Isolation and characterization of biofilm degrading bacteriophage from Gulshan Lake specific for *Escherichia coli* and *Vibrio Cholerae*

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A dissertation submitted to the Department Of Mathematics And Natural Sciences, BRAC University in partial fulfillment of the requirement for the degree of Master of Science in Biotechnology

Department of Mathematics and Natural Sciences

BRAC University, Bangladesh

May 2023

Declaration

It is hereby declared that.

1. The thesis submitted is my/our own original work while completing Maters of Science degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I/We have acknowledged all main sources of help.

Student's Full Name & Signature:

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Approval

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has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Masters in Biotechnology on 23rd May 2023.

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Ethics Statement

This material is an original work, which has not been previously published elsewhere. It is my own research and analysis in a truthful and complete manner. The paper properly credits all the sources used (correct citation).

Abstract

Bacteriophage, also known as bacterial virus, has been considered as the natural enemy of bacteria since their discovery. Bacteriophages are used to eradicate bacteria from bacterial infected regions in many medicinal situations. They are also important to control the abundance of pathogenic bacteria in the environment. Some of these bacteriophages have the potential to degrade the bacterial biofilms and research shows that this type of bacteriophage has potential influence in controlling the seasonal epidemics of diarrhea. The aim of this study was to isolate bacteriophages able to lyse pathogenic *Escherichia coli* and Vibrio Cholerae and to look for biofilms degrading capacity. Bacteriophages that degrade biofilms can increase planktonic bacterial concentration in the environment. To isolate bacteriophages lytic against *Escherichia coli* and Vibrio cholerae strain used in this study, around 7 environmental water samples were collected from Gulshan Lake in March, April, May & June. 19 bacteriophages were isolated, among them 4 specific for Vibrio Cholerae and 15 specific for *Escherichia coli*. From isolated 19 phages four were capable of degrading biofilm. Interestingly two of the bacteriophages show the ability of increasing biofilms. The characterization of those 5 bacteriophages was done. Studies on its biological characteristics may provide important information and knowledge in developing potential therapeutic agents against *Escherichia coli* and *Vibrio cholerae* infection.

This work is dedicated to

My Dear Family

Acknowledgement

First and foremost, I would like to express our sincere gratitude to Almighty God for providing me with the perseverance, patience, and knowledge needed to finish this project within the desired time span. I convey my special thanks to **Professor A. F. M. Yusuf Haider, Ph.D**., Chairman, Department of Mathematics and Natural Sciences, BRAC University, who always has tremendous support for every student. I would like to showcase my deepest and most sincere gratitude to my project supervisor, **Dr. Iftekhar Bin Naser**, Associate Professor, Department of Mathematics and Natural Sciences, BRAC University, whose expertise, constant guidance, sincere monitoring, and motivational approaches in every sphere have inspired us to conduct this project efficiently. I sincerely express my appreciation and admiration for his patience and for providing a sense of direction whenever I encounter complications throughout this phase. I feel to acknowledge my indebtedness to him for his insightful corrections and directions which shaped the present work as it is today. I would also like to extend my heartfelt appreciation to **Dr. Munima Haque** for her unwavering support and invaluable guidance throughout my thesis project. I am indebted to all the extraordinary people of the Life Science Laboratories, BRAC University, specially Rockybul Hasan. Without their all-time support this would not be complete. Lastly, I would like to thank our parents for their constant support and encouragement that further pushed us to work harder and face the difficulties during this time frame.

Sincerely,

Afroza Khanam Anika

Department of Mathematics and Natural Sciences, BRAC University

Table of Content

List of Figures

List of Tables

List of Acronyms

DNA	Deoxyribonucleic acid
ds	Double stranded
FDA	Food and Drug Administration
CTX	Cholera Toxin
CVEC	Conditionally Viable Environmental Cell
DLA	Double Layer Assay
EAEC	Enteroaggregative *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
EPS	Extracellular Polymeric Substance
ER	Endoplasmic Reticulum
ETEC	Enterotoxigenic *Escherichia coli*
ICTV	Committee on Taxonomy of Viruses
LB	Luria Bertani
OD	Optical Density
PFU	Plaque-Forming Units
RPM	Rotations Per Minute
USA	United States of America
μl	Microliter
μm	Micrometer
UV	Ultraviolet
VBNC	Viable But Not Culturable

1. INTRODUCTION

Bacteriophages or viruses that infect bacteria are extremely abundant in nature, probably the most abundant life form on Earth. They outnumber bacteria in most studied habitats including human and animal –associated microbial communities (Weinbauer, 2004). Since its discovery at the beginning of the 20th century, bacteriophages have been utilized to treat bacterial infections in humans (Sulakvelidze & Kutter, 2004). Phages have so far shown promise in cattle and aquaculture as well as in treating bacterial plant diseases in various agricultural contexts (Sulakvelidze & Barrow, 2004). Recently, scientists have been experimenting with using phage as a molecular tool for gene therapy, vaccine administration, and as a diagnostic tool to identify bacterial species in environmental and clinical samples (Clark & March, 2006).

Bacteriophages can have either a lytic or lysogenic life cycle. Lytic phages, such as T4, include the ability to lyse the host cell following virion multiplication. The host cell is not immediately lysed by lysogenic phages. They are referred to as moderate phages. Without destroying the cell, the bacteriophage genome collaborates with the host genome and reproduces alongside it. The phages initiate the regenerative cycle when conditions for the have cell deteriorate, such as when it needs nutrients. The isolation of bacteriophages for phage treatment is frequently described as a somewhat simple process that involves mixing a phage-containing test with have microorganisms, followed by a straightforward ejection of bacterial flotsam and jetsam by filtration or centrifugation the next day. (Ackermann, 2012) (Ackermann, 2009) However, among all these applications, the ability of phages to lyse specific bacterial cells, particularly those that are antibiotic resistant, and to prevent or cure bacterial infections makes phages an intriguing alternative antimicrobial agent in situations where chemically synthesized antibiotics may fail.

While bacteria are typically found in nature as single cells, they can also group together to form multicellular formations known as biofilms. Biofilms are surface-associated bacterial colonies that are encased in a matrix of complex heterogeneous extracellular polymeric components that include polysaccharides, proteins, nucleic acids, and lipids. One or more species of bacteria can be found in complex bacterial clusters called biofilms. Extracellular polymeric substances (EPS) bind them and keep them affixed to various surfaces, including pipes,

industrial equipment, food, living tissue, and medical devices (Pilar Domingo-Calap, 2020). Bacterial biofilms have been implicated as a source of infection and contamination in medical and industrial contexts, as well as in pathogen transmission via water. Pathogenic bacteria in biofilms are especially difficult to eliminate because they are resistant to antimicrobial treatments and frequently serve as a source of a high dose of the pathogen in ambient fluids. Several ecological factors, such as bacteriophages and metabolic products of other bacteria, have been postulated to influence the concentration of culturable V. cholerae in water. (Naser et al., 2017).

In this article, I will first go over phage isolation methods, including some variations on the more common ones. I then go over the general characterization of newly isolated bacteriophages. All previously obtained phages unique to *Escherichia coli* and *Vibrio Cholerae* showed variance as well as similarities in their phenotypic and genotypic traits, confirming phage diversity. Hence, an *Escherichia coli* & *Vibrio Cholerae* specific phage isolated from Gulshan Lake in Dhaka could demonstrate variation and similarities to previously isolated *Escherichia coli* & *Vibrio Cholerae* specific phages and could be added to the International Committee on Taxonomy of Viruses (ICTV) database. The basic understanding of phage biology of the isolated *Escherichia coli* & *Vibrio Cholerae* phage could be useful in the development of Diarrhea and Cholerae therapeutic agents.

1.1. RESEARCH OBJECTIVE

The main aim of this project was to isolate bacteriophage from Gulshan Lake water against pathogenic bacteria for a specific time and to identify biofilm degrading bacteriophages characteristics to determine the therapeutic potential as a therapeutic agent.

1.2. AIMS OF THE STUDY

1. To isolate Strong lytic bacteriophage against *Escherichia coli and Vibrio cholerae* from Gulshan Lake water against pathogenic bacteria for a specific time.
2. To look for biofilm degrading bacteriophages.
3. To characterize the biofilm degrading bacteriophages based on:
    a) thermal stability
    b) pH stability

2. LITERATURE REVIEW

2.1. Bacteriophage

Bacteriophages are viruses that infect and replicate only in bacterial cells additionally they are highly host specific and have the ability to grow rapidly inside bacterial cell (Clark & March, 2006). They are the most abundant as well as the most genetically diverse biological entities on Earth, with global population number estimated at 1030 to 1032 (Hemminga et al., 2010). Approximately there are 5 to 10 viruses for each bacteria (Weinbauer, 2004). They are recognized as the most abundant biological agent on earth and present everywhere in the environment.

Antibiotic resistance has little effect on bacteriophages, which can also target bacteria embedded in biofilms. The impermeability of the biofilm matrix has led to the common belief that biofilms confer bacteriophage resistance. However, bacteriophages are still much smaller than their bacterial hosts despite being much larger than chemical antibiotics. Many bacteriophages can infect bacteria within biofilms. (Harper et al., 2014a)

A certain bacteriophage will connect to a particular receptor on the bacterial cell wall and target a particular bacterial species (Edgar et al., 2008). The quantity of bacteria reduces as the

number of bacteriophages rises, and vice versa. Between March and May and September and December, less bacteriophages are present in Bangladesh, which causes bacteria to multiply more quickly and more people to contract bacterial infections. Although from June to August, the reverse pattern is seen. The number of bacteria declines as the number of bacteriophages increases. In recent years, it has become commonly accepted that bacteriophages are pervasive in the environment and have a significant impact on the biosphere (Naser et al., 2017).

Bacteriophages are thought to eliminate 20–40% of oceanic bacteria daily, are important to an ecosystem's nutrition and energy cycles, and are the source of the planet's most genetically varied 'living form' (Suttle, 2005)

Depending on how an infection develops, phages can be categorized as either virulent or lytic and temperate or lysogenic. Since they cause the host cell to lyse and do not integrate with the host genome, only lytic phages are appropriate for therapeutic use (Matsuzaki et al., 2005) . During the lytic cycle, phage particles are adsorbed on the bacterial surface; next, the genetic material of the phage permeates into the cell and takes over the host cell metabolism. This leads to a multiplication of offspring phages, cell lysis and the release of new phages into the environment. The first stage of the lytic cycle, i.e., adsorption, is facilitated by viral tail filaments that bind specific receptors on the surface of the cell. Phage receptors may include lipopolysaccharides (LPSs), corresponding proteins, sugar molecules and fimbriae.

During the lytic cycle, phage particles are adsorbed on the surface of the bacterial cell, after which the phage's genetic material permeates the cell and commandeers the metabolism of the host cell. As a result, cell lysis occurs, progeny phages multiply, and fresh phages are released into the environment. Viral tail filaments that bind to particular receptors on the cell surface enhance the initial stage of the lytic cycle, known as adsorption. Lipopolysaccharides (LPSs), matching proteins, sugar molecules, and fimbriae are examples of phage receptors. The variety of phage host species is determined by the receptors' specificity. The viral genome integrates into the host's replicon during the lysogenic cycle and stays inside the bacterium as a prophage. Up until induction, this prophage will continue to act as the lysogen, switching to the

lytic cycle and causing the system to produce additional phages. (Matsuzaki et al., 2005); (Clark & March, 2006)

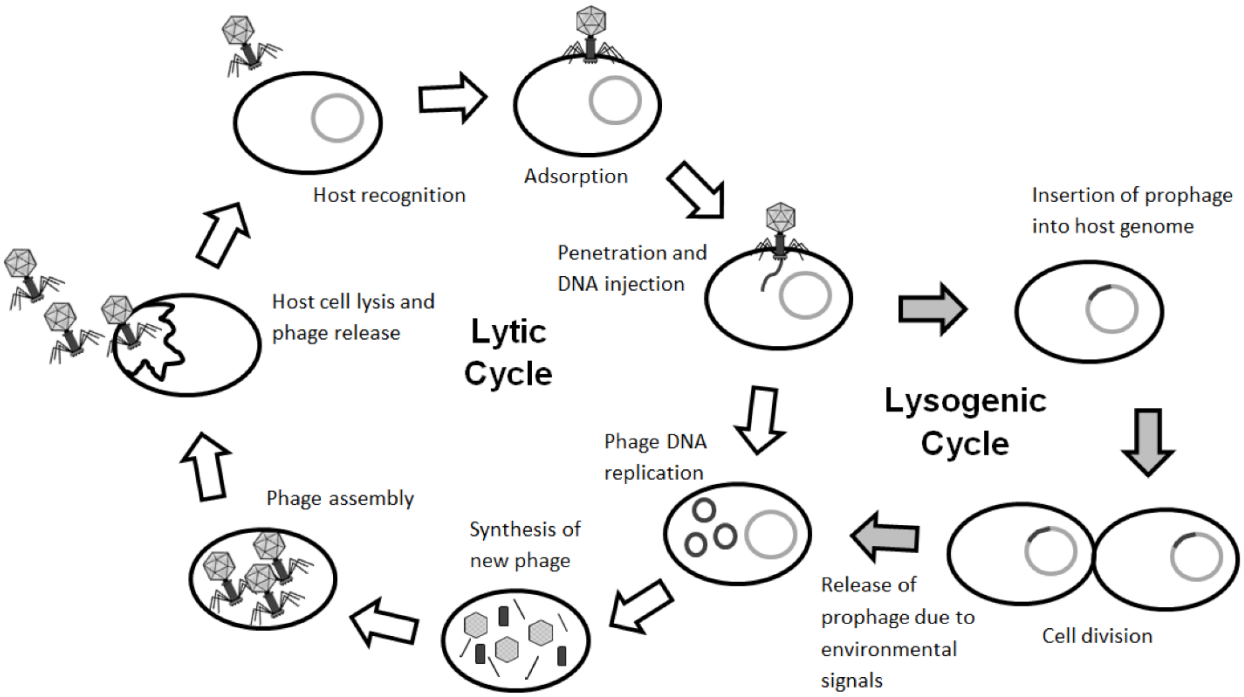

***Figure 1**: The phage life cycle. Lytic phages go through the lytic cycle, in which the host is lysed, and progeny phage are released into the environment. Temperate phage can go through the lytic or the lysogenic cycle. Some phages rely on small molecules to communicate and execute lysis–lysogeny decisions. In the lysogenic cycle, the phage genome is incorporated into the host genome; this phage DNA—now called a prophage—can be induced, leading to the expression of phage DNA and the lytic cycle (embedded from (Doss et al., 2017).*

Additionally, many bacteriophages carry enzymes on the tail of the virus particle, where they help the virus particle penetrate the bacterial cell wall. Examples of this include the T4 and HK620 bacteriophages of *Escherichia coli*. Although they might theoretically contribute to the degradation of the biofilm matrix, they frequently go unnoticed until the infection causes the tail to change its configuration, making their activity relatively confined. (Harper et al., 2014a)

2.2 Biofilm

A biofilm is a collection of microbial cells that are surface-associated and encased in a matrix of extracellular polymeric material. The first time that bacteria were seen on tooth surfaces was by Van Leeuwenhoek, who is also credited with the discovery of microbial biofilms, using his ordinary microscopes, describing microorganisms' growth attached to bare surfaces (Heukelekian & Heller, 1940). However, it was until 1978 that the theory of biofilms was proposed (Costerton et al., 1999).

Communities of bacteria grow by multiplying to attach to surfaces or to one another. They manufacture extracellular polymers by secreting polysaccharide matrix, fibrin, lipoprotein, and other chemicals. They then embed themselves in the self-made extracellular polymer matrix to create a biofilm (Tian et al., 2021). Biofilms can grow on almost any surface that is submerged in a natural aquatic environment. Biofilms are collections of cells, either eukaryotic or prokaryotic in origin, that are encased in a matrix made of extracellular polymeric substance (EPS), which is primarily or entirely created by the cells in the biofilm. Long-chain sugars, DNA, and other biological macromolecules make up this EPS, however their exact composition can vary greatly. (Santos et al., 2009). Different circumstances, such as gaseous stratifications and nutritional stratifications, produce various cell states, which result in the appearance of separate zones within the biofilm. The biofilm behaves in many aspects as a community rather than just a cluster of autonomous cells thanks to quorum sensing, in which cells signal to one another by releasing tiny chemical molecules. (Harper et al., 2014b)

The complicated yet well-controlled process of biofilm growth can be broken down into five distinct steps: (i) Surface swarming, which is enabled by the surface sensing function of planktonic bacteria's flagella; (ii) the stage of attachment, in which bacterial adhesions perform the initial reversible attachment, which indicates loosely adhering to the surface and detaching, and the subsequent irreversible attachment, which is a more precise and stable adhesion; and (iii) the excretion of EPS matrix, which indicates the formation of a biofilm and is produced by recently attached bacteria; (iv) the maturation of the biofilm, which entails bacterial cell interactions that lead to the growth of micro-colonies; and (v) the spread of the biofilm's

structure after planktonic bacteria are released, which causes the biofilm to form a new locations. (Alhede et al., 2011), (Armbruster & Parsek, 2018). In addition to the protection offered by the matrix, bacteria in biofilms can invade the host defensive mechanisms through a variety of survival strategies (Moser et al., 2017).

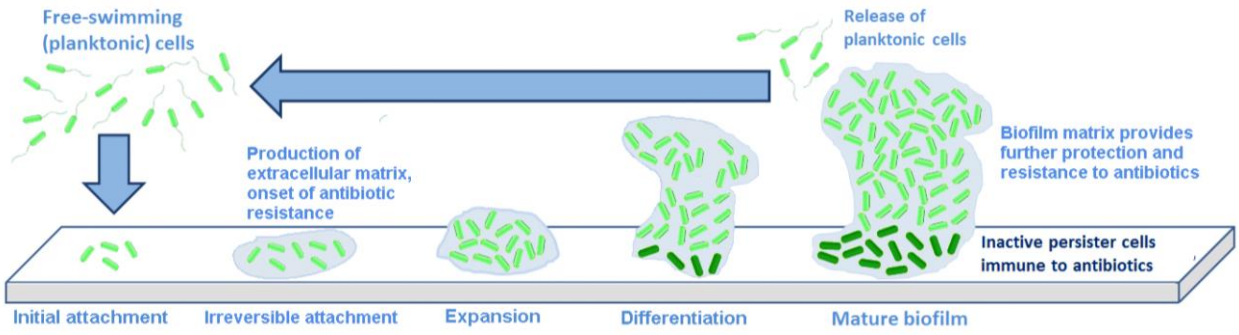

Figure 2: The life cycle of bacteria in biofilms adapted from (Harper et al., 2014a)

The fundamental mechanism of biofilm is shown in figure 2: During matrix synthesis, a variety of extracellular components participate. The extracellular DNA of other lysed bacteria can be one of these sources. This work has investigated how bacteriophage-provided free bacterial DNA contributes to the development of bacterial biofilm.

Biofilm Forming Bacteria

2.3. *Vibrio cholerae*

Devastating cholera epidemics were brought on by *Vibrio cholerae* serogroup O1 biotype classical or El Tor up to 1992. The first six pandemics, which occurred between 1817 and 1923 in the Indian subcontinent and subsequently in other parts of the world, are thought to have been triggered by the classical biotype. El Tor, a V. cholerae O1 biotype, was first observed in

1905. Unfortunately, it wasn't until the early 1960s that the sixth-pandemic V. cholerae O1 classical biotype was replaced by the V. cholerae biotype El Tor. *V. cholerae* non-O1 serovar, often known as V. cholerae synonym O139 Bengal, first appeared in Bangladesh and India in 1992. It then spread to Southeast Asia, replacing V. (Alam, Hasan, et al., 2006). People can get potentially fatal diarrheal illnesses because of the bacterium V. cholerae. There are various serogroups of V. cholerae. O1 and O139 are two of them that are known to cause significant sickness. In many developing nations of Asia, Africa, and Latin America, cholera epidemics brought on by toxigenic *Vibrio cholerae* belonging to the O1 or O139 serogroups are a serious public health issue. In the Ganges delta, which includes Bangladesh and India, cholera epidemics happen on a seasonal basis. Epidemics often happen twice a year, with the peak number of infections occurring between September and December immediately following the monsoon. In the spring, between March and May, cholera cases also reach a slightly lower peak. (Faruque et al., 2005)

Cholera is an acute dehydrating diarrhea caused by toxigenic *Vibrio cholerae* O1 and O139, and it can be epidemic or pandemic in nature. Up to seven cholera pandemics have occurred since the first one was noted in 1817. The seventh and most current cholera pandemic started in Indonesia, while previous outbreaks often started in the Gangetic delta of the Indian subcontinent before spreading to other continents. Up until the end of 1992, serovar O1 of V. cholerae, one of the 206 O serogroups, was the only one known to cause cholera. During same period, India and southern Bangladesh experienced an outbreak of acute watery diarrhea that was clinically similar to cholera. To honor its development on the Bay of Bengal coast, the bacterium that causes cholera-like diarrhea was given the designation O139 and the term "Bengal" since it was unable to agglutinate with any of the 138 V. cholerae O antisera that were then in use. At that time, O1 and O139 have been the two known serogroups responsible for cholera epidemics (Alam, Sultana, et al., 2006).

2.4. *Escherichia coli*

Escherichia coli is a gram-negative, rod-shaped member of the Enterobacteriaceae family and the Gamma proteobacteria class of bacteria. *Escherichia coli* may proliferate in around 20 minutes under ideal growth conditions. (Jang et al., 2017). E. coli is frequently used as a sign of fecal pollution of streams because it is discharged into the environment by the deposition of fecal debris. The discovery of a potential source of fecal pollution harming waterways and beaches has been the focus of research. Nevertheless, microbial source tracing is a common term for this. Yet, recent research has demonstrated that E. coli can persist for extended periods of time in the environment and may even be able to reproduce in soils, on algae, and in water in tropical, subtropical, and temperate climates (Ishii & Sadowsky, 2008).

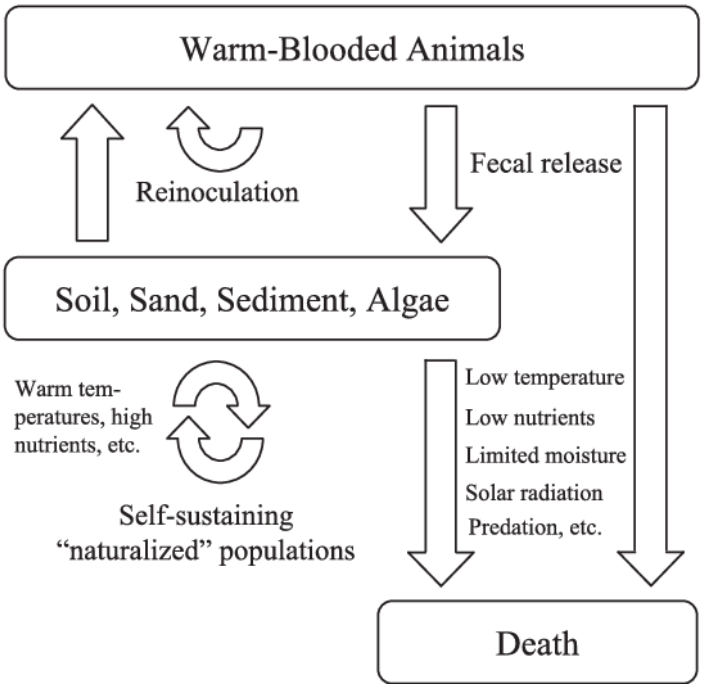

Figure 3: Life cycle of E. Coli adapted from (Ishii & Sadowsky, 2008)

Escherichia coli makes up the bulk of facultative anaerobes in the human colonic flora. The bacteria often colonize the infant's digestive tract within hours of delivery, and from that point

on, both the host and *E. coli* benefit. Yet, even typical "nonpathogenic" strains of *E. coli* can cause sickness in the weak or immunosuppressed host, as well as when gastrointestinal barriers are overcome. It can be recovered efficiently from clinical specimens on general or selective media at 37°C in aerobic conditions. To be identified, diarrheagenic *E. coli* strains must be distinguished from nonpathogenic members of the normal flora. Diarrheagenic *E. coli* strains were among the first pathogens to be identified using molecular diagnostic methods. Similarly, molecular approaches remain the most popular and dependable tools for distinguishing diarrheagenic strains from nonpathogenic components of the stool flora and differentiating one category from another. Both PCR and the development of nucleic acid-based probe technologies have made significant advances. After colonization, the pathogenetic tactics used by diarrheagenic *E. coli* strains exhibit a surprising range. There are three general paradigms by which *E. coli* can cause diarrhea, each of which is discussed in detail in the section below. ETEC and EAEC production, invasion (EIEC), and/or close contact with membrane signaling (EPEC and EHEC) (Nataro & Kaper, 1998).

3. MATERIALS AND METHODS

3.1. Place of study:

The study was done in the Biotechnology and Microbiology laboratory of the Department of Mathematics and Natural sciences, BRAC University, Dhaka, Bangladesh.

3.2. Standard laboratory practices

All the glassware, including test tubes, conical flasks, and beakers, washed twice: once with tap water and once with distilled water. Before use, culture media (both agar-based and broth-based), pipette tips, centrifuge tubes, empty test tubes for the double-layer aging method, and

autoclaved equipment were all autoclaved at 121°C at 15 psi for 15 minutes. Culture media were then stored at 4°C in an aseptic manner. To prevent contamination, experiments were carried out inside a vertical laminar flow cabinet that had previously been cleaned with 70% ethanol while wearing clean lab coats and hand gloves.

3.3. Preparation of LB:

The LB was prepared by weighing 20 grams of LB powder in 1000 ml of distilled water and then mixed well in beaker. After that we heated it for few min on Bunsen burner and later it was autoclaved at 121°C for 2 hours.

3.4. Preparation of LA:

The LB was prepared by weighing 15 grams of LA powder in 1000 ml of distilled water and then mixed well in a biker. After that we heated it for few min on Bunsen burner and later it was autoclaved at 121°C for 2 hours. Finally, we poured it on a previously prepared autoclaved plate (at 121°C for 15 min).

3.5. 70% ethanol:

To prepare 70 % ethanol, deionized water was added to the 737 ml of 95 % ethanol to make a final volume of 1000 ml.

3.6. SM buffer:

This buffer was prepared by adding 5.8-gram NaCl, 2-gram $MgSO4 \cdot 7H2O$, 50 ml Tris-Cl (1M, pH 7.5), and 5 ml Gelatin (2 % w/v) to a container and adding deionized water to make final volume

1000 ml. the buffer was put in several 50 ml falcon tube with screw cap and autoclaved at 121°C for 15 minutes. After sterilization, the buffer was stored at room temperature.

3.7. Bacterial Strains & Their Culture Conditions

All the bacterial cultures used in this project were collected from the stock of University lab. In total, 5 E. Coli and 4 Vibrio strains were used in this study. All strains were preserved in 8% soft agar at room temperature. They are E. Coli. 9A, E. Coli 9D, E. Coli. 9E, E. Coli. 9G, E. Coli. U, vibrio 7f, vibrio 7e, vibrio 9c & vibrio 13b. Bacterial samples were streaked on the fresh LA plate and incubated for 24 hours at 37°C. After checking growth, plates were wrapped with parafilm and stored at 4°C for further use. Before each experiment, bacterial samples were freshly sub-cultured and 24-hour cultures were used. By regular subculturing, viability and purity of the organisms were maintained.

Figure 4: Culture plate of E. Coli.

3.8. Isolation of Phages

Bacteriophages were isolated from water samples using the double agar overlay method. Isolated phages were purified by diluting in the SM-buffer and filtering through 0.22 µm filter. Purified lysate was further processed for analyzing its host range by using the spot method. Water is collected from Gulshan Lake using caution not to come into direct contact with the liquids since they will most definitely contain human pathogens. Then the sample is first filtered using Whatman filtration technique and then syringe filtration (0.22 µm). All of them are done within 4 hours.

For the cocktail preparation Luria broth (LB) is prepared and different strains of the same bacterial species is added in a vial containing 5ml of LB and added 1 or 2 freshly cultured colonies of the bacterial species is taken and inoculated in the broth and kept for 2 hours in the shaker incubator for lag phase. Then added 1ml sample water to the tube also Incubate in shaker incubator for 4 hours. Centrifuge with 1300 RPM with room temperature for 10 minutes. Then filtrate with 0.22 µl syringe filter & store in fridge.

3.9. Sample collection

Samples were collected from Gulshan Lake for 4 months. Twice a month.

Table-1: Sample Collection Data

Sample	**Date**	***Escherichia coli***	***Vibrio Cholerae***
Sample-1	**22nd March**	9A	7f, 13b
Sample-2	**11th April**	9A, U	13b
Sample-3	**20th April**	9A, U	13b
Sample-4	**14th May**	9A, 9D, 9E, U	
Sample-5	**28th May**	9D	
Sample-6	**12th June**	9A, 9D	
Sample-7	**30th June**	9A, 9D, 9E	

3.10. Double Layer Agar Method

The Double-Layer Agar (DLA) technique is widely used in phage research to count and identify phages, as well as isolate mutations and novel phages. Many phages create big, well-defined plaques that may be counted when plated using the DLA approach (Santos et al., 2009).

The strains were individually inoculated in separate glass vials each containing 3mL LB broth. These vials were kept in a shaker incubator for 2 hours to obtain young culture. 300µl of young culture were collected and poured in warm (54°C by using waterbath) 3 mL soft agar aliquots. Then it was mixed using a vortex. The mixtures were poured into LA plates while still warm and liquid in order to avoid bubble formation and to achieve an even distribution. Then, the plates Allow to solidify at room temperature placing the plate in a flat surface upside up. Deposit a drop of sample approximately 10 µl wait till dry then incubate overnight. After that, these were incubated overnight at 37°C. Lysis indicates presence of phage in the tested sample otherwise no lysis indicates absence of phage in the tested sample.

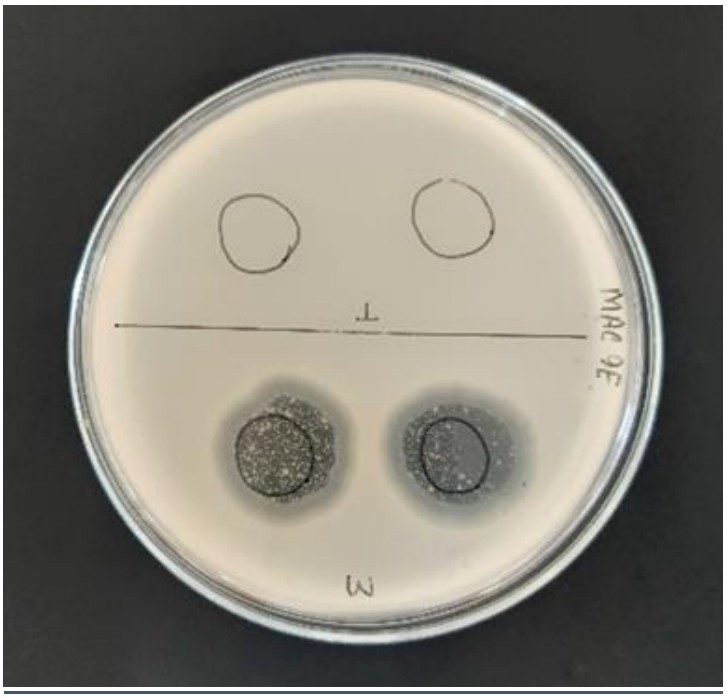

Figure 5: Double Layer Agar of Escherichia coli 9E strain by using 10 µl water sample drop.

3.11. Plaque Store

Phages are counted using direct quantitative plaque tests. The plaque assay yields result in plaque forming units (PFU). Stored plaque with 300ul saline magnesium (SM) buffer in Eppendorf (SM buffer used for routine manipulation of phage suspensions. The gelatin of SM buffer stabilizes lambda phage particles during storage). Vortex 3 minutes to release the phage from DLA. Centrifuge 1300 RPM (Revolutions Per Minute) for 5 minutes. Take only liquid portion and transfer 100 µl to two Eppendorf. Add chloroform in one Eppendorf & centrifuge for 20 sec and store in fridge. Chloroform was added to remove bacterial remnants and a virion suspension was carefully recovered. The supernatant was collected and stored in another Eppendorf for phage enrichment.

3.12. Phage Double Enrichment

At first, 10mL LB broth was inoculated with the host colony of a bacteria. It was then incubated in a shaker incubator for 2 hours at 37°C temperature and 80 rpm. Following that, 50µL phage was added and incubated again for 4 hours. Then it was centrifuged for 10 minutes at 13000 rpm. Then filtered through a 0.22 filter. Thus, single enrichment was obtained. This process was repeated for double and so on enrichment.

3.13. Serial Dilution and Phage Titer

The phage titer was determined through the double layer agar method after serial dilution of the phage stock solution and calculated using the following formula:

The PFU count for each plate is $\frac{Plaque\ count}{Dilution\ Factor \times Volume\ Plated}$.

Serial dilation of phage was required to achieve various concentration of phage in order to check their activities. To make this, initially fresh 900 µl LB broth was taken in Eppendorf and it was mixed with 100 µl phage. By using vortex in an Eppendorf and thus 10^{-1} dilution was

obtained.100µl was obtained from the vortexed mixture of phage and LB broth solution and added to another Eppendorf containing 900µl LB. So, we also have 10^{-2} dilution. The same process was repeated till the dilution concentration reached 10^{-9}.

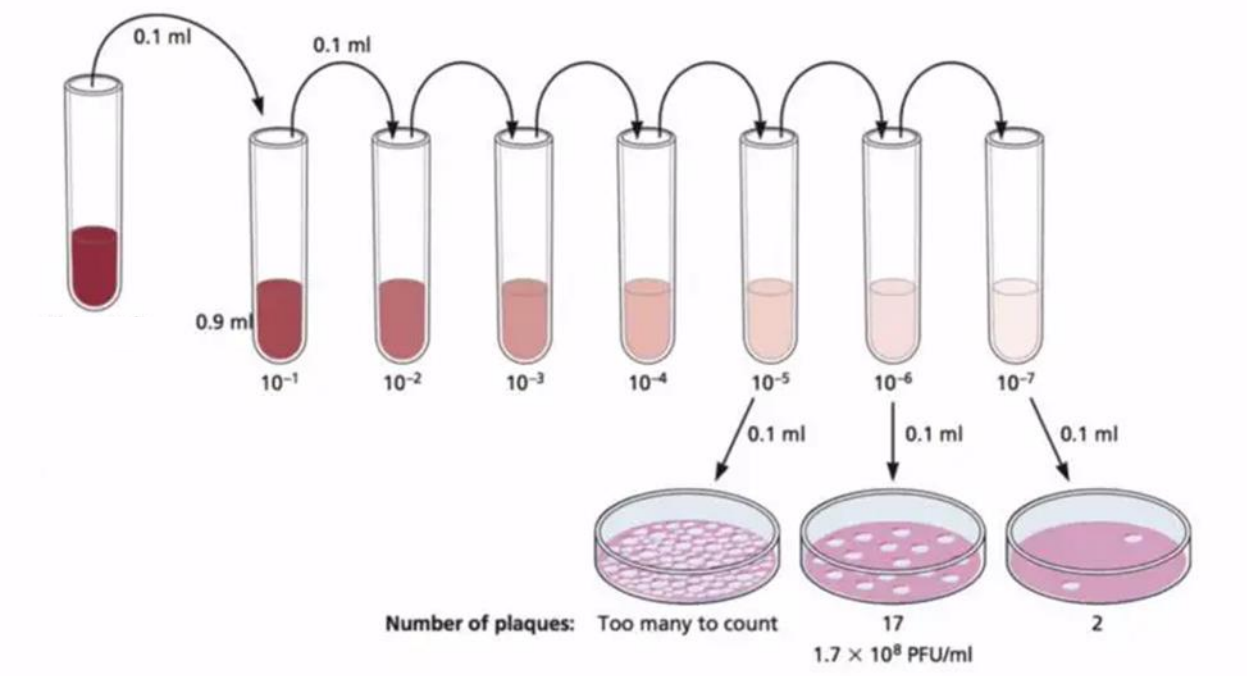

Figure 6: Serial dilution picture adapted from (Acharya Tankeshwar, n.d.)

Now, for phage titer, 3mL LB broth was inoculated with the host colony of the bacterial strain. It was then incubated in a shaker incubator for 2 hours. 300µL young host culture was added with 100µL phage in 3mL melted soft agar. The temperature of this melted agar should be neither too hot to kill the bacteria and phage, nor too cold that it gets solidified before mixing in the vortex and poring on the LA plate. After mixing, it was vortexed and poured in LA plate. After solidification of agar, the plate incubated at 37°C for 24 hours. Now, the plaque-forming units (PFU) were checked.

3.14. Biofilm Formation and Optical Density

We needed to make young culture for biofilm formation. For making a young culture, we put 3 ml of LB in a sterilized vial and added a single colony of a host bacteria. It was then placed in a shaker incubator for 3-4 hours in 37°C and 80 rpm for culture formation. Following that, we divided 500 µl of the inoculated LB among four vials. These vials were placed in a clean environment with no disturbance for 24, 48, and 72 hours respectively to form biofilm. We took the vials and rinsed them with distilled water after 24 hours, 48 hours, and 72 hours of observation. After that, we applied crystal violet and waited 90 minutes. We washed those vials again with distill water. Then we noticed the ring around the vials, which indicates the formation of biofilm. If the young culture is left undisturbed for 48- 72 hours a good biofilm forms that can be seen well on the surface.

Since phages can actively penetrate and disturb biofilms in nature, they can be used to obtain specific and improved treatments against biofilms. Phages encoding EPS-degrading enzymes are of particular interest to biofilms. For OD check after 48 hours washed the biofilm forming vials with autoclaved distilled water then added 1ml phage & store for 24 hours. Added crystal violet in another vial and put it for 30 minutes to stain the biofilm adhered to the tube wall. After 24 hours check the Optical Density (OD_{450} values) of phage solution. Then stain the biofilm with crystal violet. Pour Glacial Acetic Acid in both vials for 30 minutes then check the Optical Density (OD_{450} values) in ELISA and compare results.

3.15. Thermal stability test:

The stability of the isolated bacteriophage at different temperatures was tested. 1ml of phage solution was taken in different sterile Eppendorf tubes. These tubes were kept in the water bath for each temperature of 37°C, 55°C & 65°C for 60 minutes. Then the titer of bacteriophage after treatment was determined using double layer assay method.

3.16. pH Effect

To investigate the effect of the pH on the phage activity, each 100 µl of phage suspension (>109 PFU/mL) was added to 900 µl of SM buffer. At different pH values, ranging from 3, 7 & 9 with pH 7.5 being used as a control. 1M HCl was added drop by drop and tested by pH meter to achieve desired acidic pH and 1M NaOH was used to achieve desired basic pH. After the incubation period, phage titer was determined by double layer agar method against host bacteria.

4. RESULT

4.1. Phage Titer (pfu/ml)

Titering Phages is likely the most common phage protocol. There are numerous methods for counting phages, but here we present the two most common, which both involve serially diluting the phage sample and placing defined aliquots onto a bacterial lawn to count the resulting plaques. Titer of the 19 phages I have isolated from Gulshan Lake are given bellow:

Table 2: Phage Titer (pfu/ml)

Phages	Plaque Number	Dilution Factor	Phage Titer (pfu/ml)
9A S-1	142	10^6	1.42×10^{-9}
7f S-1	123	10^7	1.23×10^{-10}
13b S-1	147	10^7	1.47×10^{-10}
9A S-2	62	10^6	6.2×10^{-8}
U S-2	416	10^7	4.16×10^{-10}
13b S-2	75	10^7	7.5×10^{-9}
9A S-3	54	10^6	5.4×10^{-8}
U S-3	286	10^7	2.86×10^{-10}
13b S-3	52	10^7	5.2×10^{-9}
9A S-4	63	10^6	6.3×10^{-8}
9D S-4	53	10^7	5.3×10^{-9}
9E S-4	137	10^7	1.37×10^{-10}

U S-4	135	10^{7}	1.35 X 10^{-10}
9D S-5	78	10^{7}	7.8 X 10^{-9}
9A S-6	64	10^{7}	6.4 X 10^{-9}
9D S-6	51	10^{7}	5.1 X 10^{-9}
9A S-7	11	10^{6}	1.1 X 10^{-8}
9D S-7	12	10^{7}	1.2 X 10^{-9}
9E S-7	165	10^{6}	1.65 X 10^{-9}

4.2. Qualitative result

Phage titer refers to the concentration or abundance of bacteriophages (phages) in a given sample. Phage titer is determined by quantifying the number of active, infectious phage particles present in a sample. The phage titer qualitative results are given bellow in a clear and organized manner.

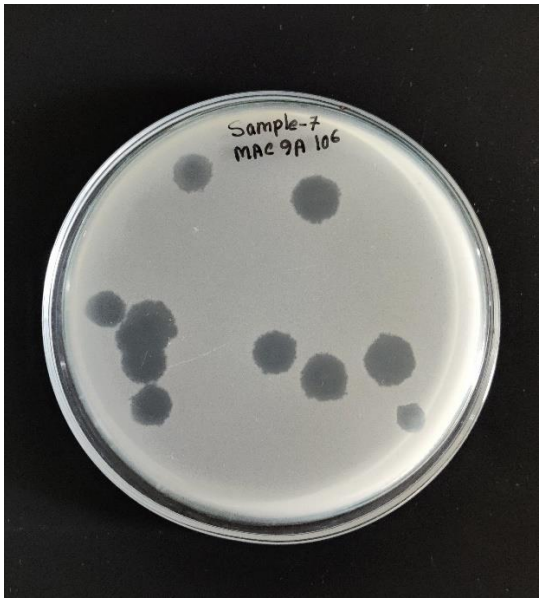

Figure 7: Phage titer assay. 10^{-6} dilution of Phage 9A S-7 *a strain of Escherichia coli gave a clear pattern.*

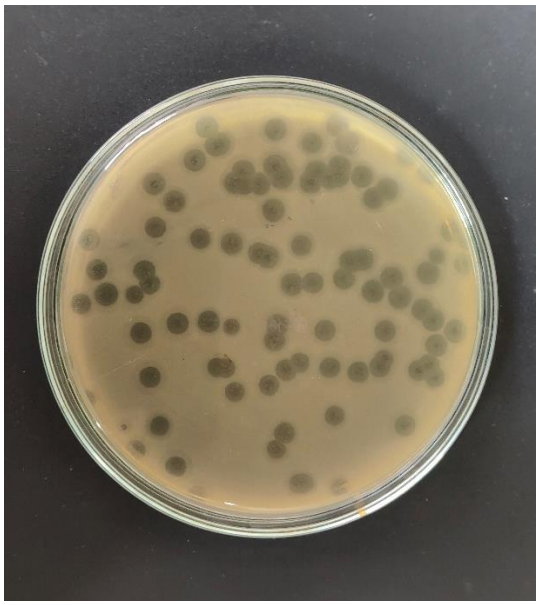

Figure 8: Phage titer assay. 10^{-7} dilution of Phage 9D S-7 *a strain of Escherichia coli gave a clear pattern.*

Figure 9: Phage titer assay. 10^{-7} dilution of Phage U S-3 *a strain of Escherichia coli gave clear pattern and pfu/ml is* 2.86×10^{-10}.

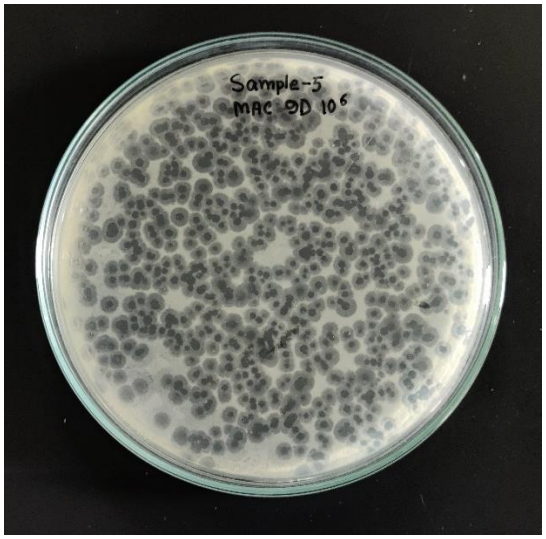

Figure 10: Phage titer assay. 10^{-6} dilution of Phage 9D S-5 *a strain of Escherichia coli gave almost clear pattern and pfu/ml is* 7.8×10^{-9}.

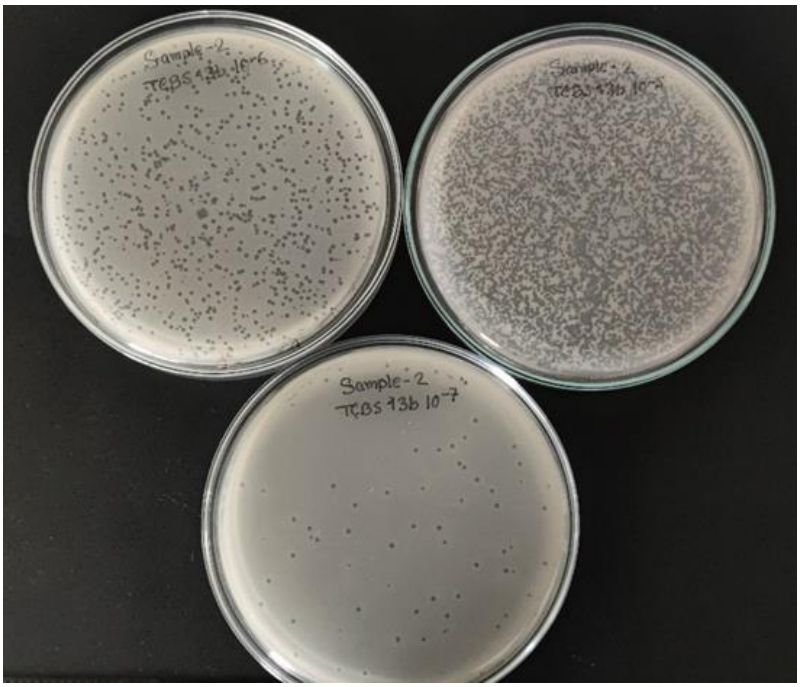

Figure 11: Phage titer assay.

(a)10^{-5} dilution of Phage 13b S-2 is uncountable.

(b) 10^{-6} dilution of Phage 13b S-2 is uncountable.

(c) 10^{-7} dilution of Phage 13b S-2 is countable & pfu/ml is 7.5×10^{-9}

4.3. Optical Density (OD)

The Optical Density (OD) was measured in this experiment using an Enzyme Linked Immunosorbent Assay (ELISA). The micro ELISA auto-reader technique is an efficient method of determining the OD of a biofilm (Mosharraf et al., 2020). ELISA is a procedure that is used in almost every immunology lab. It is based on the antigen-antibody interaction principle. The OD of this interaction can then be measured using ELISA Auto reader machines. The OD measuring property of the ELISA Auto reader was used in this experiment to obtain the OD of the biofilms formed inside ELISA plates.

Table 3: Biofilm Estimation (OD_{450} values) Using ELISA

Phages	With phage	Without phage	Control
9A S-1	0.112	0.143	0.102
9A S-2	0.071	0.143	0.093
9A S-3	0.103	0.143	0.089
9A S-4	0.136	0.143	0.083
9A S-6	0.107	0.143	0.084
9A S-7	0.051	0.143	0.083
9D S-4	0.109	0.143	0.102
9D S-5	0.076	0.143	0.093
9D S-6	0.196	0.143	0.083
9D S-7	0.086	0.143	0.086
9E S-4	0.073	0.112	0.088
9E S-7	0.104	0.112	0.091
U S-2	0.095	0.246	0.088
U S-3	0.211	0.246	0.087
U S-4	0.131	0.246	0.091
7f S1	0.149	0.124	0.083
13b S-1	0.134	0.214	0.087
13b S-2	0.096	0.214	0.089
13b S-3	0.149	0.214	0.083

4.3.1. BIOFILM ESTIMATION (OD_{450} values) IN BAR CHART

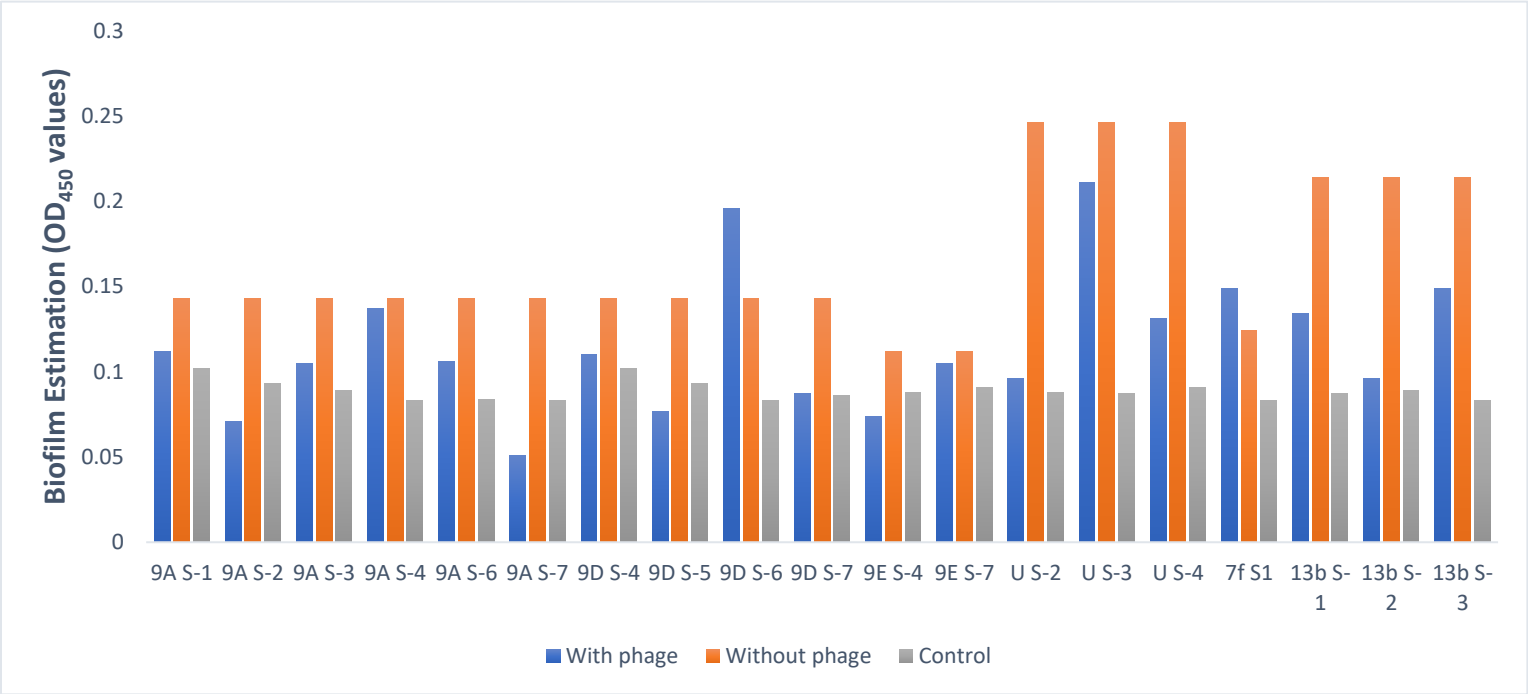

Figure 12: Biofilm estimation (OD_{450} values)

In the above chart from 19 phages, we can see that 9A S-2, 9A S-7 & 9E S-4 degrade biofilms. On the other hand, 9D S-6 & U S-3 phages seem to increase the biofilms formation. The other phages though show degradation of biofilm compared to without phages treatment, but that degradation was not significant compared to the control vials. Interestingly two of the phages significantly increase the formation of biofilms compare to control. All these results were taken from three independent experiments and statistical calculation was performed to see the difference were significant or not.

4.3.2. Qualitative Result

(a) (b) (c)

Figure 13: (a) Biofilm degradation by bacteriophage 9A S-7, (b) Biofilm degradation by bacteriophage 9A S-2, (c) Biofilm did not degrade by bacteriophage 9D S-6.

PHAGE CHARACTERIZATION

Characterization of 5 *Escherichia coli* Phages

4.3.3. Sensitivity of Phage to Temperature Treatment

The phage stock was incubated at different temperatures to assess the effect of temperature on the stability of the phage. The results revealed the phage titer did not change when it was incubated at 37°C. While the phage titers started to decrease at 55°C, until the phage completely lost its activity at 65°C. Only U S-3 survived at 65°C with pfu $6.2X10^{-10.}$

Table 4: Sensitivity of Phage to Temperature Treatment

37°C			
Phages	**Plaque number**	**Dilution factor**	**Phage count (pfu/ml)**
9A S-2	578	10^{-5}	$5.78X10^{-8}$
9A S-7	107	10^{-5}	$1.07X10^{-8}$
9D S-6	68	10^{-7}	$6.9X10^{-9}$
9E S-4	70	10^{-7}	$7X10^{-9}$
U S-3	97	10^{-8}	$9.7X10^{-10}$
55°C			
Phages	**Plaque number**	**Dilution factor**	**Phage count (pfu/ml)**
9A S-2	53	10^{-4}	$5.3X10^{-6}$
9A S-7	15	10^{-4}	$1.5X10^{-6}$
9D S-6	204	10^{-6}	$2.04X10^{-9}$

9E S-4	185	10^{-7}	$1.85X10^{-10}$
U S-3	62	10^{-8}	$6.2X10^{-10}$
65°C			
Phages	**Plaque number**	**Dilution factor**	**Phage count (pfu/ml)**
9A S-2	-	-	-
9A S-7	-	-	-
9D S-6	-	-	-
9E S-4	-	-	-
U S-3	74	10^{-5}	$7.4X10^{-6}$

4.3.4. THERMAL STABILITY IN GRAPH

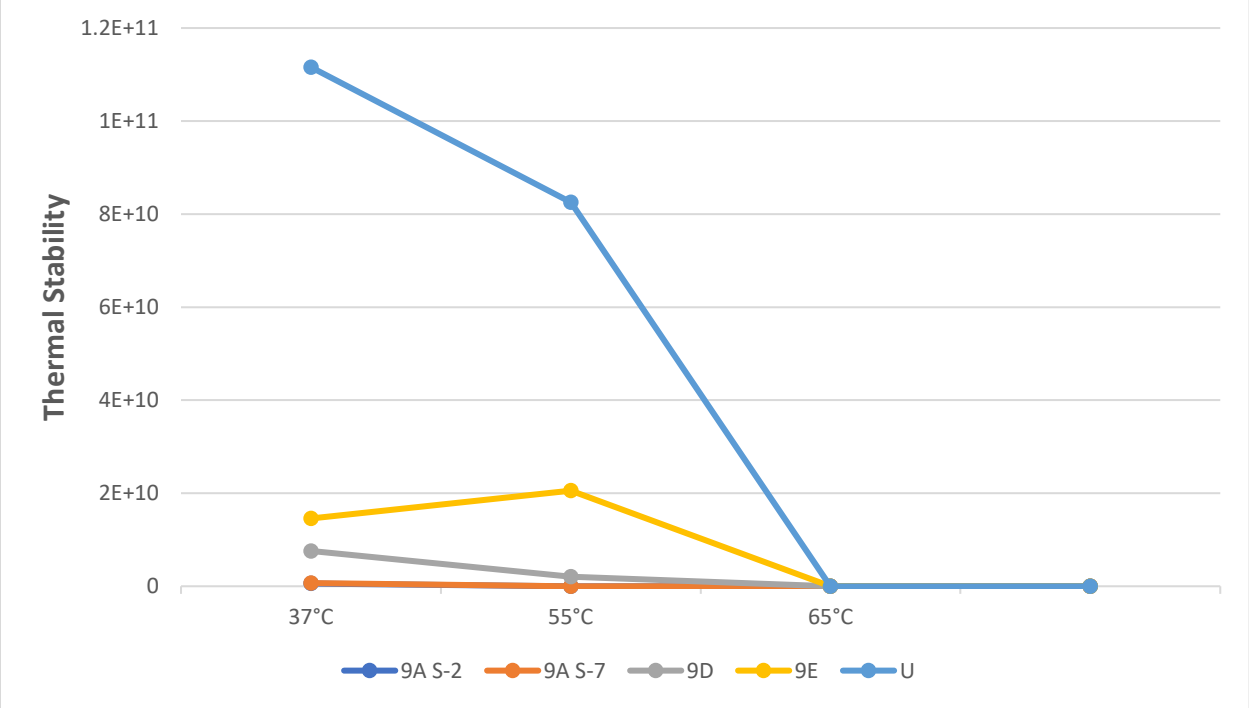

Figure 14: Thermal stability in graph

4.3.5. Sensitivity of phage to different pH

Phage sensitivity to pH was determined by incubating the phage with different pH. The results showed that the phage for 9A S-2, 9A S-7 & 9D S-6 was active at highly acidic 3 and they lost part of their activity at pH 7 also at pH 9 the phages were almost inactive.

For phage 9E S-4 & U S-3 the results showed that phages were active at highly acidic 3 pH value. On the other hand, they lost part of their activity at pH 9. At the same time, the maximum phage titer was recorded at pH 7.

Table 5: Sensitivity of phage to different pH

pH 3			
Phages	**Plaque number**	**Dilution factor**	**Phage count (pfu/ml)**
9A S-2	578	10^{-5}	$1.35X10^{-9}$
9A S-7	107	10^{-5}	$8.6X10^{-7}$
9D S-6	68	10^{-7}	$1.03X10^{-10}$
9E S-4	70	10^{-7}	$8.9X10^{-9}$
U S-3	97	10^{-8}	$4X10^{-10}$
pH 7			
Phages	**Plaque number**	**Dilution factor**	**Phage count (pfu/ml)**
9A S-2	135	10^{-6}	$7X10^{-8}$
9A S-7	86	10^{-5}	$7.3X10^{-7}$
9D S-6	103	10^{-7}	$8.8X10^{-9}$
9E S-4	89	10^{-7}	$1.03X10^{-10}$

U S-3	40	10^{-8}	$7.6X10^{-10}$
pH 9			
Phages	**Plaque number**	**Dilution factor**	**Phage count (pfu/ml)**
9A S-2	37	10^{-6}	$3.7X10^{-8}$
9A S-7	50	10^{-5}	$5X10^{-7}$
9D S-6	224	10^{-6}	$2.24X10^{-9}$
9E S-4	49	10^{-7}	$4.9X10^{-9}$
U S-3	47	10^{-8}	$4.7X10^{-10}$

4.3.6. pH STABILITY IN GRAPH

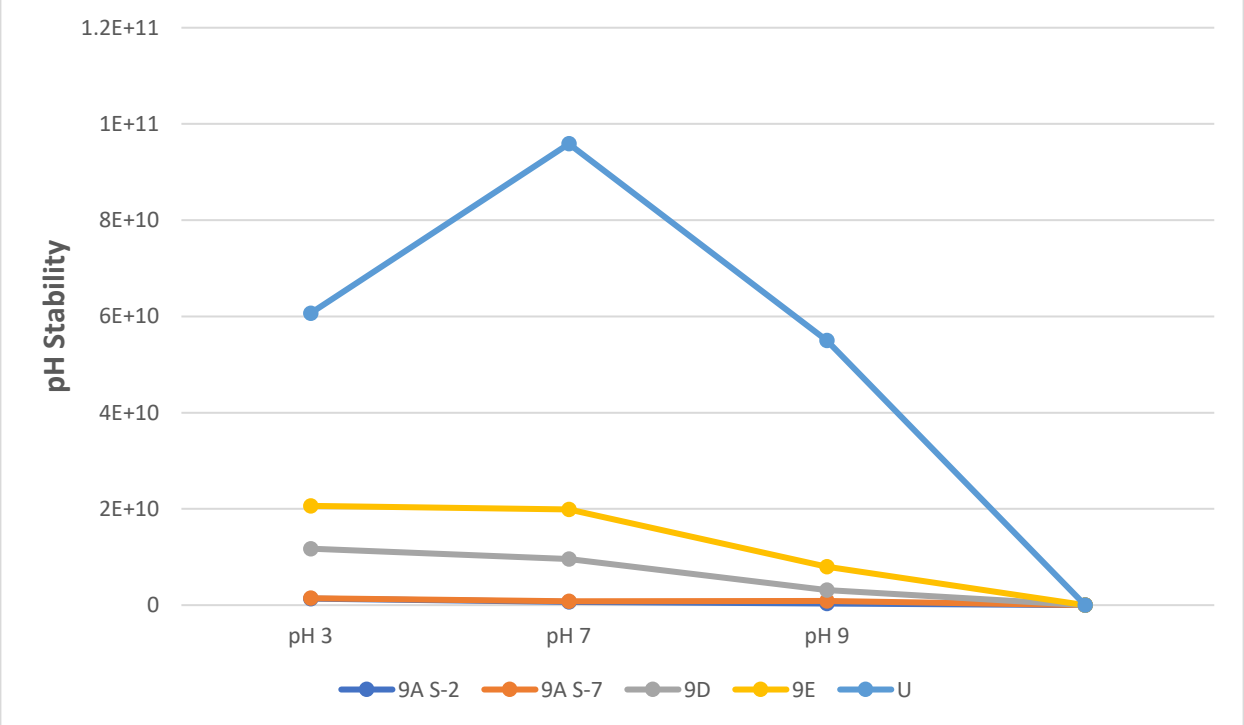

Figure 15: pH stability in graph

5. FUTURE PROSPECTS OF THE RESEARCH

Bacteriophages that degrade biofilms have the potential to play a substantial role in the epidemiology of *Escherichia coli* and Vibrio cholerae, two bacterial pathogens that build biofilms and cause serious infectious illnesses in humans. Biofilms are complex colonies of microbes that stick to surfaces and build a protective matrix, making them resistant to drugs and immunological reactions. They play an important role in the persistence and severity of bacterial infections because they can act as reservoirs for bacterial development and enhance infection dissemination. Bacteriophages, often known as phages, are viruses that infect and multiply within bacteria. Phages that target and degrade biofilms have been found and researched as potential therapeutic agents against biofilm-associated illnesses caused by *Escherichia coli* and Vibrio cholerae. In the case of *Escherichia coli*, which can cause urinary tract infections, foodborne diseases, and other infections, biofilm-degrading phages may aid in disrupting and eliminating biofilms created by *Escherichia coli* in a variety of contexts, such as medical equipment or food processing facilities. Phages may help prevent recurring infections and the transmission of *Escherichia coli* to various hosts by limiting the ability of *Escherichia coli* to build biofilms. Similarly, *Vibrio cholerae*, the causative agent of cholera, is known to form biofilms in aquatic environments, which can serve as a source of infection for humans. Biofilm-degrading phages may help to reduce the formation and stability of *Vibrio cholerae* biofilms in water sources, potentially decreasing the risk of cholera outbreaks in endemic areas. However, it's important to note that the use of phages as therapeutic agents against bacterial infections, including those associated with biofilms, is still an area of active research and development. Further studies are needed to better understand the safety, efficacy, and long-term effects of phage therapy, including biofilm-degrading phages, in the context of epidemiology and public health. Regulatory considerations and potential challenges in phage therapy, such as phage resistance and host immune responses, also need to be addressed. Nevertheless, biofilm-degrading phages hold promise as a potential tool in the fight against biofilm-associated infections caused by *Escherichia coli*, *Vibrio cholerae* and other bacterial pathogens.

6. CONCLUSION

Bacteriophages possess unique properties and show considerable promise in the control of biofilms. However, such applications are still evolving, and large-scale uses are still under development. Thus, the identification of the most effective approaches must be, at present, speculative in nature also in time and as more results are published, best practices for such uses will emerge. This work aimed to isolate and characterize *Vibrio Cholerae* & *Escherichia coli* specific lytic bacteriophage from Gulshan Lake water samples in four months in an attempt to isolate a therapeutically potential phage and to find biofilm degrading phages. This study led to the isolation of biofilm degrading bacteriophage from a water sample which was further characterized to identify its stability under the different adverse condition. The result of those experiments demonstrated that *Escherichia coli* phage is stable under pH fluctuation and can endure moderate temperature. The stability of biofilm degrading *Escherichia coli* under different conditions makes it a worthy candidate for further study to find out whether *Escherichia coli* can be used for the treatment of *Escherichia coli* related infections.

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