ISOLATION AND MOLECULAR CHARACTERIZATION OF E.COLI BACTERIOPHAGES FROM SURFACE WATER OF BANGLADESH

Submitted by

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of Bachelor of Science in Microbiology

Bachelor of Science in Microbiology

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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at BRAC University.

2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.

3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.

4. I have acknowledged all main sources of help.

5. I would like to request the embargo of my thesis for 12M from the submission date due to our attempt of publishing it in a reputed journal.

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Approval

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Ethics Statement

No human or animal model was used in this study.

ISOATION AND MOLECULAR CHARACTERIZATION OF E.COLI BACTERIOPHAGES FROM SURFACE WATER OF BANGLADESH

ABSTRUCT:

In our country Diarrheal epidemics occur seasonally. Two peaks of outbreaks agreeably 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 bacteriophages probably play an essential role in controlling the epidemics to occur or collapse. It is still not clear what factors trigger the onset of Diarrheal outbreaks. This study was design to see the effect of *E. coli* bacteriophages on the epidemics of Diarrheal disease. Routine isolation, estimation and molecular characterization reveal the prevalence *E. coli* phage. We have tried to characterize the isolated phages by analyzing the DNA using the technique called restriction fragments length polymorphism (RFLP).

Keywords: RFLP, restriction fragments length polymorphism, *E. coli*, *Vibrio cholerae*, Diarrheal.

Dedicated to

My beloved parents and siblings

Acknowledgement

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Sumaiya Tabassum

December, 2019

Contents

List of Figures

List of Tables

List of Acronyms

1. BRAC = Bangladesh Rural Advancemant Committee
2. et al = And others
3. g = Gram
4. ml = Millilitre
5. ICTV = International Committee on Taxonomy of viruses
6. *E. coli* = *Escherichia coli*
7. RFLP = Restriction fragment length polymorphism
8. µl = Microlitre
9. O/N = overnight
10. rpm = Rotation per minute
11. LA = Luria agar
12. DNase solution = Deoxyribonuclease solution
13. PCI = Phenol-Chloroform Isoamyl Alcohol
14. TE = Tris EDTA
15. EDTA = Ethylenediaminetetraacetic acid
16. DNA = Deoxyribonucleic acid

Chapter 1:

Introduction

Bacteriophages or phages for short are a group of viruses that infect specific bacteria (Abedon, 2012).They are the most abundant as well as the most genetically diverse biological entities on Earth, with global population number estimated at 10^{30} to 10^{32} (Hemminga et al., 2010). It is estimated that there are 5 to 10 viruses for each bacteria (Weinbauer, 2004). Phages are ubiquitous in nature and they are found in all environments that support bacterial proliferation (Kęsik-Szeloch et al., 2013). It is now acknowledged that phages play a crucial role in the cycling of organic matter in the biosphere and play an important role in bacterial diversity in addition with maintaining bacterial balance in the ecosystem (Chibani- Chennoufi et al., 2004; Guttman et al., 2004). Phages are thought to lyse 10-20% of the marine bacterial community every day (Suttle, 1994). Like other viruses, phages are an obligate intracellular parasite and their life cycle is completely dependent on their host bacterial cell as they lack cell structure and enzyme system required for proliferation (Carlton, 1999). By 2017, more than 25000 phage nucleotide sequence has been submitted in International Nucleotide Sequence Database Consortium (INSDC) (Adriaenssens and Brister, 2017) and researchers think that many more phages are waiting to be discovered.

E.coli is harmless and actually is an important part of a healthy human intestinal tract. However, some *E. coli* are pathogenic, meaning they can cause illness, either diarrhea or illness outside of the intestinal tract. Pathogenic *E. coli* strains are categorized into pathotypes. *Escherichia coli* (*E. coli*) are a bacterium that is commonly found in the gut of humans and warm-blooded animals. Most strains of *E. coli* are harmless. Some strains however, such as Shiga toxin-producing *E. coli* (STEC), can cause severe food borne disease. *Escherichia coli* are also part of the normal microbiota but they are opportunistic pathogens causing urinary tract infections, diarrhoea, etc. Because of the developing antibiotic resistance in bacteria, the use of antibiotics

to cure *E.coli* infections are not effective nowadays. Therefore, effective alternative therapy is in demand to cure the infections caused by antibiotic-resistant *E.coli*. Phage therapy is considered one such therapeutic alternative to cure bacterial infections.

Since the discovery of bacteriophages (phages), about a century ago, the use of phages for biological applications is increasing lately. Bacteriophages are considered as a potent antibacterial agent because they are advantageous i.e. easy availability, naturally existing, specific in their activity and they can multiply rapidly in the presence of their host. Bacteriophages are found to have a wider range of applications; in food processing industry, against plant pathogens, in water treatment plants, as a disinfectant for diagnostic tools and in therapy to treat bacterial infections. Phage therapy is the therapeutic use of lytic bacteriophages for curing bacterial infections but the use of phages for the therapeutic purpose is minimal. The use of phages in therapy is limited to countries like Russia, Georgia and Poland. Phage therapy is now getting renewed interest in the western medicine because of the developing antibiotic resistance infections. With the use of modern technology, there are large numbers of research articles and clinical studies being performed to understand the role of phages as therapeutic agents. One of the biggest complications for phage therapy is the storage of phages for long-term or simply the half-life of phages is less than a year at 4°C. Though there are different approaches being developed such as encapsulation and aerosols, lyophilization (freeze-drying) of phages is considered the most efficient method. The earlier studies that described the lyophilization of phages mainly focused on the type of stabilizers and the regimes used in freeze-drying. Further, the long-term storage (more than 20 months) of phages at lyophilized state will increase the use and application of phages in future.

Bacteriophages have been used to treat bacterial infection in human since its discovery in the early twentieth century (Sulakvelidze and Kutter, 2004). So far phages were used successfully in different agricultural settings such as treating plant bacterial disease and showed potentiality in livestock and aquaculture (Sulakvelidze and Barrow, 2004). Recently, researchers are trying to use hage as molecular tool in vaccine delivery, gene therapy Clark and March, 2006) and as a diagnostic tool to detect bacterial species in the clinical and environmental sample (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.

Till now, bacteriophages specific to *E.coli* have been isolated but the number is not very high considering the amount of diversity available in phage population. Therefore, an attempt was made in this study to isolate *E.coli* phages from surface water of different water sample across Dhaka city, Bangladesh.

The entire previously isolated phages specific to *E.coli* demonstrated variation as well as similarities in their phenotypic and genotypic characteristics which corroborate the phage diversity. Thus, E.coli specific phage isolated from the water sample in Dhaka city could possibly show variation and similarities to previously isolated *E.coli* specific phages and might have the potential to be added in the International Committee on Taxonomy of Viruses (ICTV) database. The basic understanding of phage biology of the isolated *E.coli* phage could be useful in the development of therapeutic agent against *E.coli*.

Objectives:

During my research I tried to find out the prevalence and effects of environmental *E.coli* phages and their correlation with diarrheal diseases. Phage concentrations were taken from 26th February 2019 to 28th September, 2019. Further studies are needed on this project to get a better understanding of the effect of *E. coli* phages. The main aim of this project was to isolate bacteriophage against *E.coli* and characterize them in molecular level.

Specific aims:

1. Correlation between the prevalence of environmental *E. coli* phages and diarrheal diseases.
2. To isolate Strong lytic bacteriophage against *E. coli* from the different water sample.
3. To characterize the isolated phage based on (molecular level characterization) by RFLP (restriction fragment length polymorphism)

Chapter 2:

Literature Review

2.1 Bacteriophages:

Bacteriophages or ‘phages’ for short are naturally occurring bacterial viruses which infect bacterial cells (Abedon, 2012). They are highly host specific and have 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).Phages are considered as natural killer of bacteria that can infect and lyse the host organism (Abuladze et al., 2008; Nishikawa et al., 2008). In recent times, it has been widely acknowledged that bacteriophages are abundant in the environment and they influence the biosphere extensively. 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 bacteriophages:

Bacteriophages were first observed in 1896 by a British bacteriologist, Ernest Hankin (Ackermann, 2012). In the water of Ganga and Jumna River in India, he observed the presence of an antibacterial activity against bacteria Vibrio Cholerae. He suggested that this unknown agent, being heat sensitive and could pass through porcelain filter, causing the bactericidal activity is responsible for preventing the spread of cholera disease. Two years later, while working with Bacillus subtilis, a Russian bacteriologist named Gamalaya witnessed similar phenomenon (Sulakvelidze et al., 2001). In 1901 Emmerich and Löw reported that sample from a culture which demonstrated autolysis was able to lyse different culture, was capable of curing experimentally induced infection (Summers, 2004). Almost two decades after Hankin’s observation, Frederick William Twort, a British pathologist observed a “glassy transformation” of Micrococcus colonies grown on solid agar media. He hypothesized that the unknown

substance causing the watery transformation of the bacterial colonies could be a virus. Two years later of Twort's documentation, Felix d'Herelle a French Canadian microbiologist observed similar kind of incidence. He proposed that it was 5 "ultravirus" that was causing lysis of bacterial cell in liquid media and created clear patches on the bacterial lawn which he primarily called it taches, then taches vierges, and later plaques. Felix d'Herelle also named the virus responsible for these phenomena as "bacteriophage" which derived from "bacteria" and Greek word "phagein" which means to "eat" or "devour". While Twort, for numerous reasons including financial difficulties could not pursue his findings, d'Herelle continued and devoted his research career to bacteriophage study (Calendar, 2005; Sulakvelidze et al., 2001; Summers, 2004) and he concluded bacteriophage as "exogenous agents of immunity" following the observation of an increase in phage titer in the stool sample of recovering patients suffering from dysentery and typhoid (Deresinski, 2009).

The first documented therapeutic use of bacteriophage was done by Bruynoghe and Maisin in 1921 from Louvain where they noted reduction in pain, swelling, and fever upon injection of staphylococcal phage preparation in the local region of cutaneous boils (Summers, 2004). However, the first therapeutic use of phage conducted by Felix d'Herelle at the Hospital Des Enfants-Malades in Paris in 1919 under the supervision of Professor Victor-Henri Hutinel, the hospital's Chief of Pediatrics. They administrated anti-dysentery phage preparation to a 12-year boy with severe dysentery and observed consecutive cease of symptoms and full recovery within a few days. He ran several other trails after that where patients recovered within 24 hours using only one dose of phage preparation. As the results from these trials were not published

immediately Therefore, Bruynoghe and Maisin were credited for the first stated application of phage in treating infectious disease (Sulakvelidze et al., 2001; Summers, 1999).

While several early phage therapeutic trials were considered as successful and many prominent pharmaceutical companies and research laboratories such as D'Herelle's commercial laboratory in Paris and Eli Lilly Company in the United States sold phage preparation to treat various infectious disease, they ultimately became a failure (García et al., 2008; Sulakvelidze et al., 2001; Thiel, 2004). The discovery of broad range antibiotics played the major role in declining the interest of producing phage commercially. The lack of understanding of phage biology and inadequacies in the diagnostic bacteriology techniques available at the time aided the shift in interest from phage in the western world. However, phage therapy was continued to be offered in the Eliava Institute and later by others also such as the Hirszfeld Institute of Immunology and Experimental Therapy 6 in Wroclaw, Poland (Deresinski, 2009). One the other hand phage research continued at a fundamental level in the west where the study of phage played a major role in some momentous discoveries in biological science. It led to the identification of DNA as genetic material (Van Valen et al., 2012), understanding of genetic code and phenomenon of restriction-modification and to the development of molecular recombinant technology. Phage derived proteins are now being used as diagnostics agents (Smith et al., 2001), therapeutic tools (Loeffler et al., 2001; Schuch et al., 2002) and for discovering new drug (Liu et al., 2004).

2.3 Bacteriophage classification:

Phages are enormously diverse and vary from one another based on structural, physicochemical, and biological properties. When in 1917 d'Herelle discovered bacteriophage, he presumed that there were only one species of phage containing many races. However, in 1933, Burnet showed heterogenicity among enterobacterial 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 and particle size, host range, and resistance to urea and heat which was not accepted by scientific community. Lwoff, Horne, and Tournier published a classification scheme in 1962 based on nucleic acid type and morphology. Later 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).

Shape	Nucleic acid	Virus group	Particulars	Example
Tailed	DNA, 2, L	*Myoviridae*	tail contractile	T4
		Siphoviridae	tail long, noncontractile	λ
		Podoviridae	tail short	T7
Polyhedral	DNA, 1, C	*Microviridae*	conspicuous capsomers	φX174
	2, C, S	*Corticoviridae*	complex capsids, lipids	PM2
	2, L	*Tectiviridae*	inner lipid vesicle, pseudotail	PRD1
	2, L	SHI, group*	inner lipid vesicle	SH1
	2, C	STV1 group*	turret-shaped protrusion	STIV
	RNA, 1, L	*Leviviridae*	poliovirus-like	MS2
	2, L, seg	*Cystoviridae*	envelope, lipids	Φ6
Filamentous	DNA, 1, C	*Inoviridae*	a. long filaments	fd
			b. short rods	MVL1
	2, L	*Lipothrixviridae*	envelope, lipids	TTV1
	2, L	*Rudiviridae*	TMV -like	SIR V -1
Pleomorphic	DNA, 2, C, S	*Plasmaviridae*	envelope, lipids, no capsid	L2
	2, C, S	*Fuselloviridae*	same, lemon-shaped	SSV1
	2, L, S	*Salterprovirus*	same, lemon-shaped	His1
	2, C, S	*Guttaviridae*	droplet-shaped	SNDV
	2, L	*Ampullaviridae*∗	bottle-shaped	ABV
Pleomorphic	2, C	*Bicaudaviridae*∗	two-tailed, growth cycle	ATV
Pleomorphic	2, L	*Globuloviridae*∗	paramyxovirus-like	PSV

Table 1: Overview of phage families (Adapted from Ackermann, 2007).

C Circular; L linear; S superhelical; seg segmented; 1 single-stranded; 2 double-stranded

∗ Awaiting classification

Over the years the edifice of phage classification grew slowly by addition of new families and genera. The current classification of bacteriophage by the International Committee on Taxonomy of Viruses (ICTV) consists of 1 order, 14 families, 37 genera while 5 other potential families waiting for classification (Ackermann, 2009). Over 96 % of all phages defined in the literature are tailed double-stranded (ds) DNA phage and belong to the order Caudovirales. The phages under the order Caudoviralesare further classified into three main large families, Siphoviridae, Myoviridae,and Podoviridae, differentiated by their tail length and contractile ability (Ackermann, 2004). Having long flexible tail, 61 % of the phage under Caudovirales falls in Siphoviridae; 25% are Myoviridaehaving double-layered contractile tails; and with short noncontractile tails, 15 % are Podoviridae. Other types of phages, Polyhedral, filamentous, and pleomorphic phages comprising less than 4 % of observed phages (Ackermann, 2007).

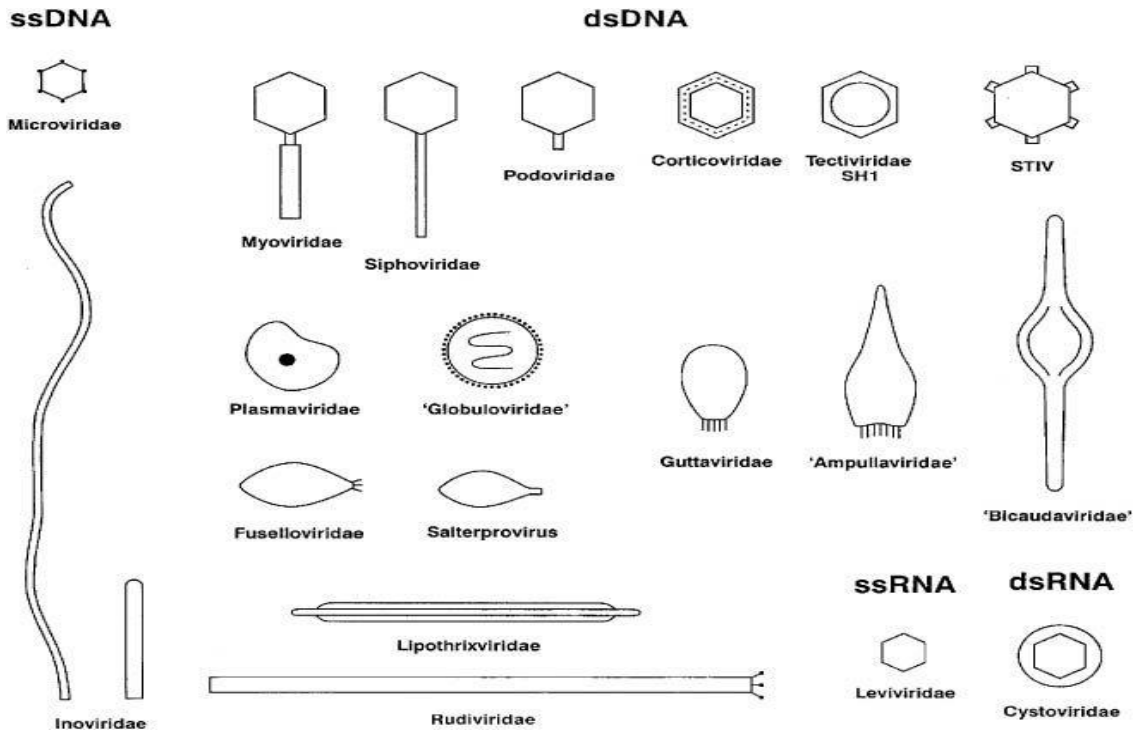

Figure 1: Morphotypes of different bacteriophage family. (Adapted from Ackermann, 2009).

The ICTV taxonomic system requires visualization of the phage structure using electron microscopy. As ICTV ignores genomic information to establish a detailed and specific classification system, its methods are currently reconsidered since it often gives rise to conflicting ideas. For instance, current classification system, on the basis of common characteristics of short tails, places both the SalmonellaP22 and T3 phage in the Podoviridae family (Ackermann et al., 2012). However, it has been demonstrated that p22 phage is gnomically much more related to λ phage (long tailed and belong to family Siphoviridae) that functional hybrid of λ-P22 genome in vivo was formed (Byl and Kropinski, 2000). These kinds of contradictory results encouraged scientists to reevaluate the current taxonomic method and to push forward a more molecular basis classification system. Forest Rohwer and Rob Edwards proposed a phage classification system in 2002 based on metagenomics data which is actually identification of phage using genomic data in a cultured independent process. This new method of classification was proposed to rectify the divergence between the current taxonomic system and available genomic data but this has been found to be problematic due to the fact that there is no universal gene specific for different existing phage family (Paul et al., 2002). As of now,there is no proof that shows metagenomics can substitute morphological classification done by electron microscopy as both of them answers a different question.

2.4 Bacteriophage abundance in the environment:

Bacteriophages are considered to be the most prominent biological entities on the planet with an estimated population size of 10^{30} or more (Chibani-Chennoufi et al., 2004). Phages have been isolated from different environmental setting such as acidic hot springs (higher than 80°C with

pH=3.0), solar salterns (10 times saltier than the ocean), alkaline lakes (pH=10), in the terrestrial subsurface (greater than 2000 m deep), below 30 m of ice in polar lakes (Breitbart and Rohwer, 2005), from soil (Ashelford et al., 2003), sewage sludge (Carey-Smith et al., 2006) and mammalian feces (O'Flynn et al., 2004).

Marine water is one of the major resources of bacteriophage and several studies indicate a greater variance in overall phage prevalence in these ecosystems. sea water is one of the major reservoirs of dense phage population (about 9×10^{8} virion ml^{-1}) and roughly 70 % of aquatic bacteria are infected by those phages (Ackermann et al., 2012). Phage abundance across aquatic system varies between less than 10^{4} $m1^{-1}$ and more than $10^{8}ml^{-1}$(Wommack and Colwell, 2000). This variation in number is generally correlated with the variation in associated host organism which ultimately depends on the productivity of the system. For instance, in marine system phage abundance is highest in the coastal environments (10^{6}–$10^{7}ml^{-1}$), intermediate in offshore surface water (10^{5}–$10^{6}ml^{-1}$) and lowest in the deep sea (10^{4}–10^{5} ml-1). It has also been demonstrated that the phage abundance decreases along the transaction from coastal to offshore (Weinbauer, 2004).

Bacteriophage prevalence in freshwater is higher than in marine water. In sea ice, phage abundance was reported 10 to 100 times higher than in surrounding water (Maranger et al., 1994). The phage abundance variation also exists inthe fresh water system. A study done by Tapper and Hicks (1998) documented that phage prevalence in first 20 μmor the surface microlayer of Lake Superior was 2 to 15 times higher than in 20 m depth. Though benthic viruses were found to be 10 to 1000 times higher than in the overlaying water column, the phage abundance decreased with sediment depth (Weinbauer, 2004).

2.5 Life cycle of bacteriophages:

Bacteriophages have multiple possible life cycles which determine their role in bacterial or archaeal biology. Two major phage life cycles are the lytic and lysogenic cycle. Both the life cycle includes 2 common steps: i) absorption of phage i) penetration of genetic material (Salmond and Fineran, 2015). Phage at first will interact with the receptors expressed on the surface of the bacterial cell. Some phages require a cluster of one specific receptor for proper absorption. Whereas, for some phage absorption stage requires different stage involving the different set of receptors such as T4-like phages (Guttman et al., 2004). After irreversible attachment of the phage to the bacterial surface, it injects its genome into the bacterial cell involving mechanism specific for each phage.

Those replication strategies following the successful penetration of phage genetic material dictate the virulent or temperate nature of bacteriophage. In lytic cycle injected phage genome take command of the bacterial replication machinery and produce necessary components for new progeny phage. When all the essential components are manufactured, the phage particles are assembled into infective virions. This followed by lysis of the host cell, where the new progeny phages are liberated from the bacterial cell via disruption of the cell wall and cell membrane. In tailed phages, this process is accomplished by two protein lysin and holing. Holin is a natural timer protein which assembles pores in the membrane at anappropriate time to allow lysin to reach peptidoglycan layer and initiate cell lysis. The lytic life cycle results in the destruction of the host cell (Guttman et al., 2004).

Lysogenic cycle is another phage proliferation technique. Here, new phage particles are not immediately produced and the host cell is not destroyed. In this process, phage genome is either

integrated into the host genome or prevail as a plasmid within the cell. In lysogenic relationship lytic genes are inhibited by the phage gene product termed a repressor. Due to lytic inhibition the phage gene will remain integrated intothe host chromosome which is called a prophage and will replicate along with the host replication process (Little, 2005).

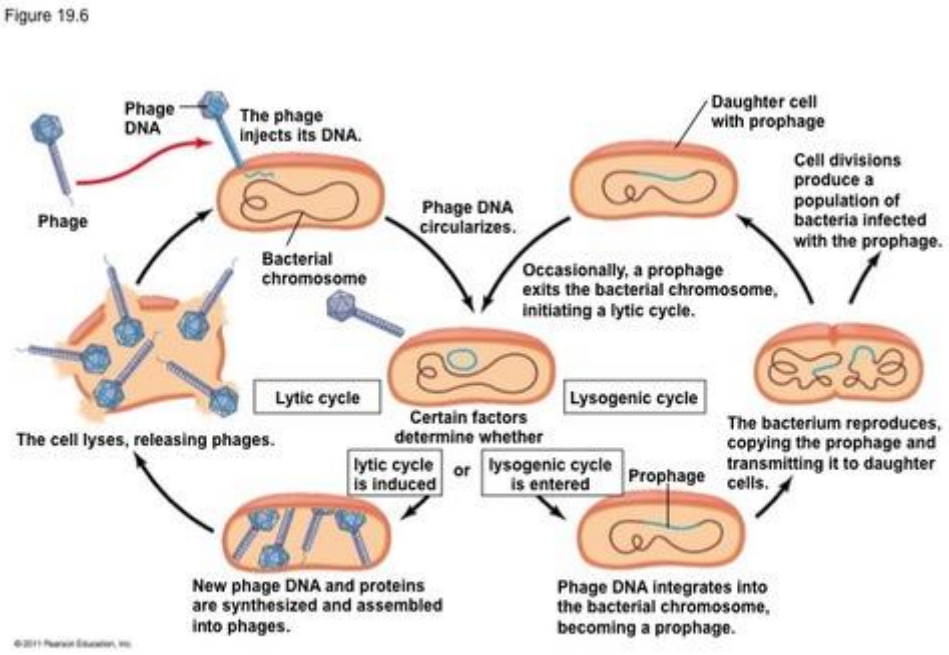

Figure 2: Lytic and Lysogenic replication cycle of bacteriophages (Adapted from Salmond and Fineran, 2015).

Though lysogenic phase is really stable it can be switched to initiate the lytic cycle. Temperate phages such as λ phages are able to proliferate via both lytic and lysogenic cycle (Little, 2005). This introduction of lytic cycle in prophage can be induced by various physical and chemical agents such as poly-aromatic hydrocarbons, mitomycin C, hydrogen peroxide, temperature,

pressure and UV radiation (Williamson et al., 2001). In order for virulent phages to replicate and survive in the environment, the rate of phage-host encounter required to exceed the virus decay and inactivation rate. However, the temperate phages are not dependent on the host cell density, rather requires a small number of lysogenic carrier cell and occasional induction of lytic cycle and release of free phages (Wommack and Colwell, 2000).

2.6 Phage impact on bacterial population:

In recent times, studies on marine biodiversity have shown that bacteriophages influence their host bacterial organism in a density-dependent manner, basically targeting and infecting a few bacterial 13 species at any one time (Ventura et al., 2011). This is in harmony with "kill-the-winner" model where the predation is directed towards "winner" (abundant) bacterial population in that environment (Rodriguez-Valera et al., 2009).As a result, the nutritional resources are more accessible to other bacterial species and provides the opportunity for a new bacterial species to become abundant. Studies involving genome size distribution have demonstrated that over a period of time specific virus become abundant, then reduce to an undetectable level and then again become abundant (Wommack et al., 1999). A recent study conducted by Middelboe et al(2009) have demonstrated how phages can drive Flavobacterium strain diversification in sea water. Bacteriophages also play a key role in bacterial population inside animal intestinal tract. A Study involving horse feces have shown that diversity and abundance of E.coli strains in horse gut are directly correlated to the relative abundance of specific coliphages (Golomidova et al., 2007). Barr et al. (2013) documented that phages adhered to mucus (BAM) of the human gut can provide an innate protection to the underlying epithelial tissue from outside pathogens.

Moreover, the presence of prophage in bacteria residing in gut flora provides a competitive advantage to the host which makes the pathogenic organism hard to compete commensal organism hence, maintain the stability of human gut microbiome (Ventura et al., 2011).

2.7 The impact of bacteriophages on host fitness:

Bacteriophage plays a key role in bacterial evolution and augmenting survivability by altering genetic combination. Temperate phages can carry bacterial genes from one cell to another via transduction method. Many temperate phages integrate at a specific site in bacterial genome and during the transition to lytic growth, they often mistakenly cut out a portion of bacterial DNA along with prophage which may later get incorporated into another bacterial cell. Hence changing the cells genomic composition by specialized transduction process. Other phages are able to alter host genome by generalized transduction method where prophages are integrated randomly in the host cell genome and then always carry some host DNA with them (Guttman et al., 2004). This phage-mediated gene mobility increases the possibility of gene maintenance in bacterial population (Miller, 2001) and increases the chance of genes spread that are costly but advantageous such as gene rendering antibiotic resistance and gene for xenobiotic compound degradation (Abedon and LeJeune, 2007).

Bacterial Host	Bacteriophage	Virulence Factor (gene)	Function of gene product	Reference
S. mitis	SM1	Coat protein (pblA, pblB)	Required for host attachment	(Bensing et al., 2001)
S. flexneri	Sf6	O-antigen (oac)	Alter antigenic recognition	(Clark et al., 1991)
S. pyogenes	H4489A	Hyaluronidase (hylP)	Involved in cellular invasion	(Hynes and Ferretti, 1989)
E. coli	λ	OMP (bor)	Required for intracellular survival	(Barondess and Beckwith, 1990)
C. botulinum	C1	Neurotoxin (c1)	Extracellular toxins	(Barksdale and Arden, 1974)
C. diphtheriae	ß-phage	Diphtheria toxin (tox)	Extracellular toxins	(Freeman, 1951)
V. cholerae	CTXΦ	Cholera toxin (ctxAB)	Extracellular toxins	(Waldor and Mekalanos, 1996)
E. coli	H-19B	Shiga toxin (stx-1)	Extracellular toxins	(Neely and Friedman, 1998)
S. enterica	Fels-1	Neuraminidase (nanH)	Putative virulence factors	(Figueroa-Bossi et al., 2001)

Table 2: Examples of bacteriophage encoded virulence factors involved in various stages of bacterial pathogenesis (Adapted from Fidelma Boyd, 2004).

It has been documented several times that various bacterial virulence factors agents contributing to the emergence of infectious disease in eukaryotes are encoded by integrated prophage (Brüssow et al., 2004). In this phage conversion process,bacteriophage encoded genes convert nonpathogenic host bacteria to pathogenic one or amplify their existing virulence by providing a mechanism for invasion of host tissue and the avoidance of host immunity system (Fidelma Boyd, 2004). One of the most known examples of this phage conversion occurs in Vibrio cholerae, a generally harmless bacteria that become highly virulent by incorporating phage cholera toxin (ctxAB) genes in the genome (Waldor and Mekalanos, 1996). There are other human bacterial infectious diseases such as botulism, diphtheria, and disease associated with Shiga Toxigenic Escherichia coli(STEC) caused by endotoxin respectively botulism toxin, diphtheria toxin, and Shiga toxin which are expressed from phage-encoded genes (Abedon and LeJeune, 2007). Many other examples of virulence inducing genes are known including genes that help in host attachment, altering antigenic recognition, cellular invasion, and intracellular survival (Table 2).

2.8 Bacteriophage application:

Once the potential of bacteriophage as antibacterial agents was realized following the initial discovery and characterization in the early twentieth century, there was a flurry of interest in phages using them as therapeutic tools. However, 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). Bacteriophages are now again reconsidered as an antimicrobial tool due tothe 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 bio-control agent, uses in the development of phage-derived vaccine and in phage display technique.

Chapter 3:

Materials and Methods

3.1 Study Area:

The study was conducted at BRAC University in Dhaka, Bangladesh. The laboratory processing, analysis of data and the overall experimental work were done in the Microbial & Environmental Biology Research Laboratory of the Department of Mathematics and Natural Sciences of BRAC University.

3.2 Period of Study:

The research work was carried out from January 2019 till October 2019.

3.3 Sample collection:

E. coli strains were collected from the environmental water samples and *E. coli* strain 1532, 1685 strain 2, strain 3 and strain 4 were collected from BRAC Universities Mathematics and Natural Sciences Departments Laboratory. These *E. coli* strains were used in the plaque assays and also for isolation and culture of *E.coli* phages from the environmental water samples. Throughout 20 samplings were done around the time of this project. The water samples were collected on a routine basis from different points of Buriganga River, Turag River, Dhanmondi Lake and from Gulshan Lake.

1. Sampling procedure:

These water samples were quickly taken into our laboratory at Mohakhali and filtered to remove the dirt and debris from the water. For growth and isolation of bacterial cells, the sample waters were filtered with Whatman filter paper and 5 ml of filter water was mixed with 5 ml LB and

placed in shaker incubator at 37 degrees Celcius and subjected to shaking at 120 rpm for 4 hours. After four hours of incubation 300 micro liters of bacterial solution was taken and it was serially diluted to 10^{-7} times. From these diluted bacterial solutions 300 micro liters sample were spread on LA agar plates at different concentrations. From the different plates, the number of bacteria obtained after overnight incubation was counted and a few of them were stocked from each sample. On the other hand, to isolate phages from the environmental water samples, the filtered water was further filtered by a 0.22-micron syringe filter to exclude all the bacteria. E. coli host cells were cultured to reach their log phase and these young culture cells were mixed with 5ml the syringe filtered water. The mixture was placed in the shaker incubator for four hours at 37 degrees Celcius and subjected to shaking at 120 rpm for the enrichment of the phages. To check the presence of phage in the enriched mixture, soft agar plaque assay was used where presence of phages leads to clear zones in the lawn commonly known as a plaque. The phages obtained was then later further enriched and stocked.

2. Soft Agar Plaque Assay:

In order to quantify the number of phage particles we have used the soft agar plaque assay method which was previously described by Faruque et al. (Faruque, et al., 2005). Suitable indicator strains have been used in this process. To measure the susceptibility of various E. coli bacterial strains to their corresponding bacteriophages, nascent bacterial cells were grown (cells grown in fresh LB for 3 to 4 hours) in a shaker (around 160 rpm) at 37°C. Then 500 µl of each nascent bacterial strain were taken and mixed with soft agar of 3.5ml volume (soft agar contains 0.8% Bactoagar, Difco along with Nutrient broth). After that, 100 µl phage solution was added

to the previous mixture which contained around 3.2×10^2 phage and the mixture was laid quickly on top of a Luria agar plate before the soft agar solidified. The culture plates were then incubated overnight at 37°C. After incubation the number of plaques was counted to measure the degree of phage susceptibility of the different bacterial strains.

3. Enrichment Method:

For enriching the phages, nascent bacterial cells were grown (cells grown in fresh LB for 3 to 4 hours) in a shaker (around 160 rpm) at 37°C. Then after that, 100 µl stored phage was added and again it was given in the shaker for 3 hours at 37°C. Finally the solution was further filtered by a 0.22-micron syringe filter and stored in a refrigerator.

4. Phage confirmation test or Spot test:

Phage confirmation test is done to see whether the collected or stored phage solution is viable or not. For this process nascent bacterial cells were grown (cells grown in fresh LB for 3 to 4 hours) in a shaker (around 160 rpm) at 37°C. Then 500 µl of each nascent bacterial strain were taken and mixed with soft agar of 2 ml volume (soft agar contains 0.8% Bactoagar, Difco along with Nutrient broth). the mixture was laid quickly on top of a Luria agar plate before the soft agar solidified and after that 20 µl phage solution was dropped in the centre of the solidified agar. Finally, the culture plates were then incubated overnight at 37°C.

3.4 Phage DNA isolation procedure:

1. Lawned specific host bacteria containing bacteriophage on LA plate (300µl bacteria + 100µl phage) and incubated over night at 37^0C.
2. Collected the upper soft agar layer containing bacteria and phages using spreader (add LB if required).
3. Culture had been taken using tips in falcon tube.
4. Centrifugation was done at 10,000 rpm for 10 mints at room temperature.
5. Collected the supernatant and filtered through 0.22 µm filter.
6. The solution had been taken into fresh Eppendorf tube.
7. Added DNase at 1unit/100µl ratio. Then added DNAse 10X buffer in the solution at 1:10 ratio, mixed well and incubated at 37^0C overnight.
8. After that, added proteinase-K at 50µl/ml ratio and then 10X proteinase-K buffer in the solution at 1:10 ratio, finally incubated at 37^0C overnight.
9. Next added equal volume of Phenol-Chloroform Isoamyl Alcohol and gently mixed 15 minutes by inverting tubes.
10. Centrifuged at 14000 rpm for 15 mints and collected the upper aqueous layer in fresh tubes.
11. Then added double volume of absolute ethanol (cold at -20^0C) and kept at -20^0C O/N
12. Again centrifuged at 14,000 rpm for 15 min at 4^0C
13. And removed the supernatant keeping the pellet.
14. Finally dissolved the pellet in Tris-EDTA buffer.

3.5 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 PST-I, Bgl- I, BamH-I, Xho-I.

1. Two reactions were set according to the table, all 7 phages were followed for group 1 reaction and only 5 phages were followed for group 2 reaction.

Table 3: Calculation for RFLP of phage DNA

Group 1 RFLP		**Group 2 RFLP**	
Reagent	Amount	Reagent	Amount
Distilled Water	11µl	Distilled Water	10µl
DNA	5µl	DNA	7µl
10x buffer	2µl	10x buffer	2µl
PST-I	2µl	Bgl-I	1.25µl
Total	20µl	BamH-I	1.25µl
		Xho-I	1.25µl

		Total	20.25µl

Total 7 phage DNA sample was digested according to the calculation 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.

3.6 Agarose gel electrophoresis procedure:

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.

Chapter 4:

Results

4.1 Result of sampling throughout the project:

January		February		March		April		May		June		July		August		September		October	
Phages found in sampling 01 and 02		Phages found in sampling 03 and 04		Phages found in sampling 05 and 06		Phages found in sampling 07 and 08		Phages found in sampling 09 and 10		Phages found in sampling 11 and 12		Phages found in sampling 13 and 14		Phages found in sampling 15 and 16		Phages found in sampling 17 and 18		Phages found in sampling 19 and 20	
-	+	-	+	+	-	-	-	+	+	+	+	-	+	+	+	+	-	-	-

Table 03: Prevalence of *E.coli* bacteriophages

A total number of 20 samplings were done throughout this entire project. Above presented chart shows the absence and presence of bacteriophages during every sampling procedure. Two sampling occurred for each month thus giving a number of 20 samplings. (+) indicates the presence of phages for that which took place successfully and (-) indicates absences of phages from that sampling took place around that time.

4.2 Result of Phage confirmation Test:

Figure 3: E.coli strain sample (picture-01)

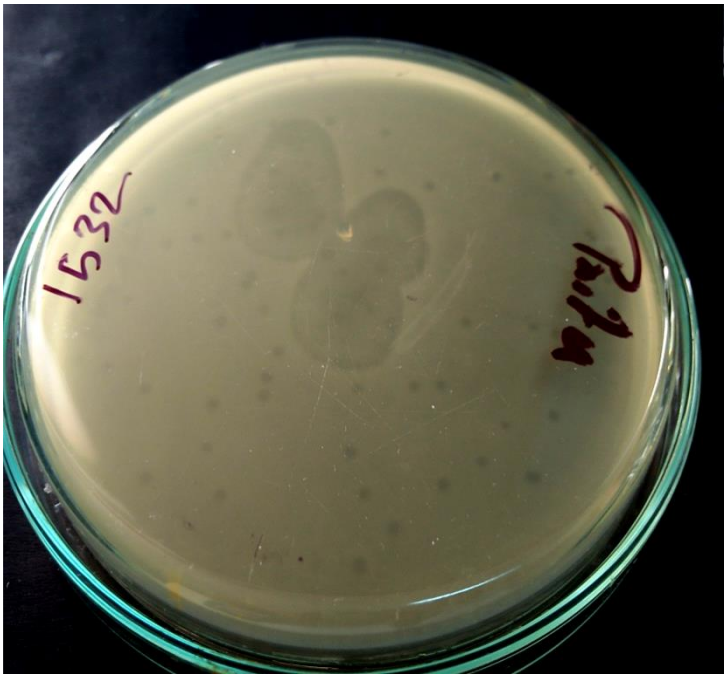

Figure 4: E.coli strain sample (picture-02)

Figure 5: Ecoli strain sample (picture-03)

After overnight incubation the phages were taken under observation among 10 plates 7 plates showed visible plaques referring that 8 different phages were viable. These were S-2;S-5 (Strain-2, Sample-5), S-2; S-16 (Strain-2, Sample-16), S-2;S-10 (Strain-2,Sample-10), S-3;S-10 (Strain-3, Sample-10), S-3;S-11 (Strain-3, Sample-11) , S-3;S-16 (Strain-3, Sample-16), S-1532;S-12 (Strain-1532, Sample-12), S-4; S-14 (Strain-4, Sample-14).

4.2 Result of phage undigested DNA detection:

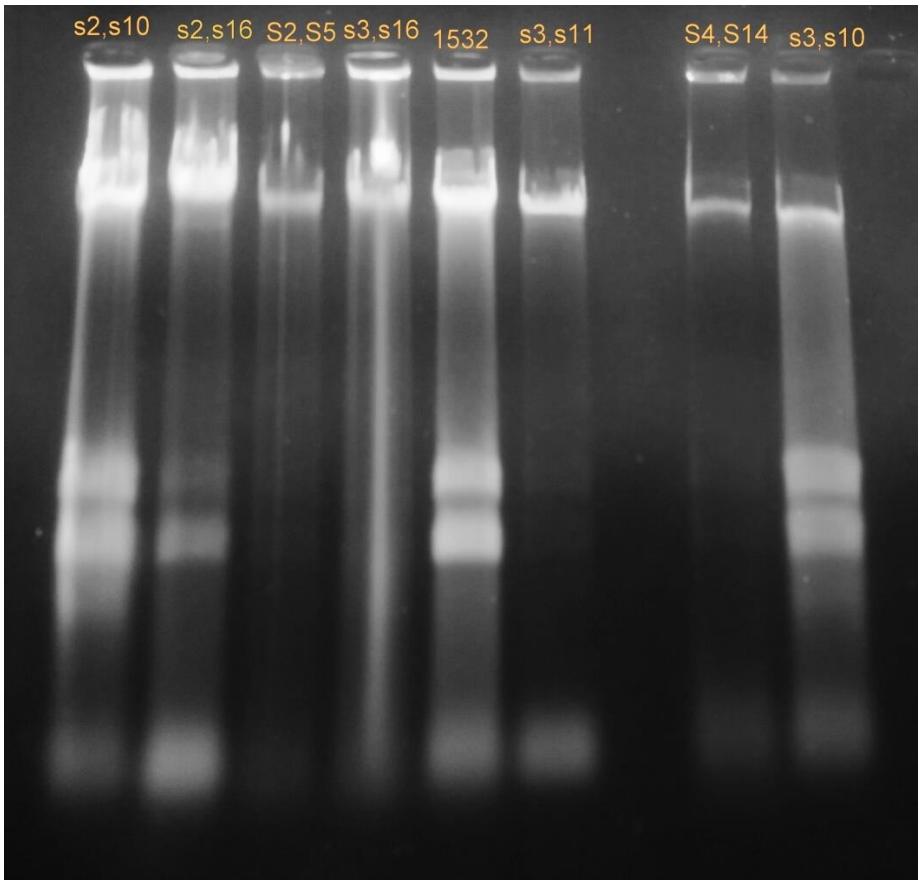

Figure 6: Gel electrophoresis of E.coli bacteriophage DNA

Phages that went through confirmation and then the phages which showed positive results or were visible went for DNA isolation after successfully isolation phage DNA they went through gel run to ensure whether phage DNA bands were visible or not. After a successful gel run the DNA were visible at the time of observation. A total of 8 DNA phages were clearly visible among 14 DNA phages.

4.3 Result for RFLP of phage digested DNA:

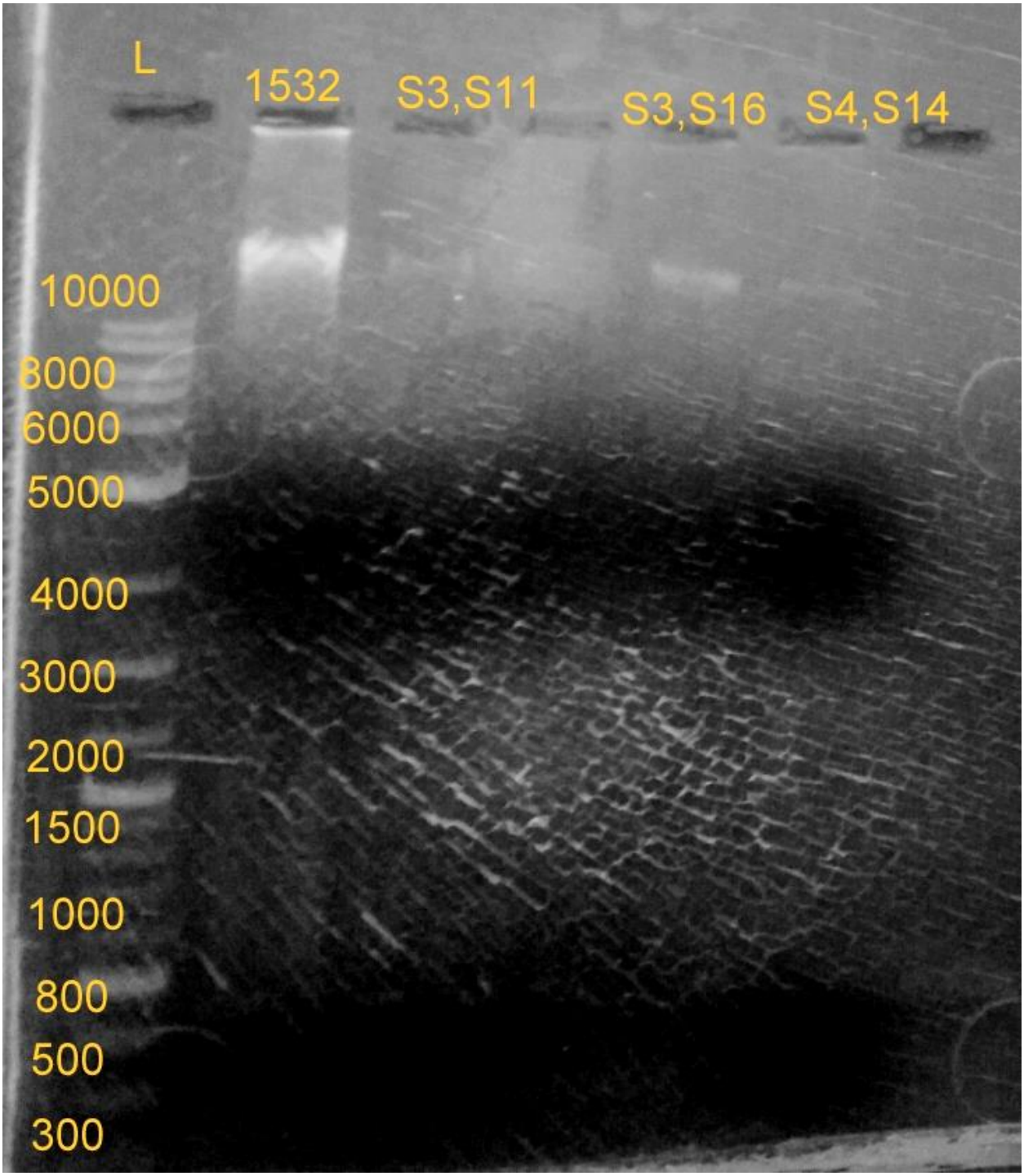

Figure 7: Group 1 RFLP (using PST-I enzyme)

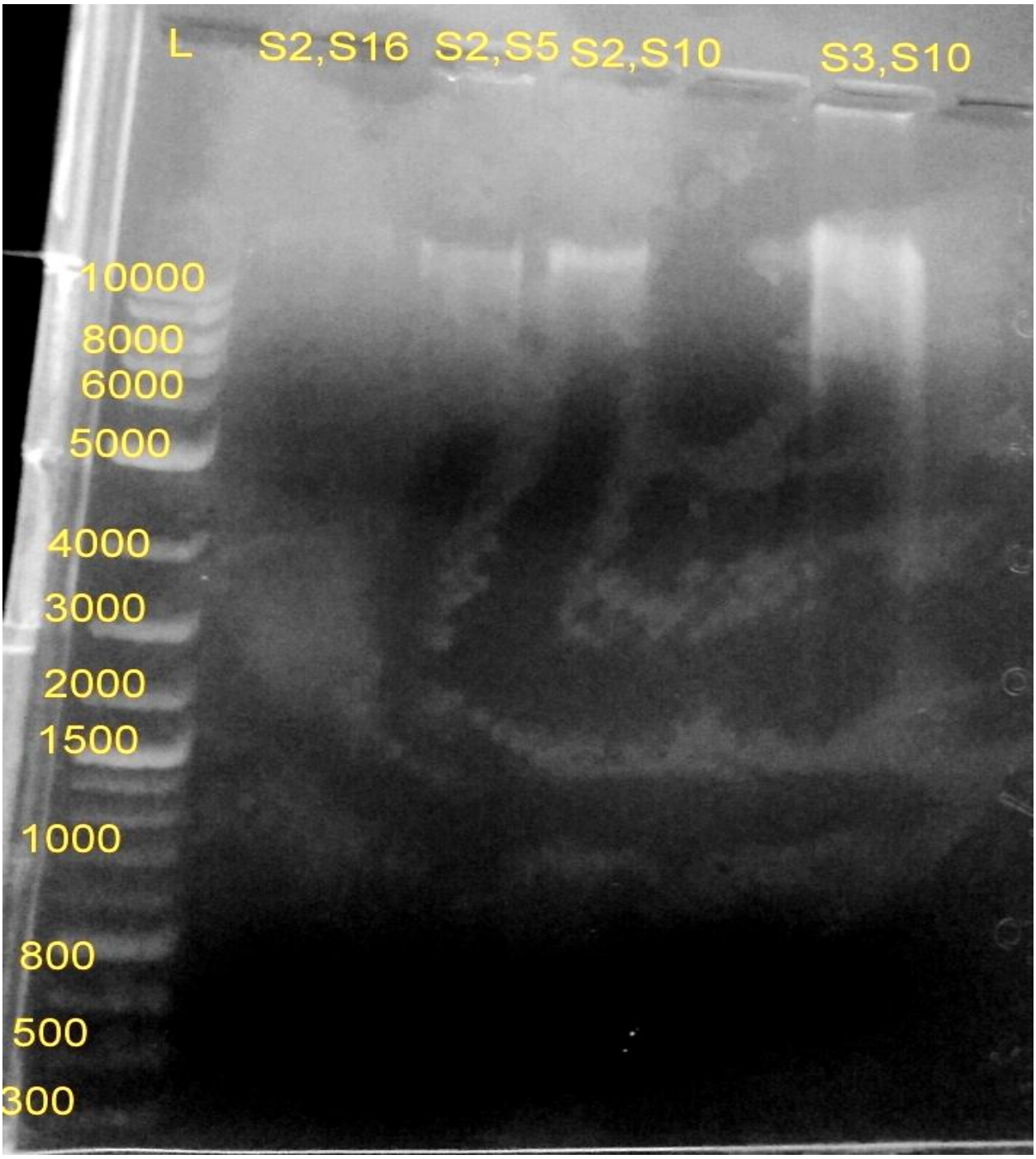

Figure 8: Group 1 RFLP (using PST-I enzyme)

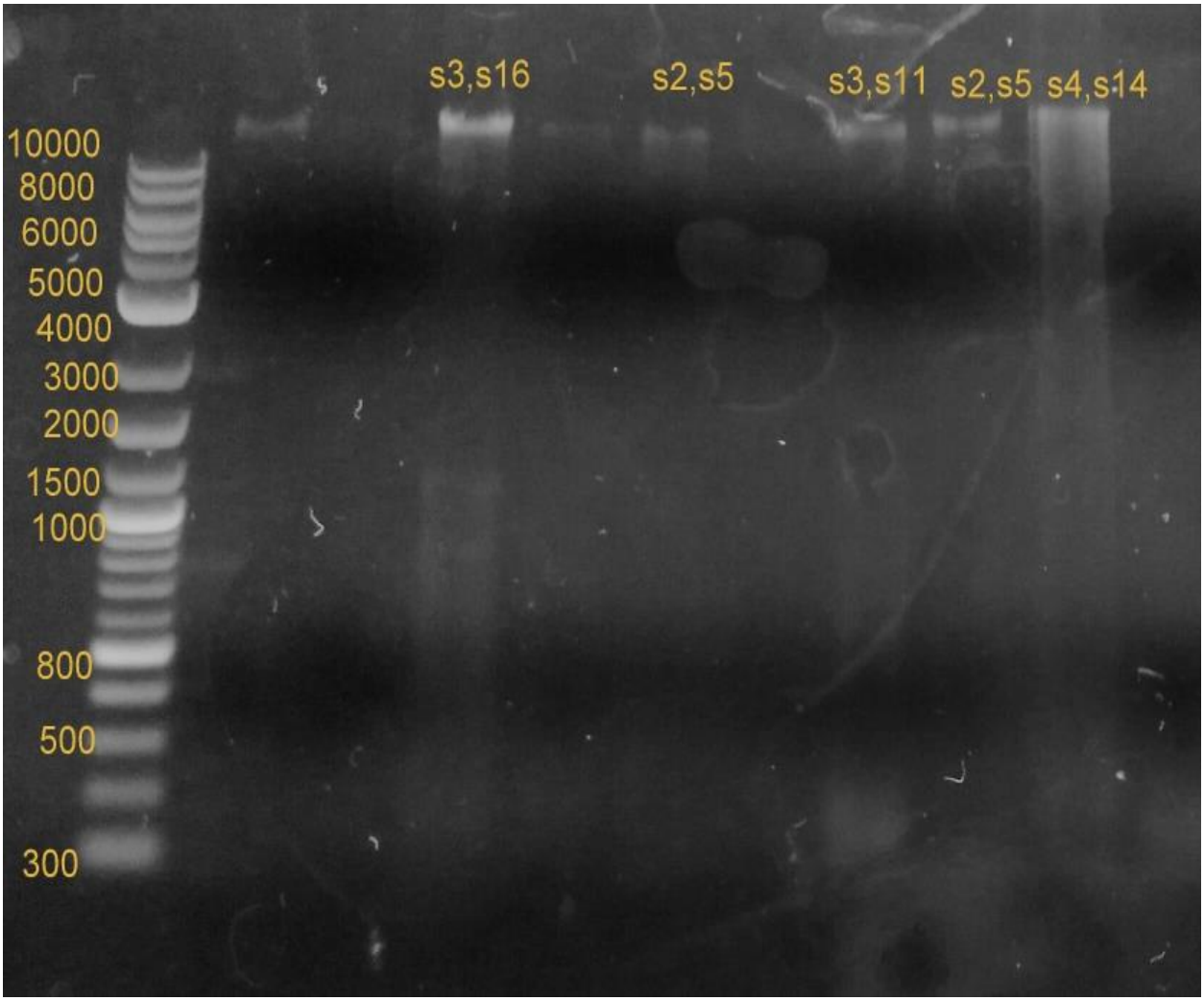

Figure 9: Group 2 RFLP (using BamH-I,Xho-I,Bgl-I enzymes)

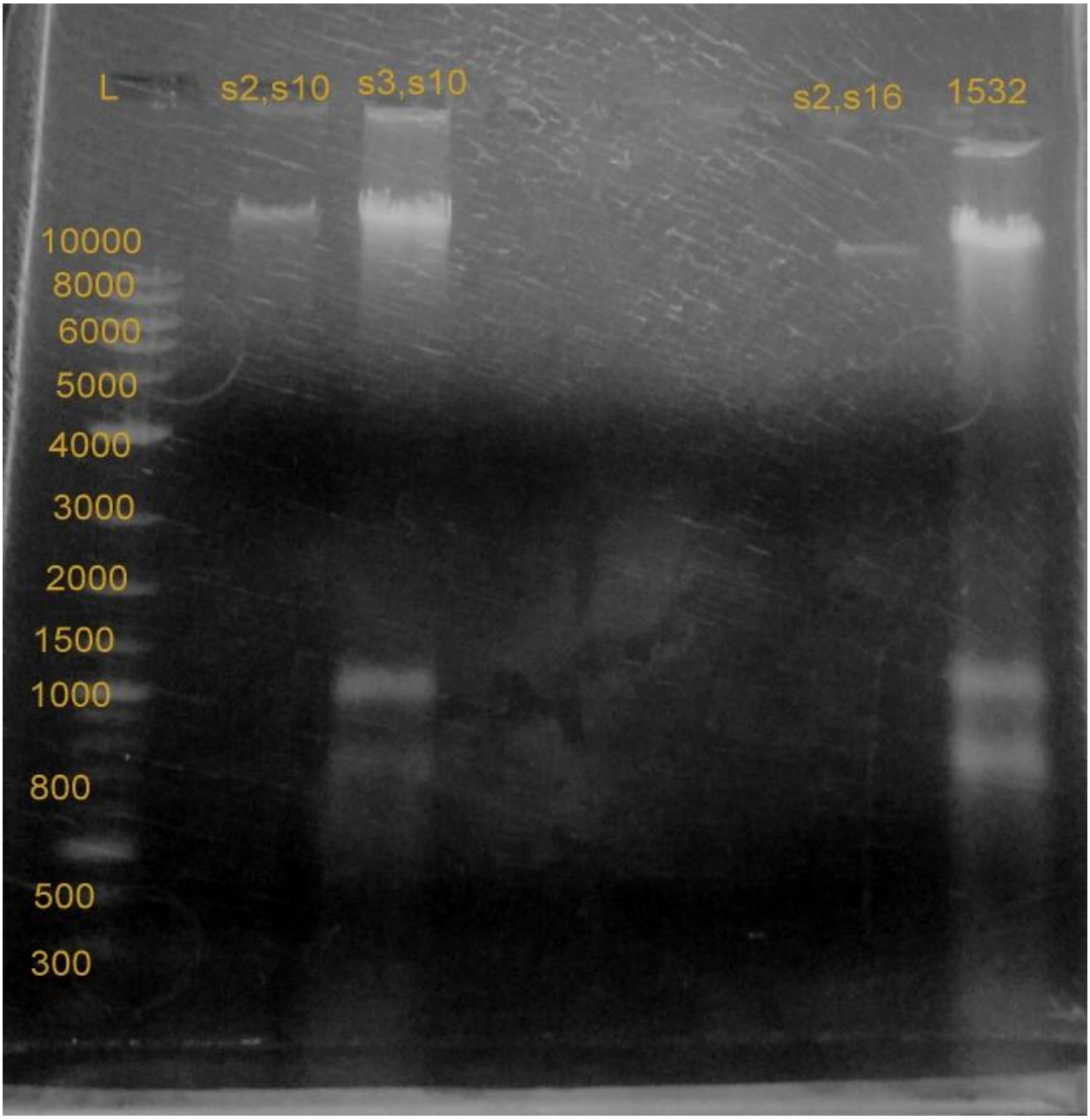

Figure 10: Group 2 RFLP (using BamH-I,Xho-I,Bgl-I enzymes)

For 8 different phage DNAs, RFLP was done. In two groups RFLP was done, one group of RFLP was done using PST-I enzyme and another group of RFLP was done using three restriction enzymes BamH-I, Xho-I, Bgl-I. Then after gel electrophoresis the above pictures were obtained. The goal was to identify whether those phage DNA were same or different in

nature according to their cutting pattern after adding the restriction enzymes. From the images above it is visible that all the phages are not same. Phages S-2;S-16, S-2;S-10, S-3;S-16 S-3; S-10, and S-1532;S-12 are different as their restriction patterns are different according to their different strains in both group of RFLPs. In both cases restriction enzyme didn't cut S-4;S-14 DNA. In case of S-3;S-11 and S-2;S-5 DNA, those were not visible enough to ensure that those DNA were fragmented in every picture. But it can be concluded saying that phages found through unknown samples were different as they showed different cutting patterns according to specific bacterial strains. And for knowing their specific genomic sizes and for further research purposes next level molecular characterization should be done.

Chapter 5:

Discussion

The main reason of diarrheal diseases is the involvement of enteropathogenic bacteria such as *E. coli* responsible for it. 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 of these organisms 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. On the other hand, phages play an important role in the evolution and prevalence of pathogenic cholera species in the environment. There is a report which state that "the presence of bacterial viruses such as *E. coli* bacteriophages acting on *E. coli* inversely correlates with the occurrence of viable *E. coli* in the aquatic environment and the number of locally reported diarrheal disease cases pointing to this. Their ebundancy in the environment is becoming same as enteropathogenic bacteria. Therefore bacteriophages in the environment are playing important role in the prevalence and distribution of pathogenic bacteria which could cause deadly epidemics like many diarrheal diseases.

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 enteropathogenic phages could play important role in controlling the epidemics of many diarrheal diseases. Here we study the possible correlation of E. coli phages with many diarrheal diseases as much as enteropathogenic phages to influence these epidemics. The *E. coli* bacteriophages were 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 pathogenic *E. coli* phages which were collected from environmental surface water sample 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 when early cases were studied or taken into a count. Therefore it seems too 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 auto-inducer in the environment. This auto-inducer in turn resuscitates the dormant enteropathogenic bacteria persist as biofilm in environment to cause the epidemics of diarrheal disease. There are possibly other mechanisms which could lead to this type of effect.

As the DNAs of different phages sample were found. Then it went through gel run to see whether the phages were concentrated enough or not, after getting a positive result. After that it was necessary to if they are the same phages or different phages. To know this, RFLP was done. Different kinds of restriction enzymes were mixed together and they were applied on the DNA with some other components. 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 It worked except one sample which was sample of S4;S14. DNA concentration of S4;S14 phage was very high that it was difficult for restriction enzymes to cut them. After the experiment, we got 6 different phages. Phages were different from each other because they give different cutting patterns under giving them specified restriction enzymes. So based on the cutting patterns in RFLP we separate them in different groups. We tried to find out if there is any different pattern of fragmenting DNA in case of pathogenic phages. But, no such pattern was found. For getting confirmation about the types of *E. coli* DNA and also to know about the band sizes sequencing needs to be done which will give more accurate result for further studies on this project. Also careful measurements should be taken in every step of the project in order to avoid contaminations and unnecessary mistakes the might interfere with the results. Before going for the final process of DNA extraction and RFLP, accurate phage confirmation test and proper enrichment should be preceded of these phages which were collected through the process of rigorous sampling. These procedures are needed to ensure whether the phages which were found through environmental samples were viable or not to go through further processes. But before that careful measurements should be taken to avoid the unnecessary contaminations the might hamper with the whole project and might interrupt the whole result.

Finally, this whole project should be run throughout the year to get the exact accurate results for this project to be successful enough for publishing procedures and to give people the better understanding of the issue.

Chapter 6:

Conclusion

We have successfully isolate bacteriophages from the surface water of Dhaka city. The prevalence was checked. The types of bacteriophages were determined by RFLP. Few different types of Phages were detected from the samples. But there are still certain limitations and recommendations which are given for further researches which can be helpful to conduct upcoming project or ventures. These are given below:-

- **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.

- **Recommendations for further research:**

1. This project was continued from January ’19 to October ’19. The result that is obtained needs to be more effective. So, it should be continued for more months like the whole year to get confirmation.
2. This research mention above only helps to know that pathogenic *E. coli* phage probably has same effects on epidemic of diarrheal diseases as pathogenic *E. coli* phage. But in which way they influence the epidemic, needs further studies and does not discuss about non-pathogenic *E. coli*.
3. Further research can be done on non-pathogenic *E. coli* phages.
4. 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 and it could also be a subject of further studies or researches.

Chapter 7:

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