Therapeutic Effect of Bacteriophage Against Antibiotic Resistance Bacteria

A Dissertation

Submitted in the partial fulfillment of the requirement for
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DECLARATION

I hereby declare that the work which is being presented in dissertation entitled "**Therapeutic effect of Bacteriophage against Antibiotic Resistance Bacteria**" submitted by me for the award of the degree of Master of Science in Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala, is true and original record of my own independent and original research work carried out under the supervision of **Dr. Prangya Ranjan Rout**, **Dr. Dwarikanath Ratha** and **Dr. Manoj Baranwal**, , Thapar Institute of Engineering & Technology, Patiala. Further, I declare that no part of this dissertation has been submitted to any other University/Institute for the award of any degree in India or abroad.

Place: Patiala

Date: 21.07.2022 Gurleen Singh

CERTIFICATE

This is to certify that the project entitled "**Therapeutic Effect of Bacteriophage Against Antibiotic Resistance Bacteria**" submitted by **Gurleen Singh** in the partial fulfilment of the requirement for the award of degree of Master of Science in Biotechnology to Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala, is a record of student's own work carried by him. The report has not been submitted for the award of any degree or certificate in this or any other University or Institute**.**

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ABSTRACT

With the increase in antibiotic resistant microbes in wastewater that causes a serious threat to public health, the demand and search for an alternative form of therapy is increasing. Bacteriophage may be explored as an alternative to antibiotics due to its natural ability to kill bacteria. But, the practical research surrounding the concept of bacteriophage therapy is lacking. Thus, current research project aims to determine the effects of bacteriophage on the antibiotic resistant bacteria isolated from wastewater in hopes to provide an alternative to antibiotics as a way to curb the rise of antibiotic resistant bacterial population in wastewater. Bacteria isolated from wastewater were screened for antibiotic resistance with four broad spectrum antibiotics (Ampicillin, Kanamycin, Streptomycin, Azithromycin). *Escherichia coli* bacteriophage MS2 was used to determine the effect on multi-antibiotic resistant strains of bacteria. Three out of four bacteria isolated from waste water were found to be antibiotic resistant. 16S rRNA sequencing was done for two bacteria in which one was identified as *Pseudomonas stutzeri* and other as *Shewanella putrefaciens.* Formation of transparent zones or plaques in culture plates indicated successful lysis of multi-antibiotic resistant bacterial cells by bacteriophage which suggests that bacteriophage can successfully infect and kill antibiotic-resistant bacterial hosts. The findings of this project will be helpful for further research in developing treatment system engaging the bacteriophages for controlling or eliminating antibiotic resistance bacteria from wastewater of different origin.

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Chapter 1: Introduction

Introductory comments - With the increase in use of antibiotics, Antibiotic resistant bacteria (ARBs) are becoming a serious public health problem. There is an extreme need for an effective form of therapy that not only helps in inhibit or kill pathogenic bacteria but also curb the rise in new drug resistant bacterial strains.

1.1 Background

Due to increased usage of antibiotics and the rising trends in the population of antibiotic-resistant bacterial strains, the world needs an alternative form of therapy. Browne *et al* estimated that since 2000 the global antibiotic consumption rate expressed in defined daily doses (DDD) has increased 46% (9.8 billion DDD in 2000 to 40.2 billion DDD per 1000 population per day in 2018) (Browne *et al*., 2021). Some examples of antibiotic-resistant bacteria over the years include methicillin-resistant *Staphylococcus aureus* (MRSA) whose spread is related to mainly public places such as hospitals, schools etc (David and Daum, 2010) and strains of Tuberculosis (TB) that were resistant to streptomycin, para-aminosalicylic acid (PAS), and isoniazid (INH) were discovered in 1956 in Great Britain and were eventually named as multi-drug resistant TB or MDR-TB (Vilchèze and Jacobs, 2014). This exponential rise in antibiotic resistant microbes have raised the need for an effective alternative that steer the human population away from a possible antibiotic resistance pandemic (Anderson, 1999).

Complex viruses such as bacteriophages have been of great interest in regard to combating bacterial infections. Bacteriophages have been shown to be effective in treating staphylococcal lung infections, *Pseudomonas aeruginosa* infections in cystic fibrosis patients, eye infections, neonatal sepsis, urinary tract infections, and surgical wound infections (Sulakvelidze *et al*., 2001). Studies conducted in Bangladesh (2012) and Switzerland (2005) involved phage preparation targeted against *Escherichia coli* for the treatment of diarrheal disease were performed in Phase I trials. The trials also included a combination of healthy and diseased children when they were done in Bangladesh in 2017. There were no adverse effects found by physical and laboratory testing as well as self-reporting when phage preparations were orally administered. (Gordillo Altamirano and Barr, 2019). Phage therapy has also been successful in plants in controlling *Xanthomonas* species that causes plant diseases such as citrus canker, bacterial spot of peach, onion blight etc (Balogh *et al*., 2010). Considering these facts, bacteriophages have been explored for its effects against antibiotic resistant bacteria in the current study.

1.2 Gap Analysis

The overuse or misuse of antibiotics in animals and humans has been detrimental to the public health by doing more harm than good. This has given bacteria a fighting chance. Certain bacterial strains such as *Salmonella typhi* have been able to grow resistant towards antibiotics that were effective once such as Gentamycin, Azithromycin (da Silva *et al*., 2022) which is not only a region or a country wide problem. These antibiotic strains can spread throughout the globe leading to an effective antibiotic shortage on a global scale (Aslam *et al*., 2018). Phages have been a crucial element in the modernization of molecular biology and moreover, the countries that continued with phage therapy have assimilated much more clinical and research experience (Hanlon, 2007). Because of these reasons, research on phage therapy and its subsequent effect on antibiotic resistant bacteria is needed. Only a few papers have been published that discuss such a crucial subject (Hatfull *et al*., 2022), (Kortright *et al*., 2019), (Gupta and Prasad, 2011). So further research is required to shed a light on the applications of bacteriophage on antibiotic resistant bacteria.

1.3 Objectives

- Screening and identification of bacteria for antibiotics resistance bacteria isolated from wastewater
- Effect of bacteriophage against antibiotics resistance bacteria

Remarks - This study aims to use bacteriophage and its ability to lyse bacterial cell in hopes to decrease the spread of antibiotic resistance around the globe that can become a serious problem and threat to public health.

CHAPTER 2 - REVIEW OF LITERATURE

Introductory Comments - Bacteriophage therapy has been used to treat bacterial epidemics in the past where there was lack of resources or an effective antibiotic. Due to the increase in antibiotic resistant bacteria, the need to further explore bacteriophage therapy treatments has become dire.

2.1 Bacteriophage and its life cycle

Bacteriophages are the natural parasites of bacteria and are considered to be the most abundant organisms (approximately ten times more than their bacterial hosts) in the biosphere which ranges between 10^{30}–10^{31} (Kiani *et al*., 2021). Bacteriophages can have DNA or RNA as genetic material and can be either single stranded or double-stranded varying from species to species. Bacteriophages affect microbial populations by two distinct life cycles - lytic and lysogenic.

Lytic or temperate phages take over the host's cell machinery to make phage components. After that, they dismantle, or lyse, the host cell, releasing phage progenies (Parker *et al*., 2016).

The lytic cycle involves the following stages (Figure 1):-

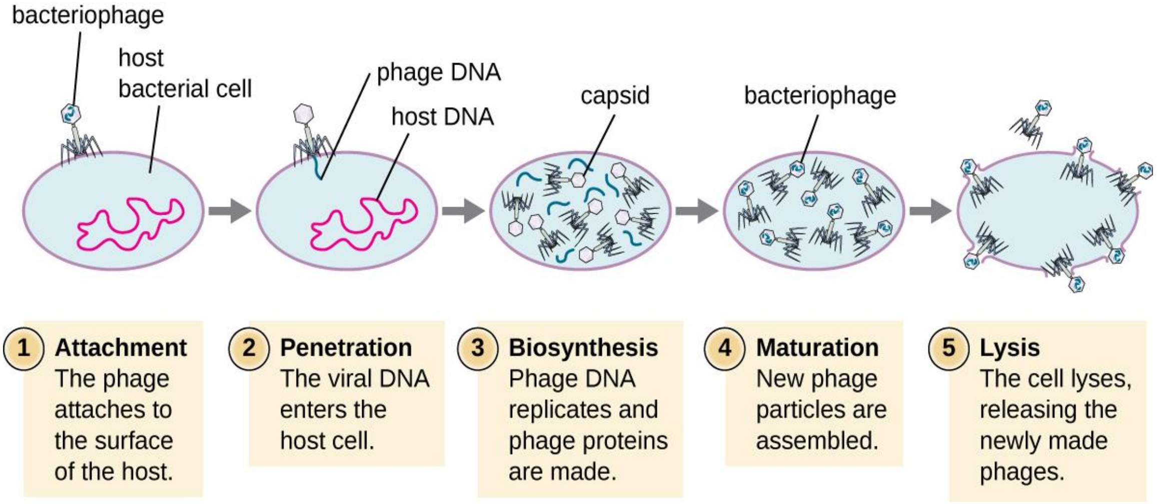

Fig 1: Lytic cycle of Bacteriophage (Parker *et al*., 2016)

A) Adsorption – A phage attaches itself to particular receptors present on the host cell that conforms to proteins of phage tail. A phage cannot attach to a host cell that does not contain any receptors that can adhere to the phage tail proteins.

B) Penetration – The fibers of the phage tail shrink and the baseplate settles on the surface of the cell. The phage infiltrates into the cell wall of the host cell an enzyme called as phage lysozyme and proceeds to push the core of the tail through the hosts' cell membrane. The genetic material of the phage gets injected into the cytoplasm of the host cell. The phage ghost comprising of phage capsid and tail is left on the outside the host cell.

C) Biosynthesis - Upon entry into the host cell, the phage genome manipulates the metabolism of the host cell. It manipulates the cellular machinery of the host cell for the formation or production of proteins and nucleic acid material that are required to assemble hundreds or even thousands of phage progenies. During this stage, a brief eclipse period occurs where a complete phage cannot be observed.

D) Maturation - Phage components such as phage DNA or RNA, tail and capsid proteins are produced. After this, phage assembly occurs. The phage's genetic material is injected into the capsid and subsequently the tail is adhered. The appearance of the first complete phage marks the end of the eclipse phage.

E) Lysis - The lysis of the host cell is triggered by the phage lysozyme consequently releasing the phage particles which further infect the neighbouring bacterial cells. The whole cycle can be over in mere 15 min for some phages.

By contrast, Lysogenic or Virulent phages integrate their genetic material into the host cell's genome and replicates together forming a unit without the killing of the host cell. The first two stages i.e. the attachment of the phage to the host cell surface and injection of genetic material of phage into the host cell are same in lysogenic cycle as that in lytic cycle. However, upon the entry of genetic material of phage into host cell, the host cell machinery is not manipulated immediately to make phage copies. Instead, it integrates itself into the bacterial or host cell genome. The recombined phage genetic material is now known as prophage, which is not active, which means that its genes aren't being actively expressed. That is, no new phage particles are being made. But, every time the bacterial or host cell multiplies, the prophage is also replicated along with the genetic material of the host, hitchhiking. The lysogenic cycle (Figure 2) is more longer than the lytic one but nevertheless another means for phage to reproduce and survive. Although after induction due to change in certain conditions the lysogenic cycle can be changed into a lytic one. (Parker *et al*., 2016)

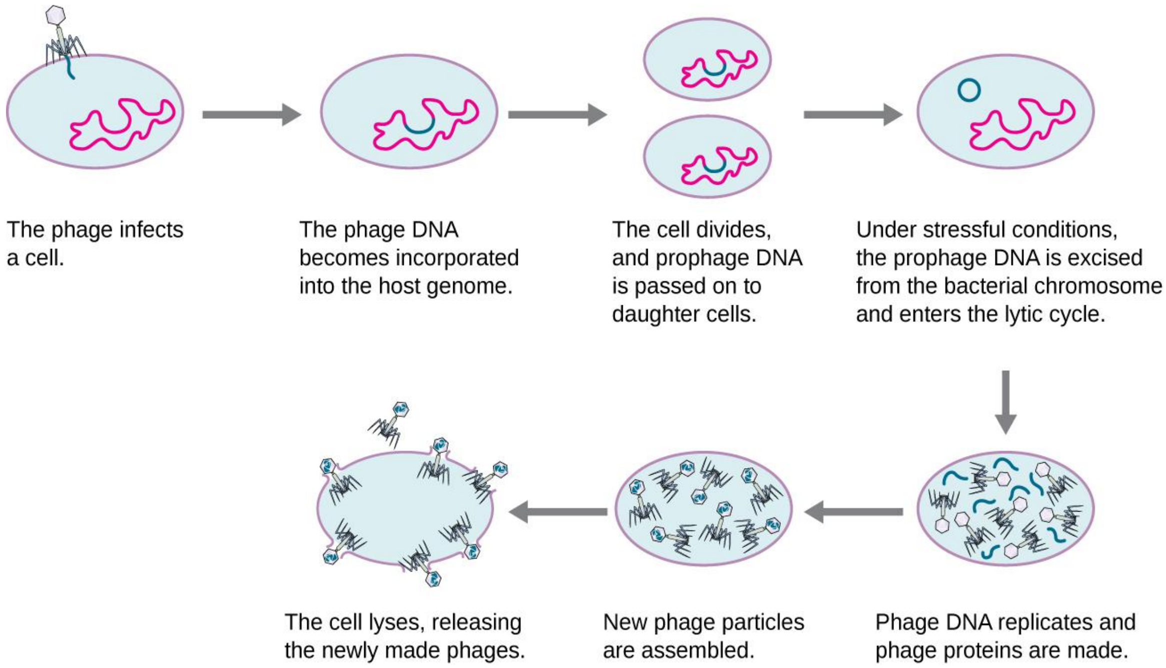

Fig 2: Lysogenic life cycle of phage (Parker *et al*., 2016)

Other life cycles include, chronic infection and pseudolysogeny. Chronic infection involves the production of phage progenies continuously but without any apparent host cell destruction. In pseudolysogeny, a bacteriophage enters the host cell but neither manipulates host cell replication machinery nor incorporates into the host genome. Pseudolysogeny usually occurs when the host cell experiences unfavourable growth conditions which indirectly plays an important part in phage survival by promoting the preservation of the phage genetic material until favorable host growth conditions become available again (Editors of Britannica Encyclopaedia, 2018).

2.1 The bacteriophage phenomenon

The earliest speculations about antibacterial activity against *Vibrio cholera* by particles that passed through fine pore filters and was heat sensitive were made by Ernest Hankin, a bacteriologist in 1896 (Figure 3). He made such observations after visiting the waters of Ganga and Jamuna rivers in India.

Several others also speculated phenomenon related to bacteriophage such as Gamaleya (bacteriologist) who was working with transmissible lysis of *Bacillus anthracis*. (Bardell, 1982) But it was not until a bacteriologist with medical training, Frederick Twort who reported a similar phenomenon 20 years after Hankin did but also built up the hypothesis that the phenomenon among other possibilities may have been caused by a virus (Sulakvelidze *et al*., 2001)

For some reason, Twort couldn't advance his research due to which it took another couple of years before bacteriophages were discovered 'officially' by a microbiologist from Institut Pasteur in Paris, Felix d' Herelle. Although he made the observations regarding bacteriophage while studying the inhibition of an epizootic of locusts by microbiological means in 1910 in Mexico, these findings are also associated with the severe outbreak of dysentery in Paris among soldiers posted at Maisons-Laffitte in 1915. While working with agar cultures regarding his research on dysentery he observed presence of clear, transparent zones which he at first, stated as taches or taches vierges and later on named them as plaques. The term 'bacteriophage' was also decided by d' Herelle and his wife Marie the day before their youngest daughter's birthday (18 October, 1916). The name is a combination of 'bacteria' and 'phagein' which means "to eat" (Sulakvelidze *et al*., 2001). Even though he was aware of the findings by Twort, d' Herelle still maintained that his discovery was different from that of the phenomenon described by Twort. Meanwhile, d' Herelle worked on bacteriophage studies actively and stated that bacteriophages were living viruses instead of 'enzymes' which was earlier speculated by his fellow scientists. Eventually, the independent discovery was accepted by many researchers, stating the discovery of bacteriophages as "Twort-d' Herelle phenomenon". Later, this phenomenon was simply called as "bacteriophage phenomenon" (Sulakvelidze *et al*., 2001).

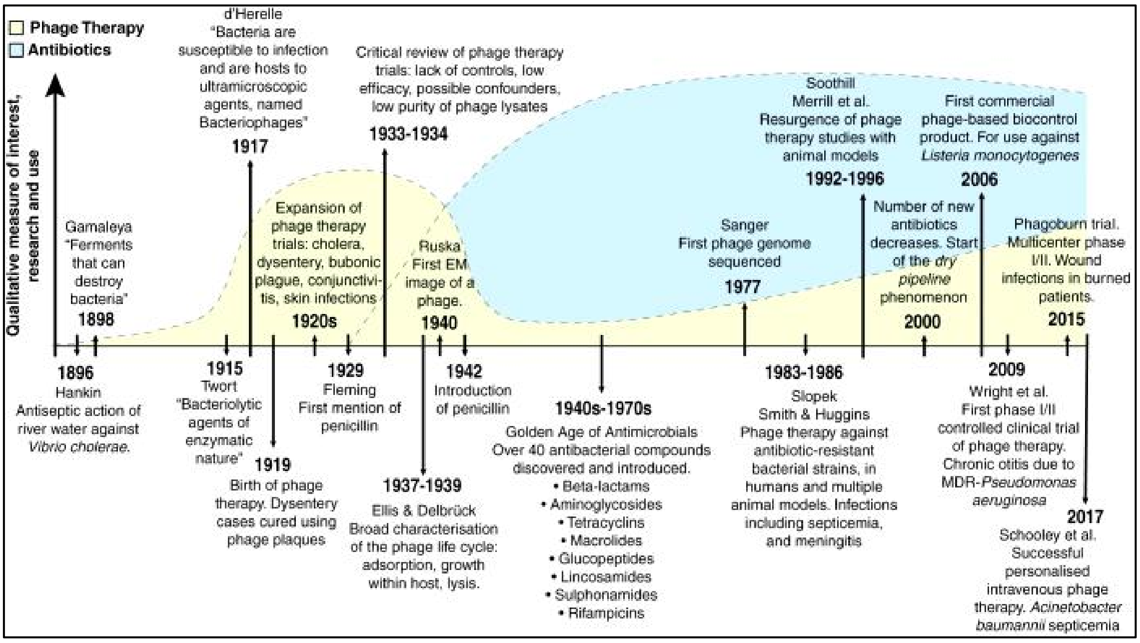

Fig 3: Timeline of major phage related events including phage discovery, phage therapy, phage sequencing. The background curves qualitatively depicts a measure of research as well as usage of antibiotics (blue) and phage therapy (yellow) (Gordillo Altamirano and Barr, 2019).

2.2 Composition of wastewater

Many research studies have reported the advantages of bacteriophages as indicators or trackers for the presence of microbes mainly bacteria in sewage treatment plants. Nevertheless, the role of microbes present in wastewater treatment systems is not well understood (Withey *et al*., 2005).

Wastewater is mainly composed of water with contamination of organic (microorganisms) and inorganic (heavy metals, antibiotics) compounds.
Wastewater composition may vary according to the source it was produced from such as hospital effluents may contain more microbial or drug load than municipal effluents (Novo and Maniaia, 2010). That said, a pandemic may change that.

The fear of COVID-19 pandemic coupled with the absence of awareness regarding the usage of antibiotics has directly impacted the access to readily available retail antibiotics, to a great deal in developing countries with bare minimum measures to curb antibiotics as well as a crippled healthcare system. In connection to this, it was outlined that usage of antibiotics such as Azithromycin and Ceftriaxone prior hospitalisation was reported by 68.9% of COVID-19 patients and the rate of self-medication was 33.0% (Ruiz, 2021).

The wastewater from hospitals either gets directed into municipal sewer system or first passes into hospital sewage system but eventually reaches its final destination i.e. wastewater treatment plant before being treated. Post-treatment, it is discharged into local water bodies or used up for irrigation. Some studies have mentioned that effluents from hospitals were linked to the rising trends of antibiotic resistant bacteria. The cause of hospital wide epidemics is antibiotic resistant Klebsiella strains. 90% Klebsiella strains were found to be resistant to ampicillin and 6% showed multiple antibiotic resistance when isolated from a sewage treatment plant (Gautam *et al*., 2007).

Developing nations in Asia and Africa might not have proper wastewater treatment resources. It was found that the main reason for the presence of drug resistant microbes in the water bodies of Pakistan were untreated hospital and municipal sewage. Akter *et al*. also stated that the prevalence of antibiotic resistant bacteria in Bangladesh was majorly due to the dissemination of under processed drugs present in hospital and agriculture wastewater leading to environmental pollution (Aukidy *et al*., 2017)

Drugs are developed considering how they will interact with the human body but most of them remain unmetabolized. Major groups of antibiotics for example, sulfonamides, quinolones and nitroimidazoles have low biodegradability. Out of these, Sulfonamides are far less biodegradable than pentachlorophenol (Gautam *et al*., 2007). Total concentrations of sulfonamides, trimethoprim, macrolides, tetracyclines, quinolones was found to be more than 63,000 ng/L in Hong Kong sewage. Ciprofloxacin was present in the highest concentrations (48,000 ng/L) followed by clarithromycin (39,551 ng/L). High levels of norfloxacin and anhyro-erythromycin were also found in Indian sewage (Riquelme *et al*., 2021).

In some cases, around 90% of the incompletely dissolved or unchanged active drug compounds are excreted out into municipal or hospital sewage system. Historically, emissions from manufacturing sites were not considered. But in some developing countries, it has been found that a tremendous amount of emissions are emitted by manufacturing sites (Larsen *et al*., 2013).

It was found that Antibiotic-resistant genes (ARGs) are found in higher numbers in Asian sewages than sewages of Europe or US which is in line with the levels of diligence in policies regarding antibiotic usage in the above mentioned countries. The lowest levels of antibiotic resistant genes were present in wastewater from Sweden, consistent with their various measures to curb unnecessary usage of antibiotics. Whereas, countries like the Philippines, Hong Kong, and India are densely populated with prescription less and illegal sales of antibiotics (Riquelme *et al*., 2021).

A recent study published by Lancet suggests that south Asian countries such as Bangladesh, India and Pakistan continue to be a problematic hub for antibiotic-resistant *Salmonella typhi*, and these emerging antibiotic-resistant strains, having no concept of borders spread across the continents. The study also states that although muti-drug resistant microbial strains are trending downwards in most South Asian regions but they are being displaced with Ceftriaxone resistant (highly drug-resistant) strains, strains extensively resistant against Floroquinone or Azithromycin which are instead overriding the decline in the overall population size of *Salmonella typhi*. (da Silva *et al*., 2022)

Higher concentrations (100 μg/L) of drugs have been found in hospital effluents than in municipal wastewater (20 μg/L). However, households are the key source of drugs in the water bodies w.r.t total load and volume. Other minor sources include landfills as expired pharmaceuticals are dumped along with municipal waste. Trace amounts of illicit drugs have also been detected in wastewater (Larsen *et al*., 2013). Large quantities of pharmaceuticals have been excreted out by animals and humans since the 1980s. Distribution of such pharmaceutical compounds has been through the application of manure, sewage etc (Boxall, 2004).

Some researchers saw the issue of antibiotic resistance bacteria on the emergence decades ago. Hirsch *et al* stated that there is an increasing number of bacterial infections that are untreatable by present known antibiotics because of rising trends of resistance in bacterial strains which is being induced by antibiotic residues causing a danger to the health of human beings (Gautam *et al*., 2007).

Resistance measures quantified in wastewater probably replicate both cutting-edge and ancient operators, which include antibiotic usage related to past patterns and selection pressures which could have happened within the human gut, that together form the resistome. But, antibiotic concentrations can best mirror usage on sampling time (Riquelme *et al*., 2021).

There is a dire requirement for further research regarding wastewater treatment especially hospital wastewater treatment. A tremendous amount of research is needed to know the adverse effects of waste produced from multi-speciality hospitals or clinical laboratories. It is doubtful that anyone will bat an eye towards this issue until it is assured that there is a situation developing right beneath our homes. Drug dumping in water could definitely use more research. It is important that we know how problematic existing concentrations of drugs are to the environment and what humans can do to curb it (Gautam *et al*., 2007).

2.3 The dawn of Bacteriophage therapy

Using bacteriophage as a therapeutic agent is not just a decade old idea. Infact, usage of bacteriophage as a therapeutic agent dates back to the 20th century (Larkum, 1926). Speculations about bacteriophage applications were made throughout the decades such as the effect of *Bacillus typhosus* bacteriophage up on peritonitis produced in guinea pigs and white mice, by means of the intraperitoneal injection of *Bacillus typhosus* (Larimore and Harris, 1929), treatment with bacteriophage against enteric tract infections (d'Herelle, 1930), the prospect for using bacteriophage as a form of therapy in Western medicine (Merril *et al*., 2003).

It was not too long after it was discovered that attempts were made to use bacteriophage therapeutically. Félix d' Herelle used bacteriophages as a form of therapy to treat dysentery in 1919 the with the help of Chief of Pediatrics, Professor Victor-Henri Hutinel at a hospital for sick children in Paris. In order to make sure that it was safe for a twelve year old patient anguishing from dysentery, they (including a few hospital interns) ingested a phage preparation. The recovery was promising as the patient's condition improved after just a single dose of phage preparation concocted by d' Herelle. Later, it was proved not to be just a one hit wonder as conditions of several other bacterial dysentery patients started to improve after 24 h of dose administration. But because these findings were not reported immediately, the first official account of bacteriophage application for treatment of pathogenic disease were published by Bruynoghe and Masin in 1921. They utilized phages for the treatment of skin infection caused by Staphylococcus bacterial species. The treatment included preparing and injecting bacteriophages into bacterial lesions that were opened surgically. The treatment started showing favorable effects after 24-48 h (Sulakvelidze *et al*., 2001).

Majority of the past research about bacteriophage therapy is linked to two institutes: the Eliava Institute which is located in Tbilisi, Georgia and the Hirszfeld Institute situated in Wroclaw, Poland. The Hirszfeld Institute worked on the development of an individualized approach to create bacteriophage therapy preparations. Whereas, the Eliava Institute has worked on the production of phage preparations or mixtures for treatment purposes which target particular pathogenic bacteria. The above mentioned institutes have a history of research studies on phage therapy with promising success rates but without the conduction or establishment of properly controlled clinical trials (Kakasis and Panitsa, 2019). These promising studies led to several scientists around the globe to consider or use bacteriophage as a source of fighting bacterial infections or diseases. Several organisations also started producing bacteriophages to be used against pathogenic bacteria commercially.

The Institute of Vaccine and Sera, Tbilisi, Georgia, developed the first and foremost a phage cocktail against cholera and it was found to be successful in the control of rapidly spreading diseases troubling the southeast occupations of the USSR (Union of Soviet Socialist Republics). It was found to be quite helpful as the mortality rates of cholera dipped down to 10% in India after the application of the phage preparation. D' Herelle published these findings in his book 'Bacteriophage and the Phenomenon of Recovery' in the Russian language in 1935 in Tbilisi, Georgia. Cholera epidemics ravaged the regions of Punjab, India in 1927. Patients were subjected to oral treatment which included cholera phages (2 ml) diluted in water (20 ml) but the titer of phages used is unknown. If vomiting was experienced by a patient, they were subjected to a repeated and more diluted dosage (5 ml phage in 100 ml of water) was given slowly with a teaspoon. A control group was also established which only used folk medicine involving plant extracts as a form of treatment. Out of 14,450 people belonging to 9 villages of the region of Punjab, only 73 were treated with phages because people in India weren't open to or trusted new treatments and rarely relied on western medicine when other relied treatments failed to work as described by d'Herelle in his book. Therefore, only those patients were administered with phage preparations who were desperate to save themselves from the disease. This study resulted in high lethal rates in the control group (62.7%) as compared to the experimental group which experienced almost $1/10^{th}$ of the control group's mortality rate (6.8%). Out of 118 people in the control group, 74 had lethal outcomes, whereas the experimental group only had 5 lethal cases out of 73. These promising studies led to several scientists around the globe to consider or use bacteriophage as a source of fighting bacterial infections or diseases. Several organisations also started producing bacteriophages to be used against pathogenic bacteria commercially (Chanishvili, 2012).

2.4 Commercial production of phages

'Bacteriophage and the Phenomenon of Recovery' by d' Herelle also stated the setting up of two industrial centres for the development of anti-cholera phage preparations in India in 1931. The commercial laboratory set up by d' Herelle in Paris developed around 5 bacteriophage preparations against numerous bacterial pathogens. These preparations were named as Bacte´-pyo-phage, Bacte´-rhino-phage, Bacte´-staphy-phage, Bacte´-coli-phage and Bacte´-intesti-phage. These phage preparations were commercialised by a company that is now popular by the name L'Ore´al (Chanishvili, 2012).

To curb the spread of dysentery in Latin American countries, antidysentery phage preparations were developed in The Oswald Cruz Institute in Rio de Janeiro, Brazil in 1924. Around 10,000 vials of bacteriophage preparations were produced and were distributed to the hospitals over Brazil (Chanishvili, 2012).

The United States also played their hand in this alternative form of therapy to curb bacterial pathogens. Eli Lilly Company in Indianapolis, IN, developed around 7 products with phage incorporated in them for public use, such as phage preparations specific to combat *Escherichia coli*, *Staphylococci* and *Streptococci* as well as other bacterial infections. The phage preparations were composed of bacteriologically sterile broth cultures of the target bacterial pathogen that was phage-lysed (eg - Staphylo-lysate, Colo-lysate etc) or jelly base coupled with the same phage preparations (eg - Staphylo-jel, Colo-jel). These phage preparations were made to tackle numerous infections as well as vaginitis, mastoid infections, abscesses, upper respiratory tract infections and purulent wounds. But, the potency of these phage treatments was controversial. The reasons could range from viability of phages to specificity of the phage or low phage titer which was the case in phage preparations prepared against Staphylococcus. Due to these unpromising results the commercialisation of phages as a form of therapeutic agent was abandoned in several Western countries. However, the opposite was happening in the Soviet Union where the Bacteriophage Institute in Tbilisi, Georgia was founded by d' Herelle and his close friend as well as colleague, George Eliava (Chanishvili, 2012).

The institute managed to survive the iron curtain and outlived its founders and later on, became one of the largest institutes around the globe for the development of phage preparations for therapeutic purposes. During their busiest times, the institute developed phage preparations (usually several tons a day) against numerous pathogenic bacterial species, including *Proteus*, *Staphylococci*, *Pseudomonas* and many enteric bacterial pathogens (Chanishvili, 2012).

2.5 Advantage of treatments with phages

Phage characteristics that are advantageous over antibiotics usage can contribute towards making phage therapy a good treatment alternative.

1) Single-dose effectiveness - Many studies involved presence of effective results just after single dosage of phage preparations due to the ability of phage to replicate 'actively' meaning phage replication via auto dosing (ability of phage to multiply especially in the presence of hosts) which results in increased bacterial cell death (Carrillo and Abendon, 2011).

2) Inability to induce cross-resistance with antibiotics - Because the workings of phage infection is distinct from that of antibiotics, mechanisms of antibiotic resistance don't translate into phage resistance mechanisms (Carrillo and Abendon, 2011).

3) Biofilm penetration - Antibiotics are unable to penetrate biofilm which is a solid-liquid interphase constituting of microbial aggregation encased in a matrix of extracellular polysaccharide layer (EPS) (Flemming and Wingender, 2010). However, phages have the ability to penetrate certain biofilms via lysing bacterial layers, one at a time or due to presence of depolymerases that might aid in biofilm degradation (Carrillo and Abendon, 2011).

4) Low potential for affecting normal microbiome - Due to their comparatively high specificity towards species and strains (Lin *et al*., 2017) phages tend to impact normal flora bacterial species minimally (Carrillo and Abendon, 2011).

5) Discovery - Discovery of phages against several pathogenic bacteria can be done from sewage and wastewater that has usually high consortium of pathogenic bacteria (Carrillo and Abendon, 2011).

6) Cost effective - Phage culturing generally involves a compatible host growth and subsequent purification which is less costlier than antibiotics (Carrillo and Abendon, 2011).

7) Inherently less toxic - As phages usually consists of nucleic acids and proteins, they are not toxic (Carrillo and Abendon, 2011).

8) Impact on environment - Due to their specific host range and composition of nucleic acids and proteins, phages are unlikely to impact the environment (Carrillo and Abendon, 2011).

Remarks - Due to tremendous advantages proven through earlier research, the need for further experimentation to test those advantages against antibiotic resistant bacteria is required.

Chapter 3 - Material and Methods

3.1 Preparation of media

Luria Agar (LA) and Luria Bertani broth (LB) were mainly used for experimentation. The composition of both these media are same with the exception of Agar in Luria Agar. The rest of the contents include Yeast extract, Tryptone, Sodium Chloride ($NaCl$). Usually standard concentrations of 2 g LB and 2 g Agar is recommended per 100 ml distilled water.

For this research, LB broth was prepared with varying volumes but standard concentrations of the components (Table 1) were followed. For LA, two different concentrations of Agar were incorporated i.e. 2 g LB and 2 g Agar dissolved in 100 ml distilled water (for standard plating), 2 g LB and 0.5% (w/v) Agar in 100 ml distilled water (for bacteriophage culturing). The flask was covered with a cotton plug before autoclaving. Standard autoclaving method was followed for sterilization of media - 121 ℃ for 15 min at 15 psi. For plating of Luria Agar, 20 ml of prepared media was poured into Petri plates with the exception of bacteriophage culturing methods which only require 15 ml of the Luria Agar to be poured.

Table 1: Composition of Luria Bertani broth

Composition	Amount per 1000 ml
$NaCl$	10 g
Peptone	10 g
Yeast extract	5 g
Distilled water	1 L

3.2 Revival and Culturing of Bacteriophage

Escherichia coli bacteriophage MS2 (ATCC 15597-B1) was obtained from American Type Culture Collection (ATCC). The host, *Escherichia coli* C-3000 (ATCC 15597) was also obtained from ATCC. The experiments were done under the sterile environment of a Biosafety Cabinet.

For revival of the bacteriophage, fresh dilutions were made in LB media. From the bacteriophage mother culture, 100 µl was pipetted out and inoculated into an Eppendorf containing 900 µl of LB media and labelled it as 10^{-2}. 100 µl of media from 10^{-2} labelled Eppendorf was inoculated into an Eppendorf containing 900 µl of LB media and labelled it as 10^{-3}. Similarly 10^{-4} dilution from the previous dilution was made until 10^{-8} dilution was reached.

Adam's Overlay method for culturing of bacteriophage was used with a few modifications (Adams, 1959). Prepared a 18-24 h old fresh culture of the ATCC recommended bacterial host (*Escherichia coli*) by determining the total volume needed and inoculating appropriate of mother culture into fresh LB media. The culture was then incubated in an incubator shaker at 37℃ until it reaches early log phase (0.1 to 0.4 at OD_{600}). LA culture plates for bottom as well as top agar was prepared. For top agar or overlay agar, lower concentration of agar was used. The soft agar was kept at 45-50℃ in water bath until use. The calculated amount of fresh host culture into the soft agar was added. 3 ml of soft agar was poured over the solidified bottom agar. After the top agar solidified, 100 µl of bacteriophage from 10^{-2} dilution was spotted. The spots were dried before sealing the culture plates with cleanfilm or parafilm. Similarly, culture plates with earlier prepared 10^{-4}, 10^{-6}, 10^{-8} bacteriophage dilutions were prepared to check the gradual decrease in phage infection and subsequent increase in distinct zones of infection. The plates were kept in an incubator at 37℃ for 24-48 h. Observation was made to look for zones of infection on the culture plates after the incubation period.

An alternative method for bacteriophage culturing by broth for better results was also incorporated (ATCC). In a 250 ml flask, an appropriate amount of Luria broth was added. A small amount of 24 h old bacterial host into the flask was added and incubated at 37℃ until the OD_{600} was 0.1 to 0.4 (early log phase).The volume of phage to be added into the bacterial host culture for infection by the formula was determined as follows:

Volume of phage to add (ml) = ($8x10^8$ x total culture volume in ml x OD_{600} x MOI) / phage titer (PFU/ml).

The calculated amount of phage was added and incubated in an incubator shaker at 120 rpm at 37℃ for 24 h.

3.3 Purification of Bacteriophage

There are two methods for purification of phage depending on the type of method used for phage culturing.

Culture plate method

After the recommended incubation period of Adam's overlay method, purification of bacteriophage from culture plates was carried out. Under the sterile environment of laminar hood or biosafety cabinet, the culture plate was unwrapped and with an inoculation loop, a loopful of top or soft agar was taken which had clear zones of infection from the culture plate into an appropriate sterile container such as a centrifugation tube. Much of the top or soft agar with the inoculation loop was taken without disturbing the bottom layer. The tubes were centrifuged at 1000 rpm for 25 min and transferred the supernatant into a fresh tube.The bacterial debris was filtered out by passing the supernatant through a 0.22 µm filter. The purified bacteriophage was stored at 4℃ until further use.

Broth culture method

After the incubation period recommended for broth method of phage culturing. The culture in centrifuge tubes was centrifuged at 4500 g for 20 min. The obtained supernatant was transferred into a sterile centrifuge tube and centrifuged again at 10,000 g for 20 min. The supernatant was transferred into a centrifuge tube and centrifuged at 4500 g for 20 min. The clear supernatant was pipetted out and transferred into a sterile Eppendorf. The obtained supernatant was filtered out via a 0.22 µm filter into an Eppendorf tube. It was stored at 4℃ until further use.

3.4 Isolation of Antibiotic-resistant bacteria from Wastewater

Wastewater was collected from the inlet of sewage treatment plant at Thapar Institute of Engineering and Technology.

3.4.1 Preparation of Media

For bacterial growth purposes, LA (Luria Agar) and LB (Luria broth) were used for making bacterial culture plates and suspension cultures respectively. For a 100 ml media, 2 g of LB in 100 ml distilled water was dissolved. The same concentration was used for a 100 ml LA media with the addition of equal amounts of Agar.

3.4.2 Preparation of dilutions from wastewater

Wastewater collected from the inlet of sewage treatment plant at Thapar Institute of Engineering and Technology was used to make dilutions with distilled water. LA media was prepared in a 250 ml flask and autoclaved. The culture plates were made by pouring 20 ml of media into each plate. They were then kept undisturbed until solidification and were labelled accordingly before usage. 9 ml distilled water was taken and added into 1 ml of wastewater and was mixed well. It was labelled as 10^{-2} dilution. 1 ml from 10^{-2} dilution was inoculated into 9 ml distilled water and was labelled as 10^{-3} dilution. Similarly dilutions till 10^{-8} were made. 1 ml from 10^{-6} dilution was taken and inoculated over a culture plate. With the help of a spreader, the inoculated solution was spread properly until the inoculated solution on the plate dried out. The spreader was heat sterilized before and after usage and was kept in alcohol until needed. 1 ml of 10^{-7} dilution was taken and inoculated over another culture plate. Using a spreader, the inoculated dilution was spread until the inoculated solution on plate dried out. Similarly the above step was repeated with 10^{-8} dilution. Duplicates of all the plates were made to increase the chances of diversified bacterial colonies. Also, a control was prepared by only inoculating distilled water over it and spreading it. The plates were sealed with clingfilm and incubated for 24-48 h at 37 ℃ . After the incubation period, the plates were observed for different bacterial colonies.

3.5 Sub-culturing

To isolate purified colonies of specific bacterial strain on separate plates, sub-culturing is done. Here, sub-culturing was performed by quadrant streaking method to allow passaging of cells.

For initial streaking, a colony was picked from the bacterial culture plate prepared from wastewater dilutions that had several different bacterial colonies with the help of a heat sterilised inoculation loop and inoculated over a quadrant in a circular fashion on a freshly prepared culture plate. Heat sterilization of the inoculation loop was done over burner until it turned red hot. It was cooled down a bit, and three to four parallel lines were drawn extending the circle over the quadrant. Heat sterilization of the loop was done and cooled down before further use. The spread lines from the first quadrant were extended into the second quadrant. Heat sterilization of the loop was done again and cooled down before further use. Similarly, the above step was repeated again, and the parallel lines containing the bacterial colony were extended from second to third and further into the fourth quadrant as well. These steps were repeated for each different bacterial colony to be isolated from the culture plate containing various different bacterial colonies. After streaking was done, all the prepared plates were sealed with clingfilm and incubated in an incubator for 24 h at 37℃. After incubation, obtained bacterial colonies were streaked again by quadrant streaking method, creating another set of culture plates for different bacterial isolates. The plates were incubated at 37℃ for 24 h in an incubator. To ensure pure strain isolation of bacterial colonies, a third streaking from the previously incubated plates was done. Again, the plates were incubated at 37℃ for 24 h in an incubator.

Around 10-20 ml Luria broth cultures were prepared in test tubes and autoclaved.

After incubation period of previously streaked plates, a colony from one of the third streaking culture plates was taken with a sterilised inoculation loop and inoculated into the freshly prepared liquid culture. This step was repeated with all the different bacterial colony samples. The liquid cultures were incubated in an incubator shaker at 120 rpm at 37℃ for 24 h.

After the incubation period, an inoculation loop was heat sterilised and cooled down and then dipped into the obtained liquid cultures and streaked onto an Agar plate by quadrant streaking method. This step was repeated for each individual bacterial culture made. The plates were sealed with parafilm and incubated in an incubator at 37℃ for 24 h.

3.6 Gram staining of the isolated bacterial colonies

The objective of the gram staining technique is to differentiate gram positive bacteria from gram negative bacteria with the help of the contents (Table 2) on the basis of presence or absence of a thick peptidoglycan layer respectively resulting in purple colored bacterial colonies if gram positive whereas red or pink colored bacterial colonies if gram negative. This step also ensures that the isolated colonies are not contaminated with another bacterial species. (Smith and Hussey, 2005)

Table 2: Contents of Gram staining

Primary Stain	Crystal Violet
Mordant	Gram's Iodine
Decolorizer	Ethanol
Counter or secondary stain	Safranin

Procedure - A clean glass slide was taken and wiped with alcohol to kill any contaminants. A bacterial culture plate or liquid culture was taken and with a sterile inoculation loop, a smear at the centre of the slide was made. The smear was hit fixed over a flame carefully for approximately 30 sec over a burner. Crystal violet was poured over the smear with resting time for 1 minute and then washed off with distilled water for 5 sec. The slide was flooded with Gram's iodine with a waiting time of 1 minute and then washed off with distilled water in a gentle and indirect stream for 5 sec.

Dropwise, decoloriser was added with a wait time of 15 sec to remove excess color. The slide was flooded with safranin for 30-45 sec and then washed off with distilled water. The slide was left to dry and then a cover slip was applied over the smear. The glass slide was observed under a light microscope at 10x first and then 40x. Notes were taken for the morphology and stain color of the bacteria to identify them. Immersion oil was used before applying coverslip for microscopy at 100x.

3.7 Biochemical Characterization of Isolated Bacteria

The isolated bacteria were subjected to biochemical characterization by using KB002 HiAssorted^{TM} Biochemical Test Kit which comprised of 12 different biochemical tests. Figure gives a brief description of 7 traditional biochemical tests and 5 carbohydrate utilization tests. The tests are based on the change in pH and utilization of the substrate. The results were indicated by change in color 24 hours after the inoculation of prepared suspension. The suspension was prepared by taking the liquid bacterial cultures and inoculating the appropriate amount into 2-3 ml sterile saline solution and the density was adjusted to $\geq$0.1 OD_{620} . The results were interpreted with reference to the interpretation chart given in the kit (Table 3).

Table 3: A brief description of 12 biochemical tests performed

No.	Test	Reagents to be added after incubation	Principle	Original colour of the medium	Positive reaction	Negative reaction
1	Citrate utilization	-	Detects capability of organism to utilize citrate as a sole carbon source	Green	Blue	Green
2	Lysine utilization	-	Detects Lysine decarboxylation	Olive green to Light purple	Purple/Dark Purple	Yellow
3	Ornithine utilization	-	Detects Ornithine decarboxylation	Olive green to Light purple	Purple/Dark Purple	Yellow
4	Urease	-	Detects Urease activity	Orangish yellow	Pink	Orangish yellow
5	Phenylalanine Deamination	2-3 drops of TDA reagent	Detects Phenylalanine deamination activity	Colorless	Green	Colorless
6	Nitrate Reduction	1-2 drops of sulphanilic acid and 1-2 drops of N,N-Dimethyl-1-Napthylamine	Detects Nitrate reduction	Colorless	Pinkish	Colorless
7	H_2S production	-	Detects H_2S production	Orangish yellow	Black	Orangish yellow
8	Glucose	-	Glucose utilization	Pinkish red/ Red	Yellow	Pinkish red/ Red
9	Adonitol	-	Adonitol utilization	Pinkish red/ Red	Yellow	Pinkish red/ Red
10	Lactose	-	Lactose utilization	Pinkish red/ Red	Yellow	Pinkish red/ Red
11	Arabinose	-	Arabinose utilization	Pinkish red/ Red	Yellow	Pinkish red/ Red
12	Sorbitol	-	Sorbitol utilization	Pinkish red/ Red	Yellow	Pinkish red/ Red

3.8 Screening of bacteria for antibiotics resistance

To check for resistance against antibiotics, antibiotic susceptibility testing was done. The tests usually can be done by 3 ways mainly -

a) Disc diffusion method - Also called Kirby Bauer disc diffusion method, small circular discs dipped in the antibiotic of interest is used against the bacterial culture plate. A presence or absence of a zone of inhibition indicates the effectiveness of the antibiotic.

b) Turbidity method - the antibiotic is added into a bacterial broth culture and spectrophotometric analysis is done with time intervals to check for log or death phase analysis.

c) Agar well diffusion method - this method involves using a borer to make wells on the bacterial culture plate and subsequent inoculation of antibiotic of interest into the wells. A presence or absence of a zone of inhibition indicates the effectiveness of the antibiotic. (Syal *et al.,* 2017)

In this research, Agar well diffusion method was used to check antibiotic susceptibility of isolated bacterial colonies against various broad spectrum antibiotics. The antibiotics in use were Ampicillin, Kanamycin, Streptomycin and Azithromycin.
Working antibiotic solutions from stock solutions were made by using the formula $C_1V_1 = C_2V_2$. From a bacterial culture broth, 100 µl was pipetted out onto the culture plate. Using a clean, sterile spreader bacterial broth was spread on to the plate. Flame sterilization of spreader was done before and after use and kept in alcohol until further use. After the spreading, wells were made into the culture plate with the help of a clean and sterile borer. Flame sterilization was done before and after use and kept it in alcohol until further use. 20 µl of antibiotic was pipetted out into the wells, and without disturbing the wells, the plate were sealed with clingfilm. The petri plates were incubated in an incubator at 37℃ for 24 h. These steps were repeated for each antibiotic as well as bacterial colony to test it against with.

3.9 Effect of bacteriophage on Antibiotic-resistant bacteria

Adam's overlay assay (with a few modifications) (Adams, 1959) (ATCC) for bacteriophage culturing was incorporated to observe the effect of bacteriophage on isolated colonies of antibiotic resistant bacteria. Bottom agar was prepared for making culture plates and soft or top agar for overlaying and kept at 45-50℃ until further use. Soft agar was equally divided according to different bacterial isolates and inoculated with the calculated amount of bacteria into the soft agar. The soft agar was overlayed over solidified bottom agar and was left to solidify as well. 100 µl of bacteriophage was spotted over the top agar. Similarly, culture plates were prepared with overlayed soft agar for all the bacterial isolates and 100 µl of bacteriophage was spotted on them as well. Also, two control plates were prepared with one of them containing only bacterial growth and the other containing only the media used to make plates. The plates were sealed carefully with a clingfilm and incubated in an incubator at 37℃ for 48-92 h.

3.10 Purification of bacteriophage from antibiotic resistant bacteria

After the recommended incubation period, the previously made bacteriophage was observed for any changes. Upon observation of any transparent zones of infection, one of the infection zones was picked with an inoculation loop under the sterile environment of the biosafety cabinet and inoculated in a flask containing freshly made Luria broth. It was sealed tightly with a cotton plug and incubated in an incubator shaker at 120 rpm at 37℃ for 24 h. After 24 h, centrifugation of the culture at 10,000 rpm for 15 min was done. The supernatant was transferred into a sterile falcon tube and the pellet was discarded. The supernatant was clear otherwise it would have required another centrifuge step. The supernatant was filtered out into a fresh tube via a 0.22 µm filter and stored at 4℃ or less. For a longer time, -80℃ would have been sufficient.

3.11 Isolation of Bacteriophage from wastewater and its effect on isolated Antibiotic resistant bacteria

An alternative method was employed which involved observation of the effect of bacteriophage (isolated from wastewater) on antibiotic resistant bacteria.
The procedure followed for isolation was taken from (Qamar *et al*., 2019) and adam's overlay assay for bacteriophage culturing was followed with a few modifications. Fresh inlet sample of wastewater was taken from the sewage treatment plant at Thapar Institute of Engineering and Technology. In a 100 ml flask, 40 ml sewage water was mixed with 5 ml fresh Luria broth and 5 ml *Escherichia coli* in early log phase. The flask was sealed with a cotton plug and kept in an incubator shaker at 37℃ for 48 h at 120 rpm. After the incubation period, the contents of the flask were added into a centrifuge tube and centrifuged at 5000 rpm for 15 min. The centrifugation step was repeated until a clear supernatant was observed. After that, the obtained supernatant was transferred to a fresh tube and was filtered out into a new tube via a 0.22 µm filter. The filtered solution was kept at 4℃ until further use. The purified bacteriophage was used to make dilutions in fresh Luria broth. 100 µl of bacteriophage was added into 900 µl of Luria broth in an Eppendorf and was labelled as 10^{-2}. Following the similar method, dilutions were made till 10^{-9}. Previously stated steps for Adam's Overlay assay were followed for bacteriophage culturing and the culture plates were kept for incubation in an incubator at 37℃ for 48 h.

Remarks - The presence of plaques against majority of the isolated antibiotic resistant bacterial isolates indicates that bacteriophage therapy can be a promising form of treatment.

CHAPTER 4 - RESULTS AND DISCUSSION

4.1 Isolation of bacteria from wastewater

Four different bacterial colonies were isolated based on morphology from the culture plates made from wastewater. The bacterial colonies were labelled as T, O, Y, R (Figure 4).

The bacterial colonies were chosen on the basis of difference in morphology. The bacterial colonies labelled T and Y were yellowish in color but transparent and opaque respectively. Whereas, the bacterial colonies labelled O and R were colored orangish and red respectively.

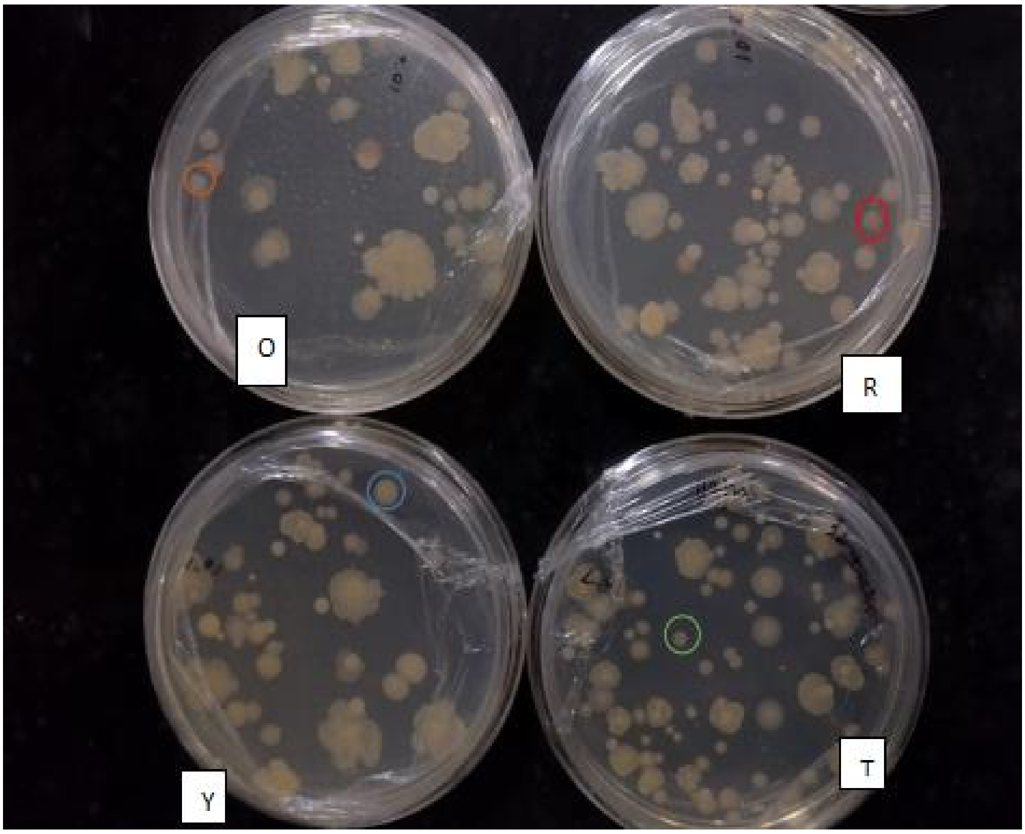

Fig 4: Presence of different bacterial colonies isolated from wastewater

Afterwards, each chosen colony was picked up one by one onto separate Agar plates for sub-culturing repeatedly to achieve pure bacterial strain. For sub-culturing, quadrant streaking was performed to obtain isolated colonies (Figure 5).

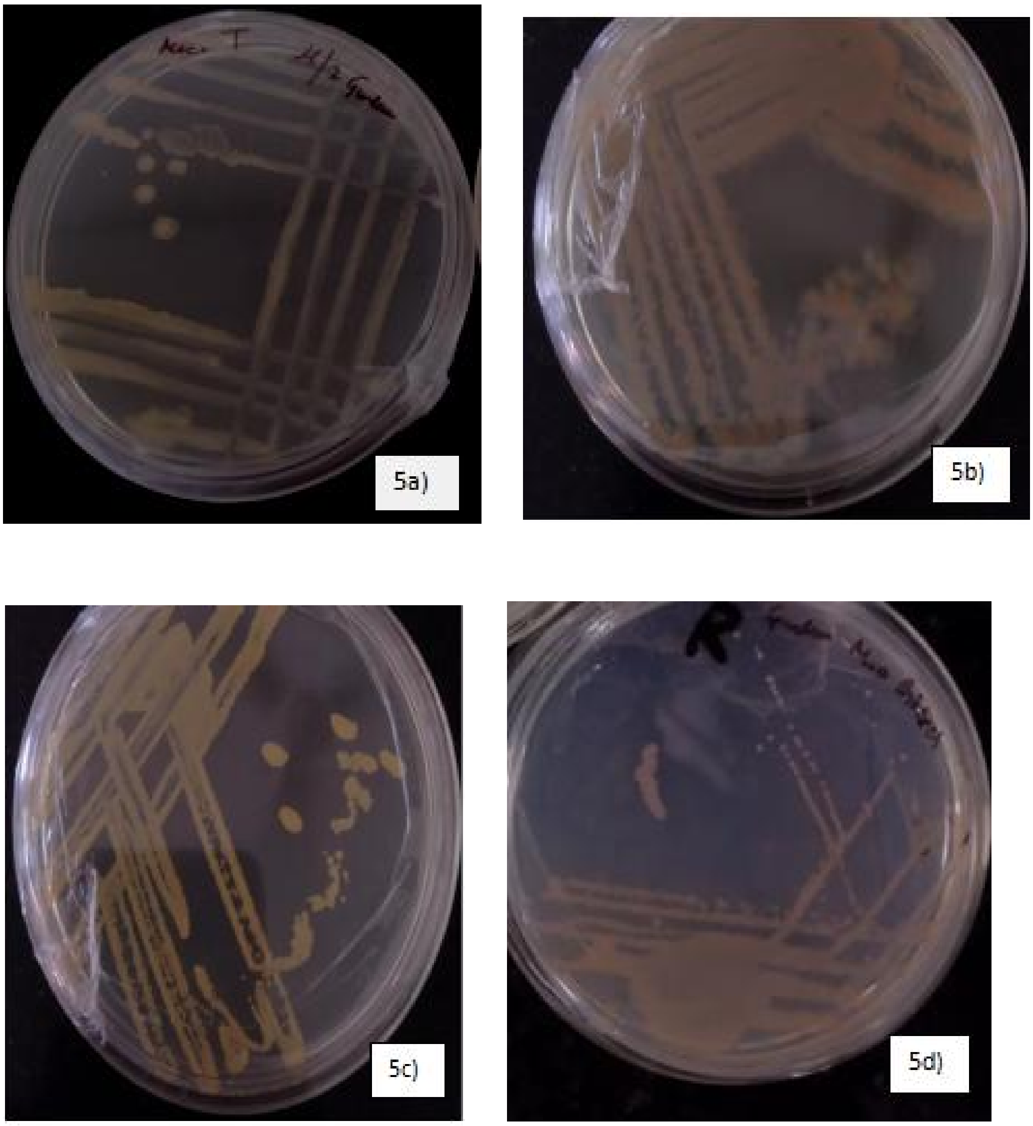

Fig 5 a, b, c and d: Sub-cultured bacterial colonies T, Y, O and R in respective order

Biochemical characterization tests results were produced after 24 hours of incubation at 35℃. The results (Table 4) were indicated by color change (for a positive reaction) and no color change (for a negative reaction) with reference to the interpretation chart (Table 3).

Table 4: Results of biochemical characterization tests where '+' sign represents a positive reaction and '-' sign represents a negative reaction. (T, O, Y, R represents the labels assigned to isolated bacteria)

No.	Test	T	O	Y	R
1	Citrate utilization	+	-	-	-
2	Lysine utilization	-	-	-	+
3	Ornithine utilization	-	-	-	+
4	Urease	-	-	-	-
5	Phenylalanine Deamination	-	-	-	-
6	Nitrate Reduction	-	+	-	+
7	H_2 S production	-	-	-	-
8	Glucose	-	+	+	-
9	Adonitol	+	-	+	-
10	Lactose	-	+	+	-
11	Arabinose	-	+	-	+
12	Sorbitol	+	+	-	-

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These results depict that 'T' is a Citrate, Glucose and Sorbitol utilizing bacteria and 'O' other than being able to reduce nitrate can also utilize most of the carbohydrates out of all the bacterial species tested. Whereas 'Y' can also utilize multiple carbohydrates and 'R' can utilize Lysine, Ornithine and Arabinose as well as reduce nitrate.

The bacterial isolates were also gram stained to identify the gram stain as well as to check for any contamination or unwanted microbial growth and it was found that bacterial cultures labelled as T and R were gram-negative as they stained pink in color whereas cultures labelled as O and Y were gram-positive as they stained red in color.

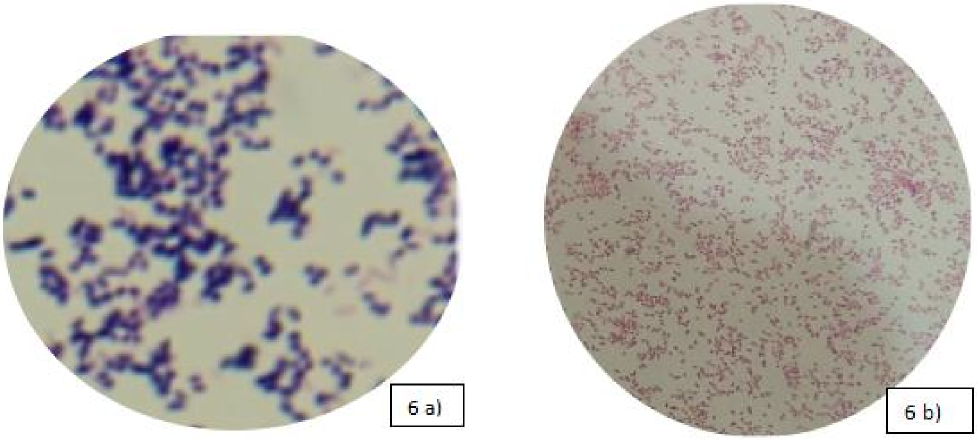


Fig 6 a and b: Gram positive rods (left) and Gram negative rods (right)

4.2 Screening of bacteria for Antibiotic resistance

The bacterial isolates were tested against various antibiotics - Ampicillin, Kanamycin, Streptomycin and Azithromycin at increasing concentrations. It was found that Y, O and T were antibiotic-resistant and R was susceptible to antibiotics. As a result, the culture plates labelled Y, O and T didn't show any signs of bacterial inhibition by the antibiotics whereas culture labelled R showed susceptibility to all the above mentioned antibiotics in the form of lack of bacterial growth and zones of inhibition (Figure 6). The resulting zones of inhibition were measured with a ruler (Table 3)

Fig 7 a and b: Antibiotic Susceptibility tests results exhibiting bacterial culture plates with absence of any zones of inhibition and presence of zones of inhibition in respective order

Table 5: Outcome of Antibiotic Susceptibility Tests

Antibiotics (30 µg/ml)	Susceptible	Resistant
Ampicillin	R- 7-10 mm	T,Y, O
Streptomycin	R- 22-25 mm	T, Y,O
Kanamycin	R- 15-17 mm	T, Y,O
Azithromycin	R- 15-27 mm	T,Y,O

4.3 Identification of Antibiotic Resistant Bacteria

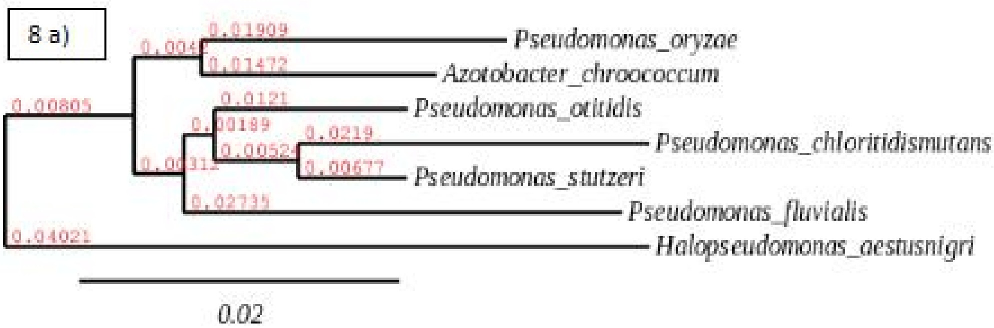
16S rRNA sequencing services were acquired through National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune. Two out of four bacteria were sent for sequencing out of which one was antibiotic resistant and the other one was antibiotic susceptible. Identification of one antibiotic susceptible and one antibiotic resistant bacteria was done to observe bacteriophage infection differences (if any) with respect to one antibiotic susceptible host and antibiotic resistant host. Phylogenetic trees were generated for the sequenced strains with the help of Phylogeny.fr tool by performing multiple sequence alignment of the obtained strains with closely related species. The phylogenetic trees below represent evolutionary relationship between the sequenced bacterial species and related bacterial species. Figure 7a represents the evolutionary relationship of *Pseudomonas stutzeri* suggesting that it shares a common ancestor with Pseudomonas chloritidismutans whereas Figure 7b represents the evolutionary relationship of *Shewanella putrefaciens* indicating that it is closely related to *Shewanella morhuae.* The branch length (indicated in red color) represents evolutionary change or divergence between species.

The resulting bacteria were as follows:

T = *Pseudomonas stutzeri*

O = *Shewanella putrefaciens*

Pseudomonas stutzeri



Shewanella putrefaciens

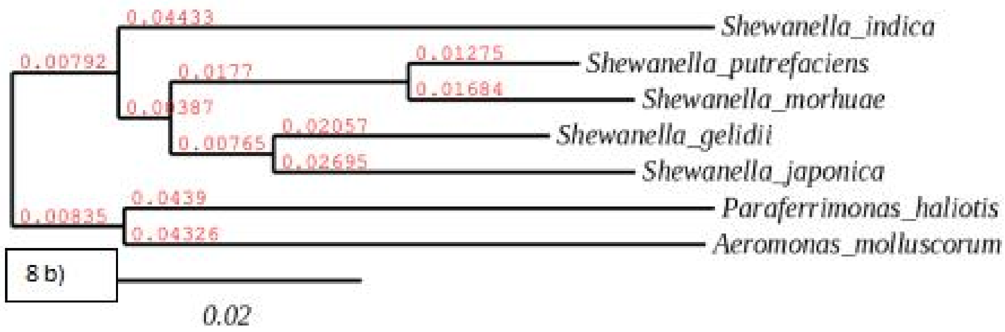


Fig 8 a and b: Distance-matrix neighbor-joining tree, representing the phylogenetic relationships of sequenced bacterial species of *Pseudomonas* and *Shwanella* respectively (generated by Phylogeny.fr, http://www.phylogeny.fr/)

4.4 Effect of Bacteriophage on Antibiotic Resistant Bacteria isolated from wastewater

The bacteriophage isolated from wastewater was kept at 4℃ until further use. The culturing resulted in plaque formation or transparent zones that suggested presence of bacteriophage in some bacterial culture plates, but there seemed to be some unwanted microbe involved in the bacteriophage isolation process that didn't get removed during the purification process because the culture plates did contain some unwanted microbial growth.

As the bacteriophage culture plates were contaminated with an unknown microbial species, further purification from the culture plates wasn't performed.

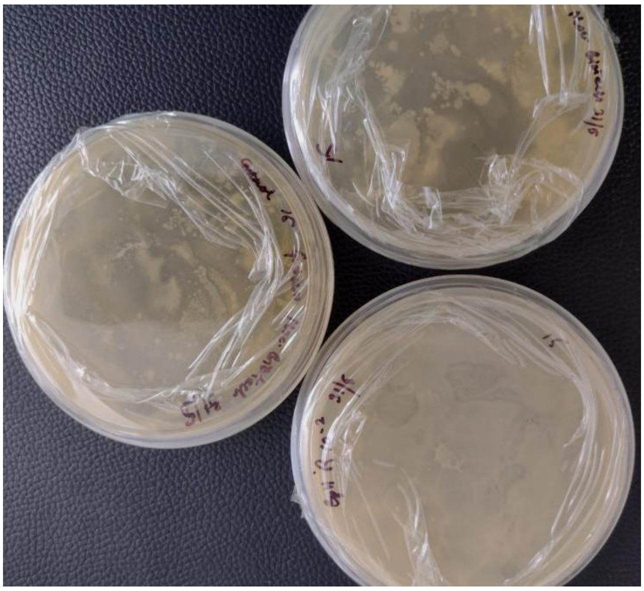
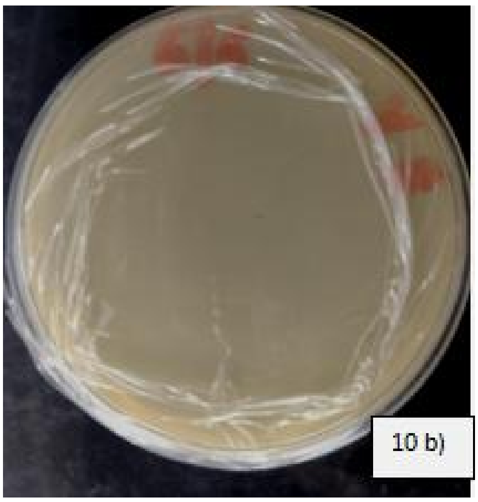


Fig 9: Presence of zones of infection indicating infection of isolated bacterial colonies by bacteriophage but with presence of unwanted microbial growth

4.5 Effect of *Escherichia coli* Bacteriophage MS2 on Antibiotic Resistant Bacteria

All cultures except culture plate labelled as Y showed formation of plaques with varying degrees indicating the bacteriophage affected each bacteria differently. This indicates that bacteriophage not only succeeds in infecting antibiotic susceptible bacterial species but also shows promising results against antibiotic resistant bacterial species. The findings also indicate that *Escherichia coli* bacteriophage MS2 is specific towards its bacterial hosts. Also, the development of plaques varied with different bacterial hosts. With *Escherichia coli* and *Shewanella putrefaciens* the initial signs of infection were presented within 24 h but the same was not true with *Psuedomonas stutzeri* and culture plate labelled O. The incubation period varied from 48 to 72 h for initial signs of bacteriophage infection to develop which suggests that bacteriophage infection time varies from species to species.

Purification of bacteriophage was performed under the contamination free environment of biosafety cabinet. The purified phage was kept at 4℃ at first then transferred to -80℃.



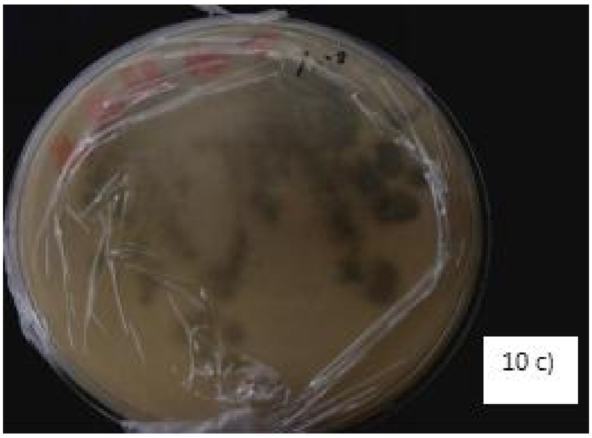


Fig 10 a, b and c: Presence of zones of infection or plaques indicating bacteriophage infection bacterial culture plates (a and c) Absence of plaques on bacterial culture plate labelled Y (b)

4.6 Discussion

To combat the prevalence of antibiotic resistant bacteria, there is a dire need for alternative form of therapies to combat pathogenic bacterial infections. With the help of bacteriophage therapy that can be possible as shown by this study. Although not all bacterial isolates were inhibited but that may be subjected to the bacteriophage specificity (Koskella and Meadon, 2013).

Its only a matter of time before these problematic multi-drug resistant strains spread around the world because of improper control measures. Due to the misuse of antibiotics and lack of control measures because of which antibiotic resistant bacteria are rampant in developing countries (da Silva *et al*., 2022), (Aukidy *et al*., 2017). A 2017 study showed promising results with bacteriophage therapy comprising of a phage cocktail prepared against a lethal multi-drug resistant bacteria *Acinetobacter baumannii* (Schooley *et al*., 2017). Bacteriophages have also been used in conjunction with antibiotics in an attempt to lower the antibiotic load and increase treatment effectivity (Terwilliger *et al*., 2021), (Pirnay *et al*., 2022). There needs to be a united effort towards creating proper measures to treat wastewater effluents around the world, implementation of strict and necessary measures for usage and sales of antibiotics and incorporation of bacteriophage therapy in treating bacterial infections. Together, these measures would help lower the spread and development of antibiotic resistance among bacterial species. For implementation of an alternative form of therapy that helps in reducing the negative effects of antibiotics, a collective front is required to establish clinical trials and studies exploring this promising field. Further research is required to explore bacteriophage therapy in the fight against antibiotic resistance and multi-drug resistant bacterial pathogens (Kaur *et al*., 2021).

CONCLUSION

The presence of such large amount of different multi-drug resistant bacterial species such as *Psuedomonas stutzeri* and bacterial cultures labelled O and Y suggest that the bacterial species have evolved to evade or resist the effects of multiple broad spectrum antibiotics such as Azithromycin, Kanamycin, Streptomycin and Ampicillin which calls for an urgent need for an alternative form of therapy especially in those countries which have underdeveloped wastewater treatment systems that are incapable to control spread of antibiotic resistant bacteria.

The presence of zones of infection or plaques of bacteriophage in antibiotic resistant bacteria culture plates proves that using bacteriophage as a therapeutic agent is a promising and an effective way to infect not only antibiotic susceptible but antibiotic resistant bacteria that survived high antibiotic concentrations of multiple antibiotics in-vitro. The findings of this study reflected upon the ability of bacteriophage to be specific towards its bacterial hosts as it was unable to infect the Y labelled bacterial culture plate.

This study sheds a necessary light on the usage of bacteriophage as a therapeutic agent against antibiotic susceptible as well as antibiotic-resistant bacterial strains. To explore this concept from a clinical outlook further studies are required which might help the world prevent an effective antibiotic shortage.

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APPENDIX I

APPENDIX II

Curiginal

Document Information

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