Method Optimization for Isolation of Klebsiella Bacteriophage from Soil Samples

By

Zumana Hayat Khan
16146053

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Bachelor of Pharmacy (Hons)

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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing 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 have acknowledged all main sources of help.

Student's Full Name & Signature:

Zumana Hayat Khan
16146053

Approval

The thesis titled “Method Optimization for Isolation of Klebsiella Bacteriophage from Soil Samples” submitted by Zumana Hayat Khan (16146053) of Spring, 2016 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy on 27.02.2020.

Examining Committee:

Supervisor:

Dr. Mohd. Raeed Jamiruddin
Assistant Professor, Department of Pharmacy
Brac University

Program Coordinator:

Dr. Hasina Yasmin
Professor, Department of Pharmacy
Brac University

Departmental Head:

Dr. Eva Rahman Kabir
Professor and Chairperson, Department of pharmacy
Brac University

Ethics Statement

The study does not involve any kind of animal trial and human trial.

Abstract

In case of antibiotic-resistance bacteria, bacteriophages are widely used as a substitute for many antibiotics in the field of medical science. In this study, the objective was to segregate and point out bacteriophages against reference *Klebsiella pneumoniae* bacteria using four types of procedures. In addition, these procedures had been optimized to find a best possible outcome. Three types of soil samples were collected near the different hospitals, lakes, ponds, and the river from Old Dhaka (Boali), Tongi and Comilla. To isolate the bacteriophage plaque double layer agar plate method was used. This study can help to minimize costs, reliance on antibiotics and destroy antibiotic-resistant bacteria as the world is confronting a major encounter in managing various resistant strains.

Keywords: Bacteriophages; *Klebsiella pneumonia*; Antibiotic-resistance; Soil sample; Isolation; Optimization

Dedication

Dedicated to my parents and supervisor

Acknowledgement

I wish to begin by thanking the Almighty God, our creator our source of life and energy, our knowledge and wisdom, for the blessings and mercy. This work would not have been done without the support of people recognized here luckily.

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List of Acronyms

ICTV	International Committee for the Taxonomy of Viruses
EU	European Union
DM	Diabetes Mellitus
DLA	Double Layer Agar
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
dsDNA	Double Stranded Deoxyribonucleic Acid
dsRN	Double Stranded Ribonucleic Acid
p.f.u	Plaque Forming Unit
PBS	Phosphate Buffer Saline

Chapter 1

Introduction

1.1 Bacteriophages

Viruses are extraordinarily small infectious particles that are not detectable in a regular microscope and can migrate through delicate filters of porcelain. These occur in a wide range of ways and affect any biological system such as animals, plants, insects and microbes(Orlova, 2012). Among these viruses, bacteriophages are the richest biological entities in the world and influence any ecosystem. These are the viruses which infect as well as kill bacteria. They are used in laboratory research for almost a century and present in all habitats where there are prokaryotes, from the human mouth to the aquatic hydrothermal vents (Pelzek et al., 2013). The word "phage" originates from the Greek term "phagein" which means "to devour"(Sultana, 2017). So, "bacteriophage" means eating bacteria, and is so-called since noxious bacteriophages can interfere with lysis throughout a prone bacterial culture (Orlova, 2012). The growth of pathogenic resistant bacteria, but not all antimicrobial agents currently available, has become a critical issue in contemporary treatment, especially as a result of the corresponding upsurge in compromised immune systems patients. The concern that humanity may re-enter the "pre-antibiotics" era has become very real and alternative modalities have emerged for infection. Therefore, bacteriophages can be an alternative to this (Lin et al., 2017).

1.2 Origin and Bacteriophage Discovery

The history of phage detection can be broken down into two eras. At first in 1896, a British bacteriologist Ernest Hanbury Hankin, who serves as Chemical Inspector and

Bacteriologist in India, found that the waters of the Ganga and Yamuna waterways covered with organic materials which demolished cholera-actuating microbe's communities. These materials could move through multiple channels, which are known to have the potential of carrying larger microorganisms, for example, microscopic organisms. His dissertation was presented in the Pasteur Institute (Wittebole et al., 2014).After that in 1951, Twort, a professionally trained British bacteriologist, brought back the topic after approximately 20 years after Hankin's suggestion is to note a similar phenomenon and advance the theory that a virus is the cause of this phenomenon. However, owing to various reasons he did not pursue his finding (Alisky et al., 1998; Rattanachaikunsopon & Phumkhachorn, 2010; Sharp, 2001; Wittebole et al., 2014). The most important person in the second era of bacteriophage discovery was the microbiologist Felix d' Herelle, at the Institute de Pasteur, Paris. Besides, being the first person to use the word "bacteriophage," his works formed the firm base for modern phage research (Alisky et al., 1998; Rattanachaikunsopon & Phumkhachorn, 2010). Again, Felix d'Herelle, who extracted these agents from the stool samples of the dysentery patients, named them bacteriophages and developed the phage assays which persist in use to this day. In early 1920s, he also conducted the first experiment during phage therapy. Back in the late 1940s, phage therapy research in the West was overshadowed because of the advent and widespread active use of antibiotics in treatment. On the other side, bacteriophage treatment was commonly declined due to unreliable and unexpected outcomes, a problem that was at the time related to the comparatively poor interpretation of phage genetics. In fact, many of the diseases handled up to the middle of the twentieth century with phage preparations stayed unlikely to have a bacterial origin. As a result, the effects of bacteriophage treatment usually appeared to

be lower than those observed for antibiotic treatment, while the latter had a broader clinical scope and therefore did not require practitioners to have advanced bacteriological expertise to be effective in administration. The utilization of phages to the diagnosis of diseases has recently received consideration in Western region. Mainly because of the trend of growing incidences regarding antibiotic resistance. Also as a result of the fact that phage biology, the relationship of phage bacteria and hence the field for bacterial diseases are much greater realized than in the middle of the twentieth century, while antibiotic therapies eclipsed phage therapy (Elbreki et al., 2014; Sharp, 2001).

1.3 Sources of Phages

Phages can be found in nearly all Earth conditions, varying from the ground, sediments, water and plants, live or dead species(Elbreki et al., 2014; Sharp, 2001). Phages can be purified from almost any bacterial growth-enhancing material. The estimated population size of the global phage is enormously high. For example, marine systems are estimated to have a cumulative phage community is over 10^31. Furthermore, most natural habitats have been displayed that they contain 10^7 pathogens per gram of soil. In wastewater, it is estimated to accommodate between 10^8 to 10^10 phages per cubic centimeter (Elbreki et al., 2014).

1.4 Categorization of Bacteriophages

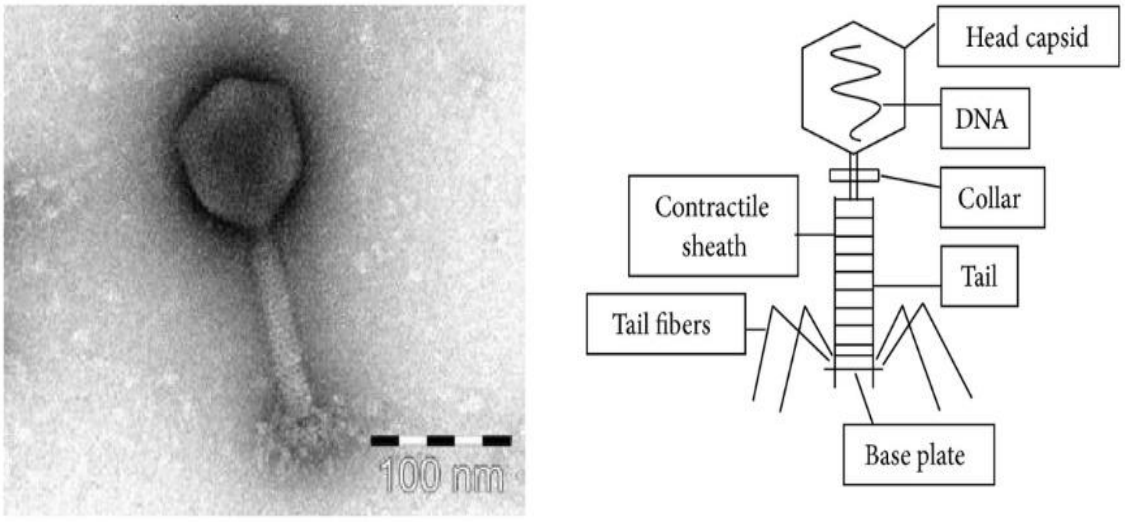
A range of forms, sizes, capsid uniformity, and construction have evolved in Phages. These are all made up of nucleic acid compressed by a protein called capsid coat. The phages may be double or single-stranded DNA or double or single-stranded RNA. In addition, capsids were found in several ways, ranging from small, three-dimensional six membered configurations to fibers to extremely complicated head and tail

frameworks (Figure: 1). Over the years, the International Committee for the Taxonomy of Viruses (ICTV) has established a complex phage categorization process to compensate for the variety. Bacteriophage taxonomy was originally organized in accordance with its morphological traits, nucleic acid shape and envelope or lipid presence or absence and was arranged in fourteen different families according to this approach (Figure: 2). Recently, the relevance of comparing the sequence of the genome in phage was also recognized. Some of the communities on bacteriophage were assembled into orders. For instance, three-tailed phage communities such as Siphoviridae, Myoviridae and Podoviridae are the member of Caudoviralesorder. In contrast, archaea-infecting phages Lipothrixviridae and Rudiviridae are the member of Ligamenvirales (Figure: 2). Moreover, Inoviruses (family Inoviridae) consist of an unwrapped pole of protein fibers surrounding a rounded single stranded DNA genome. Micro viruses (family Microviridae) have an undeviating, a single stranded DNA with a non-enveloped, icosahedron capsid. All Tectiviruses of Tectiviridae family and Corticoviruses of Corticoviridae family, have exterior icosahedron capsids with directly below the lipid layer. Such 2 families vary in case of the structure of the capsid plus the genome. While the genome of corticovirus is spherical and strongly supercoiled, the genome of tectivirus is undeviating with repeats upturned from terminals. In comparison, plasmaviruses of the family Plasmaviridae, have an exterior lipid shell, pleomorphic arrangement, and a spherical genome. They are recognized only on behalf of infesting the *Acholeplasma* mycoplasmic family. The cystoviruses of the family of Cystoviridae, have an undeviating, disjointed and double stranded RNA. Double capsid is the distinguishing element on them and which has a lipid ring around them. Leviviruses of the family of Leviviridae, contains a spherical, positive-stranded, single stranded RNA with a sphere-shaped capsid that is not enveloped

(Table: 1; Figure: 3)(Ackermann, 2003; Elbreki et al., 2014; Hatfull & Hendrix, 2011).

Table 1 Bacteriophage families established on the features of morphology plus nucleic acid (Elbreki et al., 2014).

Family	**Morphology**	**Nucleic Acid**
Myoviridae	Non-enveloped, contractile tail	Undeviating, double stranded DNA
Siphoviridae	Non-enveloped, no contractile tail (long)	Undeviating, double stranded DNA
Podoviridae	Non-enveloped, no contractile tail (short)	Undeviating, double stranded DNA
Lipothrixviridae	Enfolded, rod like shape (infect Archea)	Undeviating, double stranded DNA
Rudiviridae	Non-enveloped, rod like shape (infect Archea)	Undeviating, double stranded DNA
Ampullaviridae	Enfolded, bottle like shape	Undeviating, double stranded DNA
Bicaudaviridae	Non-enveloped, lemon like shape	Spherical, double stranded DNA
Clavaviridae	Non-enveloped, rod like shape	Spherical, double stranded DNA
Corticoviridae	Non-enveloped, isometric	Spherical, double stranded DNA
Cystoviridae	Enfolded, spherical	Fragmented, double stranded RNA
Fuselloviridae	Non-enveloped, lemon like shape	Spherical, double stranded DNA
Globuloviridae	Enfolded, isometric	Undeviating, double stranded DNA
Guttavirus	Non-enveloped, ovoid	Spherical, double stranded DNA
Inoviridae	Non-enveloped, filamentous	Spherical, double stranded DNA
Leviviridae	Non-enveloped, isometric	Undeviating, double stranded DNA
Plasmavirida	Enfolded, pleomorphic	Spherical, double stranded DNA
Tectiviridae	Non-enveloped, isometric	Undeviating, double stranded DNA
Microviridae	Non-enveloped, isometric	Spherical, double stranded DNA



(a) (b)

Figure 1 (a) Electron micrograph of a negative stained Acinetobacter baumannii phage and (b) generalized tailed phage structure (Elbreki et al., 2014)

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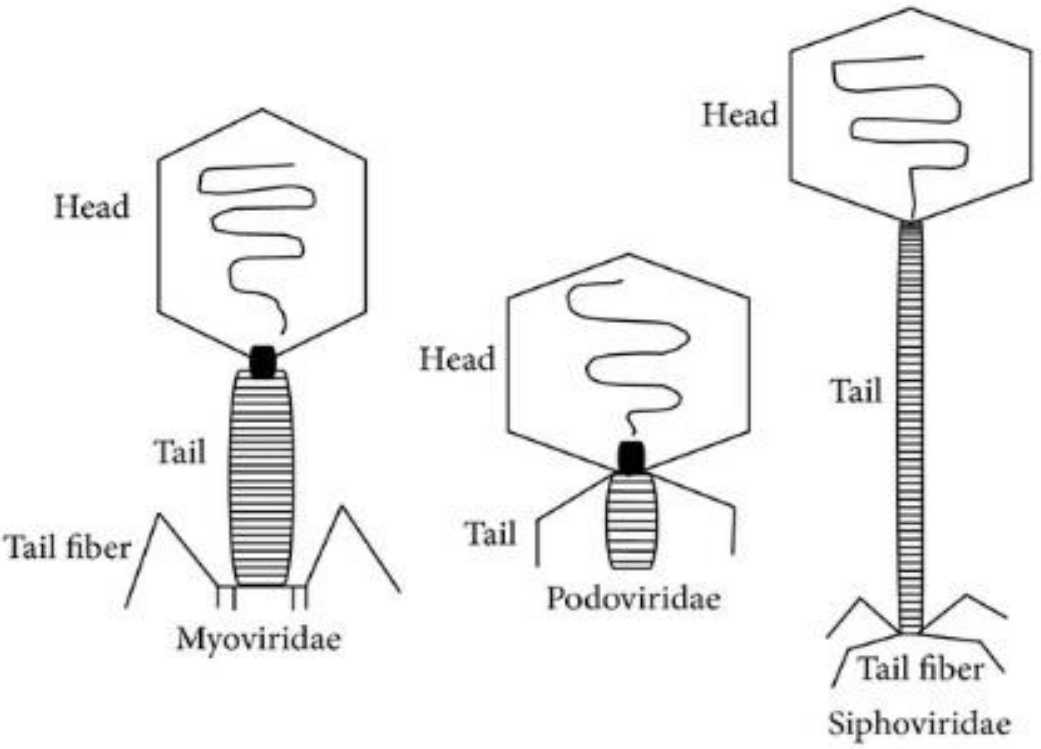


Figure 2 The three tailed phage families (Elbreki et al., 2014)

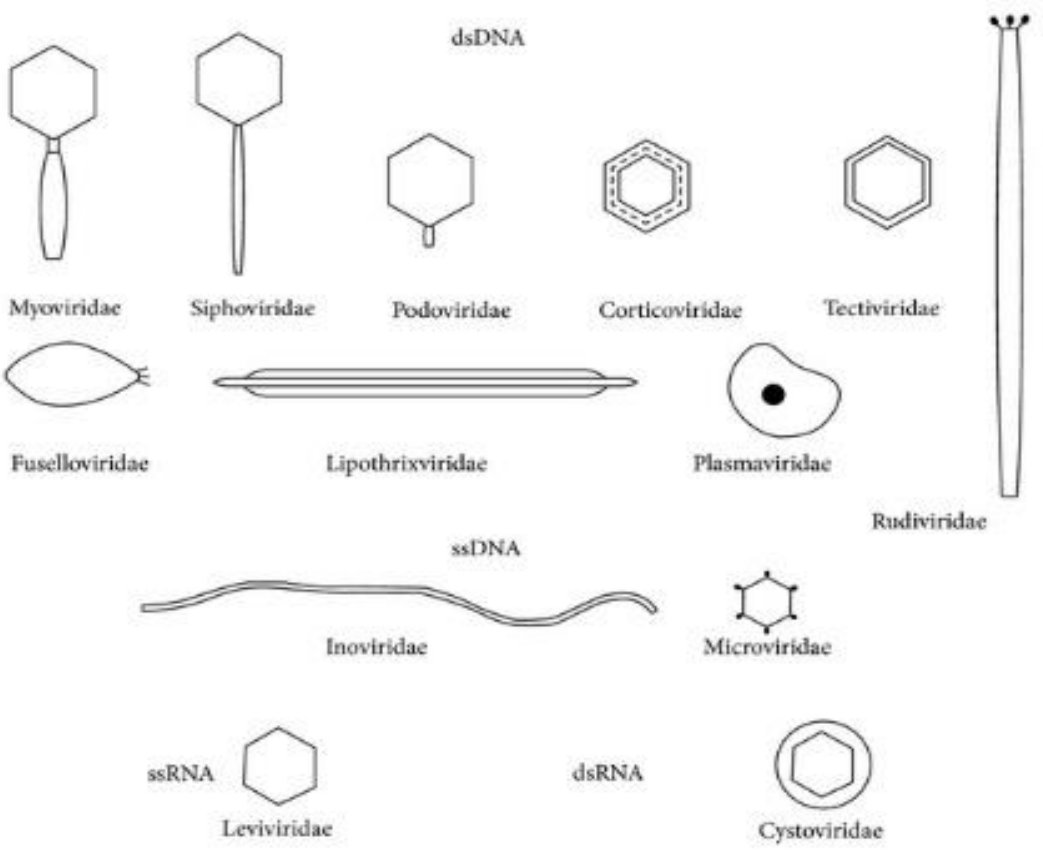


Figure 3 The 14 phage families based on the features of morphology and genome. For more info, see Table 1. (Elbreki et al., 2014)

1.5 The life cycle of Bacteriophages

Phages consist of two life cycles. One is lysis and another one is the lysogenic cycle. They can be considered as virulent and temperate. Virulent phage goes through the lysis cycle. On the other hand, temperate phages that initiate a lysis or lysogenic cycle at host entry to duplicate (Doss et al., 2017; Pelzek et al., 2013). In the lysis cycle, the bacteriophages will always kill the infected bacterial cells and discharge new phages (Connerton et al., 2011). In addition, phage binds precisely on a receptor originate on the surface of the microbes to the bacterial host, and inoculates its gene inside the chamber. The host organism offers the necessary molecules plus enzymes for replicating the gene of the phage and generating the phage of the offspring. Phage-encoded proteins for instance, endolysin and holin destroy the host cell. Later on, the

phage can contaminate and destroy all adjacent bacteria in the external environment. this type of phage making in large numbers of offspring is an advantage when the phage is used in bacteriophage remedy (Doss et al., 2017). In contrast, the lysogenic life cycle encompasses their DNA progression into the host genome is usually inappropriate for bacteriophage treatments which can cause the host bacterium to become immune to further infection by producing a phage-programmed repressor. In addition, lysogenic bacteriophage infection can lead to the allocation and diffusion of DNA programming pathogenic characters among its host organism (Connerton et al., 2011). In reality, the host cell is not immediately destroyed through a temperate phage in the lysogenic process; rather, the genetic material is introduced into the host chromosome at particular locations. This phage DNA is entitled as pro-phage in the host genome, whereas the host cell which comprises the prophage is termed as lysogen. The prophage is simulated with the bacterial host genome, and a steady bond is established (Doss et al., 2017).

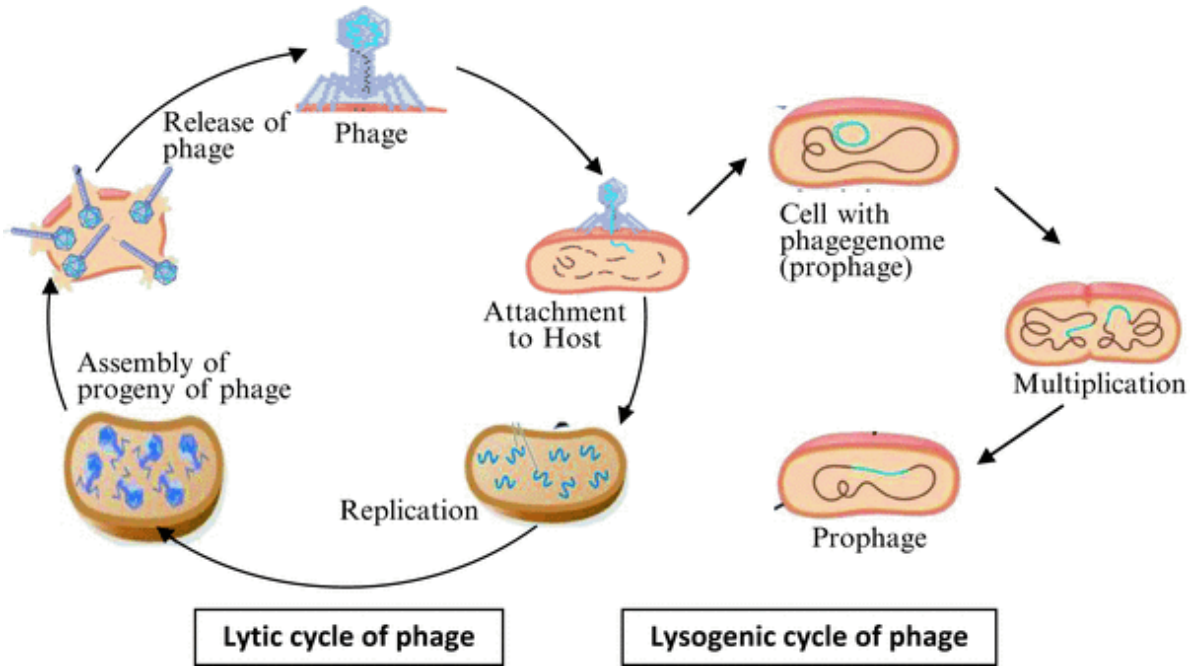


Figure 4 Lytic and Lysogenic Cycle of Bacteriophage(Kalia, 2016)

1.6 Importance of Phage Therapy

In the sense of wide-reaching antibiotic resistance and a cumulative awareness of the significance of human microbes, the possible usage of bacteriophages for therapeutic tenacities, known as phage therapy, has been resurgent (Malik et al., 2017). Since the encounter of phages a span ago, the history of phage therapy has been filled with contradictory results, misinterpretations and imperfect under-standing, all part of normal science (Summers, 2012). After their discovery about 100 years ago, they exert therapeutic effects by killing bacteria and have been successfully applied against various bacterial infections. However, the United States or the European Union (EU) have not approved a phage therapeutic approach for human use. So far, no serious phage therapy adverse events have been recorded which justify their use against antibiotic resistance of bacterial disease. More recently, clinical trials and case reports have shown promising results on a variety of indications. Nevertheless, the regulatory and legal systems are major hurdles towards the implementation of bacteriophage therapy in the Occidental culture. Present regulations that take up to be met for a decade or longer. There is an urgent need to improve phage therapy supply. Nonetheless, detailed studies of the bacteriophages are restricted to a few well-considered illustrations, and there are enormous numbers of phages which have not ever been identified (Trend et al., 2017). Additionally, a community of usually multidrug-resistant bacteria has been called ESKAPE. This is an acronym for the species *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter.* Here, bacteriophage therapy could provide a promising alternative to current antibiotic therapies to counter against these bacteria which are resistant to multiple drugs

(Alisky et al., 1998; Moelling et al., 2018). Furthermore, cellular activity and genome stayed unstudied for many that have been isolated and identified. As a result, this is a portion of what makes phages more electrifying for future latent therapy; that there are huge untapped biological resources from which the subsequent chapter in medicinal therapy will derive. Again, modern expertise has enabled scientists to study the molecular and genomic alterations in the host organism following phage septicity, thus growing our understanding of phage replication environmental science (Trend et al., 2017).

1.7 *Klebsiella pneumoniae*

The microorganism *Klebsiella pneumonia*e, is a major cause of worldwide morbidity and mortality (Anand et al., 2019; Tsai et al., 2010). It is the facultative anaerobic bacteria and it is gram negative (Anand et al., 2019; Struve et al., 2008; Tsai et al., 2010). After *Escherichia coli K. pneumoniae* is the subsequent communal reason of gram-negative bacteremia (Juan et al., 2019; Tsai et al., 2010). In a population-based survey in Canada, the case fatality rate was 20 per cent. *K. pneumoniae* bloodstream infections typically occur as a result of focal urinary, gastrointestinal, or respiratory tract infections although they may occur occasionally without a definable source (Meatherall et al., 2009; Struve et al., 2008). In fact, the most significant features of these infections are secondary infections – for example, the pyogenic brain swelling, endophthalmitis, plus meningitis (Anand et al., 2019; Tsai et al., 2010). Besides, some underlying diseases like malignancy, biliary tract disorders, cirrhosis, diabetes mellitus (DM), and alcoholism that weaken the defenses of a person and raise the danger of this infection (Tsai et al., 2010). Even the infections caused by these bacteria are often life-threatening in children, elderly and immunosuppressed patients. In turn, the rise of antibiotic-resistant of these strains, prolonged spectrum β-

lactamase fabricators (ESBLs) and carbapenem impervious strains have emerged as a worldwide source of great concern. Therefore, in the current antimicrobial resistance situation a hunt for antibiotic substitutes is ongoing and phage treatment has added considerable attention (Anand et al., 2019; Juan et al., 2019).

1.8 Objective of the study

The objective of the research is to isolate bacteriophage from soil samples of different areas and observe the effects of bacteriophage over *Klebsiella pneumoniae* show the multi-drug resistance characteristics. In fact, it developed resistance against many known antibiotics, for instance, Ampicillin (96%), Cephalothin (90%), Amox-Clav (90%), Cefuroxime (90%), Aztreonam (87%), Ceftriaxone (85%), Cefepime (80%), Trim-Sulfa (82%) and Ceftazidime (80%)(Alsanie, 2020). So, therefore, the research is to optimize and evaluate four different methods to sequestrate bacteriophage for that specific bacteria.

Chapter 2

Materials and Methodology

2.1 Study Place

The study was carried out at Pharmaceutical Microbiology Lab, the department of Pharmacy of BRAC University.

2.2 Soil Sample Collection

Soil samples were collected from three different places in Bangladesh. Two samples were collected around the Dhaka city. Among these two samples, one collected from nearby hospitals and lake or pond near the river type area. The samples were collected in a specific manner. Firstly, three plastic bottles were taken and were rinsed was collected from Old Dhaka (Boali) (Figure 6) and one from Tongi (figure7). And the other sample which was outside Dhaka was collected from Comilla (Figure8). The samples were properly. Secondly, then after drying 300ml sterile PBS (Phosphate Buffer Saline) was poured into the bottles carefully and sealed the bottles properly with the caps. This process was done in laminar air flow to avoid contamination. Finally, the bottles were taken to the collection area and the soils inside the bottles were poured carefully and the bottles were sealed again properly.

The soil samples were obtained using hand gloves to avoid contamination. In addition, some other properties of the soil were measured such as longitude, latitude, moisture content, area temperature, etc. (Table 2).

Table 2 Physical properties of the soil samples (types, temperature, moisture content, longitude and latitude)

Sample number	Area of soil sample	Type of soil	Temperature	Moisture content	Longitude	Latitude
1.	Old Dhaka(Boali)	Wet, loamy	23◦c	44.64%	90.3662695	23.7543795
2.	Tongi	Wet, Silty	15◦c	37.73%	90.4016748	23.8818311
3.	Comilla	Wet, bit sandy	27◦c	19.48%	91.1792889	23.4675189

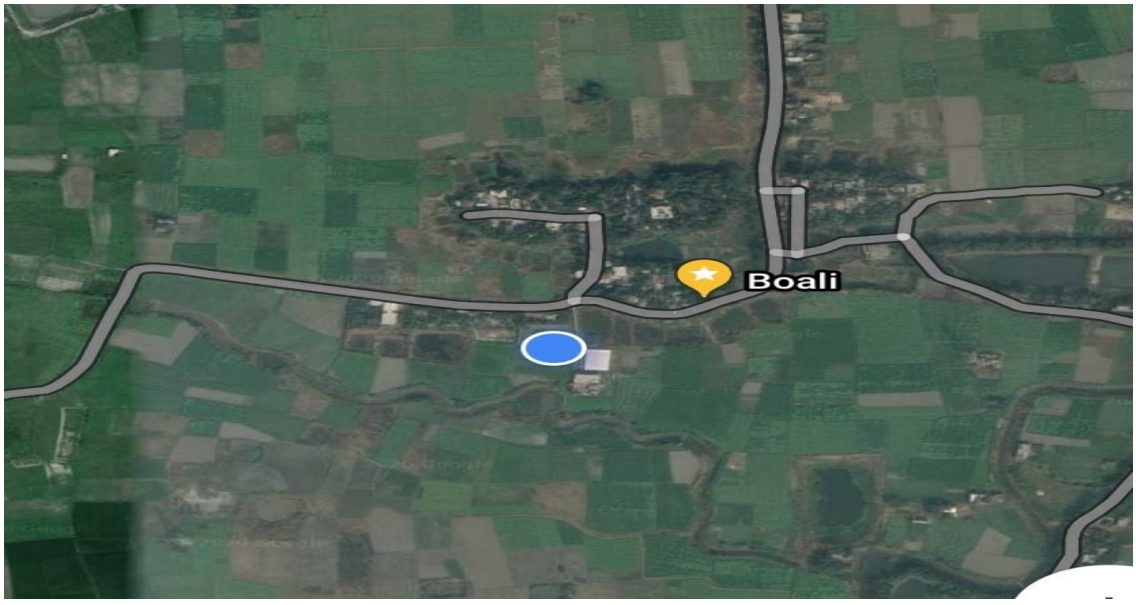


Figure 5 Location of the soil sample collection; Old Dhaka (Boali) (Google Maps, 2020)

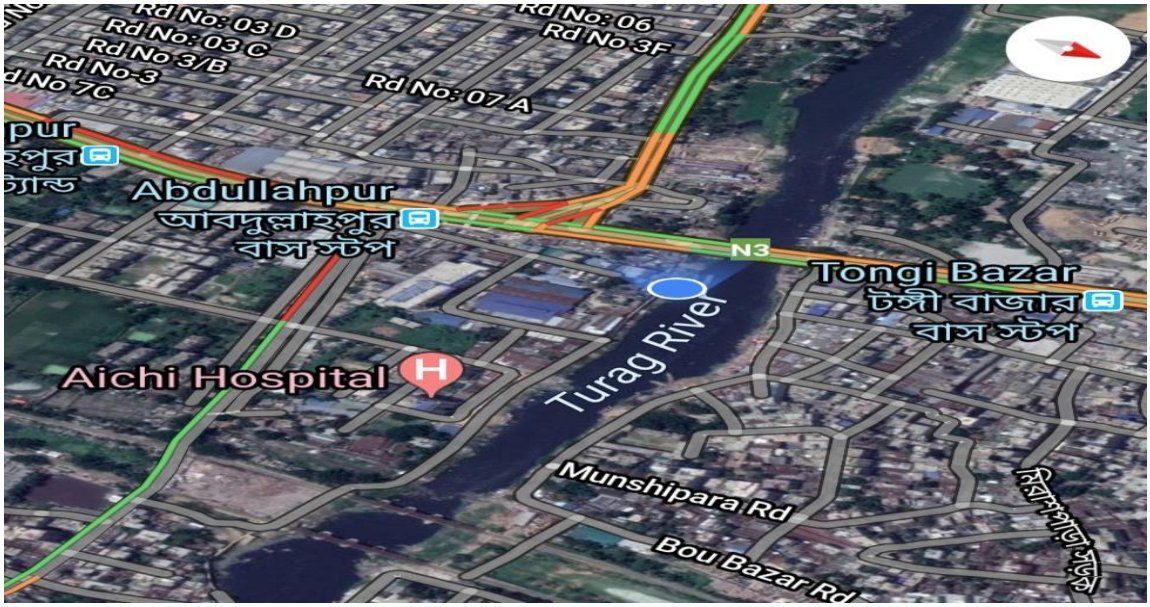


Figure 6 Location of the soil sample collection; Tongi (Google Maps, 2020)

Figure 7 Location of the sample collection; Comilla (Google Maps, 2020)

2.2 Culture of *Klebsiella pneumoniae* strains

The *Klebsiella pneumonia**e*** strains were cultured in a streaking method. At first, the nutrient agar of 2% w/v concentration was prepared. Then the agar preparation and petri dishes were autoclaved at 120°C for 45 minutes. When the autoclave was finished then the agar was allowed to cool down and after the agar comes into 50-60°C temperature, 20 ml nutrient agar was poured in each petri dish. Next, the agar was allowed to set for 20 minutes. When the agar is perfectly settled down, with the

help of a loop *Klebsiella pneumoniae* strains were distributed in three areas of the plate. The petri dishes were then placed for incubation at 37°C for 24 hours (Figure 9) (Sanders, 2012).

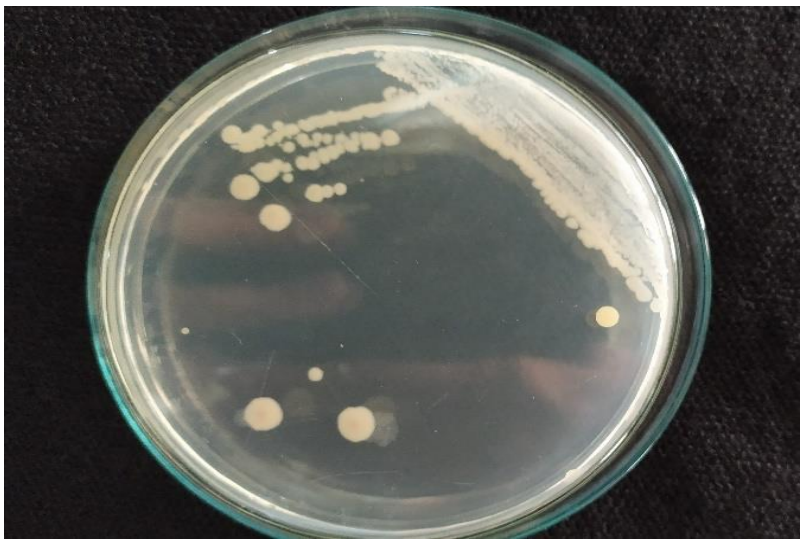


Figure 8 Klebsiella pneumoniae culture by streaking method.

2.3 Bacterial broth culture preparation

First of all, the 260mg of nutrient broth was taken for the 10ml of distilled water and autoclaved at 120 °C for 45 minutes. The broth was then allowed to cool off and when it comes into 50-60°C, the 2.2 bacterial culture (Figure 9) was taken with the help of a loop. After that, it was mixed cautiously into the test tube comprising nutrient broth and located inside the incubator for 24 hours at 37 ° C so that the bacteria could spread.

2.4 Double Layer Agar Plate Method

Phages isolation was achieved with the assistance of the system of the double layer agar plate. As determining the functional concentration of bacteriophage particles (titer), typically stated as plaque-forming units (p.f.u)/ml, is the basic protocol for those of dealing with bacteriophages. This requires combining phage suspension

dilutions with host bacteria in a diluted agar and spreading this mixture to solidify on a regular agar plate. Plaques are visualized as clearing zones (or diminished growth) in the bacterial lawn after incubation, usually overnight. Visualizing individual plaques allows for far more than pure enumeration. It is the base for the isolation of phages (Chhibber et al., 2018; Pelzek et al., 2013; Sanders, 2012).

2.5 Bottom and top agar preparation

10ml bottom and 5ml top agar at a concentration of 1.5% was prepared. Here, the amount of top agar was half of the bottom agar. After preparing the agars, they were autoclaved at 120 ° C for 45 minutes. Then the petri dish had the bottom agar dispensed and allowed to solidify. Once the hard agar was solidified, it was tipped onto the soft agar containing diluted phage solution. After that the plates were placed in the incubator at 37 ° C when they were prepared.

2.6 Method 1

2.6.1 Bacteriophage culture from soil samples

In this process, at the beginning the soil sample is filtered with a paper filter two times and then it was filtered with .22 micro-filter so that merely virus can pass through this filter in a test tube which was autoclaved at 120 ° C. And so, it retrieved the bacteriophage from the soil sample. Then the bottom and top agar were prepared in the above-mentioned manner (2.5). After the autoclave was completed, the top agar was held at 55 ° C in the water bath so that it would not solidify. Next to the *Klebsiella pneumoniae* broth, culture from 2.3 was taken, and 2.5 ml of bacterial broth was taken into an autoclaved beaker with the aid of an autoclaved 5ml pipette.2.5 ml of filtered phage was also taken with another pipette which was also

autoclaved and poured in the same beaker to mix the phage and bacteria. The mixture was then placed in the incubator for 15 minutes at 37 ° C so that the phages can attach to the bacterial cell surface. Thereafter the mixture was mixed with the 5 ml of top agar and effused over the bottom agar then allowed the mixture to set for 10 to 20 minutes in the laminar. The concentration of bacteria and phage mixture volume was equal to the top agar volume so that the top agar concentration became half to the bottom agar concentration. After setting down of the top agar the petri dish was held inside the incubator at 37 ° C for 24 hours to see the result.

After 24 hours of incubation, a large amount of bacteriophages occurred which was difficult to isolate. So, to get a proper and pure clear zone of bacteriophage, dilution was necessary. For that a sterilized toothpick was taken. Using it some phage was scraped from the petri dish and dissolved into 10 ml of broth. The broth concentration was 260 mg, and was autoclaved before use. The mixture then was held inside the incubator at 37 ° C for 24 hours.

2.6.2 Isolation and purification of bacteriophage

In this step, at first the broth culture of bacteriophage from 2.6.1 was filtered two times with paper filter then then filter with .22 micro-filter to make sure that no bacteria or other microorganism present in the supernatant. All the instruments were autoclaved for filtration before being used at 120 ° C. Then PBS (Phosphate Buffer Saline) was taken which was also autoclaved previously for the dilution of the bacteriophage supernatant. The sample was diluted to 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} concentrations. Additionally, the top and bottom agar was prepared in the same manner mentioned in the 2.5. Then the top agar was kept in the water bath at 55° C. And then the diluted phage was mixed in the same amount as the bacterial broth

described in the 2.6.1 and mixed with the top agar. Then the top agar was overlaid to the bottom agar and allowed to settle down. After 10 to 20 minutes the petri dish was retained for incubation at 37 ° C temperature for 24 hours.

2.7 Method 2

The first steps of this method are exactly similar to those of the steps 2.6.1 and 2.6.2. But there is one difference in these two methods. And that is only a few drops of chloroform are applied to the 2.6.1 phage broth solution before filtration. As chloroform helps to remove contamination and eradicates bacteria in the solution (Hyman, 2019; Pelzek et al., 2013; SCHMIDT & STANIER, 1965). After adding the chloroform the filtration is done likewise 2.6.2 and the rest of the process was also done in the same way alluded in 2.6.2.

2.8 Method 3

2.8.1 Bacteriophage filtration from soil sample

At first, some amount of sample was taken into a test tube. It was allowed to centrifuge at 4000rpm intended for 20 minutes. Then the sample was strained with a paper filter two times in another test tube so that no soil particles are left in the sample.

2.8.2 Bacteriophage broth culture preparation

5ml of the filtered sample from 2.8.1 was mixed 5ml of previously autoclave nutrient broth. The sample was then kept in the incubator for 15 minutes. After that it was filtered with .22 micro-filter to avoid bacteria in the sample.

2.8.3 Preparation of the agars and phage sample dilution

Agars were prepared in a similar way which has been mentioned in 2.5. Then the bacteriophage sample was diluted with the help of PBS. The sample was diluted to 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} concentrations.

2.8.4 Overlay of the top agar containing diluted phage culture

After dilution 2.5ml of diluted phage was mixed with a 2.5ml of the bacterial broth just as 2.6.2 and allowed to incubate for 20 minutes and then it was mixed with the top agar. After that tipped over the bottom agar. Finally, after settling down of the top agar, it was placed for incubation at 37° C for 24 hours.

2.9 Method 4

2.9.1 Bacteriophage filtration

First of all, in this method some sample was taken into a test tube and centrifuged at 4000rpm speed for 20 minutes. Then the sample was filtered with two paper filters and then with .22 micro-filter to remove the possible amount of bacteria. All the test tubes used in the filtration were previously autoclave to avoid maximum levels of contamination.

2.9.2 Mixture of soil with bacterial broth culture

In this step, a bit of soil was added to the 5ml of *Klebsiella pneumoniae* broth culture. Then it was centrifuged for 20 minutes at 4000rpm speed. This step has been done to increase the amount of phage.

2.9.3 Mixture of supernatant and soil containing broth

5ml of supernatant from 2.9.1 was mixed with 2.9.2 which was the centrifuged soil broth.

2.9.4 Filtration and dilution of the mixture

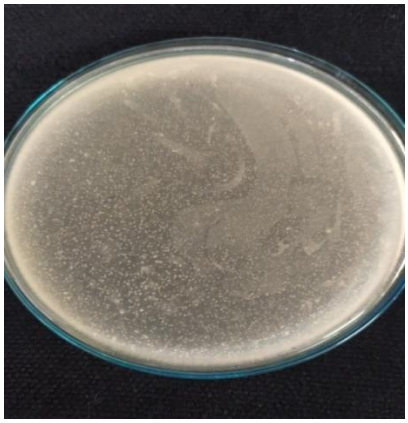
The mixture from 2.9.3 was again centrifuged at 400o rpm for 20 minutes. Then a few drops of chloroform was added to the mixture to kill all the bacteria from the mixture (Hyman, 2019; Pelzek et al., 2013; SCHMIDT & STANIER, 1965). Then the mixture was filtered with paper filters two times and with micro-filter afterwards to remove the chloroform and bacteria. Next, the sample was diluted with PBS to 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} concentrations.

2.9.5 Overlay of the top agar comprising of phage mixture

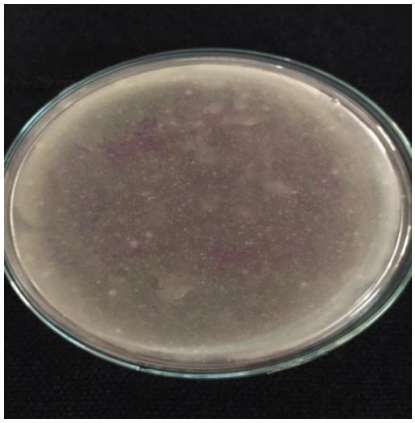
In this step, the diluted sample was mixed with bacteria culture and then added to top agar was poured over bottom agar in the corresponding system and amount from 2.8.4. Finally, it was placed for incubation at 37° C for 24 hours.

Chapter 3

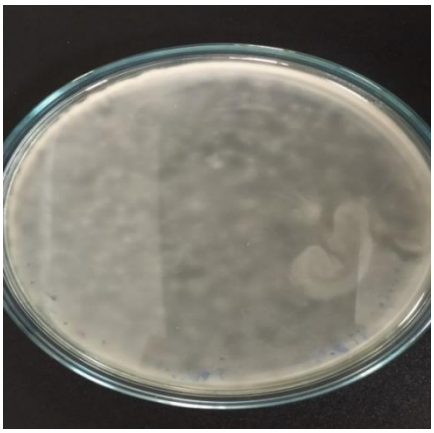
Result



Sample No. 1



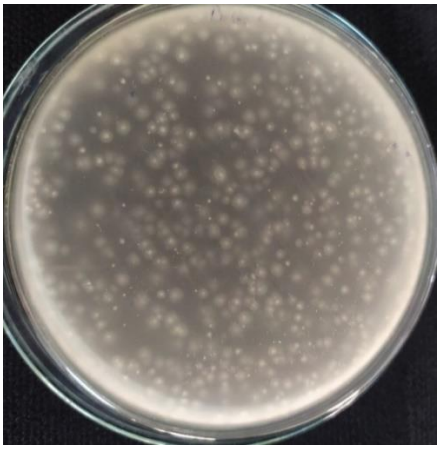
Sample No. 2

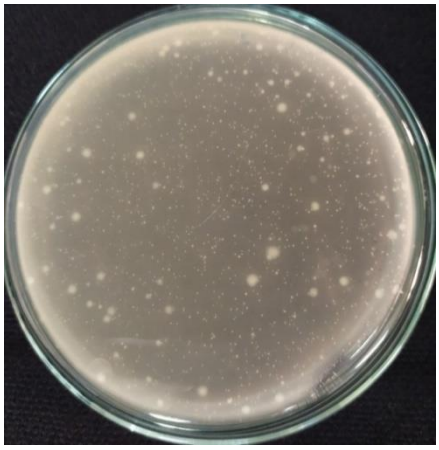


Sample No. 3

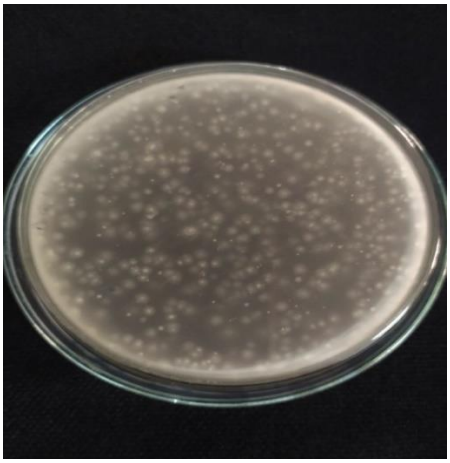
Figure 9 Final results of Method 1(no proper clear zone, turbid zones appeared and bacterial growth occurred)

In the above figure, the result of method one has been shown. In this process no proper clear zone appeared and bacterial growth has appeared. This also means that contamination occurred. But some blur areas occurred in all three samples because of the bacteriophage lysis process.





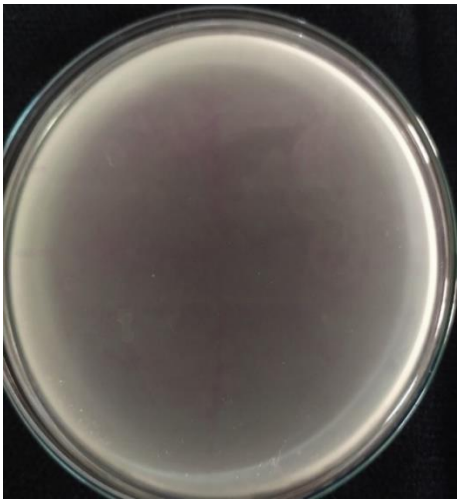
Sample No. 1 Sample No. 2



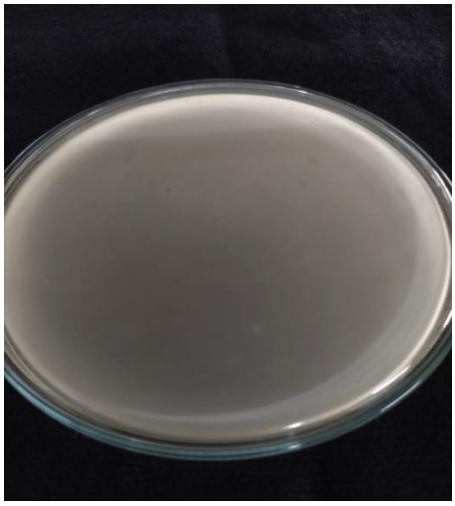
Sample No.3

Figure 10 Final results of Method 2(bacteriophage plaque formed in sample 1 and 3 but turbid, bacterial growth occurred in all three samples)

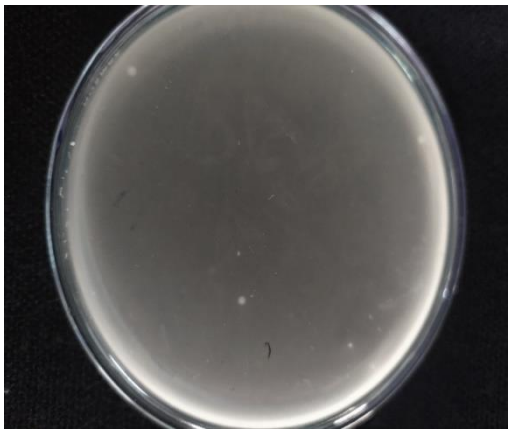
In the above mentioned figure it can be stated that in sample 1 and 3 there are presence of phage plaques but not very clear as bacterial growth has occurred. On the other hand in sample 2 there is no presence of clear zones. But the sample plates are more clear than the above samples of method one (Figure 10) due to the use of chloroform. As chloroform used to remove contamination. Yet contamination has occurred in all three samples, may be due to some errors and for that reason no perfect clear zone has occurred.



Sample No. 1



Sample No. 2

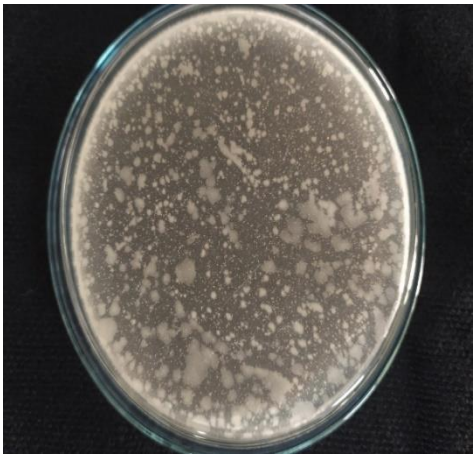


Sample No. 3

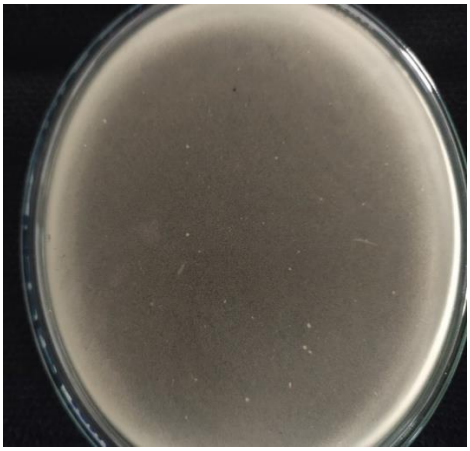
Figure 11 Final results of method 3(no presence of bacteriophages)

With method 3 the expected result did not come which is clearly seen in the above figure. Here, no clear zone appeared at all due to some errors. The level of contamination and bacterial growth, however, is very smaller than the findings of method one and two (Figure 10 and Figure 11). In addition, though in this process no chloroform is used but the amount of contamination occurred. There could be many possible reasons for this negative results such as the weakening of the phages, the lesser amount of phages in the sample, unexpected errors, etc.

Sample No. 1



Sample No. 2



Sample No. 3

Figure 12 Final results of method 4(plaque formation in sample 1 and 2 but not in 3 and bacterial growth occurred)

In this method in the first two samples some bacteriophage colonies appear, but due to huge amounts of bacterial growth it is not clear. However, in sample 3 no clear zone appeared which means no presence of bacteriophage. Yet bacterial growth is less extensive. Moreover, from the following pictures, it can be stated that the result of any of the methods is not satisfactory. No perfect clear zone has been seen and bacterial growth occurred. Many reasons can be behind this problem such as the lesser amount of bacteriophages in the samples, contamination, keeping the sample in the

refrigerator for many days, collection time of the samples, faults in method steps, not enough dilution and so on.

Table 3 Findings of all three samples with four different methods

Method number	**Sample number**	**Sample name**	**Bacteriophage plaque formation**	**Bacterial growth formation**
1	1	Old Dhaka(Boali)	Negative	Positive
1	2	Tongi	Negative	Positive
1	3	Comilla	Turbid plaque	Positive
2	1	Old Dhaka(Boali)	Turbid plaque	Positive
2	2	Tongi	Negative	Positive
2	3	Comilla	Turbid plaque	Positive
3	1	Old Dhaka(Boali)	Negative	Negative
3	2	Tongi	Negative	Negative
3	3	Comilla	Negative	Positive
4	1	Old Dhaka(Boali)	Turbid plaque	Positive
4	2	Tongi	Turbid plaque	Positive
4	3	Comilla	Negative	Positive

Chapter 4

Discussion

From the above result, it has been clear that the following results are not satisfactory. There could be various reasons for not getting the proper results from any of those following methods. One of the probable problems could be that the amount of phage which effects the *Klebsiella pneumoniae* strains were very less as the choice of isolation host is the most critical part of the isolation process, since the isolation host will restrict isolated phage types (Abdulsattar et al., 2020; Hyman, 2019). In addition, changes in environmental conditions in different seasons resulting in differences in bacteriophage concentration, as more bacteriophages are available in the spring, summer and autumn, but less in winter (Drucker & Dutova, 2009). Here, the soils were collected in the winter season when the soils become dry due to low amounts of moisture and nutritional value (Germida & Casida, 1981). As a consequence, the amount of phages decreased. Which could be a possible reason for the lesser amount of bacteriophages into the soil sample. Also may be due to the soil types which are taken is not appropriate for bacteriophages. As a result, very few pages have been found from these three samples (Williamson et al., 2003). In addition, the sample which has been collected from Tongi is a very contaminated area with different types of chemical wastes. Sometimes the presence of toxic materials decreases the amount of microorganisms into the environmental samples(Maurice et al., 2010). Therefore, no phage plaque was seen at all in method three and no clear plaque was created in the other three methods.

Once again, the results show that almost all the plates had bacterial growth and that in some plates, the phage plaques were also turbid. The possible reason for this could be

that the incubator of the lab was not working properly for some days so the temperature was not maintained properly. Therefore, after making the bacterial broth it was kept into the incubator for three days but in various temperatures as the incubator was not working. So, there could be another reason for contamination and bacterial growth into plates.

In method two and four chloroform has been used, but still there was the presence of bacterial growth. Yet chloroform is typically used to extract bacteria from bacteria and destroys the bacteria and prevents pollution. Furthermore, sometimes chloroform can kill some phages also (Lanning & Williams, 1982; Pelzek et al., 2013; Williamson et al., 2003). As a result, phage plaques did not form properly. Although the outcomes of two and four methods are not favorable, there is one positive thing about both methods. And because of the use of chloroform, the plates consist of less bacterial growth (Figures 11 and 13) than one and three plates of the process (Figures 10 and 12). If the amount of chloroform was given in the right manner, maybe the bacterial growth had not occurred like this.

The double layer agar (DLA) plaque amount is a well-well-known tool inn case of bacteriophage evaluation, isolation and identification and isolation of phage-resistant mutants. The double layer agar is a common approach for plaques of both bulky and tiny sizes. Nevertheless, the accurate record of tiny plaque forming phages is very problematic due to large areas of study and very little distinguishability, which can lead to error inn case of evaluation of phages (Chhibber et al., 2018; Cormier & Janes, 2014). This can also be a reason for not getting the proper results from any of the methods.

Moreover, due to mistakes of handling the apparatuses properly or not maintaining the aseptic techniques in case of collecting the soil sample and in the lab can also be a reason for this type of results. And problems in filtration and dilution can be another problem for not getting the proper result. Another important reason is that the sample was collected nearly a month ago, and kept in the fridge for a long time. For this reason may be the phages become weak or dead and decrease in concentration. So due to this plaques did not form.

Though from any of the methods no proper result has been achieved but among all four methods, method two is better. In future by modifying this process better results can be expected. On the other method four can also be used in the future. Because in these two methods more or less some blur plaques are formed but due to bacterial growth the plaque is not clear. But these two methods have the potential to get proper plaques. In both techniques proper enrichment media has been used and also sterilization process has been used. Both these methods need some modification and with that they can give perfect results in the future. And by solving the above problems better results can be found in the future time.

Chapter 6

Conclusion

In this project, our main aim is to segregate and classify the bacteriophages as microorganisms immune to antibiotics progressively become strong and responsive to traditional antibiotics. So, we collected some samples around Bangladesh and tried to isolate and classify bacteriophages. The bacteriophages can invade and destroy antibiotic-resistant bacterial strains, and further reduce antibiotic costs and dependency. So, saving lives and saving the world, researchers should prioritize such project.

Chapter 7

Future Work

In future, we would like to do apply method 2 and method 4 with other samples to see whether those to methods are valid or not. In addition, we would collect the sample in the seasons when the amount of getting bacteriophages from environmental sample is higher. Also, we would modify some steps such as, increasing the amount of dilution, appropriate use of chloroform, etc. Moreover, we would try to take more precautions so that contamination can be avoided to the highest possible extent.

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