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 Requirement For he Degree Of Bachelor Of science In Biotechnology

Department of Mathematics and Natural Sciences

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Declaration

It is hereby declared that

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

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Ethics Statement

The entire research work has no conflict of interest. No human and animal models were used in this experiment.

Abstract

Bacterial infections and its treatment has been a concern for researchers and scientists for centuries. At first, the invention and treatment with antibiotics seemed like a promising and reliable solution to the problem. However, with an increasing bacterial population gaining antibiotic resistance through horizontal gene transfer, it is now crucial to find a replacement for antibiotic treatment against different bacteria. Bacteriophage therapy has been considered as a potential candidate for the substitution. Therefore, the objective of our study was to evaluate the potentiality of phage infectivity on different strains of a specific bacteria and on the same strains transformed with antibiotic resistant genes to consider the impact of horizontal gene transfer. We focused on 5 native infectious (O1) strains of *Vibrio cholerae* and 2 specific antibiotics: ampicillin and kanamycin. We also used another strain of *Vibrio cholerae* as the control for the experiment. We extracted ampicillin resistant gene containing pGLO plasmid from *E. coli* DH5alpha and kanamycin resistant gene containing chromosomal DNA from 1877 strain of *Vibrio cholerae*. Then transformed these genes into the 5 native strains separately to achieve the effect of horizontal gene transfer *in vitro*. As a result, we conducted the experiment on 16 strains of *Vibrio*: 5 native strains, 5 native strains transformed with ampicillin resistance gene, 5 native strains transformed with kanamycin resistance gene, and one control strain (1877 strain of *Vibrio*). Then we carried out a spot test on each of the strains with 6 phages from our lab stock and observed the result to detect any impact of the transformation (Horizontal gene transfer) on the infectivity of the phages.

Dedication

To our loving and encouraging family and friends, and to us…..

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List of Acronyms

V. cholerae	*Vibrio cholerae*
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
TCBS	Thiosulfate-citrate-bile-sucrose
HGT	Horizontal Gene Transfer
AMR	Antimicrobial resistance
FDA	Food and Drug Administration
MDR	Multidrug-resistant
MSSA	Methicillin-sensitive Staphylococcus aureus
SDS	Sodium dodecyl sulfate
Rpm	Rotations per minute
UV	Ultraviolet
ml	Milliliter
µl	Microliter
CT	Cholerae Toxin

ARGs	Antibiotic Resistance Genes
TCP	Toxin-Coregulated Pilus

Chapter 1

Introduction

Antibiotics is a remarkable discovery in the medical sector , since it has provided the success that was never possible with any other medicine to treat bacterial infections and saving endless patients' lives. But the question arises when bacteria become resistant to many antibiotics which eventually lead to a severe drawback in public health. To overcome this challenge of fighting against bacterial infections ,scientists are researching many alternative ways to address antibiotic resistance, among them development of diverse tactics for work against bacterial strains that have been resistant against certain antibiotics. Phage therapy is a light of hope amongst the approaches since it gives promising and favorable outcome with viable answers against antibiotic resistant bacteria.While going through and winning against this challenge , phage therapy treatment has been emphasized as a light of hope for future against antibiotic resistance since we can see that , antibiotics has become huge concern from being as a blessing against bacterial infections.

From learning about the life cycle of bacteriophages, phage therapy has grabbed scientists attention to employ phage against bacterial infection. Since then phage therapy is being researched as a significant approach to fight against bacterial infections across many organisms.

Phage therapy is emphasized as one of the glorious research on ongoing research into infectious disease worldwide (Abedon et al., 2011). Antibiotics have been working against bacterial diseases for a long time. With the evolution of antibiotic resistance bacteria, the challenge became finding new ways to deal with infectious diseases. After a lot of research, they discovered phage therapy, a promising method to tackle bacteria-causing infections. Bacteriophages are tiny viruses that precisely target and kill bacteria for its own purpose. They employ a variety of techniques to successfully penetrate into bacterial cells and also within biofilms and it has been tested and proven to be successful against many resistant bacteria (Singh et al., 2022).Not only they can be used as a treatment against antibiotic resistant microbes but also they can be bacterial strains.The scientific research community has conducted numerous studies on the treatment of infectious diseases using phages. Moreover, particular importance

has been assigned to the research on phage utilization in treating and preventing cholera, driven by the rise in antimicrobial-resistant strains of *V. cholerae*.

Cholera has been a terrible disease, with several medications, including ampicillin and kanamycin, being used for therapy; nevertheless, the cholera bacteria has developed resistance to many of them. As a result, over the years, different methods of treating cholera have been tested.Because of the developing antibiotic resistance, research into phage therapy for cholera is critical, as it offers a potential option to work against this difficult and chronic health concern.Concern regarding the rise of multi-drug resistant strains in *V. cholerae* has lately increased.(Das et al., 2019). In many countries, Phage therapy is still being researched as a potential cholera treatment. Horizontal gene transfer (HGT) is used in bacteriophage-based therapies to eliminate pathogenic bacteria at every stage of their lytic life cycle. The aim of this study is to investigate the susceptibility of native, antibiotic-resistant *Vibrio cholerae* strains to bacteriophage infection. Finding suitable substitutes is crucial, as worries about antibiotic-resistant *Vibrio cholerae* are becoming more and more pressing. Finding efficient bacteriophages to treat these resistant bacteria may have uses in public health, environmental biotechnology, and medicine.

Chapter 2

Literature review

2.1 *Vibrio cholerae*

A gram-negative bacterium known as *Vibrio cholerae* is widely known to cause the potentially fatal diarrheal illness cholerae. This particular type of bacteria is found in the order Vibrionales, family Vibrionaceae, phylum Proteobacteria, class GammaProteobacteria, and kingdom Bacteria. It is mainly found near coasts and in salty seas. Cholera is mostly caused by a toxin-producing strain of this bacterium.Other *Vibrio* species have been linked to gastroenteritis and infections outside the intestines, most typically affecting the ears, soft tissues, and circulation. This bacterium often enters the human body after consuming contaminated water or food. Once inside, it adheres to the intestinal walls and secretes a poison. This toxin is responsible for the watery diarrhea associated with cholera, which can lead to severe dehydration and even death if not treated promptly.

The likelihood of a cholera pandemic increases when people are forced to live in congested areas with poor sanitation due to poverty, conflict, or natural disasters (Somboonwit et al., 2017). Since its discovery in 1817, cholera has caused seven outbreaks worldwide. There are two varieties of *Vibrio cholerae* bacteria: O1 and O139. Both of these bacteria have the ability to cause severe illness. The O1 type in its most basic form was the source of the first six epidemics. The greatest and longest-lasting outbreak to date started in 1961 in Indonesia and is currently in its seventh year. It is linked to the El Tor biotype, a unique variation of the O1 type. (Faruque & Mekalanos, 2012). *Vibrio cholerae* bacteria belonging to non-O1 or O139 groups have a very unique genetic makeup. Only a small percentage of these non-O1/non-O139 strains are able to produce cholera toxin. Severe cases of gastroenteritis have been associated with these strains. The illness caused by *V. cholerae* can be diagnosed using a variety of methods. Cultivating the bacteria in TCBS, a unique kind of growth media, is one common method. Depending on their ability to ferment sucrose, *Vibrio* species grow colonies on TCBS (Thiosulfate Citrate Bile Salts Sucrose) agar that can be either green or yellow in color. These bacteria ferment sucrose, which

gives the colonies their yellow color. Additional approaches include Darkfield microscopy, which is particularly useful for detecting *V. cholerae*, commercial kits that may swiftly identify the antigens of the bacteria, and PCR (Polymerase Chain Reaction) methods for identifying the bacterial DNA. Having access to clean water, keeping up proper hygiene, and handling food safely are all preventative precautions against cholerae. Getting your water from a safe source and cooking your meals properly are two good ways to prevent infection.Vaccines are available to offer temporary protection against cholera in locations where the disease is prevalent.Rehydration—either intravenously or orally, depending on the severity of dehydration—is the mainstay of treatment for cholera. Antibiotics are not always required, although they can be used to reduce the length and intensity of the sickness.

The prevention of cholera outbreaks caused by *Vibrio cholerae* involves improving the water and sanitation systems, educating the population on good hygiene practices, and responsible volunteers to work in the event of an outbreak. Despite these efforts, cholera remains a severe hazard to public health in many parts of the world, particularly in places with limited access to sanitation and clean water.

2.1.1 Clinical Manifestation

The symptoms of cholera can develop rapidly after consuming food or water tainted with the *Vibrio cholerae* bacteria. Early symptoms of *Vibrio cholerae* include abdominal pain, rumbling stomach noises, and vomiting. It worsens and complicates when patients lose a lot of fluids and electrolytes from diarrhea. The most severe cases of cholera are frequently caused by two types of *Vibrio cholerae* bacteria: the O1 and O139 strains. The O1 strain contains two variations, with the El Tor strain being the most common cause of cholera outbreaks worldwide. The O139 strain, discovered later, causes severe illness equivalent to the O1 strain. (Finkelstein, 1996)

When someone ingests food or water contaminated with the *Vibrio cholerae* bacterium, the symptoms of cholera develop quite quickly. The duration of the incubation period, which is the interval between the bacterium's exposure and the appearance of symptoms, usually spans from a few hours to five days. Usually, two to three days following exposure is when symptoms first

appear. Because of its sudden onset of symptoms, cholera can be especially hazardous if treatment is delayed.(Clemens et al., 2017).

2.1.2 Patients with Diarrhea

When someone suffers from cholera, they can experience extremely rapid and severe symptoms, particularly in the form of acute, watery diarrhea. This can start within 6 to 12 hours of the first clinical indications appearing. The diarrhea grows increasingly watery, finally becoming a hazy, rice-like material with a fishy odor. This fast loss of fluids and electrolytes can cause dehydration, which, if not addressed immediately, can be deadly.

In severe cases, the rate at which an adult with cholera loses fluid through diarrhea can be astonishing – up to 1 liter per hour. It is possible for youngsters suffering from acute cholera to lose between 10 and 20 cc/kg/hour of feces. (Harris et al., 2011). Since cholera causes significantly more fluid loss than other diarrheal illnesses do on average, it is extremely dangerous and potentially fatal if proper medical attention is not received promptly. Serious health issues can arise from cholera, particularly in the elderly. These include stroke and kidney damage, most especially damage to the portion of the kidney that filters blood. Low blood pressure and inadequate blood flow throughout the body are common causes of these problems. Vomit-related lung infections are another possible consequence. Severe dehydration is nearly invariably the cause of mortality in cholera patients. (Waldor & Ryan, 2015). Cholera can cause major gastrointestinal issues in addition to diarrhea. Vomiting is a common symptom that commonly occurs together with watery diarrhea. This vomiting may begin prior to the onset of diarrhea or after it has begun. Patients may also get cramps in their stomachs. However, cholera typically doesn't cause the intense stomach pain that other severe gastrointestinal disorders are known to cause, unlike dysentery. These additional indicators hasten the loss of fluids and electrolytes, raising the possibility of severe dehydration.(Jafet A,2022).

If cholera is not treated, it can be extremely deadly. The death rate for those who don't receive treatment can be as high as 50-70% (Lindenbaum, 2022). Children are up to ten times more likely than adults to die from cholera in regions where the disease is prevalent. Moreover, cholera during pregnancy carries a high risk to unborn children, with a 50% possibility of fetal

death in the third trimester. (Hirschhorn et al., 1969).This highlights the importance of urgent and effective treatment for cholera to prevent these severe outcomes.

2.1.3 Virulence Factor of *Vibrio cholerae*

The cholera-causing agent, *Vibrio cholerae*, uses a number of virulence factors to establish infection and cause the disease's distinctive symptoms. Cholera toxin (CT) and toxin-coregulated pilus (TCP) are the main virulence factors of toxigenic *V. cholerae*, and they are each separately responsible for the pathogenesis of the infection.(Faruque et al., 2003).

2.1.4 Cholera Toxin (CT):

A single A subunit and five B subunits make up the complex protein known as cholera toxin. Monosialoganglioside (GM1) is one of the specific receptors on the surface of host cells that the B subunits are in charge of binding to.The adenosine diphosphate-ribosylating enzyme that makes up the A subunit is sometimes referred to as the toxic domain.(Kawshik,b.&Nirmal,K.,2011) The A subunit is able to enter the host cell once the B subunits connect to GM1. After entering the cell, it catalyzes the transfer of ADP-ribose from nicotinamide adenine dinucleotide (NAD) to a protein in the host cell, impairing normal physiological processes. Intestinal Epithelial Cells Are Affected: Cyclic AMP (cAMP) is more concentrated intracellularly when adenylate cyclase is activated as a result of CT-induced ADP-ribosylation of host cell proteins. Increased levels of cAMP in turn cause anion secretion and prevent the absorption of electroneutral NaCl. The intestinal epithelial cells lose water, salt, and chloride as a result of this disruption in ion transport, which results in the characteristic cholera diarrhea.(Wernick, et. al ,2010)

2.1.5 Toxin-Coregulated Pilus (TCP):

One of the most important colonization factors for *V. cholerae* is TCP. It helps the bacterium adhere to the intestinal epithelium, which encourages the spread of infection. The CTX phage, which carries the cholera toxin gene, binds to TCP as a receptor. The coordinated activity of several virulence factors in the pathogenesis of *V. cholerae* is highlighted by this link. It contributes to the bacterium's rapid spread by facilitating in the development of microcolonies on

the intestinal mucosa. In addition to its function in secreting fluid and ions, CtxA, an amino acid of cholera toxin, has been associated with the disruption of the intestinal barrier. This interference helps the toxin interact with with target cells and aids in its translocation.(Sharma,et.al.,2008)

The toxicity of *Vibrio cholerae*, the bacterium responsible for cholera, operates through a complex yet fascinating biological mechanism. This process primarily involves the toxin secreted by the bacteria, known as the cholera toxin (CT). (Oki,et.al., 2013)

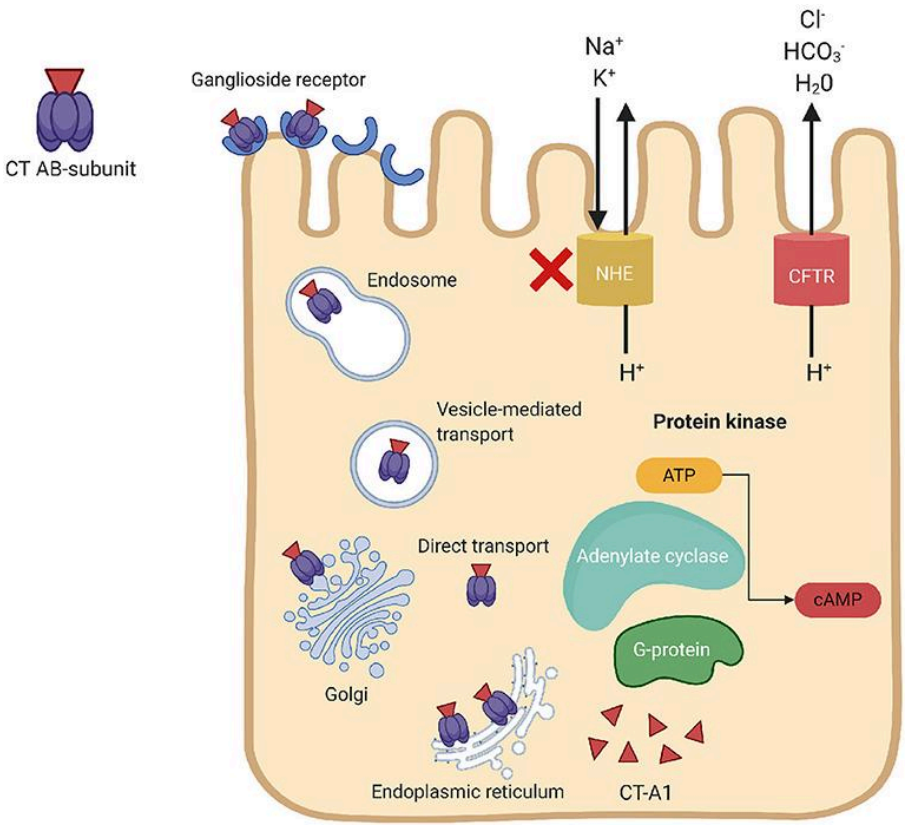

Figure 1 : Mechanism of Cholerae toxin (Thandavarayan,et.al., 2020)

Vibrio cholerae has a remarkably intricate molecular mechanism that underlies its toxicity. The process starts when the cholera toxin (CT) attaches itself to certain receptors on the surface of host epithelial cells in the intestine. These receptors are known as ganglioside receptors. This

binding initiates the process known as endocytosis, that enables the poison to be absorbed into the cell.(Thandavarayan.et.al.,2020). The cholera toxin is transported via the endosome, a toxin-containing vesicle, to the Golgi apparatus and eventually to the endoplasmic reticulum (ER) once it is inside the cell. The toxin has to travel via this intracellular pathway in order to function. The catalytic CT-A1 polypeptide, the key component of the toxin, is broken down and transferred into the cytosol in the ER.(Holmes et.al.,2006). The ER-associated degradation process enables this movement, which is commonly referred to as retro-translocation. The guanine nucleotide-binding regulatory protein (Gs protein) is a cellular protein that gets activated by CT-A1 after it enters the cytoplasm. An enzyme known as adenylate cyclase (AC) indicates a significant rise in activity upon Gs protein activation. An essential signaling molecule called cyclic adenosine monophosphate (cAMP) is produced by this enzyme from ATP.(Huang et. al., 2017)

Protein kinase A (PKA) is then activated by the increased cAMP levels. PKA then adds a phosphate group to several proteins, including the chloride channel proteins that are part of the cystic fibrosis transmembrane conductance regulator (CFTR). These channels' activity is changed by this phosphorylation. The CFTR channels are changed, causing the cells to release more chloride ions. In the intestinal lumen, which is bicarbonate (HCO3), sodium (Na+), potassium (K+), and water (H2O) are secreted simultaneously. As a result, NHE transporters are unable to absorb sodium chloride (NaCl) as normally. Massive fluid release into the intestines as a result of these changes in ion transport eventually results in severe, watery diarrhea. This is the classic cholera sign that causes the electrolyte imbalances and dehydration associated with the disease.*Vibrio cholerae* O1 and O139 serogroups have been identified to produce epidemic cholera, but other serious diseases, such as diarrhea, can also be caused by non-O1/non-O139 strains of the virus. Compared to the epidemic strains, these non-epidemic strains typically are considered as non-pathogenic, even if some of them may be linked to human illness.

Cholera toxin and pilus which are coregulated by the toxin combine to cause virulence in V. cholerae. The cholera toxin causes severe diarrhea by causing changes in ion transport through host cell signaling pathways. At the same time, TCP promotes the first colonization of the intestinal mucosa, thus establishing the basis for the subsequent development of infection. The

combination of these virulence factors demonstrates the complex strategies used by V. cholerae to spread and infect the host.

2.2 Global Spread of Antibiotic Resistant *V. Cholerae*

Antibiotics were the greatest discovery of the 20th century, but because bacteria have multiple resistance genes and methods of horizontal gene transmission, their exceptional genetic ability has gotten better from humans' frequent consumption of antibiotics. Because of this misuse, every antibiotic that is introduced for use in medicine, agriculture, or other settings has given rise to a variety of resistance mechanisms.(Davies J & Davies D, 2010). Either the acquisition of foreign DNA or mutations in the present genome can lead to the development of antibiotic resistance.(Larsson D G & Flach C F ,2021)The ability of pathogenic bacteria to evolve resistance against different antibiotics is an ongoing threat in every aspect of medical therapy.(Davies, J., & Davies, D. ,2010).

In the past, antibiotics have been used to treat cholera caused by *Vibrio cholerae*, and some of these medicines have proven to be effective towards this bacterium. The antibiotics erythromycin, fluoroquinolones, and tetracycline are frequently used to treat cholera. However, strains of *Vibrio cholerae* have been observed to be resistant to these drugs.There are challenges to treating cholera due to the variable levels of antibiotic resistance that *Vibrio cholerae* has shown throughout the world. Antibiotic resistance is the result of bacteria developing defense mechanisms against antibiotic exposure, which reduces the potency of the medications. According to the most recent evaluation, almost all clinical isolates of *Vibrio cholerae* showed resistance to widely used antibiotics, which is an alarming pattern. In comparison with Asia or the Americas, cholera outbreaks and cases have been occurring more frequently in African nations lately. Large-scale cholera outbreaks have affected many countries in eastern and southern Africa, sometimes leaving a large number of people ill or even dead. This began in the 1970s when *Vibrio cholerae* O1 serotype El Tor, a type of bacteria that causes cholera, first appeared and spread throughout West African nations.(Mwansa et al., 2007).

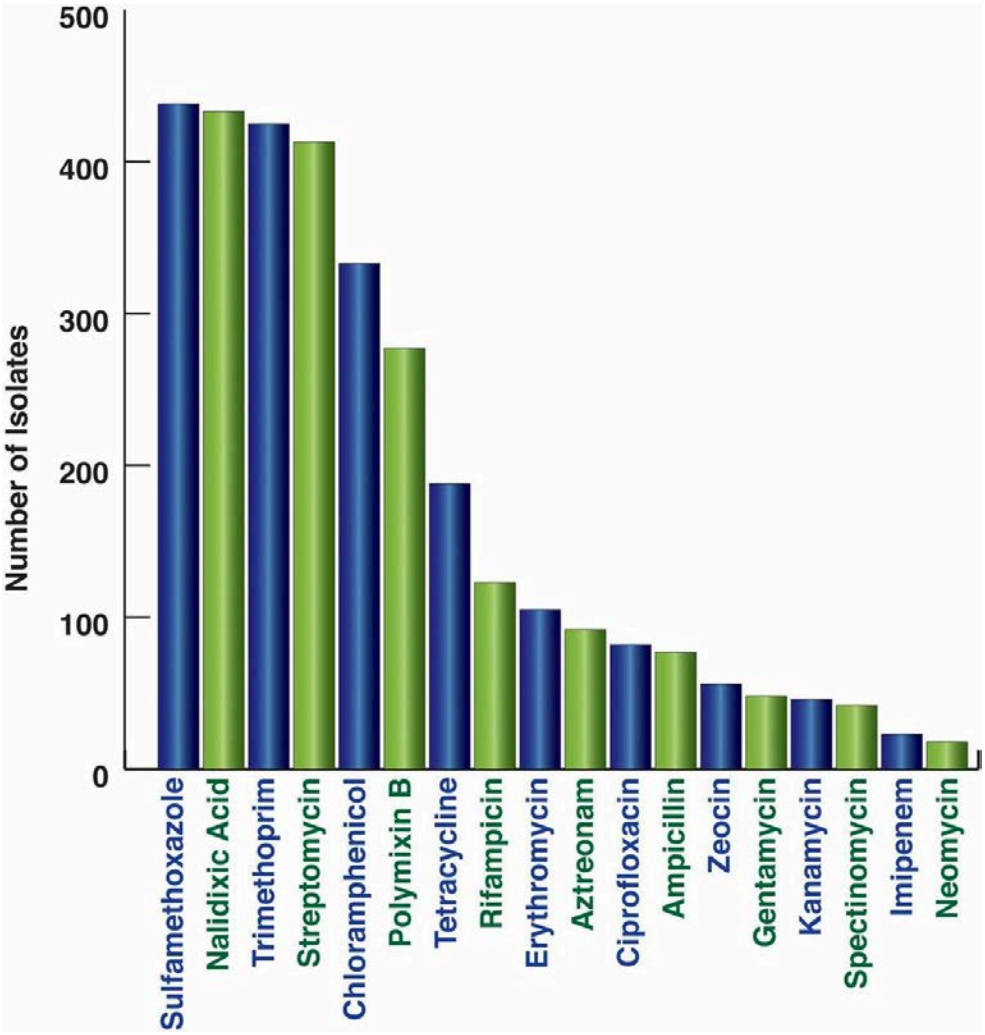

Figure 2: Antibiotic resistance and Sensitivity of *Vibrio cholerae* (Verma, et.al,2019)

Certain strains of the *V. cholerae* bacterium naturally resist various antibiotics. Researchers looked at how different antibiotics affected them and discovered that *V. cholerae* O1, a particular strain, had a higher diversity of resistance than other types. Using their level of resistance as a guide, they divided the bacteria into three categories. Nearly all strains of *V. cholerae* exhibited resistance to two or more antibiotics, and some even exhibited resistance to ten or more. The most prevalent resistance was to the bacterial growth-inhibiting drug sulfamethoxazole.(Verma, et.al,2019)

Geographic variations are present in antibiotic resistance in *Vibrio cholerae*. Different antibiotics may cause distinct levels of resistance in various parts of the country. Understanding the changing environment of resistance patterns in *Vibrio cholerae* requires constant surveillance and monitoring of antibiotic resistance. Developing attempts to slow the spread of resistant strains and modifying treatment guidelines are made easier with the application of surveillance.

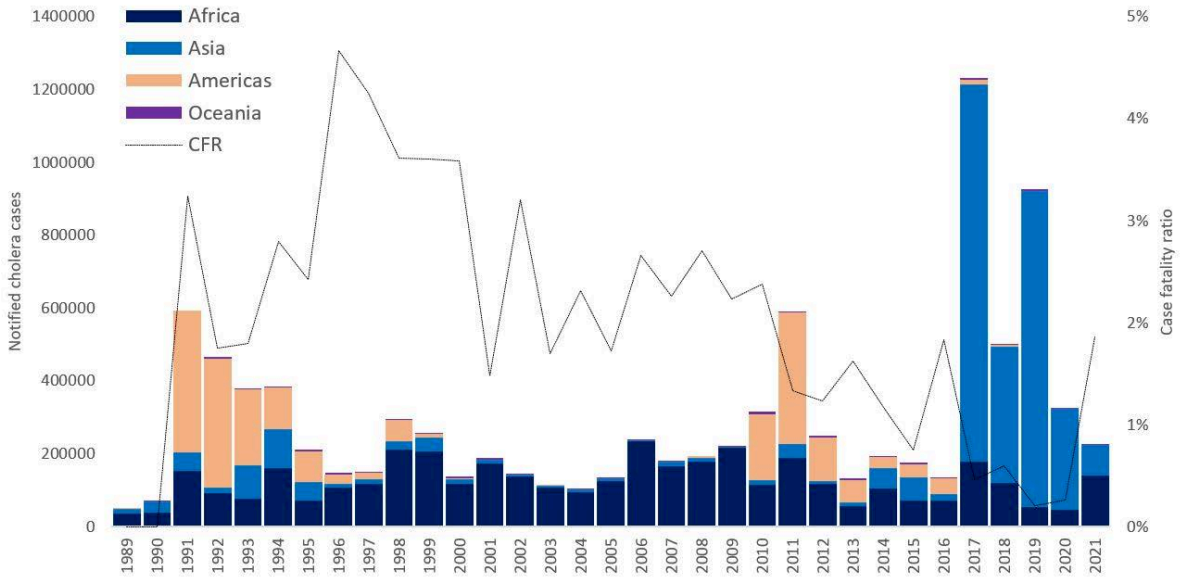

Figure 3: Cholerae cases from 1989 -2021 (Cholera worldwide overview,2022).

A complicated and alarming narrative of the spread of cholera from 1991 to 2022 has irreversibly changed the landscape of global public health. Yemen has been a major source of cholera cases, accounting for 84% and 93% of cases in 2017 and 2019, respectively. Still, this regional supremacy is a component of a larger global trend. Globally, the number of cholera cases has significantly increased since 2021; in 2022, outbreaks were reported in over 29 nations, an increase from the 23 countries in 2021. This comeback of the current seventh cholera pandemic, which started in 1961, is especially concerning because it involves countries that had not reported cholera cases in a number of years.(Cholera worldwide overview,2022).

16 countries reported extended outbreaks as of November 30, 2022, showing the variety of issues involved. Many of these countries face complicated humanitarian issues and weak health systems, making their problems worse due to climate change. Moreover, the worldwide lack of

resources, such as an insufficient supply of the oral cholera vaccine, puts pressure on the ability to react quickly worldwide. The increase in case mortality ratios (CFR)—the greatest in more than ten years—highlights the critical nature of the situation. The global average reached 1.9% in 2021 and peaked in Africa at 2.9%. Unprecedented difficulties result from the simultaneous spread of many cases of cholera and the stressed public health and medical staff managing multiple disease outbreaks at the same time.The uncertain nature of estimates and incomplete and neglected data complicate a thorough understanding. The total data from 1991 to 2022 presents an ever-changing and ongoing threat, hence demanding immediate, focused, and well-resourced international efforts to tackle the complex issues raised by cholera.Improving the infrastructure related to water and sanitation is the main strategy for cholera control. The need for antibiotics for treatment may rise in places with poor access to sanitary facilities and clean water, which may accelerate the emergence of resistant strains of the disease.The fact that *V.cholerae* is developing resistance to numerous antibiotics has been repeatedly observed, and this has become a significant global public health concern(Mwansa et al., 2007). The advice is to pick the right antibiotics for treating cholera based on the resistance patterns of the local strains in each area. This way, we can better resolve the issue of antibiotic resistance and make sure treatments work effectively for the specific strains in each region.

2.2.1 Microbes Growing Resistant to Multiple Antibiotics

Bacteria have faced several challenges from other bacteria, bacteriophages (viruses), and predators throughout their evolutionary journey. Bacteria have developed advanced defense mechanisms in response to these challenges. These defense systems, which were initially created as adaptive responses to external threats, are now necessary for protecting bacteria against antibiotics and other therapeutic interventions. (Smith, W. P. J et.al,2023)Antibiotic-resistant bacteria have a variety of defense mechanisms, such as the ability to break down the antibiotic, alter the part of their bodies that the antibiotic targets, regulate what enters them, and develop new strategies for survival when the antibiotic doesn't target the area of their bodies that it attacks.(Kapoor, G., Saigal, S., & Elongavan, A. 2017). Innate resistance refers to a person's lack of sensitivity and is the most fundamental type of resistance. Within a species, strain, or bacterial population, this innate characteristic is always present. In the context of antibiotics, innate

resistance is the idea that a particular microorganism's inherent insensitivity keeps it immune to some antibiotic families.(Urban-Chmiel & et. al 2022).

As a result of their unique genetic adaptability, bacteria may react to a wide range of environmental dangers, including antibiotics that may threaten their existence. Bacteria have developed innate resistance mechanisms in environments shared with species that produce antibiotics, which enable them to survive in the presence of harmful antibiotic compounds. Two main genetic techniques were developed by evolution in response to antibiotic attacks: horizontal gene transfer (HGT) to obtain foreign DNA and mutations in drug-associated genes.

2.2.2 Mutational resistance

When an antibiotic is present, a portion of bacterial cells that have developed mutational resistance experience changes in the drug's action that protect their ability to survive. As populations at risk are destroyed, resistant mutations proliferate. Although antimicrobial resistance-affecting mutations often compromise cell homeostasis, they survive when drugs are present. Changes to antibacterial targets, reduced medication absorption, activation of release mechanisms, or global alterations in metabolic pathways are a few examples of resistance mechanisms.(Munita, J.M.&caser,A.A.,2016)

2.3 Horizontal gene transfer

One of the main reasons behind the evolution of bacteria and the development of antibiotic resistance is horizontal gene transfer. Since it has intrinsic genetic resistance factors, the environmental resistance is a rich source of antibiotic resistance genes to treat significant microorganisms. Three methods are used by bacteria to obtain foreign genetic material: transformation, transduction, and conjugation. Conjugation is a common process in hospitals, particularly in the gastrointestinal tracts of patients receiving antibiotic therapy. Effective genetic information transfer is greatly assisted by mobile genetic elements like transposons and plasmids. Integrons, which are site-specific recombination mechanisms, are particularly effective at accumulating genes that promote resistance to antibiotics. This method allows additional genes to be incorporated into bacterial chromosomes. This mechanism is a strong way to achieve genetic exchange and a major reason behind the evolution of bacteria. In conclusion, the way

that bacteria respond to antibiotics involves both horizontal gene transfer and mutations that modify their DNA, both of which aid in the development and spread of antibiotic resistance in species that are relevant to medicine.(Munita, J.M.&caser,A.A.,2016)

Antibiotic resistance is a serious worldwide issue that puts at risk progress in food production, healthcare, and living. It is a global issue that is not limited to a particular region. The issue is that antibiotic resistance spreads rapidly due to the ease with which humans, animals, and various other objects may cross worldwide. This implies that the medicine we use to treat infections may not be effective at all or not as well. It presents a significant challenge for modern medicine and has an impact on not just our general well-being but also agriculture and health. This shows how important it is for people to work together globally in order to find solutions for antibiotic resistance.

2.4 Bacteriophage and its Discovery

Bacteriophages are viruses that recognize and infect specific bacteria to take advantage of their metabolism to produce their own stable progenies that survive to attack new hosts in their surroundings. (Sanz-Gaitero et al., 2021) They are also known as phages. All bacteriophages contain a nucleic acid genome as their genetic material which is encapsulated inside capsid proteins encoded by the phage. As the most prevalent biological agents on the planet, they are widely distributed across the environment. Their size, morphology, and genetic structure display great diversity. Most importantly, bacteriophages are host-specific meaning; a phage only attacks its specified bacteria. Typically, a phage infects only one species of bacteria or certain strains of a bacterial species. Therefore, phages have no infectious effect on human cells. (Kasman & Porter, 2022)

The function and effect of bacteriophage was first identified by English bacteriologist, aeronautical theorist, and naturalist Ernest Hanbury Hankin in 1896. (Bacteriophage.news, 2024) He reported his observation of antibacterial activity against *Vibrio cholerae* in the Ganges and Jumna rivers in India. Similar antibacterial activity was observed on *Bacillus subtilis* by Russian bacteriologist Gamaleya in 1898. However, it was Frederick Twort who reestablished and further

researched the phenomenon after almost 20 years. After observing similar results like before, he hypothesized that the responsible being for such an event could be a virus. Unfortunately, he could not continue his studies due to various reasons. It was Felix d'Herelle who finally discovered phage through his studies and experiments on hemorrhagic dysentery outbreak among French troops. He observed clear zones, named as *plaques*, on agar cultures. The agar culture contained a mixture of *Shigella* strains extracted from the patients and bacteria-free filtrate of fecal samples of the patients, which were incubated and then spread on agar media. He proposed that a virus capable of infecting bacteria was responsible for this phenomenon. He then coined the term "Bacteriophage" from 'bacteria' and 'phage' which derived from the Greek meaning 'to eat or devour', indicating that phages eat bacteria. This discovery led to the belief that phages are live viruses rather than enzymes with antibacterial capabilities which was thought by many researchers. (Sulakvelidze et al., 2001)

2.4.1 Bacteriophage Characteristics and Diversity

The population of bacteriophages can be stated as the largest population of all organisms including bacteria because more than 10^{31} phages are present on the earth. These phages can be divided into two categories; virulent phages which go through the lytic cycle for replication and temperate phages which go through the lysogenic cycle for replication. (Bacteriophage.news, 2024)

14,244 entire phage genomes had been sequenced as of January 2021. Phages that infect a limited number of bacterial genera account for the majority of the INPHARED (INfrastructure for a PHAge REference Database) data set; only 30 bacterial genera are the target of 75% of the phage isolates. This database consists of more genomes of lytic phages (about 70%) and fewer genomes of temperate phages (about 30%). Among these temperate phages, about 54% of genomes are derived from 3 host genera. (Cook et al., 2021) The genomes of phages vary greatly in size; the ~3,300 nucleotide ssRNA viruses of *Escherichia coli* are much smaller than the over 500 kbp genome of *Bacillus megaterium* phage G. Though there are wide variations in size, the smallest dsDNA-tailed phage genomes are approximately 11.5 kbp for *Mycoplasma* phage P1, 21 kbp for *Lactococcus* phage c2, and approximately 30 kbp for *Pasteurella* phage F108,

belonging to the *Podoviridae*, *Siphoviridae*, and *Myoviridae*, respectively. (Hatfull & Hendrix, 2011)

In order to protect the phages from degradation, the structure of the protein capsids is highly symmetrical which stores the genome of the phage. Based on the type of the phage, the genome can be single-stranded DNA, double-stranded DNA, single-stranded RNA, or double-stranded RNA. Capsids of various shapes can be observed in different phages, like icosahedral, helical, spherical, etc. As phages are host-specific, they require a host cell recognition mechanism. One or more capsid proteins may function for host cell recognition for different phages. In addition to components intended for genome transfer, host recognition, and genome protection, more complex phage particles could also include proteins for environmental sensing, binding to appropriate matrices where host bacteria are likely to be found, and other purposes. (Sanz-Gaitero et al., 2021)

2.4.2 Classification of Bacteriophages

Three families, *Myoviridae* with a contractile tail, *Podoviridae* with a short tail, and *Siphoviridae* with a non-contractile long tail, make up the *Caudovirales* order, which accounts for about 96% of all documented bacteriophages. (Kaliniene et al. 2017) Grouped into ten tiny families, filamentous, cubic, and polymorphic phages makeup approximately 3.6% of all bacteriophages now in existence. They are all members of the same order. Phage morphologies are illustrated in Table 1. (Ackermann 2009)

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,

Table-1: Classification of Bacteriophage (Giri, 2021)

2.4.3 Life Cycle of Bacteriophage

When a phage infects a bacterial cell, its genetic material enters the cell which generates a production cycle of the phage. Upon the insertion of the phage genome, the bacterial cell reprograms and functions as a phage production factory. Therefore, the biosynthetic machinery (ribosomes, ATP generators) dedicated to the reproduction of the bacteria is reprogrammed to reproduce and replicate the phage. After infection, the phage mRNAs translate into phage-specified proteins that start different pathways of reprogramming the bacterial cell. (Campbell, 2003)

All bacteriophages mainly go through 2 types of replication systems; either the lytic cycle (virulent phage) or the lysogenic cycle (temperate phage). (Bacteriophage.news, 2024)

When the phages infect bacteria, they first recognize the host cell surface receptors (like lipopolysaccharides, fimbriae, flagella, outer membrane protein, etc.) and reversibly attach through adsorption with the help of tail fibers or tail spikes. Due to viral specificity, the phage attaches to only its specified bacterial strain. The bacterial cell wall's polysaccharides can be depolymerized by some of the tail fibers because they have enzymatic activity. (Prokhorov et al. 2017). After that, the tail fibers contract due to the structural rearrangement of the base plate which makes the attachment. Using various carbohydrate depolymerase and lysins, the phage penetrates the host cell while the tail fiber merges with the host cytoplasmic membrane creating a transmembrane channel. Through this channel, the phage injects its nucleic acid (DNA or RNA) into the host cytoplasm. (Giri, 2021)

Virulent phages go through the lytic cycle. After the entry of the genetic material into the bacterial host cytoplasm, the phage DNA or RNA uses the replication machinery of the host to generate its own particles like capsid proteins, tails, nucleic acid, etc. Through various enzymatic activities, the viral particles are assembled and synthesized. When phage DNA reaches maturity, its head and tail proteins come together and encase each piece in a protein coat. During maturation, the head and tail proteins of phage assemble, and each nucleic acid (DNA or RNA) is enveloped by a protein coat. Lastly, virions are formed and the bacterial cell is lysed which releases many phage progenies ready to infect new hosts. (Bacteriophage.news, 2024)

Temperate phages go through the lysogenic cycle for replication. Instead of producing virions inside the host cell, these phages incorporate their genetic material with the bacterial genome known as prophage. Becoming a part of the host's genetic material, the phage DNA or RNA replicates as well without causing any harm to the host. (Howard-Varona, 2017) A lysogenic bacteriophage can, however, transition from the lysogenic cycle to the lytic cycle in response to pressure or threats to its existence. This process causes the phages to multiply quickly and rupture from the host cell. (Bacteriophage.news, 2024)

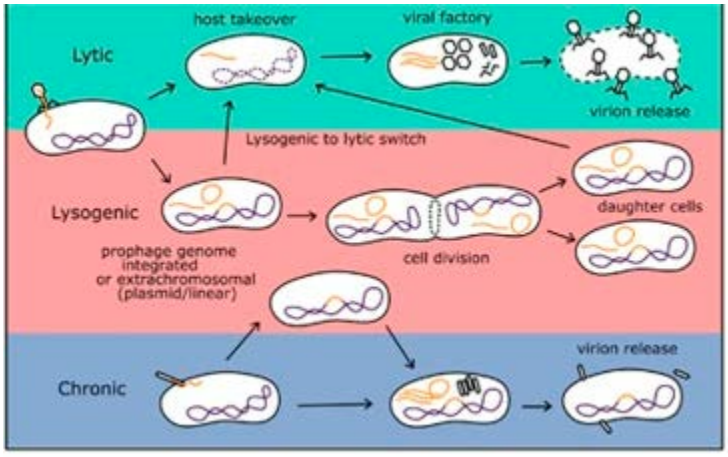

Figure-4: Life cycles of phage

Two other life cycles occur rarely; pseudolysogeny and chronic infection. When a host cell experiences unfavorable development conditions, pseudolysogeny takes place. However, the phage does not fuse its genetic material with the host's. This process seems to be crucial for phage survival because it allows the phage genome to be preserved until the host growth conditions are favorable once more. Long-term, continual production of new phage particles occurs in chronic infections, yet there is no apparent cell death. (Britannica, 2023)

2.5 Phage Therapy

Phage therapy is an approach to treating bacterial infections with bacteriophages. Due to viral specificity, phages only attack bacteria and cause no harm to other living organisms like humans, plants, insects, etc. (Iftikhar, 2019) Phages are ubiquitous and of various kinds. Antibiotics have been widely used for the treatment and elimination of bacterial infections for a long time.

Although antibiotics work against harmful bacteria and destroy them, they can eliminate several beneficial bacteria from the microbiota which can lead to further problems. On the other hand, phages have evolved to be more specific in their target bacterial strain or species. (Barron, 2022) The specificity of phages arises due to the phage-host receptor surface, the phage nature, the genetic and physical defense systems of the host, etc. (Hibstu et al., 2022) Therefore, phage therapy only targets the pathogen without disrupting the microbiome. As a result, it is an appealing substitute for treating infections because of its specificity, particularly when dealing with multi-drug resistant (MDR) microorganisms. (Barron, 2022) It has been around for 100 years but it has not been widely used.

2.5.1 Mode of Action

Virulent or lytic phages, bioengineered phages, and purified phage lytic proteins are used as the delivery agents of phage therapy. (Hibstu et al., 2022) The mechanism of phage therapy is killing the bacteria by lysing the cell. The phage infects the bacteria and injects its genetic material (DNA or RNA) into the host cell. Several virion copies are produced inside the bacterial cell and lastly lyses the cell releasing thousands of copies of the phage. After that, the phages can stay dormant until they find new bacteria in their surroundings and kill them with the same mechanism. (Iftikhar, 2019) To treat a bacterial infection with phage therapy, the first step would be isolating active phages specific against that bacterium. Next, the phages need to be amplified and delivered to the patient in a way that they contact the infectious agents. The phages will proliferate as long as they can detect bacteria in the area. The phages will be degraded after the bacteria are removed because they require a host to survive. (Brives & Pourraz, 2022)

2.5.2 Strategies of Phage Therapy

Various therapeutic options have been developed for phage-based treatments against bacterial infection. Some of them are briefly described below:

Phage Cocktail

If phage therapy is conducted by administering a single phage type then it is known as monophage treatment. However, a patient may not always be infected by a single bacteria type, rather multiple infections may occur. In that case, monophage therapy is not suitable. Moreover,

over the years, bacteria have evolved and mutated to modify their cell surface receptors or modify restriction enzymes that can degrade phages. (Liu et al., 2022) Therefore, to improve treatment results and to increase the host infectivity range, a mixture of different types of phages can be used instead of monophage treatment. So, phage cocktails aim to target diverse bacterial types. Moreover, it can suppress the evolution potential of bacteria to grow resistant against phages. (Abedon et al., 2021)

Phage-Antibiotic Synergy

Recent studies suggest that combining phage with antibiotics leads to increased and more efficient eradication of the targeted bacterial population, thus increasing the mortality rate of the bacteria. As administered in combination with antimicrobial medications, it has been suggested that phages can significantly reduce biofilm viability as compared to each treatment on its own, indicating either synergy or facilitation. (Liu et al., 2022) Nonetheless, the growing knowledge of antagonistic behavior has prompted some in vitro evaluations of phage-antibiotic activity before therapy in order to identify synergistic pairings. In some instances, a customized strategy has produced positive therapeutic results. (Gu Liu et al., 2020)

Genetically Engineered Phages

Modifications can be made in the phages by genetic engineering to achieve a wider host range, increase bactericidal activity, enhance biofilm degradation capacity, etc. Mechanisms for altering the phage may include gene mutation, replacing a gene, or integration of a foreign desired gene. Mutation of a gene or substitution usually aims to enhance the host range. The products of gene introduction impose great bactericidal activity. (Guo et al., 2021)

Phage Derived Enzymes

Phages produce a lot of bacterial biofilm-degrading enzymes like lysins, depolymerases, lipases, DNases, etc. that not only lyse existing biofilm but can also restrict the development of biofilms. As the enzymes derived from phages display antibacterial activity, they can be used as therapeutic agents for the treatment of bacterial infections. By degrading the extracellular polymerase substances (EPS), phages can disrupt and enter through the biofilm into the deeper

layer of the bacterial structure. Phages that produce extracellular polysaccharide depolymerases can conduct this action. (Liu et al., 2022)

Combining Phages with Other Agents

Other synergistic approaches suggest combining phages with chemical disinfection, biological nanoparticles, etc. to improve the treatment and to increase efficiency. (Liu et al., 2022)

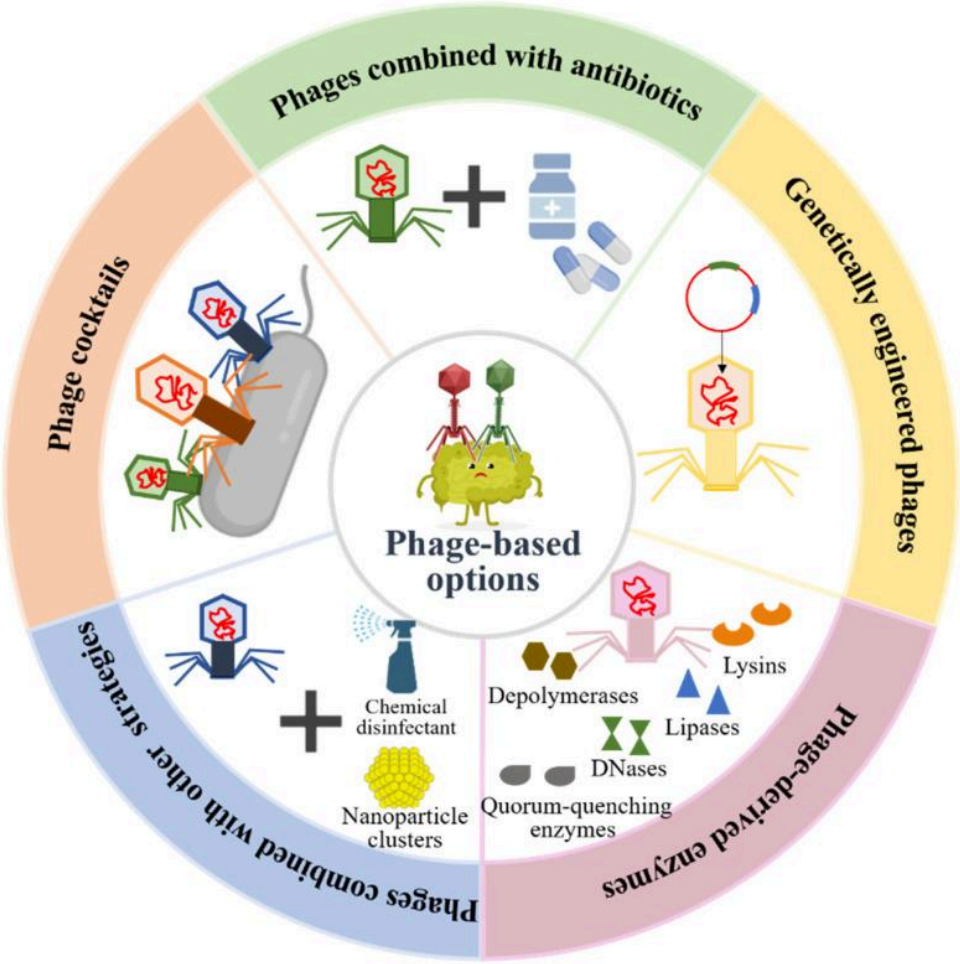

Figure-5: Strategies of Phage Therapy (Liu et al., 2022)

2.5.3 Advantages of Phage Therapy

Phage therapy has been proposed as a substitution for various beneficial properties of phages. Although more extensive research and experiments are being conducted, here are a few advantages of phage therapy: (Loc-Carrillo & Abedon, 2011)

· As phages possess bactericidal activity, their target host bacteria are killed rather than suppressed. As a result, they cannot become viable again. On the other hand, antibiotics are bacteriostatic. Therefore, they suppress the growth of bacteria which can contribute to the evolution of antibiotic resistance.

- · As phages keep reproducing and infecting bacteria as long as they can find their hosts, they themselves help in setting the phage dosage, which can be termed as auto "dosing". However, few phages depend on bacterial densities and require higher density to reproduce.
- · Due to viral specificity, phages target only its dedicated bacterial strain and do not affect strains out of their host range. So, introducing phages into patients has no or very low impact on their normal flora. But with a broad-spectrum activity, antibiotics tend to have an impact on the normal flora leading to further problems.
- · Biofilms have more potential to become antibiotic resistant than planktonic bacteria. Phage-borne enzymes like lysins, depolymerases, lipases, DNases, etc. have the capacity to degrade extracellular polymerase substances and penetrate through biofilms. Phages that produce these enzymes can degrade some biofilms.
- · Antibiotics tend to have an impact on the environment. Discarding antibiotics in the environment may lead to the development of resistance among the surrounding bacteria and the chemical used may have an effect as well. However, phages are constituted of nucleic acids and proteins. It is unlikely that they will have a bad impact on the environment. At worst, they can affect a tiny portion of bacteria found in the environment. Rapid inactivation can also occur in phages that are not acclimated to degradative environmental conditions like sunshine, desiccation, or extremely high or low temperatures.

- · Similar to antibiotics, phages can be developed in a variety of ways, including in combination with other antibiotics. Not only are they appropriate for the majority of administration routes, but they also come in a variety of application forms, such as liquids, creams, and impregnated into solids. Additionally, distinct phages can be combined as a cocktail to enhance their characteristics, usually leading to an overall wider range of antibacterial activity.

2.5.4 Current State of Phage Therapy

Due to bacteria's constant evolution to resist both existing and new antibiotics, newly developed antibiotics will inevitably run into drug-resistant mutants. Resistance to a novel antibiotic can quickly spread to other harmful bacteria through horizontal gene transfer. For instance, resistance to ceftazidime-avibactam was noted shortly after its introduction in the US and is now commonplace. Consequently, the FDA has started to approve phage therapy for compassionate use in order to address antibiotic failure in chronic infections. These infections include those that are frequently found in 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)

Approval of phage therapy as compassionate use in multi-drug resistant bacterial infection and biofilm-based infections has resulted in some successes through clinical trials. A chronic prosthetic knee-joint infection caused by methicillin-sensitive *Staphylococcus aureus* (MSSA) was successfully treated with phage therapy as it was a biofilm-based infection that had poor outcomes with antibiotic treatment. By conducting a two-stage exchange procedure, this 61-year-old patient was successfully treated after the second course of phage therapy. (Ramirez-Sanchez et al., 2021) In another study, a specific phage cocktail was used in a patient with a pancreatic pseudocyst infected with multi-drug resistance *A. baumannii* causing necrotizing pancreatitis which resulted in drastic improvement in the patient's health. (Schooley et al., 2017)

2.5.5 Clinical Trial of Phage Therapy

Adapted from (Giri, 2021)

Trial	Infection	Treatment Group	Phage Dose and Application	Outcome
1	*Pseudomonas aeruginosa* otitis	12 individuals had received phage cocktail	10^9 PFU was intra-aurally delivered (single dose)	From each group three individuals found to have undetectable levels of *P. aeruginosa* at the finale of the trial
2	*Escherichia coli* diarrheal diseases	40 individuals had received phage cocktail M, 39 individuals had received phage cocktail T	1.4×10^9 PFU cocktail M or 3.6×10^8 PFU cocktail T delivered orally three times per day for 4 days (12 doses) in oral rehydration solution	No substantial difference observed between placebo group and phage treatment group

3	*Pseudomonas aeruginosa* burn wound infection	12 individuals received a phage cocktail	2×10^7 PFU (expected) 200–2,000 PFU (actual) topically applied one time per day up to 7 days (seven doses)	Trial halted, insufficient efficacy; may be due to considerably lower applied dose of phage than estimated
4	Several multidrug-resistant bacteria	157 patients was orally, intrarectally or intravaginally administrated to Hirszfeld Institute phage collection	10- 20 ml of phage collection were administrated 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 were applied on 65 patients	1×10^9 Phage cocktail were topically applied on wound infection once a week for 12 weeks	Different wound healing rates with time (differential wound size reduction over time)

Table 2: Clinical Trial of Phage Therapy (Giri, 2021)

Phage has several potential applications in dentistry, veterinary medicine, human health, and agriculture. In addition to antibiotics, bacteriophages are used in many other industries, such as the food and cattle industries.

2.5.6 Phage therapy in treating *V.cholerae* infection:

Phage treatment involves the use of viruses known as phages, which are specifically targeted to particular types of bacteria, as a means to address problematic and uncontrolled microorganisms. These microorganisms are often associated with infectious diseases. Unlike antibiotics that broadly affect various bacteria, phages are highly specific, targeting particular bacterial strains. When a bacterial infection occurs, phages can be employed to infect and destroy the harmful bacteria while leaving beneficial bacteria and human cells unharmed (Loc-Carrillo & Abedon, 2011). This precision makes phage treatment an appealing option for combating bacterial infections, offering a targeted and potentially more tailored approach compared to traditional antibiotic therapies. The use of phages in treatment aligns with the growing interest in alternative and personalized strategies to address bacterial infections, particularly in cases where antibiotic resistance poses a challenge.Given the urgency to find effective treatments, there's renewed interest in bacteriophage (phage) therapy, a century-old approach. Unlike antibiotics, which may only reduce diarrhea and bacterial shedding, managing diseases like cholera requires additional support through robust fluid and electrolyte supplementation. The search for alternative therapies like phage therapy is crucial in addressing the challenges posed by antibiotic resistance in developing nations.The majority of *V. cholerae* in endemic regions are currently resistant to the widely used medications (Dengo-Baloi et al., 2017; Rijal et al., 2019; Verma et al., 2019; Chatterjee et al., 2020).

Phages have the ability to kill a large number of bacteria, making them a valuable tool in developing phage therapy for cholera. This technique is effective in treating cholera patients, preventing the spread of the illness and reducing the harmful bacteria load. In the initial report on phage therapy, large doses of Anti-Cholera phages (ranging from 100 to 200 phages) were administered to combat the cholera bacterium. However, the phages were unable to effectively perform their job due to frequent replication and amplification challenges in the presence of *Vibrio* (Fazil & Singh, 2011).

When patients with illnesses were treated with bacteriophage therapy, along with tetracycline and fluid replacements, it was noticed that extremely high doses of phages were administered. The phage treatment showed a similar reduction in the amount of harmful *Vibrios* in feces, comparable to the effects of tetracycline. However, this reduction did not lead to overall clinical

improvement, such as a shorter duration of diarrhea or faster healing. Challenges were observed in evaluating phage therapy for cholera due to the diversity of *Vibrios*' serotypes and other hindrances. Two key factors were noted: varying susceptibility of these bacteria to phages, and the rapid passage of phages through the digestive tract in people with cholera. This rapid passage might have played a role in the lower frequency of infection treatment and possibly affected the significance of a second phage infection.

In various parts of the world, researchers have explored the potential of lytic cholera phages as a preventive measure. Studies, conducted using a newborn mouse infection model, examined how lytic phages influenced the growth of *Vibrio cholerae*. The findings indicated that the phages were effective in significantly reducing the amount of *Vibrio cholerae* in the small intestine (Jaiswal et al., 2012) Phage therapy, using viruses that target harmful bacteria, has been attempted to treat or prevent cholera, with varying success.A single type of phage given an hour before exposing neonatal rabbits to *Vibrio cholerae* prevented cholera symptoms (Dutta et al.,2012). Another study showed a cocktail of five phage types given 6 or 12 hours before exposing adult rabbits to *Vibrio cholerae*, resulting in a slight reduction in diarrhea severity but no significant decrease in bacterial load. However, the same phage cocktail successfully reduced the bacterial load when administered 6 or 12 hours after the *Vibrio cholerae* challenge. (Jaiswal et al.2012)

As the field of phage therapy advances, the targeted and specific nature of phages makes them a promising avenue for addressing bacterial infections, especially in the context of rising antibiotic resistance. The use of bacteriophages in treating cholera showcases their potential in reducing bacterial loads and preventing the spread of illness. However, challenges persist, including issues with replication and amplification, varying susceptibility of bacteria to phages, and the rapid passage of phages through the digestive tract. Despite these challenges, the urgency to find effective treatments, particularly in the face of antibiotic resistance, has sparked renewed interest in phage therapy. Researchers are continuing to explore and refine the application of phages as a potential alternative or complementary strategy in the global fight against infectious diseases, offering a tailored and precision-based approach to treatment.

Chapter 3

Methods and Materials

3.1 Place of Study

Exploring the Biotechnology and Microbiology Laboratory at BRAC University, Dhaka, Bangladesh.

The presented research study was conducted at the esteemed BRAC University, situated in the vibrant city of Dhaka, Bangladesh. Specifically, the investigation took place within the well-equipped and specialized Biotechnology and Microbiology Laboratory, which falls under the purview of the esteemed Department of Mathematics and Natural Sciences. This choice of location was deliberate, as it provided the necessary infrastructure and resources to carry out the rigorous scientific inquiry required for this study

3.2 Lab Protocol Essentials: A guide to best practices

While performing the research, we ensured the implementation of rigorous safety protocols. Our team diligently followed proper laboratory procedures by wearing clean lab coats and using hand gloves. We were aware of what we were doing at all times. Additionally, various equipment such as pipette tips, falcon tubes, microcentrifuge tubes, culture media (both agar-based and broth-based), and empty test tubes for the spot test method were subjected to autoclaving at 121°C and 15 pounds of pressure for approximately two hours. Any autoclaved media that required storage was kept at 4°C. To maintain cleanliness, all glassware utilized in the project, including conical flasks, beakers, and test tubes, underwent thorough washing with both tap water and distilled water. To ensure a sterile working environment, 70% ethanol was employed when working at the laminar airflow station, as well as at the general working station, to prevent any potential contamination. We paid attention to unfamiliar smells and substances and contacted the lab officer if such an incident happened. These stringent measures were implemented to uphold the integrity and reliability of our research findings.

3.3 Preparation of Culture Media, Reagents and Solutions

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

TCBS agar is a highly selective medium used for the isolation and cultivation of *Vibrio cholerae* and its related species. In this particular project, TCBS agar played a crucial role in facilitating the growth and identification of *Vibrio cholerae*. The composition of TCBS agar includes agar, Ox bile, Yeast extract, sodium thiosulfate, peptone, sodium citrate (Na3C6H5O7), NaCl, sucrose, bromothymol blue, ferric citrate, thymol blue, and distilled water. It's worth noting that ready-made TCBS agar, readily available in the laboratory, was utilized for this research endeavor, ensuring the reliability and consistency of the experimental results.

By employing TCBS agar, the project aimed to specifically target and cultivate *Vibrio cholerae*, as *Vibrio* changes the color of the media after growth by fermenting the sucrose. TCBS itself is a green-colored media. *Vibrio* produces yellow colonies after culture.

Preparation of TCBS Agar

To prepare the TCBS agar, a weight of 8.908 g of the powdered medium was measured using an electronic balance. According to the manufacturer's instructions, 89.08g of the powder media is required to produce 1000 ml of TCBS agar media. The measured powder media was then mixed with 100 ml of distilled water in a conical flask. The mixture was stirred using a glass rod and heated on a Bunsen burner until bubbles appeared, ensuring proper mixing of the media. To maintain sterility and prevent contamination, the media was poured into sterile petri dishes within a laminar airflow environment.

For this particular project, medium-sized petri dishes were predominantly used, with approximately 20-25 ml of media poured into each plate. It is important to note that TCBS media does not require autoclaving due to the presence of Ox bile, a component derived from bile salts. Autoclaving can potentially affect the sensitivity of Ox bile and inhibit the growth of gram-positive bacteria.

By following these meticulous preparation steps, optimal conditions for the growth and isolation of *Vibrio cholerae* on TCBS agar was ensured. This standardized approach guarantees the reliability and consistency of experimental outcomes, contributing to the scientific understanding of *Vibrio cholerae* and its related species.

Luria-Bertani, Miller Broth (LB)

LB is a widely utilized growth medium for cultivating bacteria due to its favorable composition. It consists of yeast extract, NaCl, and tryptone. For this project, a pre-prepared LB medium from the laboratory was employed.

LB, or Luria-Bertani, serves as a versatile and nutrient-rich medium that supports the growth and proliferation of various bacterial species. Its formulation includes yeast extract, which provides essential vitamins, minerals, and amino acids necessary for bacterial metabolism. Additionally, NaCl is incorporated to maintain the osmotic balance, ensuring optimal growth conditions. Tryptone, derived from casein, acts as a source of nitrogen and peptides, further promoting bacterial growth.

By utilizing the ready-made LB medium available in the laboratory, the project benefited from the convenience and reliability it offers. This standardized medium provides a consistent and controlled environment for the cultivation of bacteria, facilitating accurate experimental results and allowing for a comprehensive understanding of microbial behavior.

Preparation of Luria-Bertani, Miller Broth (LB)

To prepare a 100 ml batch of LB media, 2 g of powder media was carefully measured on a foil paper using an electronic balance. Following the manufacturer's instructions, the measured powder media was combined with 100 ml of distilled water in a conical flask. The mixture was thoroughly stirred using a glass rod and then heated on a Bunsen burner until clear, ensuring proper mixing of the media components.

To maintain sterility and prevent contamination, the media was subsequently autoclaved at 121°C for approximately 2 hours. Autoclaving is a crucial step in the preparation process as it effectively eliminates any potential contaminants, ensuring the purity and integrity of the LB media. After the autoclave cycle was completed, the flask was set aside to cool, signifying that the LB media was now ready for use.

Luria Agar (LA)

Luria agar, also known as LA agar, is a widely employed non-selective growth medium utilized for bacterial culture. It provides a nutrient-rich environment that supports the growth and proliferation of various bacterial species. The high nutrient content of this medium is essential for the optimal growth and development of bacteria.

In this project, LA medium was utilized for streaking the inoculated stock bacteria and also for sub-culturing. Streaking involves the technique of transferring a small amount of bacteria onto the surface of the agar plate in a pattern that allows for the isolation of individual colonies. By employing LA medium for this process, it ensured that the bacteria had access to the necessary nutrients required for their growth and survival.

Luria Agar (LA) preparation

To prepare a 100 ml batch of LB media, 4 g of LA powder media was carefully measured on a foil paper using an electronic balance. Following the manufacturer's instructions, the measured powder media was combined with 100 ml of distilled water in a conical flask. If commercially ready-made LA was not available in the laboratory, a combination of Luria Bertani (LB) Miller broth and agar powder was utilized. In order to produce 100 ml of LA media, 2 g of LB powder media and 1.5 g of agar powder (1.5% agar concentration) were carefully measured using an electronic balance.

The measured quantities of the media were then added to a conical flask, followed by the addition of 100 ml of distilled water. The mixture was thoroughly stirred using a glass rod and heated on a Bunsen burner until bubbles appeared, ensuring proper mixing of the media components.

To maintain sterility and prevent contamination, the media was subsequently autoclaved at 121°C pressure for approximately 2 hours. Once the autoclave cycle was completed, the media was carefully poured into sterile petri dishes within a laminar airflow hood, minimizing the risk of contamination. The media was then allowed to solidify, creating a suitable surface for bacterial growth.

Preparation of LA media containing Ampicillin

In this project, it is necessary to prepare an ampicillin selective plate for the transformed strains. After the preparation of the Luria agar (LA) media and the completion of the autoclave process, the flask containing the media is set aside to allow it to cool down. This cooling period is crucial to ensure the survival and effectiveness of the antibiotic, ampicillin, which will be added.

Once the flask is cool enough to be touched with bare hands, the ampicillin supplement is introduced. According to the manufacturer's instructions, 0.5 ml of ampicillin supplement should be added to 99.5 ml of media. Therefore, we prepared a stock of ampicillin solution with concentration of 2mg/ml by adding 10 mg of ampicillin powder with 5mL of dH_2O. The required concentration of ampicillin needs to be 0.01 mg/ml. For each 99.5ml of LA media, we added 0.5ml of the solution from the stock.

The addition of the ampicillin supplement to the media provides selective pressure, allowing only the desired strains to grow while inhibiting the growth of non-resistant strains. This selective plate preparation technique is crucial in experiments and research involving specific strains and their response to ampicillin.

By accurately following these steps, we can ensure the successful creation of ampicillin selective plates, which will aid in the isolation and identification of target strains in our project.

Preparation of LA media containing Kanamycin

In this project, it is necessary to prepare a kanamycin selective plate for the transformed strains. Similar to the process described earlier for ampicillin, the preparation of the Luria agar (LA) media is followed by the autoclaving step. After the autoclave is done, the flask containing the media is set aside to allow it to cool down. This cooling period is important to ensure the survival and effectiveness of the antibiotic, kanamycin, which will be added.

Once the flask is cool enough to be touched with bare hands, we add the kanamycin. We prepared a stock of kanamycin solution with concentration of 5mg/ml by adding 500 mg of kanamycin powder with 100ml of dH_2O. The required concentration of kanamycin needs to be 0.05 mg/ml. For each 99 ml of LA media, we added 1 ml of the solution from the stock.

The addition of kanamycin to the media provides selective pressure, allowing only the desired strains to grow while inhibiting the growth of non-resistant strains. This selective plate preparation technique is crucial in experiments and research involving specific strains and their response to kanamycin.

By following these steps accurately, we ensure the successful creation of kanamycin selective plates, which will aid in the isolation and identification of target strains in our project.

Soft Agar Preparation

To perform the spot test and to prepare stocks for the strains, it was necessary to prepare soft agar with a concentration of 0.6% and 0.8% respectively. For this purpose, a measured amount of 2 g of Luria Bertani broth powder and 0.6 g of agar powder for spot test and 0.8g agar powder for stock media were carefully weighed on a foil paper using an electronic balance. These precise measurements ensure the accuracy of the agar composition.

Next, 100 ml of distilled water was added to a conical flask along with the measured powders. The mixture was stirred using a glass rod and heated on a Bunsen burner until bubbles started to form. This heating step is crucial to thoroughly mix the media and ensure its homogeneity.

Once the soft agar media was well-mixed and bubbles were observed, it was carefully poured into clean test tubes for the spot test using a glass pipette. Each test tube was filled with 5-6 ml of the media. These test tubes containing the soft agar were then subjected to autoclaving at 121°C and pressure for approximately 2 hours. Autoclaving is a standard sterilization process that eliminates any potential contaminants, ensuring the purity of the soft agar. And for stock, 1 ml of the autoclaved soft agar media was poured into clean MCTs and set aside to cool down to be stabbed with organisms later.

The preparation of soft agar with the specified concentration is crucial for the spot test, as it provides a suitable medium for the growth and observation of bacterial colonies. By following these precise steps, we ensure the reliability and consistency of our experimental results.

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* DH5a strain on LA Plates:

The pGLO plasmid of *E. coli* DH5a strains contain a GFP gene, and a gene for metabolizing arabinose. In the presence of arabinose, the GFP gene inside the plasmid gets expressed which leads to fluorescence under UV light. Therefore, arabinose is added in LA plates during the culture of *E. coli* DH5a to confirm and identify the presence of pGLO plasmid.

For 20% Arabinose solution, 2 g of arabinose powder is added in 100 ml of distilled water.

For each 100 ml of LA media, 3 ml of arabinose solution is mixed.

For 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 3: Preparation of Alkaline Solution I

After the reagents were measured, they were put into one conical flask and mixed with distilled water. NaOH/HCl was used to balance the pH.

Alkaline Solution II : For 30 ml 10N NaOH Solution: 12 g of NaOH mixed in 30 ml distilled water. 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: 14.72 g of Potassium Acetate was mixed with distilled water to create a 5M solution. Molecular weight of potassium acetate is 98.15 g/mol. pH was adjusted by drop by drop addition of glacial acetic acid.

For Competent Cell Preparation:

Preparation of 30 ml CaCl2 Solution:

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

Table 4: 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: 7.38 g of sodium acetate powder mixed with distilled water. Molecular weight of sodium acetate is 82.0343 g/mol.

HCl was added drop by drop to reach the pH of 5.2.

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

For Chitin Transformation:

Preparation of 0.9% Saline: 0.9 g of NaOH was mixed with 100 ml of distilled water and autoclaved.

Buffers:

TE Buffer: pH: 8

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 5: Preparation of TE Buffer

The reagent powders were mixed with distilled water and HCl/NaOH was used to adjust the pH.

50X TAE Buffer: pH:8

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 6: Preparation of 50X TAE Buffer

The reagent powders were mixed with distilled water and HCl/NaOH was used to adjust the pH.

1X TAE Buffer: For 100 ml Solution:

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

Table 7: Preparation of 1X TAE Buffer

3.4 Preparation of Different *Vibrio* strains

Native strain

In our study, we obtained a total of six strains of *Vibrio cholerae*, namely 1877, WT042, WT443, WT032, WT006, and WT331. To ensure the accuracy of our strain selection, all of these strains were cultured on TCBS (thiosulfate citrate bile salts sucrose) media. Following an appropriate incubation period, the presence of yellow colonies served as a positive indicator for the strains of interest.

By utilizing TCBS media, we were able to selectively cultivate *Vibrio cholerae* strains and confirm their identity based on the distinctive yellow coloration of the colonies. This method allowed us to focus specifically on the strains we intended to study, ensuring the validity and relevance of our research.

The collection and verification of these specific strains are essential for our investigation into the characteristics and behavior of *Vibrio cholerae*. Through this rigorous selection process, we can confidently analyze and compare the different strains, gaining valuable insights into their genetic, phenotypic, and pathogenic variations.

Culture on Non Selective Media

Once we obtained our strains of interest, we proceeded with our subsequent experiments using LA (Luria Agar) media. This particular media formulation was chosen due to its ability to provide the necessary nutrients required for robust bacterial growth. By offering an optimal nutritional environment, LA media facilitated the proliferation of the bacterial colonies, enabling us to achieve significant growth rates.

The selection of LA media as our growth medium was a deliberate choice, as it is widely recognized and utilized in microbiological research. Its composition ensures the availability of essential nutrients, such as amino acids, vitamins, and carbohydrates, which are vital for the metabolic activities and replication of bacterial cells.

Through the utilization of LA media, we aimed to create an environment that would support the expansion of our bacterial strains, allowing us to obtain sufficient biomass for subsequent analyses. The successful growth of the bacterial colonies is crucial for the accurate characterization and investigation of their physiological and genetic properties.

By employing LA media as our growth medium, we were able to provide the necessary nutrition for our bacterial colonies, facilitating their growth and enabling us to conduct comprehensive studies on our strains of interest.

3.5 Preparation of Ampicillin Resistant Strains

3.5.1 pGLO Plasmid Isolation from *E.coli* DH5α

1. The desired plasmid-harboring bacterial cells were cultured overnight in 25 ml of LB medium supplemented with appropriate antibiotics.

2. Subsequently, the cells were harvested through centrifugation at 10,000 Xg for 2 minutes, repeated three times.

3. The resulting pellet was then resuspended in 200 µl of Solution 1, followed by the addition of freshly prepared Solution 2 (400 µl), which was gently mixed by inversion.

4. The cells were incubated at room temperature for 5 minutes to induce cell lysis.

5. Following this, an ice-cold solution of Solution 3 (300 µl) was added, mixed by inversion, and incubated on ice for 10 minutes.

6. The mixture was then subjected to centrifugation at 12,000 Xg for 15 minutes.

7. The supernatant containing the desired components was carefully collected in a fresh tube.

8. To this, an equal volume of phenol: chloroform: IAA (25:24:1) was added, vortexed, and subsequently centrifuged at 12,000 Xg for 2 minutes.

9. The resulting supernatant was collected, and an equal volume of chloroform: IAA (24:1) was added, mixed by vortexing, and centrifuged at 12,000 Xg for 5 minutes.

10. The supernatant was once again collected, and an equal volume of isopropanol was added, mixed by inversion, and subjected to centrifugation at 12,000 Xg for 15 minutes.

11. The resulting DNA pellet was washed with 70% ethanol, air-dried, and finally resuspended in 200 µl of TE buffer.

12. The plasmid was then stored at -20 °C for future use.

These steps outline the protocol employed for the extraction and purification of the desired plasmid from the bacterial cells. Following these procedures ensures the isolation of high-quality plasmid DNA suitable for various downstream applications.

3.5.2 Competent Cell Preparation

1. Single colonies of each native strain of *Vibrio cholerae* (WT006, WT032, WT042, WT331, WT443) were inoculated separately in 5 ml tubes of LB media and incubated overnight at 37°C on a shaker.

2. A 1 ml aliquot of the cell cultures were then separately inoculated into 50 ml of LB media.

3. The cultures were placed in a shaker incubator at 37°C and periodically checked until the optical density (OD) reached 0.4, with measurements taken every 2 hours.

4. Once the cultures reached an OD of 0.4, They were transferred to separate MCTs and placed in an ice bucket for 10 minutes.

5. The cultures were then centrifuged at 5000 rpm for 10 minutes at 4°C.

6. From each MCT, the media was removed.

7. Next, 0.8 ml of CaCl2 solution was added to the resuspended cells, which were incubated in an ice bucket for 30 minutes, followed by centrifugation at 5000 rpm for 10 minutes.

8. The media was again removed, and the cells were resuspended with 0.8 ml of CaCl2 solution. This resuspension was incubated in an ice bucket for 20 minutes and then centrifuged at 5000 rpm for 10 minutes.

9. Finally, from each MCT the media was removed, and 200 µl of glycerol was added to the cells. The cells were then stored at -20°C for future use in the transformation experiment.

These steps outline the process of preparing *Vibrio cholerae* cells for use in a transformation experiment. Following these procedures helps ensure the viability and quality of the cells for successful transformation.

3.5.3 Transformation with pGLO Plasmid

1. The component cell cultures were removed from storage and placed on ice.

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

3. The mixtures were then subjected to a heat shock by placing it in a hot water bath at 42°C for precisely 90 seconds.

4. Immediately after heat shock, the mixtures were transferred back to ice for 2 minutes to close the cell membrane pores.

5. The mixtures were then combined with 1 ml of LB media and cultured for 1 hour at 37°C.

6. Finally, 100µl of the cultured mixtures were plated on LB media plates under various conditions to assess the presence of transformed cells.

These steps describe the process of transforming the component cells with a plasmid. By following these procedures, researchers can introduce the desired genetic material into the component cells and subsequently assess the success of the transformation

3.6 Preparation of Kanamycin Resistant Strains:

3.6.1 Bacterial Chromosomal DNA Extraction:

1. 1ml overnight culture of 1877 *Vibrio* strain was transferred to a 1.5ml MCT.

2. The MCT with bacterial culture was centrifuged at 8000 rpm for 10 minutes at room temperature (25°C).

3. 200 µL TE buffer was added to the tubes and the bacterial pellet was dispersed by vortexing.

4. The tube was heated at 100°C for 10 minutes and then was immediately incubated for another 10 minutes at -4°C.

5. 20 µL of 10% SDS solution was added to the tube and was mixed gently. Then it was incubated at 55°C for 1 hour until the mixture was observed to become clear and viscous.

6. Equal volume of PCI (220 µL) was added and mixed by vortexing. Then the tube was centrifuged at 13200 rpm for 10 minutes.

7. The upper aqueous layer was carefully collected in fresh tubes.

8. Equal volume of CI was added and mixed by vortexing. Then the tube was centrifuged at 13200 rpm for 10 minutes.

9. The upper aqueous layer was carefully collected in fresh tubes.

10. 0.1x volume of 3M sodium acetate and 2.5x volume of chilled absolute ethanol were added and gently mixed by vortexing. Then the tube was incubated at -20°C for 1 hour.

11. The tube was centrifuged at 13200 rpm at -4°C for 30 minutes.

12. The supernatant was discarded and the DNA was dissolved in 20 µL TE buffer. The DNA was stored at -20°C.

These steps describe the process of extracting DNA from a bacterial culture. By following these procedures, researchers can obtain DNA for further analysis or experimentation.

3.6.2 Chitin Induced Transformation Protocol:

Day 1:

1. The selected bacteria from stocks were streaked on LA plates or bacterial colonies were inoculated in LB medium from fresh plates and incubated overnight.

Day 2:

1. A volume of 1ml of the overnight culture was transferred to 9 ml of fresh LB medium to create a young culture of 10ml. This culture was incubated in a shaker incubator for approximately 1.5 to 2 hours.

2. The culture was then centrifuged at 5000 RPM for 10 minutes.

3. The cell pellet was washed with 5 ml of 0.9% NaCl (saline) by first re-pipetting and then centrifuging. The washing process was carried out using the centrifuge conditions of 5000 RPM for 2 minutes.

4. The saline was discarded, and if necessary, the washing step was repeated twice.

5. To the washed bacterial cell pellet, 5 ml of saline and autoclaved chitin flakes (50-80mg) were added, followed by re-pipetting.

6. The mixture was thoroughly mixed and incubated at 30 degrees Celsius overnight.

Day 3:

1. Centrifugation was performed at 3000 rpm for 5 minutes, and the supernatant was subsequently discarded.

2. A volume of 5ml of saline was added, along with the appropriate amount of previously isolated genomic DNA. The mixture was then incubated for 2, 4, 6, or 24 hours.

3. The shrimp shells were transferred to a fresh Falcon tube.

4. An adequate amount of saline was added, ensuring that the shells were fully submerged. The minimal volume of saline required was determined to be 1.5ml.

5. Vigorous vortexing of the tube for 5 minutes facilitated the detachment of bacteria from the chitin.

6. The tube was left undisturbed until all the flakes settled down.

7. The supernatant, the liquid above the settled flakes, was carefully collected.

8. If the supernatant exhibited significant turbidity, it was diluted with fresh LB. Otherwise, dilution was unnecessary.

9. The diluted or undiluted supernatant was spread onto LA plates or LA plates supplemented with Kanamycin.

3.7 Gel Electrophoresis:

3.7.1 Preparation of Agarose Gel

1. A flask was taken and 0.5 g of agarose was measured and added to it, along with 50 ml of 1X TAE buffer. The solution was then placed in the microwave on a hot plate until the agarose was dissolved and the solution became clear.

2. The solution was cooled down a little and then 2 µl of EtBr was added.

2. The solution was poured into the gel tray and left to cool at room temperature. The comb was placed into the gel tray before the solution cooled completely. The comb was positioned about 1 inch from one end of the tray and was placed vertically, with the teeth about 1-2 mm above the surface of the tray.

These steps describe the process of preparing a 50 ml, 1% agarose solution for gel electrophoresis. It's important to follow these steps carefully to ensure the agarose is properly dissolved and the gel is set up correctly.

3.7.2 Gel Electrophoresis:

1. The comb was gently removed, and the gel tray was placed in the electrophoresis chamber. The tray was then covered with an electrophoresis buffer, approximately 1 L of TAE buffer, the same buffer used for agarose preparation. The cover was placed just until the wells were submerged.

2. For the preparation of samples for electrophoresis, 2 µl of gel loading dye was added for every 8 µl of DNA sample.

3. The gel was run at a voltage of 90 V, and it may have taken approximately 45 minutes for the run to be completed.

These steps outline the procedure for running gel electrophoresis. It is important to adhere to these steps meticulously to ensure accurate results.

3.8 Bacteriophage Identification & Purification

3.8.1 Bacteriophage Identification from Stock Solution

As part of this experimental procedure, a total of six bacteriophages were obtained from the stock, namely JSF2, JSF6, JSF7, JSF10, JSF25, JSF30. Each phage was individually collected in sterile falcons, with a volume of 10 ml for each sample. The purpose of this study was to investigate the host specificity of these phages.

3.8.2 Bacteriophage Enrichment:

After collecting the phages from stock, they were enriched through the following steps:

1. Fresh culture of the host bacteria for each phage was prepared in LA plates.

2. 1 or 2 colonies were taken and inoculated in a fresh 15 ml falcon containing LB media.

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

4. The desired bacteriophage was added to the culture.

5. The culture was incubated in the shaker incubator at 37°C for 4 hours.

6. Then, the culture was centrifuged at 13000 rpm at 4°C for 10 minutes.

7. The supernatant was filtered into a new fresh falcon using a 0.22 um filter.

The resulting clear suspension was free from bacterial cells and contained the desired bacteriophage. The falcon tubes were appropriately labeled, such as "V.Cholerae 1877(JSF2)," and securely wrapped with parafilm. These tubes were then stored at 4 degrees Celsius. In order to enhance the phage quantity in case of complete consumption, the enrichment procedure for the isolated phage was repeated.

3.8.3 Phage Infectivity Test on Different *Vibrio* Strains:

For the phage infectivity test on different Vibrio strains,

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

2. The following day, the single colonies of each strain were inoculated into separate falcon tubes containing 5 ml of LB media. The tubes were then incubated at 37 degrees

Celsius to cultivate a young culture, characterized by a slightly hazy appearance where light can pass through.

3. Once the young culture was prepared, a lawn of bacteria was created by pouring 6 ml of soft agar (0.6% agar) mixed with 100 microliters of the bacterial young culture onto an LA plate.

4. Sufficient time was allowed for the layer of agar to solidify on the LA plate.

5. Two LA plates were designated for each bacterial strain, clearly marked for the placement of phage droplets.

6. Using a micropipette, 10-15 microliters of each phage was dropped onto the bacterial lawn, and ample time was given for the diffusion of the phages through the soft agar.

7. Finally, a total of 12 plates were incubated at 37 degrees Celsius for a duration of 12-13 hours.

8. The areas on the plates where clear zones were observed around the droplets were identified as potential phages for those specific bacteria.

To assess the host specificity, a spot test was conducted using native bacterial strains. This test involved the application of small droplets of the phage samples onto agar plates previously inoculated with the respective bacterial strains. The plates were then incubated under appropriate conditions to allow for the growth and interaction between the phages and the bacterial hosts.

The spot test served as a preliminary screening method to observe the presence or absence of clear zones, known as plaques, which indicate the lysis of bacterial cells by the phages. The formation of plaques suggests that the phages have the ability to infect and replicate within the specific bacterial strains tested. This information is crucial in understanding the host range and potential applications of these bacteriophages.

3.8.4 Phage Infectivity Test on Different Antibiotic Resistant *Vibrio* Strains:

To test ampicillin resistant strains, LA media plates were used for subculture, LB media for young culture, and LA plates for the spot test. The media was made selective by adding an adequate amount of ampicillin supplement (0.5ml ampicillin for 100ml media).

Similarly, for kanamycin resistant strains, LA media plates were used for subculture, LB media for young culture, and LA plates for the spot test. The media was made selective by adding an adequate amount of kanamycin (0.5ml kanamycin for 100ml media).

For both antibiotic resistant strains, the same steps were followed and plaques were observed.

These steps outline the procedure for testing the infectivity of the phage on different *Vibrio* strains. By using selective media, the test specifically targets ampicillin resistant and kanamycin resistant strains. This research contributes to the understanding of phage-host interactions and the development of strategies for combating antibiotic-resistant bacteria.

Chapter 4

Result

After completing all experiments, the results were gathered and organized for comprehensive analysis. The experiments were repetitively undertaken to ascertain the accuracy of the results. The following *V. cholerae* strains of serotype 01 were selected as sample in this experiment: WT042, WT443, WT006, WT331, and WT032. We selected from an array of 15 diverse *V. cholerae* strains, 15 strains were tested for sensitivity against ampicillin and kanamycin antibiotic and those 5 strains were chosen for sensitivity to both ampicillin and kanamycin.The selected native strain samples are given below :

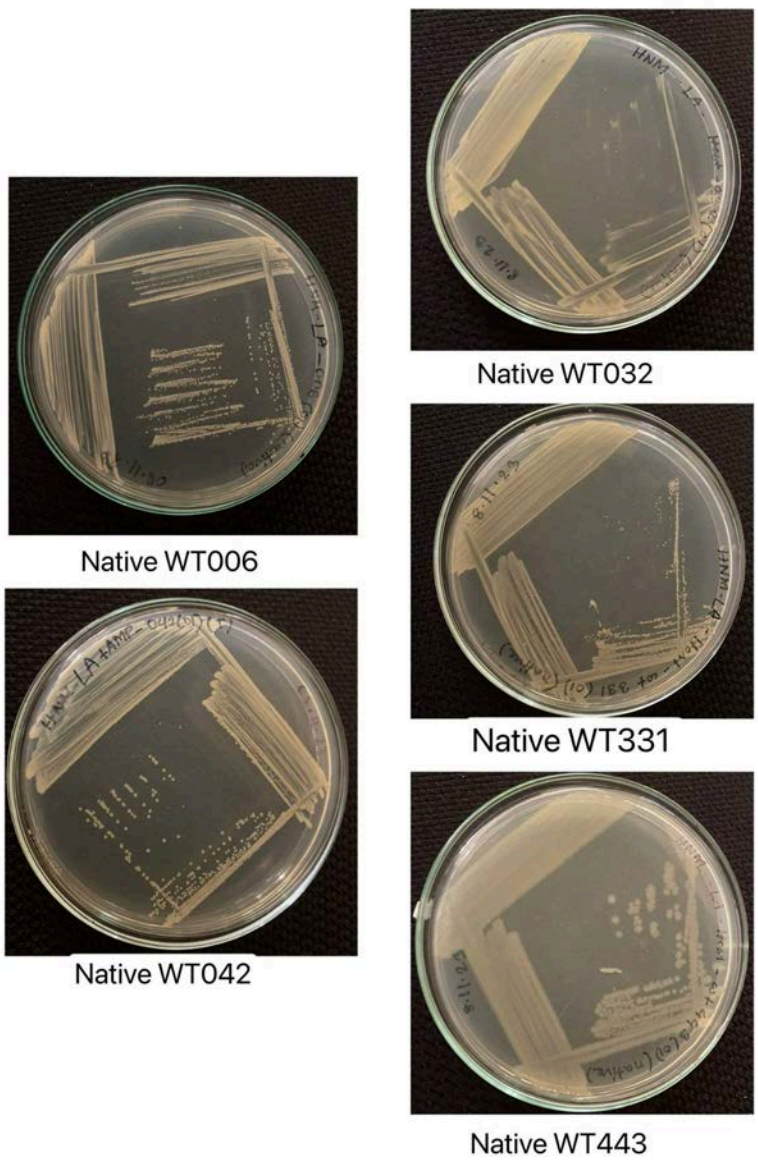

Figure 6: 5 native *Vibrio* 01 serotype strains named WT042, WT443, WT032, WT331, WT006. All of these strains are sensitive to ampicillin and kanamycin antibiotics.

These native strains went under horizontal gene transfer to make two copies of each strain, one with ampicillin resistance gene and another batch with kanamycin resistance gene. For Ampicillin resistant gene , E.coli DH5a was used. pGLO plasmid had been isolated from E.coli DH5a. And kanamycin gene was isolated from the chromosomal DNA of *Vibrio* 1877 strain that natively contain kanamycin resistant gene in itself .After transforming ampicillin resistant gene

containing pGLO plasmid and kanamycin resistant gene containing DNA from 1877 into the native strains, we got 2 transformed copies of each of the 5 native strains that we have labeled as "T"

Ampicillin Resistant Gene Containing pGLO Plasmid Transformed Strains

The figure shows that all the strains have successfully accepted pGLO plasmid. After transformation , the strains were cultured on LA media with ampicillin ,and the growth of the strains after HGT are very usual ,which indicated that these new strains became resistant to ampicillin. These new strains are shown below

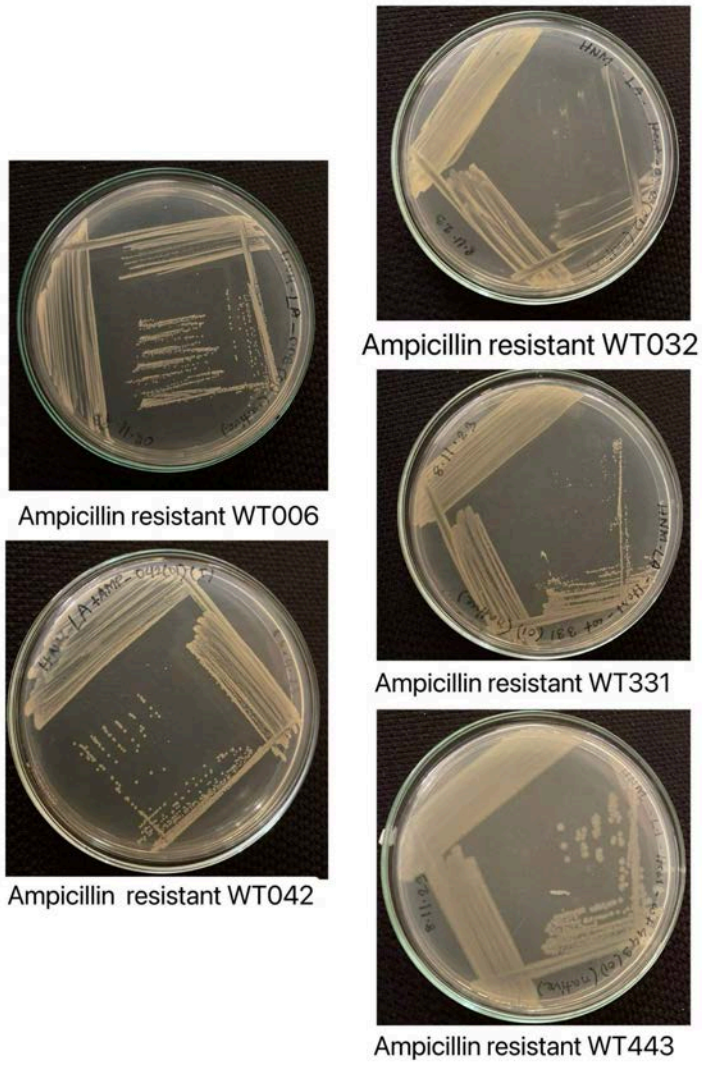

Figure 7: Growth of transformed strains WT042, WT443, WT032, WT331, WT006 with ampicillin resistance gene on LA media containing ampicillin .

Kanamycin Resistant Gene Containing DNA Transformed Strain

The figure shows that, kanamycin resistant gene containing DNA has been transferred to each strain successfully and the growth of the transformed strains is also usual. Since, their growth is usual on LA media containing kanamycin , that indicates this new batch of strains are successfully transformed and has became resistant to kanamycin

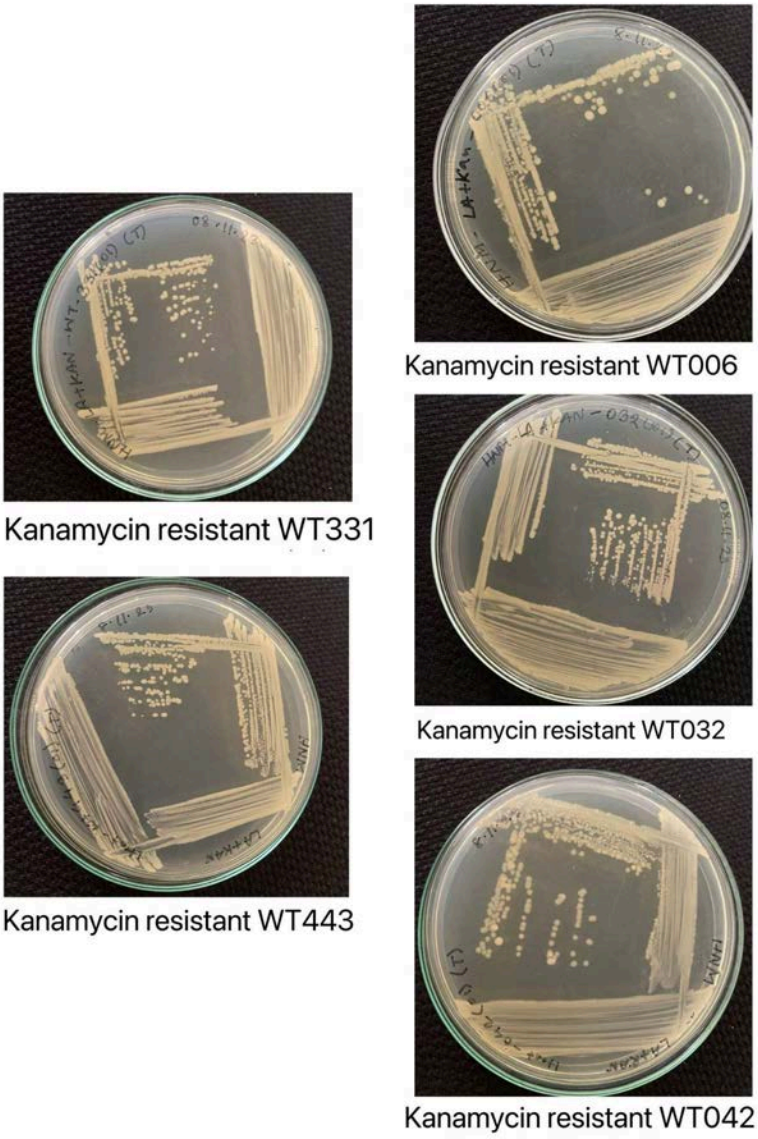

Figure 8: Growth of transformed strains WT042, WT443, WT032, WT331, WT006 with kanamycin resistance gene on LA media containing kanamycin .

Spot Test:

Following the formation of these groups, they underwent phage treatment by spot test to examine the impact on the original strain and the transformed strains. The control strain 1877 was used to confirm the phage activity following spot tests using 6 phages: JSF2, JSF6, JSF7, JSF10, JSF25, and JSF30. The outcomes were observed after a 12-13 hour incubation period, attached below.

Spot Test Result Analysis on Native Strains along with Control

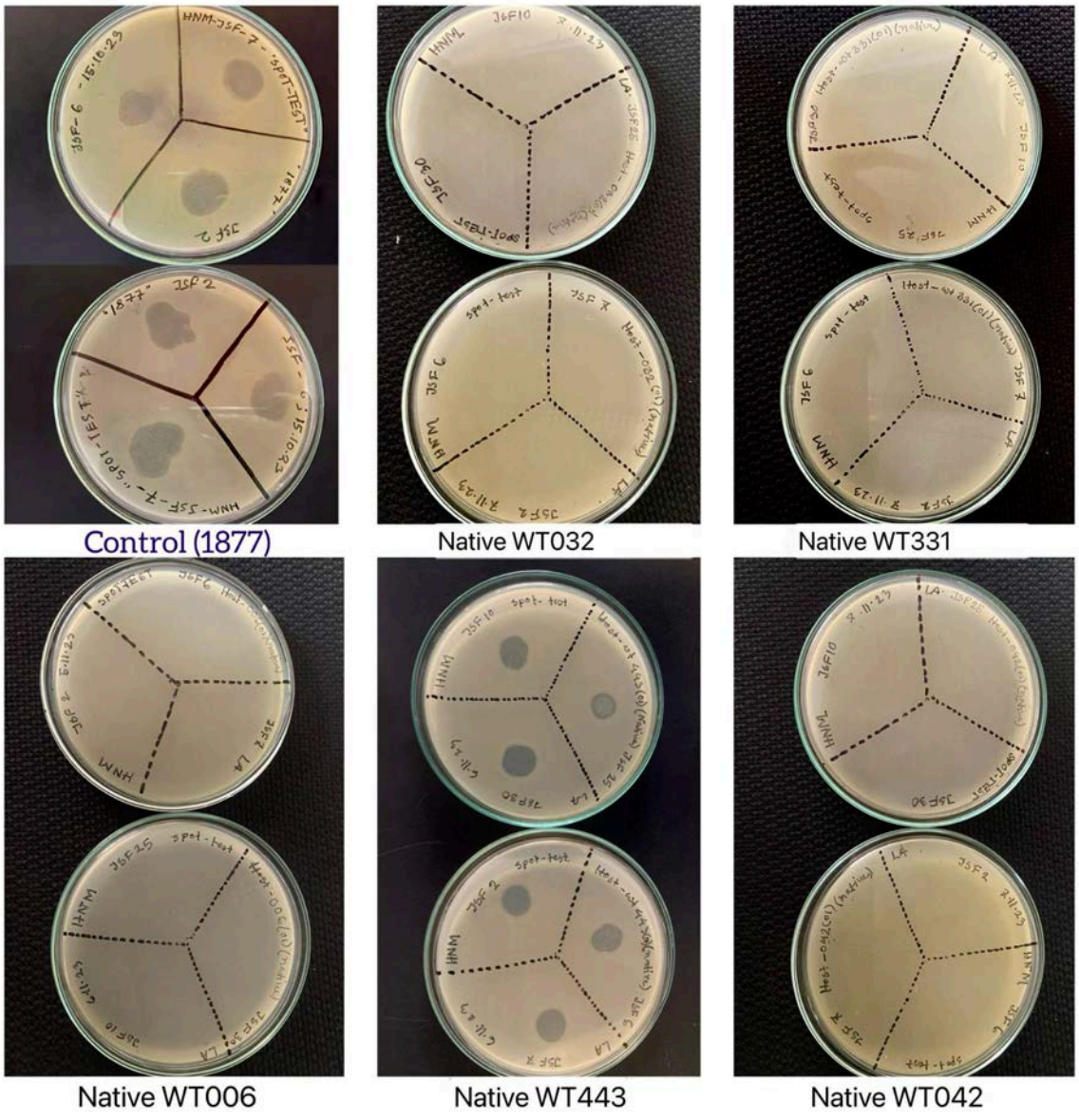

Figure 9: Spot test on native strains WT042, WT443, WT032, WT331, WT006 with control 1877

A tabular presentation of the result of effect of phage on native strain

Infection	✔
No infection	✘

Strain Name	Phage Number	JSF2	JSF6	JSF7	JSF10	JSF25	JSF30
1877 (control)		✔	✔	✔	✔	✔	✔
WT 006		✘	✘	✘	✘	✘	✘
WT032		✘	✘	✘	✘	✘	✘
WT042		✘	✘	✘	✘	✘	✘
WT331		✘	✘	✘	✘	✘	✘
WT443		✔	✔	✔	✔	✔	✔

Table 8 : The effect of 6 different phages on 5 different native *Vibrio* strains WT042, WT443, WT032, WT331, WT006 of 01 serotype with one control 1877 .

In our experiment, as clear zones were observed on 1877 strain for each of the phages, it indicated that the test was conducted properly and successfully and also that the phages were efficient. Both the native WT443 and control 1877 produced clear zones when tested against JSF2, JSF6, JSF7, JSF10, JSF25, and JSF 30.However, the other native strains—WT006, WT032, WT042, and WT33 —didn't show any visible clear zone when tested with these phages. Which means phages could not infect and create plaques on WT006, WT032, WT042, and WT331. Therefore, the result says native WT042, WT331, WT032, WT006 are resistant, whereas native WT443 strain is sensitive towards the phages JSF2, JSF6, JSF7, JSF10, JSF25, JSF30 as clear zones can be observed in the spot test. It seems like only the native WT443 strain can be effectively treated with these phages, offering alternatives to antibiotics.

Spot test result : Effect of Phage on Ampicillin Resistant Strains:

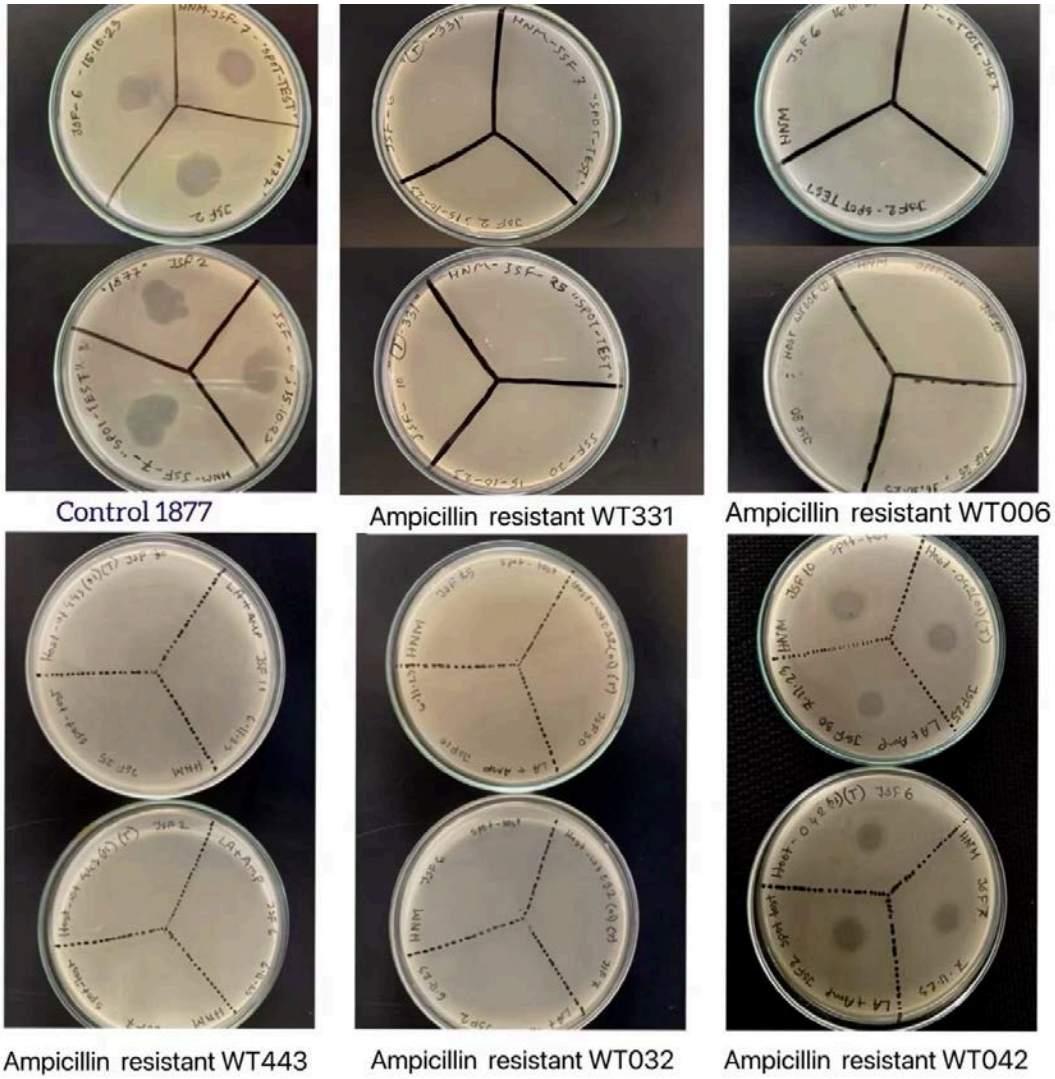

Figure 10: Spot test on transformed ampicillin resistance strains WT042, WT443, WT032, WT331, WT006 with control 1877

Tabular presentation of the result

Infection	✔
No infection	✘

Strain Name	Phage Number	JSF2	JSF6	JSF7	JSF10	JSF25	JSF30
1877 (control)		✔	✔	✔	✔	✔	✔
WT 006		✘	✘	✘	✘	✘	✘
WT032		✘	✘	✘	✘	✘	✘
WT042		✔	✔	✔	✔	✔	✔
WT331		✘	✘	✘	✘	✘	✘
WT443		✘	✘	✘	✘	✘	✘

Table 9 : The effect of 6 different phages on control 1877 and 5 different *Vibrio* strains WT042, WT443, WT032, WT331, WT006 of 01 serotype transformed with ampicillin resistance gene

The results of spot testing on the transformed ampicillin resistant strains. In this set of spot tests , only one strain along with control are infected by the phages. Moreover , control 1877 and transformed ampicillin resistant WT042 have shown clear zones against phage JSF2, JSF6, JSF7, JSF10, JSF25, JSF30 . And the rest of the transformed strain WT006, WT032, WT331, WT443 strain show negative results and have not shown any clear zone on them. Therefore, after HGT of the ampicillin resistant gene , WT042 has become sensitive and surprisingly WT443 became resistant.The effect of phage has been changed on WT443 and WT042. While in native strain phage created plaques on WT443 but could not create plaque on WT042 , but after being transformed with ampicillin resistant gene , these two strains have the opposite effects against phages, which implies that horizontal gene transfer has an impact on phage therapy.

Spot test result : Phage Effect on Kanamycin Resistant Strains:

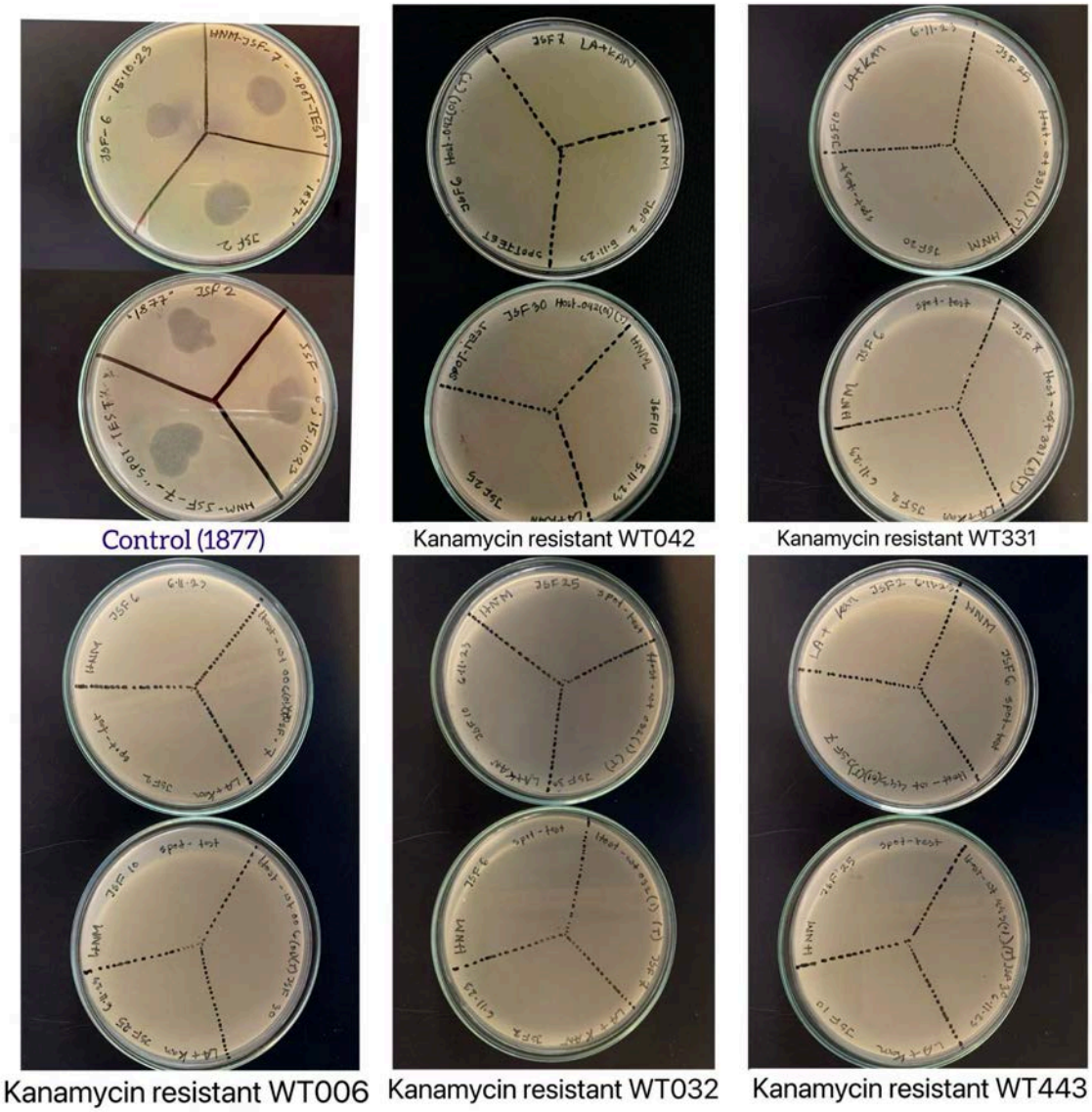

Figure 11: Spot test on transformed kanamycin resistance strains WT443, WT042,WT032,WT331, WT006 with control 1877.

A tabular representation of phage therapy on transformed kanamycin resistant strains

Infection	✔
No infection	✘

Strain Name	Phage Number	JSF2	JSF6	JSF7	JSF10	JSF25	JSF30
1877 (control)		✔	✔	✔	✔	✔	✔
WT 006		✘	✘	✘	✘	✘	✘
WT032		✘	✘	✘	✘	✘	✘
WT042		✘	✘	✘	✘	✘	✘
WT331		✘	✘	✘	✘	✘	✘
WT443		✘	✘	✘	✘	✘	✘

Table 10: The effect of 6 different phages on control 1877 and 5 different *Vibrio* strains WT042, WT443, WT032, WT331, WT006 of 01 serotype transformed with kanamycin resistance gene.

After the horizontal transfer of the kanamycin gene into strains WT006, WT032, WT042, WT331, and WT443, the impact of phages varied. Surprisingly, there was no discernible effect of phages on any of the strains except for the control strain 1877.*Vibrio* strains WT006, WT032, WT042, WT331, and WT443 all exhibited negative results in spot testing against phages JSF2, JSF6, JSF7, JSF10, JSF25, and JSF30. Compared to native strain with kanamycin gene transformed strain , effect of phage on WT443(01) has changed from positive to negative result, since phage has created plaque on native WT443 strain but failed to create plaque on kanamycin transformed strain of WT443(01).

Cross Comparative Result Analysis

Effect of Phage	✔
No effect of Phage	✘

Strains	Effect of phage on native strains	Effect of phage after transformation with ampicillin resistance gene	Effect of phage after transformation with kanamycin resistance gene
WT032(01)	✘	✘	✘
WT042(01)	✘	✔	✘
WT331(01)	✘	✘	✘
WT443(01)	✔	✘	✘
WT006(01)	✘	✘	✘

Table 11: Comparative results of phage effect on native WT032, WT042, WT331, WT443, WT006 and Ampicillin resistance gene transferred strains and Kanamycin resistance gene transferred strains .

We used 1877 strain as the control for each spot test because it is sensitive to all of the phages used. Interestingly, the phage impact was changed in the case of *Vibrio* strains WT042 and WT443, after the horizontal gene transfer. To elaborate, WT443 (previously sensitive to the phages) gained resistance to all 6 phages after transformation with ampicillin and kanamycin resistant genes. And for WT042, transformation with ampicillin resistant gene resulted in susceptibility to the phages, whereas, kanamycin resistant strain remained resistant similar to the native strain.

Chapter 5

Discussion

We conducted this experiment intending to observe the impact of horizontal gene transfer on bacteriophage therapy. Due to the rise of antibiotic resistance among bacteria owing to horizontal gene transfer, it is necessary to find an alternative to antibiotic treatment for bacterial infection. Because, in the future, no antibiotic may work on patients as bacteria are becoming multi-drug resistant. Researchers have been focusing on using bacteriophages as therapeutic agents as a substitution for antibiotics due to their availability and specified host range. However, whether phages that infect and lyse a bacterial strain will be able to infect and lyse the same bacterial strain with antibiotic resistance is a concern. Horizontal gene transfer has been causing evolution and diversity in bacterial populations by transferring antibiotic resistance genes (ARGs) from one species to another. Therefore, phages need to kill hosts with ARGs in order to consider phage therapy as an alternative as resistant bacteria are becoming more and more abundant.

For this experiment, we took 5 infectious native strains (O1) of *Vibrio cholerae* (WT006, WT032, WT042, WT331, and WT443) from our lab stock. Then we transferred each strain with either of two antibiotic resistance genes: Ampicillin or Kanamycin. We obtained Ampicillin resistant gene from *E. coli* DH5α strain and Kanamycin resistant gene from the 1877 strain of *Vibrio cholerae*. Gel Electrophoresis was done after each extraction to confirm the result and make sure no contamination of other organisms occurred. We observed the effect of 6 phages (JSF2, JSF6, JSF7, JSF10, JSF25, and JSF30) on the 16 strains (native. ampicillin-resistant, kanamycin-resistant, 1877 as control) through spot test. Most spot tests resulted in no difference between the native and resistant strains. However, 2 strains showed different results. Whereas plaques were observed for native WT443 in spot test for all 6 phages, no plaques were observed for ampicillin and kanamycin-resistant strains. Indicating that although native WT443 is susceptible to those 6 phages, the antibiotic-resistant strains are phage-resistant. On the other hand, although the native and kanamycin-resistant strains were seen to be all phage-resistant, the ampicillin-resistant strain was shown to be phage-sensitive. Therefore, HGT has a probable effect on phage infectivity.

The reasons behind this change in spot test are not confirmed. However, based on previous knowledge of HGT and its effects on the genotype and phenotype of the transferred organism, some of the following issues may be the reasons behind it:

- · The host cell receptor of phages may be eliminated due to HGT. During transformation, homologous chromosome exchange occurs. The gene encoding for the host cell receptor may be cut out and exchanged with the desired ARG. Resulting in, the strain becomes resistant to the phages as there is no receptor for the phage to bind.
- · The host receptor surface may become modified owing to HGT resulting in susceptibility or resistance towards the phages. The gene acquired from HGT can modify proteins or may produce enzymes that can change the receptor site. This alteration can facilitate or interfere with the binding of the phage.
- · HGT may encourage the inheritance of characteristics through epigenetics. (Dalia, 2019) Therefore it can have an impact on the binding of the phage. Moreover, DNA methyltransferases were transferred from bacteria to eukaryotes through HGT during early evolution. (Arkhipova et al., 2023) Therefore, bacteria may inherit epigenetic factors during HGT like DNA methyltransferase. It can later methylate sequences related to phage interaction causing a change of susceptibility to the phage.
- · During horizontal gene transfer, other genes can be incorporated into the bacteria along with ARG. These genes can code for enzymes, proteins, or other factors. (Dougherty, 2014) Some of these may degrade phages. Therefore, native strains sensitive to phage can acquire other genes that encode phage-degrading factors after transformation with ARG. As a result, the transformed strain will become resistant to the phage.
- · Horizontal gene transfer promotes environmental adaptation. (Daubin & Szöllősi, 2016) Therefore, the bacterial strains may alter their biological structure to adapt to the environment and survive. Thus, HGT may encourage phage resistance for the survival of the bacteria.

These may be some of the probable causes of the effect of HGT on phage infectivity. However, the distinct reason cannot be confirmed because we do not know the change and effect on the gene sequence of the bacteria due to transformation.

If we could conduct this experiment on more *Vibrio* strains with more phages, then we could construct a stronger hypothesis for this project. However, by working with such a short amount of samples, it is hard to establish a strong ground. Moreover, we could not conduct sequencing for any of the strains. If we could, then the reason behind such a result would have been much clearer.

We aim to extend our research and experiment further by sequencing the genome of the native and transformed bacterial strains. That way we will be able to compare and differentiate between the sequences and identify the exact reason for the change in phage infectivity. By observing the changed sequence, we may find out the newly expressed or repressed enzymes and proteins or other factors that may have caused the changed infectivity in WT042 and WT443. Furthermore, it is our goal to continue this research on more *Vibrio* strains with more phages to establish a strong and reliable case of effect of horizontal gene transfer on phage infectivity.

Chapter 6

Conclusion

Antibiotic resistance has been a major issue for the last few decades regarding combating bacterial infections. Emerging phage therapy can be a potential solution to this issue if it can surpass the effects of horizontal gene transfer. Due to HGT, bacteria are gaining ARGs for different drugs and as a result, phages specific against a certain bacteria need to be infectious against horizontally transferred strain of that bacteria. As we are focusing on *Vibrio cholerae* for this study, our focus was on whether the candidate phages against these *Vibrio* strains would be able to display the same efficiency against the same strains containing antibiotic resistance genes. Although most spot test results were the same for the native strains and ampicillin and kanamycin resistant strains, 2 strains had different results. Whereas, native and kanamycin resistant strains of WT042 were resistant to all 6 phages, the ampicillin resistant strain of WT042 was sensitive to all 6 phages. Moreover, the native strain of WT443 was sensitive to all phages, however, both ampicillin and kanamycin resistant strains of WT443 became resistant to all of the phages. This implies that, the transformation meaning the insertion of ARGs into these strains rendered a change in them that resulted in the alteration of their state of infectivity to phages compared to their native strains. Therefore, we cannot completely ignore the fact that HGT may have a probable impact on the infectivity of bacteriophages. However, if we could continue this study on further bacteria and phages combined with sequencing of the strains with changed results, we could establish a valid and well grounded issue with confirmed reasons.

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