Targeted bacteriophage application to control water contamination by Escherichia coli, Vibrio and Salmonella

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Declaration

It is hereby declared that;

1. The thesis submitted is our own original work while completing the Bachelor of Science in Biotechnology 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. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;

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

5. All main sources of help have been acknowledged.

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The thesis titled "***Targeted bacte*riophage a*pplication to control water contamination by Eshcheria coli, Vibrio and Salmonella***" submitted by Anika Raina (ID: 18136024), Lamisa Shaik (ID: 18136018), Sadia Zaman Shetu (ID: 18136026) and Salwa Khair (ID: 18136049) of Spring 2018, has been accepted as satisfactory in partial fulfillment of requirement for the degree of Bachelors in Biotechnology.

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Dedication

We would like to dedicate this thesis to our beloved families for their love, prayers, support, and sacrifices.

Acknowledgement

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Abstract:

Water contamination and scarcity of pure water is a major issue of concern in our country. Water borne epidemics caused by different strains of *Escherichia coli, Salmonella* or *Vibrio* have become more frequent in many developing and underdeveloped countries. Over the years, the application of bacteriophage as bacterial control tools in various sectors of medicine, food and biotechnology has grown to be immensely popular. This study was conducted to shed light on the potential effects of *Escherichia coli, Vibrio and Salmonella* specific bacteriophages as bacterial control tools to control water pollution. In this study, after collecting 16 samples from three surface water sources in Dhaka city, isolation of bacteria and bacteriophages was done. Eventual confirmation of bacteriophage and its hosts led to comparative analysis of the change in the lag phase of bacterial growth in presence or absence of phage. The current research may provide information on how bacteriophages are used as bacterial control tools, and how they help with bacterial control in water pollution.

Chapter 1: Introduction

E. coli, Salmonella or *Vibrio* can be found in water sources contaminated with excrement from diseased humans or animals, such as private wells, lakes, rivers, and other bodies of water. Sewage overflows, malfunctioning sewage systems, dirty storm water runoff, and agricultural runoff are just a few of the ways waste can reach the water bodies. Ingestion of coliforms such as *Escherichia coli* (E. coli), Salmonella species (*Salmonella spp.*), and *Vibrio* polluted water can cause major consequences such as diarrhea, enteritis, and even death, according to studies, resulting in high economic losses. The Vibrio genus of bacteria is one of the natural inhabitants of aquatic habitats, and it plays an important role in maintaining the aquatic ecosystem. Vibrio are gram-negative organisms that have straight or curved rod-like forms and measure between 1.4 and 2.6 meters in length.

Salmonella bacteria are Gram-negative, flagellated, facultatively anaerobic bacilli with antigens O, H, and Vi. There are over 1800 identified serovars, each of which is classified as a different species under current categorization. Salmonella has been found in surface waters including rivers, lakes, and ponds, however groundwater has a better microbiological condition for irrigation. Culture methods remain the gold standard for detecting, isolating, and identifying Salmonella in foods and water to this day. Other methods for detecting Salmonella in water include most probable numbers, ELISA, and PCR, in addition to culture. Escherichia coli is a Gram-negative, non-spore-forming bacteria that is normally motile by peritrichous flagella. Because, E. coli is an excellent marker of fecal pollution, it was introduced into water bacteriology and has since become an essential marker in food and water hygiene. The capacity of E. coli to develop as a biofilm will surely enhance its survival in natural situations.

Bacteriophages are bacteria-infecting viruses and Bacteriophage (phage) research is a fast-growing field with significant future possibilities. (Doss et al., 2017)

In temperate climatic zones, lake or river ecosystems are intensively exploited for recreational and daily domestic needs. Anthropogenic activities are the leading cause of contamination in all ecosystems, according to several studies (Yunus et al., 2020). Water contamination is one of the most important environmental challenges in many countries throughout the world (Xu et al., 2020). Water contamination has increased as a result of decades of development, mostly due to

agricultural runoff and hazardous industrial effluents (Purohit et al., 2020). In the suburbs, many industries develop, and they generate a considerable amount of sewage each year. Secondary water pollution is also caused by ineffective sewage treatment (Shi et al., 2020).

For our study, 16 water samples were collected in winter (November 21'- December 21') from three sources in Dhaka. These are **Dhanmondi lake, Hatirjheel lake and Buriganga river.** Samples were collected by the group mates early in the morning and then brought to the lab for filtration.

Water scarcity is a pressing issue that must be addressed. Drinking water safety is also getting increasing attention. The major purpose of this thesis is to look at bacteriophage's potential as a bacterial control tool that may possibly be utilized to control contaminated water.

The potential applications and limitations of phage use in water pollution prevention are also investigated in this study. Also, it was observed, how phages can be used to lyse bacteria, track pollution sources, and possibly control water pollution. Next some of the limitations of phage-based technologies were discussed and possible solutions were provided.

1.1 Background

Bacteriophages, often known as phages, are a type of virus that infects bacteria (Abedon, 2012). They are the most numerous and genetically varied biological beings on the planet. Each bacteria is thought to have between 5 and 10 viruses (Weinbauer, 2004). Phages are abundant in nature and can be found in any setting that promotes bacterial growth (Ksik-Szeloch et al., 2013). It is now well known that phages play a significant role in the biosphere's organic matter cycling and bacterial diversity, as well as maintaining bacterial balance in the ecosystem (Chibani- Chennoufi et al., 2004; Guttman et al., 2004). Every day, phages are thought to lyse 10-20% of the marine bacterial community.

Phages, like other viruses, are obligate intracellular parasites that rely on their host bacterial cell for their entire life cycle since they lack the cell structure and enzyme system essential for multiplication (Carlton, 1999). Since their discovery in the early twentieth century, bacteriophages

have been utilized to treat bacterial infections in humans. Bacteriophages are thought to be a powerful antibacterial agent because they have several advantages, including easy availability, natural existence, activity specificity, and the capacity to replicate rapidly in the presence of their host.

Therefore, bacteriophages have been utilized to treat bacterial infections in humans whereas phage therapy is the therapeutic use of lytic bacteriophages to treat bacterial infections, though phages are rarely used for this reason.

1.1.1 Life Cycle of Bacteriophage:

Bacteriophages have two life cycles: lytic and lysogenic

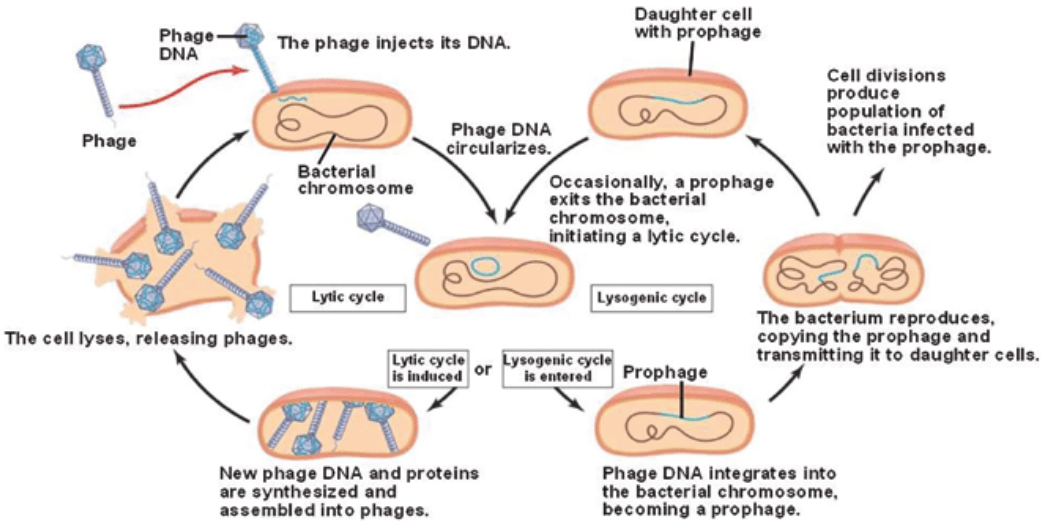

Fig: Life cycle of Bacteriophage, (source: P. (2022, March 29). *The Viruses- classification, structure and diseases*. PCSSTUDIES. https://pcsstudies.com/the-viruses-classification-structure-and-diseases/)

1.1.2 Lytic Cycle

Bacterial cells are broken open (lysed) and destroyed by lytic phages after the virion has been replicated. The phage progeny can find new hosts to infect as soon as the cell is destroyed. T4, which infects E. coli in the human digestive tract, is an example of a lytic bacteriophage. Phage therapy is better with lytic phages.

When extracellular phage concentrations are high, some lytic phages experience a condition known as lysis inhibition, in which finished phage offspring do not instantly lyse out of the cell.

1.1.3 Lysogenic cycle

The lysogenic cycle, on the other hand, does not result in the host cell being lysed right away. Temperate phages are phages that have the ability to undergo lysogeny. Their viral genome will integrate with host DNA and multiply harmlessly alongside it, or it may even establish itself as a plasmid. The virus remains dormant until the host's circumstances deteriorate, maybe due to food depletion; at that point, the virus's endogenous phages (known as prophages) become active. They begin the reproductive cycle at this moment, causing the host cell to lyse. The virus is replicated in all the cell's children because the lysogenic cycle allows the host cell to survive and proliferate. The accumulation of phage-encoded lysozyme during late protein synthesis results in bacteriophage lysis.

1.1.4 Bacteriophage therapy

Bacteriophage therapy is another name for phage therapy. It treats bacterial infections with viruses. Phages solely kill bacteria; they are not harmful to humans, animals, or plants. Hence, Bacteriophage therapy refers to the use of natural or modified aggressive lytic bacteriophages to treat bacterial infections.

Bacteriophages are bacteria's natural enemies. Bacteriophage is a word that means "bacteria eater." Bacteria can be found in soil, sewage, water, and other locations. In nature, these viruses aid in the control of bacterial development.

1.1.5 Mechanism

Bacteriophages cause bacteria to rupture or lyse, killing them. When the virus connects to the bacteria, this occurs. A virus infects bacteria by injecting its genes into the organism (DNA or RNA).

The phage virus replicates (copies) itself inside the bacteria. Each bacterium can produce up to 1000 additional viruses as a result of this. Finally, the virus ruptures the bacteria, allowing new bacteriophages to emerge.

Bacteriophages can only reproduce and flourish within the confines of a bacterium. They'll stop reproducing until all of the bacteria have been lysed (killed). Phage viruses, like other viruses, can go inactive (hibernate) until new bacteria appear.

1.1.6 Bacteriophage isolation

The isolation of bacteriophages for phage therapy is frequently depicted as a simple process of combining a phage-containing sample with host bacteria, followed by a simple removal of bacterial detritus the next day using filtration and/or centrifugation. *(Melo et al., 2014*)

1.1.7 Bacteriophage enrichment

The purpose of bacteriophage enrichment is to increase the number of phages that can infect the target host. This is accomplished by extracting the endogenous bacteria from the sample and inoculating it with bacterial culture media and a growing culture of chosen host.

1.1.8 Bacteriophage presence in environmental surface water

During this study, the presence of Bacteriophage was confirmed in the sample water. Surface Water was collected as a sample from three sources (surface water) of Dhaka city i.e Buriganga river, Hatirjheel and Dhanmondi lake. Hence it can be assumed that there is presence of both bacteria and bacteriophage at the same time in Dhaka city’s surface water.

1.1.9 Literature review

Bacteriophage (phage) may lyse a bacterial cell with pinpoint precision, allowing for the treatment of a specific bacterial illness without disrupting the native microflora of the host.

According to a recent study by (*Jun et al., 2016*), it was found that, the isolation and characterization of pSs-1, a pathogenic *Myoviridae* phage. The biological features of pSs-1 were investigated, and it was discovered to have effective bacteriolytic activity against both *S. flexneri* and *S. sonnei*. Furthermore, its genome was sequenced in its entirety and compared to that of similar phages.

Hence, in this study the possibility of phage as a biocontrol agent for Shigella-infected water was described and also it was intended to isolate a virulent phage infecting *S. sonnei* strains in order to create the optimal mix of diverse phages for use in a phage cocktail, as the isolation of *S. sonnei* phage was previously mentioned.

Chapter 2: Materials and Methods

2.1 Standard laboratory practices

- Excess amount of agar should not be prepared without asking the supervisor/mentor.
- After plating agar into Petri plates, the remaining agar should not be discarded. Remaining agar must be poured into existing Petri plates. If pouring the remaining amount of agar into existing Petri-plates is not possible, the container can be sealed properly and must be kept in the refrigerator for future use.
- Culture plates and fresh plates must not be stored in the refrigerator for more than two weeks.
- Culture plates cannot be kept in the incubator for more than two days.
- Track of prepared and culture media must be kept.
- Proper labeling must be done on the media plates and containers: name, media name, date of preparation.
- After weighing the reagents,

  a. Weighing machine, spatula, surrounding areas should be cleaned before and after use.

  b. The reagent must be kept to its assigned place.

c. The cap of the reagent container must be closed otherwise; the reagent will be solidified in contact with air.

- The laminar hood surface and surrounding must be cleaned before and after work.
- One should wear a lab coat while conducting laboratory work.
- Use of lab coats outside the laboratory must be avoided.
- Burner should be turned off after finishing work.
- If anything breaks down while conducting research work, lab attendants must be informed immediately.
- One should be careful while using a spirit lamp.
- Adjustment of the micropipette volume parameter after work should be done.
- Dry discards should not be kept in the wet discard.
- Hair should be tied up before entering the laboratory.
- If any glassware/plasticware is broken, laboratory assistants should be informed.
- Proper initial treatment needs to be taken after any cut or burn; otherwise, one will get infected with laboratory microorganisms.
- Ethanol containers should not be kept near the burner or flame. Open workbench should be cleaned with disinfectant (ethanol, Hexi sol) before and after work.

2.2 Materials

2.2.1 Reagents for media preparation

- Distilled Water
- Mac CONKEY agar
- TCBS agar
- SS agar/XLD agar
- Luria Broth medium
- Luria Bertani Agar
- Sodium Chloride
- Yeast extract
- Tryptone

- ❖ Bacterial agar

2.2.2 Apparatus

- ➢ Micropipette
- ➢ Pipette
- ➢ Glass spreader
- ➢ Test tubes
- ➢ Glass vials
- ➢ Conical flasks
- ➢ Falcon tubes
- ➢ Eppendorf tubes
- ➢ Needle, Loop
- ➢ Ethanol
- ➢ Spirit lamp
- ➢ Aluminum foil paper
- ➢ Micropipette tips
- ➢ Shaker incubator
- ➢ Water bath
- ➢ Centrifuge machine
- ➢ Weighing machine
- ➢ Cuvettes
- ➢ 0.22 Micron filter
- ➢ Syringe
- ➢ Vortex machine
- ➢ Spectrophotometer
- ➢ Paraffin oil, parafilm paper
- ➢ Sterile Petri plates

2.3 Sample collection

E. coli, *Vibrio* , *Salmonella* strains were collected from the environmental water samples. *E. coli* strain *SHIGATOXIN-PRODUCING ESCHERICHIA COLI* and *Vibrio* strain WT 324 were

collected from BRAC University Mathematical and Natural Sciences Department laboratory. These *E. coli , Vibrio, Salmonella* strains were used in the plaque essay, isolation, and enrichment of phages from environmental water samples. Throughout 16 samples were done from 14/11/21 to 18/01/22. The water samples were collected on a routine basis from different points of Dhanmondi Lake, Hatirjheel Lake, Buriganga River.

Table 1 : Sample collection with source

Sample number	Sample collection Date	Source
1	14.11.21	Dhanmondi
2	16.11.21	Dhanmondi
3	21.11.21	Dhanmondi
4	23.11.21	Buriganga
5	28.11.21	Buriganga
6	29.11.21	Hatirjheel
7	01.12.21	Buriganga
8	07.12.21	Hatirjheel
9	08.12.21	Buriganga
10	08.12.21	Dhanmondi
11	26.12.21	Buriganga
12	27.12.21	Dhanmondi
13	27.12.21	Hatirjheel
14	28.12.21	Hatirjheel
15	04..01.22	Hatirjheel
16	18.01.22	Dhanmondi

2.3.1 Filtration of water

These water samples were quickly taken into our laboratory at Mohakhali and filtered to remove the dirt and debris from the water. The sample waters were filtered with Whatman filter paper

placing it in a funnel .100 ml filtered water was stored in 3 large falcon tubes. 10 ml of filtered water was taken from the tube and mixed with 3 ml Luria Broth (prepared earlier and autoclaved).The (LB+water) were kept in an incubator(37 degree Celsius) for about 4 hours.

2.3.2 Phage separation by micron filter

The filtered water was taken through a syringe and then installed in a 0.22ul micron filter. a 0.22 ul micron filter was used to isolate phage from sample water. 10 ml of water containing raw phage was stored in small eppendorf tubes. The eppendorf tubes were sealed with parafilm paper and kept in 4'c culture fridge. These phage will be enriched by further procedures.

2.4 Preparation of culture media, reagents, and solutions

2.4.1 Saline Preparation:

Saline is a mixture of 0.9% sodium chloride (NaCl) and water.

i. 200 ml of distilled water was measured and taken into a Duran bottle .

ii. The reagent was measured through an analytical balance machine. 1.8 gm of NaCl reagent powder was taken and measured on a foil paper.

iii. Reagent was mixed into the distilled water by shaking the flask in circular motion until it dissolved.

iv. Afterwards, it was autoclaved at 15 degree Celsius and 121 PSI for 2 hours to sterilize it.

vi. After 2 hours of autoclave, the LB was taken out of the machine and kept outside to cool it down for use.

2.4.2 Luria Broth preparation

Luria-Bertani (LB) is a nutrient-rich liquid broth for growing bacteria. LB contains Tryptone, NaCl, and Yeast Extract. We used the ready to made LB which was available in the laboratory.

i. 500 ml of distilled water was measured and taken into a conical flask .

ii. The reagent was measured through an analytical balance machine. 10 gm of LB reagent powder was taken and measured on a foil paper.

iii. Reagent was mixed into the distilled water by shaking the conical flask in circular motion until it dissolved.

iv. The conical flask was kept on a burner and was given a good stir.

v. After a slight boil, it was kept in an autoclave machine at 15 degrees Celsius and 121 PSI for 2 hours to sterilize it.

vi. After 2 hours of autoclave, the LB was taken out of the machine and kept outside to cool it down for use.

2.4.3 McConkey Agar Preparation

McConkey agar is a selective and differentiating agar that only grows gram-negative bacterial species particularly members of the family Enterobacteriaceae and the genus *Pseudomonas*. Peptone (Pancreatic digest of gelatin), Proteose peptone (meat and casein), Lactose monohydrate, Bile salts, Sodium chloride, Neutral red, Crystal Violet, Agar are the components of Mac CONKEY agar.

The ready to make reagent (dehydrated medium) was available in the laboratory and it was used instead.

Preparation-

i. 25.765 grams of dehydrated medium were weighed on a foil paper through a balance machine.

ii. 500 ml of distilled water was measured and taken in a conical flask.

iii. Suspension 49.53 grams of dehydrated medium was done in 500 ml distilled water.

iv. Heat was applied to boil and dissolve the medium completely.

v. Sterilization was done by autoclaving the media for 2 hours.

vi. Cooled to 45-50°C.

vii. Media was mixed well before pouring into sterile Petri plates.

2.4.4 Thiosulfate-citrate-bile salts-sucrose (TCBS) Agar Preparation:

TCBS Agar is a selective differential medium for isolating and cultivating Vibrio and its other species. Proteose peptone, Yeast extract, Sodium thiosulphate, Sodium citrate, Bile, Sucrose, Sodium chloride, Ferric citrate, Bromothymol blue, Thymol blue and agar are the components of TCBS agar.

The ready to make reagent was used here which was available in the laboratory.

Preparation-

i. 44.54 grams of dehydrated medium were weighed on a foil paper through a balance machine.

ii. 500 ml of distilled water was measured and taken in a conical flask.

iii. Suspension 44.54 grams of dehydrated medium was done in 500 ml distilled water.

iv. Heat was applied to boil and dissolve the medium completely.

v. No autoclave needed.

vi. Media was mixed well before pouring into sterile petri plates.

2.4.5 SS Agar preparation

SS Agar (Salmonella Shigella Agar) is a differential selective media used for the isolation of *Salmonella* and some *Shigella* species from pathological specimens. Proteose peptone, Lactose, bile salts mixture, Sodium thiosulphate, Ferric citrate, Brilliant green, Neutral red, Agar are the components for SS Agar.

The ready- made reagent was used here available in the laboratory.

Preparation-

i. 31.51 grams of dehydrated medium were weighed on a foil paper through a balance machine.

ii. 500 ml of distilled water was measured and taken in a conical flask.

iii. Suspension 31.51 grams of dehydrated medium was done in 500 ml distilled water.

iv. Heat was applied to boil and dissolve the medium completely.

v. No autoclave needed.

vi. Media was mixed well before pouring into sterile petri plates.

2.4.6 XLD preparation

Xylose Lysine Deoxycholate (XLD) agar is a selective growth medium used for the isolation of *Salmonella* and *Shigella* species from clinical samples and from food. Yeast extract, L- Lysine, Lactose, Sucrose, Xylose, Sodium Chloride, Sodium deoxycholate, Ferric ammonium citrate, Phenol red agar are the components of XLD agar .Ready-made media was used as per availability in laboratory .

i. Suspension of 28.34 grams of dehydrated media in 500 ml distilled water was done.

ii. Heating was done with frequent agitation until the medium boils.

ii. No need *to* autoclave.

iv. After cooling, media was poured into sterile Petri plates.

2.4.7 Luria Agar (LA) Preparation

For streaking of the inoculated stock bacteria, the use of Luria Agar (LA) media is necessary. LA media is a non-selective powdered media mainly used to routinely culture members of the *Enterobacteriaceae* family and for coliphage plaque assays. The medium is nutritionally rich for the growth of pure cultures of strains. Readymade LA media was used in this case which contains a mixture of tryptone, yeast extract, sodium chloride and 1.5% agar.

The media was prepared beforehand to minimize delay. To prepare 400ml media, 16gm of the powder was measured in an electronic balance machine and then suspended in 400ml of distilled water. The mixture was then heated to boiling on a Bunsen burner to dissolve the medium completely. It was sterilized by autoclaving for 2 hours. After cooling to 45-50°C, the media was poured into sterile petri dishes inside a laminar hood and allowed to solidify for a while. Since the media will be used for streaking, large plates were used. 16 large plates were prepared each time and the required amount was used and the rest of the plates were kept in the 4-degree Celsius fresh refrigerator.

2.4.8 Soft Agar Preparation:

Soft agar is an essential media for performing spot tests or plaque assay. 100ml of soft agar was prepared beforehand each time. To prepare 100ml of soft agar, 1gm of NaCl and Tryptone, 0.5gm of yeast extract and 0.6gm bacteriological agar was measured in the weighing machine, and then suspended in 100ml of distilled water. The mixture was then heated to boiling on a Bunsen burner to dissolve the medium completely. It was then poured into clean test tubes in 3 ml aliquots. Finally, the test tubes containing soft agar were sterilized by autoclaving for 2 hours. The required amount was used, and the rest of the plates were kept in the 4 degree Celsius fresh refrigerator.

2.4.9 Salt Magnesium (SM) Buffer Preparation

For 50ml SM buffer, 0.2gm NaCl, 0.1gm $MgSO_4$, 1M 2.5ml Tris-HCl and 0.5gm gelatin is required. To prepare the SM buffer, at first Tris-HCl needs to be prepared (approximately 30ml). From that, 2.5ml Tris-HCl had been used for the SM buffer.

For Tris-HCl preparation,

Tris base needs to be prepared and pH needs to be checked and lowered to 8 from 10 by adding drops of HCl.

Since 1ml Tris-HCl is 121.14 g/mol, 3.63gm Tris was added in 30ml distilled water to prepare the base. pH value was kept at 8 before autoclaving as after autoclave, it will lower to 7.5.

Now, 0.2gm NaCl, 0.1gm MgSO4, and 0.5gm gelatin was measured in the weighing balance and 47.5ml distilled water was added to it. Then, from the Tris-HCl, 2.5ml was added and this is how the 50ml SM buffer was prepared. Finally, autoclave the Tris-HCl and SM buffer and store at room temperature.

2.5 Spread Plate method

The spread plate method involves plating a liquid sample containing bacteria so that they may be counted and isolated easily. A successful spread plate will contain a countable number of isolated bacterial colonies dispersed equally around the plate.

Before the spread plate method, serial dilution was carried out to find out the cell counts of the sample by counting the number of colonies that are cultured.

2.5.1 Serial Dilution

A serial dilution was done up to 10^{-3} for the filtered sample water with saline .The dilution was done in Eppendorf tube with 900 μl saline and 100 μl filtered sample water.1 single eppendorf was labelled as raw as it contained 100 ul of filtered sample water only. For each sample water, there was 4 eppendorf tubes : raw ,10^{-1},10^{-2} ,10^{-3} . This dilution was done to continue the spread plate method.

After the 4 hours incubation of (10 ml filtered water + 3 ml LB) , a serial dilution was done upto 10^{-6}.For each sample there were 7 eppendorf tubes : raw, 10^{-1},10^{-2},10^{-3},10^{-4},10^{-5},10^{-6} where “Raw” tube contained 100ul of (water + LB) solution. The dilution was also done with 900 ul saline and 100 ul (water + LB) solution in this case. To perform spread plate method, this serial dilution was done as well.

Procedure

i. A dilution series from the sample was done.

ii. 100 ul of each diluted solution was taken through micropipette and poured onto the center of the surface of an agar plate.

iii. The L-shaped glass spreader was dipped into alcohol.

iv. Glass spreader was flamed over a Bunsen burner.

v. Each diluted solution was spread evenly over the surface of agar using the sterile glass spreader, carefully rotating the Petri dish underneath at the same time.

vi. Incubation of the plates were done at 37°C for 24 hours. Calculation of CFU value of the sample was done.

2.5.2 Colony Count

The visible single colonies were counted from the petri plates after the 24 hours incubation period. Spread plate method was done for 3 different types of differential and selective media plates. For each sample, spreading was done on a total 18 plates.6 plates contained MacConkey agar,6 plates contained TCBS agar and 6 plates contained SS/XLD agar.

The colony count was done according to the visible color of bacterial colonies on agar plates.

E coli gives pink to dark pink color on the MacConkey agar plate and its surrounded by a dark pink area of precipitated bile salts. So, counts were taken from each plate and noted to find out the CFU. Each plate was labelled with specific dilution. Pink colonies were counted only for Mac CONKEY agar plates.

Similarly yellow colored single colonies were counted from TCBS agar plates. Strains of *Vibrio* produce yellow colonies on TCBS Agar because of fermentation of sucrose. Count was taken and noted.

From SS agar plate, single colony count was taken and noted. *Salmonella* and *Shigella* gives colorless with black center/colorless colonies.

2.5.3 Stock preparation

Luria Broth is a widely used bacterial culture medium. Luria agar is a non-selective powdered media .The medium is nutritionally rich for the growth of pure cultures of strains. Tryptone, yeast extract,

sodium chloride and bacterial agar was needed as reagents of stock solution. Here readymade LA was not used to prepare the media as it is more solid. Instead, manually it was prepared for better results.

Recipe

Tryptone-2.5 grams

Yeast extract-1.25 grams

Sodium Chloride-2.5 grams

Bacterial agar-3.76 grams

Distilled water- 250 ml

Procedure

- The reagents were measured on a weighing machine and taken to mix it all together in a 250 ml conical flask.
- 250 ml distilled water was added to it and stirred until the media mixed properly.
- Then, the media was taken to give it a good boil and autoclave was done afterwards.
- After the autoclave was done ,the media was poured into glass vials.
- 2.5 ml of media was poured in each vial. Within a few moments, the media was solidified .
- 100 glass vials were filled with 2.5 ml media each and stored in 4'c fresh fridge.

2.5.4 Stock inoculation

Single bacterial colonies were taken from the petri plates and stabbed into the media inside vial through a needle. For each sampling,12 vials containing media were stabbed with bacteria. To keep track, each vial was labelled with sample number and media name. After stabbing, the vials were wrapped with parafilm paper to keep it airtight and was put into incubator for bacterial growth. Bacterial growth was observed in a semisolid media the next day and paraffin oil was

given around 200 ul in each vial. The again the vials were sealed with parafilm paper and stored in normal room temperature. In this way 3 types of bacteria were stocked from 16 sample.

2.6 Sample bacteriophage enrichment

The goal of phage enrichment is to enrich the sample for phages capable of infecting the desired host. Enrichment procedures are required to amplify the number of phages as it is difficult to isolate specific phages *(van Twest et al., 2009*). Phage enrichment is usually done by using a bacterial growth medium such as Luria Broth (LB). The prefiltered water sample or unconfirmed phage sample that is to be screened for phages against a given target bacterium is enriched with the growth medium and amended with that target bacteria prior to centrifugation and filtration. *(Chan., 2016).* A 0.22-micron filter is used during the process so any organism bigger than 0.22 µm will retain on the membrane pore. Thus, if a phage capable of infecting the inoculated bacteria is present in the given sample, then that phage will propagate and increase to a number which can be detected by plating techniques.

This step enhances the adsorption of the bacterial host and the phages, thereby amplifying the numbers of associated bacteriophages during growth in the growth medium. Enrichment in this way allows screening for phages in a much larger volume of sample (typically 15–40 mL), thus increasing the probability of isolating rare phages, and allows the combination of different target hosts (e.g., different strains of a specific bacteria of interest) in the same incubation, again increasing the possibility of phage isolation. (*Chan., 2016*).

As mentioned, phage separation from the 16 samples was done previously, which is why the enrichment of the sample phages is needed to possibly find and isolate phages capable of infecting host bacteria. Thus, the phage enrichment of all 16 samples were done accordingly to amplify the number of phages if present.

2.6.1 Streaking for isolated bacterial colonies:

To perform phage enrichment, firstly, streaking was done to get isolated single bacterial colonies of the collected stock *E.coli, Vibrio* and *Salmonella*. This procedure was done the previous day as an overnight incubation period was required. For each of the three bacteria, 3 large LA plates were

taken and divided into sectors accordingly so that 10 strains of one bacteria can be streaked. From the stock bacteria kept in vials/eppendorfs, at least 7-8 stock vials each of *E.coli, Vibrio* and *Salmonella* were taken to be streaked. These stock bacteria were not all from the same sample, rather stock from other samples were taken if it fell short. Moreover, the 10 strains of one bacteria could either be different or one strain might be streaked twice.

Each sector of the plate was labeled according to the bacteria that was going to be streaked in that specific sector. Then, bacteria were taken from the stock vials with a loop and streaked accordingly. This streaking procedure was done for *E.coli, Vibrio* and *Salmonella* plates and thus there were 9 plates in total each time. After overnight incubation at 37°C, the plates were checked for growth of isolated colonies.

2.6.2 Cocktail preparation (mixture of 10 bacteria):

Luria broth (LB) was prepared, and inside a laminar hood,10ml fresh LB was added in 3 different autoclaved falcon tubes. The falcon tubes were labelled with *E.coli*, *Vibrio* and *Salmonella.* Then, 10 single colonies of the different strains of the same bacterial species were inoculated in the falcon tube containing broth. This was done for all three bacteria and that mixture is called a cocktail of 10 bacteria. After that, the falcon tubes were kept for 1.5-2 hours in the shaker incubator at 37°C.

2.6.3 Sample bacteriophage enrichment process:

After incubation, the turbidity of the bacterial solution was checked since slight turbidity ensures logarithmic phase cells. 1.5ml pure phage solution of the same sample was added to each culture of the 10 bacteria cocktails. The phage solution was the raw stock phage that was isolated from water samples and stored. The three falcon tubes were then incubated for 3.5-4 hours at 37°C in the shaker incubator. During this time, the phages infect the host bacterial cells and increase their number.

After incubation, the solution was centrifuged at 13,000 rpm for 10 minutes. This separates the bacterial cells as pellets and the enriched phages suspend in the supernatant. The eppendorf tubes were labeled beforehand with the bacteria name and sample number, such as "*E.coli* S1" etc. The supernatant of each falcon tube was then collected in the fresh eppendorf tubes accordingly after

being filtered using a 0.22-micron syringe filter. Care has to be taken to not contaminate the 0.22 micron filter. The eppendorfs were sealed with parafilm paper and stored at 4°C. This procedure was repeated until the phage enrichment of all 16 samples was complete. The enriched phage stock was always stored at 4°C to be used further for spot testing.

2.7 Phage detection using soft agar overlay spot test:

After the primary sample phages have been enriched, it is required to detect the presence of bacteriophages through an overlay method spot test. A spot test is a quick way to verify if a phage sample can infect a bacterium by putting a small drop or "spot" of phage on a bacterium-inoculated plate. This experiment determines whether bacteria will be infected by the putative phage. The bacterium inoculated plates are prepared by mixing susceptible bacterial cells in several milliliters of soft agar, which are then overlaid and spread onto agar plates containing regular nutrient media. The “top agar” is called a soft agar as it contains 0.6% concentration of agar in comparison to the 1.5% agar present in regular nutrient media. This lower concentration of agar in soft agar allows phage to diffuse easily through the media to infect the bacterial cells while allowing consistent plaque formation. Hard agar often causes uneven formation of plaque due to uneven absorption.

If phage infects the bacteria, then a clear zone of lysed cells or plaque will be formed within the lawn of bacteria where the spot of phage was placed. It is presumed that the plaques are initiated from a point source, which is, usually from a single phage virion or a phage-infected bacteria. (Abedon, 2018)Each phage has its particular host range, the range of bacteria it can infect. Typically, the concentration of phage allows many plaques to fuse together to form a bacterial death zone the size of the drop area.

2.7.1 Spot test:

For phage detection, a wide variety of bacterial strains were used. The selected bacterial strains were streaked beforehand and were individually inoculated in 3ml of fresh LB. After that, the vials containing LB were kept in shaker incubation for 1.5 hours at 37°C and 130 rpm. During this time large LA plates were taken accordingly and labelled with selected bacterial strains. The soft agar aliquots stored in the refrigerator were melted by boiling and kept carefully at 52°C to prevent solidification of the agar. Do not remove it from the water bath until immediately before use.

After the incubation period, a soft agar aliquot was retrieved from the water bath and after waiting 15 seconds, 200 µl of the young individual bacterial culture was ejected into it. Care was taken to not touch the inside of the test tube with pipette tip. Then the solution was mixed properly. The appropriate LA plate was taken, and the contents of the tube were poured onto the LA agar surface to create a bacterial lawn. The plate was swirled gently to distribute the molten agar across the plate and the plate was kept aside for 40 mins to let the top agar solidify. This process was repeated for the rest of the plates.

After the soft agar layer had dried, the plates were marked giving numbers of each enriched bacteriophage sample number. Stock *Vibrio* phages JSF-7, JSF-5, JSF-35, and JSF-13 were taken from the biotechnology and microbiology laboratory of BRAC University to be used as controls for the *Vibrio* plates. Then 15 µl (one drop) of the phage suspensions were deposited onto the top agar layer, on marked corresponding labels and the plate was kept for drying for 30/40 mins. After the drops dried, the plates were kept at 37° C overnight . The next day the presence of clear zones of lysis was checked.

2.8 Purification of visible phages:

Phage mixtures must be purified to produce pure phage isolates suitable for characterization. Upon phage confirmation, purification of the visible phages was carried. Firstly, 200 µl aliquots of SM buffer and $1/10^{th}$ of the SM buffer, i.e. 20 µl chloroform was pipetted into the same eppendorfs. Then the clear plaques were selected from the lawn and picked off from the agar using a tip of sterile small white tip. This was dispersed and re-pipetted gently into the SM buffer + chloroform mixture. The tip of the small pipette tip was cut before inserting into the plaque. The suspended plaque was vigorously vortexed for 1 minute to release the phages from the plaque agar. These purified phages were then preserved at 4°C until processing. During storage, the chloroform will destroy any remaining bacteria if present.

2.9 Spot test for confirmation of phage:

Spot test was done again with the purified phages for the bacterial strains which showed visible plaques. These strains were individually inoculated in 3ml of fresh LB. After that, the vials containing LB were kept in shaker incubation for 1.5 hours. During this time large LA plates were

taken accordingly and labelled with selected bacterial strains. The soft agar aliquots stored in the refrigerator were melted by boiling and kept carefully at 52°C.

After the incubation period, a soft agar aliquot was retrieved from the water bath and after waiting 15 seconds, 200 µl of the young individual bacterial culture was ejected into it. Then the solution was mixed properly. The appropriate LA plate was taken, and the contents of the tube were poured onto the LA agar surface to create a bacterial lawn. The plate was swirled gently to distribute the molten agar across the plate and the plate was kept aside to let the bacterial lawn solidify. This process was repeated for the rest of the plates.

After the soft agar layer had dried, the plates were marked giving numbers of bacteriophage sample numbers. 15 µl of the purified phage suspensions kept in SM buffer, were deposited onto the top agar layer, on marked labels and the plate was kept for drying for 30/40 mins. After the drops dried, the plates were kept at 37° C overnight. The next day the presence of clear zones of the samples was checked. The isolated phages and their hosts strains were confirmed which are summarized in the table below;

Table 1 : Host strains and its bacteriophages

Host bacterial strains	**Bacteriophage (sample no.)**
Shigatoxin-producing Escherichia coli	SEC5, SEC9
WT 324	VW8 ϕ1, VW8 ϕ2
Salmonella 7g	SL9
Salmonella 7f	SL4 ϕ1, SL4 ϕ2
Salmonella 15c	SL14
Salmonella 15f	SL7

E.coli 15c	EC10 ϕ1, EC10 ϕ2
Vibrio 12c	VI8 ϕ1

2.9.1 Enrichment of isolated phage:

After confirmation of the bacteriophage through the Spot test, it is isolated and enriched for future utilization. Like sample bacteriophage enrichment, the isolated phages are also enriched in the same process. After culturing host bacterial cells(in 3ml LB) in the shaker incubator for 1.5 hour, 50 microliter of isolated phage that were previously stored in SM buffer at 4 degree Celsius were added and further incubated in the shaker incubator for 4 hours. After incubation, the solution was centrifuged at 13,000 rpm for 10 minutes to separate the bacterial cells as pellets and the enriched phages suspended in the supernatant. The eppendorf tubes were labeled beforehand with the host bacteria name and its respective phage(given in bracket), such as "*E.coli* 15C (10a)" etc. The supernatant of each falcon tube was then collected in the fresh eppendorf tubes accordingly after being filtered using a 0.22-micron syringe filter. Care has to be taken to not contaminate the 0.22 micron filter.

The enrichment process of the isolated phage was repeated in order to increase the volume of the phage if all used up. In addition to this, further steps of calculating the multiplicity of infection, double layer assay requires enriched phage solution for better outcomes. The enriched phage stock was always stored at 4°C for future usage.

2.9.2 Double Layer Assay (DLA):

Double Layer Assay or plaque assay is such an extensive technique in phage research where the pure bacteriophage population can be isolated and identified .This technique is considered as the standard for determination of phage titer. Through phage titration, concentration of bacteriophages can also be obtained. By using the DLA, the titer of each phage which is expressed as plaque forming units (pfu), was determined *(Santos et al; 2009)* Moreover, phage titer is determined for

bacteriophage application by quantitating viable bacteriophages precisely and reproducibly so that their potencies can be studied for further research *(Anderson et al., 2011).*

The premise of that conventional phage assay (PA) includes the interaction of a single lytic phage molecule and a lenient bacterium, which comes about within the host bacterium's lysis and the release of recently formed phage progeny. The host bacterial cells resume their growth when mixtures of phage and their host bacteria in molten soft agar are poured in nutrient agar enabling bacterial growth. The agar plate where phage is not present, lawns in soft agar overlay and the bacteria goes to the stationary phase of its growth curve *(Anderson et al., 2011).* Again, the areas where phage is present, produce a clear zone in the soft agar overlay where there are innumerable freed phages, and the zone can be seen with naked eyes. And this clear zone or the circular area is termed 'plaque'.

The plaques are counted, and the phage concentration/titer is commonly expressed as the number of plaque-forming units (PFU)/mL of the measured preparation.

Phage titer (PFU/ml) = Number of plaques (PFU) / (Dilution factor x Volume of phage added to plate)

Before performing DLA, through spot test, host bacteria and its specific phages are obtained. The host bacteria that we got from the 16 samples we worked on, were-

- *Shigatoxin-producing Escherichia coli*
- WT 324
- Salmonella 7g
- Salmonella 7f
- Salmonella 15c
- Salmonella 15f
- E.coli 15c
- Vibrio 12c

To measure the susceptibility of various bacterial strains to their corresponding phages, nascent bacterial cells are grown (in fresh LB) in a shaker incubator at 37 degrees Celsius(160 rpm) for 1.5 hours. This is called young culture.

For performing DLA, at first young culture of the host bacteria is prepared whose plaques are desired to be counted. Then the respective phages of the host bacteria are subjected to serial dilution in saline up to 10^{-6} and in some specific cases upto 10^{-8} dilution. After dilution of phage, 100 microliters of the phage solution is taken and 300 microliters of the young culture of the host bacteria is taken in 3 ml of molten soft agar kept in a sterile test tube. The temperature of the molten soft agar is maintained carefully at 52 degrees Celsius otherwise it would solidify sooner. The contents (phage solution, young culture of host bacteria and molten soft agar) are then mixed properly and poured in Luria-Bertani agar plates. While pouring, care should be taken so that there is no bubble formation as bubbles in the agar plate could be misinterpreted as plaques. The plates are left to solidify and then kept in an incubator at 37 degree Celsius overnight. After overnight incubation, the plates are checked thoroughly for plaque formation. After obtaining plaques, PFU/ml is calculated. Generally, 30-300 plaques are considered standard and counted if plaque is formed in this range.

2.9.3 Spot titer:

Spot titer is an alternative method to DLA through which phage titer can also be determined. This technique is a bit different to DLA. In this technique, a lawn of bacteria is made and then droplets of different dilution phage solution is given over the bacterial lawn. Lawn of bacteria is made by pouring 3 ml soft agar with 200 microliter of bacterial culture(prepared in 3ml LB and incubated in a shaker incubator for 1.5 hour) in an LA plate. Similarly, like DLA, phage dilution is done and droplets of 15 microliter of phage solution are given over the bacterial lawn on the LA plate.
In several cases, due to some limitations expected results of DLA do not come. In that case, spot titer can prove to be an alternative in counting plaques or measuring phage titer.

2.9.4 Optical Density Measurement for measuring bacterial load

Optical density (OD) measurement is needed to assess microbial growth, and it is one of the most central techniques used in microbiology. *(Stevenson et al., 2016).* It is a measure of absorbance of light that passes through a culture and the degree of light scattering caused by the bacteria within the culture. OD value obtained is proportional to the concentration of the sample in accordance

with the Beer-Lambert law. *(Stevenson et al., 2016).* This means the OD will decrease as the bacterial density decreases. OD of bacteria is commonly measured at a wavelength of 600nm or OD600 using a spectrophotometer and is measured in relation to a blank control *(JoVE Science Education Database, 2022).* 600 nm is the optimum wavelength for measuring optical density as it minimizes cell damage.

Here, OD measurement was necessary to figure out the colony forming unit(CFU/ml) of the samples. Out of the 8 hosts, the optical density of WT 324, *Shigatoxin-producing Escherichia coli*, Salmonella 7f, Vibrio 12c and E.coli 15c were measured. To do so, young culture of the bacteria was prepared by growing in LB and incubating in a shaker at 37° C. After that, serial dilution up to 10^(-6) was done in glass vials by taking 2.7ml saline and 300 µl bacterial culture. Cuvettes were cleaned with ethanol beforehand and labeled with all the dilution numbers, with one cuvette being labeled as 'B' for blank. The transparent part of the cuvette should not be touched as light passes through that part. After serial dilution was done, each dilution was transferred gently to the respective labeled cuvettes accordingly.

No bubbles should be present inside. In the blank cuvette, 3ml saline was transferred.

The spectrophotometer was turned on and the settings were adjusted to 600 nm wavelength. The OD reading of 6 samples (including blank) could be taken together in the spectrophotometer, so 6 cuvettes were placed together to get the OD measurement of each. OD was measured 4 times to reduce the chances of anomalies or machine errors. The OD of each dilution was noted down. This process was repeated for all the selected bacteria.

To calculate viable bacterial load by CFU/ml, spread plating was done in large LA plates. From each dilution tube, 50 µl was taken and spreading was done, and the plates were kept for overnight incubation at 37° C. The plates were checked the following day to count single bacterial colonies and calculate CFU/ml of each dilution. Generally, 30-300 colonies are considered as standard to calculate CFU. For this work, if the standard OD of one type among Vibrio, E.coli and Salmonella is measured, then that standard and bacterial load can be used for further MOI.

2.9.5 Multiplicity of Infection (MOI):

In each infection medium, Multiplicity of infection or MOI represents the ratio of the numbers of virus particles to the numbers of the host cells (Laskin et al., 2012). It is a frequently used term in

virology. The objective of MOI was to characterize the relative concentration of virus and bacteria that would lead to a controlled number of infections per bacterium. This accepted that all virions were irresistible and that all cells were susceptible (Shabram & Aguilar-Cordova, 2000).

Calculating MOI:

The value of MOI is 1, if the same number of host cell bacteria and its phage is taken. For example, if one million virions are added to one million cells, the value of MOI is 1. Again, the value of MOI is 10, when the number of virions added is 10 times more than that of the host bacteria cell. For example, if 10 million virions are added to one million cells, then MOI is 10.
And if the number of virions is 10 times less than the number of host bacterial cells, then MOI is 0.1. For example, if 100000 virions are added to one million cells, then MOI is 0.1.

For calculating the MOI, respective steps need to be done.

- **Water filtration**
- **Young culture preparation**
- **OD check**
- **Preparation of known concentration of phage and bacteria**
- **Dilution**
- **Spotting in an LA plate.**

Water filtration:

At first, taking water from the environment source, two way filtration is done. Firstly, water is filtered through Whatman filter paper placing it in a funnel. Secondly, the Whatman filtered water is again filtered through a 0.22 micron filter. In this case, the filtered water is taken through a syringe and then installed in a 0.22 micron filter. After that, after filtration through a micron filter, it is kept in a falcon tube.

Bacteria culture:

We take 3ml of LB and inoculate a single colony from host bacteria in it and keep it in shaker incubation for half an hour.

OD check:

The OD of the host cell bacteria was previously measured and considered as a standard value. There are three standard values measured for three bacteria- *E.coli, Salmonella* and *Vibrio*. After half an hour of incubation of host cell bacteria, OD is measured after dilution(dilution is done taking 2.7ml saline and 300 microliter bacterial culture). The dilution which matches with the standard OD value, is taken.

Preparation of known concentration of phage and bacteria:

After measurement of OD, the dilution of the host bacteria and previously stored enriched isolated phage is taken. Calculation is done for the known concentration of virus and bacteria. After calculation, the required amount of phage and host cell from that specific dilution is taken in a falcon tube. 4 falcon tubes are taken for each ratio of MOI and labeled accordingly. Then it is kept in a shaker incubator for 4 hours.

Dilution and spreading:

After 4 hours of incubation, the prepared solution of bacteria and phage is diluted up to 10^{-8}. After dilution, spreading of different dilution eppendorf is done in the LA plates. 100 microliters of the diluted solution is taken by pipette and spread on respective LA plates. After this, the LA plates are kept for overnight incubation.

Chapter 3: Result

3.1.1 Spread plate results:

The figures 3.1.1 (a,b,c,d) shows spread plate results of *E.coli, Salmonella* and *Vibrio* in their respective selective media. In the below figure number 3.1.1(c) and 3.1.1(d), bacterial growth is seen with LB for growth enhancement.

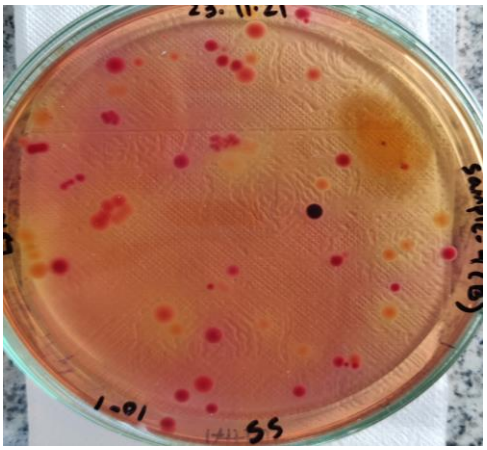

Figure 3.1.1 (a)

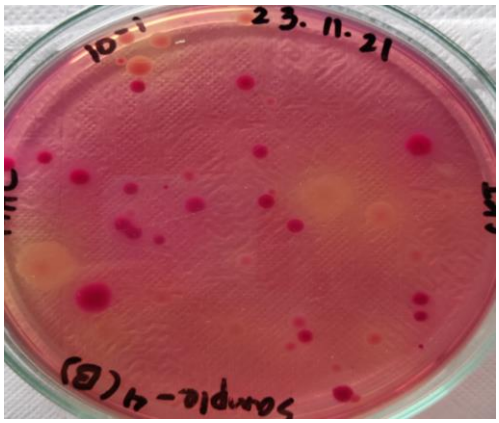

Figure 3.1.1 (b)

Figure 3.1.1 (c)

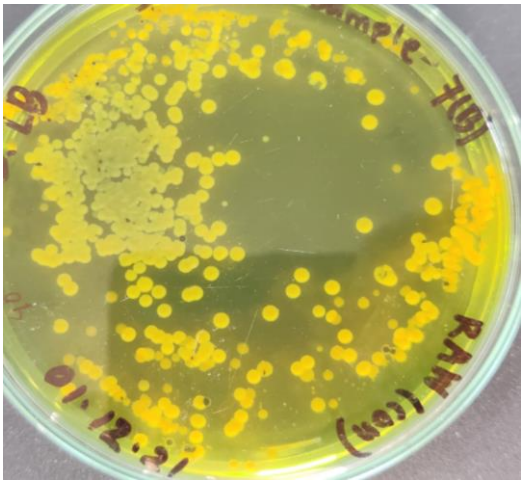

Figure 3.1.1 (d)

In figure 3.1.1 (a), visible black centered colony on 10^{-1}SS agar plate can be seen. The black single colony which may indicate the presence of Salmonella. The Figure 3.1.1 (b) shows pink colonies of E.coli on Mac CONKEY agar plate (10^{-1}). This indicates the presence of E coli. Besides, in figure 3.1.1 (c) black centered colonies on XLD agar plate is seen. This indicates the presence of Salmonella. Finally, Fig 3.1.1(d) shows yellow single colonies of Vibrio on TCBS agar plate(raw) from. This indicates the presence of Vibrio.

Table 2: Sample-wise calculation of CFU/ml

Media	MAC	TCBS	SS
Sample No.	CFU/ml	CFU/ml	CFU/ml

1	2.00E+01	0.00E+00	3.00E+01
2	4.60E+02	4.10E+02	2.60E+02
3	1.10E+02	0.00E+00	3.90E+02
4	4.00E+01	6.00E+01	5.00E+01
5	3.50E+02	5.50E+02	2.00E+01
6	1.80E+02	2.40E+02	7.00E+02
7	2.40E+02	4.10E+02	8.00E+01
8	1.20E+02	6.00E+01	6.00E+01
9	3.30E+02	7.00E+01	4.30E+02
10	3.00E+01	3.00E+01	1.20E+02
11	1.00E+02	2.70E+02	5.10E+02
12	4.00E+02	2.50E+02	1.10E+03
13	2.10E+02	2.90E+02	1.50E+02
14	8.00E+01	2.70E+02	6.00E+02
15	1.80E+03	1.50E+02	0.00E+00
16	5.00E+01	0.00E+00	0.00E+00
Total	4.52E+03	3.06E+03	4.50E+03
Average	2.83E+02	2.35E+02	3.21E+02

3.2 Optical density results

The OD results of the 3 bacteria are shown in the table below along with the colony count. The absorbance decreases as the concentration decreases. The highlighted region indicates the standard value. *(TNTC indicates too numerous to count, when the colonies are more than 300 then TNTC is written.)*

Table 3: Measurement of absorbance for bacterial colony count

Bacteria Name	Dilution	Absorbance	Colony Count
	10^{-1}	0.005A	TNTC
	10^{-2}	0.017A	76

Vibrio (WT 324)	10^{-3}	0.012A	15
E.coli (*SHIGATOXIN-PRODUCING ESCHERICHIA COLI* 9)	10^{-1}	0.111A	TNTC
	10^{-2}	0.017A	TNTC
	10^{-3}	0.007A	70
	10^{-4}	0.005A	174
	10^{-5}	0.004A	100
	10^{-6}	0	No Bacteria
Salmonella (S7f)	10^{-1}	0.088A	TNTC
	10^{-2}	0.009A	>300
	10^{-3}	0.008A	>300
	10^{-4}	0.003A	200
	10^{-5}	0.002A	100
	10^{-6}	0	No bacteria

3.2.1 OD Measurement for MOI

Table 4.1, 4.2 and 4.3 depicting the measurement of OD of the host bacteria for taking the standard dilution for MOI. The dilution which is taken as standard is highlighted in the table below.

Table 4.1 : *E.coli OD*

Bacteria Name	**Dilution**	**Absorbance**
Ecoli (*SHIGATOXIN-PRODUCING ESCHERICHIA COLI* 9)	10^{-1}	0.034A
	10^{-2}	0.008A
	10^{-3}	0.004A
	10^{-4}	0.002A
	10^{-5}	0.001A
	10^{-6}	0.001A
E.coli	10^{-1}	0.071A
	10^{-2}	0.009A

(***SHIGATOXIN-PRODUCING ESCHERICHIA COLI*9**)	10^{-3}	0.008A
	10^{-4}	0.004A
	10^{-5}	0.002A
	10^{-6}	0.001A
Escherichia coli 15c	10^{-1}	0.037A
	10^{-2}	0.010A
	10^{-3}	0.002A
	10^{-4}	0.001A
	10^{-5}	0
	10^{-6}	0

Table 4.2: *Salmonella* (7f) OD

Bacteria Name	**Dilution**	**Absorbance**
	10^{-1}	0.047A

Salmonella 7f	10^{-2}	0.002A
	10^{-3}	0.001A
	10^{-4}	0.001A
	10^{-5}	0
	10^{-6}	0
Salmonella 7f	10^{-1}	0.042A
	10^{-2}	0.006A
	10^{-3}	0.010A
	10^{-4}	0.002A
	10^{-5}	0.001A
	10^{-6}	0
	10^{-1}	0.047A
	10^{-2}	0.006A

Salmonella 7f	10^{-3}	0.011A
	10^{-4}	0.003A
	10^{-5}	0.001A
	10^{-6}	0

Table 4.3: *Vibrio* (WT) OD

Bacteria Name	**Dilution**	**Absorbance**
Vibrio (WT 8a)	10^{-1}	0.019A
	10^{-2}	0.006A
	10^{-3}	0.008A
	10^{-4}	0.002A
	10^{-5}	0.013A
	10^{-6}	0
	10^{-1}	0.028A

Vibrio (WT 8a)	10^{-2}	0.012A
	10^{-3}	0.004A
	10^{-4}	0.001A
	10^{-5}	0
	10^{-6}	0
Vibrio (WT 8a)	10^{-1}	0.022A
	10^{-2}	0.012A
	10^{-3}	0.010A
	10^{-4}	0.004A
	10^{-5}	0.003A
	10^{-6}	0.002A

3.3 Results of phage detection spotting tests:

16 water samples were collected and enriched for Vibrio, E.coli and Salmonella bacteriophage, among them multiple samples showed clear zones (shown in figures 3.3 (a,b,c) below). The

assessment of this enriched sample water by a double-layer assay spot test led to an individual plaque. Some samples also showed turbid circular regions which do not indicate a positive result.

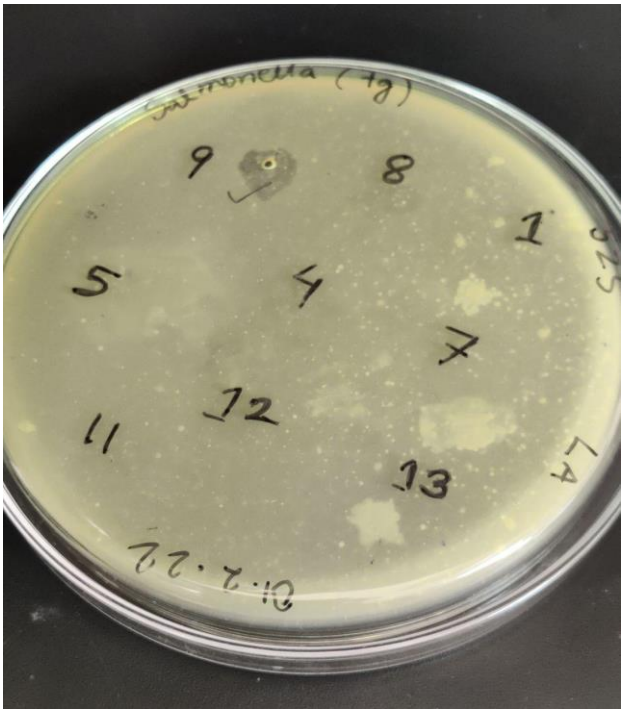

Figure 3.3 (a)

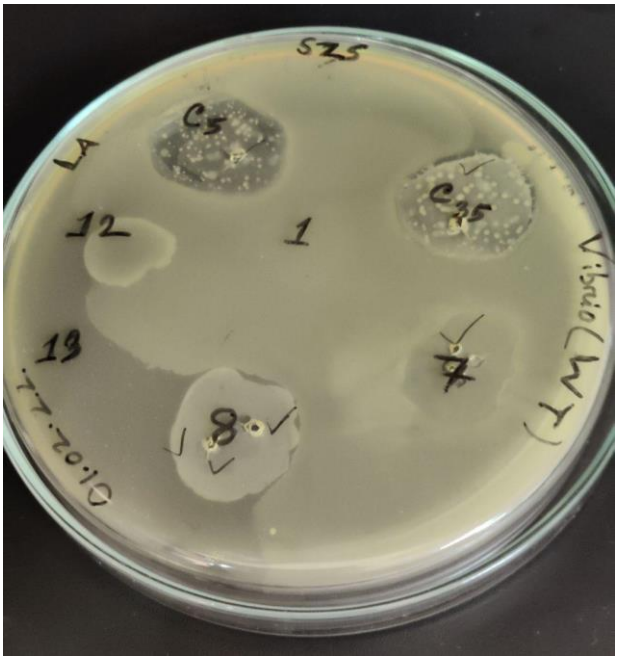

Figure 3.3 (b)

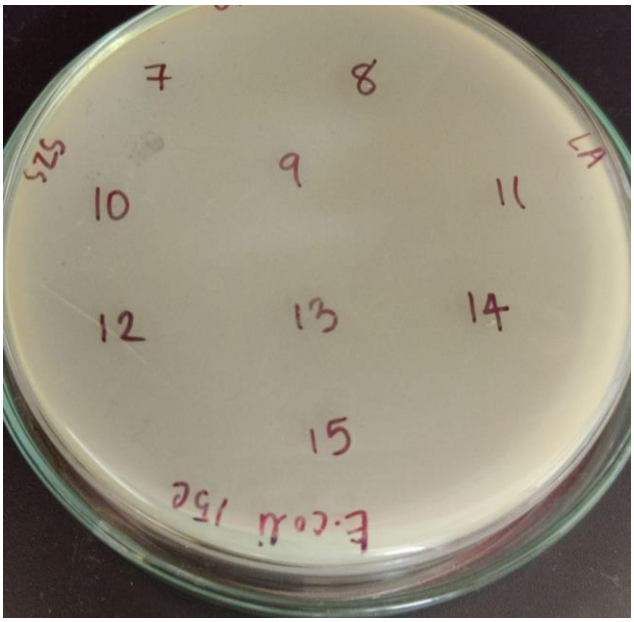

Figure 3.3 (c)

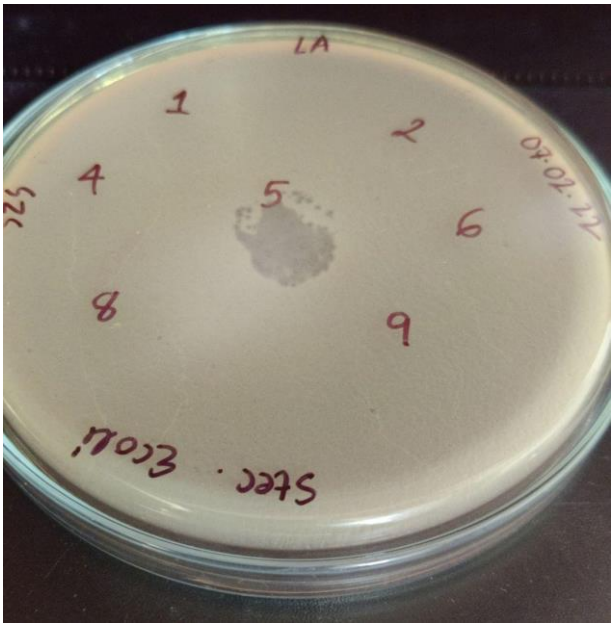

Figure 3.3 (d)

In Figure 3.3(a) clear zone or plaque shows the presence of bacteriophage from the enriched sample (9). Individual plaque formation of Salmonella (7g strain) on double-layer agar plates. Figure 3.3(b) shows clear zones or plaques show the presence of bacteriophage

from the enriched sample 7,8, and stock JSF-5, JSF-35. Individual plaque formations of Vibrio (WT 324) on double-layer agar plates. Figure 3.3(c) shows clear zone or plaque show the presence of bacteriophage from the enriched sample 10. Individual plaque formation of E.coli (15c strain) on double-layer agar plates. Figure 3.3(d) shows clear zone shows the presence of bacteriophage from the enriched sample 5 and 9. Individual plaque formation of Shiga toxin-producing Escherichia coli strain on double-layer agar plates.

Among the 16 water sample phages enriched for *Vibrio, E.coli* and *Salmonella* bacteriophage, multiple samples showed clear zones which are summarized in the table below.

Table 5: Phage detection results (Spot test)

Bacteria name	**Samples positive for phages**
E.coli	7,10
Shigatoxin-producing Escherichia coli (Shiga-toxin producing E.coli)	2,5,6,9
Salmonella	4-10,14
Salmonella typhi	None
Vibrio	7,8, JSF-5, JSF-35
WT 324	7,8, JSF-7, JSF-35, JSF-5

3.4 Results of phage confirmation spotting tests:

After detecting phages and isolating them, through another spotting test those phages were confirmed. The results revealed 8 host bacterial strains and 7 sample phages. Individual clear zones or plaques confirmed the presence of these bacteriophages.

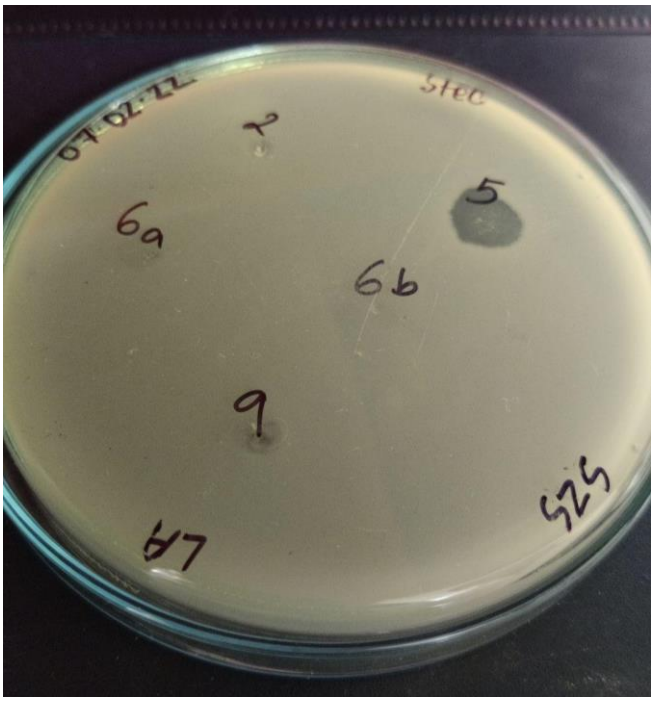

Figure 3.4 (a)

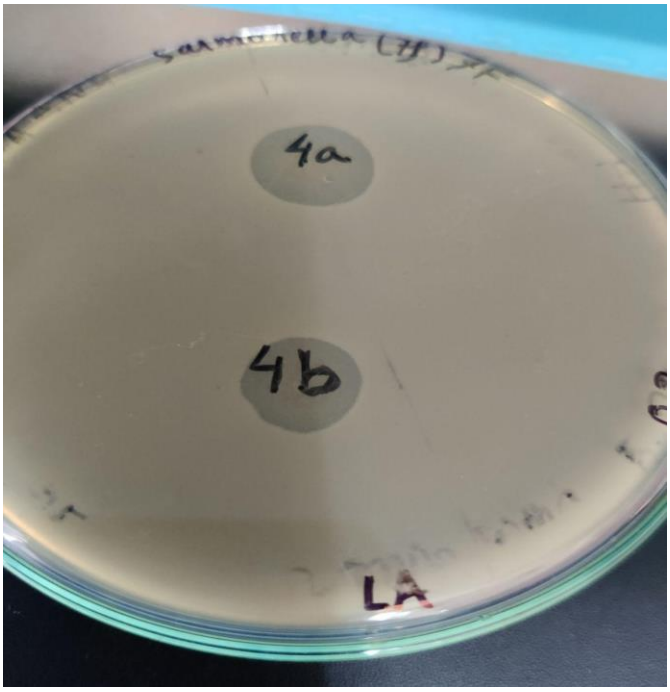

Figure 3.4 (b)

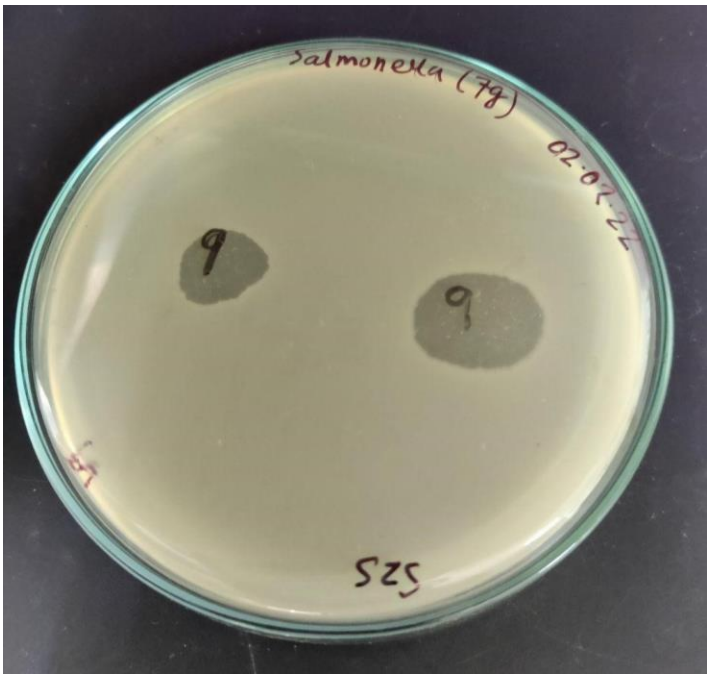

Figure 3.4 (c)

Figure 3.4(a) shows clear zone of plaque seen in sample 5 and 9 of Shiga toxin -producing *Escherichia coli* plate. In figure 3.4(b), clear zone of plaque seen in sample 10 of Salmonella 15c plate. Finally, figure 3.4(c) shows clear zone of plaque seen in sample 8 of Salmonella 7g plate.

Among the 16 water sample phages enriched for *Vibrio, E.coli* and *Salmonella* bacteriophage, a summary of the host bacterial strains and their specific bacteriophages are given below:

Table 6: Host bacteria and its bacteriophages

Host bacterial strains	**Bacteriophage**
Shigatoxin-producing Escherichia coli	SEC5, SEC9
WT 324	VW8 ϕ1, VW8 ϕ2
Salmonella 7g	SL9
Salmonella 7f	SL4 ϕ1, SL4 ϕ2
Salmonella 15c	SL14
Salmonella 15f	SL7
E.coli 15c	EC10 ϕ1, EC10 ϕ2
Vibrio 12c	VI8 ϕ1

3.5 DLA Results

The double layer assay was performed in 8 host bacteria to confirm the presence of plaques. After overnight incubation, out of 8 host bacterial strains, countable single plaques were obtained in four. The strains *Salmonella 7f, WT-324, E.coli 15c* and *Shiga toxin-producing Escherichia coli* showed visible single plaques (shown in figures 3.5 a, b,c) from few to uncountable in number. Moreover, it is also observed that, number of plaque varies in different dilutions.

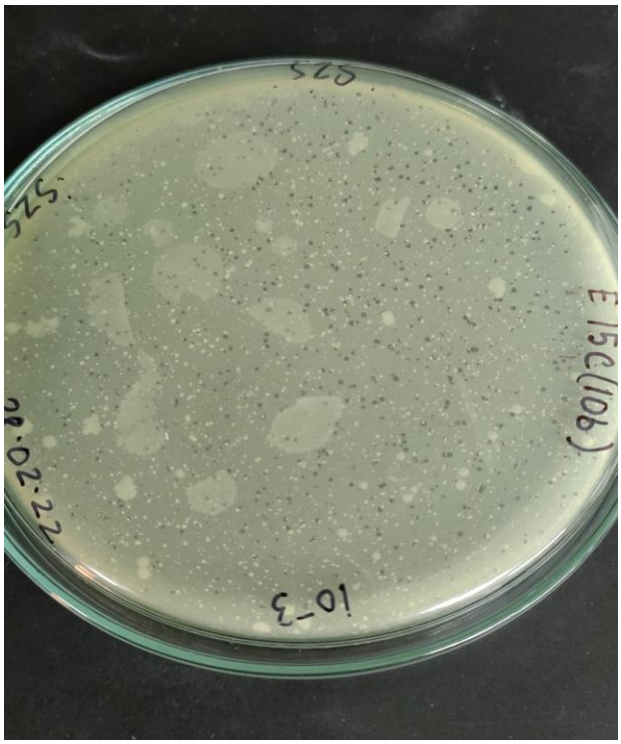

Figure 3.5 (a)

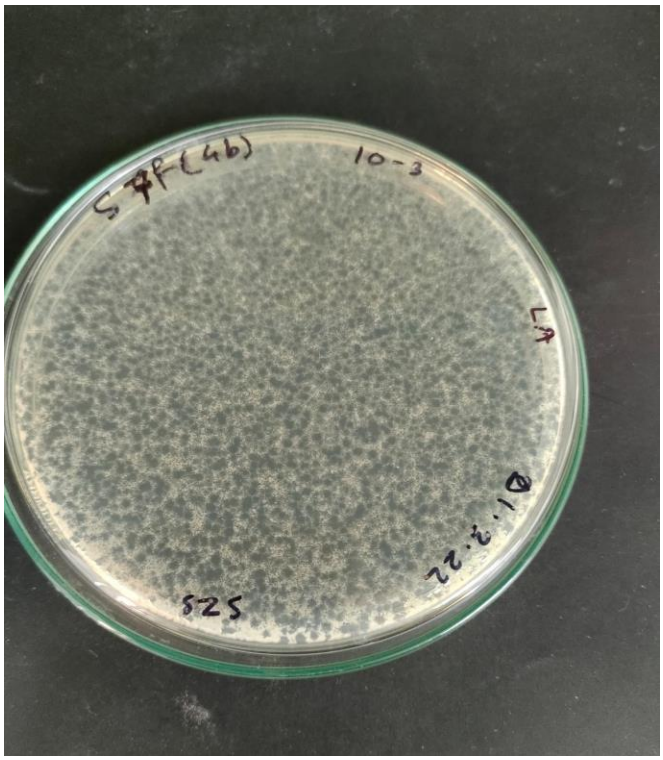

Figure 3.5 (b)

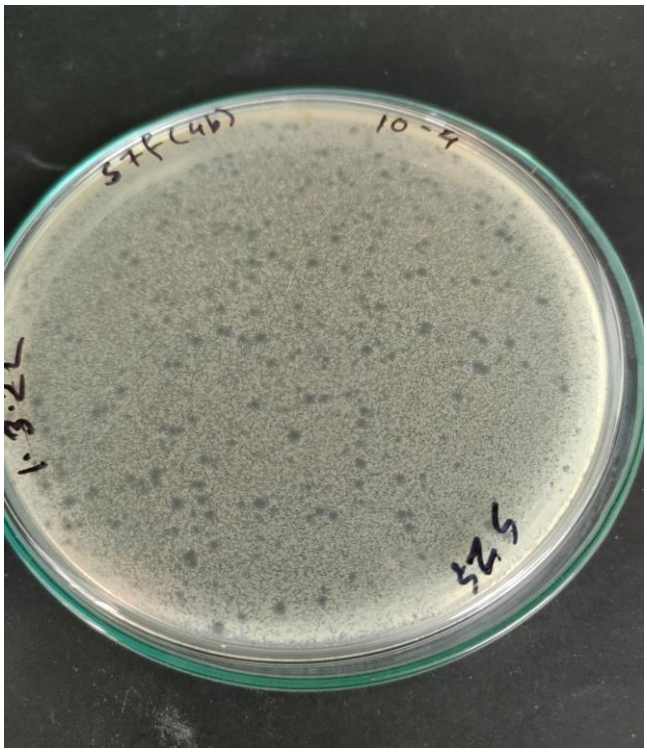

Figure 3.5 (c)

In Figure 3.5(a), single countable plaques are seen in 10-3 dilution plate of E.coli 15c. Then in Figure 3.5(b), uncountable plaques seen in 10−3 dilution plate of Salmonella 7f. Finally, the figure 3.5(c) shows countable single plaques seen in 10−4 dilution plate of Salmonella 7f.

3.6 MOI Results

Table 7.1- 7.9 depicts the MOI result of the host bacteria and its phage, obtained in four different MOI values/ ratios in dilution upto 10^{-8}.

In MOI, we determine four values or ratios. They are- 1. In MOI, we determine four values or ratios. They are- 1) Raw (no phage is added) 2) MOI value 0.1 or 1:10 (bacteria is 10 fold more than the phage) 3) MOI value 1 or 1:1 (phage and bacteria is of equal volume) 4) MOI value 10 or 10:1 (phage is 10 fold more than the phage).

Table 7.1: *E.coli* (*SHIGATOXIN-PRODUCING ESCHERICHIA COLI* 9) MOI

The tables below depicts the colonies in different dilutions of four different ratios (MOI value) of the three bacteria.

Name Of Bacteria	Ratio (phage: bacteria)	Dilution	Number of Colonies
	Raw (no phage)	10^{-1}	No Single Colony
		10^{-2}	No Single Colony
		10^{-3}	TNTC
		10^{-4}	6
		10^{-5}	14
		10^{-6}	9
		10^{-7}	6
		10^{-8}	9
(*SHIGA TOXIN-PRODUCING ESCHERICH*		10^{-1}	TNTC
		10^{-2}	13

***IA COLI* 9)**	1:1	10^{-3}	10
		10^{-4}	29
		10^{-5}	8
		10^{-6}	13
		10^{-7}	9
		10^{-8}	0
	1:10	10^{-1}	No Single Colony
		10^{-2}	No Single Colony
		10^{-3}	TNTC
		10^{-4}	TNTC
		10^{-5}	16
		10^{-6}	14
		10^{-7}	15

		10^{-8}	5
	10:1	10^{-1}	5
		10^{-2}	6
		10^{-3}	5
		10^{-4}	5
		10^{-5}	8
		10^{-6}	4
		10^{-7}	3
		10^{-8}	3

Table 7.2: *E.coli* (*SHIGATOXIN-PRODUCING ESCHERICHIA COLI* 9) MOI

Bacteria	Ratio	Dilution							
	Phage: bacteria	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}

E coli 15 c (10 b)	Raw (no phage)	TNTC	TNTC	TNTC	30	4	1	0	0
	1:10	TNTC	TNTC	TNTC	30	3	0	0	0
	1:1	TNTC	TNTC	TNTC	TNTC	31	0	0	0
	10:1	0	0	0	0	0	0	0	0

Table 7.3: *E.coli* (*SHIGA TOXIN-PRODUCING ESCHERICHIA COLI* 9) MOI

Bacteria	**Ratio Phage: bacteria**	**Dilution**							
E.coli (*SHIGA TOXIN-PRODUCING ESCHERICHIA COLI* 9)		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
	Raw (no phage)	TNTC	TNTC	9	1	0	0	0	0
	1:10	TNTC	TNTC	7	0	0	0	0	0

	1:1	TNTC	TNTC	9	0	0	0	0	0
	10:1	40	4	2	0	0	0	0	0

Table 7.4: MOI

Bacteria	Ratio (phage: bacteria)	Dilution							
Salmonella (S7f 4b)		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
	Raw (no phage)	TNTC	TNTC	TNTC	39	10	2	0	0
	1:10	contamination	TNTC	TNTC	28	9	4	1	0
	1:1	1	3	0	0	0	0	0	0
	10:1	0	4	1	1	0	0	0	1

Table 7.5: MOI

Bacteria	Ratio (phage: bacteria)	Dilution							
Salmonella (S7f 4b)		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
	Raw (No phage)	TNTC	TNTC	TNTC	20	3	0	0	0
	1:10	10	TNTC	TNTC	17	3	1	0	0
	1:1	0	3	1	0	0	0	0	0
	10:1	0	0	0	0	0	0	0	0

Table 7.6: MOI

Bacteria	Ratio (Phage: bacteria)	Dilution							
Salmonella 7f (4b)		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
	Raw (no phage)	TNTC	TNTC	TNTC	2	0	0	0	0
	1:10	TNTC	TNTC	TNTC	12	3	0	0	0
	1:1	TNTC	TNTC	TNTC	35	0	0	0	0
	10:1	0	0	0	0	0	0	0	0

Table 7.7: MOI

Bacteria	Ratio (phage: bacteria)	Dilution							
Vibrio(Wt 8a)		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
	Raw(no phage)	TNTC	TNTC	TNTC	12	0	0	0	0
	1:10	1	0	1	0	0	0	0	0
	1:1	3	0	0	0	0	0	0	0
	10:1	5	10	5	0	0	0	0	0

Table 7.8: MOI

Bacteria	Ratio(phage: bacteria)	Dilution							
Vibrio(Wt 8a)		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}

	Raw (no phage)	TNTC	TNTC	TNTC	TNTC	TNTC	uncountable	1	0
	1:10	TNTC	TNTC	TNTC	TNTC	7	3	0	0
	1:1	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	10:1	TNTC	TNTC	0	0	0	0	0	0

Table 7.9:MOI

Bacteria	Ratio(phage: bacteria)	Dilution							
Vibrio(wt 8a)		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
	Raw (no phage)	Uncountable	Uncountable	Uncountable	Uncountable	Uncountable	0	0	Uncountable

	1:10	Uncountable	Uncountable	Uncountable	Uncountable	Uncountable	Uncountable	Uncountable	Uncountable
	1:1	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	10:1	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC

MOI(Multiplicity of infection) were conducted for the three bacteria Escherichia coli, Vibrio, Salmonella in four different ratios or MOI values (0.1, 1, 10). In different dilutions, single colonies of the bacteria were observed and compared within the different MOI values.

Calculations:

MOI calculation:

- CFU calculation
- PFU calculation

- CFU/ml = (no. of colonies x dilution factor) / volume of culture plate
- Phage titer (PFU/ml) = Number of plaques (PFU) / (Dilution factor x Volume of phage added to plate)

(1) *E.coli*:

***Shigatoxin-producing Escherichia coli*(9):**

The standard value of absorbance that is considered is 0.007A. The CFU of the *E.coli* at standard absorbance is 2000 CFU/ml.

The average count of *E.coli*= 282 CFU/ml

So, for 5ml water, total bacteria=282*5= 1410.

In 1:10 ratio, for 1410 bacteria, the required volume= 1410/2000= 0.7 ml

In 1:10 ratio, for phage, the required volume= (141*100)/70 = 201.4 micro litre.

In 1:1 ratio, the required volume of bacteria= 0.7 ml

In 1:1 ratio, the volume required of phage = (1410*100)/70 = 2014.2 micro litre.

In 10:1 ratio, the required volume of bacteria= 0.7 ml

In 10:1 ratio, the required volume of phage = (14100*100)/70 = 20 ml.

This calculation of *Shigatoxin-producing Escherichia coli* (9) is repeated for taking another reading.

***E.coli* 15c (10b):**

The standard value of absorbance that is considered is 0.007A. The CFU of the *E.coli* at standard absorbance is 2000 CFU/ml.

The average count of *E.coli*= 282 CFU/ml

So, for 3ml water, total bacteria=282*3= 846.

In 1:10 ratio, for 846 bacteria, the required volume= 846/2000= 0.42 ml

In 1:10 ratio, for phage, the required volume= (84.6*100)/58 = 145.86 micro litre.

In 1:1 ratio, the required volume of bacteria= 0.42 ml

In 1:1 ratio, the volume required of phage = (846*100)/58 = 1458.6 micro litre.

In 10:1 ratio, the required volume of bacteria= 0.42 ml

In 10:1 ratio, the required volume of phage = (8460*100)/58 = 14.58 ml.

(2) *Salmonella*:

***Salmonella* 7f (4b)**

The standard value of absorbance that is considered is 0.007A. The CFU of the *Salmonella* at standard absorbance is 4000 CFU/ml.

The average count of *Salmonella*= 321 CFU/ml

So, for 3ml water, total bacteria=321*3= 963.

In 1:10 ratio, for 846 bacteria, the required volume= 963/2000= 0.48 ml

In 1:10 ratio, for phage, the required volume= (96.3*100)/55 = 175 micro litre.

In 1:1 ratio, the required volume of bacteria= 0.48 ml

In 1:1 ratio, the volume required of phage = (963*100)/55 = 1750 micro litre.

In 10:1 ratio, the required volume of bacteria= 0.48 ml

In 10:1 ratio, the required volume of phage = (9630*100)/55 = 17.5 ml.

This is repeated twice for taking two more readings.

(3) *Vibrio*:

The standard value of absorbance that is considered is 0.012A. The CFU of the *Vibrio* at standard absorbance is 300 CFU/ml.

The average count of *Vibrio* = 235 CFU/ml

So, for 3ml water, total bacteria=235*3= 705.

In 1:10 ratio, for 705 bacteria, the required volume= 705/300= 2.35 ml

In 1:10 ratio, for phage, the required volume= (70.5*100)/80 = 88 micro litre.

In 1:1 ratio, the required volume of bacteria= 2.35 ml

In 1:1 ratio, the volume required of phage = (705*100)/80 = 881 micro litre.

In 10:1 ratio, the required volume of bacteria= 2.35 ml

In 10:1 ratio, the required volume of phage = (7050*100)/80 = 8.81 ml.

This is repeated twice for taking two more readings.

Chapter 4: Discussion

4.1 Isolation of bacteriophages:

Water contamination and scarcity of safe drinking water has become a major challenge over the years. Water borne epidemics have become more frequent in many developing and under developed countries due to this reason. Over the years, the application of bacteriophage as bacterial control tools in various sectors of medicine, food and biotechnology has grown to be immensely popular. The aim of this thesis is to shed light on bacteriophage's potential as a bacterial control tool to possibly control water contamination. For this study, 16 water samples were collected in winter (November 21'- December 21') from three groundwater sources in Dhaka. These are Dhanmondi lake, Hatirjheel lake and Buriganga river. In the environment, numerous bacteria and bacteriophages persist together. Season and climate have an influence on the abundance of phage communities, which is why there is a possibility of lower prevalence of phage during winter. However, the bacteriophages that were used for this study and their host bacteria were isolated from these surface water sources.

Phage of different samples were isolated and after enrichment was plated with different host strains that are as follows;

- *Shiga toxin-producing Escherichia coli*
- WT 324
- *Salmonella* 7g

- *Salmonella* 7f
- *Salmonella* 15c
- *Salmonella* 15f
- *E.coli* 15c
- *Vibrio* 12c

The appearance of a circular transparent zone or plaques in the plates where drops of phage samples were placed, indicates the existence of phage. Plaque formation was seen in agar plates after overnight incubation and these plaques indicate bacterial lysis. The outcome indicates the existence of phage from various samples, which may be affected by season and climate.

Among all phage samples, samples 4 (Buriganga river), 5 (Buriganga), 7(Buriganga), 8 (Hatirjheel lake), 9 (Buriganga), 10 (Dhanmondi lake), 14 (Hatirjheel lake) had bacteriophages in spot test and the rest failed to show positive results. The difference in the abundance of host bacteria in sample collection sites might be a reason for not getting clear zones. If the host bacteria are present in lower density in source water, there is less chance that host-specific phages will interact with each other. Furthermore, there were some plates in which concentrated circular regions were seen which may indicate contamination. Overlap of multiple drops of phage also plays a significant role in the formation of these clouded regions.

4.2 Comparative study of the ramification of phage concentration in bacterial load:

After phage isolation Plaque assay was done to count and identify phages, as well as isolate mutations and novel phages. When plated using the DLA approach, many phages form large, well-defined single plaques that are easily viewed and counted.

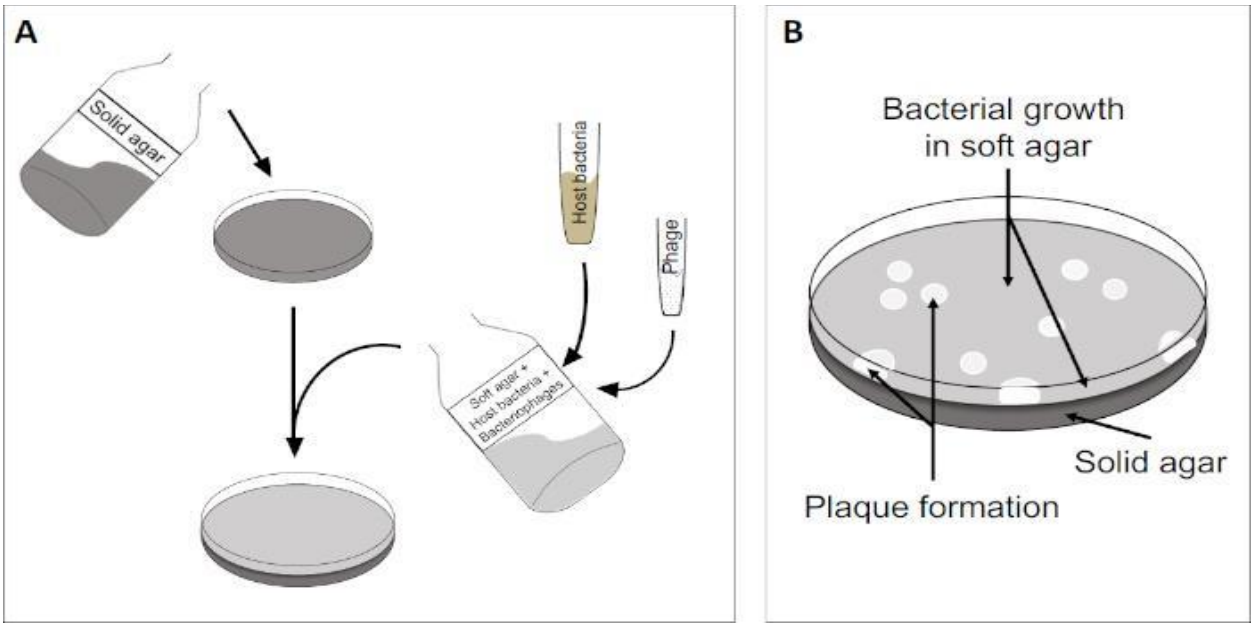

Fig: DLA procedure

As the thesis work was based on water samples, this technique was used to enumerate, isolate, and detect bacteriophages in water samples. Therefore, the aim of using this technique was to obtain single countable plaques (clear zone) .The plaques are counted, and the phage concentration/titer is commonly expressed as the number of plaque-forming units (PFU)/mL of the measured preparation. The number of plaque forming units per volume (i.e., PFU/mL) of a sample, can thus be determined from the number of plaques generated.

However, in this study after the plaque assay experiment, it was observed that out of eight bacterial host strains the PFU of only 5 bacterial host strains were obtained. These are namely: S7f, WT, S15c, E15c and *Shiga toxin-producing Escherichia coli* (**Shiga toxin-producing *Escherichia coli)***.

The reason for obtaining PFU from these particular bacterial strains is that- phage lysis of those bacteria might have taken place. Multiple neighboring cells become infected with each lysed cell. A clear zone (a plaque) can be seen on the turbid plate after a few cycles, confirming the formation of a single bacteriophage particle. Plaques can be identified as clearing zones in the bacterial lawn, either with complete clearance or partial re-growth caused by the formation of resistant bacteria, due to lysis of susceptible host cells.

On the other hand, since phage lysis did not take place, PFU was not obtained from the three other bacterial strains. Moreover, another reason for not obtaining plaques might be due to contamination in petri-plates and sometimes multiple plaques might occasionally overlap and form a cluster, making individual plaque identification difficult.

4.3 Bacterial Load and MOI:

Through MOI, the bacterial load is counted. MOI reading ensures relative concentration of bacteria and phage. Moreover, MOI calculation gave us an idea of bacterial load and how it is changed with the increasing phage number.

After collecting the sample water from 16 sources, its average bacterial load has been calculated and expressed in the following graph.

Chart 1: Bacteria vs CFU/ml

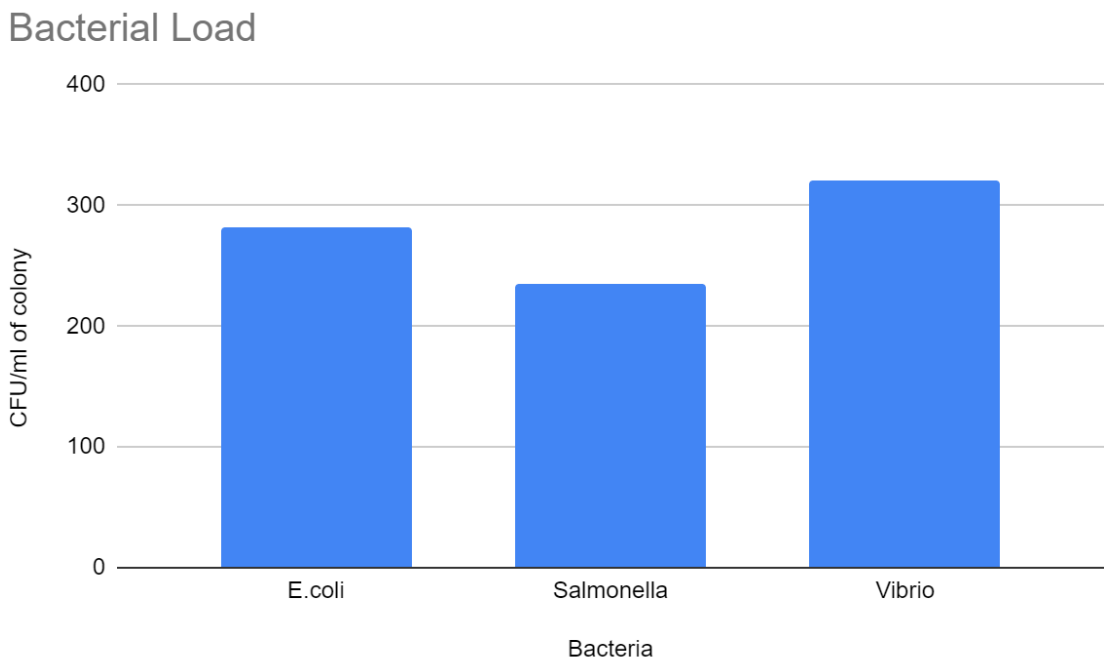

Figure 4.3 (a): Bacteria vs CFU/ml Chart

It is seen that, the average bacterial load of *E.coli* is 282.5 CFU/ml, *Salmonella* is 321 CFU/ml and *Vibrio* is 235 CFU/ml.

In the results of MOI, it is seen that as the phage number is increased, the bacterial count decreases. In addition to this, the bacterial count also decreases with the increasing number of dilution. Too many colonies were seen in 10^{-1}, 10^{-2} but in 10^{-8} the bacterial cell was too less comparatively. Single colonies were clearly observed more in 10^{-4}, 10^{-5}, 10^{-6}dilutions.

Again, when the MOI value is 10, it shows better results than the other ratios as in this ratio, the number of phage has increased 10 fold. When different ratios of MOI were compared, it has been observed that, in MOI value 1, the bacterial count of the bacteria- *Ecoli, Salmonella* is the highest. Here, the concentration of bacteria and phage remains the same. From the graph, it can be depicted that the phage has lysed some of the bacteria but since the concentration of bacteria was even, the phage lysis did not occur properly. Because of this, the bacterial count increases. Whereas, in MOI value 0.1, the bacterial count is seen to have decreased and further decreased in MOI value 10. In the ratio, where no phage was given the bacterial count increases than that of the MOI value 0.1 and 10. This is because, due to absence of phage, no lysis of bacteria occurs and thus the bacterial count did not decrease.

Graph 1: *E.coli* MOI

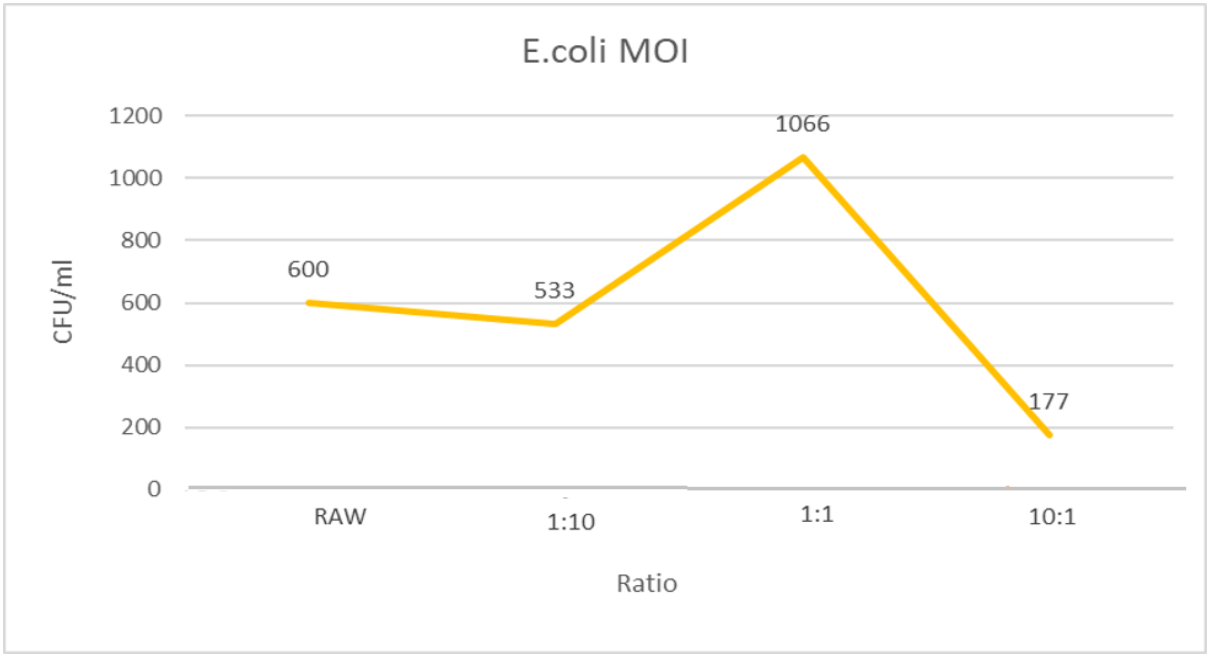

Figure 4.3 (b) : *E.coli* MOI with CFU/ml vs Ratio

Graph 2: *Salmonella* MOI

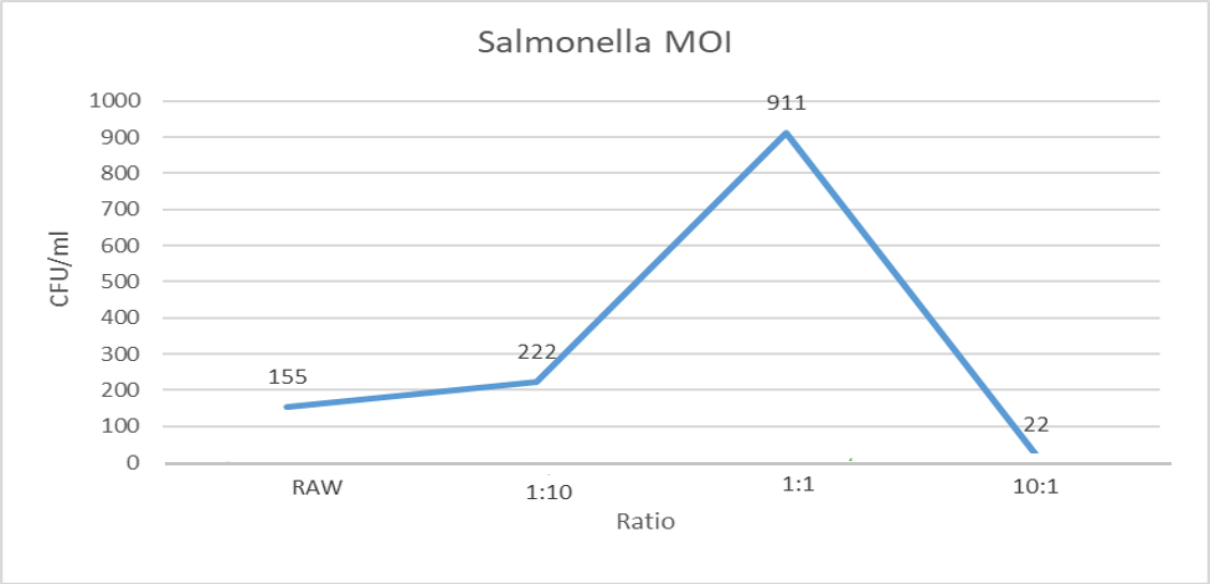

Figure 4.3 (c): *Salmonella* MOI with CFU/ml vs Ratio

An exception is seen in the case of vibrio. In this case, in MOI value 1, the bacterial count is seen to have the lowest value. And then when the MOI value is 10, the bacterial count suddenly increases. Since the volume of phage is more in MOI value of 10, the bacterial count should have decreased. But this did not happen in case of vibrio. Contamination may happen in the process. While measuring the OD, several problems were faced. The volume of the bacteria was more in case of Vibrio which caused problems in dilution. The dilution for measuring the absorbance had to be repeated to match with the required volume of the bacteria as the total limit of the cuvette(in measuring absorbance) was 3ml. Thus, the expected result of Vibrio did not come.

Graph 3: *Vibrio* MOI

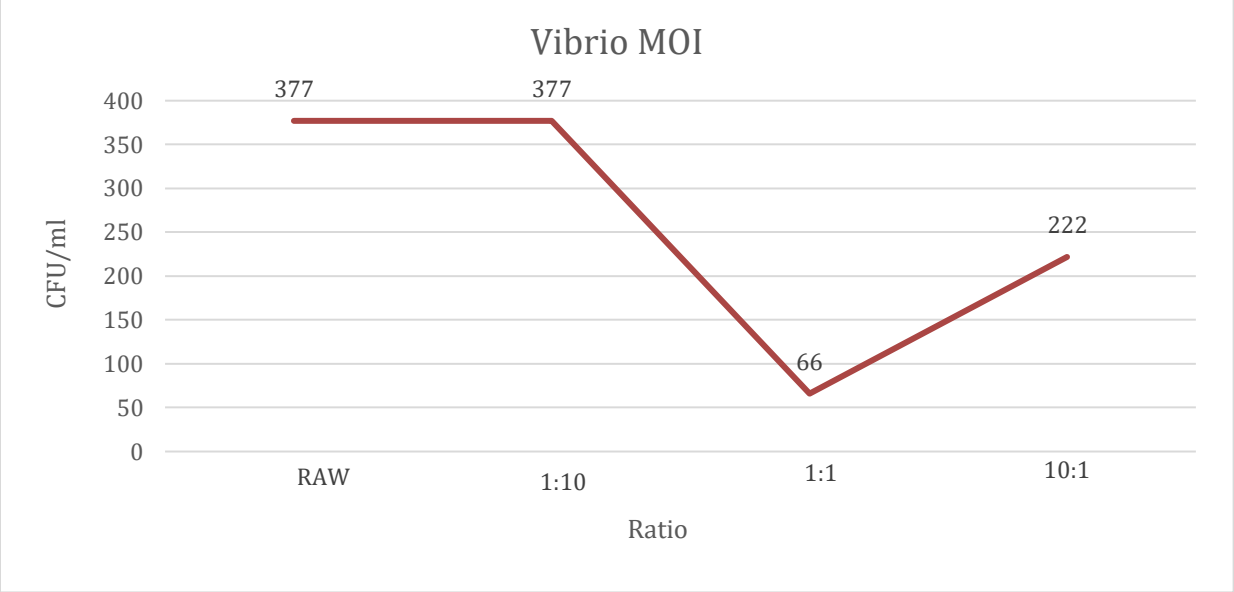

Figure 4.3 (d): *Vibrio* MOI with CFU/ml vs Ratio

Therefore, the decrease of bacterial count in the MOI value 10 indicates the bacteriophage's potential as a bacterial tool to control water contamination. In addition, it also indicates that the phage has lysed most of the bacteria and is effective in its application.

4.4 Limitations and Recommendations

The most common difficulty seen in this study was high bacterial contamination in the double-layer assay and spotting plates. Despite maintaining an aseptic environment for as long as possible during various experiments, different bacterial colonies would occasionally appear on the assay plate surface, often covering a potential plaque, resulting in a false plaque count that could affect the result's reliability and accuracy. Cleaning the micropipette with ethanol and centrifuging bacterial cells from the phage stock, followed by filtering through a 0.22m syringe filter, eliminated the potential of bacterial contamination.

During the double layer assay and spotting experiment, multiple plaques would sometimes overlap and form a cluster, making it difficult to distinguish individual plaques. Greater dilution would reduce the overall number of plaques, reducing the chance of plaque overlap, because the average

plaque size was relatively large. Furthermore, if the soft agar used in these experiments is too hot then, the inoculated bacteria or phage may die.

Due to non-productive lysis, it's possible to get false positives, much like with spot testing. Early infections may cause cell debris to bind to and inactivate free phages, interfering with subsequent infections.

Additionally, during serial dilution, volumetric errors related to the use of micropipettes could occur. Inaccuracies in the transfer process results in a less exact and precise transfer. The highest dilution has the most inaccuracies and the least accuracy as a result. Because serial dilution is done in a stepwise fashion, it takes a longer time to complete, limiting the method's efficiency.

Faulty pipette, cuvette mishandling, faulty machine can be the reason for anomalies in OD reading. So, OD was checked 4 times and the average value was taken from it as OD readings do not increase linearly as cell concentration increases.

Chapter 5: Future Perspectives

Phage based technology in controlling water contamination has massive potential in recent times. They can control pathogenic or other infectious bacteria through different ways such as; selective lysis of target bacteria, destroying biofilms that protect bacteria (Mathieu et al., 2019). Thus, the most fit application for phage in water systems is as antimicrobial tools where antimicrobials are partially effective and selective bacterial targeting is confirmed (Mathieu et al., 2019). The application of phages as microbial tools might be improved by combining with other agents to reduce phage resistance or by simply using a combination of phages (Pires et al., 2020). However, further research needs to be made to fulfill the potential of phage-based biocontrol to reduce bacterial problems in water.

5.1 Conclusion

Bacteriophages, also known as bacterial-specific viruses, are the most prominent biological form on the planet. Bacteriophages are found in every bacterial species, and their specificity of infection makes them an attractive option for bacterial control and environmental safety as a biotechnological tool against harmful bacteria.

Despite the fact that bacteriophages have yet to gain widespread recognition as a bacterial control tool, bacteriophage-based environmental technologies are currently being researched. Bacteriophage infections can be found in the same habitats as their bacterial hosts, or were present there, and that phages are consumed on a daily basis by humans. Understanding the life cycles of phages and how they interact with their hosts might help reduce or eliminate refractory bacterial populations.

One of the challenges faced during the thesis work was that there was less chance of obtaining phages from the surface water because season and climate have an influence on the abundance of phage communities. So, the drawback was, the research work was carried out in winter due to which there was a risk of lower prevalence of bacteriophages.

Another limitation is the long-term phage preservation, or simply the fact that phages have a half-life of less than a year at 4°C, which is one of the most difficult aspects of phage therapy.

Furthermore, this research looked into the impacts of particular bacteriophages for Escherichia coli, Vibrio, and Salmonella as bacterial control tools for water pollution control in Dhaka city. As a result of this research, bacteriophage lysis has been identified as an optimal, natural, and cost-effective strategy for controlling pollution of surface water. As mentioned earlier, sample collection was done from November '21 to 'December 21 which was in winter. Hence for obtaining more effective and precise results, the sample must be collected in other seasons except winter. However, the findings of the current study unquestionably provide a significant breakthrough for future research of bacteriophages as a potential alternative.

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