

BACTERIOPHAGE MEDIATED INTER & INTRASPECIES CHROMOSOMAL GENE TRANSFER

By

**MD. RAKUNUZZAMAN KHAN
ID: 18236020**

**TANZIA KAISAR MAYURI
ID: 18236022**

**MD. ASHRAFUL HAQUE ABIR
ID: 18236019**

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

Biotechnology Program,
Department of Mathematics and Natural Sciences
BRAC University
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Declaration

It is hereby declared that

1. The thesis titled “**BACTERIOPHAGE MEDIATED INTER AND INTRASPECIES CHROMOSOMAL GENE TRANSFER**” is our own original work while completing the Bachelor of Science in Biotechnology 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. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
4. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution;
5. All main sources of help have been acknowledged.

Student’s Full Name and Signature:

**TANZIA KAISAR
MAYURI**
ID: 18236022

Bachelor of Science,
Biotechnology.

Department of Mathematics
and Natural Sciences,
BRAC University, Dhaka,
Bangladesh

**MD.
RAKUNUZZAMAN
KHAN**
ID: 18236020

Bachelor of Science,
Biotechnology

Department of Mathematics
and Natural Sciences,
BRAC University, Dhaka,
Bangladesh

**MD. ASHRAFUL
HAQUE ABIR**
ID: 18236019

Bachelor of Science,
Biotechnology

Department of Mathematics
and Natural Sciences,
BRAC University, Dhaka,
Bangladesh

Approval

The thesis “**Bacteriophage mediated inter and intraspecies chromosomal gene transfer**” submitted by Tanzia Kaisar Mayuri (ID:18236022), Md. Rakunuzzaman Khan (ID:18236020) and Md. Ashraful Haque Abir (ID:18236019) of Spring-22, 2022 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Biotechnology on April 2023.

Examining Committee:

Supervisor:
(Member)

Dr. Iftekhar Bin Naser

Associate Professor
Department of Mathematics and Natural Sciences
BRAC University

Program Director:
(Member)

Dr. Munima Haque

Associate Professor
Department of Mathematics and Natural Sciences
BRAC University

Departmental Head:
(Chair)

Dr. A F M Yusuf Haider

Professor and Chairperson
Department of Mathematics and Natural Sciences
BRAC University

Ethics Statement

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

Title

“Bacteriophage Mediated Inter and Intra-species Chromosomal Gene Transfer”

Abstract

Although many different methods of horizontal gene transfer have already been identified, and the effect of bacteriophages on horizontal chromosomal gene transfer is yet to be well studied. Bacteriophages are linked to controlling the number of bacteria in the environment. During specific seasons the concentration of phages increases in the environment which could influence the availability of free DNA in the environment. Our study focus is to check whether the abundance of free DNA induces by increased bacteriophages has any effect on horizontal gene transfer. According to research done to date, plasmid DNA can be passed to other bacteria via horizontal gene transfer, whereas chromosomal genes can also be transmitted but need some genetic modifications. In our study, we used bacteriophage to lyse the host bacterial cell so that it could release the genetic material inside the experimental environment and introduced the targeted bacteria to the same environment so that it could take up the released genetic material and be modified. The result showed that the antibiotic resistance chromosomal gene is transferred from antibiotic-resistant bacteria to antibiotic-sensitive bacteria. Thus, bacteriophage-induced chromosomal gene transformation could be the novel mechanism of HGT.

Keywords: Bacteriophage, HGT, antibiotic resistance gene, interspecies, intra-species, chromosomal gene transfer.

Dedication

We would like to dedicate this thesis to our beloved families for their love, prayers, support, and sacrifices.

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List of Abbreviations

<i>E. coli</i>	<i>Escherichia coli</i>
STEC	Shiga toxin-producing <i>Escherichia coli</i>
<i>V. cholerae</i>	<i>Vibrio cholerae</i>
Phage	Bacteriophage
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
HGT	Horizontal gene transfer
AMR	Antimicrobial resistance
ARGs	Antibiotic resistance genes
CLSI	Clinical and laboratory Standards Institute
ZOI	Zone of Inhibition
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i> .
F-plasmid	Fertility Plasmid
ETEC	Enterotoxigenic <i>Escherichia coli</i>
EPEC	Enteropathogenic <i>Escherichia coli</i>
EAEC	Enterotoxigenic <i>Escherichia coli</i>
DAEC	Diffusely-adherent <i>Escherichia coli</i>
EIEC	Enteroinvasive <i>Escherichia coli</i>
UV	Ultra Violet
FDA	Food and Drug Administration
WHO	World Health Organization
ICTV	International Committee on Taxonomy of Viruses
US	United States
DLA	Double-Layer Agar
PFU	Plaque-forming unit
ml	Milliliter
μl	Microliter
psi	Pounds per square inch
rpm	Rotations per minute
SDS	Sodium dodecyl sulfate
PCI	Phenol-Chloroform Isoamyl Alcohol
EDTA	Ethylene-diamine-tetra acetic acid
TE	Tris EDTA
TCBS	Thiosulfate-citrate-bile-sucrose
et al.	And others
MEM-LAB	Molecular and Environmental Microbiology Laboratory

CHAPTER 1: INTRODUCTION

1.1 Background of the study:

Bacteriophages, usually referred to as phages, are the biological organisms that are frequently found in the natural environment, which is a ubiquitous feature of prokaryotic existence on the planet (Clokiet al., 2011). They are basically viruses that have the capability to infect bacteria, and around 10^{23} to 10^{25} bacterial cells are believed to be killed by bacteriophages every second (Deresinski, 2009; Keen et al., 2017). Additionally, bacteriophages are able to hijack the bacteria's metabolic mechanisms and replicate inside the host bacteria; then, the phage typically meets one of two lytic (virulent) or lysogenic (temperate) life cycles, through which it can moderate the DNA replication and protein synthesis of the host cell, which subsequently leads to the host cell's death and bursts out phage copies (Feiner et al., 2015). Although the phage can infect plasmid-containing bacteria, it is still unidentified whether these bacteria's plasmids are destroyed by the phage during infection or released as intact plasmid copies after phage lysis. If this is the case, then bacteria's encoded resistance could be acquired and manifested by other bacteria through the transformation method, which is one of the most common methods of horizontal gene transfer (Keen et al., 2017).

Bacteria can acquire antibiotic resistance through chromosomal mutations or by acquiring genetic material from other bacteria or the environment via horizontal gene transfer. The latter mechanism is mostly driven by mobile genetic elements such as plasmids, transposons, or bacteriophages, which play an important role in microbial evolution and ecology by regulating the intra-species and interspecies transfer of genetic information (Keen et al., 2017). While these mobile genetic elements can be transferred via transformation, transduction, or conjugation, it is considered to be the most effective method for exchanging genetic material among bacteria (Courvalin, 1994; von Wintersdorff et al., 2016). Bacteria can acquire free DNA from their environment in case of the transformation method of horizontal gene transfer. Instead of direct gene transfer from bacteria to another cell, which is what is meant by the term of conjugation, whereas the transduction refers to the movement of genes by bacteriophages.

Recent studies based on cutting-edge genomic technologies indicate that bacteriophages play a more significant role in the mobilization and activation of Antibiotic Resistance Genes (ARGs) than previously expected (Balcázar, 2018). As a consequence, phages have emerged as prime suspects in bacterial evolution and modification by promoting the transmission of genetic material. These features make them suitable vehicles for the acquisition, preservation, and transmission of antibiotic resistance genes. Although plasmids containing antibiotic-resistance genes (ARGs) and other genes have been established to be transferred between bacteria by phages for more than 60 years via transduction, the potential role of the bacteriophage during bacterial transformation has received less attention (Zinder & Lederberg, 1952). Though plasmids usually contain antibiotic-resistance genes (ARGs); their fate during phage infection and lysis may have significant implications for evolution, ecology, and biomedicine (Keen et al., 2017).

Although transduction is a horizontal gene transfer mechanism associated with phages, recent research demonstrated that certain phages (lytic phages) can also promote transformation, which involves the uptake of free DNA and recombination (Keen et al., 2017; Balcázar, 2018). Usually, homologous recombination and DNA repair mechanisms restrict the feasibility and effectiveness of transforming DNA from closely related bacteria; however, ARGs may be spread via broad-host-range plasmids without the need for recombination (Thomas & Nielsen, 2005). It should be emphasized that many bacteria contain plasmids, extra-chromosomal genetic components that frequently encode the antibiotic resistance genes, which can be released during phage infection and then acquired from other bacteria through transformation. These research results indicate that phages may play a significant role in the acquisition and spread of ARGs into the environment.

In this research, we studied whether chromosomal DNA can also be able to contain the drug-resistance gene without any genetic modifications. For these, we develop an easily performable phage-mediated transformation experiment procedure to demonstrate that the antibiotic resistance gene (ARG) can be transferred from one bacteria to another. For these experiments, the target bacterium has been cultured with the host bacteria after the host has been lysed by its unique bacteriophage. To evaluate the methodology and the results of the phage-mediated transformation experiment, we used both inter- and intra-species comparisons. Thus, both intra-species and inter-species were used, and the experiment was carried out according to our established study protocol.

We have performed experiments with *Vibrio cholera*, *E. coli*, and STEC for inter-species situations, and other strains of *Vibrio cholera* have been used for intra-species situations. In the instance of inter-species interactions, our host was *Vibrio cholera* with kanamycin resistance gene in the chromosome and the targets were *E. coli* and STEC, respectively, and as a consequence, we obtained an antibiotic-resistance gene. In a similar manner, in the case of intra-species transfer, our host was *Vibrio cholera* and the targets were different strains of *V. cholera*, and as a result, we got an antibiotic-resistance gene. Finally, we isolated the plasmids and transformed them into competent cells to evaluate the predicted results indirectly as well as evaluate the results through Antibigrams and Spot tests.

1.2 Research Objective:

The purpose of this research is to find a phage-mediated or induced mechanism of intra- and interspecies horizontal gene transfer through which chromosomal genes could be transferred.

1.3 Specific Aims:

To study the potential function of bacteriophage to induce HGT.

1.4 Novelty of this research:

It will be the novel mechanism of Horizontal gene transfer because in this study we will try to show the transfer of chromosomal antibiotic resistance gene (ARG) from *Vibrio cholera* to *E. coli* and also to the different other strains of the *Vibrio cholera* by bacteriophage.

1.5 Research Question:

According to Keen et al., (2017) and many other researcher bacteriophage can induce plasmid to be transferred the gene horizontally in between the same and different species in the nature. But is it possible to transfer the chromosomal gene by HGT in between the same and different species in the nature?

CHAPTER 2: LITERATURE REVIEW

2.1. Introduction to *Escherichia coli* and *Vibrio cholerae*

2.1. a. *Escherichia coli*

Escherichia coli, the most prevalent aetiological cause of urinary tract infections and urinary tract sepsis, is ubiquitous in the intestinal microbiota of most warm-blooded animals. It is a gram-negative, facultatively anaerobic, non-spore-forming coliform bacterium. This bacterium is widespread throughout the human gastrointestinal system, but it exists in very small amounts and has no detrimental effect on host health except in immunodeficient people (**Köhler & Dobrindt, 2011**). In 1885, Theodor Eschrich first described the bacteria as "bacterium coli commune" a prevalent intestinal inhabitant of neonates and infants.

2.1. b. *Vibrio cholerae*

Vibrio cholerae, the aetiological agent that causes severe secretory diarrhea, is a naturally occurring component of the aquatic ecosystem. It is a gram-negative, comma-shaped aerobic or facultative anaerobic bacillus. This bacterium is widely distributed in the aquatic environment, but only a tiny proportion of the environmental strains can cause cholera (**Faruque et al., 1998**). In 1884, Robert Koch first identified the bacteria as the cause of cholera, and in 1959, Sambhu Nath De isolated the species' key virulence factor, the cholera toxin, and established that the toxin was responsible for cholera. Cholera is an acute diarrheal disease caused by a bacterial infection in the intestine with *Vibrio cholerae* serogroups O1 or O139.

2.1.1. Global health concerns induced by *E. coli* and *V. cholerae*

Escherichia coli is the leading cause of diarrheagenic disease in the world, as well as the most common causative agent of both simple and severe urinary tract infections. Although the majority of the strains are harmless, six pathogenic strains, namely enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), enteroinvasive *E. coli* (EIEC), and enterohemorrhagic *E. coli* (STEC), are frequently associated with food-borne infections. In 2011, the yearly frequency of 31 pathogenic strains in the United States was found to be around 6.6–12.7 million infected, with 700–2300 fatalities (**Heiman et al., 2015**). The infectious types cause food poisoning by producing toxins, the most lethal of which is STEC's Shiga-like toxin (**Poole, 2015**). Aside from foodborne illnesses, *E. coli* is the most common agent causing neonatal meningitis, which has a high mortality rate globally. The fatality rate in neonatal meningitis varies between 15 to 50%, and survivors are said to have severe neurological damage for the rest of their lives (**Donnenberg, 2017**).

Vibrio cholerae, the most well-known enteric pathogen, has been responsible for numerous outbreaks of cholera throughout history. In the past, outbreaks were prevalent worldwide; however, infection is now mainly concentrated in underdeveloped or developing nations with inadequate hygiene and sanitary measures. According to WHO reports, there are an expected 1.3–4 million cases of cholera

each year, with an estimated 21,000–143,000 fatalities worldwide. (Ali et al., 2015). Though many nations now have endemic illnesses, but during the 19th century, cholera spread throughout the whole world from its natural reservoir in the Ganges delta, resulting in six fatal pandemics across all continents. Currently, approximately 200 *Vibrio cholerae* serogroups have been identified; however, only *V. cholerae* O1 and *V. cholerae* O139 genetic variants have been linked to pandemic cholera. (WHO; cholera, 2020). Because transmission happens via fecal-oral routes, the humanitarian crisis, such as insufficient hygiene and overcrowded shelters, increases the frequency of outbreaks and epidemics.

2.1.2. Bacteriophage

Bacteriophages (also known as phages) are naturally occurring viruses that have the ability to infect bacteria and replicate solely inside the host (bacterial) cells (Clark and March, 2006; Hagens and Loessner, 2007; Hanlon, 2007; Nishikawa et al., 2008; Viazis et al., 2011; Abedon, 2012; Kasman et al., 2022). Bacteriophages are the most abundant and ubiquitous infectious acellular organisms, with an estimated 10^{31} phage particles in the biosphere, where their existence is still elusive (Keen, 2015). They are obligatory intracellular pathogens that can be found everywhere wherever a bacteria can grow (Suttle, 2005). Bacteriophages play a substantial role in nature because they are used as carriers for gene transfer (Gómez-Gómez et al., 2019), and are a ubiquitous feature of prokaryotic existence (Clokier et al., 2011).

Phages have been extracted from every habitat where bacteria exist, and it is claimed that at least one variety of phage, and most likely many more, infects every form of bacteria, and our current understanding is only the tip of the iceberg (Keen et al., 2017). According to Cook et al., there are 14,244 complete phage genomes that have been sequenced and stored in GenBank (as of January 2021), many of which have not been studied experimentally, but there are believed to be many trillions of phage species in natural environments.

Bacteriophages are considered as natural killer of bacteria that can infect and lyse the host organism (Abuladze et al., 2008; Nishikawa et al., 2008). When a phage invades a susceptible bacterial cell, the phage (or at least its nucleic acid) penetrates the cell and initiates phage replication cycle. Phages, like other viruses, are obligate intracellular parasites, and their life cycle is entirely reliant on their host bacterial cell because they lack the cell structure and enzyme system required for replication (Carlton, 1999).

2.1.3. History of bacteriophages

Bacteriophages were identified more than a century ago. In 1896, a British bacteriologist named Ernest Hankin first witnessed the bacteriophages in the water of the Ganga and Jumna rivers in India, which had unexpected heat-sensitive antibacterial properties against bacteria (*Vibrio cholera*), and this water could pass through a very fine porcelain filter and retain this distinguishing feature (Ackermann, 2012; Hankin, 1896). However, Hankin did not pursue this finding further, but two

years later, a Russian bacteriologist named Gamalaya also observed a similar phenomenon while working with *Bacillus subtilis* (Sulakvelidze et al., 2001). In 1901, Emmerich and Löw reported that a sample from an autolytic culture could lyse various cultures and cure an experimentally caused infection (Summers, 2005). Later on, the British bacteriologist Frederick Twort observed a "glassy transformation" of *Micrococcus* colonies grown on solid agar media, and in 1915 he found a small agent that killed bacterial colonies in growing cultures. From there, he speculated that the unknown substance causing the bacterial colonies' watery transformation could be a virus. Then he published the results, but the subsequent work was interrupted by the beginning of World War I and a shortage of funding (Calendar, 2005; Sulakvelidze et al., 2001; Summers, 2005).

Two years after Twort's documentation, Felix d'Herelle observed a similar kind of incidence and discovered the agent killing bacteria independently at the Pasteur Institute in France. He proposed that it was an "ultravirus" that was causing lysis of bacterial cells in liquid media and created clear patches on the bacterial lawn, which he primarily called it *taches*, then *taches vierges*, and later *plaques*. Felix d'Herelle also named the virus responsible for these phenomena as "bacteriophage" which derived from "bacteria" and the Greek word "phagein" which means to "eat" or "devour" (Fruciano & Bourne, 2007). Finally, he concluded bacteriophage as "exogenous agents of immunity" following the observation of an increase in phage titer in the stool samples of recovering patients suffering from dysentery and typhoid (Deresinski, 2009).

In 1923, the Eliava Institute was opened in Tbilisi, Georgia, to study bacteriophages and develop phage therapy. Since then, many scientists have been involved in developing techniques to study phages and using them for various purposes. In 1969, Max Delbrück, Alfred Hershey, and Salvador Luria were awarded the prestigious Nobel Prize in Physiology and Medicine for their discoveries of the replication of viruses and their genetic structure.

2.1.4. Bacteriophage abundance 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). It is estimated that each bacteria is thought to have between 5 and 10 viruses (Weinbauer & Rassoulzadegan, 2004). Phages are abundant in nature and can be found in any setting that promotes bacterial growth (Ksik-Szeloch et al., 2013). 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 faeces (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). Moreover, phages are thought to kill and lyse between 15-40% of the marine bacterial community every day.

The abundance and distribution of phages are highly correlated with their specific host organisms, as phages must require specific host to survive. Although we 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 1.2×10^{29} , 2.6×10^{29} , 3.5×10^{30} , and $0.25\text{--}2.5 \times 10^{30}$ cells, respectively (Whitman et al., 1998). Bacteria and archaea are significant for both humans and other animals because they can provide many niche environments. In several cases, these microorganisms have evolved into essential symbionts (Carey-Smith et al., 2006). Bacteria become much more important when they are associated with humans, especially in a disease or food-producing context where bacteria are the causes of disease or where we must rely on bacteria. Therefore, while addressing these human effects, bacteriophages have significant importance that can infect these bacteria.

As bacteriophages are regarded as the most prevalent biological entities on earth and are widely distributed throughout the environment. Their size, appearance, and genetic structure are incredibly varied (Simmonds & Aiewsakun, 2018). All, however, include a nucleic acid genome wrapped in a coating of phage-encoded capsid proteins that mediate the passage of the genetic material into the following host cell (Hatfull & Hendrix, 2011). Several different phage kinds, including those that seemed to have "heads," "legs," and "tails," may now be seen in detail because of the electron microscopy. Despite their outward mobility, phages rely on Brownian motion to travel to their prey (Barr et al., 2015).

2.1.5. Features of Bacteriophages

2.1.5.1 Specificity: Bacteriophages are highly specific to the host bacteria they infect. Each phage is capable of infecting only a limited number of bacterial strains or species. The first step in the interaction between a host and a phage is the attachment of the phage to specific receptors on the cell surface. These receptors can vary depending on the type of bacteria, such as proteins, lipopolysaccharides, teichoic acids, or other structures on the surface of the cell (Hymen & Abedon, 2010). The receptors could be located in the cell wall, bacterial capsules, slime layers, pili, or flagella. Once the phage is bound to the receptor, it becomes irreversible (Etz et al., 2001).

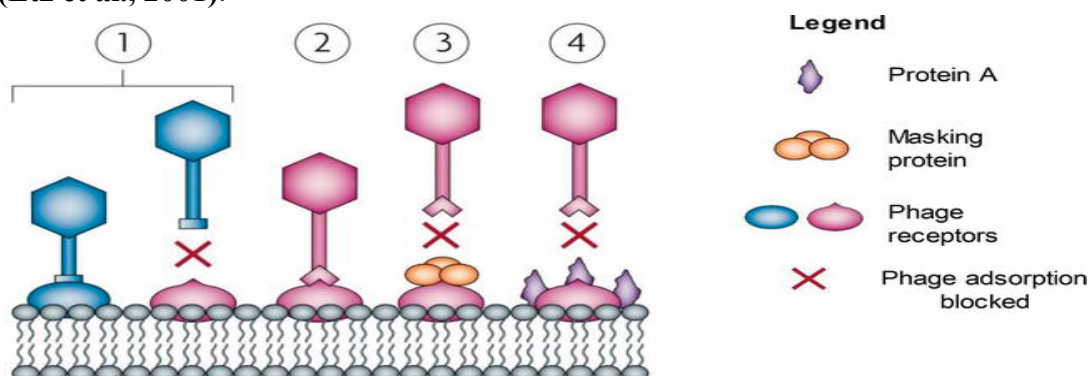


Figure 2.1: The interaction between bacteriophages and the host bacteria is specific (Adapted from Orzechowska & Mohammed, 2019)

2.1.5.2 Structure: Bacteriophages have a diverse range of structures, but they typically consist of a head or capsid containing the genetic material, and a tail used for attaching to and injecting the genetic material into the host bacterium. Other than they have spikes to seat on the host so that the injecting process would be easier.

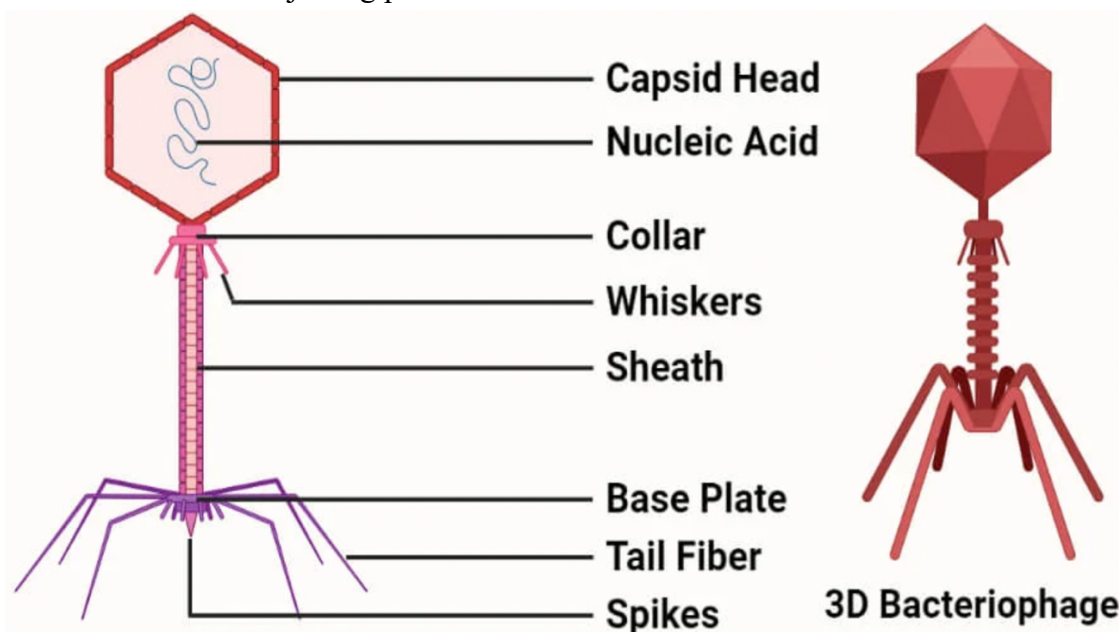


Figure 2.2: A Complete Structure of Bacteriophages (Created with BioRender.com and Adapted from: Sapkota, 2022)

2.1.5.3 Genetic material: Bacteriophages can have either DNA or RNA as their genetic material, which can be either single-stranded or double-stranded depending on the specific phage species. Phage DNA is often circular, although linear forms can also be found, whereas phage RNA is typically single-stranded (**Birge, 2013**). The genetic material of a phage contains all the information necessary for the virus to replicate itself and produce more phage particles, as well as to direct the process of infecting and taking over the host bacterium. Some phages also have additional genetic elements, such as plasmids, that can integrate into the bacterial host chromosome or replicate independently. Understanding the genetic material of bacteriophages is crucial for the development of phage therapy, a potential alternative to antibiotics for treating bacterial infections (**Lin et al., 2017**).

2.1.6. Classification of bacteriophages

Phages are enormously diverse and vary based on their structural, physicochemical, and biological properties. In 1933, Burnet observed heterogeneity among different enterobacterial phages, and in 1943, Ruska observed three morphological types of six bacteriophages, which evoked the necessity of proper classification of phages (**Ackermann, 2012**). Holmes proposed a classification system of phages based on plaque morphology and particle size, host range, and resistance to urea and heat, which was not accepted by the scientific community. Later on, the International Committee on

Taxonomy of Viruses (ICTV) classified phages based on nucleic acid and gross morphology and grouped them into six genera (Ackermann, 2004).

Shape	Nucleic Acid	Virus Group	Particulars	Example
Tailed	DNA	Myoviridae	Tail Contractile	T4
		Siphoviridae	Tail Long, Non-Contractile	λ
		Podoviridae	Tail short	T7
Polyhedral	DNA, 1, C	Microviridae	Conspicuous Capsomers	ϕ X174
	2, C, S	Corticoviridae	Complex Capsids, Lipids	PM2
	2, L	Tectiviridae	Inner Lipid Vesicle, Pseudotail	PRD1
	2, L	SHI, group*	Inner Lipid Vesicle	SH1
	2, C	STV1 group*	Turret-shaped Protrusion	STIV
	RNA, 1, L	Leviviridae	Poliovirus-like	MS2
	2, L, seg		Envelope, Lipids	Φ 6
Filamentous	DNA, 1, C	Inoviridae	a. long filaments	fd
			b. short rods	MVL1
	2, L	Lipothrixviridae	Envelope, Lipids	TTV1
	2, L	Rudoviridae	TMV-like	SIRV-1
Pleomorphic	DNA, 2, C, S	Plasmaviridae	envelope, lipids, no capsid	L2
	2, C, S	Fuselloviridae	same, lemon-shaped	SSV1
	2, L, S	Salterprovirus	same, lemon-shaped	His1
	2, C, S	Guttaviridae	droplet-shaped	SNDV
	2, L	Ampullaviridae*	bottle-shaped	ABV
	2, C	Bicaudaviridae*	two-tailed, growth cycle	ATV
	2, L	Globuloviridae*	paramyxovirus-like	PSV

Table 2.1: Overview of phage families (Ackermann, 2007); C Circular; L linear; S superhelical; seg segmented; 1 single-stranded; 2 double-stranded

The current classification of bacteriophages by the International Committee on Taxonomy of Viruses (ICTV) contains 1 order, 14 families, and 37 genera, while 5 other potential families are waiting for classification (Ackermann, 2009). Among them, over 96% of all phages are tailed double-stranded (ds) DNA phages and belong to the order Caudovirales, which is further classified into three main large families, Siphoviridae, Myoviridae, and Podoviridae, differentiated by their tail length and

contractile ability (**Ackermann, 2004**). With a long flexible tail, 61% of the phage under Caudovirales falls into Siphoviridae; 25% are Myoviridae, having double-layered contractile tails; and with short noncontractile tails, 15% are Podoviridae. Other types of phages, polyhedral, filamentous, and pleomorphic phages, comprise less than 4% of observed phages (**Ackermann, 2007**). The ICTV taxonomic system requires visualization of the phage structure using electron microscopy.

2.1.7. Bacteriophage Replication Process

Viruses (i.e., Phage) do not encode for all of the enzymes necessary for viral replication, so they have to depend on the host cell for their reproduction. Bacteriophage replication has been broadly categorized into two replication methods: lytic and lysogenic. Interchange between these two methods is an important decision for bacteriophages as well as all the biological entities associated with them (**Bohannon & Lenski, 2000; Weinbauer & Rassoulzadegan, 2004**). Bacteriophages most commonly replicate through these two major phage life cycles, though they have multiple possible life cycles, which determine their role in bacterial, or archaeal biology. Both the lytic and lysogenic life cycle includes 2 common steps: i) absorption of phage ii) penetration of genetic material (**Salmond and Fineran, 2015**). So, phages at first interact with the receptors expressed on the surface of the bacterial and get absorbed followed by the injection of its genome into the bacterial cell involving mechanism specific for each phage (**Guttman et al., 2004**).

2.1.8. Life Cycle of Bacteriophage

Viruses enter the host cells in order to proliferate, which causes the host cell to contract a variety of illnesses. The virus's lifecycle includes the processes of entering the host cell, replicating, and exiting. Bacteriophages, like all viruses, follow a similar pattern, by infecting the host cell, bacteriophages can replicate or grow. Bacteriophage requires a specific host cell for replicating and enumeration (**Kasman & Porter, 2019**). There are two types of bacteriophage lifecycles that differ in how DNA replication occurs. In one type, the DNA replicates separately (Lytic Cycle), while in the other, the viral DNA merges with the host DNA. These lifecycles may occur independently or in different types of bacteriophages.

Moreover, bacteriophages are classified into two distinct categories: lytic bacteriophages and temperate bacteriophages. Bacteriophages that reproduce via the lytic life cycle are known as lytic bacteriophages because they lyse the host bacterium as part of their regular life cycle. The term "temperate phage" refers to bacteriophages that can undergo lysogenic cycle. A temperate phage can either integrate its DNA into the host bacteria's DNA and become a non-infectious prophage, or it can reproduce through the lytic life cycle and kill the host bacterium (**Kaiser, 2022**). Now let's discuss how bacteriophages go through their lytic and lysogenic life-cycles.

2.1.8.1 Lytic Cycle: Bacteriophages that replicate through the lytic life cycle are called lytic bacteriophages. Specific strains of bacteriophages can only adsorb to a specific strain of host bacteria, which is known as viral specificity (**Keen, 2015**). This cycle is most often seen in

virulent phages, which cause the infected cell membrane to be destroyed during the release of viral particles.

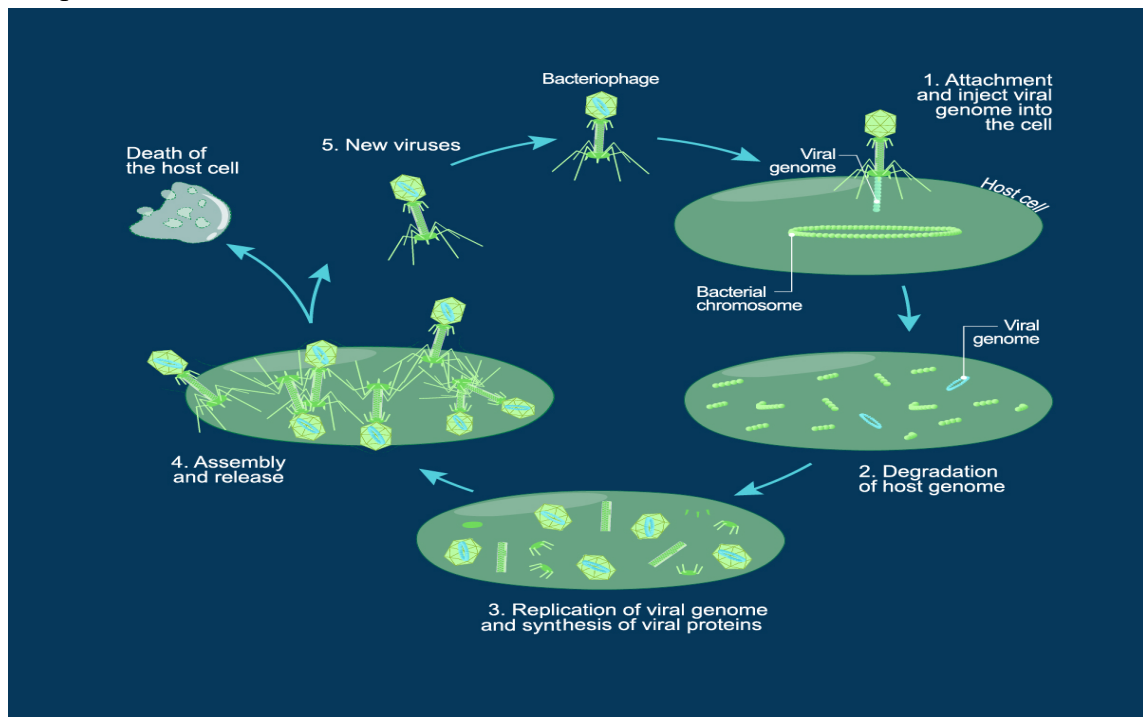


Figure 2.3: Lytic cycle of Bacteriophage (Adopted from Steward, 2018)

In the lytic cycle, the injected phage genome takes control of the bacterial replication machinery and produces the necessary components for a new progeny phage. When all the essential components are manufactured, the phage particles are assembled into infective virions, followed by lysis of the host cell, where the new progeny phages are liberated from the bacterial cell via disruption of the cell wall and cell membrane. Then, the newly released phage progenies are ready for reinfection (**Kaiser, 2022**). Consequently, at the end of the lytic lifecycle results in the destruction of the infected bacterial host cell and cell membrane (**Guttman et al., 2004; Steward, 2018**).

2.1.8.2 Lysogenic Cycle: Similar to the lytic cycle, Lysogenic cycle is another phage proliferation technique, where the bacteriophage adheres to and injects its genetic material into the host bacterium's genome. In this phase, the infected bacterial cells can continue to grow and replicate normally after bacteriophage replication. However, in the lysogenic cycle, the phage does not immediately kill the host cell but instead uses it as a shelter where it exists in a dormant state, i.e., it causes no harm to the host cell but rather quietly uses its resources (**Howard-Varona et al., 2017; Doss et al., 2017**). In lysogenic relationship, lytic genes are inhibited by the phage gene product termed a repressor (**Abedon, n.d.**). Due to lytic inhibition, the phage gene will remain integrated into the host chromosome which is termed as a prophage and will replicate along with the host replication process (**Little, 2005**) or is left free as a plasmid and becomes latent within the bacterium (**Ackermann, 2012**).

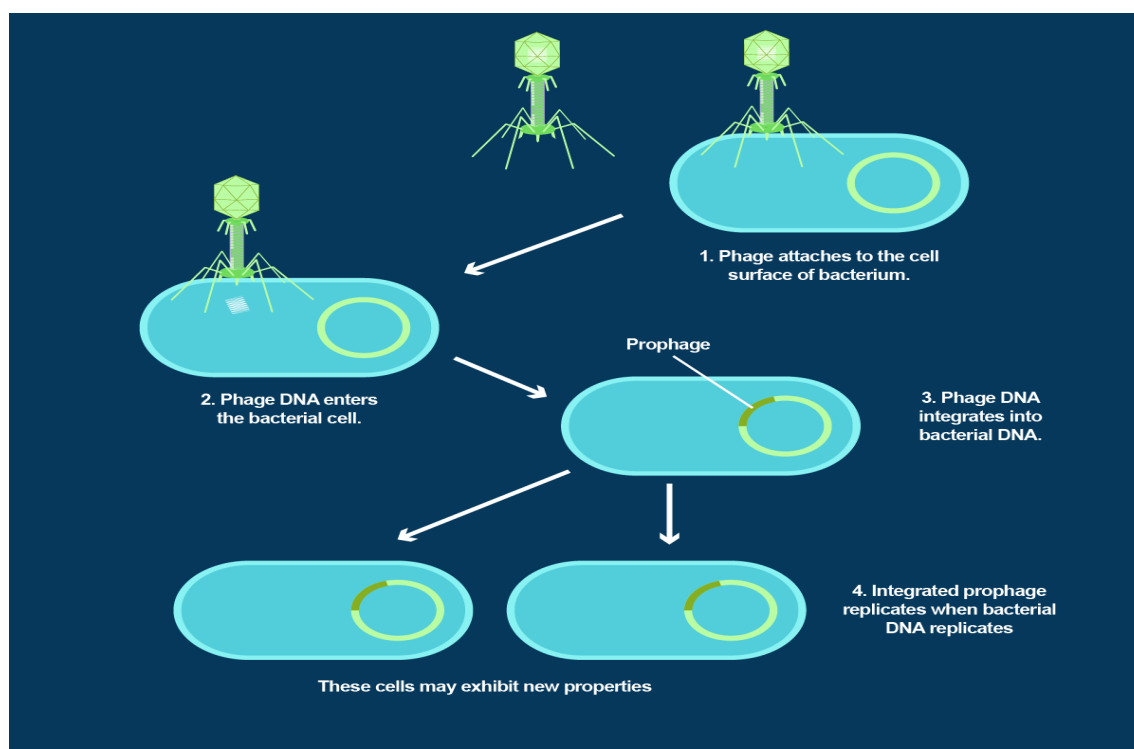


Figure 2.4: Lysogenic cycle of Bacteriophage (Adopted from Steward, 2018)

However, the lysogenic cycle may shift to the lytic cycle due to various stressors agents such as physical stressors (e.g., UV radiation) or chemical stressors (e.g., nutrient deficiency), poly-aromatic hydrocarbons, mitomycin C, hydrogen peroxide, temperature, and pressure leading to transcription of viral DNA and viral protein production (**Williamson et al., 2001**). Then, the viral proteins and genome are then assembled to form complete viral particles that are released from the host cell through cell lysis (**Oppenheim et al., 2005; Kassa, 2021**). For instance, temperate phages such as λ phages are able to proliferate via both lytic and lysogenic cycle (**Little, 2005**). In order for virulent phages to replicate and survive in the environment, the rate of phage-host encounter required to exceed the virus decay and inactivation rate. However, the temperate phages are not dependent on the host cell density, rather requires a small number of lysogenic carrier cell and occasional induction of lytic cycle and release of free phages (**Wommack and Colwell, 2000**). Regeneration of progeny phages requires time ranging from around 30 minutes to 55 minutes depending on their complexity.

2.1.9. Usefulness of Phages

Phages have been studied as model microorganisms to obtain insight into fundamental genetic concepts such as viral gene expression. Bacteriophage research contributed significantly to improving our understanding of viruses and many basic concepts in molecular biology.

2.1.9.1 Bacteriophage Application in Biotechnology and Research: In modern biotechnology, bacteriophages are considered as Natural Killers of bacteria, and because of the selectively targeted antibacterial capacity of bacteriophages to infect and disrupt harmful bacteria, they have been given enough importance to be used as therapeutic agents or can act as potential antibiotic substitutes for treating bacterial illnesses. However, due to a lack of understanding of the biological mechanism of phage activity and the subsequent discovery and widespread use of broad-spectrum antibiotics in the late 1930s and early 1940s, interest in the therapeutic use of bacteriophages has declined, and it has been considered only a research tool in molecular biology for many years (**Clark and March, 2006**). Bacteriophages have recently been re-considered as an antimicrobial tool because of the present upward trend of bacterial resistance and the availability of the required molecular methods and tools to accurately evaluate the safety and effectiveness of phage use as a result of the advancements of modern biotechnology. Phages can be used in a variety of applications, including agriculture, the petroleum industry, the food industry, and, most significantly, clinical research.

Bacteriophages have been commercially manufactured at larger scales through some renowned biotechnological companies for implementation in several industries. Phages have recently been used to treat localized infections in humans (**Morozova et al., 2018**), and "Pyophage and Sekstaphage" technology was applied for the treatment of wound infections. Moreover, several phage cocktail trials were conducted for three years in France, Belgium, and Switzerland for the treatment of burn wound infections (**Nikolich & Filippov, 2020**). Additionally, they are now under consideration as a gene therapy delivery mechanism, as a biocontrol agent, uses in the development of phage-derived vaccines, and in the phage display method. Bacteriophages have been used as vaccine delivery vehicles in two ways: 1) by vaccinating with phages that express vaccine antigens on their surface, and 2) by integrating a DNA vaccine expression cassette into the phage genome and using the phage particle to transport that DNA cassette (**Clark and March, 2004**). Several clinical trials were conducted, and it was proved to be highly effective.

2.1.9.2 Bacteriophage Therapy: Bacteriophage therapy is another name for phage therapy. The use of bacteriophages to cure multiple bacterial infections is known as phage therapy. It treats bacterial infections with viruses. Phages solely kill bacteria; they are not harmful to humans, animals, or plants. Hence, Bacteriophage therapy refers to the use of natural or modified aggressive lytic bacteriophages to treat bacterial infections. Bacteriophages are bacteria's natural enemies, and the idea of using phages clinically was first convicted by Felix d'Herelle in 1919 to treat bacterial dysentery cases (**D'Hérelle, 1931**). Unfortunately, those trials were not successful because of poor understanding of phages activity in human body but currently phage treatment is reconsidered because of the availability of necessary molecular techniques and better understanding the phage activity on the host organisms.

Moreover, FDA (Food and Drug Administration) also approved the first US clinical trial of intravenously administered bacteriophage therapy in 2019 (**Strathdee, 2023**).

Phage therapy is now used to treat various bacterial infections such as *Staphylococcus aureus*, *Enterococcus*, *Proteus*, and *Pseudomonas aeruginosa*. Antibiotics are often related to phage therapy, and it has been recognized that phage therapy has many benefits over antibiotics. The use of bacteriophages to kill bacterial cells involved in infections is the fundamental concept of phage therapy as a potential means of treatment and prevention of bacterial infection. The administration of phages is the first step in using phages as a method of therapy. Within 1-3 hours after an oral injection, the phages enter the bloodstream, and after 10 hours, they are present in the internal organs (**Strathdee, 2023**). The phages' bactericidal behavior is the product of viruses replicating via the lytic cycle within the host cell.

2.1.9.3 Mechanism of Phage (Bacteriophage) Therapy: Bacteriophages cause bacteria to burst or lyse by killing them, when the virus binds to the bacteria. A virus infects bacteria by injecting its genes (DNA or RNA) into the organism. The phage virus replicates (makes copies) itself inside the bacteria. As a result, each bacterium can produce up to 1000 additional new viruses (**Golkar et al., 2014**). Finally, the virus ruptures the bacterial cell, allowing to release new bacteriophages to emerge. Bacteriophages can only replicate and grow inside the bacterial cell. When all the host bacteria are burst out (death), phage will stop replicating. Phage viruses, like other viruses, can lay dormant (inactivate or hibernate) until new bacteria appear. Bacteriophages are termed as “predators” and Bacteria as “prey”. So, this “predator–prey” relation is exploited by the scientists to treat many infectious diseases (**Maciejewska et al., 2018**).

Phage therapy uses lytic phages, which have a bactericidal effect and are host-specific. Phage cocktails, which contain multiple phages, can increase the range of target cells. However, the selection of appropriate phages is crucial for the successful elimination of clinically relevant pathogens while avoiding adverse effects, such as anaphylaxis, which is an adverse immune reaction and with the rise of antimicrobial resistance and the limitations of current treatments, phage therapy has regained attention as a potential alternative or complement to antibiotics.

2.1.9.4 Phage Therapy Reports Over the Years: Phages have the ability to selectively target the specific pathogen responsible for an infection, which allows for the preservation of beneficial bacteria in the microbiota. A study showed that (Figure 2.5) over the last 10 to 15 years, an increasing number of more detailed case reports have emerged (Figure 2.5.A), as evidenced by the quantity of literature available on PubMed.gov (**Strathdee, 2023**). Although case reports have a selection bias, more than half of the described cases are related to pulmonary infections and infections in implanted vascular and orthopedic devices.

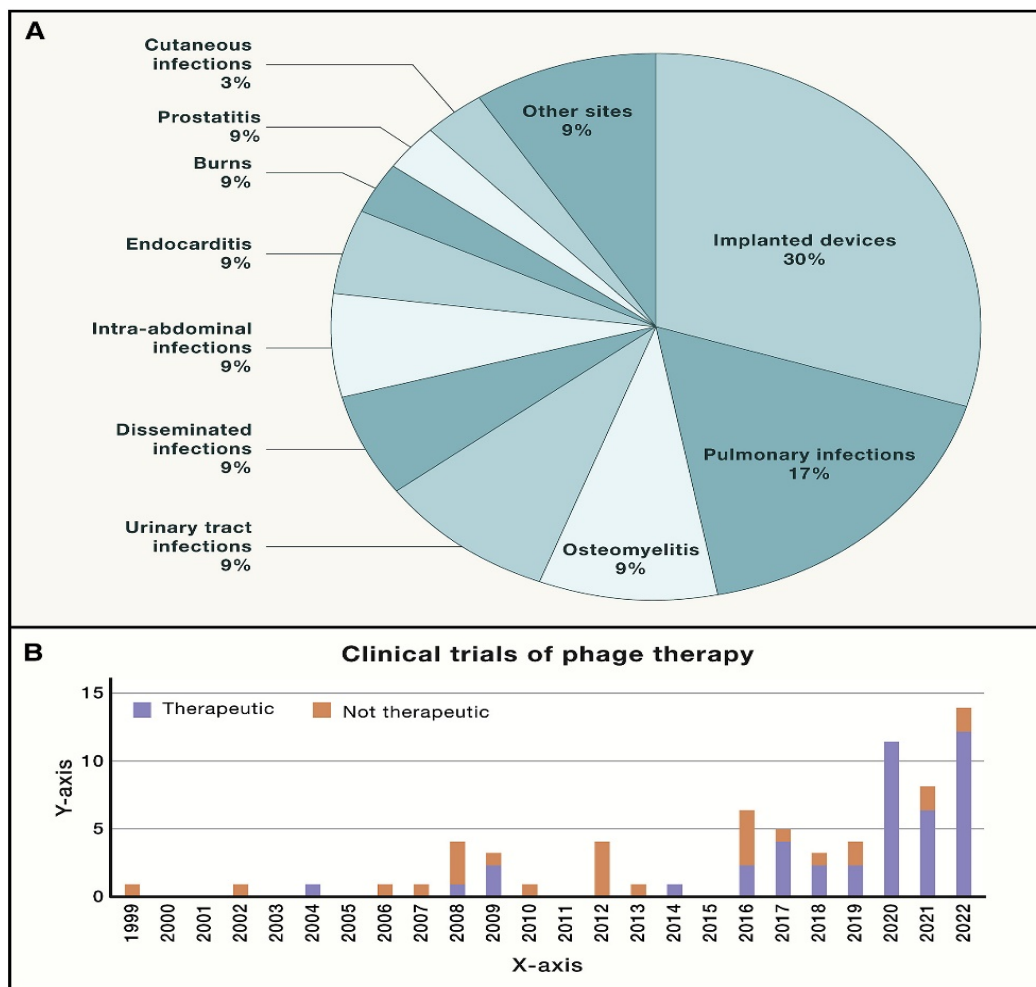


Figure 2.5: Phage therapy reports and phage studies by year listed (Adapted from Strathdee, 2023). (A) Case reports of phage therapy since 2000. A PubMed search was performed on September 22, 2022, using the search terms “(bacteriophage) AND (therapy) AND (case report).” Sites of infection in each of the 70 cases reported in 53 manuscripts are depicted. (B) Clinical trials of phage therapy reported to [ClinicalTrials.gov](https://clinicaltrials.gov) since 1999. The registry was queried using the key word “phage” on September 9, 2022.

Recent research has suggested utilizing phages for antimicrobial treatment. Currently, technological advancements have allowed the preparation of high-quality therapeutic phages, enabling the increasing use of intravenous delivery of phage therapy. A recent review of phage administration safety concluded that it is generally well-tolerated, regardless of the delivery route but as phages may act as a vehicle to carry the antibiotic resistance and virulence genes so for safety reasons, biosafety assessment of the phage should be done before it use (Liu, et al., 2021). However, non-parenteral delivery routes may require less rigorously prepared phages, which may compromise efficient delivery to the site of infection (Jerne & Avegno, 1956). For instance, treating enteric infections with phages requires formulations that can protect phages from gastric acid in the stomach (Strathdee, 2023). So, the importance of these bacteria in human health is still being explored.

However, the success of phage therapy in achieving this potential depends on the development approach chosen in the therapy's models (**Brives & Pourraz, 2020**).

Bacteriophages are one of the most significant microorganisms in the field of molecular and cellular biology because they are used as gene transfer vehicles (**Gómez-Gómez et al., 2019**). Bacteria have the ability to acquire foreign genes through bacteriophages, which play a crucial role in the process of horizontal gene transfer (HGT) (**Ackermann, 2011; Cui et al., 2017**). Bacteriophages can mobilize genetic material from the same or distantly related bacterial species (**Lorenzo-Daz et al., 2017**), and the foreign genes tend to retain the characteristics of the donor bacterium in the recipient host bacterium, and they may differ significantly from the native genes of the recipient bacterium in terms of optimal codon usage (**Chakraborty et al., 2009**). Bacteriophages, and horizontally transmissible genetic elements are known to have a significant role in the epidemiology and evolution of the pathogen (**Faruque, 2013**). Thus, horizontal gene transfer between phage-host interactions contributes to the genetic diversity and evolution of the bacterial population (**Chibani-Chennoufi et al., 2004**).

2.2 Horizontal Gene Transfer:

HGT, abbreviated as horizontal gene transfer, is the transmission of genetic material between organisms that are not related by descent. It is also known as the lateral gene transfer, and this process allows organisms to acquire new genetic material that can provide a selective advantage in their environment. HGT has been observed in bacteria, archaea, and eukaryotes, and it plays a significant role in the evolution of these organisms.

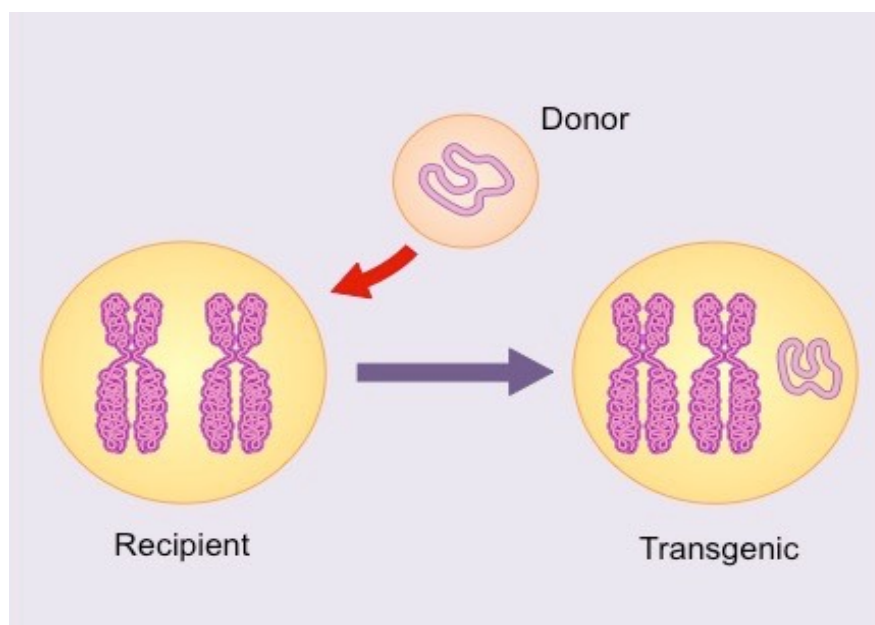


Figure 2.6: Horizontal gene transfer in between two different organisms. (Adapted from BioNinja, n.d)

2.2.1. Types of Horizontal Gene Transfer

Horizontal Gene Transfer (HGT) occurs through three well-understood genetic mechanisms which are transformation, conjugation, and transduction.

2.2.1.1 Transformation: In 1928, Griffith conducted an experiment on *Streptococcus pneumoniae*, which demonstrated the first direct genetic exchange between different strains of the bacterium (**Griffith, 1928**). He found that certain bacteria were able to uptake, integrate, and express functional extracellular DNA fragments through a process called transformation. This process occurs naturally in some bacteria (naturally competent) and is also used in the laboratory (through physical or chemical substances) to genetically modify bacteria. In nature, transformation can occur when bacteria release DNA into the environment, and other bacteria take up this DNA. This process can lead to the transfer of beneficial genes, such as antibiotic resistance genes, between bacteria.

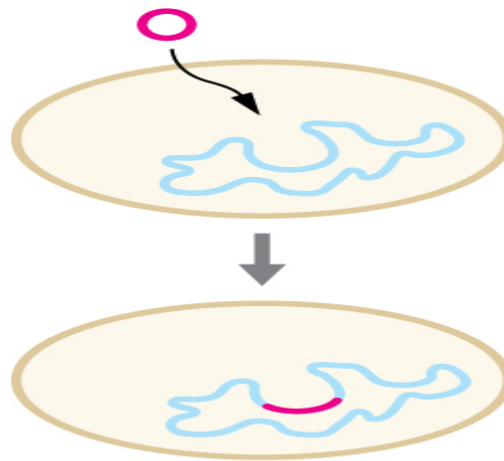


Figure2.7: Transformation (Adapted from Parker et al., 2016)

In this figure, it is shown that in case of transformation bacterial cell takes up DNA directly from their environment. The DNA may remain separate as a plasmid or be incorporated into the host genome. It has been discovered that many bacteria have cell surface proteins that can help to recognize DNA from similar species of bacteria so that foreign DNA from dead bacteria can be transported into the cell and incorporated into its genome.

2.2.1.2 Transduction: Transduction is the process by which genetic material is transferred between the recipients to the donor bacteria through bacteriophage. Transduction is the process of transfer of genes from. During transduction, a virus infects a bacterium and incorporates some of the bacterium's DNA into its own genome. When the virus infects another bacterium, it can transfer the incorporated DNA from the original bacterium to the new bacterium. Transduction can be beneficial to bacteria because it can allow for the transfer of beneficial genes, but it can also be harmful if it leads to the transfer of virulence genes.

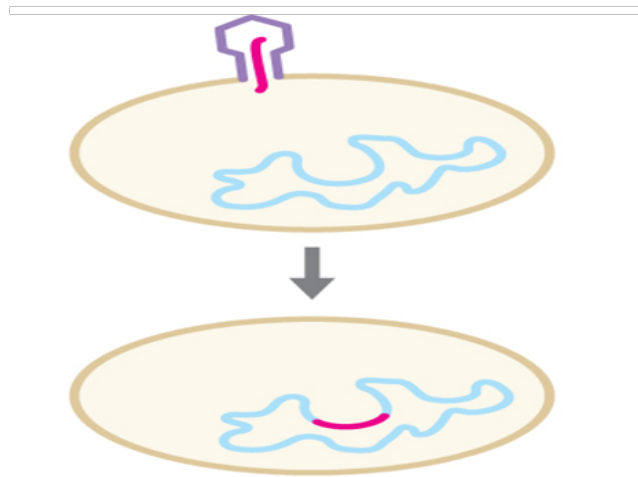


Figure 2.8: Transduction (Adapted from Parker et al., 2016)

In transduction, a bacteriophage may transfer bacterial chromosomal DNA from a previously infected donor cell to the recipient cell. This process is also used in the laboratory as a technique to introduce new genes into bacteria for research.

2.2.1.3 Conjugation: Conjugation is the process by which genetic material is transferred between bacteria by direct cell-to-cell contact. During conjugation, a donor bacterium transfers a copy of its plasmid (a small, circular piece of DNA) to a recipient bacterium. The plasmid can contain genes that confer a selective advantage, such as antibiotic resistance genes.

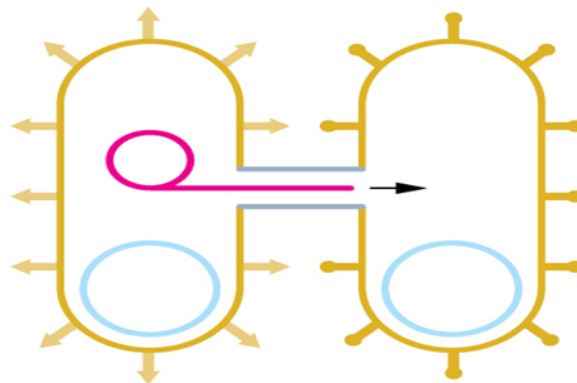


Figure 2.9: Conjugation (Adapted from Parker et al., 2016)

Conjugation can occur between bacteria of the same species or between different species. It is an efficient way for bacteria to exchange genetic material and acquire new traits. Bacteria that can undergo conjugation must possess a specific plasmid called the F plasmid. The F stands for “fertility” due to deliberate comparisons with exchange of genetic material in animal reproduction. However, the difference between the process of bacterial conjugation and sexual reproduction is that the products of bacterial conjugation do not create new cells, but rather transfer genetic information between two established cells.

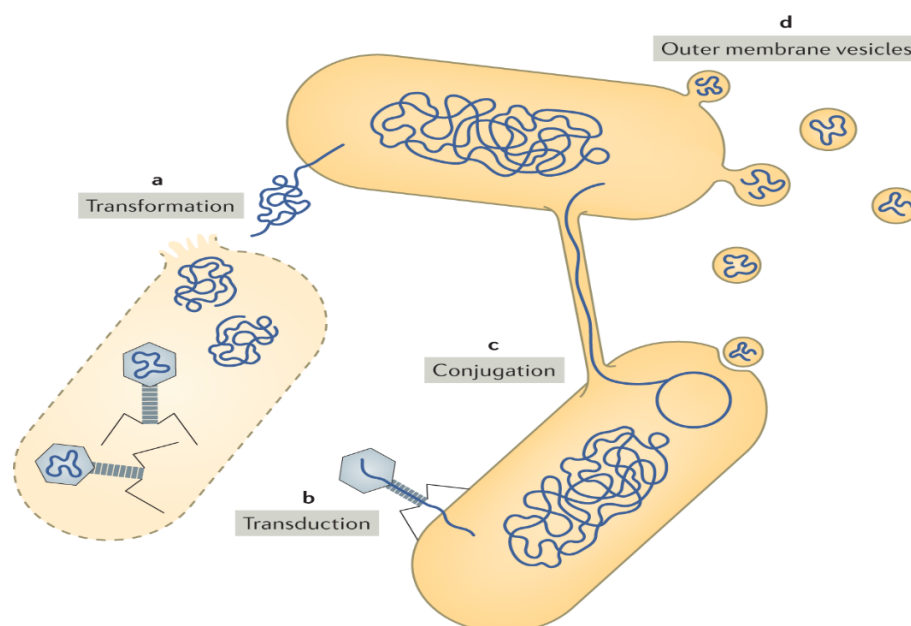


Figure 2.10: General routes of Horizontal Gene Transfer within natural communities, (Adapted from- Brito, 2021).

This schematic diagram basically shows the different mechanisms of horizontal gene transfer, where **(a)**. Transformation involves the uptake of naked DNA from lysed cells in the environment; **(b)**. During transduction, genetic material is introduced from a phage into bacterial genomes; **(c)**. Conjugation involves the transfer of DNA through conjugative pili and is the predominant mechanisms by which DNA is transferred between bacteria, and **(d)**. Additional mechanisms involve outer membrane vesicles and DNA packaged into virus-like particles (not shown), although their contribution to overall horizontal gene transfer is unknown.

In summary, transformation, transduction, and conjugation are the three main types of HGT. Each type of HGT has its own unique mechanism of transfer and can lead to the transfer of beneficial or harmful genetic material between organisms. Understanding the different types of HGT is essential for understanding the evolution of bacteria and other microorganisms.

2.3 Antimicrobial Resistance (AMR)

Antimicrobial resistance (AMR) is a natural phenomenon in which microorganisms, such as bacteria, viruses, fungi, and parasites, develop the ability to resist the effects of antimicrobial agents, including antibiotics, antivirals, antifungals, antiparasiticides, etc. and this resistance can arise through genetic mutations as well as the acquisition of resistance genes from other bacteria through horizontal gene transfer (HGT). The development of AMR is accelerated by the misuse and overuse of antimicrobial agents, which can lead to the selection and spread of resistant microorganisms (**World Health Organization, 2021; González-Villalobos, 2021**). AMR is an encyclopedic threat to public health,

as it reduces the effectiveness of antimicrobial agents, leading to prolonged illness, disability, and death, as well as increased healthcare costs (Wairimu et al., 2021). Also, the emergence of antibiotic resistance against bacteria is a significant threat to public health due to the lack of new antimicrobial compound discovery (Cui et al., 2017).

2.3.1. Correlation between HGT and AMR:

The Antimicrobial resistance (AMR) can occur through various mechanisms, such as mutations in the bacterial genome or through horizontal gene transfer, where bacteria acquire new genetic material, including genes that confer antimicrobial resistance (AMR). Basically, HGT can facilitate the spread of antimicrobial resistance genes between different bacterial strains or species, leading to the emergence and dissemination of multidrug-resistant organisms.

Transformation allowed bacteria to exchange ARGs, enabling them to evade antibiotics. Hotchkiss continued Griffith's work in 1951 by inducing penicillin and streptomycin resistance in previously sensitive strains of *Streptococcus pneumonia* (*S. pneumonia*) through the exposure to DNA from resistant strains (Hotchkiss, 1951). After that, Alexander and Leidy built on this work by showing that streptomycin resistance could be transferred not only within the same species of bacteria but also between different species of Haemophilus bacteria, including *H. influenzae*, and *H. parainfluenzae* (Alexander and Leidy, 1953; Alexander et al., 1956).

The DNA sequences that can be transferred from one organism to another include those found in chromosomes as well as mobile genetic elements like plasmids, transposons, and genomic islands (Brown-Jaque et al., 2015). There have been several documented cases of antibiotic resistance genes being transferred between bacterial species through processes like transduction, where bacteriophages carry and transfer the genes. For instance, erythromycin resistance has been transferred via transduction in the past, as well as tetracycline and multiple resistances in *S. pyogenes*, and β -lactamase genes in *E. coli* and Salmonella carried by bacteriophages. Antibiotic resistance plasmids have also been transferred in MRSA (Methicillin-resistant *Staphylococcus aureus*), and make MRSA Super Bug, which is extremely difficult to treat because of resistance to some antibiotics (Varga et al., 2012).

Moreover, a microbe that has acquired a resistance gene by HGT can quickly transfer that gene to other microorganisms, which causes resistance to spread quickly. It can also happen in natural microorganism reservoirs including soil, water, or animals that might act as sources of resistance genes for human infections. These events can take place within an individual patient or in a population. Furthermore, the Antimicrobial Resistance (AMR) and Horizontal Gene Transfer (HGT) have major implications because they reduce the efficacy of currently available antimicrobial medications and raise the likelihood that infectious illness treatments will be unsuccessful. Thus, it may result in lengthier hospital admissions, higher rates of morbidity and mortality, and higher medical expenses. Furthermore, the development of multidrug-resistant organisms might reduce the range of available treatments for infectious diseases, making it challenging to manage outbreaks.

CHAPTER 3: MATERIALS & METHODS

3.1 Place of Study

The 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.

3.2 Experimental Design

1. Sample organisms are collected from the BRAC University MEM Laboratory.
2. Identified isolates from glycerol stock were revived on the NA or LA plate.
3. Antibiotic susceptibility testing using the Kirby-Bauer disk diffusion method.
4. Bacteriophage collection from BRAC University MNS thesis laboratory stock
5. Phage enrichment and determination of the phage titer by the DLA method
6. Recipient and Donor organism selection by the Spot test and Kirby Bauer disk diffusion method.
7. Phage-mediated chromosomal inter- and intra-species gene transfer.
8. Screening of the transformed colonies on their specific agar plate.
9. Spot test and AntibioGram for Modified and Native strains
10. Plasmid DNA Extraction of modified strains and preparing competent cells.
11. Transformation of the modified strain plasmid into competent cells.
12. Evaluate the Results

3.3 Standard Laboratory Practice:

The glass items, such as test tubes, conical flasks, and beakers, were washed two times, using tap water and distilled water, to ensure cleanliness. Culture media, which included agar-based and broth-based media, pipette tips, centrifuge tubes, empty test tubes for the double-layer agar method, and sterilized equipment were all autoclaved at 121°C and 15 PSI for 15 minutes before use. To maintain sterility, the culture media were stored at 4°C, and experiments were performed inside vertical laminar flow cabinets that had been cleaned with 70% ethanol. Additionally, to prevent contamination, lab coats and hand gloves were worn during the experiments.

3.4 Materials:

3.4.1 Reagents for Media, Buffer & Solution preparation

- ✧ Distilled Water
- ✧ Bacteriological Agar
- ✧ Luria Broth medium
- ✧ Luria Bertani Agar
- ✧ MacConkey agar
- ✧ TCBS agar
- ✧ Sodium Chloride

- ❑ Yeast extract
- ❑ Tryptone
- ❑ Glucose
- ❑ Tris-HCL
- ❑ EDTA
- ❑ NaOH
- ❑ SDS
- ❑ Potassium Acetate
- ❑ Glacial Acetic Acid
- ❑ Phenol
- ❑ Chloroform
- ❑ Iso-amyl-alcohol
- ❑ Ethanol
- ❑ Isopropanol
- ❑ CaCl₂
- ❑ Glycerol
- ❑ Kanamycin Sulfate
- ❑ Ampicillin Sodium

3.4.2 Apparatus

- ❑ Micropipette
- ❑ Pipette
- ❑ Glass spreader
- ❑ Test tubes
- ❑ Glass vials
- ❑ Conical flasks
- ❑ Falcon tubes
- ❑ Eppendorf tubes
- ❑ Needle, Loop
- ❑ Ethanol
- ❑ Spirit lamp
- ❑ Aluminum foil paper
- ❑ Micropipette tips
- ❑ Shaker incubator
- ❑ Water bath
- ❑ Centrifuge machine
- ❑ Balance machine
- ❑ Cuvettes
- ❑ 0.22 Micron filter
- ❑ 0.45 Micron filter
- ❑ Syringe
- ❑ pH meter
- ❑ pH strip
- ❑ Vortex machine
- ❑ Spectrophotometer
- ❑ Paraffin oil, Parafilm Paper
- ❑ Sterile Petri Plate.

3.5 Sample Collection:

No	Strain	Source
1	<i>E. coli</i> E0001	Brac University
2	STEC	Brac University
3	1877	Brac University
4	O31	Brac University
5	WT324	Brac University
6	WT331	Brac University
7	WT332	Brac University
8	WT334	Brac University
9	WT346	Brac University
10	WT386	Brac University
11	WT391	Brac University
12	WT406	Brac University

3.6 Bacteriophage Collection:

No	Strain	Source
1	<i>E. coli</i> Phage	Brac University
2	JSF2 Phage	Brac University

3.7 Preparation of Culture media:

3.7.1 Luria Bertani Broth Preparation: Luria Bertani broth has been used for getting the rapid and good quality growth of the Enterobacteriaceae family members. It is composed by combining Tryptone 10gram, Yeast extract 5gram & NaCl 10gram powders.

- ✧ Measured of 1000 ml of distilled water and place it in a conical flask.
- ✧ Using an analytical balance equipment, the reagent was measured. A foil paper was used to weigh out 25 g of LB reagent powder.
- ✧ Prior to adding to the distilled water, the reagent was dissolved by shaking the conical flask in a circular motion.
- ✧ The conical flask was stirred thoroughly while being heated.
- ✧ Following a brief boil, the mixture was sterilized in an autoclave machine at a temperature of 121°C and a pressure of 15 PSI for 2 hours.
- ✧ After undergoing two hours of autoclaving, the LB was taken out of the autoclave machine and left to cool before use.

3.7.2 Luria Bertani Agar Preparation: Luria Bertani Agar has been used for giving the Enterobacteriaceae family members a good and solid surface to grow. It composes by combining the LB along with the bacteriological agar.

- ✧ Measured of 1000 ml of distilled water and place it in a conical flask.
- ✧ Using an analytical balance equipment, the reagent was measured. A foil paper was used to weigh out 25 g of LB reagent powder and 15g of Agar powder.
- ✧ Prior to adding to the distilled water, the reagent was dissolved by shaking the conical flask in a circular motion.
- ✧ The conical flask was stirred thoroughly while being heated.
- ✧ Following a brief boil, the mixture was sterilized in an autoclave machine at a temperature of 121°C and a pressure of 15 PSI for 2 hours.
- ✧ After undergoing two hours of autoclaving, the LA was taken out of the autoclave machine and ready to pour it in the Patri dish.

3.7.3 MacConkey Agar Preparation: MacConkey agar is a specialized type of culture medium that has both selective and differential properties and is utilized to separate and characterize non-demanding gram-negative rods, mainly belonging to the family Enterobacteriaceae and the Pseudomonas genus. It composes of Peptone 17gm, Proteose peptone 3gm, Lactose monohydrate 10gm, Bile Salts 1.5gm, Sodium Chloride 5gm, Neutral Red 0.03gm, Crystal Violet 0.001gm, Agar 13.5gm.

- ✧ Dissolve 49.53 grams of dehydrated medium in 1000 ml of demineralized water by heating it to the boiling point.
- ✧ Ensure complete dissolution and sterilize by subjecting the mixture to 15 PSI pressure (121°C) in an autoclave for 2 hours.
- ✧ Then, allow it to cool down to 45-50°C before mixing well and pouring into sterile Petri plates.

3.7.4 Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar Preparation: TCBS (Thiosulfate-Citrate-Bile-Sucrose) agar is a selective and differential culture medium for the preferential separation of *cholera vibrios* and *Vibrio parahaemolyticus* from diverse clinical and nonclinical examples. *Vibrio* types are primarily recognized for their part in human intestinal illnesses, and cholera and *V. parahaemolyticus* diarrhea are crucial worldwide. All disease-causing *Vibrio spp.*, except *Vibrio hollisae*, will cultivate on TCBS Agar. And it composes of sucrose (20.0 gm), di-peptone (10.0 gm), sodium citrate (10.0 gm), sodium thiosulfate (10.0 gm), sodium chloride (10.0 gm), yeast extract (5.0 gm), oxbile (oxgall) (5.0 gm), sodium cholate (3.0 gm), ferric citrate (1.0 gm), bromothymol blue (0.04 gm), thymol blue (0.04 gm), and agar (15.0 gm).

- ✧ Dissolve 88.1 gm of the dried medium in one liter of distilled or deionized water.
- ✧ Heat gradually with constant stirring until fully dissolved,
- ✧ There is no need to sterilize by autoclaving.
- ✧ Lower the temperature to 50°C and transfer the solution into sterile Petri dishes for further use.

3.7.5 Mueller Hinton Agar (MHA) Preparation: Mueller Hinton Agar (MHA) is a media which is used for the routine susceptibility testing of non-fastidious microorganism by the Kirby-Bauer disk diffusion technique. It comprises of beef extract (2.00 gm), acid hydrolysate of casein (17.50 gm), starch (1.50 gm), agar (17.00 gm), and distilled water (1000 ml).

- ✧ Dissolve 38 grams of dehydrated medium in 1000 ml of demineralized water by heating it to the boiling point.
- ✧ Ensure complete dissolution and sterilize by subjecting the mixture to 15 PSI pressure (121°C) in an autoclave for 2 hours.
- ✧ Then, allow it to cool down to 45-50°C before mixing well and pouring into sterile Petri plates for further use.

3.7.6 4% Soft Agar Preparation: Soft agar is a necessary medium for carrying out plaque assays or spot tests.

- ✧ Measured of 1000 ml of distilled water and place it in a conical flask.
- ✧ Using an analytical balance equipment, the reagent was measured. A foil paper was used to weigh out 25g of LB reagent powder and 4g of bacteriological agar.
- ✧ Prior to adding to the distilled water, the reagent was dissolved by shaking the conical flask in a circular motion.
- ✧ The conical flask was stirred thoroughly while being heated.
- ✧ Following a brief boil, the mixture was sterilized in an autoclave machine at a temperature of 15°C and a pressure of 121 PSI for 2 hours.
- ✧ After undergoing two hours of autoclaving, the 4% soft agar was taken out of the autoclave machine and left to cool before use.

3.7.7 Preparation of LA media containing Ampicillin: For this study, it is necessary to create a plate that selectively uses ampicillin for some experiments. This involves first preparing LA media and allowing it to cool after autoclaving, so that the ampicillin antibiotic can withstand the heat. Once the media has cooled enough to be touched, we add the ampicillin supplement. As our concentration of Ampicillin was 100mg/ml, so according to S1V1 and S2V2 on 120ml of media, we added 120µl of Ampicillin stock solution.

3.7.8 Preparation of LA media containing Kanamycin: To create a kanamycin selective plate for experiment in this study, we first prepare the LA media and set it aside after autoclaving to cool down. This is done to ensure that the kanamycin antibiotic we will be adding can withstand the heat. Once the media has cooled enough to be touched, we add the kanamycin. As our concentration of Kanamycin was 100mg/ml, so according to S1V1 and S2V2 for 120ml of media, we added 120µl of Kanamycin stock solution.

3.8 Preparation of Solutions:

3.8.1 Kanamycin Stock Solution Preparation (50 mg/ml): Kanamycin sulfate is a type of antibiotic known as an aminoglycoside, which has the ability to hinder the development of various bacteria types such as Gram-positive, Gram-negative, and mycoplasmas when used in cell cultures. The working concentration of Kanamycin is 50 µg/mL. So, we prepared 50 mg/ml of kanamycin solution.

- ❏ Add 5gm Kan powder to 100ml of distilled water.
- ❏ Mix well by shaking or swirling until the powder dissolves completely.
- ❏ Filter the solution through a sterile filter with 0.22µm pores to sterilize it.
- ❏ Divide the solution into 1-milliliter portions and transfer each portion into a 1.5-milliliter tube.
- ❏ Freeze the tubes at a temperature of -20° Celsius for storage.

3.8.2 Ampicillin Stock Solution Preparation (100mg/ml): Ampicillin sodium is a type of β-lactam antibiotic that belongs to the extended-spectrum family and has a chemical structure similar to penicillin. This antibiotic is commonly used to select antibiotic-resistant cell hybrids and exhibits a potent activity against numerous types of gram-negative bacteria. The working concentration of Ampicillin is 100 µg/mL. So, we prepared 100 mg/ml of Ampicillin solution.

- ❏ Add 1 gram of Ampicillin powder to 10 milliliters of distilled water.
- ❏ Mix well by shaking or swirling until the powder dissolves completely.
- ❏ Filter the solution through a sterile filter with 0.22µm pores to sterilize it.
- ❏ Divide the solution into 1-milliliter portions and transfer each portion into a 1.5-milliliter tube.
- ❏ Freeze the tubes at a temperature of -20° Celsius for storage.

3.9 Prepare Solution for Plasmid Isolation:

3.9.1 Solution I Preparation: Solution-I is made by mixing three different things together and they are –

1. **1 M Tris-Cl stock solution (50 mL):** To prepare a stock solution of 1 M Tris-HCl in a 50 mL volume, dissolve 6.057 grams of Tris base in 50 mL of sterilized deionized water. Adjust the pH to the desired level by adding concentrated HCl.
2. **0.5 M EDTA stock solution (100 mL):** A 0.5 M EDTA stock solution in a 100 mL volume can be made by dissolving 14.612 grams of EDTA in 100 mL of sterilized deionized water. The pH should be adjusted to 8.0 using NaOH
3. **1 M glucose stock solution (50 mL):** A solution of 1 M glucose in a 50 mL volume can be prepared by dissolving 9 grams of glucose in 50 mL of deionized water that has been sterilized. The solution should be filtered through a 0.20 µM membrane Millipore to ensure sterility. The glucose solution can be used immediately or stored in a 4° C cabinet for preservation.

Before mix all those three things together the mixer of Tris and EDTA has been autoclaved together and then glucose has been added to avoid the caramelization of the glucose. To prepare 100ml of

solution 1, 2.5ml of Tris-Cl, 1ml of EDTA, 5ml of Glucose from stock solution has been mixed along with the 90.5 ml of deionized water.

3.9.2 Solution II Preparation: Solution-II is comprised of NaOH and SDS.

1. **10 N NaOH stock solution (50 mL):** To create a 10 N NaOH stock solution with a volume of 50 mL, dissolve 20 grams of NaOH in 50 mL of sterilized deionized water.
2. **1% SDS stock solution (30 mL):** A stock solution of 1% SDS with a volume of 30 mL can be prepared by dissolving 0.3 grams of SDS in 30 mL of sterilized deionized water.

As solution 2 is always need to be prepared freshly before doing the work so most of the time there is no need to prepare the stock solution. Rather, 0.12g of NaOH (0.2N) powder and 0.15gm of SDS (1%) has been dissolved in 15 ml of distilled water.

3.9.3 Solution III Preparation: Solution-III contains potassium acetate and glacial acetic acid.

1. **5 M potassium acetate stock solution (100 mL):** To create a 5N potassium acetate stock solution with a volume of 50 mL, dissolve 49.071 gram of potassium acetate in 100 mL of sterilized deionized water. And then add glacial acetic acid until the pH become 5.2. and after it get ready store the solution at -20°C.

3.9.4 Phenol: Chloroform: Isoamyl Alcohol Preparation: Took Amber bottle and mix 25ml of phenol, 24ml chloroform and 1ml of Isoamyl alcohol inside it and cover up the bottle as all of them are light sensitive. To avoid the reduction of chloroform TE buffer was added on the top layer of the prepared solution.

3.9.5 70% Ethanol Preparation: For preparing 20ml of 70% ethanol, 14ml of absolute ethanol has been added with 6ml of distilled water.

3.9.6 TE Buffer Preparation: Dispense 1 ml of 1M Tris-Cl solution (with pH value of 8.0) into a Duran bottle with a capacity of 100 milliliters. Measure out 0.2 milliliters of 0.5 molar EDTA solution (with pH value of 8.0) and transfer it to the same Duran bottle. Complete the solution to a total volume of 100 milliliters by pouring 98.8 milliliters of distilled water into the Duran bottle. Seal the bottle with its cap and gently invert it several times to ensure thorough mixing.

3.10 Prepare Solution for Competent Cell preparation:

3.10.1 100mM CaCl₂ Preparation: For preparing 100ml of 100mM CaCl₂ stock solution dissolve 1.4701gm of CaCl₂ powder into 100 ml of distilled water and store it at -20°C.

3.10.2 CaCl₂ + 15% Glycerol Solution: From 100ml of 100mM CaCl₂ stock solution took out 17ml of CaCl₂ and added 3ml of 15% of Glycerol to make 20ml of CaCl₂ + 15% Glycerol solution.

3.11 Bacterial Cell Culture by Streaking Method: After getting the desired strains of bacteria, it has been streaked on the LA plate so that the bacterial will grow there and with the help of that we do our further work. Most importantly the streaking has been done one day before the next experiments being done because streaked plate need an incubation period to let the bacteria grow.

- i. Firstly, the fresh plate labeled with important information like group name, strain name, date of streaking, and the name of the media.
- ii. Secondly, with the help of a loop stab on the bacterial stock and streak on the fresh plate
- iii. Incubate the plate at 37°C temperature for overnight.

3.12 Bacterial Liquid Cell Culture: Preparing liquid bacterial cultures involves transferring a small number of bacteria from a solid agar plate into a sterile liquid growth medium, such as nutrient broth or LB (Luria-Bertani) broth. The bacteria are then allowed to grow in the liquid medium under controlled conditions, such as temperature, agitation, and aeration.

Once the bacteria have grown to the desired density or phase of growth, they can be harvested and used for experiments. This allows researchers to obtain a large number of bacterial cells that are in a uniform state of growth and physiological condition, which is important for reproducibility and accuracy in experiments.

3.13 Pour Plate Method: The pour plate method allows for the detection and enumeration of bacterial cells present in the original liquid culture. As the agar and bacterial mixture is poured into the petri dishes, the bacterial cells become distributed throughout the agar, resulting in the formation of colonies both within and on top of the agar.

Here, a small amount of the bacterial liquid culture is taken and mixed with sterile water or saline to dilute it. This is done to obtain a known concentration of bacterial cells. After the autoclaved agar has been sterilized and cooled to a temperature that will not kill the bacterial cells, a known amount of the diluted bacterial culture is added to the agar mixture and mixed thoroughly. The agar and bacterial mixture are poured into sterile petri dishes. Once the agar has been poured into the petri dishes, it needs to be allowed to solidify. Finally, the plates need to be incubated under the appropriate conditions to allow the bacterial cells to grow and form visible colonies within and on top of the agar.

3.14 Spread Plate Method: In the spread plate method, the bacterial cells are spread evenly on the surface of the agar, which allows for the formation of isolated colonies on the surface of the agar. After preparing a liquid culture of bacteria it dropped on the solid surface of the agar plate and with the help of a spreader the culture spread on the liquid media till it got sticky.

3.15 Bacterial Stock Preparation (T₁N₁): Luria Broth is a commonly utilized culture medium for bacteria, and Luria agar is a non-selective powdered media that is nutritionally rich for the growth of pure strains. The stock solution of the medium requires reagents such as Tryptone, sodium chloride, and bacterial agar. In order to achieve better results, readymade LA was not used to prepare the medium as it is more solid. Instead, the medium was manually prepared using the given recipe and procedure.

- » Tryptone = 10g
- » NaCl = 10g
- » Agar = 20g
- » Distilled water = 1000ml

The required reagents were measured and mixed together in a conical flask, containing distilled water and stirred until the media was mixed properly. The media was then boiled and autoclaved before being poured into glass vials. Each vial was filled with 2.5 ml of media, which solidified within a few moments and stored in a fresh fridge at 4°C. Then with the help of a loop the desired bacteria have been taken and directly stab in the T₁N₁ media containing vials, and let them incubate. It can be store for 6 months. To store for 1 year paraffin oil has been added on the top layer of the vial.

3.16 The Kirby-Bauer Antibiotic Sensitivity test: It is also known as the Disk Diffusion method, is a standard laboratory procedure used to determine the susceptibility of bacterial strains to antibiotics. Here are the general steps involved in the Kirby-Bauer method:

- » **Prepare the bacterial culture:** A pure culture of the bacterial strain to be tested is grown in a liquid growth medium until it reaches the appropriate growth phase.
- » **Inoculate the agar plate:** Using a sterile swab or loop, the bacterial culture is streaked onto a Mueller-Hinton agar plate in a uniform pattern.
- » **Place the antibiotic disks:** Once the bacterial culture has been inoculated onto the agar plate, antibiotic disks are placed onto the surface of the agar. Each disk contains a specific antibiotic in a known concentration.
- » **Incubate the plate:** The agar plate with the antibiotic disks is then incubated under appropriate conditions to allow bacterial growth and antibiotic diffusion.
- » **Measure the zones of inhibition:** After incubation, the zone of inhibition around each antibiotic disk is measured and recorded. The size of the zone of inhibition is an indicator of the effectiveness of the antibiotic against the bacterial strain.
- » **Interpret the results:** The results are interpreted by comparing the size of the zones of inhibition to standard values set by regulatory agencies. The bacterial strain is classified as

susceptible, intermediate, or resistant to each antibiotic based on the size of the zone of inhibition.

3.17 Bacteriophage Enrichment test: To increase the volume of isolated and characterized phages, the enrichment process was repeated within 2 months. Enriched phage solution was necessary for accurate results in subsequent steps such as calculating the multiplicity of infection and conducting a double layer assay. The enriched phage stock was stored at a temperature of 4°C for future use.

- » **Young culture:** Took 3 or 5 ml of LB and inoculate the specific bacterial strain in it and incubate into the rotatory shaker until OD600 0.4 (usually 2-3 hours).
- » **Introducing the phage:** Add 50 µl of into the falcon of young culture and put it back to the rotatory shaker incubator for 4 Hours.
- » **Centrifugation:** After that the bacteria and phage containing falcon need to be centrifuge at 13,000 rpm for 10 minutes to separate the bacterial cells as pellets and the enriched phages suspended in the supernatant.
- » **Filtration:** Used a 0.22-micron syringe filter to collect the sterile phage from the supernatant.
- » **Store:** The enriched phage has to be stored on 4°C temperature.

3.18 Bacteriophage Spot Test: The enriched phages were subjected to a spot test with bacterial strains that had previously shown visible plaques.

- » Each strain was inoculated in 3ml of fresh LB and incubated on a shaker for 1.5 hours.
- » Large LA plates were prepared and labeled accordingly while soft agar aliquots stored in the refrigerator were melted by boiling and kept at 52°C.
- » After incubation, a soft agar aliquot was mixed with 200µl of the young individual bacterial culture and poured onto the surface of the LA agar plate to create a bacterial lawn.
- » The plates were then marked with bacteriophage sample numbers and 15µl of purified phage suspensions were deposited onto the top agar layer.
- » After the plates were dried, they were kept overnight at 37°C and checked for the presence of clear zones.
- » The results confirmed the isolated phages and their host strains.

3.19 Phage Titer Determination by Double Layer Agar Assay: The Double Layer Assay, also known as plaque assay, is a widely used technique in phage research to isolate and identify pure bacteriophage populations. It is considered the standard method for determining phage titer and concentration. By using this technique, the titer of each phage can be determined and expressed as plaque forming units (pfu). This allows for precise and reproducible quantification of viable bacteriophages, which is important for further research on phage applications.

The conventional Phage Assay (PA) involves the interaction of a single lytic phage molecule and a susceptible bacterium, resulting in the lysis of the host bacterium and release of phage progeny. When mixtures of phage and host bacteria are poured onto nutrient agar with a molten soft agar overlay, bacterial growth is allowed to resume. Areas without phage present will form a bacterial lawn in the soft agar overlay, while areas with phage present will produce a clear zone where the newly formed phages have been released, called a "Plaque".

The number of plaques is counted and the phage concentration/titer is commonly expressed as the number of plaque-forming units (PFU/mL) of the measured preparation. The formula for calculating phage titer (PFU/mL) involves dividing the number of plaques by the dilution factor multiplied by the volume of phage added to the plate.

$$\text{Titre (PFU/ml)} = \frac{\text{Number of plaque (PFU)}}{\text{dilution} \times \text{volume of phage added to plate (ml)}}$$

To perform the Double Layer Agar Assay (DLA),

- » The first step is to prepare a young culture of the host bacteria whose plaques need to be counted.
- » Then, the corresponding phages of the host bacteria are diluted serially in LB up to 10^{-6} or in some cases up to 10^{-8} dilution.
- » After diluting the phage, 100 microliters of the phage solution are mixed with 600 microliters of the young culture of the host bacteria let the adsorption occur between bacteria and phage have done at least for 10-15mins.
- » It mixed with 5ml of molten soft agar kept in a sterile test tube. Care should be taken to maintain the temperature of the molten soft agar at 52° Celsius, otherwise, it would solidify sooner.
- » The contents are mixed thoroughly and poured into Luria-Bertani agar plates, avoiding bubble formation.
- » The plates are left to solidify and then incubated overnight at 37° Celsius.
- » After incubation, the plates are checked for plaque formation and PFU/ml is calculated. A standard count of 30-300 plaques is considered and counted if plaques are formed within this range.

3.20 Phage Mediated Chromosomal Gene Transfer Protocol: It is the main working protocol to transfer the kanamycin resistance Chromosomal gene from host to the kanamycin sensitive bacteria for making it resistant.

3.20.1 Protocol for Inter-species gene transfer (1877 to *E. coli* and STEC): As 1877 *Vibrio cholerae* strain and *E. coli* is not belong to same specie so it is known as inter-species protocol.

1. Take one to two colony of *vibrio cholera* 1877 stain and dissolve in 10 ml of Luria Bertani broth.
2. Put it on the shaker incubator for 2 hours.
3. Usually, young culture contains 10^6 cells per ml. Mix 100 micro-L JSF2 phage to it.
4. Put it on the shaker incubator for 4 hours.
5. Now take 3ml LB. Add 300 micro-L of 100mg/ml concentrated ampicillin and mixed properly for *E. coli* as according to susceptibility test it is ampicillin resistant. But in case of STEC there is no need to add ampicillin.
6. Mix *E. coli* and STEC colony accordingly to 3ml LB falcon tubes and put it on the shaker incubator for 2 hours.
7. Take 3 ml of *E. coli* and STEC young culture and mixed it accordingly to the falcon tubes that contain 1877 and JSF2 phage.
8. Put it on the rotatory shaker for overnight.
9. Do the serial dilution with help of LB up to 10^{-6} times.
10. Put 100 micro-L of dilution sample to the Ampicillin and kanamycin containing McConkey plates and Kanamycin containing McConkey plates accordingly for *E. coli* and STEC, spread it by the spreader until it feels sticky.
11. Incubate the plates overnight.
12. If the red colony found on plate it indicates that gene of Kanamycin resistance of *Vibrio cholerae* is transformed into the *E. coli* and STEC.

3.20.2 Protocol for Intra-species gene transfer (1877 to WT331, WT332 and WT391): As 1877 *Vibrio cholerae* strain and WT331, WT332 and WT391 are also belong to same specie so it is known as intra-species protocol.

1. Take one to two colony of *Vibrio cholera* 1877 stain and dissolve in 10 ml of Luria Bertani broth containing three different falcons
2. Put it on the shaker incubator for 2 hours.
3. Usually, young culture contains 10^6 cells per ml. Mix 100 micro-L JSF2 phage to it.
4. Put it on the shaker incubator for 4 hours.
5. Now take 3ml LB into 3 different falcon tubes.
6. Mix WT331, WT332 and WT391 colony accordingly to 3ml LB falcon tubes and put it on the shaker incubator for 2 hours.
7. Take 3 ml of WT331, WT332 and WT391 young culture and mixed it accordingly to the falcon tubes that contain 1877 and JSF2 phage.
8. In case of intra species protocol, the culture of JSF2 and 1877 have to filter with the help of 0.22-micron filter.
9. Put it on the rotatory shaker for overnight.
10. Do the serial dilution with help of LB up to 10^{-6} times.
11. Put 100 microliter of dilution sample to the kanamycin containing TCBS plates and spread it by the spreader until it feels sticky.
12. Incubate the plates overnight.
13. If the yellow colony found on plate it indicates that gene of Kanamycin resistance of *Vibrio cholerae* is transformed into the WT331, WT332 and WT391.

3.21 Plasmid DNA Isolation from Native and Modified strains of bacteria by Miniprep Protocol:

1. Take *E. coli*, Modified *E. coli*, 1877, STEC, Modified STEC cells and inoculate in 3ml LB of 5 different falcon tube. For the modified strains Kanamycin solution must be mix with the LB and culture for overnight
2. Take 1 or 2 ml [1ml is most preferable but if it gives very little amount of pellet then add another 1ml] of culture and centrifuge at 10000rpm for 3min. This step can repeat one more time if required.
3. Remove the supernatant and on the pellet add solution 1 (50 mM glucose and 25 mM Tris-HCl, pH 8, 10 mM EDTA) 200microliter and dissolve the pellet and then add solution 2 (0.2 N NaOH and 1% SDS) 400microliter. Solution 2 need to be freshly prepared every time.
4. Let them incubate the room temperature or on ice for 5 mins (exactly for 5mins not more than that)
5. Now add solution 3 (5M Sodium acetate pH 5.2, Glacial acetic acid, distilled water) 300microliter on that and mix properly by inversion.
6. Let it be incubated on ice for 10mins.
7. Centrifuge at 12000 rpm for 15mins.
8. The supernatant is collected at fresh Eppendorf
9. Now add equal volume of phenol: chloroform: IAA (25:24:1) and mix properly and centrifuge at 12000 rpm for 2mins.
10. Collect the supernatant and add 2 volumes of ice cold 100% Ethanol or ice-cold equal volume of isopropanol and keep it at -20°C for 2hrs, 4hrs or for overnight and centrifuge at highest speed (12000rpm) for 15min.
11. DNA pellet wash with 70%ethanol (centrifuge at 12000 rpm, 2mins) air dried and resuspended in 10-15microliter of TE buffer.
12. Store the plasmid at -20°C.

3.22 Preparing DH5α Competent Cell:

1. Inoculate a single colony of DH5α strain in a 5ml LB media containing tube and place in a shaker incubator at 37°C.
2. Next day Inoculate 50ml LB medium with 500microliter of saturated overnight culture and put on Shaker incubator at 37°C until OD600=0.4 (usually 2-3 hours)
3. Place in an ice bath for 10 minutes [After this point the cells should never touch anything that is warm]
4. Transfer 1ml of the culture into pre-chilled Eppendorf (place the Eppendorf on ice bucket)
5. Centrifuge at 5000rpm for 10 minutes at 4°C
6. Remove the medium, resuspend the cell pellet with 1ml ice cold CaCl₂ by swirling on ice gently and incubate on fridge for 30 minutes and Centrifuge at 5000rpm for 10 minutes at 4°C
7. Remove the medium, resuspend the cell pellet with 0.8ml ice-cold 100mM CaCl₂ by on ice gently. Incubate on ice for 20 minutes and Centrifuge at 5000rpm for 10 minutes at 4°C

8. Remove the supernatant and add 100 microliter ice-cold 15% glycerol and CaCl_2 , mix and store in -80°C

3.23 Transformation protocol of extracted plasmid to competent cell DH5 α :

1. Take competent cell solution and plasmid out of storage and place it on ice
2. Take 100microliter of Competent cell with 10 microliters of isolated plasmid in Ice for 10mins.
3. Place in hot water bath at 42°C for exactly 90 Seconds to apply heat shock.
4. After that place on ice immediately. For 2mins to close cell membranes pores.
5. Mix 1ml LB media and culture for 1hr at 37°C .
6. Place 100microliter of culture in large LA plates under to check for presence of transformed cells.

CHAPTER 4: RESULT INTERPRETATION

4.1 The Kirby-Bauer Disk Diffusion Susceptibility Test Result of Native bacterial strains: There were total 12 different strains of bacteria have been used in this experiment. and this test has been done for each and every strain. The result is shown below with a help of table (4.1).

Table 4.1: The Kirby-Bauer Disk Diffusion Susceptibility Test Result [R= Resistance, I= Intermediate, S= Sensitive]

Strain	Used Antibiotics				
	Ampicillin-25	Kanamycin-30	Streptomycin-25	Erythromycin-15	Tigecycline-15
1877	15mm (S)	R	13mm(I)	22mm(I)	25mm(S)
<i>E. coli</i>	R	18mm(S)	17mm(I)	R	20mm(S)
STEC	18mm(S)	21mm(S)	20mm(S)	15mm(I)	25mm(S)
WT331	21mm(S)	24mm(S)	17mm(I)	31mm(S)	29mm(S)
WT332	25mm(S)	20mm(S)	R	21mm(I)	26mm(S)
WT391	22mm(S)	20mm(S)	19mm(S)	29mm(S)	25mm(S)
WT334	R	R	15mm(I)	R	21mm(S)
WT324	R	19mm(S)	18mm(I)	R	22mm(S)
WT346	R	20mm(S)	20mm(I)	11mm(R)	20mm(S)
WT406	R	25mm(S)	23mm(S)	21mm(I)	27mm(S)
031	R	19mm(S)	19mm(I)	R	21mm(S)
WT386	R	19mm(S)	20mm(I)	25mm(S)	25mm(S)

The table 4.1 is illustrate with the help of a column Chart below:

Antibiotic Susceptibility Test

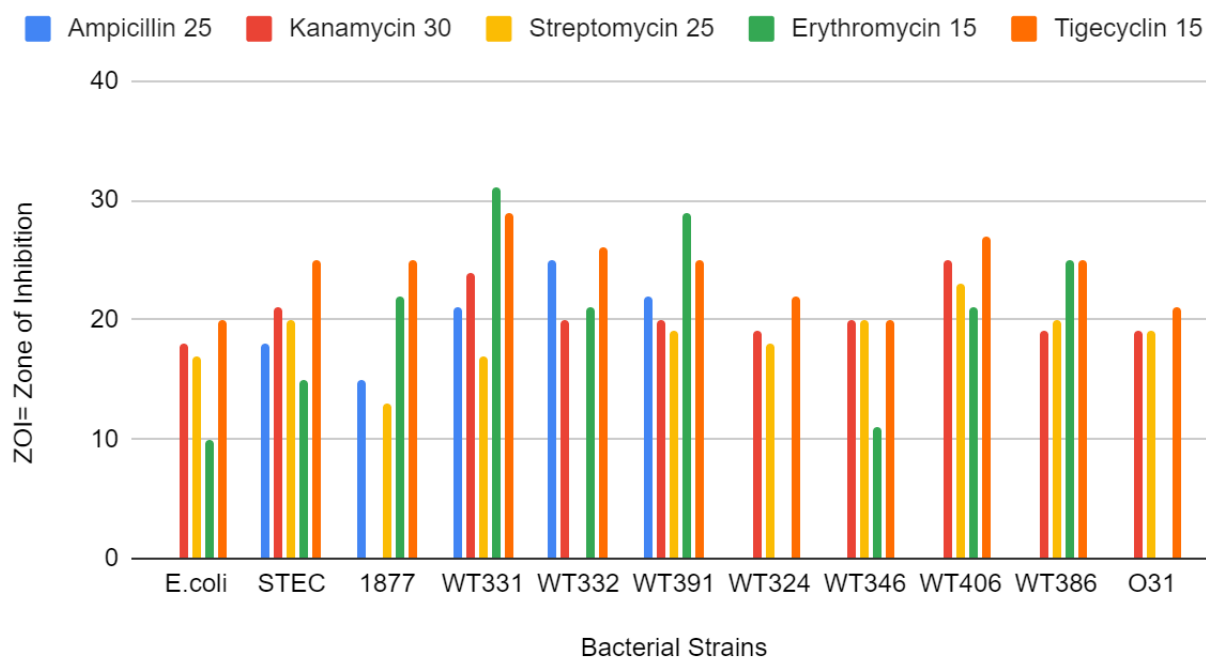


Figure 4.1: Antibiotic Activity on Different Strains

According to the CLSI guideline interpret the result:

Antibiotics	Resistance (mm)	Intermediate (mm)	Sensitive (mm)
Ampicillin 25	11	12-13	>13
Kanamycin 30	13	14-17	>17
Streptomycin 25	14	15-20	>20
Erythromycin 15	13	14-22	>22
Tigecycline 15	15	15-17	>18

4.1.1 Antibiotic susceptibility test for *E. coli* E0001: From the given chart, the *E. coli* is Kanamycin sensitive because of having 18mm Diameter clear zone. On the other hand, this strain of *E. coli* is Ampicillin Resistant (Fig 4.2).

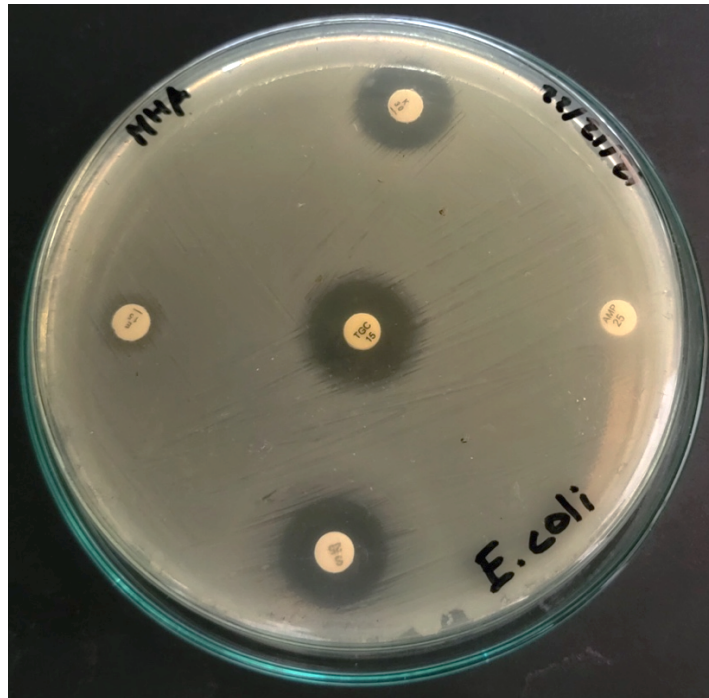


Figure 4.2: The Kirby-Bauer Disk Diffusion Susceptibility Test on *E. coli* strain

4.1.2 Antibiotic Susceptibility test for STEC: From the given chart, the STEC is both Kanamycin and Ampicillin sensitive because of having accordingly 21mm and 18mm diameter clear zone (Fig 4.3).



Figure 4.3: The Kirby-Bauer Disk Diffusion Susceptibility Test on STEC.

4.1.2 Antibiotic Susceptibility Test for 1877: From the given chart, the 1877 is Kanamycin resistant and Ampicillin sensitive because of having 15mm diameter clear zone (Fig 4.4).

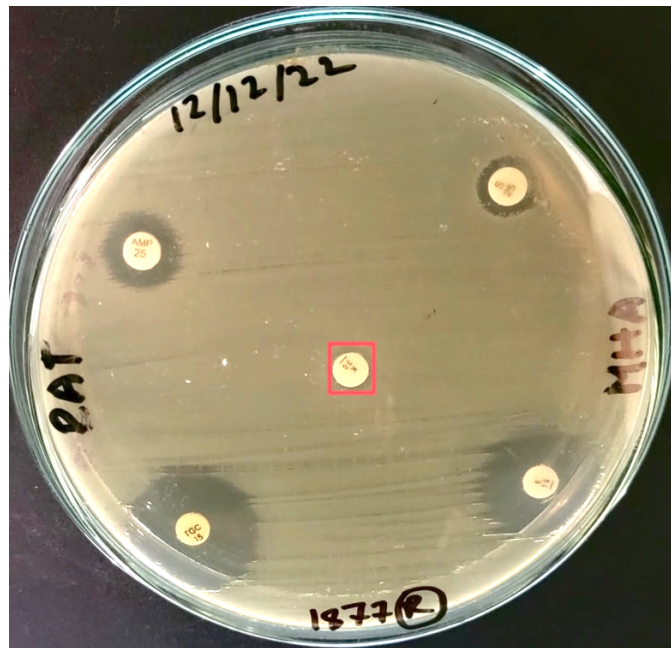


Figure 4.4: The Kirby-Bauer Disk Diffusion Susceptibility Test on 1877.

4.1.3 Antibiotic Susceptibility Test for WT331, WT332, WT391: From the given chart, the WT331 is both Kanamycin and Ampicillin sensitive because of having accordingly 24mm and 21mm diameter clear zone. Moreover, the WT332 is also both Kanamycin and ampicillin sensitive because of having accordingly 20mm and 25mm diameter clear zone. Furthermore, the WT391 is also both Kanamycin and ampicillin sensitive because of having accordingly 20mm and 22mm diameter clear zone (Fig 4.5).

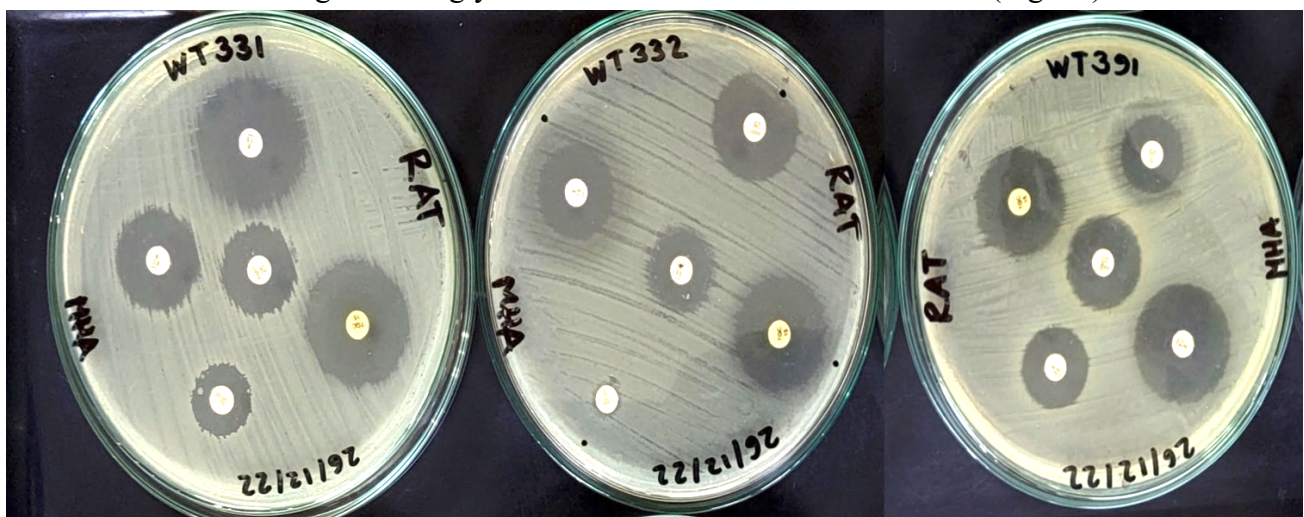


Figure 4.5: The Kirby-Bauer Disk Diffusion Susceptibility Test on WT331, WT332, WT391

4.2 Spot test for the confirmation of Plaque: There were total 12 different strains of bacteria have been used in this experiment. and this test has been done for each and every strain. The result is shown below with a help of table 4.2-

Table 4.2: The Result of Spot Test [+ indicates Ø susceptibility, - indicates Ø resistance]

Strain	JSF2 Ø	E. coli Ø
1877	+	-
E. coli	-	+
STEC	-	-
WT331	-	-
WT332	-	-
WT391	-	-
WT334	-	-
WT324	+	-
WT346	+	-
WT406	-	-
O31	-	-
WT386	-	-

Here the highlighted boxes shows that which bacteriophages have been used and able to create a clear zone or plaque on which bacterial culture.

4.2.1 Spot test result of *E. coli* phage positive & negative strain: There are two types of phages have been used in this study and they are JSF2 and *E. coli* phage. *E. coli* phage can only cut the *E. coli* strain and create a clear zone on the agar media (Fig 4.6). On the other hand, *E. coli* phage cannot create clear zone for the rest 11 strains that have been used in this study.

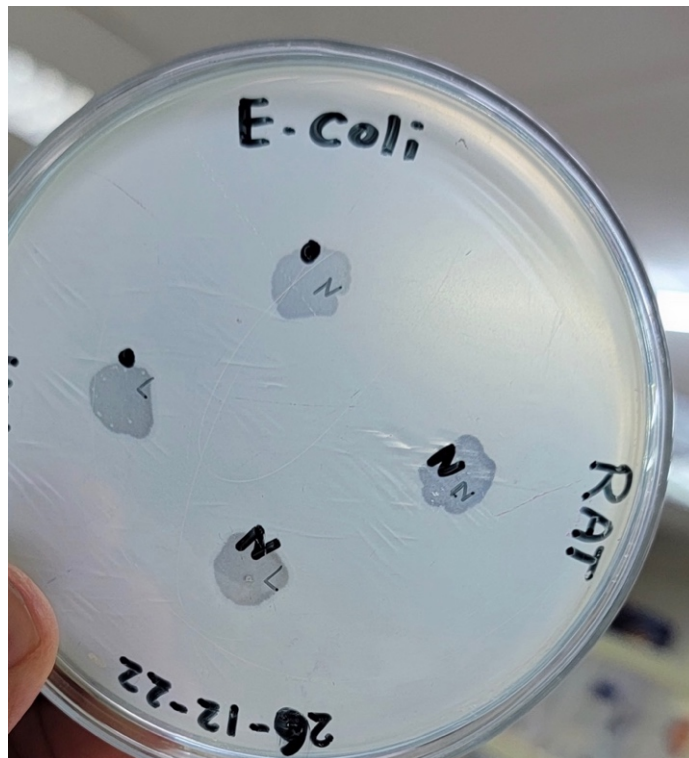


Figure 4.6: *E. coli* phage create a clear zone on native *E. coli* strain.

4.2.3 Spot test result of JSF2 phage positive strain: There are only three strains found that are sensitive to the JSF2 phage. And they are 1877, WT324, WT346 (Fig 4.7).

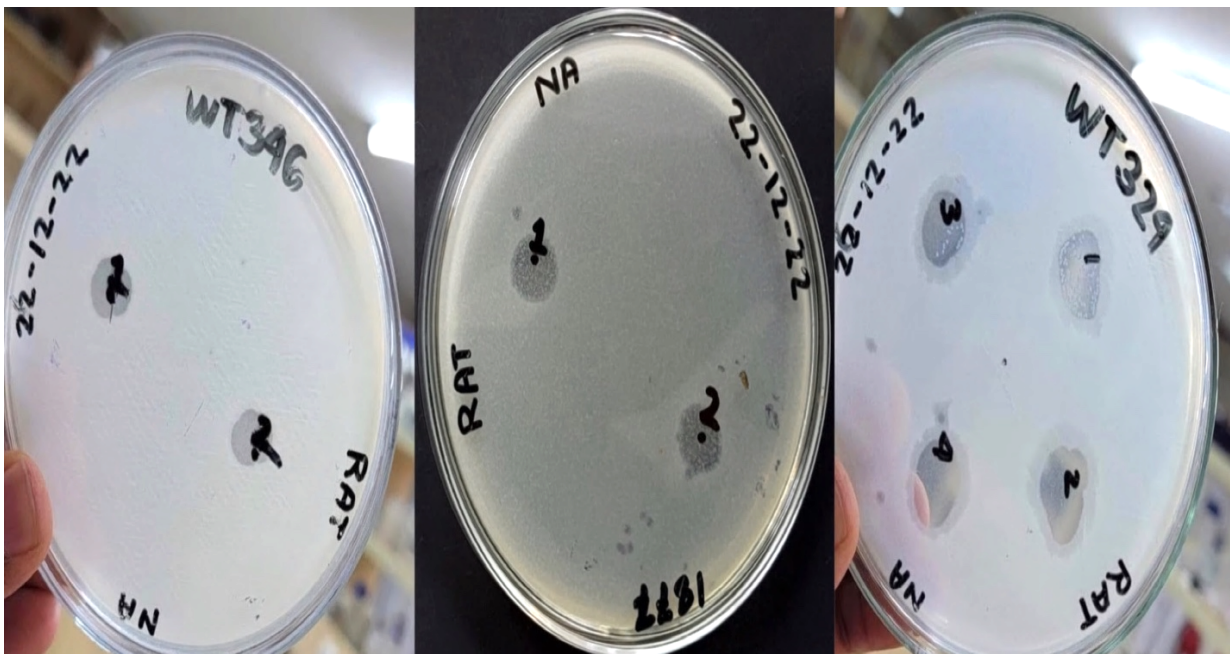


Figure 4.7 JSF2 positive result

4.2.4 Spot test result of JSF2 phage negative strain: There are 9 strains were found where JSF2 phage was not able to make clear zone.

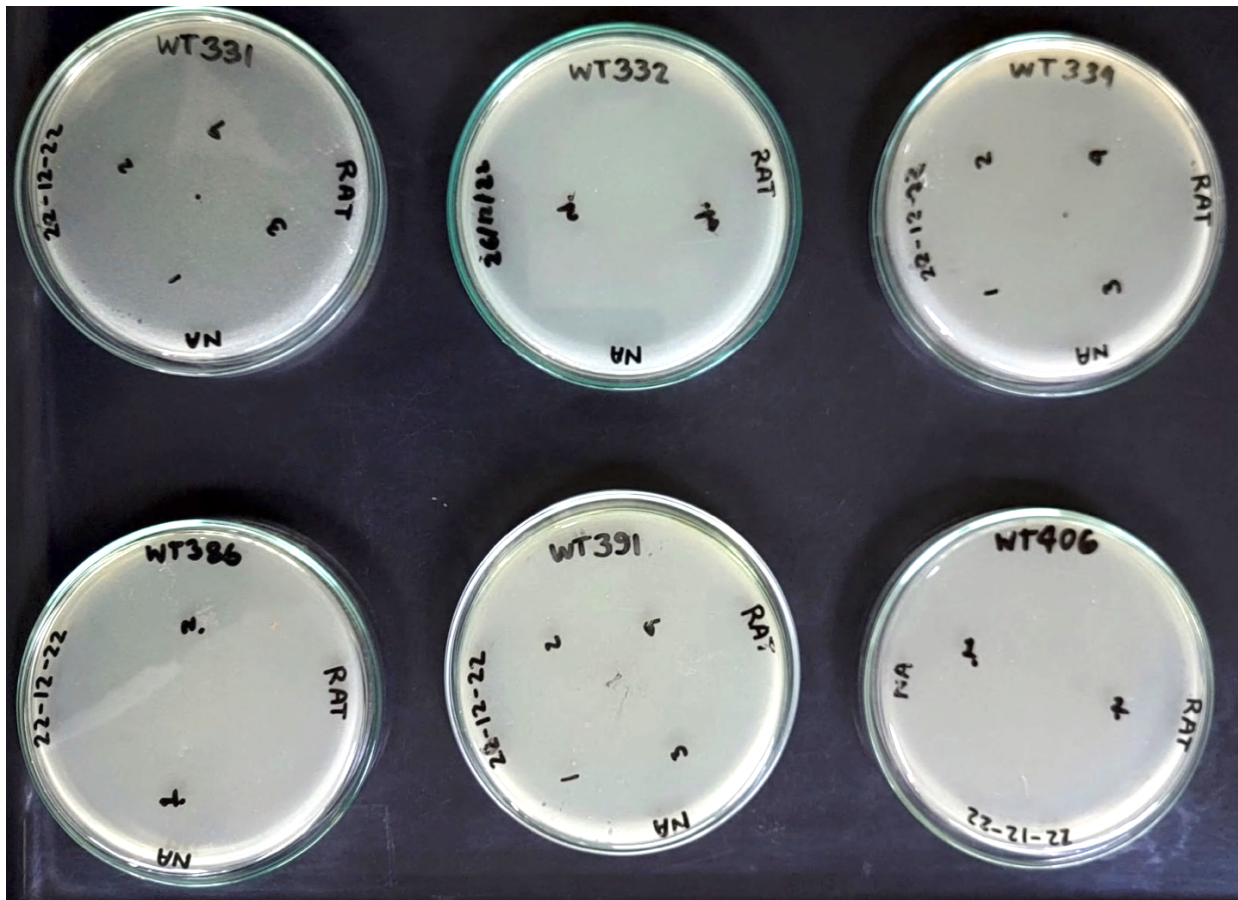


Figure 4.8 Negative strains of JSF2 phage

4.3 Double Layer Agar Assay: We have already got to know JSF2 and *E. coli* phage is accordingly virulent to 1877, WT324 and WT346 and *E. coli* native strains. So, for conducting DLA, 1877 and *E. coli* native strains have been used.

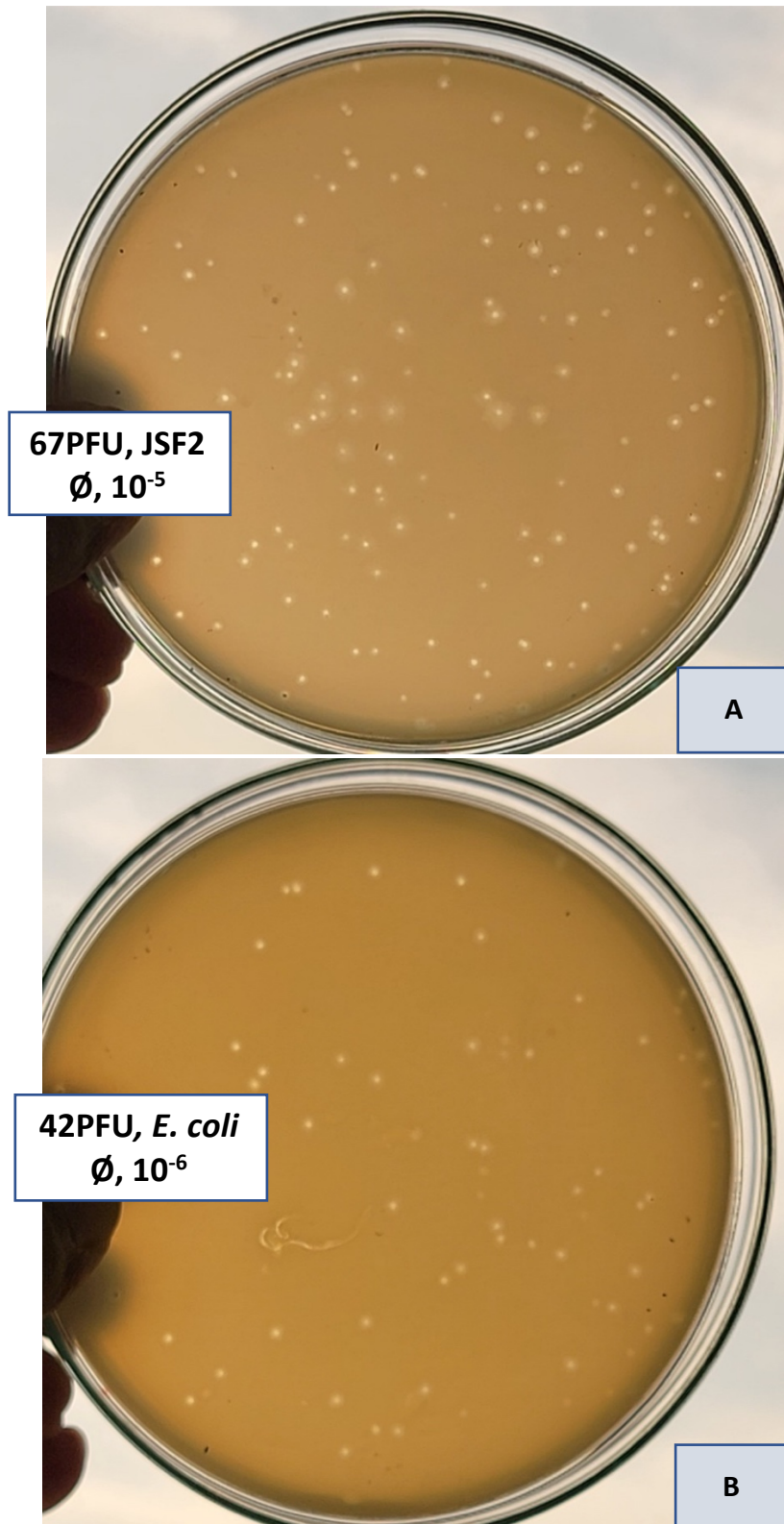


Figure 4.9: DLA of JSF2 (A) and *E. coli* Phage (B)

Results DLA of JSF2, *E. coli* phage are shown below (Table 4.3) which was conducted in between two months during the research period.

Table 4.3 Results of Double Layer Agar Assay

Enrichment numbers	JSF2		<i>E. coli</i>	
	Dilution	PFU	Dilution	PFU
1	10^{-2}	32	10^{-3}	42
2	10^{-6}	40	10^{-4}	46
3	10^{-4}	58	10^{-5}	52
4	10^{-5}	67	10^{-6}	62
5	10^{-7}	107	10^{-7}	102

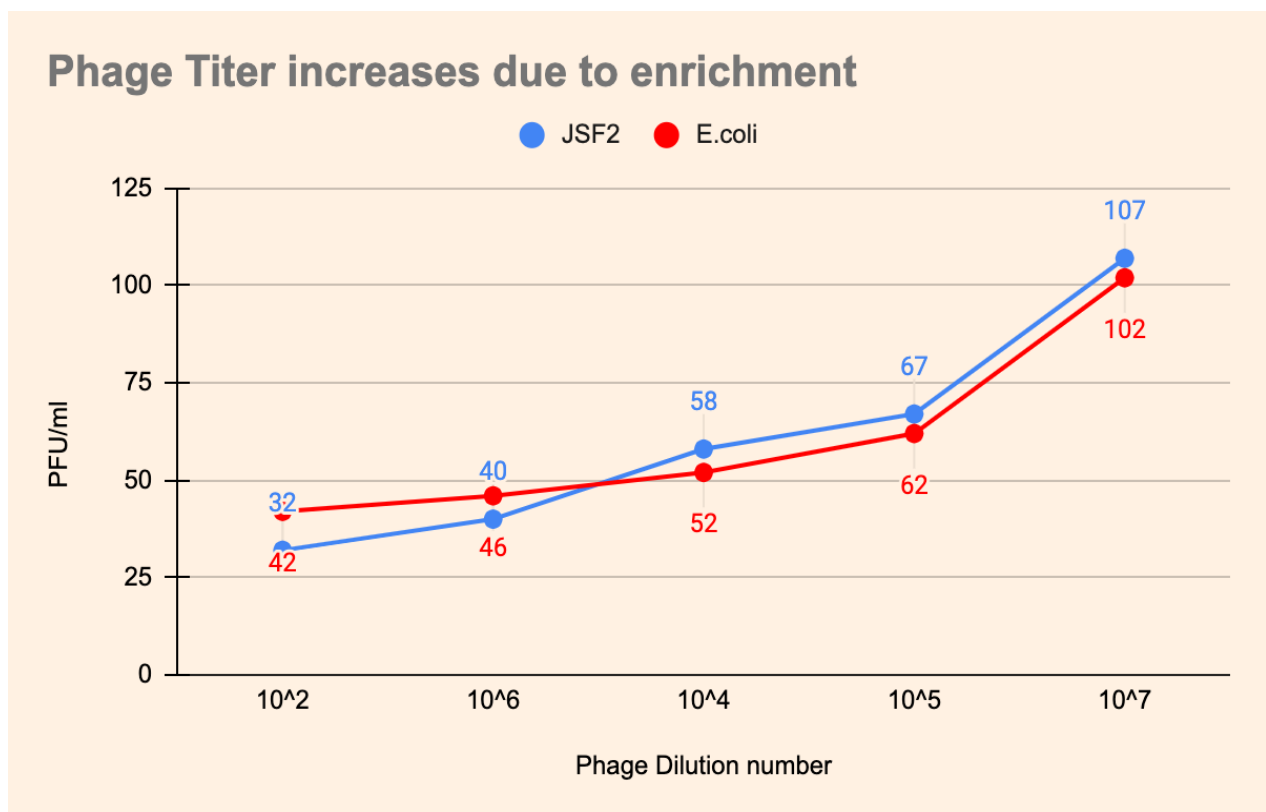


Figure 4.10: Compare DLA result of JSF2 and *E. coli* Phage

4.4 Phage Mediated Chromosomal Gene Transfer Results:

4.4.1 1877 to *E. coli*: 1877 is Kanamycin resistance strain from where the kanamycin resistance chromosomal gene has been transferred to the *E. coli*.

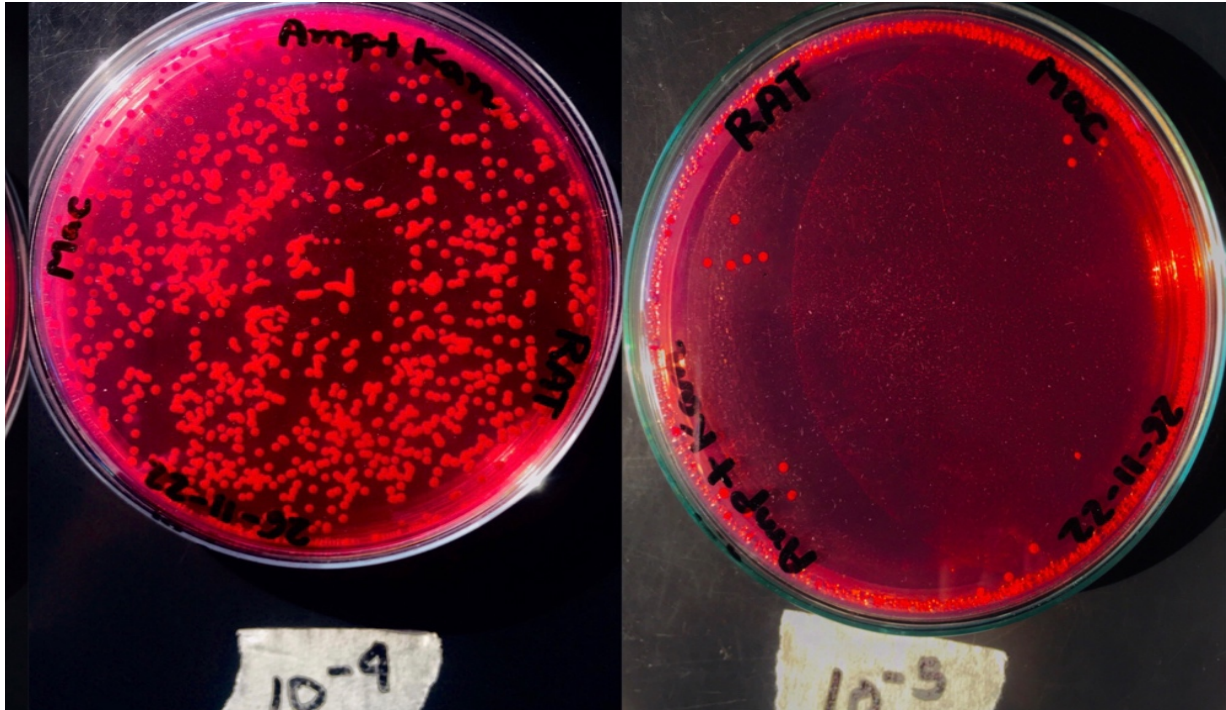


Figure 4.11: Interspecies chromosomal gene transfer from 1877 to *E. coli*

4.4.2 1877 to STEC: 1877 is Kanamycin resistance strain from where the kanamycin resistance chromosomal gene has been transferred to the STEC.

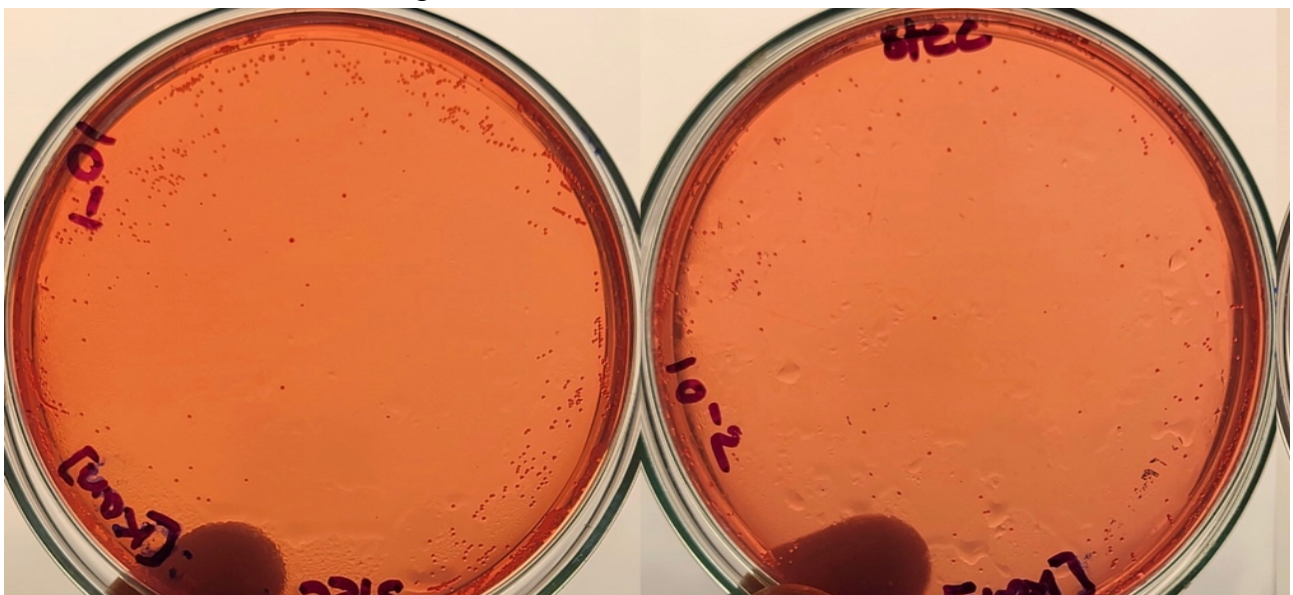


Figure 4.12: Interspecies chromosomal gene transfer from 1877 to STEC

4.4.3 1877 to WT331: 1877 is Kanamycin resistance strain from where the kanamycin resistance chromosomal gene has been transferred to the WT331.

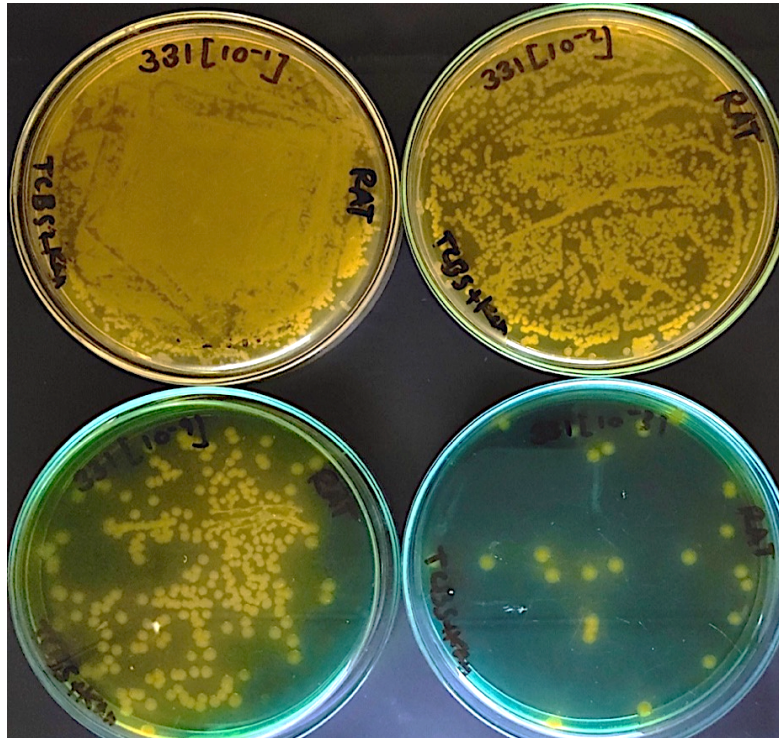


Figure 4.13: Intraspecies chromosomal gene transfer from 1877 to WT331

4.4.4 1877 to WT332: 1877 is Kanamycin resistance strain from where the kanamycin resistance chromosomal gene has been transferred to the WT332.

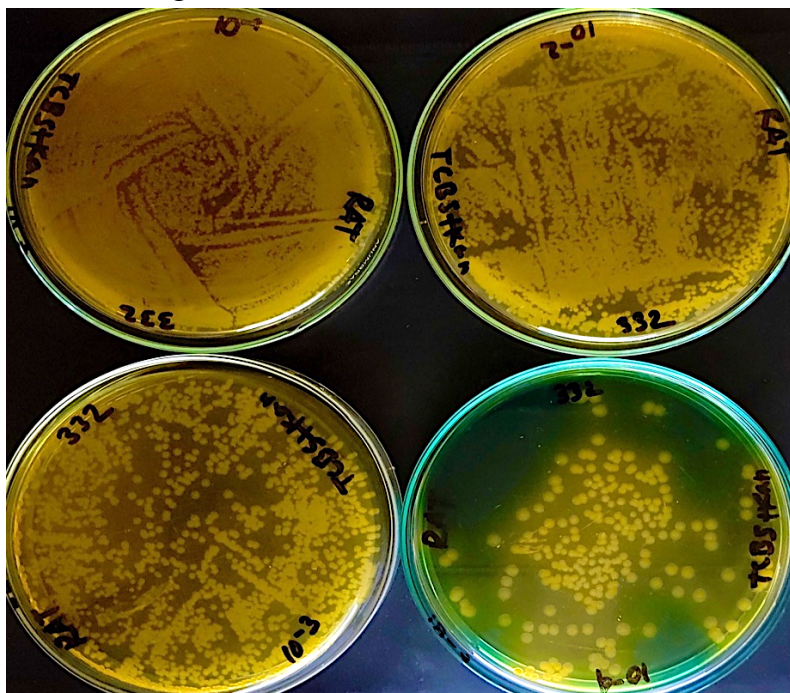


Figure 4.14: Intraspecies chromosomal gene transfer from 1877 to WT332

4.4.3 1877 to WT391: 1877 is Kanamycin resistance strain from where the kanamycin resistance chromosomal gene has been transferred to the WT391.

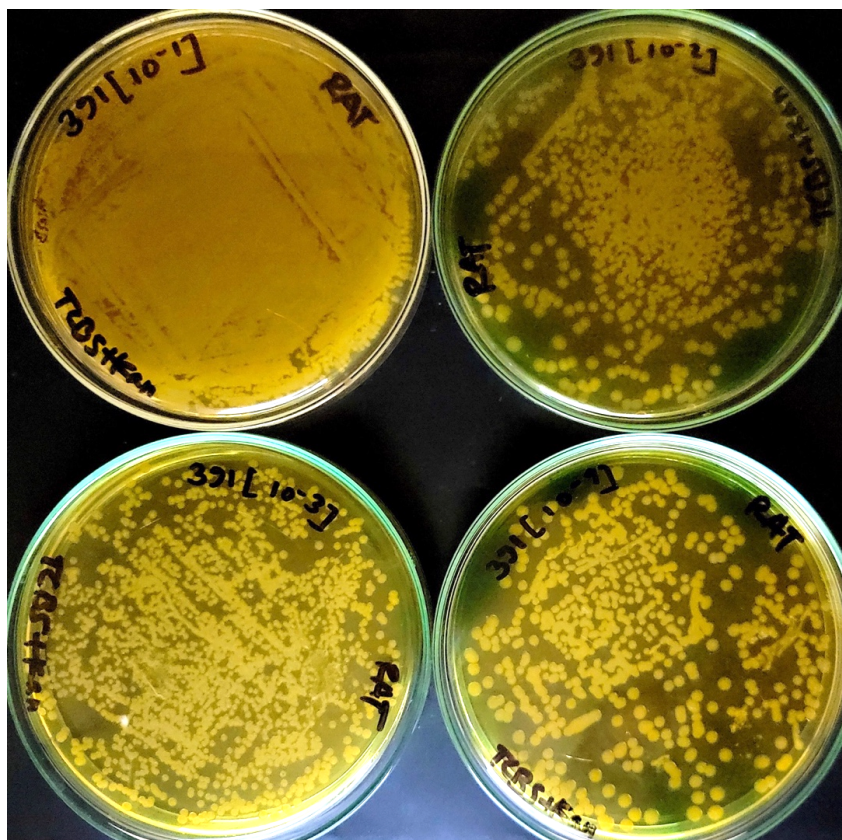


Figure 4.15: Intraspecies chromosomal gene transfer from 1877 to WT391

4.4.4 Transformation Rate: Interspecies and Intraspecies transformation rate after conducting the Phage Mediated Chromosomal Gene Transfer experiment.

Table 4.4 Transformation Rate

Modified Strains	Transformation Rate (CFU/13 ml of reaction)	Transformation Rate (CFU/ml of reaction)	Scientific Notation Transformation Rate (CFU/ml of reaction)
<i>E. coli</i>	9.74×10^{-6}	749230.7692	7.49×10^{-3}
STEC	2.45×10^{-4}	1884.615385	1.88×10^{-5}
WT331	3.43×10^{-5}	263846.1538	2.64×10^{-6}
WT332	2.76×10^{-6}	212307.6923	2.12×10^{-3}
WT391	4.9×10^{-6}	376923.0769	3.77×10^{-5}

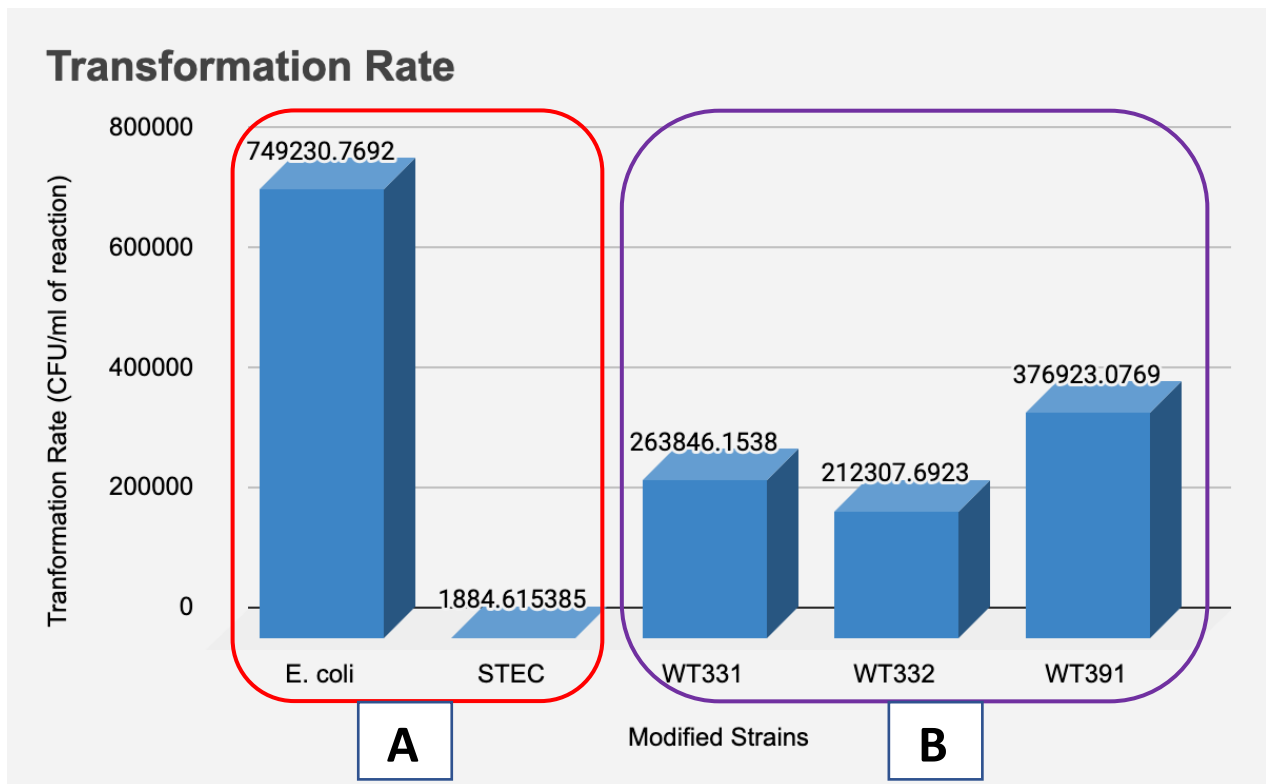


Figure 4.16: Transformation Rate (CFU/mL)
(A) Interspecies Transformation Rate, (B) Intraspecies Transformation Rate

4.5 Compare the Kirby-Bauer Disk Diffusion Susceptibility Test and between Native and modified bacterial strains:

4.5.1 Native *E. coli* vs Modified *E. coli*: Native *E. coli* was kanamycin sensitive and *E. coli* phage were able to cut the native strain. On the contrary, Modified Strain of *E. coli* become kanamycin resistant and won't be able to cut by the *E. coli* phage.

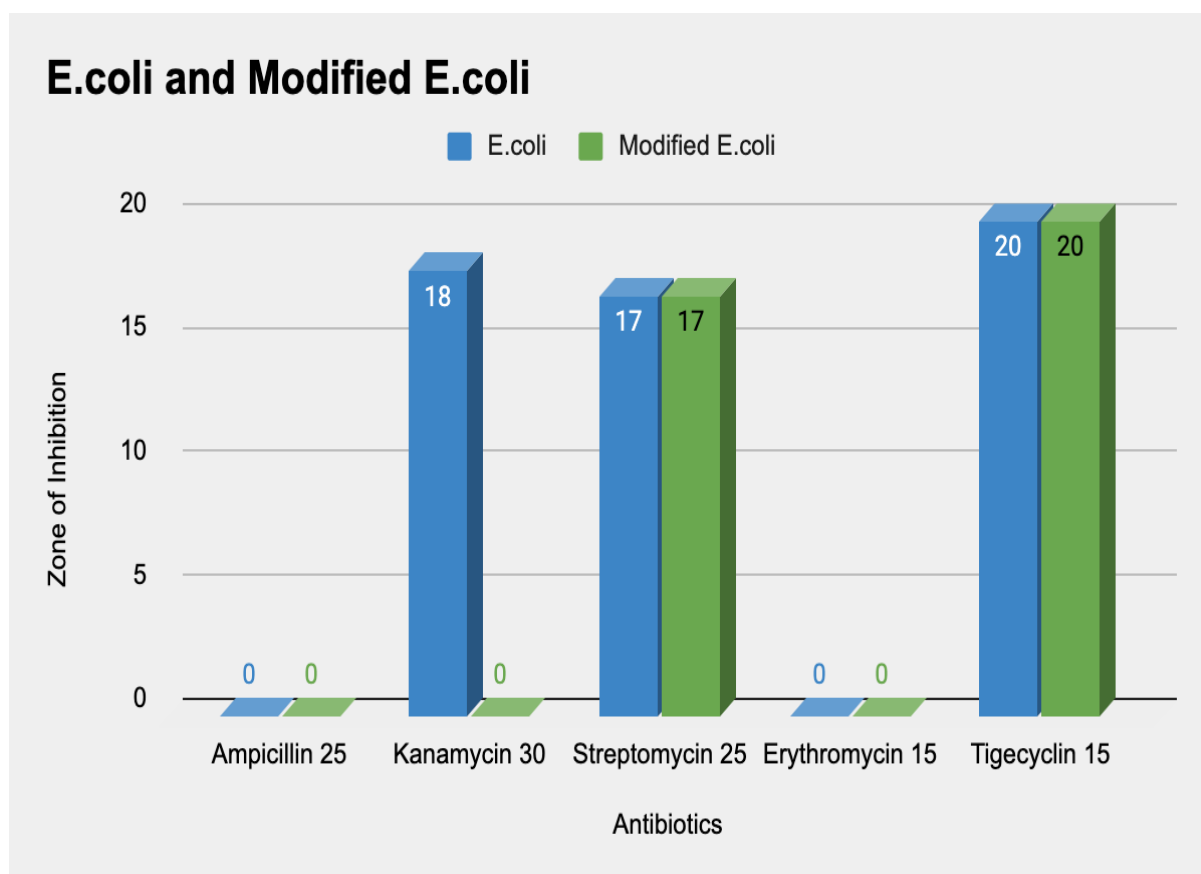
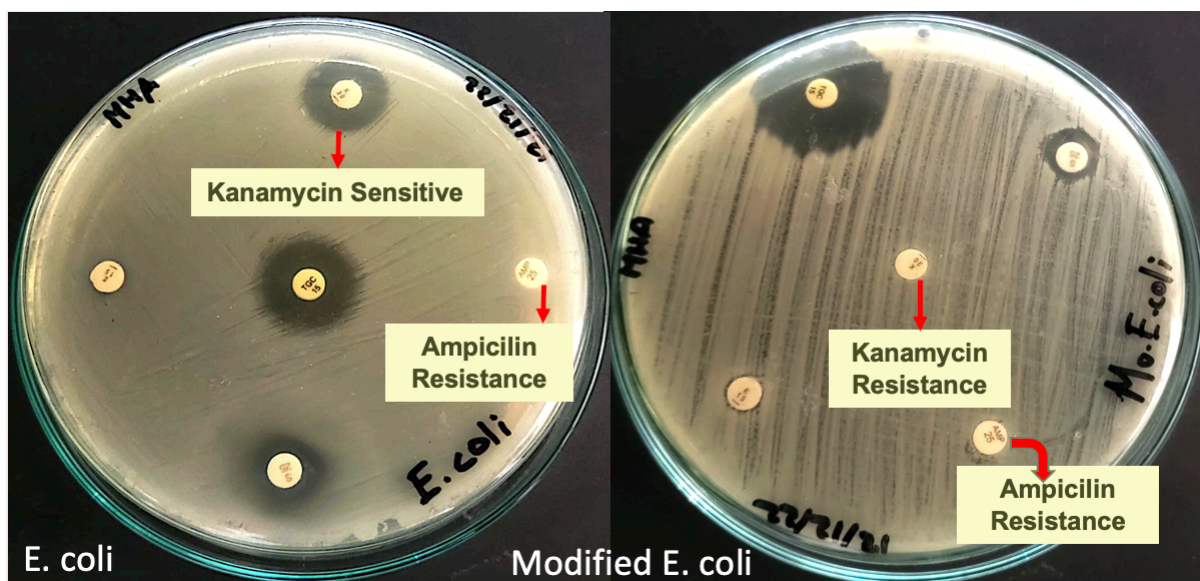


Figure 4.17 Antibigram result of Native vs Modified *E. coli* Strain

4.5.1 Native STEC vs Modified STEC: Native STEC was kanamycin sensitive. On the contrary, Modified Strain of STEC become kanamycin resistant.

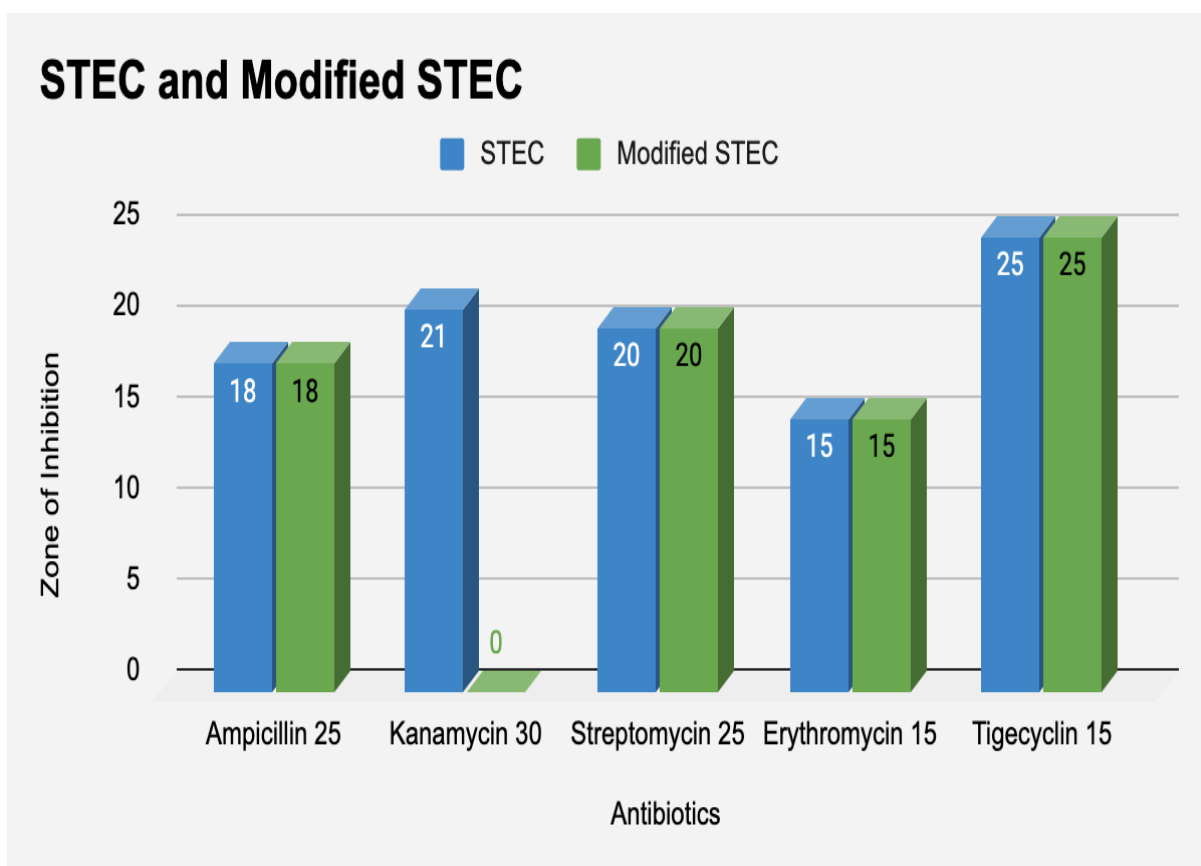
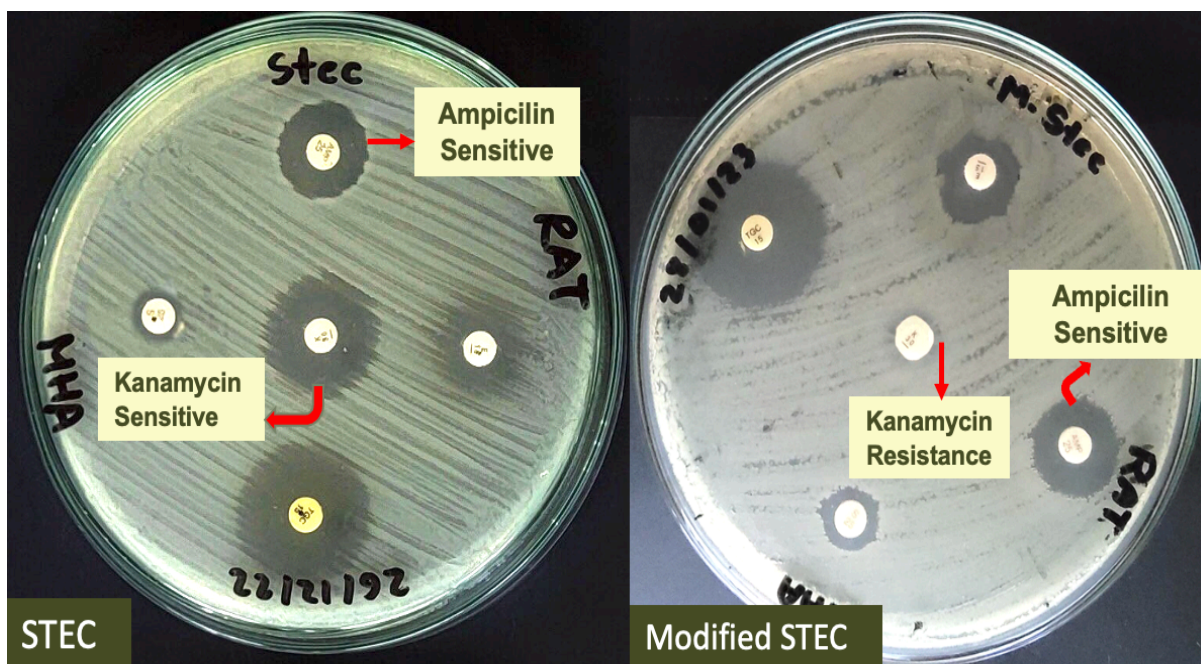
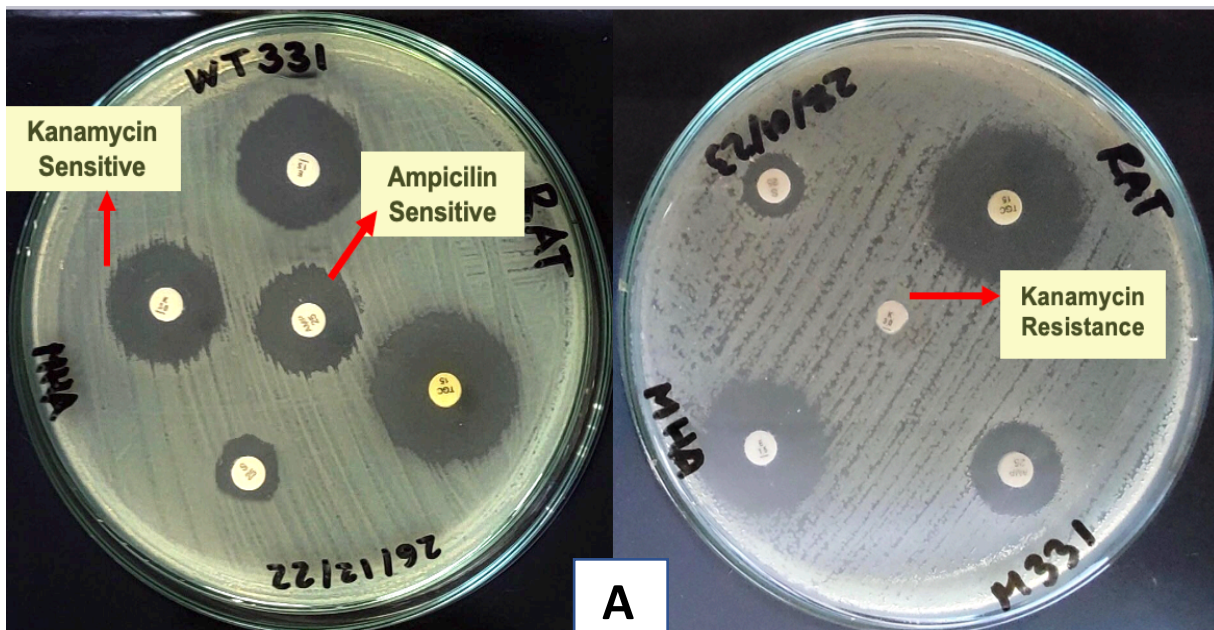


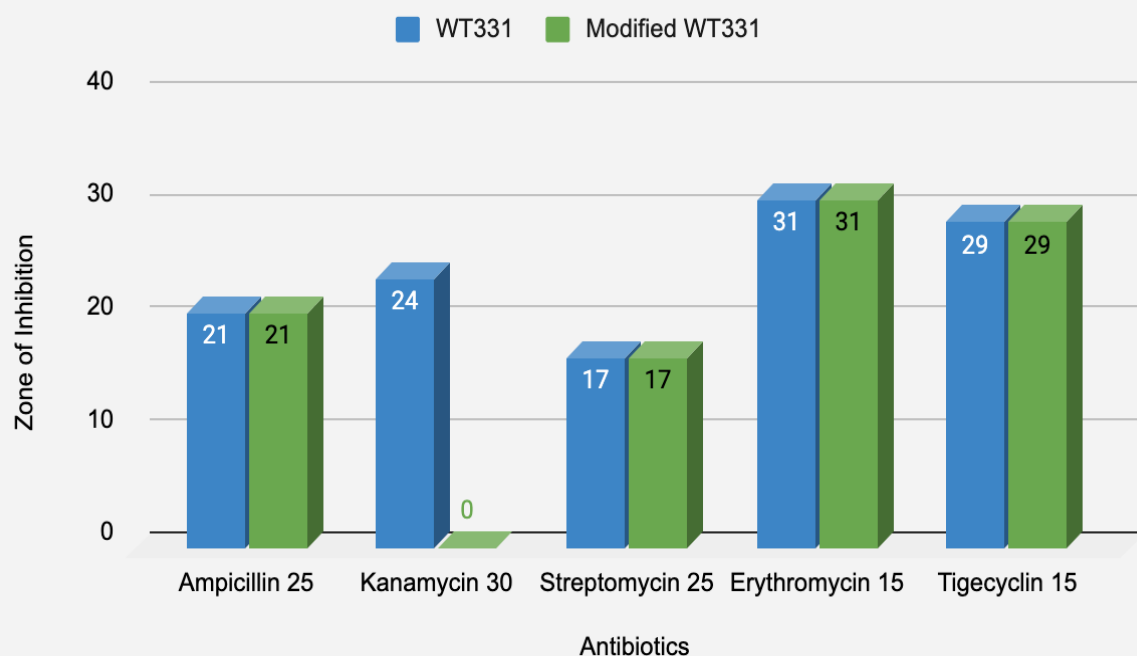
Figure 4.18 Antibiogram result of Native vs Modified STEC Strain

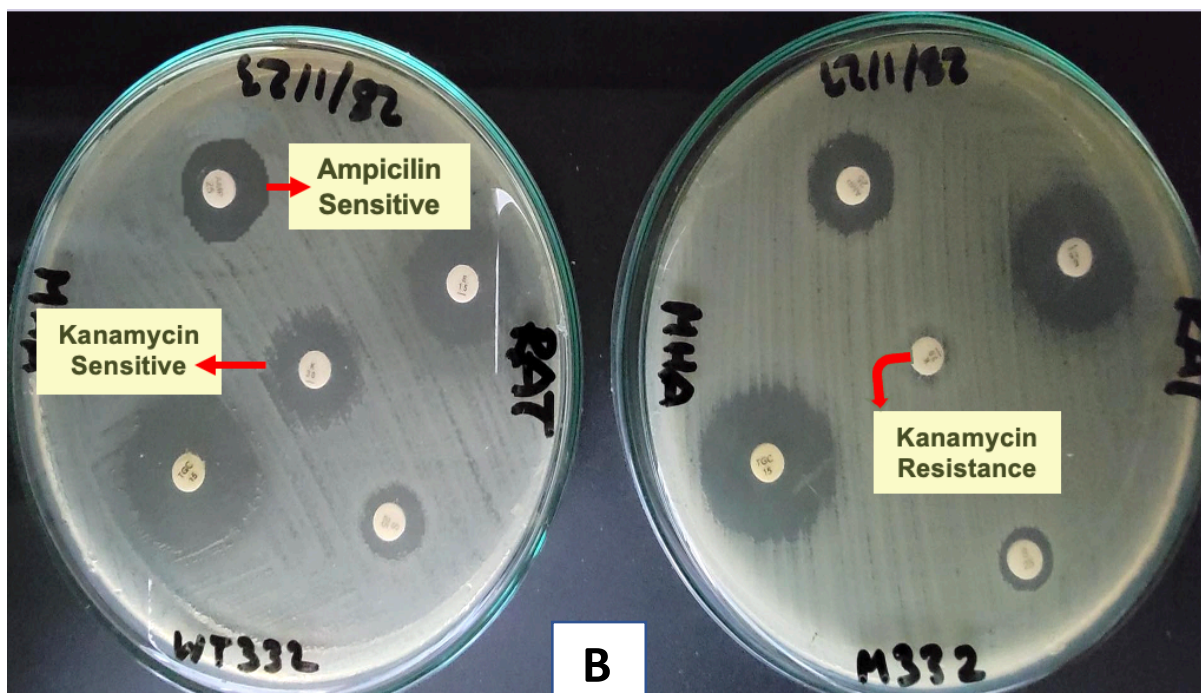
4.5.3 Native WT331, WT332, WT391 vs Modified WT33, WT332, WT391:

Native WT331 was kanamycin sensitive and JSF2 phage wasn't able to cut the native strain. On the contrary, Modified Strain of WT331 become kanamycin resistant and will not be able to cut by the JSF2 phage.

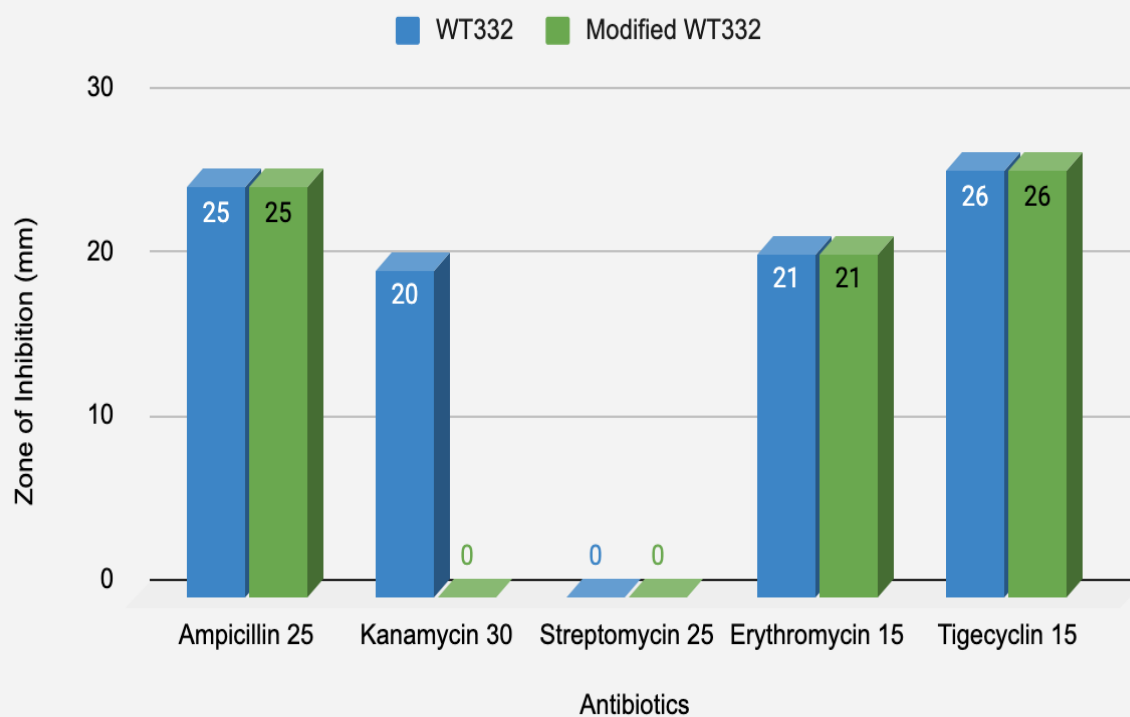


WT331 and Modified WT331





WT332 and Modified WT332



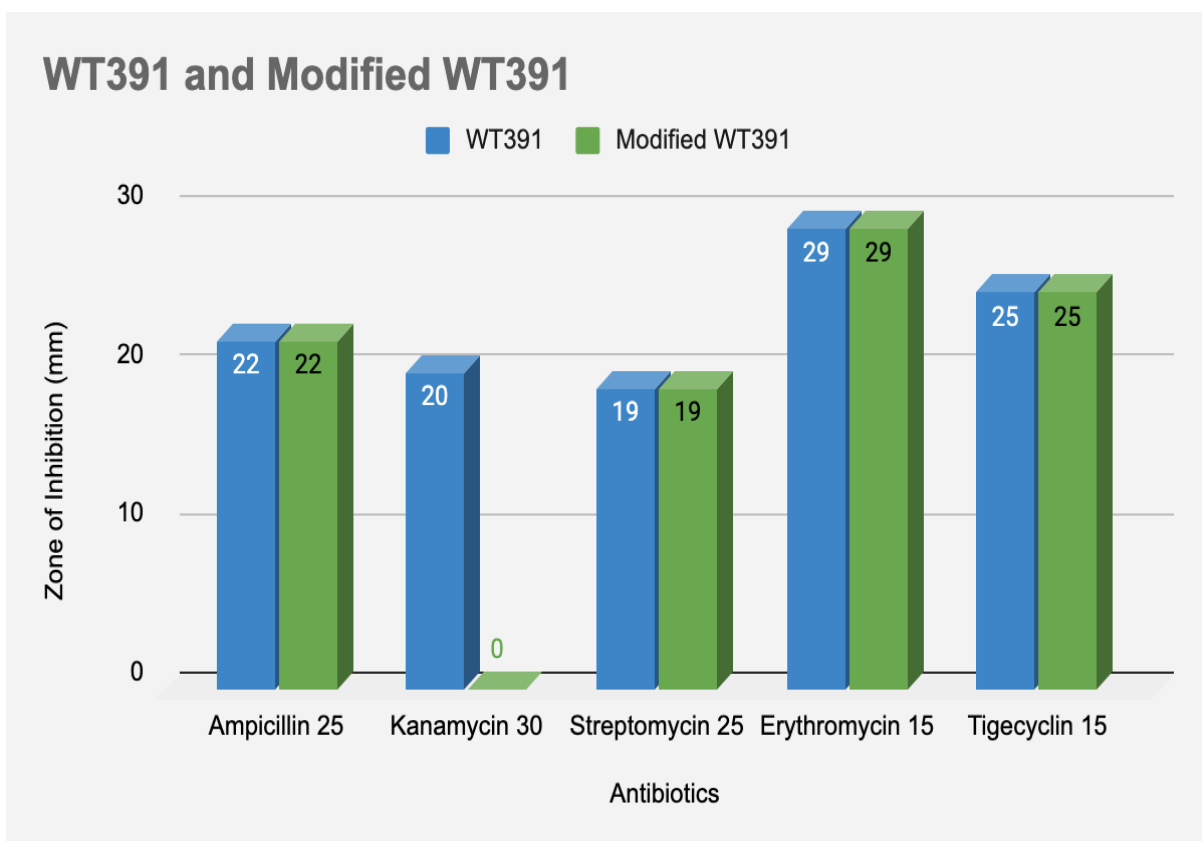
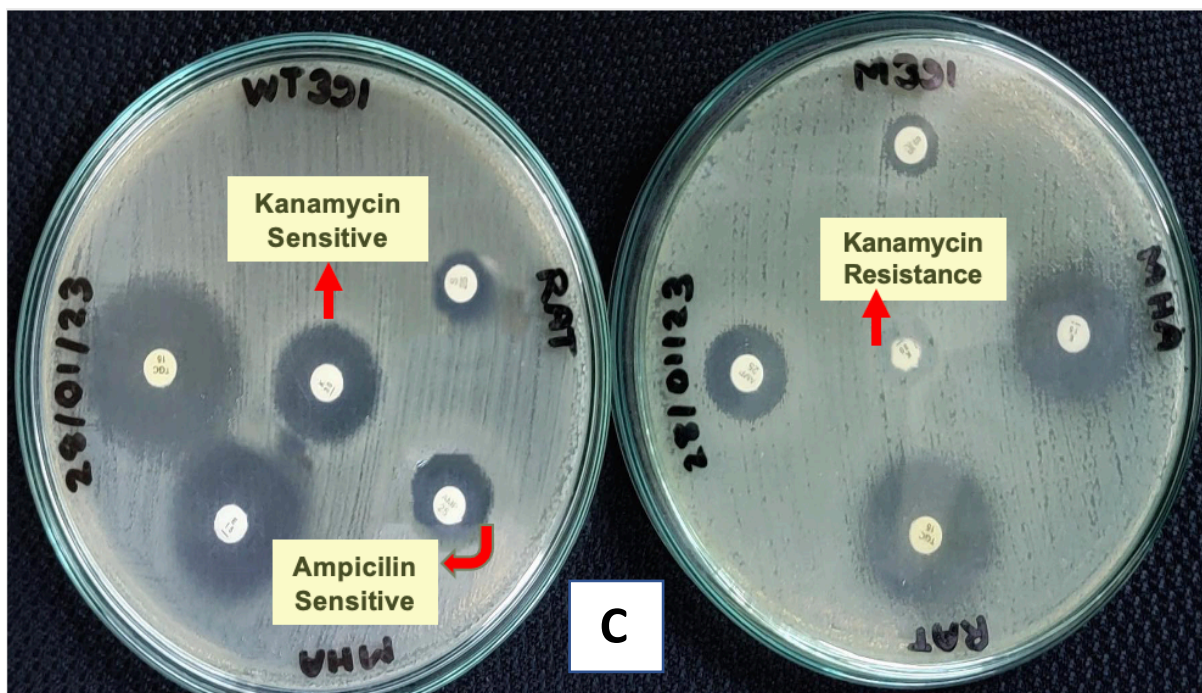


Figure 4.19: Antibiotogram result of Native and modified strain
 (A) WT331 vs Modified WT331, (B) WT332 vs Modified WT332, (C) WT391 vs Modified WT391

4.6 Compare the Spot Test for the confirmation of Plaque in between Native and Modified bacterial strains

4.6.1 Native vs Modified *E. coli*: Though the native strain was virulent to the *E. coli* phage, kanamycin resistance modified *E. coli* strain is non-virulent to the *E. coli* phage

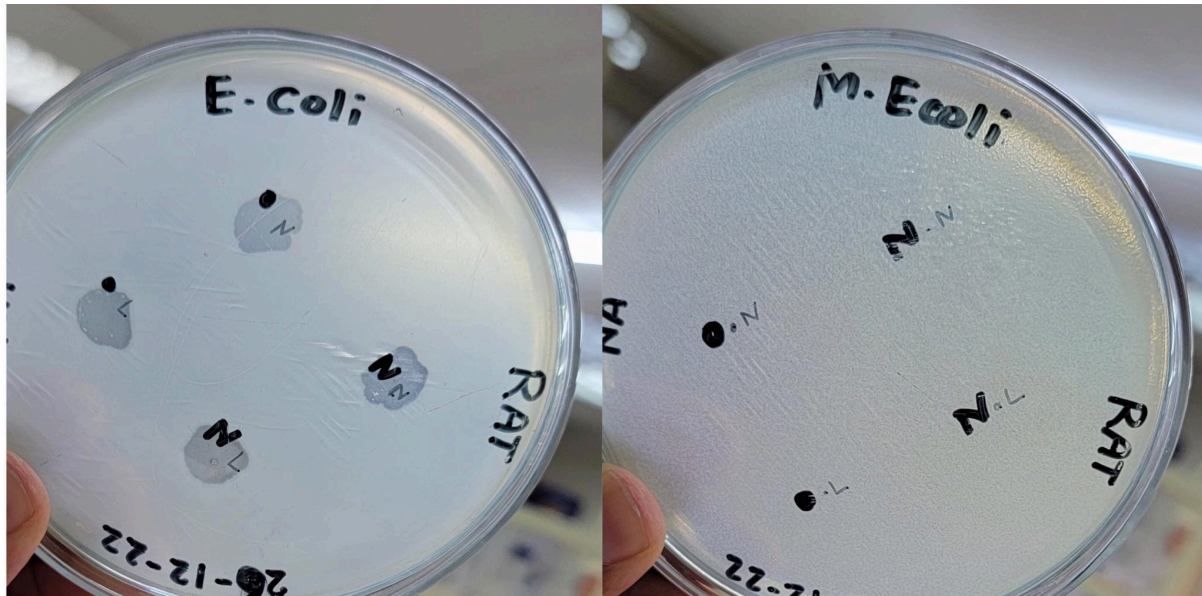
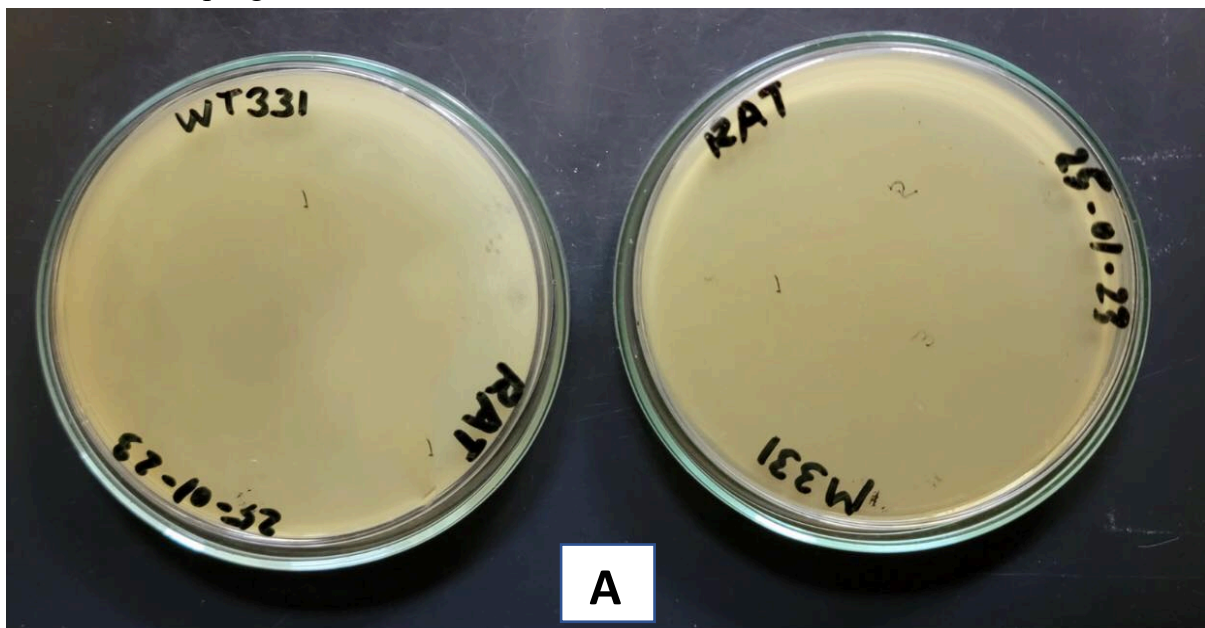


Figure 4.20: *E. coli* phage create plaque only on native *E. coli*.

4.6.2 Native WT331, WT332, WT391 vs Modified WT33, WT332, WT391: In case of intra-species transformation there is no change happened in case of virulency against bacteriophage.



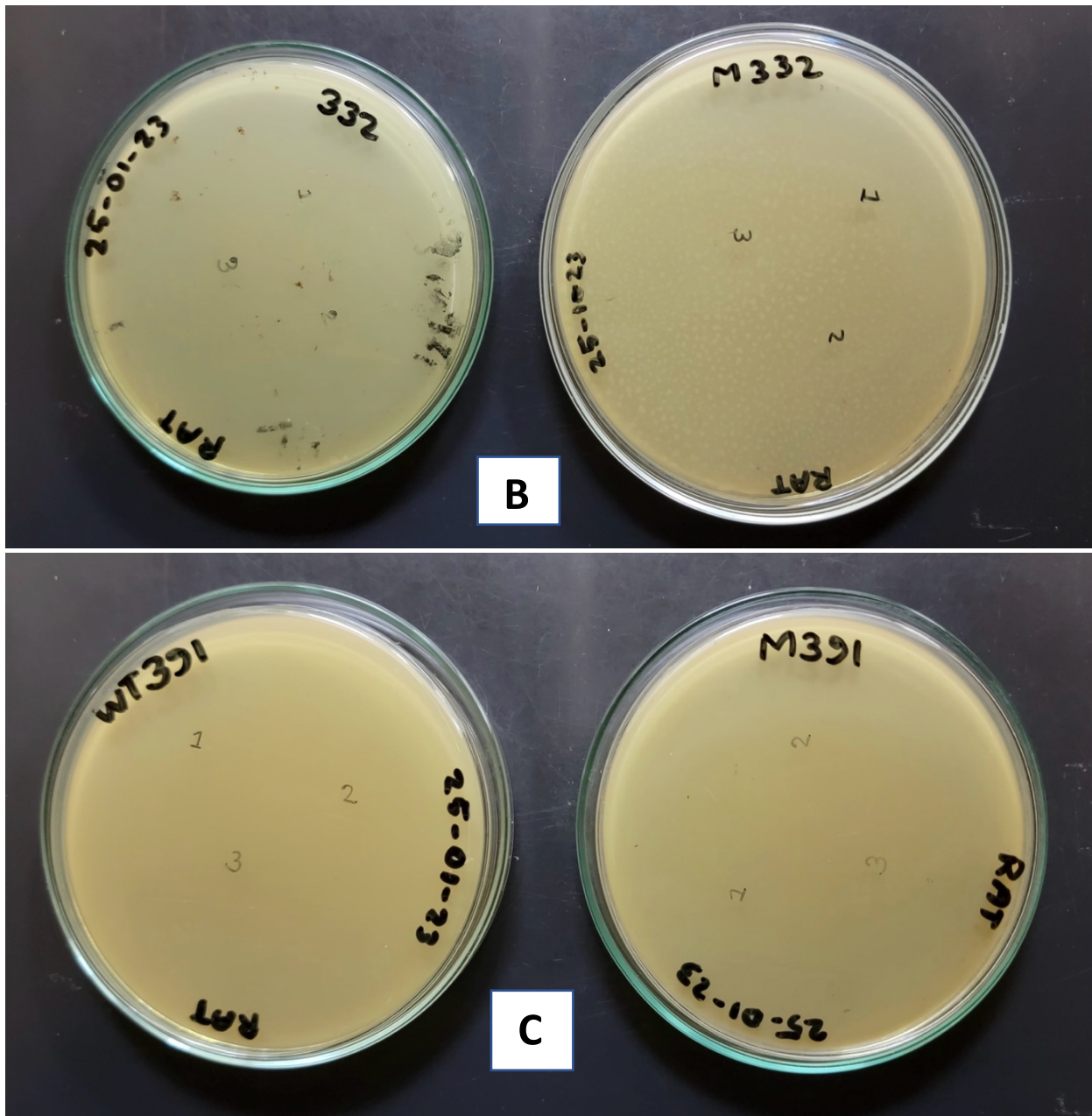


Figure 4.21: Spot test result of Native and Modified strains

(A) WT331 vs Modified WT331, (B) WT332 vs Modified WT332, (C) WT391 vs Modified WT391

4.7 Transformation of competent *E. coli* cells with modified bacterial Plasmid:

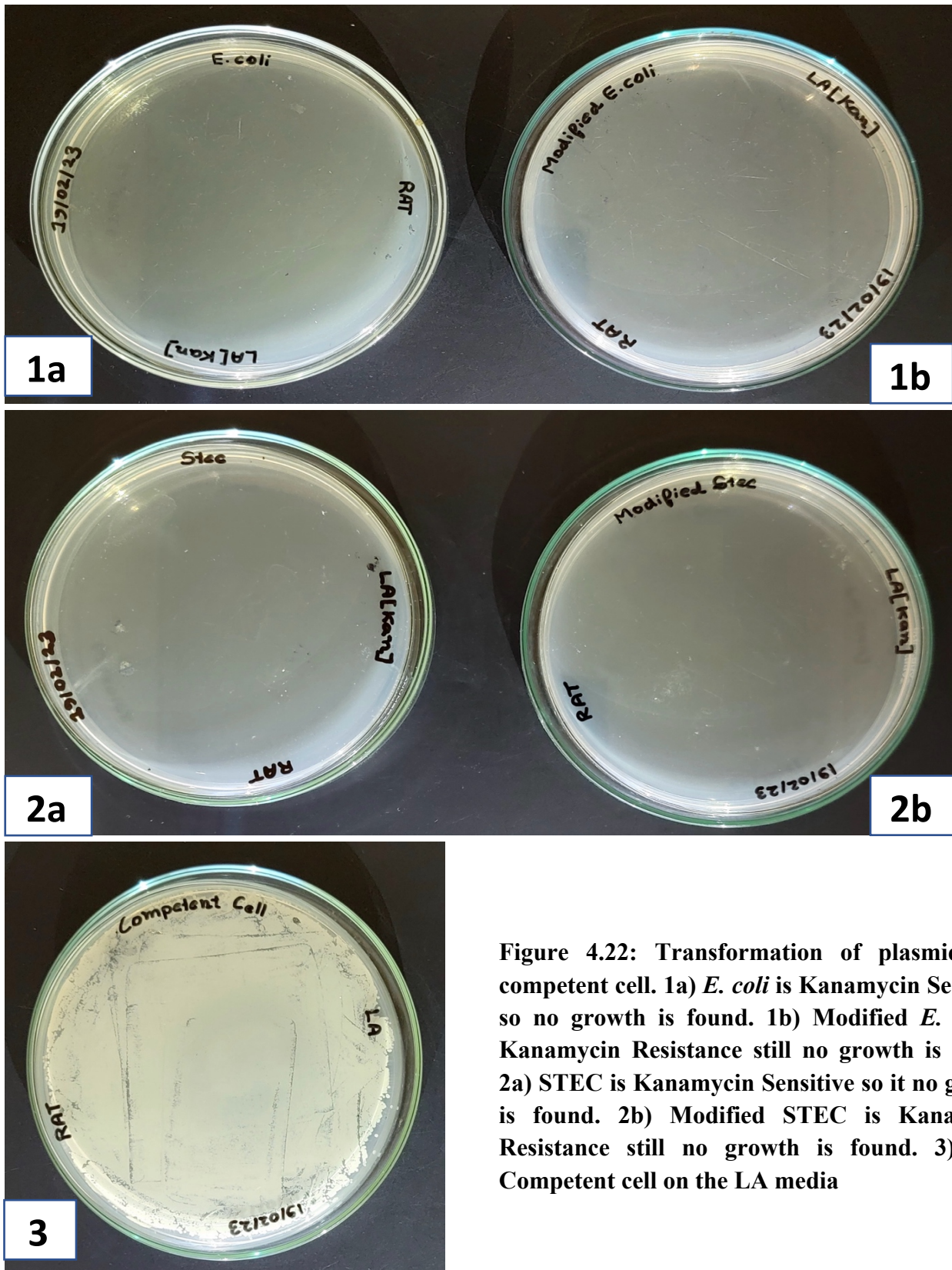


Figure 4.22: Transformation of plasmid into competent cell. 1a) *E. coli* is Kanamycin Sensitive so no growth is found. 1b) Modified *E. coli* is Kanamycin Resistance still no growth is found. 2a) STEC is Kanamycin Sensitive so it no growth is found. 2b) Modified STEC is Kanamycin Resistance still no growth is found. 3) Raw Competent cell on the LA media

CHAPTER 5: DISCUSSIONS

Bacteriophage is one of the most important tools for transferring the foreign genetic material into the target bacterium and it always considered as the transduction. But it also can be worked in different-way except transduction. In this study, bacteriophage use as a tool to lyse the host bacteria so that it could release the genetic material to come out of it so that another bacterium present on the system could capture it. To support the statement different experiments has been conducted, and they are explained with reasons below.

5.1 The Kirby-Bauer Disk Diffusion Susceptibility Test for Native

Strain: This test determines the susceptibility of bacteria isolated from a patient's infection to clinically approved antibiotics which is mean this culture-based microbiology assay used in diagnostic and drug discovery laboratories (Sharma, 2021). Antibiotic is an antimicrobial chemical that fight against bacterial infections and it produced by microorganisms against other microorganisms. Most importantly, bacteria respond in two different ways to antibiotics even within the same species. Like some bacteria are resistance to some antibiotics that means they can grow in the presence of that specific bacteria. But in some cases, for that specific antibiotic some bacteria are sensitive means they can't grow in the presence of that antibiotic (National Library of Medicine, n. d.).

From the result of susceptibility test we can see that *E. coli* is Kanamycin sensitive and Ampicillin Resistant. Moreover, *STEC* is both Kanamycin and Ampicillin sensitive. Furthermore, *1877* is Kanamycin resistant and Ampicillin sensitive. In addition, *WT331*, *WT332*, *WT391* are both Kanamycin and Ampicillin sensitive.

Here we have used the published CLSI guidelines and these charts are different for different organisms. From the chart we got to know that for each drug, indicate on the recording sheet whether the zone size is susceptible (S), intermediate (I), or resistant (R) based on the interpretation chart (CLSI, 2020).

The *E. coli* showed the Zone of inhibition for Ampicillin-25 is 0mm that means it is resistant. Moreover, for Kanamycin-30 the ZOI is 18mm. On the other hand, *STEC* showed the ZOI for Ampicillin-25 and Kanamycin-30 is accordingly 18mm and 21mm. The *1877* strain showed ZOI for Ampicillin-25 and Kanamycin-30 accordingly 15mm and 0mm. In addition, *WT331*, *WT332*, *WT391* showed the ZOI against Ampicillin accordingly 21mm, 25mm, 22. Furthermore, they manifest the ZOI against Kanamycin-30 accordingly 24mm, 20mm, 20mm.

From the standard guideline of CLSI, we can say that Though *E. coli* strain is Ampicillin-25 resistance, *1877*, *WT331*, *WT332*, *WT391* are Ampicillin-25 sensitive. On the other hand, rather than *1877* all the other strains like *E. Coli*, *WT331*, *WT332*, *WT391* are Kanamycin sensitive.

From figure-4.1 we can see that different strains of bacteria shows different level of resistance or sensitivity against the same or different bacteria. For example, among all the strain WT331 is comparatively one of the most sensitive strains against Erythromycin. On the other hand, *E. coli* is less sensitive. Moreover, both WT324 and 031 are Ampicillin and Erythromycin resistance. Furthermore, 1877 and WT332 are kanamycin resistance. In addition, it is noticeable that all the strains are Tigecycline sensitive (Fig 4.1).

Here, the organism contain sample made with LB for maintaining the osmotic pressure and adjust the turbidity of this suspension. Then the culture inoculated into the MHA agar because it is a non-selective, non-differential medium. As it contains starch the media can absorb toxins released from bacteria, so that they cannot interfere with the antibiotic's properties. It also mediates the rate of diffusion of the antibiotics through the agar because it is a loose agar.

Following incubation, measure the zone sizes to the nearest millimeter using a ruler or caliper; include the diameter of the disk in the measurement. During measuring zone diameters, always round up to the next millimeter. All measurements are made with the unaided eye while viewing the back of the petri dish. Hold the plate a few inches above a black, nonreflecting surface illuminated with reflected light.

Finally, based on this this test, we primarily do confirm and select which strain should be the host and which one would be the target.

5.2 Spot test for the confirmation of Plaque: The spot test method is used to ascertain virus's host range and minimum infectious titer, which differs depending on the strains (Magin et al., 2019). This assay is usually done before the titer determining double-layer agar assay. It helps to know how much the bacteriophage is virulent to infect the strain and what is the life cycle of that phage for that specific strain of host.

Here, in this study we have used two different kind of phages that are JSF2 and *E. coli* Phages. And we have conducted the spot test with both of the phages for all the 12 strains that have been used in this study. And from the table-4.2 we already know that JSF2 is virulent for 1877, WT346, WT324 strains where it is follows the lytic life cycle. Moreover, *E. coli* phage is one and only virulent for *E. coli* native strain and it follows the lytic life cycle here.

In this experiment, for each of the bacterial strains, 3 ml of freshly prepared LB was added individually, and the vials were incubated on a shaker for an additional 2 hours. After this incubation period, large LA plates were obtained and labeled with the corresponding bacterial strain. Soft agar aliquots, which had been stored in a refrigerator, were melted by boiling at 52°C. Once the soft agar had melted, a 200 µl sample of the young individual bacterial culture was added to it after a 15 second delay. The resulting solution was then mixed thoroughly. To create a bacterial lawn, the appropriate

LA plate was taken and the contents of the tube were poured onto the LA agar surface. The plate was gently swirl to ensure even distribution of the mixture. The plates were marked with numerals representing the bacteriophage sample numbers after the soft agar layer had dried. The top layer of agar was covered with 15 µl of the purified phage and the plate was left to dry for 30–40 minutes. The plates were held at 37° C overnight after the drops had dried. The following day, the samples' clean zones were examined.

Finally, this experiment also helps to determine which strain would the best fit as target and host for our study.

5.3 Phage titer determination by Double Layer Agar Assay: In phage research, the Double Layer test, sometimes referred to as the plaque test, is a frequently used method for isolating and identifying pure bacteriophage populations. This method is frequently advised for determining phage titer. Plaques on the plates are counted first to determine the titer of the particular phage, and the titer is then calculated using the PFU equation.

Therefore, the 1877 strain has been chosen as the target bacteria from all the antibiogram and spot test results. In particular, 1877 is virulent to the JFS2 phage and resistant to kanamycin. Additionally, STEC and *E. coli* both come from different families than in 1877. So, we used these two to create the interspecies environment. Furthermore, WT331, WT332, WT391 and 1877 both of them are belong to same species so they are used to conduct the intraspecies modification experiment.

5.4 Phage Mediated Chromosomal Gene Transfer Protocol: The major experiment for our investigation is this one. In this case, the bacteriophage was indispensable in transmitting the chromosomal gene for kanamycin resistance from the host bacterium 1877 to the target bacteria, including *E. coli*, STEC, WT331, WT332, and WT391. The modification is characterized as an "Interspecies Modification" (Figure: 4.10 & 4.11) in the case of *E. coli* and STEC and an "Intraspecies Modification" (Figure: 4.12-4.14) in the case of WT331, WT332, and WT391. In this experiment, the host bacteria and the bacteriophage were cultured together in a liquid medium so that the bacteriophage could lyse the host and release the genetic material into the medium. The target bacteria are then added, and they are cultured together so that the target bacteria can absorb the foreign genetic material. The changed bacteria are then cultured separately on a particular medium, and additional antibiograms and spot tests are carried out to confirm the altered characteristics compared to the native strain.

After conducting the experiment, the number of colonies from various dilution plates for each of the targeted strains has been counted to create a diagram that actually shows the number of colony-forming bacteria per milliliter (CFU/mL) of each host and target sample solution. Figure 4.16 shows that in the case of interspecies, the transformation of *E. coli* is higher than that of STEC, whereas a stable transformation rate has been observed for intraspecies.

5.5 Compare the Kirby-Bauer Disk Diffusion Susceptibility Test Result between native and modified bacterial strains:

The disk diffusion susceptibility test for the modified bacterial colonies that we had obtained from the particular agar medium revealed only one difference between the modified and native strains. Figures 4.10–4.14 shows that all modified strains, such as modified *E. coli*, modified STEC, modified WT331, altered WT332, and altered WT391, have been transformed into kanamycin-resistant strains. Furthermore, the graphical displays make it abundantly evident that the changed strains only displayed modification in the case of the antibiotic kanamycin. It serves as an extremely simple yet crucial proof of the successful phage-mediated chromosomal gene modification.

5.6 Compare Spot Test for the confirmation of Plaque between native and modified bacterial strains:

For additional investigation, we performed the bacteriophage spot in order to determine whether the strains are still virulent to their particular phage or not. Figure 4.18 demonstrated that interspecies transformation occurred when the bacterial strain changed because the *E. coli* phage no longer recognizes the altered *E. coli*. Additionally, we were unable to conduct this experiment for STEC because we are unable to locate the STEC-specific phage in our lab. Furthermore, in the case of intraspecies transformation (Figure 4.19) JSF2 phage was specific for the native and modified strains.

5.7 Transformation of competent *E. coli* cells with modified bacterial Plasmid:

For the purpose of the research illustration, we have decided to obtain the plasmid of the modified bacterial strains. Because then the plasmid of the modified strains inserted into a competent cell and evaluate the transformation of that.

5.7.1 Plasmid Extraction: During plasmid isolation from the modified bacterial strains, we have used solution 1, 2, 3. Solution 1 contains Tris, EDTA, Glucose. Solution 2 contains NaOH and 1% SDS. Solution 3 contains Potassium Acetate and Glacial acetic acid. For extracting the plasmid many acidic as well as basic solution used so balancing the pH the tri buffer solution used here. For example, NaOH actually increase the pH up to 14 which leads to denature the DNA completely but it avoided by the Tris buffer solution of solution I. Moreover, glucose maintains the osmotic pressure of the experiment environment. Furthermore, solution III played a vital role here and it helped to renature the plasmid DNA faster than the chromosomal DNA because the chromosomal DNA attach to the salt and got precipitated. In addition, for extracting the purified circular DNA we did Phenol: Chloroform: Isoamyl alcohol (25:24:1) treatment. This step helps to remove the protein contamination and chloroform and isoamyl alcohol increase the gravitation force and increase the layer separation. The separated layer then collected and washed with the isopropanol so that the

remaining salt contamination removed from the plasmid DNA and store the extracted plasmid on TE Buffer at -20°C.

5.7.2 Competent Cell Preparation: At the same time, we treated competent cells with CaCl₂ to prepare them. This enables the bacterial cell to absorb plasmid DNA or other foreign DNA molecules. The process of treatment entails cultivating the bacteria until the mid-log phase, at which point they are harvested by centrifugation. In order to get the cells ready for DNA absorption, a CaCl₂ solution is used to wash the cells. The cells are then resuspended in a cold CaCl₂ solution that contains the desired DNA. To further increase the permeability of the bacterial cell membrane, the suspension is next incubated on ice and subjected to a heat shock procedure. The membrane is momentarily damaged by the heat shock procedure, allowing the DNA to enter the cell. This process enables the bacterium to take up DNA from the environment or from a transformation experiment and incorporates it into their genome.

5.7.3 Transformation of the Modified Bacterial Plasmid: When the competent cells were prepared, the extracted circular DNA was combined with them in the presence of divalent cation calcium chloride, which facilitate the uptake of foreign DNA into the bacterial cell. A brief heat shock treatment, typically at 42°C for 60–90 seconds, is applied to the mixture of competent cells and DNA. This procedure helps to improve the permeability of the bacterial cell membrane, enabling the foreign DNA to enter the cell. The foreign DNA introduced antibiotic resistance genes that have the opportunity to express themselves when the converted cells increase their cells number in a nutrient-rich media like Luria Bertani Broth. After that, the modified cells can be chosen by plating them onto media with a specific antibiotic, which will only allow antibiotic resistance genes containing cells to survive. The transformed cells can be isolated by plating them onto media containing the appropriate antibiotic that only supports the development of cells that have incorporated foreign DNA.

But from Figure 4.20 it is clear that the kanamycin resistance gene won't be modified into the plasmid of the bacteria rather the modification happens into the chromosomal DNA. Because the isolated and transformed plasmid won't show the growth on the LA plate which contain the kanamycin antibiotics. *E. coli* & STEC both are Kanamycin Sensitive so it shows no growth on the kanamycin containing LA plate. Moreover, Modified *E. coli* & STEC both are Kanamycin Resistance but still no growth is found.

In a nutshell, the chromosomal gene of the target bacterium is transformed when the native kanamycin-sensitive bacteria ingest the kanamycin-resistant gene that is released by the bacteriophage lysis to the liquid culture of the experimental environment.

CHAPTER 6: CONCLUSION

The Bacteriophage is a widely distributed invader that can serve as a tool for both inter- and intra-species horizontal gene transformation. When the transformation occurs within the same species of bacteria, it is known as intraspecies transformation, while transformation between different species, such as from Vibrionaceae to Enterobacteriaceae, is referred to as interspecies transformation. The natural environment commonly seen the incorporation of genes through various means, including transformation, transduction, and conjugation. Bacteriophage-mediated gene transfer is another method of gene incorporation that is proved to exist by this study. It is allowing for the transfer of chromosomal genes between both the same and different species of bacteria. The experiment was designed with a specific goal in mind. The host bacterial strains were co-cultured with phages to enable the cutting and release of genetic material, which the target strain could then incorporate. Looking to the future, phage-mediated chromosomal gene transfer might be introduced as a novel mechanism with potential applications in, evolutionary, ecological gene therapy, and biomedical implications.

CHAPTER 7: REFERENCES

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CHAPTER 8: APPENDIX

Appendix – I: Media Compositions

All media used in this research were prepared according to the manual, and all these media components mentioned here were autoclaved at 121°C, 15 PSI for 15 minutes, except TCBS, for which no autoclave was needed. The composition of all the media used in the study is given below:

Nutrient Agar

Component	Amount (g/L)
Peptone	5.0
Sodium Chloride	5.0
Beef Extract	3.0
Agar	15.0
Final pH	7.0

Nutrient Broth

Component	Amount (g/L)
Peptic digest of animal tissue	5.0
Sodium Chloride	5.0
Beef Extract	1.5
Yeast Extract	1.5
Final pH	7.4 ±0.2 at 25°C

Luria Bertani Agar (LB Agar)

Component	Amount (g/L)
Casein enzymatic hydrolyte	10.0
Yeast Extract	5.0
Sodium Chloride	15.0
Agar	10.0
Final pH	7.5 ±0.2 at 25°C

Luria Bertani Broth (LB Broth)

Component	Amount (g/L)
Yeast Extract	5.0
Sodium Chloride	10.0

Tryptone	10.0
Final pH	6.9-7.1

MacConkey Agar

Component	Amount (g/L)
Peptic digest of animal tissue	1.5
Casein enzymatic hydrolysate	1.5
Pancreatic digest of gelatin	17.0
Lactose	10.0
Bile Salt	1.50
Crystal Violet	0.001
Neutral Red	0.03
Agar	15.0
Final pH	7.1 $\pm$ 0.2 at 25°C

TCBS Agar

Component	Amount (g/L)
Proteose Peptone	10.0
Yeast Extract	5.0
Sodium Thiosulphate	10.0
Sodium Citrate	10.0
Oxgall	8.0
Sucrose	20.0
Sodium Chloride	10.0
Ferric Citrate	1.0
Bromothymol Blue	0.04
Thymol Blue	0.04
Agar	15.0
Final pH	8.6 $\pm$ 0.2 at 25°C

Muller Hilton Agar

Component	Amount (g/L)
Beef, dehydrated infusion form	300
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH	7.3 $\pm$ 0.1 at 25°C

Appendix – II: Reagents and Buffers

Reagents:

Ampicillin supplement: 0.5ml ampicillin supplement per 99.5 ml LA media.

Kanamycin : 0.5ml kanamycin supplement per 99.5 ml LA media.

**Phenol: chloroform : isoamyl alcohol = 25:25:1,v/v Phenol:
chloroform=1:1**

Solutions:

Alkaline Solution I: pH 8.0. For 25 ml solution:

1M Tris-HCL	12.114%	3.03g
0.5M EDTA	14.6122%	3.65
1M Glucose	18.0156%	4.504

Solution was prepared using distilled water. In order to balance the pH NaOH was used.

Alkaline Solution II: For 10ml solution:

10N NaOH	40%	4ml
1% SDS	1%	100ul

Alkaline Solution III: pH 5.2 : For 25 ml solution

5M Sodium acetate	41.0172%	10.26g
Distilled water	-	14.74ml
Glacial Acetic Acid	-	Added drop by drop for pH- adjustment

1M CaCl₂ (Stock Solution): For 10ml solution:

CaCl ₂	1.4702g
Distilled water	8.5298ml

0.1M CaCl₂ (Working Solution):

1M CaCl ₂ stock solution	1 ml
Distilled water	9ml

Buffers:

TE Buffer: pH 8.0. For 25 ml solution:

10 mM Tris	0.12114%	0.0303g
1mM EDTA	0.029%	0.00735g
Distilled water	-	24.96 ml

0.5M EDTA: pH 8.0. For 100ml solution:

EDTA	18.61g
Distilled water	81.39ml

Appendix – III: Instruments Used

Autoclave	Wisd Laboratory Instruments, Made in Rep. of Korea. TOMY ES-315, Japan.
Electronic Balance	Model: WTB 200, RADWAG Wagi ELEktroniczne; Model: XINGYUN JA-503, China.
Incubator	Model: DSI 3000; Digisystem Laboratory Instruments Inc. Made in Taiwan.
Microcentrifuge	Model: MC-12, Benchmark Scientific; MiniSpin Plus 5433, Eppendorf, North America.
Refrigerated Microcentrifuge	Model: ScanSpeed 1730R Labogene
Refrigerator	Brand: Samsung, Siemens.
Shaking Incubator	Model: JSSI-1000C; JS RESEARCH INC. Made in Rep. of Korea
Spectrophotometer	Model: UVmini-1240 UV-VIS Spectrophotometer SHIMADZU Corp.
Syringe Filter	MS® MCE Syringe Filter Membrane Solutions, LLC
Micropipettes	Finnpipette® F3, Thermo Fisher Scientific Inc., USA; Eppendorf, Germany.
Micropipette Tips	Finntip Flex, Thermo Fisher Scientific Inc., USA; Eppendorf, Germany.
Microcentrifuge tubes	Eppendorf, Germany.
Bacti-Cinerator/ Sterilizer Loop-	Model: HM-3000A, Hangzhou Allsheng Instruments Co., Ltd. China
Vortex Mixer	Model: VM-2000; Digisystem Laboratory Instruments Inc. Made in Taiwan
Water Bath (WiseBath®)	Model: WB-11 Wisd Laboratory Instruments; DAIHAN Scientific Co., Made in Rep. of Korea
Laminar Airflow	Model-SLF-V, vertical, SAARC group Bangladesh Cabinet