Isolation of *E. coli* phages from environmental water samples and analysis of their effect on periodic epidemics of cholera

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

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DECLARATION

This is to declare that the research work embodying the results reported in this thesis entitled "**Isolation of *E. coli* phages from environmental water samples and analysis of their effect on periodic epidemics of cholera"** has been carried out by the undersigned under supervision of Dr. Iftekhar Bin Naser, icddr, b and Romana Siddique, Senior Lecturer, Biotechnology and Microbiology program, Department of Mathematics and Natural Sciences, BRAC University. It is further declared that the research work presented here is original and submitted in the partial fulfilment for the degree of Bachelors of Science in Biotechnology, BRAC University, Dhaka and has also been submitted in icddr,b for a degree or diploma.

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Dedicated to

My beloved parents

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I perceive this opportunity as a big milestone in my career development. I will strive to use the gained skills and knowledge in the best way possible.

Navila Sultana

Abstract:

Cholera is a deadly infectious disease caused by the pathogen *Vibrio cholerae*. In our country cholera epidemics occur seasonally. Two peaks of outbreaks nicely coincide with dry season and monsoon rain. Several factors control the outbreaks to occur and collapse. Bacteriophages are one of them which have been reported to trigger the collapse of the outbreaks. The concentration of the *Vibrio cholerae* specific bacteriophages is inversely correlated with the concentration of *Vibrio cholerae* in the environment. Therefore these bacteriophages probably play an important role in controlling the epidemics to occur or collapse. It is still not clear what factors trigger the onset of cholera outbreaks. There are numerous amounts of other species of bacteria and viruses in the environment that could influence the cholera epidemic to start. This study was design to see the effect of *E. coli* bacteriophages on the epidemics of cholera. Routine isolation and estimation of *E. coli* phages reveal that pathogenic *E. coli* phage concentration is proportional to O1 *Vibrio cholerae* (pathogenic) phage concentration. The concentration of nonpathogenic *E. coli* phages does not oscillate like pathogenic *E. coli* phages. Therefore the oscillating abundance pattern of pathogenic *E. coli* phages might control the concentration of pathogenic *E. coli* which in turn controls the *Vibrio cholerae* count by some yet unknown mechanism.

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LIST OF ABBREVIATION

BRAC	Bangladesh Rural Advancemant Committee
et al	And others
g	Gram
ml	Millilitre
ICTV	International Committee on Taxonomy f viruses
E. coli	*Escherichia coli*
RFLP	Restriction fragment length polymorphism
µl	Microlitre
O/N	overnight
rpm	Rotation per minute
Pfu	Plaque forming unit
LA	Luria agar
DNase solution	Deoxyribonuclease solution
RNase solution	Ribonuclease solution
PCI	Phenol-Chloroform Isoamyl Alcohol
TE	Tris EDTA
EDTA	Ethylenediaminetetraacetic acid
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
F primer	Forward primer
R primer	Reverse primer
dNTPs	Deoxynucleotide Triphosphates
EPEC	Enteropathogenic *E. coli*
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EAEC	Entero adherent *E. coli*
ETEC	Enterotoxigenic *E. coli*
CRISPR	Clustered regularly interspaced short palindromic repeats
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid

1. 1. Introduction:

Bacteriophage which is also named as phage is a virus that causes infection and replication inside a bacterium. The term “phage” is derived from Greek word “phagein” which means “to devour”. Phages are made up of proteins which encapsulate DNA or RNA genome. These genomes may encode as four genes or hundred genes. Phages are found wherever bacteria exist.(Mc Grath and van Sinderen ,2007).Locations which are populated with bacterial hosts, for example soil or the intestine of animals are largely distributed by phages. In sea water there are up to 9×10^8 virions per ml which is found in microbial mats at the surface(Wommack and Colwell .,2000)and up to 70% marine bacteria is infected by phages (Prescott ,1993). In the former Soviet Union, central Europe, France, phages are utilized as a substitute of antibiotics for over 90 years. These phages can possibly be used as a cure for multi- drug- resistant strains of many bacteria (Keen, 2012).Bacteriophages of inoviridae complicate biofilms in pneumonia and cystic fibrosis and it gives shelter bacteria from drugs to decrease diseases and promote persistent infection (Goldman, 2015).

1.2. History:

According to Ernest Hanbury Hankin, in 1896 something was found in Ganges and Yamuna rivers in India which had marked antibacterial action against cholera having the characteristic to pass through a very fine porcelain filter (Hankin, 1896).British bacteriologist Frederick Twort invented a small agent that infected and killed bacteria. He assumed that, the small agent is,

- A phase of bacteria life cycle
- Bacteria produced enzyme
- A virus which grew on and killed bacteria (Twort, 1915).

Because of World War 1 and lacking of funding Twort’s work was interrupted. On 3 September 1917 a, French-Canadian microbiologist Félix d'Hérelle invented an invisible, antagonistic microbe of the dysentery bacillus. For d’Hérelle, there was no question as to the nature of his discovery: "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 called the virus a bacteriophage or bacteria-eater (Félix d'Hérelles, 1917).Introduction of

phage therapy and much research in bacteriophage had been conducted by D'Hérelle (Keen, 2012).

For the discoveries 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.

1.3 Classification:

In the biosphere bacteriophages are very much available. On the basis of morphology and nucleic acid the **International Committee on Taxonomy of Viruses (ICTV) classified phages,**

Table 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 dsDNA	
	Clavaviridae	Nonenveloped, rod-shaped	Circular dsDNA	
	Corticoviridae	Nonenveloped,	Circular dsDNA	

Unassigned		isometric		
	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	

1.4.1Enterobacteria phage T4:

It's a bacteriophage which infects *E. coli* bacteria-even phages is a group which have enterobacteriophages like T2 and T6. T4 phage belongs to this group. It undergoes lytic lifecycle but avoid lysogenic cycle.

1.4.2. Genome and Structure:

The DNA genome of T4 phage is double stranded and about 169 kbp long (Millerand Kutter *et al.,2003)*. It encodes for 289 proteins. T4 genome first replicated as a unit and then all other genomic units recombined end to end to form a concatemer.When they are packaged the concatemer are separated at unspecific positions of the same length which leads to several genomes (Madigan and Martinko,2006).T4 genome bears intron sequences.

1.4.3. Translation:

In early genes of bacteriophages the shine-dalgarno sequences GAGG dominates. On the other hand, GGAG is a target for T$ and nuclease RegB which initiates the early mRNA degradation.(Malys, 2012)

1.4.4. Virus particle structure:

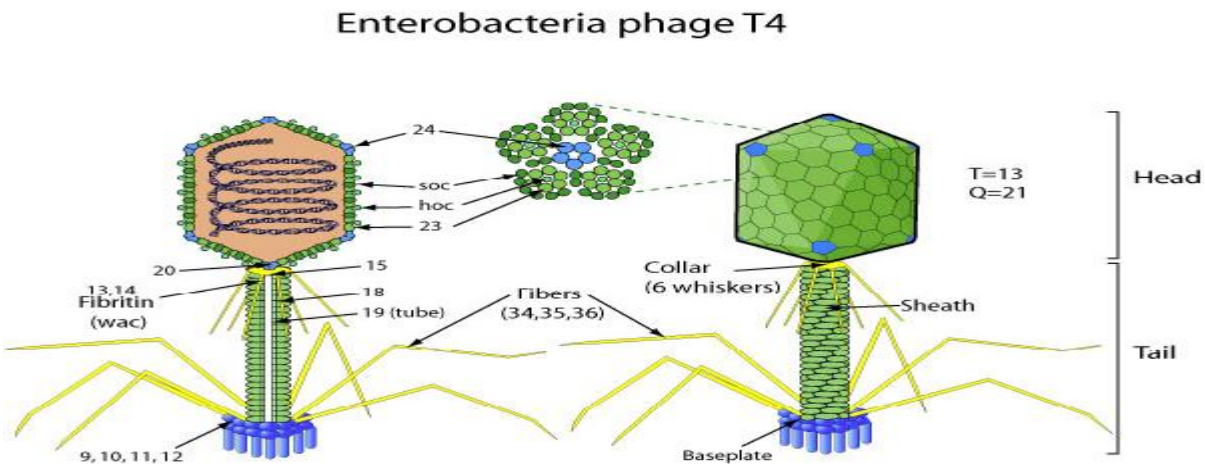


Figure 1: T4 virus (Fokine A and Chipman PR.*et al.*2004)

T4 phage is 90 nm wide and 200 nm long which make it a large phage.Capsid, known as the anicosahedral head of DNA genome**.** Tail is hollow so the phage can pass the nucleic acid into the cell. Tail fibres help tail to attach to a host cell. These fibres also recognize host cell surface receptor to determine whether the bacterium is within the phage's host range (Ackermann and Krisch, 2014).In atomic detail, it is described that the 6 megadalton T4 baseplate is comprised of 127 polypeptide chains of 13 different proteins. The tail tube protein gp54 and the main tube protein gp19 form an atomic model of proximal region, tape measure protein of gp29 is found in the baseplate- tail tube complexes could not be modeled (Taylor and Nicholas M.I. *et al.*, 2016).

1.5. Bacteriophage Infection:

By infecting the host cell bacteriophages reproduce. Steps involve in this cycle are called lifecycle of the phage. Phages like T4 phage reproduce by lytic cycle but others switches between lytic and lysogenic cycle. Lambda phage can infect *E. coli* and switch between lytic and lysogenic cycle.

1.5.1. Lytic cycle:

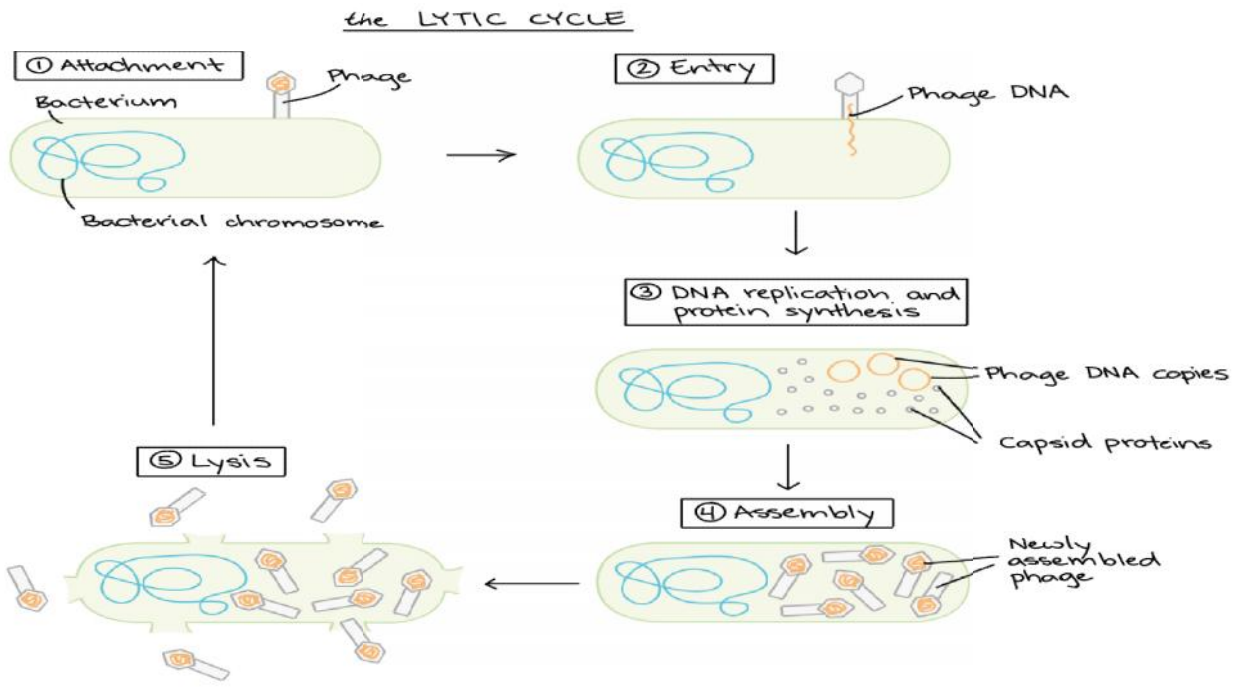


Figure 2: Lytic cycle (www.khanacademy.org/science/biology/biology-of-viruses/virus-biology/a/bacteriophages)

In lytic cycle phage infects host cell, uses its resources and produce lots of new phages and at the end lyse(burst) the cell .

1.5.2. Lysogenic cycle:

Phage reproduces other phages without killing the host. First two steps are like lytic cycle but in it when phage DNA is inside the cell it recombines with a particular region of the bacterial chromosome. Thus the DNA integrated into the chromosome.

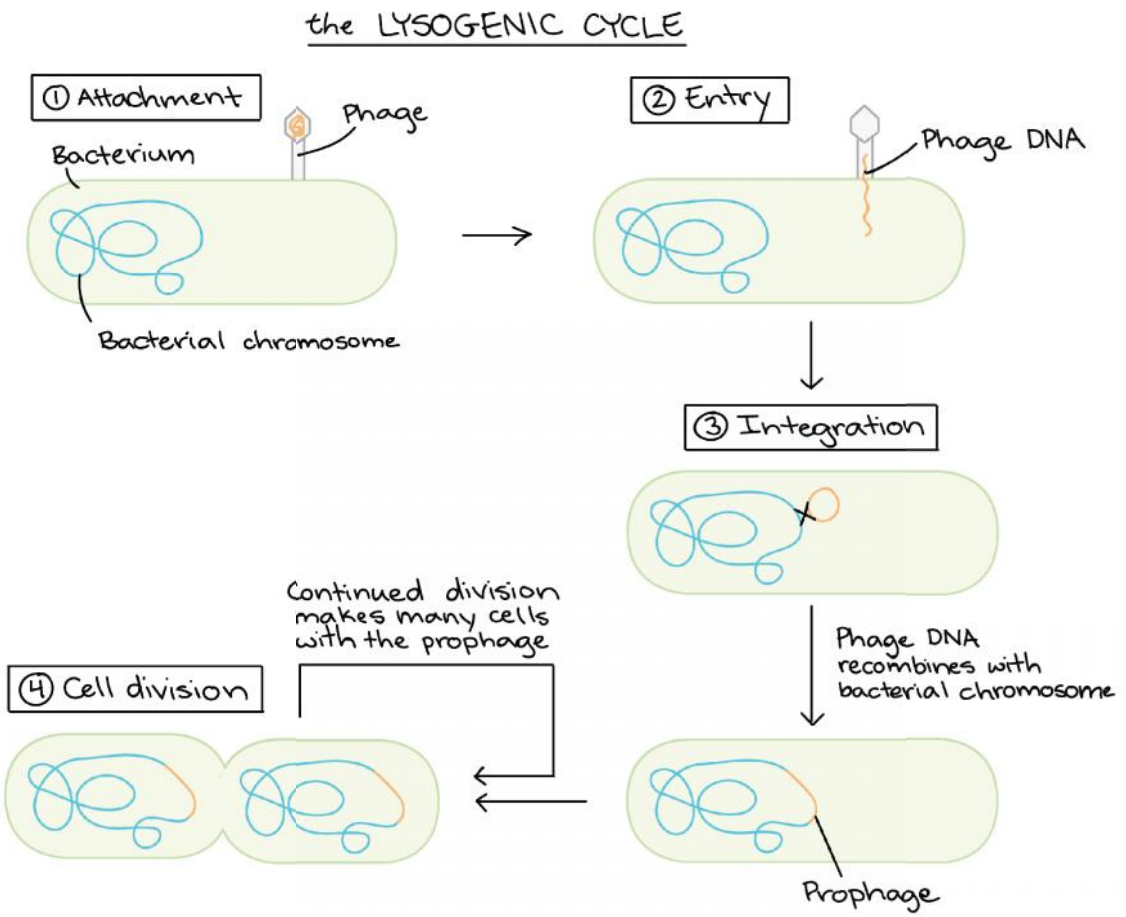


Figure 3: Lysogenic cycle (www.khanacademy.org/science/biology/biology-of-viruses/virus-biology/a/bacteriophages)

1.6. Environmental existence of bacteriophages:

Toxigenic *Vibrio cholerae*, the causative agent of the epidemic diarrhoeal disease cholera interacts with numerous bacteriophages both in the aquatic environment and inside the human intestine. Phages which infect *Vibrio cholerae* (vibriophages) also contribute to the evolution of this pathogen by mediating horizontal transfer of genes and genomic rearrangements Cholera epidemics are known to be self-limiting in nature, since the

epidemics subside after reaching a peak, even without any active human intervention. Among other factors, lytic phages that kill *Vibrio cholerae* have been shown to play a significant role in modulating the course of epidemics presumably through their inherent bactericidal activity. In this latter process, bacterial mutants that are able to resist phage predation (for example, those that have lost cell surface receptors required for phage infection) presumably enjoy a survival advantage. However, phages frequently utilize receptors that are crucial to the pathogenicity of *Vibrio cholerae* such as cell surface lipopolysaccharide O side chain polysacchardies. Furthermore, an antagonistic interaction between a *Vibrio cholerae* chromosomal island that encode phage resistance, and phage encoded CRISPR (clustered regularly interspaced short palindromic repeats) that attacks the island's DNA sequence, provides an additional example of the 'arms race' that occurs between *Vibrio cholera* and its phages.

In the aquatic environment *Vibrio cholerae* is known to exist mostly as biofilms which are comprised of densely packed cells embedded in an exopolysaccharide matrix, or as aggregates of dormant cells referred to as conditionally viable environmental cells (CVEC). Likewise, the colonization of the human gut by *Vibrio cholerae* leads to a state of high bacterial cell density in the intestinal lumen and mucosal surface. The stools of cholera victims are replete with clumps of *Vibrio cholerae* suggesting that bacterial cells may indeed interact closely with each other at high densities during the latter stages of the infection cycle. Independent evidence for cell-cell interactions *in vivo* has also been obtained in a *Vibrio cholerae* animal model through Tn-seq mutational analysis.

Gene expression dependent on bacterial cell density referred to as "quorum sensing" is known to regulate metabolic processes that may influence bacterial survival under unfavorable conditions. The regulatory pathways which control cell density dependent metabolic responses in *Vibrio cholerae* include two autoinducers (CAI-1 and AI-2) and their cognate receptors CqsS and LuxPQ respectively, along with a signal transduction cascade that involves phosphorylation and de-phosphorylation of transcriptional regulatory proteins, non-coding small RNAs, and RNA chaperons. A recent study has proposed the existence of two additional autoinducer sensors in *Vibrio cholerae*, namely VpsS and CqsR, but the signals sensed by these sensors are presumably different from the

two canonical autoinducers, CAI-1 and AI-2. Since the density of bacterial population may be a risk parameter for increased exposure to phages, study shows that quorum sensing could modulate sensitivity of *Vibrio cholerae* to phage predation. Quorum sensing does indeed modulate the sensitivity of *Vibrio cholerae* to phage infection through several ways that include extracellular phage inactivation by haemagglutinin protease (HAP) as well as modulation of the function or accessibility of phage to the LPS O-antigen receptor. (Mozammel and Naser.*et.al*, 2016).

Therefore quoram sensing through autoinducers might play a role to control the prevelance of pathogenic *Vibrio cholerae* in the environment. Different bacteriophages could control the concentration of the bacteria/s that produces autoinducers probably an important factor in this concern. Here we investigate the prevalence of the *E. coli* bacteriophages and there possible correlation with cholera bacteriophages in controlling the pathogenic *Vibrio cholerae* in environment.

1.7. Objectives:

According to a research reported by molecular genetics laboratory of icddr, b, ”Seasonal epidemics of cholera inversely correlate with the prevalence of environmental cholera phages”. That means if number of *Vibrio.cholerae* increases then phage concentration decreases .Again, if *Vibrio cholerae* decreases, then phage concentration increases. During my research project I tried to find out the prevalence and effects of environmental *E. coli* phages on seasonal epidemics of cholera. Phage concentrations were taken from 22nd December, 2016 to 26th July, 2017. Further studies are needed on this project to get a better understanding of the effect of *E. coli* phages. The objectives of this study are,

1. Correlation between the prevalence of environmental *E. coli* and *Vibrio cholerae* phages.
2. Characterization of *E. coli* phages by RFLP (restriction fragment length polymorphism)
3. pH stability of *E. coli* phages
4. Temperature stability of *E. coli* phages

2. Material and Method:

2.1. Place of study:

The study was carried out in Molecular Genetics Laboratory, International Centre for Diarrhoeal Disease Research Bangladesh.

2.2. Sample collection: Sample was collected from Turag river of Bangladesh.

2.3. Preparation of template DNA of *E. coli* strains:

The *E. coli* strains used to isolate phages from sample water were tested to know if they are pathogenic or non-pathogenic by PCR,

1. In order to identify whether the isolated *E. coli* strains are pathogenic or non-pathogenic by multiplex PCR, boiled supernatant was prepared. As 24 *E. coli* was used in this experiment, their supernatant was produced.
2. Respective strains were incubated in 2gm LB media for overnight growth.
3. After incubation 1200µl solution was taken in the tube
4. Then centrifuged at 10000rpm for 5 minutes and supernatant was discarded.
5. 1ml of PBS was added and the pellet was dissolved. Again, the solution was centrifuged at 10000rpm for 5 minutes and supernatant was discarded.
6. This above process of adding PBS was repeated and denatured in boiling water bath for 10 minutes.

   After 10 minutes, the tubes were plunged immediately into ice and kept it for 10 minutes. The supernatant was collected which is a 10x template. It was made 1x by mixing 20 µl supernatant with 180µl distilled water.

2.4. Multiplex PCR of environmental *E.coli* samples:

PCR is used to amplify specific regions of a DNA strand (the DNA target). This can be a single gene, a 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.

1. A basic PCR set up requires several components and reagents. These components include:
2. DNA template that contains the DNA region to be amplified.
3. Two primers, which are complementary to the DNA regions at the 5' or 3' ends of the DNA region.
4. Taq polymerase to amplify the DNA
5. Deoxynucleoside triphosphates (dNTPs), the building blocks from which the DNA polymerases synthesize a new DNA strand.
6. Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.

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

Denaturation step: This 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 DNA-DNA 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.

(http://www.srmuniv.ac.in/downloads/bi0311_genetic_engineering_lab_manual.pdf)

Procedure for PCR:

Preparation of master mix:

- As we were indentifying whether this E.coli samples are ETEC, EPEC, EAEC and EIEC, we had to use reverse and forward primer of *lt, st, eae, bfp, aata , aaic* and *ipah.*

Table2: Calculation for reagents and enzymes of master mix

Reagents and Enzyme	**µl** x n	**Amount (µl)**
Buffer	4×26	104
MgCl2	1.6×26	41.6
dNTPs	.4×26	10.6
lt F	.5×26	13
lt R	.5×26	13
st F	.5×26	13
st R	.5×26	13
aaic F	.5×26	13
aaic R	.5×26	13
aata F	.5×26	13
aata R	.5×26	13
bfpa F	.5×26	13
bfpa R	.5×26	13
eae F	.5×26	13
eae R	.5×26	13
ipah F	.5×26	13
ipah R	.5×26	13
Taq polymerase	.1×26	2.6
Pcr water	4.9×26	127.4
Total		468.2

- After the master mix was made, 18µl of master mix added with 2µl DNA of that environmental *E.coli* strains.5 *E. coli* strains were used as positive control.
- The components were mixed gently and placed in a thermo cycler.

- The denaturation temperature of cycler is 95°C for 1 minute , annealing is 54°Cfor 30 seconds and elongation is 70°C for 1 minute .
- After the PCR was completed 10µl of solution was loaded in the agarose gel and followed the below-mentioned process of gel electrophoresis.

2.5. Detection and isolation of *E. coli* Phages:

At first, to detect *E. coli* phages and culture *E. coli* processing of environmental water sample was done within three hours of collection. For phages, logarithmic phase cells (50µl) of each of 10 hosts *E. coli* strains in nutrient broth were mixed in 50ml aliquots of nutrient broth.

1. After mixing those bacteria strains in 50 ml nutrient broth, they were incubated for 1-1.5 hours at 37 °C.
2. When the incubation was done, 10ml of environmental water sample was added and again incubated at 37 °C for o/n.
3. After incubation, the incubated sample was centrifuged at 13000 rpm for 10 minutes and filtered with the help of .22 µm pore filter and collected into fresh tube.
4. Then plaque assay was done to obtain phage concentration.

2.6. Plaque assay for phage concentration:

Plaque is an area in a monolayer displaying acytopathic effect for instance, appearing round and darker under the microscope than other cells or looks like white spot. The center of the plaque doesn't have any cell as it is lysed by virus. Plaque forming unit is a virus or group of viruses causing a plaque.

1. For plaque assay young culture of 10 *E. coli* strains were made. Then 10 LA media plates were prepared.
2. Soft agar was prepared and it was ensured that soft agar was in accurate temperature.

3. Then 10 empty and sterile bottles were taken and 100µl of 10 different *E. coli* strains which were used in making mixture were kept in those bottles individually. The LA plates and the empty plates were marked accurately in order to avoid any mistakes.
4. After the marking process, 3.9 ml of soft agar were added in the bottles and poured the whole mixture on the LA plate after vortexing.
5. The filtered solution of phage was diluted 10 times and then 2µl of each diluted sample was added on the LA plates.
6. When the drops of phage samples became dry, LA plates were kept in 30°C for overnight incubation.

2.7. Determining viral titers:

A sample is scored positive if plaque is observed on the plate. Plaques that were formed counted and the concentration of phage in the sample was determined by the equation that is mentioned below:

$$\text{Pfu/ml} = \frac{\text{Number of plaque}}{D \times V}$$

D= Dilution; V= Volume of diluted virus added to the plate.

2.8. DNA isolation from phage strains:

1. Phages from the representative single plaque were collected to and used for high titer stocks. The single plaque was stored in SM buffer. This solution was used for DNA isolation.
2. 10 different bacteria for 10 different phage solutions were selected. Then with the above-mentioned process plaque assay was done.
3. After overnight incubation, it was observed that so many plaques were formed on the LA plates.
4. 10 falcon tubes were taken and marked. Then 2ml of reaction buffer 2 was added on each LA plates and vortexed.

5. With the use of spread glass tubes soft agar part of LA plates were separated and kept in the falcon tubes. Each tubes were vortexed for 2 minutes.
6. Then each tube was centrifuged at 10000 rpm for 2 minutes. After centrifugation two layers were formed in the tube. By using a 3ml syringe the aqueous layer in falcon tube was separated and filtered with .22 μm pore filter.
7. After that in each of those 10 filtered solution 15μl of DNAs and 100μl of RNAs solution was added and mixed them by inverting. They were kept in 37°C water bath for overnight.
8. Then 50μl of pk buffer and 15μl of pk solution were added in eppendorf tube and mixed them by inverting and kept them for overnight in the water bath.
9. After that equal volume of PCI solution was added in the tube and inverted for 15 minutes. Then centrifuged at 13000 rpm.after centrifugation with a cut tips the upper aqueous layer was separated. Again equal volume of PCI was added and the whole above process was repeated.
10. In the aqueous layer double volume of absolute ethanol was added and kept in -20°C for overnight.
11. Then the solution was centrifuged at 13000 rpm for 15 minutes and after that the supernatant was separated. and 600μl of 70% ethanol was added and after ethanol wash a white pellet was formed.
12. The pellet was dried in the vacuum dryer for 5 minutes. Then 20μl of TE buffer was added and mixed well by pipetting. Then OD of the DNA pellet was taken and stored in -20°C.

2.9. Restriction Fragment Length polymorphism (RFLP) of phage DNA:

DNA is cut into specific sites or restriction recognition sequences by a restriction enzyme. Most of the restriction recognition sequences are palindromic and vary in lengths between 4 and 8 nucleotides. Restriction enzyme makes two incisions, once through each sugar-phosphate backbone (i.e. each strand) of the DNA double helix. Some restriction enzymes cut the double stranded DNA in two different positions and generate ends that are staggered, with 5’ or 3’ protruding terminal nucleotides; others cut at the same position and produce blunt ends.

In these process phage DNA were digested with EcoRI, BglI, HindIII, XbaI, ClaI and SpeI

1. Two reactions were set according to the table, phages of 25^{th}, $26^{th,}$ 27^{th} ,28^{th} ,29^{th} , 31^{st},32^{nd},34^{th} batch were followed group 1 reaction and 30^{th}, 33^{rd} batch were followed group 2 reaction.

Table 3: Calculation for RFLP of phage DNA

Group 1 RFLP		Group 2 RFLP	
Reagent	Amount	Reagent	Amount
BSA	2µl	BSA	2µl
10xbuffer	2µl	10xbuffer	2µl
RNAs	2µl	RNAs	2µl
Bgl1	1µl	HindIII	1µl
EcoR1	1µl	XbalI	1µl
DNA	9µl	ClaI	1µl
Distilled water	3µl	SpeI	1µl
Total	20µl	DNA	10µl
		Total	20µl

Other 8 phage DNA sample was digested according to the calculation mentioned above.

2. The added reagents were mixed gently and kept in 37°C for overnight.

3. Then gel electrophoresis was done to see the fragments of DNA.

2.10. Agarose gel electrophoresis:

1. To analyze DNA gel electrophoresis is done. Agarose is linear polymer. Pore size can be altered by changing the gel concentration. Smaller the pore size, higher the gel concentration.
2. For making agarose gel a conical flask was taken and .8g agarose was measured and kept in the flask.
3. After that, 80 ml,.5x TBE buffer and 20 ml distilled water , 1.2 µl ethidium bromide were added. Heat was given until the solution started boiling.
4. Then the solution was poured into the gel plate and the comb was set.
5. After sometime when the gel was cool down 20µl of 10 different samples were loaded in the gel. Before loading bromophenol blue (the tracking dye) was added.

6. Once the sample was loaded in to the well, the cathode (Black negative terminal) connected towards the top end of the gel and the anode (Red positive terminal was connected towards the bottom end of the gel.. The electrophoresis is started by switching on the D. C. Power pack. The gel is run at 40v/cm.
7. As the bromophenol blue (the tracking dye) had moved 1 cm above the bottom end, the current is switched off, the power supply is disconnected and the gel along with the platform was taken.
8. Then with the help of a gel doc machine DNA bands were seen and a photograph was taken.

2.11. Temperature and pH stability test:

1ml aliquot of 4 different phage sustentions were incubated at temperatures of normal, 50°C, 60°C and 70°C for 2 hours. Phage Infectivity was then assayed by plaque assay technique mentioned above. The pH stability of the phage was identified in SM buffer adjusted to different ph values like normal, 3, 5, 9, 10. After incubating for 2 hours at room temperature the surviving phage was diluted and plaque forming capacity was measured by plaque assay technique.

3. Result:

3.1. Multiplex PCR of environmental *E. coli* samples:

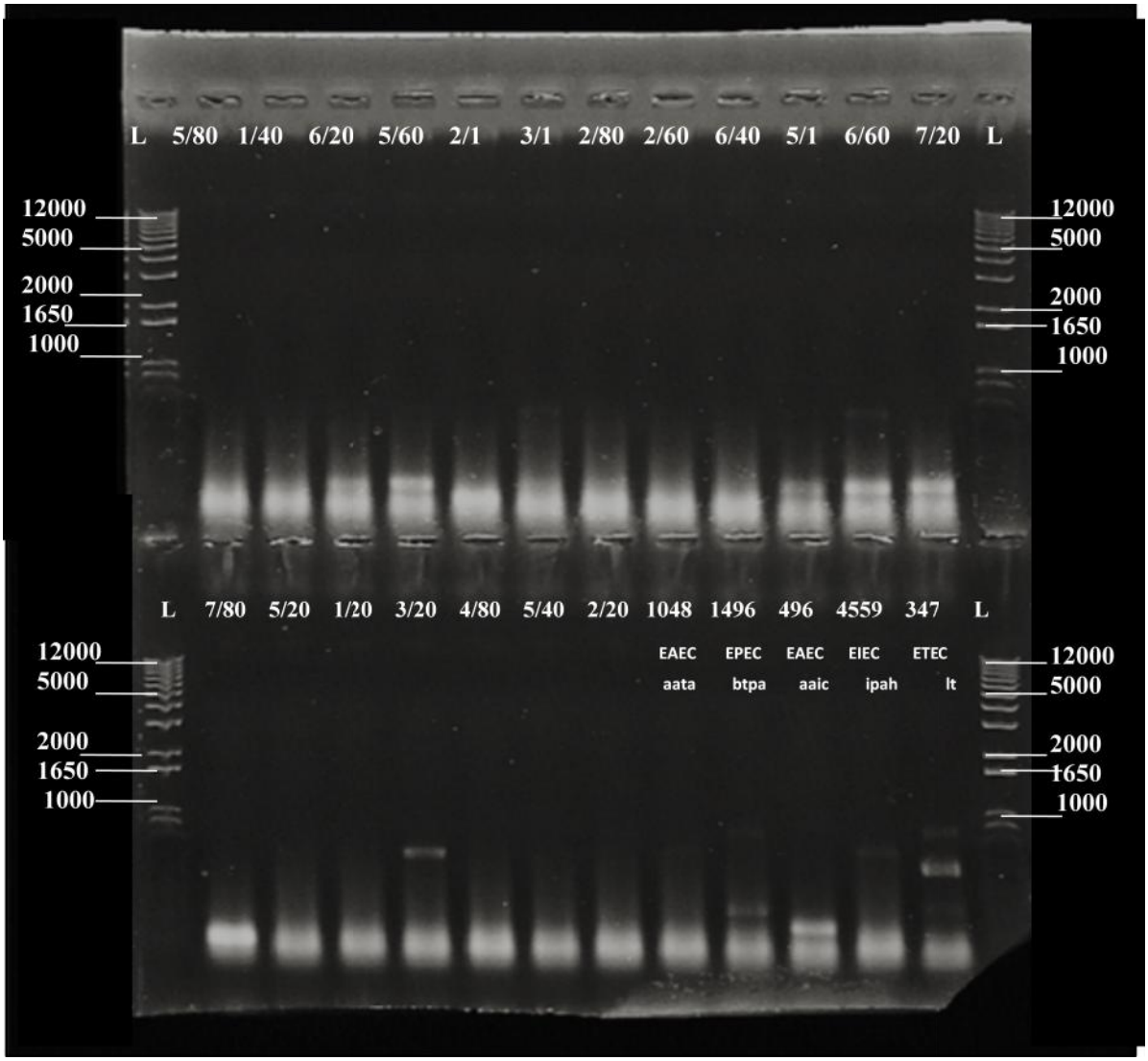


Figure 4: Multiplex PCR of *E. coli* strains

Multiplex PCR was done to determine pathogenic and non-pathogenic *E. coli.* After gel electrophoresis of PCR product the above image was obtained. ETEC contains *lt* and *st* gene and their sizes are 508bp and 147bp.EPEC contains *eae* and *bfp* and their sizes are 881 and 300 bp. EAEC contains *aata* and *aaic* and their sizes are 650 and 25bp. EIEC contains *ipah* gene and size is 423. These five strains were used as positive control for the sample strains.

The result is given below,

Sample number	Specific gene	Type of *E. Coli*	Pathogenic(+), Non pathogenic(-)
1048	*aata*	EAEC	+
1496	*btpA, eae*	EPEC	+
496	*aaic*	EAEC	+
4559	*ipaH*	EIEC	+
347	*lt*	ETEC	+
5/80	-		-
1/40	-		-
6/20	-		-
5/60	*aaic*	EAEC	+
2/1	*st*	ETEC	+
3/1	*aata*	EAEC	+
2/80	-		
2/60	-		
6/40	-		
5/1	-		
6/60	*aata , aaic*	EAEC	+
7/20	*aaic*	EAEC	+
7/80	*st*	ETEC	+
5/20	-		
1/20	-		
3/20	*ipaH*	EIEC	+

4/80	-		
5/40	-		
2/20	-		

Table: 4 : Result of the multiplex PCR of E.coli strains.

3.2.1. Correlation between *E. coli* and *Vibrio cholerae* O1 phage:

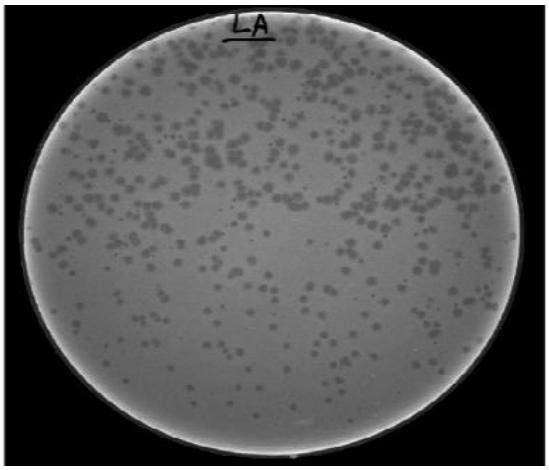


Figure 5: *E. coli* phage plaque

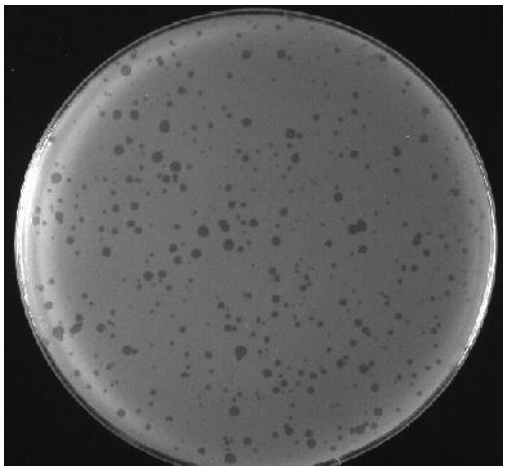


Figure 6: *Vibrio cholerae* phage plaque

From the pictures given above we can differentiate between *E. coli* and *Vibrio cholerae* phage plaque.

According to the chart mentioned below, *E. coli* phage shows stable concentration .All It's concentrations from December'16 to July'17 are above 7.Only at the end of January and July it shows low concentration. However, *Vibrio cholerae* O1 phage does not show as stable concentration as *E. coli* phage. From December'16 to February'17 it shows low concentrations .But from April, concentrations become high and it continues till May and again low from June.

Figure 7: Correlation between *E. coli* and *Vibrio cholerae* O1 phage:

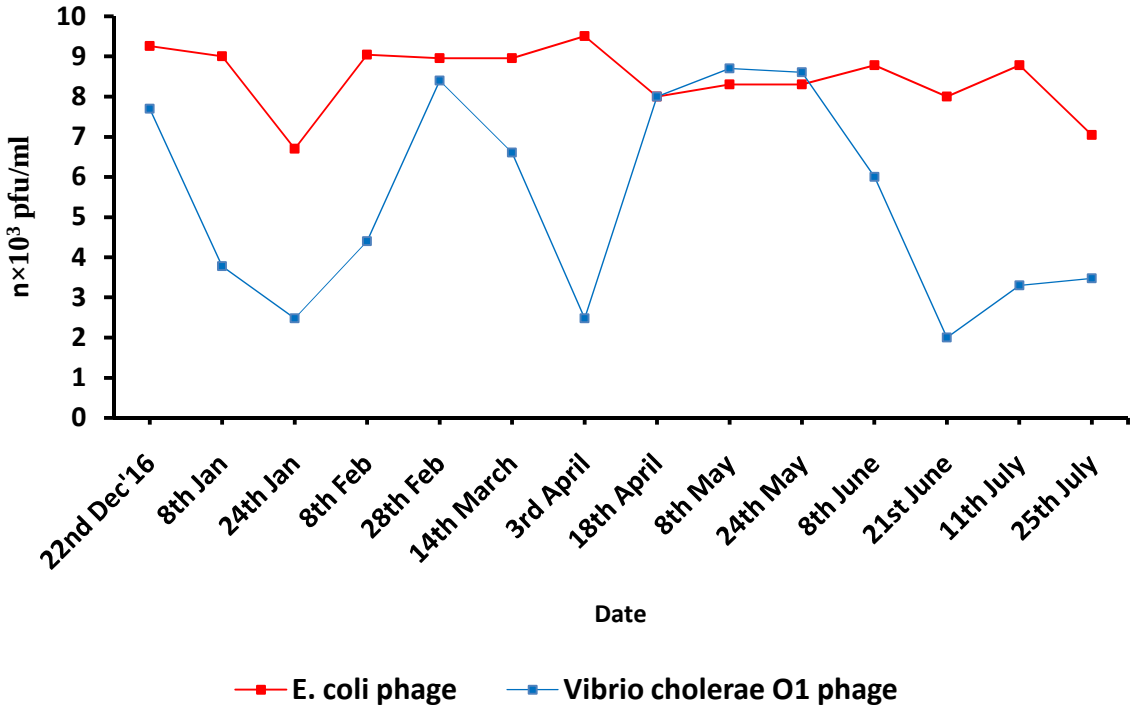


Table 5: Data of *E. coli* and *Vibrio cholerae* phage concentrations

Date	*E. coli* phage concentration(pfu/ml)	*Vibrio cholerae* phage concentration (pfu/ml)
22nd Dec '16	1.8×10^{9}	5×10^{7}
8th Jan	1×10^{9}	6×10^{3}
24th Jan	5×10^{6}	3×10^{2}
8th Feb	1.1×10^{9}	2.5×10^{4}
28th Feb	9×10^{8}	2.5×10^{8}
14th March	9×10^{8}	4×10^{6}
3rd April	3.2×10^{9}	3×10^{2}
18th April	1×10^{8}	1×10^{8}
8th May	2×10^{8}	5×10^{8}
24th May	2×10^{8}	4×10^{8}
8th June	6×10^{8}	1×10^{6}
21st June	1×10^{8}	1×10^{2}
11th July	6×10^{8}	2×10^{3}
25th July	1.1×10^{7}	3×10^{3}

3.2. 2.Correlation between pathogenic and non-pathogenic *E. coli* phage:

Figure8: Pathogenic and non-pathogenic *E. coli* phage correlation

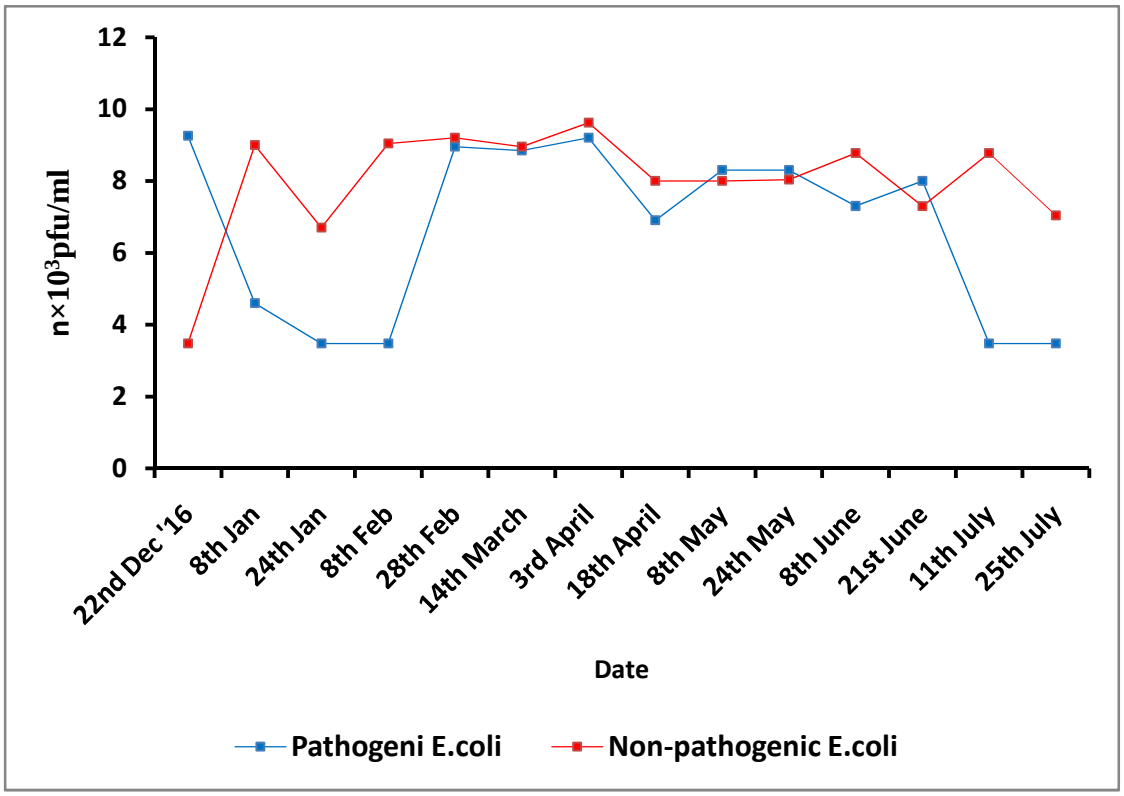


Table 6: Data of pathogenic and non-pathogenic *E. coli* phage concentrations

Date	Pathogenic *E. coli* phage concentration (pfu/ml)	Non-pathogenic *E. coli* phage concentration (pfu/ml)
22nd Dec '16	1.8×10^{9}	3×10^{3}
8th Jan	4×10^{4}	1×10^{9}
24th Jan	3×10^{3}	5×10^{6}
8th Feb	3×10^{3}	1.1×10^{9}
28th Feb	9×10^{8}	1.6×10^{9}
14th March	7×10^{8}	9×10^{8}
3rd April	1.6×10^{8}	4.2×10^{9}
18th April	8×10^{6}	1×10^{8}
8th May	2×10^{8}	1×10^{8}
24th May	2×10^{8}	1.1×10^{8}
8th June	2×10^{7}	6×10^{8}
21st June	1×10^{8}	2×10^{7}
11th July	3×10^{3}	6×10^{8}
25th July	3×10^{3}	1.1×10^{7}

The above chart shows the difference between pathogenic and non-pathogenic *E. coli* phage concentration. From December ’16 to February’17 both types of phage show different concentrations. If pathogenic phage increases, then non-pathogenic phage decreases. However, from March to June they show stable and comparatively same result. But from July pathogenic phage decreases and non-pathogenic phage increases.

3.2.3. Correlation between pathogenic *E. coli* and *Vibrio cholerae* O1phage:

Figure 9: Pathogenic *E. coli* and *Vibrio cholerae* phage correlation:

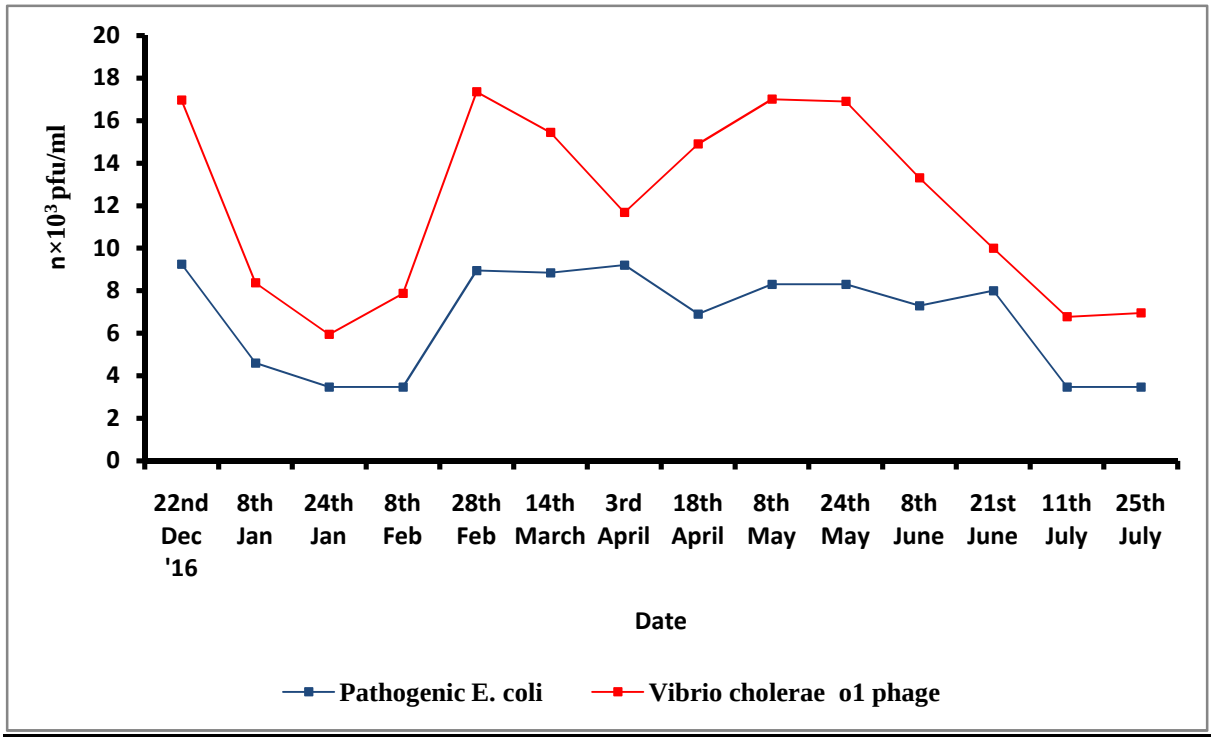


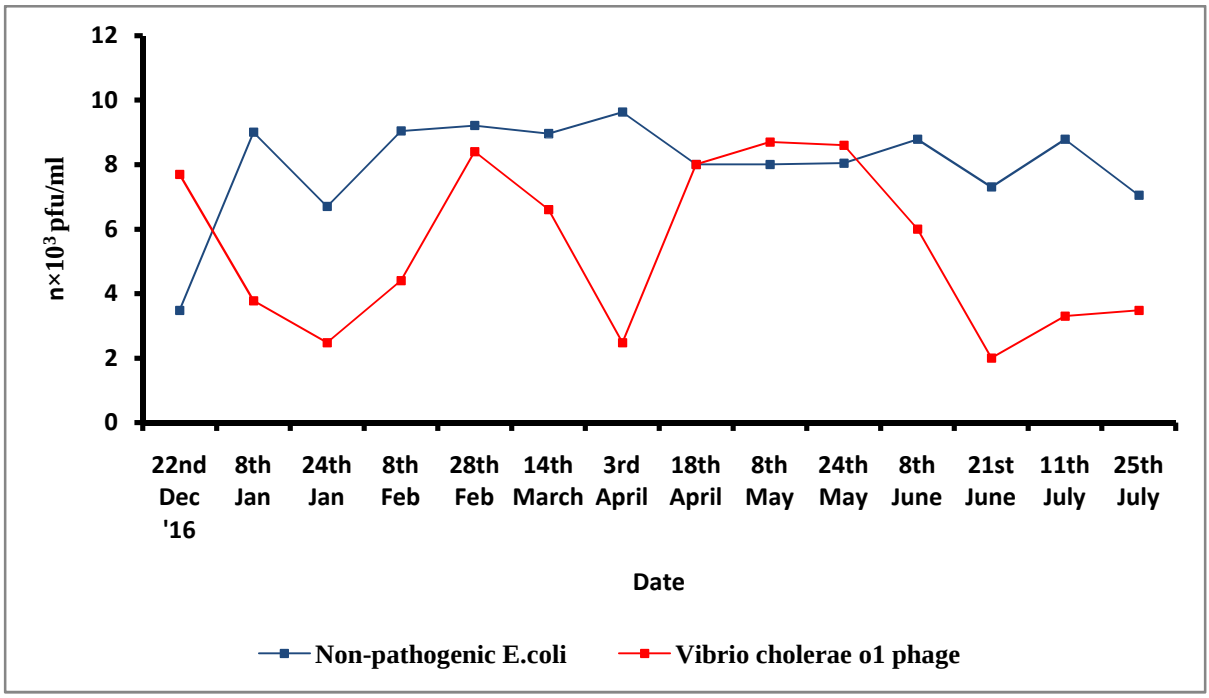
Table 7:Data of pathogenic *E. coli* and *Vibrio cholerae* O1 phage concentrations

Date	Pathogenic *E. coli* phage concentration(pfu/ml)	*Vibrio cholerae* phage concentration(pfu/ml)
22nd Dec '16	1.8×10^{9}	5×10^{7}
8th Jan	4×10^{4}	6×10^{3}
24th Jan	3×10^{3}	3×10^{2}
8th Feb	3×10^{3}	2.5×10^{4}
28th Feb	9×10^{8}	2.5×10^{8}
14th March	7×10^{8}	4×10^{6}
3rd April	1.6×10^{8}	3×10^{2}
18th April	8×10^{6}	1×10^{8}
8th May	2×10^{8}	5×10^{8}
24th May	2×10^{8}	4×10^{8}
8th June	2×10^{7}	1×10^{6}
21st June	1×10^{8}	1×10^{2}
11th July	3×10^{3}	2×10^{3}
25th July	3×10^{3}	3×10^{3}

According to the data visible in the chart, pathogenic *E. coli* phage shows same result as O1 *Vibrio.cholerae* phage which is also pathogenic. That means, concentrations of pathogenic *E. coli* phage are proportional to pathogenic O1 *Vibrio cholerae* phage.

3.2.4. Non-pathogenic *E. coli* and *Vibrio cholerae* O1 phage:

Figure 10:Non-pathogenic *E. coli* and *Vibrio cholerae* O1 phage correlation



.Table 8: Data of Non-pathogenic *E. coli* and *Vibrio cholerae* phage concentrations

Date	Non-pathogenic *E. coli* phage concentration(pfu/ml)	*Vibrio cholerae* phage concentration(pfu/ml)
22nd Dec '16	3×10^{3}	5×10^{7}
8th Jan	1×10^{9}	6×10^{3}
24th Jan	5×10^{6}	3×10^{2}
8th Feb	1.1×10^{9}	2.5×10^{4}
28th Feb	1.6×10^{9}	2.5×10^{8}
14th March	9×10^{8}	4×10^{6}
3rd April	4.2×10^{9}	3×10^{2}
18th April	1×10^{8}	1×10^{8}
8th May	1×10^{8}	5×10^{8}
24th May	1.1×10^{8}	4×10^{8}
8th June	6×10^{8}	1×10^{6}
21st June	2×10^{7}	1×10^{2}
11th July	6×10^{8}	2×10^{3}
25th July	1.1×10^{7}	3×10^{3}

According to the chart, non-pathogenic *E. coli* phage is inversely correlated to pathogenic O1 *Vibrio cholerae* phage. When *E. coli* phage concentration decreases cholera phage increases and when *Vibrio cholerae* phage decreases *E. coli* phage increases

3.3. RFLP of phage DNA:

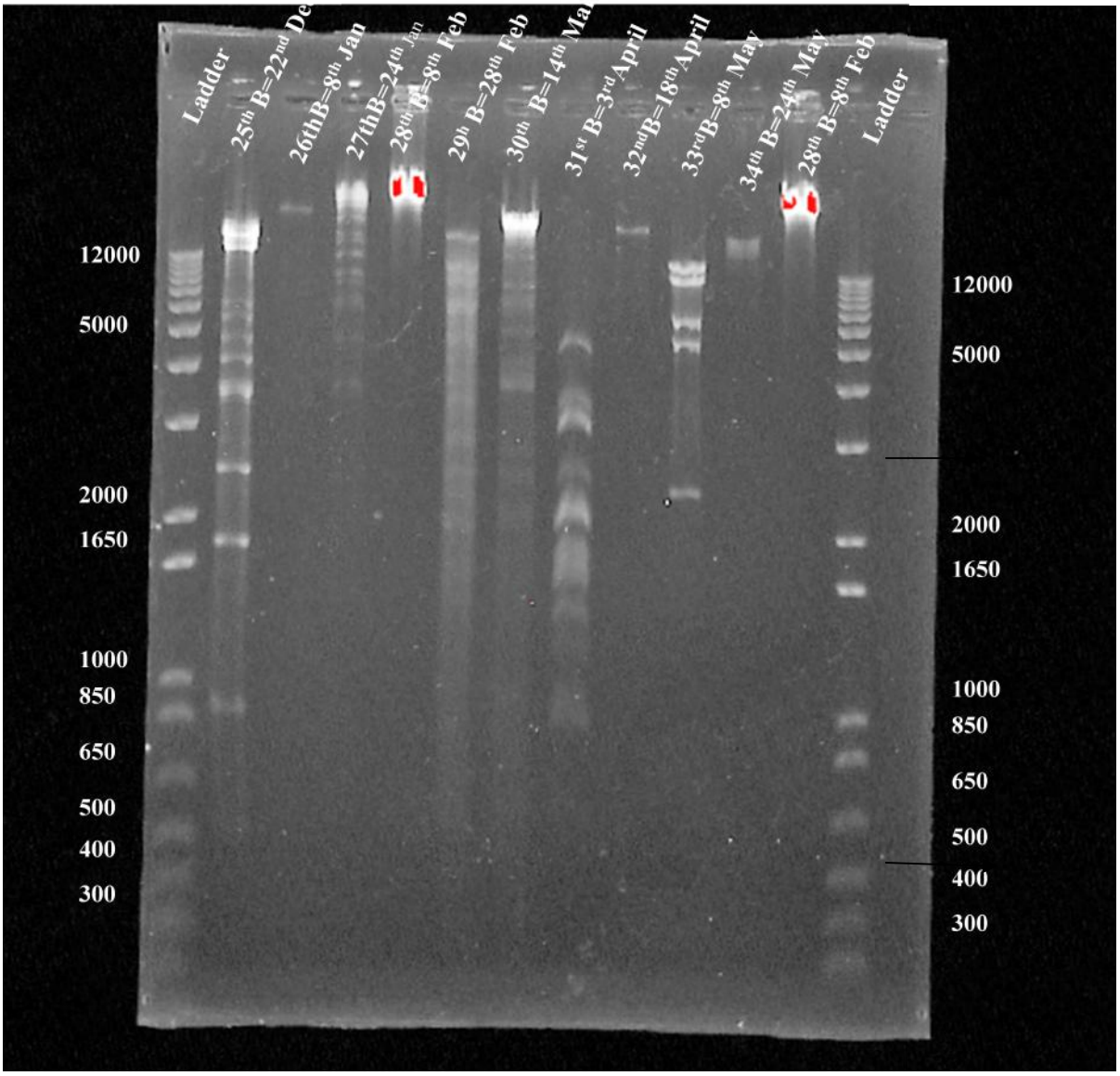


Figure11: RFLP of Phage DNAs

For 10 different phage DNAs, RFLP was done. Then after gel electrophoresis the above picture was obtained. The goal was to identify whether those phage DNA were same or different in nature. From the image above it is visible that all the phages are not same. Phages of 25th, 27th, 29th, 30th, 31st, 33rd and 34th batch are different as their restriction patterns aredifferent. Restriction enzyme didn't cut 28th batch DNA. In case of 26th and 32nd batch DNA, it is not visible enough to ensure that the DNA was fragmented.

3.4. Temperature stability test of *E. coli* phages of 27th, 29th and 33rd and 34thbatch:

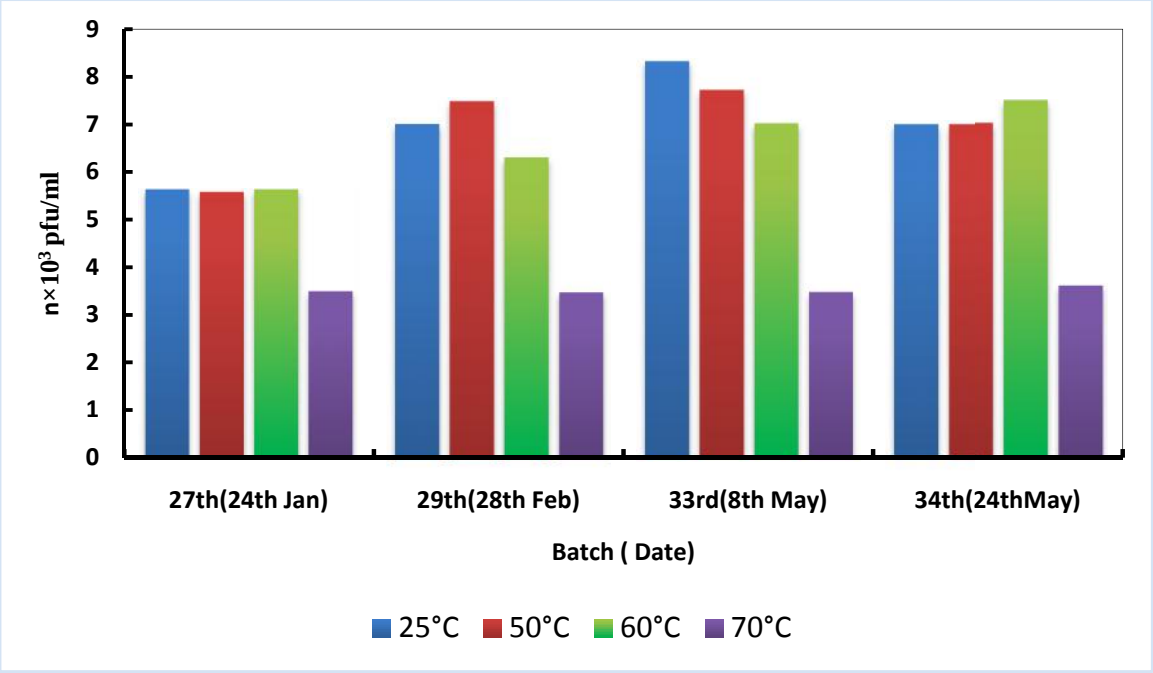


Figure12: Temperature stability of different phages

Table 9: Data of *E. coli* phage concentrations at different temperatures:

Batch (Date)	Phage concentration at 25°C (pfu/ml)	Phage concentration at 50°C(pfu/ml)	Phage concentration at 60°C(pfu/ml)	Phage concentration at 70°C(pfu/ml)
27th (24th Jan)	4×10^5	3.5×10^5	4×10^5	3×10^3
29th(28th Feb)	1×10^7	3×10^7	2×10^6	3×10^3
33rd(8th May)	2×10^8	5×10^7	1×10^7	3×10^3
34th(24th May)	1×10^7	1×10^7	3×10^7	4×10^3

According to the above-mentioned chart, phage concentration of 29th and 33rd batch is high at 50°C and 28 °C and moderate at 60°C and low at 70°C.However, phage concentration of 34thand 27thbatch is same at 28°Cand 50°C.They show moderate concentration at these two temperatures. At 60°C, both of these two phage batches show high concentration. Like other batches their concentration is low 70°C. So, *E. coli* phages are highly stable between 28°C to 60°C.The temperature of the environment rises up to 38°C.That means, *E. coli* phages are very much stable in our environment.

3.7. pH stability test of *E. coli* phages of 27th, 29th, 33rd and 34thbatch:

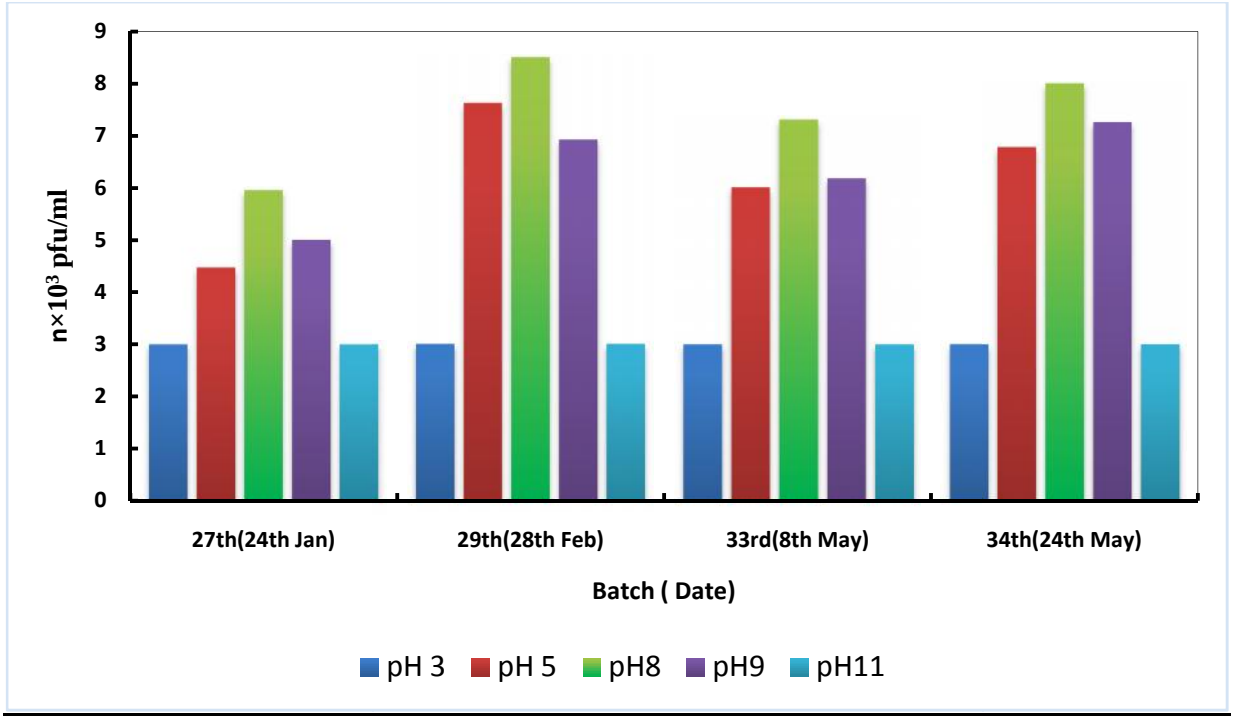


Figure 13: pH stability of different phages

Table10: Data of *E. coli* phage concentrations at different pH

Batch (Date)	Phage concentration at pH3 (pfu/ml)	Phage concentration at pH5 (pfu/ml)	Phage concentration at pH8 (pfu/ml)	Phage concentration at pH9 (pfu/ml)	Phage concentration at pH11 (pfu/ml)
27th(24th Jan)	1×10^{3}	3×10^{4}	9×10^{5}	1×10^{5}	1×10^{3}
29th(28th Feb)	1×10^{3}	4×10^{7}	3×10^{8}	8×10^{6}	1×10^{3}
33rd(8th May)	1×10^{3}	1×10^{7}	2×10^{7}	1.5×10^{6}	1×10^{3}
34th(24th May)	1×10^{3}	6×10^{6}	1×10^{8}	1.8×10^{7}	1×10^{3}

Again, phage concentration of 27th, 29th, 33rdand 34th batches is high at pH8, moderate atpH5 and pH9 and very low at pH3and pH11.So, *E.coli* phage is highly active between pH5 to pH9.As, pH of the environment is close to 8 , these phage is very much available in the environment.

4. Discussion:

The main reason of cholera epidemics is the involvement of *Vibrio cholera* with serogroups of O1 and O139. Usually, these epidemics occur twice in a year during the dry season and monsoon rain. In dry season salt concentration of the surface water increases which favors the colonization *Vibrio cholerae* and in the rainy season flood water allows these bacteria to contaminate the sources of drinking water. In many developing countries it's a major public health problem. Phages play important roles in the evolution and prevalence of pathogenic cholera species in the environment. There is a report which state that "the presence of bacterial viruses acting on *Vibrio cholerae* O1 or O139 (cholera phages) inversely correlates with the occurrence of viable *Vibrio cholerae* in the aquatic environment and the number of locally reported cholera cases. Therefore bacteriophages in the environment are playing important role in the prevalence and distribution of pathogenic bacteria which could cause deadly epidemics like cholera.

In the environment numerous numbers of bacteria and bacteriophages persist together. But only few of them cause the infectious disease in the form of epidemics. Therefore there are possibilities that interaction between these microbes allow the clonal amplification pathogenic microbes that can cause the disease. As reported earlier vibriophages could play important role in controlling the epidemics of cholera here we study the possible correlation of *E. coli* phages with vibriophages to influence the epidemics. The phage was isolated from the environmental surface water. Collection of water sample was done twice in a month to get the accurate result. Sample was enriched to increase the phage concentration otherwise it would have been difficult to isolate the result. During the process of phage isolation, it's important to filter the solution carefully to make it bacteria free. After isolation, plaque assay was done. The chances of contaminations are very high in the plaque assay. Therefore, the tips were handled carefully during serial dilution and pouring soft agar in the small bottles to avoid contamination. . Plaque assay gave us a precise idea of the concentration of the phage

in the water samples. Single plaque was collected and further processed for the DNA isolation.

The strains that were used to enrich the phage samples were separated into pathogenic and non-pathogenic *E. coli* strains. It was done to identify the pathogenic or non-pathogenic phages of *E. coli*. The results show that nonpathogenic *E. coli* phages are present in the environment in a uniform concentration. We always isolate nonpathogenic phages from environment in a same frequency therefore it indicates that it probably has nothing to do with the oscillating nature of the cholera outbreak. On the other hand the pathogenic *E. coli* phages show a nice oscillating behavior in terms of their abundance in the environment. The frequency of their isolation nicely matches with that of pathogenic cholera phages. Therefore it seems to reasonable that these phages might have some effect on the seasonal epidemics of cholera in Bangladesh. How these *E. coli* phages influence the cholera epidemics is a subject of further study. One possibility is that, theses phages may control the concentration of *E.coli* strains in the environment which could control the concentration of autoinducer in the environment. These autoinducer in turn resuscitate the dormant *Vibrio cholerae* persist as biofilm in environment to cause the cholera epidemics. There are possibly other mechanisms which could lead to this type of effect.

As, the DNA of different phage sample were found, it was important to know if they are the same phages or different phages. To know this, RFLP was done.. Different kinds of restriction enzymes were mixed together and the applied on the DNA. It worked except one sample which was sample of 28^{th} batch. DNA concentration of 28^{th} batch phage was very high that it was difficult for restriction enzymes to cut them. Then the gel electrophoresis was done in a very low voltage as DNA was getting cranked. This process needs to be run in a slow speed to get more effective result. After the experiment, we got 6 different phages. Phages were different from each other because they give different band size. So based on the band size we separate them in six different groups. We tried to find out if there is any different pattern of fragmenting DNA in case of pathogenic and non-pathogenic phages. But, no such pattern was found. For getting confirmation about the types of *E. coli* sequencing should have to be done which will give more accurate result.

Temperature and pH sensitivity tests of *E. coli* were done. For these two tests phage solution of four different types were taken to get accurate results. Phage solutions were kept in water bath of different temperatures. After that plaque assay was done carefully and concentration was taken to get the exact result. Phages were active between 25°C to 60 °C and inactive at 70°C.Again, in between pH 5-9 phages were active but in case of pH 3 and pH 11 they give negative results .That means these *E. coli* phages are very much active in our environment causing numerous problems.

Limitations:

1. For isolation of phage DNA, at least 7 days are needed. So, if there is any gap between those days the chances of occurring contaminations are very high.
2. During plaque assay it's good to use the soft agar for 5-6 plates at once as it becomes cold easily. If the soft agar becomes slightly cold, the plaque isn't formed.
3. To avoid contamination tips were used carefully.
4. During filtration of the enriched culture, it was done carefully as it can pour on the floor or dress and spoil the whole process.
5. When RFLP was done, restriction enzymes were mixed carefully. As some enzymes were not available at that time, some DNA was not fragmented properly.
6. Gel electrophoresis was done in low voltage. As DNA got crank; the experiment was repeated to get a good view of the restriction enzyme fragmented DNA. It took so much time for instance 6 to 7 hours to be done as the voltage was slow.
7. For multiplex PCR, several kinds of primers were needed. It took time to collect those primers as they were not available.

Recommendations for further research:

1. This project was continued from December '16 to July '17. The result that is obtained needs to be more effective. So, it should be continued for more months to get confirmation.

2. The obtained result was only compared with O1 cholerae phage. Non O1 phage can be compared which may lead to invention of another topic to study further.
3. From the paper published before it was found out how cholera phage affects epidemics of cholera. This research mention above only helps to know that pathogenic *E. coli* phage probably has same effects on epidemic of cholerae as pathogenic cholerae phage. But in which way they influence the epidemic, needs further studies.
4. More research can be done on non-pathogenic *E. coli* phages.
5. If non-pathogenic *E. coli* phages didn't show same result as pathogenic phages of it might be interesting to know the reason behind this.

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