ISOLATION OF BACTERIOPHAGES FROM SURFACE WATER FOR SOME DISTINCT BACTERIAS AND THEIR CHARACTERIZATION AS POTENTIAL BIOCONTROL AGENTS.

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

Department of Mathematics and Natural Sciences

Brac University
December, 2020

Declaration

It is hereby declared that

1. The thesis submitted is our original work while completing Microbiology and Biotechnology degree at BRAC University.

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

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

4. We have acknowledged all sources of help.

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Approval

The thesis/project titled "**ISOLATION OF BACTERIOPHAGES FROM SURFACE WATERS FOR SOME DISTINCT BACTERIAS AND THEIR CHARACTERIZATION AS POTENTIAL BIOCONTROL AGENTS**." has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Microbiology and Biotechnology on November, 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:

E. coli of many different kinds, most of which are part of the intestine's normal flora and are thought to be comparatively harmless. However, some strains have developed pathogenicity systems, which means they can cause disease in humans and livestock. Like this harmful strain, there are some other harmful pathogenic Bacteria are found in sewage water or wastewater. Through this sewage water, this microbes can contaminate our drinking water. Which might become the cause of different intestinal disease. These can be intestinal (diarrhoea) or extraintestinal (UTI), septicaemia and pneumonia. 75% to 95% Urinary tract infection is caused by a certain type of *E. coli.* Some types of *E. coli* can produce Shiga toxin which damages the line of the intestine. This type of *E. coli* strain is called STEC which is short for "Shiga Toxin producing *E. coli"*. O157: H7 is another effective strain of *E. coli* which typically causes abdominal cramps, vomiting and bloody diarrhoea when it is consumed through contaminated food or water. *Shigella dysenteriae* spread by contaminated water causes the most severe dysentery because of its potent and deadly Shiga toxin. Typhoid fever is caused by *Salmonella typhi* bacteria, it infects the intestinal tract and blood. *Vibrio* causes vibriosis or cholera. The primary objective of this project was to isolate bacteriophage against *E. coli* and to recognize its different characteristics as an antimicrobial agent to determine the therapeutic potential. In this study, specific bacteriophage for all these six bacteria was isolated and characterized to develop a therapeutic agent. A total number of 60 samples were collected for the study of this experiment. The samples were collected from the wastewater of different cities of Dhaka city. The samples were initially filtered and the double layer- agar method was used for the isolation of bacteriophages. For the characterization of bacteriophages, host range specificity test, Heat stability and pH stability were checked. After examination of the host range, the most prominent bacterial hosts were ETEC, EAEC, Shiga-toxin producing *E. coli* (STEC), O157:H7, *Klebsiella pneumonia* and *Shigella dysenteriae* were lysed by *E. coli* 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 *E. coli*. Studies on its biological features may provide helpful data and understanding to identify prospective therapeutic agents against infection with *E. coli.*

Dedication:

We would like to dedicate our Father and our Mother to their love, prayers, supports, and sacrifices for educating us and preparing us for our future.

Acknowledgement

First of all, we would like to express our deepest gratitude to Almighty Allah for everything he has blessed us with in our whole life and for giving us patience and strength to make this project a success.

We would like to thank Professor **A F M Yusuf Haider, Ph.D**., Professor and Chairperson of Department of Mathematics and Natural Sciences, BRAC University for allowing me to continue my work at the BRAC University microbiology laboratory. We express our heartiest gratitude to **Late Professor A. A. Ziauddin Ahmad,** former Chairperson of Department of Mathematics and Natural Sciences, BRAC University for his valuable advice, enthusiastic encouragement and the support he gave me throughout my undergraduate study.

We are obliged to our senior lecturer **Mr. Mahbubul H. Siddique** Department of Mathematics and Natural Sciences, BRAC University for believing in us and giving us support whenever I needed. We would like to again thank him for his constant encouragement and guidance throughout this project.

We would like to especially thank Professor **Dr. Iftekhar Bin Naser**, Department of Mathematics and Natural Sciences, BRAC University for the inspiration and guidance he provided us as a student. We would also like to show thank to Professor **Dr. Mahboob Hossain**, professor of the Biotechnology and microbiology program, Department of Mathematics and Natural Sciences, BRAC University for his exemplary guidance and encouragement during his short stay with us.

We also thank all the other Teaching Assistants the Lab Officers and the Lab Assistants who helped us to go through the complexities of laboratory work.

We especially thank our friends and classmates **Tushar Ahmed Shishir, Ayesha Mubarak, Naima Fatema, Progga Rosette Gomes** and all other classmates for giving me company and helping me throughout my undergraduate study.

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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 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 depends enti rely 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.

Pathogenic *E. coli* strains associated with intestinal diseases and it has been classified into six different main groups in the basis of epidemiological evidence, phenotypic traits, clinical feature of the disease and specific virulence factors: enteropathogenic: EPEC, enterotoxigenic: ETEC, enteroaggregative: EAggEC, enteroinvasive: EIEC, enterohaemorrhagic: EHEC and diffusely adherent:DAEC.Various strains of *E.coli* are responsible for various intestinal and extratestinal disease by virulence, which affect a wide variety of cell processes.

The primary objective of this project was to isolate bacteriophage against the type strains of *E. coli* ,STEC and O157:H7 and to recognize its different characteristics as an antimicrobial agent to determine the therapeutic potential.

1.1 Bacteriophages:

Bacteriophages, which are also known as 'phages' for are basically happening bacterial infections which attack bacterial cells (Abedon, 2012). They are very distinctive and have the evolved to multiply inside bacterial cell (Clark and Walk, 2006; Hagens and Loessner, 2007; Hanlon, 2007; Nishikawa et al., 2008; Viazis et al., 2011). Phages are considered as characteristic executioner of microscopic organisms that can taint and lyse the have living being (Abuladze et al., 2008; Nishikawa et al., 2008). In later times, many of its other attributes are discovered and it is then widely recognized that bacteriophages are plenteous within the environment and they have a huge impact in the biosphere. The various other discoveries of Bacteriophages include it being assessed to slaughter between 20-40 % of maritime bacteria every day, playing a key part in supplement and vitality cycle of an environment and shaping the pool of most genetically assorted

'life form' on soil (Suttle, 2005).

1.2 Early history of bacteriophages:

Ernest Hankin, a British bacteriologist, first discovered bacteriophages in 1896 and started working on it, (Ackermann, 2012). Within the water of Ganga and Jumna Waterway in India, the nearness of an antibacterial activity against microscopic organisms *Vibrio Cholerae* was discovered by him. Thus, he proposed that this obscure operator, that is heat sensitive and seem to pass through porcelain channel, causing the prevention of the spread of cholera infection by bactericidal action. For a long time afterward, whereas working with *Bacillus subtilis*, a Russian bacteriologist named Gamalaya seen comparative wonder (Sulakvelidze et al., 2001). In 1901 Emmerich and Low detailed that test from a culture which demonstrated autolysis was able to lyse diverse culture, was able of curing tentatively initiated infection (Summers,2004). Nearly two decades after Hankin'sperception, Frederick William Twort, a British pathologist watched a "glassy transformation" of Micrococcus colonies developed on solid agar media. He hypothesized that the obscure substance causing the watery change of the bacterial colonies may be a infection.

For a long time afterward of Twort's documentation, Felix d'Herelle a French Canadian microbiologist observed the comparative kind of rate and he proposed that it was "ultravirus" that

was causing lysis of bacterial cell in fluid media and made clear patches on the bacterial grass which he fundamentally called it taches, at that point achesvierges, and afterward plaques. As the infection capable for these wonders, Felix d'Herelletoo named it as "bacteriophage" which is derived from the Greek words "bacterium" and "phagein" which suggests to "eat" or "devour". Whereas Twort, for numerous reasons was facing money related challenges, thus could not seek after his discoveries, d'Herelle continued and given his investigate career to bacteriophage consider (Calendar, 2005; Sulakvelidze et al., 2001; Summers, 2004) and so, he concluded bacteriophage as "exogenous specialists of immunity" after the perception of an increase in phage titer within the stool test of recouping patients suffering from loose bowels and typhoid (Deresinski, 2009).

In 1921, The primary archived helpful utilize of bacteriophage was done by Bruynoghe and Maisin in 1921 from Louvain where they famous lessening in torment, swelling, and fever upon injection of staphylococcal phage planning within the local locale of cutaneous bubbles (Summers, 2004). However, the primary helpful utilize of phage conducted by Felix d'Herelle at the Clinic Des Enfants-Malades in Paris in 1919 beneath the supervision of Teacher Victor-Henri Hutinel, the hospital's Chief of Pediatrics. They administrated anti-dysentery phage arrangement to a 12-year boy who was suffering from serious diarrhea and watched successive desist of indications and full recuperation within a few days. He then ran a few other trails after that where patients recouped inside 24 hours using only a single dosage of phage planning. As the result that comes about from these trials were not published immediately, subsequently, Bruynoghe and Maisin were then credited for the primary expressed application of phage in treating irresistible malady (Sulakvelidze et al., 2001; Summers, 1999). Whereas a few early phage helpful trials were considered as fruitful and numerous prominent pharmaceutical companies and investigate research facilities such as D'Herelle's commercial research facility in Paris and Eli Lilly Company within the Joined together States sold phage arrangement to treat different infectious disease, they eventually turned out to be a disappointment (García et al., 2008; Sulakvelidze et al., 2001; Thiel, 2004). The disclosure of wide extendantimicrobials played the most important part in declining the intrigued of producing phage for commercial purposes. The need of understanding of phage science and insufficiencies in the demonstrative bacteriology strategies accessible at the time helped the move in intrigued from phage in the western world. In any case, phage treatment was kept on be advertised within the Eliava Institute and afterward by others moreover such as the

Hirszfeld Founded of Immunology and Test Treatment in Wroclaw, Poland (Deresinski, 2009). One the other hand phage inquire about proceeded at a fundamental level within the west where ponder of phage played a major part in a few momentous discoveries in organic science. It driven to the recognizable proof of DNA as hereditary fabric (Van Valen et al., 2012), understanding of hereditary code and wonder of restriction-modification and to the development of atomic recombinant innovation. Phage determined proteins are presently being utilized as diagnostics operators (Smith et al., 2001), helpful instruments (Loeffler et al., 2001; Schuch et al., 2002) and for finding unused medicate (Liu et al., 2004).

1.3 Bacteriophages classifications:

Phages are hugely ordered and shifted from one another based on basic, physicochemical, and natural properties. Back in 1917, D'Herelle observed bacteriophage, he thought that there were as it were one species of phage containing numerous races. In any case, in 1933, Burnet showed hetero genicity among entero bacterial phages and in 1943, Ruska discovered three morphological types of bacteriophage which evoked the need of appropriate classification of phages. Holmes proposed a classification framework of phages based on plaque and molecule measure, haveextend, and resistance to urea and warm which was not acknowledged by logical community. Lwoff, Horne, and Tournier diversified a classification conspire in 1962 based on nucleic corrosive and morphology. Lastly, the Universal Committee on Scientific classification of Infections (ICTV) classified phages based on nucleic corrosive and net morphology and divided them into six genera (Ackermann, 2004).

1.4 Bacteriophage abundance in the environment:

Bacteriophages are considered to be the foremost distinctive organic elements on the planet with an estimated populace estimate of 1030 or more (Chibani-Chennoufi et al., 2004). Phages have been collected from distinctive natural setting such as acidic hot springs (higher than 80°C with pH=3.0), sun oriented salterns (10 times saltier than the ocean), alkaline lakes (pH=10), within the terrestrial subsurface (more noteworthy than 2000 m profound), underneath 30 m of ice in polar lakes (Breitbart and Rohwer, 2005), from soil (Ashelford et al., 2003), sewage slime

(Carey-Smith et al., 2006) and mammalian faeces (O'Flynn et al., 2004) of dense phage population (about $9\times10_8$ virion ml_{-1}) and around 70 % of aquatic bacteria are infected by those phages (Ackermann et al., 2012). Phage abundance across aquatic system varies from less than 10_4 m1_{-1} to more than 10_8 ml_{-1} (Wommack and Colwell, 2000). This variation in number is usually correlated with the variation in associated host organism which ultimately depends on the productivity of the system. For example, in aquatic system phage abundance is highest in the coastal environments (10_6–10_7 ml-1), intermediate in offshore surface water (10_5–10_6 ml-1) and lowest in the deep sea (104–105 ml-1). It has also been demonstrated that the phage abundance decreases along with transect from coastal to offshore (Weinbauer, 2004).

Bacteriophage has abundancebin freshwater than in marine water. In ocean ice, phage abundance was detailed 10 to 100 times higher than in encompassing water (Maranger et al., 1994). The phage abundance variety too exists within then water framework. A ponder done by

Tapper and Hicks (1998) reported that phage predominance in to begin with 20 μm or the surface micro layer of Lake Superior was 2 to 15 times higher than in 20 m profundity. In spite of the fact that benthic infections were found to be 10 to 1000 times higher than within the overlaying water column, the phage wealth diminished with sediment depth (Weinbauer, 2004).

The abundance and distribution of phages is hugely dependent on that of their host organisms, as phages need a host to survive. Therefore to find the path of this viral abundance, one need to understand where the majority of their hosts exist. Although we are mostly concerned about bacterial pathogens, most of the Earth's Bacteria and Archaea can actually be seen in the open ocean, the soil and in ocean sediments, and terrestrial sub-surfaces where there are nearly 1.2×10^{29}, 2.6×10^{29}, 3.5×10^{30} and $0.25–2.5 \times 10^{30}$ cells respectively, Bacteria and Archaea have significance on humans and animals which can assure many niche environments within them, often in many cases these micro-organisms have eventually turned into an essential symbiont. Bacteria holds much more importance if associated with humans, particularly either in a disease, or a food producing context when bacteria are the reasons of causing disease, or where we ought to depend on bacteria. Therefore, while discussing human impact, bacteriophages have paramount importance which can infect these bacterias.

1.5 Phages as 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).

Furthermore, 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 coliphages are at least as resistant to environmental stresses and to chlorination as enteroviruses (Kott et al., 1974; Settler, 1984).

E.coli 0157:H7 - The gram-negative bacteria Escherichia coli O157:H7 and other enterohemorrhagic E. coli (EHEC) usually cause severe bloody diarrhea, which mostly lead to hemolytic-uremic syndrome. Symptoms are abdominal cramps and diarrhea which can be grossly bloody. Fever is not prominent. Diagnosis is done by stool culture and through toxin assay. Treatment is usually suggested and antibiotic use is not recommended.

STEC- There are kinds of E coli that causes disease by making a poison called shiga toxin. The bacteria which make these toxins are called shiga toxin producing E coli or STEC in short. People of any age can be infected. But young children and the elderly are more likely to develop more severe illness and hemolytic uremic syndrome (HUS) than others, but even healthy older children and young adults can become seriously ill. The symptoms of STEC infections vary from person to person but often include severe stomach cramps, diarrhea (often bloody), and vomiting. If there is fever, it usually is not very high (less than 101˚F/less than 38.5˚C). Usually, most people get better within 5–7 days. Some infections are very mild, but there are others that are severe or even life-threatening.

Vibrio- Enteric illness appears suddenly after a period of 15 to 24 hours of incubation and the manifestations include cramping abdominal pain large amounts of watery diarrhea (stools may be

bloody and contain polymorphonuclear leukocytes) tenesmus,weakness and sometimes nausea,vomiting and low grade fever.symptoms subside spontaneously in 24-48 hours.

Salmonella species are Gram-negative, flagellated facultatively anaerobic bacilli containing more than 2600 different serovars that can be divided into typhoidal and non-typhoidal Salmonella serovars. Even those these two groups are genetically similar, they elicit very different diseases and distinct immune responses in humans. Salmonella ingested via food passage through the gastric acid barrier and invade the mucosa of the small and large intestine and produce toxins. As a result, inflammatory cytokines are releases which induce an inflammatory reaction. This inflammatory reaction causes diarrhea and may lead to ulceration and destruction of the mucosa. The disease caused by Salmonella is called salmonellosis which includes several syndromes that include gastroenteritis, enteric fevers, septicemia, focal infections and an asymptomatic carrier state. Particular serovars can produce particular syndromes, for example, Salmonella typhi, and Salmonella paratyphi-A produces enteric fever, where Salmonella choleraesuis produces septicemia or focal infections. However, any serotype can produce any of the syndromes occasionally.

Shigella are Gram-negative, nonmotile, facultative anaerobic, non-spore forming rods that can be differentiated from the closely related Escherichia coli on the basis of pathogenicity, physiology (ability to ferment lactose or decarboxylate lysine) and serology. The genus is divided into four serogroups with multiple serotypes. They are,

1. Shigella dysenteriae, 12 serotypes
2. Shigella flexneri, 6 serotypes
3. Shigella boydii, 18 serotypes
4. Shigella sonnei, 1 serotype

Infection of Shigella is initiated by the ingestion of it, usually through fecal-oral contamination. As an early symptom, diarrhea may occur (possibly due to enterotoxin and/or cytotoxin) as the organisms pass through small intestine. The disease caused by Shigella called “shigellosis”, is a bacterial invasion of the colonic epithelium and inflammatory colitis. It is an interdependent process amplified by local release of cytokines and by the infiltration of inflammatory elements. Shigella dysenteriae serotype 1 expresses Shiga toxin, which is an extremely potent cytotoxin that inhibits protein synthesis in susceptible mammalian cells. This toxin also has an enterotoxic activity, but its role in human diarrhea is unclear, because Shigella apparently express a number

of enterotoxins. This cytotoxin causes capillary destruction and focal hemorrhage that exacerbates dysentery when experimentally infected rhesus monkeys with Shigella dysenteriae 1 and a Shiga toxin-negative mutant. Moreover, Shiga toxin is associated with the hemolytic-uremic syndrome, a complication of infections with Shigella dysenteriae 1.

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 brothbased), 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.

2.3 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, 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 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 in order to prepare 70%ethanol.

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

  In order 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 was 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.

2.4 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 subculture and 24 hours cultures were used. Viability and purity of the organisms were maintained by regular sub-culturing.

2.5 Bacteriophage isolations:

To isolate bacteriophage specific to *E. coli* a total number of 19 samples were collected for the study of this experiment. The samples were collected from various corners of the 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, Banani lake, Gulshan lake, Korail, Dhanmondi Lake, Wari 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 STEC and O157:H7)**:** 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 drop wise 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 no: 1 Sources of sample collection:

	Serial numbers	Date of collection	Location	
	1	24.04.2019	Korail	
	2	4.03.2019	Buriganga	
	3	17.04.2019	Gulshan	
	4	24.04.2019	Korail	
	5*	17.04.2019	Gulshan	
	6	04.03.2019	Buriganga	
	7	31.01.2019	Police Plaza	
	8	24.04.2019	Gulshan	
	9*	18.04.2019	Korail	
	10*	24.04.2019	Korail	
	11	28.11.2018	Buriganga	
	12	05.03.2019	Hatirjheel	
	13	18.04.2019	Korail	
	14*	18.04.2019	Korail	
	15*	18.04.2019	Hatirjheel	
	16*	17.04.2019	Gulshan	
	17*	24.04.2019	Hatirjheel	
	18	24.04.2019	Buriganga	
	19	30.04.2019	Buriganga	

2.5.7 Bacteriophage enrichment process:

Freshly grown bacteria were inoculated into 10ml of LB and incubated for 2 hours in 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 syringe filter. Finally, by spot testing, we ensured the existence of phages.

2.6 Phage Characterization:

2.6.1 Bacteriophage host range determination:

2 strains of *E. coli* were used in this study, where one is Shiga toxin Producing Escherichia coli (STEC) and another is O157:H7. Host range of fifteen bacteriophages was determined by spotting 10µL of bacteriophage preparation on lawn culture of fifteen pathogenic bacterial strains. After incubation at 37 ° C for 16 to 24 hours, plates were observed for the appearance of clear zone.

2.6.2 Heat stability of bacteriophages:

For the heat treatment, eppendroff tubes with a volume of 1.5ml were used.

First, in this study the titre of the initial bacteriophage was determined. In order to ensure heat resistance, tubes full of bacteriophages were placed in water bath for a period of 30 minutes at each (30 ° C, 40 ° C, 50 ° C, 60 ° C, 70 ° C, 80 ° C, 90 ° C) temperature to maintain the stability.

2.6.3 pH stability of bacteriophages:

Bacteriophage samples were mixed into a number of tubes for pH stability tests that contain different LB broth and pH adjusted by NaOH or HCl, which were incubated at 37° C for 2 hours. All titers of bacteriophages were measured by the double-layer agar plate method.

Chapter 3: Results

3.1 Revive Table:

Bacteriophages that are stocked by the previous students, had been gone through the process of revival and we could successfully revive many of them. Here's the list of organisms that we could revive:

Table 2: revive

Serial number (v1774)	Place	Date of collection
1	Gulshan	17.04.19
2	Hatirjhil 2	
3	Korail 3	
4	Hatirjhil	14.04.19
5	Gulshan	17.04.19
6	K (3)	
7	Gulshan	17.04.19
8	Jahajbari	12.03.19
9	Gulshan	24.04.19
10	Badda	05.03.19
11	Badda	06.03.19
12	Korail	
13	Hatirjhil	18.04.19
14	Hatirjhil (2)	
15	Korail	18.04.19
16	K (3)	
17	Hatirjhil	24.04.19
18	H (2)	
19	Gulshan lake (3)	24.04.19
20	Hatirjhil	24.04.19
21	Hatirjhil	24.04.19

Serial number (0157:H7)	Place	Date of collection
1	Mohakhali	24.07.19

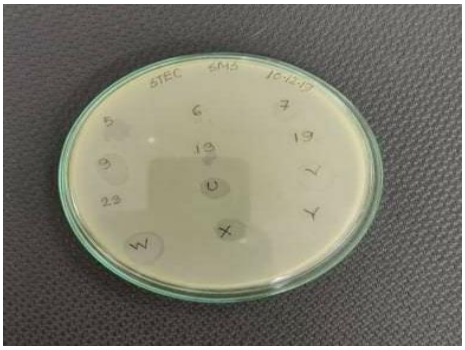
2	Mohakhali	30.07.19
3	Mohakhali	24.07.19
4	Niketon	17.06.19
5	Korail	24.04.19
6	Korail	18.04.19
7	Burigonga	04.03.19
8	Burigonga	28.11.19

Serial number (STEC)	Place	Date of collection
1	Mohakhali	19.06.19
2	Mohakhali	19.06.19
3	Mohakhali	19.0619
4	Mohakhali	24.07.19
5	Gulshan	19.05.19
6	Burigonga	24.03.19
7	Burigonga	30.04.19
8	Gulshan	17.04.19
9	Gulshan	24.04.19

Serial number (shigella)	Place	Date of collection
1	Mohakhali	24.07.19
2	Hatirjhil	24.07.19
3	Korail (new)	24.07.19
4	Hatirjhil	25.05.19
5	Korail	20.05.19
6	Hatirjhil (new)	24.04.19
7	Hatirjhil	24.04.19
8	Korail (new)	24.04.19
9	Gulshan	24.04.19
10	Gulshan(2)	24.04.19
11	Hatirjhil (new)	24.04.19
12	Korail	24.04.19
13	Gulshan(2)	24.04.19
14	Gulshan	24.04.19
15	Hatirjhil (new)	24.04.19
16	Mohakhali	30.06.19
17	Niketon	17.06.19
18	Hatirjhil	18.04.19
19	Gulshan	17.04.19
20	Korail	04.04.19
21	Korail	18.04.19

Serial number (salmonella)	Place	Date of collection
1	Mohakhali	24.07.19

2	Gulshan	24.04.19
3	Gulshan	24.04.19
4	Gulshan	04.03.19
5	Mohakhali	17.06.19
6	Dhanmondi	17.06.19
7	Gulshan	17.04.19
8	Postokhola	25.03.19
9	Sadar	



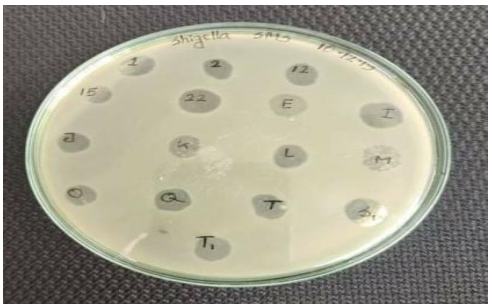
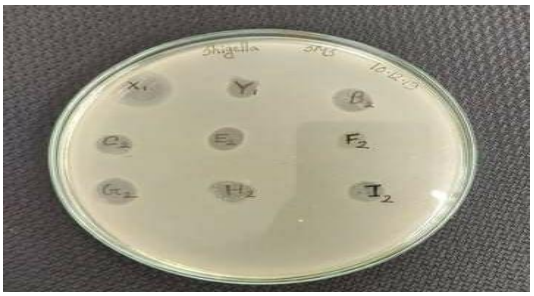
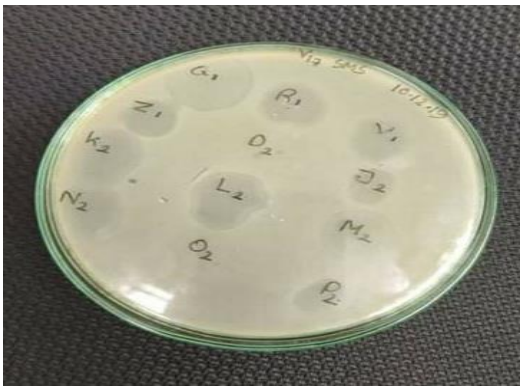





Figure: Revival of bacteriophages

3.2 Host Range:

The host ranges of seventeen bacteriophages were determined by using fifteen pathogenic strains of bacteria. Among all the bacteriophages few of them possessed wide host range compared to the others.

Table 3: Host range spectrum of *Shigella dysenteriae* against different bacterial cultures:

Sample	Vibrio cholerae (OGAWA)	Pseudomonas aeroginosa	ETEC	EAEC	STEC	0157:H7	Enterococcus faecalis	Enteropathogenic (atypical)	Salmonella typhi	Sigella dysentariae	Shigella flexneri	Klebsiella pneumoniae	Salmonella paratyphi	Bacillus cereus	Staphylococcus aureus
1	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-

10	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
12	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-
13	-	-	-	-		-	-	-	-	-	-	-	-	-	-
14	-	-	-	-		-	-	-	-	-	-	-	-	-	-
15	-	-	-	-		-	-	-	-	-	-	-	-	-	-
16	-	-	-	-		-	-	-	-	-	-	-	-	-	-
17	-	-	-	-		-	-	-	-	-	-	-	-	-	-
18	-	-	-	-		+	-	-	-	-	-	-	-	-	-
19	-	-	-	-		+	-	-	-	-	-	-	-	-	-
20	-	-	-	-		-	-	-	-	-	-	-	-	-	-
21	-	-	-	-		-	-	-	-	-	-	-	-	-	-

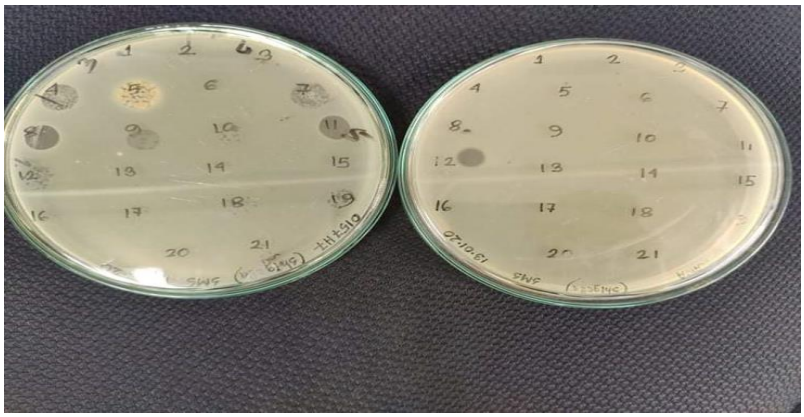


Figure: 0157:H7 colonies in the plate of *Shigella dysenteriae*

Table 4: Host range spectrum of *Salmonella typhi* against different bacterial cultures:

Serial number	OGAWA	Pseudomonas Aeroginosa	ETEC	EAEC	STEC	0157:H7	Enteococcus faecalis	Enteropathogenic (atypical)	Samonella typhi	Shigella dysentariae	Shigella flexnari	Klebsiella pneumoniae	Salmonella paratyphi	Baillus cereus	Staphyloccus aureus
1	-	-	-	-	-	+	-	-	+	+	+	-	-	-	+
2	-	-	-	-	-	-	-	-	-	+		-	-	-	-
3	-	-	-	-	-	-	-	-	-	-		-	-	-	-
4	-	-	-	-	-	-	-	-	-	+		-	-	-	-
5	-	-	-	-	-	-	-	-	-	+		-	-	-	-
6	-	-	-	-	-	-	-	-	-	+		-	-	-	-
7	-	-	-	-	-	-	-	-	-	+		-	-	-	-
8	-	-	-	-	-	-	-	-	-	-		-	-	-	-
9	-	-	-	-	-	-	-	-	-	-		-	-	-	-

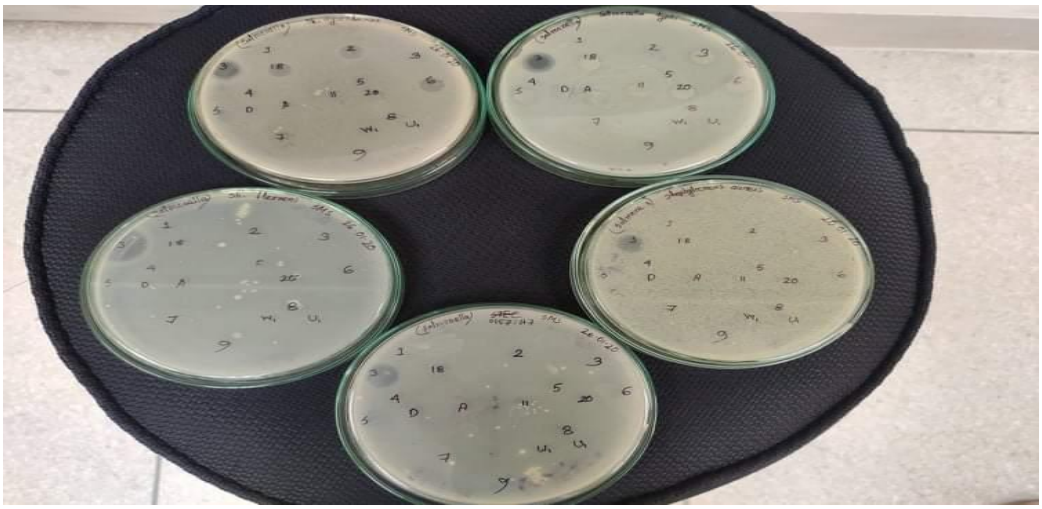


Figure: Bacteriophagee colonies in the plate of *Salmonella typhi*

Table 5: Host range spectrum of *0157:H7*against different bacterial cultures:

Serial no.	OGAWA	Pseudomonas aeroginosa	ETEC	EAEC	STEC	0157:H7	Enterococcus faecalis	Enteropathogenic (atypical)	Salmonella typhi	Shigella flexnari	Klebsiella pneumoniae	Salmonella paratyphi	Bacillus cereus	Shigella dysentariae	Staphylococcus aureus
1	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-
7	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-

8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

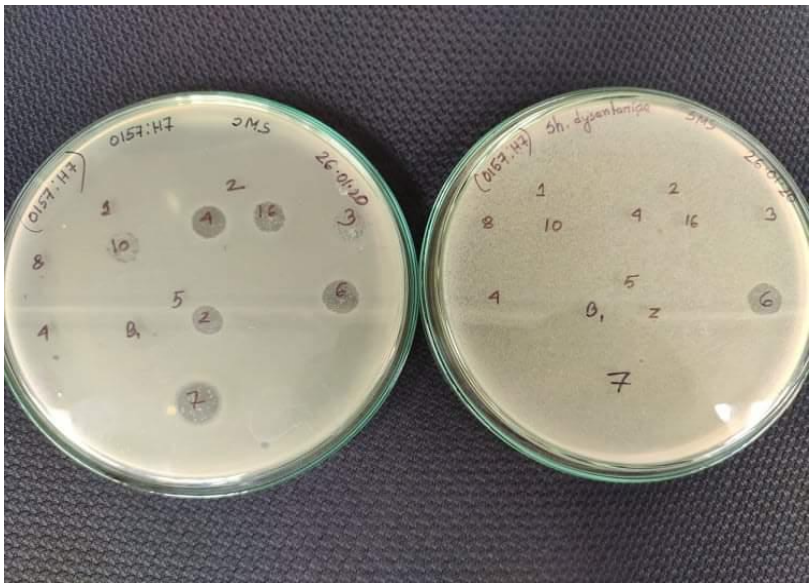


Figure: Host range plate of 0157:H7

Table 6: Host range spectrum of *STEC* against different bacterial cultures:

Serial no	OGAWA	Pseudomonas Aeroginosa	ETEC	EAEC	STEC	0157:H7	Enterococcus Faecalis	Enteropathogenic (atypical)	Salmonella typhi	Shigella dysentariae	Shigellaflexnari	Klebsiella pneumoniae	Salmonella paratyphi	Bacillus cereus	Staphylococcus aureus
1	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

4	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Figure: Host range plate of STEC

Table 7: Host range spectrum of V1774 against different bacterial cultures:

Serial no	OGAWA	Pseudomonas aeroginosa	ETEC	EAEC	STEC	0157:H7	Enterococcus faecalis	Enteropathogenic (atypical)		Salmonella typhi	Shigella dysentariae	Klebsiella pneumoniae	Salmonella paratyphi	Shigella flexnari	Bacillus cereus	Staphylococcus aureus
1	+	-	-	-	+	-	-	-		-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-
5	+	-	-	-	-	-	-	-		-	+	-	-	-	-	-
6	-	-	-	+	+	-	-	-		-	+			+		
7	-	-	-			-	-	-		-	-	-	-	-	-	-
8	+	-	-	+		-	-	-		-	-	-	-	-	-	-
9	-	-	-	+		-	-	-		-	+	-	-	-	-	-
10	-	-	-			-	-	-		-	-	-	-	+	-	-
11	-	-	-	+		-	-	-		-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-
14	+	-	-	-	-	-	-	-		-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-
16	+	-	-	-	+	+	-	-		-	+	-	-	-	-	-
17	+	-	-		+	+	-	-		-	+	-	-	-	-	-

18	+	-	-	+	+	+	-	-		-	+	-	-	+	-	-
19	-	-	-				-	-		-	-	-	-	-	-	-
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21	-	-	-				-	-		-	+	-	-	-	-	-

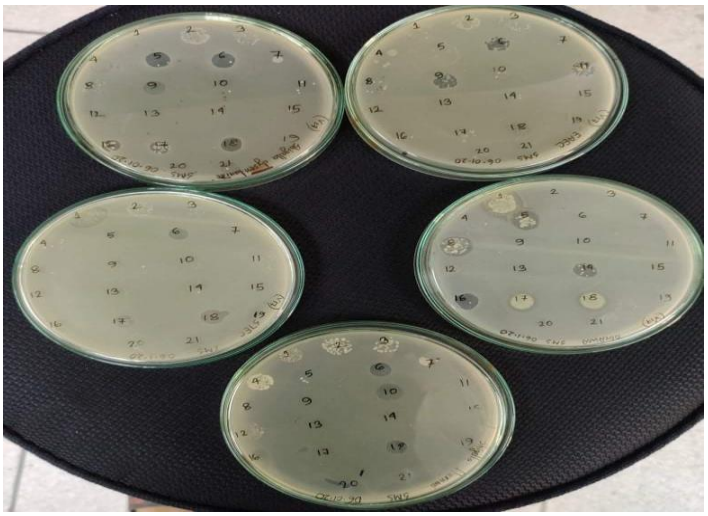

Figure: Host range plate of V1774 strain

Chapter 4: Discussion

Bacteriophages were isolated from the sewage water of different areas in Dhaka city. The areas that were covered for the phage isolation were, Gulshan lake, Hatirjheel, Korail, Buriganga River and Police plaza. The sample water was transported into laboratory as soon as possible right after the collection for the sampling process. Sample water was filtered twice. Firstly with using Watman filter paper and then syringe filter (0.22 µm). Bacteria such as two strains of *E. coli*, STEC and O157: H7 were cultured in 3ml LB broth in a vial individually for 2.5 hours for young culture. Cocktail (mixture of both strains of *E. coli*) was prepared in 3ml LB broth in a vial and cultured for 2 hours and added the sample water into it. The ratio of the preparation was 2:1. After the mixture of water sample with cocktail, the preparation was kept into incubation for further 4hours. Bacterial young cultures were mixed with soft agar for double layer method and LB agar medium were used. Cocktail was centrifuged in 13000rpm and syringe filtered (0.22 µm). The supernatant were collected and diluted up to $10^{8.}$ 10µm of the diluted sample is added drop wise 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. After 24hrs incubation, plaque was collected using sterile tips and stored by adding 200 ml of saline and 20µl of chloroform and stored at -4ºC. Host range of seventeen bacteriophages was determined by spotting 10µL of bacteriophage preparation on lawn culture of fifteen pathogenic bacterial strains. The targeted fifteen pathogenic bacterial strains were *Vibrio cholarae*, *Pseudomonas aeroginosa*, ETEC, EAEC, STEC, O157:H7, *Enterococcus faecalis, Entero pathogenic E. coli(*a typical*), Salmonella typhi, Shigella dysenteriae, Klebseilla pneumonia, Salmonell paratyphi, Bacillus cereus*. STEC, O157:H7, *Shigella dysenteriae, Klebsiella pneumonia*, EAEC and ETEC created clear zone against *E. coli* and plaque were

observed. The titer of the initial bacteriophage was determined in order to ensure heat resistance, tubes full of bacteriophages were placed in water bath for a period of 30 minutes at each (30 ° C, 40 ° C, 50 ° C, 60 ° C, 70 ° C, 80 ° C, 90 ° C) temperature to maintain the stability. pH stability tests were proceed by the tubes containing different pH in LB broth and pH adjusted by NaOH or HCl, which were incubated at 37° C for 2 hours. All titers of bacteriophagus were measured by the double-layer agar plate method.

4.1 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.

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