ASSESSING THE IMPACTS OF BACTERIOPHAGE COINFECTION ON PHAGE INFECTIVITY, HOST SPECIFICITY, AND THEIR COMPETENCE USING PHAGE COCKTAIL

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

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Declaration

It is hereby declared that

1. The thesis submitted is our own original work while completing the 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.
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Ethics Statement

This thesis does not include any human or animal subject.

Abstract

The MDR or Multi-drug resistant pathogens for example, *Vibrio cholerae*, represent an emerging serious health problem since these bacteria have natural capabilities for adaptation to environmental stresses and antibiotics. This adaptability arises through genetic evolution under the pressure of natural selection. Interactions with bacteriophages are among the most important factors influencing bacterial evolution in natural environments. These are nature's predators of bacteria; therefore, they also interact in complicated co-evolutionary dynamics with their hosts. Over time, this could lead to a phage that either infects or loses the ability to infect its original bacterial host. Besides, the co-infection phenomenon, whereby a single bacterium is invaded by multiple viruses, can give rise to new strains of viruses with wider host ranges or increase competency, some of which we have seen in this study. These events may be infrequent, but they do bear great relevance in regard to phage therapy as an approach for the treatment of bacterial diseases. Herein, we attempt to dissect what happens in co-infection with phages, particularly with the use of phage cocktails in infecting Vibrio cholerae hosts, with our focus being mainly on changes with respect to phage infectivity, host specificity, and competence.

Dedication

This thesis is dedicated to our families for their endless love, support and sacrifices.

Acknowledgement

We would like to commence after expressing our earnest gratitude to the Almighty Allah for endowing us with the opportunity of this research project and then providing us with the boldness needed throughout this journey to fulfill it successfully. This research could not have been completed without the support of many people, who are gratefully acknowledged here. First and foremost, we would like to express our deepest gratitude and appreciation to our esteemed supervisor, **Dr. Iftekhar Bin Naser**, Associate Professor, Biotechnology Program, BRAC University, without whom our instinct to work on some important issues would not be feasible. Our supervisor's constant effort and encouragement towards this research project allowed us to grow as researchers during this period, and we could not be more grateful towards him. His extraordinary research skills have allowed us to solve various erratic conditions with ease. As a result of this, we were able to collect a significant amount of resources within a short period of time and multiple research materials that enabled us to run new research projects.

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List of Acronyms

1. BAM: bacteriophage adherence to mucin
2. ICTV: The International Committee on Taxonomy of Viruses
3. MEM: Molecular and Environmental Microbiology Laboratory
4. MEB: Molecular and Environmental Biology Laboratory
5. DLA: Double Layer Assay
6. PSI: Pound per Square Inch
7. LB: Luria Bertani
8. NaCl: Sodium Chloride
9. TCBS: Thiosulfate-Citrate-Bile-Sucrose
10. SM: Salt Magnesium
11. MgSO4: Magnesium sulfate
12. OD: Optical Density
13. PFU: plaque-forming units
14. MCT: Microtubes

CHAPTER 01: INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Vibrio cholerae, the causative agent of the infectious disease 'Cholerae', is a gram negative bacteria that have straight or curved rod-like shape and size 0.5-0.8 μm in width and 2-3 μm in length (Mandal & Mandal, 2014) renowned for causing severe diarrhea and dehydration in human beings. So much so, that if left untreated, it can lead to death within 48 hours (Qadri et al., 2005). According to Bhandari et al. (2021), *V. cholerae* is an extracellular pathogen of the small intestine that has been reported globally mainly in developing countries where transmission is aggravated by poor sanitation and poverty and thus is responsible for millions of deaths each year. In the 2018 World Health Organization (WHO) weekly epidemiological record, the WHO reported 1,227,391 cases worldwide and 5654 deaths in 2017 due to cholera illness (World Health Organisation, 2019). *Vibrio cholerae* can be found in water bodies like wells, ponds, lakes and rivers. The Vibrio genus of bacteria is a prominent constituent of aquatic environments, where it fulfills a significant ecological function in sustaining the equilibrium of the aquatic ecosystem, such as breaking down organic matter and recycling nutrients, thus maintaining the aquatic environment stability. (Kumarage et al., 2022) However, it is also responsible for causing water borne diseases. Water or food contaminated by *V. cholerae* can lead to significant health implications such as diarrhea, enteritis, and potentially fatal outcome. To tackle this strain of bacteria, certain viruses are useful such as bacteriophages. Bacteriophages, commonly referred to as phages, are a class of viral agents that specifically target and infect bacterial cells, and replicate within the bacterial cells. Bacteriophage is derived from the latin word 'bacteria' and 'phagos' which means 'to eat'. Phages are extremely diverse and are found in almost every habitat that is colonized by bacterias, including water bodies, soil and gastrointestinal ecosystems. (Bondy-Denomy et al., 2016) Bacteriophages have a strong influence on microbial populations, affecting the performance of food webs, nutrient cycling, biodiversity, species distribution and genetic transfer. (Suttle, 2005)

1.2 RESEARCH OBJECTIVE

The objective of this research is to explore the impact of coinfection phages on bacterial strains and assess whether such coinfection can modify the phage infectivity and host range.

1.3 SPECIFIC AIMS

To study co-infection as an evolutionary process of bacteriophages with the goal of analyzing Phages effectiveness with altered host ranges.

1.4 NOVELTY OF THE RESEARCH

The novel method of this research explores the dynamics of phage-bacteria interactions, providing a distinct viewpoint on the phages in reaction to coinfection.

1.5 LITERATURE REVIEW

1.5.1 Introduction to BACTERIOPHAGE

Bacteriophages infect bacterial cells solely and are known for over a century. To answer the question “what is life?”, bacteriophages were elected as the simplest and most logical biological systems, with research based on them becoming the cradle of molecular biology (Harada et al., 2018). Bacteriophages are classified according to their unique morphology as they come in various shapes and sizes and according to the type of genome they consist of because they can contain either an RNA or a DNA. Phage are now recognized as the most abundant biological entities on our planet and they play major roles in the ecological balance of microbial life.

Understanding and harnessing the biology and biotechnological potential of these physiologically inert particles requires extensive investigation due to the high number of bacteriophages and the even greater number of undiscovered genes they contain. Nearly all bacteria on Earth are infected by the extremely diverse group of bacteriophages (Harada et al., 2018; Catalão et al., 2013). Their genomes contain proteins that have proved helpful for biotechnology applications such as DNA delivery vehicles, phage therapy for infections caused by bacterial strains particularly resistant to antibiotics, and diagnostics for food safety, among many other pertinent technologies. After infecting one bacterial cell and replicating, phages produce new virions that infect additional cells. Bacterial populations are therefore regulated by phages, which also play a role in the transfer of genes from one bacterium to another. The evolution of genomes, bacterial adaptive evolution, DNA expression and copy mechanisms, and the potential for novel biotechnology product creation are all clarified by studying bacteriophage

particles. The unimpeded rise in bacterial resistance to traditional chemical antibiotics across the globe is reviving interest in bacteriophage (or phage) therapy, which employs lytic phage particles because these organisms lacking any metabolic machinery have no affinity for eukaryotic cells. (Harada et al., 2018; Chan and Abedon, 2012; Dąbrowska et al., 2005).

1.5.2 Discovery of Bacteriophages

William Twort made the initial discovery of bacteriophages in 1915, and Felix d'Herelle realized they might kill bacteria in 1917. Mainly due to the relative simplicity with which antibiotics could be administered, they were basically ignored as important therapeutic agents in the West after a pre-antibiotic heyday. The bacteriophages in the water of the Ganga and Yamuna rivers in India were discovered in 1896 by the British bacteriologist Ernest Hankin. This water was able to pass through a very fine porcelain filter while retaining its unique characteristic of having unexpected heat-sensitive antibacterial properties against bacteria (Vibrio cholera) (Ackermann, 2012; Hankin, 1896). Hankin did not follow up on this discovery, though, and two years later, Russian bacteriologist Gamalaya also noted a comparable occurrence when Bacillus subtilis under work (Sulakvelidze et al., 2001). Emmerich and Löw (1891) showed that an autolytic culture sample may lyse other cultures and treat an illness that was brought on during experimentation (Summers, 2005). Frederick Twort, a British bacteriologist, discovered a tiny substance in 1915 that killed bacterial colonies in developing cultures. Later, Twort noticed a "glassy transformation" of Micrococcus colonies grown on solid agar media. From there, he surmised that the agent that was unknown and caused the fluid metamorphosis of the bacterial colonies might be a virus. After that, he reported the findings, but the start of World War I and scarcity of funds delayed his further studies.(Calendar, 2005; Sulakvelidze et al., 2001; Summers, 2005).

Felix d'Herelle saw a similar type of incident two years after Twort documented it, and at the Pasteur Institute in France, he independently found the agent killing germs. First dubbed taches, then taches vierges, and finally plaques, he postulated that the lysis of bacterial cells in liquid medium caused by this "ultravirus" was responsible for the formation of distinct patches on the bacterial lawn. According to Fraciano and Bourne (2007), Felix d'Herelle also labeled the virus causing these symptoms as "bacteriophage," which is derived from the Greek words "phagein," which means to "eat" or "devour," and "bacteria." Ultimately, he came to the conclusion that

bacteriophages were "exogenous agents of immunity" after seeing a rise in phage titer in the stool samples of typhoid and dysentery patients who were on the mend (Deresinski, 2009). The Eliava Institute was established in Tbilisi, Georgia, in 1923 with the goal of researching bacteriophages and creating phage treatment. Since then, a large number of scientists have worked to create methods for studying phages and using them in many contexts. Salvador Luria, Alfred Hershey, and Max Delbrück were granted the coveted Nobel Prize in Physiology and Medicine in 1969 in recognition of their studies into the genetic makeup and reproduction of viruses.

1.5.3 The Abundance of Bacteriophages in the Environment

Bacteriophages or phages are considered as the most prominent and genetically varied biological entities on the earth with the estimated population size of 10^31 or more (Keen, 2015). Phages are abundant in nature and can be found in any setting that promotes bacterial growth (Ksik-Szeloch et al., 2013). It is estimated that each bacteria is thought to have between 5 and 10 viruses (Weinbauer & Rassoulzadegan, 2004). Although human beings are primarily concerned with bacterial diseases, the majority of Earth's bacteria and archaea can be found in the open ocean, soil and ocean sediments, and land sub surfaces, where there are approximately 1.2×1029, 2.6×1029, 3.5×1030, and 0.25–2.5 ×1030 cells of these microorganisms (Whitman et al., 1998). This creates the opportunity for a diverse combination of phage-bacteria association. Phages have been isolated from different environmental setting such as acidic hot springs (higher than 80°C with pH=3.0), solar salterns (10 times saltier than the ocean), alkaline lakes (pH=10), in the terrestrial subsurface (greater than 2000 m deep), below 30 m of ice in polar lakes (Breitbart and Rohwer, 2005), from soil (Ashelford et al., 2003), sewage sludge (Carey-Smith et al., 2006) and mammalian feces (O'Flynn et al., 2004). It is now well known that phages play a significant role in the biosphere's organic matter cycling and bacterial diversity, as well as maintaining bacterial balance in the ecosystem (Chibani- Chennoufi et al., 2004; Guttman et al., 2004). Felix D'Herelle reported that bacteriophages are a natural part of healthy animal and human microbiota. Therefore, it can be stated that bacteriophages are prevalent on almost all biotic factors and living organisms.

The abundance of bacteriophages in the human body is remarkable. According to Shkoporov and Hill (2019), the human body is the greatest reservoir of microbiota, where most of these microorganisms are found in the gut. The number of bacteriophages therefore outnumber the quantity of bacteria present in the human body by at least five to ten folds. The human body comprises mucosal surfaces, where microbes inhabit and communicate with their host directly (Dethlefsen et al., 2007). As stated by Naureen et al. (2020) Bacteriophages can bind to these mucin glycoproteins through immunoglobulin-like spikes present in their capsid via "bacteriophage adherence to mucin" (BAM). BAM plays two significant roles in regulating the bacterial-host interactions: it provides protection for host against the pathogenic bacteria by accommodating bacteriophages with lytic activity that would otherwise demolish the beneficial bacteria leading to local or systemic infection (Rodriguez-Brito et al., 2010); it provides lysogenic bacteria an environment to develop bacterial symbiotic relationship that benefits the human host (Gerken, 2004). These phages can therefore be considered as living in a mutual beneficial endosymbiosis with the human body.

Despite the fact that bacteriophages are abundant in the atmosphere of the earth, the variation amongst this species is numerous. This results from the abundance of this organism in the uneven earth's surface that provides organisms with an array of different temperatures, climates, humidity, acidity, sunlight, salinity, alkalinity, acidity and many more important yet differentiated factors. As a result, even though bacteriophages are regarded as the most prevalent biological entities on earth and are widely distributed in the environment, their size, appearance, and genetic structure incredibly varies (Simmonds & Aiewsakun, 2018).

1.5.4 Unique Features of Bacteriophage

Bacteriophages have unique features such as their capability of self-assembling inside the host bacterial cell, and its ability to densely pack the genetic material inside into a small capsid head.

1.5.4.1 Specificity

Bacteriophages are very species-specific about their hosts and usually only infect a single bacterial species or even specific strains within a species (Kasman & Porter, 2022). Many phages seem to be specific to a single bacterial species, and are often specific to only a few strains

within that species. Studies have shown that highly pathogenic bacterial toxins are encoded by bacteriophage genomes, such that the host bacterium is only pathogenic when lysogenized by the toxin-encoding phage. Examples are: the cholera toxin in *Vibrio cholerae* and the diphtheria toxin in *Corynebacterium diphtheriae* (Clokie et al., 2011).

1.5.4.2 Structure

The most studied group is that of tailed phages with a dsDNA genome, and it also represents the largest group. The tailed phages have three major components: a capsid where the genome is packed, a tail that serves as a pipe during infection to secure transfer of genome into host cell and a special adhesive system (adsorption apparatus) at the very end of the tail that will recognise the host cell and penetrate its wall. (White & Orlova, 2020) The capsids of the dsDNA phages often have fivefold or icosahedral symmetries.

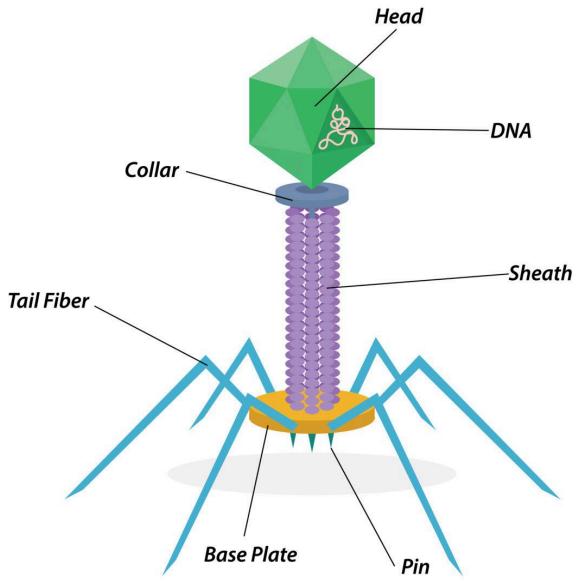


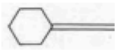
Figure 1: Structure of T4 Bacteriophage Virus (Naureen et al., 2020)

1.5.4.3 Genetic material

Phages contain genetic material that can be single- or double-stranded, packaged in a protein capsid. The three types of phage structures are tailed icosahedral head, tailless icosahedral head, and filamentous forms (Sapkota, 2023; Naureen et al., 2020; Vos et al., 2009). As in bacteria, the genetic material of bacteriophages is also protected by the capsid protein. Most phages also have tails that contain proteins which can be recognized via receptors on the bacterial surface (Moineau and Tremblay, 2017). There are some phages that have plasmids which might either become part of the bacterial genome or replicate separately. It is important to know the entire genetic composition of bacteriophages because it plays a critical role in developing phage therapy, which provides hope for treating bacterial infections without the use of antibiotics (Lin et al.,2017).

1.5.5 Classification of Bacteriophages

Table 1: Classification of phages (Ackermann, 2004)

Shape	Order or family	Nucleic acid, particulars, size	Member	Number[a]
	Caudovirales	dsDNA (L), no envelope		
	*Myoviridae*	Tail contractile	T4	1312
	*Siphoviridae*	Tail long, noncontractile	λ	3262
	*Podoviridae*	Tail short	T7	771
	*Microviridae*	ssDNA (C), 27 nm, 12 knoblike capsomers	ϕX174	38
	*Corticoviridae*	dsDNA (C), complex capsid, lipids, 63 nm	PM2	3?
	*Tectiviridae*	dsDNA (L), inner lipid vesicle, pseudo-tail, 60 nm	PRD1	19
	*Leviviridae*	ssRNA (L), 23 nm, like poliovirus	MS2	38
	*Cystoviridae*	dsRNA (L), segmented, lipidic envelope, 70–80 nm	ϕ6	3
	*Inoviridae*	ssDNA (C), filaments or rods, 85–1950 x 7 nm	fd	66
	*Plasmaviridae*	dsDNA (C), lipidic envelope, no capsid, 80 nm	MVL2	5

[a] From reference 1. C, circular; L, linear.

In 1962, Scientist Lwoff, Horne and Tournier published a system of virus based on its morphology and nucleic acid type. Later in 1967, six basic phage types were introduced, these are tailed phages, filamentous phages, isohedral phages with single-stranded DNA or single-stranded RNA. The International Committee on Taxonomy of Viruses (ICTV) has classified the phages on six genera. They are T4, λ, φX174, MS2, fd, PM2 phages (Ackermann, 2004). At present, ICTV recognizes 1 order, 13 families, and 31 genera of phages

1.5.6 Life Cycle of Bacteriophages

The life cycle of bacteriophages is divided into two cycles:

1.5.6.1 Lytic Cycle

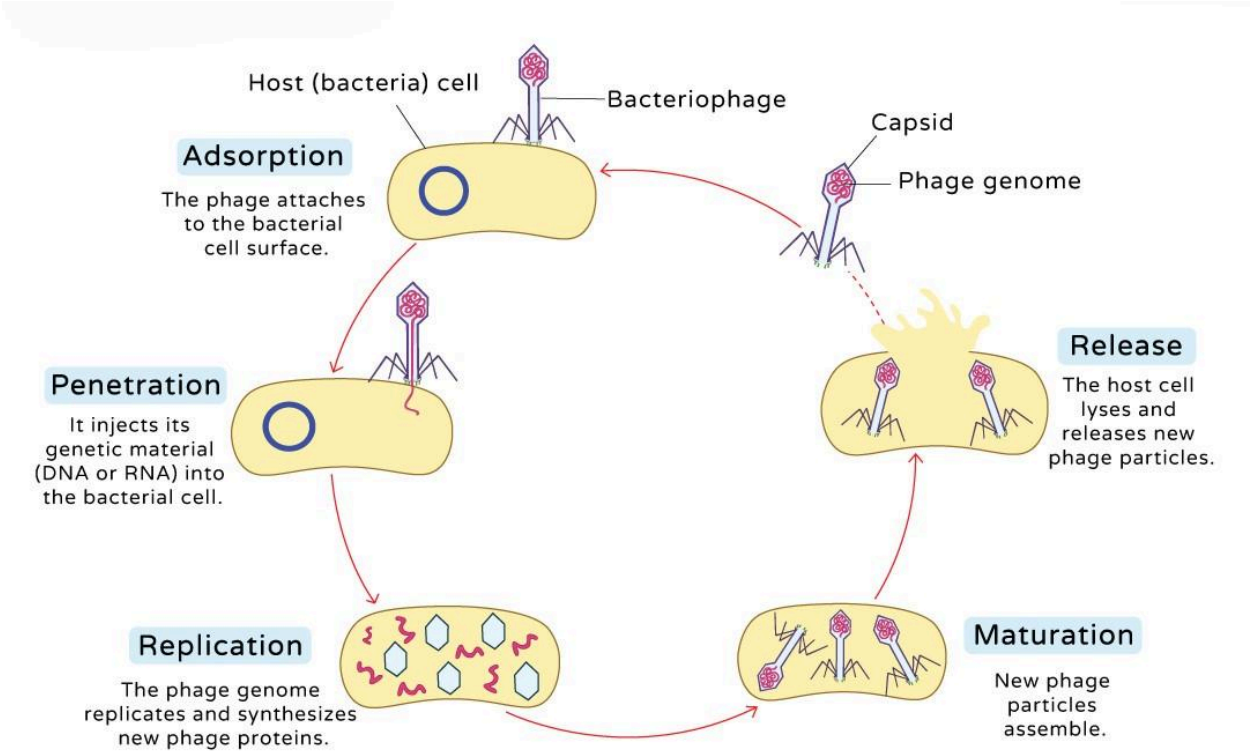


Figure 2: Lytic Cycle of Bacteriophage (Mukherjee, 2023)

At first, the phage must identify a susceptible and suitable host bacterial cell to which it is able to attach, then the phage attaches itself to the bacterial cell surface, then it injects the genetic material, DNA or RNA into the bacterial cell. Biosynthesis starts in this phase, phage DNA synthesis of viral components by the host cell and maturation occurs by viral components assembled into virions. Lastly, release of new phage particles and host cell lysis. In this way, the lytic cycle of bacteriophage continues.

1.5.6.2 Lysogenic Cycle:

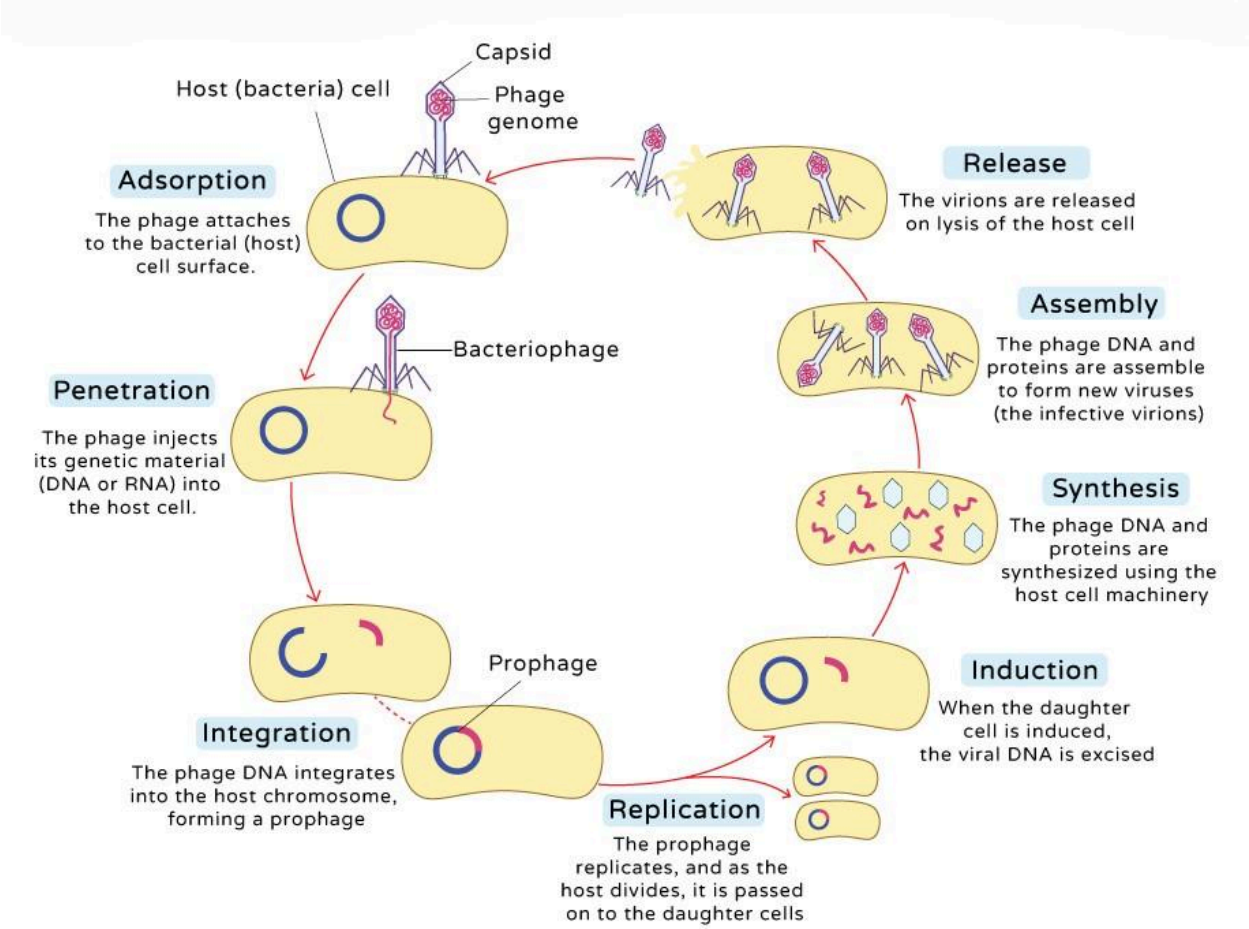


Figure 3: Lysogenic cycle of Bacteriophage (Mukherjee, 2023)

Lysogenic cycle often referred to as non-virulent infection, it does not kill the host cell but uses it as a refuge where it can exist in a dormant state. The bacteriophage attaches itself to the bacterial host cell surface, and injects its genetic material, DNA or RNA into the host cell. Phage genetic material integrates itself into the host chromosome and forms a prophage. The prophage genome is then replicated passively along with the host genome as the host cell divides for as long as it remains there and does not form the proteins required to produce progeny. (Steward, 2024) When the daughter cell is induced, the viral DNA is excised and synthesis of phage DNA and proteins using host cell machinery. The phage DNA and proteins are assembled to form new infective virions and released on host cell lysis.

1.5.7 Bacteriophage Mechanism

Bacteriophages kill bacteria by causing them to burst or lyse. This happens when the virus interacts with the bacteria, then the virus infects bacteria by introducing its genes (DNA or RNA) into the living thing. Inside the bacteria, the phage virus duplicates (copies) itself. This allows each bacterium to produce up to 1000 more viruses. Finally, the bacteria are ruptured by the virus, allowing fresh bacteriophages to produce (Golkar et al., 2014). When inside a bacteria, the bacteriophages can proliferate and thrive, until all of the bacterias have been lysed , they will not cease the replication process. Similar to other viruses, bacteriophages can hibernate or go dormant until fresh bacteria develops.

1.5.7.1 Correlation of Vibrio Cholerae and Vibrio Phages

Vibrio Phages are viruses infecting bacteria from the *Vibrionaceae* family (Bischoff et al., 2021). Where these bacteria from the *Vibrionaceae* family represent a genetically and metabolically diverse group of heterotrophic bacteria. They mainly serve as suitable hosts for the proliferation of vibrio phages. Vibrio Phages have been known to play an important role in virulence of *V. cholerae* and in controlling the seasonal outbreaks of cholera (Impact of Phages on the Vibrio Cholerae Life Cycle in Bangladesh, n.d.). It was discovered in the 1930s that cholera cases were positively correlated with the isolation of vibrio phages in the aquatic environment (Pasricha et al.,1931). A model developed by Jensen *et al*. predicts that if an outbreak is initiated by an increase in *V. cholerae* in the environment, vibriophages will subsequently increase in density, thereby promoting a decline in the outbreak of the epidemic. Epidemiological and environmental

observations of a cholera outbreak in Dhaka, Bangladesh, suggest that lytic bacteriophage specific for *V. cholerae* may limit the severity of cholera outbreaks by killing bacteria present in the reservoir and in infected individuals. Therefore it can be deduced that there is an undeniable relationship between vibrio phages and the occurrence of cholerae in developing countries such as the one mentioned. This has been illustrated by the recent studies conducted at the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), where scientists led by Faruque et al. have isolated about 36 different cholera phages, known as the JSF series based on their RFLP analysis (G Balakrish Nair et al., 2014).

Epidemics of cholera caused by toxigenic *Vibrio cholerae* belonging to the O1 or O139 serogroups are a major public health problem in many developing countries of Asia, Africa, and Latin America (Faruque et al., 1998). According to a study conducted by Faruque et al. (2005), bacterial viruses, so called bacteriophages, significantly influence the evolution of pathogenic bacteria, including *Vibrio cholerae*. For instance, cholera toxin genes are transferred to non-toxigenic strains through a lysogenic filamentous phage called CTXΦ. However, the presence of phages infecting *V. cholerae* O1 or O139 is negatively associated with the number of viable *V. cholerae* in aquatic environments and the reported cases of cholera, which in turn serves as a therapeutic target. “We found that the majority of water samples showed an inverse correlation between the presence of vibrio phages capable of lysing a given serogroup of *V. cholerae* and the presence of a strain of that same serogroup”, said Faruque et al. (2005). This result has been demonstrated on the table below:

Table 2: Presence of specific vibriophages and susceptible *V. cholerae* strains belonging to the O1 and O139 serogroups in two major rivers and a lake in Dhaka, Bangladesh, between January 2001 and November 2003 Faruque et al., (2005).

		No. of water samples (%)				
Source of water	No. of water samples analyzed* (A)	Phage-positive and *V. cholerae*-negative (B)	Phage-negative and *V. cholerae*-positive (C)	Samples positive for either phage or *V. cholerae* (B + C)	Both phage- and susceptible *V. cholerae*-positive (D)	*P* value‡
Gulshan Lake	63	26 (41.2)	19 (30.1)	45 (71.4)	1 (1.5)	0.0001
Buriganga River	62	30 (48.3)	12 (19.3)	42 (67.7)	2 (3.2)	0.004
Turag River	96	31 (32.2)	18 (18.7)	49 (51.0)	2 (2.0)	0.02
Total samples	221	87 (39.3)	49 (22.1)	136 (61.5)	5 (2.2)	<0.00001

The results from table 2 infer that, since the frequency of water samples containing both a vibrio phage and an epidemic *Vibrio cholerae* strain that was susceptible to these phages was much lower than the expected value, the null hypothesis is rejected. This result suggests that if a phage capable of lysing epidemic strain such as *V. cholerae* O1 was in fact present in a water sample, then that sample was unlikely to contain *V. cholerae* O1 strains susceptible to the phage given that the significance level of $P < 0.00001$.

1.5.8 Important Applications of Bacteriophages

Bacterial viruses, phages, have several scientific applications including replacement of antibiotic medications, serving as research models and in diagnostics. In addition to this, the natural predators of bacteria are used as vehicles for DNA and protein vaccines, as well as for the detection of pathogenic bacterial strain, as a display system for various proteins and antibodies. Furthermore, bacteriophages can be used as biocontrol agents in agriculture and petroleum industry. According to Haq et al. (2012) bacteriophages are a diverse group of viruses which are easily manipulated and therefore they have potential uses in biotechnology such as in research, and therapeutics. Thus bacteriophage research has played a crucial role in promoting the understanding of not only viruses but also biotechnology as a whole and has significantly contributed to the development of basic concepts in biological sciences. Some of the most mentionable applications of bacteriophages would be their use as antimicrobial agents, in therapeutic purposes and in biotechnological research. These ideas have been further discussed in the following subtopics.

1.5.8.1 Bacteriophages as Antimicrobial Agents

Bacteriophages, when replicating through a lytic life cycle, destroy their host cells in the process. While on the other hand, some phages that are called temperate phages, can replicate without killing their respective host. These phages can integrate their genetic material into the host's chromosome, allowing their genome to replicate along with the host's for multiple generations. This does not kill the host initially but eventually when the virus production is stimulated and proliferation of the viruses overwhelms the host to allow the new prophages to burst out as mature viruses. After the discovery of bacteriophages in the early 20th century many researchers pondered upon their potential of killing bacteria, which could undoubtedly make them possible therapeutic agents (Haq et al., 2012). But after World War II when antibiotics were discovered, this natural potential therapeutic agent got little attention and was only considered as a research tool for many years (Clark & March, 2006). In recent years, the rise in antibiotic resistance has led to scientists turning towards probable alternatives, in this case bacteriophages exhibiting antimicrobial properties.

The main advantage of phages is their specificity for target bacteria which reduces the damage to normal flora of the host greatly (Haq et al., 2012) which is contradictory to the use of antibiotic agents. The bacteria to be targeted must be identified first or otherwise a cocktail of phages should be used. Another mainstream advantage of bacteriophage over antibiotics is that if the bacteria become resistant to phages then phages do evolve naturally to infect the now resistant bacteria, hence minimizing the chances of bacterial escape. Although the concept of using bacteriophages as antimicrobial agents is utterly promising, there are certain drawbacks that need to be overcome when considering in vivo conditions. To illustrate, after the administration of phages, they can dissipate very quickly throughout the body reaching almost every organ; but the immune system swiftly clears systemic phages which pose a threat to their acceptance as therapeutic agents as narrated by Dabrowska et al. (2005). It has been discovered that an antimicrobial compound called 'lysins' is present in bacteria killing viruses. The spectrum of efficiency of natural lysins is generally limited to Gram-positive bacteria; however, recombinant lysins have shown an ability to destabilize the outer membrane of Gram-negative bacteria as well and ultimately lead to rapid death of the target bacteria (Kakasis & Panitsa, 2019). A few mentionable examples of successful bacteriophage antimicrobial efficiency include treatment of staphylococcal lung infections (Ioseliani et al., 1980), *P. aeruginosa* infections in cystic fibrosis patients and infections of the eye (Proskurov, 1970).

1.5.8.2 Bacteriophages in Research sectors

Bacteriophages are indispensable in molecular biology and the research of biotechnology, where they are used in genetic engineering, protein expression, and for manipulating bacterial genomes. For example, lambda phage has been extensively scrutinized for its significant impact on genetic studies. In addition to this, bacteria infecting viruses are now under consideration as a gene therapy delivery mechanism, as a biocontrol agent, used in the development of phage-derived vaccines, and in the phage display method.

Phage display is a molecular technique used for synthesizing polypeptides with novel characteristics (Haq et al., 2012). The DNA that encodes the polypeptide is fused with phage coat protein genes, and the desired protein is expressed on the surface of the phage particle. Phage display libraries are useful for identifying and isolating peptides that specifically bind to

target proteins. These peptides can be utilized in drug design, serving as tools for understanding molecular recognition and reducing the chances of receptor mimicking (Sidhu, 2000). The use of these protein constructs in therapeutics can be as receptor-ligand interaction inhibitor or acting as agonist.

Moreover these proteins can be used for the detection of pathogens and agents that are considered to be a potential threat to the environment (Petrenko & Vodyanoy, 2003). Directed evolution of proteins can be used to enhance the enzymatic activity and binding properties (Fernandez-Gacio et al., 2003). Phage display has significantly escalated the identification of peptide molecules that bind to various receptors. It has also played a vital role in the development of binding sites within specific scaffolds, and the production of high-affinity antibodies without the need for immunization (Fernandez-Gacio et al., 2003). Although Phage display is a relatively recent addition to the field of molecular detection methods, it is mentionable due to its rapid progress in the creation of molecular probes for targeting multiple biological compounds (Petrenko & Vodyanoy, 2003). These probes can be selected from extensive libraries containing numerous recombinant phage clones, each displaying a wide range of peptides and proteins on their surface.

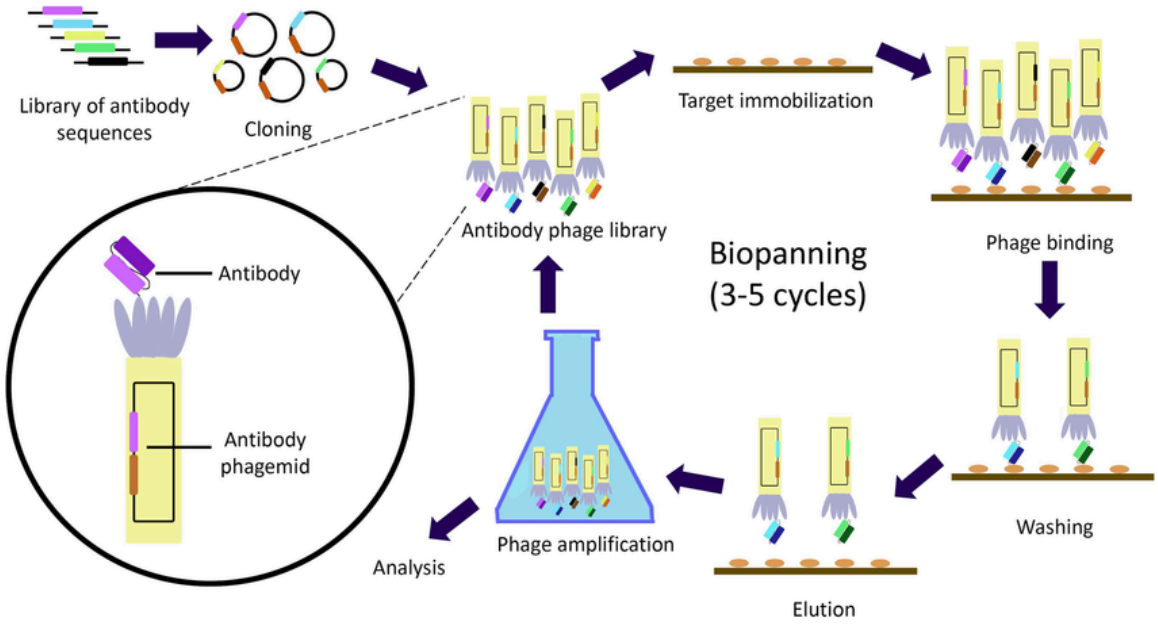


Fig 4: The Overview of Phage Display Technology (Yeoh et al., 2021)

Phage display is initiated by inserting a gene of interest inside the genetic material of bacteriophage so that the protein or peptide sequence that has been inserted can be expressed on the phage surface. Then a library is created by inserting a diverse collection of different genes into the phage. A library consists of millions of phages each containing a unique sequence of protein or peptide. Afterwards, the phage library is exposed to a target molecule, namely a specific protein or antigen. The displayed peptides or proteins are designed to bind to the molecule that has been presented. If the peptide or protein on the phage surface has a strong affinity for the target molecule, only then it will bind to it. The next step requires the washing of all unbound phages and it is followed by the elution of the bound phages. These eluded phages are then amplified on a target bacteria that produces more of the selected phages. Finally, the genes from the selected phages are sequenced, and the corresponding peptides or proteins are analyzed which have been manipulated to be expressed. This allows researchers to identify which sequences are specific to the target molecule binding. This technique can potentially contribute to diagnostics in the medical sector as well.

1.5.8.3 Bacteriophages in Therapeutics (Phage Therapy)

The use of bacteriophages in therapeutic purposes has now acquired a well known term “Phage Therapy”. It is a promising proposition given the current situation of Antibiotic resistance which has significantly reduced the effectiveness of multiple drugs used against bacterial diseases. Phage therapy is particularly intriguing because it offers an alternative to traditional antibiotics. For instance, Current research on the use of phages and their lytic proteins, specifically against multidrug-resistant bacterial infections, suggests phage therapy has the potential to be used as either an alternative or a supplement to antibiotic treatments (Lin et al., 2017). Although much is still unknown about the interactions between phage, bacteria, and human host, the time to take phage therapy seriously seems to be rapidly approaching.

An overview of how phage therapy proceeds is as follows:

i. In the beginning, bacteriophages are isolated from environments like soil, water or sewage, where they are naturally found. These phages are then tested against a particular bacteria in order to find its efficacy in killing the bacteria of interest.

ii. Once the appropriate phages are identified, they are then grown in labs to produce a large quantity in bulk.
iii. The prepared phages are administered to the patient, usually by direct application to the infection site, ingestion, or intravenous procedures.
iv. Once inside the patient, the bacteriophages infect and kill the target bacteria by injecting their genetic material into the bacterial cells, which then hijack the bacterial machinery of reproduction. The bacteria eventually burst open as the number of phages surpass a certain threshold, releasing new phages that can infect other bacteria inside the patient's body.

Phage therapy is not as recent as most would understand it to be. Bacteriophages have been used as a treatment against pathogens such as *Shigella dysenteriae* as early as 1919 (Chanishvili, 2012). In a 1931 trial conducted in India to test phage therapy for cholera, there were 118 control subjects and 73 experimental subjects who received phage treatment. In this particular experiment, D'Herelle noted a 90% reduction in mortality, with 74 deaths in the control group and merely 5 deaths in the experimental group as reported by Chanishvili (2012).

Apart from direct use of bacteriophages in clinical trials, bacteria killing viruses have also shown to produce promising results when administered as a second line of treatment in association with antibiotics as narrated by Altamirano et al. (2020). In this method, phage therapy involves using phages with lytic activity to target bacteria, targeting the development of phage-resistant strains. For an MDR *A. baumannii* infection (characterized by encapsulated red cells in Fig 5) that usually resists initial antibiotic treatments, phage therapy can be used as a second-line treatment. The phages will kill the bacteria initially, and this will then lead to the emergence of phage-resistant mutants (such as unencapsulated red cells in Fig 5). These resistant strains can be targeted using a third-line treatment strategy, which may include readministration of antibiotics, using different phages, or other immune system interventions. This multi-step approach aims to achieve a second round of bacterial killing to reduce the infection thereby resulting in a better clinical outcome. This methodology has been illustrated in the following diagram (Fig 5).

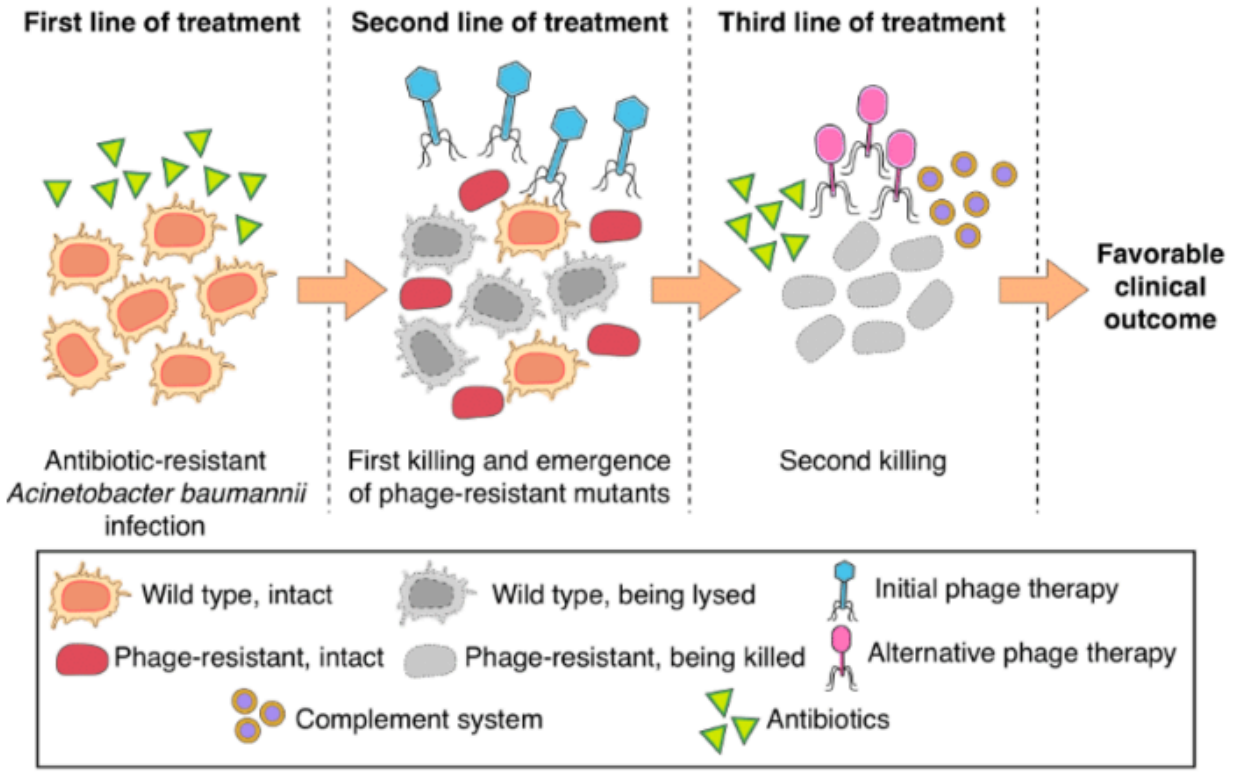


Fig 5: An approach toward phage therapy utilizing lytic activity of phages (Altamirano et al., 2020)

Recent investigations using animal models have explored phage treatment against a range of clinically significant pathogens. One mentionable example would be an animal model study: when diseased with gut-derived sepsis due to *P. aeruginosa*, oral administration of phage saved 66.7% of mice from mortality compared to 0% in the control group (Watanabe et al., 2007). For assessing the effectiveness of phage therapy in human trials, the Eliava Institute has comprehensively used phage in preclinical and clinical treatment of common bacterial pathogens such as *S. aureus*, *P. aeruginosa*, *Proteus* spp., *Streptococcus* spp., *E. coli*, *S. dysenteriae*, *Salmonella* spp., and *Enterococcus* spp (Kutateladze & Adamia, 2008). Furthermore, innovations in the gene editing tool CRISPR/Cas have created novel opportunities for phage therapy. One praiseworthy example of the usefulness of this recent technology in phage therapy is the use of bioengineered phage to deliver a CRISPR/Cas that has been programmed to disrupt antibiotic resistance genes and destroy antibiotic resistance plasmids (Yosef et al., 2015). In conclusion, it can be deduced that even though "Phage Therapy" holds multiple limitations, they can still be overcome and triumph over the global emergency situation of antibiotic resistance.

Chapter - 02: Materials, methods and procedures

2.1 Standard Laboratory Practices of BRAC University MNS Laboratory.

This research was conducted at BRAC University in Dhaka, Bangladesh, at the Molecular and Environmental Microbiology Laboratory (MEM), Molecular and Environmental Biology Laboratory (MEB) of the Department of Mathematics and Natural Sciences. Certain guidelines of this particular laboratory were strictly followed. They are mentioned below:

- Aseptic measures such as sterilization of the workplaces and apparatus are to be followed at all times.
- Protective measures such as wearing a Lab coat is mandatory inside the laboratory.
- The Laminar hood cabinet must be cleaned twice, once before and once after the work.
- Burner should not be left burning, it must be turned off after each use.
- Wasting of reagents or any useful material of the Lab is strictly prohibited.
- All apparatus used directly in the experiment must be autoclaved at 121 degrees celsius for 15 minutes prior to use. This ensures that no other experiments bear the risk of contamination.
- Any broken utensil or apparatus must be noted to the Lab attendants immediately.
- Use of common apparatus such as the weighing machines, require it to be cleaned both before and after the use of the machine.
- Culture plates are not to be kept in the refrigerator for more than two days.
- Petri dishes should be discarded and washed within a week of receiving them by requisition.
- Fresh plates must not be stored in the refrigerator for more than a week as it increases the chances of contamination.
- Reagents and apparatus used must be put back in their original shelves.
- The micropipette must be adjusted back to its maximum capacity once it has been used.

2.2 Design of the Experiment is as follows:

1. Specific bacterial strains collection from BRAC University MNS Thesis Laboratory.
2. The bacterial strains are revived by streaking them onto LA plates. To ensure these bacteria have not been contaminated, the strains are then streaked on TCBS agar plates that are selective for the strains of interest only.
3. Bacteriophage collection from BRAC University MNS Thesis Laboratory.
4. Enrichment of the Bacteriophages.
5. Spot test of the bacteriophages for the infection profiling.
6. Preparation of the phage cocktail and allowing for coinfection by the DLA method.
7. collection of the plaques from coinfected plates.
8. Spot test of the collected plates on all strains of test bacteria.
9. Evaluation of the results to identify any possible exceptions.

2.3 Materials used in this Research

2.3.1 Reagents

- Luria Bertani Agar
- Luria Broth medium
- Sodium Chloride
- Yeast Extract
- Tryptone
- Agar
- Gelatin
- Magnesium Sulfate
- Tris-HCl
- Chloroform
- Sodium Hydroxide pellets
- Ethanol
- Distilled Water

2.3.2 Apparatus

- Petri plates
- Test tubes
- Glass vials
- Conical flasks
- Beakers
- Falcon tubes
- Micro centrifuge tubes
- Micropipette
- Pipette
- Micropipette tips
- Syringes
- 0.22 Micron filters
- Inoculating loops
- Inoculating needles
- Spirit lamps
- Vortex machine
- Centrifuging machine
- Shaker incubator
- Water bath
- Aluminum foil paper
- Parafilm paper
- Bunsen burner

2.4 Preparation of the Culture Media

2.4.1 Saline Preparation

The saline solution used in this research is 0.9% sodium chloride in distilled water. The steps done in the preparation of this saline solution are as follows:

1. 0.9 grams of sodium chloride was measured using an electronic balance and transferred to a conical flask with the use of aluminum foil paper.
2. 100 ml of distilled water was measured using a measuring cylinder and then transferred to the conical flask.
3. The two reagents were mixed together until the solute dissolved in the solvent.
4. Then the solution was autoclaved at 121 degrees celsius and a pressure of 15 PSI for 2 hours.

2.4.2 Luria Broth preparation

Luria broth (LB), also known as Luria Bertani Broth, is a nutrient rich liquid broth that is commonly used for the culture of Enterobacteriaceae members as well as for the phage plaque assay. The broth consists of Tryptone, sodium chloride and yeast extract. In this research, the ready to make LB powder was used as it is available in the laboratory reagents.

1. 10 grams of LB powder was measured using an electronic balance and transferred to a conical flask with the use of aluminum foil paper.
2. 500 ml of distilled water was measured using a measuring cylinder and then transferred to the conical flask.
3. The two reagents were mixed together until the solute dissolved in the solvent. Heat was often used for better solubility.
4. Then the solution was autoclaved at 121 degrees celsius and a pressure of 15 PSI for 2 hours.
5. The LB broth was then ready to be used.

2.4.3 Luria Agar (LA) Preparation

Luria Agar (LA), is basically LB medium which has agar added to it in order for the media to solidify. LA was used for streaking the inoculated stock bacteria as it is a non selective media which allows the culture of Enterobacteriaceae members and for the coliphage plaque assays. In addition to this, the media is also effective for the growth of pure cultures of recombinant strains which was the prior object of interest of this particular study. For the preparation of LA media, the ready to make powdered form was used which is available in the laboratory.

1. 20 grams of LA powder was measured using an electronic balance and transferred to a conical flask with the use of aluminum foil paper.
2. 500 ml of distilled water was measured using a measuring cylinder and then transferred to the same conical flask.
3. The two reagents were mixed together until the solute dissolved in the solvent. Heat was used for increasing the solubility by using a bunsen burner.
4. Then the solution was autoclaved at 121 degrees celsius and a pressure of 15 PSI for 2 hours.
5. The LA was then ready to be poured into sterile petri dishes. This was conducted under a laminar hood and the plates were then allowed to solidify.

2.4.4 Soft Agar Preparation

Soft agar is a growth medium for bacteria with a lower percentage of agar than normal and it is essential for performing experiments such as spot test or Double Layer Assay for the analysis of Bacteriophages. The components required for the preparation of soft agar are sodium chloride, tryptone, yeast extract and bacteriological agar.

1. For each 100 ml of soft agar to be prepared, 1 gram of NaCl, 1 gram tryptone, 0.5gm of yeast extract and 0.6gm bacteriological agar was measured using an electronic balance and transferred to a conical flask with the use of aluminum foil paper.
2. 100 ml of distilled water was measured using a measuring cylinder and then transferred to the same conical flask.

3. All the reagents were mixed together until the solute dissolved in the solvent. Heat was used for increasing the solubility by using a bunsen burner.
4. The soft agar solution was then poured into sterile test tubes, 6 ml of soft agar was transferred to each test tube.
5. Then the test tubes were sealed in a large beaker and autoclaved at 121 degrees celsius and a pressure of 15 PSI for 2 hours.

2.4.5 Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar Preparation:

TCBS (Thiosulfate-Citrate-Bile-Sucrose) agar is a specialized culture medium that is used in microbiology laboratories to isolate *Vibrio* species. TCBS Agar is a selective differential medium for isolating and cultivating *Vibrio cholerae* and other *Vibrio* species from clinical specimens and other materials (Aryaal, 2023). It is composed of protease peptone, yeast extract, sodium thiosulfate, sodium citrate, bile, sucrose, sodium chloride, ferric citrate, bromothymol blue, thymol blue and agar.

1. For each 1000 ml of TCBS media, 88.1gm of the dried media was measured using an electronic balance and transferred to a conical flask with the use of aluminum foil paper.
2. 1000 ml of distilled water was measured using a measuring cylinder and then transferred to the same conical flask.
3. All the reagents were mixed together until the solute dissolved in the solvent. Heat was used for increasing the solubility by using a bunsen burner.
4. Sterilization by autoclaving is not required
5. The media was then ready to be poured into sterile petri dishes. This was conducted under a laminar hood and the plates were then allowed to solidify.

2.5 Salt Magnesium (SM) Buffer Preparation

Salt Magnesium buffer was used to store the isolated phage plaques so that the media does not alter the optimum conditions, namely the pH, required by the microorganism of interest. Buffers do so by resisting the change in pH as it consists of a weak acid and a conjugate base of the weak acid.

1. For each 50 ml of SM buffer, 0.29gm NaCl, 0.1gm MgSO4, 1M 7.88 grams Tris-HCl and 0.2 gram gelatin was measured using an electronic balance and transferred to a conical flask with the use of aluminum foil paper.
2. 10 ml of distilled water was measured using a measuring cylinder and then transferred to the same conical flask.
3. All the reagents were mixed together until the solute dissolved in the solvent.
4. pH of the solution was then measured and adjusted to 8 by the addition of either sodium hydroxide pellets or dilute hydrochloric acid.
5. The remaining volume of the solution was then made 50 ml.
6. The buffer was then autoclaved at 121 degrees celsius and a pressure of 15 PSI for 2 hours.

2.6 Bacterial Cell culture by the process of streaking

The test bacteria strains were streaked onto Luria Bertani Agar plates that provide suitable nutrients for the optimum growth of bacteria. This is ideally done the day before further experiments to be able to work with fresh culture of bacteria.

The steps required for this procedure are as follows:

1. The LA plates are labeled according to the group name, date of streaking and strain of the bacterium.
2. Using a cool sterile loop, bacteria is picked up from the stock by stabbing.
3. The bacteria is then streaked onto the fresh plates and incubated at 37 degrees celsius overnight.

2.7 Bacterial Liquid Culture by the process of cell suspension

Liquid culture is prepared by inoculating the bacteria onto liquid nutrient media, namely Luria Bertani broth (LB broth), and then incubating at 37 degrees celsius in a shaker incubator for around one hour to one and a half hours.

This is carried out just before performing experiments such as 'Spot Test' and 'Double Layer Assay' as this provides live bacterial suspension at an optimum density that is in a very juvenile state and so it cannot resist the activity of bacteriophages if it does not already have an innate ability to do so. This suspension culture is called a 'Young Culture'.

2.8 Bacteriophage Enrichment

To amplify the phage volume, the enrichment process is repeated over a period of two weeks. An enriched phage solution is crucial for precise results in subsequent procedures, including determining the multiplicity of infection and performing a double-layer assay. The resulting enriched phage stock is preserved at a temperature of 4°C for future utilization. The steps of bacteriophage enrichment are as follows:

1. Young culture is prepared by inoculating 5 ml of LB broth with the specific bacterial strain, and incubating into the shaker incubator until OD600 0.4. This usually takes a time of around 1 hour to 1 and a half hours.
2. To this young culture, 100 µl phage is added and incubated again in the shaker incubator for 4 hours.
3. Afterwards, the bacteria and phage containing falcon tube needs to be centrifuged at 13,000 rpm for 10 minutes to separate the bacterial cells as pellets and the enriched phages suspended in the supernatant.
4. The phages are then filtered through a 0.22-micron syringe filter and pure phage collected in another labeled falcon tube.
5. The fresh phages are stored in the refrigerator at 4 degrees celsius until further use.

2.9 Spot Test for Bacteriophage

Spot test is carried out to check whether a test phage sample is able to infect a bacterium. It is done by placing a small drop of phage sample on a plate that has been lawned by the bacteria strain in question. If the sample phage is effective against the test bacteria, then a small 'Spot' of clear zone appears on the turbit lawn of the inoculum. The steps of this procedure are as follows:

1. Each strain of bacteria is inoculated in 3 ml of fresh LB and incubated in a shaker for 1.5 hours. This prepares the young culture for the test.
2. LA plates are prepared, and labeled with host strain of bacteria and numerals representing bacteriophages, while soft agar aliquots stored in the refrigerator are melted by boiling and kept at 52°C in a water bath.
3. After incubation, 8 ml of soft agar aliquot is mixed with 150 µl of the young individual bacterial culture and poured onto the surface of the LA agar plate to create bacterial lawns.
4. The soft agar mixture is then allowed to sit until it solidifies completely.
5. Afterwards, 15 µl of phage suspension is placed on top of the bacterial lawn of soft agar.
6. The plates are then left to rest for 20-30 minutes until the phage suspension dries off completely. Then the plates are incubated at 37 degrees celsius for 16 hours.

2.10 Double Layer Assay for Bacteriophage Titer Analysis

The Double Layer Assay, also known as plaque assay, is a widely used technique in phage research to isolate and identify pure bacteriophage populations and it is considered as the standard method for determining phage titer and concentration. The number of plaques is counted and the phage concentration/titer is estimated as the number of plaque-forming units (PFU/mL) of the measured preparation. According to Mocé-Llivina et al. (2004), these assays are based on the use of solid or semisolid culture media, utilizing the principle that each viable virus multiplies to produce a localized area of cells destroyed or killed by viral action. These discrete clones are known as plaques; each plaque is considered to be derived from an infectious virus particle. The formula for calculating the titer (PFU/ml) is as follows:

$$\frac{\text{\# of plaques}}{\text{Dilution factor X volume of diluted virus added}} = \text{PFU/ml}$$

The steps for performing Double Layer Assay are given below:

1. The host strain of bacteria is inoculated in 3 ml of fresh LB and incubated in a shaker for 1.5 hours. This prepares the young culture for the test.
2. The corresponding phages of the host bacteria are serially diluted in LB up to 10^{-5} by adding 100 µl of phage solution in 900 µl of LB broth for each consecutive dilution.
3. Afterwards, 100 µl of the phage solution and 150 µl of the young culture of host bacteria are added to a test tube containing 6 ml of liquid soft agar at 55 degrees celsius.
4. The test tube cap is closed and the container inverted a few times before the mixture can be poured into medium sized LA plates.
5. The plates are then allowed to solidify. This takes around 10-15 minutes.
6. The plates are then incubated at 37 degrees celsius for 16 hours after which the results can be interpreted.

2.11 Preparation of Phage Cocktail by Enrichment followed by Double layer Assay (Co-infection)

This procedure of coinfection of bacteriophages was conducted by combining the processes of bacteriophage enrichment, double layer assay and plaque collection. At first, cocktail phage was prepared by enriching 100 µl of each phage (JSF-2 & JSF-25), simultaneously in a single glass vial. Then the cocktail phage solution was filtered and used in double layer assay to allow co-infection of the host bacterial strain.

Cocktail bacteriophage enrichment:

1. The host strain of bacteria is inoculated in 3 ml of fresh LB and incubated in a shaker for 1.5 hours. This prepares the young culture for the test.
2. To this young culture, 100 µl each phage (JSF-2 & JSF-25) is added and incubated again in the shaker incubator for 4 hours.
3. Afterwards, the bacteria and cocktail phage containing falcon tube needs to be centrifuged at 13,000 rpm for 10 minutes to separate the bacterial cells as pellets and the enriched phages suspended in the supernatant.

4. The phages are then filtered through a 0.22-micron syringe filter and pure phage collected in another labeled falcon tube.

Cocktail bacteriophage coinfection by Double Layer Assay:

1. The host strain of bacteria is inoculated in 3 ml of fresh LB and incubated in a shaker for 1.5 hours. This prepares the young culture for the test.
2. The cocktail phage solution is serially diluted in LB up to 10^{-5} by adding 100 µl of phage solution in 900 µl of LB broth for each consecutive dilution.
3. Afterwards, 100 µl of the phage solution and 150 µl of the young culture of host bacteria are added to a test tube containing 6 ml of liquid soft agar at 55 degrees celsius.
4. The test tube cap is closed and the container inverted a few times before the mixture can be poured into medium sized LA plates.
5. The plates are then allowed to solidify. This takes around 10-15 minutes.
6. The plates are then incubated at 37 degrees celsius for 16 hours after which the coinfected phage plaques can be collected for further analysis.

Coinfected phage plaques collection:

1. 10 single plaques are collected using 10 different sterile micropipette tips.
2. Each plaque is individually transferred to eppendorf microtubes (MCT) containing 750 µl SM buffer.
3. The plaques are squashed in the MCT tubes using the specific micropipette tip that was used to transfer it.
4. 75 µl of chloroform was then added to each MCT tube. Here, Chloroform is used for disruption of bacterial cells present with the agar collected from the coinfection plates.
5. The mixture was vigorously vortexed for uniform distribution of the contents.
6. The MCT tubes were then centrifuged at 13,000 rpm for 10 minutes to separate the bacterial cells and other debris as pellets and the enriched phages suspended in the supernatant.
7. The coinfected phages are then filtered through a 0.22-micron syringe filter and coinfected phage collected in correspondingly labeled MCT tubes.
8. The phages are stored in the refrigerator at 4 degrees celsius until further use.

2.12 Phage Coinfection Evaluation by Spot Test

The Phage Coinfection Evaluation by Spot Test is the most important step in this study as it was used in assessing the outcomes of the coinfection experiment. In this process, selected phage samples obtained from coinfection trials are applied as spots on bacterial lawns of 14 different strains of bacteria, namely: WT-032, 042, 050, 331, 389, 006, 428, 429, 439, 443, 351, 371, 388, 1877. This evaluation determines whether or not there have been any significant changes in the coinfected bacteriophages that could allow it to alter its effectiveness against a particular strain of bacteria.

The steps are as follows:

1. Each strain of bacteria is inoculated in 3 ml of fresh LB and incubated in a shaker for 1.5 hours. This prepares the young culture for the test.
2. LA plates are prepared, and labeled with the particular strain of bacteria and numerals representing bacteriophages, while soft agar aliquots stored in the refrigerator are melted by boiling and kept at 52°C in a water bath.
3. After incubation, 8 ml of soft agar aliquot is mixed with 150 µl of the young individual bacterial culture and poured onto the surface of the LA agar plate to create bacterial lawns.
4. The soft agar mixture is then allowed to sit until it solidifies completely.
5. Afterwards, 10 µl of coinfected phage suspension is placed on top of the bacterial lawn of soft agar.
6. Each plate of bacteria had 12 spots in total (10 from the coinfected phage and 2 from the native JSF-2 and 25).

The plates are then left to rest for 20-30 minutes until the phage suspension dries off completely. Then the plates are incubated at 37 degrees celsius for 16 hours.

Chapter 3: Result

3.1. Profiling results of spot test of Bacteriophage JSF- 2 & JSF-25

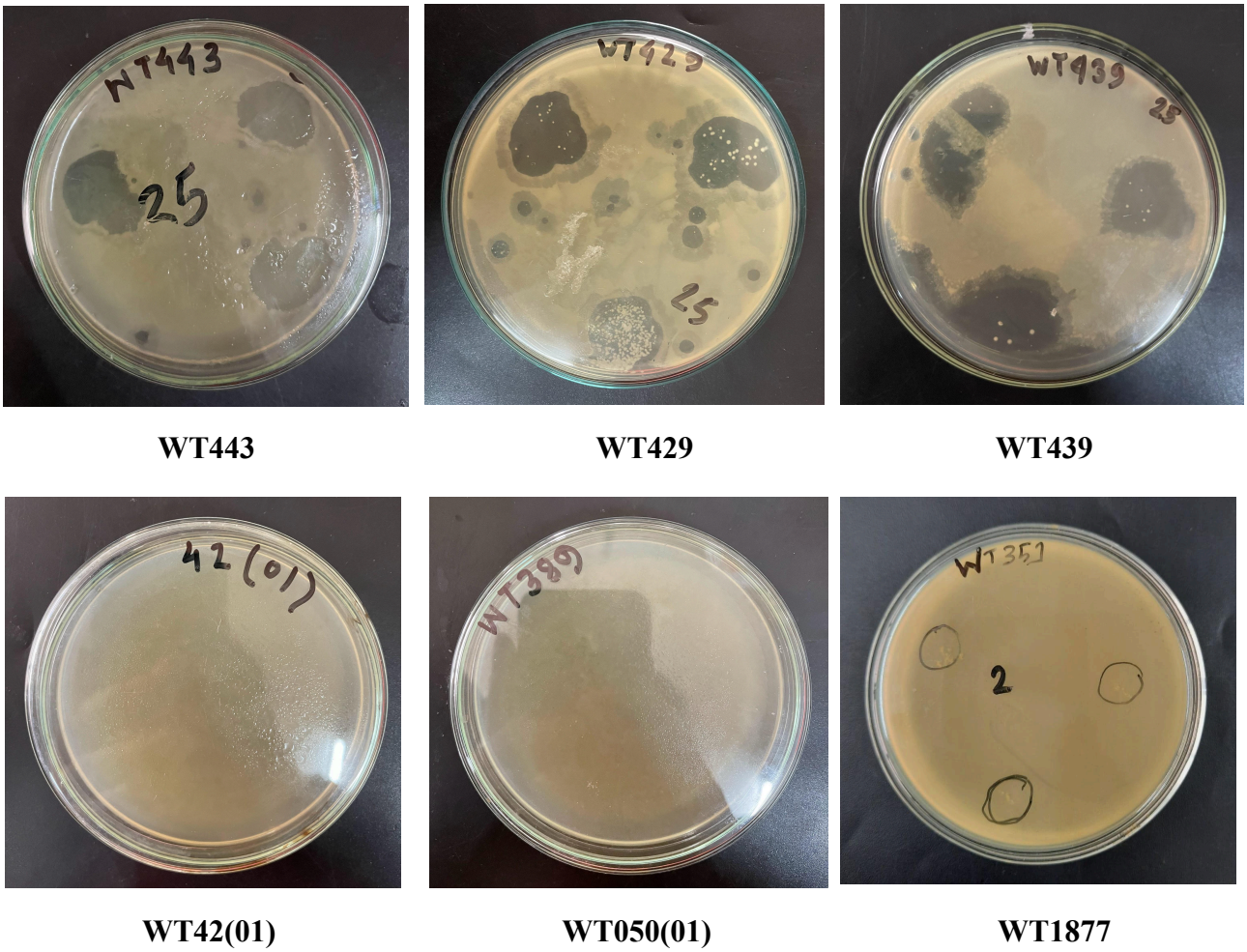


Figure 6: The profiling result of *Vibrio cholerae* strains by JSF-2 and JSF-25 in early trial experiments.

Table 3. Profiling results of Bacteriophage JSF-2 & JSF-25 on *Vibrio cholerae* strains

SL no.	Bacterial Strains	JSF-25	JSF-2
1	WT 032 (01)	Negative	Negative
2	WT 42 (01)	Negative	Negative
3	WT 050 (01)	Negative	Negative
4	WT 331 (01)	Negative	Negative
5	WT 389 (01)	Negative	Negative
6	WT 006 (01)	Negative	Negative
7	WT 428 (01)	Positive *(resistant colony)*	Positive
8	WT 429 (01)	Positive *(resistant colony*	Positive
9	WT 439 (01)	Positive *(resistant colony)*	Positive
10	WT 443 (J)	Positive	Positive
11	WT 351 (N)	Negative	Negative
12	WT 371 (N)	Negative	Negative
13	WT 388 (01)	Negative	Negative
14	WT 1877	Positive *(resistant colony)*	Positive *(resistant colony)*

The above table shows that, in JSF-25, all the results were positive but it was hard to distinguish whether it was resistant colony or contamination. And in JSF-2, almost all the results were negative except for a few. Lastly, WT1877, the control bacteria shows positive in both phages.

3.2. Co-infection DLA result

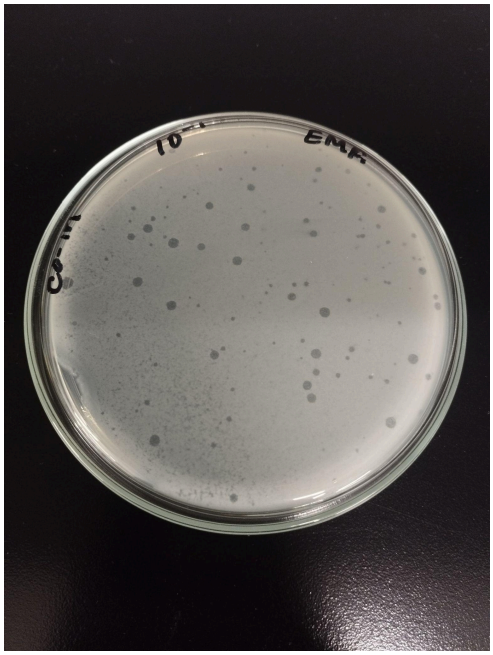


Figure 7: Co-infection DLA plaques obtained from 10^{-1} dilution using phage JSF-2 and JSF-25

Colony count of cocktail phage:

143 / (10^{-1} times 0.1) =14300 PFU/ml.

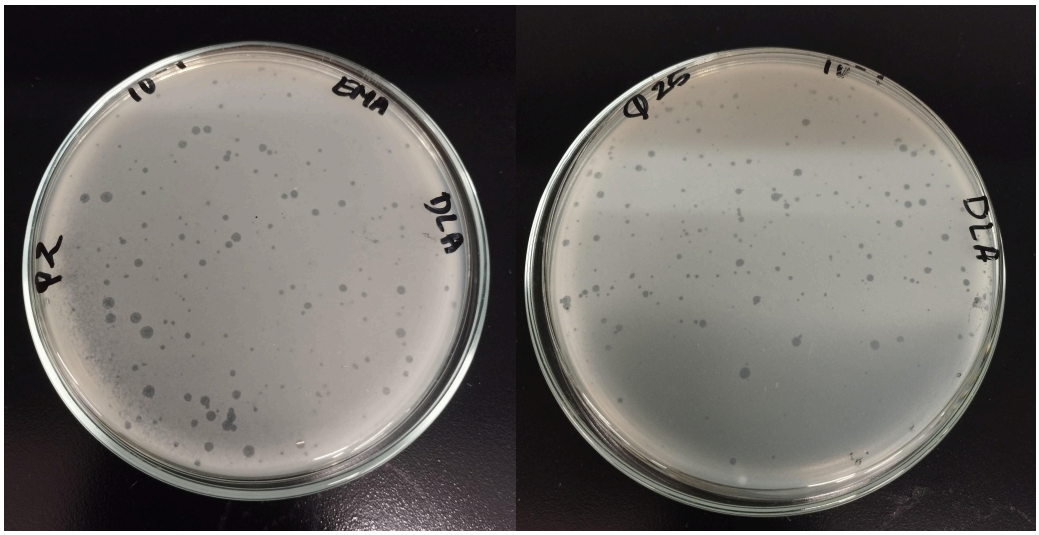


Figure 8: Double Layer Assay result of native bacteriophages at a dilution factor of 10^{-1}

Colony count of phage JSF-2:

173 / (10^{-1} times 0.1) = 17300 PFU/ml

Colony count of phage JSF-25:

161 / (10^{-1} times 0.1) = 16100 PFU/ml

3.3. Spot test first trial results using collected plaques from Co-infection DLA

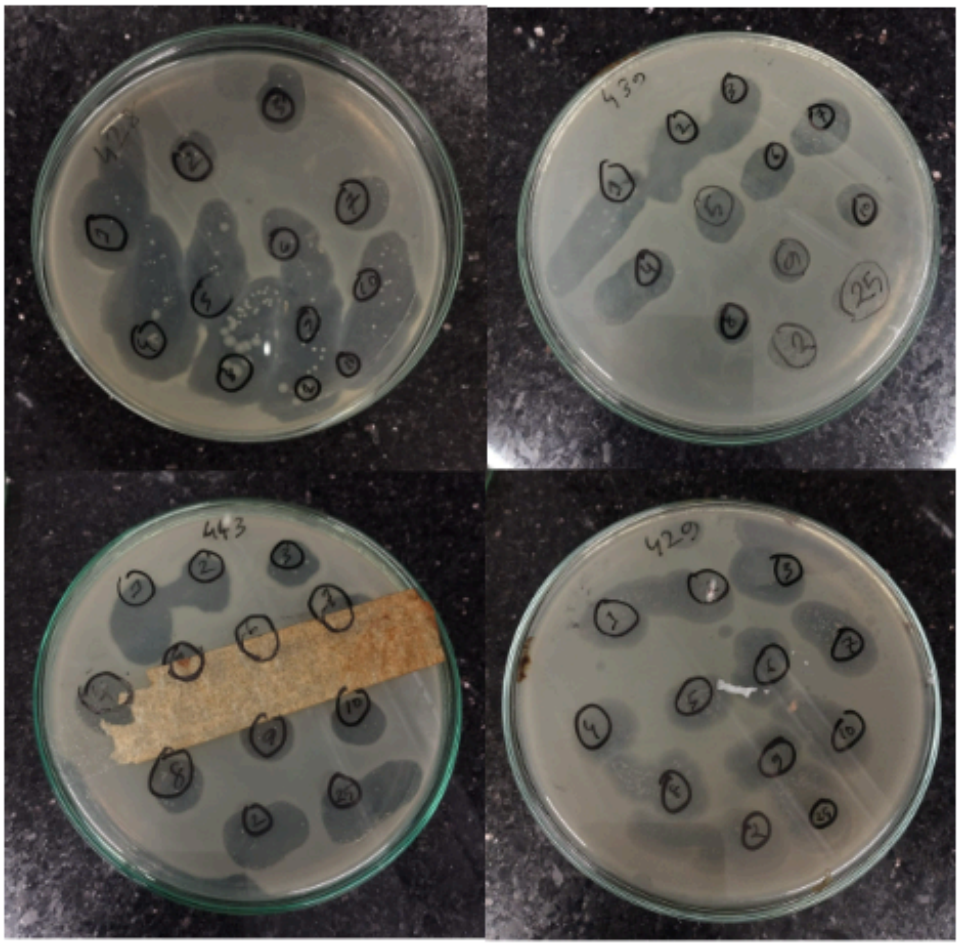


Figure 9: spot test results by using plaques from Co-infection DLA

The above bacterial strain of *Vibrio cholerae*, WT1877 including WT443, WT429, WT428 and WT439 shows positive results in the Co-infection experiment.

Table 4. The following table shows the results of the Co-infection spot test from Phage 1-4 in the first trial including JSF-25 and JSF-2 phage.

SL no.	Bacterial Strain	JSF-25	JSF-2	Phage-1	Phage-2	Phage-3	Phage-4
1	WT 032 (01)	-	-	-	-	-	-
2	WT 42 (01)	-	-	-	-	-	-
3	WT 050 (01)	-	-	-	-	-	-
4	WT 331 (01)	-	-	-	-	-	-
5	WT 389 (01)	-	-	-	-	-	-
6	WT 006 (01)	-	-	-	-	-	-
7	WT 428 (01)	+	+	+	+	+	+
8	WT 429 (01)	+	+	+	+	+	+
9	WT 439 (01)	+	+	+	+	+	+
10	WT 443 (J)	+	+	+	+	+	+
11	WT 351 (N)	-	-	-	-	-	-
12	WT 371 (N)	-	-	-	-	-	-
13	WT 388 (01)	-	-	-	-	-	-
14	WT 1877	+	+	+	+	+	+

Table 5. The following table shows the results of the Co-infection spot test from Phage 5-10 in the first trial.

SL no.	Bacterial Strain	Phage-5	Phage-6	Phage-7	Phage-8	Phage -9	Phage-10
1	WT 032 (01)	-	-	-	-	-	-
2	WT 42 (01)	-	-	-	-	-	-
3	WT 050 (01)	-	-	-	-	-	-
4	WT 331 (01)	-	-	-	-	-	-
5	WT 389 (01)	-	-	-	-	-	-
6	WT 006 (01)	-	-	-	-	-	-
7	WT 428 (01)	+	+	+	+	+	+
8	WT 429 (01)	+	+	+	+	+	+
9	WT 439 (01)	+	+	+	+	+	+
10	WT 443 (J)	+	+	+	+	+	+
11	WT 351 (N)	-	-	-	-	-	-
12	WT 371 (N)	-	-	-	-	-	-
13	WT 388 (01)	-	-	-	-	-	-
14	WT 1877	+	+	+	+	+	+

3.4. Spot test final trial results using collected plaques from Co-infection DLA

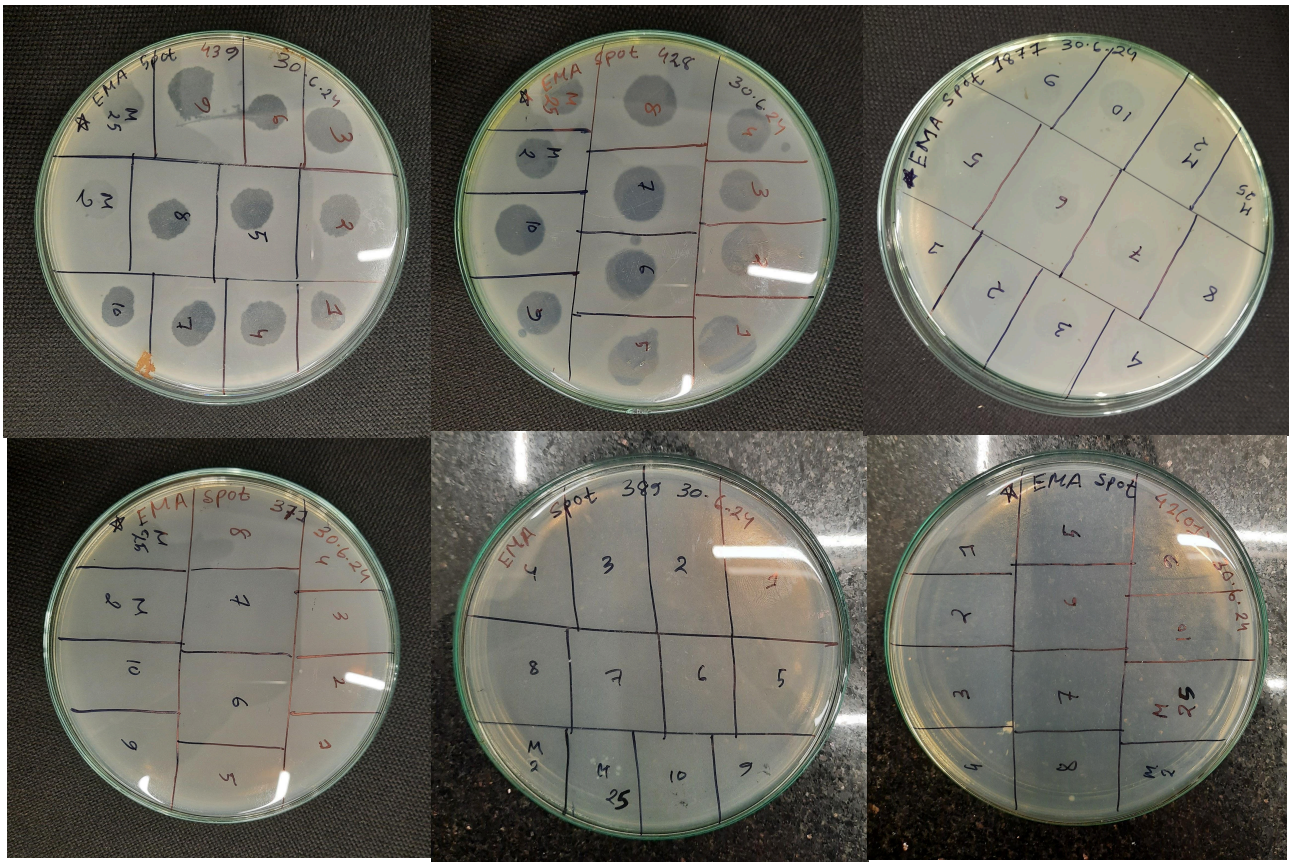


Figure 10: The following figure shows positive results in WT439, WT428, WT1877 bacterial strains, and, in WT371, WT389, WT42(01) shows negative results: no clear zone in the given spot.

Table 6. The following table shows the results of the Co-infection spot test from Phage 21-24 in the final trial including JSF-25 and JSF-2 phage.

SL no.	Bacterial Strain	JSF-25	JSF-2	Phage-21	Phage-22	Phage-23	Phage-24
1	WT 032 (01)	-	-	-	-	-	-
2	WT 42 (01)	-	-	-	-	-	-
3	WT 050 (01)	-	-	-	-	-	-
4	WT 331 (01)	-	-	-	-	-	-
5	WT 389 (01)	-	-	-	-	-	-
6	WT 006 (01)	-	-	-	-	-	-
7	WT 428 (01)	+	+	+	+	+	+
8	WT 429 (01)	+	+	+	+	+	+
9	WT 439 (01)	+	+	+	+	+	+
10	WT 443 (J)	+	+	+	+	+	+
11	WT 351 (N)	-	-	-	-	-	-
12	WT 371 (N)	-	-	-	-	-	-
13	WT 388 (01)	-	-	-	-	-	-
14	WT 1877	+	+	+	+	+	+

Table 7. The following table shows the results of the Co-infection spot test from Phage 5-10 in the final trial.

SL no.	Bacterial Strain	Phage-25	Phage-26	Phage-27	Phage-28	Phage -29	Phage-30
1	WT 032 (01)	-	-	-	-	-	-
2	WT 42 (01)	-	-	-	-	-	-
3	WT 050 (01)	-	-	-	-	-	-
4	WT 331 (01)	-	-	-	-	-	-
5	WT 389 (01)	-	-	-	-	-	-
6	WT 006 (01)	-	-	-	-	-	-
7	WT 428 (01)	+	+	+	+	+	+
8	WT 429 (01)	+	+	+	+	+	+
9	WT 439 (01)	+	+	+	+	+	+
10	WT 443 (J)	+	+	+	+	+	+
11	WT 351 (N)	-	-	-	-	-	-
12	WT 371 (N)	-	-	-	-	-	-
13	WT 388 (01)	-	-	-	-	-	-
14	WT 1877	+	+	+	+	+	+

Chapter 4: Discussion

Comparative genomics have demonstrated that bacteria and bacteriophages evolve together (BrüSsow et al., 2004). This is mostly noticeable in harmful bacteria, where many have prophages (viral DNA inserted into the bacterial DNA) or remnants of these prophages. Some of these prophages carry genes that make the bacteria even more harmful. *Vibrio cholerae* is a well known example of such bacteria. On the other hand, prophages of some bacteria like *Staphylococcus aureus,* provide benefit to the bacteria by creating a "swarm" of related prophages. Where these prophages constantly change due to recombination with other phages or available mobile DNA elements that are commonly found in plasmids. This constant change also contributes to the variation in the bacterial genome. According to BrüSsow et al. (2004), prophages make up a significant part of the unique DNA sequences in many different bacterial strains and can even cause large-scale rearrangements in the bacterial genome. Hence it can be stated that both bacterial and viral DNA alteration is a relatively common phenomenon. This study was particularly based on this phenomenon that anticipated a change in the Bacteriophage Strains of JSF-2 and JSF-25 when allowed to co-infect a host bacteria after it had been enriched and lawned together.

One of the challenges faced during the thesis work was the incubation period, after conducting Double Layer Assay (DLA) or Spot test experiment, less than 24 hours and not more than 16 hours incubation period is crucial for ideal results. If the incubation period exceeds, then overgrowth of bacteria is seen on the spot region. Moreover, the shaker incubation period needs to be strictly maintained during enrichment for co-infection spot experiment. Overtime shaker incubation period results in maturation of bacteria and less effectiveness of bacteriophage. Therefore, the results are inconsistent. In addition to that, continuous enrichment of phage was necessary in order to get consistent results in the co-infection trials.

4.1 Profiling of the Infection States of Native Phages by Spot Test

Spot test is a microbiology experiment that is used to detect and identify bacteriophages depending on their ability to lyse or break down bacterial cells. Spot test allows the study of interactions between bacteriophages and bacteria, such as understanding the mechanisms of infection and identifying whether or not a particular phage is virulent against a known bacteria. The latter has been of this specific experiment's objective.

Here, in this study two different kinds of phages that are JSF-2 and JSF-25 have been used to determine their natural ability to infect fourteen different strains of heterotrophic bacteria. These bacteria include: WT 032(01), WT 042(01), WT 050 (01), WT 331(01), WT 389 (01), WT 006 (01), WT 428 (01), WT 429 (01), WT 439 (01), WT 443 (J), WT 351 (N), WT 371 (N), WT 388 (01) and WT 1877 (this was used as the control strain known to be infected by both strains of bacteriophages). The results of this experiment have been demonstrated in Table 3.

In this experiment, 3 ml of LB media was transferred to sterilized glass vials for each of the bacterial strain inoculations separately, and then these vials were incubated at 37 degrees celsius in a shaker incubator for roughly around one hour and thirty minutes. Afterwards, these young cultures of liquid bacterial suspensions were lawned with the use of soft agars on LA plates properly labeled with the respective bacterial strain name. Upon these plates, drops of bacteriophage suspension are added and allowed to dry. After an incubation period of 16 hours at 37 degrees celsius, the results can be interpreted. If a clear zone is observed then it can be inferred that the bacteria cells have been lysed by the corresponding phage. If no clear zone is observed then it can be said that the bacteria is insensitive to the phage it has been tested for.

According to the results of Table 3, it can be noticed that, only four strains of bacteria (excluding the control strain) have shown sensitivity to both phages JSF-2 and JSF-25. The sensitive bacteria strains are WT 428 (01), WT 429 (01), WT 439 (01), WT 443 (J) and WT 1877 which is the control of the experiment. Since the control has given consistent results, it can be perceived that the experiment has valid results.

4.2 Phage Cocktail DLA Interpretation

Coinfection experiments are known to mimic natural conditions where many different phages interact with bacteria. This is aimed to induce an artificially created selection pressure on both the phages and bacteria. As a result, bacteria might develop resistance mechanisms against phage infections or these phages may evolve strategies to bypass the bacterial defenses. These alterations therefore are highly likely to bring about evolutionary changes in these microorganisms.

A phage cocktail is a mixture of multiple bacteriophages combined together, aimed for the targeting and killing a broader range of bacterial strains or to enhance the effectiveness of the phages. In this particular set of experimental trials, a series of different procedures were carried out: cocktail virus enrichment, cocktail virus coinfection by Double Layer Assay and Coinfected virus plaques collection. Initially a cocktail of phages (JSF-2 & JSF-25) was prepared by enriching 100 µl of each phage together in a glass vial. Then this cocktail phage solution was filtered and used in plaque assay to allow co-infection of the host. After an incubation period of 16 hours at 37 degrees celsius, visible clear zones of bacteriophages were observed. These plaques were isolated and purified to obtain the cocktail phages solely.

Figure 7 shows the results of visible viral plaques that were obtained from the plaque assay technique that was done using the enriched cocktail phage solution. From the numerous plaques seen on the LA plates, 10 plaques from each trial were randomly selected using sterile micropipette tips and collected in microtubes containing 750 microliters of SM buffer. These virus solutions were then vortexed in a vortex machine and then filtered using 0.22-micron syringe filters for further storage at 4°C to maintain the integrity of the isolated phages, which will later be tested via the method of spot tests.

4.3 Phage Titer determination via the Method of Double Layer Assay

Double Layer test or the plaque test, is a frequently used method for isolating and identifying pure bacteriophage populations. It is also used for determining the native phage titer. Plaques on the plates are first counted to roughly determine the titer of the phage, and the titer is then calculated using the PFU equation for better analysis and validity. The formula for calculating phage titer (PFU/mL) requires dividing the number of plaques by the dilution factor multiplied by the volume of phage added to the plate.

Figure 8 shows the titer of the two phages JSF-2 and JSF-25 on the control bacterial strain WT 1877. The phage titer for JSF-2 was found to be 17300 PFU/ml and that of JSF-25 was found to be 16100 PFU/ml. The phage titer when compared to the cocktail phage titer did not yield a significant difference as it was found to be 14300 PFU/ml. So, in conclusion it can be said that the coinfection of bacteriophages, particularly JSF-2 and JSF-25 did not alter the phage titer obtained after the plaque assay technique.

4.4 Discussion of Phage Coinfection Evaluation Spot Test

Spot test is a fundamental tool in bacteriophage research and applications, since it helps researchers understand and utilize these viruses for various purposes such as phage characterization and phage therapy. Therefore, evaluation of the spot test trials, conducted using the cocktail phage after successful completion of coinfection is the most crucial step of this paper. This is because, the series of trials in this procedure determines whether or not there have been any possible alterations in the virulence factors of the coinfected phages. In another language, it contributes to a deeper understanding of the specific phage-bacteria dynamics and any possible alterations in the host range of the phages.

For the evaluation of phage coinfection, a series of spot tests have been performed. The design of this analysis was such that, on each bacterial strain lawned on large sized LA plates, 12 drops of separate phages have been placed upon correct labeling. The first ten drops were from that of the collected plaque phages after coinfection, and the remaining two drops were of native phage strains JSF-2 and JSF-25. Plaque collection was done in a total of three trials of each set consisting of ten plaques. So in total, thirty plaques were collected and a combination of four

hundred and twenty(420) spots were analyzed given that all 30 plaques were tested for all 14 strains of bacteria: WT 032(01), WT 042(01), WT 050 (01), WT 331(01), WT 389 (01), WT 006 (01), WT 428 (01), WT 429 (01), WT 439 (01), WT 443 (J), WT 351 (N), WT 371 (N), WT 388 (01) and WT 1877. The last strain of WT 1877 was yet again used as the control of the experiment since it was known to have plaques for all drops of phages placed upon its lawn.

The exemplary results of this set of experiments have been demonstrated in figures 9 and 10. A detailed compilation of all sets of combinations have been represented in tables 4, 5, 6 and 7. In each trial, 14 trials of spot test were conducted where each plate had 12 spots of ten microliters of viral suspensions. The number of whole trials was three. So in total 420 spot test analysis was performed. This was aimed to ensure the validity of the findings and account for variability. The repeated experimentation has been designed to improve the reliability of its conclusions and produce a thorough understanding of the dynamics at play in phage-bacteria interactions during coinfection.

In order to detect any changes in host ranges or evolutionary developments, the spot test conducted by the phage cocktail was compared to its native profiling states (presented in table 4) as well as the controls that were present on each plate where a drop of JSF-2 and JSF-25 were placed. However, the results yielded no new changes or alterations. It showed consistency to its native profiling states. Therefore, it can be concluded that, there were no observable changes in the infectivity spectrum or evidence of shifts in host preference of the coinfected phages among the 14 test bacterial strains. The consistency across repeated trials resembles the reliability and reproducibility of the results. The phages JSF-2 and JSF-25 maintain a stable host range across the tested bacterial strains even after they have been enriched together in a cocktail phage solution and allowed to co-infect a host bacteria. This is a significant finding, offering valuable insights into the consistent and therefore predictable behavior of these phages in co-infection experiments, particularly of the subject microorganisms strains of this study.

4.5 Genetic Variability:

Through repeated coinfection trials, it is possible for promoting genetic diversity within phage populations to occur on a dynamic level. With the passing of time recurrence of such trials results in increased expression of mutation rates and replication errors typical to bacteriophages hence driving up genetic variability. Genetic variability may bring about emergence of mutant phage variants possessing improved genetics which could result in heightened lytic efficiency or infectivity against the strains of bacteria employed in such experiments. Such modifications usually affect pivotal genes that control various stages in the phage life cycle, including host specificity, adherence and penetration into the cell, essential during successful infection. Over time this kind mutation can considerably enhance how effective phage infection is thereby exerting a selective pressure favoring development of bacteria-viruses populations with better infective characteristics. Consequently, those ongoing cycles of co-infection not only foster diversity among viruses but also lead to concentration of highly potent viral types thereby perfecting strain samples for further research or therapeutic purposes.

4.6 Selective Pressures:

Over time, the evolving phage population in the biological models faced considerable selective pressures related to the experimental conditions including specific bacterial strains utilized and several environmental factors. Under such conditions, lytic active phage variants (having increased ability to replicate and infect the given bacterial hosts) would have thrived. As a result of their increased efficiency in counteracting the bacterial defenses, those variants would have been more successful than less effective ones leading to their increase and eventual predominance within that population. This means that due to all these interactions among phages, bacterial strains as well as environmental factors, enriched phage variants with optimized infectivity were likely encountered later during the trials than earlier ones.

4.7 Dynamics of Evolution:

The ability of phages to quickly adapt by undergoing short-generation times accompanied by high mutation rates leads to significant genetic deviations across much shorter periods. The changes observed in their population throughout the tests probably reflect these evolutionary processes; where some particular phage variants that were more suitable under specific

experimental conditions were selected for. Through time, these well-adapted varieties would gradually have surpassed others as such advantages as heightened infectivity, improved host recognition or defense mechanisms contributed to this success. As these phages multiplied in numbers, natural selection continued molding them leading to greater prevalence of such forms. This explains how environmental and biological factors can influence evolution within controlled experiments involving phage populations.

4.8 Coevolution of the Phage-Host:

Coevolutionary dynamics are more pronounced during co-infection trials because of prolonged exposure to specific bacterium stains, which resulted in both viruses and bacteria influencing one another's evolution. In this case, newly emergent lytic variants may have arisen through interactions between bacterial defenses and their infecting phages. Consequently, such coevolutionary selection might have led to the emergence of a phage population possessing increased potency in lysing its associated strain. We could have been more certain if these new variants were sequenced.

Chapter 5: Conclusion

Bacteriophage or phage are obligate intracellular parasites that multiply inside bacteria by using biosynthetic machinery. Depending upon the phage, the nucleic acid can be DNA or RNA and it can exist in various forms. The nucleic acids of phages often contain unusual or modified bases. These modified bases protect phage nucleic acid from nucleases that break down host nucleic acids during phage infection. The size of the nucleic acid varies depending upon the phage. The simplest phages only have enough nucleic acid to code for 3-5 average size gene products while the more complex phages may code for over 100 gene products. Experimental trials of coinfection are essential for understanding the complex interactions between bacteriophages and bacteria. Even though the results from the trials of this study show consistency of the native strains of the microorganisms, it is not confirmed that these tiny forms of life will do so in other forms of manipulated environments. Hence they may be trialed for other conditions to yield probable results. The insights that have been gained from the trials of this study have far-reaching implications, including therapeutic uses, environmental management, and the creation of novel approaches to address bacterial infections. However due to limitations of the study, further evaluation of the coinfected phages could not be performed such as molecular level investigations.

Despite the fact that there are certain limitations of this experiment, it offers valuable insights into how phages behave during coinfection and emphasizes the importance of further investigation into this phenomenon of phage-behavior in co-infection trials.

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