Rapid detection of viable *Vibrio Cholerae* by Bacteriophage

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

Department of Mathematics and Natural Sciences

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Declaration

It is hereby declared that,

1. The report submitted is our work while completing a degree at BRAC University.

2. The report 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 report does not contain material that has been accepted or submitted, for any other degree or diploma at a university or other institution.

4. We have acknowledged all main sources of help.

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Approval

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Dedication

“Dedicated to our friends and well-wishers

who was always there for us

blessed to have them all”

Ethics Statement

The entire research work has no conflict of interest. No human and animal models were used in this experiment.

Acknowledgment

This dissertation is very special to me since we have studied meticulously to choose this topic of research. It may have been a small step but a great leap towards fulfilling our research passion. We needed help and encouragement during repeated failures in conducting the experiments. Fortunately, several people around us provided the needed support. Firstly, we would like to thank my parents and the Almighty Allah (SWT) for their constant moral support during our research work.

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Before concluding, we would like to share that this thesis study could not be completed due to the ongoing corona lockdown, load-shedding and so few works were left pending. We are still hopeful about the cessation of the corona pandemic and may we stay safe to fulfill our long-cherished works and contribute to the betterment of humankind and the environment.

List of Acronyms

V.cholerae	Vibrio cholerae
DNA	Deoxyribonucleotide acid
RNA	Ribonucleic acid
DNAse	Solution Deoxyribonuclease Solution
RNAse	Solution Ribonuclease Solution
ds	Double stranded
ss	Single stranded
CRISPR	Clustered regularly interspaced short palindromic repeats
rpm	Rotation per minute
EDTA	Ethylenediaminetetraacetic acid
RAST	Rapid Annotation using Subsystem Technology

NCBI	National Center for Biotechnology Information
bp	Base pair

Keywords:

Cell viability. Detection methods. Foodborne pathogens. Rapid methods. Viable-but-non-culturable.

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Abstract

For the sake of food safety and public health, it is crucial to be able to quickly identify live viruses in food. Traditional methods of establishing microbial viability, known as “culture-based detection methods” can be difficult, time-consuming, and slowly produce outcomes. In recent years, several culture-free techniques to identify live viruses have been published, including both based on nucleic acids (PCR combined with the use of cell viability dyes or reverse-transcriptase PCR to detect messenger RNA) PCR/qPCR, immunoassay, or enzymatic assay to detect host, as well as phage-based (plaque assay or phage amplification and lysis plus DNA, phage offspring, or intracellular elements) techniques. When compared to culture for food testing, several of these more recent techniques, notably phage-based techniques, offer promise in terms of spathe ed, sensitivity of detection, and cost. This study examines the existing limitations and potential future applications of these innovative technologies for food testing as well as their advantages and disadvantages for identifying live pathogens in food.

Chapter 1

1.1 Introduction:

Cholera is still a major public health problem in our countries. Infected water sources, such as private wells, lakes, rivers, and other bodies of water, can contain E. coli, Salmonella, or Vibrio. Waste can enter water bodies through sewage, broken sewage systems, contaminate stormwater runoff, and agricultural runoff, to name a few. According to studies, consuming coliforms like Escherichia coli, Salmonella species, and Vibrio contaminated water can have serious consequences like diarrhea, enteritis, and even death, resulting in high economic losses. One of the naturally occurring bacteria in aquatic habitats, the Vibrio genus is crucial to the preservation of the aquatic ecosystem. C

By isolating V. cholerae from stool cultures, cholera can be diagnosed. Traditional culture techniques may take three days or longer to isolate and identify V. cholerae, and they also require a good laboratory setup and knowledgeable personnel. Ineffective public health measures, the spread of the disease, and an increase in morbidity and mortality are just a few of the negative effects of delayed cholera outbreak detection. It is essential for the prompt implementation of interventions to identify and confirm cholera cases as soon as possible.

A gram-negative, facultative anaerobe and comma-shaped bacterial species are known as ***Vibrio cholera***. The most reliable method for finding, isolating, and identifying Vibrio cholerae in food and water is still culture methods. Along with culture, other techniques for finding Vibrio cholerae in water include most likely numbers, ELISA, and PCR. It has a single polar flagellum that allows for high motility, is highly halophilic, typically has a rod-like shape that can be straight or curved, and is extremely acid sensitive. C

Frederick Twort originally detected bacteria-inhibiting particles known as phages in 1915. However, D'Herelle, who was the scientist to isolate and explain phages in 1917. Furthermore, He was able to develop first phage therapy for the treatment of chicken typhoid, which is caused by Salmonella Gallinarum. The process of developing this research on the possible application of infections which able to destroy microorganisms in treating diseases in humans and other animals has been aided by the beneficial effects of bacteriophages in combating bacterial contaminations.

Bacteriophages are viruses that infect bacteria, and research into these phages is an area that is rapidly expanding and has promising prospects. (2017) (Doss et al.) Lake or river ecosystems are heavily exploited for recreational and daily domestic needs in temperate climate zones. According to several studies, anthropogenic activities are the main source of contamination in all ecosystems (Yunus et al., 2020). One of the most significant environmental issues in many nations around the world is water contamination (Xu et al., 2020). Following decades of development, water contamination has increased, mostly as a result of agricultural runoff and hazardous industrial effluents (Purohit et al., 2020). Numerous industries grow in the suburbs, producing a sizable amount of sewage annually. Ineffective sewage also contributes to secondary water pollution.

The urgent problem of water and foods scarcity needs to be resolved. Also receiving more attention is the safety of drinking water. Examining bacteriophage's potential as a bacterial control tool that might be used to manage contaminated water is the main goal of this thesis. This study also examines the potential uses and restrictions of phages used in preventing water pollution. It was also noted that phages can be used to lyse bacteria, find the sources of pollution, and perhaps even control water source pollution. Next, some of the phage-based technologies' drawbacks were discussed, and potential fixes were offered.

1.2 Phage Therapy Benefits

Phage therapy has a beneficial way for treatment over the antibiotics for human. The main objective of phage is to select the pathogenic bacteria for isolating them from the body without harming the body’s commensal microbiome. Phages also maintains the specificity in high value while in actions. For isolation, depending on bacteria, phages are found in environments with their selected bacteria. In modern civilization, biological science and genetic engineering are developing enormously, so clinical treatment is also developing with this. the ratio of growth of microorganisms is directly proportional to the phage multiplication. It is anticipated that phages will come out form the cell without hampering, because they cannot grow inside the microorganism host.

The life cycle of bacteria cells is closely correlated with that of a group of infections known as bacteriophages. They are referred to as bacterial parasites because they lack the cell structure and chemical structure required for food uptake, protein synthesis, or the development of new particles. As a result, these creatures can only replicate within a living cell.

1.3 Bacteriophage Life Cycle

Lysogenic activity, which includes joining the bacteriophage's genetic material with the bacterial chromosome and replicating it in a characteristic of the bacterial DNA, results come of a prophage. Lytic activity, which is typical for destructive phages, is distinguished from this type of activity against the bacterial cell. Bacteriophages engage in adsorption, which includes

binding to the bacterial cell, and phage proteins which were identified receptors on bacterial cell surface, for example - teichoic and lipoteichoic acid for Gram-positive microbes or LPS for Gram-negative microbes. The bacteriophage proteins that make up the penetration also allow the hereditary material to enter the host cell through the cracks in the cell wall.

The eclipse stage follows, which involves the replication of the proteins and nucleic acids that make up the structural part of the capsid while bacterial DNA replication is suppressed. By following, the bacteriophage's structure and growth, bacterial cell's lysis, and appearance of daughter phages ready to infect different cells. The bacteriophages T1 and T4 are examples of those that go through the lytic cycle. The lysogenic cycle involves positioning the prophage, incorporating genetic material with the host genome, and directly coordinating it, bacterial chromosome. Bacteriophage's genome enters a state of inertness and replication is prevented. This state may be disrupted right away or ultraviolet rays, alkylating agents, or specific anti-microbials like mitomycin . for examples, bacteriophages with a lysogenic cycle are Escherichia coli, Mu, which moves against E. coli, Salmonella, Citrobacter, and Erwinia, MM1 S. pneumonia, and 11 S. aureus.

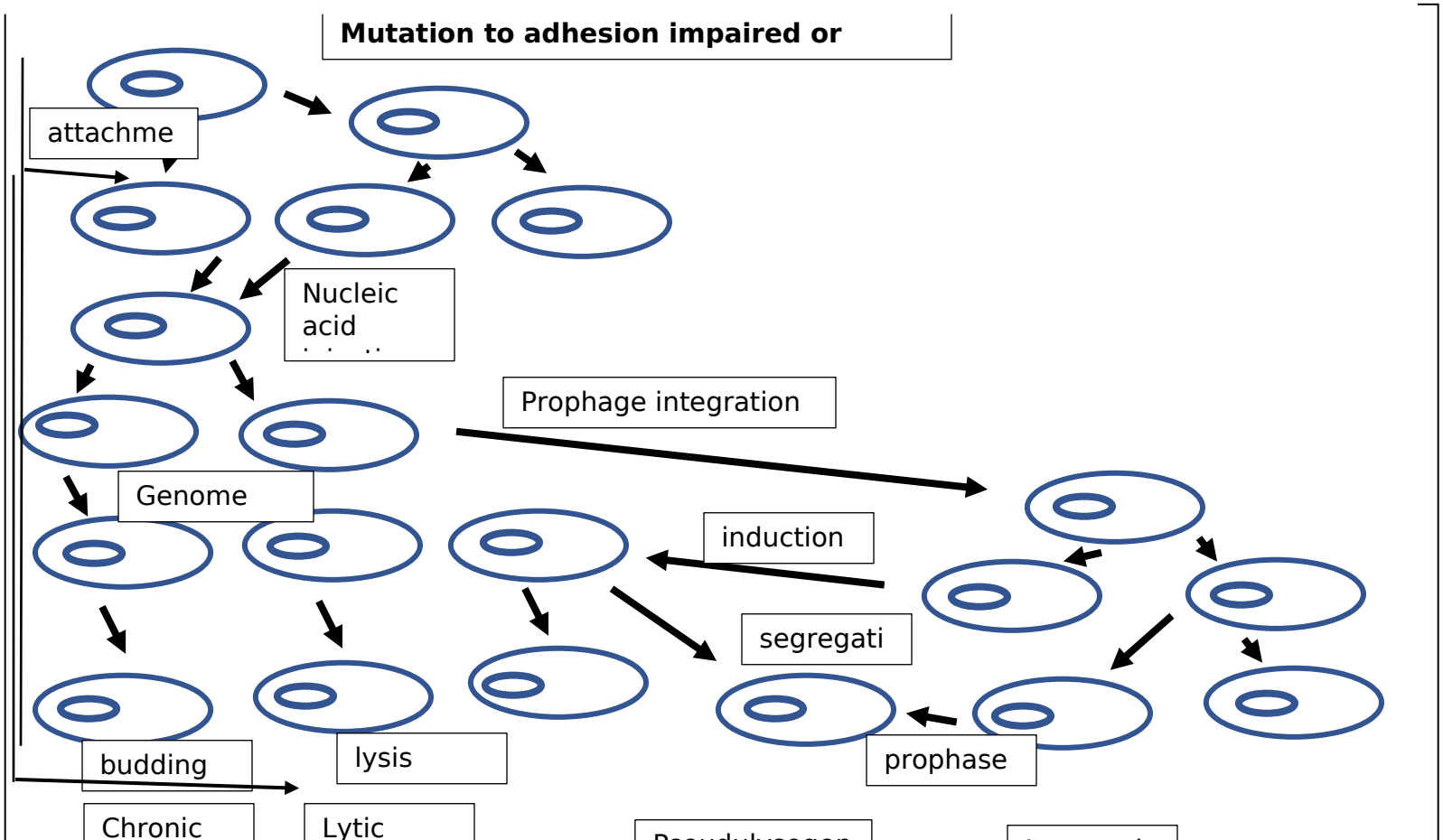


Figure 1: life cycle of Bacteriophage

1.4 Category of Bacteriophages:

Under the electron microscope, the viral nature of bacteriophages was discovered in the early 1940s. Lwoff, Horne, and Tournier published the orders Urovirales for tailed phages, Microviridae for filamentous phages, families for Inoviridae, and also X-type phages based on morphology and nucleic acid type.

The six basic phage types Bradley identified in 1967—a. tailed, b. filamentous, c. icosahedral phages with single-stranded DNA (ssDNA) or single-stranded RNA (ssRNA)—remain the cornerstone of phage classification. Later, based on morphology and different nucleic acid types, the International Committee on Taxonomy of Viruses (ICTV) divided phages into **six** genera.

1.5 Bacteriophage structure and infection:

Bacteriophages reproduce by contaminating the host cell. The life cycle of the page is the collective name for the steps in this process. While some phages alternate between the lytic and lysogenic cycles, others replicate via the lytic stage. The rotation of lytic cells and the lysogenic phase presents an opportunity for the lambda phage to infect bacteria.

1.6 Lytic and lysogenic in their modes of action

Both lytic and lysogenic cycles of bacterial contamination occurred, depends on the type of bacteriophage. Lytic bacteriophages are frequently referred to as virulent phages because they replicate viral genetic material inside the cytoplasm of their hosts to attack their metabolic processes. Due to the increased production of lytic phages, the bacterium's cells are lysed as a result.

Conversely, lysogenic bacteriophages—also known as temperature bacteriophages—replicate later on without breaking the host.

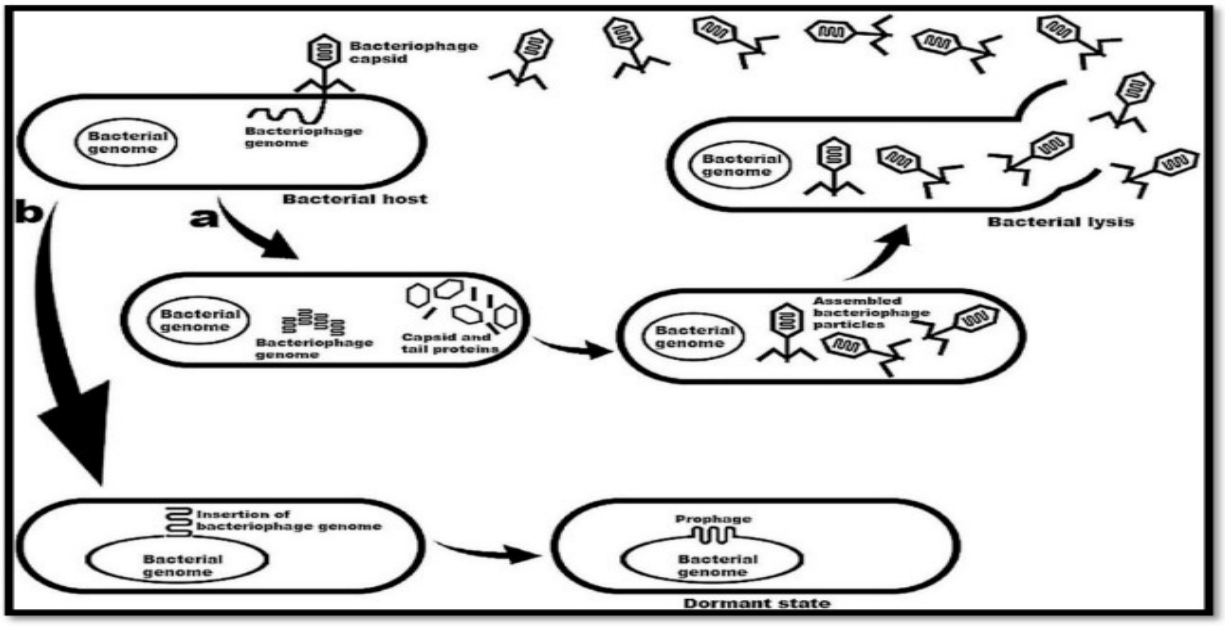


Figure 2: Lytic(a), Lysogenic(b) infection of Bacteriophage (Lilium K, 2018)

1.7 Cholera's origins and past pandemics

The contagious Vibrio cholera bacterium is the culprit behind the severe diarrheal illness known as cholera. Around the world, cholera is acknowledged as a serious public health issue, and there have already been seven cholera pandemics. Before it was recognized as a disease, Gaspar Correa, a Portuguese historian and the author of Legendary India, explained several indications as a "**moryxy**" epidemic that occurred in the Ganges Delta in 1543. Seven cholera pandemics have been reported globally in the last 50 years. According to the 2017 WHO Cholera Annual Report, the first six pandemics started on the Indian subcontinent before spreading to South Asia, Africa, the Middle East, Southern Europe, and finally South and Central America.

The seven pandemics (7P) that have occurred in the last two centuries, primarily from 1961 to the present, have their origins in Indonesia. The ongoing pandemic also includes the more recent outbreaks in Haiti and Yemen.

1.8 Possibilities of Phages

Phages investigated as model organisms to learn more about fundamental genetic ideas like viral gene expression. We have gained a great deal of knowledge about viruses and many basic principles of molecular biology thanks to research on bacteriophages. C

1.9 choosing rapid detection of phages

It is critical to quickly identify Vibrio cholerae during epidemics. Moreover, conventional methods are more time-consuming and expensive. This study's goal was to create a quick, affordable, and highly sensitive method for identifying Vibrio cholerae. To achieve this, based on PCR detection method for identifying bacterial DNA.

In remote locations with sparse laboratory resources, rapid detection tests (RDT) are promising tools for early detection of the pathogen V. cholerae. The WHO's cholera investigation kit includes RDT kits because of their significance. In suspected water/food samples, a V. cholerae test is required for epidemiological purposes. As a result of the lack of skilled labor, media, reagents, equipment, electricity, etc., culture techniques are frequently challenging to establish in cholera outbreak settings. RDT can be thought of as an investigative tool in these situations. For choosing rapid detection test because it has several features, for example - Low price, speed, ease of use, less complexity in sample processing, handling of equipment, steady supply from the manufacturer, long shelf life in ambient conditions, etc. This test gave 38% less positive compared with culture method and not consistent when other organisms prevail in the sample. moreover, the disadvantage of traditional methods for DNA isolation is that they frequently take a long time, are difficult to automate, or are ineffective for downscaling to small sample volumes. These methods are either column-based techniques or include precipitation and centrifugation steps with toxic organic solvents. Through the magnetic bead technology these main limitations can be avoided.

The polymerase chain reaction (PCR) is a popular technique for quickly producing millions to billions of copies of a specific DNA sample, enabling researchers amplify it to a level that allows for in-depth analysis. It is the most basic process for using in genetic testing and research. It also helps in ancient samples of DNA too. It is now common in every medical research of varieties

were also biomedical research and criminal forensics, infectious diseases applications, medical and diagnostic,

Through PCR, Thermal cycles, which make up the majority of the 20–40 repeated temperature changes, these are made up of two or three distinct temperature steps. The steps are – sample collection, initialization, denaturation, annealing, elongation, final elongation. In every step, temperature, time, cycles are maintained by which user demands. By amplifying multiple cycles, the targeted DNA can be made from millions copies to billions. This copying starts to calculate with the formula of 2^{n}, where n is the number of cycles. These cycles happen from the process of denaturation to elongation. Lastly, the in the final step, it cools the reaction chamber for an indefinite time by maintain the temperature between 4-15^{o}C.

For detection of V. cholerae, the presence of these organisms in environmental samples can also be determined directly molecularly using the polymerase chain reaction (PCR). These techniques work by identifying species-specific nucleotide sequences, amplifying the desired nucleic acid, and then visualizing the result on an agarose gel. Environmental factors like water temperature, salinity, conductivity, turbidity, and pH should be noted when collecting specimens.

The disadvantage of traditional DNA isolation techniques is that they frequently require the use of toxic organic solvents, require precipitation and centrifugation steps, and are not suitable for scaling down to small sample volumes. So, this Hybrid capture using magnetic particles, which relies on selective isolation of target DNA by hybridization to oligonucleotide probes linked to magnetic nanoparticles, makes the sample preparation quick and highly effective for detection. This would reduce the overall detection time, increase PCR sensitivity, and remove the majority of the inhibitors of the amplification reaction as well as excess non-target DNA.

Chapter 2

Literature Review

It is very important to have the ability to identify viable pathogens in food as fast as possible which is the sake of food safety and public health. Culture based detection method is time consuming but widely used method. Recently, different types of culture independent techniques

are used for identifying viable pathogens along with PCR combined with cell viability dyes or reverse transcriptase PCR. Also, phage-based techniques like plaque assay or phage amplification method. Sensitivity of detection and cost when compared to culture for food testing this phage-based techniques in particular show promises. (Acs. et al. 2020)

According to recent study, for the bacteriophage amplification technique a protocol was developed. For quantitative detection of viable *Listeria monocytogenes* cells using the A511 listeriophage with plaque formation was developed by end-point assay. Laser and toluidine blue O (TBO) were used for selective virucidal treatment for destruction of exogenous bacteriophage. (Oliveira et al. 2012). This method takes about 10-hour assay duration and constitutes an alternative for rapid, sensitive and quantitative detection of L. monocytogenes.

In this study, virucidal laser treatment was demonstrated better protection for the listeria cells than other agents which were previously tested. It was faster and easier to perform than other standard procedure. In food industry this protocol will be very helpful but it will be a bit costly.

There are many applicable methods but we choose to work on PCR as rapid detection protocol due to lower the cost and reduce the time period. This specific method will be applicable in food industry as molecular method cannot discriminate between viable and non-infectious phages. There are many applicable methods but it is necessary to take care of some specific factors like-infectious phage, whole phage particle or nucleic acid etc. Additionally, the concept behind the technique to avoid misinterpretations which is a major factor. (Farkas. Et al. 2015)

Chapter 3

Methodology

3.1 Materials and Method

Standard laboratory practices

- After plating into a petri dish, the remaining media must not be discarded. The extra media-filled container must be sealed properly with masking tape and should be kept in the refrigerator for future use.
- Culture plates must not be kept in an incubator for more than two days.
- Must keep track of prepared culture plates and media.
- Reagent must keep to its assigned shelf.
- One must wear a lab coat while doing laboratory work. However, wearing a lab coat outside the laboratory should be prohibited.
- The laminar hood surface must clean before and after work.
- While using a spirit lamp or any kind of flammable component, one must be very careful.
- Proper treatment must be taken as any kind of burn or cut occurred.

To start the whole process, it is important to design a proper protocol and identify the requirements for research work. The whole research was conducted in the Environmental Life Science Laboratory of BRAC University. Three Vibrio cholerae strains- WT346, WT333, WT324 and two bacteriophage strain- JSF35, JSF-7, which is a specific phage used in this project. The Vibrio species has grown in Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar and Luria Bertani agar has been used for stocks. However, to conduct this research WT346 has used mostly and the specific phage host JSF35, JSF-7 was used for purification and amplification of the phage.

3.2 Materials requirement

Solid medium and soft medium both are prepared with LB and agar media but mixed in different ratios for plate preparation and lawn culture respectively. To prepare solid media, 1.5% agar was mixed with LB. However, 0.6% agar was used to prepare soft agar media. TCBS Agar was prepared with the commercially available medium. Moreover, a .22µm syringe filter was used to separate bacteriophage from bacteria in the media.

Material	Usage
Bacteriological Agar	Culture plate preparation
Luria Broth	Growth medium
TCBS Agar	Selective media for *Vibrio Cholerae*
SM buffer	Phage stock solution

Table 1: materials requirements

3.3 Luria-Bertani, Miller Broth (LB) preparation

LB is a nutrient-rich liquid broth media used for growing bacteria. Tryptone, NaCl, and Yeast extract are the components of LB media. We used the commercially available LB which was accessible in our laboratory.

3.4 Preparation

I. Reagent was measured through a fine analytical balance machine. 10gm of LB reagent powder was taken with a fresh spatula and measured on foil paper.

II. 500ml of distilled water was measured by using the measuring cylinder and then taken into a clean conical flask.

III. Reagent was mixed with distilled water and then heated with a burner.

IV. After boiling, it was given for autoclave at 15°C and 121 PSI for two hours approximately. The purpose of this is to sterilize the media.

V. As the autoclave is done, LB media was taken out and kept outside for cooling it down so it is ready to use.

3.5 Preparation of Thiosulfate-Citrate-Bile Salt-Sucrose (TCBS) Agar

TCBS agar is differential selective media for Vibrio and its other species, to isolate and cultivate them. The components of TCBS agar are- sodium thiosulphate, sodium citrate, protease peptone,

yeast extract, bile, sucrose, ferric citrate, sodium chloride, bromothymol blue, thymol blue, and agar. Ready to make reagent was available here which was used in this project.

3.6 Preparation

I. 44.54 grams of powder medium were weighted on foil paper by a balance machine.

II. 500 ml of distilled water was measured through a measuring cylinder and taken into a conical flask to make it a suspension.

III. Stir the suspension with a glass rod and the heat was applied till boiling and dissolved the solution properly.

IV. This media does not need any autoclave.

3.7 Luria Agar (LA) preparation

LA media is necessary for the streaking of the inoculated stock bacteria. It is a non-selective powdered media that is mainly used for routine culture. This medium is rich in nutrition for the growth of pure culture strain. Commercially available readymade LA media was used which contains- tryptone, yeast extract, sodium chloride, and 1.5% agar.

3.8 Preparation

I. 16gm of LA powder was measured through an electronic balance machine and 400ml distilled water was mixed with it to prepare 400 ml media.

II. The mixture was heated on a Bunsen burner to dissolve the media completely.

III. Then it will be autoclaved for 2 hours to sterilize.

IV. As the autoclave is done, then the media is poured into a sterile Petri dish inside a laminar hood and waited till it solidifies.

3.9 Preparation of Soft Agar

To perform a spot test or phage titer count soft agar is an essential media. To prepare 100ml soft agar 1gm NaCl, 1gm Tryptone, 0.5gm of yeast extract, and 0.6gm bacteriological agar were measured in an electronic balance machine. After that, it is suspended in 100ml of distilled water. Then the mixture is heated with a Bunsen burner to dissolve the media properly. It was then poured into test tubes at 5ml approximately. Lastly, the test tubes containing soft agar were autoclaved for 2 hours.

3.10 SM Buffer Preparation

To prepare 50 ml Salt Magnesium (SM) buffer, 0.2 gm NaCl, 0.1 gm $MgSO_4$, 1M 2.5ml Tris-HCl and 0.5gm gelatin is required. To prepare SM buffer, firstly, approximately 30 ml Tris-HCl needs to be prepared. From that, 2.5ml Tris-HCl was used for the SM buffer. To prepare Tris-HCl, Tris base needs to be prepared and then pH is needed to be checked if it is lowered to 8 from 10 then a drop of HCl is added. As 1ml Tris-HCl is 121.14 g/mol, 3.63gm Tris was added to 30ml distilled water to prepare the base, and the pH value is kept at 8 before autoclave as after autoclave is done the pH will drop to 7.5. Then, 0.2 gm NaCl, 0.1 gm $MgSO_4$, and 0.5 gm gelatin were measured with an electric balance machine and added with 47.5ml distilled water. After that, 2.5 ml Tris-HCl was added and that is how 50 ml SM buffer was prepared. Lastly, Tris-HCl and SM buffer are given for autoclave and are stored at room temperature.

3.11 Bacteriophage identification from a stock sample-

Bacteria strain WT346, which is JSF-35 and JSF-7 phage specific host was streaked in an LA plate and then incubated overnight at 37°C. to prepare young culture or logarithmic phase cells of WT346, a few single colonies from the overnight culture plate were mixed with 3ml freshly prepared LB. The suspension was then positioned in a shaker incubator at 37°C for two hours. Afterward, 300µl logarithmic phase cells of WT346 were added with 5ml of 0.6% soft agar and poured on freshly prepared LA plates. It will take 10 to 15 minutes to dry up the plates. To prevent the stored phage from any kind of bacterial contamination, a .22µm syringe filter was

used to filter and prevent any kind of bacterial contamination. Lastly, 10µl of pure phage solution was topped on the specific bacteria poured LA plate and kept for 20 minutes to dry up. The plates will be kept in an incubator temperature at 37°C overnight. The whole procedure is for confirming the phage presence in the stock solution which can be sure if a clear plaque is observed on the double layer plate. (Abedon et al. 2021)

3.12 Enrichment procedure for pure phage

To enrich the pure phage JSF-35 and JSF-7, firstly the host bacteria WT346 was streaked on LA medium plate and incubated at 37°C overnight. From the overnight grown WT346 few single colonies were taken and inoculated in 3ml of LB medium and incubated for 1.5 to 2 hours in a shaker incubator at 37°C. After the incubation period, the turbidity of the bacterial solution was checked and ensured logarithmic phase cells as the suspension was slightly turbid. Then 100µl phage solution was added to the young bacterial culture of WT346 and incubated for 4 to 6 hours at 37°C in a shaker incubator. During this incubation period, the phages infect the host bacterial cells by increasing their number. As the incubation period ended, the solution was centrifuged at 13000rpm for 5 minutes. Then the supernatant was collected by a fresh syringe and filtered through a .22-micron syringe filter. This sterilized filtered clear suspension which is completely free from any bacterial cells was then stored in an autoclaved fresh falcon tube and wrapped with parafilm. To get the desired phage titer, enrichment must be done a couple of times following the same procedure. The phage-enriched solution must be stored at 4°C.

3.13 Preparation of phage stock solution

To carry out further experiments, once the bacteriophage has been confirmed and the enrichment procedure is done phage stock solution must be prepared. Firstly, take a single plaque from a bacterial lawn LA plate by using a sterile pipette tip and then place it in SM buffer (100 mM NaCl, 8.1 mM $MgSO_4$, 0.5 mM Tris-Cl [pH 7.5]). To widen the diameter, the pipette tip was cut and the cut plaque was put into SM buffer through the micropipette. This suspended plaque was then vortexed for 5 minutes to release the phages from the plaque agar medium. Then this suspension was centrifuged at 4000rpm for 5 minutes at room temperature which results in the agar to stable as pellets and the phages were suspended in the supernatant. This supernatant is

collected into a fresh microcentrifuge tube and then one-third of SM buffer's volume chloroform was added. Chloroform is added because it evaporates from the solution during storage and leaves only the pure phages within the clear suspension. However, after adding chloroform the solution was gently vortexed and this pure stock solution was stored at 4°C.

3.14 Titer determination of Bacteriophage (DLA Method)

To hold a clear idea about the bacteriophage count it is necessary to determine the titer or the concentration of bacteriophage is known as the DLA method or Double Layer Agar method. Firstly, a few single colonies of the suitable host were taken in a falcon tube containing LB medium and this will be gently mixed with vortex. This will be cultured for three hours in a shaker incubator to get the young bacteria. Then 300µl LB medium containing young culture bottlewasher mixed with fresh .8% soft agar along with, bacteriophage which was diluted until 10^{-10} and 100µl equal volume of phages were diluted in the Eppendorf tube. After this, the mixture was poured onto LA plates. Separate dilution was plated in separate plates. These plates were kept inside a laminar for 15 minutes for drying up. These dried plates were incubated at 37°C overnight. After the incubation period is over, single plaques were observed and then counted to determine the titer by using PFU (Plaque Forming Unit) formula. (Stachurska et al. 2021) Each plaque is considered as on PFU and the titer (PFU/ml) is calculated by using the given formula below-

$$\frac{\text{PFU x Dilution factor}}{\text{Volume of phage lysate (ml)}}$$

3.15 Burst out time identification of bacteriophages

For analyzing the burst out time of bacteriophage strain JSF-35, JSF-7, bacterial strain WT-346 was used as A host. A few single colonies of the host bacteria were cultured in an LB medium for 1.5 to 2 hours to get the young bacteria. 300µl of young bacteria and 100µl diluted bacteriophages were passed to a separate Eppendorf tube. Then after, the mixture was cultured in separate vials for 10, 15, 20, 25, and 30 minutes in a shaker incubator at 37°C. And from the individual vials, a 100µl mixture was taken and mixed with soft agar by a gentle vortex. It was

then poured onto an LA plate and dried for 30 minutes to get the bacteriophage absorbed. After overnight incubation at 37°C plaques can be counted at different times.

Rapid Detection of the Presence of JSF-35, JSF-7 Bacteriophage

3.2.1 DNA Extraction by Boiling Method

According to the results of phage titer count, JSF-35 and JSF-7 was prepared for DNA isolation and the PCR procedure. Subsequently, the selected bacteria WT346 were inoculated in individual vials containing autoclaved LB broth and then incubated overnight at 37°C in the shaker incubator. The next day, from each vial 500µl of the culture, was taken and mixed with 100, 110, 120, and 130µl of phages are transferred onto the individual vial and a gentle vortex is given. After about 20 minutes of incubation in the shaker incubator at 37°C, the solution was transferred into a sterile Eppendorf tube. And each quantity containing the Eppendorf tube was subjected to boiling for 50°C and 60°C one after another for about 10 minutes. Then the mixture was taken for immediate cooling at -20°C and these extracted DNA collections were stored there until further use.

3.2.2 Performance of the Polymerase Chain Reaction

To determine the rapid detection of the JSF-35 and JSF-7 bacteriophage strain PCR was carried out on the respective samples of DNA which had been previously extracted. A total of 10 pairs of forwarding and reverse primers were prepared. The sequence of the primers JSF-35 and JSF-7 in the pair was respectively: Forward primer 5'-AGACAAGCGAAGAGGGTTGA-3' and Reverse primer 5'-ACGTTGAACGGTAGGATTGC-3', Forward 5' – TGCTTTCTTTGGCTCCAAGT – 3' and Reverse 5' – GAAATGGAAGGGCATCTTCA – 3'. Firstly, 1µl of nuclease-free water was added to each Eppendorf tube to avoid DNA degradation and then 10µl of the master mix was added. The final volume of the total mixture in each PCR tube will be 20µl as templates for PCR is 5µl which was extracted previously. The whole process was performed in a thermal cycler machine.

The condition for PCR is given below-

Primer	Primer sequence	Condition for	Number of	Target band

		PCR	cycles	size
JSF-35	HMR F 5'-AGACAAGCGAAGAGGGTTGA-3' HMR R 5'-ACGTTGAACGGTAGGATTGC – 3'	94.0°C for 4 minutes 94.0°C for 30 seconds 52.0°C for 30 seconds 72.0°C for 60 seconds 72.0°C for 10 minutes	30 cycles	500bp
JSF-7	F 5' – TGCTTTCTTTGGCTCCAAGT – 3' R 5' – GAAATGGAAGGGCATCTTCA – 3'			

Table 1: condition for PCR

3.2.3 Preparation of Gel Electrophoresis

PCR amplicons will be viewed by 1% agarose gel electrophoresis. For the sizing and quantification of the PCR products, a 1kb DNA ladder was used. The whole procedure was performed in Biometra Electrophoresis Machine. For visualization under the UV

spectrophotometer 8µl of Ethidium Bromide was added with the loading gel. For running buffer 1X concentration of TAE buffer was used. No extra loading dye was needed because the master mix was green-colored dye. The whole process of gel run was conducted for 45 minutes and 70 volts were given. Later on, the results were noted down.

Chapter 4

Result

4.1 JSF-35 and JSF-7 Phage identification From the Stored Sample

With the specific host WT-346, JSF-35 and JSF-7 phage was used and the soft agar overlay method was conducted which showed clear plaque. The given picture below supports the confirmation of JSF-35 and JSF-7 phage.

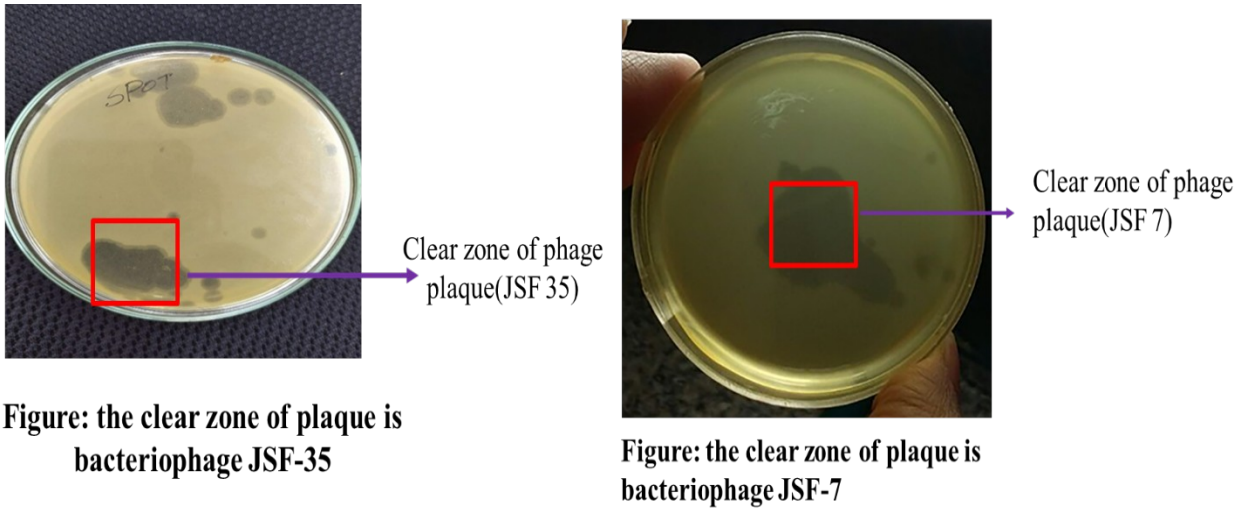


Figure: the clear zone of plaque is bacteriophage JSF-35

Figure: the clear zone of plaque is bacteriophage JSF-7

Figure 3: Phage JSF-35, presence is checked in the stored sample shown in the figure

4.2 Phage DLA result

A couple of enrichment, phages showed increased PFU/ml. The DLA assay was conducted on host bacteria (WT-346) strains, and countable plaques were obtained in one strain that is- WT-

346. Several plaques varied in different dilutions and few to an uncountable number of plaques are observed.

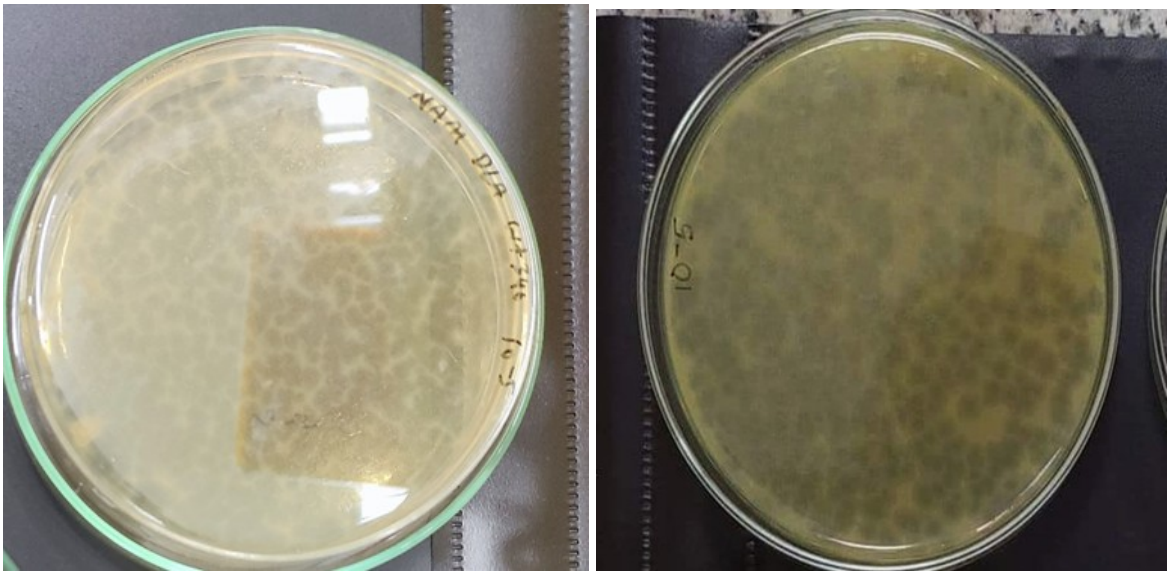


Figure: 4.2(A)

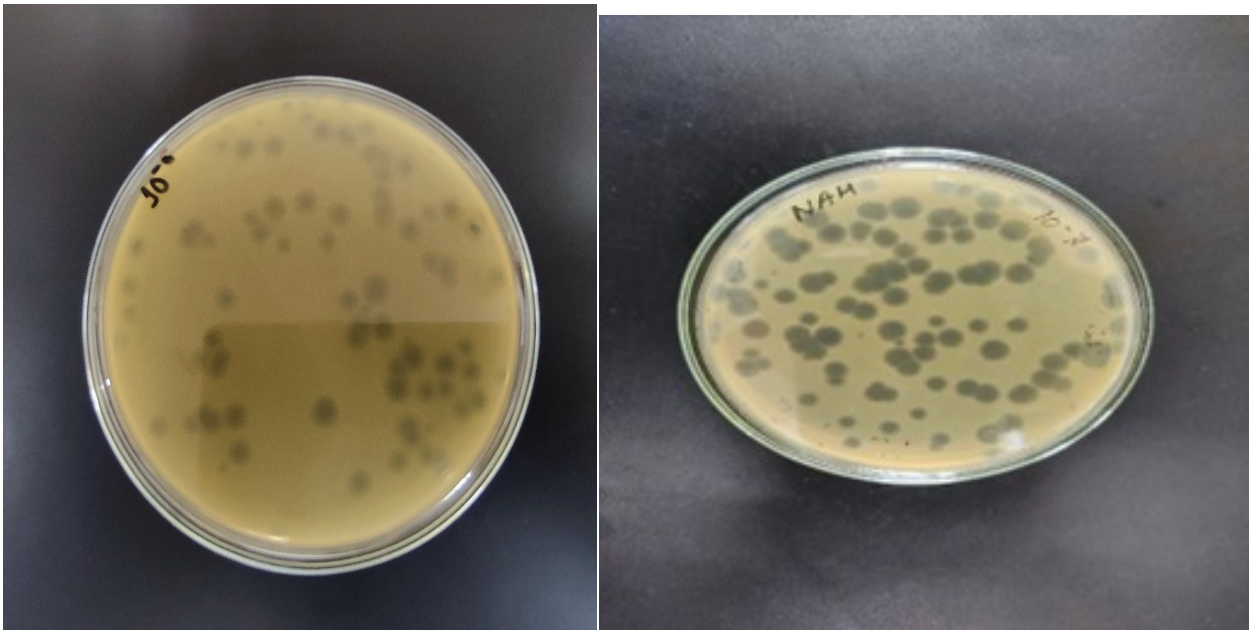
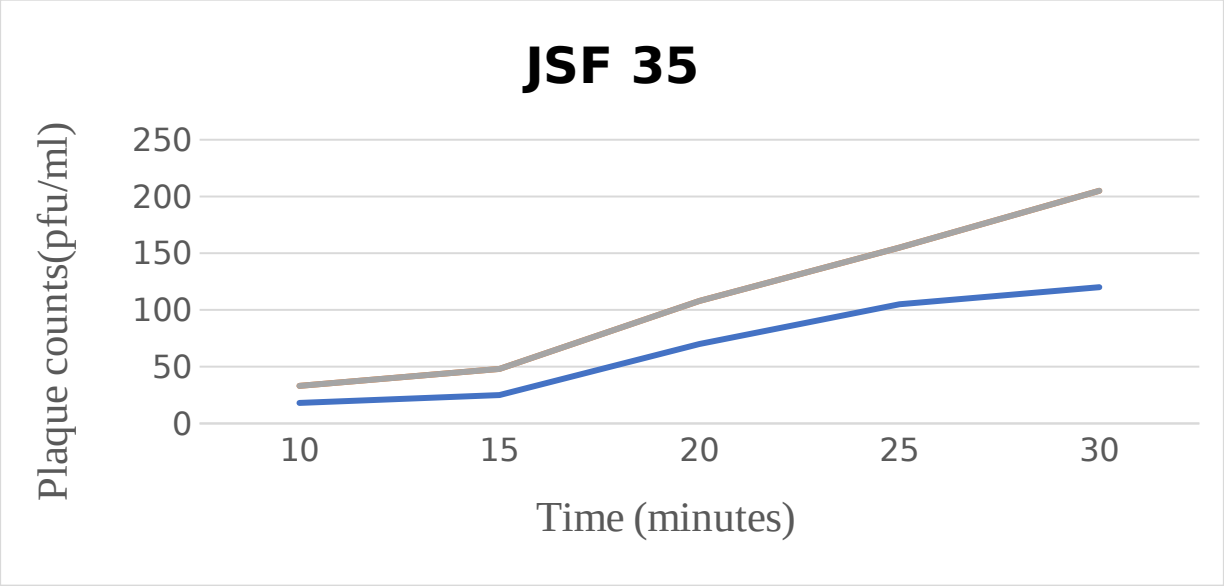


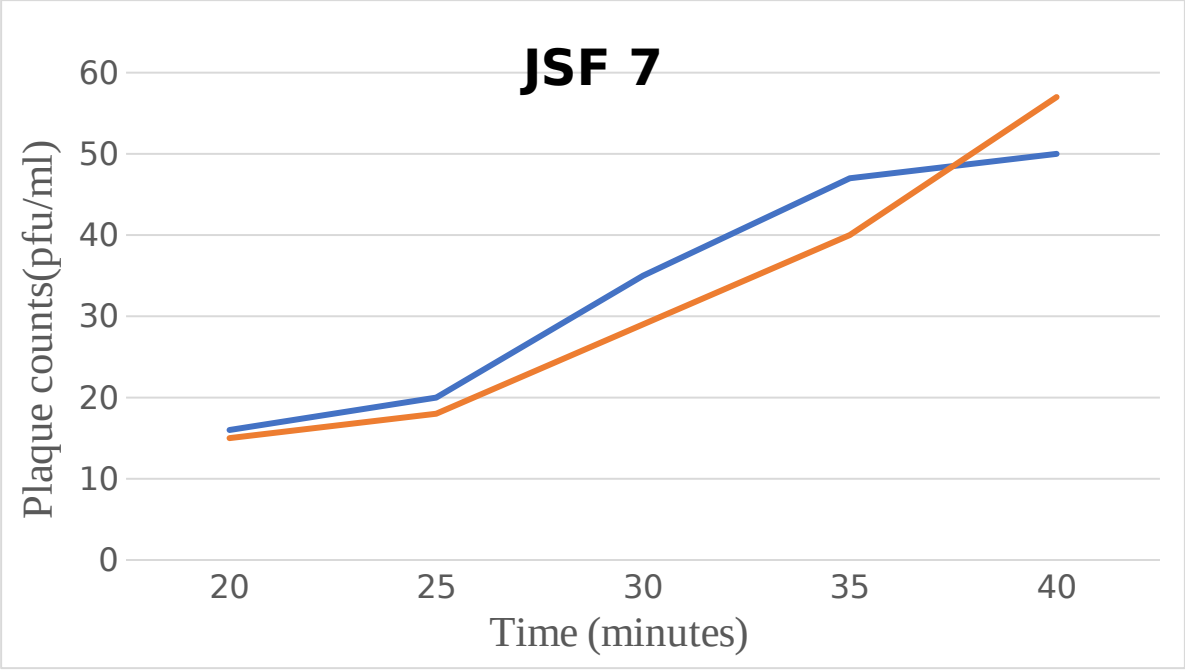
Figure: 4.2(B)

Figure 4: (A, B), different dilutions of Phage JSF-35 and JSF-7 are visible where the host bacteria are WT-346. In the Fig- 4.2 (A), there is an uncountable number of phages JSF-7. And in the 10 -6 and 10 -7 dilutions plate of JSF-35, there are 200 and 135 plaques respectively.

4.3. Burst out time



Graph: Brust out time (JSF-35)



Graph: Burst out time (JSF-7)

Results showed that an incubation time of 20 minutes for amplification was enough to complete an infection cycle of JSF-35. The average PFU was 18 at the initial phase of the study.

However, for JSF-7 multiplication time was little longer as results showed that between 30 to 35 minutes was their multiplication time.

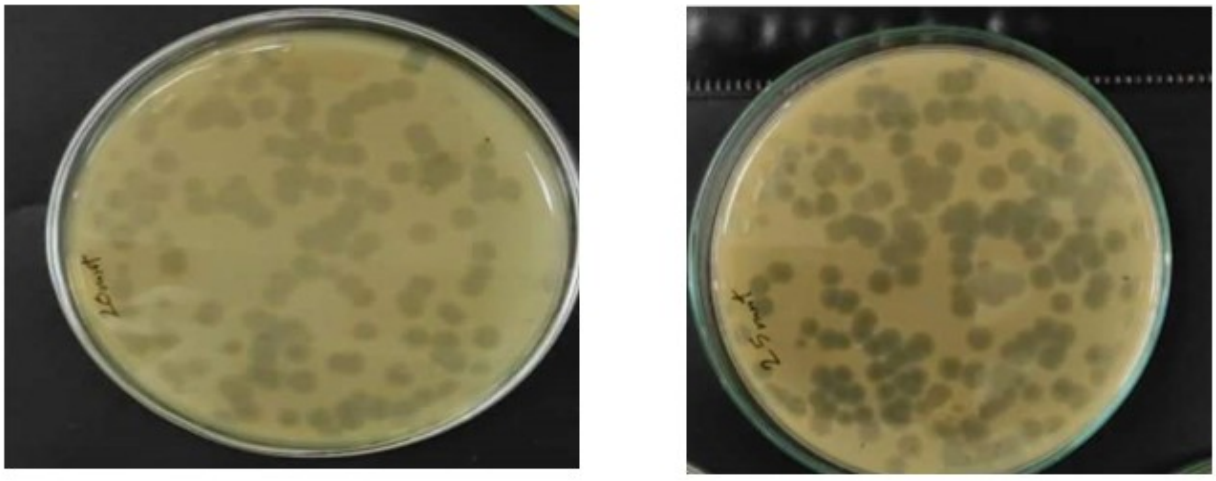


Figure 5: Brust out time of JSF-35

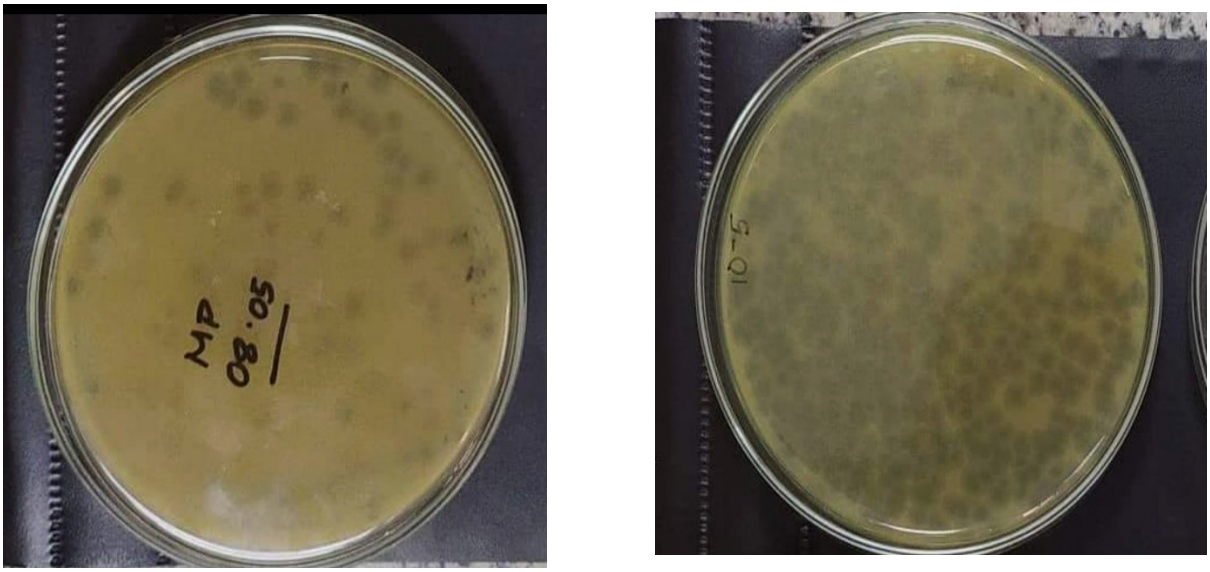


Figure: Burst out time for JSF-7

4.4 Gel run outcome

The purpose of this study is to moderate the detection method and to rapidly detect bacteriophages from any solution we have conducted PCR test. After the gel run the whole method was found to be appropriate as there are clear bands visible.

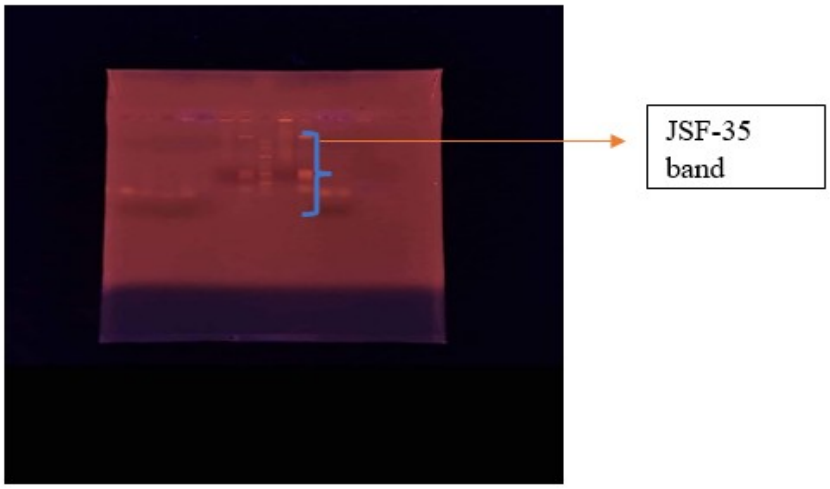


Figure 6: clear PCR band of JSF-35

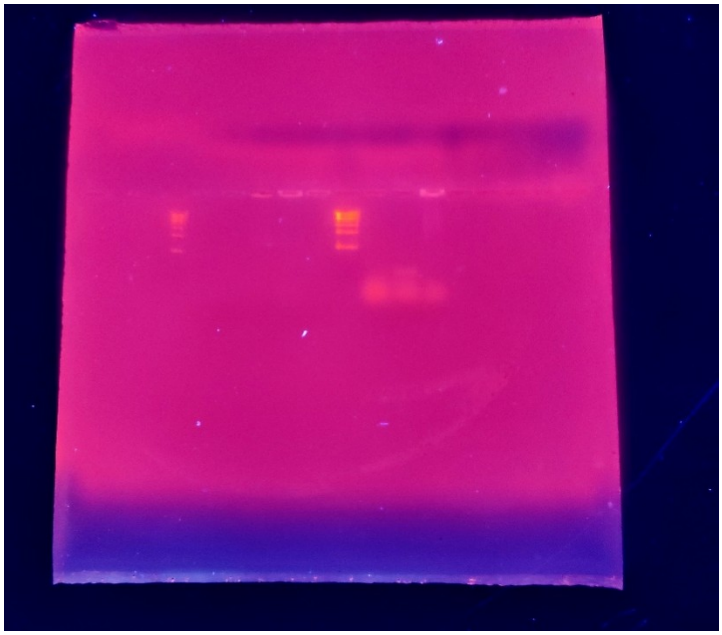


Figure: In JSF-7, no bands were observed

Discussion

Over the years, the bacteriophage is used for pathogenic bacterial detection in various sectors of food and biotechnology. This thesis aims to rapidly detect pathogenic bacteria by bacteriophage which can be a solution to prevent possible food contamination. As this is a controlled study, we did not isolate any organism from the environment. However, the bacteriophage that was used for this specific study and their host bacteria were taken from the stock sample. Here, only one *Vibrio Cholerae* strain was used that is WT-346.

In this thesis, we have found that bacteriophage regeneration time greatly varies on their culture time which must be specified. Infection and phage lyses are very important considerations for JSF-35 band phage amplification assay as the burst out time of the lytic cycle of phage JSF-35 and JSF-7 were investigated through this. And for JSF-35 it was 20 minute and for JSF-7 it was 30-minute absorbance time. So, we have conducted the PCR test and our desired band was observed in JSF-35. Which just proved that JSF-35 can lysis the specific bacteria WT-346. As in JSF-7 no bands were observed which indicates that JSF07 could not lyse the bacteria which is why it was unable to detect the bacteria. According to *Oliveira et al. (2012),* sufficient time for infection before the virucidal addition but to achieve good results time of infection should not be too long. Faster phage-based detection can be achieved by combining the lytic part of the plaque assay and an alternate detection method, which can be immunological or molecular. So, we have decided to choose the molecular method of Polymerase Chain Reaction, PCR. For rapid, specific, and sensitive standard PCR detection method was used alone where it did not give any indication about the viability of the detected cells. In this method, phages only replicate within viable cells and ultimately lyse these cells to release progeny phages within an agar lawn to form plaques. It is a 24-hour test produces countable plaques giving quantitative results. (*Farkas et al. 2015*) But by detecting with PCR we can reduce the time period and the whole procedure will take only 5 hours to complete.

As we were thinking of something faster so we went for PCR. It is a rapid, one-day test where there is an option to detect released phages or the host DNA to demonstrate that lysis has occurred. We have also monitored that here only, viable cells lyse so, potentially it is a quantitative assay. But there are some limitations that, it is important to detect if DNA is released into as small a volume as possible to maximize detection sensitivity. There is also the integrity of the cell membrane is not always a reliable indicator of the viability of cells because some evidence suggests that there might be some cells that remain intact even if they do not show any metabolic activity which may lead to false positive results. These assays are demonstrated on broth culture but they can be also appropriate for water and milk. This pilot method is appropriate for detecting Enteropathogenic bacteria with this specific phage JSF-35.

This detection method of phages observed in laboratory conditions can be greatly used in food systems. There are some limiting factors which we have faced during this whole experiment. Reduced diffusion rate, adverse factors like- temperature, pH, inhibitory compounds are included. To ensure proper phage performance, it is important to isolate phages from same habitat as they are better adapted to replicate and can survive those conditions. Bacterial fitness and physiological status contain clear influence in case of phage infection rate.

Future Aspect and Conclusion

Bacteriophage may serve as an alternative for antibiotics, for instance, multi-drug resistant pathogens and also it has the potential to work as antimicrobial agents. For community-acquired and nosocomial infection which is caused by drug-resistant Staphylococcus, can be a possible cure by using phage therapy. Additionally, it can be a good candidate for prevention of bacterial contamination in industry and animal husbandry. Most importantly, it can work as detection tool for bacterial presence in food industry. Fields of application has comprised of water, food safety, agriculture and animal health such as- food manufacturing industry, EBI Food Safety recently marketed ListexTM P100 for controlling *Listeria* in cheese and meat (**Carlton *et al.* 2005**). Along with this, to enhance microbiological safety, their relatively easy handling and specific antimicrobial activity Bacteriophage based biocontrol measurements have a great potential.

As we know that, *Staphylococcus aureus, Vibrio Cholerae* often cause food poising. They are also a major concern for dairy industry and important source of milk contamination. This rapid detection method can be applied in this sector to detect bacterial contamination in the shortest period of time. Nonvirulent, genetically well characterized bacteria is advisable to develop because it can be a way of safe large scale production process establishment. The antimicrobial activity of phages observed in laboratory conditions could be reduced in food systems. Current research is at the experimental stage despite that the increasing number of publications and emerging companies in this area are making its way for the future of phage-based detection technologies and methods. It can be a landmark for the next step towards commercial application so that consumers feel confident about safety.

To conclude, there is always a need for faster and more accurate testing to identify live infections in food. Culture-based techniques are becoming to time- and labor-intensive to use, and they may only be able to detect pathogens in a VBNC condition if they are present in food. Testing for molecules a possible answer is mRNA-based diagnostics, in particular. To quickly find living microbes. Though, the transient nature of mRNA still poses a challenge to the Reverse transcriptase PCR is widely used for food testing purposes. Various lytic phage-based techniques have during the past two decades, which are displaying high levels of sensitivity to various foodborne pathogens detection in numerous various matrices, such as food and water. The combination of phage amplification and lysis with PCR/qPCR, immunoassay, or enzyme assay endpoint detection approaches seems to be the most promising rapid alternative to cultural methods for the detection of viable pathogens in food. Providing host cell metabolism is occurring, phage amplification will take place and pathogen cells will eventually burst to release measurable intracellular components such as ATP, enzymes, host DNA, or progeny phages.

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