Combination of Phages and Antibiotic Therapy to Control and Revert Antimicrobial Resistance of *Salmonella* Enteritidis Isolated from Poultry Farms in Kiambu and Nairobi Counties, Kenya

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A research thesis submitted in partial fulfillment of the requirement for the award of a Master of Science degree in Tropical and Infectious diseases at the University of Nairobi

December 2022

DECLARATION

This research thesis is my original work and has not been presented for a degree award in any other university.

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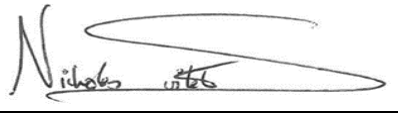
Signature  Date 29/11/2022

DEDICATION

This work is dedicated to my parents, Mr. and Mrs. Guantai, my siblings, and my son Kai for their love, support, and continuous encouragement.

ACKNOWLEDGEMENT

I would like to sincerely thank my supervisors, Dr. Moses Masika and Dr. Nicholas Svitek for their support, and mentorship and for patiently guiding me through my research.

My sincere appreciation goes to the University of Nairobi for offering me a scholarship to pursue my master's degree, Dr. Julius Oyugi for believing in me, and IDRC for funding this research through the Phages for One Health Project, and the Animal, and Human Health Program at ILRI for their stimulating research environment. I am grateful to my colleagues Dr. Angela Makumi for helping come up with research ideas, Josiah Odaba and Amos Mhone for their entire kindness, support, and advice, and Dr. Arshnee Moodley for guidance in performing the Minimum Inhibitory Concentrations (MIC) experiments. I also wish to thank the entire ILRI fraternity for guidance and the opportunities to present my work at conferences and training. I very much appreciate Dr. Nicholas Svitek and Dr. Dishon Muloi for assisting me with data analysis. I was privileged to work with such a helpful, hands-on team.

I also would like to extend my appreciation to my family and friends for all their prayers and moral support.

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LIST OF ABBREVIATIONS AND ACRONYMS

AMP Ampicillin

AMR Antimicrobial resistance

AZI Azithromycin

BIMS Bacteriophage insensitive mutants

BPW Buffered Peptone Water

CHI Chloramphenicol

CIP Ciprofloxacin

CLSI Clinical and Laboratory Standards Institute

CRISPR/Cas9 Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9

CRO Ceftriaxone

DNA Deoxyribonucleic acid

FOX Cefoxitin

GEN Gentamicin

LPS Lipopolysaccharide

MRD Multidrug-resistant

NAL Nalidixic acid

NTS non-typhoidal *Salmonella*

PAS Phage antibiotic synergism

PCR Polymerase chain reaction

RNA Ribonucleic acid

STR Streptomycin

STX Trimethoprim/sulfamethoxazole

TET Tetracycline

TSB Tryptic Soya Broth

PFU Phage forming units

WHO World health organization

ABSTRACT

Multi-drug resistant (MDR) *Salmonella enterica* Enteritidis (*S.* Enteritidis) is an important cause of food-related illness globally. MDR *Salmonella* infections have complicated the current chemotherapeutic options. Bacteriophages (phages) can be used as an alternative to control Antimicrobial resistant (AMR) infections. Furthermore, phage-antibiotic combination therapy has been suggested as a strategy to revert antibiotic resistance while also decreasing the occurrence of bacteriophage-insensitive mutants (BIMs). This study tested the ability of two newly isolated *S.* Enteritidis-specific phages to reverse antibiotic resistance in six Kenyan field strains of *S.* Enteritidis. We isolated 44 BIMs using six AMR *S.* Enteritidis strains exposed to two broad host range phages. First, we observed that most BIMs demonstrated lowered phage adsorption. Their antibiotic susceptibility profile was then assessed using 23 antibiotics. Comparing the relative antibiotic sensitivities of the BIMs to the parental host, we observed a higher proportion of BIMs becoming susceptible to the antibiotics to which the parent was previously resistant (25/44), whereas 12/44 had maintained their relative sensitivities to the antibiotics. A small number of the BIMs (4/44) became resistant to antibiotics the parent had been susceptible to. Remarkably, one BIM from isolate Sal 572 had drastic increases in nalidixic acid, gentamicin, and tetracycline susceptibilities. We further tested whether a panel of AMR genes known to be present in the parental strains could still be detected in the BIMs. Interestingly, the BIMs isolated from Sal 572 had lost *tetA*, *tetB*, *strA*, and *strB* genes. Finally, after carrying out kill curves with antibiotics and phages used in combination, we observed a more significant bacterial load reduction than phages or antibiotics used alone in four isolates. In conclusion, our study demonstrates the potential use of a combination of antibiotics and phages for therapy against MDR *Salmonella* from Kenya.

Keywords: *Salmonella* Enteritidis**;** AMR; MDR; BIMs; Combination therapy; phages; antibiotics

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Background information

An increase in the occurrence of bacteria that are resistant to antibiotics in animals, humans, and the environment due to decades of antibiotic use, misuse, and or abuse is a global concern. Multi-drug-resistant *Salmonella enterica* serovar Enteritidis found in poultry, pork, and other foods of animal origin have caused outbreaks of food-borne diseases in humans (Atterbury et al., 2007). Pathogenic *Escherichia coli (E.coli)* can obtain and preserve resistance genes from generic *E. coli* (prevalent commensal) and other organisms in animal populations and the environment. These organisms are of interest in AMR surveillance programs (Varga et al., 2008).

Private-sector funding for the discovery of novel antibiotics has reduced over the years due to a lack of profitability and funding for research (Domínguez & Meza-Rodriguez, 2019). Research investments have seen advancements regarding the development of new agents to fight drug-resistant organisms. This has augmented interest in other therapeutic alternatives to treat multi-drug-resistant pathogens (Chang et al., 2022).

Alternative therapies to control bacterial infections include bacteriophages, probiotics, antimicrobial peptides, prebiotics, enzymes, hyperimmune immunoglobulins, and traditional medicines. Bacteriophages are viruses that specifically infect bacteria. They are simple micro-organisms made up of a central genetic material enclosed in a protein capsid. Phages have nucleic acid which is either DNA or RNA and may be single or double-stranded. There are four basic

structural forms of phages, an icosahedral head with a tail, an icosahedral head without a tail, a filamentous form and a prolate capsid. (Hesse & Adhya, 2019).

Felix d'Hérelle first described lytic phages in 1917, and to date, phages are used in clinical practices in several countries, especially in Eastern Europe (Myelnikov, 2018). For an infection to occur, a bacteriophage attaches to a host cell and inserts its genetic material. The phage trails either virulent or temperate cycles. Virulent bacteriophages typically use the bacterial cell's machinery to replicate its components (Rohwer, Youle, & Maughan, 2014). Subsequently, the cell is lysed releasing new phage particles. Lysogenic/avirulent phages incorporate their genetic material into the chromosome of the host cell and replicate with it as one entity without killing the host cell (Ackermann et al., 1978). Under some conditions such as low nutrient conditions, UV light, or chemicals like mitomycin C, lysogenic phages may be made to follow a virulent cycle.(Berchieri et al., 1991; Rohde et al., 2018; Torres-Barceló, 2018).

Bacteriophage therapy mainly uses lytic phages because they reproduce rapidly inside and kill the host cell. Phages typically have a very narrow host range which poses certain drawbacks (Carlton, 1999). The success of bacteriophages for the control of AMR has been described in numerous cases including in the therapy of human infections including *Pseudomonas aeruginosa* infected wounds and the food industry (Fong et al., 2020).

Unfortunately, the occurrence of bacteriophage-insensitive mutants (BIMs) due to frequent exposure of bacteria to specific phage strains can occur (Valério et al., 2017). Bacteria may become resistant through numerous mechanisms such as bacterial abortive infection which are cell death systems upon phage infection which limits the replication of the virus, restriction-modification, CRISPR/Cas9 mechanisms, and frequently through mutations of attachment sites of phages

(Pouillot et al., 2012). Various genera and species of bacteria have been shown to have different fitness capabilities although not many studies have been carried out with foodborne pathogens such as *Salmonella* Enteritidis and pathogenic *Escherichia coli* (Fong et al., 2020).

When a bacterium becomes insensitive to a bacteriophage e.g., through receptor site mutations, that transformation is likely to increase the susceptibility of the bacterium to some antibiotics. Alternatively, where a bacterium is resistant to antibiotics through a mutation it might become susceptible to phages. Therefore, if the bacterium is exposed to both antibiotics and phages, the chances stand very low that any genes conferring resistance to it begins to express to promote its survival.

A study by Chaudry et al showed that combining antibiotics and phages could kill more bacteria in biofilms caused by *Pseudomonas aeruginosa* than either agent alone. They also showed that treating biofilms with phages before antibiotics could considerably increase the treatment efficacy (Chaudhry et al., 2017). Another study showed that combined phages and antibiotics treatment was effective in lowering bacteria density and controlling the emergence of BIMs in *Escherichia coli.* (Lopes et al., 2018). A study carried out *in vivo* on broiler chicken indicated a three-log reduction of *Escherichia coli* density when phages were combined with ciprofloxacin as compared to using antibiotics alone (Valério et al., 2017a). This indicates that infection management with both phages and antibiotics could aid avert the possibility of the development of bacterial resistance by improving the clinical use of antibiotics and phages. This is largely dependent on the bacteria strain , antibiotic type (mechanism of action/target site on the bacteria), and the bacterium resistant mechanism, as some antibiotics might have an antagonistic effect with phages (Chaudhry et al., 2017; Comeau et al., 2007a; Liu et al., 2020; Tagliaferri et al., 2019).

1.2 Problem Statement

Treatment of infectious diseases has become challenging with each passing year. This has been observed in many Gram-negative bacteria, especially *Escherichia coli* and *Salmonella enterica* serovar Enteritidis. *S.* Enteritidis and *E. coli* are responsible for approximately 93 million gastroenteritis infections worldwide and 150,000 related deaths yearly (Tacconelli et al., 2017).

S. Enteritidis causes bloodstream infections which pose an increased threat to HIV or Malaria-infected individuals (Morpeth et al., 2009). Invasive *Salmonella* Enteritidis infections are often fatal if left untreated. Immunosuppressed persons including individuals infected by HIV, Tuberculosis, or Malaria, infants, and children with malnutrition are mostly at risk of acquiring non-typhoidal *Salmonella* (NTS) (McDermott et al., 2018).

The magnitude of *S.* Enteritidis has only become intensely important and noticeable in recent years which could indicate an alteration in epidemiology. Antibiotic resistance has also contributed to the profound necessity for more research on *Salmonella* strains (Balasubramanian et al., 2019).

The pathogen's ability to inhabit several environments has caused their tenacity and endurance in a diversity of foods. These foods, such as dairy products (milk and meat), poultry products (eggs and meat), fresh produce, and a range of ready-to-consume foodstuffs may serve as agents of transmission (Fong et al., 2020). Given the multiplicity of transmission routes and frequency of infection, the rise of AMR in these pathogens is of great concern. WHO ranked AMR *Salmonella* as one of the top ten pathogens of significance and an imminent threat to public health (Tacconelli et al., 2017). These genera can rapidly develop resistance to multiple antibiotics. Plasmid-mediated transfer of β-lactamases is of great concern for both genera.

The worldwide surge in antibiotic resistance in the environment, animals, and humans warrants the need for alternative antimicrobial strategies while employing a one-health approach. Phages were first discovered in 1915 but used as therapeutic agents in 1917 by Felix D'Herelle and is still frequently used in Russia (Chanishvili, 2012). However, Western countries and Africa did not develop phage therapy due to the availability of antibiotics. Research and data on bacteriophage pharmacokinetics, pharmacodynamics, interaction with other drugs, *in vivo* efficacy, and development of resistance remain scarce (Oechslin et al., 2017).

Bacteriophages are DNA/RNA viruses found in the environment that can infect and then kill susceptible bacteria. Unfortunately, BIMs emergence is a major constraint to phage therapy (Uddin et al., 2019). The selection pressure of phage infection favors mutants of the bacteria that can resist phage infection (Uddin et al., 2019). Bacteria can become resistant to bacteriophages through numerous processes, such as the alteration of bacterial-surface ligands and the transformation of foreign DNA. Some mutations may affect the phage receptors including efflux pumps, porin, pili, and the LPS which can influence bacterial fitness and survival capabilities (Oechslin et al., 2017).

Several studies have revealed that phage exposure before antibiotic treatment improves bacterial killing by antibiotics (Valério et al., 2017a). The ability of phages to select against antibiotic resistance in the bacteria indicates that combining phages with antibiotics can steer an evolutionary trade-off of phages with antibiotics (Rodriguez-Gonzalez et al., 2020). A study by Carmen showed that bacteriophages offer a complementary effect by dropping the minimum inhibitory concentration (MIC) for antibiotic-resistant bacteria strains (Liu et al., 2020).

1.3 Justification

Bacteriophages are simple yet amazingly diverse organisms. They are made up of a protein capsid as the outer layer covering the nucleic acid (Atterbury et al., 2007). Although the idea of employing phages as a treatment against bacterial infections has recently become popular in response to pathogens that are insensitive to multiple antibiotics, they have been used for approximately a century (Lin et al., 2017).

There has been a renewed interest in phage therapy due to the increased AMR cases. Phage therapy is a very encouraging substitute for the control and management of bacterial infections, to be utilized singly, or by combining with antibiotics (Chaudhry et al., 2017). Lytic phages are used to control infections, primarily those instigated by multidrug-resistant bacteria (Gordillo Altamirano & Barr, 2019). There are many likely merits of employing bacteriophages to treat infections over antibiotics including self-dosing due to their self-amplification, non-toxic nature, and they are easily isolated from the environment. Bacteriophages are typically exceedingly selective to a specific strain or species of bacteria. This ensures that normal flora is minimally affected (Lopes et al., 2018). Phages have a controlling influence on ordinary bacterial colonies because they self-replicate and limit themselves, multiply rapidly with the replication of bacteria, and decrease when the bacteria number declines (Almeida et al., 2009).

Functional phage-based applications require further studies of crucial relations between bacteriophages and bacteria especially since bacterial resistance to phages is predestined (Fong et al., 2020). Efforts to optimize phage therapy have been limited mainly by the emergence of mutants that are phage resistant. BIMs compromise the performance of the phages (Fong et al., 2020).

Phages and antibiotics work as antibacterials that control bacterial growth through lysing or inhibiting them systematically (Lin et al., 2017). Combination therapy is effective not only in lowering bacterial numbers but also to reduce the emergence of antibiotic/phage-resistant mutants (Valério et al., 2017). Combination therapy is mainly effective due to the phage-antibiotic synergy whereby sub-lethal concentrations of some classes of antibiotics enhance the hosts' multiplication of phages, hence increasing the killing efficiency of the host (Comeau et al.,2007a; Chan et al., 2016b; Cairns et al., 2017; B. K.; Valério et al., 2017a ; Lopes et al., 2018; Liu et al., 2020;). There is also a substantial reduction in the development of antibiotic resistance when using combination therapy. Nevertheless, this assessment mainly conversed only concerning resistance to antibiotics, not to the emergence of bacteriophage-resistant mutants. There is the likelihood that the development of bacteriophage-insensitive mutants can cause a trade-off increasing the bacteria hosts' susceptibility to antibiotics. This study compared at least 4 antibiotics with and without phage additions and the insensitivity to the phages when antibiotics are added or not. This study proves that a combination of antibiotics and phages can be used to treat and revert antibiotic resistance in *S.* Enteritidis of poultry origin. Clinically, phage-antibiotic combinations could bring back the usage of some antibiotics which are no longer regarded as a treatment of choice for some infections by enhancing their activity.

1.4 Objectives of the Study

1.4.1 General Objective

To use lytic phages to select for phage resistance that compromises drug resistance and/or virulence

1.4.2 Specific objectives

1. To isolate *S.* Enteritidis BIMs and determine their AMR phenotype profiles.
2. To determine if AMR genes are still detected on AMR *S.* Enteritidis after exposure to bacteriophages.
3. To determine if phage ILRI_K24 works synergistically with antibiotics and restores the sensitivity of AMR *S.*Enteritidis to antibiotics.

1.5 Research Questions

1. How fast does *S.* Enteritidis develop resistance to phages after frequent exposure and does it vary among phages?
2. Can AMR genes still be detected on MDR *S.* Enteritidis after exposure to phages?
3. Does phage ILRI_K24 work synergistically with antibiotics and restore the sensitivity of AMR *S.* Enteritidis to antibiotics.

1.6 Hypothesis

Ho: Usage of both phages and lower concentrations of antibiotics has a similar effect as using either phages or antibiotics alone.

Ha: Usage of both phages and a lower concentration of antibiotics is more effective than using either phages or antibiotics alone.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Introduction

Salmonella enterica serovar Enteritidis is a Gram-negative, motile *Enterobacteriaceae* that commonly causes gastroenteritis. *Salmonella* Enteritidis colonizes the intestinal tract of a variety of animals such as pigs, poultry, cattle, wild, and companion animals. Humans acquire infection by ingesting contaminated animal products. Most illnesses are associated with the intake of poultry and poultry products such as eggs (McDermott et al., 2018). Approximations propose that *Salmonella* Enteritidis is the main cause of almost 100 million enteric infections and 150,000 related mortalities yearly. *S. enterica* serovars are known to cause invasive infections in certain geographical areas posing a specific threat to immunosuppressed individuals (Balasubramanian et al., 2019). Foodborne illnesses are mostly caused by *S.* Enteritidis and *S.* Typhimurium serovars. Antibiotic-resistant *S. enterica* serovars have arisen as a result of constant antibiotic pressure (Balasubramanian et al., 2019).

2.2 Antimicrobial Resistance

Antimicrobial resistance poses a community health challenge worldwide. It is projected that 30 years from now, drug-resistant illnesses are expected to cause mortality of about 10 million people yearly if antimicrobial resistance is not tackled (Maingi et al., 2019). Globally, antibiotics have been exponentially used in human and animal production (Poirel et al., 2018). The microbial biosphere has accrued a vast range of survival and metabolic processes that could be organized in response to outside burdens and pressure for billions of years of evolution now including

antibiotics (Aminov, 2010). The conventional method depends on the development of newly discovered, more effective antibiotics. Most antimicrobials being used today were discovered several decades ago largely from Actinomyces, unfortunately, no newer drug classes have been found outside the niche in the past two decades (Subedi et al., 2018).

Several factors are causal agents to the emergence and transmission of antimicrobial insensitivity and this problem requires the development of alternative approaches (Aminov, 2010). The antibiotic treatment choices for prevailing or emerging multiple drug-resistant bacterial infections are inadequate. This results in increased rates of mortality and morbidity (Maingi et al., 2019). Former WHO director Margaret Chan when talking about hospital-acquired infections caused by drug-resistant bacteria stated that the "post-antibiotic era means, in effect, an end to modern medicine as we know it. Things as common as strep throat or a child's scratched knee could once again kill" (M. Chan, 2012). Chan also stated that the mortality rate of those infected by drug-resistant pathogens had been shown to rise by 50% (Ferri et al., 2017).

Genes conferring antibiotic resistance can be transferred from one bacterium to another by horizontal transfer mechanisms such as plasmids, integrons, or transposons that can convey numerous resistance genes that can move within the genome (McMillan et al., 2019). Integrons can be carried in plasmids or be integrated into the chromosome as it occurs in a serotype of *Salmonella enterica* Typhimurium. Recently, new machinery of resistance has caused the instantaneous resistance development to many classes of antibiotics resulting in multidrug-resistant (MDR) strains recognized as "superbugs" which are hard to treat (McDermott et al., 2018).

Phages have been implicated in the transfer of drug-resistant genes by phage-mediated transduction (Feiner et al., 2015). Transduction happens using lytic and lysogenic phages. There are two types of transductions, specialized and generalized. Generalized transduction is defined as the process where host bacterial DNA is mispackaged in the capsid of the phage. When the phage infects another susceptible cell, the genetic material may be intergrated to the other cell by homologous recombination (McMillan et al., 2019). Specialized transduction is described as the improper excision of a prophage from the host bacteria chromosome. This causes the bacterial DNA to be packaged into phages at an increased frequency than generalized transduction. This horizontal transfer of drug resistance encoding genes contributes to the spread of antibiotic resistance globally (Rohwer, Youle, Maughan, et al., 2014)(Colavecchio et al., 2017).

In poultry production, antibiotics have been utilized for growth promotion, to prevent, and to treat infections. Most farmers, especially in developing countries, have limited knowledge of what antibiotics are, how to use them, and their effects on animals, humans, and the environment. With the increased demand for poultry and poultry products, the production of poultry is predicted to rise annually by 3.6% in developing countries until 2030 (Olaf & Dafyad Pilling, 2008). In Kenya, farming poultry is an extensive livestock venture. Several survey studies estimate that Kenya has roughly 37 million chickens. A study reported that approximately 65% of farms in Kenya keep chicken, with Kiambu and Nairobi counties having the greatest number of poultry kept per household. Previous studies have reported an increased frequency of resistance in bacteria isolated from samples from the rectum of chicken in Kenya (Maingi et al., 2019).

The World Health Organization (WHO) emphasized the actual menace of gram-negative bacteria that are no longer susceptible to several antibiotics in 2017 (WHO, 2017). Detection and development of novel and substitute therapeutical options are critical.

2.3 Phage Biology

Phages undergo different infection cycles including, the lytic infection , lysogenic/avirulent cycle, and pseudolysogeny. There are also some phages that are chronic and some can be superspreader (mutant lytic phages). During the lytic cycle, a phage attaches to a ligand(s) on the bacterium cell, inserts its genomic material into the host cell; undergoes replication of its components in the cytosol using bacteria cell machinery (Hesse & Adhya, 2019). Upon the formation of a new phage particle, they are released through the digestion of the host cell. The process is repetitive (Torres-Barceló, 2018). This highlights the advantages of bacteriophage therapy, using lytic viruses as self-multiplying antimicrobials that bind to and kill susceptible host cells may be more effective than using antibiotics (Chanishvili, 2012). Lysogenic phages assimilate inside the bacterial host genome and get transferred to the progeny cells during binary fission; nevertheless, lysogenic phages can enter the lytic cycle due to environmental changes or other physical stress. Lysogenic phages are favored in several biotechnology applications, but virulent phages are the best candidates for the development of bacteriophage therapy although some temperate phages have been used as therapeutic agents recently (Almeida et al., 2009).

It is increasingly recognized that bacteriophages are immensely diverse. Some phages are immensely specific to a single bacteria strain or species (Rohwer, Youle, Maughan, et al., 2014). Other phages naturally have a wide host range or can simply mutate to target bacterial strains and genotypes apart from the normal host (Skurnik et al., 2007).

2.4 Bacteriophage Therapy

Bacteriophages are viruses that specifically target, infect, and multiply inside bacterial cells. Being the most diverse organisms on Earth, they partake in significant roles in microbial pathogenesis, evolution, and treatment of infections (Kutter et al., 2010). Felix d'Hérelle invented the word

"bacteriophage" which means "bacterium eater" and started using bacteriophages for the control and treatment of bacterial infections in human patients in the early 1917 (Gordillo Altamirano & Barr, 2019). Bacteriophages have revealed high potential as a substitute therapy for antibiotics for the treatment of pathogenic bacteria, such as the pathogens *Salmonella* and *E. coli* (Kortright et al., 2019).

Bacteriophage therapy is described as the introduction of lytic bacteriophages to a patient or sick animal to clear and control the infection caused by a bacterial pathogen (Capparelli et al., 2010). The first reports of phage therapy were met with ardent interest although the discovery of antibiotics rendered phage therapy redundant. However, some parts of the Soviet Union continued to use phage therapy to date (Rohde et al., 2018) including the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy (Gordillo Altamirano & Barr, 2019).

Initially, the phage's ability to be highly specific for a single bacterial species or strain (Ackermann et al., 1978) was subjugated for the classification of *S. enterica* serovars. Lately, bacteriophages have been utilized for the management of contamination in poultry by *S. enterica* (Atterbury et al., 2007; Berchieri et al., 1991).

2.4.1 Advantages of phage therapy

Lytic phages have several advantages over antibiotics. Firstly, they do not allow bacteria to recuperate their viability in contrast to some bacteriostatic antibiotics such as tetracycline. Bacteriophages also establish their phage dose as they can increase in number during bacterial killing. These are defined as auto-dosing or self-amplifying (Abedon & Thomas-Abedon, 2010). Secondly, highly purified phage preparation reduces the occurrence of harmful immune responses which is enhanced by the inherently non-toxic makeup of phages as they are made up of proteins

and nucleic (Skurnik et al., 2007). Thirdly, phages exhibit a narrow host range which lowers the number of bacterial types with which selection for specific phage-resistance mechanisms can occur (Carlton, 1999a; Makumi et al., 2021)

Phages are easily isolated from the environment and they display a high formulation and application versatility such as being used in combination with certain antibiotics and could be applied in several forms (Capparelli et al., 2010). Phages have also demonstrated the ability to clear and penetrate biofilms caused by different bacteria including, *Pseudomonas aeruginosa* (Chaudhry et al., 2017)

2.4.2 Disadvantages of phage therapy

Good phages for therapy should be obligately lytic and should have the ability to reach and kill bacteria with a minimal negative impact on the environment and normal flora (Loc-Carrillo & Abedon, 2011). Lytic phages can become lysogenic which can convert phage-susceptible bacteria into resistant ones and they also may encode bacterial virulence factors such as toxins (Hanlon, 2007; Kutateladze & Adamia, 2010a).

Bacteriophages' narrow host specificity can also be a disadvantage (Loc-Carrillo & Abedon, 2011). It limits treatment courses that start before antimicrobial sensitivity testing of the pathogen. Some phages that have a wide host range are still more specific in their range of activity than normal antibiotics that have a narrow spectrum (Kutateladze & Adamia, 2010).

Nonetheless, phages can be misunderstood by the community as viruses causing infections like normal viruses in humans. Up to now, however, public resistance has not occurred and a few phage products have been approved through regulatory standards (Atterbury, 2009).

2.5 Bacteriophage Insensitive Mutants (BIMs)

The emergence of bacterial mutants that are insensitive to phages (known as bacteriophage-insensitive mutants or BIMs) has been a major cause of alarm concerning phage therapy. BIMs arise upon frequent subjection of bacterial hosts to specific strains of phages (Oechslin et al., 2017), Phage killing of bacterial cells can radically alter bacterial host population densities ultimately exerting robust selection pressure on the population. Consequently, BIMs are likely to develop and become dominant (Rodriguez-Gonzalez et al., 2020).

Bacteria acquire resistance through a variety of mechanisms including restriction-modification, manufacture of restriction endonucleases which cut and destroy phage nucleic acid, (CRISPR/Cas9) system that identifies and degrade previously encountered foreign DNA, and commonly via mutation or loss of bacterial cell receptors (Fong et al., 2020). Bacteriophage attaching to the binding site pressure the bacteria to modify or reduce expression of the receptor via mutations thus avoiding infection. Chan proposed an evolutionary-based approach that influences a genetic compromise by using phages to evolve augmented resistance to phages at the same time increasing susceptibility to antibiotics (Chan et al., 2016a)

The transformations that alter lipopolysaccharides on the bacterial cell membrane may affect bacterial fitness or virulence. Other mutations may restore virulence (Lin et al., 2017). Comprehensive knowledge of bacteria cell insensitivity to bacteriophages is crucial if bacteriophages are to be utilized further in clinical practices (Oechslin et al., 2017).

Several studies have shown that bacteria typically develop resistance to antibiotics once per six divisions and resistance to phages once per seven divisions. Thus, the chances of a bacterium developing mutation against both phages and antibiotics would likely take 10^{13} bacterial mutations

(Carlton, 1999). If a target on the cell surface serves as a binding site for both the phage and the antibiotic, increased antibiotic sensitivity may arise in phage-resistant bacteria (Chan et al., 2016).

2.6 Combination Therapy

BIMs can be controlled by utilizing combination therapy. Phage-antibiotic synergy is defined as the increased production of lytic phages by the host bacteria cell brought about by sub-lethal concentrations of some antibiotics (Ryan et al., 2012). The mechanism of Phage Antibiotic Synergism (PAS) is explained to be an outcome of antibiotic-induced mutation of the bacteria, which could enable the recognition and attachment of phages to their bacterial host target (Kirby, 2012). PAS has been previously described (Comeau et al., 2007b; Kamal & Dennis, 2015b; Kirby, 2012; Knezevic et al., 2013). It has been shown that combination therapy is an alternative that not only is efficacious in controlling bacterial numbers but also decreases the development of antibiotic and bacteriophage insensitive mutants. Other studies propose that the decreased resistance acquisition is because a strain that is not sensitive to one antimicrobial agent is susceptible to the other. For instance, antibiotics might hasten the killing of infected cells consequently allowing the phages to proliferate more quickly (Kortright et al., 2019).

A study by Comeau et al. displayed synergy among several antibiotics such as aztreonam and Cefixime and the phage MFP when subjected to a uropathogenic *Escherichia coli* strain. The combined antibiotic and phage therapy displayed an increased incidence of phage lysis on the host bacterium (Comeau et al., 2007). Adding antibiotics sequentially after phage treatment can also control the development of phage mutants. This is according to (Valério et al., 2017) who further showed that upon the addition of sub-lethal concentrations of ciprofloxacin when combined with the phage, the occurrence of phage mutants was lesser than that observed when antibiotics were not added (Valério et al., 2017).

However, some recent studies have revealed that phage and some antibiotics combination raises the frequency of phage resistance evolution (Cairns et al., 2017). The study showed that low concentrations of streptomycin raised the rate of phage-resistant evolution

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Study Sites

The study was conducted in the Biosafety Level 2 (BSL 2) laboratory at the International Livestock Research Institute (ILRI), with field *Salmonella enterica* serovar Enteritidis bacteriophage lysates and bacterial isolates collected from a previous study.

3.2 Study Design

This was an experimental study, designed to assess bacterial suppression by combination therapy of antibiotics and phages.

3.3 Sample Size

Based on previous studies *Salmonella* Enteritidis isolates in chicken droppings may not be more than 10% so it is estimated that 523 samples give enough information about bacteria distribution and their susceptibility to antibiotics and phages.

The sample size was calculated according to (Naing et al., 2006): -

$$SS = \frac{Z^2 P (1-P)}{d^2}$$

Where Z = Z statistic for a level of confidence

P = Expected prevalence of bacteria or % of picking a *Salmonella* Enteritidis isolate (5-10%) in the area, expressed in decimal.

d = The desired level of sampling error ±5%, (0.05)

SS = Sample size

Using this formula, the sample size was obtained: -

Z = 1.96, P = 50% (0.50), d = 5% (0.05)

$$\mathbf{SS} = \frac{1.96^2 \times 0.50(1-0.50)}{0.05^2}$$

$$\mathbf{SS} = \frac{3.8416 \times 0.25}{0.0025}$$

SS = 384.16 ~ 385

Six confirmed *S.*Enteritidis and two characterized broad host range phages were used for the isolation of BIMs and combination therapy efficacy test.

3.4 Study population

3.4.1 Bacteriophage and bacteria isolates

Salmonella enterica serovar Enteritidis positive bacteriophages were stored at 4℃ in the Biosafety Level 2 (BSL 2) laboratory previously collected from Nairobi and Kiambu county, Kenya. This study is an objective part of the main project that aims to use bacteriophages as a One Health approach as an alternative to antibiotics and reduction of drug-resistant nontyphoidal *Salmonella*, in poultry farms in Kenya (Svitek et al., 2019)

The phages were isolated from fecal and water samples collected from chicken farms and tested for lytic activity against a host range of different *S.* Enteritidis serovars . They were then purified, characterized, and stored at 4℃. in the ILRI laboratory.

3.4.2 Inclusion criteria

From the data collected previously, only samples collected from smallholder poultry farms with 1-1000 chickens were included in this study. Only confirmed *S.* Enteritidis and phages with broad host range and high titres were used.

3.4.3 Exclusion criteria

This study excluded other Non-typhoidal *Salmonella* and narrow host range phages.

Ethical approval

KNH-UON ethics-**P48/02/2021**

3.5 Sample Processing

3.5.1 Bacteriological Examination

About 1 g of stored fecal matter was inoculated in 5ml of Tryptic Soya Broth (TSB) (Oxoid, Hampshire, UK) and then incubated aerobically overnight at 37 °C. 50µl of the overnight culture was placed in 5 ml of Rappaport Vassiliadis Broth and Selenite fecal broth (Oxoid, Hampshire, UK) and incubated at 42 °C for 24 h. After 24 hours of culturing on primary media (MacConkey Agar) (Oxoid, Hampshire, UK), one colony from each plate was selected and subcultured on Selective media, Brilliance Green *Salmonella* Agar, XLT-4, and *Salmonella-Shigella* Agar (Oxoid, Hampshire, UK) then incubated overnight at 37ºC aerobically. Culturing was then done on Tryptone Soy Agar (TSA) from which biochemical tests and Polymerase Chain Reaction (PCR) were carried out.

3.5.2 Biochemical Identification

Biochemical identification was performed using Triple Sugar Iron agar (TSI) (Oxoid, Hampshire, UK, Urea hydrolysis test agar (Oxoid, Hampshire, UK, motility indole-lysine media (Oxoid, Hampshire, UK, and Biomeriux API test strips for confirmation of *Salmonella* (Biomériux, France).

3.5.3 Serological Identification

Serological typing of the *Salmonella sp*. strains was performed (Edwards, 1972). Isolates were serotyped using Polyvalent O and H (phase 1 and phase 11) *Salmonella* antisera. (Salmonella Agglutinating Serum, Remel Europe Ltd, Cambridge, UK).

3.5.4 Antimicrobial Sensitivity Testing

In our previous work, we carried out antibiotic susceptibility tests on 16 *Salmonella* isolates and observed that most isolates were resistant to streptomycin, tetracycline, and ciprofloxacin (Makumi et al., manuscript in preparation). We next tested whether resistance to novel phage ILRI_K24 caused a genetic trade-off between resistance to phages and antibiotic susceptibility for the benefit of phage therapy against MDR *S.* Enteritidis. In particular, we determined whether bacterial resistance to phages increased the inhibition efficiency (increased antibiotic disc diameters) of 23 antibiotics. An antibiotic sensitivity test was carried out following a disk diffusion method (Kirby-Bauer). All *Salmonella* isolates were tested for their susceptibility to the different antibiotics including cefoxitin (FOX), ceftriaxone (CRO), azithromycin (AZ), chloramphenicol (CHL), tetracycline (TET), ciprofloxacin (CIP), gentamicin (GEN), nalidixic acid (NA), ampicillin (AMP), streptomycin (ST), amoxicillin/clavulanic acid and trimethoprim/sulfamethoxazole (SXT) and interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2016). All antibiotics used in this study were purchased from OXOID,UK. A *Salmonella* ATCC strain and an *Escherichia coli* ATCC 25922 strain were used for quality control.

3.5.5 Extraction of Genomic DNA

DNA was used in PCR to detect target genes and therefore pure colonies of non-typhoidal *Salmonella* cultured overnight were subjected to DNA extraction with the Wizard Genomic DNA Purification Kit, (PROMEGA, USA, 2022). The quality and quantity control was assessed using gel electrophoresis using 1.5% agarose gel (Sigma Aldrich , A4718) prepared in 1X TAE buffer (Thermofisher scientific, B52) and run at 50V for 1 hour. The DNA bands were visualized using a gel imager (ChemiDoc, Biorad, America) DNA samples were stored at -20℃ .

3.5.6 Polymerase Chain Reaction Amplification

Polymerase Chain Reaction (PCR) was used to amplify the *inv*A gene for the isolation of *Salmonella*. The *invA* gene typically codes for a protein in the inner bacterial cell membrane that controls the invasion of the intestinal cells of the host. It has sequences that are unique and specific to *Salmonella*. (Antunes et al., 2016)

Primer	**Gene**	**Oligonucleotide sequence (5'→3')**	**Amplicon product (bp)**
invA Fwd.	InVA	GTGAAATTATCGCCACGTTCGGGCAA	284bp
invA Rev	InVA	TCATCGCACCGTCAAAGGAACC	

Table 3-1: *invA* PCR primers for the detection of *Salmonella*

Amplification was conducted in a thermocycler. The cycle was carried out at an initial denaturation of 94 ^{o}C for 1 min, followed by 35 cycles of denaturation at 94 ^{o}C for 60 s, annealing at 64 ^{o}C for 30 s, and a 7 min final extension period at 72 ^{o}C. PCR products were visualized by agarose gel electrophoresis containing 1.5 % agarose with gel red and visualized using a U.V trans-illuminator.

3.5.7 Bacteria and phage densities

Bacterial densities were determined by serial dilution in TSB and plating on TSA (Oxoid, Hampshire, UK). The plates were incubated at 37^{o}C for 18h, the number of colonies was counted, and the results were expressed as colony-forming units (CFU)/ml. The bacterial strain was

maintained in solid TSA at 4ºC. One isolated colony was transferred aseptically to 5ml of TSB for each test and incubated.

3.5.8 Isolation of BIMs

An earlier study demonstrated that non-typhoidal *Salmonella* (NTS) BIMs were frequently developed if a particular phage was exposed severally to the same bacterial strain. We, therefore, decided to isolate and characterize bacteriophage-insensitive mutants using two phages ILRI_K23 and ILRI|_K24 using an optimized method (Fong et al., 2020; Pereira et al., 2016) (Figure 3-1). Briefly, phage densities were determined by mixing serially diluted culture with an overnight culture of *Salmonella* (100ul of a 1 in 10 dilutions of a fresh overnight culture) added to 5 ml 0.7% TSA soft agar and poured onto 1.5 % TSA in Petri dishes. Phages were added at 10^9 pfu/ml at an MOI of 10. Six strains of *S*. Enteritidis (Sal 16, Sal 73, Sal 177, Sal 568, Sal 569, and Sal 572) were exposed to two phages ILRI_K23 and ILRI_K24 as follows: 100 µL of the phage (10^9 PFU/mL) and 100ul of stationary phase bacteria were added to 4ml 0.7 %TSA and poured onto 1.5% TSA. Incubation was done for 48 h at 37◦C to allow the growth of colonies. Isolated colonies were picked and inoculated in 5ml of TSB and grown for 18-24 hours at 37℃ for 18 h. The suspected mutants and a control (parental host) were streaked across a phage inoculum on TSA plates and incubated for 24 hr. at 37◦C. The process was carried out three times to prevent the selection of isolates with temporary bacteriophage resistance phenotypes. Confirmed BIMs were stocked in TSB supplemented with 20% glycerol and then kept at -80℃ for further analysis.

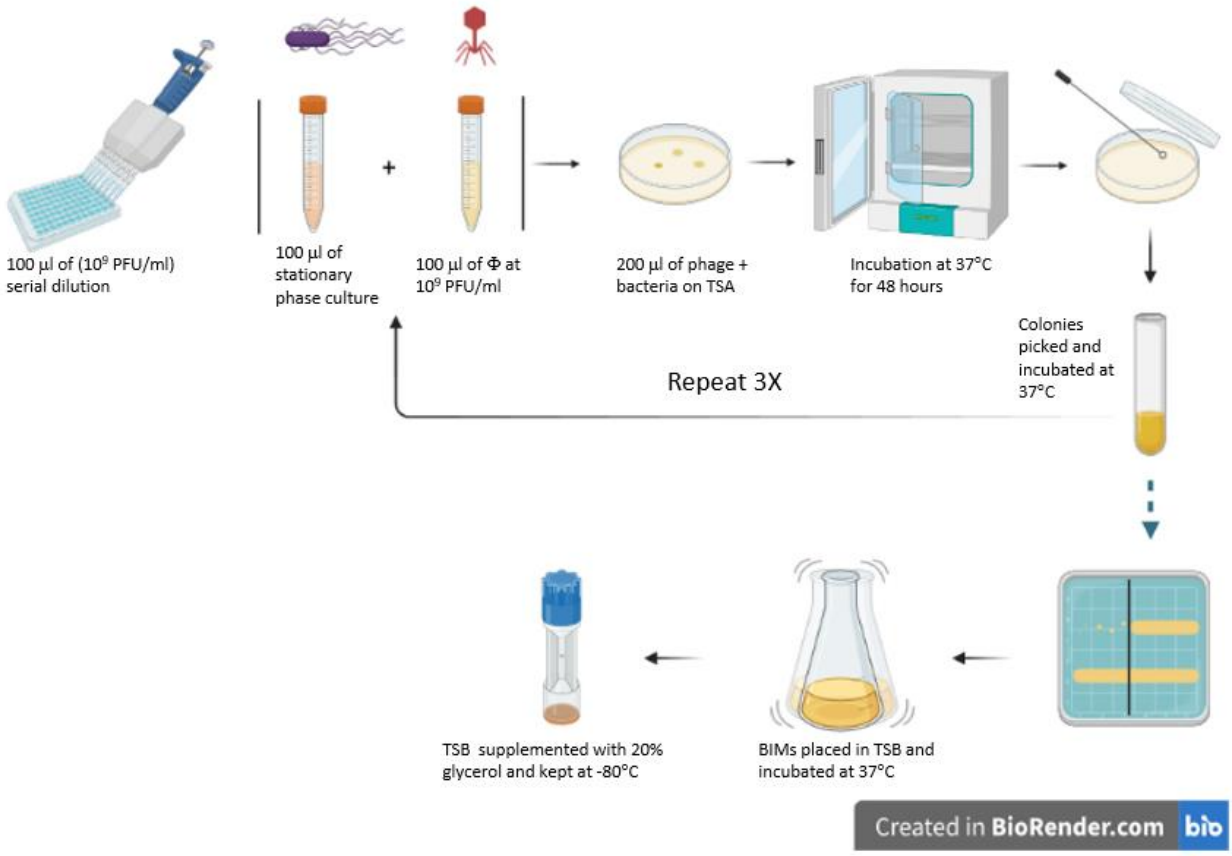

Figure 3-1: Isolation of Bacteriophage insensitive mutants

3.5.9 Detection of antimicrobial-resistant genes of *Salmonella* by PCR

Conventional PCR was used to screen for the *tetA*, *tetB*, *strA*, and *strB* antimicrobial resistance genes in the BIMs and their parental strains as described in (Langata et al., 2019). Briefly, the cycle for the *tetA* gene amplicon was carried out with an initial denaturation at 95 ^{o}C for 5 min, followed by 35 cycles of denaturation at 94 ^{o}C for 30 sec, annealing at 59 ^{o}C for 30 sec, and an extension at 72 ^{o}C for 60 sec, with a final extension for 10 min at 72 ^{o}C. The cycle for the *tetB* gene amplicon was carried out with an initial denaturation at 95 ^{o}C for 5 min, followed by 35 cycles of denaturation at 94 ^{o}C for 30 sec, annealing at 56 ^{o}C for 30 sec, and extension at 72 ^{o}C for 60 sec,

with a final extension for 10 min at 72 ºC. The cycle for the *strA* gene amplicon was carried out with an initial denaturation at 95 ºC for 5 min, followed by 35 cycles of denaturation at 94 ºC for 30 sec, annealing at 64 ºC for 30 sec, an extension at 72 ºC for 60 sec, with a final extension for 10 min at 72 ºC. The cycle for the *strB* gene amplicon was carried out with an initial denaturation of 95 ºC for 5 min, followed by 35 cycles of denaturation at 94 ºC for 30 sec, annealing at 56 ºC for 30 sec, an extension at 72 ºC for 60 sec, with a final extension for 10 min at 72 ºC. PCR products were visualized by agarose gel electrophoresis containing 1.5 % agarose with gel red and visualized using a U.V transilluminator.

3.5.10 Adsorption Test

Determination of the binding efficiency of phages to the six *Salmonella* Enteritidis versus the BIMs was done as described in (Fong et al., 2020) (Figure 3-2). Briefly, an exponential culture was prepared from an overnight culture, infected with phage ILRI_K23 and ILRI_K24 placed at room temperature for 15 minutes, and then centrifuged at 10,000g for 2min. The supernatant was serial diluted and spotting done on a double-agar layer plate then incubated at 37 ºC overnight.The phage forming units (PFU/ml) were then determined by using pour-plating method by adding 100ul of bacteria to 100ul of the supernatant in different dilutions to soft agar and poured on a TSA plate. The plates were incubated at 37 ºC ovenight and the PFU of the bacteria counted.

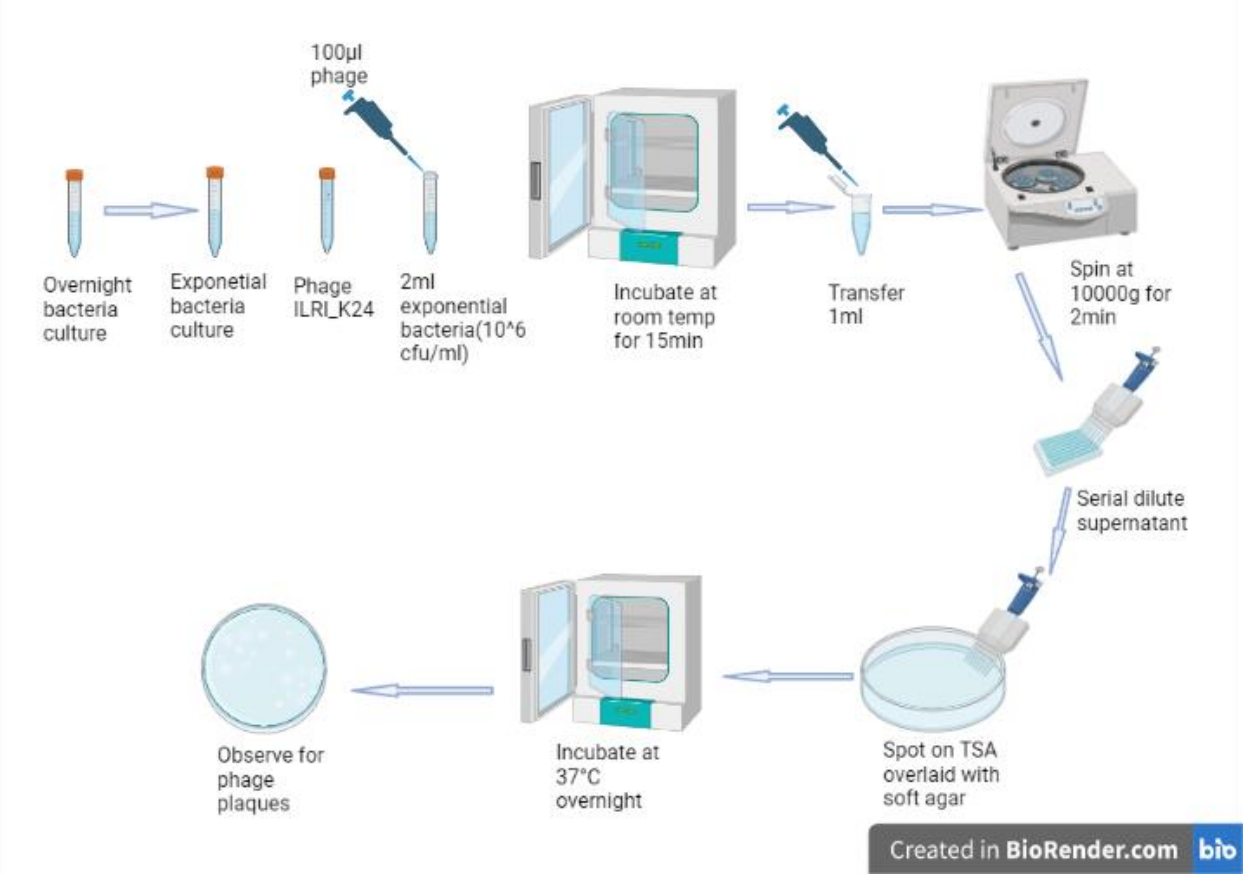

Figure 3-2: Adsorption test to determine whether BIMs lose their binding efficiency

3.5.11 Minimum-Inhibitory Concentration Assays

Minimum inhibitory concentrations (MICs) of ciprofloxacin, gentamicin, tetracycline and streptomycin against the six *S.* Enteritidis isolates, BIMs of *S.* Enteritidis, and a phage-sensitive control strain were tested using the E-test method (Wiegand et al., 2008) (Figure 3-3). The minimum concentration of each antibiotic that inhibited the growth of bacteria was considered the MIC. An E-test strip was used for each antibiotic(Biomériux,France).

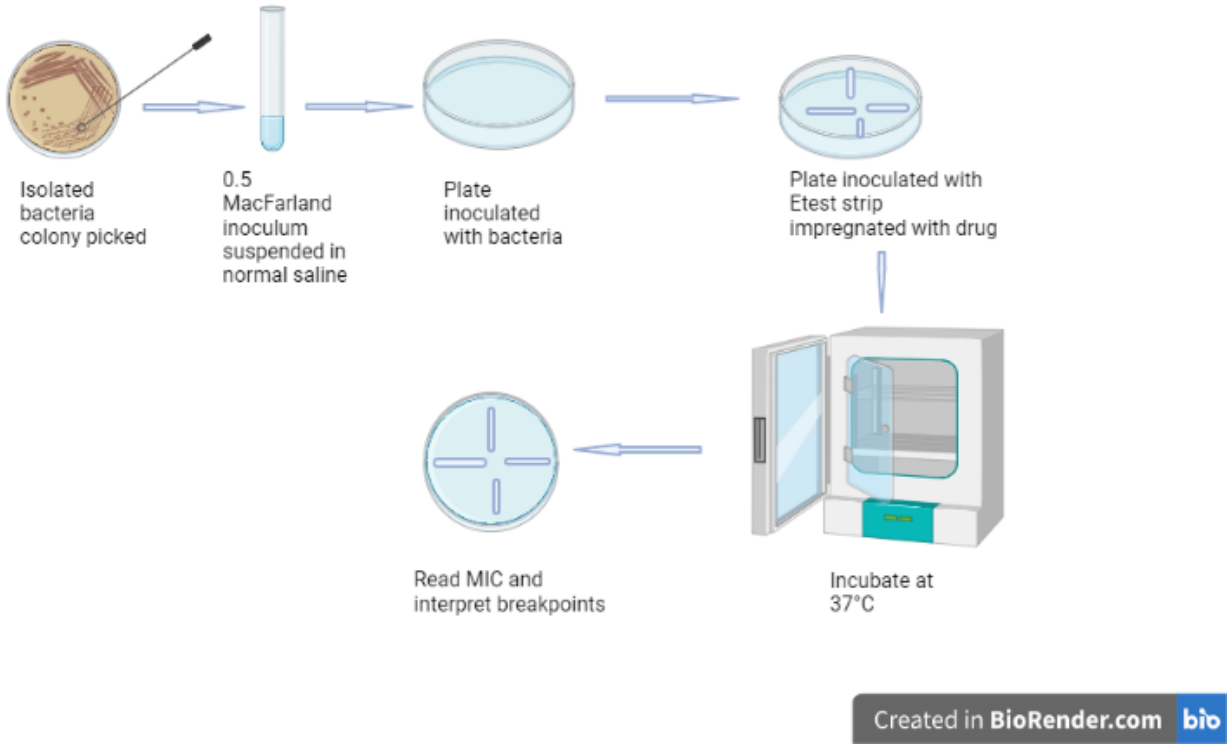

Figure 3-3: Minimum Inhibitory Concentration using E-Test Strips

3.5.12 Bacteriophage and Antibiotic kill curves in Tryptic Soy Broth

Salmonella inactivation was tested using phage ILRI_K24 and ciprofloxacin at 0.5 mg/ml, Streptomycin at 8 mg/ml, and tetracycline at 4mg/ml (Valério et al., 2017) (Figure 3-4). A multiplicity of infection (MOI) of 1 in TSB was used. 6.5 µl of 10^8 overnight bacterial culture and 12µl of 10^8 phage forming units per ml (PFU/ML) of the phage were inoculated in 96-well plates tubes with 150 µl TSB. Two controls were included, a bacteria control, and a phage control. The samples were incubated at room temperature and a revolution of 50 revolutions per minute (rpm). Aliquots were picked at 0, 1, 2, 3, 4, 5, 6, 22, and 24 hours of incubation for optical density measurement using a multimode reader (BioTEK, US).

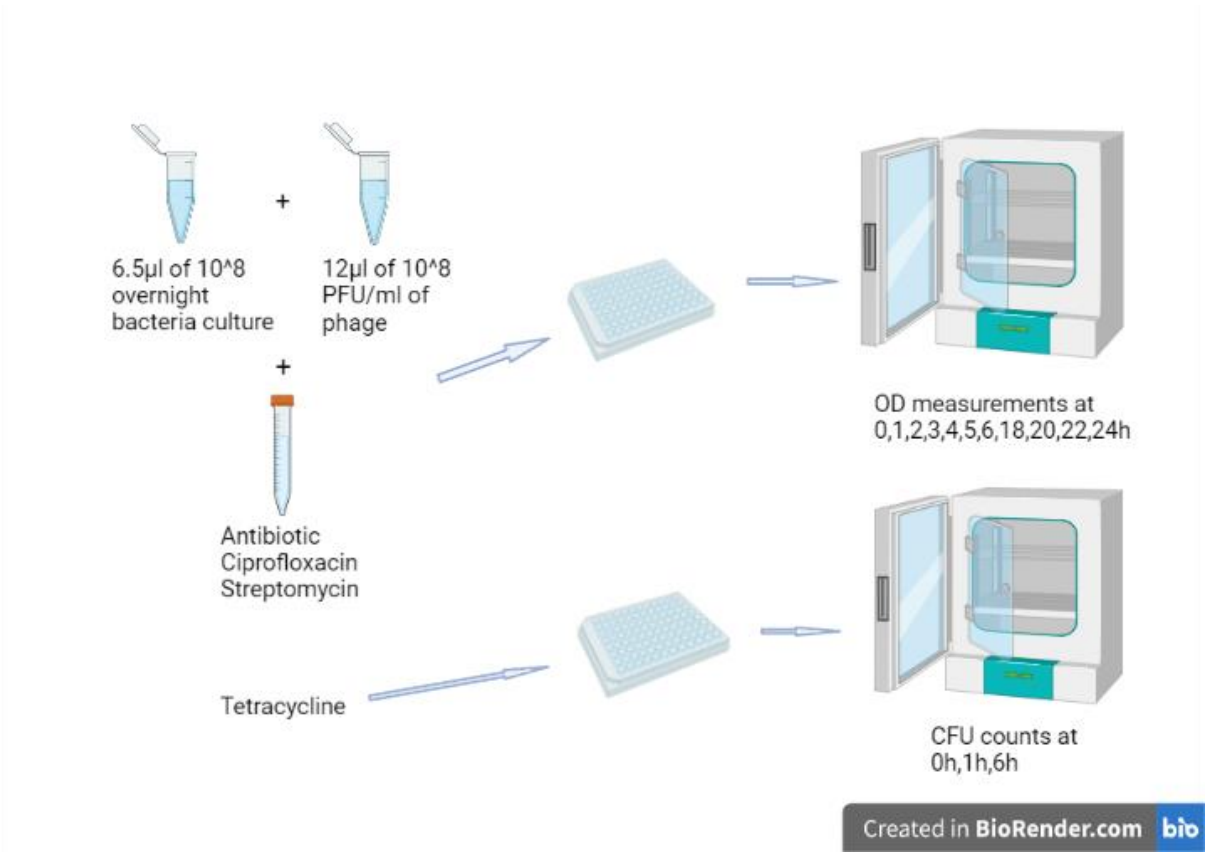

Figure 3-4: Bacteriophage and antibiotics kill curves in TSB

3.5.13 Statistical analysis

Graphpad prism software version 9.4.1 was used for data analysis and graphics.

Means obtained with the different treatment strategies were compared using two-way ANOVA to check the difference between the means of the usage of single-agent and combination therapy. A p-value of </=0.05 was considered significant (B. K. Chan et al., 2016).

CHAPTER FOUR

4.0 RESULTS

4.1 Isolation, biochemical and serological identification

Six isolates were confirmed to be *S.* Enteritidis as the colonies were non-lactose fermenters in MacConkey agar and were pale with dark centered colonies in SS, XLT-4, and dark purple in Brilliance *Salmonella* green agar (Figure 4-1). These isolates were subjected to biochemical tests using TSI and API Strips (Figure 4-3) and only urease negative colonies were picked for further confirmation. The confirmed isolates were further identified by serology using a *Salmonella* kit, Poly O,9, g, h, and I antisera (Figures (4-2,).

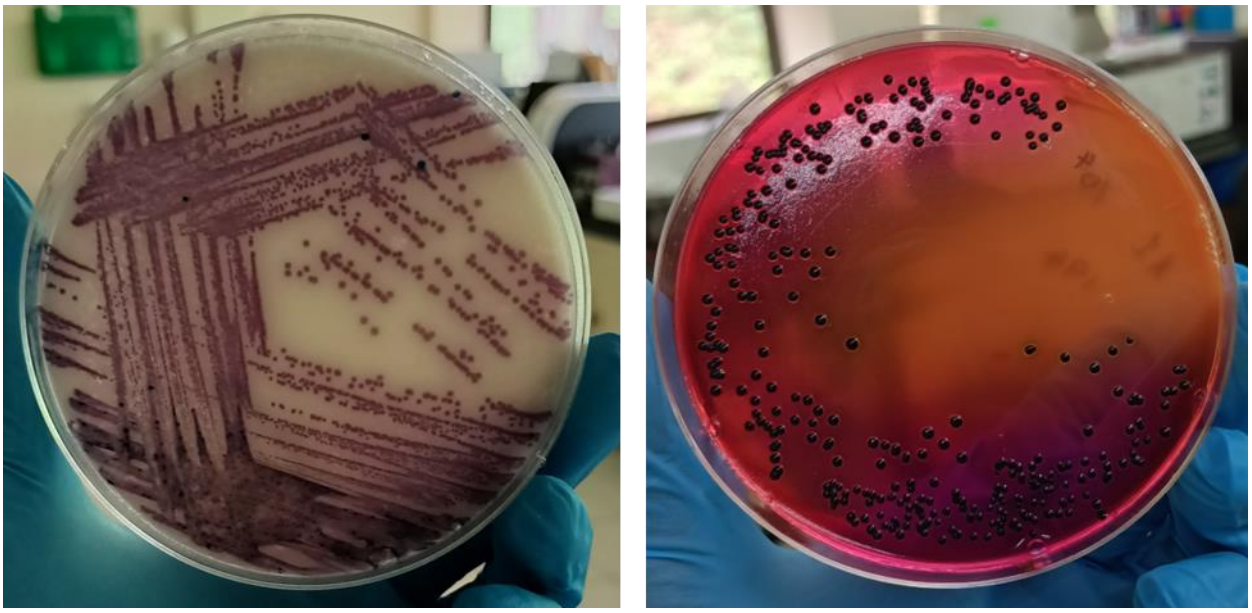

Figure 4-1: *Salmonella* Isolation at ILRI Phage laboratory

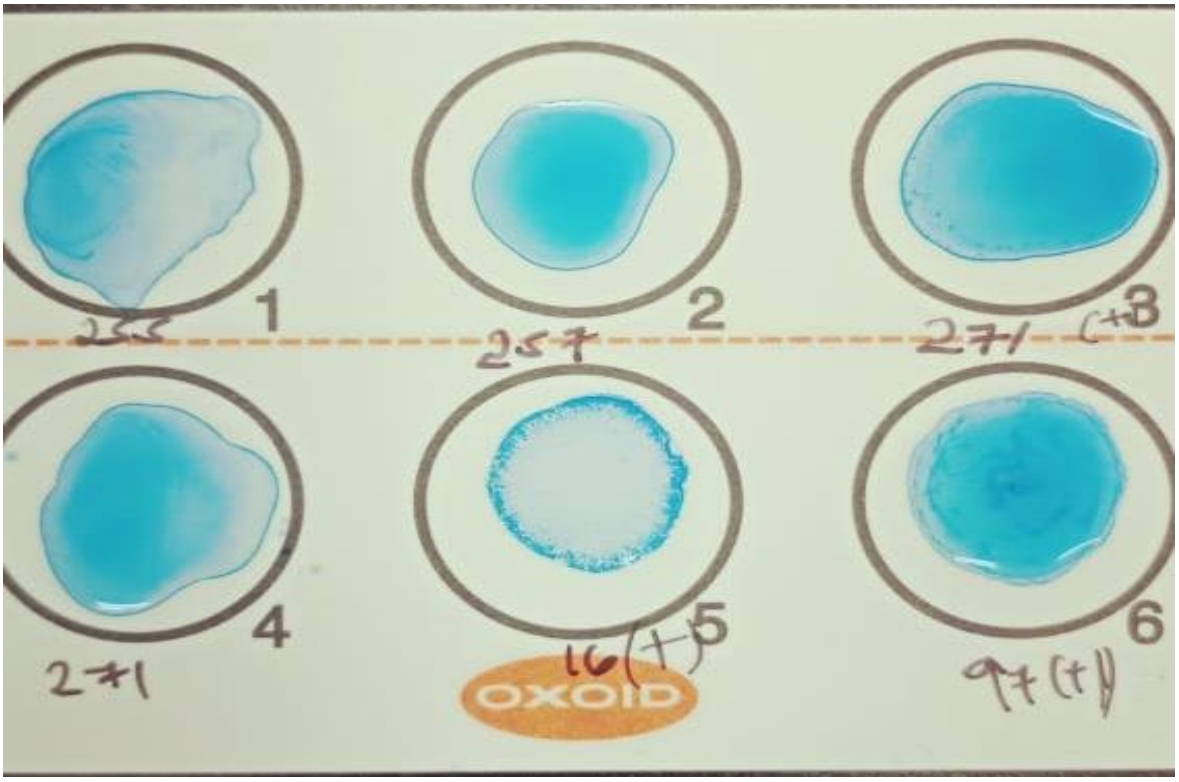

Figure 4-2: Salmonella agglutination test using *Salmonella* specific kit

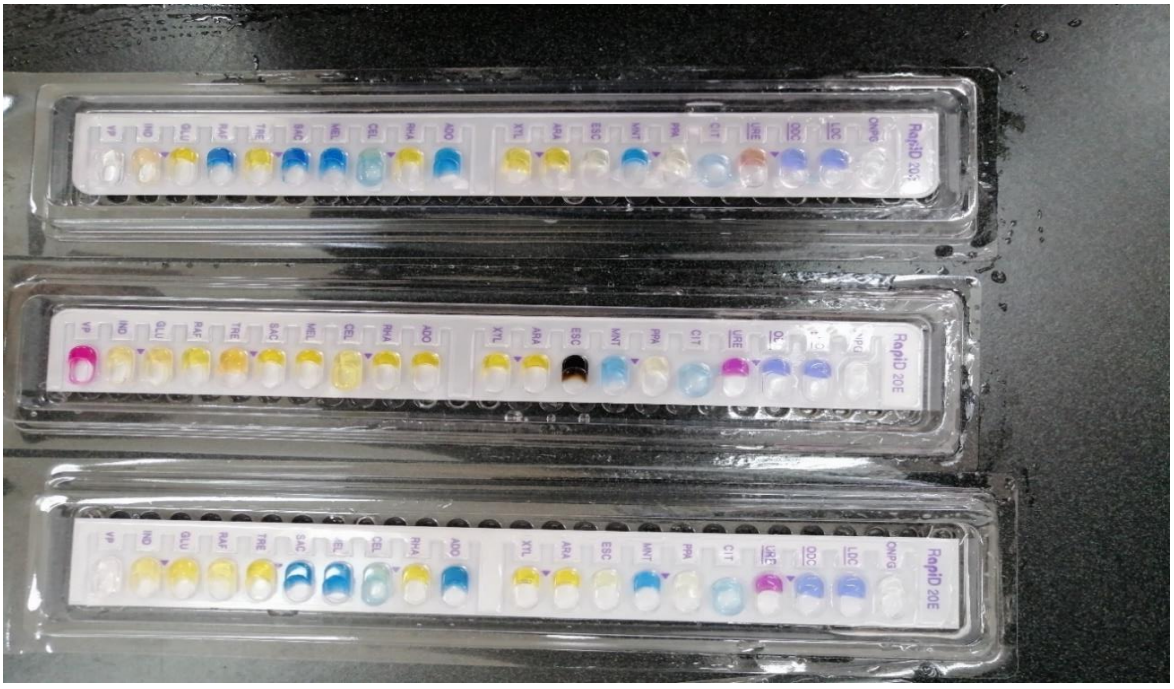

Figure 4-3: API strips used for the biochemical identification of *Salmonella*

4.2 *inv*A PCR for the confirmation of *Salmonella*

The six selected *S.* Enteritidis strains (Sal 16, Sal 73, Sal 177, Sal 568, Sal 569, and Sal 572) were confirmed using *inv*A PCR as shown in figure 4-4.

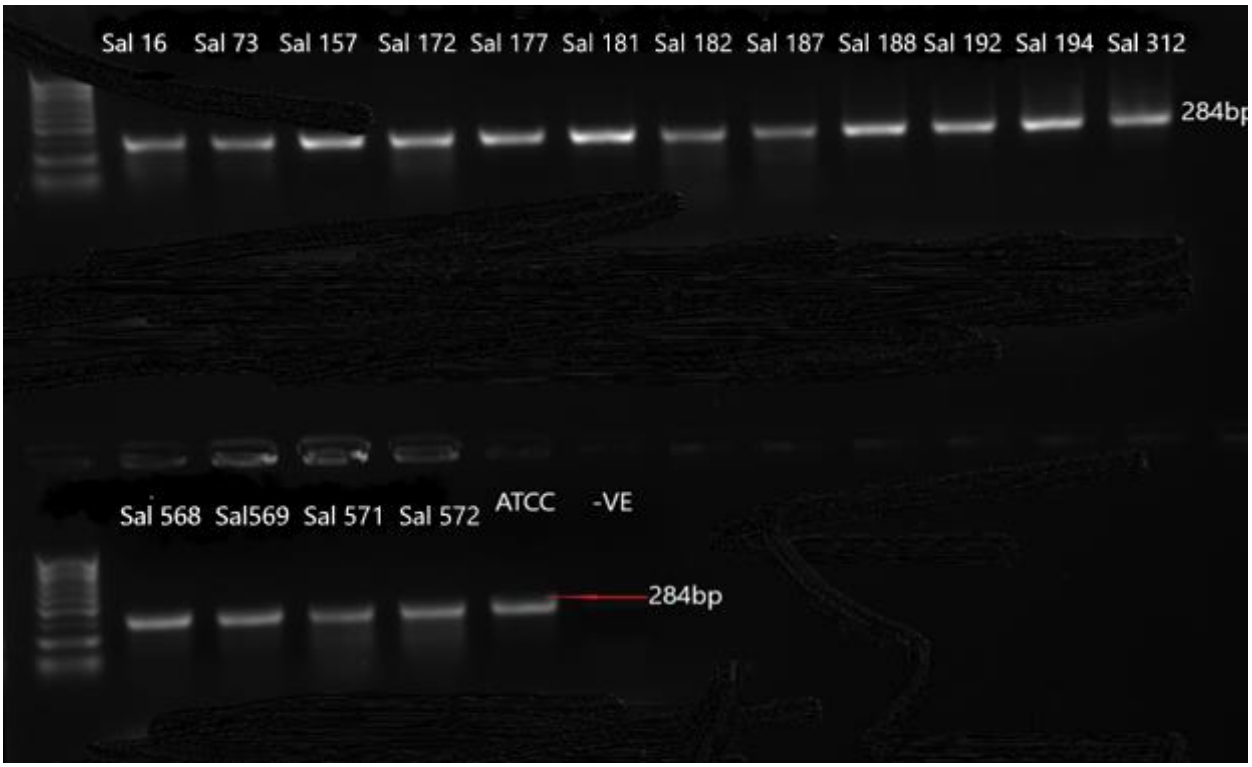

Figure 4-4: Gel image of InVA PCR products of *Salmonella* isolates run in 1.5% Agarose Gel electrophoresis for 60 minutes and viewed under ultraviolet light

4.3 Isolation of bacteriophage insensitive mutants of field strains of *Salmonella* Enteritidis

Analyses of bacterial resistance (Figure 4-5) after six passages revealed that the development of resistance varies between phages (Figure 4-6). In total, 44 BIMs of the six *S*. Enteritidis strains (Sal 16, Sal 177, Sal 569, Sal 73, Sal 568, and Sal 572) were isolated. In general, resistance development was 79.5% when phage ILRI_K24 was used as compared to 20.5% when ILRI_K23 was used (Figure 4-6). The BIMs were plated on green indicator plates to ensure that we only pick pure colonies without phages for the rest of the experiments (Figure 4-7)

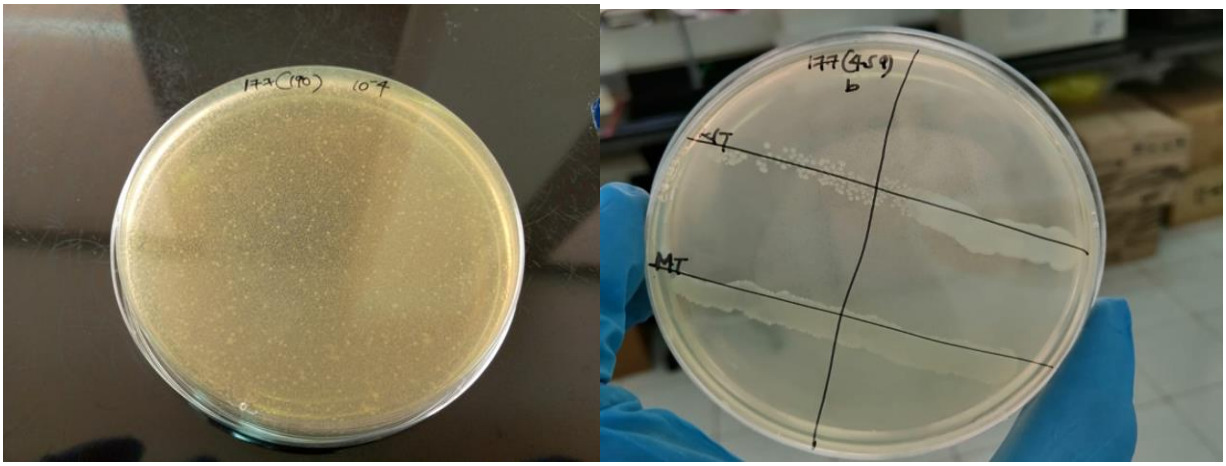

Figure 4-5: TSA plates of BIMs, Plate 1: Isolated colonies after pour plating phage and bacteria. Plate 2: A phage streaked across a plate with a mutant and a susceptible bacterium

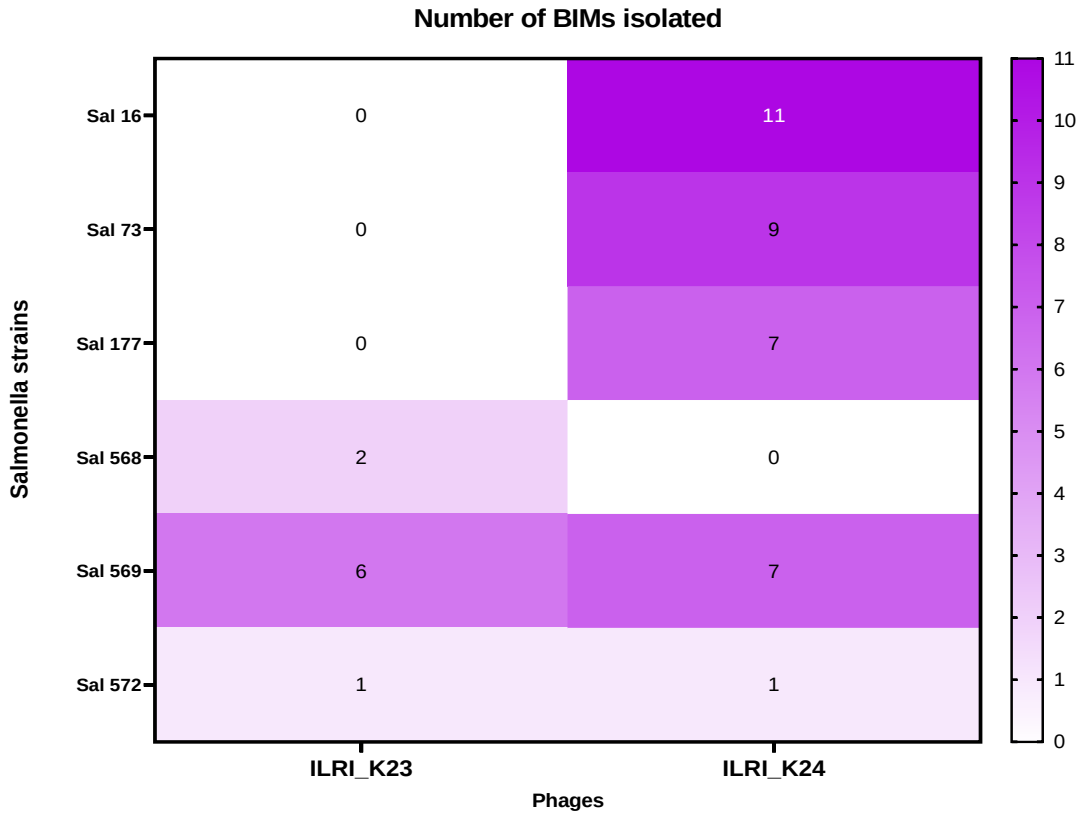

Figure 4-6: Heatmap of the distribution of BIMs isolated from two phages ILRI_K23 and ILRI_K24 subjected to six *S*. Enteritidis strains

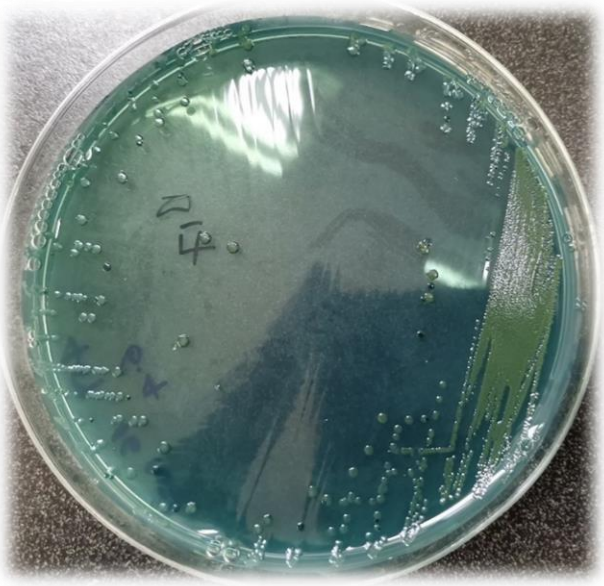

Figure 4-7: Green indicator plates used to obtain pure BIMs colonies without phages

4.4 Antibiotic Sensitivity of BIMs

As shown in figures (4-8,4-9,4-10), compared to the parental strains, sensitivity to streptomycin increased in 90% of the BIMs. BIMs did not increase their sensitivities to ceftriaxone, azithromycin, erythromycin, neomycin, chloramphenicol, gentamicin, ampicillin, streptomycin, amoxicillin/clavulanic acid, and trimethoprim/sulfamethoxazole. Susceptibility to penicillin and erythromycin did not increase after exposure to phages. Two BIMs became resistant to tetracycline and kanamycin (Figure 4-8 and Figure 4-9). Interestingly, Sal 572 was previously resistant to nalidixic acid and gentamicin, but all the BIMs isolated from 572 were now susceptible to both nalidixic acid and gentamicin (Figure 4-10)

Furthermore, more than half (56.8%) of the BIMs whose parents were resistant became susceptible. About a quarter (27.3%) of the BIMs did not change their susceptibilities to all antibiotics. Only a small percentage were no longer susceptible after exposure to phages (9.1%) or had changed in their susceptibility patterns, becoming resistant and sensitive to different antibiotics (6.8%) (Figure 4-11). In summary, all the BIMs were now susceptible to all cephalosporins, a few had a subtle reduction in sensitivity to ciprofloxacin and most resistance was observed in the aminoglycosides, including erythromycin and neomycin.

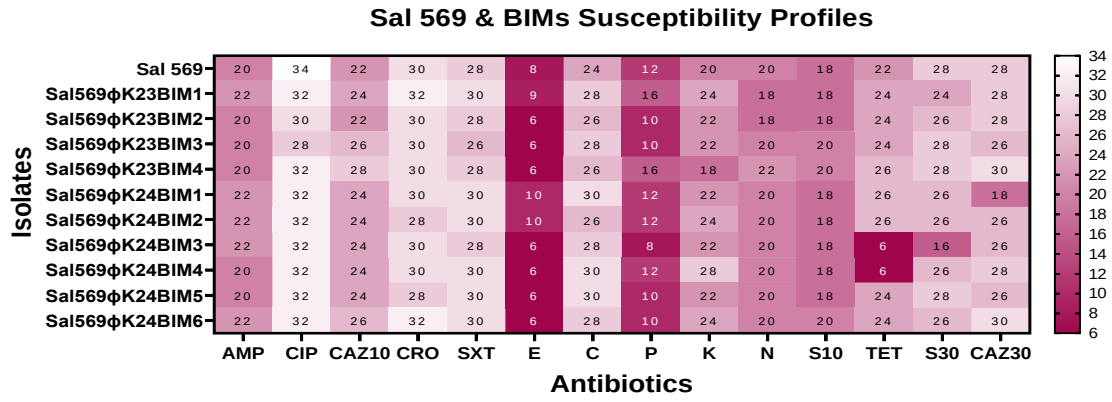

Figure 4-8: Heatmap of Sal 73 BIMs susceptibility profiles

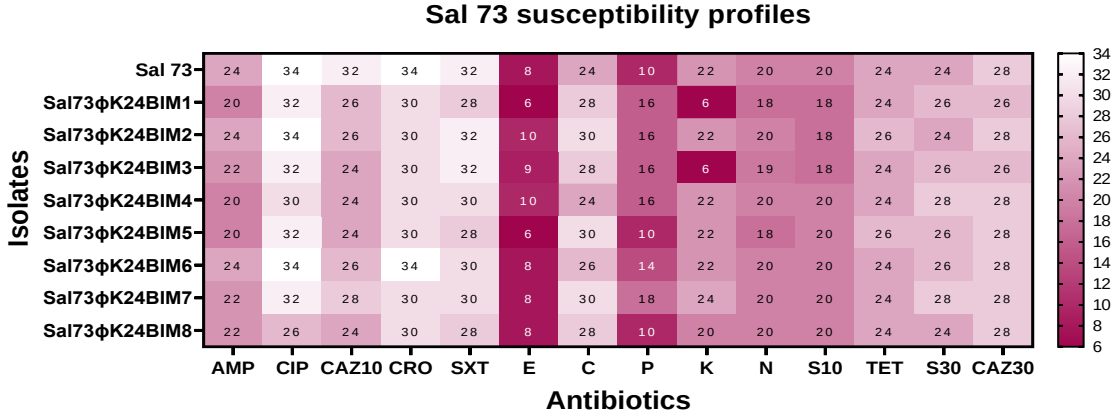

Figure 4-9: Heatmap of Sal 569 BIMs susceptibility profiles

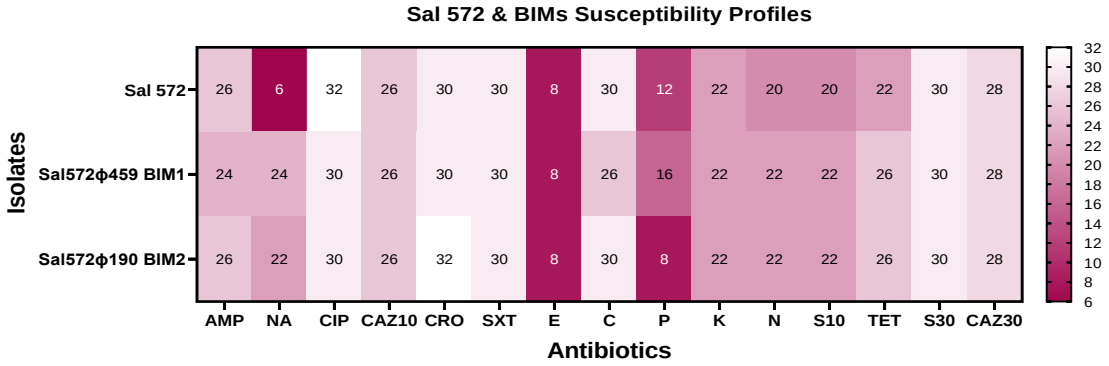

Figure 4-10: Heatmap of Sal 572 BIMs susceptibility profiles

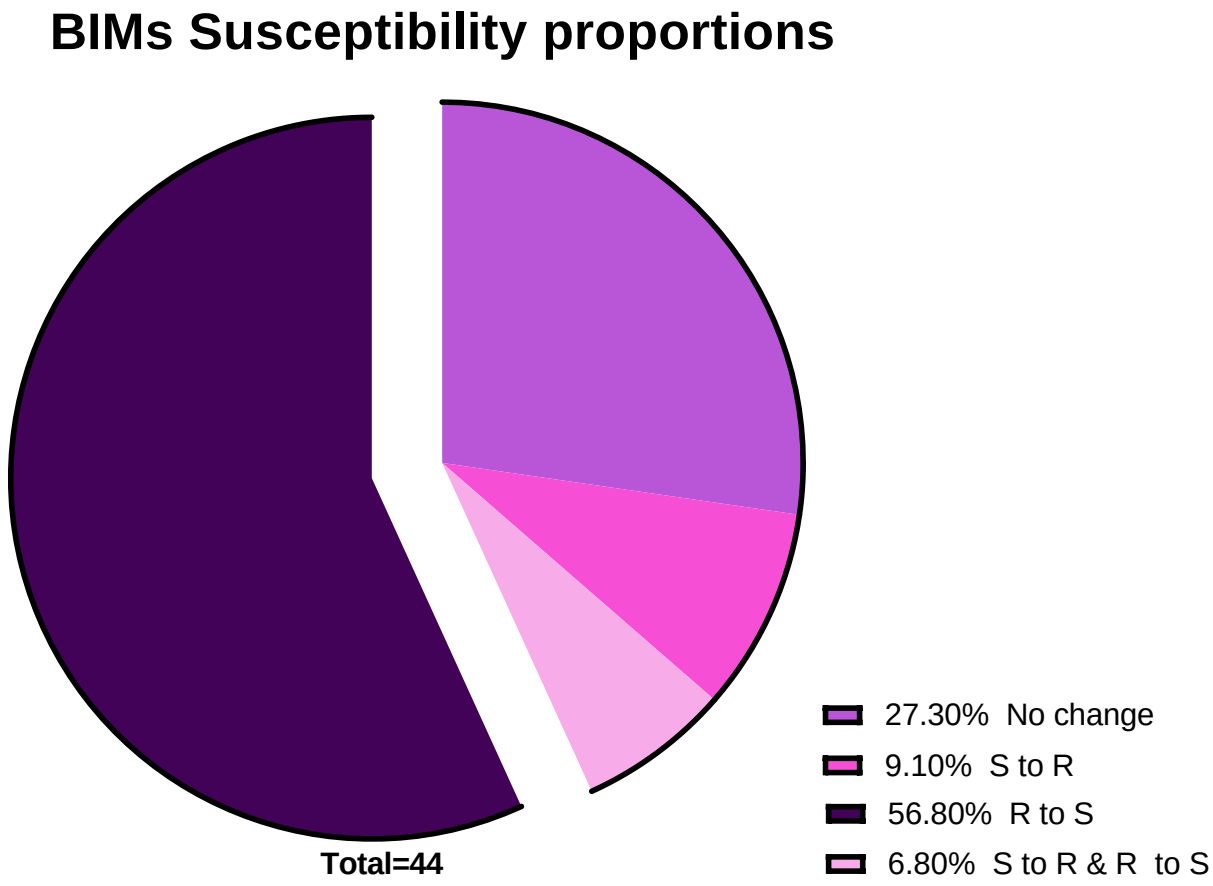

Figure 4-11: Pie chart of BIMs antibiotic susceptibility proportions

4.5 Adsorption test

4.5.1 Phages reduction of binding efficiency of selected BIMs

Since several antibiotic resistance genes encode for antibiotic efflux pumps, such as *tet*A, *tet*B in *S.* Enteritidis, *tol*C in *E. coli*, and *nor*A in *S. aureus* (Peterson & Kaur, 2018), and since they can serve as a viral receptor to some phages since they are located at the cell surface, we decided to investigate how many of the BIMs that became susceptible to antibiotics and had lost *tetA* gene had reduced capacity to bind the phages (Figure 3-2). BIMs had reduced binding efficiency as compared to the parent strains as we observed more phages presence in the supernatant of the BIMs (Figure 4-12). The binding efficiency also varied among the BIMs with some showing higher binding capacity (Figure 4-13). This can be because bacteria might have developed resistance using other mechanisms such as R-M modification or CRISPR/Cas.

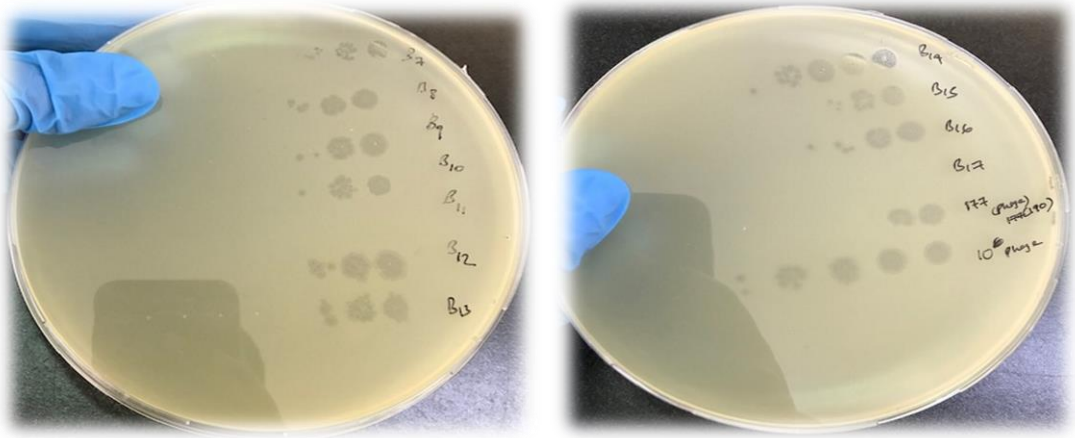

Figure 4-12: TSA plates of spot analysis of supernatant of BIMs after adsorption showing BIMs vary in their adsorption capacities

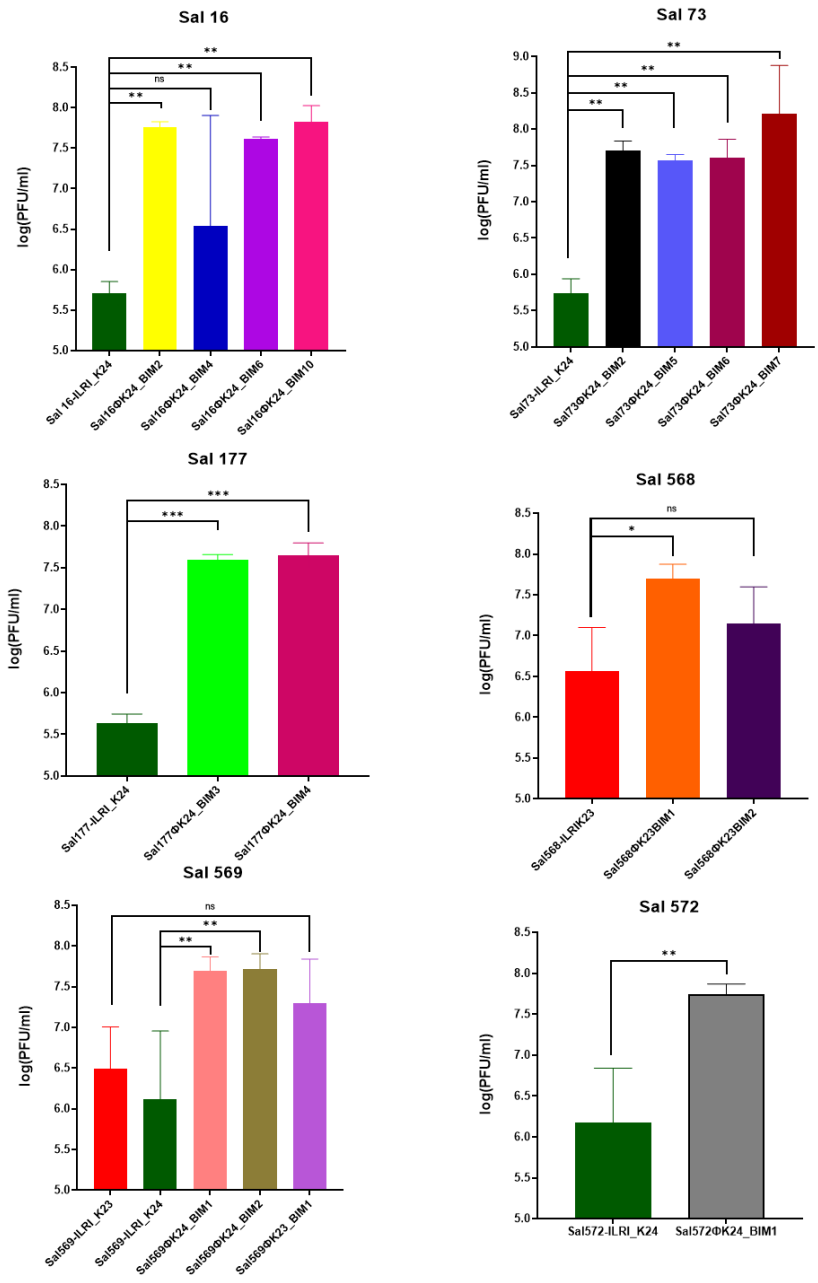

Figure 4-13: Bar graph of the adsorption capacities of 16 BIMs that had lost tet A genes versus the hosts Sal 16, Sal 73, Sal 177, Sal 568, Sal 569 and Sal 572 using M01 of 0.1 of phage ILRI_K24.

4.5.2 Loss of antibiotic resistance genes by BIMs

After carrying out an antimicrobial sensitivity test, we noticed that phage resistance caused a remarkably increased susceptibility to antibiotics such as streptomycin and tetracycline. Out of the six *S.* Enteritidis strains selected, two were resistant to streptomycin (30mcg) and two showed intermediate resistance to that antibiotic. We decided to further test for the presence of AMR genes coding for streptomycin ,tetracycline and ciprofloxacin resistance on the BIMs and the parental strain to determine if they were lost. The *tet*A, *tet*B, *str*A, *strB, aadA1, aadA2 and gyrA* genes were tested with these BIMs. As shown in Figures 4-14 and 4-15, *tet*A, *tet*B, *str*A, and *str*B and gyrA were present in the parent strain Sal 572, but all the BIMs that originated from that strain did not have these genes. The *tetA* gene was also present in Sal 16, Sal 73, and Sal 569 but absent in their BIMs.

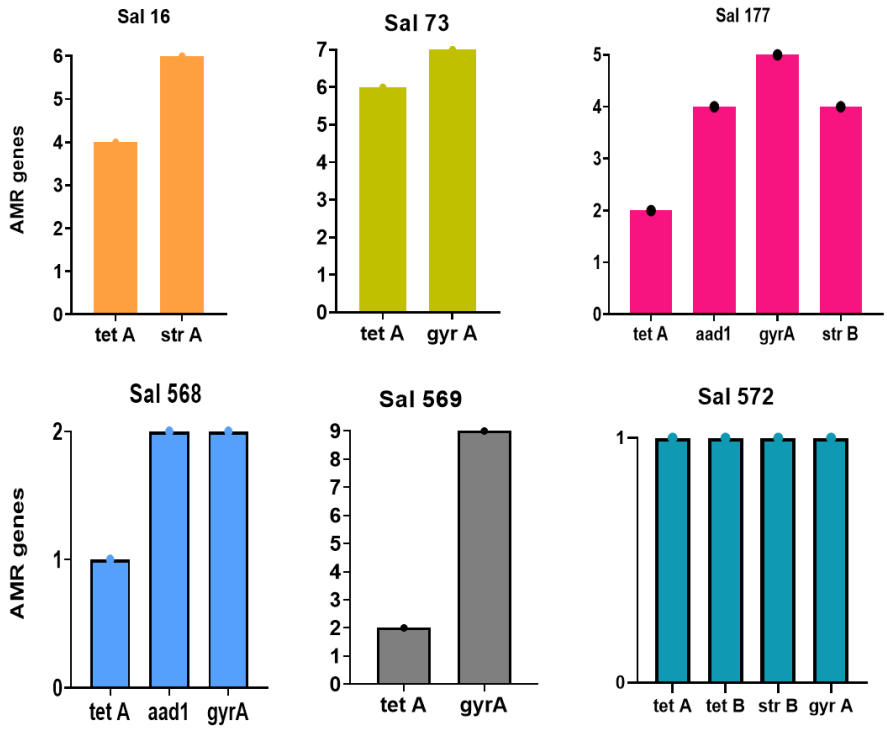

Figure 4-14: Bargraphs of AMR genes in the six S. Enteritidis versus the BIMs

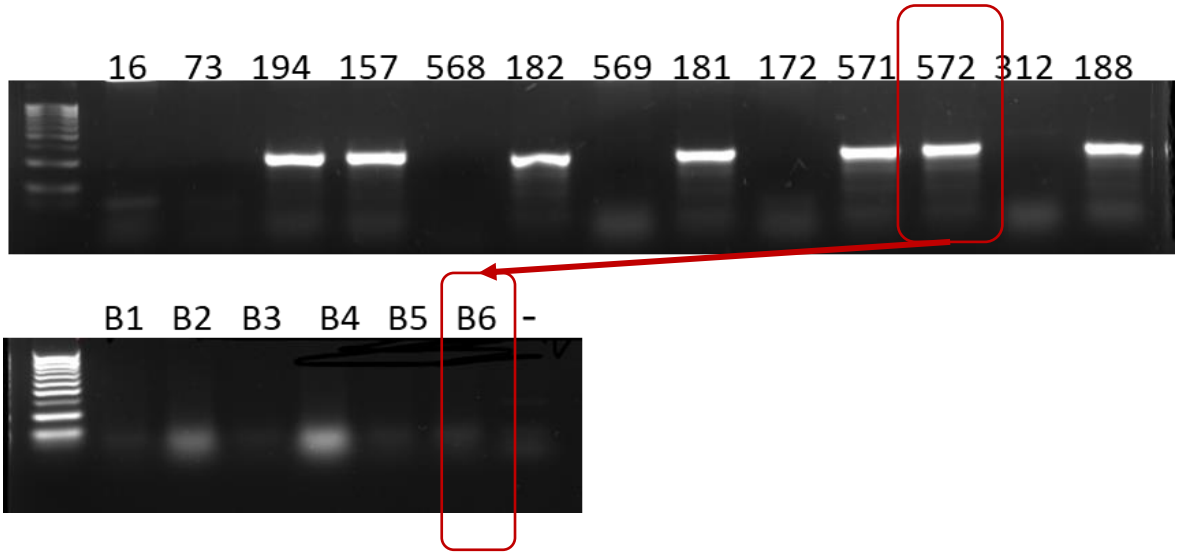

Figure 4-15:: Gel image of tetA PCR products showing loss of AMR gene in the BIMs

4.6 Minimum Inhibitory Concentrations

The assessed MIC of the six *Salmonella* isolates to ciprofloxacin ranged between 0.032 mg/ml and 8mg/ml, 1.5 -2mg/ml to tetracycline, and 3-12 mg/ml to streptomycin. A comparison of the BIMs' and host parents' MICs showed that while some BIMs developed resistance to some antibiotics such as B35 to gentamicin (Figure 4-16) most of the BIMs required a lower concentration of antibiotics as shown in Figure 4-17.

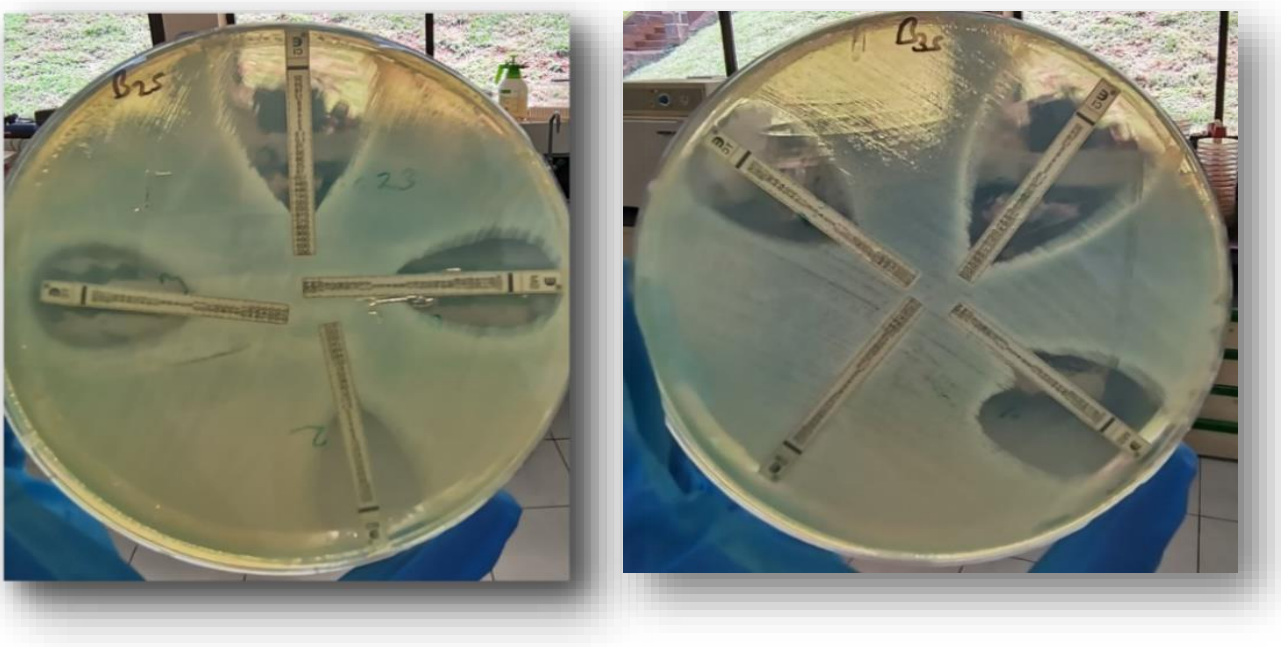

Figure 4-16:: TSA plates of the MIC of two BIMs B25 and B35 using four antibiotics E-test strips

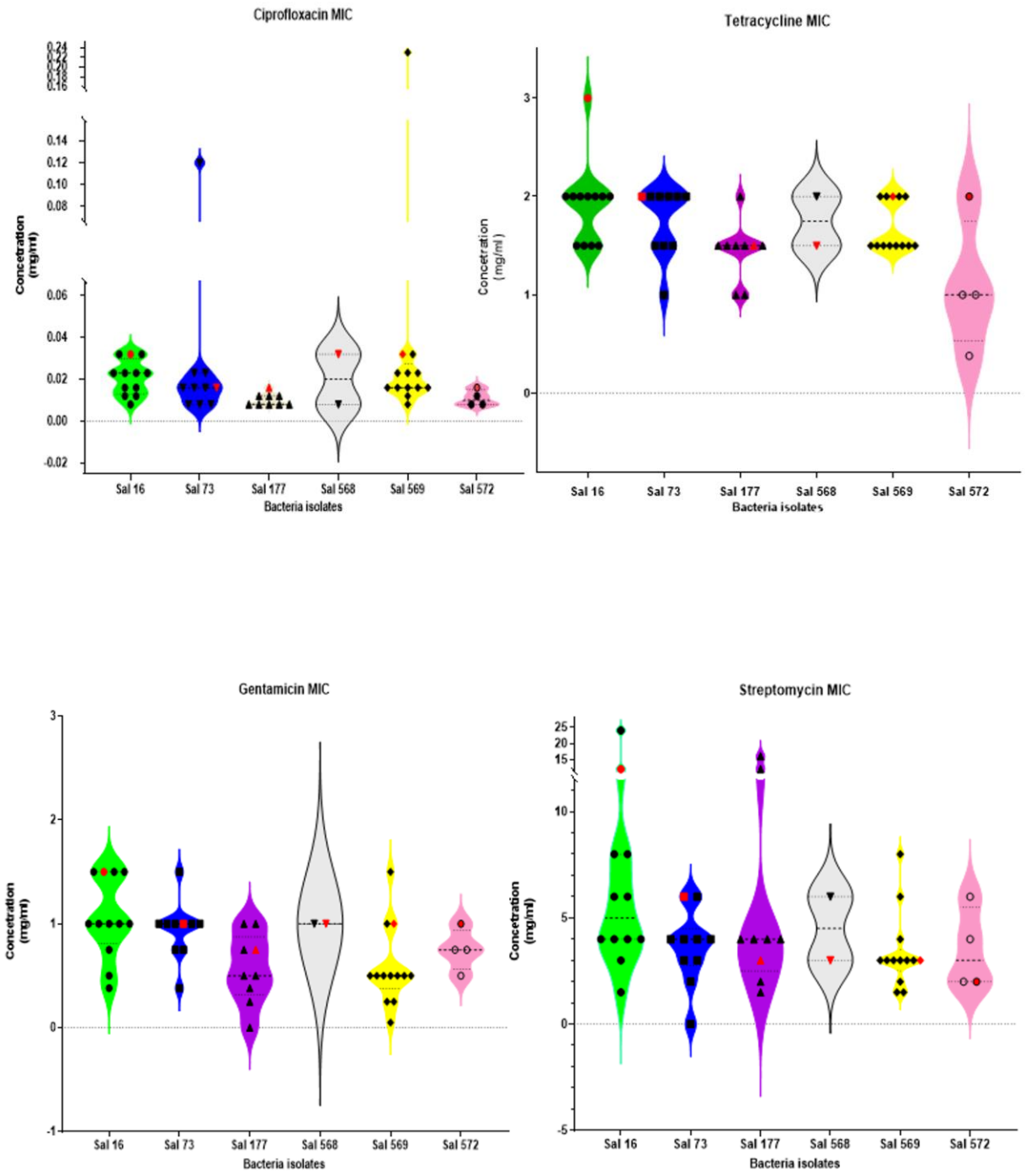

Figure 4-17: Violin plots of MIC (mg/ml) of the six *S*. Enteriditis strains to A): ciprofloxacin, B): tetracycline, C): gentamicin and D): streptomycin.

4.7 Bacteriophage and antibiotic kill curves in TSB

Salmonella inactivation was tested using phage ILRI_K24 - and ciprofloxacin at 0.5mg/ml, streptomycin at 8mg/ml, and tetracycline at 4mg/ml. Phage control (PC) density was consistent throughout the experiment (ANOVA, $p>0.05$). The phage was able to cause a decrease in the bacterial density after 6h (B+P) (ANOVA, $p<0.05$) after which a slight increase in the bacterial density is observed in all isolates.

The effectiveness of the combined therapy was different among the different antibiotics, the bacterial strain, and the time of antibiotic addition. Sal 16,73,177, 569, and 572 had a significant reduction of the bacteria density when streptomycin was added to the phage and bacteria after 6 hours (B+P+S(8) after 6 hr.) (ANOVA, $p<0.05$) as compared to when the antibiotic was added simultaneously with phage at the beginning of the experiment (B+P+S(8)) (ANOVA, $p>0.05$) (Figure 4-18).

Sal 177, 568, 569, and 572 were significantly killed when a combination of ciprofloxacin and phage (B+P+Cip (0.5)) was used as compared to the antibiotic alone (B+cip(0.5)) after 20h ANOVA,$p<0.05$). 3/6 of the isolates where significantly killed when ciprofloxacin was added 6 hours after phage infection (Figure 4-19) . Sal 177 and Sal 572 colony-forming units (CFU) were significantly reduced when a combination of phage and tetracycline (B+T+P) was used (ANOVA, $p<0.05$) Sequential treatment was also more effective than simultaneous treatment with zero bacteria growth at the end of the experiment (Figure 4-20). Measurement of tetracycline OD was difficult because of the colored tetracycline antibiotic hence we measured the differences in the treatment using CFU/ml.

Sal 569 was significantly killed when streptomycin was added simultaneously with the phage and also when streptomycin was added sequentially after six hours. When the antibiotic was added after 6h of phage addition, no regrowth of bacteria was observed in all six strains till the completion of the treatment, contrary to the observation for ((B+P, B+P+S(8), B+P+Cip(0.5))

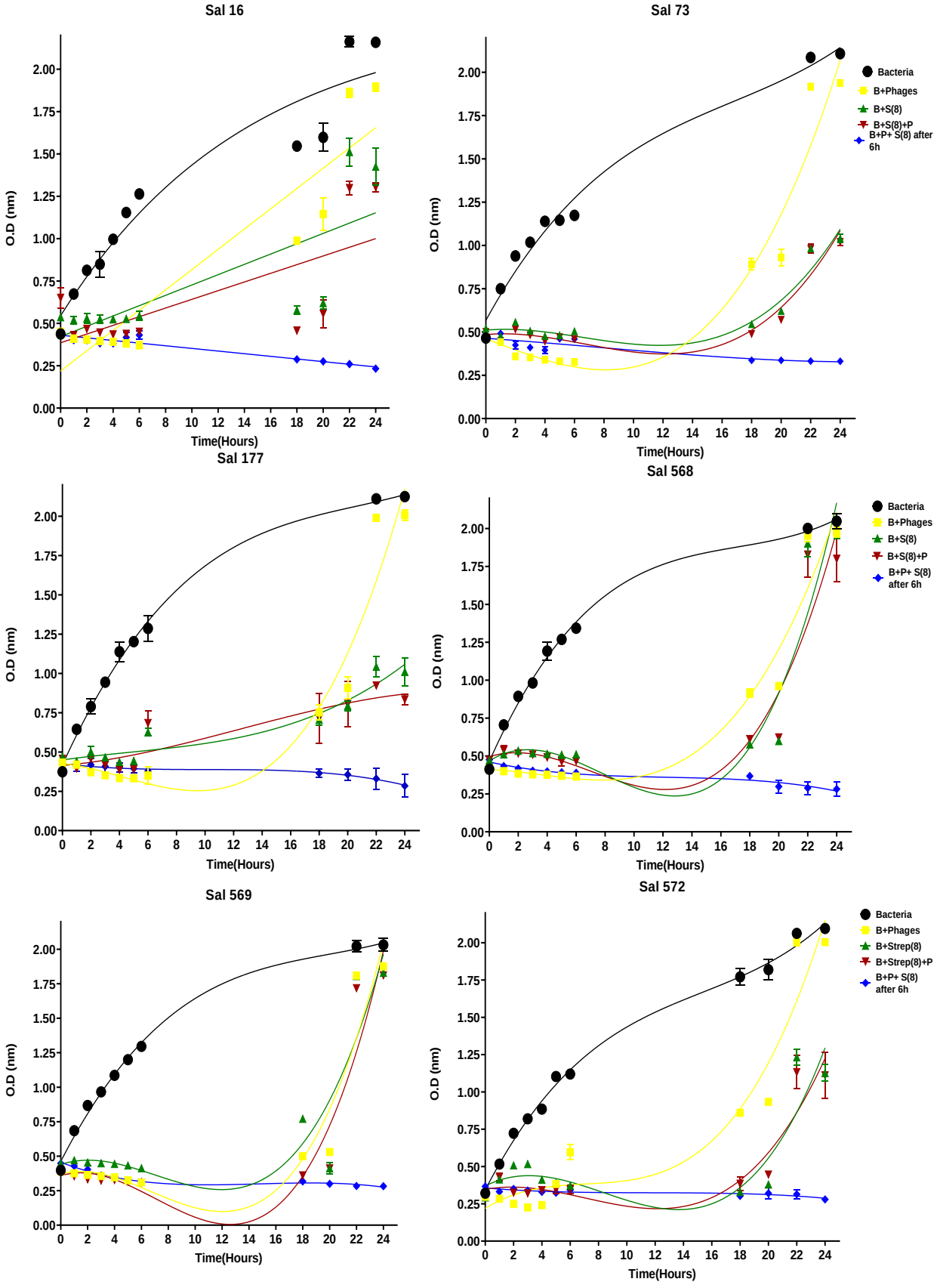
Sal 16
Sal 73
Sal 177
Sal 568
Sal 569
Sal 572
O.D (nm)
Time(Hours)
Bacteria
B+Phages
B+S(8)
B+S(8)+P
B+P+ S(8) after 6h
B+Strep(8)
B+Strep(8)+P

Figure 4-18: : Effect of antibiotic alone and of combined phage and streptomycin treatments at (8mg/ml) on the inactivation of Sal 16 in TSB over a 24h period; Bacteria; B + Phages, bacteria plus phages; PC, phage control; B+S(8), bacteria plus streptomycin : B+S+P, bacteria plus streptomycin plus phage; B+S+P after 6H.

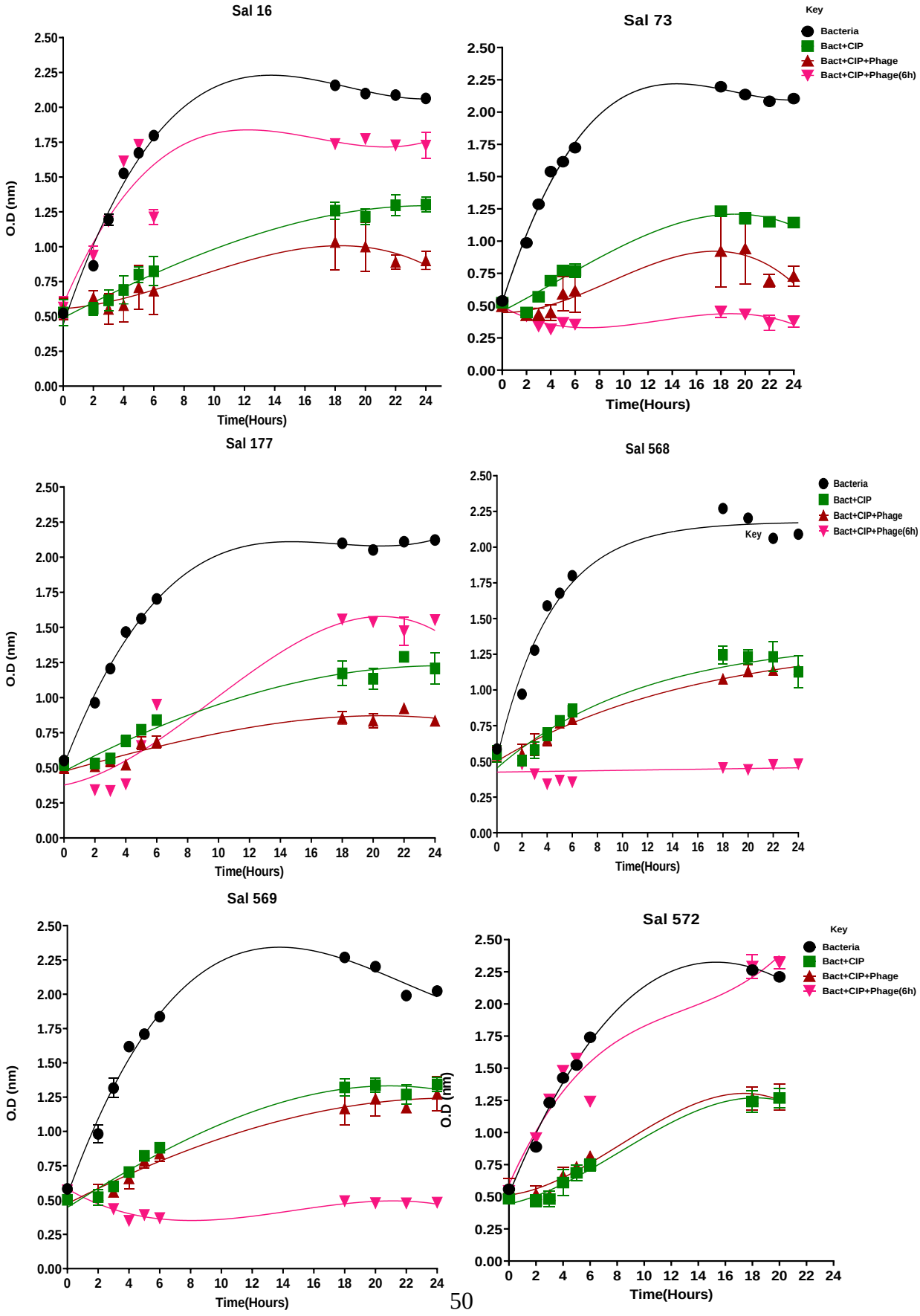
Sal 16
Sal 73
Sal 177
Sal 568
Sal 569
Sal 572
Key
Bacteria
Bact+CIP
Bact+CIP+Phage
Bact+CIP+Phage(6h)
O.D (nm)
o.b (nm)
Time(Hours)

Figure 4-19: : Effect of antibiotic alone and of combined phage and ciprofloxacin treatments at (0.5mg/ml) on the inactivation of Sal 16 in TSB over a 24h period; Bacteria; Bact + Cip(0.5),bacteria plus ciprofloxacin 0,5 mg/ml; Bact + Cip(0.5)+P, bacteria plus ciprofloxacin 0.5mg/ml plus phage.bact+cip+phage after 6H.

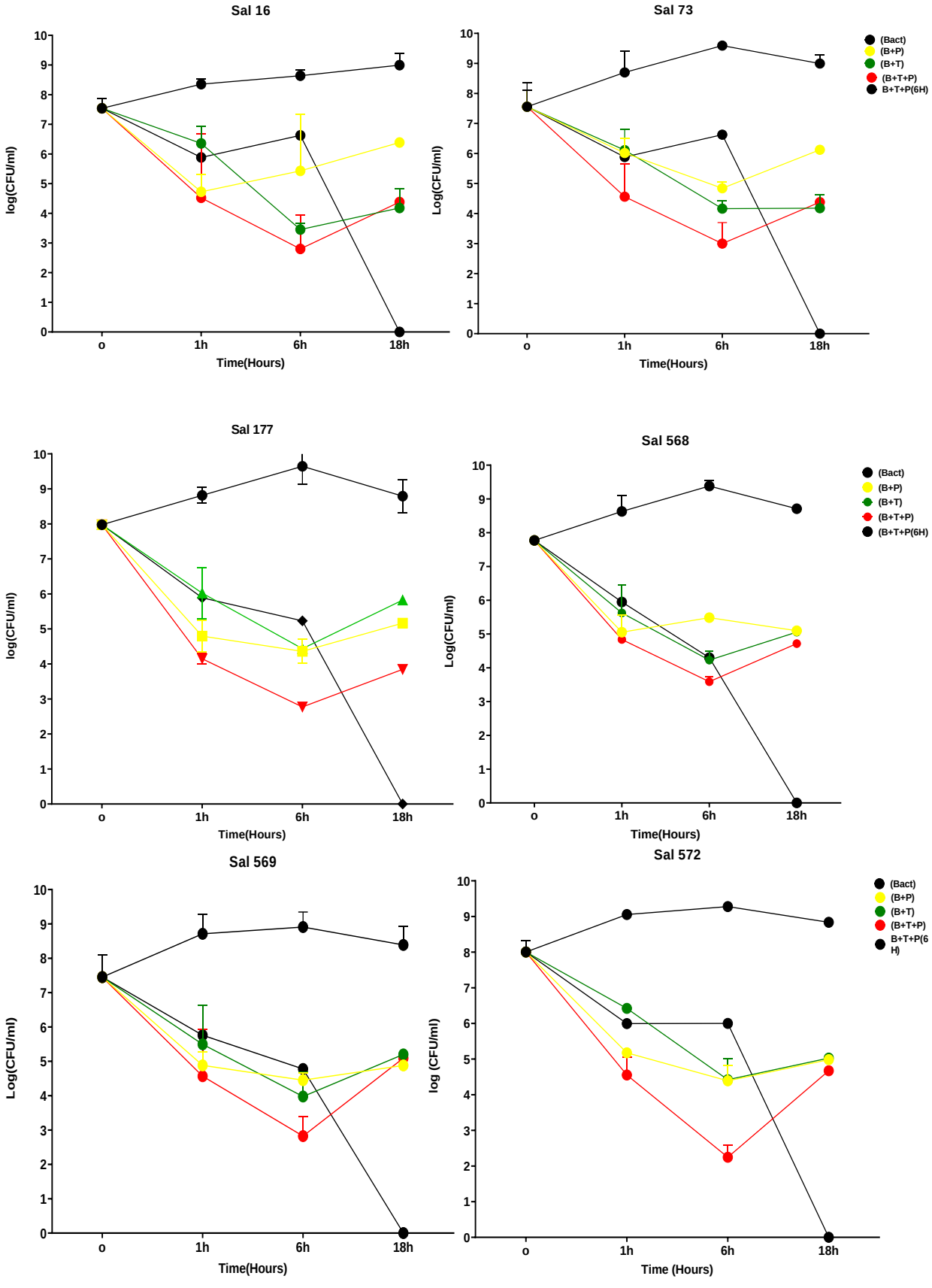
Sal 16
log(CFU/ml)
Time(Hours)
o
1h
6h
18h
Sal 73
Log(CFU/ml)
Time(Hours)
(Bact)
(B+P)
(B+T)
(B+T+P)
B+T+P(6H)
Sal 177
log(CFU/ml)
Time(Hours)
Sal 568
Log(CFU/ml)
Time(Hours)
(Bact)
(B+P)
(B+T)
(B+T+P)
(B+T+P(6H)
Sal 569
Log(CFU/ml)
Time(Hours)
Sal 572
log (CFU/ml)
Time (Hours)
(Bact)
(B+P)
(B+T)
(B+T+P)
B+T+P(6H)

Figure 4-20: Effect of antibiotic alone and combined phage and tetracycline treatments at (1mg/ml) on the inactivation of Sal 177 and Sal 572 in TSB over a 24h period; Bact, bacteria; B+P, bacteria plus phage; B+T, bacteria plus tetracycline; B+T+P, bacteria plus tetracycline plus phage.B+T+P(6H), bacteria plus phage and tetracycline added after 6H. of phage infection.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONs, AND RECOMMENDATIONS

5.1 Discussion

The complex problem of antimicrobial resistance in common bacteria such as *E.coli* and *Salmonella* sp. is spread across human and animal health, and the environment, making this a global One Health issue. Bacteria develop resistance to antibiotics using several mechanisms which can be acquired or internal. Resistance to tetracycline is primarily due to efflux pumps, while streptomycin resistance is regulated by multiple factors such as target modification, inactivating enzymes, and efflux pumps. Resistance to ciprofloxacin can arise through acquiring mutations in the genes that code the target proteins of ciprofloxacin and regulators of efflux pumps. It is crucial to develop alternative treatments to combat this problem (Lopes et al., 2018). AMR burden is high in low and middle-income countries (LMIC) caused by some factors such as irrational use of antibiotics, lack of awareness, and low-quality drugs, among others (Urbaniak et al., n.d.). Various studies have shown the ability of phages to be used to control pathogenic bacteria (Kutateladze & Adamia, 2010b; Makumi et al., 2021; Rohwer, Youle, Maughan, et al., 2014) However, the development of bacteriophage-resistant mutants is a general shortcoming (Fong et al., 2020).

Combinations of antibiotics and phages could potentially reduce the emergence of resistance to either phages or antibiotics (B. K. Chan et al., 2016b; Chaudhry et al., 2017; Kamal & Dennis, 2015a). Few studies have reported on the interaction process of phages and antibiotics in combination therapy, especially regarding bacteriophage resistance emergence and the effect on antimicrobial resistance. Hence it is important to carry out more studies to better understand the

interaction between phages and antibiotics (Chaudhry et al., 2017; Comeau et al., 2007a; Rodriguez-Gonzalez et al., 2020a)

This study aimed to understand whether phage exposure could reverse antibiotic resistance in our newly isolated *S.* Enteritidis strains with our novel *S.* Enteritidis-specific phages isolated from Kenyan poultry farms. We also aimed to test whether our phages could cause mutations or deletions of the AMR genes in the BIMs. In our study, we observed that some BIMs had lost their AMR genes. An explanation for this is that the antibiotic resistance genes could have mutated or possibly been lost, which would explain the increased susceptibility of the previously resistant bacteria to specific antibiotics.

We expected that the fitness costs associated with antibiotic and phage resistance would result in decreased resistance evolution to antibiotics. Our findings confirm previous studies showing that phage-antibiotic combinations typically result in an increased reduction of bacterial densities and less resistance than single antimicrobials. We observed that phage resistance evolution can positively affect the reversal of bacterial antibiotic-resistant phenotype. We also observed a significant decrease in bacterial density while using a combination of phage ILRI_K24 and ciprofloxacin as compared to using the antibiotic alone. Tetracycline also showed a significant synergy with the phage showing that combining the antibiotic was more efficacious than using a single agent. Interestingly, we observed that the addition of streptomycin after 6 hours of phage infection lowered the bacterial load more significantly than when both agents are added at the same time. This might be because the phages got time to replicate and proliferate therefore improving the synergy effect with antibiotics.

The results of this study demonstrated that the combined treatment reduced the emergence of antibiotic-resistant variants. Some studies have demonstrated that phage-antibiotic combinations cause equal resistance when the agents are used individually. The combination effectively reduces the development of bacterial resistance to both phages and antibiotics.

5.2 Conclusions

When exposed to phages severally, bacteria develop resistance as a survival mechanism, in the process rendering the treatment ineffective. Forty-four BIMs were isolated when two phages,

ILRI_K23 and ILRI_K24 were exposed to six *Salmonella* Enteritidis (Sal 16, Sal 73, Sal 177, Sal 568, Sal 569, and Sal 572). The development of BIMSs varied with the phage as some phages can bypass the defense mechanism set up by bacteria. More BIMs were selected with phage ILRI_K24 in our study. The development of BIMs can affect phage therapy but at the same time, it can be used to select against antimicrobial resistance factors and sometimes also virulence factors when bacteriophages are used in combination with antibiotics. When exposed to antibiotics, a huge percent of the BIMs became sensitive to antibiotics to which the parent strain was resistant, with most also showing lowered MICs of ciprofloxacin, streptomycin, tetracycline, and gentamicin required for *Salmonella* inactivation. We could no longer detect some AMR genes in the BIMs, including some efflux pump-related genes such as *tet*A and *tet*B which shows that a positive genetic trade-off had occurred selecting against the AMR genes.

Despite that there was a significant reduction in the bacteria density when combined treatment was applied when ciprofloxacin, tetracycline, and streptomycin were used, we noted that a greater reduction was observed when streptomycin was added after six hours of phage infection, therefore, it is important to note that the antibiotic concentration and the antibiotic application time are

essential factors that should be considered for one to come up with the most effective combined treatment.

5.3 Recommendations

The following are recommended based on the findings of this study

Most BIMs become sensitive to some specific classes of antibiotics, especially ciprofloxacin, tetracycline, and streptomycin. While using combined treatment the classes of antibiotics used should be considered to avoid antagonistic effects.

More studies should be carried out to understand the relationship between adsorption capacity reduction in BIMs and receptors used for attachment of BIMs and antibiotics.

Screening of more genes coding for AMR should be done to better understand the effect of phage exposure to AMR genes.

Sequential treatment of antibiotics should be considered as it is more effective than the simultaneous application of antimicrobial agents.

REFERENCE

Abedon, S., & Thomas-Abedon, C. (2010). Phage Therapy Pharmacology. *Current Pharmaceutical Biotechnology*, *11*(1), 28–47. https://doi.org/10.2174/138920110790725410

Ackermann, H. W., Audurier, A., Berthiaume, L., Jones, L. A., Mayo, J. A., & Vidaver, A. K. (1978). Guidelines for bacteriophage characterization. *Advances in Virus Research*, *23*(C), 1–24. https://doi.org/10.1016/S0065-3527(08)60096-2

Almeida, A., Cunha, Â., Gomes, N. C. M., Alves, E., Costa, L., & Faustino, M. A. F. (2009). Phage therapy and photodynamic therapy: Low environmental impact approaches to inactivate microorganisms in fish farming plants. In *Marine Drugs* (Vol. 7, Issue 3, pp. 268–313). Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/md7030268

Aminov, R. I. (2010). A Brief History of the Antibiotic Era: Lessons Learned and Challenges for the Future. *Frontiers in Microbiology*, *1*(DEC), 134. https://doi.org/10.3389/fmicb.2010.00134

Antunes, P., Mourão, J., Campos, J., & Peixe, L. (2016). Salmonellosis: The role of poultry meat. In *Clinical Microbiology and Infection* (Vol. 22, Issue 2, pp. 110–121). Elsevier B.V. https://doi.org/10.1016/j.cmi.2015.12.004

API for Microorganism Identification | bioMérieux. (n.d.). Retrieved February 16, 2022, from https://www.biomerieux-usa.com/clinical/api

Atterbury, R. J. (2009). Bacteriophage biocontrol in animals and meat products. In *Microbial Biotechnology* (Vol. 2, Issue 6, pp. 601–612). Wiley-Blackwell. https://doi.org/10.1111/j.1751-7915.2009.00089.x

Atterbury, R. J., Van Bergen, M. A. P., Ortiz, F., Lovell, M. A., Harris, J. A., De Boer, A., Wagenaar, J. A., Allen, V. M., & Barrow, P. A. (2007). Bacteriophage therapy to reduce Salmonella colonization of broiler chickens. *Applied and Environmental Microbiology*, *73*(14), 4543–4549. https://doi.org/10.1128/AEM.00049-07

Balasubramanian, R., Im, J., Lee, J.-S., Jeon, H. J., Mogeni, O. D., Kim, J. H., Rakotozandrindrainy, R., Baker, S., & Marks, F. (2019). The global burden and epidemiology of invasive non-typhoidal *Salmonella* infections. *Human Vaccines & Immunotherapeutics*, *15*(6), 1421–1426. https://doi.org/10.1080/21645515.2018.1504717

Berchieri, A., Lovell, M. A., & Barrow, P. A. (1991). The activity in the chicken alimentary tract of bacteriophages lytic for Salmonella typhimurium. *Research in Microbiology*, *142*(5), 541–549. https://doi.org/10.1016/0923-2508(91)90187-F

Cairns, J., Becks, L., Jalasvuori, M., & Hiltunen, T. (2017). Sublethal streptomycin concentrations and lytic bacteriophage together promote resistance evolution. *Philosophical*

Transactions of the Royal Society B: Biological Sciences, *372*(1712). https://doi.org/10.1098/rstb.2016.0040

Capparelli, R., Nocerino, N., Iannaccone, M., Ercolini, D., Parlato, M., Chiara, M., & Iannelli, D. (2010). Bacteriophage Therapy of Salmonella enterica: A Fresh Appraisal of Bacteriophage Therapy . *The Journal of Infectious Diseases*, *201*(1), 52–61. https://doi.org/10.1086/648478

Carlton, R. M. (1999a). Phage therapy: Past history and future prospects. *Archivum Immunologiae et Therapiae Experimentalis*, *47*(5), 267–274.

Carlton, R. M. (1999b). Phage Therapy: Past History and Future Prospects R. M. Carlton: Phage Therapy in the Past and Future. In *Archivum Immunologiae et Therapiae Experimentalis* (Vol. 47).

Chan, B. K., Sistrom, M., Wertz, J. E., Kortright, K. E., Narayan, D., & Turner, P. E. (2016a). Phage selection restores antibiotic sensitivity in MDR Pseudomonas aeruginosa. *Scientific Reports 2016 6:1*, *6*(1), 1–8. https://doi.org/10.1038/srep26717

Chan, B. K., Sistrom, M., Wertz, J. E., Kortright, K. E., Narayan, D., & Turner, P. E. (2016b). Phage selection restores antibiotic sensitivity in MDR Pseudomonas aeruginosa. *Scientific Reports*, *6*, 26717. https://doi.org/10.1038/srep26717

Chan, M. (2012). Antimicrobial resistance in the European Union and the world. In *Combating antimicrobial resistance: time for action* (p. 55). https://www.who.int/dg/speeches/2012/amr_20120314/en/

Chang, R. Y. K., Nang, S. C., Chan, H.-K., & Li, J. (2022). Novel antimicrobial agents for combating antibiotic-resistant bacteria. *Advanced Drug Delivery Reviews*, *187*, 114378. https://doi.org/10.1016/j.addr.2022.114378

Chanishvili, N. (2012). Phage Therapy-History from Twort and d'Herelle Through Soviet Experience to Current Approaches. In *Advances in Virus Research* (Vol. 83, pp. 3–40). Academic Press Inc. https://doi.org/10.1016/B978-0-12-394438-2.00001-3

Chaudhry, W. N., Concepcion-Acevedo, J., Park, T., Andleeb, S., Bull, J. J., & Levin, B. R. (2017). Synergy and order effects of antibiotics and phages in killing pseudomonas aeruginosa biofilms. *PLoS ONE*, *12*(1), 1–16. https://doi.org/10.1371/journal.pone.0168615

CLSI. (2016). *CATALOG Setting the Standard for Quality*. www.clsi.org/edu/webinars

Colavecchio, A., Cadieux, B., Lo, A., & Goodridge, L. D. (2017). Bacteriophages Contribute to the Spread of Antibiotic Resistance Genes among Foodborne Pathogens of the Enterobacteriaceae Family – A Review. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.01108

Comeau, A. M., Tétart, F., Trojet, S. N., Prère, M.-F., & Krisch, H. M. (2007a). Phage-Antibiotic Synergy (PAS): β-Lactam and Quinolone Antibiotics Stimulate Virulent Phage Growth.

PLoS ONE, *2*(8), e799. https://doi.org/10.1371/journal.pone.0000799

Comeau, A. M., Tétart, F., Trojet, S. N., Prère, M. F., & Krisch, H. M. (2007b). Phage-antibiotic synergy (PAS): β-lactam and quinolone antibiotics stimulate virulent phage growth. *PLoS ONE*, *2*(8), e799. https://doi.org/10.1371/journal.pone.0000799

Domínguez, D. C., & Meza-Rodriguez, S. M. (2019). Development of antimicrobial resistance: future challenges. In *Pharmaceuticals and Personal Care Products: Waste Management and Treatment Technology* (pp. 383–408). Elsevier. https://doi.org/10.1016/b978-0-12-816189-0.00016-0

Edwards, E. (1972). *Selected Technical Publications - Google Books*. https://books.google.co.ke/books?id=ler_i8s83ZwC&pg=PA242&lpg=PA242&dq=Edwards,+R.+and+Ewing,+H.+(1972).+Identification+of+Enterobacteriacae.+Minneapolis,+Burgess+Publishing+Co.,+PP.+709.&source=bl&ots=7-JrwCQPyB&sig=ACfU3U2f_mPIw8Br7ipEsIrqRYDEiCS0rA&hl=en&sa=X&ved=2ahUKEwij1JKTz9voAhVJyoUKHU7SCXgQ6AEwAHoECAwQJw#v=onepage&q=Edwards%2C R. and Ewing%2C H. (1972). Identification of Enterobacteriacae. Minneapolis%2C Burgess Publishing Co.%2C PP. 709.&f=false

Feiner, R., Argov, T., Rabinovich, L., Sigal, N., Borovok, I., & Herskovits, A. A. (2015). A new perspective on lysogeny: prophages as active regulatory switches of bacteria. *Nature Reviews Microbiology*, *13*(10), 641–650. https://doi.org/10.1038/nrmicro3527

Ferri, M., Ranucci, E., Romagnoli, P., Giaccone, V., Ferri, M., Ranucci, E., Romagnoli, P., & Giaccone, V. (2017). Antimicrobial resistance : A global emerging threat to public health systems. *Critical Reviews in Food Science and Nutrition*, *57*(13), 2857–2876. https://doi.org/10.1080/10408398.2015.1077192

Fong, K., Mu, K., Rheault, J. G., Levesque, R. C., Kitts, D. D., Delaquis, P., Goodridge, L., & Wang, S. (2020). Bacteriophage-insensitive mutants of antimicrobial-resistant salmonella enterica are altered in their tetracycline resistance and virulence in CaCO-2 intestinal cells. *International Journal of Molecular Sciences*, *21*(5). https://doi.org/10.3390/ijms21051883

Gordillo Altamirano, F. L., & Barr, J. J. (2019). Phage therapy in the postantibiotic era. *Clinical Microbiology Reviews*, *32*(2), 1–25. https://doi.org/10.1128/CMR.00066-18

Hanlon, G. W. (2007). Bacteriophages: an appraisal of their role in the treatment of bacterial infections. *International Journal of Antimicrobial Agents*, *30*(2), 118–128. https://doi.org/10.1016/j.ijantimicag.2007.04.006

Hesse, S., & Adhya, S. (2019). Phage Therapy in the Twenty-First Century: Facing the Decline of the Antibiotic Era; Is It Finally Time for the Age of the Phage? *Annual Review of Microbiology*, *73*(1), 155–174. https://doi.org/10.1146/annurev-micro-090817-062535

Kamal, F., & Dennis, J. J. (2015a). Burkholderia cepacia complex phage-antibiotic synergy (PAS): Antibiotics stimulate lytic phage activity. *Applied and Environmental Microbiology*,

81(3), 1132–1138. https://doi.org/10.1128/AEM.02850-14/ASSET/E8F026ED-ABB6-454B-9F9B-E746AFFA163D/ASSETS/GRAPHIC/ZAM9991160000003.JPEG

Kamal, F., & Dennis, J. J. (2015b). Burkholderia cepacia complex phage-antibiotic synergy (PAS): Antibiotics stimulate lytic phage activity. *Applied and Environmental Microbiology*, *81*(3), 1132–1138. https://doi.org/10.1128/AEM.02850-14

Kirby, A. E. (2012). Synergistic Action of Gentamicin and Bacteriophage in a Continuous Culture Population of Staphylococcus aureus. *PLoS ONE*, *7*(11), e51017. https://doi.org/10.1371/journal.pone.0051017

Knezevic, P., Curcin, S., Aleksic, V., Petrusic, M., & Vlaski, L. (2013). Phage-antibiotic synergism: A possible approach to combatting Pseudomonas aeruginosa. *Research in Microbiology*, *164*(1), 55–60. https://doi.org/10.1016/j.resmic.2012.08.008

Kortright, K. E., Chan, B. K., Koff, J. L., & Turner, P. E. (2019a). Phage Therapy: A Renewed Approach to Combat Antibiotic-Resistant Bacteria. *Cell Host and Microbe*, *25*(2), 219–232. https://doi.org/10.1016/j.chom.2019.01.014

Kortright, K. E., Chan, B. K., Koff, J. L., & Turner, P. E. (2019b). Phage Therapy: A Renewed Approach to Combat Antibiotic-Resistant Bacteria. In *Cell Host and Microbe* (Vol. 25, Issue 2, pp. 219–232). Cell Press. https://doi.org/10.1016/j.chom.2019.01.014

Kutateladze, M., & Adamia, R. (2010a). Bacteriophages as potential new therapeutics to replace or supplement antibiotics. *Trends in Biotechnology*, *28*(12), 591–595. https://doi.org/10.1016/j.tibtech.2010.08.001

Kutateladze, M., & Adamia, R. (2010b). Bacteriophages as potential new therapeutics to replace or supplement antibiotics. *Trends in Biotechnology*, *28*(12), 591–595. https://doi.org/10.1016/j.tibtech.2010.08.001

Kutter, E., De Vos, D., Gvasalia, G., Alavidze, Z., Gogokhia, L., Kuhl, S., & Abedon, S. (2010). Phage Therapy in Clinical Practice: Treatment of Human Infections. *Current Pharmaceutical Biotechnology*, *11*(1), 69–86. https://doi.org/10.2174/138920110790725401

Langata, L. M., Maingi, J. M., Musonye, H. A., Kiiru, J., & Nyamache, A. K. (2019). Antimicrobial resistance genes in Salmonella and Escherichia coli isolates from chicken droppings in Nairobi, Kenya. *BMC Research Notes*, *12*(1), 22. https://doi.org/10.1186/s13104-019-4068-8

Lin, D. M., Koskella, B., & Lin, H. C. (2017). Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. *World Journal of Gastrointestinal Pharmacology and Therapeutics*, *8*(3), 162. https://doi.org/10.4292/wjgpt.v8.i3.162

Liu, C. G., Green, S. I., Min, L., Clark, J. R., Salazar, K. C., Terwilliger, A. L., Kaplan, H. B., Trautner, B. W., Ramig, R. F., & Maresso, A. W. (2020). Phage-Antibiotic Synergy is Driven by a Unique Combination of Antibacterial Mechanism of Action and Stoichiometry.

BioRxiv, 2020.02.27.967034. https://doi.org/10.1101/2020.02.27.967034

Loc-Carrillo, C., & Abedon, S. T. (2011). Pros and cons of phage therapy. *Bacteriophage*, *1*(2), 111–114. https://doi.org/10.4161/bact.1.2.14590

Lopes, A., Pereira, C., & Almeida, A. (2018). Sequential Combined Effect of Phages and Antibiotics on the Inactivation of Escherichia coli. *Microorganisms*, *6*(4), 125. https://doi.org/10.3390/microorganisms6040125

Maingi, J. M., Musonye, H. A., Kiiru, J., & Nyamache, A. K. (2019). Antimicrobial resistance genes in Salmonella and Escherichia coli isolates from chicken droppings in Nairobi , Kenya. *BMC Research Notes*, 4326–4332. https://doi.org/10.1186/s13104-019-4068-8

Makumi, A., Mhone, A. L., Odaba, J., Guantai, L., & Svitek, N. (2021). Phages for Africa: The Potential Benefit and Challenges of Phage Therapy for the Livestock Sector in Sub-Saharan Africa. *Antibiotics (Basel, Switzerland)*, *10*(9). https://doi.org/10.3390/ANTIBIOTICS10091085

McDermott, P. F., Zhao, S., & Tate, H. (2018a). Antimicrobial Resistance in Nontyphoidal Salmonella. *Microbiology Spectrum*, *6*(4). https://doi.org/10.1128/microbiolspec.arba-0014-2017

McDermott, P. F., Zhao, S., & Tate, H. (2018b). Antimicrobial Resistance in Nontyphoidal *Salmonella*. In *Antimicrobial Resistance in Bacteria from Livestock and Companion Animals* (pp. 261–287). ASM Press. https://doi.org/10.1128/9781555819804.ch12

McMillan, E. A., Gupta, S. K., Williams, L. E., Jové, T., Hiott, L. M., Woodley, T. A., Barrett, J. B., Jackson, C. R., Wasilenko, J. L., Simmons, M., Tillman, G. E., McClelland, M., & Frye, J. G. (2019). Antimicrobial resistance genes, cassettes, and plasmids present in salmonella enterica associated with United States food animals. *Frontiers in Microbiology*, *10*(APR). https://doi.org/10.3389/FMICB.2019.00832/FULL

Morpeth, S. C., Ramadhani, H. O., & Crump, J. A. (2009). Invasive non-Typhi Salmonella disease in Africa. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *49*(4), 606. https://doi.org/10.1086/603553

Myelnikov, D. (2018). An Alternative Cure: The Adoption and Survival of Bacteriophage Therapy in the USSR, 1922–1955. *Journal of the History of Medicine and Allied Sciences*, *73*(4), 385–411. https://doi.org/10.1093/jhmas/jry024

Naing, L., Winn, T., & Rusli, B. N. (2006). Practical Issues in Calculating the Sample Size for Prevalence Studies. In *Archives of Orofacial Sciences* (Vol. 1).

Oechslin, F., Piccardi, P., Mancini, S., Gabard, J., Moreillon, P., Entenza, J. M., Resch, G., & Que, Y. A. (2017). Synergistic interaction between phage therapy and antibiotics clears Pseudomonas Aeruginosa infection in endocarditis and reduces virulence. *Journal of Infectious Diseases*, *215*(5), 703–712. https://doi.org/10.1093/infdis/jiw632

Olaf, T., & Dafyad Pilling. (2008). *FAO Animal Procuction and Health,poultry in the 21st century,Avian Influenza and Beyon*.

Pereira, C., Moreirinha, C., Lewicka, M., Almeida, P., Clemente, C., Cunha, Â., Delgadillo, I., Romalde, J. L., Nunes, M. L., & Almeida, A. (2016). Bacteriophages with potential to inactivate Salmonella Typhimurium: Use of single phage suspensions and phage cocktails. *Virus Research*, *220*, 179–192. https://doi.org/10.1016/j.virusres.2016.04.020

Peterson, E., & Kaur, P. (2018). Antibiotic resistance mechanisms in bacteria: Relationships between resistance determinants of antibiotic producers, environmental bacteria, and clinical pathogens. *Frontiers in Microbiology*, *9*(NOV), 2928. https://doi.org/10.3389/FMICB.2018.02928/BIBTEX

Poirel, L., Madec, J., Lupo, A., & Schink, A. (2018). *Antimicrobial Resistance in Escherichia coli*. https://doi.org/10.1128/microbiolspec.ARBA-0026-2017

Pouillot, F., Chomton, M., Blois, H., Courroux, C., Noelig, J., Bidet, P., Bingen, E., & Bonacorsi, S. (2012). Efficacy of bacteriophage therapy in experimental sepsis and meningitis caused by a clone O25b: H4-ST131 Escherichia coli strain producing CTX-M-15. *Antimicrobial Agents and Chemotherapy*, *56*(7), 3568–3575. https://doi.org/10.1128/AAC.06330-11

Rodriguez-Gonzalez, R. A., Leung, C. Y., Chan, B. K., Turner, P. E., & Weitz, J. S. (2020a). Quantitative Models of Phage-Antibiotic Combination Therapy. *MSystems*, *5*(1). https://doi.org/10.1128/msystems.00756-19

Rodriguez-Gonzalez, R. A., Leung, C. Y., Chan, B. K., Turner, P. E., & Weitz, J. S. (2020b). Quantitative Models of Phage-Antibiotic Combination Therapy. *MSystems*, *5*(1). https://doi.org/10.1128/msystems.00756-19

Rohde, C., Wittmann, J., & Kutter, E. (2018). Bacteriophages: A therapy concept against multi-drug-resistant bacteria. In *Surgical Infections* (Vol. 19, Issue 8, pp. 737–744). https://doi.org/10.1089/sur.2018.184

Rohwer, F., Youle, M., & Maughan, H. (2014). *Life in Our Phage World*. Wholon. https://books.google.com/books/about/Life_in_Our_Phage_World.html?id=g4HTrQEACAAJ&pgis=1

Rohwer, F., Youle, M., Maughan, H., & Hisakawa, N. (2014). *Life in Our Phage World, a centennial field guide to the Earth's most diverse inhabitants* (1st ed.). Wholon. http://2015phage.org/art.php

Ryan, E. M., Alkawareek, M. Y., Donnelly, R. F., & Gilmore, B. F. (2012). Synergistic phage-antibiotic combinations for the control of Escherichia coli biofilms in vitro. *FEMS Immunology and Medical Microbiology*, *65*(2), 395–398. https://doi.org/10.1111/j.1574-695X.2012.00977.x

Skurnik, M., Pajunen, M., & Kiljunen, S. (2007). Biotechnological challenges of phage therapy.

In *Biotechnology Letters* (Vol. 29, Issue 7, pp. 995–1003). https://doi.org/10.1007/s10529-007-9346-1

Subedi, M., Luitel, H., Devkota, B., Bhattarai, R. K., Phuyal, S., & Panthi, P. (2018). *Antibiotic resistance pattern and virulence genes content in avian pathogenic Escherichia coli (APEC) from broiler chickens in Chitwan , Nepal*. 4–9.

Svitek, N., De Haan, N., & Moineau, S. (2019). *Phages as a One Health approach for the replacement of antibiotics , and reduction of drug resistant nontyphoidal Salmonella , in poultry farms in Kenya*. *February*, 2019.

Tacconelli, E., Carrara, E., Savoldi, A., Kattula, D., & Burkert, F. (2017). *GLOBAL PRIORITY LIST OF ANTIBIOTIC-RESISTANT BACTERIA TO GUIDE RESEARCH, DISCOVERY, AND DEVELOPMENT OF NEW ANTIBIOTICS*. http://www.cdc.gov/drugresistance/threat-report-2013/

Tagliaferri, T. L., Jansen, M., & Horz, H. P. (2019). Fighting Pathogenic Bacteria on Two Fronts: Phages and Antibiotics as Combined Strategy. In *Frontiers in cellular and infection microbiology* (Vol. 9, p. 22). NLM (Medline). https://doi.org/10.3389/fcimb.2019.00022

Torres-Barceló, C. (2018). *The disparate effects of bacteriophages on antibiotic-resistant bacteria*. https://doi.org/10.1038/s41426-018-0169-z

Uddin, M. J., Kim, B., Dawan, J., Ding, T., Kim, J. C., & Ahn, J. (2019). Assessment of antibiotic resistance in bacteriophage-insensitive Klebsiella pneumoniae. *Microbial Pathogenesis*, *135*, 103625. https://doi.org/10.1016/j.micpath.2019.103625

Urbaniak, C., Sielaff, A., Frey, K., Allen, J., reports, N. S.-S., & 2018, undefined. (n.d.). Detection of antimicrobial resistance genes associated with the International Space Station environmental surfaces. *Nature.Com*. Retrieved January 19, 2022, from https://www.nature.com/articles/s41598-017-18506-4

Valério, N., Oliveira, C., Jesus, V., Branco, T., Pereira, C., Moreirinha, C., & Almeida, A. (2017a). Effects of single and combined use of bacteriophages and antibiotics to inactivate Escherichia coli. *Virus Research*, *240*, 8–17. https://doi.org/10.1016/j.virusres.2017.07.015

Valério, N., Oliveira, C., Jesus, V., Branco, T., Pereira, C., Moreirinha, C., & Almeida, A. (2017b). Effects of single and combined use of bacteriophages and antibiotics to inactivate Escherichia coli. *Virus Research*, *240*, 8–17. https://doi.org/10.1016/j.virusres.2017.07.015

Varga, C., Rajić, A., McFall, M. E., Reid-Smith, R. J., Deckert, A. E., Pearl, D. L., Avery, B. P., Checkley, S. L., & McEwen, S. A. (2008). Comparison of antimicrobial resistance in generic Escherichia coli and Salmonella spp. cultured from identical fecal samples in finishing swine. *Canadian Journal of Veterinary Research*, *72*(2 SPEC. ISS.), 181–187.

WHO. (2017). WHO PUBLISHES LIST OF BACTERIA FOR WHICH NEW ANTIBIOTICS ARE URGENTLY NEEDED. *Saudi Medical Journal*, *38*(4), 444. https://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-

which-new-antibiotics-are-urgently-needed

Wiegand, I., Hilpert, K., & Hancock, R. E. W. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols*, *3*(2), 163–175. https://doi.org/10.1038/nprot.2007.521

APPENDICES

Appendix 1: Preparation of Bacteria Culture Media

All media preparations were done according to the manufacturer's instructions. Recommended amounts were weighed and dissolved in a certain amount of distilled water and gently boiled so that the powder was homogenized.

I. MacConkey agar (Oxoid)

52g were suspended in 1 liter of distilled water. Boiled to dissolve completely then sterilized by autoclaving at 121^{o}c for 15 minutes. The media was then incubated at 50^{o}c before pouring into sterile Petri dishes.

II. Sorbitol MacConkey agar (Himedia)

50.03g were suspended in 1 liter of distilled water, boiled to dissolve then sterilized by autoclaving at 121^{o}c for 15 minutes. The media were cooled to 50^{o}c and poured into sterile Petri dishes.

III. Salmonella Shigella agar (Oxoid)

63g of the agar were suspended in 1 liter of distilled water. The media were boiled with frequent mixing and then allowed to simmer gently to dissolve the agar. The media were cooled to 50^{o}c and poured into sterile Petri dishes.

IV. Brilliant Green agar (Oxoid)

50g of agar were suspended in 1 liter of distilled water. Boiled, then sterilized by autoclaving at 121^{o}c for 15 minutes. The media were cooled to 50^{o}c and poured into sterile Petri dishes.

V. XLT-4 agar (Oxoid)

59g of the agar were suspended in 1 liter of distilled water, 4.6ml of the selective supplement were added then the medium was boiled. The media were cooled to 50^{o}c and poured into sterile Petri dishes.

VI. Tryptic Soy Agar (Oxoid)

40g of the agar were added to 1 liter of distilled water, dissolved then sterilized by autoclaving at 121^{o}c for 15 minutes.

VII. Triple sugar Iron agar (Oxoid)

Biochemical test media like TSI were heated to boil then dispensed into test tubes then subjected to autoclaving before being slanted appropriately to create appropriate slants.

VIII. Urea agar (Oxoid)

2.4g of the agar were suspended in 95ml of distilled water, boiled then sterilized by autoclaving at 115oc for 20 minutes. The media were cooled to 50^{o}c and aseptically added 5ml sterile 40% Urea solution. The mixed well was then distributed into 10ml sterile tubes and allowed to set in the slope position.

Appendix 2: Preparation of PCR Reagents

All reagents were thawed on ice. A reaction mix was prepared in 0.2ml PCR tubes. Reagents were added in the following order: PCR water, buffer, dNTPs, $MgCl_2$, forward primer, reverse primer, and Taq polymerase. These were gently mixed and then briefly centrifuged to settle tub contents. Negative and positive control were included.