ANALYSIS OF THE EFFECTS OF HORIZONTAL GENE TRANSFER ON BACTERIOPHAGE INFECTIVITY

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

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Declaration

It is hereby declared that

1. The thesis submitted is our own original work while completing the 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 research was conducted with the utmost commitment to ethical integrity and rigor. No human or animal subjects were used at any stage of the experimental process, ensuring that the study adhered to all established guidelines for non-invasive and non-human research. Additionally, all data collection, analysis, and reporting were carried out with transparency and respect for ethical norms. We also declare that there are no financial, personal, or professional conflicts of interest that could have influenced the research process, its outcomes, or the interpretation of findings presented in this report. This study was conducted with full independence, prioritizing objectivity and scientific integrity at every step.

Abstract

Bacterial infections and their treatment have been a longstanding interest for researchers and scientists throughout history. Initially, the discovery and application of antibiotics appeared to be a hopeful and dependable resolution to the issue. Nevertheless, since the bacterial population continues to grow and develop antibiotic resistance through the transfer of genes, it has become imperative to identify an alternative to antibiotic therapy for combating various bacterial strains. Bacteriophage therapy has been seen as a viable option for substitution. Hence, our study aimed to assess the capacity of phage infectivity on various strains of a particular bacterium, as well as on those same strains that have been genetically modified with antibiotic-resistant genes, in order to examine the possible effects of horizontal gene transfer. Our study specifically examined five indigenous infectious strains (O1) of *Vibrio Cholerae* and two particular antibiotics: Ampicillin and Kanamycin. Additionally, we employed another strain of *Vibrio Cholerae* as the experimental control. We isolated the pGLO plasmid from *E. coli* DH5α which contains the Ampicillin resistance gene. Additionally, we obtained the chromosomal DNA from the 1877 strain of *Vibrio Cholerae,* which carries the Kanamycin-resistant gene. Subsequently, the genes were introduced into the 5 indigenous strains individually, aiming to accomplish horizontal gene transfer in a controlled laboratory setting. Consequently, we experimented on a total of 24 strains of *Vibrio Cholerae*. These included 5 naturally occurring strains WT391, WT428, WT429, WT435, and WT439 that were genetically modified with the Ampicillin resistance gene, and also were genetically modified with the Kanamycin resistance gene, and one control strain (specifically, the 1877 strain of *Vibrio Cholerae*). Subsequently, we conducted a spot test on each strain using 6 phages JSF2, JSF6, JSF7, JSF10, JSF25, and JSF30 from our laboratory inventory. Our objective was to identify any influence of the transformation (horizontal gene transfer) on the infectivity of the phages.

Dedication

To our loving family and friends, whose unwavering support and encouragement have made this journey possible. To our mentors and colleagues, whose wisdom and guidance have inspired us to push our boundaries and strive for greatness. To the diverse communities that remind us of the importance of our work and the impact we can make. And to ourselves—here's to the countless hours of hard work, the day and night dedication, and the challenges we overcame together. This dedication honors our collective efforts, resilience, and commitment to learning. Together, we celebrate the pursuit of knowledge and the journey we have shared.

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Lists of Acronyms

Acronyms	Full Form
V. Cholerae	Vibrio Cholerae
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
HGT	Horizontal Gene Transfer
MDR	Multidrug-resistant
TCP	Toxin-Coregulated Pilus
AMR	Antimicrobial resistance
FDA	Food and Drug Administration
PCR	Polymerase Chain Reaction
CFR	Case Mortality Ratios
MSSA	Methicillin-sensitive *Staphylococcus aureus*
rpm	Rotations per minute
CT	CT Cholerae Toxin
UV	UV Ultraviolet
ARGs	Antibiotic Resistance Genes
TCP	Toxin-Coregulated Pilus

Chapter 01

Vibrio Cholerae

1.1 In Depth of *Vibrio Cholerae*

1.1.1 Introduction

Antibiotics are a significant innovation in the field of medicine, as they have achieved unprecedented success in treating bacterial infections and saving countless lives, surpassing the capabilities of any other medication. However, a concern arises when bacteria develop resistance to multiple antibiotics, resulting in a significant detriment to public health. In order to combat the problem of antibiotic resistance, scientists are currently investigating various alternative approaches to tackle bacterial infections. One of these strategies involves the development of diverse tactics to target bacterial strains that have developed resistance to specific antibiotics. Phage therapy is a promising approach that offers viable solutions against antibiotic-resistant bacteria, providing a favorable outcome and a ray of hope. During the process of overcoming this challenge, the significance of phage therapy as a potential solution against antibiotic resistance has been highlighted. It is evident that antibiotics, which were once considered a valuable tool against bacterial infections, have now become a major cause for concern.

Scientists have become interested in using phage therapy to combat bacterial infections after studying the life cycle of bacteriophages. Phage therapy is currently being investigated as a prominent method to combat bacterial infections in various organisms. Phage therapy is highlighted as a prominent area of ongoing research worldwide in the field of infectious diseases. Antibiotics have been efficacious in combating bacterial diseases for an extended duration. As antibiotic-resistant bacteria have evolved, the task has become to discover novel approaches to combat infectious diseases. Following extensive research, they uncovered phage therapy, a promising approach to combat bacterial infections. Bacteriophages are minuscule viruses that specifically target and eliminate bacteria for their own objectives. They utilize diverse strategies to effectively infiltrate bacterial cells, including those within biofilms. Extensive testing has demonstrated its efficacy against numerous antibiotic-resistant bacteria (Singh et al., 2022). Not only can they be utilized as a remedy for antibiotic-resistant microorganisms, but they can also function as bacterial strains. Phages have been extensively studied by the scientific research community for the treatment of infectious diseases. Furthermore, significant emphasis has been placed on

studying the use of phages in the treatment and prevention of cholera, motivated by the increase in strains of *V. Cholerae* that are resistant to antimicrobial drugs.

Cholera is a severe disease that has been treated with various medications such as Ampicillin and Kanamycin. However, the cholera bacteria has become resistant to many of these drugs. Consequently, various approaches to treating cholera have been experimented with over time. Due to the emergence of antibiotic resistance, it is crucial to conduct research on phage therapy for cholera. This therapy presents a promising alternative to combat this challenging and persistent health issue. There has been a recent increase in concern about the emergence of multi-drug-resistant strains in *V. Cholerae* (Das et al., 2019). Phage therapy is currently under investigation as a potential treatment for cholera in numerous countries. Horizontal gene transfer (HGT) is employed in bacteriophage-based therapies to eradicate pathogenic bacteria throughout all phases of their lytic life cycle. The objective of this study is to examine the vulnerability of indigenous, antibiotic-resistant *Vibrio Cholerae* strains to bacteriophage infection. Identifying appropriate alternatives is essential, as concerns regarding antibiotic-resistant *Vibrio Cholerae* are increasingly urgent. Identifying highly effective bacteriophages for treating these antibiotic-resistant bacteria could have applications in public health, environmental biotechnology, and medicine.

1.1.2 Literature review

Vibrio Cholerae, a gram-negative bacterium, is widely recognized as the causative agent of the potentially lethal diarrhoeal disease known as cholera. This specific strain of bacteria is located within the taxonomic order Vibrionales, family Vibrionaceae, phylum Proteobacteria, class GammaProteobacteria, and kingdom Bacteria. It is primarily located close to coastlines and within saline bodies of water. The primary cause of cholera is a strain of this bacterium that produces toxins. Additional Vibrio species have been associated with gastroenteritis and extraintestinal infections, commonly affecting the ears, soft tissues, and circulation. This bacterium commonly infiltrates the human body following the ingestion of water or food that has been contaminated. Upon entering, it attaches to the walls of the intestines and releases a toxic substance. The presence of this toxin is the cause of the watery diarrhea commonly observed in cholera cases. If left untreated, this condition can result in significant dehydration and potentially fatal outcomes.

The probability of a cholera pandemic escalates when individuals are compelled to reside in densely populated regions with inadequate sanitation as a result of poverty, conflict, or natural calamities. Cholera, which was first identified in 1817, has resulted in seven global epidemics. *Vibrio Cholerae* bacteria can be classified into two distinct varieties: O1 and O139. Both of these bacteria possess the capability to induce severe illness. The O1 strain, in its simplest manifestation, was responsible for the initial six outbreaks. The most significant and enduring epidemic to date originated in Indonesia in 1961 and is presently in its seventh year. The El Tor biotype is genetically associated with a distinct variant of the O1 type (Faruque & Mekalanos, 2012). The genetic composition of *Vibrio Cholerae* bacteria from non-O1 or O139 groups is highly distinctive. Only a minority of these non-O1/non-O139 strains possess the capability to generate cholera toxin. These strains have been linked to severe cases of gastroenteritis. Various diagnostic methods can be employed to identify the illness caused by *V. Cholerae*. One common method is to cultivate the bacteria in TCBS, which is a distinct type of growth media. Vibrio species exhibit differential growth on TCBS (Thiosulphate Citrate Bile Salts Sucrose) agar, with colonies displaying either a green or yellow color depending on their ability to ferment sucrose. The bacteria undergo sucrose fermentation, resulting in the yellow pigmentation of the colonies. Other

methods that can be used include Darkfield microscopy, which is especially effective in detecting *V. Cholerae*, commercially available kits that can quickly identify the antigens of the bacteria, and PCR (Polymerase Chain Reaction) techniques for identifying the bacterial DNA. Accessing clean water, maintaining proper hygiene practices, and ensuring safe food handling are all preventive measures against cholera. Obtaining water from a reliable source and ensuring proper cooking of meals are two effective methods to prevent infection. Vaccines are accessible for providing transient immunity against cholera in areas where the disease is widespread. The primary treatment for cholera is rehydration, which can be administered either intravenously or orally, depending on the level of dehydration. Antibiotics are not always necessary, although they can be utilized to diminish the duration and severity of the illness.

To prevent cholera outbreaks caused by *Vibrio Cholerae*, it is necessary to enhance water and sanitation systems, educate the population on proper hygiene practices, and mobilize responsible volunteers to respond to any outbreak. Notwithstanding these endeavors, cholera continues to pose a significant threat to public health in numerous regions across the globe, especially in areas with restricted availability of sanitation and potable water.

1.1.3 Clinical Manifestation

Cholera symptoms can quickly emerge following the consumption of contaminated food or water containing the *Vibrio Cholerae* bacteria. The initial manifestations of *Vibrio Cholerae* consist of abdominal pain, gastrointestinal gurgling sounds, and emesis. The situation deteriorates and becomes more complex when patients experience a significant loss of fluids and electrolytes due to diarrhea. The most severe instances of cholera are commonly attributed to two strains of *Vibrio Cholerae* bacteria: the O1 and O139 strains. The O1 strain encompasses two variants, with the El Tor strain being the predominant instigator of cholera outbreaks on a global scale. According to Finkelstein, 1996, the O139 strain, which was identified at a later time, induces a severe form of illness that is comparable to the O1 strain.

Upon consuming food or water that is contaminated with the *Vibrio Cholerae* bacterium, the symptoms of cholera manifest rapidly. The incubation period, defined as

the time between exposure to the bacterium and the onset of symptoms, typically ranges from a few hours to five days. Typically, symptoms manifest within two to three days after being exposed. Cholera can pose a significant risk if treatment is delayed due to its abrupt manifestation of symptoms (Clemens et al., 2017).

1.1.4 Patients with Diarrhea

Cholera can cause individuals to experience highly rapid and severe symptoms, especially in the form of acute, watery diarrhea. The onset of symptoms can occur within a timeframe of 6 to 12 hours after the initial clinical manifestations become apparent. The diarrhea progressively becomes more dilute, ultimately transforming into a cloudy, grainy substance with a distinct fishy smell. Rapid depletion of fluids and electrolytes can lead to dehydration, a potentially fatal condition if not promptly treated.

In extreme cases, the fluid loss through diarrhea in adults with cholera can be remarkably high, reaching up to 1 liter per hour. Young individuals afflicted with severe cholera can excrete between 10 and 20 cubic centimeters per kilogram per hour of fecal matter (Harris et al., 2011). Due to its tendency to cause a greater amount of fluid loss compared to other types of diarrhoeal illnesses, cholera is highly perilous and can potentially result in death if immediate medical attention is not sought. Cholera can lead to severe health complications, especially in older individuals. These complications encompass stroke and renal damage, particularly affecting the segment of the kidney responsible for blood filtration. Hypotension and insufficient systemic blood circulation are frequent underlying factors for these issues. Another potential outcome is the development of pulmonary infections caused by vomiting. Cholera patients almost always die as a result of severe dehydration (Waldor & Ryan, 2015). Cholera can result in significant gastrointestinal problems, along with diarrhea. Emesis is a prevalent symptom that frequently accompanies aqueous diarrhea. Emesis may occur before or after the initiation of diarrhea. Patients may also experience abdominal cramps. Contrary to dysentery, cholera generally does not induce the severe abdominal pain associated with other serious gastrointestinal disorders. These supplementary indicators accelerate the depletion of fluids and electrolytes, increasing the likelihood of severe dehydration (Jafet A, 2022).

Untreated cholera can be highly lethal. The mortality rate among individuals who do not receive treatment can reach up to 50-70% (Lindenbaum, 2022). In regions where cholera is prevalent, children have a significantly higher risk of dying from the disease compared to adults, with the likelihood being up to ten times greater. Furthermore, pregnant women who contract cholera face a significant danger to their unborn babies, as there is a 50% chance of fetal mortality in the third trimester. This underscores the significance of prompt and efficient intervention for cholera in order to avert these grave consequences.

1.1.5 Virulence Factor of *Vibrio Cholerae*

Vibrio Cholerae, the pathogen responsible for cholera, employs various virulence factors to initiate infection and induce the characteristic symptoms of the disease. Cholera toxin (CT) and toxin-coregulated pilus (TCP) are the primary factors that contribute to the virulence of toxigenic *V. Cholerae*. Each of these factors independently plays a role in the development of the infection (Faruque et al., 2003).

1.1.6 Cholera Toxin (CT):

The cholera toxin is composed of one A subunit and five B subunits. Monosialoganglioside (GM1) serves as a specific receptor on the surface of host cells, which is targeted by the B subunits for binding. The A subunit of the enzyme, which is responsible for adenosine diphosphate-ribosylation, is commonly known as the toxic domain (Kawshik, B. & Nirmal, K., 2011) Once the B subunits bind to GM1, the A subunit can enter the host cell. Upon entering the cell, it initiates the process of transferring ADP-ribose from nicotinamide adenine dinucleotide (NAD) to a protein in the host cell, thereby disrupting regular physiological processes. Intestinal epithelial cells experience an impact: When adenylate cyclase is activated by CT-induced ADP-ribosylation of host cell proteins, the concentration of cyclic AMP (cAMP) increases intracellularly. Elevated concentrations of cAMP subsequently induce anion secretion and hinder the uptake of electroneutral NaCl. The disruption in ion transport leads to the loss of water, salt, and chloride by the intestinal epithelial cells, resulting in distinct diarrhea observed in cholera (Wernick, et. al, 2010).

1.1.7 Toxin-Coregulated Pilus (TCP):

TCP is a crucial colonization factor for *V. Cholerae*. It facilitates the attachment of the bacterium to the intestinal epithelium, thereby promoting the dissemination of infection. The CTX phage, which contains the cholera toxin gene, attaches to TCP as a receptor. This link emphasizes the synchronized action of multiple virulence factors in the development of *V. Cholerae*'s pathogenesis. It aids in the proliferation of bacteria by promoting the formation of small colonies on the lining of the intestines, leading to their rapid spread. CtxA, an amino acid found in cholera toxin, not only secretes fluid and ions but also contributes to the disruption of the intestinal barrier. This interference facilitates the interaction of the toxin with target cells and assists in its translocation (Sharma, et al., 2008).

The virulence of *Vibrio Cholerae*, the bacterium accountable for cholera, functions through an intricate yet captivating biological mechanism. This process primarily involves the secretion of the cholera toxin (CT) by the bacteria (Oki et al., 2013).

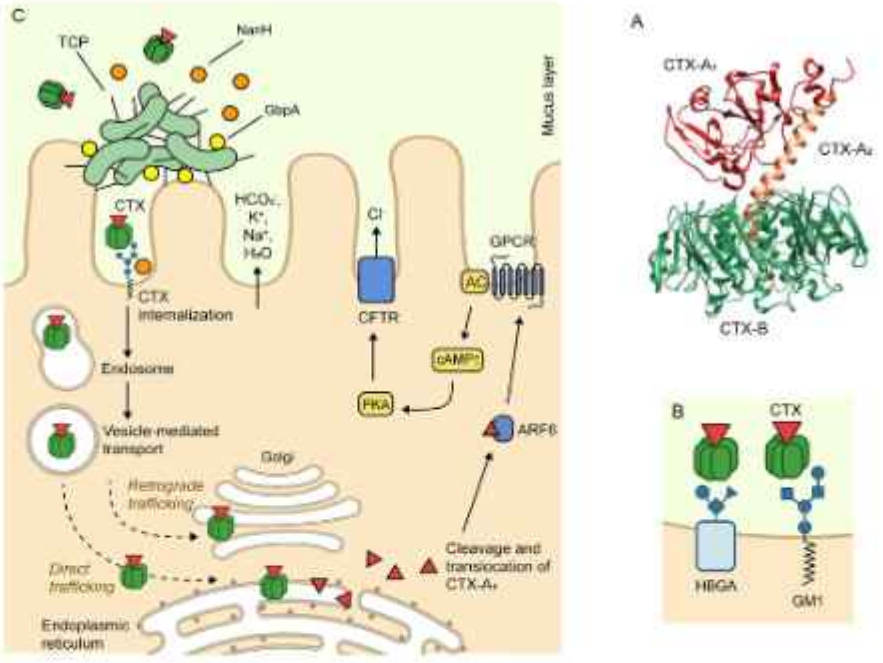


Figure 1.1: Mechanism of Cholerae toxin adapted from (*Mechanism of Action of Cholera Toxin. (A) The Crystal Structure of CTX,.*)

Vibrio Cholerae possesses a highly complex molecular mechanism that is responsible for its toxicity. The process initiates when the cholera toxin (CT) binds to specific receptors on the surface of host epithelial cells in the intestine. The receptors in question are referred to as ganglioside receptors. The binding of this substance triggers the process called endocytosis, which allows the poison to enter the cell. The cholera toxin is conveyed through the endosome, a vesicle that contains the toxin, to the Golgi apparatus and subsequently to the endoplasmic reticulum (ER) after entering the cell. In order to exert its effects, the toxin must be transported through this intracellular pathway. The catalytic CT-A1 polypeptide, which is the essential element of the toxin, undergoes degradation and is transported into the cytosol within the endoplasmic reticulum (ER). Retro-translocation is the term commonly used to describe the movement facilitated by the ER-associated degradation process. The Gs protein, a cellular protein, is activated by CT-A1 upon its entry into the cytoplasm. Upon activation of the Gs protein, there is a notable increase in the activity of an enzyme called adenylate cyclase (AC). This enzyme synthesizes cyclic adenosine monophosphate (cAMP), a crucial signaling molecule, from adenosine triphosphate (ATP).

Increased levels of cyclic adenosine monophosphate (cAMP) activate protein kinase A (PKA). PKA phosphorylates multiple proteins, including the chloride channel proteins found in the cystic fibrosis transmembrane conductance regulator (CFTR). The phosphorylation alters the activity of these channels. The CFTR channels undergo alterations, resulting in an increased release of chloride ions by the cells. Bicarbonate (HCO3), sodium (Na+), potassium (K+), and water (H2O) are all secreted simultaneously in the intestinal lumen. Consequently, NHE transporters are incapable of effectively absorbing sodium chloride (NaCl). The changes in ion transport ultimately lead to a significant discharge of fluid into the intestines, causing the development of intense and liquid diarrhea. This is the typical manifestation of cholera, characterized by electrolyte imbalances and dehydration commonly observed in individuals with the disease. *Vibrio Cholerae* O1 and O139 serogroups are known to cause epidemic cholera, but non-O1/non-O139 strains of the virus can also cause other severe diseases, such as diarrhea. When compared to the epidemic strains, these non-epidemic strains are generally considered non-pathogenic, although some of them may still be associated with human illness.

The virulence of *V. Cholerae* is caused by the combined action of cholera toxin and pilus, which are co-regulated by the toxin. The cholera toxin induces profound diarrhea by modulating ion transport via host cell signaling pathways. Simultaneously, TCP facilitates the initial colonization of the intestinal mucosa, thereby laying the foundation for the subsequent progression of infection. *Vibrio Cholerae* employs a variety of virulence factors to effectively disseminate and infect the host, showcasing its intricate strategies.

1.2 Global Spread of Antibiotic Resistant *V. Cholerae*

Antibiotics, which were the most significant breakthrough of the 20th century, have been further enhanced by the exceptional genetic capabilities of bacteria. This is due to the presence of multiple resistance genes in bacteria and their ability to transfer genes horizontally. The frequent consumption of antibiotics by humans has contributed to this improvement. As a result of this improper use, the introduction of any antibiotic for medical, agricultural, or other purposes has led to the emergence of various mechanisms of resistance. (Davies J & Davies D, 2010). The development of antibiotic resistance can occur through either the acquisition of foreign DNA or mutations in the current genome (Larsson D G & Flach C F, 2021). The capacity of pathogenic bacteria to develop resistance against various antibiotics poses a persistent danger in all areas of medical treatment (Davies, J., & Davies, D., 2010).

Historically, antibiotics have been employed for the treatment of cholera caused by *Vibrio Cholerae,* and certain medications have demonstrated efficacy against this bacterium. Cholera is commonly treated with the antibiotics erythromycin, fluoroquinolones, and tetracycline. Nevertheless, certain strains of *Vibrio Cholerae* have been observed to exhibit resistance to these medications. Treating cholera poses challenges due to the fluctuating levels of antibiotic resistance exhibited by *Vibrio Cholerae* worldwide. Antibiotic resistance occurs when bacteria acquire defense mechanisms to counteract the effects of antibiotics, thereby diminishing the effectiveness of these medications. The latest assessment reveals that nearly all clinical strains of *Vibrio Cholerae* exhibited resistance to commonly prescribed antibiotics, indicating a concerning trend. Cholera outbreaks and cases in African nations have been more prevalent recently when compared to Asia or the America. Significant

cholera epidemics have impacted numerous countries in eastern and southern Africa, often resulting in a substantial number of individuals falling ill or perishing. The emergence and dissemination of *Vibrio Cholerae* O1 serotype El Tor, a bacterium responsible for cholera, originated in the 1970s and rapidly spread across various West African countries (Mwansa et al., 2007).

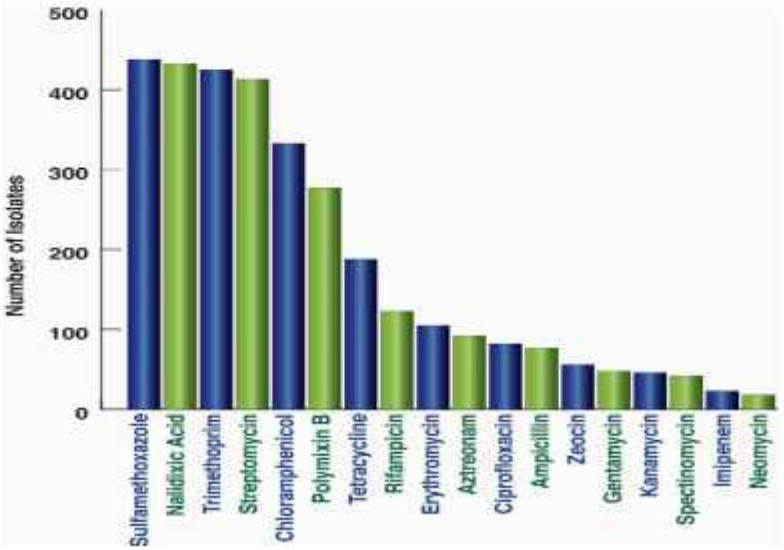


Figure 1.2: Antibiotic resistance and Sensitivity of Vibrio Cholerae Adapted from (Verma, et.al,2019)

Specific strains of the *V. Cholerae* bacterium possess inherent resistance to different antibiotics. Scientists examined the impact of various antibiotics on *V. Cholerae* O1, a specific strain, and found that it exhibited a greater range of resistance compared to other strains. Based on the bacteria's level of resistance, they classified them into three categories. Almost all strains of *V. Cholerae* displayed resistance to two or more antibiotics, and some even demonstrated resistance to ten or more. The most common form of resistance observed was towards the bacteriostatic drug sulfamethoxazole (Verma, et al., 2019). Variations in antibiotic resistance in *Vibrio Cholerae* are observed across different geographical regions. Various antibiotics can induce varying degrees of resistance in different regions of the country. To comprehend the evolving landscape of resistance patterns in *Vibrio Cholerae*, it is imperative to consistently engage in surveillance and monitoring of antibiotic resistance. The application of surveillance facilitates efforts to slow the spread of resistant strains and modify treatment guidelines in order to combat them.

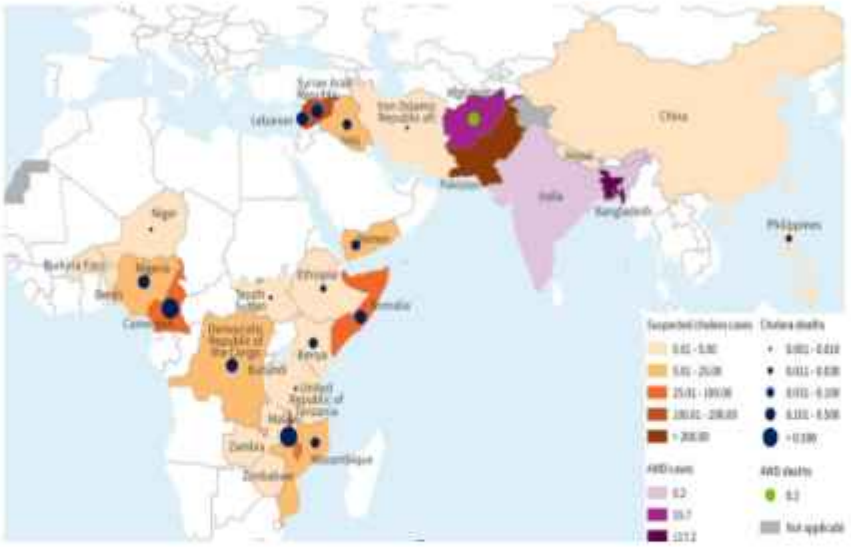


Figure 1.3: Incidence of cholera cases per 100,000 population reported to WHO (1Jan-30 Nov, 2022) adapted from (*Cholera – Global Situation*, 2022.)

Since 2021, there has been a global surge in cholera cases, accompanied by an expansion in their geographic spread. In 2021, cholera outbreaks were reported in 23 countries, primarily in the WHO Regions of Africa and the Eastern Mediterranean. In 2022, cholera cases or outbreaks have been reported in more than 29 countries (Figure 1), indicating a persistent trend. As of November 30, 2022, 16 of these have been experiencing prolonged outbreaks. A significant number of those countries documented an increase in both the number of cases and the case fatality ratio (CFR) compared to previous years. The global reported case fatality rate (CFR) for cholera in 2021 was 1.9%, with Africa experiencing a higher rate of 2.9%. These rates are significantly above the acceptable threshold of less than 1% and represent the highest recorded CFR in over ten years.

Global cholera cases and deaths have significantly increased this year, reversing a previous trend of decline. The outbreaks in 13 countries that did not report any cholera cases in 2021 are of particular concern. Among these, several countries had not documented any instances of cholera outbreaks for a significant period, ranging from three to 30 years. Additionally, several of these countries are not classified as having a high incidence of cholera. I, II The present circumstances signify a reemergence of the persistent seventh cholera pandemic that commenced in 1961.

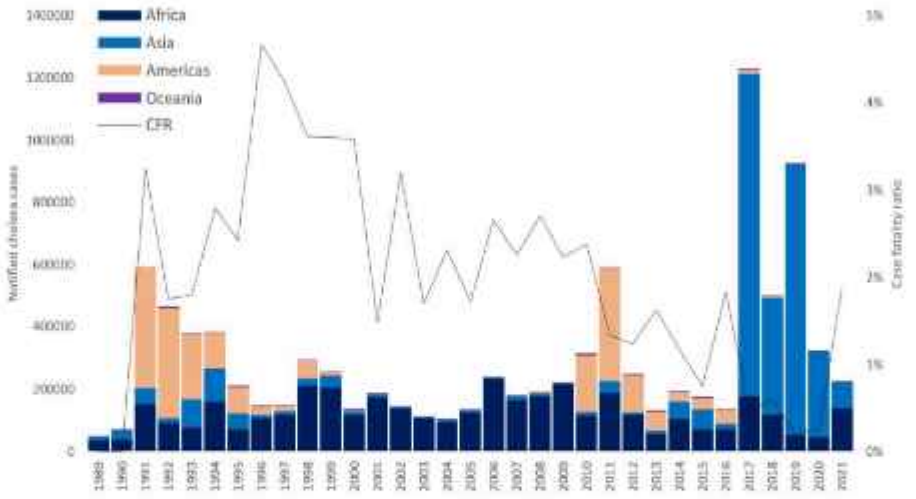


Figure 1.4: Worldwide cholerae cases from (1989 -2021) adapted from (*Cholera – Global Situation*, 2022.).

An intricate and concerning account of the transmission of cholera from 1991 to 2022 has permanently altered the state of worldwide public health. In both 2017 and 2019, Yemen accounted for the majority of cholera cases, specifically 84% and 93% of cases, respectively. However, this regional dominance is part of a broader global pattern. On a global scale, there has been a substantial rise in the incidence of cholera cases since 2021. In 2022, outbreaks were documented in more than 29 countries, which marks an increase compared to the 23 nations affected in 2021. The resurgence of the ongoing seventh cholera pandemic, which commenced in 1961, is particularly alarming due to its occurrence in countries that had previously not documented any cholera cases for several years.

As of November 30, 2022, 16 countries have reported prolonged outbreaks, indicating the diverse range of problems involved. Several of these nations encounter intricate humanitarian challenges and fragile healthcare infrastructures, exacerbating their predicaments as a result of climate change. Furthermore, the global scarcity of resources, including an inadequate supply of the oral cholera vaccine, hampers the capacity to respond promptly on a global scale. The surge in case mortality ratios (CFR), which is the highest in over a decade, underscores the severity of the situation. In 2021, the global average reached 1.9% and Africa experienced the highest peak at 2.9%. The occurrence of numerous cholera cases, combined with the burden on public health and medical personnel dealing with multiple disease outbreaks concurrently,

leads to unprecedented challenges. The ambiguity of estimations and the presence of insufficient and disregarded data hinder a comprehensive comprehension. The cumulative data spanning from 1991 to 2022 highlights a persistent and evolving danger, necessitating urgent, targeted, and adequately funded global initiatives to address the intricate challenges posed by cholera. The primary approach for controlling cholera is to enhance the water and sanitation infrastructure.

In areas with limited access to proper sanitation and clean water, there is an increased demand for antibiotics as a form of treatment. This can contribute to the rapid development of drug-resistant strains of the disease. It has been repeatedly observed that *V. Cholerae* is developing resistance to multiple antibiotics, which has become a significant global public health concern (Mwansa et al., 2007). It is recommended to select appropriate antibiotics for the treatment of cholera based on the resistance patterns exhibited by local strains in each specific region. By adopting this approach, we can more effectively address the problem of antibiotic resistance and ensure that treatments are successful in combating the specific strains prevalent in each geographical area.

1.2.1 Microbes Growing Resistant to Multiple Antibiotics

Bacteria have encountered numerous obstacles from other bacteria, bacteriophages (viruses), and predators during their evolutionary progression. Bacteria have evolved sophisticated defense mechanisms in order to adapt to these challenges. The defense systems, originally developed as adaptive measures against external threats, are now essential for safeguarding bacteria from antibiotics and other therapeutic interventions (Smith, W. P. J et al., 2023). Antibiotic-resistant bacteria possess a range of defense mechanisms, including the capacity to degrade the antibiotic, modify the targeted region of their bodies, regulate the substances that enter them, and devise novel survival strategies when the antibiotic fails to attack the intended area (Kapoor, G., Saigal, S., & Elongavan, A. 2017). Innate resistance is characterized by an individual's inherent insensitivity and is considered the most fundamental form of resistance. This inherent trait is consistently found within a species, strain, or bacterial population. In the realm of antibiotics, innate resistance refers to the concept that certain microorganisms possess inherent insensitivity, which

renders them immune to certain families of antibiotics.

Due to their exceptional genetic adaptability, bacteria can respond to various environmental hazards, including antibiotics that pose a threat to their survival. Bacteria have evolved inherent resistance mechanisms in environments where they coexist with antibiotic-producing species, allowing them to endure the presence of detrimental antibiotic compounds. Evolution has developed two primary genetic techniques in response to antibiotic attacks: horizontal gene transfer (HGT) to acquire foreign DNA, and mutations in drug-associated genes.

1.2.2 Mutational resistance

In the presence of an antibiotic, a subset of bacterial cells that have acquired mutational resistance undergo alterations in the drug's mechanism, which safeguard their capacity to survive. As vulnerable populations are eradicated, the number of resistant mutations increases rapidly. Antimicrobial resistance is caused by mutations that disrupt cell homeostasis, but these mutations allow the cells to survive in the presence of drugs. Resistance mechanisms can include changes to antibacterial targets, decreased medication absorption, activation of release mechanisms, or overall changes in metabolic pathways.

1.3 Horizontal gene transfer

Horizontal gene transfer is a significant factor contributing to the evolution of bacteria and the emergence of antibiotic resistance. The presence of inherent genetic resistance factors in the environment makes it a valuable reservoir of antibiotic-resistance genes for combating important microorganisms. Bacteria employ three mechanisms to acquire exogenous genetic material: transformation, transduction, and conjugation. Conjugation is a frequent occurrence in hospitals, especially in the gastrointestinal tracts of patients undergoing antibiotic treatment. Mobile genetic elements such as transposons and plasmids greatly facilitate the efficient transfer of genetic information. Integrons, as site-specific recombination mechanisms, excel at accumulating genes that enhance antibiotic resistance. This technique enables the integration of extra genes into bacterial chromosomes. This mechanism is a potent

method for facilitating genetic exchange and a significant factor contributing to the evolution of bacteria. Ultimately, the response of bacteria to antibiotics is influenced by both horizontal gene transfer and DNA mutations. These mechanisms play a crucial role in the emergence and dissemination of antibiotic resistance in medically relevant species.

Antibiotic resistance is a significant global problem that jeopardizes advancements in food production, healthcare, and quality of life. This is a worldwide problem that is not restricted to a specific geographical area. The rapid spread of antibiotic resistance is facilitated by the ease with which humans, animals, and various other objects can travel globally. This suggests that the medication we employ to combat infections may be ineffective or less effective. It poses a substantial obstacle for contemporary medicine and affects not only our overall health but also agriculture and well-being. This highlights the significance of global collaboration in addressing antibiotic resistance.

1.4 Bacteriophage and its Discovery

Bacteriophages are viruses that selectively target and invade particular bacteria, exploiting their metabolic processes to generate viable offspring, which then propagate to infect new hosts in their vicinity (Sanz-Gaitero et al., 2021). Phages are an alternative term used to refer to them. Bacteriophages consist of a nucleic acid genome as their genetic material, which is enclosed within capsid proteins that are encoded by the phage. Being the most abundant biological organisms on Earth, they are extensively found throughout the environment. They exhibit significant diversity in terms of their size, morphology, and genetic structure. Crucially, bacteriophages exhibit host-specificity, which means that a phage exclusively targets the bacteria it is designed to attack. Usually, a phage only infects a single species of bacteria or specific strains within a bacterial species. Thus, phages do not exert any infective influence on human cells (Kasman and Porter, 2022).

In 1896, Ernest Hanbury Hankin, an English bacteriologist, aeronautical theorist, and naturalist, was the first to identify the function and effect of bacteriophage. He documented his findings of antibacterial activity against *Vibrio Cholerae* in the

Ganges and Jumna rivers in India. In 1898, the Russian bacteriologist Gamaleya observed a comparable antibacterial activity on *Bacillus subtilis*. Nevertheless, it was Frederick Twort who restored and conducted additional research on the phenomenon after an interval of nearly two decades. Upon witnessing consistent outcomes resembling previous instances, he postulated that a virus could be the causative agent behind this occurrence. Regrettably, he was unable to pursue his studies due to a multitude of reasons. Felix d'Herelle made the ultimate breakthrough in the discovery of phage by conducting extensive research and experiments on a hemorrhagic dysentery epidemic that affected French soldiers. He noticed distinct areas, referred to as plaques, on agar cultures. The agar culture consisted of a combination of Shigella strains obtained from the patients and a bacteria-free filtrate of their fecal samples. This mixture was incubated and subsequently spread onto agar media. He hypothesized that a bacteriophage was the causative agent for this phenomenon. Subsequently, he introduced the term "Bacteriophage" by combining the words "bacteria" and "phage," which originated from the Greek language and signifies "to eat or devour." This term was chosen to convey the idea that phages have the ability to consume bacteria. This discovery prompted the belief among researchers that phages are indeed living viruses, as opposed to enzymes with antibacterial properties, which was previously hypothesized. (Sulakvelidze et al., 2001).

1.4.1 Bacteriophage Characteristics and Diversity

The number of bacteriophages is the largest among all organisms, including bacteria, as there are an estimated 1031 phages on Earth. These phages can be classified into two categories: virulent phages, which undergo the lytic cycle for replication, and temperate phages, which undergo the lysogenic cycle for replication.

As of January 2021, a total of 14,244 complete phage genomes have been sequenced. The INPHARED (INfrastructure for a PHAge REference Database) data set primarily consists of phages that infect a small number of bacterial genera. Specifically, 75% of the phage isolates in the database target only 30 bacterial genera. This database primarily contains a higher proportion of genomes from lytic phages (approximately 70%) and a lower proportion of genomes from temperate phages (approximately 30%). Approximately 54% of the genomes of these temperate phages originate from three

specific host genera (Cook et al., 2021). The sizes of phage genomes exhibit significant variation. For instance, the genomes of *Escherichia coli* phages consist of approximately 3,300 nucleotides of single-stranded RNA, which is considerably smaller than the genome of *Bacillus megaterium* phage G, which spans over 500 kilobase pairs. The smallest genomes of dsDNA-tailed phages exhibit considerable size differences, with *Mycoplasma* phage P1 measuring approximately 11.5 kbp, *Lactococcus* phage c2 measuring 21 kbp, and *Pasteurella* phage F108 measuring around 30 kbp. These phages belong to the Podoviridae, Siphoviridae, and Myoviridae families, respectively (Hatfull & Hendrix, 2011).

To safeguard the phages from degradation, the protein capsids possess a highly symmetrical structure that serves as a protective container for the phage's genome. The genome of a phage can be classified into four types: single-stranded DNA, double-stranded DNA, single-stranded RNA, or double-stranded RNA. Different phages exhibit capsids of diverse shapes, such as icosahedral, helical, spherical, and others. Phages, being host-specific, necessitate a mechanism for recognizing host cells. Multiple capsid proteins may serve as host cell recognition factors for various phages. Phage particles can contain not only components for genome transfer, host recognition, and genome protection, but also proteins for environmental sensing, binding to specific matrices where host bacteria are commonly found, and other functions (Sanz-Gaitero et al., 2021).

1.4.2 Classification of Bacteriophages

The Caudovirales order consists of three families: Myoviridae, which have a contractile tail; Podoviridae, which have a short tail; and Siphoviridae, which have a non-contractile long tail. These families collectively represent approximately 96% of all known bacteriophages (Kaliniene et al., 2017). There are ten small families of bacteriophages, which are filamentous, cubic, and polymorphic in shape. These phages make up about 3.6% of all bacteriophages currently known. All of them belong to the same order.

The phage morphologies are depicted in the below:

Table 1.1: Classification of Bacteriophage (Giri, 2021)

Family	Morphology	Nucleic acid	Characteristic
Myoviridae		Linear dsDNA	contractile tail,Non-enveloped
Siphoviridae		Linear dsDNA	Long non-contractile tail,Non-enveloped
Podoviridae		Linear dsDNA	Short non-contractile,Non-enveloped tail
Tectiviridae		Linear dsDNA	Isometric , Non-enveloped
Corticoviridae		Circular dsDNA	Isometric,Non-enveloped,
Lipothrixviridae		Linear dsDNA	rod-shaped,Enveloped
Plasmaviridae		Circular dsDNA	Pleomorphic, Enveloped
Rudiviridae		Linear dsDNA	Rod-shaped , Enveloped
Fuselloviridae		Circular dsDNA	lemon shaped , Non-enveloped
Inoviridae		Circular ssDNA	Filamentous,Non-enveloped
Microviridae		Circular ssDNA	Isometric,Non-enveloped
Leviviridae		Linear ssDNA	Isometric, Non-enveloped
Cystoviridae		Segmented dsDNA	Spherical , Enveloped,

1.4.3 Life Cycle of Bacteriophage

When a bacteriophage infects a bacterial cell, its genetic material is introduced into the cell, initiating a replication cycle of the bacteriophage. When the phage genome is inserted into the bacterial cell, the cell undergoes reprogramming and starts functioning as a factory for producing phages. Consequently, the biological apparatus (ribosomes, ATP generators) responsible for the replication of the bacteria is altered to reproduce and duplicate the phage. Following infection, the phage messenger RNAs are translated into proteins that are specific to the phage. These proteins initiate various pathways that reprogram the bacterial cell (Campbell, 2003).

Bacteriophages primarily undergo two types of replication systems: the lytic cycle, which is characteristic of virulent phages, and the lysogenic cycle, which is characteristic of temperate phages. Upon infecting bacteria, phages initially identify the host cell surface receptors, such as lipopolysaccharides, fimbriae, flagella, outer membrane proteins, etc. They then attach to these receptors through reversible adsorption, facilitated by tail fibers or tail spikes. For its viral specificity, the phage exclusively binds to its designated bacterial strain. Some tail fibers possess enzymatic activity that allows them to depolymerize the polysaccharides of the bacterial cell wall (Prokhorov et al., 2017). Subsequently, the tail fibers undergo contraction as a result of the structural reorganization of the base plate, facilitating attachment. The phage enters the host cell by utilizing different carbohydrate depolymerase and lysins. Simultaneously, the tail fiber of the phage combines with the host cytoplasmic membrane, resulting in the formation of a transmembrane channel. The phage introduces its nucleic acid (DNA or RNA) into the host cytoplasm via this channel (Giri, 2021).

Virulent bacteriophages undergo the lytic cycle. Once the genetic material enters the cytoplasm of the bacterial host, the phage DNA or RNA utilizes the host's replication machinery to produce its components such as capsid proteins, tails, and nucleic acid. The viral particles are synthesized and assembled through a variety of enzymatic activities. Upon reaching maturity, the phage's DNA is enveloped by a protein coat formed by the assembly of its head and tail proteins. During the process of maturation, the head and tail proteins of the phage come together, and each nucleic acid molecule (DNA or RNA) is enclosed by a protective protein coat. Finally, virions are

produced and the bacterial cell undergoes lysis, releasing numerous phage progenies that are prepared to infect new hosts.

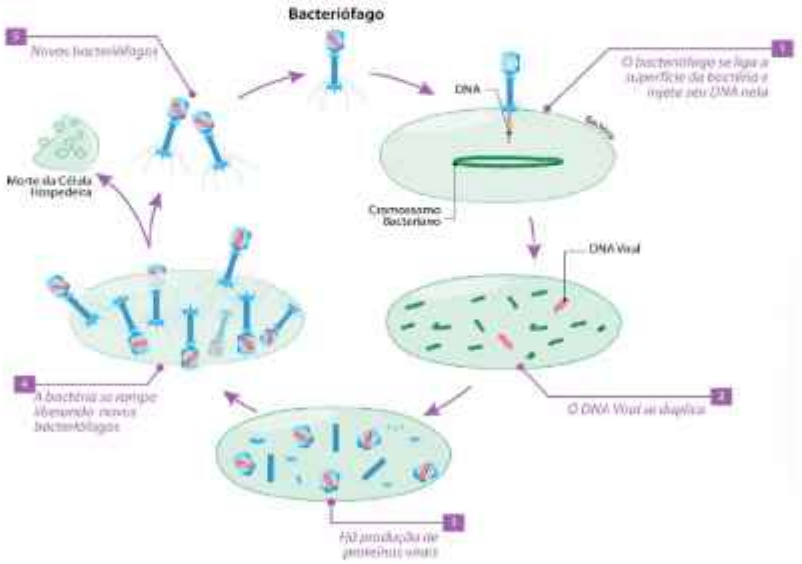


Figure 1.5: Life cycle of Phages adapted from (Lins, 2021)

Temperate phages undergo the lysogenic cycle to replicate. Rather than generating virions within the host cell, these phages integrate their genetic material into the bacterial genome, referred to as prophage. The phage DNA or RNA integrates into the host's genetic material and replicates without causing any harm. (Howard-Varona, 2017) A lysogenic bacteriophage can switch from the lysogenic cycle to the lytic cycle when faced with pressure or threats to its survival. This process results in rapid phage multiplication and subsequent rupture from the host cell.

Two additional life cycles occur infrequently: pseudolysogeny and chronic infection. Pseudolysogeny occurs when a host cell encounters unfavorable developmental conditions. Nevertheless, the phage refrains from merging its genetic material with that of the host. This process is essential for the survival of phages as it enables the preservation of the phage genome until the host growth conditions become favorable again. In chronic infections, there is a sustained and ongoing production of new phage particles over a long time, without any observable cell death. (Britannica, 2023)

1.5 Phage Therapy

Phage therapy is a method of treating bacterial infections by using bacteriophages. Phages exhibit viral specificity, targeting only bacteria and posing no threat to other organisms such as humans, plants, and insects. (Iftikhar, 2019) Bacteriophages are omnipresent and exist in diverse forms. Antibiotics have been extensively employed for the treatment and eradication of bacterial infections over a significant time. While antibiotics effectively combat harmful bacteria by eradicating them, they can also eliminate several beneficial bacteria from the microbiota, which can subsequently result in additional complications. In contrast, phages have developed a greater degree of specificity in targeting particular bacterial strains or species. The specificity of phages is determined by factors such as the phage-host receptor surface, the inherent characteristics of the phage, and the genetic and physical defence mechanisms of the host. (Hibstu et al., 2022) Thus, phage therapy specifically focuses on eliminating the pathogen while leaving the microbiome undisturbed. Consequently, it is an attractive alternative for treating infections due to its specificity, especially when dealing with multi-drug resistant (MDR) microorganisms.

1.5.1 Mode of Action

Phage therapy utilizes virulent or lytic phages, bioengineered phages, and purified phage lytic proteins as delivery agents (Hibstu et al., 2022). The mechanism of phage therapy involves the eradication of bacteria by causing cell lysis. The phage invades the bacteria and introduces its genetic material (DNA or RNA) into the host cell. Multiple virion copies are generated within the bacterial cell and ultimately cause the cell to rupture, liberating numerous phage copies. Subsequently, the phages can remain in a state of dormancy until they encounter fresh bacteria in their environment and eliminate them using the same mechanism. (Iftikhar, 2019) In order to utilize phage therapy for the treatment of a bacterial infection, the initial stage would involve the isolation of active phages that specifically target the particular bacterium. Subsequently, the phages must undergo amplification and be administered to the patient in a manner that facilitates their interaction with the infectious agents. The phages will multiply as long as they can detect bacteria in the vicinity. The phages will undergo degradation once the bacteria are eliminated as they rely on a host organism for their survival.

1.5.2 Strategies of Phage Therapy

Several therapeutic strategies have been devised for the use of bacteriophages in treating bacterial infections. Below, a few of them are succinctly outlined:

Phage Cocktail

Monophage treatment refers to the administration of a single phage type in phage therapy. However, a patient can be infected by more than one type of bacteria, rather than just a single type. Therefore, monophage therapy is not appropriate in that scenario. In addition, bacteria have undergone evolutionary changes and mutations over time to alter their cell surface receptors or modify restriction enzymes capable of breaking down phages. (Liu et al., 2022) Hence, in order to enhance treatment outcomes and expand the range of hosts that can be infected, a combination of diverse phage types can be employed instead of using a single phage for treatment. Phage cocktails are designed to specifically target a wide range of bacterial species. Furthermore, it can inhibit the evolutionary capacity of bacteria to develop resistance against phages (Abedon et al., 2021).

Phage-antibiotic synergy

Recent research indicates that the combination of phage and antibiotics results in enhanced and more effective elimination of the specific bacterial population, thereby elevating the mortality rate of the bacteria. When used together with antimicrobial medications, phages have been proposed to greatly decrease the viability of biofilms compared to each treatment alone, suggesting either synergy or facilitation. (Liu et al., 2022) However, the increasing understanding of hostile behavior has led to the initiation of laboratory tests to assess the combined effectiveness of phages and antibiotics before treatment, intending to identify advantageous combinations. Occasionally, a tailored approach has yielded favorable therapeutic outcomes (Liu et al., 2020).

Genetically engineered phages

Phages can be genetically engineered to expand their ability to infect a broader range of hosts, enhance their ability to kill bacteria and improve their capacity to degrade biofilms. Possible mechanisms for modifying the phage include gene mutation,

gene replacement, or integration of a desired foreign gene. Gene mutation or substitution typically seeks to expand the host range. Therefore, the introduction of genes results in significant bactericidal activity of the products (Guo et al., 202).

Enzymes derived from bacteriophages

Bacteriophages generate a multitude of enzymes, such as lysins, depolymerases, lipases, and DNases, which are highly effective in breaking down bacterial biofilms. These enzymes not only destroy existing biofilms but also impede the formation of new ones. Phage-derived enzymes exhibit antibacterial properties, making them suitable for use as therapeutic agents in the treatment of bacterial infections. Phages can degrade the extracellular polymerase substances (EPS), which allows them to disrupt the biofilm and penetrate the deeper layers of the bacterial structure. Phages capable of producing extracellular polysaccharide depolymerase can perform this action (Liu et al., 2022).

Integration of bacteriophages with other agents

Additional synergistic strategies propose the integration of phages with chemical disinfection, biological nanoparticles, and other methods to enhance treatment efficacy and efficiency (Liu et al., 2022).

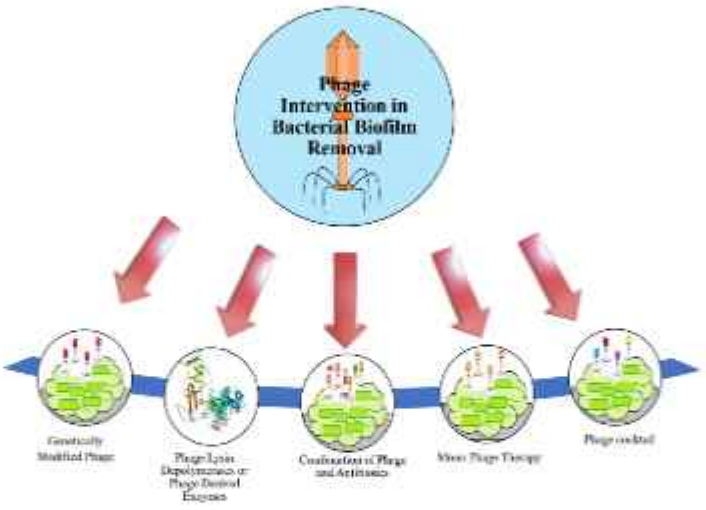


Figure 1.6: Phage Therapy strategies adapted from (Singh et al., 2022)

1.5.3 Advantages of Phage Therapy

Phage therapy has been suggested as a replacement for the diverse advantageous characteristics of phages. Despite ongoing research and experiments, there are a few benefits of phage therapy (Loc-Carrillo & Abedon, 2011).

- Due to their bactericidal activity, phages kill their target host bacteria instead of merely suppressing them. Consequently, they are unable to regain viability. However, antibiotics can inhibit the growth of bacteria. Consequently, they inhibit the proliferation of bacteria, thereby impeding the development of antibiotic resistance.

- Phages contribute to the regulation of phage dosage by continuously reproducing and infecting bacteria as long as suitable hosts are available. This process can be referred to as auto "dosing". Nevertheless, only a small number of phages rely on bacterial densities and necessitate a higher density in order to replicate.

- Phages exhibit viral specificity, meaning they exclusively target their dedicated bacterial strain and do not have any impact on strains that fall outside of their host range. Therefore, the introduction of phages into patients has minimal or negligible effect on their normal flora. However, due to their wide-ranging effectiveness, antibiotics tend to disrupt the balance of normal microorganisms, resulting in additional complications.

- Biofilms possess a greater propensity to develop antibiotic resistance compared to planktonic bacteria.

- Phage-borne enzymes such as lysins, depolymerases, lipases, and DNases are capable of breaking down external polymer substances and infiltrating biofilms. Phages capable of producing these enzymes can break down certain biofilms.

- Antibiotics typically exert an influence on the environment. Disposing of antibiotics in the environment can potentially result in the emergence of resistance among the nearby bacteria, and the chemical itself may also have an impact. Phages are composed of nucleic acids and proteins. Their potential negative impact on the environment is improbable. In the most extreme

scenario, they have the potential to impact only a small fraction of the bacteria present in the surrounding environment. Phages that are not adapted to degradative environmental conditions such as sunlight, desiccation, or extreme temperatures can also experience rapid inactivation.

- Phages, like antibiotics, can be developed through various methods, including in conjunction with other antibiotics. They are suitable for most administration routes and are available in various application forms, including liquids, creams, and solid impregnation. In addition, different phages can be mixed as a cocktail to augment their properties, typically resulting in a broader spectrum of antibacterial effectiveness.

1.5.4 Current State of Phage Therapy

As bacteria continually evolve to develop resistance against current and novel antibiotics, newly created antibiotics will inevitably encounter drug-resistant mutants. Resistance to a new antibiotic can rapidly disseminate to other pathogenic bacteria via horizontal gene transfer. An example of this is the widespread occurrence of resistance to ceftazidime-avibactam, which was observed shortly after its introduction in the US. As a result, the FDA has initiated the approval of phage therapy for compassionate use as a solution to combat antibiotic resistance in long-lasting infections. These infections encompass commonly encountered cases such as prosthetic joint infections, patients with solid organ transplants, hematological transplants, mechanical circulatory device implants, complicated surgical infections, and long-term immunosuppression (Verma et al., 2022).

The authorization of phage therapy for compassionate use in cases of multi-drug-resistant bacterial infection and biofilm-based infections has led to certain achievements in clinical trials. A persistent infection in the knee joint, caused by methicillin-sensitive *Staphylococcus aureus* (MSSA), was effectively treated using phage therapy. This infection was characterized by the presence of biofilms and had unfavorable results when treated with antibiotics. The 61-year-old patient was effectively treated after undergoing a two-stage exchange procedure and receiving a second course of phage therapy. In a separate investigation, a particular combination of phages was administered to a patient suffering from a pancreatic pseudocyst that was

infected with multi-drug resistant *A. baumannii*, leading to necrotising pancreatitis. This treatment resulted in a significant and positive change in the patient's overall well-being (Schooley et al., 2017)

1.5.5 Clinical Trial of Phage Therapy

Table 1.2: Clinical Trial of Phage Therapy (Giri, 2021)

Trial	Infection	Treatment Group	Phage Dose and Application	Outcome
1	*Pseudomonas aeruginosa otitis*	12 received a phage cocktail	109 PFU was intra-aurally delivered (single dose)	Three individuals from each group had undetectable *P. aeruginosa* levels at the trial's end
2	*Escherichia coli* diarrheal diseases	40 received phage cocktail M, 39 had cocktail T	1.4 X 109 PFU cocktail M or 3.6 X 108 PFU cocktail T delivered orally three times per day for 4 days in oral rehydration solution	No substantial difference was observed between the placebo group and the phage treatment group
3	*Pseudomonas aeruginosa burn*	12 received a phage	2 X 107 PFU (expected)	Trial halted, insufficient

	wound infection	cocktail	200– 2,000 PFU (actual) topically applied one time per day up to 7 days	efficacy; may be due to considerably lower applied dose of phage than estimated
4	Several multidrug-resistant bacteria	157 patients were administered phages from the Hirszfeld Institute collection via oral, rectal, or vaginal routes	10- 20 ml of phage collection was administered thrice daily for 12 weeks.	No antagonistic events; phage therapy provided good response up to 40% rate
5	*Escherichia coli; Staphylococcus aureus; Pseudomonas aeruginosa*	Intralytix phage cocktail WPP-201 was applied to 65 patients	1 X 109 Phage cocktail was topically applied on wound infection once a week for 12 weeks	Different wound healing rates with time (differential wound size reduction over time)

Phage exhibits numerous potential applications in the fields of dentistry, veterinary medicine, human health, and agriculture. Bacteriophages are utilized in various industries, including the food and cattle sectors, in addition to antibiotics.

1.5.6 Phage therapy in treating *V.cholerae* Infection

Phage treatment utilizes phages, which are viruses that specifically target certain bacteria, to effectively combat problematic and uncontrolled microorganisms. These microorganisms are frequently linked to contagious illnesses. Phages exhibit a high degree of specificity, selectively targeting specific bacterial strains, in contrast to antibiotics which have a broader impact on various bacteria. Phages can be utilized to infect and eradicate harmful bacteria during a bacterial infection while sparing beneficial bacteria and human cells from harm (Loc-Carrillo & Abedon, 2011). The accuracy of phage treatment makes it an attractive choice for fighting bacterial infections, providing a focused and potentially more customized approach in comparison to conventional antibiotic therapies. The utilization of bacteriophages in therapy is in line with the increasing fascination with alternative and individualized approaches to combat bacterial infections, especially in situations where antibiotic resistance presents difficulty. Due to the pressing need to discover efficient treatments, there is a revived fascination with bacteriophage (phage) therapy, an approach that has been in use for a century. In contrast to antibiotics, which may only have a limited effect on reducing diarrhea and bacterial shedding, the management of diseases such as cholera necessitates additional assistance in the form of strong fluid and electrolyte supplementation. Exploring alternative therapies such as phage therapy is essential for tackling the difficulties presented by antibiotic resistance in developing countries. Currently, most *V. Cholerae* strains in regions where the disease is common have developed resistance to commonly prescribed medications.

Phages possess the capacity to eliminate a substantial quantity of bacteria, rendering them a valuable instrument in the advancement of phage therapy for cholera. This technique is efficacious in the treatment of cholera patients, inhibiting the transmission of the disease, and diminishing the burden of harmful bacteria. In the preliminary study on phage therapy, high quantities of Anti-Cholera phages (ranging from 100 to 200 phages) were administered to combat the cholera bacterium. Nevertheless, the phages were hindered in their ability to carry out their task effectively due to frequent difficulties in replicating and amplifying in the presence of *Vibrio Cholerae*.

During the treatment of patients with illnesses, bacteriophage therapy was used in combination with tetracycline and fluid replacements. It was observed that the patients received very large amounts of phages. The phage treatment exhibited a commensurate decrease in the quantity of deleterious Vibrios in fecal matter, akin to the impacts of tetracycline. Nevertheless, this decrease did not result in any significant enhancement in clinical outcomes, such as a reduced duration of diarrhea or accelerated healing. The evaluation of phage therapy for cholera faced challenges due to the diverse serotypes of Vibrios and other obstacles. Two primary factors were observed: the differing vulnerability of these bacteria to phages, and the swift transit of phages through the gastrointestinal tract in individuals with cholera. The swift progression of this process may have contributed to the decreased occurrence of infection treatment and potentially influenced the importance of a subsequent phage infection.

Researchers in different regions of the world have investigated the potential of lytic cholera phages as a preventive intervention. The growth of *Vibrio Cholerae* was investigated in studies using a newborn mouse infection model, focussing on the impact of lytic phages. The results suggested that the phages were successful in substantially decreasing the quantity of *Vibrio Cholerae* in the small intestine. Phage therapy, which involves the use of viruses that specifically target harmful bacteria, has been employed to treat or prevent cholera, yielding inconsistent outcomes. Administering a specific type of phage to neonatal rabbits one hour before their exposure to *Vibrio Cholerae* effectively prevented the development of cholera symptoms, as demonstrated in a study conducted by Dutta et al. in 2012. A separate investigation demonstrated that administering a combination of five phage variants to adult rabbits 6 or 12 hours before exposing them to *Vibrio Cholerae* led to a minor alleviation in the intensity of diarrhea, but did not result in a notable reduction in the amount of bacteria present. Nevertheless, the identical phage mixture effectively decreased the amount of bacteria when given 6 or 12 hours after the *Vibrio Cholerae* infection.

Phage therapy is progressing in the field, and the precise and focused characteristics of phages make them a hopeful approach to treating bacterial infections, particularly in the face of increasing antibiotic resistance. The utilization of bacteriophages in the treatment of cholera demonstrates their capacity to diminish bacterial quantities and hinder the transmission of disease. Nevertheless, there are still obstacles that need to be overcome, such as problems related to the replication and

amplification of phages, the differing susceptibility of bacteria to phages, and the quick passage of phages through the digestive tract. However, the need to discover effective treatments, especially due to the rise of antibiotic resistance, has generated a renewed interest in phage therapy. Scientists are actively investigating and improving the use of phages as a potential alternative or additional method in the worldwide battle against infectious diseases. This approach provides a customized and precise treatment option.

Chapter 02

Methods and Materials

2.1 Place of Study

Investigating the Biotechnology and Microbiology Laboratory at BRAC University, located in Dhaka, Bangladesh. The research study took place at BRAC University, located in Dhaka, Bangladesh. The investigation was conducted in the Biotechnology and Microbiology Laboratory, which is part of the Department of Mathematics and Natural Sciences. The decision to select this location was intentional, as it offered the essential infrastructure and resources needed to conduct the thorough scientific investigation demanded by this study.

2.2 Lab Protocol Essentials: A guide to best practices

During the research process, we made sure to incorporate stringent safety protocols. Our team adhered to rigorous laboratory protocols by donning pristine lab coats and employing hand gloves. We were constantly cognisant of our actions. In addition, a range of equipment including pipette tips, falcon tubes, microcentrifuge tubes, culture media (both agar-based and broth-based), and empty test tubes for the spot test method underwent autoclaving at a temperature of 121°C and a pressure of 15 pounds for approximately two hours. All autoclaved media that needed to be stored was kept at a temperature of 4°C. In order to ensure cleanliness, all glassware used in the project, such as conical flasks, beakers, and test tubes, were thoroughly washed using both tap water and distilled water. In order to maintain a sterile working environment, a solution of 70% ethanol was used both at the laminar airflow station and the general working station to prevent any possible contamination. We diligently observed unfamiliar odors and substances and promptly notified the laboratory supervisor in the event of such occurrences. These rigorous measures were implemented to maintain the integrity and dependability of our research findings.

2.3 Preparation of Culture Media, Reagents and Solutions

2.3.1 TCBS Agar (Thiosulfate-Citrate-Bile-Sucrose Agar)

TCBS agar is a specialized medium that is specifically designed to isolate and grow *Vibrio Cholerae* and other closely related species. TCBS agar was essential in promoting the cultivation and detection of *Vibrio Cholerae* in this specific project. TCBS agar is formulated with agar, Ox bile, Yeast extract, sodium thiosulphate, peptone, sodium citrate (Na3C6H5O7), NaCl, sucrose, bromothymol blue, ferric citrate, thymol blue, and distilled water. It is important to mention that this research used pre-made TCBS agar, which was easily accessible in the laboratory. This choice was made to ensure the reliability and consistency of the experimental results.

The project utilized TCBS agar to selectively isolate and culture *Vibrio Cholerae*, as this bacterium alters the color of the growth medium through sucrose fermentation. TCBS is a media that has a green color. Vibrio exhibits the formation of yellow colonies upon cultivation.

Preparation of TCBS Agar

An electronic balance was used to measure a precise weight of 8.908 g of the powdered medium for the preparation of TCBS agar. As per the instructions provided by the manufacturer, a quantity of 89.08g of the powder media is necessary to create a 1000 ml volume of TCBS agar media. The powder media that was measured was subsequently combined with 100 ml of distilled water in a conical flask. The solution was agitated with a glass rod and subjected to heat from a Bunsen burner until the formation of bubbles, ensuring thorough homogenization of the substances. To preserve sterility and avoid contamination, the media was carefully poured into sterile Petri dishes in a controlled laminar airflow environment.

In this specific project, primarily medium-sized petri dishes were utilized, with an approximate volume of 20-25 ml of media poured into each plate. It is worth mentioning that TCBS media does not need to be autoclaved because it contains Ox bile, a substance derived from bile salts. The process of autoclaving has the potential to impact the sensitivity of oxbile and hinder the growth of gram-positive bacteria. By

meticulously following these preparation steps, we ensured the optimal conditions for the growth and isolation of *Vibrio Cholerae* on TCBS agar. By employing this standardized approach, the reliability, and consistency of experimental outcomes are ensured, thereby enhancing the scientific comprehension of *Vibrio Cholerae* and its associated species.

2.3.2 Luria-Bertani, Miller Broth (LB)

Luria-Bertani (LB) is a commonly employed nutrient-rich medium for bacterial cultivation, owing to its advantageous composition. The composition comprises yeast extract, sodium chloride, and tryptone. In this project, a pre-prepared LB medium obtained from the laboratory was used. LB, also known as Luria-Bertani, is a highly adaptable and nutrient-dense substance that facilitates the development and multiplication of different types of bacteria. The formulation contains yeast extract, which supplies vital vitamins, minerals, and amino acids required for bacterial metabolism. In addition, NaCl is added to maintain osmotic equilibrium, thus ensuring optimal conditions for growth.

Tryptone, which is obtained from casein, serves as a nitrogen and peptide source, thereby enhancing bacterial proliferation. The project was advantageous because it used the pre-prepared LB medium provided in the laboratory, as it offered convenience and reliability. The use of this standardized medium ensures a uniform and regulated setting for the growth of bacteria, which enables precise experimental outcomes and facilitates a thorough comprehension of microbial behavior.

Preparation of Luria-Bertani, Miller Broth (LB)

In order to prepare a 100 ml batch of LB media, 2 g of powdered media was accurately measured on foil paper using an electronic balance. According to the instructions provided by the manufacturer, the powder media was mixed with 100 ml of distilled water in a conical flask. The solution was vigorously agitated with a glass rod and subsequently heated on a Bunsen burner until it became transparent, ensuring the complete blending of the different components of the mixture. To ensure sterility and avoid contamination, the media was subsequently subjected to autoclaving at a

temperature of 121°C for approximately 2 hours. Autoclaving is an essential step in the preparation process as it efficiently eradicates any possible contaminants, guaranteeing the purity and integrity of the LB media. Once the autoclave cycle finished, the flask was left to cool, indicating that the LB media was now prepared for use.

2.3.3 Luria Agar (LA)

Luria agar, also referred to as LA agar, is a commonly used growth medium that is not selective and is used for bacterial culture. It creates a nourishing environment that facilitates the growth and multiplication of different bacterial species. The abundant nutrient content of this medium is crucial for the optimal proliferation and maturation of bacteria.

The LA medium was used in this project for streaking the inoculated stock bacteria and for sub-culturing. Streaking is a method used to transfer a small quantity of bacteria onto the surface of an agar plate in a specific pattern that enables the separation of individual colonies. By utilizing the LA medium, it ensured that the bacteria had sufficient access to the essential nutrients needed for their proliferation and survival.

Luria Agar (LA) preparation

In order to prepare a 100 ml batch of LB media, 4 g of LA powder media was accurately measured on foil paper using an electronic balance. According to the instructions provided by the manufacturer, the measured powder media was mixed with 100 ml of distilled water in a conical flask. If commercially prepared LA was unavailable in the laboratory, a mixture of Luria Bertani (LB) Miller broth and agar powder was used. To prepare 100 ml of LA media, 2 g of LB powder media and 1.5 g of agar powder (with a concentration of 1.5% agar) were accurately measured using an electronic balance. The measured quantities of the media were subsequently introduced into a conical flask, and then 100 ml of distilled water was added. The solution was vigorously agitated using a glass rod and subjected to heat from a Bunsen burner until the formation of bubbles, ensuring effective blending of the various components of the medium.

In order to preserve sterility and avoid contamination, the media was sterilized using an autoclave at a temperature of 121°C and under pressure for approximately 2

hours. After the autoclave cycle concluded, the media was cautiously transferred into sterile Petri dishes inside a laminar airflow hood, thereby reducing the likelihood of contamination. Subsequently, the media underwent solidification, resulting in the formation of an appropriate substrate for bacterial proliferation.

2.3.4 Preparation of LA media containing Ampicillin

For this project, it is essential to create a plate that selectively allows the growth of strains that have been transformed with Ampicillin. Once the Luria agar (LA) media has been prepared and the autoclave process is finished, the flask containing the media is left to cool. The cooling period is essential to guarantee the viability and efficacy of the antibiotic, Ampicillin, which will be introduced.

While the flask has reached a temperature that allows it to be handled without any protective covering, the Ampicillin supplement is added. As per the manufacturer's instructions, it is recommended to add 0.5 ml of Ampicillin supplement to 99.5 ml of media. Thus, we created a supply of Ampicillin solution with a concentration of 2mg/ml by combining 10 mg of Ampicillin powder with 5 mL of dH2O. The Ampicillin concentration must be precisely 0.01 mg/ml. We added 0.5 ml of the stock solution to each 99.5 ml of LA media. The inclusion of the Ampicillin supplement in the media creates a selective pressure, enabling the growth of only the desired strains while suppressing the growth of non-resistant strains. The utilization of this meticulous plate preparation technique is essential in experiments and research that focus on particular strains and their reaction to Ampicillin.

By meticulously adhering to these steps, we can guarantee the triumphant fabrication of Ampicillin selective plates, which will facilitate the isolation and identification of target strains in our project.

2.3.5 Preparation of LA media containing Kanamycin

For this project, it is essential to create a Kanamycin selective plate for the modified strains. Just like the method explained earlier for Ampicillin, the Luria agar (LA) media is prepared and then autoclaved. Once the autoclave cycle is complete, the

flask containing the media is placed in a separate location to facilitate the cooling process. The cooling period is crucial to guarantee the viability and efficacy of the antibiotic, Kanamycin, that will be introduced.

After the flask has reached a temperature that allows it to be handled without any protective covering, we proceed to introduce the Kanamycin. We created a supply of Kanamycin solution with a concentration of 5mg/ml by combining 500 mg of Kanamycin powder with 100ml of dH2O. The Kanamycin solution must have a concentration of 0.05 mg/ml. We added 1 ml of the stock solution to each 99 ml of LA media. Kanamycin is added to the media to exert selective pressure, thereby promoting the growth of desired strains while suppressing the growth of non-resistant strains. The utilization of this discriminating plate preparation technique is essential in experiments and research that focus on particular strains and their reaction to Kanamycin.

By meticulously adhering to these steps, we guarantee the triumphant fabrication of Kanamycin selective plates, which will facilitate the isolation and identification of target strains in our project.

2.3.6 Soft Agar Preparation

In order to conduct the spot test and create stocks for the strains, it was essential to prepare soft agar with concentrations of 0.6% and 0.8% respectively. To accomplish this, a precise quantity of 2 grams of Luria Bertani broth powder and 0.6 grams of agar powder for spot test, as well as 0.8 grams of agar powder for stock media, were accurately measured using an electronic balance and placed on a foil paper. The accuracy of the agar composition is ensured by these precise measurements. Subsequently, 100 ml of distilled water was introduced into a conical flask, together with the measured powders.

The solution was agitated with a glass rod and subjected to heat from a Bunsen burner until the formation of bubbles commenced. The heating process is essential for effectively blending the media and guaranteeing its uniformity. After thoroughly mixing the soft agar media and noticing the presence of bubbles, it was cautiously transferred into sterile test tubes using a glass pipette for the spot test. The media was poured into each test tube, filling them with a volume of 5-6 ml. The test tubes

containing the soft agar were sterilized by autoclaving at a temperature of 121°C and pressure for about 2 hours. Autoclaving is a commonly used method of sterilization that effectively removes all possible contaminants, thus guaranteeing the purity of the soft agar. For the stock, a volume of 1 ml of the sterilized soft agar media was poured into clean MCTs and left to cool before being inoculated with organisms.

Ensuring the correct concentration of soft agar is essential for the spot test, as it creates an appropriate environment for the growth and examination of bacterial colonies. By adhering to these meticulous procedures, we guarantee the dependability and uniformity of our experimental findings.

Reagents

Ampicillin Stock: 10 mg Ampicillin supplement per 5 ml of distilled water.

Ampicillin Supplement: 0.5ml Ampicillin stock solution per 99.5 ml LA media.

Kanamycin Stock: 250 mg Kanamycin supplement per 50 ml of distilled water.

Kanamycin Supplement: 1 ml Kanamycin stock solution per 99 ml LA media.

Phenol: Chloroform: Isoamyl alcohol - 25:24:1,v/v

Chloroform: Isoamyl alcohol - 24:1, v/v

Solutions: 20 % Arabinose Solution for Detection of pGLO Plasmid inside *E. coli* DH5α strain on LA Plates. The *E. coli* DH5α strains harbor a pGLO plasmid that carries a GFP gene and a gene responsible for metabolizing arabinose.

When arabinose is present, the GFP gene within the plasmid is activated, resulting in the emission of fluorescence when exposed to UV light. Hence, arabinose is included in LA plates while cultivating *E. coli* DH5α to verify and ascertain the existence of the pGLO plasmid. To prepare a 20% Arabinose solution, 2 grams of arabinose powder are dissolved in 100 ml of distilled water, and 3% of arabinose solution is mixed with LA.

2.4 Preparation for the Transformation of Antibiotic Resistance Strains

2.4.1 Reagents of pGLO Plasmid Isolation:

Alkaline Solution I: pH 8.0. For 30 ml solution:

Reagent	Molecular Weight (g/mol)	Calculated Weight for 30ml (g)
1M Tris-HCL	121.14	4.72
0.5 M EDTA	292.2438	4.38
1M Glucose	180.156	5.40

Table 2.1: Preparation of Alkaline Solution I

Once the reagents were measured, they were combined in a single conical flask and diluted with distilled water. Sodium hydroxide (NaOH) and hydrochloric acid (HCl) were utilized to adjust the pH.

Alkaline Solution II: For 30 ml 10N NaOH Solution: 12 g of NaOH mixed in 30 ml distilled water. The molecular weight of NaOH is 40 g.

For 10 ml 1% SDS Solution: 0.1g SDS mixed with 10 ml of distilled water.

Alkaline Solution III: pH 5.2. For a 30 ml solution: A solution with a concentration of 5M was prepared by mixing 14.72 g of Potassium Acetate with distilled water. The molecular weight of potassium acetate is 98.15 grams per mole. The pH was modified by gradually adding glacial acetic acid, drop by drop.

2.4.2 Reagents of Competent Cell Preparation:

Preparation of 30 ml CaCl2 Solution:

Reagent	Molecular Weight (g/mol)	Weight/Volume for 20 ml
0.1 M CaCl2	110.98 0.33 g	0.33 g
Distilled Water	-	20 ml

Table 2.2: Preparation of CaCl2 Solution

Preparation of 0.1M CaCl2 + 15% Glycerol Solution:

8.5 ml 0.1M CaCl2 solution was mixed with 1.5 ml Glycerol.

For Chromosomal DNA Extraction:

For 30 ml 3M Sodium Acetate Solution: pH 5.2: A solution was prepared by mixing 7.38gm of sodium acetate powder with distilled water. The molecular weight of sodium acetate is 82.0343gm per mole.

Hydrochloric acid (HCl) was incrementally added until the desired pH of 5.2 was achieved.

10% SDS Solution: 1 g of SDS powder was mixed with 10 ml of distilled water.

2.4.3 Reagents of Chitin Transformation Preparation:

Preparation of 0.9% Saline: A solution containing 0.9 grams of NaOH was prepared by mixing it with 100 milliliters of distilled water and then subjecting it to autoclaving.

Buffers:

TE Buffer: pH- 8: pH was adjusted using HCl/NaOH.

Reagent	Molecular Weight (g/mol)	Calculated Weight for 250 ml (g)
10mM Tris Base	121.14	0.30
1mM EDTA	292.2438	0.09

Table 2.3: Preparation of TE Buffer

The reagent powders were combined with distilled water, and the pH was adjusted.

50X TAE Buffer: pH- 8: pH was adjusted using HCl/NaOH.

Reagent	Molecular Weight (g/mol)	Calculated Weight/Volume for 30 ml
2M Tris Base	121.14	7.27 g
1M Glacial Acetic Acid	-	1.72 ml
0.5M EDTA	292.2438	0.56 g

Table 2.4: Preparation of 50X TAE Buffer

The reagent powders were combined with distilled water, and the pH was adjusted.

1X TAE Buffer: For 100 ml Solution:

Reagent	Volume (ml)
50X TAE Buffer	2
Distilled Water	98

Table 2.5: Preparation of 1X TAE Buffer

2.4.4 Preparation of Native *Vibrio Cholerae* strains

Native strain

We acquired a total of six strains of *Vibrio Cholerae* for our investigation, specifically 1877, WT 391, WT 428, WT 429, WT 435, and WT 439. In order to guarantee the precision of our strain selection, all of these strains were cultured on TCBS (thiosulphate citrate bile salts sucrose) media. After a suitable period of incubation, the existence of yellow colonies acted as a confirmation for the desired strains. Through the use of TCBS media, we successfully cultivated specific strains of *Vibrio Cholerae* and verified their identity by observing the unique yellow coloration of the colonies. By employing this method, we were able to concentrate exclusively on the strains that were the subject of our study, thereby guaranteeing the accuracy and significance of our research. Acquiring and confirming these particular strains is crucial for our study of the attributes and actions of *Vibrio Cholerae*. By implementing this rigorous selection process, we can effectively examine and contrast the various strains, acquiring valuable knowledge about their genetic, phenotypic, and pathogenic differences.

Culture on Non-Selective Media

After acquiring the strains we were interested in, we conducted our subsequent experiments using LA (Luria Agar) media. The selection of this specific media formulation was based on its capacity to supply the essential nutrients needed for vigorous bacterial proliferation. The LA media provided an ideal nutritional environment, which promoted the rapid growth of bacterial colonies, resulting in significant growth rates.

The decision to use LA media as our growth medium was intentional, as it is widely acknowledged and employed in microbiological research. The composition of this substance guarantees the presence of crucial nutrients, including amino acids, vitamins, and carbohydrates, which are necessary for the metabolic processes and reproduction of bacterial cells. By employing LA media, our objective was to establish a conducive environment for the growth of our bacterial strains, enabling us to acquire an ample amount of biomass for subsequent analyses. The successful proliferation of

the bacterial colonies is essential for the precise characterization and investigation of their physiological and genetic properties.

By utilizing LA media as our growth medium, we supplied the essential nutrients required for the proliferation of our bacterial colonies, thereby facilitating their growth and allowing us to carry out extensive investigations on our desired strains.

2.5 Transformation of Ampicillin Resistant Strains

2.5.1 pGLO Plasmid Isolation from E.coli DH5α

1. The bacterial cells containing the desired plasmid were grown overnight in 25 ml of LB medium with the addition of suitable antibiotics.

2. Afterwards, the cells were collected by spinning them in a centrifuge at a force of 10,000 times the acceleration due to gravity for a duration of 2 minutes, which was done three times.

3. The pellet obtained was then mixed with 200 µl of Solution 1, and freshly prepared Solution 2 (400 µl) was added and gently mixed by inversion.

4. The cells were exposed to room temperature for a duration of 5 minutes to cause the breakdown of cell membranes.

5. Subsequently, a frigid solution of Solution 3 (300 µl) was introduced, thoroughly mixed by inversion, and left to incubate on ice for 10 minutes.

6. The mixture was subsequently centrifuged at a force of 12,000 times the acceleration due to gravity (Xg) for a duration of 15 minutes.

7. The liquid portion containing the desired components was meticulously transferred to a new tube.

8. An equivalent amount of phenol: chloroform: IAA (25:24:1) was added to the mixture, and then vigorously mixed using a vortex. The resulting mixture was then subjected to centrifugation at a speed of 12,000 Xg for a duration of 2 minutes.

9. The supernatant that was obtained was collected, and an equal amount of chloroform:

IAA (24:1) was added. The mixture was then mixed vigorously and centrifuged at a speed of 12,000 Xg for a duration of 5 minutes.

10. The liquid portion that remained after the solid particles settled was collected again. An equal amount of isopropanol was added to it, mixed by turning the container upside down, and then spun in a centrifuge at a force of 12,000 times the acceleration due to gravity for a duration of 15 minutes.

11. The DNA pellet obtained was rinsed with 70% ethanol, dried by exposure to air, and ultimately dissolved in 200 µl of TE buffer.

12. The plasmid was subsequently preserved at a temperature of -20 °C for future utilization.

The following steps delineate the protocol utilized for extracting and purifying the desired plasmid from the bacterial cells. By adhering to these procedures, one can guarantee the isolation of plasmid DNA of exceptional quality that is appropriate for a wide range of subsequent uses.

2.5.2 Competent Cell Preparation

1. Individual colonies of each indigenous strain of *Vibrio Cholerae* (WT 391, WT 428, WT 429, WT 435, and WT 439) were separately inoculated into 5 ml tubes containing LB media and incubated overnight at 37°C on a shaker.

2. Subsequently, a 1 ml portion of the cell cultures was individually introduced into 50 ml of LB media.

3. The cultures were placed in a shaker incubator at a temperature of 37°C and were regularly monitored until the optical density (OD) reached 0.4. Measurements were taken every 2 hours.

4. When the cultures reached an optical density (OD) of 0.4, they were moved to individual microcentrifuge tubes (MCTs) and kept in an ice bucket for a duration of 10 minutes.

5. The cultures were subsequently subjected to centrifugation at a speed of 5000

revolutions per minute for a duration of 10 minutes at a temperature of 4 degrees Celsius.

6. The media was extracted from each MCT.

7. Subsequently, 0.8 ml of CaCl2 solution was introduced to the resuspended cells, which were then placed in an ice bucket for a duration of 30 minutes. Afterward, the cells were subjected to centrifugation at a speed of 5000 revolutions per minute for 10 minutes.

8. The media was once again eliminated, and the cells were suspended again using 0.8 ml of CaCl2 solution.

The resuspension was placed in an ice bucket and incubated for 20 minutes, followed by centrifugation at a speed of 5000 rpm for 10 minutes.

9. Ultimately, the media was extracted from each MCT, and 200 µl of glycerol was introduced to the cells. Subsequently, the cells were preserved at a temperature of -20°C for future utilization in the transformation experiment.

The following steps delineate the procedure for preparing *Vibrio Cholerae* cells for utilization in a transformation experiment. Adhering to these procedures is crucial for ensuring the viability and quality of the cells, which is essential for a successful transformation.

2.5.3 Transformation with pGLO Plasmid

1. The cell cultures that make up the components were taken out of storage and placed on ice.

2. A 100µl mixture of the component cell culture and 10µl (50ng) of plasmid was prepared for each strain. The mixture was then placed on ice for 10 minutes to allow for temperature adjustment with the plasmid.

3. Subsequently, the mixtures underwent a heat shock by immersing them in a hot water bath set at a temperature of 42°C for an exact duration of 90 seconds.

4. Following the heat shock, the mixtures were promptly returned to the ice for 2

minutes in order to seal the pores of the cell membrane.

5. The mixtures were subsequently mixed with 1 ml of LB media and incubated for 1 hour at 37°C.

6. Ultimately, 100µl of the cultured mixtures were spread onto LB media plates under different conditions to evaluate the existence of transformed cells.

The following steps outline the procedure for genetically modifying the individual cells using a plasmid. By adhering to these protocols, scientists can introduce the intended genetic material into the constituent cells and subsequently evaluate the efficacy of the modification.

2.6 Transformation of Kanamycin Resistant Strains

2.6.1 Bacterial Chromosomal DNA Extraction

1. A 1ml culture of the 1877 *Vibrio Cholerae* strain that had been grown overnight was moved to a 1.5ml microcentrifuge tube (MCT).

2. The MCT containing the bacterial culture was subjected to centrifugation at a speed of 8000 revolutions per minute for a duration of 10 minutes, at a temperature of 25°C.

3. A volume of 200 µL of TE buffer was introduced into the tubes, causing the bacterial pellet to disperse through vortexing.

4. The tube was subjected to heating at a temperature of 100°C for 10 minutes, followed by immediate incubation at a temperature of -4°C for an additional 10 minutes.

5. A volume of 20 µL of a solution containing 10% SDS was added to the tube and mixed with care. Next, the mixture was subjected to incubation at a temperature of 55°C for 1 hour, during which it was observed to transform into a clear and viscous state.

6. A PCI solution with a volume of 220 µL was added and thoroughly mixed by vortexing. Next, the tube was subjected to centrifugation at a speed of 13200 revolutions per minute for a duration of 10 minutes.

7. The upper aqueous layer was meticulously transferred into new tubes.

8. An equivalent volume of chloride ions (Cl) was added and thoroughly mixed by vortexing. Next, the tube underwent centrifugation at a speed of 13200 revolutions per minute for a duration of 10 minutes.

9. The upper aqueous layer was meticulously transferred into new tubes.

10. A volume of sodium acetate with a concentration of 3M that is 0.1 times the original volume and a volume of absolute ethanol that is 2.5 times the original volume were added. The mixture was gently mixed by vortexing. Subsequently, the tube was subjected to incubation at a temperature of -20°C for 1 hour.

11. The tube underwent centrifugation at a speed of 13200 revolutions per minute at a temperature of -4°C for a duration of 30 minutes.

12. The liquid portion above the sediment was removed and the DNA was dissolved in 20 µL of TE buffer. The DNA was preserved at a temperature of -20°C.

The following steps outline the procedure for isolating DNA from a bacterial culture. By adhering to these protocols, researchers can acquire DNA for subsequent analysis or experimentation.

2.6.2 Chitin Induced Transformation Protocol:

Day 1:

1. The chosen bacteria from the available supply were spread on LA plates or bacterial colonies were introduced into LB medium from newly prepared plates and left to grow overnight.

Day 2:

1 ml of the overnight culture was added to 9 ml of fresh LB medium to produce a 10 ml young culture. The culture was cultivated in a shaker incubator for approximately 1.5 to 2 hours.

2. The culture was subsequently subjected to centrifugation at a speed of 5000

revolutions per minute for a duration of 10 minutes.

3. The cell pellet was rinsed with 5 ml of 0.9% NaCl (saline) by initially using a pipette and then subjecting it to centrifugation. The washing process was conducted under centrifuge conditions at a speed of 5000 RPM for 2 minutes.

4. The saline solution was disposed of, and if needed, the rinsing process was repeated twice.

5. To the bacterial cell pellet that had been washed, 5 ml of saline and autoclaved chitin flakes weighing between 50-80 mg were added, and then the mixture was re-pipetted.

6. The mixture was completely blended and left to incubate at a temperature of 30 degrees Celsius for the entire night.

Day 3:

1. The process of centrifugation was carried out at a speed of 3000 revolutions per minute for 5 minutes, after which the liquid portion above the sediment was removed and discarded.

2. A 5ml volume of saline was introduced, along with a suitable quantity of previously isolated genomic DNA. The mixture was subsequently subjected to incubation periods of 2, 4, 6, or 24 hours.

3. The exoskeletons of the prawns were moved to a new Falcon tube.

4. A sufficient quantity of saline solution was added, guaranteeing complete submersion of the shells. The minimum volume of saline required was determined to be 1.5ml.

5. Intense agitation of the tube for 5 minutes helped separate the bacteria from the chitin.

6. The tube remained undisturbed until all the flakes had settled.

7. The liquid above the settled flakes, known as the supernatant, was meticulously gathered.

8. If the liquid above the sediment showed noticeable cloudiness, it was mixed with a

new LB solution to reduce its concentration. Alternatively, dilution was superfluous.

9. The supernatant, whether diluted or undiluted, was evenly distributed onto LA plates or LA plates that had been supplemented with Kanamycin.

2.7 Gel Electrophoresis

2.7.1 Preparation of Agarose Gel

1. A flask was obtained and 0.5gm of agarose was measured and introduced into it, along with 50 ml of 1X TAE buffer. The solution was subsequently heated in the microwave on a hot plate until the agarose was completely dissolved and the solution achieved clarity.

2. The solution was slightly cooled and then 2 µl of EtBr was introduced.

3. The solution was transferred into the gel tray and allowed to cool at ambient temperature. Before the solution reached complete cooling, the comb was inserted into the gel tray. The comb was positioned approximately 1 inch from one end of the tray and was placed in a vertical orientation, with the teeth elevated about 1-2 mm above the surface of the tray.

The following steps outline the procedure for preparing a 50 ml solution of agarose with a concentration of 1% for gel electrophoresis. It is crucial to meticulously adhere to these steps to guarantee the complete dissolution of agarose and the correct setup of the gel.

2.7.2 Performing Gel Electrophoresis

1. The comb was delicately extracted, and the gel tray was inserted into the electrophoresis chamber.

Next, the tray was coated with an electrophoresis buffer, specifically around 1 liter of TAE buffer, which is the same buffer utilized for agarose preparation. The cover was positioned temporarily until the wells were completely submerged.

2. To prepare samples for electrophoresis, 2 µl of gel loading dye was added to every 8 µl of DNA sample.

3. The gel was electrophoresed at a voltage of 90 V, and it likely required around 45 minutes for the electrophoresis to finish.

The following steps provide a concise overview of the process for conducting gel electrophoresis. It is crucial to strictly follow these steps with great attention to detail to guarantee precise outcomes.

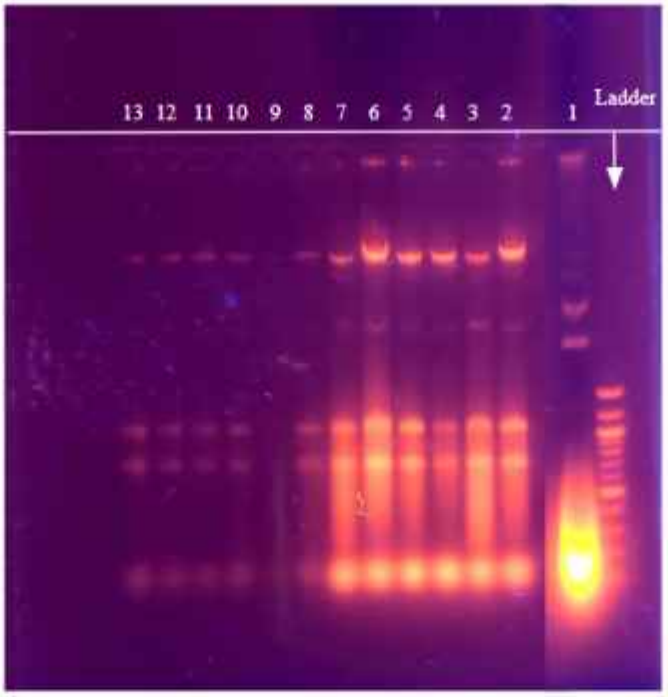


Figure 2.1: Plasmid Isolation Conformation through Gel Electrophoresis

2.8 Bacteriophage Identification & Purification

2.8.1 Bacteriophage Identification from Stock Solution

As part of this experimental procedure, a total of six bacteriophages were acquired from the stock, specifically JSF2, JSF6, JSF7, JSF10, JSF25, and JSF30. Every phage was separately gathered in sterile falcon tubes, with a volume of 10 ml for each sample. This study aimed to examine the host specificity of these phages.

2.8.2 Bacteriophage Enrichment

Following the collection of the phages from stock, they underwent enrichment through the subsequent procedures:

1. A new culture of the host bacteria was prepared for each phage using LA plates.

Two colonies were selected and transferred into a new 15 ml falcon tube containing LB media.

The host bacteria culture was prepared by incubating the culture for a maximum of 2.5 hours.

4. The intended bacteriophage was introduced into the culture.

The culture was cultivated in the shaker incubator at a temperature of 37°C for a duration of 4 hours.

6. Next, the culture was subjected to centrifugation at a speed of 13000 revolutions per minute at a temperature of 4°C for a duration of 10 minutes.

7. The liquid portion was passed through a 0.22 um filter into a new, sterile falcon tube.

The resulting transparent solution was devoid of bacterial cells and contained the intended bacteriophage. The falcon tubes were accurately labeled, for example, with the designation "*V. Cholerae* 1877(JSF2)," and tightly sealed with parafilm. Subsequently, these tubes were preserved at a temperature of 4 degrees Celsius. To increase the number of phages in the event of complete consumption, the process of enriching the isolated phage was repeated.

2.8.3 Phage Infectivity Test on Different *Vibrio Cholerae* Strains

In the phage infectivity test on various *Vibrio Cholerae* strains,

1. The LA media plate was utilized to subculture all six indigenous strains and the 1877 *Vibrio Cholerae* strain as the control.

2. On the next day, the individual colonies of each strain were introduced into separate falcon tubes containing 5 ml of LB media. The tubes were subsequently placed in an incubator set at a temperature of 37 degrees Celsius to foster the growth of a juvenile culture, which was distinguished by a slightly cloudy appearance allowing the passage of light.

3. After the young culture was ready, a layer of bacteria was formed by pouring a mixture of 6 ml of soft agar (0.6% agar) and 100 microlitres of the bacterial young culture onto an LA plate.

4. Adequate time was allocated for the layer of agar to solidify on the LA plate.

5. Two LA plates were assigned to each bacterial strain, with clear markings indicating where the phage droplets should be placed.

6. Utilizing a micropipette, a volume of 10-15 microlitres of each phage was dispensed onto the bacterial lawn, and sufficient time was allowed for the phages to spread through the soft agar via diffusion.

7. Ultimately, a total of 12 plates were placed in an incubator set at a temperature of 37 degrees Celsius for 12-13 hours.

8. The regions on the plates where transparent areas were noticed around the droplets were designated as potential phages for those particular bacteria.

To evaluate the host specificity, a spot test was performed utilizing indigenous bacterial strains. This experiment entailed applying small droplets of the phage samples onto agar plates that had been previously inoculated with the corresponding bacterial strains. The plates were subsequently placed in an environment that was suitable for the growth and interaction of the phages and the bacterial hosts.

The spot test functioned as an initial screening technique to detect the existence or nonexistence of distinct regions, referred to as plaques, that signify the destruction of bacterial cells by the phages. The presence of plaques indicates that the phages possess the capability to invade and reproduce within the particular bacterial strains that were examined. This information is essential for comprehending the spectrum of hosts and possible uses of these bacteriophages.

2.8.4 Phage Infectivity Test on Different Antibiotic Resistant *Vibrio Cholerae* Strains

Ampicillin-resistant strains were tested using LA media plates for subculture, LB media for young culture, and LA plates for the spot test. The media was rendered selective by incorporating an appropriate quantity of Ampicillin supplement (0.5ml Ampicillin per 100ml media). Similarly, Kanamycin-resistant strains were subcultured on LA media plates, young cultures were grown on LB media, and spot tests were conducted on LA plates. The media was rendered selective by incorporating an appropriate quantity of Kanamycin (0.5ml Kanamycin per 100ml media). The identical procedures were employed for both strains that exhibited resistance to antibiotics, resulting in the observation of plaques.

The following steps provide a clear procedure for testing the infectivity of the phage on various *Vibrio Cholerae* strains. The test employs selective media to identify strains that are resistant to Ampicillin and Kanamycin specifically. This study enhances our comprehension of the interactions between phages and their hosts, as well as the creation of tactics to combat bacteria that are resistant to antibiotics.

Chapter 03

Results

3.1 Native Strains Conformation:

Upon the completion of all experiments, the results were collected and systematically arranged for analysis. The experiments were repeatedly conducted to verify the precision of the outcomes.

The experiment included the selection of the following *V. Cholerae* strains of serotype 01 as samples: WT 391, WT 428, WT 429, WT 435, and WT 439. From a pool of 24 distinct *V. Cholerae* strains, we assessed the sensitivity of each strain to Ampicillin and Kanamycin antibiotics. Out of the 24 strains, 5 were found to be sensitive to both Ampicillin and Kanamycin and were selected for further analysis. The following samples are of native strains that have been selected:

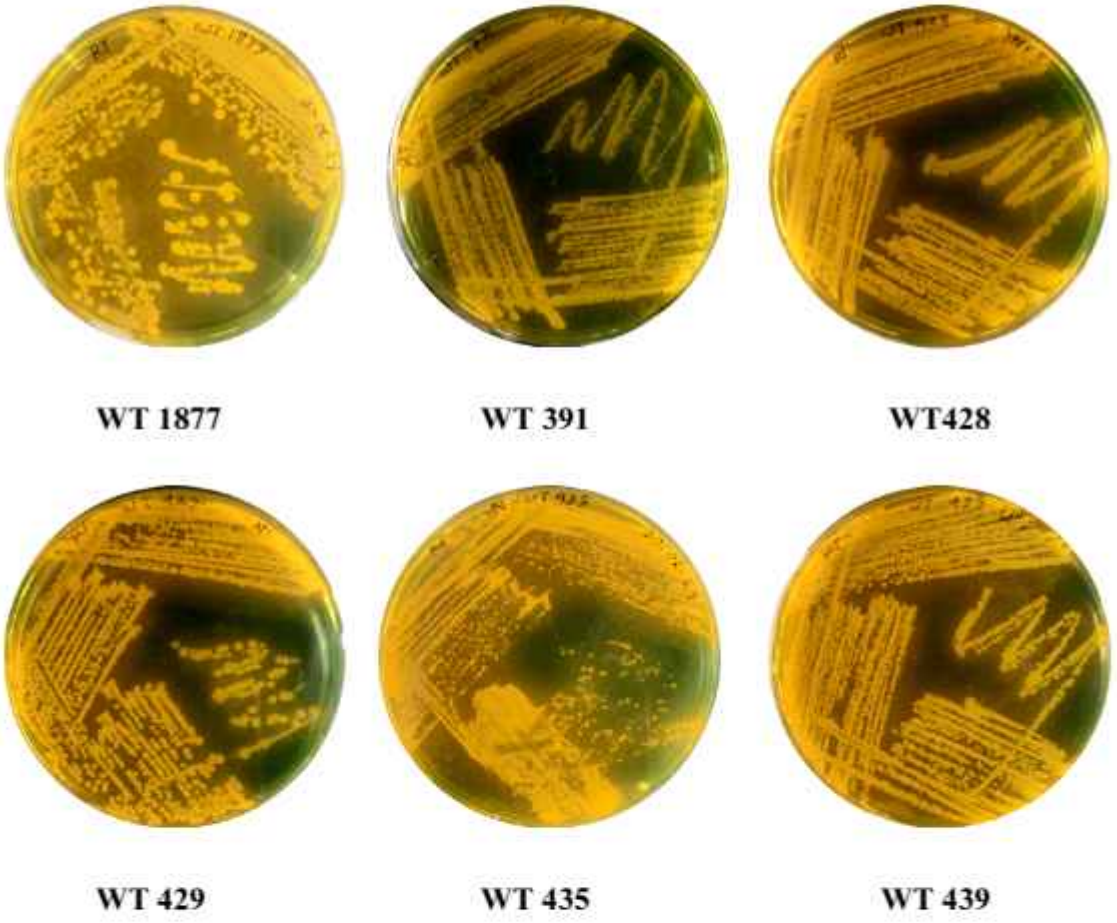


Figure 3.1: 5 native *Vibrio Cholerae* (O1) serotype strains named WT 391, WT 428, WT 429, WT 435, and WT 439 on TCBS. All of these strains are sensitive to Ampicillin and Kanamycin antibiotics.

The native strains underwent horizontal gene transfer, resulting in the creation of two copies of each strain. One copy contained the Ampicillin resistance gene, while the other copy contained the Kanamycin resistance gene. E.coli DH5α was utilized for the Ampicillin-resistant gene. The pGLO plasmid was extracted from E.coli DH5α. The Kanamycin gene was extracted from the chromosomal DNA of the *Vibrio Cholerae* 1877 strain, which naturally possesses a Kanamycin-resistant gene. After introducing the Ampicillin-resistant gene from the pGLO plasmid and the Kanamycin-resistant gene from isolated DNA of 1877 into the original strains, we obtained two copies of each transformed strain. These transformed strains have been labeled as "T".

3.2 Transformed Ampicillin Resistant Gene Conformation

Ampicillin Resistant Gene Containing pGLO Plasmid Transformed Strains

The data indicates that all of the strains have effectively incorporated the pGLO plasmid. Following transformation, the strains were cultured on LA media supplemented with Ampicillin. The growth of the strains after horizontal gene transfer (HGT) was observed to be typical, suggesting that these newly acquired strains have developed resistance to Ampicillin. The following are the newly transformed strains:

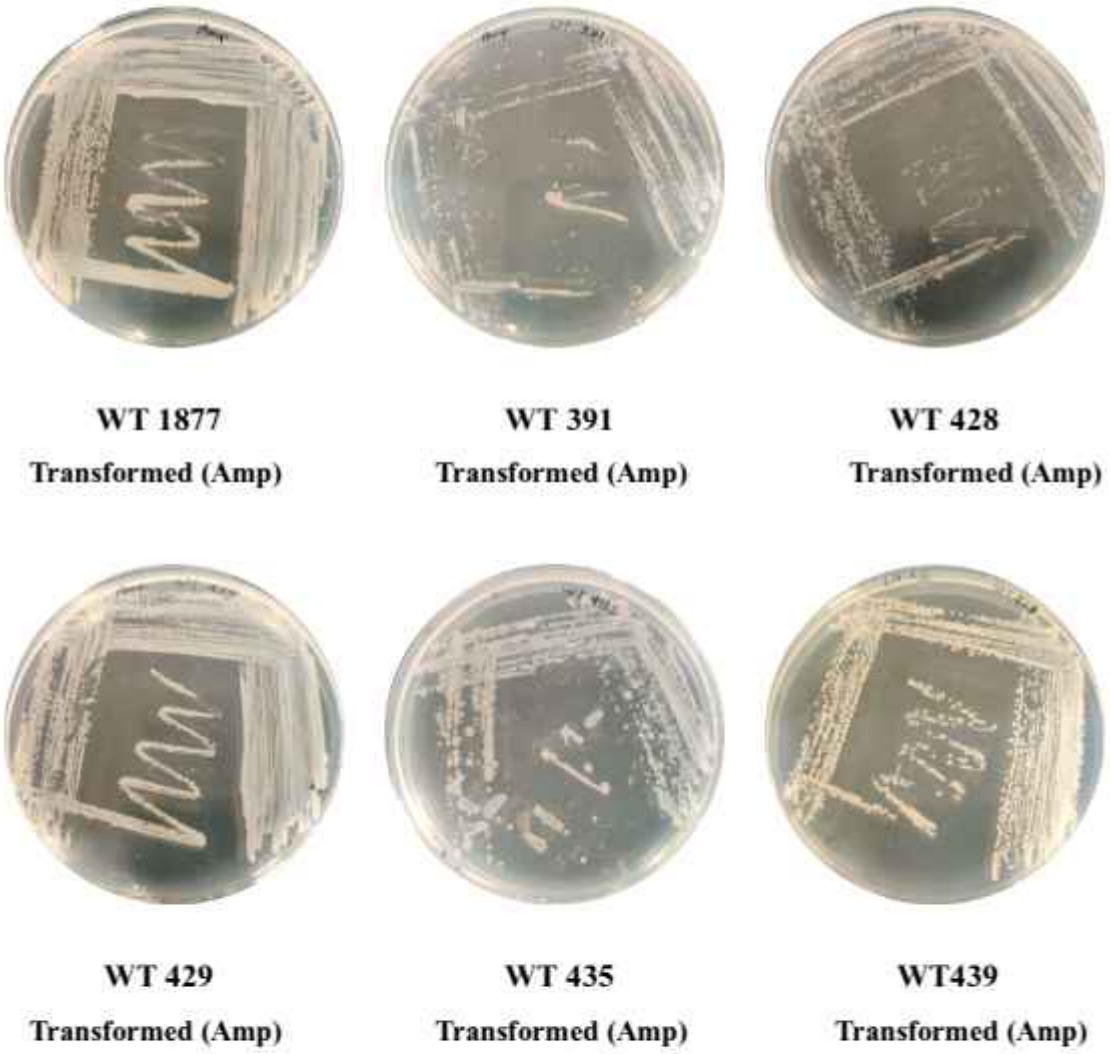


Figure 3.2: Growth of transformed strains WT 1877, WT 391, WT 428, WT 429, WT 435, and WT 439 with Ampicillin resistance gene on LA media containing Ampicillin.

3.3 Transformed Kanamycin Resistant Gene Conformation

Kanamycin Resistant Gene Containing DNA Transformed Strain

The figure demonstrates the successful transfer of DNA containing the Kanamycin-resistant gene to each strain, resulting in normal growth of the transformed strains. Since their growth is typical on LA media containing Kanamycin, this indicates that the new batch of strains has been successfully transformed and has acquired resistance to Kanamycin.

Figure 3.3: Growth of transformed strains WT 391, WT 428, WT 429, WT 435, and WT 439 with Kanamycin resistance gene on LA media containing Kanamycin.

3.4 Spot Test:

After the formation of these groups, they were subjected to phage treatment using a spot test to assess the effects on both the Native strain and the transformed strains. The control strain 1877 was utilized to validate the phage activity through spot tests involving 6 phages: JSF2, JSF6, JSF7, JSF10, JSF25, and JSF30. The results were observed following a 12-13 hour incubation period, provided below.

3.4.1 Spot Test Result: Analysis of Native Strains along with Control

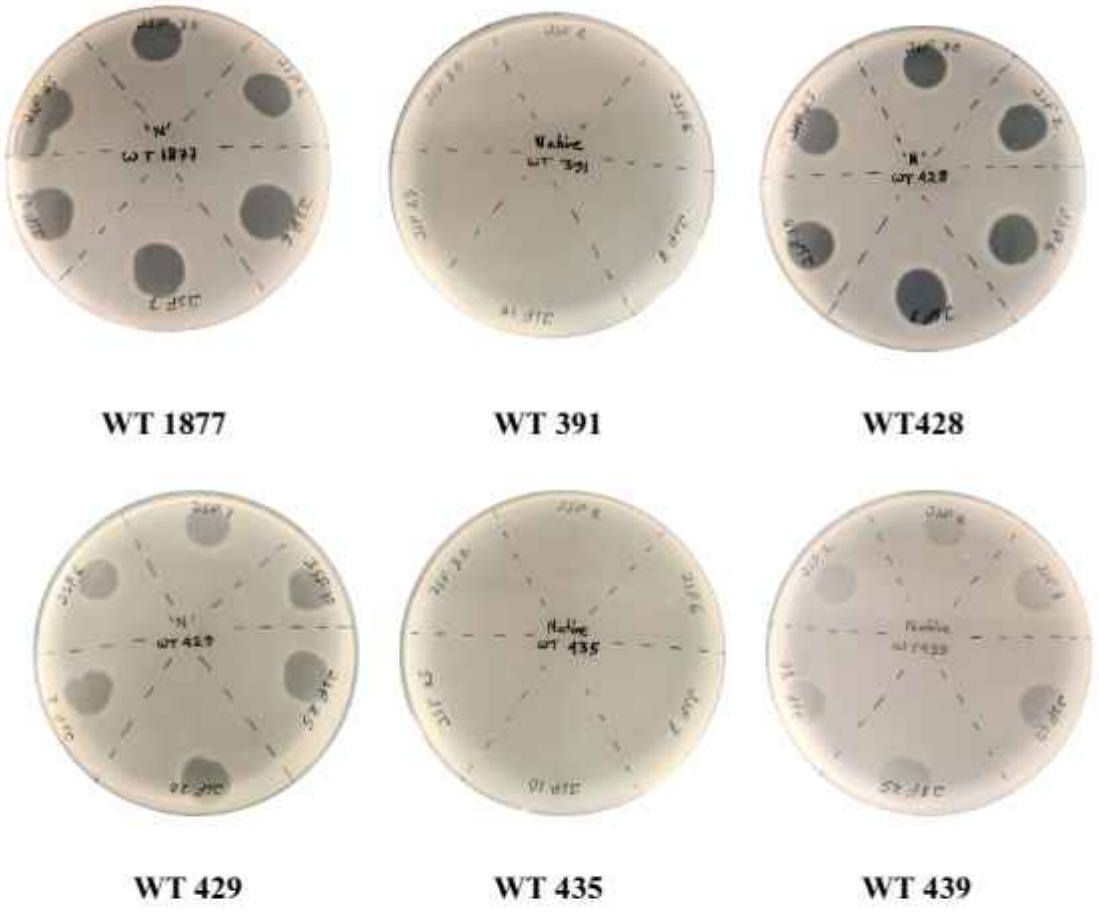


Figure 3.4: Spot test on Native strains WT 391, WT 428, WT 429, WT 435, and WT 439 with control WT 1877

A tabular presentation

The result of the effect of phage on Native strains:

Infection	✅
No infection	❌

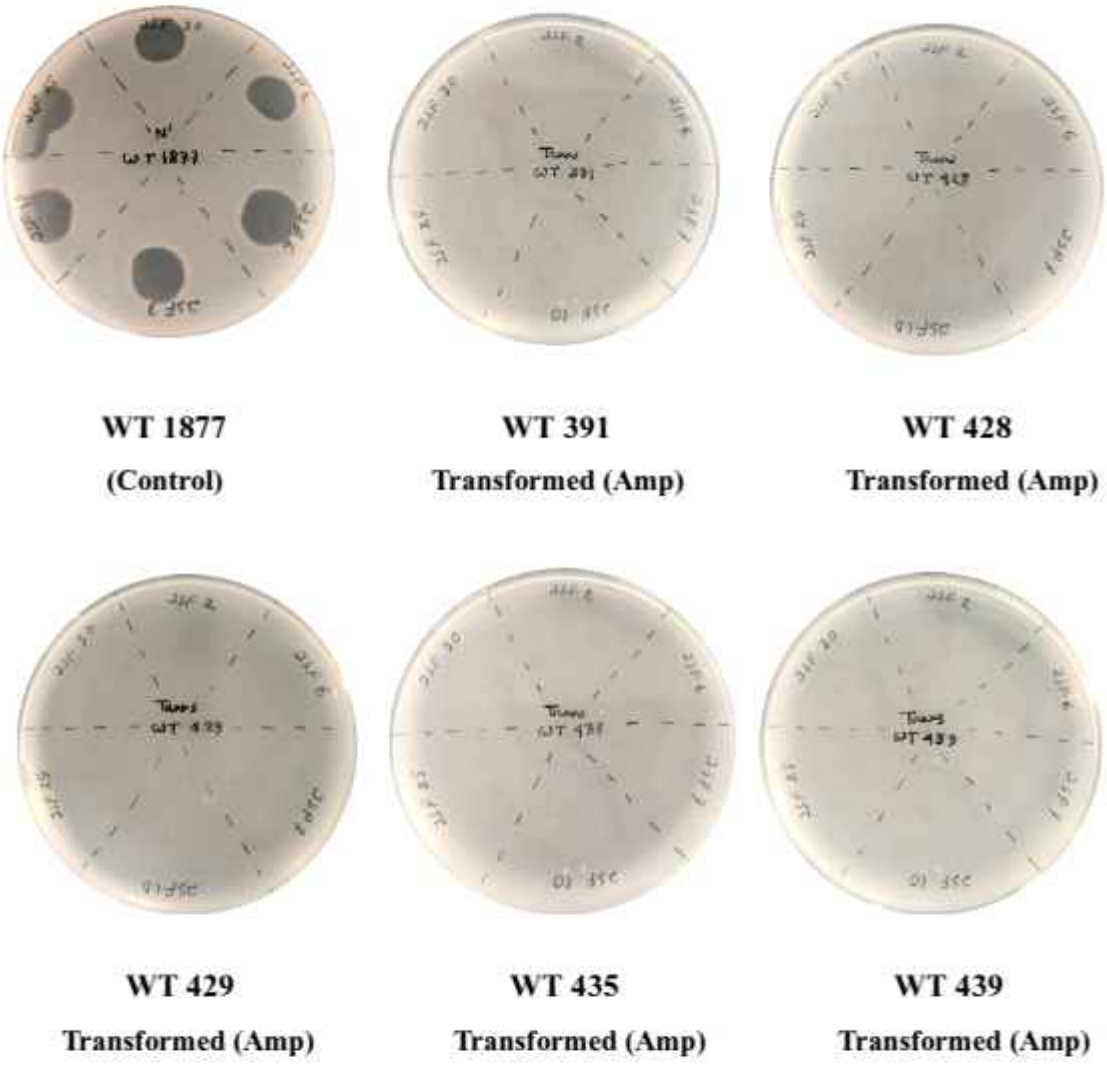
Table 3.1: The effect of 6 different phages on 5 different native *Vibrio Cholerae* strains WT 391, WT 428, WT 429, WT 435, and WT 439 of O1 serotype with one control WT 1877.

Strains	JSF 2	JSF 6	JSF 7	JSF 10	JSF 25	JSF 30
WT 1877	✅	✅	✅	✅	✅	✅
WT 391	❌	❌	❌	❌	❌	❌
WT 428	✅	✅	✅	✅	✅	✅
WT 429	✅	✅	✅	✅	✅	✅
WT 435	❌	❌	❌	❌	❌	❌
WT 439	✅	✅	✅	✅	✅	✅

The presence of clear zones on the 1877 strain for each of the phages in our experiment indicates that the test was conducted correctly and successfully, demonstrating the efficiency of the phages. The native WT 428, WT 429, WT439, and control 1877 produced clear zones while tested against JSF 2, JSF 6, JSF 7, JSF 10, JSF 25, and JSF 30. On the other hand, the other native strains WT 391, and WT 435 could not show any visible clear zone while tested with these phages. Consequently, phages were unable to infect and generate plaques on WT 391, and WT 435. Hence, the outcome indicates that the natives WT 391, and WT 435 are resistant. The native WT 428, WT 429, and WT 439 strains are susceptible to the phages JSF 2, JSF 6, JSF 7, JSF 10, JSF 25, and JSF 30, as evidenced by the presence of clear zones in the spot test. These phages appear to be effective in treating the native WT 428, WT 429, and WT 439 strains, providing antibiotic alternatives.

3.4.2 Spot Test on Transformed Ampicillin Strains

Effect of Phage on Ampicillin Resistant Strains:



WT 1877
(Control)

WT 391
Transformed (Amp)

WT 428
Transformed (Amp)

WT 429
Transformed (Amp)

WT 435
Transformed (Amp)

WT 439
Transformed (Amp)

Figure 3.5: Spot test on transformed Ampicillin resistance strains WT 391, WT 428, WT 429, WT 435, and WT 439 with control WT 1877

A tabular presentation:

The result of the effect of phages on Ampicillin Resistance strains:

Infection	✅
No infection	❌

Table 3.2: The effect of 6 different phages on control 1877 and Ampicillin resistance transformed *Vibrio Cholerae* strains.

Strains	**JSF 2**	**JSF 6**	**JSF 7**	**JSF 10**	**JSF 25**	**JSF 30**
WT 1877 Control	✅	✅	✅	✅	✅	✅
WT 391 (Amp)	❌	❌	❌	❌	❌	❌
WT 428 (Amp)	❌	❌	❌	❌	❌	❌
WT 429 (Amp)	❌	❌	❌	❌	❌	❌
WT 435 (Amp)	❌	❌	❌	❌	❌	❌
WT 439 (Amp)	❌	❌	❌	❌	❌	❌

The phages exhibited diverse effects on the transformed Ampicillin-resistant strains WT 391, WT 428, WT 429, WT 435, and WT 439. Notably, phages exerted no discernible effect on any of the strains, except for the control strain 1877. The *Vibrio Cholerae* strains WT 391, WT 428, WT 429, WT 435, and WT 439 exhibited no growth inhibition when assessed against phages JSF 2, JSF 6, JSF 7, JSF 10, JSF 25, and JSF 30. The influence of the phages on the WT 428, WT 429, and WT 439 strains has transitioned from beneficial to detrimental when contrasting the native strains with those transformed by the Ampicillin resistance gene. The phage successfully formed a plaque on the native WT 428, WT 429, and WT 439 strains, but failed to do so on the Ampicillin-transformed strains of WT 428, WT 429, and WT 439.

3.4.3 Spot Test on Transformed Kanamycin Strains

Phage Effect on Kanamycin Resistant Strains:

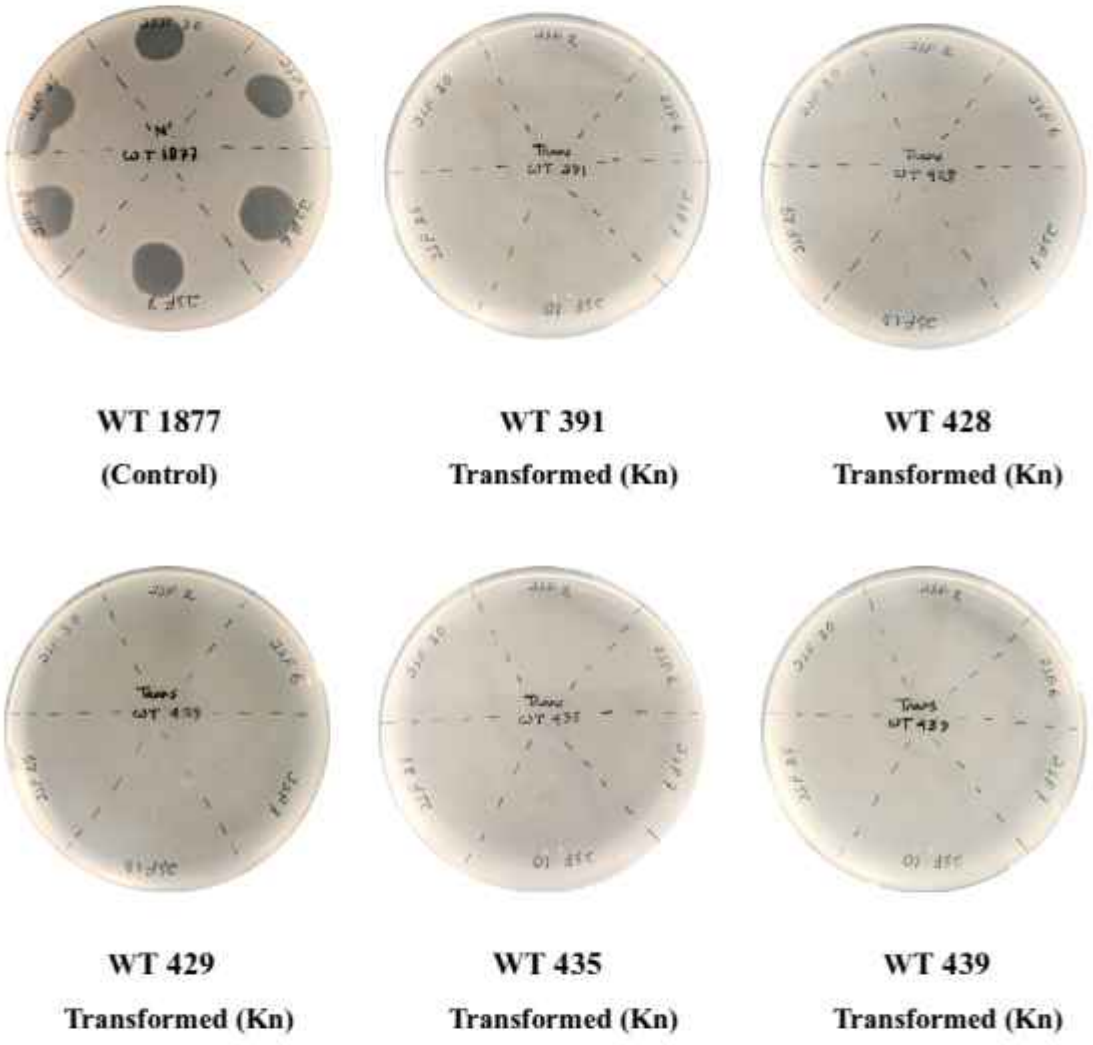


Figure 3.6: Spot test on transformed Kanamycin resistance strains WT 391, WT 428, WT 429, WT 435, and WT 439 with control 1877.

A tabular presentation:

The result of the effect of phages on Kanamycin Resistance strains:

Infection	✅
No infection	❌

Table 3.3: The effect of 6 different phages on control 1877 and 5 different *Vibrio Cholerae* strains of O1 serotype transformed with Kanamycin resistance gene

Strains	JSF 2	JSF 6	JSF 7	JSF 10	JSF 25	JSF 30
WT 1877 (Control)	✅	✅	✅	✅	✅	✅
WT 391 (Kn)	❌	❌	❌	❌	❌	❌
WT 428 (Kn)	❌	❌	❌	❌	❌	❌
WT 429 (Kn)	❌	❌	❌	❌	❌	❌
WT 435 (Kn)	❌	❌	❌	❌	❌	❌
WT 439 (Kn)	❌	❌	❌	❌	❌	❌

The phages had varying effects after the horizontal transfer of the Kanamycin gene into strains WT 391, WT 428, WT 429, WT 435, and WT 439. Remarkably, there was no noticeable impact of phages on any of the strains, with the exception of the control strain 1877. The *Vibrio Cholerae* strains WT 391, WT 428, WT 429, WT 435, and WT 439 all showed no growth inhibition while tested against phages JSF 2, JSF 6, JSF 7, JSF 10, JSF 25, and JSF 30. The impact of the phages on the WT 428, WT 429, and WT 439 strains has shifted from positive to negative when comparing the native strains with the Kanamycin gene-transformed strains. This is because the phage was able to form a plaque on the native WT 428, WT 429, and WT 439 strains, but was unable to form a plaque on the Kanamycin-transformed strains of WT 428, WT 429, and WT 439.

3.5 Cross-Comparative Result Analysis

A tabular presentation comparing the effect of phages on Ampicillin and Kanamycin Resistance strains:

Effect of Phage	✓
No effect of Phage	✗

Table 3.4: Comparative results of phage effect between the Native, Ampicillin resistance gene transferred strains, and Kanamycin resistance gene transferred strains.

Strains (O1)	Effect on Native strains	Effect on Ampicillin resistance strains	Effect on Kanamycin Resistance Strains
WT 391	✗	✗	✗
WT 428	✓	✗	✗
WT 429	✓	✗	✗
WT 435	✗	✗	✗
WT 439	✓	✗	✗

We employed the 1877 strain as the control for each spot test due to its sensitivity to all utilized phages. The phage effect altered for *Vibrio Cholerae* strains WT428, WT429, and WT439 following the plasmid transfer and horizontal gene transfer. WT428, WT429, and WT439, which were previously susceptible to the phages, acquired resistance to all six phages following transformation with Ampicillin and Kanamycin resistance genes. While the strains WT391, and WT435 stayed resistant as native strains even after transformation with Ampicillin and Kanamycin resistance genes.

Chapter 04

Discussions

4.1 Analysis of the Overall Result

This experiment was conducted to observe the effects of horizontal gene transfer on bacteriophage therapy. The development of antibiotic resistance in bacteria, attributed to horizontal gene transfer, necessitates the exploration of alternatives to antibiotic therapy for bacterial infections.

In the future, antibiotics may become ineffective for patients due to the emergence of multi-drug-resistant bacteria. Researchers have concentrated on employing bacteriophages as therapeutic agents instead of antibiotics, owing to their accessibility and specific host range. Nonetheless, the ability of phages that infect and lyse a bacterial strain to also infect and lyse the same strain exhibiting antibiotic resistance is a concern. Horizontal gene transfer has facilitated evolution and diversity in bacterial populations by transferring antibiotic resistance genes (ARGs) between species. Consequently, phages must eliminate hosts possessing antibiotic resistance genes (ARGs) to validate phage therapy as a viable alternative, given the increasing prevalence of resistant bacteria.

For this research, we utilized five infectious native strains (O1) of *Vibrio Cholerae* (WT391, WT428, WT429, WT435, and WT439) from our laboratory stock. Subsequently, we introduced each strain with one of two Antibiotic-resistance genes: Ampicillin and Kanamycin. We extracted the Ampicillin-resistant gene from the *E. coli* DH5α strain and the Kanamycin-resistant gene from the 1877 strain of *Vibrio Cholerae*. Gel electrophoresis was conducted following each extraction to verify the results and ensure the absence of contamination from other organisms. We examined the impact of six phages (JSF2, JSF6, JSF7, JSF10, JSF25, and JSF30) on sixteen strains (native, Ampicillin-resistant, Kanamycin-resistant, with 1877 serving as the control) using a spot test. In the spot tests, the two native stains WT 391 and WT 435 indicated no distinction between the native and resistant strains. Nevertheless, the three strains exhibited divergent outcomes. Plaques were detected for native WT 428, WT 429, and WT 439 in the spot test for all six phages, whereas no plaques were observed for the Ampicillin and Kanamycin-resistant WT 428, WT 429, and WT 439. Indicating that while native WT 428, WT 429, and WT 439 is susceptible to the six phages, conversely, Antibiotic and Kanamycin-resistant WT 428, WT 429, and WT 439 exhibit phage resistance. Besides, the two native strains WT 391 and WT 435, and their

Ampicillin and Kanamycin-resistant strains both exhibited phage resistance. Consequently, it has been found that horizontal gene transfer (HGT) likely influences phage infectivity.

4.2 Analysis of the Effects of Phages

The rationale for this alteration in the spot test remains unverified. Nonetheless, drawing from a prior understanding of horizontal gene transfer (HGT) and its implications for the genotype and phenotype of the recipient organism, the subsequent issues may elucidate the underlying causes:

The host cell receptor of phages may be eradicated due to horizontal gene transfer (HGT). Homologous DNA exchange transpires during transformation. The gene responsible for the host cell receptor may be excised and replaced with the desired ARG. The strain becomes resistant to the phages due to the absence of a receptor for phage binding. The host receptor surface may be altered due to horizontal gene transfer, leading to either susceptibility or resistance to phages. The gene obtained through horizontal gene transfer can alter proteins or generate enzymes that modify the receptor site. This modification can enhance or obstruct the phage's binding.

HGT may promote the transmission of traits via epigenetics (Dalia & Dalia, 2019). Consequently, it may influence the binding of the phage. Furthermore, DNA methyltransferases were acquired by eukaryotes from bacteria via horizontal gene transfer during early evolution. (Arkhipova et al., 2023) Consequently, bacteria may acquire epigenetic factors such as DNA methyltransferase during horizontal gene transfer. It can subsequently methylate sequences associated with phage interaction, resulting in altered susceptibility to the phage.

During horizontal gene transfer, additional genes may be integrated into the bacteria alongside antibiotic-resistance genes (ARG). These genes can encode enzymes, proteins, or other elements. Dougherty, 2014 Certain factors may deteriorate phages. Consequently, native strains susceptible to phage can assimilate additional genes that encode phage-degrading factors following transformation with ARG. Consequently, the modified strain will exhibit resistance to the phage.

Horizontal gene transfer facilitates environmental adaptation. Daubin and Szöllősi, 2016 Consequently, the bacterial strains may modify their biological structure to acclimate to the environment and endure. Consequently, horizontal gene transfer may promote phage resistance for bacterial survival. These may be some of the potential causes of the impact of horizontal gene transfer on phage infectivity. Nevertheless, the specific reason remains unverified as the alterations and impacts on the bacterial gene sequence resulting from transformation are unknown.

Conducting this experiment on additional *Vibrio Cholerae* strains with a greater variety of phages would enable us to formulate a more robust hypothesis for this project. Nevertheless, working with a limited number of samples makes it challenging to establish a robust foundation. Furthermore, we were unable to perform sequencing for any of the strains. Had we been able to do so, the rationale for such an outcome would have been significantly clearer.

Therefore, we intend to advance our research and experimentation by sequencing the genomes of both native and transformed bacterial strains. This approach will enable us to compare and distinguish between the sequences, thereby identifying the precise cause of the alteration in phage infectivity. By analyzing the altered sequence, we can identify the newly expressed or repressed enzymes, proteins, or other factors that may have influenced the altered infectivity in WT 428, WT 429, and WT 439. Moreover, our objective is to extend this research to additional *Vibrio Cholera* strains with various phages to substantiate a robust and credible correlation between horizontal gene transfer and phage infectivity.

5. Conclusion

Since the beginning of the previous century, antibiotic resistance has been one of the most significant challenges in the fight against bacterial infections. Emerging phage therapy has the potential to be a solution to this problem, provided that it can achieve results that are superior to those of horizontal gene transfer. Bacteria are acquiring ARGs for various drugs as a result of horizontal gene transfer (HGT), and as a consequence, phages that are specific to a particular strain of bacteria need to be infectious against a strain of that bacteria that has been horizontally transferred. Considering that the *Vibrio Cholerae* strains are the primary focus of this investigation, our primary concern was determining whether or not the candidate phages that were used against these *Vibrio Cholerae* strains would be able to demonstrate the same level of effectiveness against the same strains that contained antibiotic resistance genes. Three of the strains had different results, even though the majority of the results of the spot tests were the same for the native strains as well as the Ampicillin and Kanamycin-resistant strains. The native strains of WT 428, WT 429, and WT 439 were susceptible to all of the phages. On the other hand, transformed Ampicillin and Kanamycin resistance strains of WT 428, WT 429, and WT 439 were resistant to all of the phages. The fact that this is the case suggests that the transformation, which is defined as the introduction of ARGs into these strains, caused a change in them that led to a change in their state of infectivity to phages in comparison to their native strains. Consequently, we are unable to completely disregard the possibility that HGT may have an effect on the infectious potential of bacteriophages given the circumstances. On the other hand, if we were to carry on with this research on additional bacteria and phages, in addition to sequencing the strains with different results, we would be able to establish a valid and well-grounded issue with confirmed reasons.

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