Isolation of bacteriophage from sewage watersamplesspecific for*Vibriocholerae*

SUBMITTED BY

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A Thesis submitted to the Department of Mathematics and Natural Science in partial fulfillment of the requirements for the degree of Bachelor of Science in microbiology.

Department of Mathematics and Natural Science
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Declaration

Itisherebydeclaredthat

1. The thesis submitted is my/our own originalwork while completingdegree at BracUniversity.
2. Thethesisdoesnotcontainmaterialpreviouslypublishedorwrittenbyathirdparty, except where thisis appropriatelycited through full and accuratereferencing.
3. Thethesisdoesnotcontainmaterialwhichhasbeenaccepted,orsubmitted,foranyotherdegreeordiplomaatauniversityorotherinstitution.
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Approval

The thesis/project titled "Isolation and characterization of Bacteriophage fromsurfacewaterspecificfor*Vibriocholerae*"issubmittedbyNabilEbneyKamalofSpring,2015,hasbeenacceptedassatisfactoryinpartialfulfillmentoftherequirementfor thedegreeof Bachelor ofScienceinBiotechnologyon

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Ethicsstatement

Nohumanoranimalmodelwasusedinthisstudy.

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Abstract

The severe diarrheal disease cholera is caused by the bacterium *Vibrio cholera*.Toxigenic *Vibrio cholerae* strains belonging to O1 and O139 serogroups are thecausativeagentsofepidemicandpandemiccholera.The*Vibrio*bacteriuminteractswithnumerous phages in the aquatic ecosystem and in the intestine of cholera patients.Generally, two peaks of cholera outbreak are observed to coincide within the drysummerandmonsoonrain.Choleraepidemicisamajorpublichealthconcern.Severalfactors control the seasonal epidemics. Bacteriophages are one of the vital triggerswhich have been reported to collapse the outbreaks. Hence, the predatory nature ofbacteriophagehasahugeimpactonthepopulationfortheircorrespondinghosts.Thisstudywasdesignedtoisolate*Vibriocholera*specificbacteriophagesfromenvironmentalsamples.Theisolatedbacteriophageswerecategorizednotonlyaccordingtotheirphysiologicalcharacteristics(HostRange,pHstability,temperaturestabilityandorganic solvent sensitivity) but also on a molecular level using techniques like RFLPandPCR.

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ListofAbbreviation

etal	Andothers
g	Gram
ml	Milliliter
ICTV	InternationalCommitteeonTaxonomyofviruses
E.coli	*Escherichiacoli*
V.cholerae	*Vibriocholerae*
RFLP	Restrictionfragmentlengthpolymorphism
µL	Microliter
µm	Micrometer
O/N	Overnight
rpm	Rotationperminute
pfu	Plaqueformingunit
LA	LuriaBertaniAgar
LB	LuriaBertaniBroth
DNasesolution	Deoxyribonucleasesolution
RNasesolution	Ribonucleasesolution
PCI	Phenol-ChloroformIsoamylAlcohol
TE	TrisEDTA
EDTA	Ethylenediaminetetraaceticacid
PCR	Polymerasechainreaction
Fprimer	Forwardprimer
Rprimer	Reverseprimer
dNTPs	DeoxynucleotideTriphosphates
ds	Doublestranded
ss	Singlestranded
CRISPR	Clusteredregularlyinterspacedshortpalindromicrepeats
DNA	Deoxyribonucleicacid
RNA	Ribonucleicacid
ICP1	InternationalCentreforDiarrhoealDisease Research,Bangladesh choleraphage1
ICP2	InternationalCentre for Diarrhoeal Disease Research, Bangladeshcholeraphage2
ICP3	InternationalCentreforDiarrhoealDiseaseResearch,Bangladeshcholera phage3
Icddrb	InternationalCentreforDiarrhoealDiseaseResearch,Bangladesh

Chapter 1Introduction

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} (Hemmingaetal., 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 havebeen submitted in Internatio nal 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 potentialto 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 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.

V. 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 infect ions on global health.

Specific aims:

1. To isolate Strong lytic bacteriophage against *Vibirio Cholerae* from the differentwater sample.

Objectives:

The main aim of this project was to isolate bacteriophage against *Vibirio cholera* and to identify its various characteristic to determine the therapeutic potentialas a therapeutic agent.

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 (Sulakvelid ze 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'Héré e, 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'Hére es, 1917). d'Hérelle named the virus a bacteriophage or bacteria-eater (Félix d'Hére es, 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'Héré e'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 biologica l 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).

2.2.2 Bacteriophage infection

Bacteriophage requires a host for replicating and enumeration. Without a host the phage cannotgrow or multiply. There are to main types of infection of phage. Theseare very briefly given below,

- **Lytic Cycle**

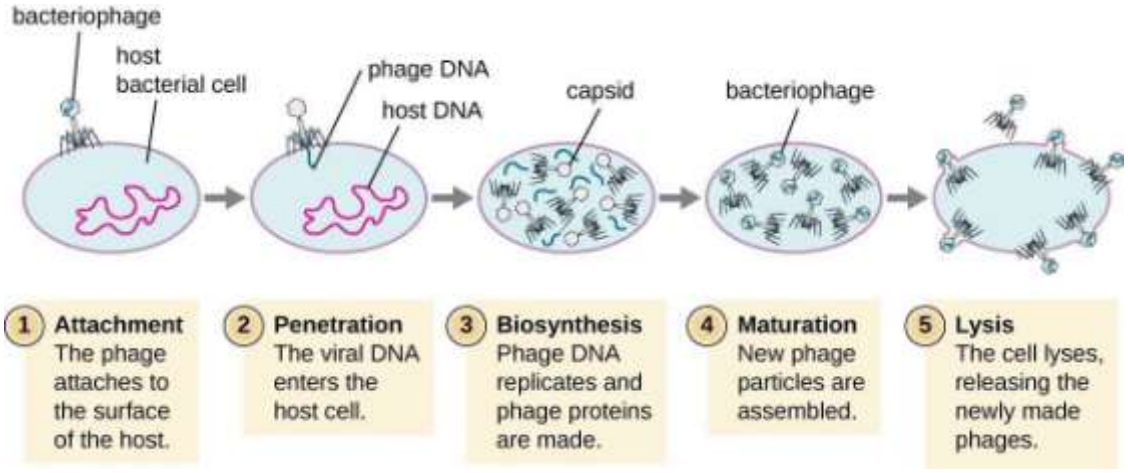

Fig-2.2.2 ***Lytic cycle of Bacteriophage***

- **Lysogenic cycle**

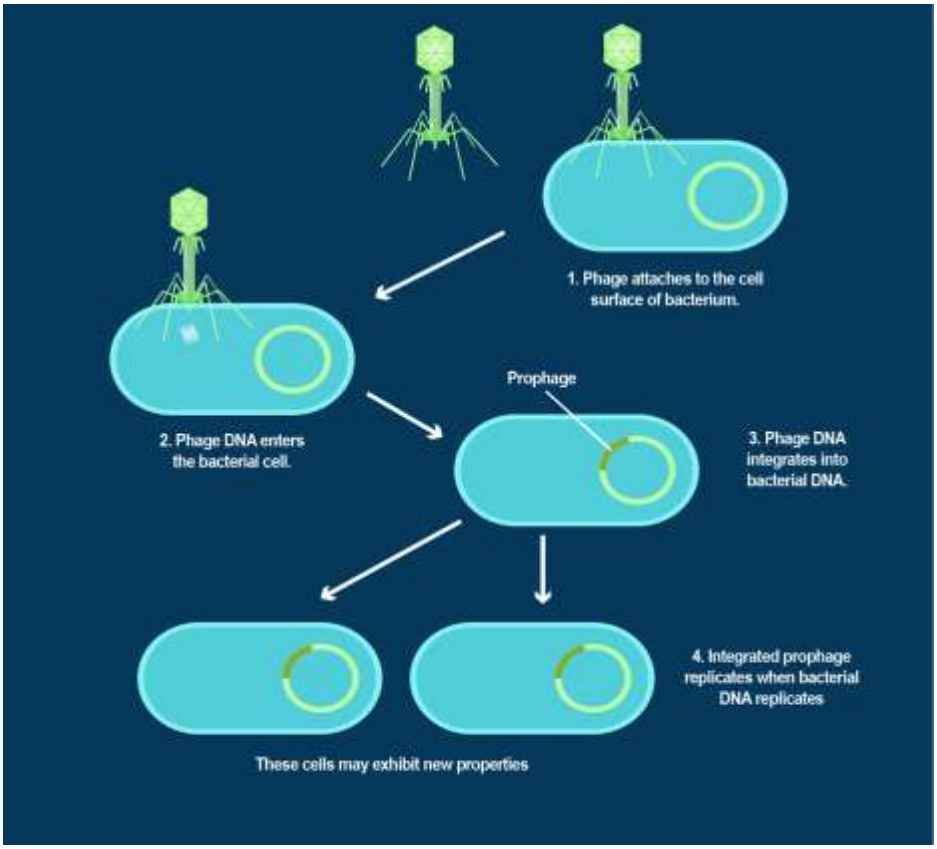

Fig-2.2.2 ***Lysogenic cycle of Bacteriophage***

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 hardto identify and harder to isolate.

2.3 Cholera

Cholera is an infectious disease caused by a bacterium called *Vibrio cholerae*. Thebacteria typically live in aquatic environments with warm and salty setup such as estuariesand 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 cellpole 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.

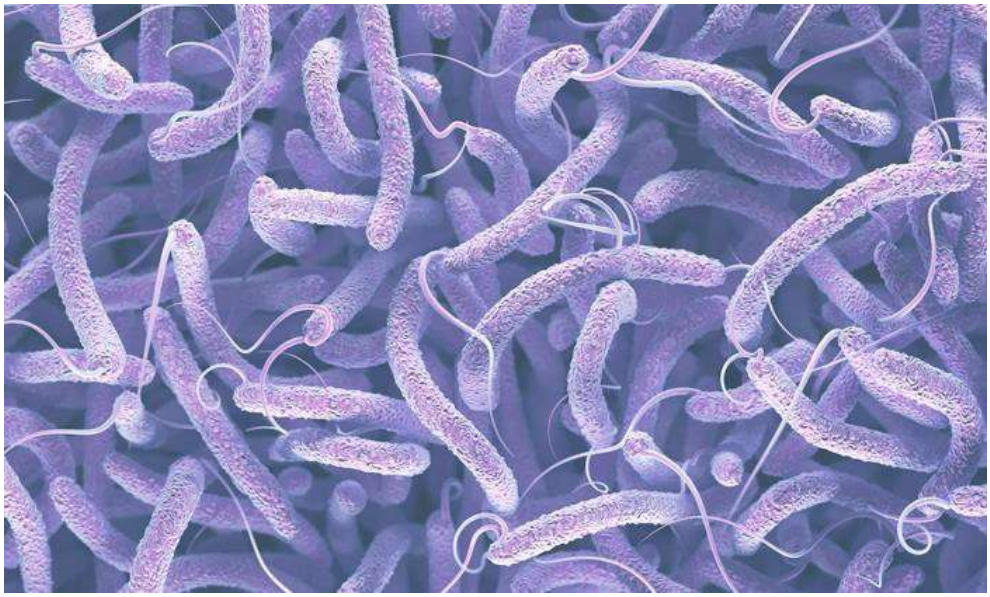

Fig-2.2.3 *Vibrio Cholera*

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 buryingthe 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.

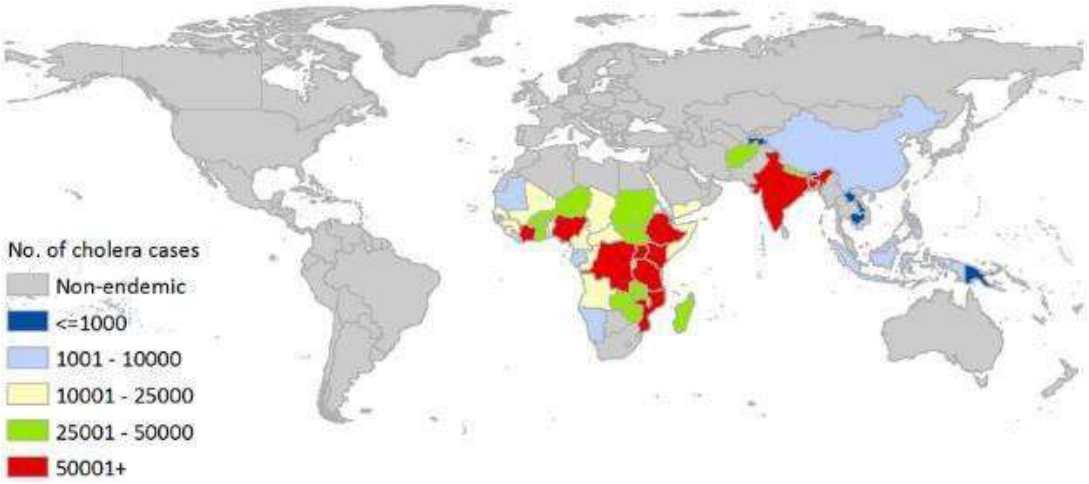

Fig- 2.4.1 Cholera endemic regions

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 occurrence 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 greatlyto understanding cholera epidemiology.

In the recent studies, the modern molecular techniques were used in a study at the international Centre for Diarrheal Disease Research, Bangladesh (ICDDRB), Faruque et al. have isolated at least 36 different cholera phages, known as the JSF series depending on their RFLP analysis. 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.

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.

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).

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).

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; Kodikaraet 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

Methods and materials

3.1 Place of study:

The study was done in the Biotechnology and Microbiology laboratory of the Department ofMathematics and Natural sciences, BRAC University, Dhaka, Bangladesh.

3.2 Standard laboratory practice:

All the glassware was washed first time with tap water followed by second time wash with distilled water. For example: test-tube, conical flask, beakers. Before using the Culture media (both agar based and broth), pipette tips, centrifuge tubes, empty test-tube for double-layer ager method were autoclaved at 121°C at 15 psi for 15 minutes. After the work done, culture media stored at 4°C and autoclaved equipment's are in aseptic condition. clean lab coat was worn while experiment is performing. Hand gloves were also used and the experiments were done inside a vertical laminar flow cabinet which was cleaned with 70 % ethanol to avoid contamination.

3.3 Preparation of culture media, reagents and solution:

3.3.1 Bacterial Culture:

The bacterial cultures which were used in this project collected from the stock of biotechnology and microbiology laboratory. Bacterial samples were streaked on freshly made LA plate and incubated them overnight at 37°C. After checking

the growth of the bacteria, the plates were kept at 4°C for further use. Before every experiment, bacterial samples were freshly sub cultured and the cultures were used for 24 hours. By regular sub-Culturing we can maintain the viability and purity of the organism.

3.3.2 Preparation of LB:

The LB was prepared by weighing 20 grams of LB powder in 1000 ml of distilled water and then mixed well in beaker. After that we heated it for few min on Bunsen burner and later it was autoclaved at 121°c for 2 hours.

3.3.3 Preparation of LA:

The LB was prepared by weighing 15 grams of LA powder in 1000 ml of distilled water and then mixed well in a biker. After that we heated it for few min on Bunsen burner and later it was autoclaved at 121°c for 2 hours. Finally, we poured it on previously prepared autoclavedplate (at 121°c for 15 min).

3.3.4 70% ethanol:

To prepare 70% ethanol, deionized water was added to the 737ml of 90% ethanol to make a final volume of 1000ml.

3.4 Collection of water samples:

Sewage water is collected from various points of the city by maintaining caution not to cometo direct contact with the water as they will most definitely contain human pathogens.

3.4.1 Location:

The Areas from which the samples were collected are inside Dhaka city are; Hatirjheel, Rampura, Police Plaza,Gulshan lake, Korail, Dhanmondi Lake, Bosila and Buriganga river.

3.4.2 Processing of water in the laboratory (including enrichment):

After collection of the water samples, working need to be started within 3 hours otherwisebacteriophage may die. then, the water is first filtered using watman filter paper

and then again filter with syringe filter.

3.4.3 Cocktail preparation (mixture of Salmonella typhi and Shigella dysenteriae):

Luria broth (LB) was prepared and pour 3ml in a vial then different strains of the same bacterial species is added in the vial. 1 or 2 freshly cultured colonies of the bacterial speciesinoculated in the LB broth and kept for 2.5 hours in the shaker incubator for lag phase.

3.5 Isolation of phage plaques:

After 2.5 hours' incubation then 1ml of the sewage water sample is added with the cocktail mixture in the vial and again kept for 4 hours in the shaker incubation.

In the meantime, individual organisms are inoculated in the broth and kept 2 hours for thegrowth of organisms. After 4 hours of shaker incubation, centrifuge them for 5 minutes at13000 rpm and filtered using syringe filtration technique.

Next, we diluted the filtered cocktail up to 10-10.

3.5.1 Preparation of plaque assay:

The individual bacterial culture (0.5ml) is mixed with 3ml of soft agar and poured over the LA plate and quickly rotated the plate to let the soft agar spread evenly over the LA and keptfor sometimes to let it dry. Next, 10µm of the diluted sample is added dropwise including the diluted cocktail and kept for overnight incubation. The dilution number 10-1, 10-3, 1-5, 10-6, 10-7 and 10-8 was taken respectively.

Storage and resuscitation of phages: After 24-48h incubation, plaque is collected using sterile tips and stored by adding 200 ml of saline and 20µl of chloroform andstored at 4℃.

3.5.2 Phage stock preparation

The next day phage lysate is enriched in the same phage isolation process and centrifuged at13000 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 4ºC.

3.5.3 Phage titer determination

Titer means the concentration or number of bacteriophage that can be assumed. The process is done by double layer method. The phage stock was diluted up to 10^-8 and in some case itwas 10^-16 for initial concentration. Each dilution was taken into Eppendorf tubes and diluted accordingly. A sterile vial was fill up with 3ml of molten 0.4% agar with 5ul of diluted phage stock. The contents were mixed carefully and pour on to fresh Luria Bertani agar plate. While pouring on the plate bubble could not be formed because it could misinterpret the result. Then waited to solidified the agar layer and incubated them at 37ºC. After overnight incubation, the plates were checked for plaque formation. The plates which have plaques were selected for titer determination.

The formula for titer determination is given below:

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

3.6 Bacteriophage DNA Isolation

Single plaque was stored in SM buffer. They were used for forming titer stocks. Phageenrichment and DNA isolation was done using this solution.

For double layer plaque assay we need 300ul host bacteria and 1 ml of bacteriophage onLuria-Bertani agar plates and incubate it overnight at 37℃.

The upper layer of the soft agar containing the bacteria and the phage. Phage is collected bysterilized spreader. Luria broth was added to enhance the process. The culture was collectedin a sterilized falcon tube. The suspension was centrifuged at 10,000 rpm for 10 minutes at room temperature. Then the supernatant was collected and filtered through 0.22um syringe filter. Dnase was added at 1unit/100ul ratio of the supernatant. To create a working solution of Dnase 10x buffer was added in the solution at 1:10 ratio. Then the solution was incubatedat 37℃ overnight. Later, Proteinase-K was added at 5mg/ml ratio. The solution is then incubated at 37℃ 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. Then centrifuged the solution at 14000rpm for 15 minutes and then the upper aqueous layer was collected in sterile falcon tubes. Phenol- Chloroform and iso-amyl alcohol was added again in same ratio and volume and centrifugedunder the same conditions.

Then, double volume of absolute ethanol (cold at -200℃) was added to the supernatant andincubated overnight at -200℃.

Next, the solution was then centrifuged at 14,000 rpm for 15 minutes at 40℃. The supernatant was removed and the pellet was washed with 70% cold ethanol. Finally, thepellet is dissolved in Tris-EDTA buffer for storage.

3.7 Bacteriophage Identification

3.7.1 Plaque assay:

Plaque assay is a standard method which is used to determine the concentration of virus. In is process suspension of phage is spread over the susceptible bacterial cells. Then the phageattaches to the bacterial cell and replicate inside it also killed it during lytic release. A clearzone or plaque formed where the lysis of the bacteriophage occurred 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.

3.7.2 Gel electrophoresis

Gel electrophoresis is a process which is used to separate DNA fragments based on their charge and size. DNA is negatively charged so the electric force migrates it across the gel topositive electrode. The smaller molecules go faster than the large molecules. Agarose is a powder that is insoluble in room temperature water (or buffer) at room temperature but becomes liquid in boiling water. When it cools it ends up like a gel. Higher concentration ofagarose creates firm gel so, small molecules of DNA can pass through it and the larger DNAmolecules cannot easily pass through gel.

3% agarose gel preparation process:

1. Measuring with analytical balance .4 (1%) grams of agarose powder.
2. Add the agarose powder with 40ml TAE buffer and heated in microwave for 1 minuteto dissolve the powder and the buffer to give a clear solution.
3. The heated molten agar was allowed to cool. Then it would be made into a gel whichwas 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 theDNA 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 abackground 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 6xloading dyes and loaded in the wells.
- Electricity was applied to the apparatus and let run for 50 minutes-1 hour, as perrequirement at 70 volts.
- Once the electrophoresis was over, the gel was visualized under UV light and the resultsare interrupted accordingly.

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 6 strains used for screening vibrio phages were WT-1774, WT-006, WT-333, WT-324, WT-1667, WT-1532. vibrio *cholerae* specific bacteriophage were obtained inthe 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 gavethe clearest and isolated individual bacteriophage, these were selectedfor characterization, identification and further study. The process of sampling commenced fromJanuary 2019- August 2019.

Table-4.1.1 Bacteriophage isolation from environmental samples

Date for Water Collection	Location	Bacteriophages isolated from the specific strains of Vibrio cholerae
28th January'19	Gulshan Lake	WT-1774
05th february'19	Bosila	-
10th February'19	Dhanmondi Lake	WT-1774, WT-1532
16th february'19	Turag	-
10th march'19	Gulshan Lake	WT-1774
12th march'19	Bosila	WT-1774, WT-006, WT-324, WT-1667
13th March'19	Dhanmondi Lake	-
17th March'19	Turag river	-
2nd April'19	Gulshan Lake	WT-1774
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	-

The isolated phages were named with smaller initials in order they were easier toidentify orrefer 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.

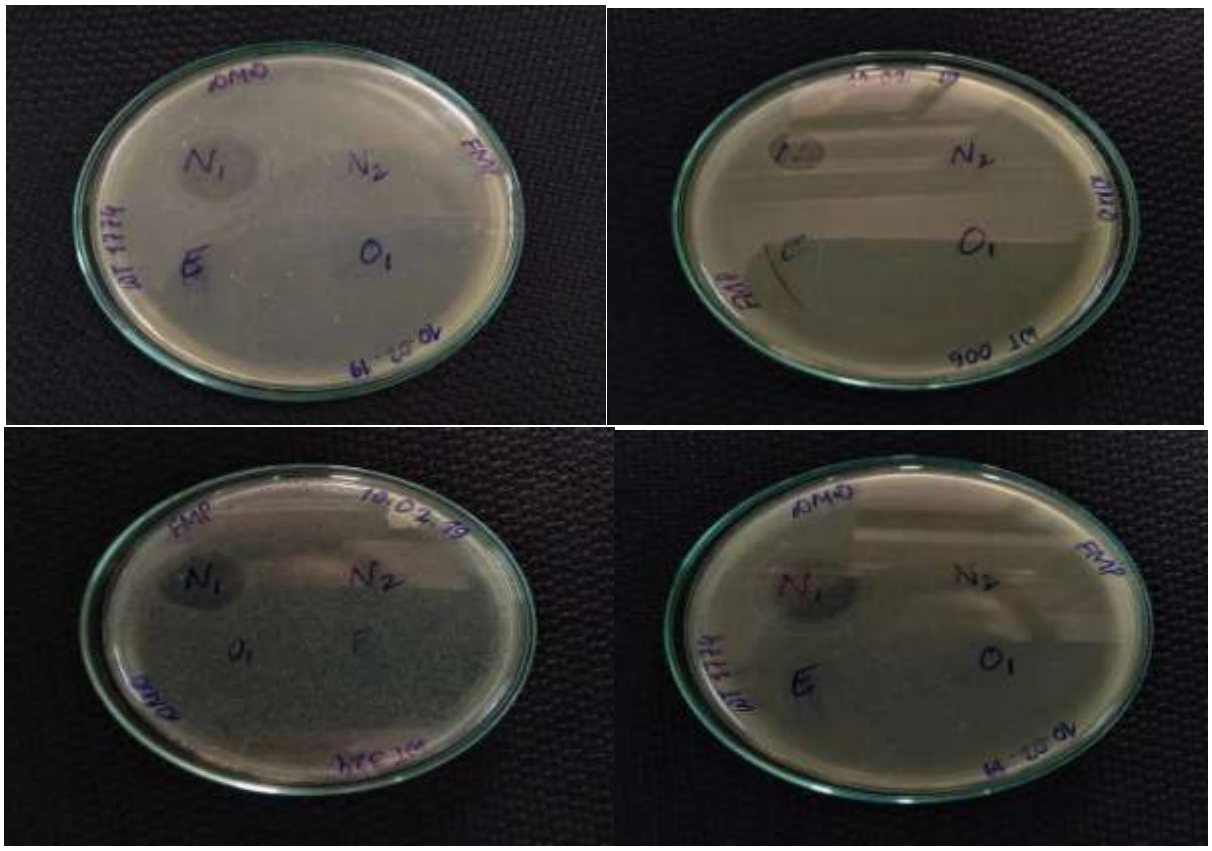

Fig-4.1.1 ***Plaque Assay from initially collected water sample***

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_1774had 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 determinedby DLA method and gave up to 106, 108, 1015 and 103 pfu/ml for phages P1_324, P2_1667, P3_1774 and P4_006 respectively.

4.2 Bacteriophage DNA Isolation and Detection:

4.2.1 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 electrophoresisheld the result,

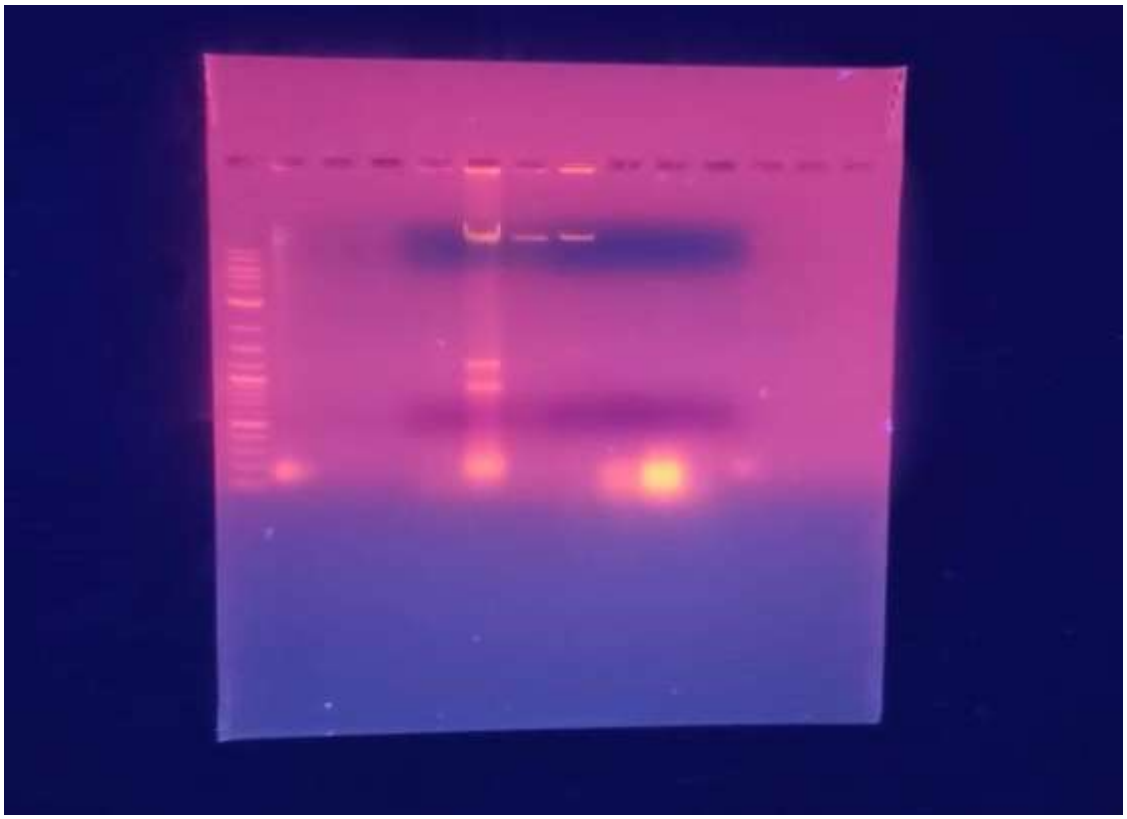

Fig-4.2.1 ***Gel Electrophoresis for detecting isolated DNA***

Chapter 5

Discussio

n

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 five of them gave *Vibrio cholerae* infective bacteriophage in plaque assay while another one isolated phage 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 follow ing 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 alongwith 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.

Limitations

Tall bacterial defilement happened within the double-layer assay plates was the foremost predominant issue experienced in this investigate. In spite of the fact that the aseptic condition was kept up as long as conceivable amid different tests, distinctive bacterial colonies would in some cases show up on the measure plate surface, regularly covering a potential plaque coming about in a wrong plaque check that may influence the unwavering quality and precision of the result. The source of these contaminations was followed back to the micropipettes utilized within the explore that were not externally cleaned utilizing ethanol some time recently the try was begun and regularly sullied within the phage stock arrangement.

The risk of bacterial debasement was lit up by cleaning the micropipette by implies of ethanol and ousting bacterial cells from the phage stock by centrifugation, taken after by filtration by implies of a 0.22 μm syringe channel. In development, to expect bacterial debasement, chloroform can be included to the phage stock course of action (Cotton and Lockingen, 1963). Different plaques would in a few cases cover each other in the midst of the twofold layer test and outline a cluster that would make it uncommonly troublesome to recognize individual plaques. Since the typical plaque degree was decently gigantic, more vital debilitating would decrease the full numberof plaques that would reduce the credibility of plaque cover.

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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