Isolation of bacteriophages from surface waters specific for *Shigella dysenteriae* and their characterization as potential biocontrol agents.

Submitted By,

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A Thesis Submitted To The Department Of Mathematics And Natural Science In Partial Fulfillment Of The Requirements For The Degree Of Bachelors Of Science In Microbiology

Microbiology Program

Department of Mathematics and Natural Sciences

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Declaration

I, Ayesha Mubarak, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research. I confirm that:

1. This work was done wholly or mainly while in candidature for bachelors in Microbiology at this University;
2. 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;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Either none of this work has been published before submission, or parts of this work have been published as: [The references are cited at the end of this thesis]

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Approval

The thesis/project titled "**Isolation of bacteriophages from surface waters specific for *Shigella dysenteriae* and their Characterization as potential biocontrol agents**" submitted by Ayesha Mubarak (14126011), 2019 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Microbiology on 25th July 2019.

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Ethics Statement:

Ethics is the discipline dealing with what is good and bad and with moral duty and obligations. In this research work, ethical approval was not necessary.

Abstract/ Executive Summary:

Shigella is a low-infectious dose pathogen that can cause disease in healthy people. It causes a range of diseases from mild watery diarrhea to severe dysentery (shigellosis). The primary objective of this project was to isolate bacteriophage against *Shigella dysenteriae* and to recognize its different characteristics as an antimicrobial agent to determine the therapeutic potential. In this study, *Shigella dysenteria* specific bacteriophage was isolated and characterized to develop a therapeutic agent. A total number of 20 samples were collected for the study of this experiment. The samples were collected from different areas of Dhaka city. The samples were initially filtered and the double layer- ager method was used for the isolation of bacteriophages. For the characterization of bacteriophages, host range specificity tests were checked. After examination of the host range, the *Shigella dysenteriae* phage stated that it had a wide host range as it was able to lyse 5 out of 13 distinct bacterial cultures. The most prominent bacterial hosts were ETEC, EAEC, Shiga-toxin producing *E.coli* (STEC), *Entero pathogenic E.coli* (a typical) and *Shigella dysenteriae* were lysed by *Shigella dysenteria* phage. In the case of bacteriophage isolations, clear zones were observed which indicates the presence of phages. This research, therefore, adds to the increasing amount of isolated bacteriophages, especially the particular bacteriophage of *Shigella dysenteriae*. Studies on its biological features may provide helpful data and understanding to identify prospective therapeutic agents against infection with *Shigella dysenteriae.*

Dedication:

I would like to dedicate this thesis to my Mother, Father and my Brother to their love, prayers, supports, and sacrifices for educating me and preparing me for my future.

Acknowledgment:

First and Foremost, praise and thanks to the Almighty Allah for showering His blessings throughout my research work to complete the research successfully.

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Ayesha Mubarak

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Chapter 1: Introduction

Bacteriophages, phages, are viruses that predate bacteria that are responsible for producing and transmitting their progeny through bacterial metabolism and reproduction machinery. The phage genetic material comprises of single or double-stranded RNA or DNA. They are the most abundant as well as the most genetically diverse biological entities on Earth, with an estimated worldwide population of 1030 to 1032 (Hemminga et al., 2010). Each bacteria is predicted to have 5 to 10 viruses (Weinbauer, 2004). It is now recognized that phages play a vital part in the cycling of organic matter in the biosphere and, in addition to keeping the bacterial equilibrium in the ecosystem, and holds a significant part in nutrient diversity (Chibani-Chennoufi et al., 2004; Guttman et al., 2004).

Like other viruses, phages are an obligatory intracellular parasite and their life cycle depend entirely on their host bacterial cell as they lack the proliferation cell structure and enzyme system (Carlton, 1999).

The life cycle of bacteriophage is either lytic or lysogenic. Lytic phages such as T4, after virion replication, lyses the host cell. The progeny of the phage will then be discharged to locate new hosts. The host cell is not instantly lysed by lysogenic phages. These phages are known as temperate phages. The genome of bacteriophage is integrated and replicated with the host genome without destroying the cell.

Shigella spp. are Gram-negative rod-shaped bacilli mostly connected with mammalian or avian hosts, categorized as *Shigella* within the Enterobacteriaceae family (The et al., 2016). It is transmitted through the fecal-oral route. *Shigella* spp. can cause symptomatic infection with an exceptionally small dose of infection.

Shigella is a low-infectious-dose pathogen that can cause disease in healthy individuals. *Shigella* spp infection causes a range of illnesses from mild watery diarrhea to severe dysentery (shigellosis). The disease dysentery phase correlates with comprehensive bacterial colonization of the colonic mucosa and colonic mucosal destruction caused by bacterial invasion (Schroeder and Hilbi, 2008). *Shigella* spp. are common etiological agents of diarrhea among travelers to less developed regions.

In Central America, South and South East Asia and sub-Saharan Africa, pandemic waves of Shiga dysentery (*S. dysenteriae* type 1) have emerged since the early 1960s. Shigellosis can

also be endemic with inadequate hygienic environments in many institutional locations (prisons, mental hospitals, nursing homes). When it comes to pandemic *S. dysenteriae* type 1 strains invade these susceptible groups, assault levels are high and dysentery is often the leading cause of death (Kotloff et al. 1999). *Shigella dysenteria* type 1 impacts all age groups, but in developing areas most frequently. Shigellosis epidemics in these countries have a significant impact on kids under 5 years of age and account for 61% of all shigellosis-related fatalities in this age group (Germani and Sansonetti 2003; Emch et al. 2008).

Shigella is classified into four species and at least 54 serotypes based on their biochemical and/or LPS O-antigen component structure on the outer membrane of the cell wall: *S.flexneri* (subgroup B with 17 serotypes and sub-serotypes), *S. dysenteriae* (subgroup A with 16 serotypes). Boydii (20 serotypes subgroup C) and *S. Sonnei* (1 serotypes subgroup D) (Simmons and Romanowska 1987; Talukder and Asmi 2012).

Shigella spp. have a very small dose of infection, leading to bacillary dysentery, a clinical pathology comprised of cramps, painful straining to pass stools (tenesmus), and frequent, small-volume bloody mucoid diarrhea. The disease follows 1-4 days of incubation with fever, malaise, anorexia, and sometimes myalgia. Stool assessment with the presence of a big number of leukocytes shows watery diarrhea. When diarrhea becomes bloody, it declares dysentery. Symptoms will subside in 2-5 days in healthy adults. Loss of water and electrolytes can lead to dehydration and even death in kids and the elderly.

1.1 Phages as an indicator:

Guelin (1948) was the first to acknowledge the capacity of bacteriophage as an indicator and there have been countless reports of the capacity of bacteriophage / coliphage as indicators of microbiological water quality (Hilton and Stotzky, 1973; Grabow et al., 1987; Kennedy et al., 1985; Borrego et al., 1987). They proposed that coliphages could be used in the therapy of drinking water and wastewater as indicators of water pollution and as feasible models for enteroviruses (Hilton and Stotzky, 1973; Kott et al., 1978; Scarpino, 1978).

In addition, Coliphages have been suggested to be better indicators of enteroviruses as they have been found to be removed at comparable rates with enteroviruses during treatment processes, to exhibit a seasonal variation similar to that of enteroviruses and certain coli phages are at least as resistant to environmental stresses and to chlorination as enteroviruses (Kott et al., 1974; Settler, 1984). Reports indicate that during the therapy process, coli phages and

enteroviruses are removed at comparable levels and that coliphage exhibits a seasonal variation similar to that of enteroviruses (Berg, 1974; Kott et al., 1974; Simokova and Cervenka, 1981).

Chapter 2: Materials and Methods

2.1 Place of study:

The research was conducted at the Department of Mathematics and Natural Sciences ' Biotechnology and Microbiology Laboratory, BRAC University, Dhaka, Bangladesh.

2.2 Standard laboratory practice:

All glassware such as test tube, conical flask, beakers were cleaned once with tap water and cleaned with distilled water for the second time. Culture media (both agar-based and broth-based), pipette tips, centrifuge tubes, empty test tubes, vials were autoclaved. Clean laboratory coat was worn while performing experiments and hand gloves were used and the tests were conducted inside a vertical laminar flow cabinet, which was initially cleaned with 70% ethanol to prevent contamination.

2.5 Preparation of culture media, reagents and solutions:

- **Preparation of LB:**

  According to the manufacturer's instruction, to produce 1000ml of LB, 20gms of powder needs to be added with 1000ml distilled water. Keeping this ratio constant, the required amount of powder is measured using an electronic balance machine before adding to a flask containing distilled water. Next, it needs to be stirred well and heated on a Bunsen burner until it is clear and bubbles are produced. Then the opening of the flask was covered with aluminum foil and autoclaved for about an hour at 121°C. After sterilization, it was stored at 4°C.

- **Preparation of LA:**

  For the preparation of 1000ml of LA media, 20gms of LB and 15gms of Nutrient agar were added with distilled water after measuring them in an electronic balance machine. Next, it was heated on a Bunsen burner and the opening was covered with foil. The media was autoclaved for an hour at 121°C.

  After sterilization, the media was poured onto sterilized Petri dishes on a vertical laminar flow cabinet and kept them to solidify. After solidification, the required amount of plates were used and rest were stored at 4°C.

- **Preparation 70% ethanol:**

  Deionized water was added to 737ml of 95% ethanol to make a final volume of 1000ml to prepare 70% ethanol.

- **Preparation of 4% soft Nutrient agar:**

  To perform double-layer agar assay, 0.4% soft ager was prepared separately. For the preparation of 100ml, 2.5 LB and o.4gm Nutrient agar were added in a flask containing 100ml of distilled water. Next, it was heated on a Bunsen burner and the opening was covered with foil. The media was autoclaved for an hour at 121°C. After sterilization, it was stored at 4°C.

2.3 Bacterial Culture:

The bacterial cultures used in this project were collected from the stock of biotechnology and microbiology laboratory. Bacterial samples were streaked on a fresh LA plate and incubated at 37°C for overnight. After checking the growth, plates were stored at 4°C for further use. Before each experiment, bacterial samples were freshly subcultured and 24 hours cultures were used. The viability and purity of the organisms were maintained by regular sub-culturing.

2.4 Bacteriophage isolations:

To isolate bacteriophage specific to *Shigella dysenteriae*, a total number of 20 samples were collected for the study of this experiment. The samples were collected from different areas of Dhaka city.

Sewage water contains microorganisms that are shed in the feces repeatedly. In particular, these microorganisms could develop antibiotic resistance and pose a significant threat to human health. The aim of this work is therefore to isolate bacteriophage which can infect isolated bacteria.

2.5.1 Collection of water samples:

Sewage water is collected from various points using caution not to come into direct contact with the liquids since they will most definitely contain human pathogens. The

areas which were covered inside Dhaka city are; Hatirjheel, Rampura, Police Plaza, Gulshan lake, Korail, Dhanmondi Lake, Bosila and Buriganga river.

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

After collection of the water samples, water needs to be transported into the laboratory within 3 hours. Next, the sample is first filtered using Watman filter paper and then syringe filtration (0.22 µm) was done.

2.5.3 Cocktail preparation (mixture of *Salmonella typhi* and *Shigella dysenteriae*)**:** Luria broth (LB) was prepared and different strains of the same bacterial species is added in a vial containing 3ml of LB. 1 or 2 freshly cultured colonies of the bacterial species is taken and inoculated in the broth and kept for 2.5 hours in the shaker incubator for lag phase.

2.5.4 Isolation of phage plaques: After 2.5 hours 1ml of the sewage water sample is added with 2ml of the cocktail mixture and again kept for 4 hours in the shaker incubation. In the meantime, individual organisms are inoculated in the broth and kept for 2 hours allowing them to grow. After 4 hours of shaker incubation, centrifugation is done for 5 minutes at 13000 rpm and filtered using syringe filtration technique.
Next, we diluted the filtered cocktail up to 10^{-10} .

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

2.5.6 Storage and resuscitation of phages: After 24-48h incubation, plaque is collected using sterile tips and stored by adding 200 ml of saline and 20µl of chloroform and stored at 4^{o}C.

Table 1: Sources of sample collection:

Serial numbers	Date of collection	Location
1*	24-4-19	Hatirjheel
2*	17-4-19	Gulshan
3	24-4-19	Hatirjheel
4	18-4-19	Korail
5	18-4-19	Korail
6*	17-4-19	Gulshan
7	24-4-19	Korail
8*	24-4-19	Gulshan
9	17-4-19	Gulshan
10	24-4-19	Gulshan
11*	24-4-19	Hatirjheel
12*	24-4-19	Gulshan
13	18-4-19	Korail
14	17-4-19	Gulshan
15	24-4-19	Korail
16*	18-4-19	Hatirjheel
17*	24-4-19	Hatirjheel
18	17-4-19	Gulshan
19	24-4-19	Hatirjheel
20*	24-4-19	Korail

2.5.7 Bacteriophage enrichment process: Freshly grown bacteria were inoculated into 10ml of LB and incubated for 2 hours in a shaker incubator for further enrichment of bacteriophages. Then 1ml of phage solution from the phage stock was introduced and kept for 4 hours in shaker incubator. After that, it centrifuged for 2 min at 10000 rpm and the collecting supernatant was filtered using a syringe filter. Finally, by spot testing, we ensured the existence of phages.

2.6 Phage Characterization:

2.6.1 Host Range:

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.

Each phage has its particular host range, the range of bacteria it can infect. While some phages can infect only one or few bacterial strains within a limited range of host, others can infect many species or even bacteria of distinct genera with a wider range of host.

This experiment will determine whether phage will be propagated by the putative plaque.

2.6.2 Procedure:

For phage detection, a wide variety of host strains were used. Most of these strains on the host detect phage groups. Therefore, it is important to use standardized host strains that de tect comparable phage groups.

To determine the host range, 14 bacterial strains were selected. These bacterial strains consisted of *Vibrio cholera* (OGAWA), *Pseudomonas aeruginosa*, ETEC, EAEC, Shiga-toxin producing *E.coli* (STEC), O157: H7, *Enterococcus faecalis, Entero pathogenic E.coli* (a typical), *Salmonella typhi, Shigella dysenteriae, Shigella flexneri, Klebsiella pneumonia, Salmonella paratyphi*, and *Bacillus cereus*. Bacteriophage lysis assay was done using the double layered-ager method.

The selected bacteria were individually inoculated in 3ml of LB and kept for 2 hours in shaker incubation. After 2 hours the young individual bacterial culture (0.5ml) was mixed

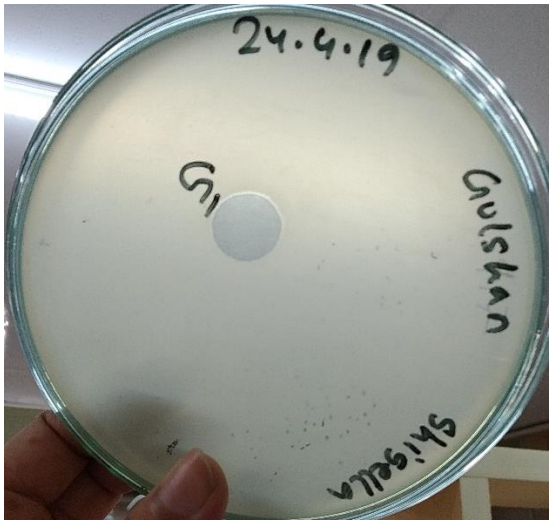
with 3ml of soft agar and it was vortexed and poured onto the bottom layer (LB agar). The top layer of the soft agar was allowed to solidify at room temperature. The plates were marked giving numbers of each 20 isolated bacteriophage suspensions and then the phage suspensions (10 µl) were pipetted on the top agar layer and kept them to dry. After drying the plates inverted and kept at 37° C overnight and the next day the presence of clear zones of lysis was seen.

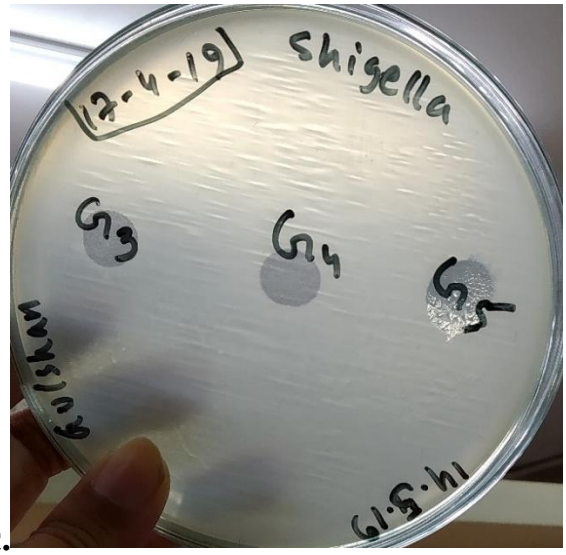
Chapter 3: Results

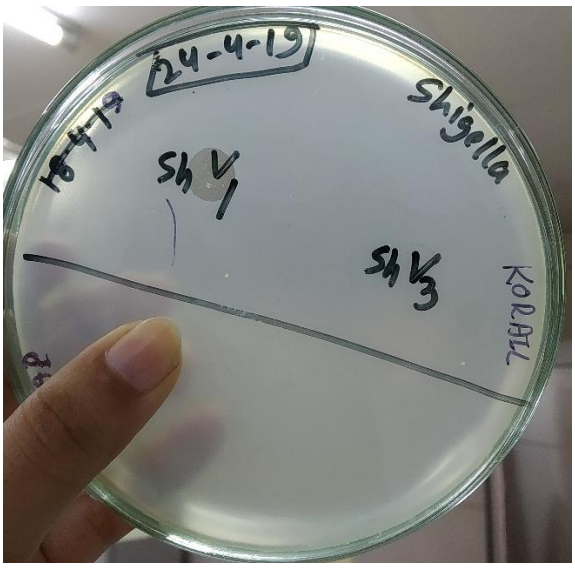
3.1 Isolation and purification of bacteriophages:

3.1.1 Spot tests: The twenty water samples were collected and enriched for *Shigella dysenteriae* bacteriophage, all of them showed clear zones. The assessment of this enriched sample water by a double-layer assay led to an individual plaque.

a. 

b. 

c. 

d. 

e. 

f. 

Figure 1: Clear zones or plaques shows the presence of bacteriophage *Shigella dysenteriae* from the enriched water samples. Individual plaque formations of *Shigella dysenteriae* on double-layer agar plates.

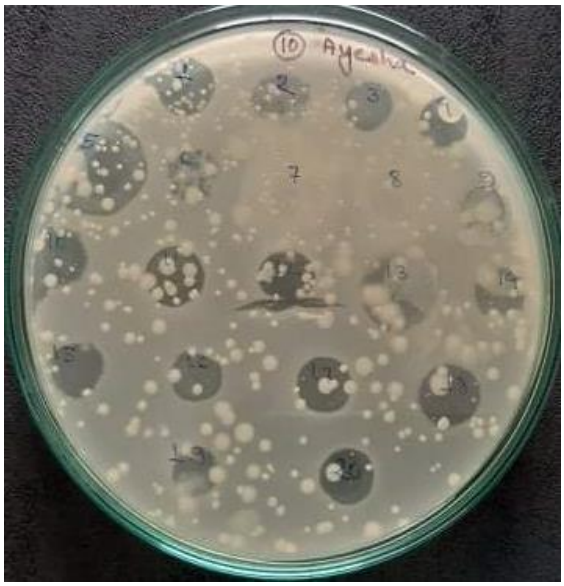
3.2.2 Host Range: The capacity of the *Shigella dysenteriae* bacteriophage to infect and produce a clear zone against various bacteria was assessed by spot testing and 5 of the bacterial strains were able to infect among 14 bacterial strains and the remaining were unable to create any definite area. 5 of the strains lysed and showed different results which made us assume that each phage shows different spectrum activity which is either broad or narrow. The bacterial strain ETEC lysed 12 phages, *Shigella dysenteriae* lysed 16 phages, Entero pathogenic *E.coli* (a typical) lysed 3 phages, Shiga-toxin producing *E.coli* (STEC) lysed 4 and EAEC lysed 4 phages. After observing the results, it can be said that the phage has both wide and narrow host specificity.

Table 2: Host range spectrum of *Shigella dysenteriae* against different bacterial cultures:

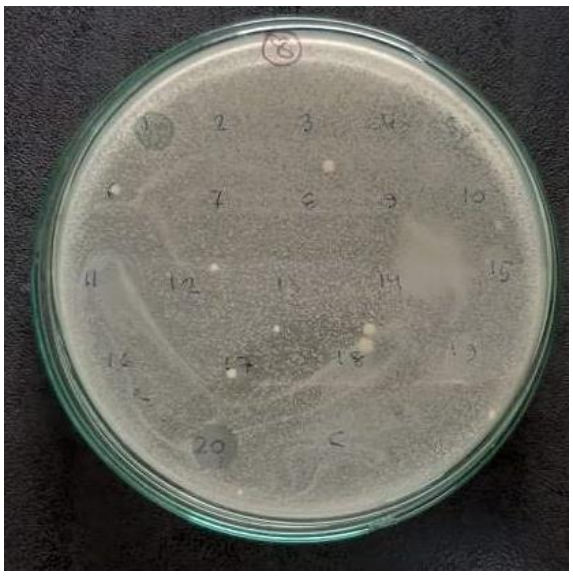
Name of pathogenic strains	**Specific phage for *Shigella***	**Host activity**	**Results from phage samples**	**Broad-spectrum activity**
1. *Vibrio cholerae* (OGAWA)	*Shigella dysenteriae*	-		
2. *Pseudomonus aeruginosa*		-		
3. ETEC		+	1,3-5, 7, 8,11,12,17-20	
4. EAEC		+	1, 8, 20,17	1, 8, 11, 12, 17, 18, 19, 20.
5. STEC		+	8,11,12,16	
6. 0157: H7		-		
7. *Enterococcus faecalis*		–		
8. Entero pathogenic (atypical)		+	1, 17, 20	
9. *Salmonella typhi*		–		
10. *Shigella dysenteriae*		+	1-6,10-20	
11. *Shigella flexneri*		–		
12. *Klebsiella pneumonia*		–		
13. *Salmonella paratyphi*		–		
14. *Bacillus cerus*		-		
15. *Staphylococcus aureus*		–		

(+) = presence of plaque, (-) = absence of plaque

a. Shiga-toxin producing E.coli (STEC)



b. *Shigella dysenteriae*



c. Entero pathogenic E.coli (a typical)

d. ETEC

Figure 2: Host range specificity of Shigella dysenteriae phage against **a.** STEC, **b.** Shigella dysenteriae, **c.** Entero pathogenic E.coli (a typical) and **d.** ETEC.

Chapter 4: Discussion

1.1 Isolation of bacteriophages:

Phage was isolated from sewage water and was plated with bacteria (*Shigella dysenteriae*) that served as a phage host. The appearance of a transparent area or plaques in the plates indicates the existence of bacteriophages. Phage-induced plaques vary in size and are distinguished by the circular cone. Plaque formation was seen in agar plates after 24hrs of incubation. These plaque formations indicate bacterial lyses or the existence of bacteriophage in the Luria-Bertani Agar plate. The outcome indicates the existence of phage from various sources, which may be affected by the season and climate. During water movement, this phage may also spread and destroy fecal coliform.

Samples were collected from different areas of the Dhaka city which includes, Hatirjheel, Rampura, Police Plaza, Gulshan Lake, Korail, Dhanmondi Lake, Bosila and Buriganga River. Among them, Hatirjheel, Gulshan Lake and Korail.gave *Shigella dysenteriae* infective bacteriophages in spot test and rest failed to show clear zones. The difference in the abundance of host bacteria in sample collection sites might be a reason not finding 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. The sampling site was open water body and here UV light may have played a significant part as the water samples were collected from surface water during the summer season when the intensity of sunlight is maximum. The abundance of phages was zero during the winter, from which it is evident that the phages have a seasonal influence.

4.2 Host determination:

Shigella dysenteriae was examined against multiple bacterial strains to identify its host range specificity. Plaques were observed against five of the bacterial strains which include ETEC, EAEC, Shiga-toxin producing *E.coli* (STEC), *Entero pathogenic E.coli* (a typical) and *Shigella dysenteriae.* After observing the results, it can be said that the phage has both wide and narrow host specificity. However, the outcome appears to show a wide variety of host species and this correlates with the concept that some phages have strain specificity while others have a wider

variety of host species infecting only various strains of one species with several closely associated species.

4.3 Limitations:

High bacterial contamination occurred in the double-layer assay plates was the most prevalent issue experienced in this research. Although the aseptic condition was maintained as long as possible during various experiments, different bacterial colonies would sometimes appear on the assay plate surface, often covering a potential plaque resulting in a false plaque count that could affect the reliability and accuracy of the result. The source of these contaminations was traced back to the micropipettes used in the experiment that was not externally cleaned using ethanol before the experiment was started and frequently contaminated in the phage stock solution.

The risk of bacterial contamination was solved by cleaning the micropipette via ethanol and removing bacterial cells from the phage stock by centrifugation, followed by filtration via a 0.22 μm syringe filter. Besides, to prevent bacterial contamination, chloroform can be added to the phage stock solution (Cotton and Lockingen, 1963).

Multiple plaques would sometimes overlap each other during the double layer assay experiment and form a cluster that would make it very difficult to identify individual plaques. Since the average plaque size was relatively large, greater dilution would reduce the total number of plaques that would decrease the possibility of plaque overlap.

A total of fifteen bacterial strains were used to define the *Shigella dysenteriae* phage host range, but only five of them showed host specificity. It requires a wide variety of hosts to use phage as an antibacterial agent. Despite the *Shigella dysenteriae* indicating a wide variety of host, a bigger pool of strain-specific *Shigella dysenteriae* and other *Shigella* species together with certain antibiotic-resistant strain bacteria need to be more accurately determined so that *Shigella dysenteriae*'s therapeutic potential can be accurately assessed.

Supposition:

This research was directed at isolating and characterizing particular bacteriophage of *Shigella dysenteriae* from various environmental water samples in an attempt to identify a future therapeutic phage. This research resulted in the isolation of *Shigella dysenteriae* bacteriophage from a water sample further characterized to identify its spectrum of the host. The phage shows a wide variety of host. Whether *Shigella dysenteriae* can be used to treat *Shigella dysenteriae* associated disease is a worthy candidate for further research.

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