifuge it, and dissolve the bacterial pellet in $NaHCO_3$ buffer.

Mouse Groups	**Bacteria (1877) CFU/ml – 10^8**	**Number of Mice examined**	**Study Period**
Adult Mouse	✓	10	24 Hours
Infants (P7 - P10)	✓	10	24 Hours
Infants (P4 – P5)	✓	10	24 Hours

Table 1: Preparation of Cholera Infection Model

3.3.2 Bacterial Infection Latency Analysis

- Inoculate *Vibrio cholerae* 1877 into LB broth and incubate overnight at 37°C with shaking.
- Transfer overnight bacterial culture into a centrifuge tube, centrifuge it, and dissolve the bacterial pellet in $NaHCO_3$ buffer.
- Administer the required amount of bacterial culture to a group of mice and evaluate hourly.

3.3.3 Temporal Dynamics of *Vibrio cholerae* Infection and Intestinal Bacterial Load

- Prepare bacterial inoculum as per the method previously described.
- Administer the required amount of bacterial culture to a group of mice.

Examined Mice (Aged 4-5 Days)	**Bacteria (1877) CFU/ml – 10^8**	**Number of Mice examined**	**Study Period**
Group 1	✓	10	4 hours
Group 2	✓	10	12 hours
Group 3	✓	10	18 hours

Table 2: Observation of temporal dynamics of cholera

- Sacrifice the mice after specific hours of infection introduction.
- Surgically remove the intestines.
- Grind the intestines and collect the supernatant.
- Prepare serial dilutions of the supernatant ranging from 10^{-1} to 10^{-3}.
- Spread 100 µl aliquots from each dilution onto TCBS agar plates.

3.3.4 Comparative Evaluation of Treatment Strategies for Cholera Infection in Mice: Intestinal Bacterial Load Analysis over time.

- Prepare bacterial inoculum as per the method previously described.
- Administrate the bacterial inoculum to each group, and give each group the required treatment after an hour.
- Mice were dissected the required hours post-treatment to assess bacterial growth.

Examined Group	Bacteria (1877) CFU/ml – 10^8	Antibiotic (Ampicillin 5gm/kg)	Bacteriophage cocktail	Number of Mice examined	Study Period
Group 1	✓	✗	✗	**6**	4 hours
Group 2	✓	✓	✗	**6**	4 hours
Group 3	✓	✗	✓	**6**	4 hours
Group 4	✓	✓	✓	**6**	4 hours

Table 3: Comparative treatment strategies for cholera infection (4-hour incubation)

Examined Group	Bacteria CFU/ml – 10^8	Antibiotic (Ampicillin-5gm/kg)	Bacteriophage cocktail	Number of Mice examined	Study Period
Group 1	✓	✗	✗	**6**	12 hours
Group 2	✓	✓	✗	**6**	12 hours
Group 3	✓	✗	✓	**6**	12 hours
Group 4	✓	✓	✓	**6**	12 hours

Table 4: Comparative treatment strategies for cholera infection (12-hour incubation)

Examined Group	Bacteria CFU/ml – 10^8	Antibiotic (Ampicillin 5gm/kg)	Bacteriophage cocktail	Number of Mice examined	Study Period
Group 1	✓	✗	✗	**6**	18 hours
Group 2	✓	✓	✗	**6**	18 hours
Group 3	✓	✗	✓	**6**	18 hours
Group 4	✓	✓	✓	**6**	18 hours

Table 5: Comparative treatment strategies for cholera infection (18-hour incubation)

- Sacrifice the mice following the required incubation period post-treatment.
- Surgically remove the intestines.
- Grind the intestines, and take the supernatant.
- Prepare serial dilutions ranging from 10^{-1} to 10^{-3}, and spread on TCBS agar plates.

3.3.5 Assessment of Treatment Efficacy in Promoting 48-Hour Survival of Cholera Infected Mice

- Prepare bacterial inoculum as per the method previously described.
- Administrate the bacterial inoculum to each group, and give each group the required treatment after an hour.

Examined Group	Bacteria CFU/ml – 10^8	Antibiotic (Ampicillin 5gm/kg)	Bacteriophage cocktail	Number of Mice examined	Study Period
Group 1	✓	✗	✗	8	48 hours
Group 2	✓	✓	✗	8	48 hours
Group 3	✓	✗	✓	8	48 hours
Group 4	✓	✓	✓	8	48 hours

Table 6: Assessment of treatment efficacy in survival

- Sacrifice the mice following the required incubation period post-treatment.
- Surgically remove the intestines.
- Grind the intestines, and take the supernatant.
- Prepare serial dilutions ranging from 10^{-1} to 10^{-3}, and spread on TCBS agar plates.

3.3.6 Evaluation of Critical Antibiotic Concentration for Survival of Infected Mice

- Prepare bacterial inoculum as per the method previously described.
- Administrate the bacterial inoculum to each group, and give each group the required treatment after an hour.

Examined Group	Bacteria CFU/ml – 10^8	Ampicillin 5gm/kg	Ampicillin 500mg/kg	Ampicillin 50mg/kg	Number of Mice Examined	Study Period
Control	✗	✗	✗	✗	3	18 hours
Control (Only Bacteria)	✓	✗	✗	✗	3	18 hours
Group 1	✓	✓	✗	✗	8	18 hours
Group 2	✓	✗	✓	✗	8	18 hours
Group 3	✓	✗	✗	✓	8	18 hours

Table 7: Evaluation of critical antibiotic concentration

- Sacrifice the mice following an overnight incubation period post-treatment.
- Surgically remove the intestines.
- Grind the intestines, and collect the supernatant.
- Prepare serial dilutions of the supernatant ranging from 10^{-1} to 10^{-3}.
- Spread 100 µl aliquots from each dilution onto TCBS agar plates.

3.3.7 Investigating Synergistic Effects of Bacteriophage Cocktail and Low-Dose Antibiotics in Combating Infection

- Prepare bacterial inoculum as per the method previously described.
- Administrate the bacterial inoculum to each group, and give each group the required treatment after an hour.

Examined Group	Bacteria CFU/ml – 10^8	Ampicillin 500mg/kg	Ampicillin 50mg/kg	Bacteriophage cocktail	Number of Mice examined	Study Period
Control (0)	✓	✕	✕	✕	3	18 hours
Control (1)	✕	✕	✕	✕	3	18 hours
Group 1	✓	✓	✕	✓	8	18 hours
Group 2	✓	✕	✓	✓	8	18 hours

Table 8: Synergistic effect of bacteriophage cocktail and low dose antibiotic in combating infection

- Sacrifice the mice following an overnight incubation period post-treatment.
- Surgically remove the intestines.
- Grind the intestines, and collect the supernatant.
- Prepare serial dilutions of the supernatant ranging from 10^{-1} to 10^{-3}, and spread on TCBS agar plates.

Chapter 4

Result and Analysis

4.1 In-Vitro Experiment

4.1.1 Plaque Assay or Spot Assay

The plaque assay experiment shows that JSF bacteriophages of our interest, JSF2, and JSF25 can infect our bacterial strain of interest *Vibrio cholerae* 1877. The clear plaques indicate the positive results.

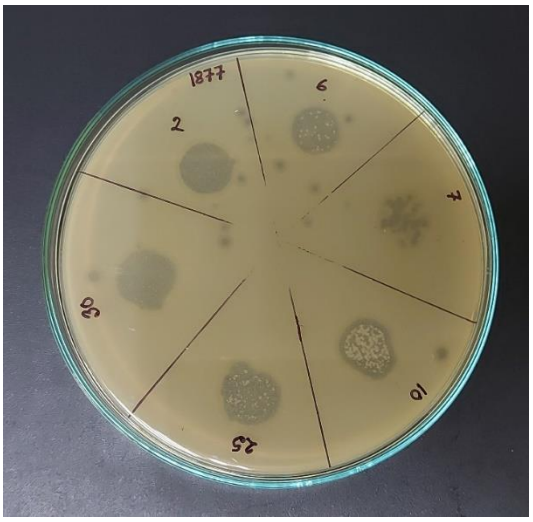

Figure 4: Spot test result of V. cholerae 1877 with JSF bacteriophages

4.1.2 Double Layer Agar Assay after Enrichment of the Specific Bacteriophages:

After the confirmation of the infection, enrichment of the bacteriophages was necessary to increase the efficiency of the bacteriophage stocks.

JSF2, Phage Titer (PFU/ml) = $\frac{Number\ of\ Plaques\ X\ Dilution\ Factor}{Volume\ of\ Phage\ added\ to\ the\ Soft\ Agar\ (ml)}$

$= \frac{38\ X\ 10^5}{0.1}$

$= 3.8\ X\ 10^7$

JSF 25, Phage Titer (PFU/ml) = $\frac{Number\ of\ Plaques\ X\ Dilution\ Factor}{Volume\ of\ Phage\ added\ to\ the\ Soft\ Agar\ (ml)}$

$= \frac{47\ X\ 10^5}{0.1}$

$= 4.7\ X\ 10^7$

4.1.3 Double Layer Agar Assay after PEG Precipitation of Bacteriophages

PEG-NaCl have been utilised to increase the bacteriophage titer even more and finally the required bacteriophage titer has been achieved.

JSF 2, Phage Titer (PFU/ml) = $\frac{Number\ of\ Plaques\ X\ Dilution\ Factor}{Volume\ of\ Phage\ added\ to\ the\ Soft\ Agar\ (ml)}$

$= \frac{52\ X\ 10^8}{0.1}$

$= 5.2\ X\ 10^{10}$

JSF25, Phage Titer (PFU/ml) = $\frac{Number\ of\ Plaques\ X\ Dilution\ Factor}{Volume\ of\ Phage\ added\ to\ the\ Soft\ Agar\ (ml)}$

$= \frac{62\ X\ 10^8}{0.1}$

$= 6.2\ X\ 10^{10}$

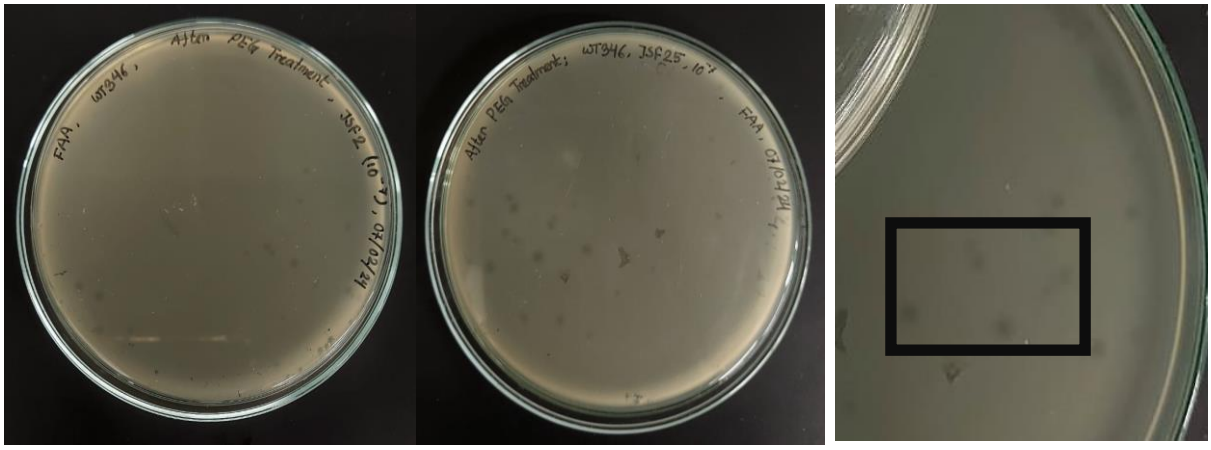

Figure 5: DLA result of bacteriophage JSF7, and JSF25 after PEG precipitation

4.1.4 Bacterial growth Curve:

The bacterial growth curve has been analyzed to maintain constant concentration of bacterial culture used as the inoculum for cholera infection. It shows that the culture requires around 18 hours to reach the concentration of 10^9 CFU/ml.

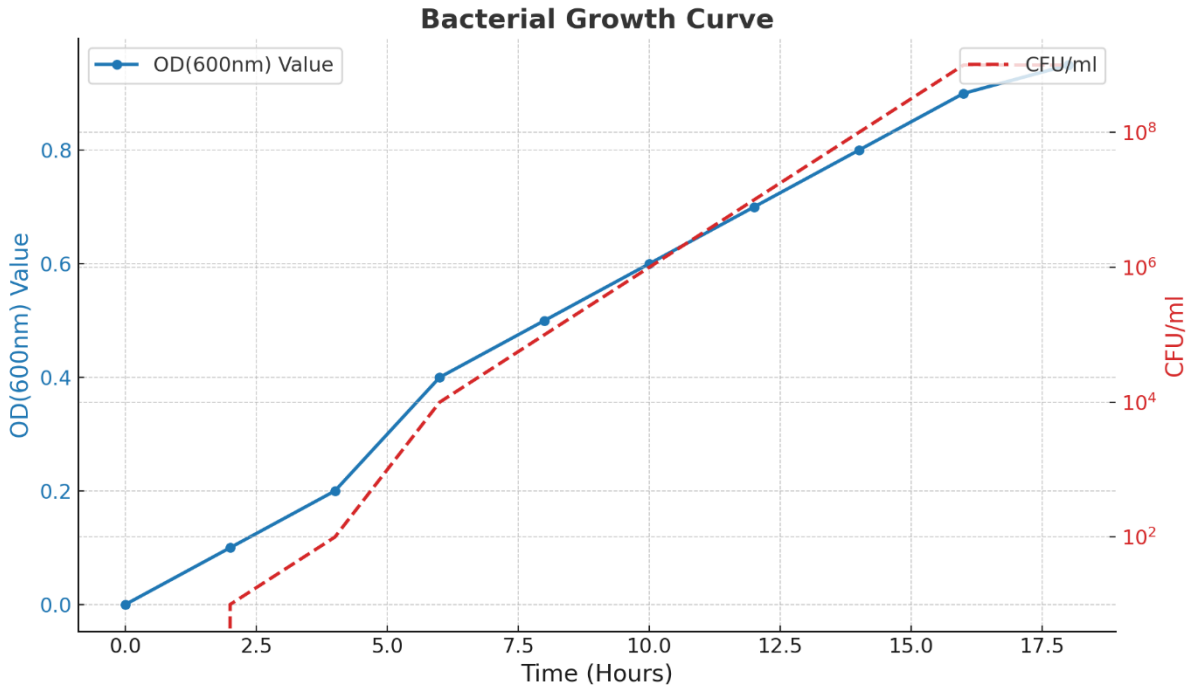

Figure 6: Growth curve of V. cholerae 1877

4.1.5 Selection of Antibiotic

After analyzing the antibiotic zone, we selected Ampicillin for our experiment.

4.2 In Vitro Experiments

4.2.1 Infection Model Preparation

Mouse Groups	Observations
Adults	Completely healthy.
Infants (P7 – P10)	Completely healthy
Infants (P4 – P5)	Presence of yellow watery stool, reduced body weight, flatulency, signs of dehydration, and death due to infection.

Table 9: Observations of infection models

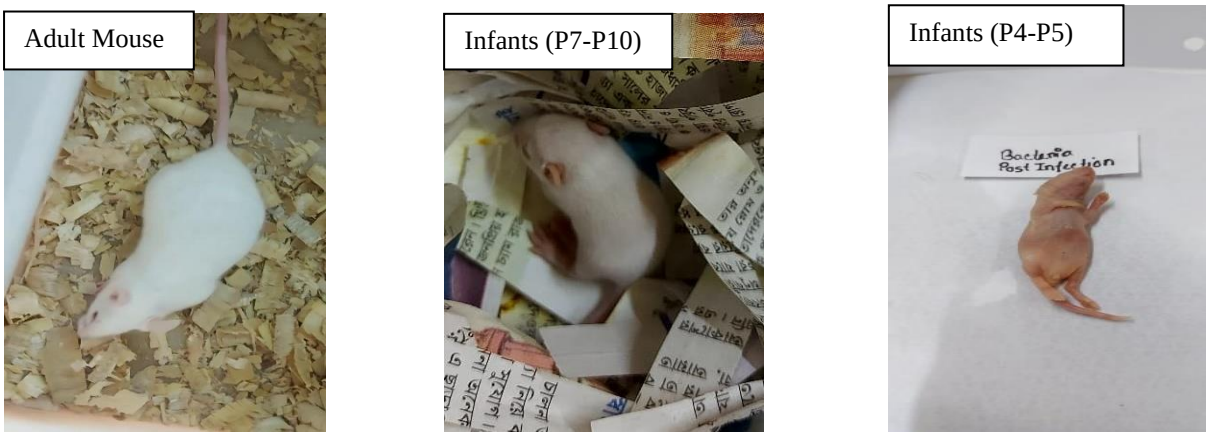

Figure 7: Establishment of Infection Model. The adult mouse and the infant aged 7-10days old are perfectly healthy after the bacterial administration. The mouse aged 4-5days died after the bacterial administration.

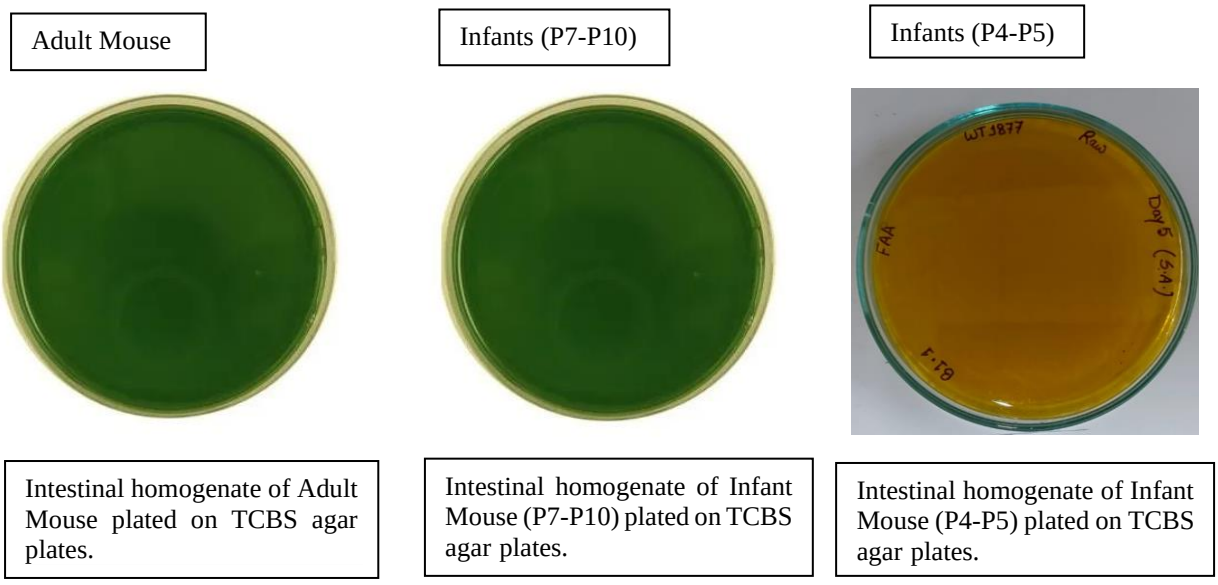

Figure 8: Intestinal homogenate of adult, and infant mice plated on TCBS agar plates

To start the in-vitro analysis, successful infection model establishment has been done. Mice of different age groups have been taken and bacteria has been introduced.

Only the infant mice of post-natal Day 4 to post-natal Day 5 have shown signs of successful infection (100%) out of the three categories.

4.2.2 Bacterial Infection Latency Analysis

All the mice (10) were observed every hour and compared with the characteristics of the successful infection model. The average infection initiation time is found to be 66 minutes.

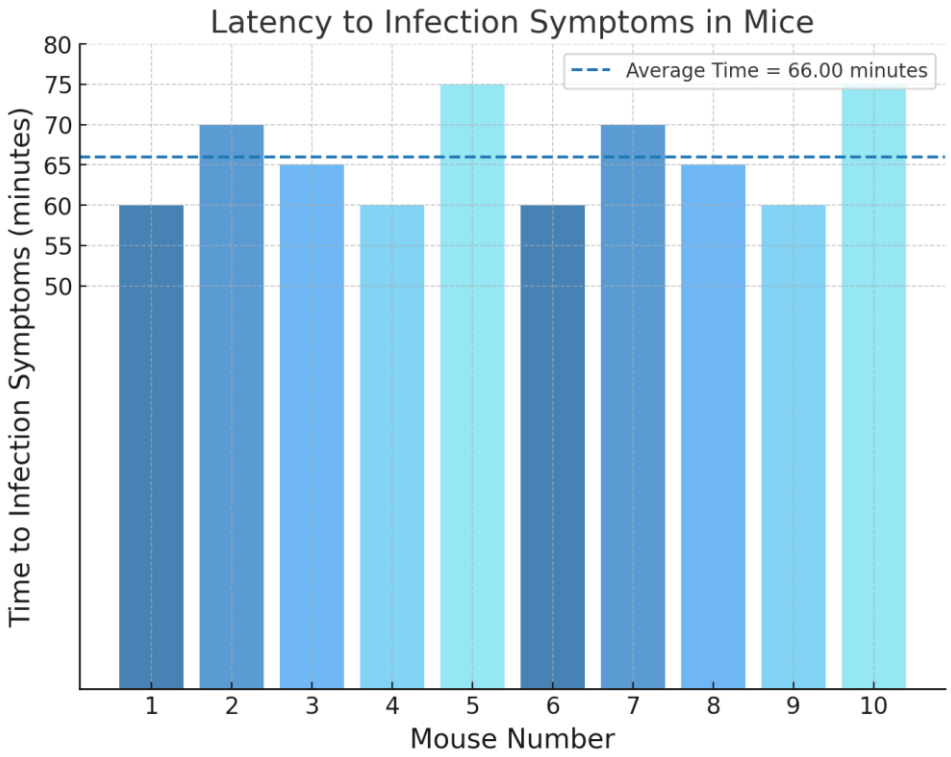

Figure 9: Average bacterial infection latency

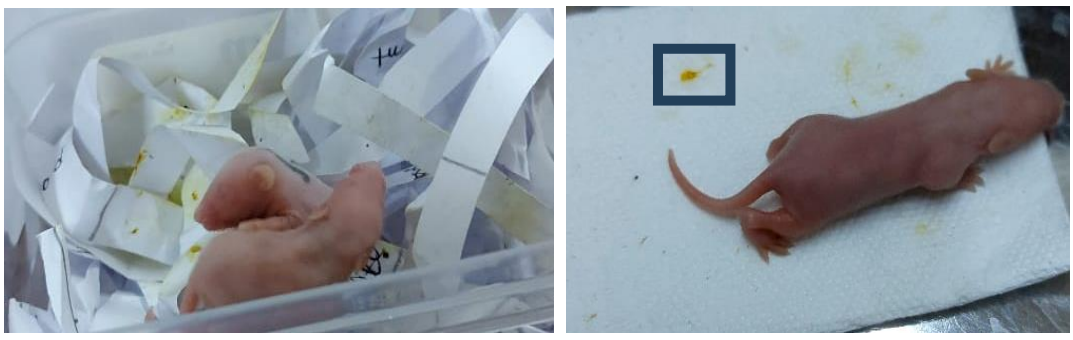

Figure 10: Presence of watery stool after bacterial inoculum had been given to the mice

4.2.3 Temporal Dynamics of *Vibrio cholerae* Infection and Intestinal Bacterial Load

Examined Groups	Study Period (Hours)	Death Rate (in Percentage)
Group 1	4 hours	100%
Group 2	12 hours	100%
Group 3	18 hours	100%

Table 10: Temporal dynamics of cholera in mice

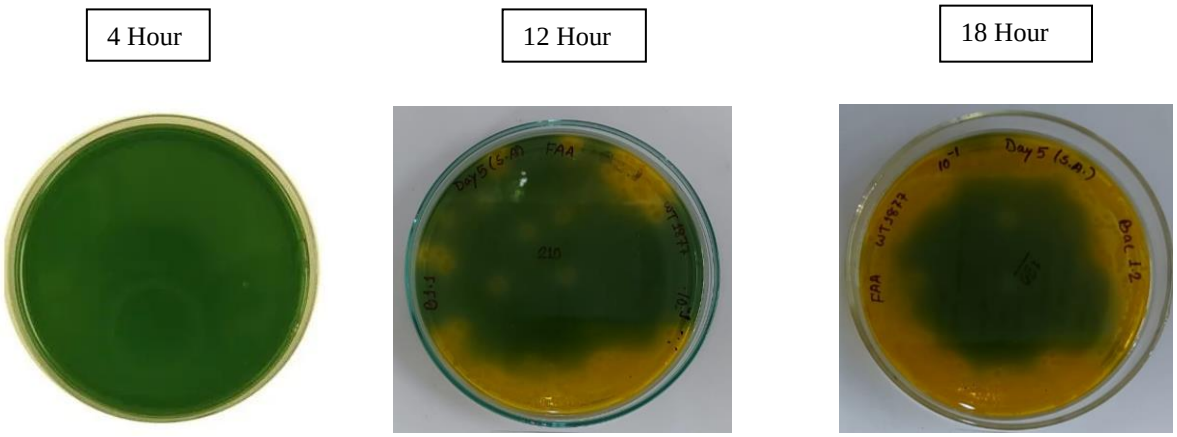

Figure 11: Bacterial counts after temporal analysis of cholera in mice. After 4 hours incubation no bacterial growth had been observed, whereas 12hours and 18 hours incubation showed countable bacterial colonies in TCBS agar plates.

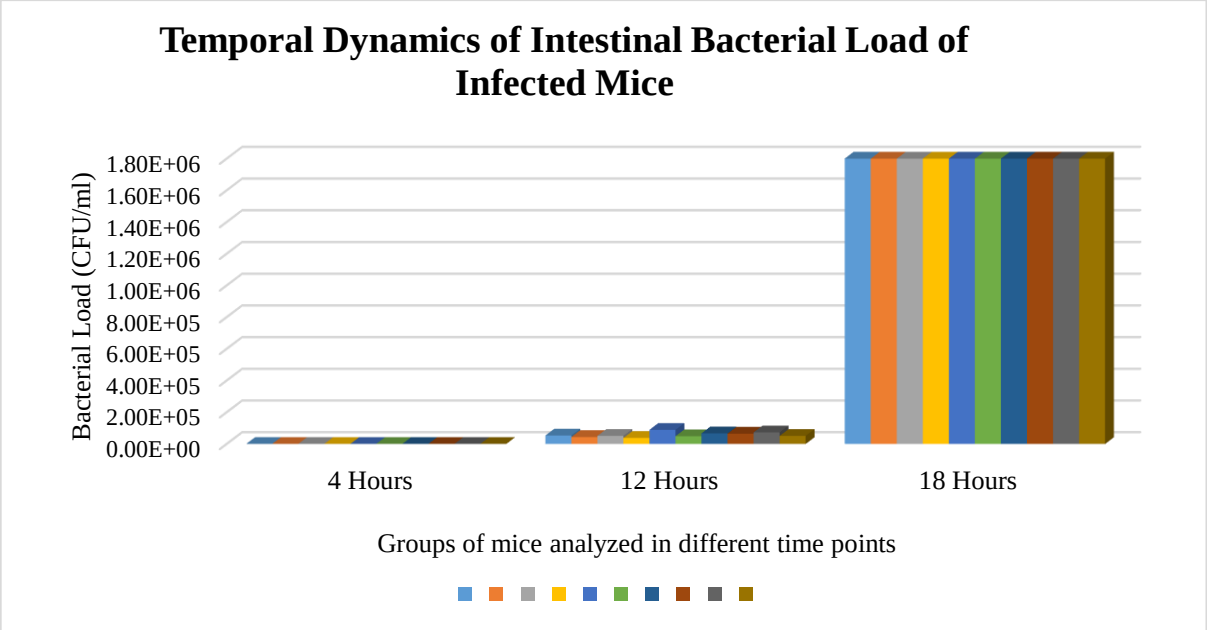

Figure 12: Intestinal bacterial loads of mice incubated for different time period.

Though after 4 hours the mice were dead, the intestinal homogenate did not show any bacterial presence in agar plates. The mice group that was sacrificed after 12 hours showed a bacterial count on average of 5.80×10^4 in TCBS agar plates. Whereas, the group of mice sacrificed after 18 hours or overnight showed a bacterial count on average of 7.832×10^6.

4.2.4 Comparative Evaluation of Treatment Strategies for Cholera Infection in Mice: Intestinal Bacterial Load Analysis over time.

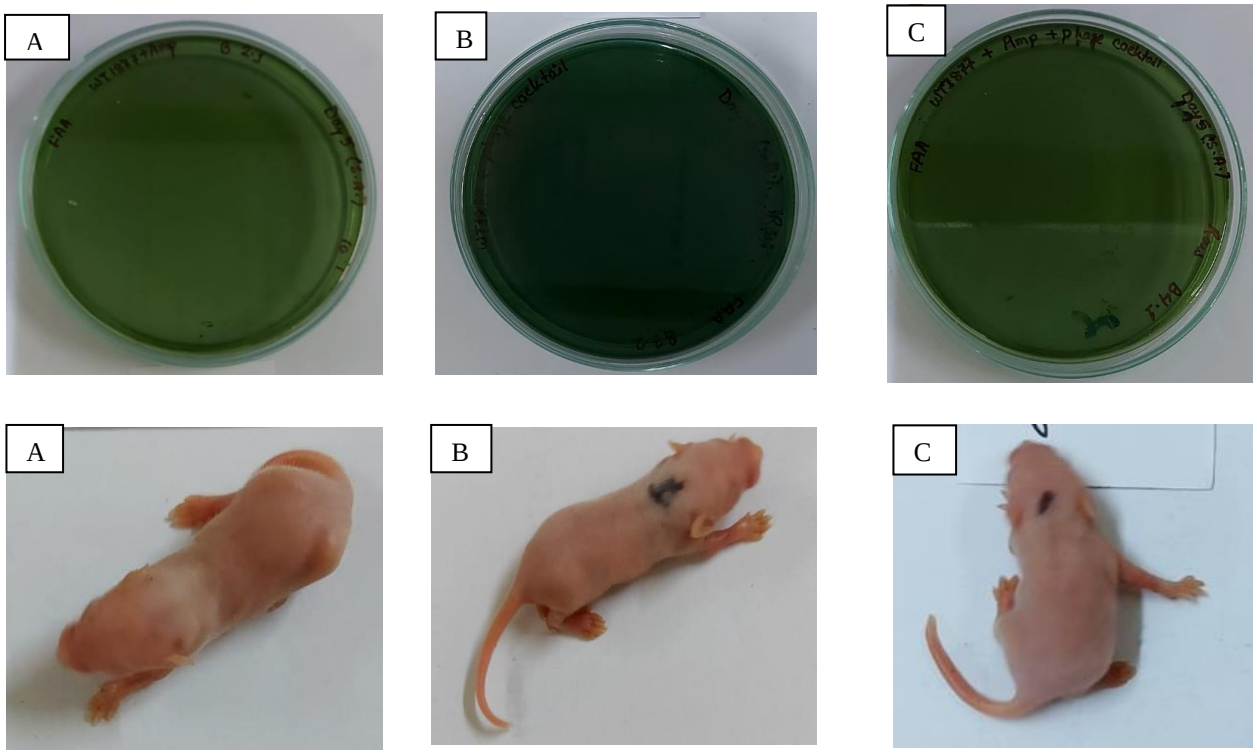

Figure 13: Comparative evaluation of treatment strategies for cholera. A, B, and C denotes treatment with Bacteriophage cocktail, Antibiotic, Bacteriophage cocktail + antibiotic. All 3 mice survived after the treatments were given. Moreover, the intestinal homogenate did not show presence of V. cholerae.

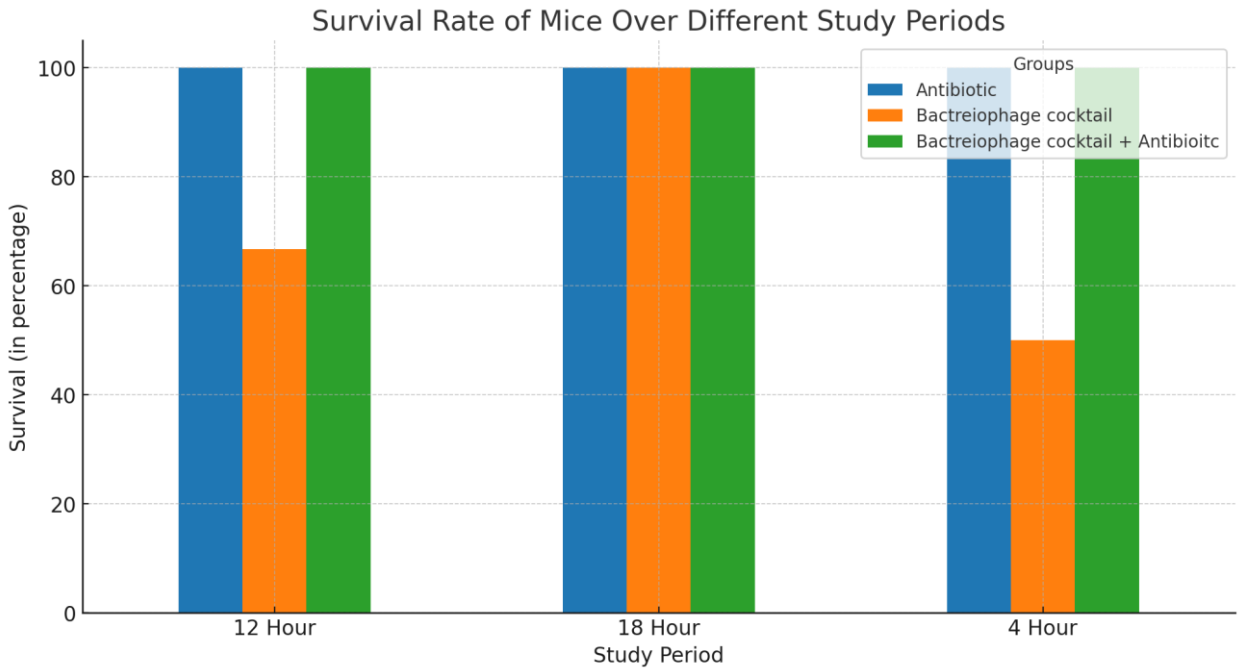

Figure 14: Survival rate of infected mice after treatments over different study periods.

This time course evaluation clearly showed complete eradication of bacterial infection immediately after introducing the antibiotic. However, bacteriophages require time to increase and eradicate infection. Usually, it takes overnight to increase its amount and start lysing bacteria.

4.2.5 Assessment of Treatment Efficacy in Promoting 48-Hour Survival of Cholera Infected Mice

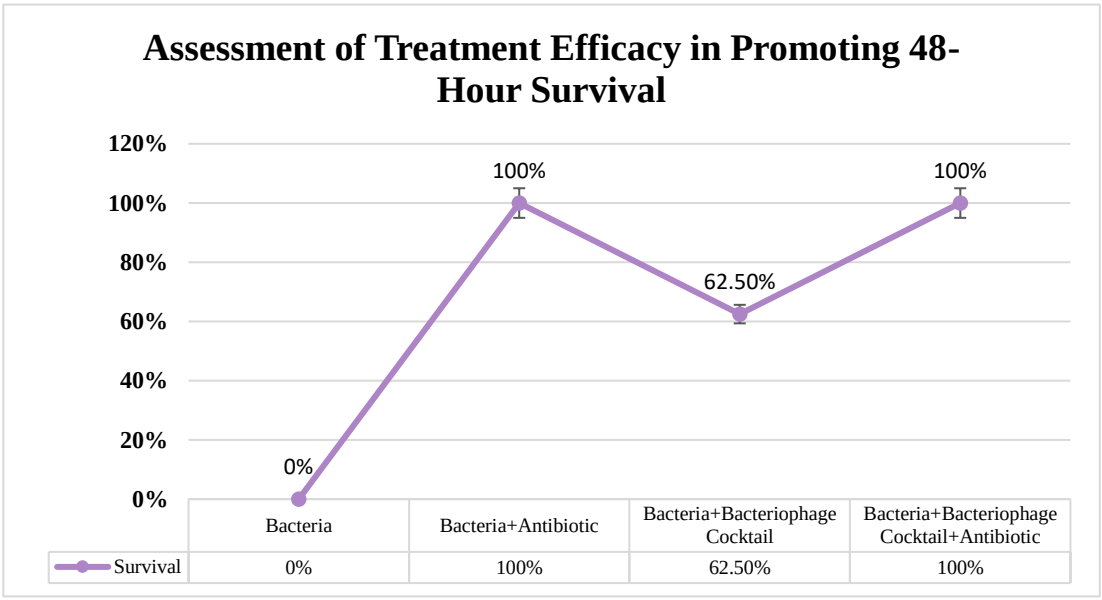

Figure 15: Assessment of treatment efficacy in survival. After 48 hours of observation, 100% of the mice treated with antibiotic and bacteriophage cocktail + antibiotic survived. However, bacteriophage cocktail alone cured 62.50% of the infected mice.

4.2.6 Evaluation of Critical Antibiotic Concentration for Survival of Infected Mice

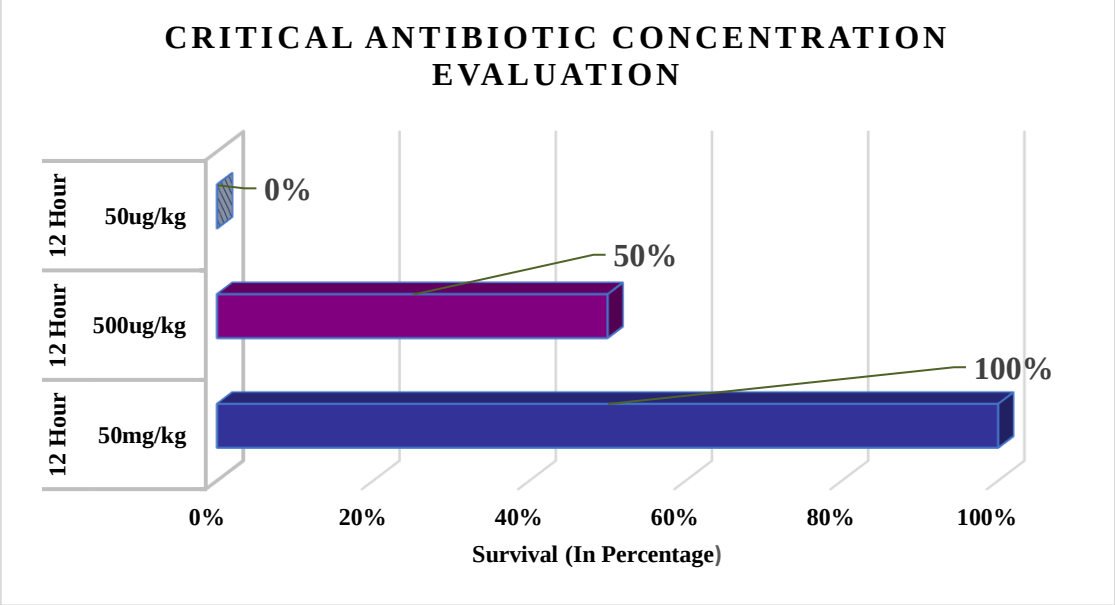

Figure 16: Critical antibiotic concentration (required for complete bacterial eradication In-vivo) determination. Only 50mg/kg concentration could cure 100% of the infected mice, whereas 500ug/kg cured only 50%, and 50ug/kg could not cure any mice.

4.2.7 Investigating Synergistic Effects of Bacteriophage Cocktail and Low-Dose Antibiotics in Combating Infection

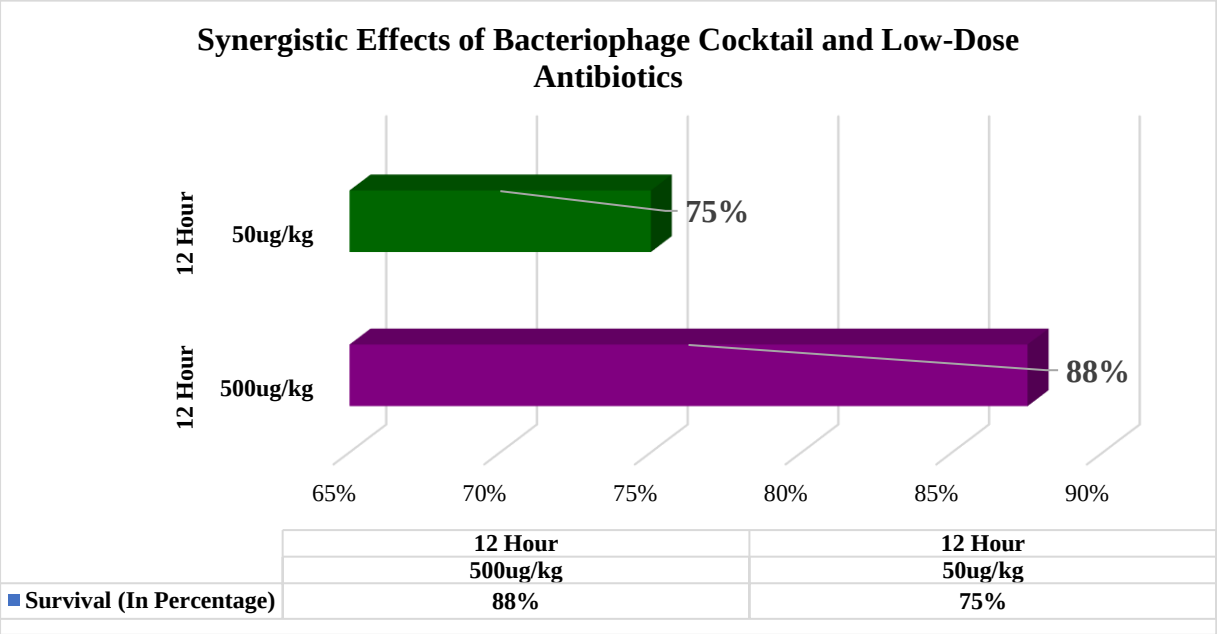

Figure 17: Evaluation of synergistic effect of bacteriophage cocktail and antibiotic in treatment of infected mice. 50ug/kg antibiotic work with bacteriophage cocktail and cured 75% of the infected mice. And 500ug/kg antibiotic cured 88% of the infected mice by working with bacteriophage cocktail.

4.2.8 Statistical Analysis

In this experiment, we aimed to evaluate whether a lower concentration of antibiotic combined with a bacteriophage cocktail could effectively treat cholera infection in mice. The motivation behind this study was to explore the potential of reducing antibiotic dosage to mitigate the risk of antibiotic resistance while maintaining therapeutic efficacy through the synergistic action of bacteriophages.

Experimental Design

Three groups of mice (8 mice per group) were infected with cholera. Each group was treated with a different antibiotic concentration:

- **Group 1**: 50mg/kg antibiotic (high dose) — Positive control

- **Group 2**: 500µg/kg antibiotic (moderate dose)
- **Group 3**: 50µg/kg antibiotic (low dose)

Post-treatment survival was monitored. The survival rates were as follows:

- **50mg/kg antibiotic**: 100% survival (8/8 mice)
- **500µg/kg antibiotic**: 50% survival (4/8 mice)
- **50µg/kg antibiotic**: 0% survival (0/8 mice)

Following this, two additional groups were treated with a combination of the antibiotic and a bacteriophage cocktail (10^9 pfu/ml):

- **50µg/kg antibiotic + bacteriophage cocktail**: 75% survival (6/8 mice)
- **500µg/kg antibiotic + bacteriophage cocktail**: 88% survival (7/8 mice)

Statistical Analysis

A. Chi-Square and Fisher's Exact Test

To determine whether the addition of bacteriophage significantly improved survival compared to antibiotic treatment alone, Chi-Square and Fisher's Exact tests were performed.

i. **Comparison between 500µg/kg Antibiotic Alone vs. 500µg/kg Antibiotic + Bacteriophage Cocktail**

Contingency Table:

Group	Survived	Died
500µg/kg Antibiotic Alone	4	4
500µg/kg Antibiotic + Phage Cocktail	7	1

Chi-Square Test:

$$\chi 2=1.163, (p=0.281)$$

Interpretation: The p-value of 0.281 suggests that the difference in survival rates between the 500µg/kg antibiotic alone group and the combination therapy group is not statistically significant. However, the higher survival rate in the combination therapy group indicates a positive trend.

ii. Comparison between 50µg/kg Antibiotic Alone vs. 50µg/kg Antibiotic + Bacteriophage Cocktail

Contingency Table:

Group	Survived	Died
50µg/kg Antibiotic Alone	0	8
50µg/kg Antibiotic + Phage Cocktail	6	2

Fisher's Exact Test:

p=0.007

Interpretation: The p-value of 0.007 indicates a statistically significant improvement in survival when bacteriophage is added to the low-dose antibiotic treatment.

B. Odds Ratios and Confidence Intervals

The odds ratio (OR) provides a measure of the strength of association between treatment and survival.

i. Odds Ratio for 500µg/kg Antibiotic Alone vs. 500µg/kg Antibiotic + Bacteriophage Cocktail

$$\text{Odds Ratio} = \frac{\text{Odds of Survival in Antibiotic Alone}}{\text{Odds of Survival in Combination Therapy}} = \frac{7/1}{4/4} = 7$$

95% Confidence Interval:

$$\text{CI} = \exp\left[\ln(7) \pm 1.96 \times \sqrt{\frac{1}{7} + \frac{1}{1} + \frac{1}{4} + \frac{1}{4}}\right] = 0.79, 62.03$$

Interpretation: The odds ratio of 7 suggests that the combination therapy group had 7 times higher odds of survival compared to the antibiotic-alone group. The wide confidence interval indicates variability in the estimate, and since it crosses 1, the result is not statistically significant.

ii. Odds Ratio for 50µg/kg Antibiotic Alone vs. 50µg/kg Antibiotic + Bacteriophage Cocktail

Using a small continuity correction due to zero survivors in the antibiotic-alone group:

$$\text{Adjusted Odds Ratio} = \frac{6/2}{0.5/8} = 48.0$$

95% Confidence Interval:

$$CI = \exp\left[\ln(48) \pm 1.96 \times \sqrt{\frac{1}{6} + \frac{1}{2} + \frac{1}{0.5} + \frac{1}{8}}\right] = 2.81, 820.12$$

Interpretation: The very high odds ratio of 48.0 indicates a strong association between combination therapy and survival. The wide confidence interval reflects uncertainty in the estimate but is still statistically significant as it does not cross 1.

The results suggest that adding a bacteriophage cocktail to a low-dose antibiotic regimen significantly improves survival rates in cholera-infected mice, particularly at the 50µg/kg antibiotic dose. The combination therapy with 500µg/kg antibiotic also showed a positive trend but was not statistically significant.

Chapter 5

Discussion

Cholera, a life-threatening disease with epidemic and pandemic potential, continues to devastate many countries worldwide. Although various treatment options have been identified, rehydration therapy remains one of the most effective methods. However, it falls short for severely ill patients. To address this, antibiotics are often administered alongside rehydration therapy. Unfortunately, the unsystematic and uncontrolled use of antibiotics has led to the emergence of antibiotic-resistant *Vibrio cholerae*. In 2016, antibiotic resistance was declared "the greatest and most urgent global risk". As an alternative, phage therapy, a revolutionary approach, was introduced. However, cases of bacteriophage resistance soon emerged, complicating the situation. To combat these challenges, we designed a study to evaluate the combined effects of antibiotics and bacteriophages in treating cholera while mitigating negative outcomes. The approach involved using antibiotics at concentrations lower than the minimum inhibitory concentration (MIC) and employing a bacteriophage cocktail to prevent antibiotic and bacteriophage resistance.

We selected a clinical strain of *V. cholerae* 1877, along with specific bacteriophages JSF2 and JSF25, which can infect this bacterial strain. Ampicillin was chosen as the antibiotic, as strain 1877 is sensitive to it. To assess the in vivo effects, it was essential to maintain specific concentrations of bacteria, bacteriophages, and antibiotics. *V. cholerae* causes infection at a concentration of 10^7–10^9 CFU/ml. To match this, we used bacteriophages at a concentration of 10^9 PFU/ml, creating a MOI of 1. PEG precipitation was used to achieve the required concentration of bacteriophages.

The combined effect was evaluated in Swiss-albino mice. We initially attempted to establish an infection model in adult mice, but this was unsuccessful. Subsequent trials in mice at postnatal days 7–10 also failed. Finally, we achieved a successful cholera infection model in mice at postnatal days 4–5. This model exhibited all the characteristics of cholera infection, likely due to age-related differences in susceptibility. Research suggests that, intestine of the mouse infants of post-natal day 4 to day 5 is similar to human intestine, and it directly correlate with our finding (Matson, 2018).

To evaluate treatment efficacy, we first identified the onset of infection and visible symptoms to determine the optimal time for initiating treatment. Bacterial infection latency analysis showed that infection typically began within 66 minutes, highlighting the need for immediate treatment. We correlated the temporal dynamics of infection with intestinal bacterial load, finding that while mice succumbed to the infection within hours, bacteria were only detectable on selective plates after a certain period. The delayed appearance of bacterial colonies in the intestinal homogenate after infection could be due to several factors. Initially, the bacterial load might have been below the detection limit, as the bacteria were likely in a lag phase, adapting to the new environment before beginning exponential growth. This could explain the absence of detectable colonies at 4 hours, with detectable colonies appearing by 12 and 18 hours as the bacteria multiplied (Kudva et al., 1999).

Additionally, the host's immune system may have initially suppressed bacterial growth, keeping the bacterial count low, but as the infection progressed, the immune response might have been overwhelmed, allowing bacterial proliferation (Thiel et al., 2005).

To assess the potential of combined treatment, we included control groups receiving either bacteria alone, bacteriophage cocktail alone, or the combined bacteriophage cocktail and antibiotic treatment. Our results showed that 100% of the infected mice recovered with

antibiotic treatment, and similarly, 100% recovery was observed with the combined treatment across different temporal groups (4 hours, 12 hours, 18 hours). In contrast, the bacteriophage cocktail alone showed variable results, requiring 18 hours or overnight to fully eradicate the infection. This variability is likely due to the bacteriophages' need for a host to multiply, with their numbers increasing over time to eventually eliminate all bacteria in the intestine. The success of the combined treatment may be attributed to the presence of the antibiotic.

To evaluate overall survival efficacy, different groups were treated and observed for 48 hours. The control group, left untreated, did not survive beyond 48 hours. However, 100% of the mice treated with the combined bacteriophage cocktail and antibiotic survived, while only 66% of those treated with the bacteriophage cocktail alone survived.

To further understand the synergistic effect, we reduced the antibiotic concentration tenfold and added the bacteriophage cocktail. The standard concentration of ampicillin for mice is 50 mg/kg, so we reduced it to 500 µg/kg and 50 µg/kg. Our results showed that 50 mg/kg ampicillin successfully treated 100% of infected mice, while 500 µg/kg treated only 50%, and 50 µg/kg was ineffective. However, when the bacteriophage cocktail was combined with 500 µg/kg and 50 µg/kg ampicillin, 88% and 75% of infected mice, respectively, were cured within 12 hours, demonstrating a clear synergistic effect.

In conducting this experiment, several challenges were encountered that could impact the overall findings and suggest areas for further study. First, the sample size of mice could be increased to enhance the statistical power of the results and ensure more robust conclusions. Additionally, while we analyzed the survival and bacterial counts at 4, 12, and 18 hours post-infection, it would have been beneficial to include an 8-hour time point. Unfortunately, this was not feasible due to time constraints and lab scheduling limitations. Preparing the infection model itself was a complex and time-consuming process requiring significant effort and

precision. Another challenge was maintaining the consistency of the mice's age throughout the experiment due to time constraints. These challenges highlight the need for careful planning and consideration of logistical factors in future research.

Chapter 6

Conclusion and Future Direction

The findings from this study align with our hypothesis regarding the synergistic relationship between bacteriophage cocktails and antibiotics in treating cholera infections. However, to fully elucidate the extent of this synergistic effect, it will be essential to conduct further studies with a larger sample size. Increasing the number of samples will enhance the statistical power of our results, allowing for more robust conclusions.

Additionally, expanding the range of antibiotic concentrations tested will provide a more comprehensive understanding of the dose-response relationship and help identify the most effective treatment regimens. In this research, we focused on a single clinical strain of *Vibrio cholerae* and employed two specific bacteriophages. Future studies should consider a broader spectrum of bacterial strains and bacteriophages to determine the generalizability of our findings and to explore potential variations in treatment efficacy across different bacterial and phage combinations.

Given that cholera remains a significant public health concern in Bangladesh, the insights gained from this study could have a profound impact on future treatment strategies. By optimizing the use of bacteriophage therapy in conjunction with antibiotics, our research has the potential to contribute to more effective and sustainable approaches to combating cholera, addressing both the immediate therapeutic challenges and the long-term issue of antibiotic resistance.

Chapter 7

References

Abdulhadi, S. K., Tukur, A. D., & Ahmed, B. O. K. (2018). Contemporary understanding of Vibrio cholerae and cholera outbreaks. *J Infectious Disease Med Microbiol. 2018; 2 (3): 1-6. J Infectious Disease Med Microbiol 2018 Volume 2 Issue, 3*.

Abedon, S. T., Kuhl, S. J., Blasdel, B. G., & Kutter, E. M. (2011). Phage treatment of human infections. *Bacteriophage, 1*(2), 66-85

Ackermann, H. W., & Prangishvili, D. (2012). Prokaryote viruses studied by electron microscopy. Archives of Virology, 157(10), 1843-1849. https://doi.org/10.1007/s00705-012-1387-4

Ali, M., Nelson, A. R., Lopez, A. L., & Sack, D. A. (2015). Updated Global Burden of Cholera in Endemic Countries. *PLoS Neglected Tropical Diseases, 9*(6), e0003832. https://doi.org/10.1371/journal.pntd.0003832

Almagro-Moreno, S., Pruss, K., & Taylor, R. K. (2015b). Intestinal colonization dynamics of vibrio cholerae. *PLOS Pathogens, 11*(5). https://doi.org/10.1371/journal.ppat.1004787

Alves, D. R., Gaudion, A., Bean, J. E., Perez Esteban, P., Arnot, T. C., Harper, D. R., Kot, W., Hansen, L. H., Enright, M. C., & Jenkins, A. T. (2014). Combined use of bacteriophage K and a novel bacteriophage to reduce Staphylococcus aureus biofilm formation. *Applied and Environmental Microbiology*, *80*(21), 6694-6703.

Bertozzi Silva, J., Storms, Z., & Sauvageau, D. (2016). Host receptors for bacteriophage adsorption. FEMS Microbiology Letters, 363(4), fnw002. https://doi.org/10.1093/femsle/fnw002

Bhandare, S., Colom, J., Baig, A., Ritchie, J. M., Bukhari, H., Shah, M. A., Sarkar, B. L., Su, J., Wren, B., Barrow, P., & Atterbury, R. J. (2018). Reviving phage therapy for the

treatment of cholera. *The Journal of Infectious Diseases*, *219*(5), 786–794. https://doi.org/10.1093/infdis/jiy563

Boyd, E. F., & Brüssow, H. (2002). Common themes in the concerted evolution of phage genomes and host genomes. *Microbiology and Molecular Biology Reviews*, 66(3), 435-450. https://doi.org/10.1128/MMBR.66.3.435-450.2002

Calendar, R. (2006). The Bacteriophages (2nd ed.). Oxford University Press.

Catalano, C. E. (2005). Viral genome packaging machines: an overview. In Catalano, C. E. (Ed.), *Viral Genome Packaging: Genetics, Structure, and Mechanism* (pp. 5-10). Springer.

Chan, B. K., Abedon, S. T., & Loc-Carrillo, C. (2013). Phage cocktails and the future of phage therapy. *Future Microbiology*, *8*(6), 769–783. https://doi.org/10.2217/fmb.13.47

Chan, B. K., Sistrom, M., Wertz, J. E., Kortright, K. E., Narayan, D., & Turner, P. E. (2016). Phage selection restores antibiotic sensitivity in MDR Pseudomonas aeruginosa. *Scientific Reports*, *6*, 26717.

Chibani-Chennoufi, S., Bruttin, A., Dillmann, M. L., & Brüssow, H. (2004). Phage-host interaction: an ecological perspective. Journal of Bacteriology, 186(12), 3677-3686.

Cockburn, T. A., & Cassanos, J. G. (1960). Epidemiology of endemic cholera. *Public health reports*, *75*(9), 791

Cohen, M. B. (2022). Bacterial, viral, and toxic causes of diarrhea, gastroenteritis, and anorectal infections. *Yamada's Textbook of Gastroenterology*, 2947–3005. https://doi.org/10.1002/9781119600206.ch144

Comeau, A. M., & Krisch, H. M. (2008). The capsid of the T4 phage superfamily: the evolution, diversity, and structure of some of the most prevalent proteins in the biosphere. Molecular Biology and Evolution, 25(7), 1321-1332.

Comeau, A. M., Tétart, F., Trojet, S. N., Prère, M. F., & Krisch, H. M. (2007). Phage-antibiotic synergy (PAS): beta-lactam and quinolone antibiotics stimulate virulent phage growth. *PLoS ONE, 2*(8), e799.

Devault, A. M., Golding, G. B., Waglechner, N., Enk, J. M., Kuch, M., Tien, J. H., Shi, M., Fisman, D. N., Dhody, A. N., Forrest, S., Bos, K. I., Earn, D. J. D., Holmes, E. C., & Poinar, H. N. (2014). Second-pandemic strain of*vibrio cholerae*from the Philadelphia cholera outbreak of 1849. *New England Journal of Medicine*, *370*(4), 334–340. https://doi.org/10.1056/nejmoa1308663

Donlan, R. M. (2009). Preventing biofilms of clinically relevant organisms using bacteriophage. *Trends in Microbiology*, *17*(2), 66-72.

Faruque, S. M., Albert, M. J., & Mekalanos, J. J. (1998). Epidemiology, genetics, and ecology of toxigenic *vibrio cholerae*. *Microbiology and Molecular Biology Reviews*, *62*(4), 1301–1314. https://doi.org/10.1128/mmbr.62.4.1301-1314.1998

Faruque, S. M., et al. (2003). Epidemiology, genetics, and ecology of toxigenic Vibrio cholerae. *Microbiology and Molecular Biology Reviews*, 67(4), 653-703.

Federspiel, F., & Ali, M. (2018). The cholera outbreak in Yemen: lessons learned and way forward. *BMC Public Health*, *18*(1). https://doi.org/10.1186/s12889-018-6227-6

Fischetti, V. A., Nelson, D., & Schuch, R. (2006). Reinventing phage therapy: Are the parts greater than the sum? *Nature Biotechnology*, *24*(12), 1508–1511. https://doi.org/10.1038/nbt1206-1508

Global Task Force on Cholera Control. (2017). Ending Cholera: A Global Roadmap to 2030. *WHO*.

Górski, A., Międzybrodzki, R., Węgrzyn, G., Jonczyk-Matysiak, E., Borysowski, J., & Weber-Dąbrowska, B. (2017). Phage therapy: current status and perspectives. *Medycyna Doswiadczalna i Mikrobiologia, 69*(4), 299-307.

Harris, J. B., LaRocque, R. C., Qadri, F., Ryan, E. T., & Calderwood, S. B. (2012). Cholera. *The Lancet, 379*(9835), 2466-2476.

Huq, A., et al. (1983). Ecological relationships between Vibrio cholerae and planktonic crustacean copepods. *Applied and Environmental Microbiology*, 45(1), 275-283.

Hyman, P., & Abedon, S. T. (2010). Bacteriophage host range and bacterial resistance. *Advances in Applied Microbiology*, 70, 217-248. https://doi.org/10.1016/S0065-2164(10)70007-1

Hynes, A. P., Villion, M., & Moineau, S. (2014). Adaptation in bacterial CRISPR-Cas immunity can be driven by defective phages. *Nature Communications*, 5, 4399. https://doi.org/10.1038/ncomms5399

Khan, M. Y., Isa, F., Saeed, M. F., Fawwad, A., Khan, M., & Ahmed, N. (2020). Efficacy of bacteriophage therapy in controlling Vibrio cholerae infections: A review. *Journal of Infection and Public Health, 13*(7), 1042-1049. doi:10.1016/j.jiph.2020.06.003

Krupovic, M., & Bamford, D. H. (2008). Virus evolution: How far does the double beta-barrel viral lineage extend? Nature Reviews Microbiology, 6(12), 941-948. https://doi.org/10.1038/nrmicro2033

Kudva, I. T., Hatfield, P. G., & Hovde, C. J. (1999). Escherichia coli O157 in microbial flora of sheep. *Journal of Clinical Microbiology*, 37(3), 679-688.

Kutter, E., De Vos, D., Gvasalia, G., Alavidze, Z., Gogokhia, L., Kuhl, S., & Pirnay, J. P. (2010). Phage therapy in clinical practice: treatment of human infections. *Current Pharmaceutical Biotechnology, 11*(1), 69-86.

Kutter, E., Kellenberger, E., Carlson, K., Eddy, S., Neitzel, J., Messinger, L., North, J., Guttman, B., & Carlson, K. (1995). Evolution of T7 and its relatives. FEMS Microbiology Reviews, 17(3), 369-378. https://doi.org/10.1111/j.1574-6976.1995.tb00212.x

Legros, D. (2018). Global Cholera Epidemiology: Opportunities to Reduce the Burden of Cholera by 2030. *˜the œJournal of Infectious Diseases (Online. University of Chicago Press)/˜the œJournal of Infectious Diseases*, *218*(suppl_3), S137–S140. https://doi.org/10.1093/infdis/jiy486

Levine, M. M., Nalin, D. R., Craig, J. P., Hoover, D., Bergquist, E. J., Waterman, D., Hornick, R. B., Young, C. R., & Sotman, S. (1978). Immunity to cholera in man: relative role of antibacterial versus antitoxic immunity. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 72*(1), 12-16.

Mandal, S., Mandal, M. D., & Pal, N. K. (2011). Cholera: A great global concern. *Asian Pacific Journal of Tropical Medicine*, *4*(7), 573–580. https://doi.org/10.1016/s1995-7645(11)60149-1

Matson, J. S. (2018). Infant mouse model of vibrio cholerae infection and colonization. *Methods in Molecular Biology*, 147–152. https://doi.org/10.1007/978-1-4939-8685-9_13

Molineux, I. J. (2006). Fifty-three years since Hershey and Chase; much ado about pressure but which pressure is it? *Virology*, 344(1), 221-229. https://doi.org/10.1016/j.virol.2005.09.041

Montero, D. A., Vidal, R. M., Velasco, J., George, S., Lucero, Y., Gómez, L. A., Carreño, L. J., García-Betancourt, R., & O'Ryan, M. (2023b). Vibrio cholerae, classification, pathogenesis, immune response, and trends in Vaccine development. *Frontiers in Medicine*, *10*. https://doi.org/10.3389/fmed.2023.1155751

Montero, D. A., Vidal, R. M., Velasco, J., George, S., Lucero, Y., Gómez, L. A., Carreño, L. J., García-Betancourt, R., & O'Ryan, M. (2023b). Vibrio cholerae, classification, pathogenesis, immune response, and trends in Vaccine development. *Frontiers in Medicine*, *10*. https://doi.org/10.3389/fmed.2023.1155751

Newsom, S. (2006). Pioneers in infection control: John Snow, Henry Whitehead, the Broad Street pump, and the beginnings of geographical epidemiology. *˜the œJournal of Hospital Infection/Journal of Hospital Infection*, *64*(3), 210–216. https://doi.org/10.1016/j.jhin.2006.05.020

Piarroux, R., Moore, S., & Rebaudet, S. (2022). Cholera in Haiti. *˜La œPresse Médicale*, *51*(3), 104136. https://doi.org/10.1016/j.lpm.2022.104136

Prouty, M. G., & Klose, K. E. (2014). Vibrio cholerae: the Genetics of Pathogenesis and Environmental Persistence. In *ASM Press eBooks* (pp. 309–339). https://doi.org/10.1128/9781555815714.ch23

Ptashne, M. (2004). *A Genetic Switch: Phage Lambda Revisited*. Cold Spring Harbor Laboratory Press.

Rakonjac, J., Bennett, N. J., Spagnuolo, J., Gagic, D., & Russel, M. (2011). Filamentous bacteriophage: biology, phage display and nanotechnology applications. *Current Issues in Molecular Biology*, 13(2), 51-76.

Sack, D. A., et al. (2004). Cholera. *The Lancet*, 363(9404), 223-233.

Sarker, S. A., Berger, B., Deng, Y., Kieser, S., Foata, F., Moine, D., ... & Brüssow, H. (2016). Oral application of *Escherichia coli* bacteriophage: safety tests in healthy and diarrheal children from Bangladesh. *Environmental Microbiology*, 18(6), 2196-2206.

Sarker, S. A., Sultana, S., Reuteler, G., Moine, D., Descombes, P., Charton, F., ... & Brussow, H. (2016). Oral phage therapy of acute bacterial diarrhea with two coliphage preparations: a randomized trial in children from Bangladesh. *EBioMedicine*, *4*, 124-137. doi:10.1016/j.ebiom.2015.12.023

Schooley, R. T., Biswas, B., Gill, J. J., Hernandez-Morales, A., Lancaster, J., Lessor, L., ... & Strathdee, S. A. (2017). Development and use of personalized bacteriophage-based

therapeutic cocktails to treat a patient with a disseminated resistant Acinetobacter baumannii infection. *Antimicrobial Agents and Chemotherapy, 61*(10), e00954-17.

Sharifi-Mood, B., & Metanat, M. (2014). Diagnosis, clinical management, prevention, and control of cholera; a review study. *International Journal of Infection*, *1*(1). https://doi.org/10.17795/iji-18303

Smith, G. P. (1985). Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science*, 228(4705), 1315-1317. https://doi.org/10.1126/science.4001944

Smith, G. P., & Petrenko, V. A. (1997). Phage display. *Chemical Reviews*, *97*(2), 391-410. doi:10.1021/cr960065d

Taylor, N. M. I., van Raaij, M. J., & Leiman, P. G. (2016). Contractile injection systems of bacteriophages and related systems. Molecular Microbiology, 60(2), 233-249. https://doi.org/10.1111/j.1365-2958.2006.05031.x

Thiel, A., Hartung, A., Kurzai, O., Roesler, U., Muller, M., & Einsele, H. (2005). Host immune responses in a murine model of Candida albicans esophagitis: Impact of the infection route and relevance to host immunity. *Infection and Immunity*, 73(5), 3212-3221.

Torres-Barceló, C., Arias-Sánchez, F. I., Vasse, M., Ramsayer, J., Kaltz, O., Hochberg, M. E., ... & Ghoul, M. (2018). A window of opportunity to control the bacterial pathogen *Pseudomonas aeruginosa* combining antibiotics and phages. *PLoS One*, 13(9), e0194551.

UNICEF. (2020). Water, sanitation and hygiene (WASH). *UNICEF*

Weinbauer, M. G. (2004). Ecology of prokaryotic viruses. FEMS Microbiology Reviews, 28(2), 127-181.

Weinbauer, M. G. (2004). Ecology of prokaryotic viruses. FEMS Microbiology Reviews, 28(2), 127-181.

World Health Organization (WHO). (2017). *Cholera outbreak: assessing the outbreak response and improving preparedness*. WHO Press.

World Health Organization. (n.d.). *Cholera*. World Health Organization. https://www.who.int/news-room/fact-sheets/detail/cholera

Yen, M., Cairns, L. S., Camilli, A. (2017). A cocktail of three virulent bacteriophages prevents *Vibrio cholerae* infection in animal models. *Nature Communications*, 8(1), 14187.

Young, R. (2014). Phage lysis: Three steps, three choices, one outcome. Journal of Microbiology, 52(3), 243-258. https://doi.org/10.1007/s00203-014-0964-1

Zhou, S., Liu, Z., Song, J., & Chen, Y. (2023). Disarm the bacteria: What temperate phages can do. *Current Issues in Molecular Biology*, *45*(2), 1149–1167. https://doi.org/10.3390/cimb45020076