Method optimization for isolation of Pseudomonas bacteriophage from soil samples

By

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A thesis submitted to the Department of Pharmacy in partial fulfillment of the requirements for the degree of
Bachelor of Pharmacy (Hons.)

Department of Pharmacy
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Declaration

It is hereby declared that

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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.
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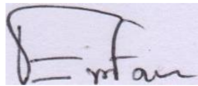

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Approval

The thesis/project titled “Method optimization for isolation of Pseudomonas bacteriophage from soil samples” submitted by Md. Erfanul Islam (14346024) of Summer, 2014 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy on July16, 2020.

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Ethics Statement

This project does not involve any kind of animal and human trial.

Abstract

Bacteriophages as antibiotic are very useful against antibiotic resistant bacteria in the medical fields. Bacteria like *pseudomonas aeruginosa* are resistant to many types of antibiotics and can cause severe diseases to human and animals. In our research project the goal is to identify and isolate these pseudomonas specific bacteriophages from the environment through different methods. In this process we collected soil samples from different region of the country like boali, comilla and tongi and Isolated bacteriophages were applied on the hard agar medium along with soft agar and respective bacterial strains to observe the plaques as bacteriophages. The methods we used to isolate pseudomonas specific bacteriophage didn't show any promising result except method four which showed a trace amount of plaque with one sample from Boali. This research may help to reduce the cost, dependency on antibiotics and to kill the bacteria those are resistant to antibiotics. This research will help the world in the long run to control the resistant bacteria from spreading to newborn to older individuals.

Keywords: Bacteriophages; antibiotic resistant bacteria; isolation; control; soil sample

Dedication

I dedicate this work to my parents for their endless love and support.

Acknowledgement

First of all, I am very thankful to Almighty Allah for his blessing to me. I believe that he gives me the devotion and strength that helps me to fulfil this project and defeated all the difficulties that accompanied with it. It cannot be possible to do all the work without his blessing.

Secondly, to my supervisor Dr. Mohd. Raeed Jamiruddin, Assistant Professor, Department of Pharmacy, Brac University for his constant assist and help regarding any problems that I faced or any questions that I had. I am extremely grateful to him for his instruction, support and encouragement during my project work. His linguistic skill helped me to build up the capacity of expressing thought in an ordered manner.

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List of Acronyms

DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
PBS	Phosphate Buffered Saline
ICTV	Intenational Committee of Texonomy of Viruses

Chapter 1

Introduction

1.1 Bacteriophage

Bacteriophages are viruses and these species are found in abundance on earth. These entities are basically made up of DNA or RNA wrapped in their body by proteins. “Capsid” is the part that encapsulate the genetic material of bacteriophages and it is attached to the fibrous tail that works as a receptor to bind with bacteria (Ul Haq et al., 2012). These are the entities that really plays a very important role in regulation of bacterial population on earth (Lin et al., 2017). Since 1959 over 5600 bacteriophages are examined (Dabrowska, Switala-Jelen, et al., 2005). Most of the phages are polyhedral except the one with the filamentous appearance. These phages attack bacteria and propagate in two distinct ways, one is lytic and the other one is lysogenic (Ul Haq et al., 2012). Bacteriophages can also be attacked by viruses. Phages have one of the interesting subjects to the scientists. The scientists use these phages as the tool of molecular science to understand the nature and molecular biology of the bacterium (Clokie et al., 2011). Bacteriophages were first discovered by William Twort in 1915. In 1915 another scientist named Felix d’Herelle found out that these phages have the potential to kill bacteria. In early 90’s bacteriophages were mainly used to identify the genetic materials of organisms and restriction enzymes. However, at that time the scientist were doing research only on few bacteriophages but as the time goes by and bacteria started to dominate the natural environment the biology of the bacteriophages becomes one of the major concern for the researchers (Clokie et al., 2011).

So, in today’s world of antibacterial resistance and multidrug resistance bacteriophages become one of the vital concerns for the researchers to fight against these untreatable problems.

1.2 Historical background of bacteriophage

We already mentioned earlier that bacteriophage are discovered by Fredrick W. Twort but it was an accidental discovery. He was trying to grow *vaccinia virus* on agar plate and found something other than bacteria which degenerated the bacteria into granules (*Bacteriophages: Biology and Applications - Google Books*, n.d.). Researchers from then started to examine more about the bacteriophage entity as it shows the ability to degrade or kill the bacteria. Though the therapeutic value of bacteriophage was identified but it was still a controversial topic for the scientists. His name was Felix d'Herelle who first find out that bacteriophage can kill bacteria and can be used as an antibiotic and he also given the name of the antibacterial entity a name which we all know now as Bacteriophage (Dabrowska, Switała-Jelen, et al., 2005).

People in USA on that time was dying because of many bacterial diseases which was uncurable as no antibiotic was invented on that time. This phage therapy was used on many patients. The therapeutic use of the bacteriophages were used to treat many bacterial diseases including cases of bacterial dysentery (*Phage therapy: An alternative to antibiotics in the age of multi-drug resistance*, n.d.).

However, the lack of knowledge about genetic materials (DNA & RNA) stops the fulfill understanding of bacteriophages. Furthermore, it was discovered that phages are kind of viruses that can be harmful for human body and can worsen the bacterial disease if used as an antimicrobial agent (Wittebole et al., 2014).

1.2.1 Bacteriophage in modern days

Beckwith in 1969 first exploit bacteriophages for technological application and finds out that these entities can purify and isolate cellular genes (Summers, 2019). This approach makes the researchers to research more on phages. Now a days, bacteriophages are used in treating many bacterial diseases in animals. This therapy is showing bizarre results in treating neonatal

enterotoxigenic *E. coli* infection in lambs, pigs and in calves. However, more and more research is required to find out the true potential of phages which includes the mode of administration (Johnson et al., 2008). As the increase incident of antibiotic resistance and multidrug resistance is now a days a major problems scientists are looking forward to use bacteriophages as an alternate drug to kill resistant bacteria (Lu & Koeris, 2011). This enormous number of phages in the environment can not only be exploited to bacterial identification but also be used as a therapeutic biocontrol agent. Phages are now exploited to design biosensor using chemical and physical functionalization to detect bacteria like *Staphylococcus aureus.* Commonly lytic phages are used to detect these bacterial contamination in foods (Singh et al., 2013). In addition, lots of distinct strategies were figured out to counter microorganisms causing diseases using bacteriophage. Numerous bacteriophages were identified and distinguished by research. However, plating technology is yet a successful route for the exploration of them all (Keen, 2015). Lastly, the future of bacteriophage research helps in divers' fields from diagnostic to biotechnological therapeutically application in plant, humans and animal species. Still several challenges remain untouched to realize the true potential of phages but the outcomes both in fundamental research and therapeutic application have highly significant potential (Singh et al., 2013)

1.3 Structure of bacteriophage.

In 1940, first picture of bacterium was released infected by bacteriophage. The picture was released after the invention of first electron microscope. Like other viruses bacteriophages do not contain many genetic information and mostly multiply in host cell (Wurtz, 1992). Bacteriophages have genetic material in the form of DNA or RNA which is incapsulated by the protein layer. They usually have a tail. Most of the phages are polyhedral but some are filamentous (Ul Haq et al., 2012).

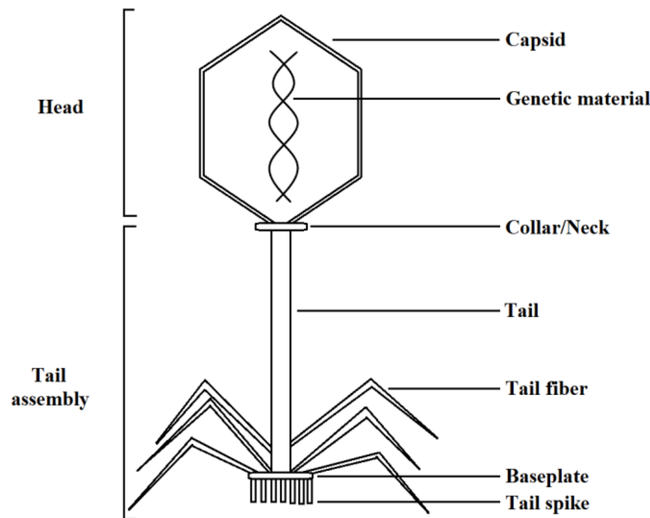

Figure 1: Structure of T4 bacteriophage and its different parts

Bacteriophage is consisting of two basic parts and these are its head and tail. Head contain the genetic material and the tail works as a receptor to bind with bacterium. Phages are of different size and shapes but the most studied phages are tailed with dsDNA genome. Phages have three major components: capsid, tail and special adhesive system. Capsid contain the genetic material, tail serve as pipe to transfer genetic material into bacterium during infection and finally the adhesives recognizes the bacterium and bonds with it (E. White & V. Orlova, 2019).

1.4 Classification of bacteriophage

in early 90's researchers only descovered one bacteriphages and that was *Bacteriophagum intestinale*. so, they decided to classify phages in suborder, a single family, and a single genusof the order *Virales*. this calssification was premature as classifying viruses according to symptom of disease was forgotten earlier. after that ICTV intenational committee of texonomy of viruses classified pghages in total six classes and these are T4, λ, φX174, MS2, fd and PM2. in recent days ICTV classified phages into three orders, sixtyone families and 241 genera (Ackermann, 2003)

Table1: classification of bacteriophage according to physical structure (Ackermann, 2003)

Shape	**Charectaristics**
Tailed	usually consist of a head and helical tail inclosed with protein.
Polyhedral	these phages contain RNA or DNA and polyheadral shape head. these phages are classified into four genre.
Filamentous	this group consist of 4 genere and have different host range. these phages have long, rigid and filamentous tails.
Pleomorphic	these phages are not usually found in the nature and have no capsid but has envelop and dense nucleoprotine granules.

1.5 Life cycle of bacteriophage

Bacteriophages infect bacteria in two different ways. One is called lytic cycle and another is lysogenic cycle. Basically, lytic phase refers to the lysis of bacterial cell by propagation of bacteriophage. On the other hand, lysogenic phase refers to propagation of phage without disrupting the cell wall of bacterium (Ul Haq et al., 2012).

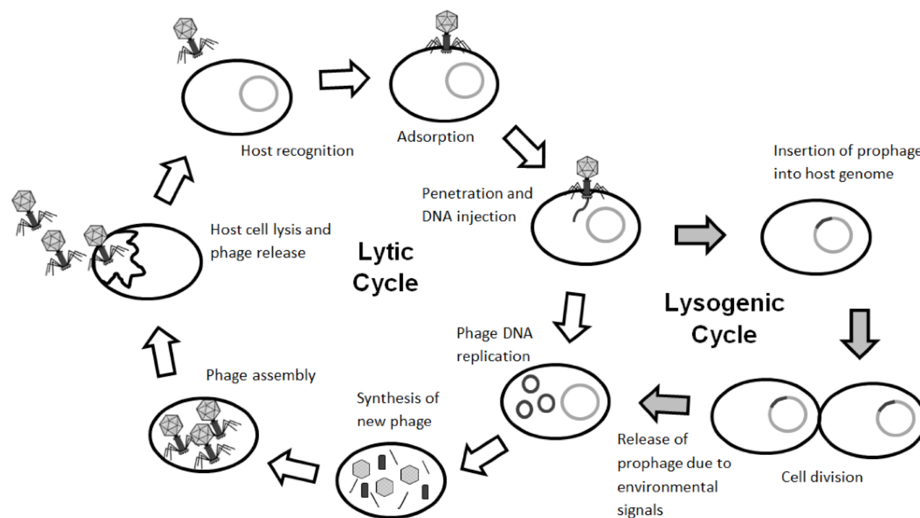

Figure 2: Lytic and Lysogenic lifecycle of bacteriophage (Doss et al., 2017)

a) Lytic cycle: lytic phage not only destroy bacteria but also kills neighboring bacteria in the process of propagation. Lytic phages are used in phage therapy as it has narrow host range and attacks specific bacteria (Doss et al., 2017).

b) Lysogenic Cycle: in this process phages do not lyse the bacterium immediately. Here, phages insert their genetic material in host cell and it replicate along with the host cell and remain dormant for many years. Temperate phages are not used in phage therapy as it can remain in host cell being dormant (Doss et al., 2017).

1.6 Ecological environment of bacteriophage

The largest reservoir of virus and bacteriophage is undoubtedly the oceans. Bacteriophages are found in enormous amount in marine environment (Burbano-Rosero et al., 2011). These viral and bacterial species encompass 90% of the biomass of the ocean. It is also estimated that phages are the most abundant entity in the planet. Phages which belong to the order *Caudovirales* are mostly found in the surface layer of the ocean. Even in the extreme environment of the ocean including hydrothermal vents, cold temperature, high pressure bacteriophage are seen and found (Batinovic et al., 2019).

Soil bacteriophages are relatively understudied than the marine one. Bacteriophage in soil maintain the number of bacteria and regulate the optimum nature of the soil. Different type of soil contains different types of bacteriophages and it's totally depends on the bacterium present in the soil. Different phages play different role in individual soil types. These soil phages are also used in biocontrol protocol of controlling plant disease (Batinovic et al., 2019).

1.7 Isolation and culture of *Pseudomonas aeruginosa* bacteriophage

Bacteriophages are natural antimicrobial agents' fount in nature that don't just attack and kill bacteria but also reproduce which helps in curing many infectious diseases. *Pseudomonas aeruginosa* is a gram negative bacteria that is present natural environment even in nonliving surfaces (Adnan et al., 2020). P. aeruginosa is a diverse cause of many infectious diseases. It has the potential to form biofilm that is resistant to several antibiotics (Kwiatek et al., 2017). The biofilm formed by these bacteria is very dangerous and can weaken the immune system as well. Pathogenic strains of this bacterium commonly cause pneumonia in hospital patients (Adnan et al., 2020). As it is very difficult to treat these infectious disease caused by P. aeruginosa, bacteriophage can be the most relevant alternatives therapeutic option we can think of now (Kwiatek et al., 2017).

Collection of samples: Soil sample were collected from different places around Bangladesh. As bacteriophage tend to live in dam soil so we collected soil near ponds and lakes. Soil was collected approximately 20 grams in 300ml of PBS phosphate buffer saline in a bottle and kept in a dry and cool place.

Bacterial strains: in this research we use *Pseudomonas aeruginosa* to isolate bacteriophage from the collected samples.

Chapter 2

Components

2.1 Reagents and tools

1. The strains of the respective bacteria for culture and isolation of bacteriophage
2. Cultural medium: 1. Nutrient broth 2. Nutrient agar
3. Filtered bacteriophage from reserve
4. Phosphate Buffer Saline
5. Chloroform
6. 0.22 µm size syringe filters
7. Several numbers of sterile beakers in different sizes
8. Several numbers of sterile vials
9. Sterile clean cotton
10. Ethanol (70%) as disinfectant
11. 90 mm sterile plates
12. Several numbers of sterilized test tubes
13. Different sizes conical flask
14. Foil paper of aluminum
15. Whatman filter paper

2.2 Apparatus (Machines)

1. Incubator for bacterial growth
2. Refrigerator used to reserve and store the samples, cultured plates and reagents
3. Laminar air flow
4. Incubator with shaker
5. Centrifugation machine
6. Autoclave machine as steam sterilizer
7. Hot water bath for soft agar

Chapter 3

Methodology

3.1 Method 1

3.1.1 Streaking and preparation of broth-bacterial suspension in test tube

a) First of all, several sterile plates were made ready for the pure culture of the bacteria on medium.

b) Then 2.5% w/v nutrient agar was prepared in the flask and autoclaved it at 121°C for 20 minutes.

c) After autoclaving agar was poured in to the plates when the temperature is down to 55°C and let to solidify.

d) After that strains of *pseudomonas aeruginosa* was streaked on the agar mediums and kept into the incubator for 24 hours at 37°C for the growth.

e) Several test tubes were prepared with nutrient broth (0.13 gm/5 ml) and respective strains of the bacteria that cultured previously were added to the test tubes and kept in the incubator for 24 hours at 37°C for the preparation of broth-bacterial suspension.

3.1.3 Filtration of collected samples

a) As the soil sample was collected in the PBS (Phosphate Buffer Solution) it's then filtered three times with Whatman filter paper.

b) After that the filtered sample was again filtered with 0.2μm microfilter to make sure no bacterium was present in the sample.

3.1.4 Preparation of the phage containing soft agar

a) 2.5 ml of bacterial broth was mixed with 2.5 ml of filtered soil sample and the mixture was incubating at 37°C for 15 minutes. This step allows the bacteriophage to bind with the bacteria.

b) After that this mixture was mixed with 5ml of soft agar and poured on to the top of the 10ml hard agar and let it set for 10-15 minutes.

c) Finally, the plate was kept in the incubator at 37°C for 24 hours.

3.1.5 Preparation of broth containing bacteriophage

a) Small portion from the clear zone of the previously made plate was cut using loop and mixed with 10 ml broth (0.13 gm/5 ml).

b) This broth was then kept in the incubator for 24 hours for the bacteriophage to grow.

3.1.6 Filtration and dilution of bacteriophage broth.

a) After 24hrs the broth with the bacteriophage was filtered once with paper filter and with micro filter.

b) Secondly, the filtered solution was diluted ranging from 10^{-6} to 10^{-8} times.

c) After that the diluted solution was mixed with bacterial broth and kept in the incubator for 15 minutes.

d) Finally, the bacteriophage and bacterial mixture is mixed with soft agar and poured on to the top of hard agar.

e) The Petri dishes were then kept in the incubator for 24 hours.

3.2 Method 2

3.2.1 Bacterial broth preparation and streaking

a) Several plates were prepared with 2.5% nutrient agar for the streaking of *Pseudomonas aeruginosa*.

b) Agar was poured into the plate and kept in the laminar for several minutes to solidify.

c) After solidification the bacteria was streaked into the plate and kept in the incubator for 24 hours.

d) After 24 hours, 0.13/5 ml of nutrient broth was prepared and bacteria was inoculated in them. Finally, those broths were kept in the incubator for the bacterium to grow.

3.2.2 Filtration of collected sample

a) Soil sample collected in PBS was filtered three times with paper filter to remove any soil particle.

b) After paper filtration, the sample was filtered with micro filter to eliminate any bacteria present in it.

3.2.3 Preparation of double layer plate

a) 10ml/plate of nutrient agar was prepared and poured into several plates to solidify.

b) 2.5ml of bacterial broth and 2.5 ml of filtered phage sample was mixed and kept in the incubator for 15 minutes.

c) After 15 minutes, the mixture was again mixed with 5ml soft agar and poured onto the bottom agar. Those plates were kept in the incubator for 24 hours.

3.2.4 Broth preparation for bacteriophage

a) 10 ml of nutrient broth was prepared (0.13/5ml) in several test tubes.

b) A small portion of agar was cut from the previous plates and mixed with the broth.

c) Those mixtures were kept in the incubator for 24 hours for the phages to grow.

3.2.5 Filtration of the broth containing phages

a) After 24 hours of incubation, the broths were treated with CHCl3 chloroform to eliminate any remaining bacteria present in the broth.

b) Then the broth was centrifuged for 10 minutes at 10 thousand rpm.

c) Before going to the dilution, the broths were filtered one time with paper filter and one time with micro filter.

3.2.6 Dilution and final double layer plate preparation

a) Dilution was done using PBS from 10^{-6} to 10^{-8} time.

b) 9.9ml of PBS was taken in test tubes and 100 micro liter sample was mixed with it to dilute the sample 10^{-2} time. This process was done three to four times.

c) Final diluted sample was taken 2.5ml in a beaker and 2.5ml of bacterial broth as well.

d) This mixture after incubation for 15 minutes was mixed with 5 ml of soft agar and finally poured onto the top of bottom agar in the plates.

e) Those plates were kept in the incubator for 24 hours for the plaques to form.

3.3 Method 3

3.3.1 Filtration of soil sample

a) Approximately 10ml of soil samples in PBS was taken in test tubes and centrifuged for 10 minutes at 10 thousand RPM to separate the small particles as sediment in the bottom.

b) After that the centrifuged samples are filtered using paper filter two times respectively.

3.3.2 Preparation of bacterial broth and mixing with filtered phage sample.

a) *Pseudomonas aeruginosa* was cultured in test tubes with broth in it and kept in the incubator for 24hrs.

b) After 24 hrs. 2.5ml of bacterial broth and same amount of phage sample is mixed together in a 50 ml beaker.

c) This mixture was then kept in incubator for 20 minutes. This process helps bacteriophages binding with bacteria.

d) Finally, this incubated mixture was filtered once with the help of 0.2µ micro filter.

3.3.3 Agar preparation for double layer

a) Bottom layer nutrient agar was prepared (1.5 w/v) 30ml, 10ml for each plate.

b) For the upper layer 3ml agar was prepared with the same concentration for each plate.

3.3.5 Dilution and double layer plate preparation

a) The bacteria and phage mixture were diluted up to 10^{-8} times using micropipette.

b) 100 microliters were sample was withdrawn and mixed with 9.9 ml of PBS to dilute the sample. This process was continued till 10^{-8}.

c) After that 1ml of diluted sample and 1ml of bacterial broth was taken in a beaker and kept in the incubator for 15 minutes at optimum temperature.

d) At last, the phage and bacterial mixture was mixed with 3ml of top agar and poured over the bottom layer. Those plates were kept in incubator for 24hrs for the bacteriophage to grow.

3.4 Method 4

3.4.1 Preparation of bacterial broth suspension in test tubes

a) 10ml of broth is prepared in test tubes with the concentration of 0.13gm per 5 ml.

b) Bacterial strains are swabbed from the dish and inoculate in the broth using a loop.

c) Then the broths are kept in the incubator for 24 hours at 37°C.

3.4.2 Preparation of soil and bacterial broth mixture.

a) Small amount of soil from the collected samples are mixed with bacterial broth.

b) This mixture was the centrifuged for 15 minutes at 15 thousand RPM.

3.4.3 Preparation of supernatant.

a) Soil sample collected in PBS was transferred into a test tube.

b) Secondly, the test tubes were centrifuged for 10 minutes at 15 thousand RPM.

c) After centrifuge the sample was filtered once with paper filter and once with micro filter to obtain the supernatant.

3.4.4 Filtration and dilution to obtain pure phage sample.

a) First of all, supernatant and bacterial broth with soil sample were mixed together in an adequate amount and centrifuged for 15 minutes at 15thousand RPM.

b) After centrifuge few drops of chloroform (CH3Cl) was added in to the mixture to eliminate any bacteria present in the solution.

c) Then the solution was filtered twice with paper filter and once with micro filter to completely free it from any bacteria; contamination.

d) Finally, the sample was diluted up to 10^{-8} times to gain pure phage sample.

3.4.5 Upper layer preparation.

a) 2.5ml of pure phage sample and 2.5ml of bacterial broth was mixed together and incubate for 15 minutes.

b) After that the mixture was again mixed with 5ml soft agar.

c) Finally, the mixture was poured on top of the 10 ml hard agar let it set for 15 minutes. After 15 minutes the plates were transferred into the incubator for 24 hours.

Chapter 4

Results

4.1 Method 1

4.1.1 Sample 1: Boali, Konakhola

Date of collection: 25.01.2020

Location: Geographical position of the collected sample source as latitude is 23.7543795 and 90.3662695 as longitude.

Figure 3: Boali, Konakhola

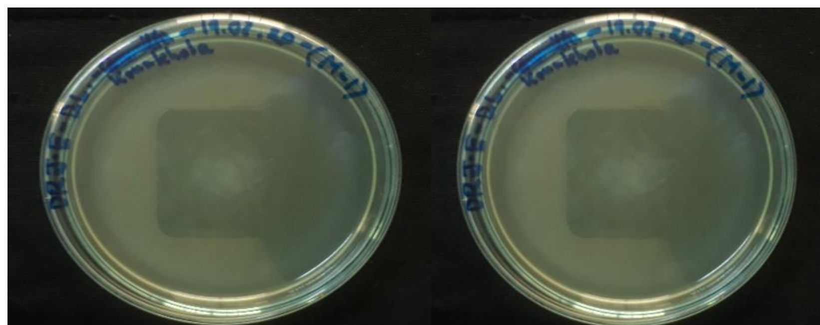

Figure 4: Bacteriophage from Boali

Observation: No clear plaques have been observed with this sample.

4.1.2 Sample 2: Tongi

Date of collection: 30.01.2020

Location: Geographical position of the collected sample source as latitude is 23.8818311 and 90.4016748 as longitude.

Figure 5: Tongi

Figure 6: Bacteriophage from Tongi

Observation: No clear plaques have been observed with this sample.

4.1.3 Sample 3: Comilla

Date of collection: 21.01.2020

Location: Geographical position of the collected sample source as latitude is 23.4675189 and 91.1792889 as longitude.

Figure 7: Comilla

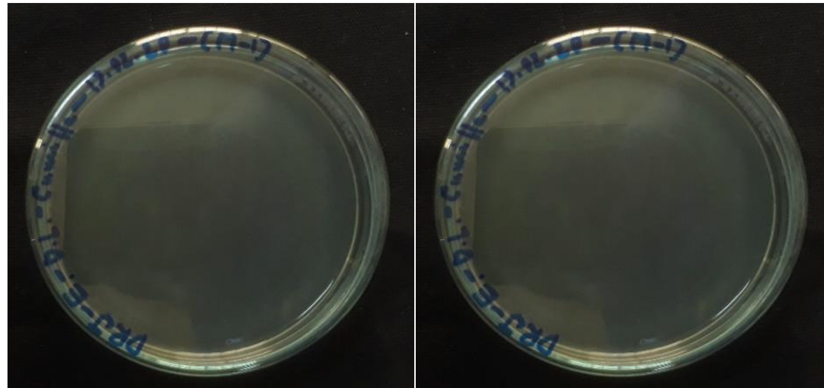

Figure 8: Bacteriophage from Comilla

Observation: No clear plaques have been observed with this sample.

4.2 Method 2

4.2.1 Sample 1: Boali, Konakhola

Date of collection: 25.01.2020

Location: Geographical position of the collected sample source as latitude is 23.7543795 and 90.3662695 as longitude.

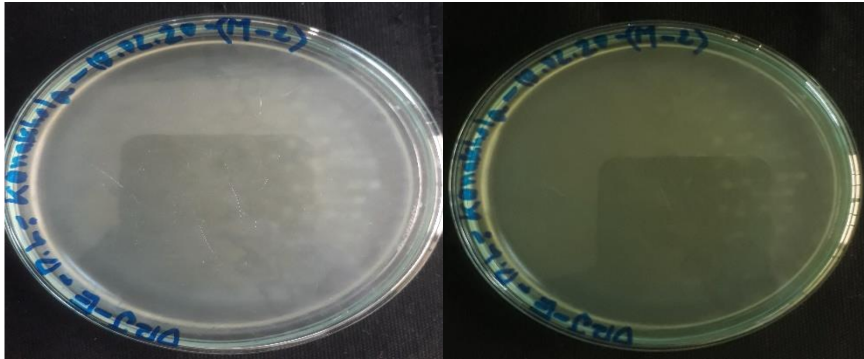

Figure 9: Bacteriophage from Boali

Observation: No clear plaques have been observed with this sample but bacterial growth is seen.

4.2.2 Sample 2: Tongi

Date of collection: 30.01.2020

Location: Geographical position of the collected sample source as latitude is 23.8818311 and 90.4016748 as longitude.

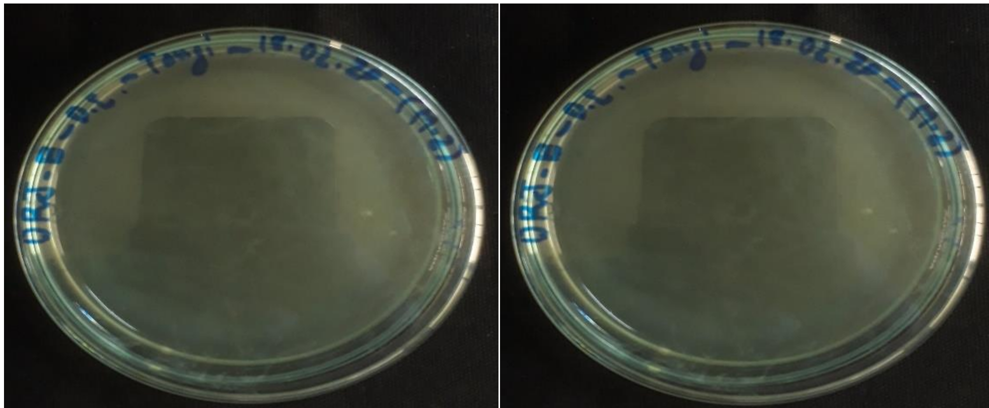

Figure 10: Bacteriophage from Tongi

Observation: No clear plaques have been observed with this sample.

4.2.3 Sample 3: Comilla

Date of collection: 21.01.2020

Location: Geographical position of the collected sample source as latitude is 23.4675189 and 91.1792889 as longitude.

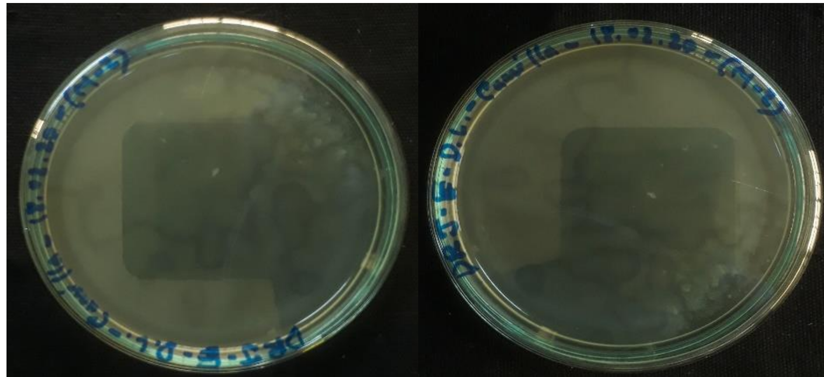

Figure 11: bacteriophage from Comilla

Observation: No clear plaques have been observed as the plate got contaminated.

4.3 Method 3

4.3.1 Sample 1: Boali, Konakhola

Date of collection: 25.01.2020

Location: Geographical position of the collected sample source as latitude is 23.7543795 and 90.3662695 as longitude.

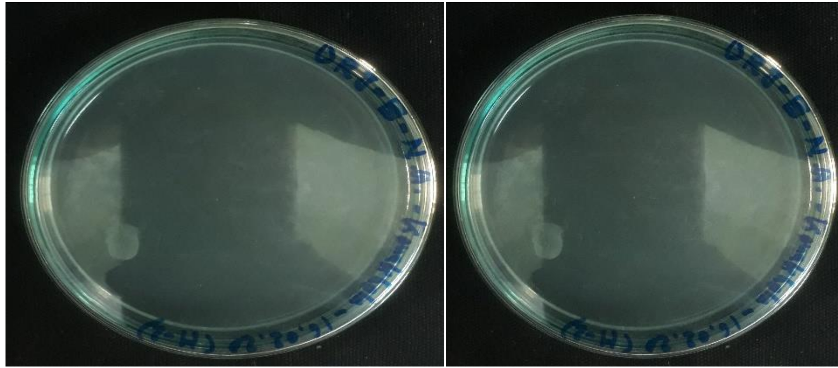

Figure 12:Bacteriophage from Boali

Observation: No plaque has been observed with this sample.

4.3.2 Sample 2: Tongi

Date of collection: 30.01.2020

Location: Geographical position of the collected sample source as latitude is 23.8818311 and 90.4016748 as longitude.

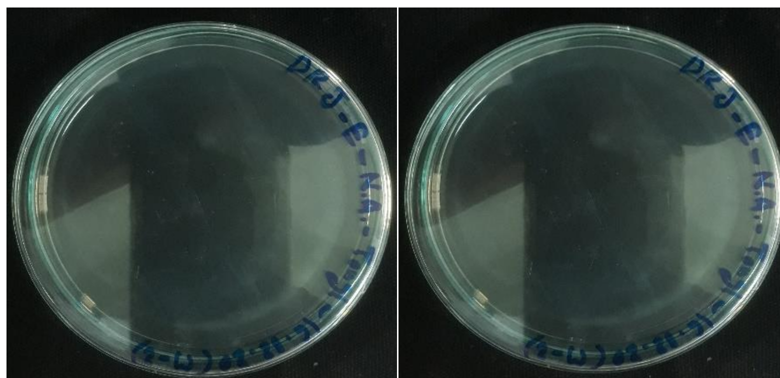

Figure 13:Bacteriophage sample from Tongi

Observation: This sample also doesn't show clear plaques.

4.3.3 Sample 3: Comilla

Date of collection: 21.01.2020

Location: Geographical position of the collected sample source as latitude is 23.4675189 and 91.1792889 as longitude.

Figure 14: Bacteriophage sample from Comilla

Observation: No clear plaques have been observed with this sample.

4.4 Method 4

4.4.1 Sample 1: Boali, Konakhola

Date of collection: 25.01.2020

Location: Geographical position of the collected sample source as latitude is 23.7543795 and 90.3662695 as longitude.

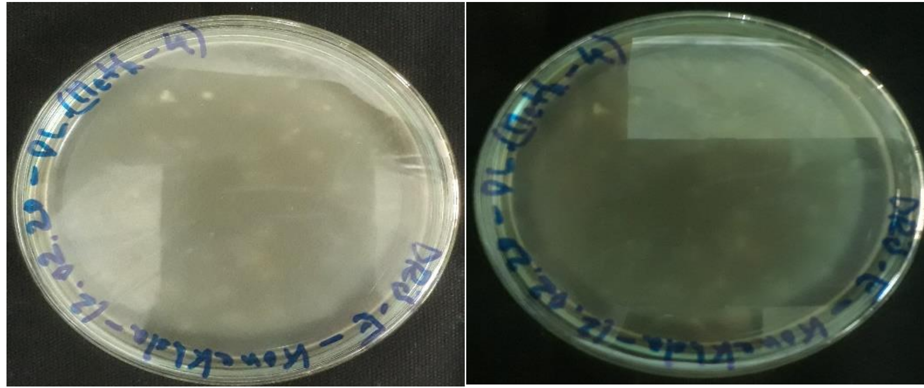

Figure 15:Bacteriophage sample from Boali

Observation: Trace amount of plaque has been observed with this sample which needed further experiment to grow.

4.4.2 Sample 2: Tongi

Date of collection: 30.01.2020

Location: Geographical position of the collected sample source as latitude is 23.8818311 and 90.4016748 as longitude.

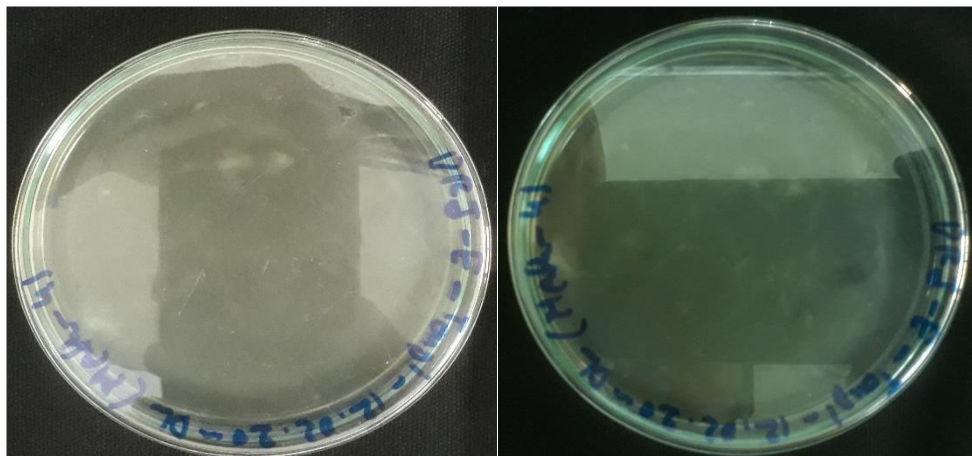

Figure 16: Bacteriophage sample from Tongi

Observation: No clear plaques have been observed with this sample.

4.4.3 Sample 3: Comilla

Date of collection: 21.01.2020

Location: Geographical position of the collected sample source as latitude is 23.4675189 and 91.1792889 as longitude.

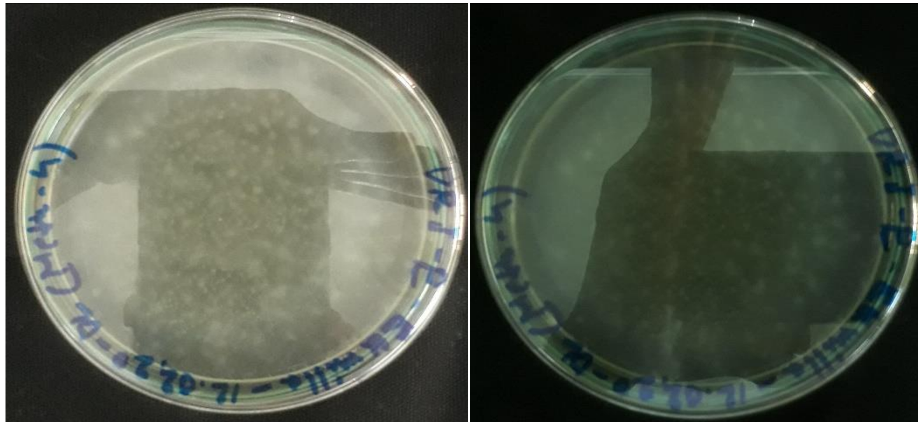

Figure 17: Bacteriophage sample from Comilla

Observation: No plaque has been observed with this sample as well.

Chapter 5

Discussion

In this experiment none of the results were very satisfactory as we didn't get any clear bacteriophage plaque. Three samples were collected from three different areas which may contain different types of bacteriophages. In this research four different methods were used to isolate and identify pseudomonas specific bacteriophages. Four methods were used to find out which one of these shows more prominent results. Every method was different from each other's, like in one of the methods chloroforms was used to eliminate any bacterial from the samples. Some method took three to four days including more dilution of the samples, used different concentration of the medium and different amount of filtration and procedures. However, none of the methods didn't give us any satisfactory results. There can be a lot of reasons that the results were not satisfactory. One of the factors that may be reason of this result can be the growth medium. Bacteriophages growth in agar medium can be divided into two different parts, one is extracellular phase and the intracellular phase which respectively shows diffusion and progeny production (Gallet et al., 2011). These two phase depends on agar density of the agar as the density rises plaques forms slowly (Clokie & Kropinski, 2009). In our research the density of the agar may be one of the reasons we didn't get any satisfactory result. The reason can be lower bacterial density in lawns which farther delays the plaque formation process (Clokie & Kropinski, 2009). Another reason the results were not satisfactory can be the dryness of the agar. Excessive dryness of the agar can reduce rate of nutrition supplement which eventually lead to formation of smaller plaques or delayed plaque formation (Clokie & Kropinski, 2009). Thickness and softness of the agar is also an important factor which helps in the visibility of the plaques(Cormier & Janes, 2014). We diluted the bacteriophage samples to different degrees reengining from 10^{-6}-10^{-8}. Phage preparation needs to be diluted properly to a certain degree to get a proper and greater amount of plaque (Dolan

et al., 2016). Temperature and time also play a great role in plaque formation. In this research the incubator we used was not working once that's why we had to use the oven instead which can also be one of the reasons of the results. Different phage-infected bacteria produce plaque in different temperatures. Some produce plaques in 10 °C, some in 4 °C and some in 22 °C or 37 °C (Dolan et al., 2016). In this experiment we constantly used 37 °C for every method. Moreover, we didn't used glycine in our growth medium and sodium azide in phage preparation where incorporation of glycine in medium and sodium azide in phage preparation can increase phage size and prevent bacterial growth (Dolan et al., 2016). Finally, bacteriophages are usually found in wet environment but we collected the soil sample in winter season where the soil was not moist enough which could be another reason, we didn't get any satisfactory result Consequently, in the event that we overhaul the cycle, control the pollution and guarantee the correct measure of time and schedule, positive outcomes can be achieved.

Chapter 6

Conclusion

Our primary objective of this project was to identify bacteriophages present in the environment which has the potency to fight against dangerous antibiotic resistant bacteria. Now a days, most of the bacteria are mutating and becoming resistant to certain antibiotics. So, we in this project collected soil samples containing bacteriophage from three different areas of Bangladesh and tried to isolate these pseudomonas specific bacteriophages using different methods and protocols. These methods show the true potency of these bacteriophages which is really important to save the lives in this planet in the upcoming future.

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