Isolation and characterization of Bacteriophages from surface water specific for Vibrio Cholerae

A DISSERTATION 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

By
Farzana Mansur Priyanka
Student ID: 15136008
30th January, 2020
Biotechnology Program
Department of Mathematics and Natural Sciences
Brac University

Declaration

It is hereby declared that

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

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Farzana Mansur Priyanka

ID:15136008

Approval

The thesis/project titled "Isolation and characterization of Bacteriophage from surface water specific for *Vibrio cholerae*" is submitted by
Farzana Mansur Priyanka of Spring,2015, has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Biotechnology on 30th January 2020

Examining Committee:

Supervisor:
(Member)

_____

Dr. Iftekhar Bin Naser
Assistant Professor
Department of Mathematics and Natural Sciences
BRAC University

Program Coordinator:
(Member)

_____

Dr. Iftekhar Bin Naser
Assistant Professor
Department of Mathematics and Natural Sciences
BRAC University

Departmental Head:
(Chair)

_____

A F M Yusuf Haider
Professor and Chairperson
Department of Mathematics and Natural Sciences
BRAC University

Ethics statement

No human or animal model was used in this study.

Abstract

The severe diarrheal disease cholera is caused by the bacterium *Vibrio cholera*. Toxigenic *Vibrio cholerae* strains belonging to O1 and O139 serogroups are the causative agents of epidemic and pandemic cholera. The *Vibrio* bacterium interacts with numerous phages in the aquatic ecosystem and in the intestine of cholera patients. Generally, two peaks of cholera outbreak are observed to coincide within the dry summer and monsoon rain. Cholera epidemic is a major public health concern. Several factors control the seasonal epidemics. Bacteriophages are one of the vital triggers which have been reported to collapse the outbreaks. Hence, the predatory nature of bacteriophage has a huge impact on the population for their corresponding hosts. This study was designed to isolate *Vibrio cholera* specific bacteriophages from environmental samples. The isolated bacteriophages were categorized not only according to their physiological characteristics (Host Range, pH stability, temperature stability and organic solvent sensitivity) but also on a molecular level using techniques like RFLP and PCR.

Dedicated to my mother and father for always believing in me

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First of all, I would like to express my deepest gratitude to Almighty Allah for everything He has enabled me to accomplish and nevertheless my parents, especially my mother who has never questioned my capability to achieve everything I ever aimed for.

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Farzana Mansur Priyanka

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List of Abbreviation

et al	And others
g	Gram
ml	Milliliter
ICTV	International Committee on Taxonomy of viruses
E. coli	*Escherichia coli*
V.cholerae	*Vibrio cholerae*
RFLP	Restriction fragment length polymorphism
µL	Microliter
µm	Micrometer
O/N	Overnight
rpm	Rotation per minute
pfu	Plaque forming unit
LA	Luria Bertani Agar
LB	Luria Bertani Broth
DNase solution	Deoxyribonuclease solution
RNase solution	Ribonuclease solution
PCI	Phenol-Chloroform Isoamyl Alcohol
TE	Tris EDTA
EDTA	Ethylenediaminetetraacetic acid
PCR	Polymerase chain reaction
F primer	Forward primer
R primer	Reverse primer
dNTPs	Deoxynucleotide Triphosphates
ds	Double stranded
ss	Single stranded
CRISPR	Clustered regularly interspaced short palindromic repeats
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
ICP1	International Centre for Diarrhoeal Disease Research, Bangladesh cholera phage 1
ICP2	International Centre for Diarrhoeal Disease Research, Bangladesh cholera phage 2
ICP3	International Centre for Diarrhoeal Disease Research, Bangladesh cholera phage 3
Icddr,b	International Centre for Diarrhoeal Disease Research, Bangladesh

Chapter 1

Introduction

Introduction

Bacteriophage, also referred to as phage, is a virus that infects and replicates inside a bacterium. "Phage" terminology has its origin from Greek word "phagein" meaning "to devour". Phages are considered the most abundant and genetically diverse biological entities on Earth, with global population number estimated to be around 10^{30} to 10^{32} (Hemminga et al., 2010). Recently it has been acknowledged that phages play a crucial role in recycling organic matter in the biosphere and hence play an essential role in maintaining bacterial diversity as well as maintaining overall balance. (Chibani-Chennoufi et al., 2004; Guttman et al., 2004). The study of phages and more researches on its predation mechanism can be proved valuable for fighting against multi drug resistant bacteria through phage therapy and in lieu of traditional antibiotics (Keen, 2012). Till 2017, more than 25000 phage nucleotide sequences have been submitted in International Nucleotide Sequence Database Consortium (INSDC) (Adriaenssens and Brister, 2017). More over researchers assume that there are so many of more to come.

Vibrio cholerae are halophilic, highly motile, curved, Gram-negative rods. *V. cholerae* is the causative agent for cholera, a profound secretory diarrhea. While *V. cholerae* is a naturally occurring member of aquatic environments, only a small portion of environmental *V. cholerae* is capable of causing cholera. The structure of its cell surface lipopolysaccharide O antigen is used to classify *V. cholerae* into more than 200 serogroups, of which only two, O1 and O139, possess the potential to cause epidemic or pandemic cholera. The O1 serogroup is further divided into two biotypes, classical and El Tor, which evolved from independent lineages and they display genotypic and phenotypic differences. (Pang B *et al*, 2007). Strains of *V. cholerae* that cause cholera harbor a filamentous bacteriophage (CTXΦ) that encodes cholera toxin, the cholera toxin is encoded by *ctxA* and *ctxB*. Most commonly known as choleragen and abbreviated to CTX, CT or CTx . It is a coordinately regulated virulence factor, the toxin co-regulated pilus (TCP), which is essential for colonization. Pandemic strains of *V. cholerae* serogroup O1 have evolved rapidly through horizontal acquisition of clusters of virulence genes including CTXΦ. In patients with cholera, *V. cholerae* is shed in prodigious quantities in the stool and vomit. Hence the amount of V. cholera is abundant in the water bodies

and general aquatic environment. Disease-causing strains of *V. cholerae* employ a variety of mechanisms to survive both in aquatic reservoirs and in the human host.

Prevalence of cholera since history has been detected in South Asia, especially the Ganges delta region. (Siddique, 2014). Phage typing has been used to identify
V. cholerae strains and has contributed greatly to understanding cholera epidemiology. Recently, there has been a new interest in cholera phage study using the modern molecular techniques. In a study, Dr. Kimberley Seed and her team tested clinical samples for phage presence by plaque assay. Three virulent phages were then identified and designated as ICP1, ICP2, and ICP3. The categories differed in the type of *v. cholera* they infected, their whole genome content as well. ICP1 was classified specific for O1 serogroup *V. cholerae*; however, the host ranges for ICP2 and ICP3 are broader and include some non-O1 serogroup *V. cholerae* strains. The study reflected on the fact, while the presence of ICP1 is more naturally occurring, the presence of ICP2 and ICP3 are observed in separate intervals. With the radicle emergence of *V. cholerae* it has developed different strategies too in order to fight phage invasion in their system.
By understanding and characterizing the molecular mechanisms of these predator-prey relationships, we can envision a fast and specific tool to reduce the burden of bacterial infections on global health.

Objectives:

The main aim of this project was to isolate bacteriophage against *Vibrio cholerae* and to identify its various physiological and molecular characteristics in order to determine its justifiability to be used as an ideal phage for further study with broader perspective.

Specific aims:

1. To isolate strong lytic bacteriophages against *Vibrio cholerae* from different water sample.
2. To characterize the isolated phage based on

a) host range specificity

b) thermal stability

c) pH stability

d) organic solvent sensitivity (ethanol and chloroform)

3. To identify the isolated phages in order to determine the specific category of the phage (ICP1, ICP2 and ICP3)

Chapter 2

Literature Review

2.1 Bacteriophage

Bacteriophages or 'phages' for short are naturally occurring bacterial viruses which infect bacterial cells (Abedon, 2012). They are highly host specific and include the ability to proliferate inside bacterial cell (Clark and March, 2006; Hagens and Loessner, 2007; Hanlon, 2007; Nishikawa et al., 2008; Viazis et al., 2011). Since its discovery, bacteriophages have been used to treat bacterial infection in human (Sulakvelidze and Kutter, 2004). Phages isolated so far were successfully used in the fields of different agricultural setups for treating plant bacterial disease. Moreover, it laid a promising foundation in maintaining livestock and aquaculture (Sulakvelidze and Barrow, 2004). Furthermore, the use of phages was also implemented in the dentistry department to clear bacterial contamination.

Recently, researchers are trying to implement phage as molecular tool in vaccine delivery, gene therapy (Clark and March, 2006) and as a specific diagnostic marker to detect bacterial species in the clinical and environmental samples (Funatsu et al., 2002). However, out of all these usages of phage, the ability to lyse specific bacterial cell especially those that are antibiotic resistant and prevent or cure bacterial infections makes phages an interesting alternative antimicrobial agent where chemically synthesized antibiotics may fail. Additionally, bacteriophages are estimated to kill between 20-40 % of oceanic bacteria every day, play a key role in nutrient and energy cycle of an ecosystem and forms the pool of most genetically diverse 'life form' on earth (Suttle, 2005).

2.2 Early history of Bacteriophage

According to history, in 1896 a discovery was made by Ernest Hanbury Hankin. He identified the existence of a microscopic individual in the Ganges and Yamuna rivers in India, which very clearly had been marked to have an antibacterial action against cholera. Additionally, having the characteristic to pass through a fine porcelain filter (Hankin, 1896). Later on, British bacteriologist Frederick Twort confirmed a small agent that infected and killed bacteria. (Twort, 1915).

Sadly, enough, Twort's work was interrupted for the World War and lack of funding. His unfinished work was somehow gained its path of discovery to detection by French-Canadian

microbiologist Félix d'Hérelle, who discovered an invisible, antagonistic microbe of the dysentery bacillus. For d'Hérelle, there was no question as to the nature of his discovery which said "In a flash I had understood: what caused my clear spots was in fact an invisible microbe … a virus parasitic on bacteria" (Félix d'Hérelles, 1917*)*. d'Hérelle named the virus a bacteriophage or bacteria-eater (Félix d'Hérelles, 1917). Keeping D' theory or research an introduction and implementation of new studies and new levels of studies regarding phage has been done by researches all over the globe. An introduction of phage therapy which can be a ground breaking research in this century are on its way for formulation and implementation on the basis of D'Hérelle's study.

Furthermore, for the discovery of the replication of viruses and their genetic structure, Max Delbrück, Alfred Hershey and Salvador Luria were awarded the Nobel Prize in Physiology or Medicine, in 1969.

2.2.1 Bacteriophage classification

Phages are enormously diverse and vary based on structure, physicochemical and biological properties. In 1933, Burnet observed heterogeneity among different phages and in 1943, Ruska observed three morphological types of bacteriophage which evoked the necessity of proper classification of phages. Holmes proposed a classification system of phages based on plaque morphology and particle size, host range, and resistance to urea and heat which was not accepted by scientific community. Later on, the International Committee on Taxonomy of Viruses (ICTV) classified phages based on nucleic acid and gross morphology and grouped them into six genera (Ackermann, 2004).

Table 2.2.1 ICTV classification of prokaryotic (bacterial and archaeal) viruses. *(Mc Grath and van Sinderen, 2007)*

Order	Family	Morphology	Nucleic acid	Example
Caudovirales	*Myoviridae*	Nonenveloped, contractile tail	Linear dsDNA	T4 phage, Mu,
	Siphoviridae	Nonenveloped, noncontractile tail (long)	Linear dsDNA	λ phage, T5 phage
	Podoviridae	Nonenveloped, noncontractile tail (short	Linear dsDNA	T7 phage, T3 phage
Ligamenvirales	*Lipothrixviridae*	Enveloped, rod-shaped	Linear dsDNA	Acidianus filamentous virus 1
	Rudiviridae	Nonenveloped, rod-shaped	Linear dsDNA	Sulfolobus islandicus rod-shaped virus 1
	Ampullaviridae	Enveloped, bottle-shaped	Linear dsDNA	
	Bicaudaviridae	Nonenveloped, lemon-shaped	Circular A dsDN	
	Clavaviridae	Nonenveloped, rod-shaped	Circular A dsDN	
	Corticoviridae	Nonenveloped,	Circular A dsDN	

Unassigned	*Cystoviridae*	Enveloped, spherical	Segmented dsRNA	
	Fuselloviridae	Nonenveloped, lemon-shaped	Circular dsDNA	
	Globuloviridae	Enveloped, isometric	Linear dsDNA	
	Guttaviridae	Nonenveloped, ovoid	Circular dsDNA	
	Inoviridae	Nonenveloped, filamentous	Circular ssDNA	M13
	Leviviridae	Nonenveloped, isometric	Linear ssRNA	MS2, Qβ
	Microviridae	Nonenveloped, isometric	Circular ssDNA	ΦX174
	Plasmaviridae	Enveloped, pleomorphic	Circular dsDNA	
	Tectiviridae	Nonenveloped, isometric	Linear dsDNA	

2.2.2 Bacteriophage infection

Bacteriophage requires a host for replicating and enumeration. Without a host the phage cannot grow or multiply. There are to main types of infection of phage. These are very briefly given below,

- **Lytic Cycle**

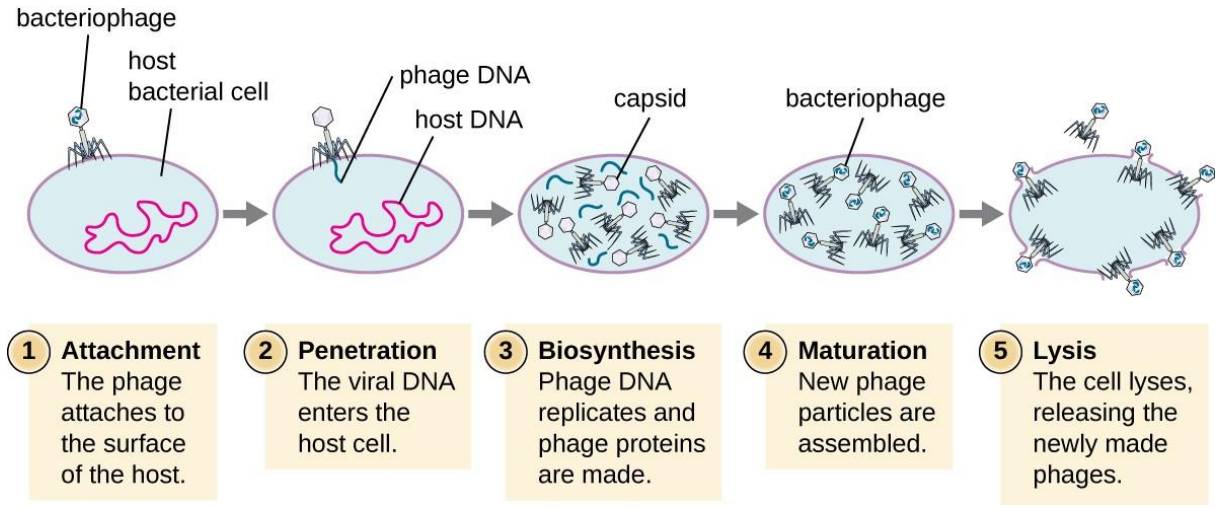


Fig 2.2.2(1)- Lytic cycle of Bacteriophage

- **Lysogenic cycle**

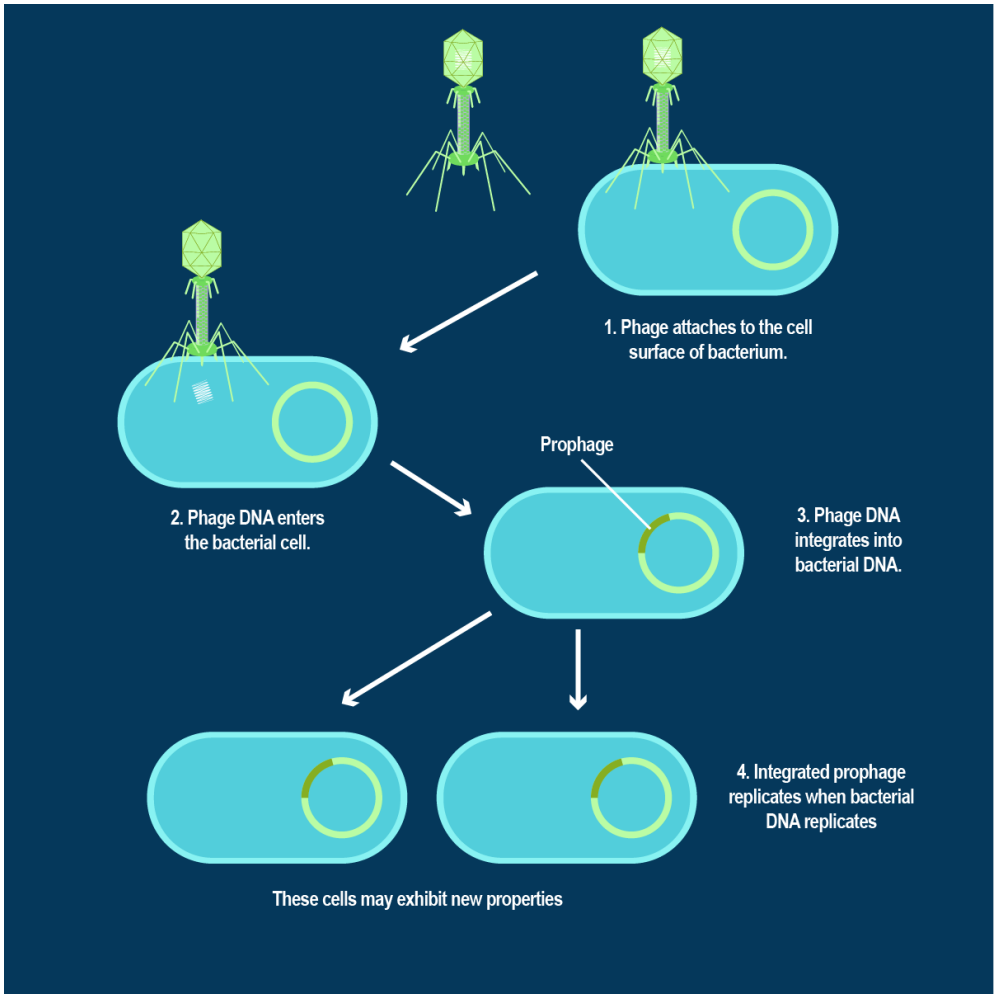


Fig 2.2.2(2)- Lysogenic cycle of Bacteriophage

The study was designed to isolate lytic bacteriophages as they gave a clear lytic zone on the agar plate and were easier to isolate. Whereas, the lysogenic phages are hard to identify and harder to isolate.

2.3 Cholera

Cholera is an infectious disease caused by a bacterium called *Vibrio cholerae*. The bacteria typically live in aquatic environments with warm and salty setup such as estuaries and waters along coastal areas. People are affected with cholera after coming in contact with *V. cholerae* after

drinking liquids or eating foods contaminated with the bacteria, such as raw or undercooked shellfish.

The causative agent for cholera, *V. cholerae* is a Gram-negative, comma-shaped bacterium. The bacterium's natural habitat is brackish or saltwater and attach themselves easily to the chitin-containing shells of crabs, shrimps, and other shellfish. Some strains of *V. cholerae* cause the disease cholera, which can be derived from the consumption of undercooked or raw marine life species. *V. cholerae* is a facultative anaerobe and has a flagellum at one cell pole as well as pili. *V. cholerae* can undergo respiratory and fermentative metabolism. When ingested, *V. cholerae* can cause diarrhea and vomiting in a host within several hours to 2–3 days of ingestion.

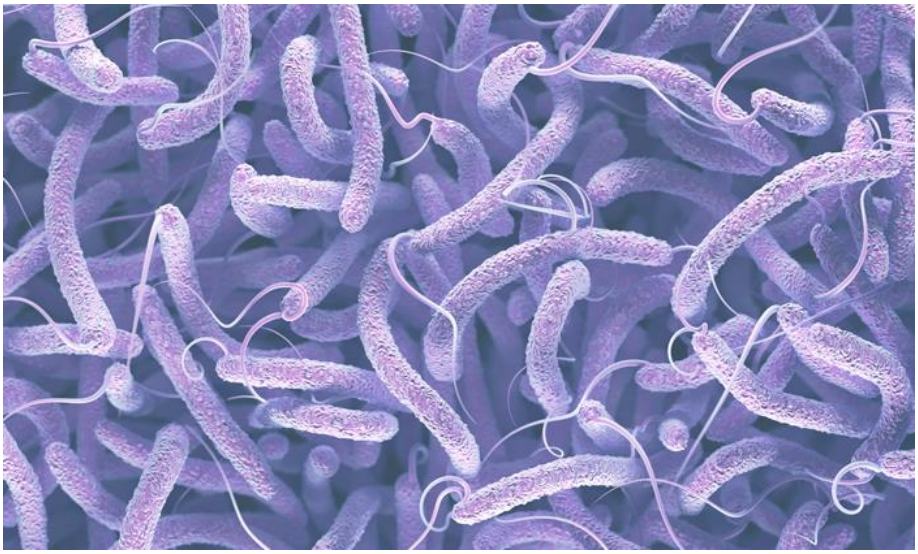


Fig 2.3- *Vibrio cholerae*

V. cholerae is a heterogeneous species with 206 serotypes identified to date based on the heat-stable somatic O antigen. Among all of them, only two serotypes, O1and O139, have been characterized as toxigenic and have caused epidemics of cholerae (Rivera et al.,2003). *V. cholerae* non O1 or O139 is isolated in abundance from aquatic environments whereas *V. cholerae* O1 is seldom recovered from the ecosystems in the inter-epidemic periods of the disease or it may be found in the non-toxigenic form, "viable but non-culturable" form or in the form of biofilms (Leal et al., 2008). *V. cholerae* O1 again, is divided into two biotypes, classical and El Tor, which are distinguished by a variety of phenotypic markers (Kaper et al., 1995). However, three variants of the El Tor biotype were recently described in Bangladesh, Mozambique and other regions of Asia

and Africa (Taneja et al., 2009). The ability of *V. cholerae* to cause disease is dependent on multiple factors that allow the pathogen to colonize on the epithelium of the small intestine and produce the respective enterotoxins that disrupts ion transport. Additionally, the expression of two virulence factors, the cholerae toxin (CT) which is a potent enterotoxin and a pilus-colonization factor known as the toxin-coregulated pilus (TCP) are also important for pathogenicity (Faruque and Mekalanos, 2003). Both virulence factors are encoded by genes that form part of larger genetic elements namely the ctxAB gene which encodes for CT and the TCP-ACF element encoding for TCP, alternatively referred to as the Vibrio pathogenicity island (Faruque and Mekalanos, 2003).

Huge number of research is being done to understand the trigger that starts the epidemic of cholerae during specific seasons.

2.4 Early history of Cholera

It is hard to point out the initiation of cholera but early texts from India (by Sushruta Samhita in the 5th century B.C.) and Greece (Hippocrates in the 4th century B.C. and Aretaeus of Cappadocia in the 1st century A.D.) describe isolated cases of cholera-like illnesses.

Among all, one of the first detailed accounts of a cholera epidemic originated from Gaspar Correa (Portuguese historian and author of Legendary India) who described an outbreak in the spring of 1543 of a disease in the Ganges Delta, which is located in the south Asia area of Bangladesh and India. The local people called the disease "moryxy," and it reportedly killed victims within 8 hours of developing symptoms. Additionally, the mortality rate of the disease was so high that the locals faced difficulty in burying the bodies.

Since then, numerous reports of cholera manifestations along the West coast of India by Portuguese, Dutch, French and British observers followed throughout the next few centuries.

2.4.1 Cholera Pandemics

Reportedly, in 1817 the first cholera epidemic emerged out of the Ganges Delta with an outbreak in Jessore and India, stemming from contaminated rice. The disease quickly spread throughout most of India, modern-day Myanmar, and modern-day Sri Lanka by traveling along trade routes established by Europeans.

By the time of 1820, cholera was widely spread in the countries of Thailand, Indonesia and the Philippines. The death toll was increased by 100,000 people on the island of Java alone from cholera. The disease made its way to China and Japan surpassing Thailand and Indonesia by infecting people who were near the coastal regions.

The wrath of cholera spread further than Asia and made its way to England in 1821. The British troops traveling from India to Oman brought cholera to the Persian Gulf. The disease eventually made its way to European territory, reaching modern-day Turkey, Syria and Southern Russia.

Sadly, the pandemic died out 6 years after it began. Seven **cholera pandemics** have occurred in the past 200 years, with the seventh pandemic originating in Indonesia in 1961. More recently, the Yemen cholera outbreak is the lastly reported cholera pandemic.

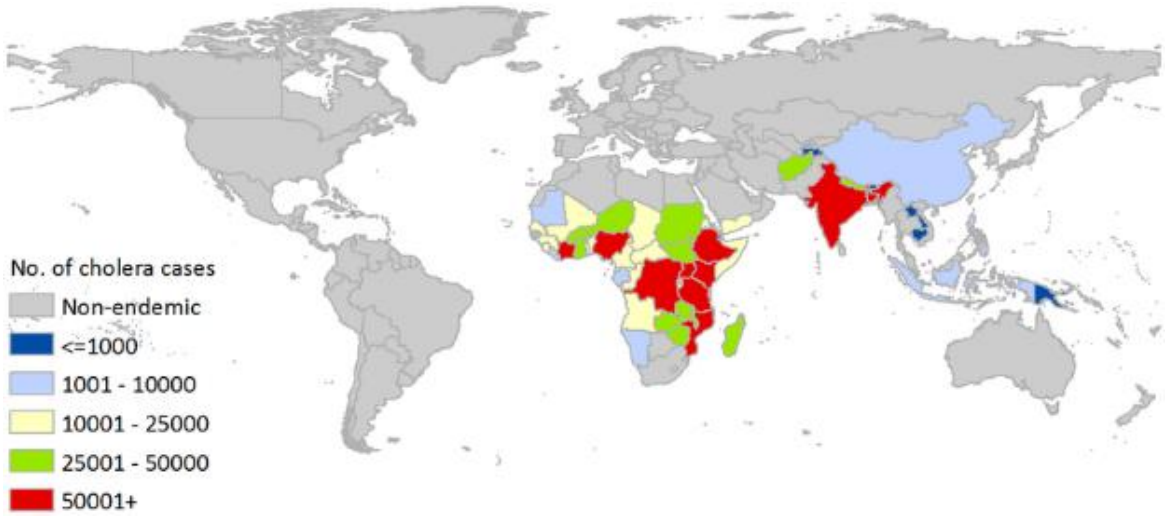


Fig 2.4.1 Cholera endemic regions

2.5 *Vibrio cholerae* and vibriophages

Recently, a correlation was theorized to have a with the existence of bacteriophages available in the environment and the cholerae epidemics. After every seasonal epidemic the causative agent vibrio cholera would disappear and then resurface with slight mutations. There are many selection pressures for which these mutations can occur and among them the occurance of bacteriophage is

considered a salient reason for the evolution of their host bacteria in a variety of ways. This phage-host interaction give rise to many mechanisms for the host to fled from phage infectivity or vice versa. These mechanisms involve, blocking of adsorption, abortive infection system, restriction modification system, and the CRISPR-Cas (clustered regularly interspersed short palindromic repeats-CRISPR-associated proteins) system.

Overtime, many bacteriophages were isolated from clinical samples as well as environmental sources in order to understand the nature of the bacteriophage. Phage typing has been used to identify *V. cholerae* strains and has contributed greatly to understanding cholera epidemiology. In the recent studies, the modern molecular techniques were used in a study at the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), Faruque et al. have isolated at least 36 different cholera phages, known as the JSF series depending on their RFLP anaylsis. Additionally, three kinds of phages were isolated from 31 Bangladeshi clinical stool samples that span a 10-year period from 2001 to 2010 and referred to as ICP Phages. The virulent phages were designated as ICP1, ICP2 and ICP3. They are divided as follows,

Table 2.5 ICP differentiation of vibriophages

Table 1. Genome characteristics of the sequenced ICP phages isolated from cholera patients at the ICDDR,B

Phage	Taxonomic family	Genome size (bp)	G+C content (%)	No. of predicted CDSs	% CDSs similar to known proteins
ICP1	*Myoviridae*	125,956	37.1	230	12
ICP2	*Podoviridae*	49,675	42.7	73	19
ICP3	*Podoviridae*	39,162	42.9	54	48

ICP1 is specific for O1 serogroup *V. cholerae*; however, the host ranges for ICP2 and ICP3 are broader and include some non-O1 serogroup *V. cholerae* strains. The prevalence of ICP1 is more in the environmental samples whereas the presence of ICP2 and ICP3 are somewhat sporadic.
(Seed K, 2010)

2.6 Bacteriophage application

For the antibacterial capacity of bacteriophage they have been given enough importance to be used as a therapeutic agent. However, for the poor understanding of the biological mechanism of phage

activity and subsequent discovery and general application of broad-spectrum antibiotics in the late 1930s and 1940s, interest in the therapeutic use of bacteriophage declined and for many years was only considered as a research tool in molecular biology (Clark and March, 2006).

Recently, bacteriophages are again reconsidered as an antimicrobial tool due to the current upward trend of bacterial resistance and availability of necessary molecular techniques and tools to precisely assess the safety and efficacy of using phage, thanks to the advancement of modern biotechnology. They are also being evaluated as a delivery vehicle for gene therapy, as a biocontrol agent, uses in the development of phage-derived vaccine and in phage display technique.

2.6.1 Phage Therapy

Using bacteriophages as therapeutic agents over antibiotics can be advantageous, but it comes with some concerns as well. Phages have been used in the treatment of plant, animal and human beings with varying degree of success. Phages host specificity acts as an advantage as it is less likely to interfere with the natural flora of host. It has been reported that after administration, phages dissipate swiftly through the body and reach most organ (Dabrowska et al., 2005). However, having protein and/or lipid structure, phages can elicit an immune response which can result in quick removal of phages from circulation. More research can be done to overcome the difficulty. Recently, phage therapy is being applied in dentistry and food industry.

2.6.2 Phage Display

In phage display method, a DNA encoding desired peptide or protein is ligated with phage coat protein gene which ultimately is expressed on the surface of bacteriophage (Clark and March, 2006). This technique can be used to generate a library and screened to isolate proteins or peptide with particular application. It can be used to isolate protein with high affinity that can act as a diagnostic tool in detection of pathogen or agents posing a biological threat (Petrenko and Vodyanoy, 2003). Phage-display library can also help in identifying the protein with enhanced enzymatic activity by screening a library of proteins with a randomly altered active site (Fernandez-Gacio et al., 2003).

2.6.3 Phages as vaccine delivery vehicle

Bacteriophages have been used as transport for vaccine delivery in two ways: 1) vaccinating with phages expressing vaccine antigens on their surface and 2) by incorporating a DNA vaccine expression cassette into phage genome and using the phage particle to deliver that DNA cassette (Clark and March, 2004). In phage-display vaccination method, the target antigen can either be generated by transcriptional fusion to coat protein or by artificially conjugating antigen protein to the phage surface which enables broad range antigen display ability (Molenaar et al., 2002). It has been demonstrated that unmodified phages deliver DNA vaccine more efficiently compared to standard DNA vaccine procedure as phage coat protein protects the DNA vaccine more efficiently and shows greater antibody response (Clark and March, 2006).

2.6.4 Phage Typing

The specificity of phages for bacterial cells enables them to be used as a diagnostics tool for detection of bacterial species and typing of the bacterial cell. For this process, several methods can be employed such as delivery of reporter gene (e.g. lux or green fluorescent protein) using phages that would be expressed after successful infection of target bacteria (Funatsu et al., 2002; Kodikara et al., 1991). Again, it can be used for detecting specific absorption of phage that had fluorescent dye covalently attached to its surface (Goodridge et al., 1999; Hennes et al., 1995). Detection of the cellular components that are released after bacterial lysis caused by phages specific to those bacteria, such as adenylate kinase provides an alternative way for identifying pathogenic bacteria (Corbitt et al., 2000).

Chapter 3

Methodology

3.1 Place of study

The study was carried out in the Biotechnology and Microbiology laboratory of the Department of Mathematics and Natural sciences, BRAC University, Dhaka, Bangladesh.

3.2 Locations of sample collection:

For conducting the study water samples were collected from four sources. The sources were differentiated according to their area, respectively as two large water bodies and two lakes, named Bosila and Turag River; Gulshan and Dhanmondi lakes.

3.3 Standard laboratory practice:

All the glass wares used during the study such as test-tube, conical flask, beakers were washed thoroughly once with tap water followed by second time rinsing with distilled water to ensure no added contamination. All medias (both agar based and broth) used for culture, pipette tips, centrifuge tubes, empty vials for conducting double-layer ager method were autoclaved at 121°C at 15 psi for 15 minutes before use and stored at 4°C. Additionally all autoclaved equipment are stored in aseptic condition. While performing experiments, clean lab coat was worn at all times and hand gloves were used. The sensitive experiments were performed inside a vertical laminar flow cabinet which was cleaned prior to perform the study with 70% ethanol and a spirit lamp was used in order to avoid all kind of contamination.

3.4 Preparation of Culture Media, Reagents and Solutions:

Preparation of Luria-Bertani Agar and Nutrient Agar for bacterial culture:

According to the manufacturer's instruction, to produce 1000 ml of Luria-Bertani agar and Nutrient agar media for bacterial culture, 25 grams and 28 grams of powder should be added respectively. Keeping ratio constant, necessary volume of Luria and nutrient agar medium was prepared each. Accurate amount of media powder was measured using an electronic balance machine before adding it to the considered amount of distilled water and mixed thoroughly. The

flask was heated on a Bunsen burner to properly mix the solution until it bubbled and gave a clear solution. After that the media flask was autoclaved at 121°C for 15 minutes being covered by an aluminum foil.

After sterilization, the media was poured on to sterilized petri dishes inside a vertical laminar flow cabinet and solidified at room temperature. Petri dishes were previously sterilized in an oven at 160°C for 1 hour. After the media became solid, they were kept in the incubator to check for contamination and used accordingly.

Preparation of 0.7 % top Soft Agar:

For performing double layer agar assay, 0.7 % soft agar was prepared separately. For the preparation of soft agar 2 grams of Luria Bertani broth powder was measured and added to 500 ml of distilled water. Then the solution was heated to form a homogenous mixture until it was clear and formed bubbles. The solution was then autoclaved at 121°C for 15 minutes. After taking them out of autoclave they were stored at 4°C.

Preparation of Luria-Bertani Broth:

The Luria Bertani broth was prepared by weighing 25 grams of Luria Bertani broth powder in 1000 ml of distilled water. The mixture was then mixed well and autoclaved at 121°C for 15 minutes. After sterilization, the solution was stored at 4°C.

SM buffer:

SM buffer was prepared by adding 5.8 gram NaCl, 2 gram $MgSO4 \cdot 7H2O$, 50 ml Tris-Cl (1M, pH 7.4), and 5 ml Gelatin (2 % w/v) to a container and adding 1000ml distilled water. The buffer was autoclaved at 121°C for 15 minutes. After sterilization, the buffer was stored at room temperature.

70 % ethanol:

To prepare 70 % ethanol, deionized water was added to the 737 ml of 95 % ethanol to make a final volume of 1000 ml.

3.5 Bacterial culture:

All the bacterial cultures used in this project were collected from BRAC University. Bacterial samples were streaked on the fresh Luria Bertani agar plate and incubated for 16 hours at 37°C. After checking growth, plates were wrapped with parafilm and stored at 4°C for further use. Before each experiment, bacterial samples were freshly sub cultured and 16-hours cultures were used. By regular sub culturing, viability and purity of the organisms were maintained to ensure the validity of the study.

3.6 Bacteriophage isolation:

3.6.1 Phage isolation and enrichment from environmental water.

A total of 20 water samples were collected for this study in order to isolate lytic bacteriophage specific for *Vibrio cholerae*. Sewage water can be considered an ideal source from which lytic bacteriophages can be isolated since it contains high numbers of diverse bacteria as well as different ecosystems including raw input sewage in order to maximize the chance to find bacteriophages specific to *Vibrio cholerae*.

- Sewage water was collected from the specific aforementioned locations using in an autoclaved water collection bottle.
- Next, the sample as filtered using Whattman filter paper followed by syringe filtration (0.22 µm) using a syringe filter.
- **Cocktail preparation:**

  For preparation of bacterial cocktail, different strains of the same bacterial species were added in a vials containing 3ml of LB along with a single colony of the freshly cultured bacterial species. The inoculated broth was incubated for 2.5 hours in the shaker incubator in order to keep the young culture consistent in its lag phase.

- **Phage Enrichment:**
  A modified version of Twest and Kropinski (2009) was adopted for bacteriophage enrichment from collected water samples. The water samples were first well shaken for a couple of minutes then they were centrifuged at 10000 rpm for 10 minutes to remove sediment, large particulates, and bacteria. After 2.5 hours of incubation, the filtered water was added to the cocktail in the ratio of 1:1 and again incubated for 4 hours in the shaker

incubator. After the incubation period, the contents of the flask were centrifuged at 13000 rpm for 15 minutes. The clear supernatant was then filtered by passing through 0.22 μm low protein binding PES syringe filter and collected in another sterile falcon tube and stored at 4°C. It was consecutively tested for the presence of bacteriophage by spot test in different dilutions. For this study, the filtrate was diluted up to 10^-7.

- **Young Culture Preparation:**
  The specific strains of *Vibrio cholerae* were grown inoculating them individually in LB broth and incubated for 2 hours.

- **Double Layer Assay:**
  The prepared young bacterial culture (0.3ml) was mixed with 3ml of soft agar and poured into LA plate in order to create an even lawn of bacterial culture to perform a double layer assay.

- **Plaque Assay:**
  5 µL of each diluted sample was added drop wise in order to isolate phage from the collected water. Saline was used a negative control and JSF35 was used as a positive control.

3.6.2 Phage stock preparation:

After 24-48h incubation the plaque was collected and stored in SM Buffer accompanied with chloroform in the ratio of 10:1 and stored at 4°C.The next day phage lysate is enriched in the same phage isolation process and centrifuged at 13000 rpm for 15 minutes. The clear supernatant was collected into multiple sterile falcon tubes by filtering the supernatant through 0.22 µm low protein binding PES syringe filter membrane and stored at 4°C.

3.6.3 Phage titer determination:

The concentration or number of bacteriophage can be assumed by measuring its titer. The process can be carried out using the double layer agar method by Adams (1959). The phage

stock was subjected to serial dilution in saline up to 10^-8 and in some special cases upto 10^-16 of initial concentration. For carrying out this process, one hundred microliters of each dilution were taken into different sterile eppendorf tubes and diluted 100 times accordingly.

Correspondingly, a sterile vial was suffused with 3 ml of molten (48-50ºC) 0.4 % top agar along with 5 µL of the diluted phage stock. The contents were mixed carefully and pour on to fresh Luria Bertani agar plate without bubble formation, as bubbles in the agar could be misinterpreted as plaques. The plates were left to solidify the top agar layer. Then they were incubated at 37ºC overnight.

After overnight incubation, the plates were checked for plaque formation and plate with an individually distinguishable plaque was selected for phage titer determination. Counted plaque number was used to determine the titer of stock solution using the formula:

$$\text{Titre (PFU/ml)} = \frac{Number\ of\ plaque\ (PFU)}{\text{dilution x volume of phage added to plate (ml)}}$$

3.7 Bacteriophage DNA Isolation:

- Bacteriophages isolated from the representative single plaque were collected to and used for forming high titer stocks. The single plaque was stored in SM buffer. This solution was used for phage enrichment and DNA Isolation.
- Double layer assay was performed using 300 µl of specific host bacteria and 1mL of the specific bacteriophage on Luria-Bertani agar plates and incubate it overnight at 37^0C
- The upper layer of the soft agar containing the bacteria and the phage is collected using a sterilized spreader. Luria Broth was added in order to facilitate the process.
- The culture was collected in a sterilized falcon tube.
- The suspension was centrifuged at 10,000 rpm for 10 minutes at room temperature.
- Next the supernatant was collected and passed through a 0.22 µm syringe filter.
- Then, DNase was added at a concentration 1unit/100µl ratio of the supernatant. For creating a working solution of the DNase, 10X buffer in the solution is added at 1:10 ratio. The solution was mixed well and incubated at 37^0C overnight.
- Later, Proteinase-K was added at 5mg/ml ratio. Again for creating a working solution, 10X Proteinase-K buffer is added in the solution at 1:10 ratio. The solution is then incubated at 37^0C overnight.

- Next, Phenol-Chloroform and Iso-amyl Alcohol was added in the ratio 25:24:1 and gently mixed for 15 minutes by inverting tubes.
- The solution is then centrifuged at 14000 rpm for 15 minutes and the upper aqueous layer was collected in fresh and sterilized falcon tubes.
- Phenol-Chloroform and iso-amyl Alcohol was added again in the same ration and volume and centrifuged under same conditions.
- Later, double volume of absolute ethanol (cold at -20^0C) as added to the supernatant and incubated overnight at -20^0C.
- The solution was then centrifuged at 14,000 rpm for 15 minutes at 4^0C.
- Next, the supernatant was removed and the pellet was washed with cold 70% ethanol.
- Finally, the pellet is dissolved in Tris-EDTA buffer for storage.

3.8 Bacteriophage characterization

3.8.1 Host range determination

Host range is defined as the number of host species that can be used by a pathogen. This study can be used to administer an understanding about pathogen epidemiology and pathogenicity. Host range conditions the transmission dynamics and survival of pathogens and can be used to predict a major function relating to their evolution. The host range of a pathogen helps to infer how diversification and speciation occur, the incorporation of new species into the number of hosts a pathogen can infect, shifting among hosts in response to biotic and abiotic environmental variation, or the ability to persist in the environment between epidemics.

The host range determination was performed according to Verma et al. (2009). Double layer assay was done by using 3mL of 0.4 % warm (48-50ºC) top agar was mixed with 300 µl of each specific bacterial culture in different sterile vial and poured on separate fresh LA plate. After the top layer of the soft agar solidified, 5 µl of the high titer phage stock was spotted dropwise along with 5 µl of sterilized saline as control. The plates were then incubated for 24 hours at 37ºC and checked for the presence of bacterial lysis. Different bacterial cultures (Table 3.8.1) were used to assess the host range of isolated bacteriophage. the bacterial strains used for determining the host range are-

Table 3.1 Bacterial strains used for host range determination

Bacterial strain	Source
Vibrio cholerae C7606(1270)	BRAC University
Vibrio cholerae WT 376 VOI.17	BRAC University
Vibrio cholerae WT335 VIII6	BRAC University
Vibrio cholerae WT376 VOI.17	BRAC University
Vibrio cholerae WT 371 VOI.17	BRAC University
Vibrio cholerae WT 375 VOI.17	BRAC University
Shigella dysentery	BRAC University
Shigella flexneri	BRAC University
Salmonella typhii	BRAC University
Salmonella paratyphii	BRAC University
Klebsiella sp.	BRAC University
E.coli 0157	BRAC University
E.coli ATCC:25922	BRAC University
Bascillus cereus	BRAC University
DH5α	BRAC University
Streptococcus aureus	BRAC University
Streptococcus pyogens	BRAC University
Pseudomonas aeruginosa	BRAC University
E.coli K12	BRAC University
E.coli ATCC:13706	BRAC University

3.8.2 Thermal stability test:

To determine the propagation of bacteriophages at different temperatures, titers of 1ml phage lysates were assayed after storage at 35 ºC, 45 ºC, 55 ºC,60 ºC,70 ºC and incubated for 2 hours. Up to Ten-fold dilutions of phage stocks were prepared in Luria Bertani Broth and spotted on the

lawn of the host strain, prepared by the double agar layer method. Efficiency of plating was assessed relative to results obtained at 37 °C.

3.8.3 pH stability test:

The effect of an acidic and an alkaline pH on phage particles were studied using Nutrient Broth adjusted with a pH 1 through pH12 according to Verma et al. (2009) with a slight modification. 1M NaOH and 1M HCl were used to adjust the pH of the Nutrient Broth to achieve the required pH for the study. One milliliter phage suspension was added with 9 ml of pH adjusted medium and incubated for 3 hours at 37ºC. After the incubation period, phage titer was determined by double layer agar method against host bacteria *Vibrio Cholerae*. After incubation, a serial 10-fold dilution were conducted and done for plating. To determine phage stability in various pH levels, phages incubated in the medium of pH 7 were used as a control.

3.8.4 Effect of Organic Solvent:

The effect of two different organic solvents: ethanol and chloroform on phage virions, were studied. Phage suspension was mixed with equal volume of organic solvent separately and incubated at room temperature for 1 hour with shaking by hand from time to time. After incubation, the mixtures were centrifuged at 10000 rpm for 10 minutes. Phage suspended in SM buffer and held in 4ºC was considered as control and the results were compared to it for relative inactivation of phage by organic solvents. In the next step, 10-fold dilutions in Saline were prepared and used for plating.

3.9 Bacteriophage Identification

3.9.1 Plaque Assay

When a suspension of an infective phage is spread over the lawn of susceptible bacterial cells the phage attaches to the bacterial cell, replicate inside it, and kills it during its lytic release. Lysis of the bacteriophage is indicated by the formation of a zone of clearing or plaque within the lawn of bacteria. On the other hand, if the phage is not a lytic bacteria rather lysogenic then it will not give a clear plaque as required.

Each plaque corresponds to the site where a single bacteriophage acted as an infectious unit and

initiated its lytic cycle. The spread of infectious phage from the initially infected bacterial cell to the surrounding cells results in the lysis of the bacteria in the vicinity, eventually forming the plaque that is large enough to be visible to the naked eye. Plaques do not continue to spread indefinitely. The size of the plaque formed depends on the virus, the host, and conditions of culture.

The number of plaques that develop and the appropriate dilution factors can be used to calculate the number of bacteriophages i.e. plaque forming units (PFU) in a sample. It is calculated as previously stated,

$$\text{Titre (PFU/ml)} = \frac{Number\ of\ plaque\ (PFU)}{\text{dilution x volume of phage added to plate (ml)}}$$

Plaque assay is an immediate and easy method for identifying the presence bacteriophage.

3.9.2 Gel electrophoresis

Gel electrophoresis is a laboratory technique used to separate charged molecules. DNA is negatively charged and therefore, under the force of an electric current, migrates across the gel towards the positive electrode, through the matrix of both agarose gel and polyacrylamide gel. Molecules separated by size, with the smaller ones moving more rapidly through the gel than the large ones. Purified agarose is a powder that is insoluble in room temperature water (or buffer) at room temperature but becomes liquid in boiling water (or buffer). As it cools, the agarose sugars undergo cross linking with each other and cause the solution to "gel". Higher concentrations of agarose give firmer gels. As a result, smaller molecules like DNA can pass through it but as the length of the DNA increase it gets difficult for the molecules to pass through the gel. Similarly, acrylamide is a non-ionic, water soluble, and biocompatible polymer that can be synthesized as a simple linear chain or as a cross-linked structure. PAM increases the viscosity of water and encourages the flocculation of particles present in water. The cross-linked polymer can absorb and retain extremely large amounts of water because the amide groups form strong hydrogen bonds with water molecules. The hydrated PAM is a soft gel that is used in gel electrophoresis and as a

super water absorbing polymer (SAP's). DNA is visualized by including in the gel an intercalating dye, ethidium bromide (EtBr). After completion of staining and destaining respectively, the gel is exposed to gel documentation machine, the DNA bands fluoresce and this was seen as an image using "Vision capt" software into a device which was directly connected with the gel documentation machine.

3% agarose gel was prepared in the process of,

1. Measuring with analytical balance .4 (1%) grams of agarose.
2. Adding the agarose powder with 40 ml of TAE buffer (1x) and heated in the microwave for 1 minute to dissolve the powder with the buffer to give a clear solution.
3. The heated molten agar was allowed to cool. Then it would be made into a gel which was produced by pouring into casting trays and a comb was set in that tray, so that wells can be produced to load samples.

For Staining the bands, ethidium bromide was added to the gel when it had cooled down before pouring it into the casting tray. It is used because upon binding of the molecule to the DNA and illumination with a UV light source, the DNA banding pattern can be visualized. The mode of binding of EtBr is intercalation between base pairs. Ethidium bromide- DNA complexes display increased fluorescence compared to the dye in solution. This means that illumination of a stained gel under UV light allows bands of DNA to be visualized against a background of unbound dye. Gel results can either photographed with an instant camera or visualized by means of commercially available image analysis tools.

The procedure for carrying out electrophoresis consisted of the following steps,

- The agarose gel and TAE buffer was prepared prior to loading samples onto the gel.
- 5 µL of each sample (isolated DNA/PCR product/Digested Product) were diluted with 6x loading dyes and loaded in the wells.
- Electricity was applied to the apparatus and let run for 50 minutes-1 hour, as per requirement at 70 volts.
- Once the electrophoresis was over, the gel was visualized under UV light and the results are interrupted accordingly.

3.9.3 Restriction Fragment Length polymorphism (RFLP)

A restriction enzyme cuts DNA in some of its specific sites or restriction recognition sequences. Most of the restriction recognition sequences are palindromic and vary in lengths between 4 and 8 nucleotides. The working mechanism of a restriction enzyme involves making two incisions. One each through the sugar-phosphate backbone (i.e. each strand) of the DNA double helix. Moreover, some restriction enzymes cut the double stranded DNA in two different positions and generate ends that are staggered, with 5' or 3' protruding terminal nucleotide whereas others cut at the same position and produce blunt ends.

In this RFLP process, the isolated raw phage DNA were digested with

The contents added for carrying out the process of RFLP are mentioned below in the table,

- The added reagents were mixed gently and incubated at 37°C, overnight.
- Later on, gel electrophoresis was performed in order to observe the fragments of DNA.

3.9.4 Polymerase Chain Reaction(PCR)

The technique of PCR is performed to amplify specific regions of a DNA strand. The target DNA strand can be any specific gene, any part of a gene, or a non-coding sequence. Most PCR methods typically amplify DNA fragments of up to 10 Kb, although some techniques allow for amplification of fragments up to 40 kb in size. A basic PCR set up requires several components and reagents. These components include:

- DNA template
- Primers, which are complementary to the DNA regions at the 5' or 3' ends of the DNA region. Two primers are used in this case, a forward and a reverse primer.
- Taq polymerase to amplify the DNA
- Deoxynucleoside triphosphates (dNTPs), the building blocks from which the DNA polymerases synthesize a new DNA strand.

- Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.

The PCR generally consists of a series of 20 to 40 repeated temperature changes called cycles; with each cycle typically consisting of 2-3 discrete temperature steps. Most commonly PCR is carried out with cycles that have three temperature steps

Denaturation step: The denaturation step consists of heating the reaction to 94-98°C for 30 seconds. It causes melting of DNA template and primers by disrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA.

Annealing step: The reaction temperature is lowered to 50-65°C for30 seconds allowing annealing of the primers to the single-stranded DNA template.Typically the annealing temperature is about 3-5 degrees Celsius below the Tm of the primers used. Stable DNADNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.

Extension/elongation step: At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction,

Final elongation: This single step is occasionally performed at a temperature of 70- 74°C for 5-15 minutes after the last PCR cycle to ensure that any remaining single stranded DNA is fully extended.

Final hold: This step at 4-15°C for an indefinite time may be employed for short-term storage of the reaction.

Preparation of master mix:

- As our aim was to differentiate whether this *Vibrio cholerae* samples were ICP1 or ICP3 type of phage, we had to use reverse and forward primer of *jsf 35* and *jsf 2* classified as the ICP3 and ICP1 phage respectively, from a study done at icddr, b by Faruque et. al. The amplification was done for no specific gene. Rather the correct amplification was supposed

to give a distinct band at approximately 200bp. The forward primer used for *jsf 2* was 5' AGC GGT AGT GTT CCA AT 3' and the reverse primer used was 5'- ATG GTC GCG TTG TTA CAT CA -3' and the forward and reverse primer used for *jsf 35* was 5'-AGA CAA GCG AAG AGG GTT GA -3' and 5'- ACG TTG AAC GGT AGG ATT GC-3' respectively.

- After the preparation of the master mix,
- The components were mixed gently and placed in a thermo cycler.
- The denaturation temperature of cycler was 95°C for 1minute, annealing temperature was 53°C for 30 seconds and elongation was 70°C for 1 minute.
- Finally, after the PCR was completed 10µl of solution was loaded in an agarose gel and the result was interpreted.

Chapter 4

Results

4.1 Isolation and purification of bacteriophage:

4.1.1 Plaque Assay

20 water samples were collected and enriched for isolation of *Vibrio cholerae* specific bacteriophage. The 10 strains used for screening vibrio phages were WT-324 V11.16,006-V1015, WT333V11.16, WT1667, WT1774,WT 373 VO1.17,WT335 V1116, WT376 VO1.17,WT371 VO1.17,WT375 VO1.17. 6 vibrio *cholerae* specific bacteriophage were obtained in the process of isolation. Among all of the isolated results there were 4 of them that gave clear lytic zone. The analysis of the enriched sample water as carried out by double layer assay, concluded by the conformation of finding individual plaque in different dilutions. The isolated plaques that gave the clearest and isolated individual bacteriophage, these were selected for characterization, identification and further study. The process of sampling commenced from January 2019-August 2019,

Table 4.4.1 Bacteriophage isolation from environmental samples

Date for Water Collection	**Location**	**Bacteriophages isolated from the specific strains of *Vibrio cholerae***
28th January'19	Gulshan Lake	-
05th february'19	Bosila	-
10th February'19	Dhanmondi Lake	WT-324 V11.16, WT 1774, WT 1667, 006-V1015
16th february'19	Turag	-
10th march'19	Gulshan Lake	-
12th march'19	Bosila	WT 327
13th March'19	Dhanmondi Lake	WT327
17th March'19	Turag river	-
2nd April'19	Gulshan Lake	-
20th May'19	Gulshan Lake	-
21st May'19	Turag River	-
17th June'19	Bosila River	-
18th June'19	Dhanmondi Lake	-
9th July'19	Gulshan Lake	-
10th July'19	Dhanmondi Lake	-
20th July'19	Turag River	-
1st August'19	Bosila River	-
2nd August'19	Gulshan Lake	-

17th August'19	Gulshan Lake	-

The isolated phages were named with smaller initials in order they were easier to identify or refer to in the rest of the results,

Bacteriophage isolated from WT324, WT1667, WT1774 and WT006 were named as P1_324, P2_1667, P3_1774 and P4_006 respectively.

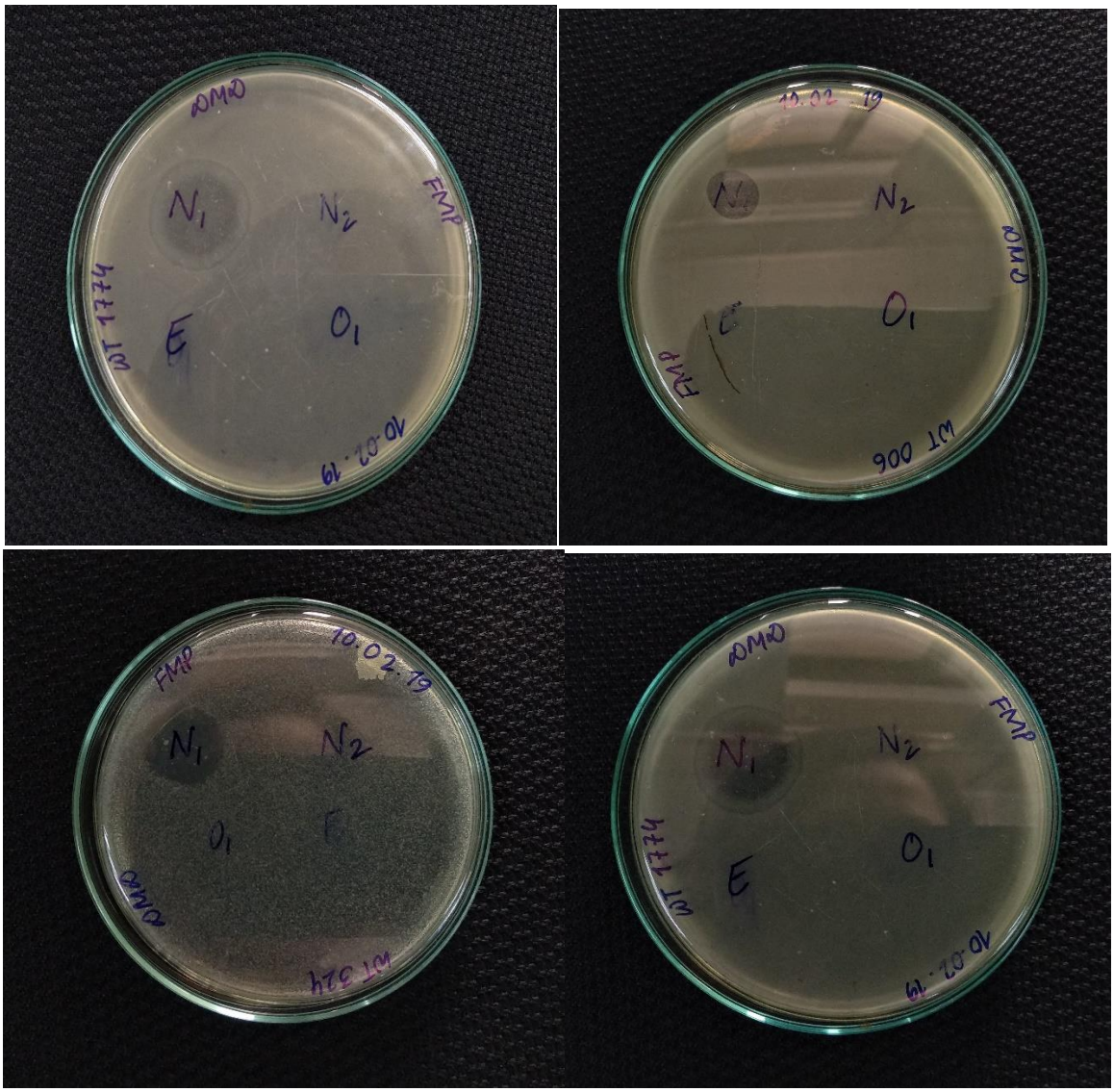


Fig 4.1.1- Plaque Assay from initially collected water sample

4.1.2 Plaque Morphology

The isolated phages as P1_324, P2_1667 and P4_006 gave circular individual plaques against host bacteria in determinant process of double layer assay. The plaques were roughly 3mm in diameter. Whereas, the plaque isolated from P4_1774 had two circular layers with an inner, translucent layer of 3 millimeters width and a completely clear center of 2 mm width.

4.1.3 Bacteriophage Titer determination

After the initial purification, the isolated age was enriched and phage titer was determined by DLA method and gave up to 10^6,10^8, 10^{15} and 10^3 pfu/ml for phages P1_324, P2_1667,P3_1774 and P4_006 respectively.

4.2 Bacteriophage characterization

4.2.1 Host range specificity:

The study of host range specificity in isolated bacteriophages P1_324, P2_1667, P3_1774 and P4_006 were performed in order to a get better understanding about their ability to infect and produce a lytic zone against different bacteria of varied of species. The study was performed by applying plaque assay on different bacterial strains (Table-3.1). The bacteriophages P1_324, P2_1667, P3_1774 and P4_006 showed varied results and some of the were incoherent. The result of host range specificity test for isolated bacteriophages P1_324, P2_1667, P3_1774 and P4_006 are as follows:

Table 4.2.1 Host specificity range for isolated phages

Bacterial strain	P1_324	P2_1667	P3_1774	P4_006
Vibrio cholerae C7606(1270)	+	+	+	+
Vibrio cholerae WT 376 VOI.17	-	-	-	-
Vibrio cholerae WT335 VIII6	-	-	-	-
Vibrio cholerae WT376 VOI.17	-	-	-	-

Vibrio cholerae WT 371 VOI.17	-	-	-	-
Vibrio cholerae WT 375 VOI.17	-	-	-	-
Shigella dysntery	-	-	-	-
Shigella flexneri	-	-	-	-
Salmonella typhii	-	-	-	-
Salmonella paratyphii	-	-	-	-
Klebsiella sp.	-	-	-	-
E.coli 0157	+	-	+	+
E.coli ATCC:25922	+	-	+	+
Bascillus cereus	-	-	-	-
DH5α	-	-	-	-
Streptococcus aureus	-	-	-	-
Streptococcus pyogens	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-
E.coli K12	-	-	+	-
E.coli ATCC:13706	-	-	-	-
V. cholerae WT 324 VOI.17	+[a]	+	-	+
V. cholerae WT 1667 VOI.17	+	+	+	+
V. cholerae WT 1774 VOI.17	+	-	+	+
V. cholerae WT 006 VOI.17	+	+	-	+

The phage P1_324 and P3_006 was able to lyse two different strains of *E. coli* and another strain of *V. cholerae*. Additionally, the phage P3_1774 was able to lyse one different strain of *V. cholerae*

and three different strains of *E. coli*. One the other hand, P2_1667 has a very specific host range that is only limited to only another strain of *V. cholerae*.

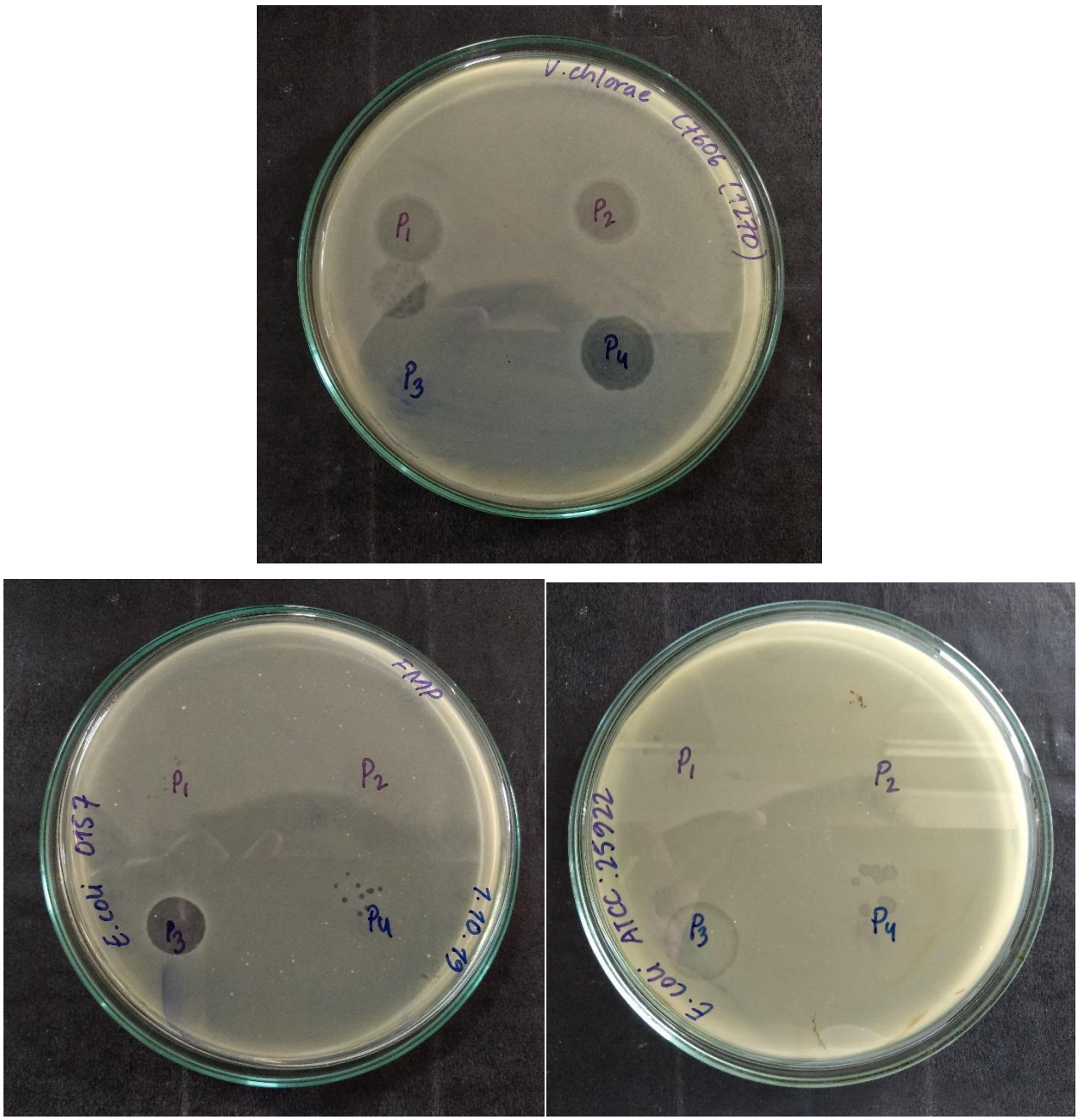


Fig 4.2.1(1) Host specificity for the isolated bacteriophages

Furthermore, when plaque assay was performed in within the four specific host from which they were initially collected, we observe, the phage P1_324 and P4_006 formed consistent plaques

among all the rest three. Consecutively, phage P2_1667 gave results for two of the *Vibrio* isolates and phage P3_1774 gave plaque for only one of the isolates.

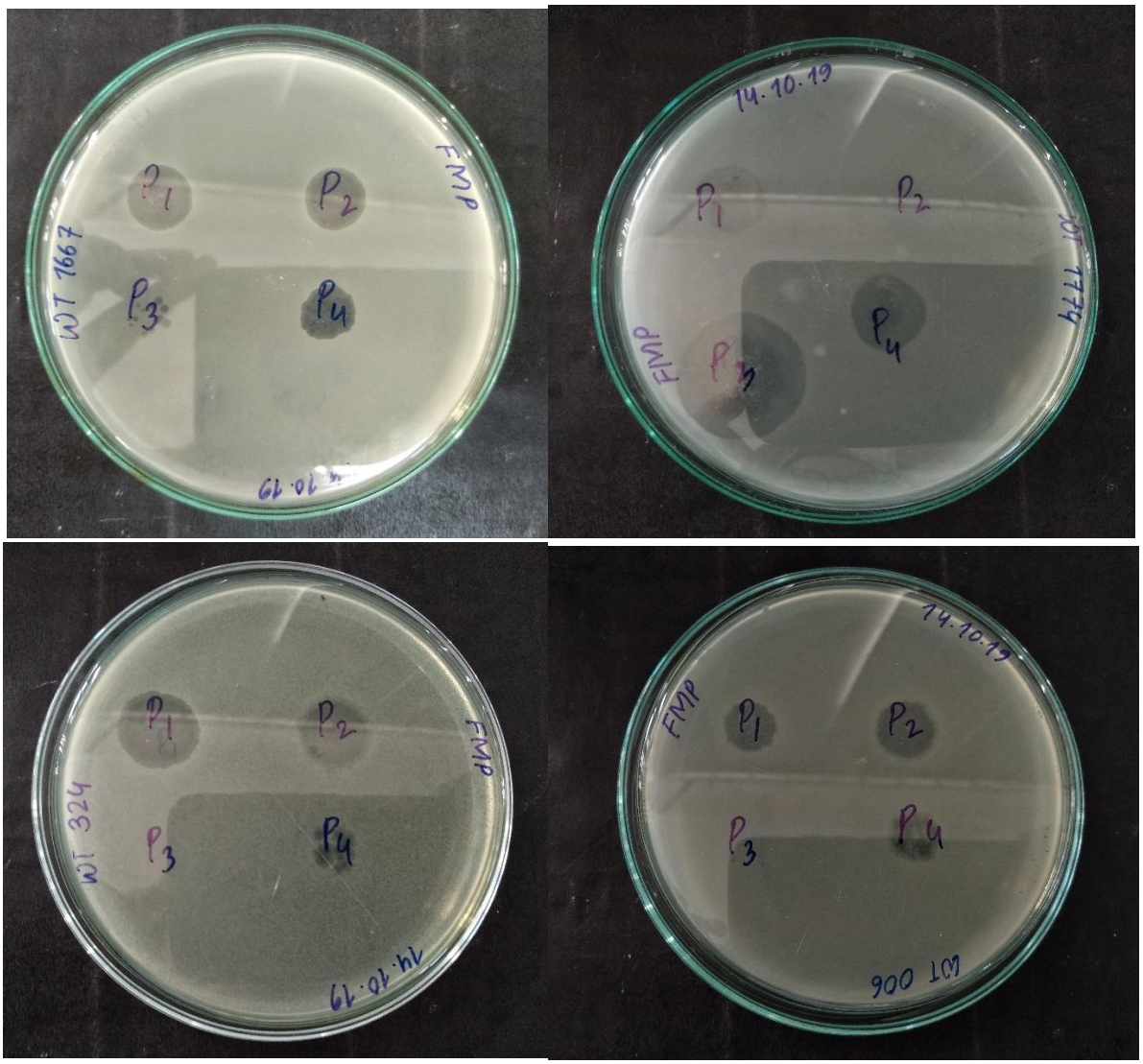
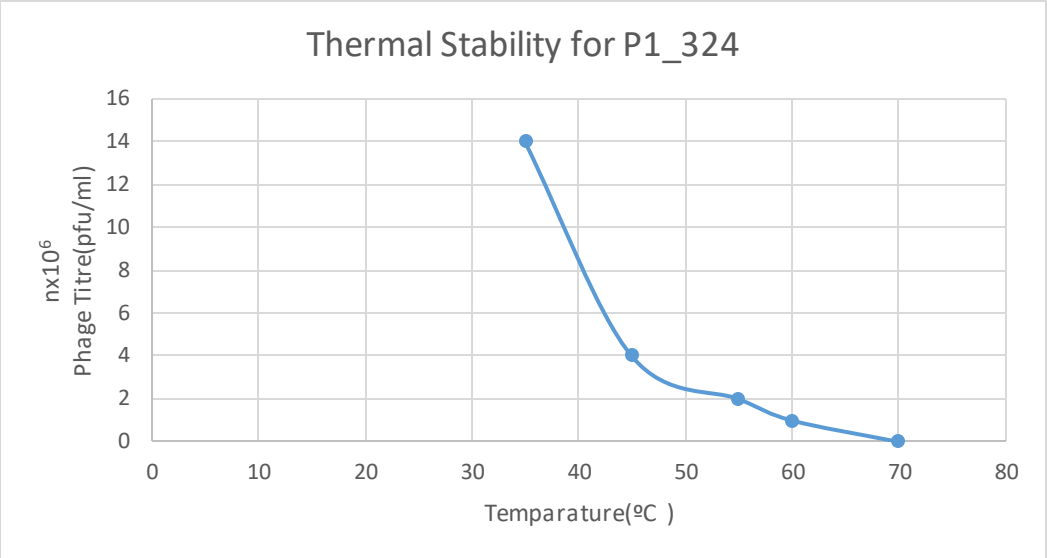


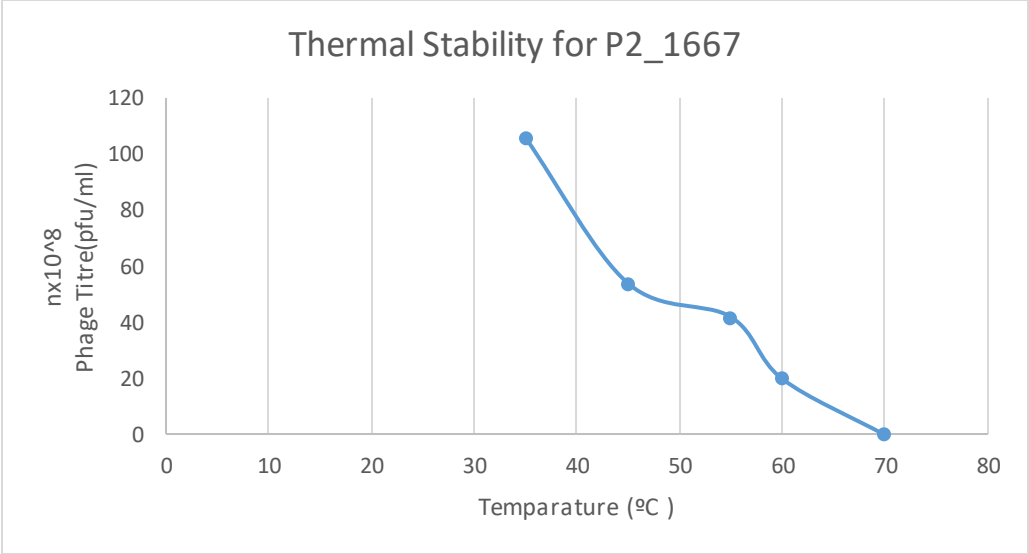
Fig4.2.1(2)- The host specificity result for particular strains from which plaques were initially isolated.

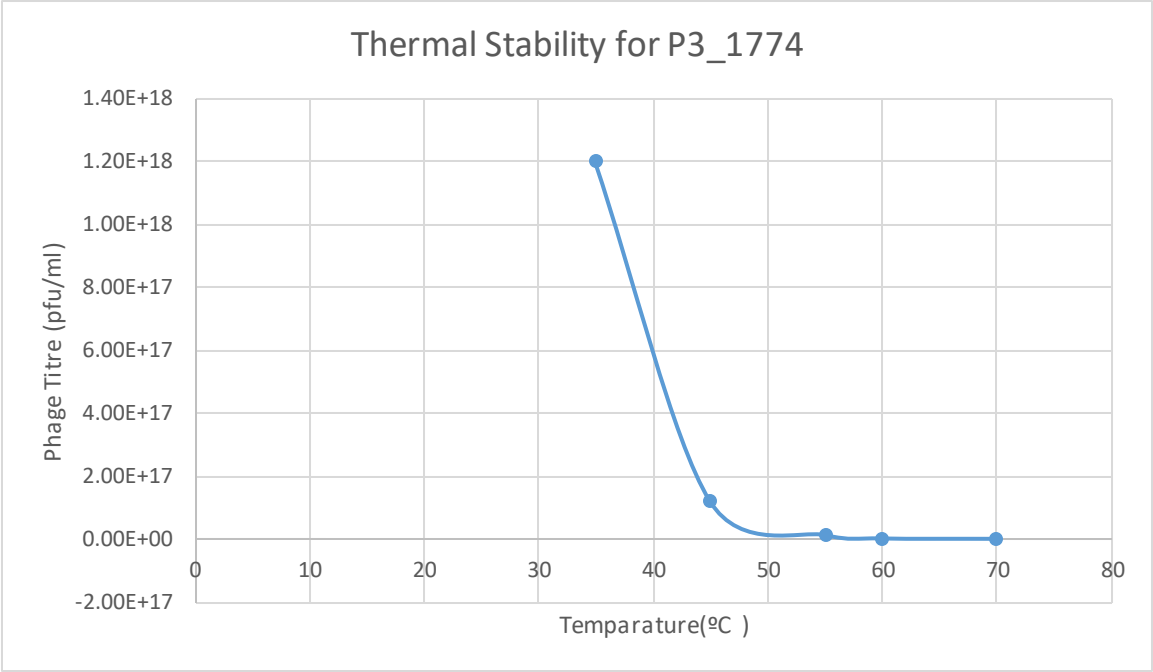
+[a] The positive result for this particular strain as nullified as the phage was initially isolated and from the specific strain of Vibrio cholerae, hence, keeping the result homogenous.

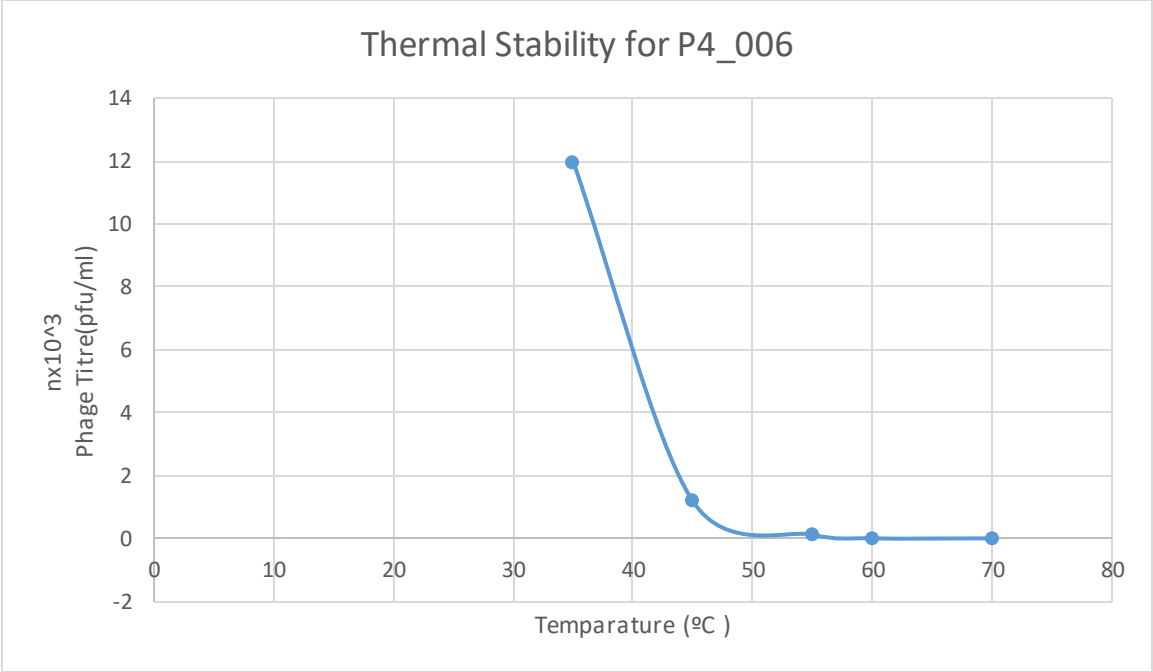
4.2.2 Thermal stability:

The thermal stability test was performed in order to measure the heat resistance capability of the phages, P1_324, P2_1667, P3_1774 and P4_006. The procedure was carried out after incubating the phages for 2 hours at the temperature 35 ºC, 45 ºC, 55 ºC,60 ºC and 70 ºC.


Thermal Stability for P1_324
nx10⁶ Phage Titre(pfu/ml)
16
14
12
10
8
6
4
2
0
0
10
20
30
40
50
60
70
80
Temparature(ºC)


Thermal Stability for P2_1667
nx10^8 Phage Titre(pfu/ml)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
Temparature (ºC)


Thermal Stability for P3_1774
1.40E+18
1.20E+18
1.00E+18
8.00E+17
6.00E+17
4.00E+17
2.00E+17
0.00E+00
-2.00E+17
Phage Titre (pfu/ml)
0
10
20
30
40
50
60
70
80
Temparature(ºC)


Thermal Stability for P4_006
14
12
10
8
6
4
2
0
-2
nx10^3
Phage Titre(pfu/ml)
0
10
20
30
40
50
60
70
80
Temparature (ºC)

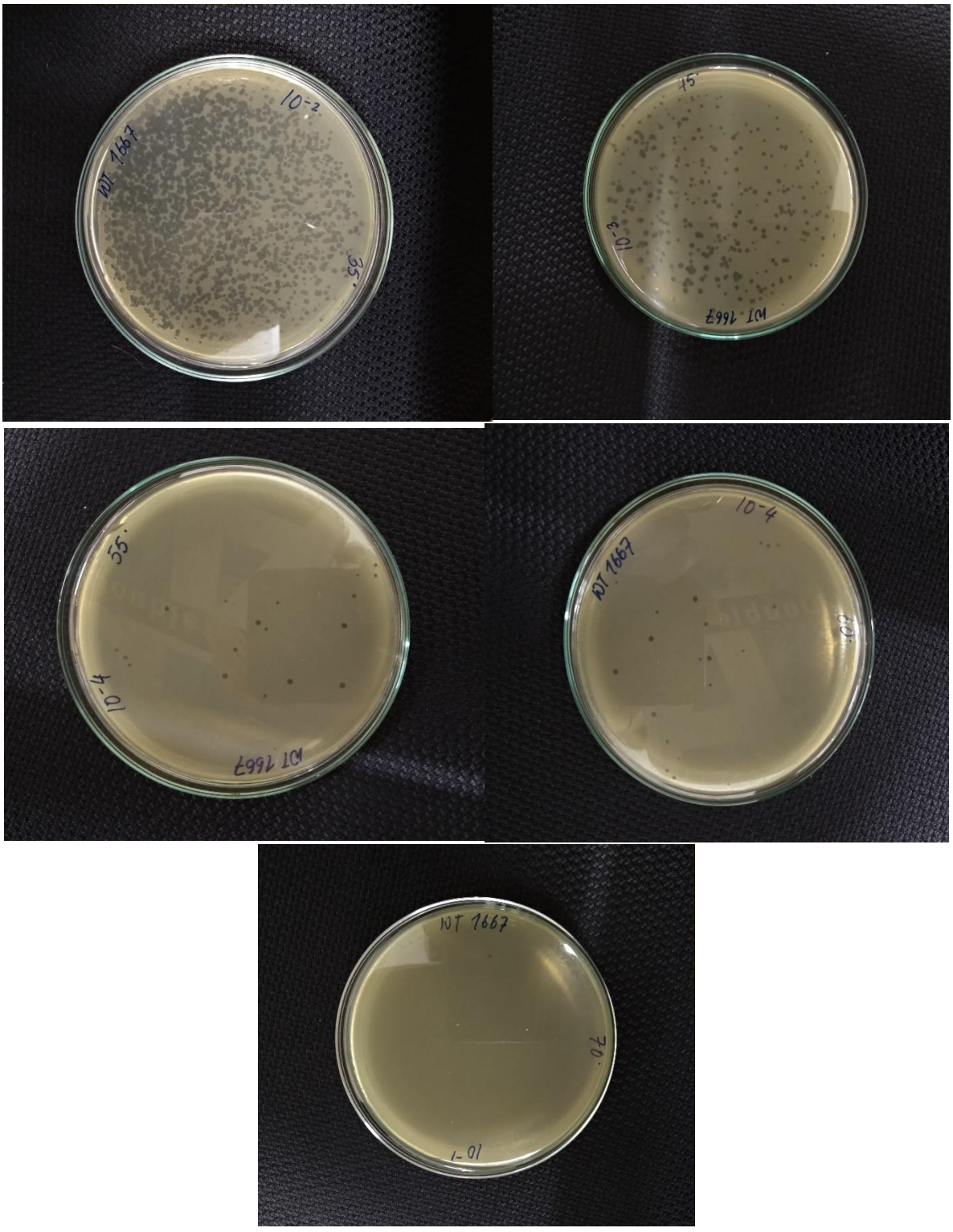


Fig 4.2.2- Thermal Sensitivity for isolated phages

Table 4.2.1 Titer determination of isolated phages at different temperatures

Temperature	**P1_324**	**P2_1667**	**P3_1774**	**P4_006**
35 ºC	$20x10^6$	$106x10^8$	1.20E+18	$20x10^3$
45 ºC	$14x10^6$	$54x10^8$	1.20E+17	$1.2x10^3$
55 ºC	$4x10^6$	$42x10^8$	1.20E+16	$.12x10^3$
60 ºC	$2x10^6$	$20x10^8$	1.20E+15	0
70 ºC	0	0	0	0

Again, the individual chart we observe that the isolated phages show a homogenous result, which is, the infectivity of the phages remain functional within 35 ºC to 55 ºC whereas with the progressing temperature up to 60 ºC all the phages show a sharp decreased pathogenicity and further approaching to 70ºC they show no infectivity at all.

Standard Deviation:

The standard deviation is calculated in order to observe the dispersion of the value from the actual number. So, to understand the deviation of the numbers we calculate the percentage of the deviation which will give us an idea how the percentage of the numbers are differentiating.

The standard deviation is calculated with the formula,

$$\sigma = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(x_i - \mu)^2}$$

Where,

μ=population mean

δ= standard deviation

N=total population

x_i= each value from the population

After normalizing the values in order for easier calculation, the standard deviation from the isolated phages P1_324, P2_1667, P3_1774 and P4_006 were,

$\delta_{P1\_324} = \sqrt{\frac{296}{5}} = 8.60$

$\delta_{P2\_1667} = \sqrt{\frac{8072.162}{5}} = 40.18$

$\delta_{P3\_1774} = \sqrt{\frac{}{5}} = 5.24181E+17$

$\delta_{P4\_006} = \sqrt{\frac{310.54}{5}} = 8.81$

Now, for calculating the percentage deviation to that of the general titer to get the percentage of increase or decrease of the isolates we take the difference of the titer and the standard deviation and divide the substrate to the product of the titer and 100. So, the percentage deviation of the isolates was,

% δ P1_324= -5.7%

% δ P2_1667=-6.20%

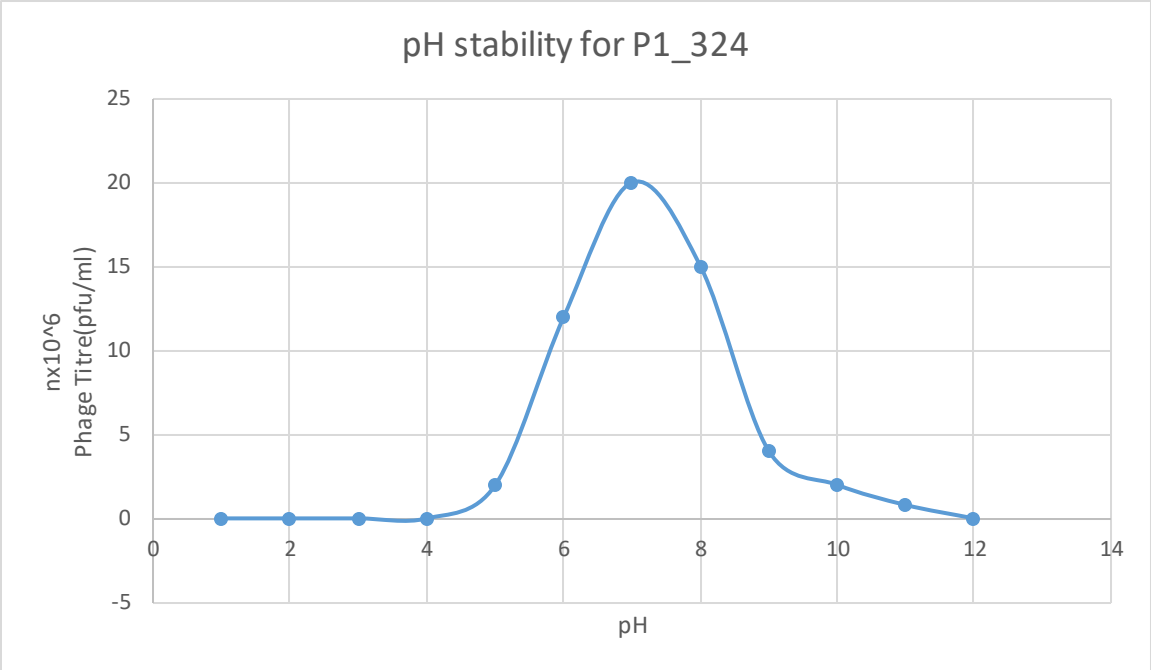
% δ P3_1774= -6.29 %

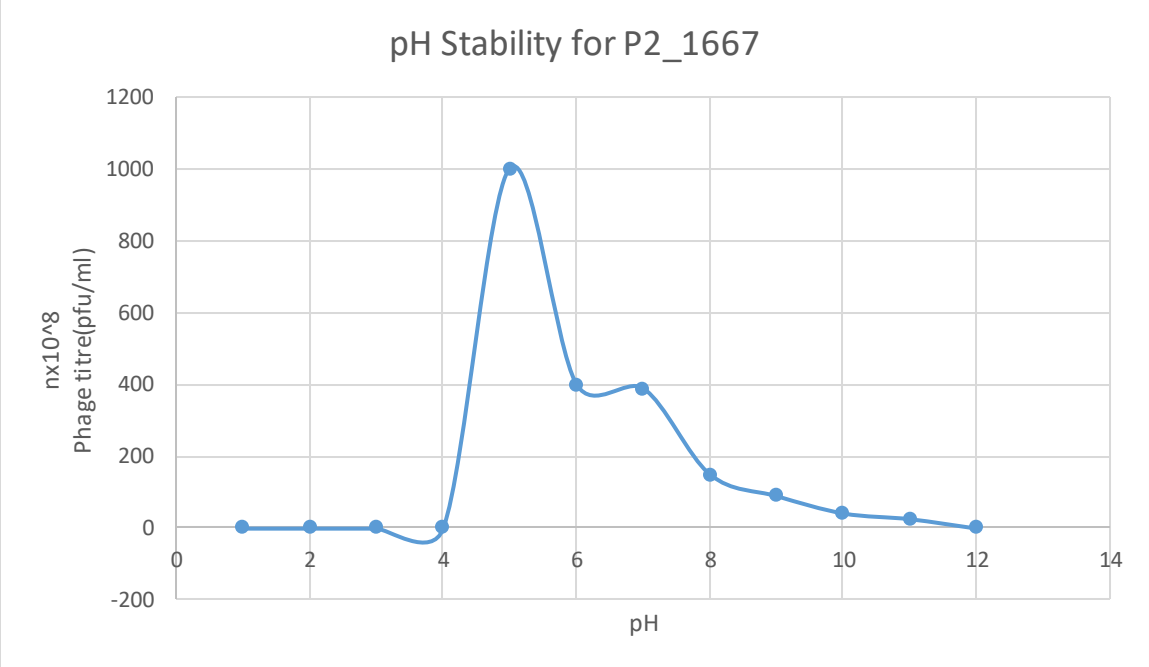
% δ P4_006= -5.59%

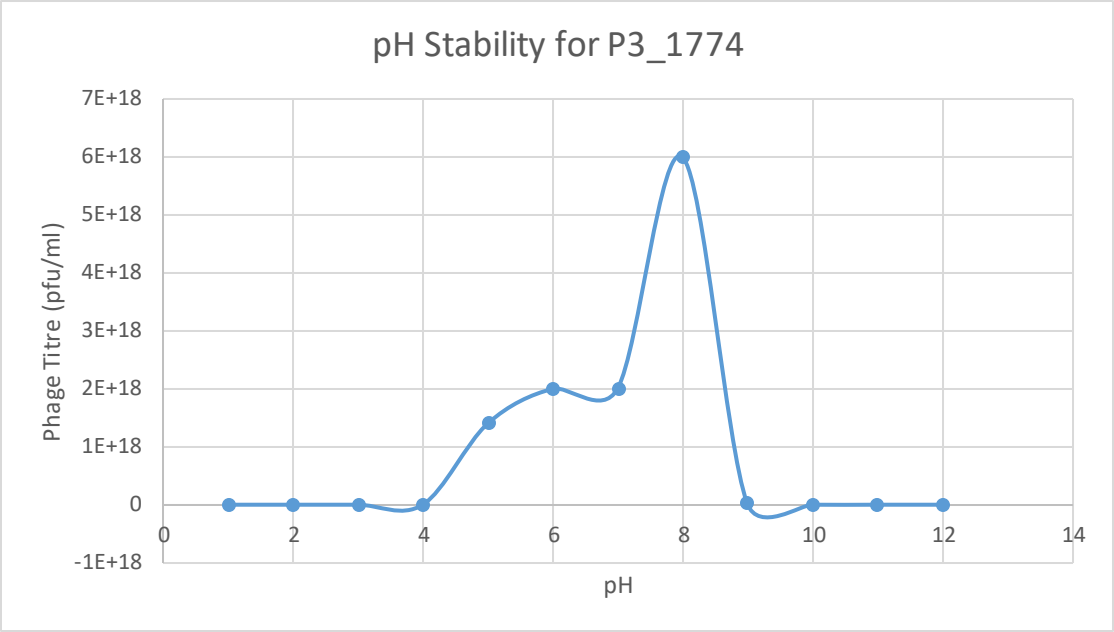
From the minus (-) sign we understand the number is decreasing and the number denotes the percentage of decrease. Hence, from the percentages we observe that the number of the titer is decreasing in the isolates by the aforementioned percentages.

4.2.3 pH stability

pH stability test as performed to determine activity of the isolated phages in a large array of pH. For pH stability test, the isolated phages were incubated in the adjusted nutrient broth for 3 hours. The separate graphs for each isolated phage is given below,


pH stability for P1_324
nx10^6
Phage Titre(pfu/ml)
25
20
15
10
5
0
-5
0
2
4
6
8
10
12
14
pH


pH Stability for P2_1667
nx10^8
Phage titre(pfu/ml)
1200
1000
800
600
400
200
0
-200
0
2
4
6
8
10
12
14
pH



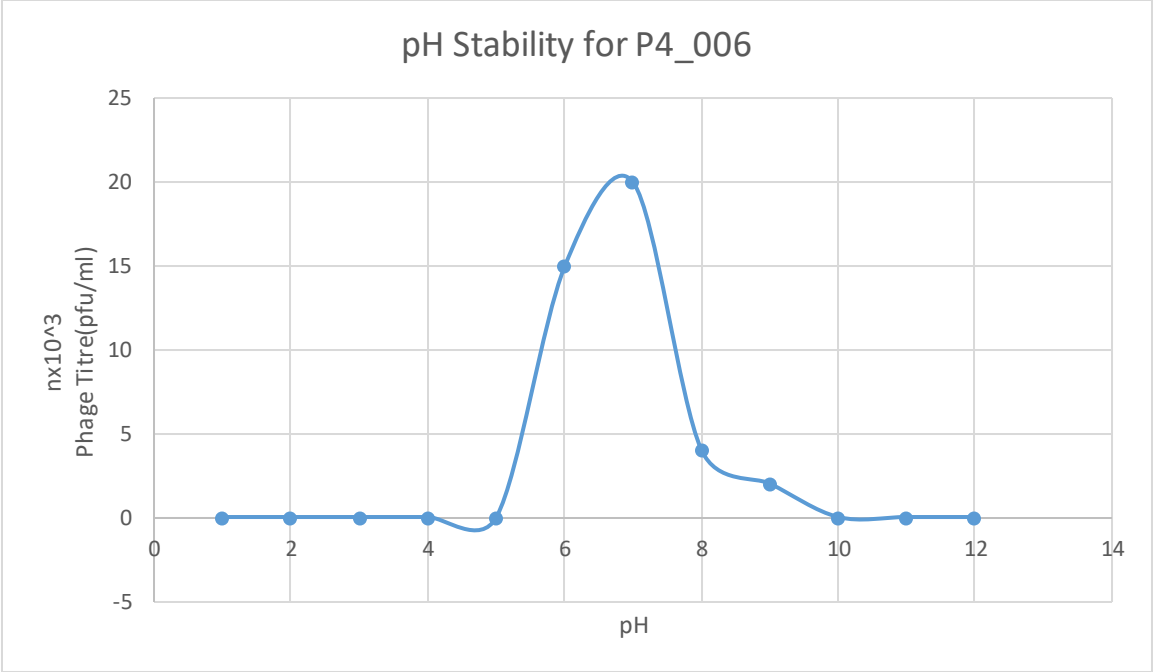


Table 4.2.3 Titer determination of isolated phage at different pH

pH	P1_324	P2_1667	P3_1774	P4_006
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	$0.006x10^8$	2.00E+12	0
5	$2x10^6$	$1000x10^8$	1.40E+18	0
6	$12x10^6$	$400x10^8$	2.00E+18	$15x10^3$
7	$20x10^6$	$106x10^8$	2.00E+18	$20x10^3$
8	$15x10^6$	$150x10^8$	6.00E+18	$4x10^3$
9	$4x10^6$	$90x10^8$	2.00E+16	$2x10^3$
10	$2x10^6$	$42x10^8$	6.00E+18	0
11	$0.8x10^6$	$26x10^8$	2.00E+14	0
12	0	0	0	0

From the pH test it was evident that the isolated phages were stable within the pH range 6.0-8.0. The isolated phages have no reactivity at all in lower pH within pH1-3 and extreme pH of pH12. Although the reactivity of the phages decline after pH9 but it is not fully destroyed. The phages P1_324, P2_1667 and P3_1774 has a high titer value even at pH11, which can be beneficial for its study. Hence we can interpret that, the phage isolated are actively portraying their pathogenicity within pH7-11.

Standard Deviation

The standard deviation of the values for the pH variation for the isolates are calculated below,

$$\delta_{P1\_324}=\sqrt{\frac{534.12}{12}}=6.968$$

$$\delta_{P2\_1667}=\sqrt{\frac{907207.03}{12}}=290.76$$

$$\delta_{P3\_1774}=\sqrt{\frac{572}{12}}=2.2698E+18$$

$$\delta_{P4\_006}=\sqrt{\frac{504.95}{12}}=6.78$$

For determining the percentage deviation, the same formula is used. The percentage deviation of the isolates is given below,

$\% \delta_{P1\_324} = -6.52\%$

$\% \delta_{P2\_1667} = 0.017\%$

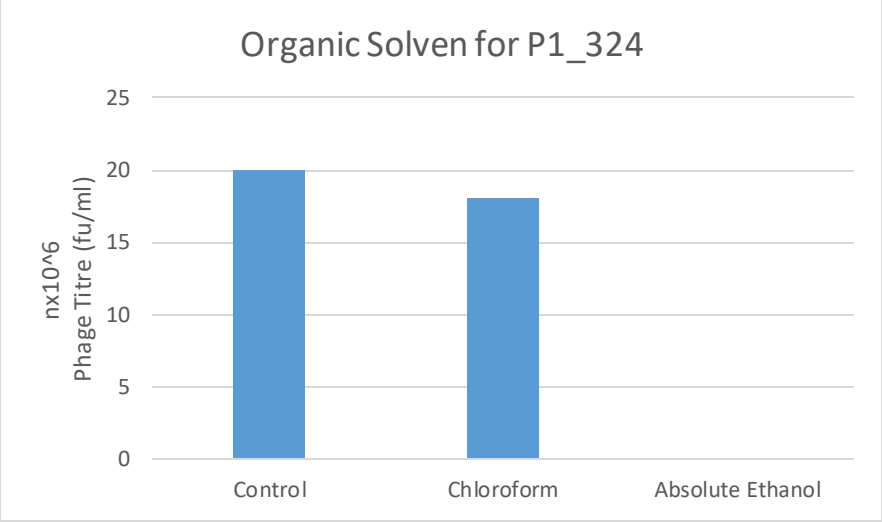
$\% \delta_{P3\_1774} = -6.21\%$

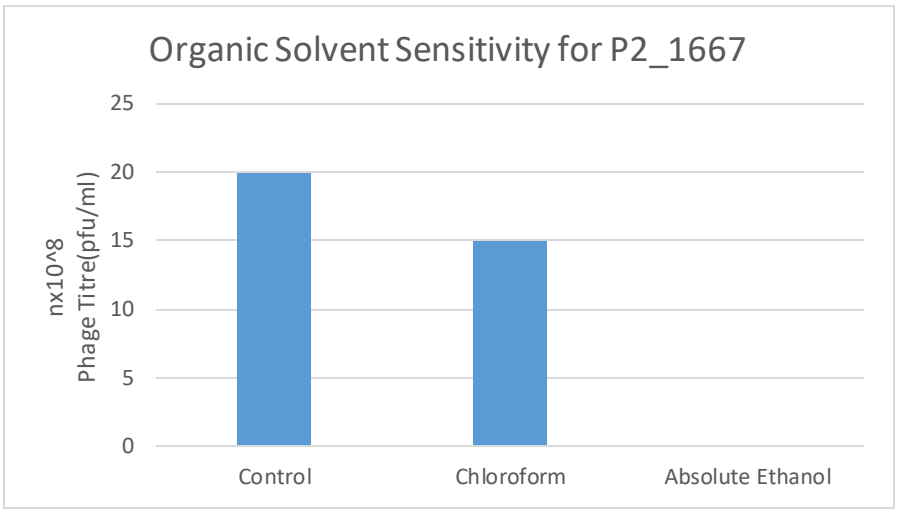
$\% \delta_{P4\_006} = -6.61\%$

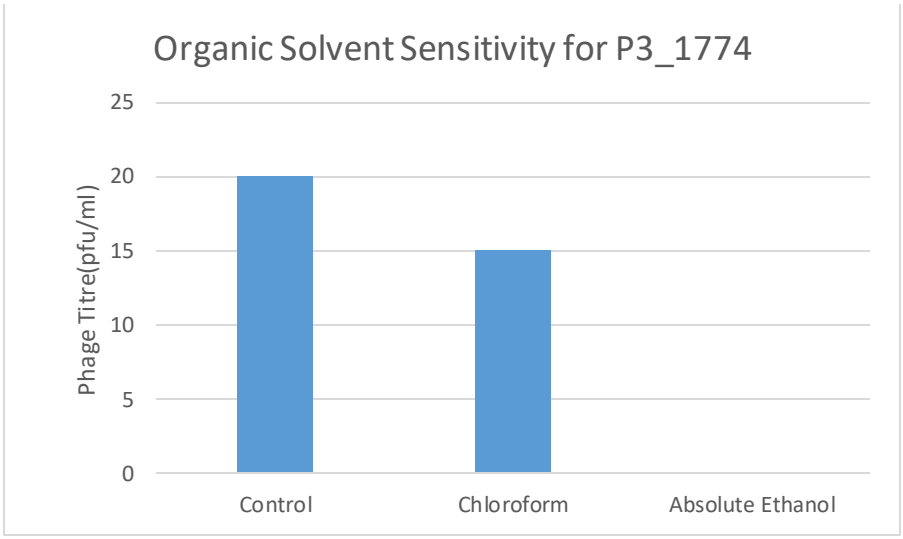
Hence, the negative sign denotes the decrease and the number denotes the percentage in which the isolate titers are decreasing.

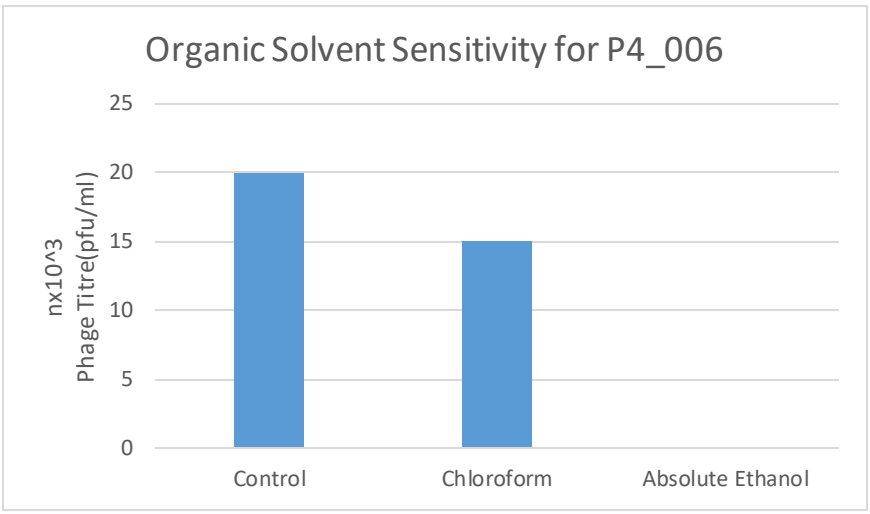
4.2.4 Organic solvent sensitivity

The sensitivity of the isolated phages was observed against the organic solvents chloroform and ethanol. The isolated were incubated for 60 minutes in chloroform and the results are,




Organic Solvent Sensitivity for P2_1667
nx10^8
Phage Titre(pfu/ml)
25
20
15
10
5
0
Control
Chloroform
Absolute Ethanol


Organic Solvent Sensitivity for P3_1774
Phage Titre(pfu/ml)
25
20
15
10
5
0
Control
Chloroform
Absolute Ethanol



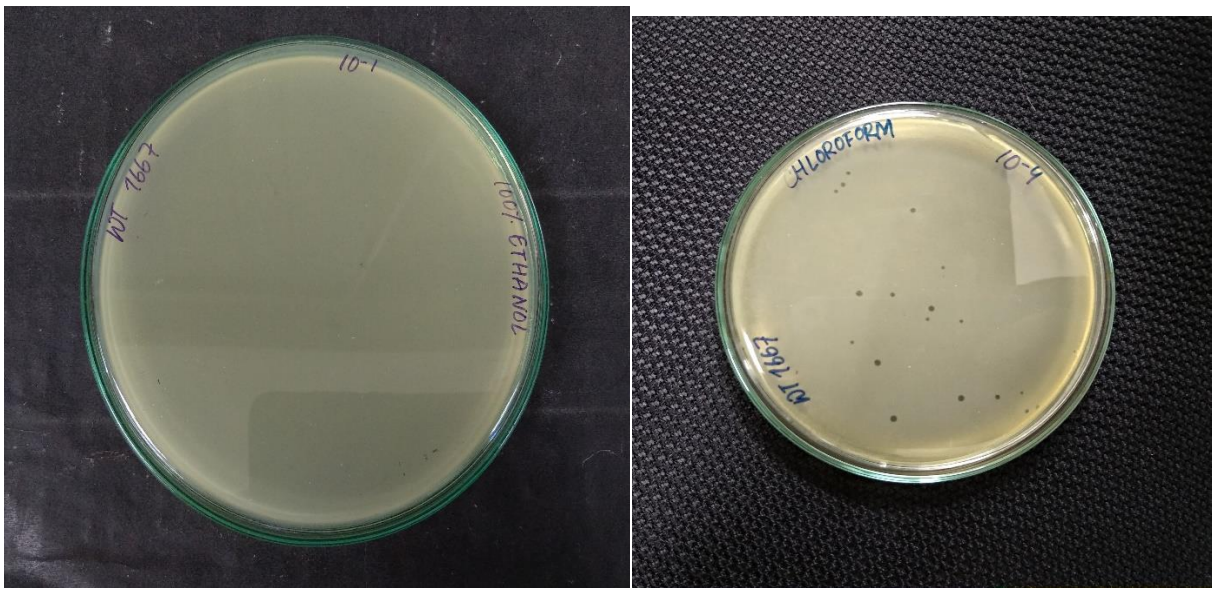


Fig 4.2.4 Organic solvent stability test results (Chloroform and Absolute Alcohol) for isolate P2_1667

Here, we observed no significant change in titer compared to control, whereas the incubation with ethanol for the exact same amount of time resulted in the complete inactivity of the isolated phages.

4.3 Bacteriophage DNA Isolation and Detection

4.3.1 Gel Electrophoresis

After performing the process of DNA isolation in order to further categorize the isolated phages, gel electrophoresis was done to ensure the presence of DNA. By ensuring the presence of DNA

we were able to carry out the molecular bio techniques as RFL and PCR as for to get the validity and proper justification that the phage isolated was hence, a *Vibro* phage. The gel electrophoresis held the result,

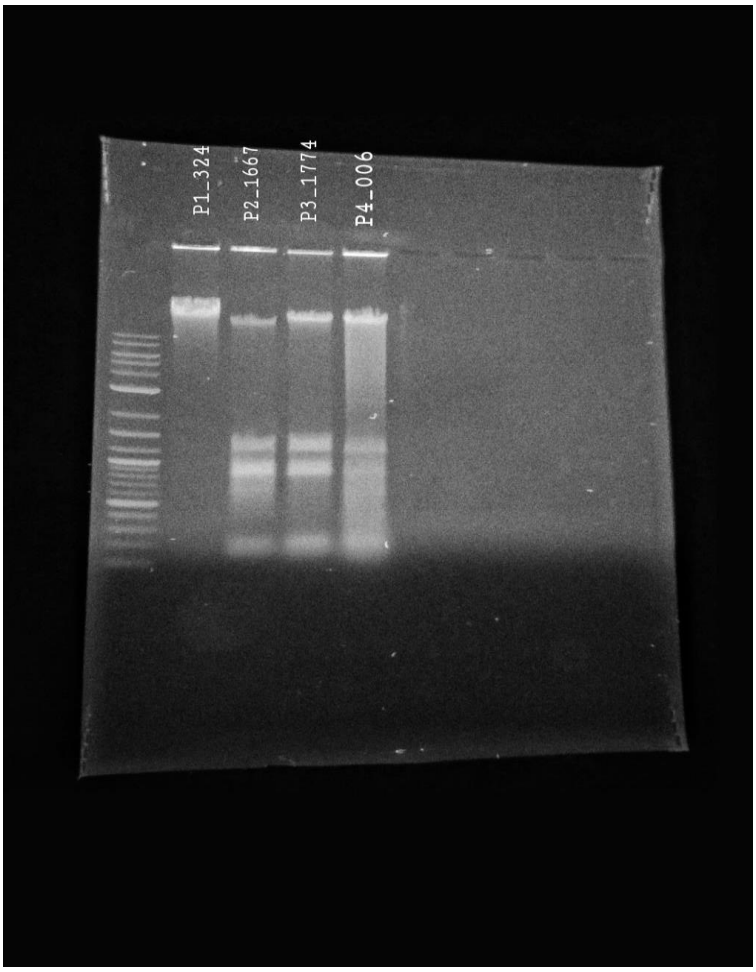


Fig 4.3: Gel Electrophoresis for detecting isolated DNA

As a primary inference from the gel electrophoresis it can be though that the phages P2_1667 ,P3_1774 and P4_006 are of the same category as they follow somewhat a same banding pattern where the phage P1_324 follows a different banding pattern from the other a different type but maybe the same type after all. The entire molecular identity of the phages can only be identified when PCR and RFLP is done.

4.3.2 Restriction Fragment Length Polymorphism

The aim of performing RFLP was to determine whether all the phages were of the same kind or different. Different restriction enzymes were added to the whole genome and the expected to cut them in particular restrictions sites that would result into distinct fragments. The result of the RFLP process is attached below,

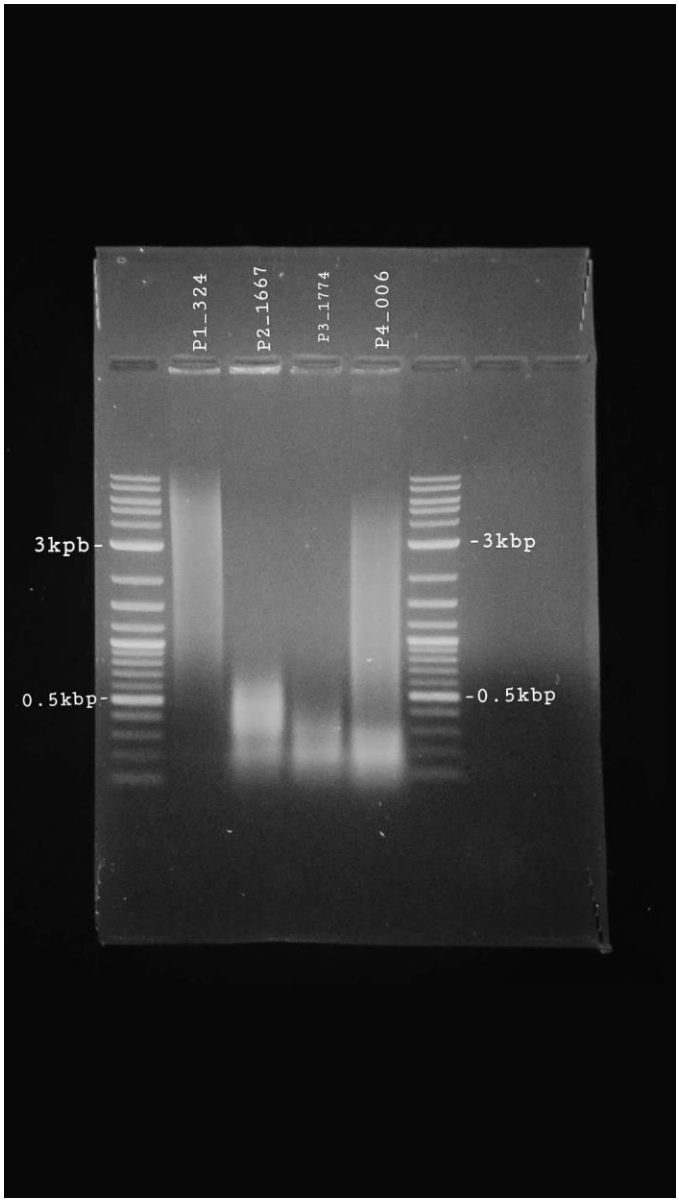


Fig 4.3.2 RFLP results for isolated phages

As it can be observed from the image, the whole DNA goes through some changes and give a different result after the addition of the restriction enzymes. After the addition of the restriction

enzymes, DNA smears were seen to have appeared in the gel. There might be many factors that influence the DNA smearing and all of them were optimized solely or altogether. After optimizing all the factors for multiples times the smearing of the DNA continued hence we turned to perform the process of PCR which would help us to classify all the isolated phages in different classes.

4.3.3 Polymerase Chain Reaction

Through the process of PCR, any fragment of the DNA is amplified using a definite primer. If the DNA amplifies and give us a band at any certain length hence e could conclude that the PCR process was a success. The PCR result is attached below,

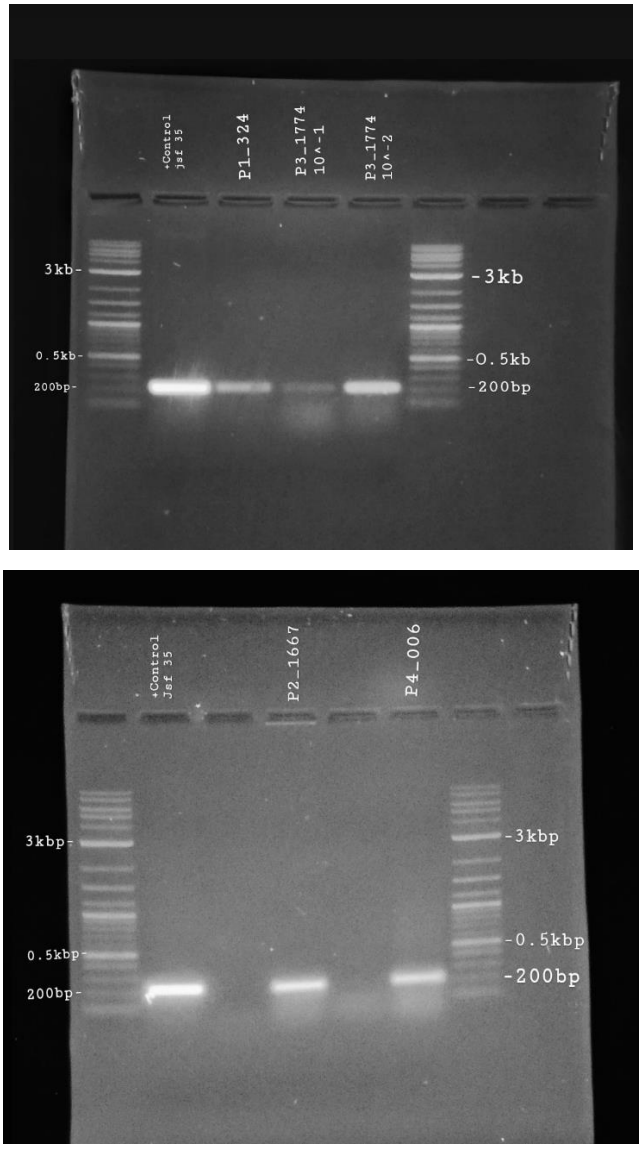


Fig 4.3.3: PCR results for the isolated phages

From the results above we can observe that all the isolated bacteriophages are giving a distinct band at 200bp corresponding to the positive control of the *jsf 35* which is classified as ICP3 phage in a study previously mentioned. In the first image, P3_1774 10^{-1} gives a faint band whereas P3_1774 10^{-2} gives a very vibrant band at 200bp confirming it a ICP3 phage.

Therefore, from the process of PCR we confirmed all the isolated phages to be ICP3 class.

Chapter 5

Discussion

The aim of the study was to isolate bacteriophage that had *Vibrio cholerae* as their specific host and characterize them specifically in order to treat them as an ideal phage for further research.

5.1 Bacteriophage Isolation

Bacteriophage isolation was the most crucial part of the study as the aim of the experiment was to isolate phage that gave clear lytic zones. Out of the six phages isolated, only four of them gave *Vibrio cholerae* infective bacteriophage in plaque assay while other 2 isolated phages failed to produce any lytic zone. Initially while isolation, somewhat hazy lytic zones were observed in the negative isolated phage, which were later extracted and used for further enrichment but following the enrichment process did not sum to any results and the reason might be the isolation of lysogenic phages. As for the categorization of the replication of the phages the isolated negative phages might be of lysogenic phages which hardly gives any clear lytic zones. On another hand, the routine enrichment of phage is necessary in order to keep the phage count to a minimum. Bacteriophages predate bacteria and they need a host in order to keep them viable. With a routine enrichment the phage gets sufficient number of host to predate and hence, increase their number. Therefore, another possibility of the negative phages might be the lack of proper enrichment hence hindering the proper enumeration of the phages. In other words, slowly proliferating phage, one which yields a low number of progeny phage, will more likely to produce a smaller plaque compared to quickly proliferating phage (Irving et al., 1990). Additionally, phages as a virus can be rendered non-infective in aquatic environment via several factors. The virucidal organism, mainly some heterotrophic bacteria that can degrade viruses considering them as another nutrient source and some protist that also destroy large viruses (Weinbauer, 2004; Fujioka et al. 1980). Again, heavy metals can also render phages non-infective by binding with them mostly in polluted water (Bitton, 1980). High energy photon can also disrupt phages (Murray and Jackson, 1993). However, the most important factor for viral decay in an aquatic environment is sunlight, specifically ultraviolet (UV) light that damages Viral genomic material to an extent which cannot be repaired (Kirchman 2012). UV-B in the sunlight has the greatest effect at about 300 nm wavelength (Caldwell, 1971; Setlow, 1974) and accounting for 50-90 % of inactivation rate of virus caused by full sunlight (Suttle & Chen, 1992).

Furthermore, the sampling for isolation of bacteriophage was carried out from January 2019-August 2019. Among this time frame, we observed a total of three seasons- Spring, Summer and Monsoon. From the detailed table we observed that the ideal time for isolation of bacteriophage was somewhere between February to March, which was the spring season whereas after the staring of the rainy season no bacteriophage was isolated at all. Although the ideal timing for isolation of bacteriophage is summer but during summer of the study, dispersed rainfall was observed. The epidemic caused by cholera is occurred with a seasonal variation in a regular pattern within the geographical location surrounding the Ganges delta region of Bangladesh and neighboring India The epidemic is caused within the regular interval of two time periods namely, just after the monsoon during September to December. Again there is a small peak of cholera pandemic within spring within March to May. Though V. cholerae is human pathogen yet it constitutes an essential part within the normal flora in aquatic environment where water is one of the prominent vehicles of cholerae transmission. As the bacteriophage follows a symbiotic relationship with its virulent host hence its occurrence within the environment is somehow related to the duration of the epidemics. Hence, the observed phage isolation cycle might give us a theory regarding the mysterious nature of seasonal epidemic of endemic cholera.

5.2 Host range specificity

The isolated phages portrayed some promising results for the host specificity range. The host specificity range of bacteriophage can be helpful to understand the spectrum of the predatory nature of the bacteriophage. According to the results accumulated, we observed the phages P1_324, P3_1774 and P4_006 showed a varied array of host that is not only limited to one species rather it had a host range of two species, which includes some strains of *E.coli* along with some strains of *V.cholerae.*

Additionally, to understand if the host specificity of the isolated phages were limited within specific strains of *V. cholerae*, the isolated phages were checked if they form lytic zones in other strains of *V. cholerae* or not and they did. Some of the isolated phages displayed a similar pattern of forming lytic zone in both *E. coli* species along with the *V. cholerae*.

Hence, from the study it was observed that the isolated bacteriophages displayed a potential to have a broad host range which not only varies from species but show promising results for different

strains too. Nevertheless, the result seems to indicate a broad host range contained which is not limited only to one species and this correlates with the idea that some phages have strain level specificity while others have broader host range infecting only multiple strains of a single species to closely related several species (Donlan, 2009). These phages could be potential candidates for designing approaches like phage therapy.

5.3 Temperature Stability

Temperature is another important factor that influences the bacteriophage stability (Hurst et al., 1980; Nasser and Oman, 1999; Yates et al., 1985). The temperature is a sensitive factor that influences the whole process of replication process which includes attachment, penetration, and multiplication (Olson et al., 2004). At suboptimal temperature, phages participate less in the process of phage replication as a less number of phage is capable to integrate its genetic material to the host hence, creating less and less phage. Additionally, temperature regulates the viability, occurrence, and storage of bacteriophage (Jończyk et al., 2011).

The isolated phages showed viability up to 50ºC with some alteration in their titer. Whereas, there was a huge reduction in phage titer somewhere more than 3 to 4 fold at 60ºC and complete reduction in phage titer at 70ºC indicate that the phage is not resistant to extreme temperature. As the isolated phages were stable between 30ºC to 55ºC it can be inferred that the isolated phages can be used as a therapeutic agent considering the average human body temperature is 37ºC hence, will not thermally deactivate its lytic activity.

5.4 pH stability

The survivability of a phage, among many factors is also dependent on the acidity or alkalinity of the environment (Krasowska et al., 2015). The isolated phage was shown to have resistance from pH range of 4.0 to 11.0 after 3 hours of incubation and became completely inactive at pH 1-2.0 and pH 12.0. The optimal pH value for most of the phages were within the range pH 6.0 to pH 8.0 where they were shown to confer stability. However, as the isolated phages demonstrated stability for a wide range of pH they can be thought to be formulated as a therapeutic agent for studying the cholera epidemic. It can be used in impregnation of bacteriological biofilm degradation.

5.5 Organic solvent sensitivity

The principle of organic solvent sensitivity of bacteriophage can be used for different therapeutic or biocontrol preparations where numerous organic solvents are involved. The stability of phage in the different organic solvents mainly depends on the stability of its protein and the stability of the protein is highly dependent on the solvents ability to keep the structure of the phage intact or to destroy certain inter and intramolecular hydrophobic and electrostatic interactions (Olofsson et al., 1998). Competition between stabilization and destabilization of different interaction exist upon exposure to organic solvents (Olofsson et al., 1998).

The isolated phages were found to be resistant upon exposure to chloroform. In a chloroform-aqueous environment which is used for the storage of the phages, keeps the phage solution from bacterial contamination as chloroform is a known antimicrobial agent and it has been used often in preparation of phage stock too (Cotton and Lockingen, 1963). On the other hand, all the isolated phages were sensitive towards absolute ethanol which correlates with the similar result found by Verma et al. (2009). Previously the study done by Olofsson et al. (1998) demonstrated that the ethanol concentration higher than 40 % would decrease the phage viability significantly. Hence, it can be said that the ethanol with 70 % concentration, which is generally used in different laboratory to maintain aseptic condition, is a good option to preserve sterile environment where the presence of any of the isolated phages are not acceptable.

5.6 Restriction Fragment Length Polymorphism

As the DNA was isolated it was essential to categorize them into categories. The aim of performing RFLP as to categorize the isolated phages if they were of the same type. The RFLP process was a failure. The DNA smears kept on appearing even after optiziming all the regulatory factors in the process. At first only one restriction enzyme was used to perform the process but the smearing kept on occurring. So, we used a mixture of three restriction enzymes to reduce the smear but the smear was still prominent. Moreover, the gel electrophoresis was done with a very minimal volt so that the DNA fragments would travel as slowly and give distinct result yet the smear kept occurring. Moreover other additional reasons for DNA smearing could be,

- Restriction enzyme(s) didn't cleave completely
- The restriction enzyme(s) is bound to the substrate DNA
- Nuclease contamination
- Salt inhibition
- Inhibition by EDTA from the TE storage Buffer
- DNA is contaminated with an inhibitor

Hence, we could not classify the bacteriophages using the process of RFLP so we used PCR to classify them.

5.7 Polymerase Chain Reaction

The process of PCR was carried out to differentiate between the various kinds of phages isolated. As the vibriophages were isolated using a specific host of *Vibrio cholerae* 01, which is responsible for causing cholera epidemic, the phages were further differentiate into ICP1, ICP2 and ICP3. ICP1 is specific for O1 serogroup *V. cholerae*; however, the host ranges for ICP2 and ICP3 are broader and include some non-O1 serogroup *V. cholerae* strains. Therefore, it is important to classify the phages into different categories to study their mechanisms. According to the PCR analysis we could successfully categorize all the isolated phages into the category of ICP3.

Three batches of PCR had to be done in order to classify the isolated phages. In the first batch all of the isolated phages gave faint or n result and that might have been occurred for the abundance of DNA. So, the DNA of the isolated phages were diluted ten folds and in case of P3_1774 two in order to carry out the process of PCR. After the dilution, all the isolated phages gave the result. Finally, they were classified as ICP3 type of bacteriophage.

6. Limitations

- The isolation of the phages and keeping them viable through enrichment is a lengthy process.
- The isolation of most phages were theoretically planned to be within the summer season but the sudden alteration of weather pattern brought unplanned hindrance for the study.

- DNA isolation of phage requires more than7 days. So, gaps are to be used very carefully in order to avoid contamination.
- Soft agar is an important component and is used to make a bacterial lawn in order to perform plaque assay. Using the soft agar can be crucial given a certain temperature is to be maintained because too warm agar can kill the bacteria and phage.
- While performing plaque assay it is important to use the tips carefully in order to avoid contamination.
- RFLP is a crucial process and a number of restriction enzymes were used carefully. The ratio of the enzymes is to be optimized as too many enzymes can give unusual results.
- The process of gel electrophoresis was done in low voltage. There is a slow run of the DNA fragments and it gives a better result.
- For carrying out PCR, several kinds of primers were needed and primer designing and acquiring them was a time consuming process.

7. Future Perspectives

- The acquired results were just specific for *Vibrio cholerae 01* and same experiments can be used for find *Vibrio Cholerae Non 01* phages and apply the same techniques to make a reference frame for it.
- The isolated phages can be used to observe they can be used to for biofilm degradation.
- The study of the isolated phages can be used to draw up a phylogenetic tree in order to understand the origin of the phage.
- The isolated phages can give a promising edge for phage therapy research given the multi drug resistant bacteria is on a high emergence.

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