EFFECTS OF HORIZONTAL GENE TRANSFER ON BACTERIOPHAGE INFECTIVITY

A thesis submitted to the Department of Mathematics And Natural Sciences, BRAC University In Partial Fulfillment Of The Requirement For The Degree Of Bachelor Of science In Biotechnology

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Declaration

It is hereby declared that

1. The thesis submitted is our own original work while completing 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.

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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

Vibrio cholerae is one of the very well-known and common pathogenic bacteria which cause mild to severe gastrointestinal diseases, and in extreme cases, patients die. Specially children are victims where proper clinical,environmental, and public health support are insufficient. As a treatment, antibiotics were promising until *vibrio cholerae* became multidrug resistant by raising a global concern. In our environment many antibiotic resistant genes are increasing day by day. Following previous successful studies, bacteriophage therapy for antibiotic resistant vibrio strains is considered as one of the potential solutions to such problems. In this study, six native strains of *vibrio cholerae* from the environment underwent antibiotic resistant transformation. The main purpose of this study is to observe the infectivity of selected bacteriophages on strains of similar genetic makeup following antibiotic resistant traits, especially ampicillin and kanamycin antibiotics. In other words, to find the potentiality of phage therapy. A positive finding will be beneficial for vibrio cholera treatment and contribute to clinical, environmental biotechnology and public health as well. Therefore, by isolating antibiotic resistant genes containing DNA, transforming those into native vibrio strains and finally double layer spot test on the prepared strains gave a conclusive and intriguing outcome of phage infectivity which favors future studies with more antibiotic resistant vibrio strains and bacteriophages.

This thesis is dedicated to our Family and Friends.

For their endless love, support and encouragement

throughout this journey.

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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
LPS	Lipopolysaccharide
HGT	Horizontal gene transfer
AMR	Antimicrobial resistance
FDA	Food and Drug Administration
MDR	Multidrug-resistant
MSSA	Methicillin-sensitive Staphylococcus aureus

CFU	Colony forming unit
SM	Salt Magnesium
SDS	Sodium dodecyl sulfate
Rpm	Rotations per minute
Psi	Pounds per square inch
UV	Ultraviolet
ml	Milliliter
µl	microliter
AR	Ampicillin Resistant
KR	Kanamycin Resistant
CT	Cholerae Toxin

Introduction

Phage treatment, or the use of phages as bactericidal agents, has been used for 90 years to treat infections caused by bacteria in both humans and other species (Abedon et al., 2011).Antibiotics' effectiveness in treating infections has decreased due to improper usage, and this has increased the prevalence of bacteria that are resistant to antibiotics. But bacteriophages can be utilized directly as biocontrol agents or indirectly to identify dangerous bacteria, according to recent investigations. Additionally, by using phage display, they may be utilized to create novel compounds for use in clinical applications, vaccine manufacturing, medication creation, and nanomedicine (El-Shibiny & El-Sahhar, 2017).

Phages are widespread viruses that infect bacteria and are actively being studied as antibiotic alternatives. Phages have been proven to be successful against many resistant nosocomial bacterial strains and use a variety of techniques to better penetrate difficult-to-reach bacterial cells in biofilms (Singh et al., 2022). According to trials in Switzerland utilizing phages to attack E. coli in human volunteers, phages are simple to make, simple to use, and safe for plants, animals, and humans (Bruttin & Brussow, 2005). Phages may also be used as a disinfectant and sanitizer to destroy bacteria that are prone to antibiotics without harming bacteria that are sensitive to antibiotics (Yosef et al., 2015).

Water, soils, food, and other host species are all possible sources of infection for many bacterial illnesses that impact human health. Some of these potential infection sources lack a sophisticated immune system that is able to remove specific foreign substances. In contrast to agricultural conditions, where the use of phage as a biocontrol is currently being explored (Weitz & Hartman, 2006). There are many studies conducted by the scientific research community in treating infectious disease by phage.Among them recently one of the most studied sectors is the use of phage in the treatment and prevention of cholera because of the increase in antimicrobial resistant strains of V. cholerae.

Cholera is still a significant burden for underdeveloped countries, particularly if hospitalization, sanitization, and the quality of the water supply are still problematic (Barman et al., 2022).Many antibiotics, including tetracycline, fluoroquinolones, and azithromycin, have been successfully

utilized over time to treat cholera patients. However, the repeated appearance of V. cholerae that is resistant to antibiotics has led to several treatment failures in recent years. Concern over the emergence of multi drug resistant strains in V. cholerae has been growing recently (Das et al., 2019). And this growing antibiotic-resistant *Vibrio cholerae* strains, a hazard to public health, has raised interest in phages as a replacement for antibiotics (Barman et al., 2022). There are several clinical studies being conducted worldwide to treat antibiotic-resistant V. cholera using bacteriophages.Following numerous fruitful animal tests. Human patient clinical studies were taken into consideration.

Bacteriophage mediated treatments are built on the basis of horizontal gene transfer (HGT). Following the lytic life cycle, final destruction of pathogenic bacterial cells is aimed in such a process. Therefore, the objective of this study is to find the sensitivity of bacteriophage infection on native and antibiotic resistant *vibrio cholerae* strains. As of current concern, antibiotic resistant *vibrio cholerae* has become quite an alarming issue, finding potential bacteriophage as a way to treat these resistant strains will be a contribution to clinical, environmental biotechnology and public health.

Chapter 2: Literature review:

2.1 Bacteriophages and its early history

Bacteriophage (phage) are viruses that only infect bacteria; as a result, infected bacterial cells eventually die and lyse. Phages are parasitic by nature and distinguished by their nanoscale size. Plage particles are usually composed of only a type of nucleic acid which is either DNA or RNA along with a protein capsid in order to protect genetic material (Moineau, 2013).The majority of phage also include a protein tail that allows for extremely selective attachment to a receptor on the surface of the host bacteria. When the adsorbed phage injects its genetic material into the cell, the phage lytic cycle begins (from the capsid through the tail and into the cell). It quickly seizes control of the bacterium, replicates its genome, and uses the host's protein biosynthetic machinery to create the building blocks of its proteins. To create mature bacterial viruses that will be released when phage enzymes rupture the cell, the structural proteins are put together and the phage genome is inserted into the capsid (Rifkind & Freeman, 2005).The cell dies as a result

of this amplification cycle, and multiple phage are released (typically between 50 and 100), ready to infect more targeted cells.

According to the research it has been found that the bacteriophages emerged in the ancient precambrian Era. (Approximately 4.5 billion years ago (Bacteriophage.news,2022). A study was published by Ernest Hanbury Hankin, an English bacteriologist, aeronautical theorist, and naturalist in 1896 and according to him the water of Ganga & Yamuna contains some antibacterial characteristics that aided in the fight against cholera.Fredrick Twort, a British bacteriologist in 1915 detected an agent that killed bacteria.Twort simply explained a possible 'ultra-microscopic virus' isolated from vaccinia virus cultures of 'white micrococcus.' The detected lytic phages appear to be bacteriophages against Staphylococcus species found in a vaccinia virus culture. However, in 1917 Felix d'Hérelle who was a French-Canadian microbiologist,, discovered bacteriophage with anti-Shigella bacillus activity from the stool of people healing from bacillary dysentery.The name bacteriophage was coined by D'Hérelle.He also performed the first clinical use of bacteriophages in 1919 and the first known usage was done in the United States in 1922.Between the early 1920s and the late 1930s, bacteriophages were increasingly used to treat many infectious illnesses all over the world (Aswani & Shukla, 2021). Human dysentery, poultry cholera, bovine hemorrhagic fever, bubonic plague, cholera, and a variety of staphylococcal and streptococcal illnesses were all treated by phage. There were spectacular achievements as well as catastrophic failures. The failures were caused by a variety of factors, including the use of crude, unregulated, inactivated, or endotoxin-containing lysates, the restricted host specificity of some phages, the lack of bacteriological standards, and simple quackery (Mahy & Marc, 2008). With the introduction of commercial antibiotics in the 1940s, the use of bacteriophages as therapeutic agents declined in Western countries.However, with the emergence of antibiotic-resistant bacteria, the medicinal potential of phages has attracted fresh interest (Aswani & Shukla, 2021).

2.1.2 Bacteriophage classification

According to Kaliniene et al. (2017), the Caudovirales order has three families of bacteriophages: the Myoviridae family, which has a contractile tail, the Podoviridae family,

which has a short tail, and the Siphoviridae family, which has a non-contractile long tail. The same order includes filamentous, cubic, and polymorphic phages, which are divided into 10 tiny

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,

Figure-1:Classification of Bacteriophage

families and account for around 3.6% of all known bacteriophages. Examples of phage morphologies are shown in Table 1 (Ackermann 2009).

2.1.3 Bacteriophage abundance in the environment:

Phages can be found in water, air, soil, and almost everywhere bacteria can be found. Some believe that the presence and unique capabilities of bacteriophages are the reason our environment has not been completely invaded by bacteria. Our world is home to about 10^{31} phages (Bacteriophage.news,2022) Phages cannot pull out most biological activities on their own and must be propagated by a living host. As a result, phages are obligate parasites of their bacterial hosts, living in nearly every environment where bacteria thrive. Bacteriophages are the most prevalent organisms on the planet.Bacteriophages of various types can be found in many ecological environments. Bacteria and phage distribution is determined by their range limitations and where their domains cross. Bacteria survive practically everywhere in all conditions, and phages exist in all environments where their hosts exist, including hypersaline habitats, polar areas, deserts, on and within organisms other than bacteria, fresh and sea water, and the soil. To detect phages from a certain environment, phages must be isolated from that environment and cultured on bacterial lawns. However, this is not always viable, as in the case of unculturable bacterial strains.In marine water, there are around 10 phages for every bacterial and archaeal cell.The ocean possesses 4 × 10^30 viruses, making it the world's largest phage reservoir. Soil phages control the nitrogen cycle as well as bacterial-plant root symbiosis (Batinovic et al., 2019).

Marine phage research began in the early 1980s. PSS2 is the first moderate marine cyanophage whose genome has been sequenced. The great majority of isolated cyanophages are obligately lytic.

According to Felix D'Herelle ,bacteriophages are a natural element of healthy animal and human microbiota. In the 1960s, electron microscope research indicated a significant concentration of phage-like particles in intestine microbial populations. The yield of total phage DNA is predicted to be10^10 ml−1. In a recent examination, 69 morphologically distinct kinds of bacteriophages were detected in one sample of horse feces out of over 200 investigated samples.

In seas, soils, and underground regions, Archaea are as prevalent as Bacteria. Archaea may survive in harsh environments such as hot springs, salt lakes, and soda lakes. Archeal viruses are thought to be as common as bacterial viruses, however only 50 phages have been identified to infect Archaea. Archaea-infecting viruses contain double-stranded DNA genomes ranging in size from 10kb to 100kb. So far, no archeal RNA virus has been discovered (Naureen et al.,2020).

Phages can be found in soils as well. Different phages have distinct functions in various soil habitats. Phages have been implicated in managing soil biogeochemical qualities by controlling bacterial abundance and hence nutrient cycling capacities, although phages found in the root-adjacent rhizosphere appear to regulate the efficiency of symbiotic relationships between plant roots and bacteria. Soil phages have also been used in biocontrol methods, which is an effective alternative to more conventional plant disease treatment methods (Batinovic et al., 2019).

2.1.4 Life cycle of bacteriophages:

Bacteriophages go through lysogenic or temperate, lytic or virulent, chronic and pseudo lysogenic cycle. In the lytic life cycle, phages infect the bacteria and thus quickly virus DNA replication occurs independently of host DNA replication and ends with the release of new phage particles where host bacteria is usually lysed. However, in the lysogenic cycle, phage DNA is incorporated into the host chromosome by site-specific recombination or transposition, sometimes known as prophage, and then viral DNA replication takes place alongside host DNA replication where the host cell is not harmed (Olszak et al., 2017).

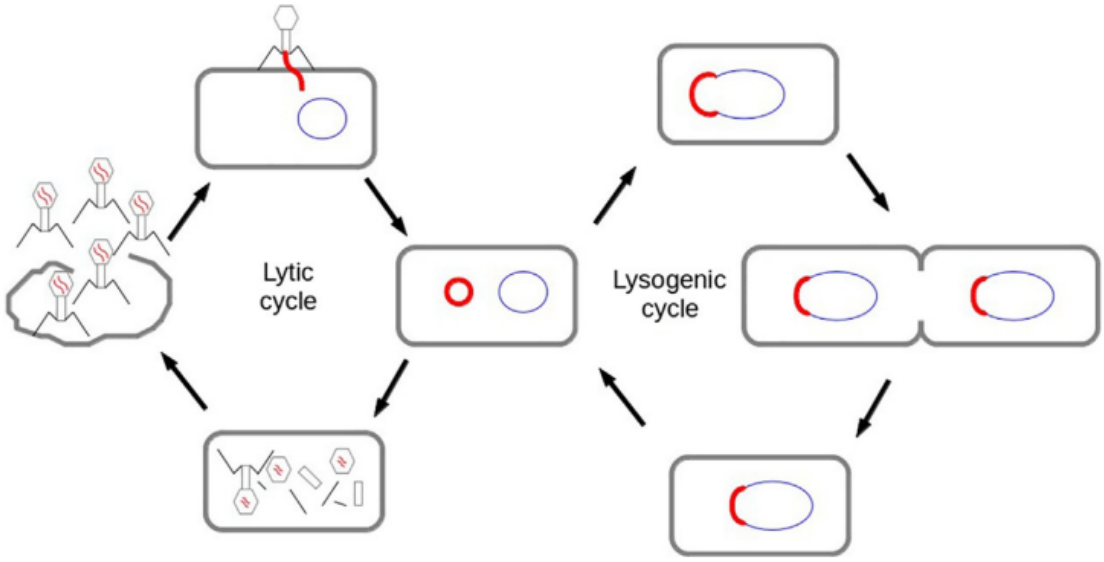

Figure 2: Lytic and lysogenic cycle of bacteriophage (Sinha et al., 2018)

Lysogenic conversion occurs when genes are expressed in an integrated situation, giving the host cell new or extra features. For instance, prophage typically transports and carries the cholera and Shiga toxins (Nanda et al.al. 2015).The prophages can remain stable for several generations until physiological or environmental factors initiate the lytic life cycle through induction.In this

instance, offspring virions leak from the cell surface slowly and constantly rather than in a single burst whereas, in lytic cycle, newly formed viruses release in one burst.

When we infect them in the laboratory, the host bacterial lawns show obvious plaques caused by lytic infection. Both the lytic and lysogenic life cycles are present in temperate phages (Giri, 2021)

Here are the steps involved in lytic cycle:

Firstly, Adsorption allows bacteriophage attachment sites to bind to host bacterial receptor sites. such as lipopolysaccharide (LPS), outer membrane proteins,flagella or fimbriae.Although some can attach to flagella or pili, most bacteriophages adhere to the bacterial cell wall. Only particular host bacterial strains can be bound by particular bacteriophage strains. It's called viral specificity.Secondly, bacteriophages that adhere to bacterial cell walls create pores in the wall using enzymes like lysins and carbohydrate depolymerase enzymes before injecting their genome into the cytoplasm of the host bacterium. To do this, certain bacteriophages form a sheath that forces the tail tube into the bacteria. The eclipse period is now underway. These tail tubes serve as entry points for the genomes of bacteriophages that attach to flagella or pili. There is no uncoating stage in either scenario since just the phage genome reaches the bacteria. After that, the replication period starts. The macromolecular (protein, RNA or DNA) production of the bacteria is stopped by enzymes that are encoded by the bacteriophage genome. The bacteriophage exploits the metabolic regulation of the host bacteria to produce bacteriophage enzymes and other structural elements. After replication, the genomes are encircled by the phage components. Matured phages are then released. In this case, the bacterial peptidoglycan is often broken down by a bacteriophage-coded lysozyme, leading to osmotic lysis and the release of the intact bacteriophages.

2.2. Horizontal gene transfer (Transformation).

Horizontal gene transfer refers to the transfer of genetic material from one organism to another without reproduction.It is distinct from vertical transformation where genetic material is passed from parent to offspring during reproduction. HGT not only occurs between similar species but also different species including archaea, bacteria, and unicellular eukaryotes and higher eukaryotes and its nucleus containing organelles (Yutin, 2013). Both prokaryotes and eukaryotes have been known to acquire new genes through horizontal gene transfer (HGT). It turns into a

significant driving factor that creates genomic heterogeneity that could help it evolve via adaptability and survivorship with the newly transferred gene (Sulaiman et al., 2019).

In most of the cases, mobile genetic elements like plasmids, transposons and bacteriophages have made horizontal gene transfer a success. Such genetic materials are transferred between species via different methods including transformation, conjugation, and transduction in prokaryotes.

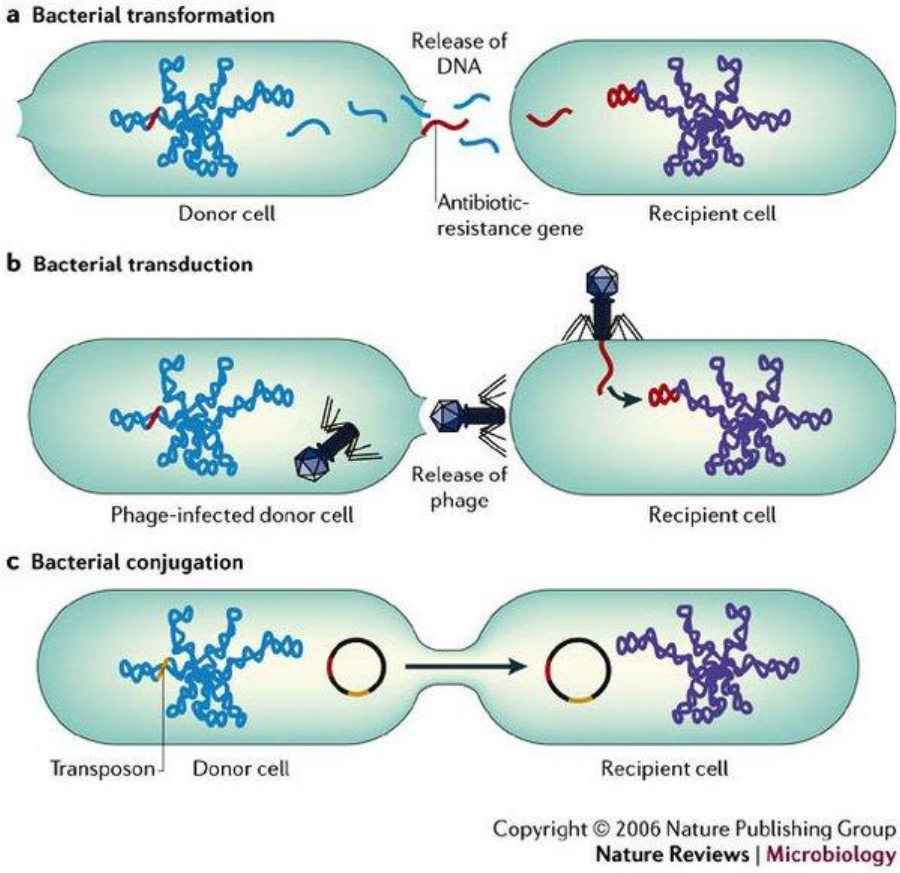

Figure-3:Mechanisms of Horizontal Gene Transfer

Transformation: In this process, prokaryotes receive DNA fragments from degraded or dead bacterium and exchanged for a portion of its own DNA.Only homologous recombination, or the Recombination of DNA sections with substantially identical nucleotide sequences is often involved in transformation. Therefore, this usually involves strains of the same bacterial species or related strains of bacteria.

Transduction: During transduction a DNA fragment is transferred by a bacteriophage from one

bacteria to another (Schneider, 2017).

Conjugation: It is the process where DNA is transferred from a living donor bacteria to a living receiver bacterium through cell-to-cell contact in a genetic recombination process. It often includes a conjugation or sex pilus in Gram-negative bacteria.

2.3 Phage therapy

Bacteriophage therapy is another name for phage therapy. It treats bacterial infections using viruses. Bacterial viruses are also referred to as phages or bacteriophages. Phages are not harmful to humans, animals, or plants (Iftikhar, 2019). Phages, also known as bacteriophages, are viruses that only destroy and target bacteria. They are the most prevalent biological entities in nature, and they have been demonstrated to fight and eliminate multi-drug resistant bacteria. Specifically, when all antibiotics fail, phages still destroy the bacteria and may save a life from disease. (What Is Phage Therapy? - IPATH, n.d.)

Bacteriophages cause bacteria to rupture or lyse. When the virus attaches to the bacterium, this occurs. A virus infects bacteria by introducing its genes into them (DNA or RNA).The phage virus replicates (copies) itself within the bacterium. This can result in up to 1000 new viruses in each bacteria. Finally, the virus ruptures the bacterium and releases the new bacteriophages.Bacteriophages can only replicate and flourish within bacteria. When all of the bacteria have already been lysed (killed), they will cease growing. Phages, like other viruses, can go inactive (hibernate) until new bacteria appear (Iftikhar, 2019).

2.3.1 Current state of phage therapy

Bacteria are constantly evolving to resist current and emerging antibiotics, and this scientific fact means that new antibiotics will quickly confront drug-resistant mutants. Antimicrobial resistance (AMR) is a well-known public health concern that poses an urgent and serious problem both locally and globally. Considering the growing risk and burden of Antimicrobial resistance bacterial infections, as well as the need to avoid constantly evolving antibiotic resistance, Phage therapy is currently being discussed progressively, resulting in phage therapy becoming more popular and intriguing.The FDA(Food and Drug Administration) has undertaken to authorize phage therapy for compassionate use to diagnose antibiotic failure in chronic infections, for

example those frequently encountered in patients with solid organ transplants, prosthetic joint infections, mechanical circulatory device implants,hematological transplants, long-term immunosuppression, and complicated surgical infections (Verma et al., 2022).

Phage therapy has been used successfully in some cases.A 68-year-old diabetic patient with necrotizing pancreatitis exacerbated by multi drug resistant *A. baumannii* infection was successfully treated with a personalized bacteriophage-based therapeutic approach (Schooley et al., 2017).Recent advances in bacteriophage therapy have enabled the effective treatment of some biofilm-based illnesses, as well as those caused by multidrug-resistant organisms, when standard antibiotic therapy has failed. After a second round of bacteriophage therapy performed through a two-stage exchange surgery, a persistent methicillin-sensitive Staphylococcus aureus (MSSA) prosthetic knee-joint infection has been successfully treated (Ramirez-Sanchez et al., 2021). During the COVID-19 pandemic period, the research community all around the world made huge attempts to develop measures to defeat the epidemic.Because of the presence of the "gut-lung axis," a group of researchers was concerned that the overuse of antibiotics during the COVID-19 epidemic may increase the formation of Antimicrobial resistance and have a detrimental impact on patient recovery. So they discussed the harmful effect of excessive antibiotic use in COVID-19 treatment and provided an outline of how phage therapy may be employed as an alternate treatment option in their review. They also discussed how the anti-inflammatory (which can target the principal cause of mortality in COVID-19) and targeted killing (narrow spectrum) capabilities of phages can be a viable alternative to antibiotics (Khan et al., 2022). Several hospitals across several nations currently provide phage therapy to cure diseases.

Table-1

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	109 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 X 109 PFU cocktail M or 3.6 X 108 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 X 107 PFU (expected) 200– 2,000 PFU (actual) topically applied one time per day upto 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 administered	10- 20 ml of phage collection were administered thrice daily for 12 weeks.	No antagonistic events; phage therapy provided good response up to 40% rate

		to Hirszfeld Institute phage collection		
5	Escherichia coli; Staphylococcus aureus; Pseudomonas aeruginosa	Intralytix phage cocktail WPP-201 were applied on 65 patients	1 X 109 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)

Phage offers a wide range of possible uses in human health, dentistry, veterinary research, and agriculture.Bacteriophages are also utilized in a variety of industries, including livestock and the food industry, in addition to antibiotics.

2.3.2 Benefits of phage therapy

The advantages of phage treatment address the limitations of antibiotics. There are several varieties of bacteriophages, just as there are numerous forms of bacteria. However, each phage will only attack one type of bacteria. It will not infect other bacteria. This indicates that phages can be employed to target disease-causing bacteria directly.A publication by (Loc-Carrillo & Abedon,2011) indicated some bacteriophage properties that may offer them an advantage in therapeutic conditions over conventional antibiotics.

- Bacteria that have been effectively infected by obligately lytic phages lose their viability. Some antibiotics, such as tetracycline, are effective against bacteria, and as a result may facilitate bacterial development towards resistance.
- Because phages are primarily composed of nucleic acids and proteins, they are naturally benign. They have the potential to cause a damaging immunological response. However, there was insufficient data to support this theory in practice.
- During the bacterial-killing process, phages can multiply particularly where hosts are present, however this has significant limits, such as a dependency on rather large bacterial

populations. The process is known as auto "dosing" since the phages themselves contribute to determining the phage dose.

- Because most phages have a very small host range, the number of bacterial species with which selection for specialized phage-resistance mechanisms can occur is limited. In contrast, most chemical antibiotics can influence a significant proportion of microorganisms. Furthermore, certain resistance mutations have a deleterious influence on bacterial fitness or virulence due to the lack of pathogenicity-related phage receptors.
- The ability of phages to infect just a few strains of a certain bacterial species or, in rare cases, a closely related bacterial genus is a desirable trait since it indicates that they will not disrupt the typical bacterial ecology. Broad-spectrum antibiotics, on the other hand, not only disturb beneficial normal flora bacteria but also frequently result in superinfection, such as Candida albicans yeast infections.antibiotic-associated *Clostridium difficile* colitis
- Because phages invade and kill employing pathways distinct from antibiotics, certain antibiotic resistance mechanisms do not convert into phage resistance mechanisms. As a result, phages can be easily used to treat antibiotic-resistant infections, such as those caused by multidrug-resistant Staphylococcus aureus.
- Many harmful bacteria phages are easily identified, frequently in sewage and other waste items with high bacterial densities. However, if the host bacteria are challenging to culture and bacteria vary in terms of the number of phage variants to which they are sensitive, isolation might be more technically demanding. Unlike antibiotics, which can be harmful, phages with low or no toxicity can be identified against the majority of target microorganisms.
- Phages can be used in a variety of formulations, including combinations with other antibiotics. In addition to being acceptable for most forms of administration, they are also diverse in application form, as liquids, creams, impregnated into solids. Moreover, different phages can be combined as cocktails to expand their characteristics, often resulting in a broader antibacterial range of action.
- It was discovered that biofilms have a considerably greater level of chemical antibiotic resistance than planktonic bacterial cells. Phages have been shown in certain instances to be able to remove biofilms, nevertheless. Some phages have the capacity to create

exopolymer-degrading depolymerase, which enables them to break down the cell's outer membrane and get access to the cell membrane with ease in order to multiply and infect more cells.

- Discarded therapeutic phages, in contrast to broad-spectrum pharmaceutical antibiotics, will at worst have an influence on only a tiny portion of environmental bacteria since they are mostly composed of nucleic acids and proteins and have very restricted host ranges. Phages that are not acclimated to environmentally damaging elements like sunlight, desiccation, or severe temperatures can also be quickly rendered inactive.
- phage manufacturing requires both host development and subsequent purification. While the cost of host growth varies based on the kind of bacteria, purifying phages seems to be getting cheaper as technology advances. While the costs of isolation and characterisation can be rather low, the costs of phage manufacture are often comparable to those of pharmaceutical production.

2.4 *Vibrio cholerae*

Vibrio Cholerae is a comma-shaped, highly mobile, gram negative bacteria that contains only one polar flagellum.It is a halophilic bacteria. It has both human and environmental stages in its life cycle. It is a facultative pathogen. Cholera is a microbial illness that is caused by the toxin producing strains of V. cholerae. It is often transmitted by polluted water. It causes dehydration and severe diarrhea. If left untreated, it may be lethal within hours. When poverty, conflict, or natural calamities compel people to live in overcrowded conditions with inadequate sanitation, the likelihood of a cholera pandemic rises (Somboonwit et al., 2017). Since the first pandemic started in 1817, there have been seven pandemics of cholera that have been documented.The enterotoxin that has the potential to start epidemics can be secreted by the two serogroups of V. cholerae, O1 and O139.The O1 serogroup's conventional biotype was responsible for the six previous pandemics.The current seventh cholera pandemic, which started in Indonesia in 1961, is the most widespread and long-lasting, and it is caused by V. cholerae El Tor biotype (Faruque & Mekalanos, 2012).The genetic diversity of non-O1/non-O139 V. cholerae strains is often very extensive. Only a few non-O1/non-O139 V. cholerae strains possess the cholera toxin-producing genes. This strain has been linked to gastroenteritis (which, at times, may be severe). For the diagnosis of the disease, V. cholerae can be isolated by culture method where selective media

(TCBS) are used, other method that are used for the isolation of V. cholerae are rapid antigen detection kit that are commercially available, PCR method, Darkdield microscopy which is very specific for v. cholerae (*UpToDate*, n.d.).

2.4.1 Clinical Manifestation:

A variety of illnesses, from asymptomatic intestinal colonization to severe diarrhea, are brought on by V. cholerae infection. Other typical symptoms include stomach pain, borborygmi, and vomiting, especially in the early stages of the illness. Most difficulties in patients with severe illness are caused by the significant volume and electrolyte loss from diarrhea. A fever is unusual.The clinical signs of cholera brought on by V. cholerae O1 and O139 are identical.

The incubation time for cholera can range from a few hours to as long as three to five days, depending on the host susceptibility and inoculum quantity (Clemens et al., 2017)

Diarrhea: Within 6 to 12 hours of the beginning of clinical signs, acute severe watery diarrhea can cause mortality from dehydration.Cholera can cause progressively waterier bowel motions that finally resemble rice water and smell fishy.In the worst circumstances, adult stools can produce as much as 1 liter each hour. The maximum rate of stool excretion in children with severe cholera is often 10 to 20 cc/kg/hour. Other causes of diarrheal sickness do not frequently exhibit this rate of fluid loss (Harris et al., 2011).

Additional gastrointestinal symptoms include vomiting is frequent and frequently accompanied by watery emesis. It can start either before or after the onset of diarrhea. Although patients may have gastrointestinal cramps, they often do not experience the intense abdominal pain that is traditionally linked to dysentery.

Cholera complications, which typically affect elderly people, can include acute renal tubular necrosis and stroke. These complications generally mirror those brought on by hypotension and hypoperfusion. Vomiting-related aspiration pneumonia is another possibility. Dehydration is nearly invariably the cause of cholera death (Waldor & Ryan, 2015).

Mortality - Untreated cholera patients may experience a 50–70% mortality rate (Lindenbaum, 2022). Children have a 10 times higher mortality risk than adults in locations where cholera is endemic.Other researches suggested a significant risk of fetal mortality related to cholera during pregnancy (up to 50% during the third trimester)(Hirschhorn et al., 1969)

2.4.2 Virulence factors of *Vibrio cholerae*:

V.Cholerae is a non-invasive intestine pathogen. The two main virulence components of toxigenic *Vibrio cholerae* are cholera toxin (CT), which is encoded by a lysogenic bacteriophage (CTX), and toxin-coregulated pilus (TCP), a crucial colonization component that also functions as the receptor for CTX (Faruque et al., 2003). Here, cholera toxin (CT) is basically an adenosine diphosphate-ribosylating enzyme which causes damage to the intestinal epithelial cells which leads to loss of water, sodium, and chloride from the body. More specifically,The primary virulence component of V. cholerae is cholera toxin, which has five B subunits and one A subunit (toxic domain) (receptor-binding domain). Figure 1 depicts the basic mechanism of CT activity. To promote internalization of CT-A, which triggers fluid loss via cAMP-mediated stimulation of anion secretion and inhibition of electroneutral NaCl absorption, secreted CT-B binds to monosialoganglioside (GM1) on the surface of host cells. Cholera's severe diarrhea is caused by the barrier-disrupting actions of CtxA in combination with excessive chloride ion secretion (Ramamurthy et al., 2020).

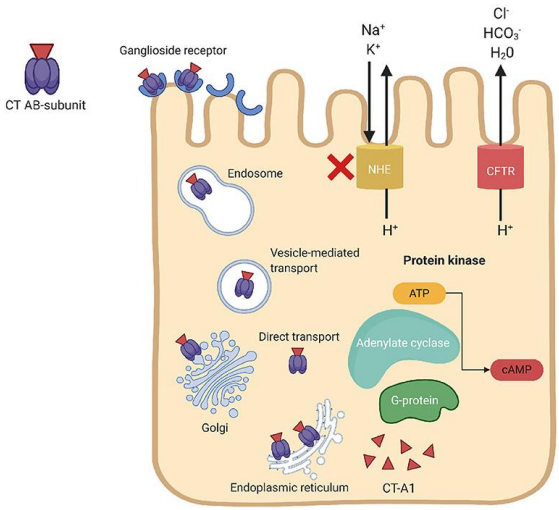

Figure 4: Mechanism of cholera toxin activity (Ramamurthy et al., 2020)

In this diagram, the holotoxin is endocytosed when CT attaches to the ganglioside receptor on the host epithelial cells. From the endosomes, the absorbed CT travels to the Golgi complex and endoplasmic reticulum (ER). Through the activity of the ER-linked degradation pathway, the catalytic CT-A1 polypeptide then moves from the ER to the cytosol by retro-translocation in

order to activate the Gs subunit of the guanine nucleotide-binding regulatory (Gs) protein. As a result of the activation of the Gs-protein, adenylate cyclase (AC) activity increases which cleaves ATP to cyclic adenosine monophosphate (cAMP). It then activates protein kinase-A. (PKA).

The cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel proteins are phosphorylated by PKA activation, which prevents NaCl from being absorbed by NHE transporters. This results in ATP-mediated outflow of chloride ions and induces the secretion of HCO3, Na+, K+, and H2O. When chloride ions are lost, the small intestine secretes a huge amount of fluid, impairing the large intestine's capacity to absorb nutrients. This causes severe watery diarrhea.

Although, V. cholerae O1 and O139 serogroups are what cause epidemic cholera, non-O1 non-O139 V. cholerae strains are those that do not agglutinate in O1 or O139 antisera and may cause severe extraintestinal infections in addition to diarrhea, especially in vulnerable hosts. Moreover, in the past, non-O1 V. cholerae, nonagglutinable (NAG) Vibrio, or non cholera Vibrio have all been used to refer to V. cholerae strains that lack the virulence characteristics required to induce epidemic cholera (also known as "non epidemic strains"). Some of these ambient, non epidemic strains have been connected to human illness, despite the fact that most of them seem to be non-pathogenic for people (Finkelstein, 2022).

2.4.3 Global spread of antibiotic resistant V. Cholerae :

When bacteria are exposed to antibiotics that were meant to kill them or stop their growth, they can develop the ability to withstand such drugs, which is known as antibiotic resistance.

The most recent trend demonstrates that nearly all clinical isolates of V. cholerae are resistant to commonly used antibiotics.

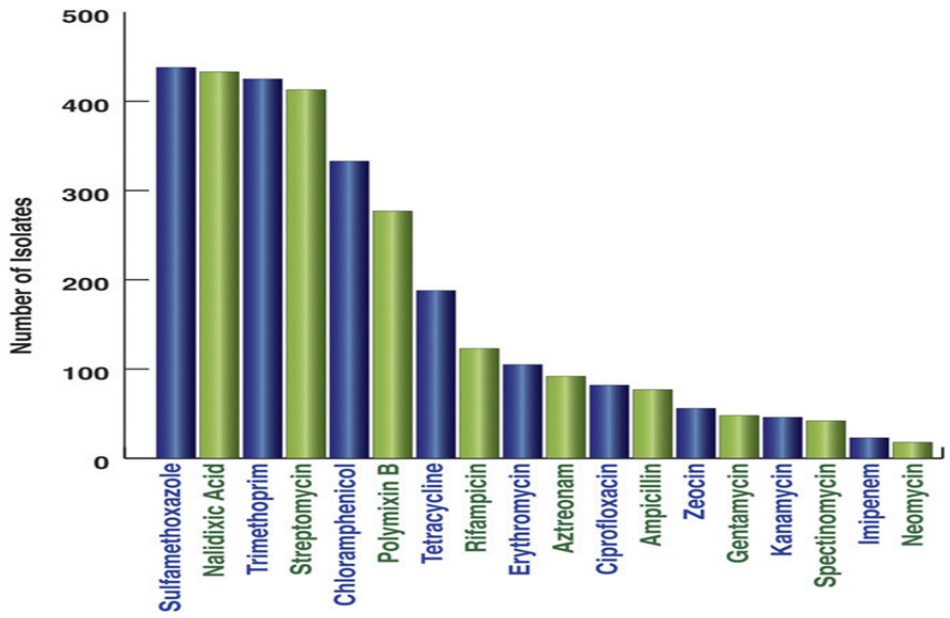

Figure-5: Resistance profile of V. cholerae against different antibiotics

Resistance profile of V. cholerae against different antibiotics. Bar graph showing the number of isolates in which resistance was detected against different antibiotics. The highest number of isolates showed resistance to sulfamethoxazole. Minimum resistance was detected against neomycin (Verma et al., 2019).

In some studies it is found that the most prevalent bacterium, V. cholerae, exhibited significant rates of resistance to several antibiotics, including furazolidone (83%), trimethoprim-sulfamethoxazole (67%), nitrofurantoin (66%), streptomycin (64%), and nalidixic acid (58%).

More cholera epidemics and cases have recently occurred in African countries than in Asia or the Americas .The vast majority of nations in the eastern and southern parts of Africa have experienced major cholera epidemics, occasionally with unusually high mortality rates, since the seventh pandemic pathogen *Vibrio cholerae* O1 biotype El Tor first reached and spread in West African countries in the 1970s (Mwansa et al., 2007).

Antibiotic resistance poses a global danger to the advancements we have made in healthcare, food production, and eventually life expectancy. Worldwide, antibiotic resistance has been documented. Antibiotic resistance can rapidly spread across borders and continents due to the ease with which people, animals, and things can now travel.

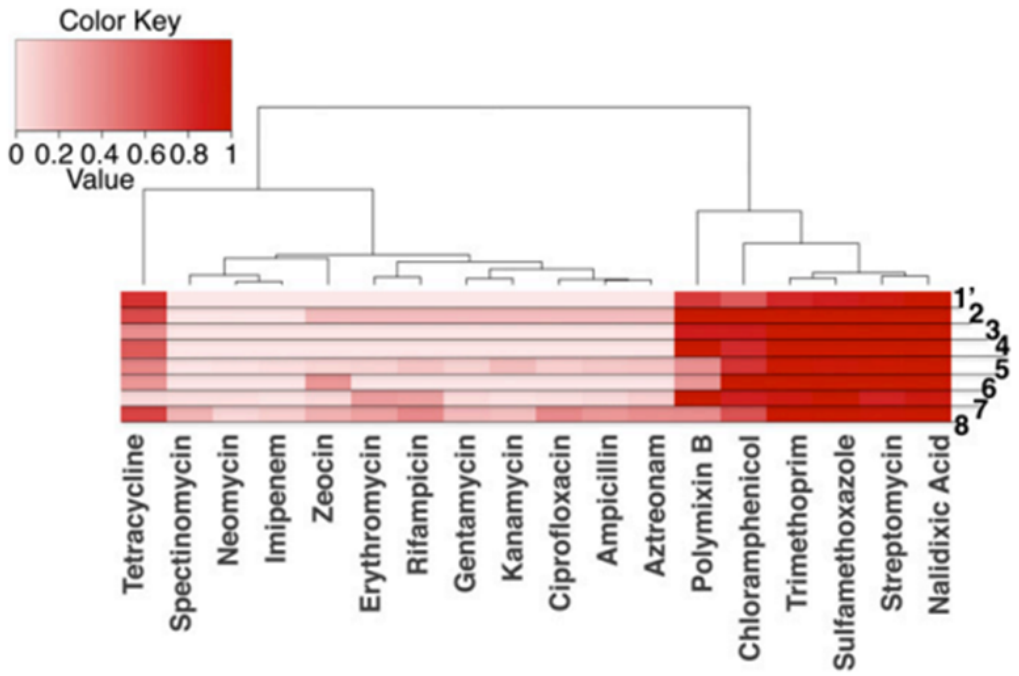

Figure-6:Resistance pattern of V. cholerae strains discovered (2008-2015)

This chart shows the year-by-year resistance pattern of V. cholerae strains discovered between 2008 and 2015. Except for 2014, resistance to polymixin B decreases year over year. Up until 2014, tetracycline resistance was also minimal. 1′, 2008; 2, 2009; 3,2010; 4, 2011; 5, 2012; 6, 2013; 7, 2014; 8, 2015 (Bagheri-Josheghani et al., 2021).

443 V. cholerae strains that were isolated between 2008 and 2015 from two sites in India—one in Kolkata (east India), where diarrheal sickness is widespread, and the other in Delhi, where it is not endemic—have had their antibiotic susceptibilities examined (north India). In order to find antimicrobial resistant genes and the MGEs that are physically connected with resistance genes, we looked into the genome sequences of isolates of V. cholerae that were both extensively drug-resistant (XDR) and multidrug-resistant (MDR). Here, we report the discovery of blaNDM-1, the gene that encodes the New Delhi metallo-beta-lactamase-1, in the chromosome of the V. cholerae strain that was isolated from patients with diarrheal illness. Prior to now, blaNDM-1 had only been identified from septicemia patients. We also discuss the molecular

makeup of various AMR(Antimicrobial resistant) traits and the MGEs that carry resistance-related genes in the V. cholerae genome (Verma et al., 2019).

Through epidemic intelligence activities, the ECDC keeps track of cholera outbreaks around the world in order to spot important epidemiological changes and alert the relevant public health authorities.

Reports are released once a month.In 2021 and 2022, cholera outbreaks were recorded in a number of African and Asian nations. It has been reported that there are significant ongoing outbreaks in Afghanistan, Bangladesh, the Democratic Republic of the Congo, Ethiopia, and Nigeria.

Approximately 39 857 suspected cholera cases, including 114 fatalities, have been reported globally since the last update on August 24, 2022.

Afghanistan, the Democratic Republic of the Congo, Iraq, Malawi, Mozambique, Nepal, the Philippines, Somalia, South Sudan, Syria, and Zambia are the nations that have reported new cases since the last update (Cholera worldwide overview,2022).

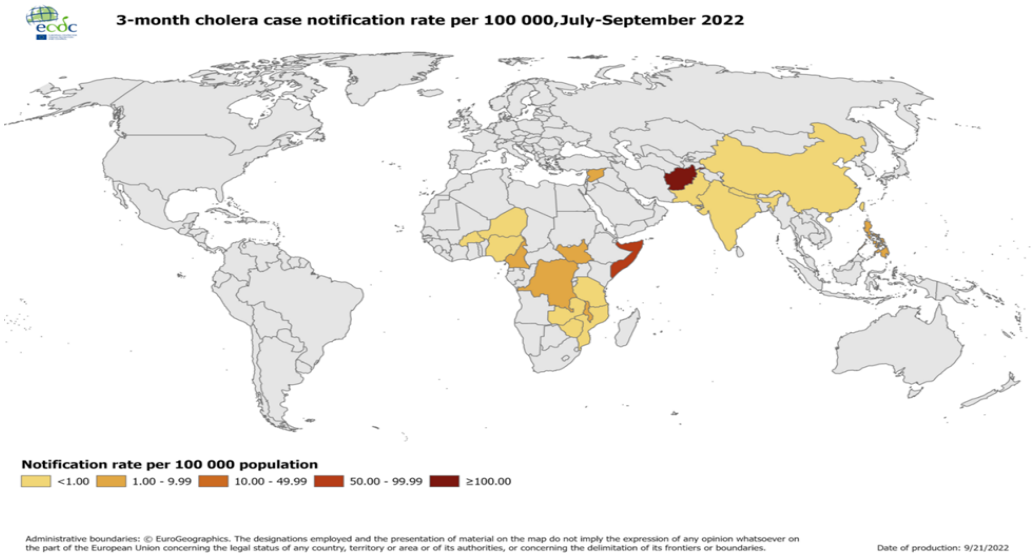

Figure-7:3 months cholera case notification rate per 100000(Cholera worldwide overview,2022)

The significant antibiotic resistance of V. cholerae strains may pose a hazard to public health worldwide, according to the findings of this meta-analysis. As a result, it is advised to choose appropriate antibiotic treatment plans for each region based on the local strains' patterns of antibiotic resistance.

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

Phage treatment is the employment of viruses (phages) that are specific to certain types of bacteria to combat undesirable and out-of-control microorganisms, such as those linked to infectious diseases (Loc-Carrillo & Abedon, 2011).

It is well established that antibiotic-resistant bacterial infections have an impact on the socioeconomic structure, food security, and healthcare systems in developing nations. The unmet demands in underdeveloped nations are even more glaring given the catastrophic state of the global antibiotic resistance epidemic. In response to the urgent need to develop alternative therapies to treat infections, the failure of conventional antimicrobials has rekindled interest in bacteriophage (phage) therapy, which has been used for a century.

Although antibiotic treatment may reduce diarrhea by 50% and the shedding of viable organisms by days, cholera management necessitates vigorous fluid and electrolyte supplementation (Harris et al., 2011). Although WHO does not advise it due to the risk from antimicrobial resistance, chemoprophylaxis within families may be helpful. 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).

Since d'Herelle first connected the drop in mortality during a cholera epidemic in India in the 1920s to phages in feces and started treating patients with oral vibriophages, phages have been used to cure cholera.(Khalid et al,1AD, January 1)

According to(Fazil & Singh, 2011),The ability of phage to kill numerous bacteria and so reduce the load of the pathogenic bacteria led to the development of phage therapy for cholera as a useful technique for the treatment of cholera patients,poison and ultimately the spread of the

illness.In the first report on phage therapy,Anti Cholera phage at large dosages (between 100 and 200 phage)cholera bacterium, but the phage was unable to do the job, as per vibrio)Replication and amplification occur frequently.

When the patient received bacteriophage therapy,hospitalized patients with illnesses who were treated with tetracycline and had fluid replacements.It was observed that extremely high-dose .Phage treatment was similar to tetracycline in lowering the amount of vibrios that feces excrete;this reduction did not, however, result in a general clinical improvement (i.e., a shorter length of diarrhea and quicker healing).The discovery of the variety of vibrios' serotypes and other issues that hindered the evaluation of phage therapy in cholera were observed.

The various degrees of these bacteria's susceptibility to the phage stocks were used, and the second was the phage's quick passage through the digestive tract.people with cholera, a fact that may have prevented the importance of the second phage infection in low frequency of infection treatment .

From (Jaiswal et al., 2012) it is found that In several places of the world, the lytic cholera phage's potential as a preventative measure has been investigated. Studies looking at how lytic phage affected the growth of V. cholerae in a newborn mouse infection model revealed that the phage could significantly lower the load of V. cholerae in the small intestine .

The therapeutic potential of cholera phages has already been investigated in human trials. Due to the limits in our understanding of bacteriophages, the results of those research were not as significant (Jaiswal et al., 2012). Phage therapy has been used in the past to treat or prevent cholera with varying degrees of success. A single phage type administered 1 hour prior to a V. cholerae exposure in a neonatal rabbit model avoided the onset of cholera symptoms, according to research by Dutta et al. A cocktail of five lytic bacteriophage types given 6 or 12 hours before a V. cholerae challenge in an adult rabbit model reduced diarrheal severity slightly but did not significantly lower the bacterial load, according to studies by (Jaiswal et al.2012); however, the same phage cocktail could successfully reduce the V. cholerae load when given 6 or 12 hours after challenge.

Chapter 3: Methods and Material

3.1 Place of study

The research was conducted at BRAC University in Dhaka, Bangladesh, at the Biotechnology and Microbiology Laboratory of the Department of Mathematics and Natural Sciences.

3.2 Standard laboratory practice

While conducting the research all safety measures were taken, clean lab coats were worn, hand gloves were used. The glassware used while working on this project such as conical flasks, beaker, test tubes were washed twice, once with tap water and once with distilled water.Before usage, pipette tips, falcon tubes, eppendorf tubes, culture media (both agar-based and broth-based), and empty test tubes for the spot test method were all autoclaved at 121°C at 15 pressure for approximately two hours. The autoclaved media, if needed, were stored at 4°C. Working station as well as while working in the laminar air flow 70 % ethanol was used to avoid any type of contamination.

3.3 Preparation of Culture Media, Reagents and Solutions

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

TCBS is highly selective for *Vibrio cholerae* and its other species. In this project for the isolation and cultivation of *Vibrio cholerae* TCBS agar was used. The ingredients that make up TCBS agar are- agar, Ox bile, Yeast extract,sodium thiosulfate, peptone, sodium citrate ($Na_3C_6H_5O_7$), NaCl, sucrose, bromothymol blue,ferric citrate, thymol blue, distilled water. Ready made TCBS agar which was available in the laboratory was used in this project.

Preparation of TCBS Agar

To prepare the TCBS agar 10.69g power medium was taken on a foil paper and the power media was measured with an electronic balance (As according to the manufacturer to produce 1000 ml of TCBS agar media 89.08g powder media need to be added).With the measured powder media 120 ml distilled water was added in a conical flask. The mixture was stirred with a glass rod and was heated on a bunsen burner to mix the media until bubbles are produced. The media is then poured in sterile petri dishes inside a laminar air flow to avoid contamination . In this project we mostly used medium petri dishes where approximately 15-20 ml media were poured in each plate. We do not need to autoclave TCBS media as it contains Ox bile, a byproduct of bile salts that can be sensitive to autoclaving and inhibit gram-positive bacteria.

Luria-Bertani, Miller Broth (LB)

LB is highly used as a microbial growth medium for the cultivation of bacteria. The components of LB are Yeast extract, Nacl, tryptone . Ready made LB which was available in the laboratory was used in this project.

Preparation of Luria-Bertani, Miller Broth (LB)

As it was mentioned by the manufacturer that to produce 1000 ml of LB media 25 g powder media need to be added so to make 500ml of LB 12.5 g of powder media was taken on a foil paper and the power media was measured with an electronic balance.With the measured powder media 500 ml distilled water was added in a conical flask. The mixture was stirred with a glass rod and was heated on a bunsen burner to mix the media until bubbles are produced. Then the media was autoclaved at 121°C, 15 psi pressure for approximately 2 hours. After the autoclave was done the flask was kept aside for cooling as it is ready for use now.

Luria Agar (LA)

Luria agar is a non selective media which is used for culture bacteria. This media contains high nutrients which are necessary for the growth of bacteria. In this project for streaking the inoculated stock bacteria LA medium was used.

Luria Agar (LA) preparation

To prepare the LA media Luria bertani miller broth and agar was used as commercially readymade LA was not available in the laboratory. To make 500ml of LA 12.5 g of LB(to produce 1000 ml of LB media 25 g powder media need to be added) powder media and 7.5 g of agar powder (1.5% agar) was taken on a foil paper , power medias were measured with an electronic balance.With the measured powder media 500 ml distilled water was added in a conical flask. The mixture was stirred with a glass rod and was heated on a bunsen burner to mix the media until bubbles are produced. Then the media was autoclaved at 121°C, 15 psi pressure for approximately 2 hours. After the autoclave was done the media was then poured in sterile petri dishes inside a laminar air flow to avoid contamination and wait until the media solidified.

Preparation of LA media containing Ampicillin

In this project we need to prepare an ampicillin selective plate for each strain . To do so, after preparing the LA media when the autoclave was done the flask was kept aside for the media to cool down so that the antibiotic which is ampicillin we are adding can survive. When we can touch the flask with our bare hands we add the ampicillin supplement. According to the manufacturer 0.5 ml ampicillin supplement needed to be added with 99.5 ml media so for 120 ml media we added 600 ul of ampicillin supplement.

Preparation of LA media containing Kanamycin

In this project we need to prepare a kanamycin selective plate for each strain . To do so, after preparing the LA media when the autoclave was done the flask was kept aside for the media to cool down so that the antibiotic which is kanamycin we are adding canasurvive. When we can touch the flask with our bare hands we add kanamycin. According to the manufacturer 0.5 ml Kanamycin needed to be added with 99.5 ml media so for 120 ml media we added 600 ul of Kanamycin.

Soft Agar Preparation

We needed to prepare soft agar to perform the spot test. Here the agar concentration was kept 0.6%. So to prepare 110 ml of soft agar 2.75 g of luria bertani broth powder and 0.66 g of agar powder were measured on a foil paper by using an electronic balance. Then With the measured powder media 110 ml distilled water was added in a conical flask. The mixture was stirred with a glass rod and was heated on a bunsen burner to mix the media until bubbles are produced.Then the media was poured in to clean test tubes with the help of a glass pipette and each test tube contains 6ml media. Then the test tubes were autoclaved at 121°C, 15 psi pressure for approximately 2 hours. Then the tubes can be stored at 4 °C.

Reagents:

Ampicillin supplement: 0.5ml ampicillin supplement per 99.5 ml LA media.

Kanamycin : 0.5ml kanamycin supplement per 99.5 ml LA media.

Phenol: chloroform : isoamyl alcohol = 25:25:1,v/v

Phenol:chloroform=1:1

Solutions:

Alkaline solution I:PH 8.0. For 25 ml solution:

1M Tris-HCL	12.114%	3.03g
0.5M EDTA	14.6122%	3.65
1M Glucose	18.0156%	4.504

Solution was prepared using distilled water. In order to balance the pH NaOH was used.

Alkaline solution II: For 10ml solution:

10N NaOH	40%	4ml
1% SDS	1%	100ul

Alkaline solution III: pH 5.2 : For 25 ml solution

5M Sodium acetate	41.0172%	10.26g
Distilled water	-	14.74ml
Glacial Acetic Acid	-	Added drop by drop for pH adjustment

1M CaCl2 (Stock solution): For 10ml solution:

CaCl2	1.4702g
Distilled water	8.5298ml

0.1M CaCl2 (working solution):

1M CaCl2 stock solution	1 ml
Distilled water	9ml

0.9% saline: To prepare 0.9% saline, 4.5g NaCl was dissolved in 500ml water and then autoclaved for sterilization.

Buffers:

TE Buffer: pH 8.0. For 25 ml solution:

10 nM Tris	0.12114%	0.0303g
1.nM EDTA	0.029%	0.00735g

Distilled water	-	24.96ml

0.5M EDTA:pH 8.0. For 100ml solution:

EDTA	18.61g
Distilled water	81.39ml

50 X TAE Buffer: pH8.0. For 100ml solution:

Tris- base	24.2g
Acetic acid	5.71ml
0.5M EDTA	10ml
Distilled water	60.09ml

1 X TAE Buffer:

50 X TAE buffer	2ml
Distilled water	98 ml

20% Arabinose: 20 ml

Arabinose	4g
Distilled water	16ml

Salt Magnesium(SM) Buffer Preparation:

For the preparation of 50 ml SM buffer 0.1 g MgSO4, 0.5 g gelatin and 0.2 g NaCl is needed. First Tris HCl needs to be prepared. (10 ml) From this 2.5 ml would be used to prepare the SM buffer.

To prepare the Tris HCl pH needs to be adjusted at 8 as after the autoclave is done the pH will drop to 7.5 . As 1 ml Tris HCl is 121.14g/mol, so to prepare 10 ml of Tris HCl 1.2114 g Tris was measured on a foil paper by using a electronic balance the measured Tris powder and 10 ml of distilled water was added in a falcon tube mixed properly then 2.5 ml of Tris HCl was added in a fresh falcon tube with it 0.2 g NaCl .0.1 g MgSO4 and 0.5 g gelatin was added finally to make final volume 50 ml 47.5 ml distilled water was added to the tube. Now we have our 50 ml buffer solution . The solution was autoclaved at 121°C, 15 psi pressure for approximately 2 hours. After sterilization, the buffer was stored at room temperature.

3.4 Preparation of different *vibrio* strains

3.4.1 Native strain

Culture on selective media: We collected six strains of *vibrio cholerae* 1877,WT404, WT406, WT048, WT382, WT386. To make sure of the strains of interest, all of them were cultured on TCBS media where after incubation, yellow colonies indicated positive results.

Culture on non selective media: Once we got our strains of interest, we continued our further work using LA media which provided adequate nutrition to the bacterial colonies in order to increase the growth

3.4.2 Antibiotic resistant strains:

Plasmid isolation from *E.coli* DH5a

1. Bacterial cells harboring desired plasmid were grown overnight in 25 ml LB medium containing appropriate antibiotics.
2. Transfer 1.0 ml cells to harvest by centrifugation at 10000 Xg for 2 min (3 times).
3.The pellet then resuspended in Solution 1 (200 ul) and to this freshly prepared solution 2(400 ul) was added and mixed by gentle inversion.
4.The cells were incubated at room temperature for 5 mins for cell lysis.
5.To this, ice cold solution 3 (300 ul) was added, mixed by inversion and incubated on ice for 10 mins.
6. The mixture was centrifuged at 12000 Xg for 15 min.
7. The supernatant was collected in a fresh tube.
8.To this, equal volume of phenol: chloroform: IAA (25:24:1) was added, mixed by vortexing and centrifuged at 12000 Xg for 2 min.
9. Supernatant was collected carefully and equal volume of chloroform: IAA (24:1) was added, mixed by vortexing and centrifuged at 12000 Xg for 5 min.
10. Supernatant was collected and then equal volume of isopropanol was added to this, mixed by inversion and centrifugation at 12000 Xg for 15 minutes.
11. The DNA pellet was washed in 70% ethanol, air-dried and resuspended in 200ul of TE buffer.
12. Store the plasmid at -20 °C.

Competent cell preparation

1. Inoculate a single colony of E. coli DH5a strain in a 5 ml LB media containing tube and place in a shaker incubate at 37°C overnight.
2. Inoculate 50 ml LB media with 1 ml cell culture aliquot
3. Place inside a shaker incubator at 37°C until OD =0.4 (Check after 2 hour intervals).

4. Place culture with OD 0.4 in an ice bucket for 10 minutes and then transfer to an eppendorf tube.

5.Centrifuge at 5000 rpm for 10 minutes at 4°C.

6. Remove media and then resuspend with pipette.

7.Add 0.8 ml $CaCl_2$ solution. incubate for 30 minutes in ice bucket and then centrifuge at 5000 rpm for 10 minutes.

8.Remove media and then resuspend with 0.8 ml $CaCl_2$ solution, incubate for 20 minutes in ice bucket and then centrifuge at 5000 rpm for 10 minutes.

9. Remove media and add 200 ul glycerol, Then store at -80°C for use in transformation experiment.

Transformation

1.Take component cell culture out of the storage and place it in ice.

2.Place 100ul of component cell with 10ul (50ng) plasmid in ice for 10 minutes (to adjust with the plasmid temperature)

3.Place in a hot water bath at 42°C for exactly 90 seconds to apply heat shock.

4.Place in ice immediately for 2 minutes to close cell membrane pores.

5.Mix with 1 ml LB media and culture for 1 hour at 37°C

6.Place 100ul of culture in LB media plates under different conditions to check for the presence of transformed cells.

3.4.3 Kanamycin resistant strain:

Bacterial chromosomal DNA isolation

1. Transfer 5 mL of ân overnight bacterial culture to an autoclaved small falcon tube. Centrifuge the filled tube for 5 minutes at 3000 rpm at room temperature. Remove the supernatant by aspiration .

2.Add 1.4 ml of TE(pH=8.0) . Disperse the bacterial pellet by vortexing twice for 20 sec each time.

3. Add 1.7 ml of 10% SDS, 1.7 ml of proteinase K(20 mg/mL in TE, pH 7.5). Incubate the bacterial lysate for 1 h at 37°C.

4. At this stage, the digested lysate is too viscous to handle. Shear the DNA by three to five passages through a 26-gauge needle. Try to minimize foaming .

5.Add 1.67 ml of a 1:1 mixture of phenol:chloroform .

6.Treat with equal volume of phenol and then with phenol:CHCl: Isoamyl alcohol. Centrifuge for 30 mins at 13200 rpm.

7.Collect the upper aqueous layer carefully in fresh tubes.

8.Precipitate with (double volume of absolute ethanol. Look for jelly like DNA in ethanol. Transfer DNA to eppendorf tube.

9.Wash with 70% ethanol (2ml) and centrifuge for 5 mins. Remove supernatant and dry pellets in the desiccator.

10. Dissolve pellet in TE buffer (100ul).

Gel Electrophoresis

Agarose Gel preparation

1. To prepare 50 ml of 0.8% agarose solution, measure 0.4 g agarose into a flask and add 50 ml 1X buffer to it. Put this solution in the microwave on a hot plate until agarose is dissolved and the solution becomes clear.

2. Pour the solution into the gel tray, then allow it to cool at room temperature. Place the comb into the gel tray before the solution becomes cool. The comb should be placed about 1 inch from one end of the tray and positioned vertically such that the teeth were about 1-2 mm above the surface of the tray.

Electrophoresis

1. To run, remove the comb gently; place the tray in the electrophoresis chamber, and cover (just until wells are submerged) with an electrophoresis buffer (about 1 L TBE buffer, the same buffer used to prepare the agarose).
2. To prepare samples for electrophoresis, add 2 ul of gel loading dye for every 8 ul of DNA sample.
4. Run the gel at 70 V (it may take approximately 1 hour for the run to be complete).
5. Stain the gel in 20 ul ethidium bromide until the DNA takes up the dye and becomes visible under short wave UV light.

Chitin Transformation

1.Each native strain inoculated in 5ml fresh LB after that overnight incubation was done at 37°C
2. 5ml LB and 50ul of overnight culture was taken to make young culture.
3.Centrifuge at 5000 rpm for 5 minutes.
4. Discard the supernatant, add saline again centrifuge at 4000 rpm for 2 minutes to wash off the pellet.
5. Discard the supernatant, add 5 ml sterile saline then re-pipette and add sterile shrimp shell (10 pieces per falcon tube)
6. Incubate in the normal incubator at 30 °C for 24 hours.
7.Centrifuge the culture tube at 1000 rpm for 5 minutes. Add 5 ml sterile saline immediately after discarding supernatant.
8.Add previously isolated DNA (200ul per tube) and incubate overnight at 30 °C.(not in the shaker incubator)
9.Collect the shrimp shell in a fresh tube.

10.Add saline necessary in the tube to submerge the shrimp shells and vortex for 10 minutes.

11.Keep the falcon tube in steady state for 30 minutes.

12. Collect the supernatant and add 100 ul of it with 900 ul of fresh LB in a fresh eppendorf tube.

13. Serial dilution was done in 5 tubes.

14.The tube containing solution of bacterial culture diluted by 10^-1,10^-3,10^-5 factor was taken (100ul) in the kanamycin plate and plating was done following the spread plate technique.

15. Incubation was done at 37°C for 24 hours.

3.5 Bacteriophage identification & Purification

3.5.1 Bacteriophage identification from stock solution:

In this procedure we collected a total of eight bacteriophages from stock e.i. JSF2,JSF5,JSF13,JSF35, S113B,S213B,S313B and S17F where 200ul of each phage was collected separately in sterile eppendorf. In order to examine host specificity of these phages, we performed a spot test, primarily on native strains.

A. Firstly, all the six native strains were subcultured on the LA media plate.
B. The next day, we inoculated the single colonies of each strain into separate falcon tubes containing 5 ml LB media and incubated at 37 degree celsius for young culture (culture having less concentration of cell and after incubation the media looks slightly hazy e.i. Light can pass through it)
C. After preparing the young culture, a lawn of bacteria was made by pouring 6ml soft agar (0.6% agar) with 200 microliter of bacterial young culture on a LA plate.
D. Let the layer solidify on the LA plate for some time.
E. Two LA plates for each bacteria strain were clearly marked on the place of the phage droplets.
F. 15 microliter of each phage was dropped on bacterial lawn by using a micropipette and waited enough to diffuse them through soft agar.
G. Lastly, 12 plates were incubated at 37 degree celsius for 12-15 hours.
H. The droplet areas having clear zones were identified as potential phages for those bacteria.

3.5.2 Visible Phage Purification:

To create pure phage isolates suitable for identification, phage mixture has to be purified. So after confirming, the phage purification of the visible phage was done. To do so, 200 ul of SM buffer was taken in a fresh eppendorf tube. Along with it 20 ul of chloroform was added to the eppendorf tube. Then plates containing the clear plaque after the spot test were selected and using a sterile blue tip the agar from the clear plaque were picked off and kept in the eppendorf tube which contains the SM buffer and chloroform. The agar was dispersed and re pipetted gently for a few times. The eppendorf tube containing the plaque was then vigorously vortexed for 1-2 minutes to release the phage from the plaque agar.Then, the purified phage was stored at 4 degree celsius for further uses.

3.5.3 Bacteriophage Enrichment:

The bacteriophage is isolated and enhanced for potential use after being confirmed by the Spot test. The separated phages go through the same enrichment procedure as the bacteriophages in the sample.First of all, phage specific host bacteria were cultured on the LA media plate. Then, young culture was prepared with 1.5ml LB-cultured host bacterial cells in a shaker incubator at 37 degree celsius for 2 hours. After that, an SM buffer with 50 microliters of isolated phage that had previously been kept at 4 degrees Celsius was added in that culture.The mixture was then incubated in a shaker incubator for a further 4 hours. The mixture was incubated, and then centrifuged at 13,000 rpm for 10 minutes to separate the enhanced phages suspended in the supernatant from the bacterial cells as pellets. After that, a new syringe was used to collect the supernatant, which was then put through a 0.22-micron syringe filter. To avoid contaminating the 0.22 micron filter, caution must be exercised.

This sterilized, filtered, clear suspension was then poured in an autoclaved, fresh falcon tube and is absolutely devoid of any bacterial cells. However, falcon tubes were named such as "*V.Cholerae* 1877(JSF2)" etc.The tubes were wrapped with parafilms. And the tubes were stored at 4°C. To enhance the amount of the phage in case it were to become completely consumed, the enrichment procedure for the isolated phage was repeated.

3.6 Phage infectivity test on different *Vibrio* strains:

The same procedure described in bacteriophage identification from stock (3.5.1) that is a spot test, was followed for ampicillin resistant and kanamycin resistant strains. The only difference was that the media was selective that time.

Ampicillin resistant strains: LA media plate for subculture, LB media for young culture and again LA plates for spot test contained an adequate amount of ampicillin supplement which made the media selective. (0.5ml ampicillin supplement for 100ml media)

Kanamycin resistant LA media plate for subculture, LB media for young culture and lastly LA plates for spot test contained an adequate amount of kanamycin which made the media selective.(0.5ml kanamycin for 100ml media)

Result:

To ensure the accuracy of the result all the experiments were performed several times. Here six strains of V. Cholera were used (1877,WT048,WT382,WT386,WT 406,WT404). First of all, these native strains were transformed into two different antibiotic resistant strains separately. One is Ampicillin resistant and another is kanamycin resistant strains. In the next part of our experiment, spot test was performed with eight bacteriophages (JSF2, JSF5, JSF13, JSF35, S113B, S213B, S313B and S17F on a total 18 strains including six native strains, six Ampicillin resistance strains and six kanamycin strains. Lastly, after 15 hours of incubation,the final outcome was observed and is shown below:

Spot test result :Native (N) Strains:

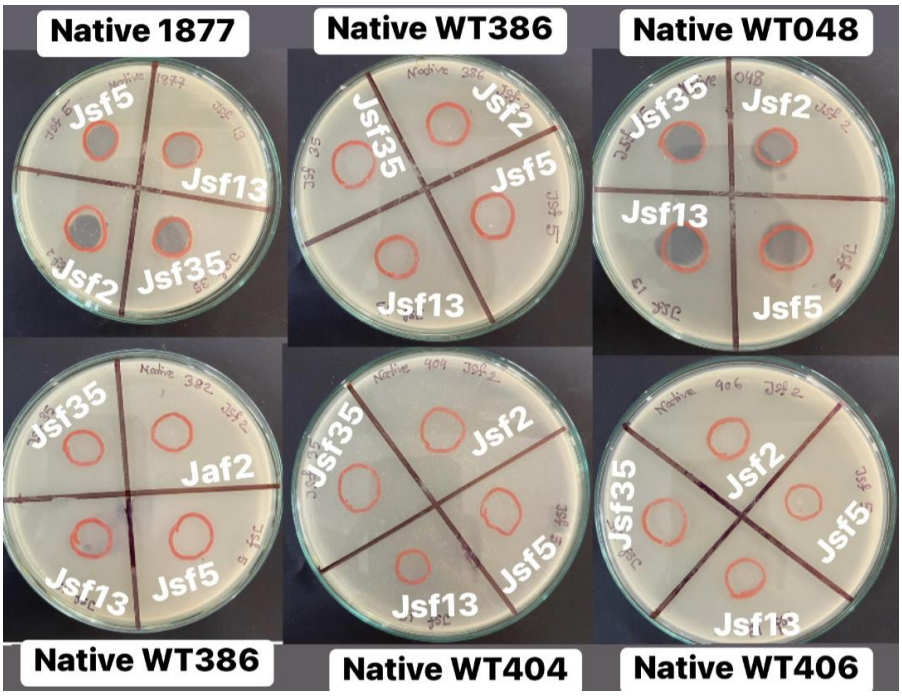

Figure-8:Spot test on native vibrio strains(Phages: JSF2,JSF5,JSF13 and JSF35)

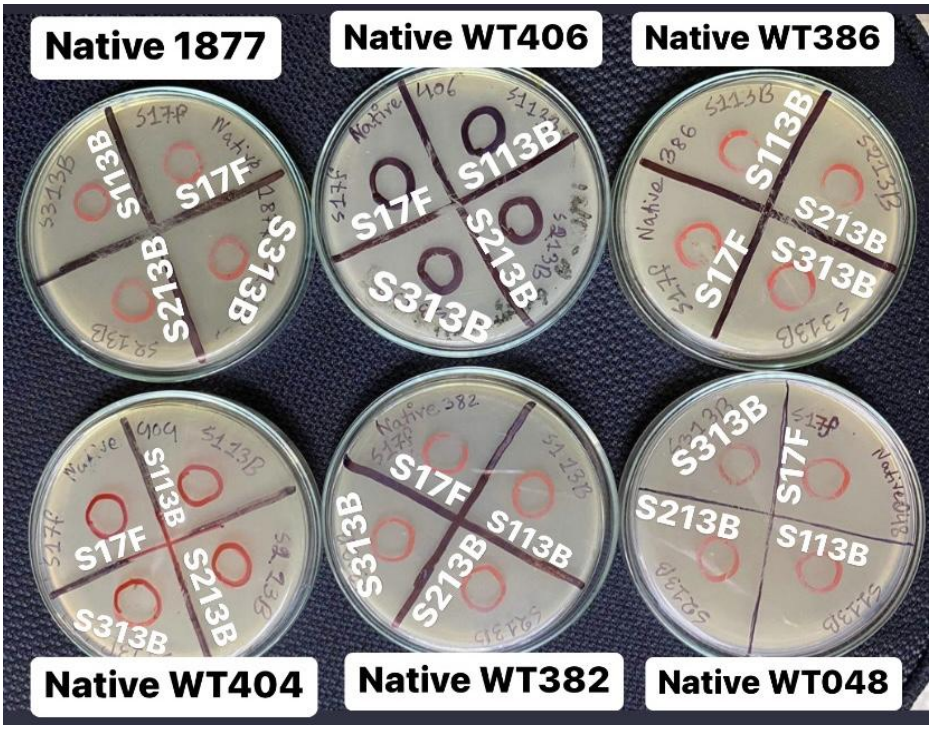

Figure:9- Spot test on native vibrio strains (Phages:S113B,S213B,S313B and S17F)

Table-2: Spot Test Result for Native Strains

Strain name	JSF 2	JSF 5	JSF 13	JSF35	S113B	S213B	S313B	S17F
1877	+	+	+	+	-	-	-	-
WT 382	-	-	-	-	-	-	-	-
WT 386	-	-	-	-	-	-	-	-
WT406	-	-	-	-	-	-	-	-
WT404	-	-	-	-	-	-	-	-
WT048	+	+	+	+	-	-	-	-

Here, all the eight bacteriophages- JSF2, JSF5, JSF13, JSF35, S113B, S213B, S313B and S17F were spot tested on the native vibrio strains we have and the result shows bacteriophage JSF2, JSF5,JSF13 and JSF35 exhibit clear zone in N1877 and N048 strains.However, S113B, S213B, S313B and S17F show no clear zone on any of the native strain.

Spot test result : Ampicillin Resistant Strains:

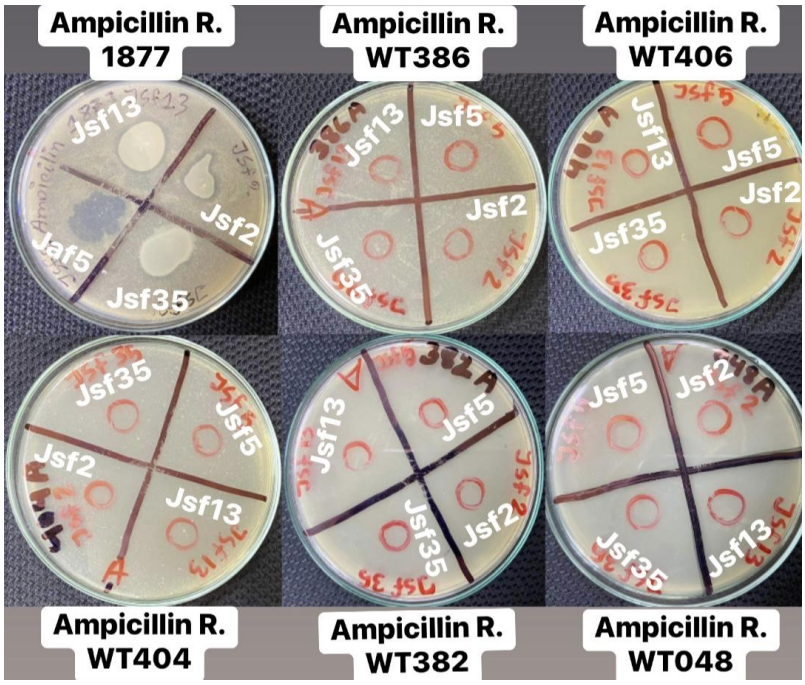

Figure:10- Spot test on ampicillin resistant vibrio strains (JSF2,JSF5,JSF13 and JSF35)

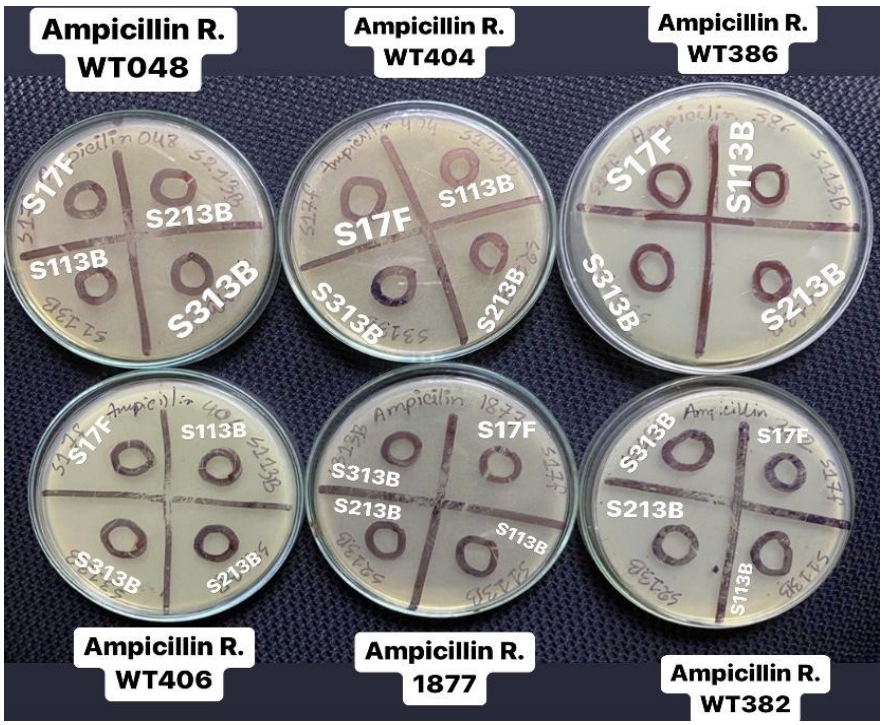

Figure:11- Spot test on ampicillin resistant vibrio strains (Phages:S113B,S213B,S313B and S17F)

Table-3:Spot Test Result for Ampicillin ResistanceStrains

Strain name	JSF 2	JSF 5	JSF 13	JSF35	S113B	S213B	S313B	S17F
1877	+	+	+	+	-	-	-	-
WT 382	-	-	-	-	-	-	-	-
WT386	-	-	-	-	-	-	-	-
WT406	-	-	-	-	-	-	-	-
WT 404	-	-	-	-	-	-	-	-
WT 048	-	-	-	-	-	-	-	-

Similar to the native strain spot test, ampicillin resistant stains were also spot tested using those eight bacteriophages. And the result shows clear zones only on the AR1877 strain for JSF2, JSF5,JSF13 and JSF35 bacteriophages. However, clear zones for JSF 2,JSF13 and JSF 35 were not clear. An extra layer seems to cover the clear zone formed before. On the other hand, the same bacteriophages did not form any clear zone on the other five strains. Similarly, S113B,S213B,S313B and S17F formed no clear zone in any of the six AR strains at all.

Spot test result: Kanamycin resistant (K.R) strains:

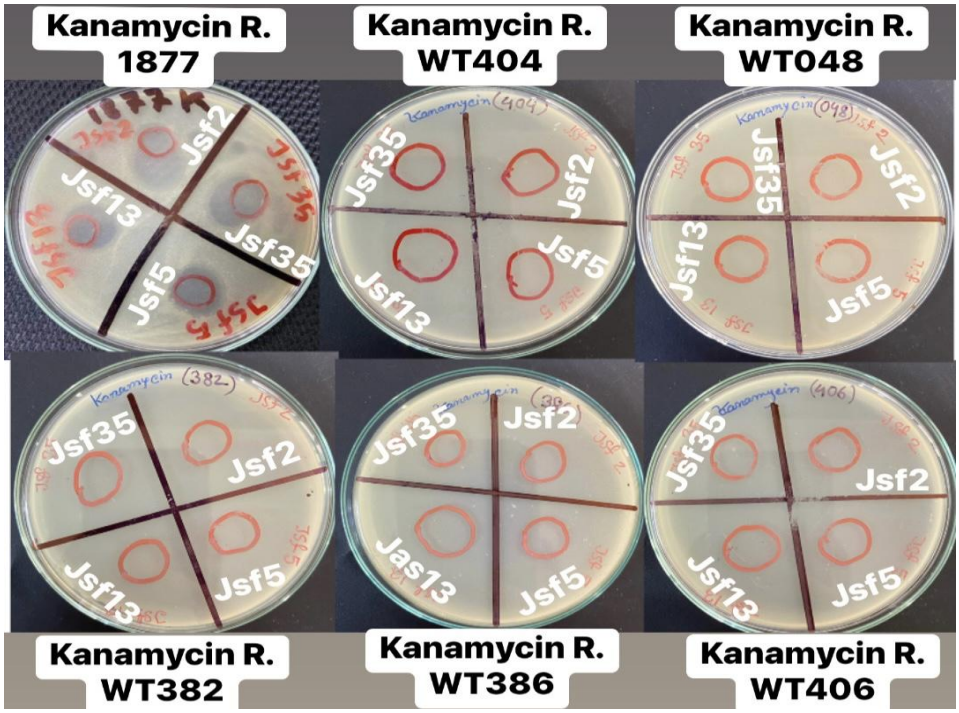

Figure:12-Spot test on kanamycin resistant vibrio strains (JSF2,JSF5,JSF13 and JSF35)

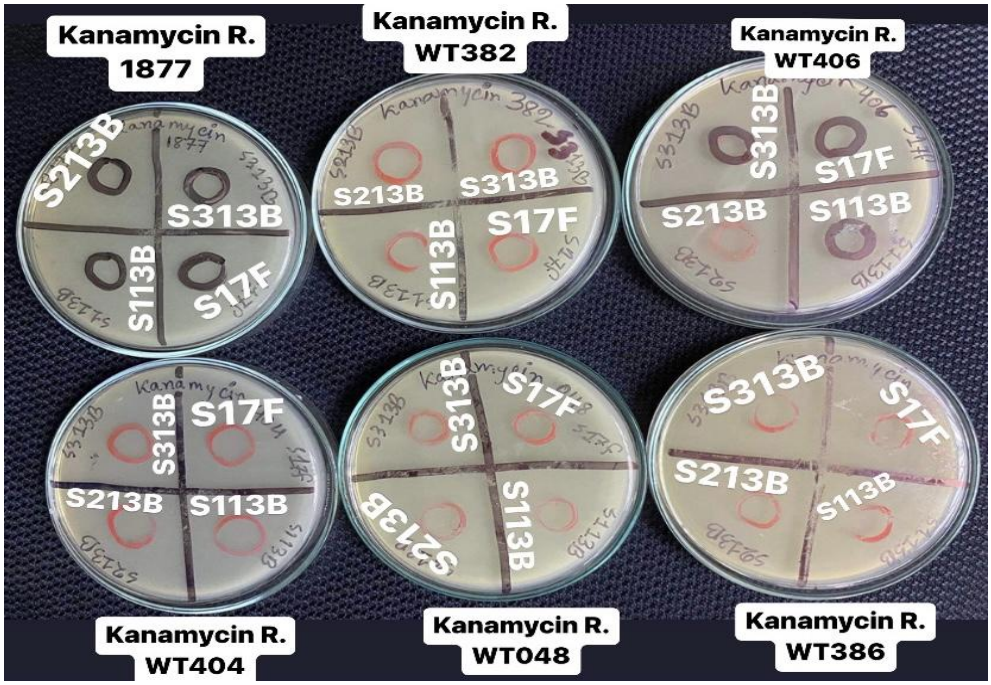

Figure:13- Spot test on kanamycin resistant vibrio strains (Phages:S113B,S213B,S313B and S17F)

Table-4:Spot Test Result for Kanamycin resistance Strains

Strain name	JSF 2	JSF 5	JSF 13	JSF35	S113B	S213B	S313B	S17F
1877	+	+	+	+	-	-	-	-
WT382	-	-	-	-	-	-	-	-
WT386	-	-	-	-	-	-	-	-
WT406	-	-	-	-	-	-	-	-
WT404	-	-	-	-	-	-	-	-
WT048	-	-	-	-	-	-	-	-

Spot test result for kanamycin resistant vibrio strains with JSF2, JSF5, JSF13, JSF35, S113B, S213B, S313B and S17F bacteriophages showed the same result as ampicillin resistant strains. But this time, Kanamycin Resistant1877 showed completely clear zones for JSF5, JSF13, and JSF35 along with JSF2.

Discussion

As more microbiological species (bacteria, viruses, fungi, and protozoa) develop resistance to the currently available therapeutic choices, difficulties with multidrug resistance (MDR) are becoming more and more widespread worldwide. As a result, problems with chronic infectious diseases, rising mortality, problems with biosecurity, animal health, and outbreaks of foodborne illnesses all become more and more frequent. Therefore, as an alternative way phage can be employed. Currently phages are considered as the most prevalent component of the human microbiome, which are thought to be essential for promoting bacterial diversity and allowing horizontal gene transfer. Phages are also thought to play a significant role in determining how the microbiome develops and employed in a variety of biotechnology and food industries for the identification of harmful microorganisms. This dissertation aims to determine the sensitivity of native and antibiotic-resistant *vibrio cholerae* strains to bacteriophage infection. As antibiotic resistant genes are circulating and increasing in our environment day by day, most *vibrio* strains are capturing those resistant genes and becoming multidrug resistant. Bacterial strains and phages are naturally found in the environment. However, for this study, we did not select antibiotic resistant vibrio strains from the environment but selected six native strains and then transformed them into antibiotic resistant strains in order to observe the effect of phage infectivity on strains of similar genetic compositions. The bacteriophage and host bacteria employed in this particular study were obtained from the stock sample.

As the results show, the final spot test exhibits clear zones for native 1877, native WT048 and ampicillin resistant 1877, it is a clear indication that these vibrio strains are sensitive to bacteriophage JSF2, JSF5, JSF13 and JSF35 which was processed following horizontal gene transfer (HGT). Transformation took place here to take full control of bacterial metabolic regulation to produce viral offsprings by the host cell lysis at the end. During incubation bacteriophages diffuse through soft agar bacterial lawn (0.6 percent agar) and easily infect the respective hosts. Then as a result of lysed cells by phages, clear zones are formed.

Comparing the phage infectivity among native, ampicillin and kanamycin resistant strains we saw that transformed native strain (ampicillin resistant WT048 and kanamycin resistant WT048) showed different results than native strain (Native WT048). However, native 1877, kanamycin

resistant 1877 and ampicillin resistant 1877 are sensitive to similar phages (although native 1877 vibrio strain is naturally kanamycin resistant, so native 1877 and kanamycin resistant 1877 show similar sensitivity). From this, two things can be implicated:

- Firstly, transformed strains (Ampicillin Resistant WT048 and Kanamycin Resistant WT048) are resistant to the phages (JSF2,JSF5,JSF13 and JSF35) but native WT048 is sensitive to the same phages.
- Secondly, native and transformed strains (Native 1877 and Ampicillin Resistant 1877) both showed sensitivity to the same phages (JSF2,JSF5,JSF13 and JSF35).

Therefore, in the first case, Horizontal gene transfer (Transformation) in vibrio native strain affected the phage infectivity but in the second case it did not.However, in ampicillin resistant 1877, three clear zones were observed with hazy overlayers (JSF2, JSF13 and JSF35) indicating contamination or resistant colonies.

Here, plasmid containing ampicillin resistant gene from *E.coli* DH5 alpha (pGLO plasmid) was isolated and transferred into native vibrio strains to make them ampicillin resistant. Gene was transformed from one bacteria to another through plasmid which is clearly horizontal gene transfer. Again, chromosomal DNA of native 1877 strain was isolated and transferred into other five native vibrio strains. After that, chitin played the key role here by acting as the only carbohydrate source for native strains where sufficient volumed isolated DNA was present. As a result, native strains transferred into kanamycin resistant as native 1877 DNA contains kanamycin resistant genes .

Then. Gel electrophoresis was performed several times after each plasmid/DNA isolation and transformation in order to ensure the successful DNA isolations and transformations. In case of ampicillin resistant strains, pGLO DNA was used as positive control and for kanamycin resistant strains, Native 1877 DNA was positive control. Not only, pure single colonies on selective agar media (Ampicillin Resistant strains on ampicillin LA plate and Kanamycin Resistant strains on kanamycin LA plates) but also DNA bands from gel electrophoresis showed transformation of native vibrio strains into antibiotic resistant strains was a success.

This pGLO plasmid is not only ampicillin resistant but also arabinose consumer which emits fluorescent green under UV light. However, after transformation,pGLO made transformed strains ampicillin resistant but they did not fluoresce under UV light. pGLO in *E.coli* DH5 alpha shows green fluorescence but in vibrio, it does not. In *E.coli* pGLO,the green fluorescence protein

(GFP) is activated by breakdown of its promoter when arabinose is present in the media but transformed vibrio cells could not utilize the presence of arabinose. As a result, ampicillin resistant strains did not glow under UV. This difference indicates that Ampicillin Resistant vibrio strains neither digest arabinose nor glow. However, about the other four phages S113B, S213B, S313B and S17F, none of them infected neither native nor antibiotic resistant strains. Thus, no changes are noted.

Therefore, observing the outcome, we can state that horizontal gene transfer mostly did not show any change in bacteriophage infectivity except for ampicillin Resistant WT048. Infectivity change in two among eighteen strains does not completely conclude the effectiveness of phage infectivity. Phage infectivity on NativeWT048, ampicillin resistant WT048 and kanamycin resistant WT048 showed that having antibiotic resistant genes, make phage therapy (with JSF2, JSF5,JSF13 and JSF35) inactive. And other strains show no change for phage infectivity despite having resistant genes in them is good as it does not change existing phage candidates. Therefore, by making more antibiotic resistant strains and testing them with more bacteriophages can hopefully bring about a stronger and reliable outcome.

Conclusion

Although genetic changes or horizontal gene transfer will inevitably result in the development of antibiotic resistance in bacteria, the misuse of antibiotics from their discovery has made the issue worse. The extensive use of antibiotics on livestock farms as growth boosters lead to slow the emergence of antibiotic resistance. Since phages have been used to limit bacterial growth in a variety of microbiomes, including those of people, animals, and other environmental contexts (such as wastewater treatment), as well as in the food industry.The numerous application possibilities in the food industry, where they may be utilized at all stages of food manufacturing, from smearing and crops to food transportation are an excellent illustration of the widespread usage of phages (Pires et al., 2020).

Since our study is focused on *vibrio cholerae*, the main goal of this study is to determine whether antibiotic resistance genes found in the environment will affect the efficiency of phages that are considered to be good candidates for phage therapy by observing the infectivity of selected bacteriophages on *V. cholerae* strains with similar genetic composition and antibiotic resistance traits. The high resistance of *V. cholerae* strains to antibiotics might be viewed as a danger to the world's public health because of the higher intake of antibiotics in V. cholerae strains. Bacteriophages, which are bacteria's natural predators, can be effectively exploited against antibiotic resistant strains.

In our study we used eight phages and 18 strains of V. cholerae(6 Native ,6 Ampicillin Resistant ,6 Kanamycin Resistant) to observe the bacteriophage infectivity on native and antibiotic resistant strains. Here, horizontal gene transfer showed no change on bacteriophage infectivity.However, as we observed one change of phage infectivity in Ampicillin Resistant WT048 strain from its native strain .This ampicillin and kanamycin resistant WT048 vibrio strain cannot be treated by JSF2,JSF5,JSF13 and JSF35 therefore these are not good candidate for phage therapy. On the other hand, it can be assumed from unchanged phage infectivity on different strains that, existence of antibiotic resistant genes does not change suitable phage candidates which makes the phage therapy a promising field. There might be some shortcomings in our findings . We only used six native stains and only eight phages to do the study so it can be considered a limitation of our study. If we had included more strains and more phages in our experiment we could expect more diverse and probably reliable results from our study. We are

hopeful that if the study limitations are overcome, reliable findings may arise if the study is further continued.

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