Using synergic relation between Bacteriophage and A bacterial species for checking the effects of antibiotic resistance genes on the lytic property of bacteriophages.

By

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

Department of Mathematics and Natural Sciences

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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at BRAC University.

2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.

3. The thesis does not contain material, which has been accepted, or submitted, for any other degree or diploma at a university or other institution.

4. I have acknowledged all main sources of help.

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Approval

The thesis “Using synergic relation between Bacteriophage and A bacterial species for checking the effects of antibiotic resistance genes on the lytic property of bacteriophages.” submitted by **Fahim Sarwar** (18276001) of Summer, 2021 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Master of Science in Biotechnology on 26th September 2019. **Examining Committee:**

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Ethics Statement

The idea of this thesis was unique and mine therefore there was no conflict of interests. Moreover, no human and animal model was involved in the experiments

Abstract

Bacterial transformation is a process of horizontal gene transfer (hgt) where a few bacteria pick up foreign genetic material from the environment. In terms of newer therapies being applied, antimicrobial resistance is getting worse. One may expect to see a return to the pre-antibiotic age of clinical treatment if discoveries slow down. Bacteriophage or phage virus is the only entity that can infect and kill harmful bacteria. However, after a century of predominant phage therapy, the public has remained unguided and ineffective in applying this technology to treating bacterial diseases. In this study, WE want to see whether the acquisition of antibiotic resistance genes has any effect on the sensitivity of the bacteria to particular bacteriophages. For this, IN some strains specific antibiotic-resistant genes were introduced, followed by plaque assay of bacteriophage.

Keywords: Horizontal gene transfer, anti-microbial resistance, transformation, bacteriophage lysis.

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Contents

1.0 Introduction

In molecular biology and recombinant DNA technology, Bacterial transformation is one of the crucial and obligatory steps. It is bacterial transformation, a process of horizontal gene transfer where a few bacteria pick up foreign genetic material from the environment (Das, Raythata and Chatterjee, 2017). The process of transferring genes with the help of the transformation process does not require an operative donor cell; rather than the process requires the inhabitance of DNA in the environment that is continual. The whole transformation process requires bacteria termed as competent cells capable of taking up free, extracellular, or exogenous genetic material.

Vibrio cholerae is a curved gram-negative bacilli, an organism widely inhabitant in aquatic environments, mostly known as a natural and free-living organism. This species of bacteria is an aetiological agent of cholera (*Encyclopedia of Food Microbiology - 2nd Edition*, no date). They are responsible for carrying specific genes such as cholera toxin (CT) and the Vibrio pathogenicity island (VPI). These genes are responsible for cholera which is characterized by severe diarrhea.

Bacteriophage, a type of virus that can infect bacteria. The word bacteriophage indicates a meaning which is bacteria eater. It is because this virus can destroy their host cells. If we have to define, we can say that viruses, those that have the capability of infecting bacteria and well-known to exist in the most abundant organisms in the biosphere and they are a ubiquitous feature of prokaryotic existence, are termed as Bacteriophages, encapsulated by a protein coat and have genetic materials in the form of either DNA or RNA (Clokie *et al.*, 2011).

During the study of this thesis, the above-stated paragraphs are linked with each other as transforming specific antibiotic sensitive bacteria to resistant bacteria was the most primary task of this thesis. Next was to compare the transformed bacteria with the non-transformed bacteria, whether the bacteriophages lyse them or not.

In terms of newer therapies being applied, antimicrobial resistance is getting worse. One may expect to see a return to the pre-antibiotic age of clinical treatment if discoveries slow down a bacteriophage may be an effective treatment for human use. However, after a century of predominantly ad hoc phage therapy, the public has remained unguided and ineffective in applying this technology to treating bacterial diseases. While some recent case studies and clinical trials indicate hope for resolving these issues, current research findings call for caution. If antibiotic resistance becomes more widely distributed and physicians attempt to find new drugs for bacterial infections, phage therapy would once again have met the age-old pledge (D *et al.*, 2019).

Bacteriophages are other experimental approaches to combat cholera. Treatment of bacteria with antiviral medication has been used in Eastern Europe and Russia. The advent of antibiotic-resistant bacteria has been used to deal with infections triggered by ***Pseudomonas*** and ***Salmonella***(AA *et al.*, 2017) as well. In contrast to antibiotic treatment, phage therapy has several benefits. The theoretical basis of our approach is on bacteriophage whether effecting antibiotic resistant bacteria or not.

1.1 Objective

There were few objectives for this research work to conduct. They are-

- To observe the effect of antibiotic resistance genes on the infectivity of the bactioriphages
- To do that we transform some bacteria with antibiotic resistant genes
- Compare the infectivity of specific bacteriophages on transformed and native bacteria

1.3 Background Information

1.3.1 Bacteriophage

Bacterial viruses, or bacteriophages, are commonly referred to as "phages." They were found twice in the early twentieth century. Frederick William Twort, a London-based British pathologist, identified the glassy transformation of "Micrococcus" colonies by a transmissible agent in 1915. He advanced multiple hypotheses, one of which was that the agent was infectious. Félix Hubert d'Hérelle, a French Canadian employed at the Pasteur Institute in Paris, discovered and identified the lysis of ***Shigella*** cultures in broth in 1917. Twort abandoned his discovery but spent decades attempting to replicate vertebrate viruses on inert media. D'Hérelle, on the other hand, recognized his agent's viral origin early on and dedicated the remainder of his research career to it. He introduced the word "bacteriophage," invented many methods that are still in use today, postulated viral intracellular replication, and pioneered phage therapy for infectious diseases (HW and HM, 1997). After some debate, the viral origin of bacteriophages was definitively established in 1940 with the advent of the electron microscope (‘Isolierung und Charakterisierung eines phagenähnlichen Bacteriocins und eines virulenten Phagen und deren therapeutische Einsatzmöglichkeiten gegen Yersinia enterocolitica-Infektionen’, no date).

1.3.2 Classification of Bacteriophage

According to d'Hérelle (On an invisible microbe antagonistic to dysentery bacilli. Note by M. F. d’Herelle, presented by M. Roux. Comptes Rendus Academie des Sciences 1917; 165:373–5, 2011), there was only one genus of bacteriophage with several races: Bacteriophagum intestinale. Holmes (‘Bergey’s manual of determinative bacteriology. By Robert S. Breed, E. G. D. Murray, and A. Parker Hitchens. Sixth edition. The Williams & Wilkins Company, Baltimore, Maryland, 1948. xvi + 1529 pp. 15 × 23.5 cm. Price, $15’, 1948) listed phages within the order Virales as a suborder, a single-family, and a single genus. His premature scheme listed viruses due to disease signs and was quickly overlooked. Lwoff, Horne, and Tournier (A, R and P, 1962) defined a virus classification scheme based on their morphology and nucleic acid type. They designated tailed phages as Urovirales, filamentous phages as Inoviridae, and φX-type phages as Microviridae. Another significant breakthrough was identifying six fundamental groups of phages: tailed phages, filamentous phages, and cubic phages with ssDNA or ssDNA(Bradley, 1967). This straightforward classification scheme continues to serve as the foundation for modern phage classification.

The ICTV, or International Committee for Virus Taxonomy, grouped phages into six genera in its initial paper, roughly referring to Bradley's specific forms. They comprised the T4, X174, MS2, and fd phage families, as well as the recently identified form PM2 (Fenner, no date; JD *et al.*, 2014; Maniloff and

Ackermann, 2014). Over time, additional phage classes were introduced. The most recent change is the creation of the order Caudovirales for tailed phages and the designation of 15 genera of tailed phages(*Virus taxonomy: classification and nomenclature of viruses. Seventh report of the International Committee on Taxonomy of Viruses.*, no date; Maniloff and Ackermann, 2014). At the time of publication, the electron microscope has been used to study approximately 5300 bacterial viruses. By contrast to other viruses (*Virus taxonomy: classification and nomenclature of viruses. Seventh report of the International Committee on Taxonomy of Viruses.*, no date), bacteriophages form the largest viral community found in nature.

(Table 1) Classification and basic properties of bacteriophages ((*Virus taxonomy: classification and nomenclature of viruses. Seventh report of the International Committee on Taxonomy of Viruses.*, no date))

Shape	Nucleic acid	Order and families	Genera	Examples	Members	Characteristics
Tailed	DNA, ds, L	*Caudovirales*	15		4950	
		Myoviridae	6	T4	1243	Tail contractile
		Siphoviridae	6	λ	3011	Tail long, noncontractile
		Podoviridae	3	T7	696	Tail short
Polyhedral	DNA, ss, C	*Microviridae*	4	φX174	40	
	ds, C, T	*Corticoviridae*	1	PM2	3?	Complex capsid, lipids
	ds, L	*Tectiviridae*	1	PRD1	18	Internal lipoprotein vesicle
	RNA, ss, L	*Leviviridae*	2	MS2	39	
	ds, L, S	*Cystoviridae*	1	φ6	1	Envelope, lipids
Filamentous	DNA, ss, C	*Inoviridae*	2	fd	57	Filaments or rods
	ds, L	*Lipothrixviridae*	1	TTV1	6?	Envelope, lipids
	ds, L	*Rudiviridae*	1	SIRV1	2	Resembles TMV
Pleomorphic	DNA, ds, C, T	*Plasmaviridae*	1	L2	6	Envelope, lipids, no capsid

ds, C, T	*Fuselloviridae*	1	SSV1	8?	Spindle-shaped, no capsid

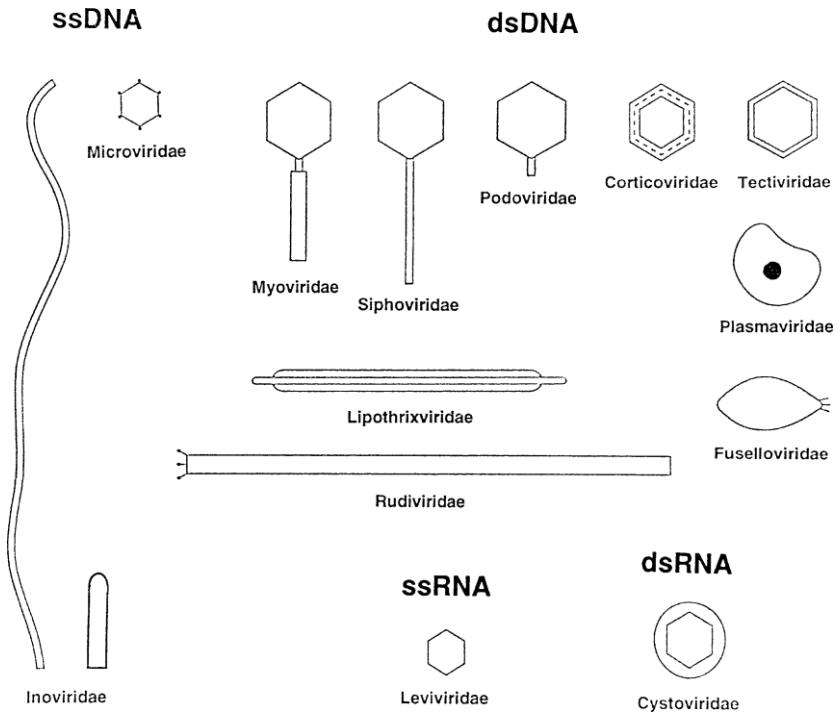


Fig. 1. Schematic representation of major phage groups; retrieved from (HW, 2003)

1.3.3 Phage classification today

The International Classification of Viruses (ICTV) currently classifies viruses into three orders, 61 families and 241 genera(*Virus taxonomy: classification and nomenclature of viruses. Seventh report of the International Committee on Taxonomy of Viruses.*, no date). Bacteriophages are classified into one order, thirteen families, and thirty genera (Table 1). Bacteriophage viruses are polyhedral, filamentous, or pleomorphic (Figure 1); while most phages contain dsDNA, few minor phage classes contain ssDNA ssRNA, or dsRNA. A few have lipid-containing envelopes or incorporate lipids into their particle walls. At the time of the most recent phage count, tailed phages accounted for at least 4950 viruses (Ackermann, 2001). They are divided into the order Caudovirales and three prominent, closely related families. Just about 190 viruses are polyhedral, filamentous, and pleomorphic (PFP) phages. They are divided into ten tiny families based on their most fundamental characteristics and include many distinct phylogenetic classes or lines of descent. Cubic symmetry capsids are icosahedra or connected bodies. Particles are either enveloped or uncased. Lipids are associated with a reduced buoyant density and a strong susceptibility to chloroform and ether. As is the case elsewhere in virology, families are characterized primarily by the nucleic acid sequence and particle morphology. There are no universally accepted standards for the classification of genera and animals. The ICTV classifies organisms based on all available properties and has followed the "polythetic species principle," which states that a species is

characterized by a collection of properties, some of which may be absent in a given member (MH, 1990). Orders, families, and genera all have Latin or Greek origins and end in -virales, -viridae, or -virus, respectively. Latinized labels are used for most genera of "cubic," filamentous, and pleomorphic phages. Until now, tailed phage genera have only had colloquial names (e.g., "T4-like viruses").

1.3.4 Evolutionary implications of host range

Phages have been identified in more than 140 bacterial genera. They are found in archaea and eubacteria, cyanobacteria, exospore and endospore producing organisms, spirochetes, and mycoplasmas. Moreover, chlamydia, in bacteria that are aerobes, anaerobes, budding, gliding, ramified, sheathed or stalked (Table 2).

Table 2- Frequency of phages in traditional host groups

Bergey section[b]	Group	*Myoviridae*	*Siphoviridae*	*Podoviridae*	PFP[c]	Total
	Gram-negative eubacteria					
1	Spirochetes	10	1			11
2	Spirilla and vibrioids	26	14		9	49
4	Rods and cocci, aerobic	303	323	230	22	878
5	Rods, facultatively anerobic	440	340	300	93	1173
6	Rods, anaerobic	4	21	5		30
7	Sulfate and sulfur reducers	1	1			2
8	Anaerobic cocci		2	2		4
9	Rickettsias and chlamydias		1	1	2	4
11	Endosymbionts			2		2
18	Anoxygenic phototrophs	3	6	3		12
19	Cyanobacteria	22	6	16		44
20	Chemolithotrophs	1	1			2
21	Budding and/or appendaged bacteria	8	90	14	8	120
22	Sheathed bacteria			1		1
23	Nonfruiting gliding bacteria	30	2		2	34
24	Myxobacteria	11		5		16
	Gram-positive eubacteria					
10	Mycoplasmas	1	2	14	21?	38
12	Cocci	44	1144	29		1217
13	Endospore producers	257	317	51	10	635
14	Nonsporing regular rods	71	213	2		286

15	Nonsporing pleomorphic rods		183	13	1	197
16	Mycobacteria	1	77			78
17, 26	Nocardioforms	1	95	1		97
28	Actinoplanes	1	4			5
29	Streptomycetes	2	121	8		131
30	Maduromycetes		3			3
31	Thermomonosporae		27			27
32	Thermoactinomycetes		3	1		4
33	Other actinomycetes		6			6
	Archaea (section 25)	7	7		18	32
Total		1243	3011	696	186	5139

Podovirus particles have also been discovered inside paramecia's bacterial endosymbionts. The presence of a normal movers in the archaeon Halobacterium halobium is particularly noteworthy. The phage, designated ΦH, resembles coliphage P2, and its prophage DNA acts similarly to that of coliphage P1(W *et al.*, 1988). Tailed phages found in Chlorella and Penicillium samples, on the other hand, are more likely laboratory toxins. Since the invention of negative staining in 1959, over 5100 phages have been examined in the electron microscope.

Table 3- Frequency of phages in bacterial phylogenetic divisions

Division or subdivision	Important host groups or genera	Tailed phages	PFP
Archaea			
Euryarchaeota	*Halobacterium, Methanobacterium*	14	4
Crenarchaeota	*Sulfolobus, Thermoproteus*		14
Eubacteria			
Bacteroides and relatives	*Cytophaga, Flavobacterium*	59	2
Chlamydiales			2
Cyanobacteria	*Anabaena, Synechococcus*	44	
Deinococcus-Thermus		14	4
Firmicutes: high G + C branch	Actinomycetes, mycobacteria, nocardioforms, streptomycetes	506	1
low G + C branch	Bacilli, lactobacilli, clostridia, Gram- positive cocci, *Listeria*, mycoplasmas	1757	31
Fusobacteria			

Proteobacteria	*Aeromonas*, enterobacteria, pseudomonads, rhizobia, vibrios	2022	128
Spirochetes		11	
Total		4427	186

Based on the organism list of the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/taxonomy/) (Database resources of the National Center for Biotechnology Information, 2015) and on phage counts from reference (Ackermann, 2001, 2007). Total phage numbers are lower than in Table 1 because rRNA data are unavailable for a number of phage hosts.

This figure does not include mutants, particulate "bacteriocins" and other defective phages, phage-like particles found in rumen or seawater without identified hosts, shadowed phages, and unpublished phages from congressional records. A 2001 study identified 4650 tailed phages (96 percent) and just 186 polyhedral, filamentous, or pleomorphic phages (3.6 percent) (Ackermann, 2001, 2007). Polyhedral or filamentous phages, such as tectiviruses, leviviruses, cystoviruses, and filamentous inoviruses, often adsorb to the sides or tips of plasmid-specified pili. This has obvious implications for host ranges. Bacteriophages are included in virtually every part of Bergey's Manual (Scheer, 1990). (Table 2). However, a comparison of phages to bacteria's phylogenetic classes (Database resources of the National Center for Biotechnology Information, 2015) (Table 3) shows that most large bacterial phyla are still devoid of phages. This indicates that several additional phages will be discovered in the future.

1.3.5 Bacteriophage genomics

Bacteriophages became the first complete genomes to be sequenced, starting with the 5386 bp single-stranded DNA (ssDNA) phage fX174 in 1977 (F *et al.*, 1977). The lambda 48 502 bp genome was the first complete sequencing of a double-stranded DNA phage (Sanger *et al.*, 1982), and the 39 936 bp phage T7 genome was published shortly afterward (Dunn, Studier and Gottesman, 1983). Nearly a decade back, the first complete sequence of a dsDNA-tailed-phage genome from a virus infecting a non-***Escherichia coli*** host was published (GF and GJ, 1993), and the amount of sequenced bacteriophage genomes has exploded since then as DNA sequencing technologies have progressed. Epifluorescence methods were applied to the enumeration of phage particles in environmental samples in the late 1980s (Bergh *et al.*, 1989; Hennes and Suttle, 1995; E and CA, 2005) resulting in significant highly bacteriophages' functions in biology and the setting. Viral densities in ocean samples range from 106 and 107 particles per milliliter, and first, the scale of the oceans and related terrestrial viral population(KE, MJ and JC, 2003), the total phage population is calculated to be about 10^{31} (Wommack and Colwell, 2000; Suttle, 2005). This is supported by separate projections of 10^{30} bacterial cells in the biosphere (Whitman, Coleman and Wiebe, 1998), and the obsto advanceal–bacterial ratios in the atmosphere usually are 5–10:1 (Wommack and Colwell, 2000; Suttle, 2007). This viral population is enormous, implying that prokaryotic viruses constitute the bulk of all biological types (RW, 2002).

Additionally, landings indicate that the population is extremely dynamic, with an average 1023 infections occurring every second globally (Suttle, 2007). Two key targets have triggered developments in bacteriophage genomics. To begin, the abundance and turnover of the phage population raises concerns about the relationships between phage genomes and their hosts, as well as the evolutionary processes that

formed this population. Second, genomics has advanced the use of phages in the advancement of genetic, biotechnological, and clinical tools, with numerous utilities and approaches identified.

1.4The Lytic and Lysogenic Cycles of Bacteriophages

1.4.1Lytic Cycle

Bacterial cells are ruptured (lysed) and killed by lytic phages after the virion's immediate replication. Once the host cell is dead, the phage progeny may begin infecting new hosts. T4, which infects E. coli present in the human intestinal tract, is an indicator of a lytic bacteriophage. Phage therapy is more appropriate for lytic phages. Certain lytic phages exhibit a mechanism called lysis inhibition, in which completed phage progeny do not spontaneously lyse out of the cell in the presence of elevated extracellular phage concentrations.

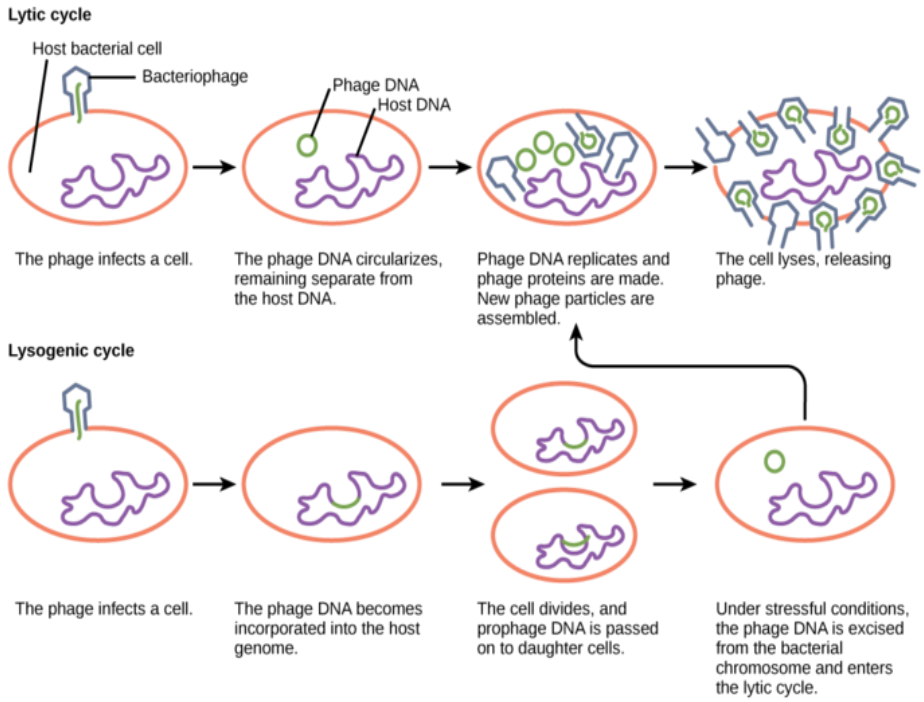


Figure-2: A temperate bacteriophage has both lytic and lysogenic cycles. In the lytic cycle, the phage replicates and lyses the host cell. In the lysogenic cycle, phage DNA is incorporated into the host genome, where it is passed on to subsequent generations. Environmental stressors such as starvation or exposure to toxic chemicals may cause the prophage to excise and enter the lytic cycle. (retrieved from (*21.2B: The Lytic and Lysogenic Cycles of Bacteriophages - Biology LibreTexts*, no date)

1.4.2 Lysogenic Cycle

By comparison, the lysogenic cycle does not immediately cause the host cell to lyse. Temperate phages are those that may undergo lysogeny. Their viral genome will insert into host DNA and reproduce harmlessly alongside it or even create a plasmid. The virus stays latent before the host environment deteriorates, possibly due to nutrient depletion; at that point, the endogenous phages (referred to as

prophages) become involved. They start the menstrual cycle at this stage, culminating in the lysis of the host cell. Because the lysogenic period enables the host cell to begin surviving and reproducing, the virus is replicated through both of the cell's progeny. The phage lambda of E. coli is an indicator of a bacteriophage that is considered to obey both the lysogenic and lytic cycles.

1.4.3 Latency Period

Viruses that invade plant or animal cells may become infected and cease developing virions for extended periods. Animal herpes viruses, such as herpes simplex viruses, form the herpes virus that causes oral and genital herpes in humans. These viruses may remain dormant in nervous tissue for extended periods without developing new virions, only to reactivate and induce lesions in the skin where the virus replicates. Although lysogeny and latency have certain parallels, the lysogenic expression period is typically reserved for bacteriophages.

1.4.5Bacteriophage Treatment

Before the development and widespread usage of antibiotics, it was proposed that bacteriophages could be used to avoid or cure bacterial infections. While early clinical trials of bacteriophages were not conducted aggressively in the United States and Western Europe, phages were also used in the former Soviet Union and Eastern Europe. The findings of these experiments were extensively presented in publications published in languages other than English (primarily Russian, Georgian, and Polish), and therefore were not immediately accessible to the western scientific community (Sulakvelidze, Alavidze and Morris, 2001).

1.4.6 Early studies of phage therapy

D'Herelle used phages to cure dysentery shortly after his observation, which was perhaps the first effort to utilize bacteriophages therapeutically. In 1919 (*Félix d`Herelle and the Origins of Molecular Biology | Yale University Press*, no date), the experiments were completed at Paris's Hôpital des Enfants-Malades under the clinical guidance of Professor Victor-Henri Hutinel, the Hospital's Chief of Pediatrics. d'Herelle, Hutinel, and other hospital interns consumed the phage preparation to ensure its protection before prescribing it the next day to a 12-year-old boy with acute dysentery. After a single dose of d'Herelle's ant dysentery phage, the patient's symptoms resolved, and the boy healed completely within a few days. Shortly afterward, the effectiveness of the phage formulation was "proven" after three subsequent patients with bacterial dysentery who were infected with one dose of the preparation began to improve within 24 hours of medication. However, since the findings of these trials were not immediately announced, the first recorded use of phages to combat infectious diseases in humans occurred in 1921, when Richard Bruynoghe and Joseph Maisin (Chanishvili, 2012)used bacteriophages to treat staphylococcal skin disease. The authors indicated that after injecting the bacteriophages into and around surgically opened wounds, the infections resolved within 24 to 48 hours. Several related positive trials followed (Rice, 1930; 'Bacteriophage Therapy in Bacillary Dysentery of the Flexner Type', 1933; J *et al.*, 2010), and inspired by these early findings, d'Herelle and others pursued therapeutic phage research [(e.g., d'Herelle used different phage preparations to cure thousands of people infected with cholera and bubonic plague in India (Summers, 1999)]. Additionally, many firms have begun commercial development of phages that are effective against various bacterial pathogens.

1.4.7 COMMERCIAL PRODUCTION OF PHAGES

D'Herelle's commercial laboratory in Paris developed at least five phage preparations against different bacterial infections. Bacté-coli-phage, Bacté-rhino-phage, Bacté-intesti-phage, Bacté-pyo-phage, and Bacté-staphy-phage were the preparations, and they were sold by what would eventually become the big French corporation L'Oréal (Summers, 1999). The United States has developed therapeutic phages. In the 1940s, the Eli Lilly Company (Indianapolis, Ind.) manufactured seven phage drugs for human use, including preparations for ***staphylococci, streptococci, Escherichia coli***, and other bacterial pathogens. These preparations included phage-lysed, bacteriologically sterile broth cultures of the desired bacteria (e.g., Colo-lysate, Ento-lysate, Neiso-lysate, and Staphylo-lysate), as well as the same preparations in a water-soluble jelly foundation (e.g., Colo-jel, Ento-jel, and Staphylo-jel). They were used for various illnesses, including abscesses, suppurating burns, vaginitis, acute and recurrent upper respiratory tract infections, and mastoid infections. However, the effectiveness of phage preparations was debated (20, 26), and industrial development of medicinal phages stopped in most Western countries with the introduction of antibiotics. Nonetheless, phages were used therapeutically in Eastern Europe and the former Soviet Union, either in conjunction with or in antibiotics. Numerous institutions in these countries were actively engaged in therapeutic phage research and development, with operations focused on the Eliava Institute of Bacteriophage, Microbiology, and Virology (EIBMV) of the Georgian Academy of Sciences in Tbilisi, Georgia, and the Polish Academy of Sciences' Hirszfeld Institute of Immunology and Experimental Therapy (HIIET) in Wroclaw, Poland.

1.4.8 EIBMV.

The Eliava Institute (http://www.geocities.com /hotsprings/spa/5386) was formed in 1923 by Giorgi Eliava and Felix d'Herelle, two well - known Georgian bacteriologists. D'Herelle spent many months in Georgia working with Eliava and other Georgian colleagues, and he decided to settle permanently in Tbilisi (a cottage designed for his use on the Institute's grounds still stands). Eliava, on the other hand, was detained in 1937 by Stalin's NKVD (forerunner of the KGB), declared a "People's Enemy," and hanged. D'Herelle never returned to Georgia, frustrated and disillusioned. Nonetheless, the Institute survived and eventually grew to be one of the world's most extensive facilities dedicated to developing therapeutic phage preparations. At its peak years, the Institute hired approximately 1,200 researchers and support staff and developed phage preparations (often multiple tons a day) against a dozen bacterial pathogens, including staphylococci, Pseudomonas, Proteus, and a variety of enteric pathogens. The majority of Soviet studies discussed in this essay used phages created and manufactured at the EIBMV (Sulakvelidze, Alavidze and Morris, 2001).

1.4.9HIIET.

Since 1957, when therapeutic phages were first used to cure **Shigella** infections, the Hirszfeld Institute (http://surfer.iitd.pan.wroc.pl/index1.htm) has been extensively engaged in phage therapy science (B. Weber-Dabrowska, personal communication). The Institute's bacteriophage laboratory was instrumental in designing and manufacturing phages for the treatment of septicemia, furunculosis, pulmonary and

urinary tract infections, and the prophylaxis or treatment of postoperative and posttraumatic infections. In several instances, phages were used to combat multidrug-resistant bacteria that were resistant to traditional antibiotic therapy.

1.4.10 PRECLINICAL STUDIES IN ANIMALS

One of the most well-known modern sequences of experiments on the usage of phages in veterinary medicine was conducted by William Smith and his colleagues (HW and MB, 1982; Smith and Huggins, 1983; HW, MB and KM, 1987b, 1987a) at the Institute for Animal Disease Research in Houghton, Cambridge shire, Great Britain. The authors mentioned successfully using phages to treat experimental ***E. coli*** infections in mice in one of their early papers (HW and MB, 1982). The investigators discovered that a single dose of a particular ***E. coli*** phage significantly decreased the number of target bacteria in the alimentary tracts of calves, lambs, and piglets contaminated with a diarrhea-causing ***E. coli*** strain in subsequent experiments (Smith and Huggins, 1983; HW, MB and KM, 1987b, 1987a). Additionally, the procedure halted the fluid loss associated with the outbreak, and all animals infected with phages survived the bacterial infection. Other reviewers (PA and JS, 1997; Carlton, 1999) analyzed these experiments and examined them using quantitative methods and predictive analysis (Levin and Bull, 2015). Additionally, the popularity of these trials rekindled curiosity in phage therapy in the West and stimulated further investigations into the impact of phages on antibiotic-resistant bacteria capable of causing human infections. For e.g., Soothill et al. (JS, 1992; Soothill, 1994)demonstrated the efficacy of phages in preventing and treating experimental disease in mice and guinea pigs infected with Pseudomonas aeruginosa and Acinetobacter, and they hypothesized that phages could also be effective in preventing infections of skin grafts used to treat burn victims. It is unknown, though, if either of these "preclinical" experiments served as a foundation for subsequent human clinical trials. Indeed, while several clinical trials were almost certainly followed by some observational research on experimental animals, there are only a few publications that document this methodology. Recent studies (GG, NN and VM, 1991; GG *et al.*, 1992) examining the effectiveness of bacteriophages in treating infections induced by ***Klebsiella ozaenae, Klebsiella rhinoscleromatis scleromatis***, ***and Klebsiella pneumoniae*** are one example. The phage preparation was reported (GG, NN and VM, 1991) to be effective in treating experimental infections in mice and (ii) nontoxic in mice and guinea pigs; i.e., no gross or histological changes were observed following intravenous (i.v.), intranasal, and intraperitoneal administration, even though mice were given a dose approximately 3,500-fold (estimated by body weight) greater than the human dose. Additionally, the authors described the ideal phage concentration and administration path, as well as other relevant information that they felt were essential for subsequent human volunteer trials. They then (GG *et al.*, 1992) evaluated the phages' protection and effectiveness in treating 109 patients with ***Klebsiella*** infections using the findings of their preclinical trials. The phage preparation was confirmed to be both successful (significant clinical changes consistent with bacteriological clearance) and nontoxic in treating ***Klebsiella*** infections.

1.4.11 TREATMENT OF BACTERIAL INFECTIONS IN HUMANS

Several hundred papers on phage therapy in humans have been reported in the international literature, with the bulk of recent publications originating from researchers in Eastern Europe with the former Soviet Union and just a few reports released in other nations. Recently, numerous phage therapy studies have been written in the English language literature. Additionally, Yale University Press recently released a systematic overview of bacteriophage discovery and phage therapy, featured on a web page (http://www.evergreen.edu/user/t4/phagetherapy/phagethea.html). It will be challenging to list all of these publications in the minireview; consequently, concentration were given upon analysis on papers written in languages other than English that are not readily available to the foreign science community.

2.0 BACTERIOPHAGES AS THERAPEUTIC AGENTS: MODE OF ACTION AND SAFETY PROFILE

2.1 Mode of Action

Despite the abundance of publications on phage therapy, there are very few studies that detail the pharmacokinetics of therapeutic phage preparations. According to the few available publications (GG, NN and VM, 1991; GG *et al.*, 1992), phages reach the bloodstream of laboratory animals (after a single oral dose) within 2–4 hours and are detected in the internal organs (liver, spleen, kidney, etc.) within approximately 10 hours. Additionally, evidence on phage persistence suggests that phages can survive in the human body for comparatively long periods of time, up to many days (EG *et al.*, 1968). Additional testing, including large-scale toxicological trials, is needed before lytic phages can be used therapeutically in the West. Regarding their bactericidal function, it was formerly thought that therapeutic phages killed their target bacteria by replicating inside and lysing the host cell (i.e., via a lytic cycle). However, subsequent research showed that not all phages reproduce equally and that the replication times of lytic and lysogenic phages are significantly different (Fig. 3). Additionally, the recent delineation of the complete sequence of the T4 phage (GenBank accession no. AF158101) and several years of elegant studies of the T4 phage replication system have shown that lytic phage lysis of host bacteria is a dynamic process requiring a cascade of events involving many structural and regulatory genes (Fig. 3). Given that the T4 phage is a normal lytic phage, it is likely that certain therapeutic phages behave similarly; however, it is also probable that certain therapeutic phages possess some special but unexplained genes or mechanisms that contribute to their ability to effectively lyse their target bacteria. For example, a group of authors from the EIBMV (Adamia *et al.*, 1990) described and cloned an anti-Salmonella phage gene that is thought to be responsible for the host strain's restriction-modification defenses. Further elucidation of these and related pathways are expected to produce knowledge useful for genetically engineering therapeutic phage preparations with the highest efficacy.

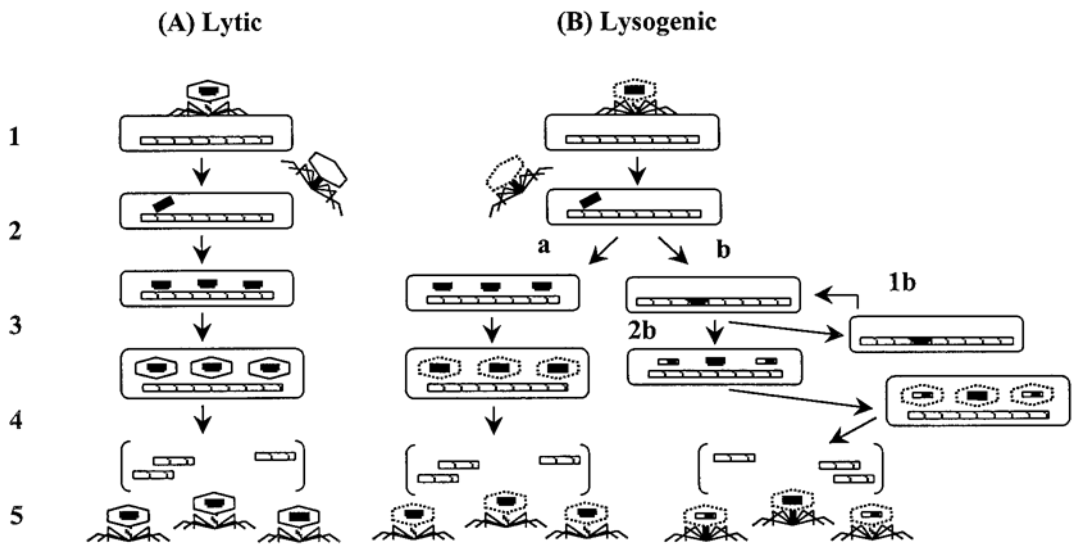


FIG. 3. Replication cycles of lytic and lysogenic phages. (A) Lytic phages: step 1, attachment; step 2, injection of phage DNA into the bacterial host; step 3, shutoff of synthesis of host components, replication of phage DNA, and production of new capsids; step 4, assembly of phages; step 5, release of mature phages (lysis). (B) Lysogenic phages: steps 1 and 2 are similar to those of lytic phages (i.e., attachment and injection, respectively); starting with step 3, lysogenic phages can, among other possibilities, initiate a reproductive cycle similar to that of lytic phages (a) or integrate their DNA into the host bacterium's chromosome (lysogenization) (b). Lysogenized cells can replicate normally for many generations (1b) or at some point undergo lysogenic induction (2b) spontaneously or because of inducing agents such as radiation or carcinogens, during which time the integrated phage DNA is excised from the bacterial chromosome and may pick up fragments of bacterial DNA

2.2 Safety

Phages prove to be harmless from a medicinal perspective. Throughout Eastern Europe and the former Soviet Union's long history of using phages as therapeutic agents (and, before the antibiotic era, in the United States), phages have been administered to humans I orally, in tablet or liquid formulations (10^5 to 10^{11} PFU/dose), (ii) rectally, (iii) locally (skin, eye, ear, nasal mucosa, etc.), in tampons, rinses, and cream (Table 5). In the United States, phage phi X174 has been used to control humoral immune function in patients with adenosine deaminase deficiency (36) and to assess the role of cell surface-associated molecules in modulating the human immune response (37) due to its apparent protection (in the latter study, phages were intravenously injected into volunteers). Additionally, phages are abundant in the atmosphere (e.g., unpolluted water contains approximately 2 *10^8 bacteriophages per ml) and are often ingested in foods. However, it would be prudent further to validate the safety of therapeutic phages before widespread use. For example, it will be essential to ensure that they do not undergo widespread transduction and (ii) have

substantial sequence homology with established significant antibiotic resistance genes, phage-encoded toxins, and other bacterial virulence factors.

TABLE (4) Comparison of the therapeutic use of phages and antibiotics

Bacteriophages	Antibiotics	Comments
Very specific (i.e., usually affect only the targeted bacterial species); therefore, dysbiosis and chances of developing secondary infections are avoided (15).	Antibiotics target both pathogenic microorganisms and normal microflora. This affects the microbial balance in the patient, which may lead to serious secondary infections.	High specificity may be considered to be a disadvantage of phages because the disease-causing bacterium must be identified before phage therapy can be successfully initiated. Antibiotics have a higher probability of being effective than phages when the identity of the etiologic agent has not been determined.
Replicate at the site of infection and are thus available where they are most needed (59).	They are metabolized and eliminated from the body and do not necessarily concentrate at the site of infection.	The "exponential growth" of phages at the site of infection may require less frequent phage administration in order to achieve the optimal therapeutic effect.
No serious side effects have been described.	Multiple side effects, including intestinal disorders, allergies, and secondary infections (e.g., yeast infections) have been reported (76).	A few minor side effects reported (17, 58) for therapeutic phages may have been due to the liberation of endotoxins from bacteria lysed in vivo by the phages. Such effects also may be observed when antibiotics are used (42).
Phage-resistant bacteria remain susceptible to other phages having a similar target range.	Resistance to antibiotics is not limited to targeted bacteria.	Because of their more broad-spectrum activity, antibiotics select for many resistant bacterial species, not just for resistant mutants of the targeted bacteria (47).
Selecting new phages (e.g., against phage-resistant bacteria) is a relatively rapid process that can frequently be accomplished in days or weeks.	Developing a new antibiotic (e.g., against antibiotic-resistant bacteria) is a time-consuming process and may take several years (16, 51).	Evolutionary arguments support the idea that active phages can be selected against every antibiotic-resistant or phage-resistant bacterium by the ever-

ongoing process of natural selection.

2.3 Prevention of cholera using phages

To meet this need, creation of a cocktail of phages that can be taken orally daily by household members before, or shortly after, exposure to ***Vibrio cholerae***. We assume that the phages can last long enough in the intestinal tract to function as a barrier against the incoming cholera bacteria. While this has been shown only in animal models of cholera, we expect that the phage mixture would also function in humans. Three benefits exist for using phages in this manner.

To begin, phages provide an immediate defense. Phages, by behaving quickly, will eradicate cholera bacteria from the intestine in a targeted manner. This is important since cholera is a highly contagious disease.

Second, phages are as effective at infecting and killing multidrug-resistant bacteria as they are at infecting and killing drug-sensitive bacteria. This is critical because the cholera bacteria have developed multidrug resistance in several areas of the world due to extensive antibiotic usage.

Thirdly, unlike antibiotics, which destroy bacteria without regard for their host species, phages are highly selective and infect only their specific host species of bacteria. Thus, when phages are used against a pathogen, they do not disrupt the beneficial bacteria found in and on the bodies of our patients, which include the microbiome. Our laboratory uses phages named ICP1, ICP2, and ICP3 that destroy only ***Vibrio cholerae*** and do not damage the beneficial bacteria in the intestinal tract. This is critical because our beneficial bacteria are essential for the body's defense against other infections and our overall diet and wellbeing.(https://theconversation.com/phage-therapy-to-prevent-cholera-infections-and-possibly-those-caused-by-other-deadly-bacteria-117058#:~:text=First%2C%20phages%20provide%20immediate%20protection,well%20as%20drug%2Dsensitive%20ones.)

2.4Phage steering

Bacterial surface factors have a critical role in the development of disease phenotypes. These components have various roles in various contexts, including adhesion, secretion, and up taking nutrition (EP, 2010; OE and K, 2012; ER and J, 2016). In the context of pathogens, surface factors are frequently referred to as virulence factors or antibiotic-resistance mechanisms, as they can facilitate host adhesion and harm, as well as antibiotic efflux. For instance, the opportunistic pathogen ***Pseudomonas aeruginosa*** contains 306 known virulence components, 45 percent of which are projected to be localized to the cell membrane by PSORTb (protein subcellular localization database) (NY *et al.*, 2010).

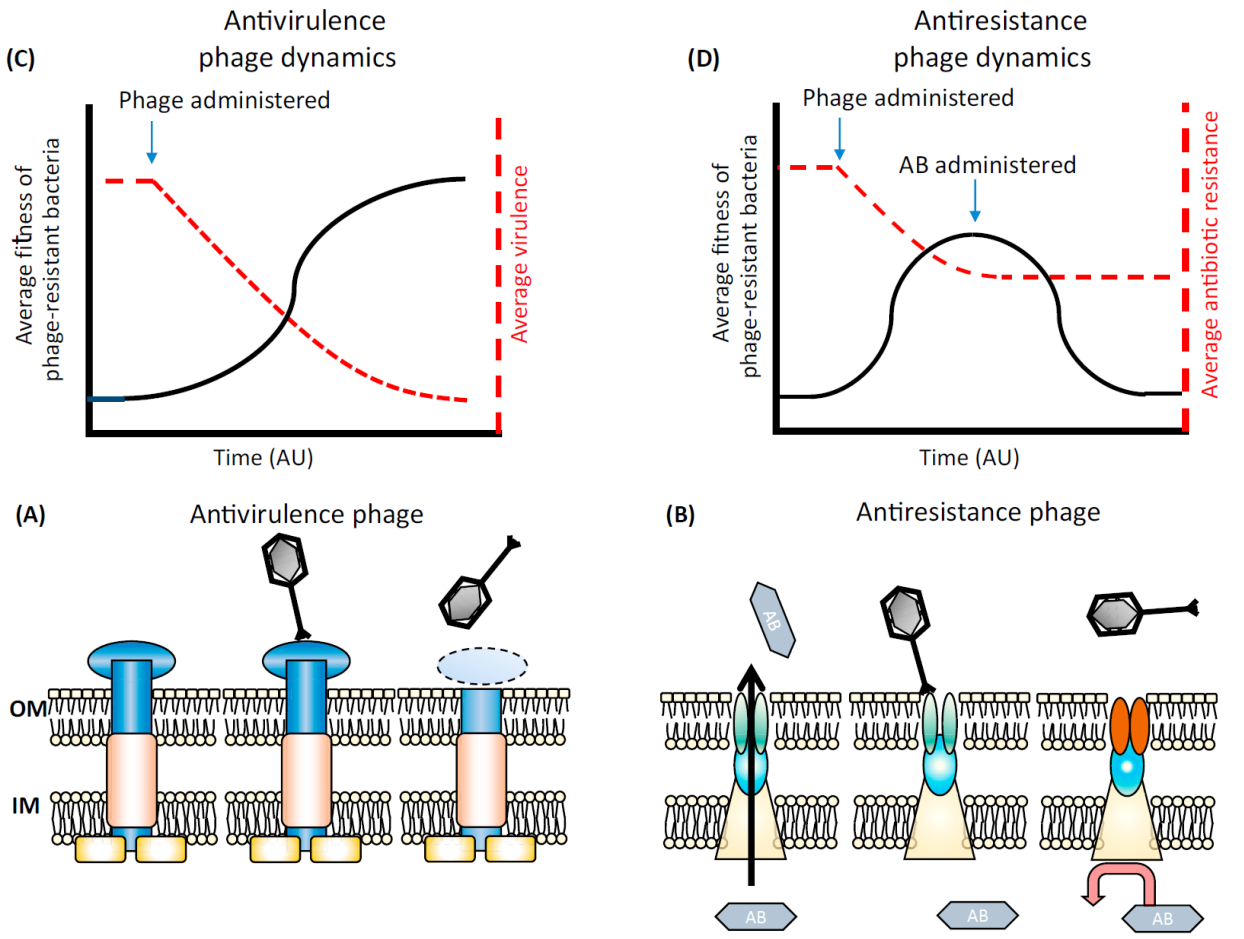


Figure 3. Phage Steering of Bacterial Virulence and Antibiotic Resistance.

Selection for bacterial resistance to phages at specific targets reduces bacterial virulence (A and C) or antibiotic resistance (B and D). As seen in C, the fitness of resistant bacteria (black line) increases due to phage pressure, while virulence (red line) declines. D shows a similar interaction, but this time focusing on antibiotic-resistance mechanisms. The fitness of phage-resistant bacteria (black line) increases as phage replicate, and there is coetaneous decline in average antibiotic resistance (red line). Subsequent administration of antibiotics would reduce the fitness of these now antibiotic sensitive cells. (Retrieved from Gurney et al., Steering Phages to Combat Bacterial Pathogens, Trends in Microbiology (2019), https://doi.org/10.1016/j.tim.2019.10.007)

Numerous constraints limit the current prospects for phage therapy; significant changes or investments in legislation, supply networks, and basic biology are required before routine clinical use. With this in mind, we present the instances in which we feel phage steering can be beneficial and hope that our debate will enlighten the field and generate additional interest and research into

these concepts. Although there are numerous ways to steer bacteria, we focus on the two that are the most well-understood and most likely to be implemented: reduced expression/activity of bacterial surface factors involved in virulence (Figure 3A, C) and reduced expression/activity of bacterial surface factors involved in antimicrobial resistance (Figure 3B, C) (Figure 3B, D) (Gurney, Brown, *et al.*, 2020).

2.5 Phage Steering to Combat Antibiotic resistance pathogen

Antimicrobial resistance is a developing global concern, prompting increased attempts to develop novel medicines. Since its discovery over a century ago, bacteriophage therapy has been used almost exclusively in Eastern Europe. One intriguing strategy is to utilize phages that reduce bacterial pathogen burdens and select phage resistance mechanisms that trade-off with antibiotic resistance – a process referred to as 'phage steering.' Recent work has demonstrated that the phage OMKO1 can interact with efflux pumps, selectively mutating the harmful bacterium ***Pseudomonas aeruginosa*** for both phage resistance and drug sensitivity. The researchers evaluated the approach's resilience against three different antibiotics in vitro (tetracycline, erythromycin, and ciprofloxacin) and one in vivo (erythromycin). The observation is that OMKO1 can reduce antibiotic resistance in ***P. aeruginosa*** (Washington PAO1) in vitro even while antibiotics are present, an effect that persists through more than 70 bacterial generations in continuous culture with phage. In vivo experiments showed that phage improved both the survival period of wax moth larvae (***Galleria mellonella***) and bacterial susceptibility to erythromycin. This improved antibiotic sensitivity was observed in both lines treated with and those not treated with the antibiotic. This discussion expands on the experimental findings and their implications for future research on this promising treatment method utilizing OMKO1 (Gurney, Pradier, et al., 2020).

2.6 Short-term selection for phage resistance leads to reduced MIC *in vitro*-

PAO1 bacteria that had become resistant to phage OMKO1 after 1 day of exposure were found to have significantly lower antibiotic resistance (Figs. 3, S1). For all three antibiotics tested, the group of phage-resistant bacteria isolates had greater than 70% lower MICs than the phage-susceptible ancestral bacteria (ciprofloxacin: t2.04 = 14.9, p = 0.004; erythromycin: t3.2 = 8.5, p = 0.013; tetracycline: t3.92 = 16.63, p = 0.004).

		Erythromycin			Tetracycline			Ciprofloxacin		
Source	d.f.	MS	F	*p*	MS	F	*p*	MS	F	*p*
Phage	1	**679.2**	**42.4**	**<0.0001**	**277.2**	**66.8**	**<0.0001**	**9.03**	**164.2**	**<0.0001**
AB	1	**1315.4**	**82.1**	**<0.0001**	9.4	2.3	0.1586	0.12	2.3	0.1514
Phage x AB	1	4.1	0.3	0.6230	<0.1	<0.1	0.9329	**0.83**	**15.2**	**0.0021**
Selection line (phage, AB)	32	192.3	0.7	0.7182	4.1	0.6	0.8077	0.05	0.9	0.5810
Residual	54	22.1			6.7			0.06		

Table (5). Analyses of variance of antibiotic resistance (minimum inhibitory concentration, MIC) for bacteria isolated from selection lines undergoing treatments with and without exposure to phage OMKO1 or each of three antibiotics (erythromycin, tetracycline, ciprofloxacin). All treatment effects were tested against the selection line effect. Significant effects in bold (Gurney, Pradier, *et al.*, 2020).

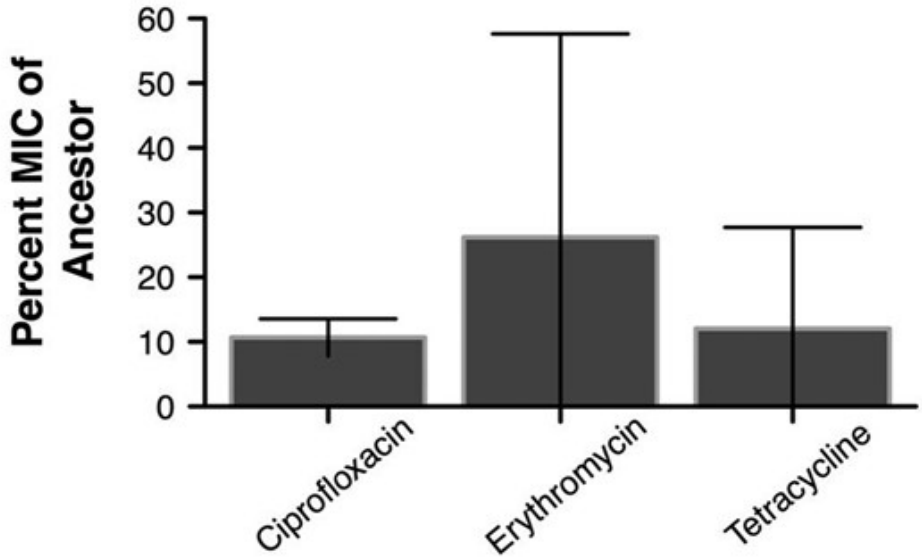


Figure 4. Percent (± 95% CI) of MIC of phage-resistant bacteria tested for three antibiotics relative to ancestral phage-susceptible bacteria. PAO1 bacteria resistant to OMKO1 were obtained after 24 h of in vitro growth in the presence of the phage. MIC levels were determined for each of three antibiotics; all three were significantly reduced from the initial ancestor level (t-test, Welch two sample test) (Gurney, Pradier, *et al.*, 2020).

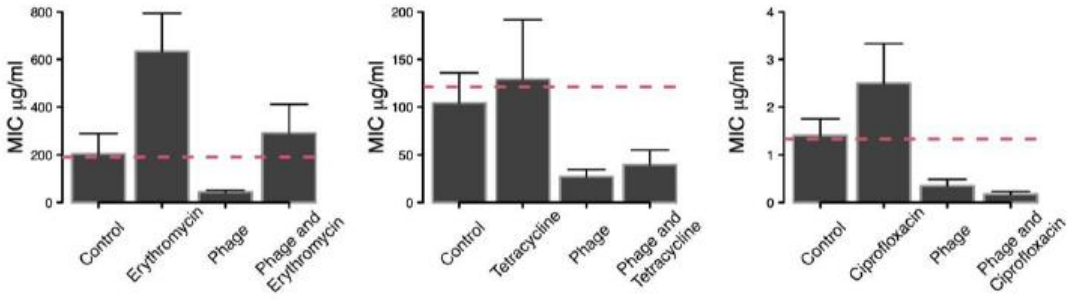


Figure 5. Levels of antibiotic resistance (minimum inhibitory concentration, MIC ± 95% CI) of bacteria evolved in the presence of an antibiotic only, phage only, or both phage and antibiotic combined. a) Erythromycin. b) Tetracycline, c) Ciprofloxacin. Bacteria were isolated after 10 serial transfers (20 days) of treatment and their MICs determined. Bacteria from antibiotic treatments were only tested against the same antibiotic; bacteria from phage-only treatments were tested against all three antibiotics. Phage OMKO1 was able to either reduce the emergence of resistance or sensitize the bacteria to the antibiotic. Dashed red line is the mean MIC for the ancestor.

2.7 Long-term exposure to phage prevents the emergence of antibiotic resistance-

Analyses of bacterial resistance (MIC) after 10 transfers revealed a general negative effect of phage treatment on MIC (significant main effects; Table 5). Bacteria that had been co-cultured with phage showed greater than 60% reductions in resistance to all three antibiotics compared to bacteria that had not been exposed to phage (Fig. 4). In contrast, there was no clear general signal of antibiotic treatments (Table 5; Fig. 4). Erythromycin significantly increased resistance (main effect of antibiotic treatment), tetracycline had no significant effect, while the effect of ciprofloxacin depended on the presence of phage (significant antibiotic x phage interaction; Table 1). Detailed pairwise treatment comparisons of MIC values showed two patterns of phage action. First, adding phage together with the antibiotic reduced bacterial resistance relative to antibiotic-alone treatments (all $p < 0.002$; Fig. 5a-c). In at least one case (ciprofloxacin), phage and antibiotic clearly had non-additive effects (significant phage x antibiotic interaction, Table 5), such that the MIC in the combined treatment was more similar to that of the phage-alone treatment (rather than the average of the two single treatments). Second, all three phage-alone treatments and two combined phage-antibiotic treatments reduced MIC levels below that of bacteria from untreated controls (all $p < 0.05$; Fig. 5). Only for erythromycin did we observe some level of antibiotic resistance evolution in the combined treatment, but this was not significant ($p > 0.05$) (Fig 5a).

Taken together, these patterns indicate that phages not only prevented antibiotic resistance evolution, but even increased susceptibility of the bacteria to ciprofloxacin and tetracycline (Fig. 5b, c).

2.8 Phage maintains selection against antibiotic resistance *in vivo*

Prior to MIC analysis, single colonies were cross-streaked against the ancestral OMKO1 and found to be resistant(Brockhurst *et al.*, 2007). Factorial ANOVA showed a highly significant overall effect of phage treatment on the MIC of evolved bacteria ($F_{1,28} = 132.7$, $P < 0.0001$), whereas the main effect of the antibiotic treatment and the antibiotic × phage interaction were small and neither were significant ($F_{1,28} = 0.44$, $P > 0.5$ and $F_{1,28} = 0.48$, $P > 0.4$, respectively). While the antibiotic alone led to only a slight increase in MIC, the action of the phage strongly reduced the MIC of the surviving bacteria (Fig. 6). Thus, like in the *in vitro* experiment, the presence of phage increased susceptibility of the bacteria to the antibiotic, resulting in lower MICs than in the untreated control lines.

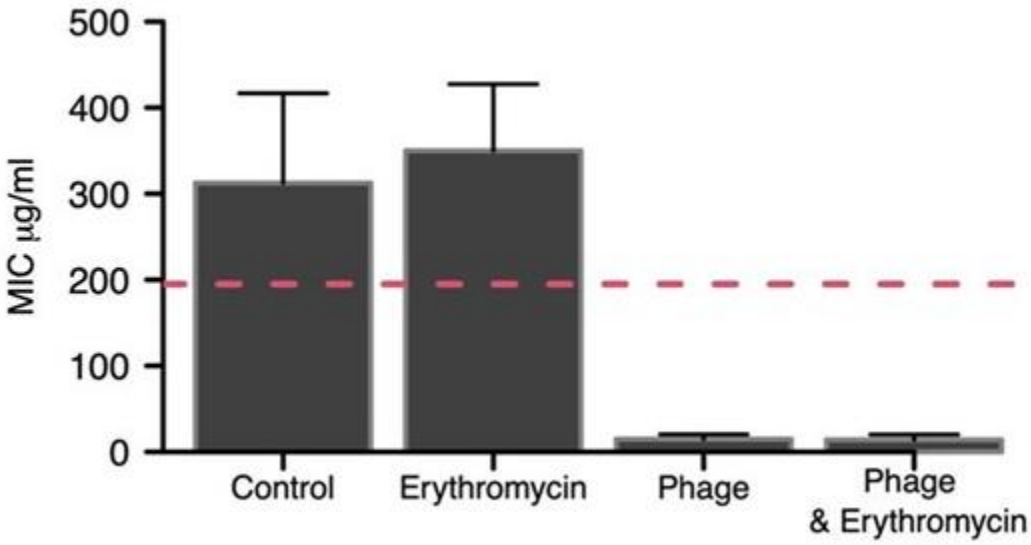


Figure 6- Mean MIC (±95% CI) of recovered PAO1 bacteria from wax moth infection assay. Bacteria were recovered from 8 wax moth larvae per treatment. Both the phage and phage + antibiotic (erythromycin) lines produced clear reductions in the MIC level post-infection. The reason for the control line MIC above the ancestor is unknown, but could be associated with

increased expression of the efflux system responsible for observed antibiotic resistance (Cornforth *et al.*, 2018). Dashed red line is the mean MIC for the ancestor

3.0 Methodology

3.1 *Vibrio cholerae* strain revival

We had 20 strains were that were stored in 0.7% soft agar, then were streaked in TCBS plate. For further workings they were streaked in LB Agar media

3.2Kanamycin Resistance test

In MFC agar strains were lawned with sterile cotton swabs,Before lawning organisms were micxd in in 0.9% saline

3.3 CHROMOSOMAL DNA ISOLATION

Incubation 50 ml Lb with bacteria at 37 °C, Centrifugation at 10000 rpm for 10 minutes, Removing supernatant by aspiration, dissolving the pellet in 2.0 ml solution 1 vortexing of pippeting, Adding 750 µl of .05 M TRIS-Cl and 750 µl of .05 M EDTA. Then adding 10 mg lysozyme to each tube. Mixing well and incubation at 40 °C for 5 minutes, Adding 2.5 ml Proteinase-K buffer and 25 µl Proteinase-K, Incubation at 45°C until the mixture is clear ,Treating with equal volume of phenol and then phenol: $CHCl_3$: Isoamyl alcohol. Centrifuging for 30 mins at 13200 rpm, Collection of upper aqueous layer carefully in fresh tubes, Precipitating with double volume of absolute ethanol; (looking for jelly like DNA in Ethanol). Transferring DNA to Eppendorf tubes, Washing with 70% ethanol and centrifuging for 5 mins, removing supernatant and drying pellet in desiccator, Dissolving pellet in TA Buffer.

3.4 *V.cholera* natural transformation-chitin flakes

Growing 20 ml culture of ***V.cholera*** to mid exponential phase in LB at 37 °C with aeration. For this, addition of 20ul of O/N culture to 20 ml LB broth and incubation at 37 °C 250 rpm for app 2.5 hours was required, Pelleting cells in 2 ml aliquots (0.8%Sea salt solution) at 5000 rpm 5 min in micro centrifuge and removing soup, Washing once with equal volume 0.8% Saline Solution and then re-suspend in 1 Ml 0.8% Saline Solution. Keeping it at room temperature, Adding 900 ul of 0.8 % Saline Solution to 50-70 mg of chitin flakes into 2 ml ep tubes. Incubating for a while (15 min at 30c), Adding 0.1 ml of resuspended bacteria to the 2 ml centrifuge tubes containing 0.8% SS and chitin. Vortexing, then Incubating at 30°C static O\N. Next day, removing 0.5 ml of soup (this depends on how much chitin you added to the tube. If added too much, then just remove 0.3-0.4 ml of sup), then adding chromosomal DNA or plasmids (the more the better) carefully, since we do not want to resuspend the bacteria that is on the chitin flakes. Incubating at 30 °C static for 2h (for plasmid) or longer (for gDNA and PCR products O/N INCUBATION is better), Vortexing the transformation reaction vigorously for 15 seconds, then let chitin flakes settle for 1-2 mins. Transferring soup to another tube and plate. Anyone can also outgrow the cells in LB for 1-2 hour if he or she wants.

3.5 Phage Enrichment

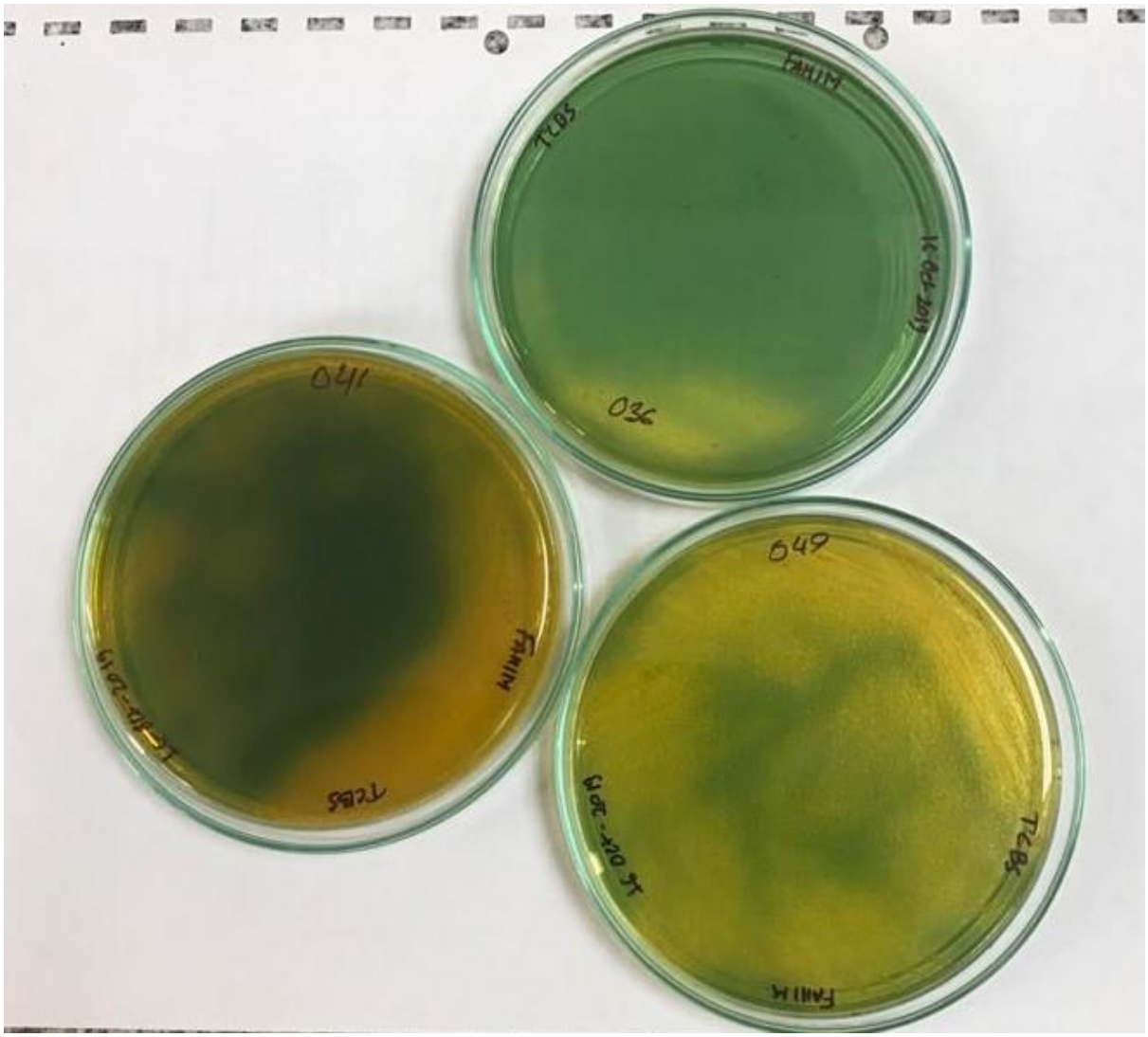
Picking a single discrete plaque from the bacterial lawn LA plate, Using sterile pipette tip and placing it in Phage or SM buffer (100 mM NaCl, 8.1 mM MgSO4, and 0.05 mM Trims-Cl [pH 7.5]). The pipette tip was cut to widen the diameter and the cut plaque was dispersed in the SM buffer by gently pulling off the micropipette. The suspended plaque was vigorously vortexed for 5 minutes to release the phages from the plaque agar. The suspension was then centrifuged at 4000 rpm for 5 minutes at 25^{O}C. This results the agar to settle as pellets and the phages remain suspended within the supernatant. The supernatant next is collected in a fresh micro-centrifuge tube and chloroform in the volume one-third of that of the SM buffer was added. The solution was gently vortexed to allow mixing and then stored at 4^{O}C. During storage, the chloroform evaporates from the solution and leaves the pure phages within the clear suspension. For enrichment of the pure phage, the host bacteria WT 346 was streaked on LA plate and incubated overnight at 37^{0}C. A few colonies from overnight grown WT 346 was inoculated in 3 ml of LB and incubated for 1.5-2 hours in the shaker incubator at 37^{O}C. After incubation, the turbidity of the bacterial solution was checked since slight turbidity ensure logarithmic phase cells. 100µl of the pure phage solution was added to the young culture of WT 346 and incubated for 4-6 hours at 37^{O}C in the shaker incubator. During this time, the phages infect the host bacterial cells and increase their number. After incubation, the solution was centrifuged at 13000 rpm for 5 minutes. This separates the bacterial cells as pellets and the enriched phages suspend in the supernatant. The supernatant was then collected in a fresh borosilicate vial and filter sterilized using 0.22-micron syringe filter. This yielded a clear suspension of phages which is completely free from any bacterial cells. This phage solution was enriched a couple of times using the same procedure to get the desired phage titer. The enriched phage stock was always stored at 4^{O}C.

3.6Phage Lysing Bacteria

Transformed and non-transformed organisms were enriched in LB broth for 3/4 hours, From enriched culture 100 µl of culture taken and it was kept in 5 ml soft agar, 5ml of soft agar (0.6%) were poured on to a LB Agar media, Waiting for some time to let the soft agar get dry Then spotting 5 µl of phage in the petri dish, Incubate at 37^{O}C for 24 hours.

4.0 Results

4.1 Strain REVIVAL some of the pictures showing organism growing on TCBS agar



4.2 Kanamycin Resistance: Among 20 given organisms 15 were revived to work with and some them were intermediate and some of them were susceptive.

Figure – Control Organism (Contains Kanamycin gene)

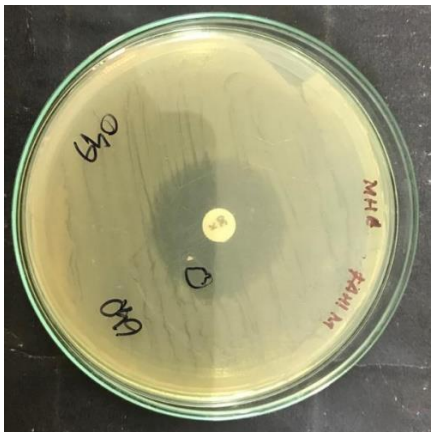


Figure- Organism sensitive to Kanamycin

Number of organisms we had worked with:

Organism Number	Kanamycin Susceptible	Kanamycin Resistant
004	(+)	(-)
005	(+)	(-)
031	(+)	(-)
033	(+)	(-)
035	(+)	(-)
036	(+)	(-)
040	(+)	(-)
041	(+)	(-)
042	(+)	(-)
048	(+)	(-)
049	(+)	(-)
050	(+)	(-)
WT- 333	(+)	(-)
WT- 324	(+)	(-)
WT- 346	(+)	(-)

Table : List of organisms

4.3 Transformation

The most crucial step of this study. If this process wasn't successful further planning's will have no future.

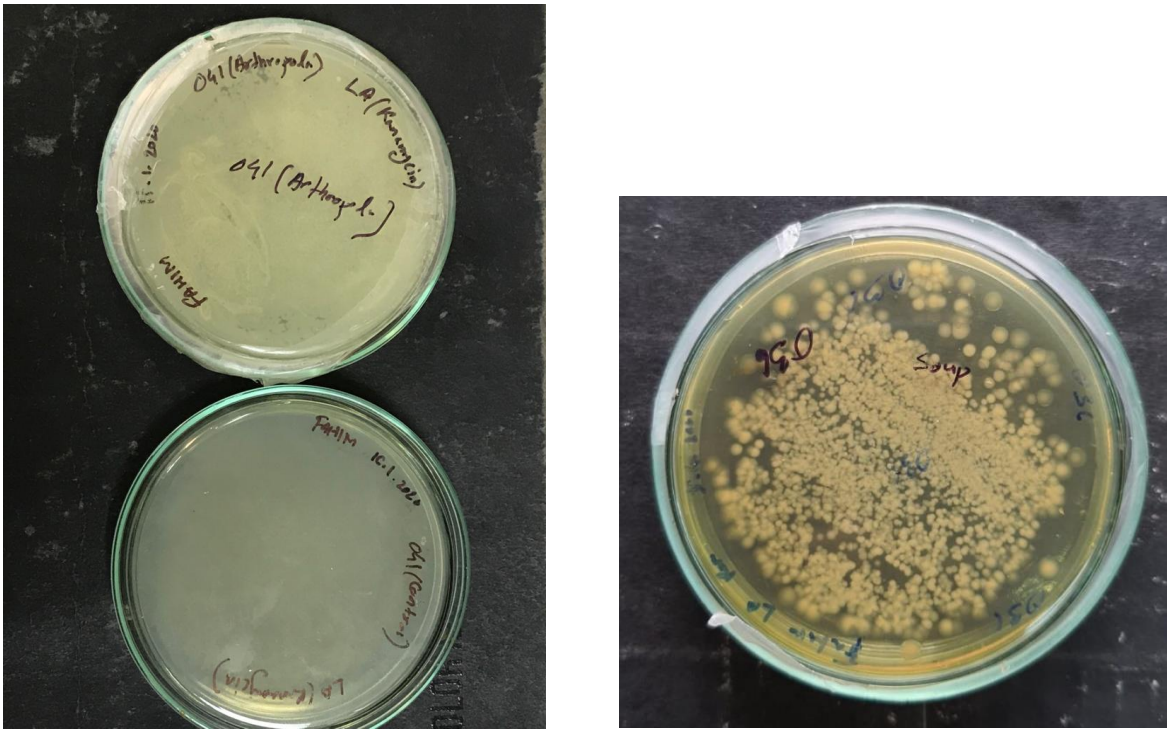


Figure: A sample that had grown on Kanamycin plate and the sample not grown is the pure culture that not been transformed

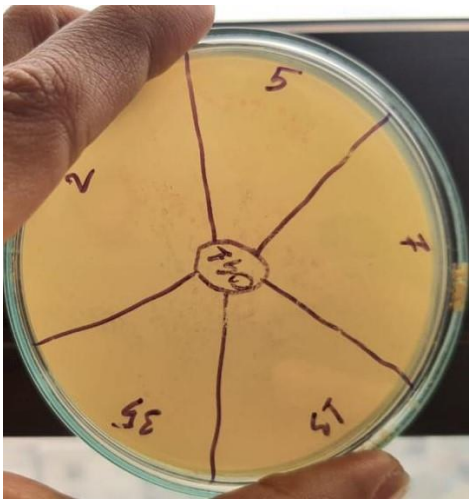
4.4 Phage Lysing Bacteria

There were 5 phages to lyse bacterial samples including both native and transformed bacterial samples

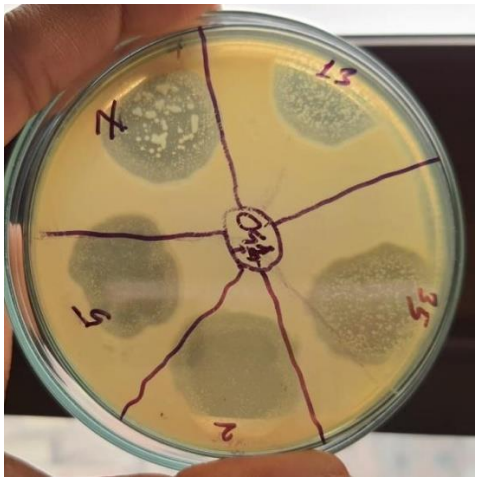
.

List of phages

Name of the phages	Number indicating the phage on petri dish
JSF-2	2
JSF-5	5
JSF-7	7
JSF-13	13
JSF-35	35



041(native)



041(transformed)

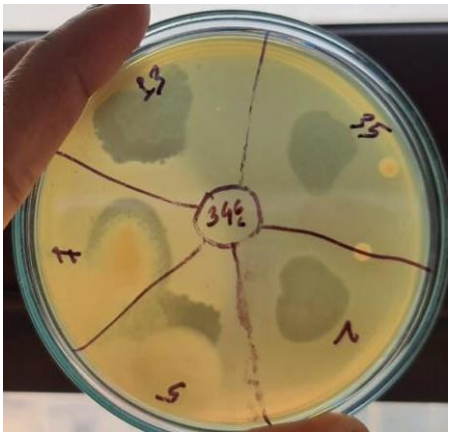
Bacteria	Bacteriophage				
	JSF-2	JSF-5	JSF-7	JSF-13	JSF-35
041(native)	(-)	(-)	(-)	(-)	(-)
041(transformed)	(+)	(+)	(+)	(+)	(+)

049(transformed)

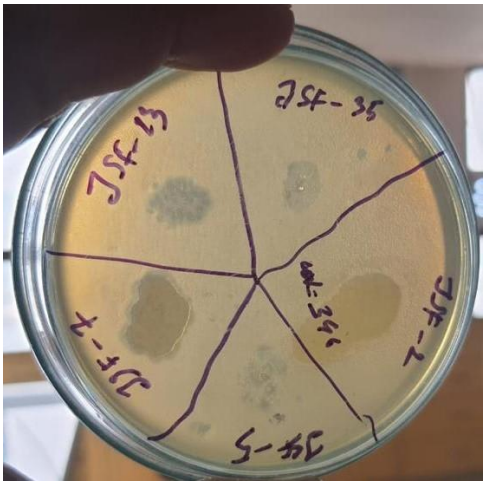
049(native)

Bacteria	Bacteriophage				
	JSF-2	JSF-5	JSF-7	JSF-13	JSF-35
049(transformed)	(-)	(-)	(-)	(-)	(-)
049(native)	(+)	(+)	(+)	(+)	(+)

WT-346(transformed)

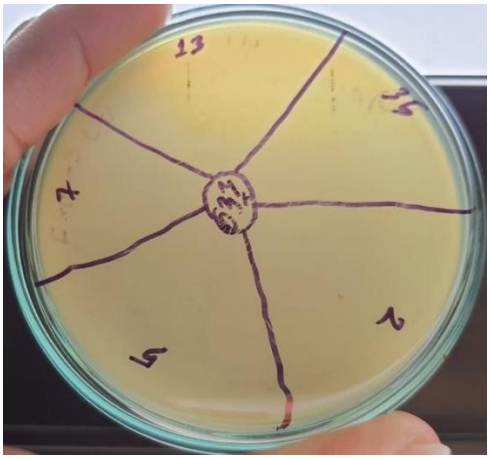


WT-346(native)



WT-346(native)

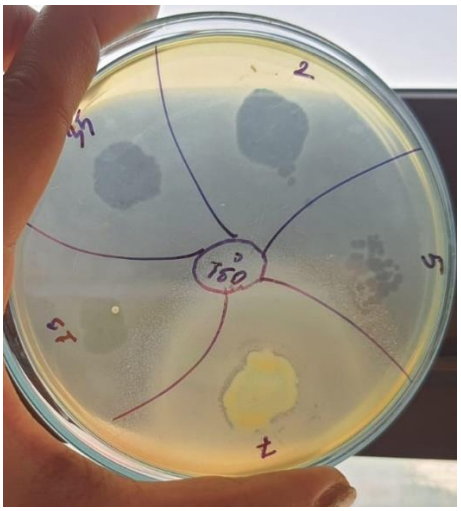
Bacteria	Bacteriophage				
	JSF-2	JSF-5	JSF-7	JSF-13	JSF-35
Wt-346(transformed)	(-)	(-)	(-)	(-)	(-)
Wt-346((native)	(+)	(+)	(+)	(+)	(+)



033(transformed)

033(native)

Bacteria	Bacteriophage				
	JSF-2	JSF-5	JSF-7	JSF-13	JSF-35
033(transformed)	(-)	(-)	(-)	(-)	(-)
033(native)	(-)	(-)	(-)	(-)	(-)



031(native)

031(transformed)

Bacteria	Bacteriophage				
	JSF-2	JSF-5	JSF-7	JSF-13	JSF-35
031(transformed)	(-)	(-)	(+)	(-)	(-)
031(native)	(+)	(+)	(+)	(+)	(+)

5.0 Discussion

During the study, we face many difficulties along with the worldwide pandemic of Corona virus or Covid-19. One of the crucial problem was that we could not think and study further. As a result it is kind of a half dead work. Though, findings are sufficient enough to work further. The primary task was to transform the bacterial species into Kanamycin resistant species. In given organisms some were intermediate and some where susceptible. It took us approximately five months to revive the bacteria followed by DNA extraction from the bacteria containing Kanamycin resistant gene and then transformation and plaque assay.

As, ***V. cholerae*** strains are very contamination prone, we had to take intensive care for keeping the strains pure. Sub-culture on a regular basis were must for us to go further. Extraction of DNA was toilsome, it is because this procedure took about three days to finish, if we follow the given protocol. In addition reagents were prepared fresh and kept in their required temperature. After transformation we had a plan to compare phage lysing Kanamycin resistant gene containing strains and Ampicillin resistant gene containing strains. Due to scarcity of time we only could transform the strains into resistance of Kanamycin.

The result of plaque assay of Kanamycin resistant strains gave raise to some questions. Why the transformed organisms were lysed and pure organism were not lysed? According to us, after addition of Kanamycin there were two explanations are involved. The first one is complete inhibition of infection development, and the second one is efficiency of infection development.

According to our hypothesis, in the second scenario we would get approximately 100 plaques in pure strains and 50 plaques in transformed strains. If it was performed and got the above stated result we could say that there is a decrement in development of infection efficiency.

For the complete inhibition of infection efficiency there would be more than 1000 plaques for pure strain plaques and zero plaques for transformed strains. Our intention was to find that antibiotic resistance genes are lysed by bacteriophage or not lysed by bacteriophage.

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