ADDIS ABABA UNIVERSITY COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE DEPARTMENT OF MICROBIOLOGY IMMUNOLOGY AND VETERINARY PUBLIC HEALTH (MIVPH)

ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGE FROM SEWAGE AND ITS THERAPEUTIC POTENTIAL FOR MULTIDRUG RESISTANT *ESCHERICHIA COLI* ON MICE

MVSc THESIS

BY

BETEMARYAM GETANEH

JUNE, 2020

BISHOFTU, ETHIOPIA

ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGE FROM SEWAGE AND ITS THERAPEUTIC POTENTIAL FOR MULTIDRUG RESISTANT *ESCHERICHIA COLI* ON MICE

A Thesis Submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University for partial fulfillment of the requirements for the degree of Master of Veterinary Science on Veterinary Microbiology

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ADDIS ABABA UNIVERSITY COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE DEPARTMENT OF MICROBIOLOGY IMMUNOLOGY AND VETERINARY PUBLIC HEALTH (MIVPH)

As MVSc research advisors, we hereby certify that we have read and evaluated this Thesis prepared under our guidance by **Betemaryam Getaneh** entitled ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGE FROM SEWAGE AND ITS THERAPEUTIC POTENTIAL FOR MULTIDRUG RESISTANT *ESCHERICHIA COLI* ON MICE

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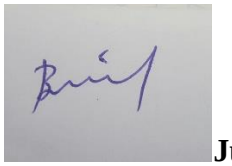

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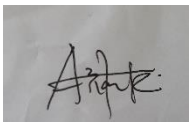
Name: Betemaryam Getaneh Signature: 

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LIST OF ABBREVIATION

AMR	Antimicrobial resistance
ARB	Antibiotic-resistant bacteria
ARG	Antibiotic-resistant genes
ATP	Adenosine triphosphate
PFU/ml	Plaque-forming units per milliliter)
BPs	Bacteriophages
CFU/ml	Colony Forming Units per ml
CLISI	Clinical and Laboratory Standards Institute protocol
DNA	Deoxyribonucleic Acid
dsDNA	Double-stranded Deoxyribonucleic Acid
dsRNA	Double-stranded Ribonucleic acid
ESBL	Extended Spectrum β-Lactamases)
HIV	human immunodeficiency viruses
ICTV	International Committee on Taxonomy of Viruses
MDR	Multi-Drug Resistant
MDROs	Multi-Drug-Resistant Organisms
MOI	Multiplicity of the infection
mRNA	messenger Ribonucleic acid
MRSA	Methicillin-Resistant Staphylococcus aureus
NVI	National veterinary Institute
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Units
RNA	Ribonucleic acid
RTE	Ready-to-Eat
SPSS	Statistical Package for Social Science
ssDNA	ingle-stranded Deoxyribonucleic Acid
TEM	Transmission electron microscopy
TSB	Tryptic soy broth
UPEC	Uropathogenic Escherichia coli

ABSTRRACT

The increasing incidence of antibiotic resistance, the emergence of virulent bacterial pathogens, and lack of new effective antibiotics, has increased interest in the use of lytic bacteriophage therapy. The aim of this study was to characterize colliphage isolated from sewage to determine their *in vitro* and *in vivo* application. This experimental study was conducted at National Veterinary Institute, Bishoftu, Ethiopia from October 2019 to May 2020. Five different locations in Bishoftu were selected for the collection of fecal and sewage samples for the isolation of *E.coli* and detection of probable phages. After isolation of seven different *E.coli* isolates antibiotic susceptibility tests were conducted and majority of the isolates were resistant to more than five out of eight drugs. Bacteriophage were isolated from sewage samples and the isolated phage were purified by diluting in the buffer and filtered through 0.22 μm filter. Purified lysate was further processed for analyzing its host range by using spot method. From these all seven isolates of *E.coli* only one isolate (*E.coli* 7) was susceptible to isolated. The Plaque forming unit of was maximum at pH 7, while the reduction was observed at both pH less than 5 and greater than 9. No plaques were observed at pH 3 and 12. The Plaque forming unit (PFU) was highest after treatment at 37°C while at 4^{o}C there is no plaque formation, when the temperature increase from 4^{o}C to 37^{o}C the PFU increase and then decrease from 37^{o}C to 42^{o}C however no lytic activity was observed after treatment above 42°C. The effect of chloroform on phage activity was cheeked then the phage produce plaque before and after the addition of chloroform. 71.4% mice have survived receiving phage therapy. However 85.7% of the mice were died in the group that only *E. coli* got. Isolation, characterization and therapeutic application of potential phages lytic against *E.coli* bacteria commonly involved in infections is a major finding of this study. Notably, we found phage lytic against multidrug-resistant *E.coli*. This promising effect against MDR pathogens has raised the probable utility of these phages for biological control of bacterial infection. Further characterization of specific phages is needed to explore the potential use of these phages for their clinical application.

Keywords: *Bacteriophage, in vitro, in vivo, MDR E. coli, mice, therapy*

1. INTRODUCTION

The extensive use of antibiotics for decades in humans, veterinary, and agricultural practices pose a significant public health threat. High levels of antibiotics in the environment leads to rapid emergence of antibiotic-resistant genes (ARGs) and bacteria (ARB), reducing the therapeutic capacity of these existing commercially available antibiotics (Levy and Marshall, 2014). These multi-drug-resistant organisms (MDROs) exhibit resistance to more than one antimicrobial agents (Magiorakos *et al*., 2012). In most of the cases, the current drugs to treat MDRO infections are expensive and have toxicity issues.

Though multidrug resistant (MDR) strains were first observed among enteric bacteria, in particular coliforms and enterococci, it was found that chief among antibiotic-resistant bacteria are Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* spp. with several other Gram-negative bacteria that are resistant to fluoroquinolones (Rizzo *et al*., 2013). These resistant bacterial strains have been identified to contain enzymes such as Carbapenemases and Extended Spectrum β-lactamases (ESBL), causing broad resistances for treatment (Queenan and Bush, 2015). The antibiotic-resistant encoding genes in bacteria are often located on the plasmids of the MDR strains and are transferred with high frequency during bacterial conjugation. In recent years, *Escherichia. Coli* (*E. coli*) has emerged as one of the major MDR strains that has become a global concern (De Kraker *et al*., 2013). For instance, the *E. coli* sequence type (ST) 131 has been consistently reported in association with urinary tract infections and bacteremia (Alhashash *et al*., 2013).

Escherichia coli are a Gram-negative bacterium of many diverse types, the majority of which are part of the normal flora of the intestine and are believed to be relatively harmless. However, some strains have evolved mechanisms of pathogenicity, meaning they can cause disease in humans and animals. Uropathogenic *E. coli* (UPEC) is one of the main bacteria causing urinary tract infections. The rates of UPEC with high resistance towards antibiotics and multidrug-resistant bacteria have increased dramatically in recent years and could difficult the treatment. Current antibiotic treatments used to treat drug-resistant or/and adherent-invasive *E.coli* can result in severe

alterations to an individual's microbiota as well as continuous relapses of disease (Langdon *et al*., 2016).

For this reason, new therapeutic strategies developed quickly use and developed. from these strategies the use of bacteriophages are ranked at top, which are viruses that infect and destroy bacteria. They possess novel mode of action compared to that of antibacterial regimens, as they selectively infect pathogenic bacteria including multidrug resistant pathogens (as it has seen both *in vivo* and *in vitro*) (Miedzybrodzki *et al*., 2012). Furthermore, they are ecologically safe and effective in lower doses and do not show adverse reactions on their application in human body (Loc-Carrillo and Abedon, 2014). To these assets, phages have garnered increasing attention in the therapeutic application in recent years.

Phage therapy is a method which used for the treatment of bacterial infections. As early as the 1920s, substantial work was performed in this field (Sulakvelidze *et al*., 2001). Bacteriophages have been used as therapeutic agent (Madhusudana Rao and Lalitha, 2015),as bio-control agents in food industry (Tan *et al*., 2014),as phage typing (Chen *et al*., 2015), and as a vector (Yanisch-Perron, 1985). Despite these great efforts, the use of phages as therapeutic agents was generally abandoned in the Western world soon after the discovery of antibiotics in the 1940s, the feasibility of phage therapy is still under investigation.

Several studies, available to date, have revealed the lytic efficacy of phages against various Susceptible organisms including *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumannii, Vibrio cholerae, Salmonella species, Staphylococcus aureus, Enterococcus* spp., and *Serratia* spp. (Carey-Smith *et al*., 2006, Matsushita *et al*., 2009, Mihu, and Martinez, 2011, Jin *et al*., 2012, Al-Fendi *et al*.,2014, Bolocan *et al*., 2016). In addition, ability of lytic phages against multidrug-resistant bacteria producing hydrolytic enzymes including extended spectrum β-lactamases (ESBL) producing *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, Methicillin-Resistant *Staphylococcus aureus* (MRSA), and Vancomycin-resistant *Enterococcus* has also been reported (Wang *et al*., 2006, Mann, 2008, Elbreki *et al*., 2014).

In Ethiopia, there is continuous increment of antimicrobial resistance among pathogenic bacterial strains of human and animals (Alemayehu *et al*., 2019). Despite the growing risk of antimicrobial resistance in the country, there is very little attention being paid for its control and weak attepts have been seen on finding the alternatives. One of the attempts could be the alteranative of phage therapy but there are newer alternatives have not been investigated yet. There are limited studies on phage isolation and its therapeutic potential, particularly, against multi-drug resistant pathogens. Efforts made in Ethiopia focus especially on isolation, and the evaluation of therapeutic value has not been investigated. Therefore the present study aimed to inverstigate therapeutic potential of locally isolated phages against multi-drug resistant *Escherichia coli* with the following objectives:

- Isolation and characterization of coliphages,
- To examine the *in vitro* and *in vivo* therapeutic potential of the isolated coliphage.

2. LITERATURE REVIEW

2.1. History of Bacteriophage

Ernest Hanbury Hankin, reported that something in the waters had antibacterial properties against *cholera* and this water could pass through a very fine porcelain filter and keep this distinctive feature (Hankin, 1896). However, Hankin did not pursue this finding. In 1915, Frederick Twort discovered agents that killed colonies of bacteria in growing cultures. He published the results but the subsequent work was interrupted by the beginning of World War I and shortage of funding. Felix d'Herelle discovered the agent killing bacteria independently at the Pasteur Institute in France in 1917. He observed that cultures of the dysentery bacteria disappear with the addition of a bacteria-free filtrate obtained from sewage. D'Herelle has published his discovery of "an invisible, antagonistic microbe of the dysentery bacillus" (d'Herelle, 1917).

In 1923, the Eliava Institute was opened in Tbilisi, Georgia, to study bacteriophages, and to develop phage therapy. In 1969 Max Delbrück, Alfred Hershey, and Salvador Luria were awarded the prestigious Nobel Prize in Physiology and Medicine for their discoveries of the replication of viruses and their genetic structure. D'Herelle has used phages to treat a boy who had bad disentheria (d'Herelle, 1917). After the administration of phages, the boy successfully recovered.

At the end of 1930s antibiotics were discovered and nearly wiped out studies on the medical use of phages. The emergence of modified pathogens such as Mycobacterium tuberculosis, Enterococcus faecalis, Staphylococcus aureus, Acinetobacter baumannii and Pseudomonas aeruginosa, and methicillin-resistant S. aureus has created massive problems in treating patients in hospitals (Coelho *et al*., 2004, Hanlon, 2007; Burrowes *et al*., 2011) and the time required to produce new antibiotics is much longer than the time of bacterial adaptation.

Current studies on the phage life cycle have revealed a way for their penetration through membrane barriers of cells. These results are important in the development of methods for using bacteriophages as a therapeutic option in the treatment of bacterial infections (Brussow and Kutter, 2005).

2.2. Bacteriophage classification

Table 1: Taxonomy of bacteriophage
Source: Krupovic *et al*., (2018)

New genus	Family	Subfamily	Type species	Number of genus-included species
Ap22virus	*Myoviridae*		*Acinetobacter virus AP22*	4
Secunda5virus	*Myoviridae*		*Aeromonas virus 25*	5
Biquartavirus	*Myoviridae*		*Aeromonas virus 44RR2*	1
Agatevirus	*Myoviridae*		*Bacillus virus Agate*	3
B4virus	*Myoviridae*		*Bacillus virus B4*	5
Bastillevirus	*Myoviridae*		*Bacillus virus Bastille*	2
Bv431virus	*Myoviridae*		*Bacillus virus Bc431*	4
Cp51virus	*Myoviridae*		*Bacillus virus CP51*	3
Nit1virus	*Myoviridae*		*Bacillus virus NIT1*	3
Wphvirus	*Myoviridae*		*Bacillus virus WPh*	1
Cvm10virus	*Myoviridae*		*Escherichia virus CVM10*	2
Kpp10virus	*Myoviridae*		*Pseudomonas virus KPP10*	3
Pakpunavirus	*Myoviridae*		*Pseudomonas virus PAKP1*	6
Rheph4virus	*Myoviridae*		*Rhizobium virus RHEph4*	1
Vhmlvirus	*Myoviridae*		*Vibrio virus VHML*	3
Tg1virus	*Myoviridae*		*Yersinia virus TG1*	2
P100virus	*Myoviridae*	*Spounavirinae*	*Listeria virus P100*	1
Kayvirus	*Myoviridae*	*Spounavirinae*	*Staphylococcus virus K*	7
Silviavirus	*Myoviridae*	*Spounavirinae*	*Staphylococcus virus Remus*	2
Rb49virus	*Myoviridae*	*Tevenvirinae*	*Escherichia virus RB49*	3
Rb69virus	*Myoviridae*	*Tevenvirinae*	*Escherichia virus RB69*	4
Js98virus	*Myoviridae*	*Tevenvirinae*	*Escherichia virus JS98*	5
Sp18virus	*Myoviridae*	*Tevenvirinae*	*Shigella virus SP18*	5
S16virus	*Myoviridae*	*Tevenvirinae*	*Salmonella virus S16*	2
Cc31virus	*Myoviridae*	*Tevenvirinae*	*Enterobacter virus CC31*	2
Cr3virus	*Myoviridae*	*Vequintavirinae* (new)	*Cronobacter virus CR3*	3
V5virus	*Myoviridae*	*Vequintavirinae* (new)	*Escherichia virus V5*	4
Se1virus	*Myoviridae*	*Vequintavirinae* (new)	*Salmonella virus SE1*	4
Pagevirus	*Podoviridae*		*Bacillus virus Page*	5
Cba41virus	*Podoviridae*		*Cellulophaga virus Cba41*	2
G7cvirus	*Podoviridae*		*Escherichia virus G7C*	8
Lit1virus	*Podoviridae*		*Pseudomonas virus LIT1*	3
Vp5virus	*Podoviridae*		*Vibrio virus VP5*	3
Kp34virus	*Podoviridae*	*Autographivirinae*	*Klebsiella virus KP34*	5
Slashvirus	*Siphoviridae*		*Bacillus virus Slash*	4
Cba181virus	*Siphoviridae*		*Cellulophaga virus Cba181*	3
Cbastvirus	*Siphoviridae*		*Cellulophaga virus ST*	1
Nonagvirus	*Siphoviridae*		*Escherichia virus 9g*	4
Seuratvirus	*Siphoviridae*		*Escherichia virus Seurat*	2
P70virus	*Siphoviridae*		*Listeria virus P70*	5
Psavirus	*Siphoviridae*		*Listeria virus PSA*	2
Ff47virus	*Siphoviridae*		*Mycobacterium virus Ff47*	2
Sitaravirus	*Siphoviridae*		*Paenibacillus virus Diva*	5
Septima3virus	*Siphoviridae*		*Pseudomonas virus 73*	5
Nonanavirus	*Siphoviridae*		*Salmonella virus 9NA*	2
Sextaecvirus	*Siphoviridae*		*Staphylococcus virus 6ec*	2
Ssp2virus	*Siphoviridae*		*Vibrio virus SSP002*	2

Table 1 continued

New genus	Family	Subfamily	Type species	Number of genus-included species
K1gvirus	*Siphoviridae*	*Guernseyvirinae* (new)	*Escherichia virus K1G*	4
Jerseyvirus (existing)	*Siphoviridae*	*Guernseyvirinae* (new)	*Salmonella virus Jersey*	6
Sp31virus	*Siphoviridae*	*Guernseyvirinae* (new)	*Salmonella virus SP31*	1
T1virus (existing)	*Siphoviridae*	*Tunavirinae* (new)	*Escherichia virus T1*	4
Tlsvirus	*Siphoviridae*	*Tunavirinae* (new)	*Escherichia virus TLS*	3
Rtpvirus	*Siphoviridae*	*Tunavirinae* (new)	*Escherichia virus Rtp*	2
Kp36virus	*Siphoviridae*	*Tunavirinae* (new)	*Klebsiella virus KP36*	3
Rogue1virus	*Siphoviridae*	*Tunavirinae* (new)	*Escherichia virus Rogue1*	8
Alpha3microvirus	*Microviridae*	*Bullavirinae* (new)	*Escherichia virus alpha3*	8
G4microvirus	*Microviridae*	*Bullavirinae* (new)	*Escherichia virus G4*	3
Phix174microvirus	*Microviridae*	*Bullavirinae* (new)	*Escherichia virus phiX174*	1
Alphapleolipovirus	*Pleolipoviridae* (new)		*Halorubrum virus HRPV-1*	5
Betapleolipovirus	*Pleolipoviridae* (new)		*Halorubrum virus HRPV-3*	2
Gammapleolipovirus	*Pleolipoviridae* (new)		*Haloarcula virus His2*	1

2.3. Bacteriophage Biology

Bacteriophages are biological entity that can be found in soil, seawater, oceanic , terrestrial surfaces, hospitals, wastewater and extreme environments, wich characterized by very high or low temperatures (Clokie *et al*., 2011).

2.3.1. Life cycle

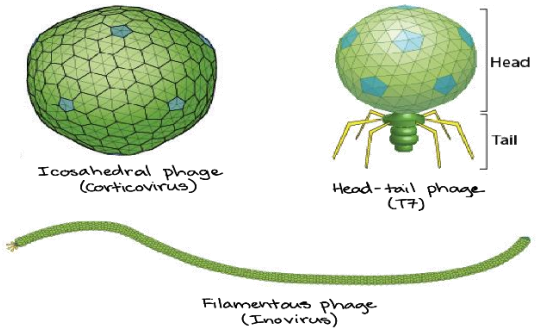

Figure 1: different shape of bacteriophage

To reproduce, bacteriophages infect a host cells and the infection process are collectively called the life cycle of the phage. The two types of life cycle of bacteriophage are lytic lifecycle, which

burst and kill their host cells and lysogenic lifecycle, which don't kill the host cell. Phage lamda is a bacteriophage that follow both lysogenic and lytic life cycle (Mason *et al*., 2011).

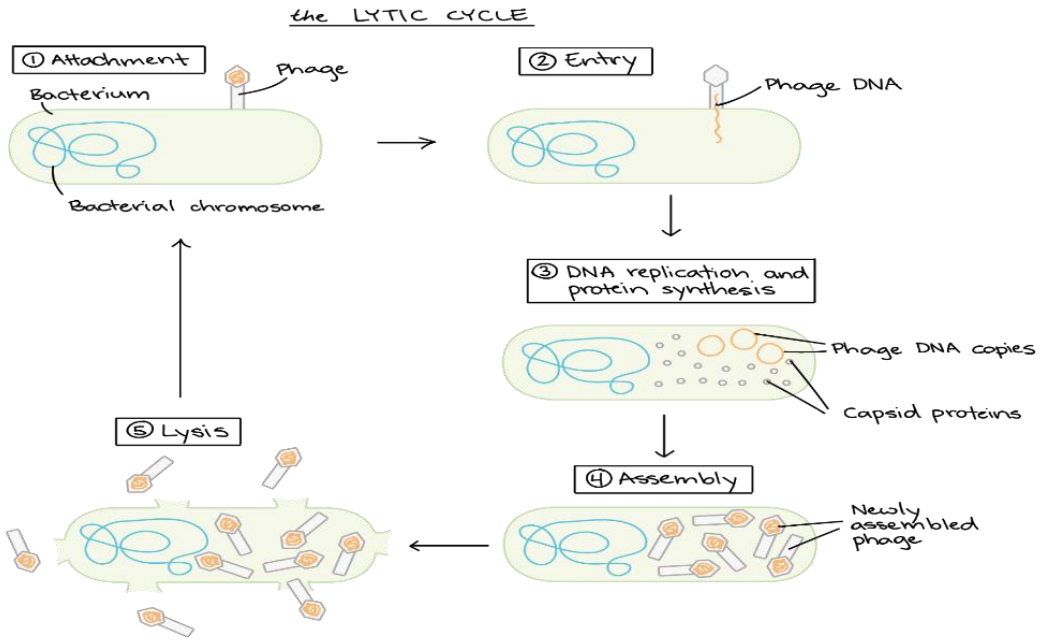

Figure 2: The lytic life cycle of bacteriophage
Source: Alberts *et al*. (2002)

The first steps of the process is attachment of the virus into the bacterial cell wall. Proteins in the "tail" of the phage bind to a specific receptor (in this case, a sugar transporter) on the surface of the bacterial cell then followed by entry. The phage injects its double-stranded DNA genome into the cytoplasm of the bacterium. After entry DNA copying and protein synthesis are followed Phage DNA is copied, and phage genes are expressed to make proteins, such as capsid proteins. After that assembly of new phage Capsids assemble from the capsid proteins and are stuffed with DNA to make lots of new phage particles. Finally Lysis of the bacteria occurred Late in the lytic cycle, the phage expresses genes for proteins that poke holes in the plasma membrane and cell wall. The holes let water flow in, making the cell expand and burst like an overfilled water balloon.

The lysogenic cycle allows a phage to reproduce without killing its host. Filamentous (long, rod-shaped) phages are secreted from the cell in a process that does not lyse or kill the cell (Rakonjac, 2012). This cycle contain Attachment. (Bacteriophage attaches to a bacterial cell), entry (Bacteriophage injects DNA into a bacterial cell), integration (phage DNA recombines with a

bacterial chromosome and becomes integrated into the chromosome as a prophage), and cell division (each time a cell containing a prophage divides, its daughter cells inherit the prophage).

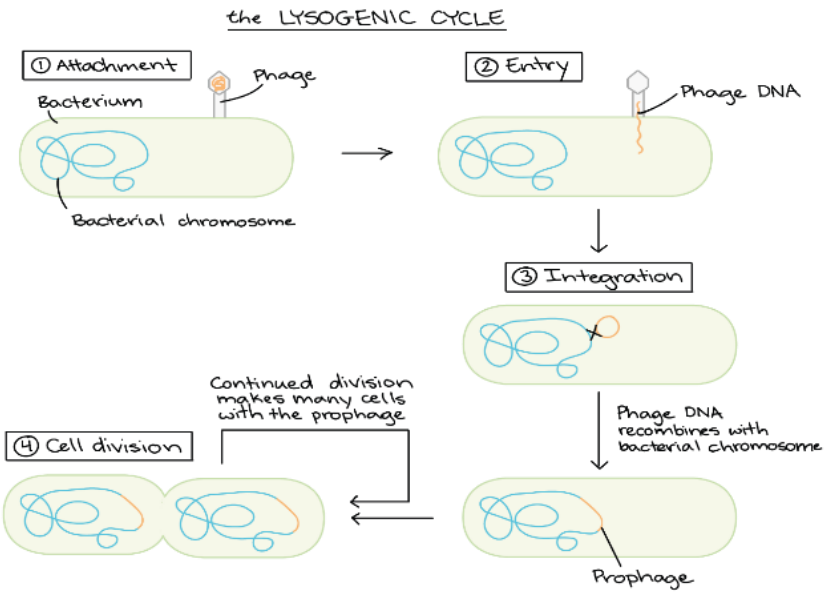

Figure 3: The lysogenic cycle of bacteriophage
Source: Alberts *et al*. (2002)

3.2.2. Properties of bacteriophage

Bacteriophages are distinguished on the bases of their morphology. Thus, electron microscopy is often the easiest and fastest way for phage identification, as well as to allocate unidentified phage to a family Ackermann, (2007), Akhtar *et al*. (2014) had working TEM to describe a collection of *Salmonella enterica* lytic bacteriophages isolated from feces and sewage samples. The phages in the assembly were mostly Siphoviridae and Myoviridae. Meanwhile, Pope *et al*. (2007) had used Cryo-electron microscopy to describe a purified cyanophage which host-specific to *Synechococcus* strain.

Bacteriophages are strictly host-specific, which infects only one bacterial species or one serotype within a species (Koskella and Meaden, 2013). However, not all bacteriophages are host-specific. Particularly, wide host range bacteriophages would be expected to be privileged relative to phages

with narrow host range, since a wide host range allows a phage to use one prey species when another is not available, or even use several simultaneously. For instance, phage (Akhtar *et al*., 2014), phage (El-Arabi *et al*., 2013) and phage (Garbe *et al*., 2011) were reported as broad host range bacteriophage against *Salmonella enterica* serovars, *Bacillus* species and *Pseudomonas aeruginosa* mutant strains, respectively. Turki *et al*. (2012) had isolated two bacteriophages, *Salmonella Zanzibar* phage and *Salmonella Anatum phage*, which lytic on more than ten *Salmonella* strains, and were able to infect *Escherichia coli* and *Citrobacter* isolates.

Upon infecting its host bacteria, most of the virulent phages act optimally at different ratios of multiplicity of the infection (MOI). the optimal MOI of a virulent bacteriophage specific for *Pseudomonas fluorescens* was determined to be 0.001 (Sun *et al*., 2012); Li and Zhang (2014) had isolated a lytic *Staphylococcus aureus* bacteriophage from wastewater of cleaning dairy cattle udders with the optimal MOI of 0.01. On the contrary, a lower MOI ratio of 0.0001 was reported for bacteriophage of *Acinetobacter baumannii* (Yang *et al*., 2010). a higher ratio of 0.1 was considered as the optimal MOI for *Escherichia* phage (Xu *et al*., 2016) and *Salmonella Typhimurium* phage (Wong *et al*., 2014).

Most of the phages normally appear to be active over a wide-ranging pH, from acidic to alkaline. The Lytic phage of the *Lactobacillus* was stable between pH 4.0 and 11.0. Bacteriophage specific for *Aeromonas sobria* was relatively stable at pH 5.0 to 10.0 and the optimal phage infectivity occurred at pH 9.0 (Ji *et al*., 2015). Likewise, *Salmonella*-infecting bacteriophages (Bao *et al*., 2015, Tiwari *et al*., 2013, Bao *et al*., 2011), were relatively stable within the pH range of 4.0 to 10.0.

Rumors showed that the viability of bacteriophages is greatly affected by temperature (Yang *et al*., 2010, Baudoux *et al*., 2012, Kesik-Szeloch *et al*., 2013, Wong *et al*., 2014) which precisely influencing in their attachment, penetration, multiplication, and the length of the latent period. Bacteriophages act optimally at temperatures between 30 -50˚C (Bao *et al*., 2015, Ji *et al*., 2015). Bacteriophages were also found to be active over the temperature range of 4-40˚C (Jun *et al*., 2013) and 46˚C (Easwaran *et al*., 2015) for *Shigella flexneri* and *Escherichia coli* phage, respectively. Significant inactivates of phages were observed at 70˚C for *Siphoviridae* (Lee *et al*., 2013), *Myoviridae* (Jamal *et al*., 2015), *Podoviridae* (Augustine *et al*., 2013). On the differing,

Escherichia coli phage (Lee and Park, 2015), *Pseudomonas aeruginosa* phage (Han *et al*., 2014), and *Weissella cibaria* phage (Pringsulaka *et al*., 2011) exhibited heat resistance at 70˚C.

2.3.3. Replication

Bacterial cells are protected by a cell wall of polysaccharides, which are significant virulence factors protecting bacterial cells against both immune host defenses and antibiotics (Drulis-Kawa *et al*., 2015). To cross the membrane, bacteriophages attach to exact receptors on the surface of bacteria, including lipopolysaccharides, teichoic acids, proteins, or even flagella. Polysaccharide-degrading enzymes, like endolysins, are virion-associated proteins to enzymatically degrade the capsular outer layer of their hosts, at the initial step of a tightly programmed phage infection process (Gabashvili *et al*., 1997).

After contacting the suitable receptor, the tail fibers bend to bring the base plate closer to the surface of the cell. This is known as changeable binding. Once attached completely, permanent binding is started and the tail contracts, possibly with the help of ATP, present in the tail, (Rakonjac, 2012). Within minutes, bacterial ribosomes start decoding viral mRNA into protein. Proteins adjust the bacterial RNA polymerase so it preferentially transcribes viral mRNA. The host's normal synthesis of proteins and nucleic acids is disrupted, and it is forced to manufacture viral products instead (Mizuno *et al*., 2019).

2.4. Application of Bacteriophage

2.4.1. Therapeutic agent

Bacteriophages have been widely used to fight various bacterial infections after their discovery in the early 20th century. For example, d'Herelle had working bacteriophages as therapeutic agents in 1929 (Madhusudana Rao and Lalitha, 2015). Although the use of bacteriophages as therapeutic agents then declined in the West, however, the developing of many antibiotic-resistant bacteria has provoked the Western world to revive the attention of phage therapy in current times (Jaiswal *et al*., 2013). Also, phage therapy has been used in animals, plants, and humans with different degrees of accomplishment (Haq *et al*., 2012).

Phage therapies are also an effective tool in eliminating bacterial infections in many species of animals. Bacteriophages have also proven effective in treating diseases in poultry. One of the objectives of phage therapy in animals is to assess the appropriateness of bacterial viruses for control of pathogens having an important influence on animal productivity and health. Phages used in treatment have been active in averting infections and in treatment of Colibacillosis in poultry (Barrow *et al*., 1998). Confident results, with a high success rate in removing pathogens, have also been obtained in fighting infections induced by numerous *Salmonella* serotypes in game fowl, such as *Enteritidis* and *Typhimurium* (Lim *et al*., 2011), as well as *campylobacteriosis* in poultry, mainly infections induced by *Campylobacter jejuni* and *Campylobacter coli* (Wagenaar *et al*., 2005)

The therapeutic success of phages is determined by their high lytic titer, the form and type of application, and period. Long-term use of phages in poultry has demonstrated to be moderately effective in reducing the number of *Salmonella* pathogens colonizing the digestive tract (Sklar and Joerger, 2001). Treatment using bacteriophages as a feed additive for chickens having contact with infected individuals led to a death rate of only 5%, as associated to 30% in the group that did not take phage therapy (Lim *et al*., 2011). The effectiveness of phage therapy may also depend on the individual antibacterial properties of a given bacteriophage and the adaptive mechanisms of the bacteria. A study by Andreatti Filho *et al*. (2007) showed that the use of particular bacteriophages in an orally administered cocktail to prevent colonization by S. Enteritidis strains in poultry was only effective for a short time (about 48 h), with no long-term protective effect, which was partly due to acquisition of resistance to the bacteriophage by the bacteria.

after inoculation of a bacteriophage suspension in the drinking water of birds at 1 week of age (10 or10^4 PFU of bacteriophages per mL) followed by air sac challenge with 10^3 PFU of coliphages. Mortality was decreased to 25% and 5%, respectively. No mortality was observed in chickens treated with 10^8 PFU of an *E. coli* bacteriophage mixture (Huff *et al*., 2002).

2.4.2. As bio-control agents in food industry

Food-borne diseases of microbial origin are serious food safety difficulties worldwide. With the current concern over the emerging of antibiotic-resistant foodborne pathogens caused from the abuse and misuse of antibiotics, bacteriophage-based bio-control may serve as an alternative

antimicrobial, which represents an economically viable field in the food industry (Henry and Debarbieux, 2012; Tan *et al*., 2014).

The efficacy of bacteriophages in food depends on the structure and chemical composition of the different food items, as well as sufficient diffusion ability of the phage particles is necessary (Guenther *et al*., 2009). Additionally, phage efficiency is also influenced by pH and activity on a solid substrate or biofilm, the emergence of resistant bacteria mutants, and the relative numbers of phages and hosts required to allow replication (Oliveira *et al*., 2015). The use of immobilized phage as packing material to maintain the safety of fresh produce is a novel concept that could allow the controlled release of bacteriophage particles into the food and reduce bacteriophage waste during food treatment using spraying (Lone *et al*., 2016). Currently, bacteriophage mixtures were introduced into prototypes of packaging materials using different techniques immobilizing on positively charged modified cellulose membranes, impregnating paper with bacteriophage suspension, and encapsulating in alginate beads followed by application of beads onto the paper, in order to enhance the safety of fresh produce and RTE meat (Lone *et al*., 2016).

2.4.3. Detection of bacterial pathogens (phage typing)

There are numerous approaches that can be used for pathogenic bacteria detection. For example, the use of bacteriophages which able to transport reporter genes (Thouand *et al*., 2008) or by means of green fluorescent protein (Piuri *et al*., 2009) that would express after infection of bacteria. Also, bacteriophages covalently attached with a fluorescent dye to their coats can be employed for the finding of specific adsorption (Goodridge *et al*., 1999).

Detection of other released components, which include adenosine triphosphate (ATP), adenylate kinase, and β D-galactosidase after the specific lysis of bacteria can also be used (Chen *et al*., 2015). On the other hand, bacteriophage amplification assays attributed an attractive different method to detect pathogenic bacteria, which retained the inherent biological specificity of the phage for its target but used an assay end-point that was not dependent upon a genetically modified virion (Schmelcher and Loessner, 2014). Therefore, it has most widely been used for the detection of *Campylobacter*, *Escherichia coli*, *Listeria*, *Mycobacterium tuberculosis*, *Pseudomonas*, and *Salmonella* (Oliveira *et al*., 2012).

2.4.4. As a vector

Both single-stranded (filamentous) and double-stranded *E. coli* phages have been exploited as cloning vectors. They coexist with the infected cells for several generations and are convenient for cloning genes that produce toxic products. Among the filamentous phages, FD, fl, and M13 have been well characterized and their genomes have been sequenced (Beck and Zink, 1981). Their gene functions and molecular mode of propagation are very similar. They infect cells via F pili, and the first mature phage appears within 15 min (Marvin and Wachtel, 1975). Phage M13 is widely used in nucleotide sequencing and site-directed mutagenesis since its genome can exist either in a single-stranded form inside a phage coat or as a double-stranded replicative form within the infected cell.

During replication, only the plus strand of the replicative form is selectively packaged by the phage proteins (Meyer, 1979). The replicative form is a covalently closed circular molecule and hence can be used as a plasmid vector and transformed into the host by the usual transformation procedures (Yanisch-Perron, 1985). The DNA fragments having non-complementary ends can be directionally cloned in this pair of vectors, and the two strands of DNA can be sequenced independently. Double-stranded phage vectors. Of the double-stranded phages, bacteriophage lambda-derived vectors are the most popular tools for several reasons: acceptance by the phage of large foreign DNA fragments, thereby increasing the chances of screening a single clone carrying a DNA sequence corresponding to a complete gene, development and availability of refined techniques aimed at minimizing the problems of background due to non-recombinants, possibility of screening several thousand clones at a time from a single Petri plate; and, finally, the ease with which the phage library can be stored as a clear lysate at 4°C for months without significant loss in plaque-forming activity (Murray, 1983).

2.5. Potential advantage of phage therapy

Specificity of action, Narrow spectrum of activity, higher safety, higher tolerability, easy administration, effect limited to the site of infection, possible additional benefits after engineering, and less expensive are the most known advantage of BPs. They have a very narrow spectrum of activity, which avoids the most important problem strictly related to the antibiotic administration, i.e., the influence on the entire microbiome with the elimination of potentially beneficial bacteria,

the overgrowth of secondary pathogens and the emergence of resistant bacteria (Domingo-Calap and Delgado-Martinez, 2018).

The use of BPs without modification of the microbiota has been reported by several studies in both animals and humans. In mice, oral administration of four T4-like BPs effective against diarrhea associated *E. coli* did not lead to any collateral damage of non-pathogenic bacteria of the same species (Chibani-Chennoufi *et al*., 2004). In humans, data confirming the specificity of BP action were shown in the study conducted by Sarker *et al*. (2012).

In contrast to antibiotics, BPs are supposed to have several other advantages. It is thought that BPs are significantly safer and better tolerated, as they replicate only in the target bacterium but cannot infect mammalian cells. This hypothesis seems supported by all the skills collected in the past in Eastern Europe and all the studies supported out more recently in experimental animals and humans, which have not reported significant adverse events following BP administration (Kakasis and Panitsa, 2018). Contrarily to antibiotics, their effect is limited to the site of infection that can be reached, even when bacteria are situated in a body organ or system in which antimicrobials can hardly penetrate (Pouillot *et al*., 2012). *in vitro* study, Lu and Collins engineered a BP affective against an *E. coli* producing biofilm to express a biofilm-degrading enzyme (Lu and Collins, 2007).

2.6. Potential limitation of phage therapy

Absence of specific activity for a given bacterial strain, Difficulty in production of BP genome without integrase genes, Reduced activity due to immune system response to BPs are limitation of phage therapy. Though, before a BP is identified as a potential therapeutic agent, it has to be established that it is specific for a given bacterial strain. This is a relatively complicated problem, as evidence of the lytic capacity of a BP can vary according to the interrelationships between the BP and bacterium and their modification with time together with the dose of virus used for the test. Additionally, the BP genome must be sequenced and not hold integrase genes, as in the lysogenic type, antibiotic-resistant genes, genes for phage-encoded toxins, or genes for other bacterial virulence factors (Mattila *et al*., 2015).

Studies indicated that the stability of preparations for clinical use is strictly BP dependent and maintenance strategies should be optimized for each BP separately (Merabishvili *et al*., 2013). This can lead to expensive and time-consuming clinical trials that could depress the pharmaceutical industry from starting research and production of preparations for use.

Alteration or loss of receptor for membrane protein modifications has been demonstrated for *E. coli, S. aureus* and *Bordetella bronchiseptica* (Liu *et al*., 2002), and *Vibrio cholera* (Seed *et al*., 2012). Secretion of extracellular polymeric substances and glycoconjugates has been described for *Pseudomonas* spp. and *Enterobacteriaceae* (Drulis-Kawa *et al*., 2012), respectively. However, all these findings designated that selection of a therapeutic BP must take into account the ability of each virus to induce bacterial resistance and the amount needed to avoid bacterial resistance development (Brabban *et al*., 2005).

Lysogenic phages consequently, might be vehicles for horizontal exchange of genetic material and play a role in the diffusion of antibiotic resistance genes . Hypothetically, due to transduction, new microbes, or even more resistant bacteria can develop (Maiques *et al*., 2007). Genes such as blaTEM (resistance to b-lactams), and (reduced susceptibility to fluoroquinolones), ermB (resistance to macrolides), sulI (resistance to sulphonamides), and tetW (resistance to tetracyclines) have been found in the virome of activated sludge and environmental water samples (Subirats *et al*., 2016) and the phage DNA fraction isolated from the intestinal mucus of wild freshwater fish species (Marti *et al*., 2014).

Lastly, numbers of phages carrying genes associated with antibiotic resistance have been detected in secretions and tissues of patients who suffer from recurrent infections due to antibiotic-resistant pathogens and had been before repeatedly treated with antimicrobial drugs. One of the best examples in this regard is CF, as evidenced by the study conducted by Fancello *et al*. (2011). This and parallel findings led to the supposition that BPs might be vehicles for the adaptation of bacteria to the CF lung environment and the emergence and selection of multidrug-resistant pathogens with chimeric repertoires (Rolain *et al*., 2011).

In several studies, conclusions were misinformed by the excessive bacterial DNA content of the studied samples. Moreover, inadequate approaches to detect ARGs in phage genomes have been used (Enault *et al*., 2017). Bacteriophages and their products are non-self-antigens, and it is not surprising that they can be recognized by the immune system and induce responses that can hypothetically decrease the importance of BP administration. In animals, BPs were taken up by phagocytic cells a few minutes after the administration and might be destroyed by these cells within 2 h (Ka´zmierczak *et al*., 2014). Moreover, examining the survival of T7 BP in the blood of healthy and immunocompromised mice, it was shown that, whereas in animals with severe combined immunodeficiency, BP titers were stable for a long time, in healthy mice 99% of BPs were eliminated within 60 min of the injection. As phage titers remained stable in the B-cell-deficient mouse, the greatest part of phage clearance from blood seemed to be due to specific antibody production (Srivastava *et al*., 2004).

2.7. *Escherichia coli* virus T4 (Coliphage)

Escherichia coli virus is a species of bacteriophages that infect *Escherichia coli* bacteria. It is a member of virus subfamily *Tevenvirinae* and includes among other strains *Enterobacteria* phage *Enterobacteria* phage, and *Enterobacteria* phage. T4 is capable of experiencing only a lytic lifecycle and not the lysogenic lifecycle. The T4 virus is double-stranded DNA genome and have about 169 kbp long (Miller, 2003) and encodes 289 proteins. When packaged, the concatemer is cut at unspecific positions of the same length, leading to several genomes that represent circular permutations of the original (Madigan and Martin, 2006).

Myoviridae phages like T4 have complex contractile tail structures with a large number of proteins involved in the tail assembly and function (Petr, 2010). The tail fibers are also important in recognizing host cell surface receptors, so they determine if a bacterium is within the virus's host range (Ackermann and Krisch, 2014). The tape measure protein is present in the baseplate-tail tube complexes, but it could not be modeled (Taylor 2016). The T4 virus initiates an *Escherichia coli* infection by binding Omp C porin proteins and lipopolysaccharide on the surface of *E. coli* cells with its long tail fibers (Malys, 2012).

2.8. Phage therapy in veterinary medicine

Phage therapy has been used in veterinary science since the beginning of the 20th century. In1919 phages were first used in France against bird typhoid fever. *Salmonella gallinarum*, as well as their bacteriophages, were isolated from the infected chicken and when tested, phages proved to be effective against *Salmonella gallinarum*. The first trial model for testing phage therapy was mice salmonellosis, mainly caused by *Salmonella typhimurium*. Phages were both administered intraperitoneally and orally, where the latter did not give a positive bactericidal result and the former only decreased microbial spread insignificantly (Topley *et al*., 1925).

This trial failed most probably because the right type of phage was not used since later scientists managed to infect typhoid bacteria *in vitro* successfully with a well-chosen anti-typhoid phage (Fisk *et al*., 1938). Many subsequent experiments in treating rabbits, Guinee pigs, mice and rats, infected with streptococci, and staphylococci have not shown to be effective. Nevertheless, there is multiple evidence of successful phage treatment of streptococcal meningitis in rabbits (Sulakvelidze and Kutter, 2012).

Today phages are used in both human and veterinary medicine. Among their advantageous characteristics, such as their specificity on bacteria, qualifies them for use in phage therapy. Modern phage medicine is based upon virulent phages of a broad range of action that are active against antibiotic-resistant bacteria. The latter phenomenon (antibiotic resistance development) has in recent decades led to less efficient bacterial disease treatment, as well as to an increase of persistent infections and latency. Antibiotics usage today is narrowed down as much as possible, to avoid, or at least, decelerate resistance growth, which provides more importance of the ecologically safe phage therapy as a modern anti-epizootic treatment, which is to be opted for (Sulakvelidze and Kutter, 2012).

Bacteriophages are known for being effective against *Enterobacteriaceae* in general and *E.coli* in specific (Huff, *et al*., 2003). An experiment has been done on colibacillosis in piglets, where T4 phage from the *Myoviridae* family was used (minimum of 10^5 phages/g body mass were applied)

providing with up to 100% protection, in all dosages; the optimal dosage has been determined to be triple treatment with 10^9 phages/1-month old piglets (Skoblikow and Zimin,2013).

2.9. Multidrug resistance

The resistance among various microbial species (infectious agents) to different antimicrobial drugs has emerged as a cause of animals and public health threat all over the world at a terrifying rate. Almost all the capable infecting agents (*e.g*., bacteria, fungi, virus, and parasite) have employed high levels of multidrug resistance (MDR) with enhanced morbidity and mortality; thus, they are referred to as "superbugs." The development of MDR is a natural phenomenon, the inappropriate use of antimicrobial drugs, inadequate sanitary conditions, inappropriate food-handling, and poor infection prevention and control practices contribute to the emergence of and encourage the further spread of MDR (Kunjachan *et al*., 2013).

2.9.1. Significance of MDR

Antimicrobial drugs have been used for several decades across the world. Surveillance in different regions of the world such as Africa, some parts of America, Eastern Mediterranean Region, Europe, South-East Asia, and Western Pacific Region has shown that many infectious microorganisms have evolved over the years and there is an alarmingly high number of antibiotic-resistant species enabling themselves to resist the inhibitory effects of these drugs. Pneumonia has become untreatable because its causative agent is resistant to cephalosporin as well as carbapenems due to extended-spectrum β-lactamases mediated mechanism (Bennett *et al*., 2010), thereby rendering all available treatment using βlactam antibiotics. In recent years, HIV drug resistance has driven the antiretroviral therapy failure to such an extent that it is charging exorbitant rates along with several side effects.

The protozoan parasite responsible for malaria had embarked on showing resistance to some of its most effective drugs, chloroquine, artemisinin, and pyrimethamine (Olasehinde *et al*., 2014). This has resulted in the replacement of these old ineffective drugs by novel drugs, which has increased the health care expenses. The emergence of resistance to antifungal drugs in invasive yeast infections, for example, Candidiasis, hassled to worldwide morbidity and mortality, contributing to global economic burden. Antimicrobial resistance (AMR) or MDR is the reason why microbes

fail to respond to standard drugs, thus, extending the duration of course of treatment further increases the health care costs which tend to worsen the situation of people who are not capable of such expenses.

2.9.2. Problem associated with MDR

Antimicrobial resistance is associated with high mortality rates and high medical costs and has a significant impact on the effectiveness of antimicrobial agents. MDR provokes obstruction in disease control by intensifying the possibility of spreading of resistant pathogens, thus, declining efficacy of treatment and, hence, resulting in a prolonged time of infection in a patient. The cost of treatment is also increased due to MDR as the pathogens have become resistant to commercially available drugs, which has triggered the use of more expensive therapies. The rate of success of present-day medical applications like organ transplantation and cancer chemotherapy has contributed immensely towards the development of MDR. Differences in the resistance profiles of bacterial and fungal pathogens as well as the quality of public hygiene also have a considerable impact on the effectiveness of antimicrobial agents. Expansion of global trade and tourism leads to an increased potential of MDR to spread all over the world and decrease in export and import of various products affecting the economy of developing countries (Fishbain and Peleg, 2010).

2.9.3. Classification of MDR

Despite the administration of appropriate doses of drugs for a specific duration of time, the survival of various microbial strains recommends the high levels of resistance developed in them. This clinical failure is due to not only the antimicrobial resistance but also the suppressed immune function, poor/deprived drug bioavailability, or increased rate of drug metabolism. Persistence of microbes after conventional/standard treatments points out different types of antimicrobial drug resistance which is an expanding problem in the medical world. MDR can be classified as primary or secondary resistance.

Primary Resistance: it occurs when the organism has never encountered the drug of interest in a particular host.

Secondary Resistance: also known as “acquired resistance,” this term is used to describe the resistance that only arises in an organism after exposure to the drug (Khalilzadeh *et al*., 2006). It may further be classified as follows. Intrinsic resistance: it refers to the insensitivity of all

microorganisms of a single species to certain common first-line drugs, which are used to treat diseases based on the clinical evidence of the patient. It is also known as multidrug resistance (MDR), for example, Mycobacterium tuberculosis to rifampicin and isoniazid or Candida spp. to fluconazole, and extensive resistance: it defines the ability of organisms to withstand the inhibitory effects of at least one or two most effective antimicrobial drugs. Also termed as XDR, this seemed to arise in patients after they have undergone treatment with first-line drugs, for example, XDR-TB resistance against fluoroquinolone (Marks *et al*., 2014).

2.9.4. Mechanism of MDR

Resistance is the term referred to as the insensitivity of a microbe to an antimicrobial drug when compared with other isolates of the same species. Although several new drugs have been introduced commercially, this development of resistance among infectious microorganisms is increasing especially in patients under prolonged drug exposure. Antimicrobial drugs generally act on the microbes either by inhibiting a metabolic pathway like nucleotide synthesis which in turn leads to the inhibition of DNA/RNA synthesis and further protein synthesis and disruption of the cell membrane or by competing with the substrate of an enzyme involved in cell wall synthesis (Chethana *et al*., 2013).

Cell wall, in both bacteria and fungi, plays a crucial role in their survival. As discussed above, drugs inhibit cell wall synthesis by binding with the peptidoglycan layer in bacteria or affecting ergosterol synthesis (He *et al*., 2013). In fungi, thus, blocking the cell growth and division. These organisms undergo certain chromosomal mutations (Alekshun and Levy, 2007) or exchange of extrachromosomal DNA elements through conjugation or transformation (horizontal gene transfer) such as in K. pneumoniae which can cause alteration in the cell membrane composition resulting in decreased permeability and uptake of drugs into the cell Mutations in the genes encoding for the target cause modifications at the molecular level and retain cellular function by reducing susceptibility to inhibition (Chethana *et al*., 2013).

Another mechanism of MDR was found to bean overexpression of drug target enzymes leading to target bypass due to modification in certain metabolic pathways (e.g., azoles and allylamines in fungi, which causes the production of alternate target molecules and interference in some protein

synthesis. Inactivation or enzymatic degradation of antimicrobials by hydrolysis of ester or amide bonds (such as resistance to β-lactams due to β-lactamases, etc.) and chemical transformation of these compounds by acetylation, phosphorylation, adenylation, glycosylation, and hydroxylation have also become increasingly apparent as causes of MDR (Alekshun and Levy, 2007). The resistant strains of clinical isolates of different microorganisms have developed the ability to oxidize or reduce the antimicrobial compounds to prevent their interaction with the respective targets (Chethana *et al*., 2013). Resistance to the inhibitory effects of drugs on the enzyme can also emerge due to any conformational changes or altered binding of substrate to the viral polymerase (Strasfeld and Chou, 2010).

MDR mediated by drug efflux pumps remains the predominant mechanism of MDR. The overexpression of genes encoding for ATP-binding cassette transporter membrane proteins (*e.g*., P-glycoprotein), also known as the multidrug efflux pumps which are responsible for the export or expulsion of drugs out of the cell (Li and Nikaido, 2009), usually generates MDR and continues cellular functions without any interference. Overexpression of P-glycoprotein, in Entamoeba spp. and Leishmania spp. membrane or multidrug-resistant proteins, affects the fluidity and permeability, leading to an ATP-dependent efflux of the antimicrobials and decreasing their intracellular concentration (Orozco *et al*., 2002).

2.9.5. Remedies of MDR

The development of MDR is a complicated issue that has become an international dreadful concern. To decrease the rise and spread of MDR, cooperative efforts are requisite because diseases that were curable earlier are becoming major causes of deaths in this era (Singh, 2013). Moreover, focusing on areas that are susceptible to inappropriate use of antimicrobials by the implementation of antibiotic stewardship (defined as coordinated interventions designed to improve and measure the appropriate use of antimicrobials) is the need of the hour (Hamilton and Fishman, 2014). Various antimicrobial stewardship programs (ASPs) are conducted nowadays to optimize antimicrobial therapy, reduce treatment-related cost, improve clinical outcomes and safety, and minimize or stabilize MDR (Owens, 2008) Interventions through ASPs are either by restricting the availability of selected antimicrobial agents, known as “front-end,” or by examining broad-

spectrum use of antibiotics and then streamlining or discontinuing it, known as "back-end" (Moehring and Anderson, 2012).

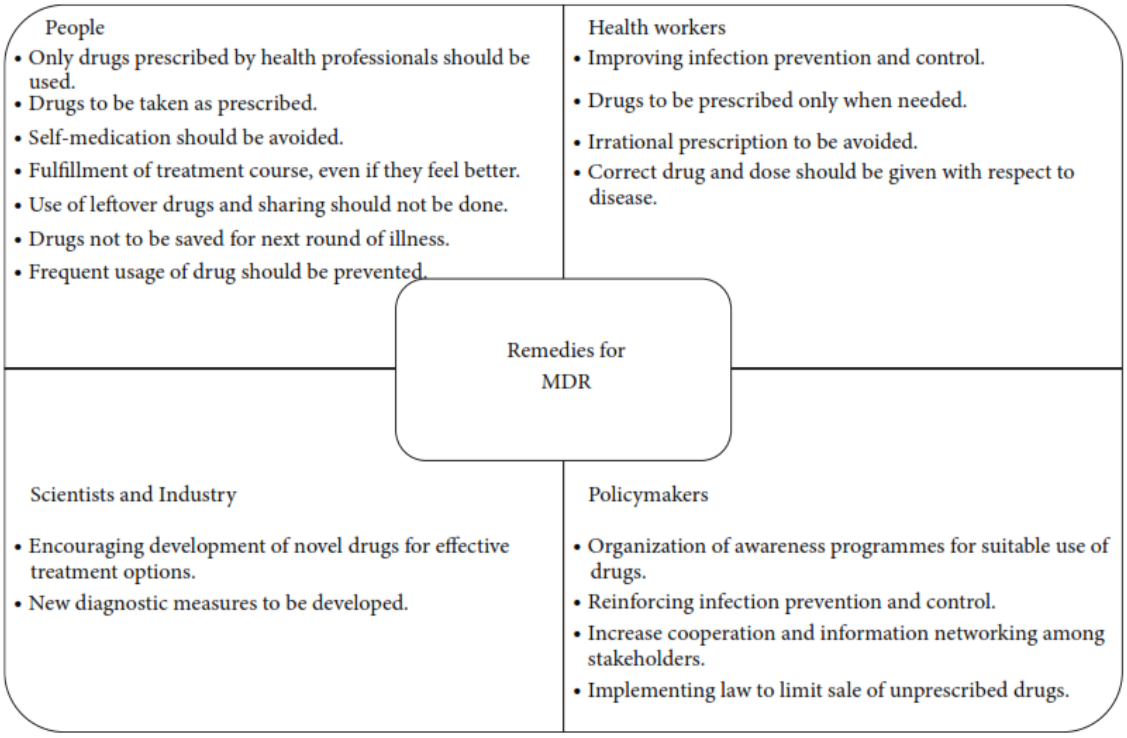

Figure 4: Remedies of MDR
Source: (Kunjachan *et al*., 2013).

3. MATERIALS AND METHODS

3.1. Study area

The study was performed at National Veterinary Institute Bishoftu, Ethiopia from October 2019 to May 2020. Bishoftu is located about 45kms south east of Addis Ababa at 9^0N latitude and 40^0E longitudes at an altitude of 1850 meters above sea level in central high land of Ethiopia. It has an annual rainfall of 866 mm of which 84% is in the long rainy season (June to September). The dry season extends from October to February. The mean annual maximum and minimum temperature are 26^0C and 14^0C respectively, with mean relative humidity of 61.3%.

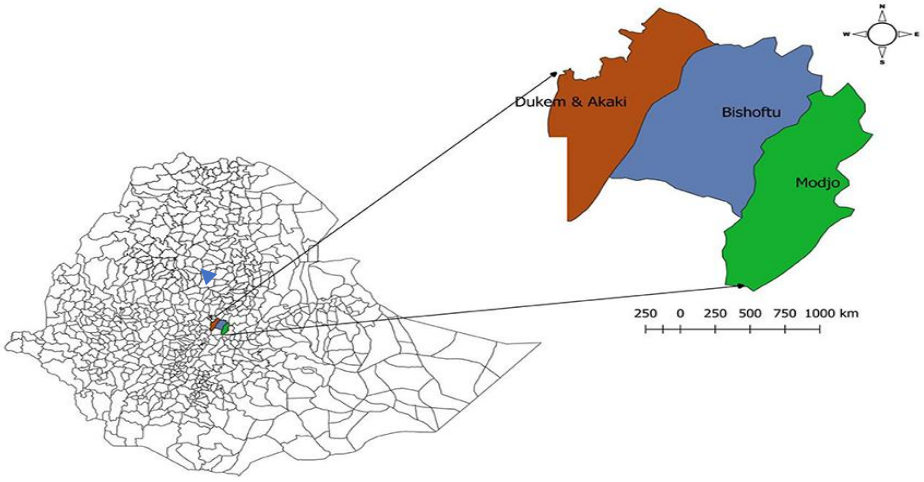

Figure 5: Map of Bishoftu town

3.2. Study design

This experimental study was carried out in the laboratory of Microbiology at the NVI was designed to isolate and characterize potent phages present in water samples from sewages and drainage found in Bishoftu and to analyze their lytic effect on MDR *E.coli* using bacterial culture(*In vitro*) and mice (*In vivo*). In the last seven months different sources of phages were identified and extensive purification and analysis of their effect on different MDR- *E.coli* were investigated.

3.3. Data collection and laboratory investigation

3.3.1. Sample collection

The sewage samples were gathered from seven different water source of sewage and drainage namely;Alema farms sewage tank, College of Veterinary Medicine and Agriculture students' toilet drainage, College of Veterinary Medicine and Agriculture Veterinary Teaching Hospital (VTH), Elfora Export Abattoir sewage tank, Zikuala condominium drainage, Genesis farm sewage tank, and College of Veterinary Medicine and Agriculture sewage tank found in Bishoftu, using sterile capped bottles and under aseptic conditions for phage and *Escherichia coli* isolation purpose. The fecal samples were collected from diarrheic cattle came to VTH. The samples were transferred to the microbiology laboratory of NVI, at 4ºC.

3.3.2, Isolation and identification of E.coli

Escherichia coli was isolated from fecal and sewage samples by standard microbiological methods suggested by American Society for Microbiology (Irwin *et al*., 2010). One milliliter of sample effluent was cultured in Lysogeny broth (LB) and incubated at 37ºC for 24 hours. One standard loop from the positive LB medium (Oxoid)was cultured in MacConkey (Himedia) agar using the streak plate method and incubated at 37ºC for 24 hours. The purified bacteria on MacConkey agar were analyzed for morphological, biochemical characteristics, and antibiotic susceptibility tests.

3.3.3. Antibacterial Susceptibility Test on Isolated E.coli for MDR E.coli isolation

Antimicrobial susceptibility against different antibiotics was tested by the disk diffusion method (modified Kirby-Bauer) on Mueller-Hinton Agar (Bauer *et al*., 1969). The antibiotics used for this test were amoxicillin (30 µg), ampicillin (10 µg), erythromycin (15 µg), nalidixic acid (30 µg), penicillin (10 µg), streptomycin (10 µg), tetracycline (30 µg), and vancomycin (30 µg). The readings for the zone of inhibition were interpreted as recommended by Clinical and Laboratory Standards Institute (CLSI, 2019).

3.3.4. Isolation of coliphages (plaque assay)

Isolation of bacteriophages specific to *E.coli* were carried out from sewage and drainage samples according to the standard protocol described by Twest and Kropinski (2009). Briefly, the samples were processed by the enrichment method in the microbiology laboratory of the National

Veterinary Institute (NVI). Ten milliliters of each sample was centrifuged at 10,000rpm for 10 minutes to remove particulate materials. The supernatants were filtered and sterilized by passing through a 0.22 µm pore membrane filter. The filtrate (50 ml) along with 5ml *E.coli* strain was then mixed with an equal volume of sterile double strength nutrient broth containing in a 250 ml flask. The flask was incubated overnight at 37 ^{O}C which contains stirrer with 150rpm for 24 hrs. After the mixture was centrifuged at 10,000 rpm for 15 minutes, the supernatant was passed through a 0.22 µm pore membrane filter under aseptic condition. The pellet discarded whereas the filtrate was held for amplification of phage. To amplify the phage from these steps, 50 ml of the filtrate mixed with an equal volume of double strength containing nutrient broth and Incubated with 5ml of the indicator strain (*E.coli*). Indicator strain (*E.coli*) at 37 ^{O}C incubator containing stirrer with 150rpm for 24 hrs. Then the mixture centrifuged at 10,000 rpm for 15 minutes. The supernatant was filtered and sterilized through a 0.22µm pore membrane under aseptic condition.

3.3.5. Purification of Isolated Bacteriophages

The amplified filtrate obtained was tested for phage activity against the *E.coli* by using spot assay as described by Kumari *et al*. (2010). The indicator cell (0.1ml) was added to sterile molten soft agar (0.75%) prepared and maintained at 45°C in a water bath and mixed quickly. The contents were poured on a previously prepared nutrient agar plate surface. Around 50-100 microliter of the amplified filtrate was spotted on each plate at different places. The plates were allowed drying at room temperature and then incubated at 37°C overnight for 24 hours. The plates were examined the following day for clearance at the spot area. All the isolate phages were purified by successive single plaque isolation until homogenous plaque obtained according to the protocol followed by Schuch *et al*. (2002). A single plaque was picked aseptically by using a sterile toothpick and transferred into a tube containing 5ml broth and fresh log-phase has grown indicator strain. Another tube containing indicator strain left as a control. Both tubes were incubated at 37°C. Phage host mixture and control preparation was centrifuged at 10,000rpm for 15 minutes. The supernatant was filtered by passing through a sterile 0.22 µm pore membrane to remove bacterial contaminants. The filtrate was serially diluted and assayed for plaque developments. The procedure was repeated for three times to ascertain the purity of isolate phage.

3.3.6. Bacteriophage Titration (in vitro test of optimal phage titer to challenge bacterium)

Titer of the phage preparation plaque-forming units per milliliter (PFU/ml) were estimated by the soft agar overlay method as described by Adams (1959). The high titer was prepared by adding phages to the early log phase host culture and incubated at 37ºC until complete clearance obtained. Then the large plaque-forming sample content was selected for the next processes. Serial dilution of the bacteriophage sample was made in sterile peptone water. A 0.1ml of bacteriophage suspension from each dilution was mixed with 0.1ml of the indicator host cells of *E.coli* and added to about 8ml of molten soft agar held at 45ºC. This thoroughly mixed without air bubbles and quickly poured over previously moisture dried nutrient agar plate. The overlays allowed solidifying upright for a few minutes at room temperature and then incubating in an inverted position at 37^0C overnight. The following day plates with 30 to 300 plaque forms were selected, counted, and calculated PFU/ml.

3.3.7. Characterization of Bacteriophages

3.3.7.1. Effect of temperatures on phage activity

To determine the effects of temperatures on phage activity, phage was incubated at a range of 4ºC to 60°C and titre of the phage was determined by the soft-agar overlay technique. The plates were incubated at 37 °C for 24 hrs. and after 24 hrs. the number of plaques were counted, PFU/ml calculated and recorded.

3.3.7.2. Effect of pH on phage activity

The pH effect on phage was determined using Harley and Prescott, (1993) method. the pH of TSB was adjusted to 3, 5, 7, 9, and 12 by the addition of either HCl or NaOH. 900 µl of the pH adjusted TSB and 100 µl of phage was mixed in 250ml glass tube and left to stand at room temperature for 18 hrs. After 18 hrs the titer of the phages were determined using the soft agar overlay technique. The plates were incubated at 37 °C for 24 hrs. and the number of plaques were counted, PFU/ml calculated and recorded.

3.3.7.3. Effect of chloroform on phage stability:

The effect of chloroform on the stability of phages was determined using a previously described method (Harley and Prescott, 1993). Optimal phage dilutions were prepared in a diluent. Aliquots of 1 ml of each dilution were treated with chloroform and stored at room temperature (25 °C) with gentle shaking for 1 hr. The suspension centrifuged at 10000 rpm for 10 minutes. The phage titer of the supernatant was determined using an overnight culture of *E. coli* as the host bacterium. A phage diluted in diluent used as a control sample. Plates incubated at 37 °C for 24 h, the effect of chloroform checked on phage activity and the result was recorded.

3.3.8. In vitro phage therapy (tube lysis test)

One well-formed plaque containing bacteriophages from each agar plate was selected for the *in vitro* assay. The plaques from agar plates diluted, and then, chloroform added to remove bacteria. The phage lysates centrifuged, and the supernatant used to test lytic activity against MDR-*E.coli*. MDR-strains of *E.coli* (1 ml) inoculated in two flasks with 150 ml Tryptic Soy Broth (TSB) media. To evaluate the *in vitro* lytic efficacy of bacteriophages, 500 µl phage transferred into test flasks containing 150 mL TSB with MDR-*E.coli*. In parallel, controls sated up with flasks containing the respective MDR-*E.coli* alone. The test and control flasks incubated in an incubator containing stirrer (150rpm) at 37 ^{0}C for 24 h. (Pallavali *et al*., 2017).

3.3.9. In vivo Therapeutic Potential of Isolated Bacteriophage against Antibiotic Resistant E.coli on mice

Standardize the dose: The dose of *E.coli* lethal to mice and dose of Phage were determined by injecting mice with varying numbers of *E.coli*, and phage. After standardizing the dose, obtained Twenty one 8 weeks old female mice from National veterinary institute animal services (Bishoftu, Ethiopia). Mice were randomly allocated to 3 cages with seven mice each and housed under standard day length, temperature, and humidity conditions. Water and pelleted feed offered, the water was changed every two days and labeled the cage into three different groups namely Phage and PBS challenged, Phage and bacteria challenged, and bacteria and PBS challenged group.

Finally checked the healthy status of the mice. The efficacy of phage therapy was evaluated in three treatment groups of mice (7 mice per group) using *E.coli*. The first group evaluated for the effect

of phage on establishing *E.coli* infection for this purpose, the mice challenged by resistant and phage intraperitoneal. The second group challenged with resistant *E.coli* and PBS intraperitoneal. The last group gave phage and PBS intraperitoneal. Infected animals and controls observed under the sterile condition for three days and the status of the animals were monitor and record by photography. During suffering and at the end of the experiment mice was euthanized using anesthesia (giving alcohol intracerebral).

3.4. Ethical Consideration

Application for animal ethics clearance for approval of proposed research involving live animals was submitted to Addis Ababa University College of Veterinary Medicine and Agriculture Animal. Research Ethical Review Committee, and all animal work was conducted according to animal research ethics. The approved certificate number was VM/ERC08/01/12/2020.

3.5. Methods of Data Analysis

All data were coded and stored in Microsoft Office Excel spread sheet and transferred to SPSS latest version (V26) 2020 for statistical analysis. All qualitative data (laboratory findings) were presented by graphs and tables. Some comparisons were also summarized by line graphs where as an association between intervention group and the outcomes variable was tested by Fisher's exact test. In all the analyses, 95% confidence level and $p \leq 0.05$ was set for significance.

4. RESULT

4.1. Bacterial Isolation

In the present investigation, *E.coli* from seven selected samples (AF, C1, C2, EA, JF, ZC, and C3) were cultured on LB and MacConkey medium for morphological characterization. After 24 hrs, visual and microscopic observations were used to characterize the bacteria. The colonies were pink in color in MacConkey while creamy yellow in LB medium. *E.coli* grown on MacConkey Agar was capable of metabolizing lactose, which produces acid by-products that lower the pH of the media. In Gram's staining, the morphology of the isolated bacteria exhibited pink coloured, small rod shaped, Gram negative bacilli. Different biochemical tests were also performed for the 7 isolates to know their biochemical characteristics. All the isolates were positive for catalase,Indole, MR and OF tests while negative for oxidase and citrate test. Reactions in TSI agar slant revealed yellow slant and butt with gas (*E.coli* 2 and *E.coli* 7) with out gas (other five *E. coli* isoleets) but no production of hydrogen sulphide gas was observed. Details of the biochemical characters of the bacteria are noted in table 2. The above results gave an idea of the morphology, colony characteristics and biochemical nature of the isolated bacteria which would aid in the identification and characterization of *E. coli* bacteria.

Table 2: Morphological and biochemical characterstics of *E. coli*

Samples Name	**Bacteria**	**Gram stain**	**Catalase Test**	**Oxidas test**	**Indole test**	**TSI test**	**MR test**	**Citrate test**	**OF test**
AF-sample	*E.coli* 1	-Ve	+ve	-ve	+ve	All yellow no gas	+ve	-ve	+ve
C1-sample	*E.coli* 2	-Ve	+ve	-ve	+ve	All yellow gas	+ve	-ve	+ve
C2-sample	*E.coli* 3	-Ve	+ve	-ve	+ve	All yellow no gas	+ve	-ve	+ve
EA-sample	*E.coli* 4	-Ve	+ve	-ve	+ve	All yellow no gas	+ve	-ve	+ve
JF-sample	*E.coli* 5	-Ve	+ve	-ve	+ve	All yellow no gas	+ve	-ve	+ve
ZC-sample	*E.coli* 6	-Ve	+ve	-ve	+ve	All yellow No gas	+ve	-ve	+ve
C3-sample	*E.coli* 7	-Ve	+ve	-ve	+ve	All yellow gas	+ve	-ve	+ve

4.2. Antibiotic susceptibility test (MDR *E.coli* Isolation)

The result of antimicrobial susceptibility test of 7 *E. coli* isolates against 8 commercially available antimicrobial agents namely Amoxicillin, Ampicillin, Erythromycin, Nalidixic acid, Penicillin, Streptomycin, Tetracycline, and Vancomycin were summarized in table 3 and 4. The degree of susceptibility ranges from 25% up to 50%. Similarly, resistance ranges from 50% up to 75% in the isolated *E. coli*. Among the eight antiprograms, the maximum resistance was seen on Erythromycin, Penicillin, and Vancomycin 7 (100%), followed by Ampicillin 6(85.7%), Amoxicillin, 5(71.4%), Streptomycin, 4 (57.1%), Tetracycline, 2 (28.6%) (Table 3). The resistance profile of the seven isolates revealed maximum resistance in *E. coli 1* and *E. coli 3* 6(75%) followed by *E.coli* **2**, **5**, 6, and **7** 5(62.5%), and, *E.coli* **4** 4(50%) (Table 4)

Table 3: Drug susceptibility test for all isolated *E.coli*

Name of bacterial isolate	AMC	AMP	E	NA	P	S	TE	VA
E.coli 1	R	R	R	S	R	S	R	R
E.coli 2	S	R	R	S	R	R	S	R
E.coli 3	R	R	R	S	R	R	S	R
E.coli 4	S	R	R	S	R	S	I	R
E.coli 5	R	R	R	S	R	S	S	R
E.coli 6	R	S	R	S	R	R	S	R
E.coli 7	R	R	R	S	R	I	S	R

Key: AMC= Amoxicillin, AMP= Ampicillin, E= Erythromycin, NA=Nalidixic Acid, P= Penicillin, S=Streptomycin, TE=Tetracycline, VA= Vancomycin R=Resistance, S= Susceptible

Table 4: The level of resistance for seven *E.coli* isolates for different drugs

Name of *E. coli* isolates	level of resistance for different drugs (n=8)	
	Resistance (%)	Sensitive (%)
E.coli 1	75%	25%
E.coli 2	62.5%	37.5%
E.coli 3	75%	25%
E.coli 4	50%	50%
E.coli 5	62.5%	37.5%
E.coli 6	62.5%	37.5%
E.coli 7	62.5%	37.5%

4.3. Isolation of coliphages (plaque Assay) from sample

Seven *E. coli* specific bacteriophages were isolated from sewage and drainage samples. The isolated phages were named as AF, C1, C2, EA, GF, ZC and C3, according to the name of sample source (Table 5). One phage (C3) revealed clear (complete lysis) plaques, and six Isolates turbid plaques were observed.

4.4 Host Range of Phages Against isolated *E. coli*

Table 5: Isolation of bacteriophage from different source and infectivity to Multi Drug Resistant *E. coli*.

Name of sample	Name of Isolated bacteria	Name of phage trial	Phages Host Range
AF-sample	*E.coli **1***	AF- phage trial	no lytic effect
C1-sample	*E.coli **2***	C1-Phage trial	no lytic effect
C2- sample	*E.coli **3***	C2-phage trial	no lytic effect
EA-sample	*E.coli 4*	EA- phage trial	no lytic effect
GF-sample	*E.coli 5*	JF- phage trial	no lytic effect
ZC-sample	*E.coli 6*	ZC-phage trial	no lytic effect
C3-sample	*E.coli **7***	C3-phage trial	Produce lytic effect

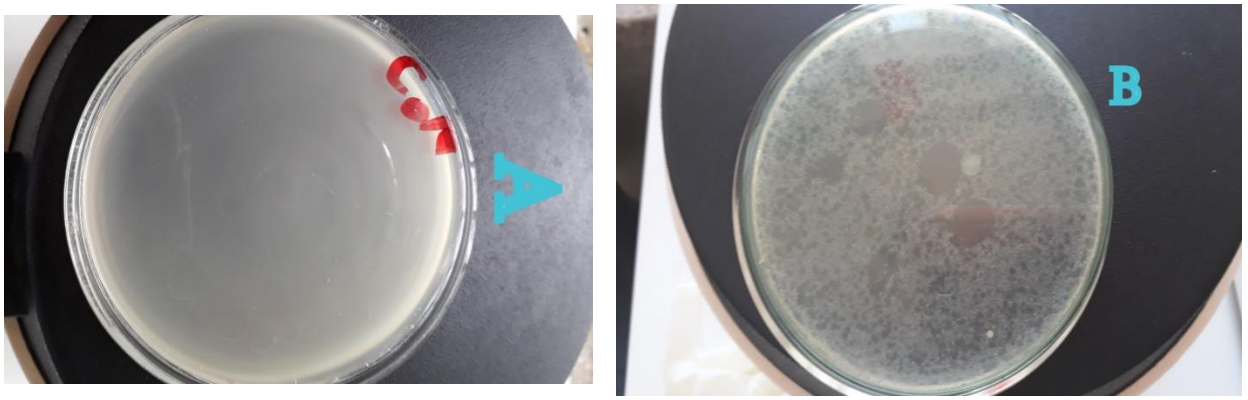

Figure 6: Representative image depicting spot test results of phage on *E. coli* 7
Key: Plate A contain only bacteria while plate B contain bacteria and Phage, lysed *E. coli 7* quickly as indicated by big, clear plaques.

4.5. Phage characterization

4.5.1. Phage Titration

Titration of phage were found by performing a serial dilution of the isolate. For *in vitro* test the optimal phage titer to challenge bacterium done the maximum number of phage forming units per ml was recorded at the fifth serial dilution (10^{-5}) with $3.2x10^7$ PFU/ml phage *(*Table below)

Table 6: Titration of phage

Plate Number	Dilution factor	plaque	PFU/ml
1	1: 10	209	2.09×10^4
2	1: 100	151	1.51×10^5
3	1: 1000	88	8.8×10^5
4	1: 10000	40	4×10^6
5	1: 100000	32	3.2×10^7
6	1:1000000	14	0
7	1:10000000	6	0
8	1:100000000	0	0
9	1:1000000000	0	0
10	1:10000000000	0	0

4.5.2. Phage activities against different Temperatures and pH

The phage survival in the adverse environment is also a desired characteristic for their use in phage therapy. The effect of different temperatures and pH on the phage was determined by agar overlay method. Phage revealed various responses when incubated for 30 min at 4°C to 60°C.The PFU/ml was ranged from 6.9 to 7.5 $\log_{10}$ PFU/ml at 4°C to 60°C. The maximum PFU/ml showed at 37°C (7.5 $\log_{10}$ PFU/ml) (Figure 7). Phage exhibited a decrease PFU/ml from 40°C to 60°C (6.9 $\log_{10}$ PFU/ml).

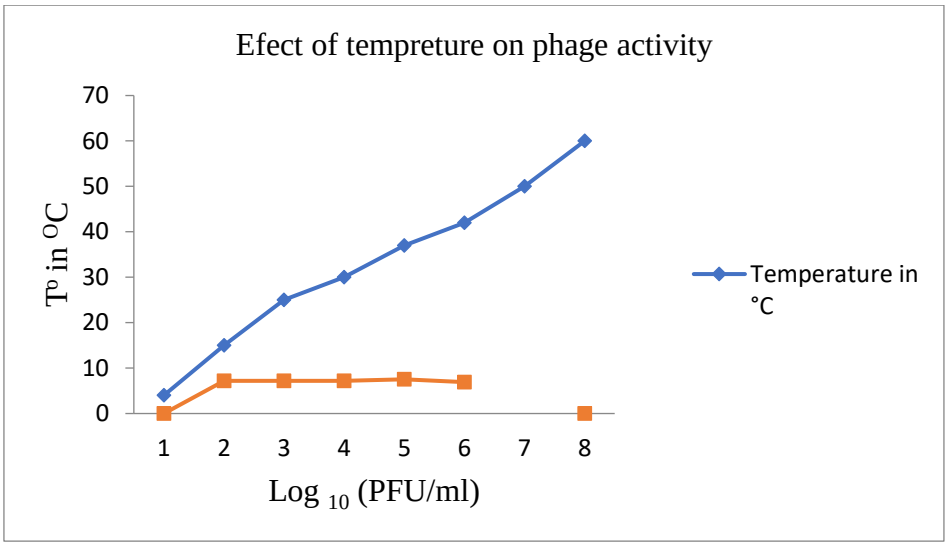

Figure 7: Phage activities againest different temperature conditions

Phage survival with in different the pH was also tested for 3, 5, 7, 9, and 12 and the response of pH on phage activity when incubated for 24hrs. (Figures 8). The PFU/ml of the phage ranged from 7.6 to 8.0 with different pH ranges The phage showed maximum PFU at pH 7.0 and progressive low effect below and above pH 7

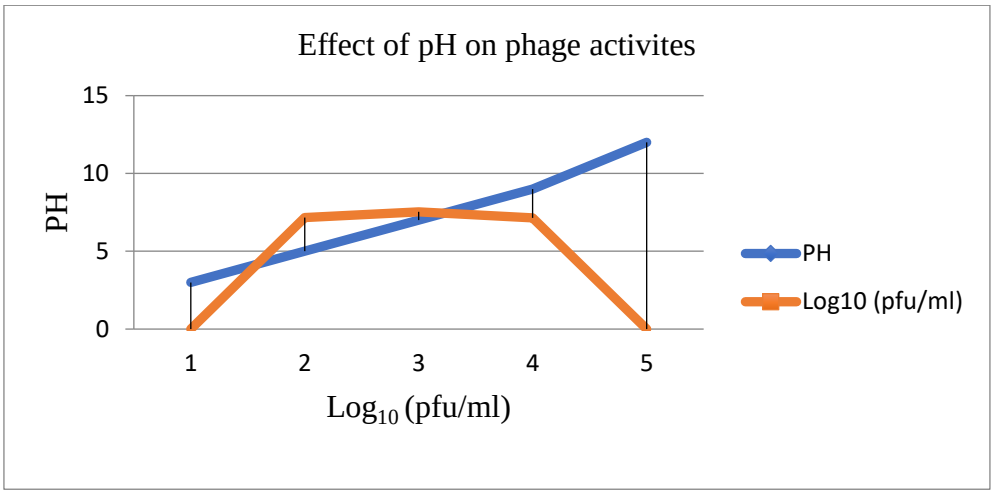

Figure 8: Phage activities againest different pH conditions

4.5.3. Chloroform

The effects of organic solvent (chloroform) on the survival, infectivity, growth and multiplication of phage was determined. Infectivity of the phage was not affected by chloroform treatment at 37°C as determined by the ability of the phages to form plaques similar to those formed by

untreated control phages on agar plate assays. The percentage survival and infectivity of phage after treatment with chloroform were seen to be relatively higher and the phages infectivity were found to be almost complete (figure 9)

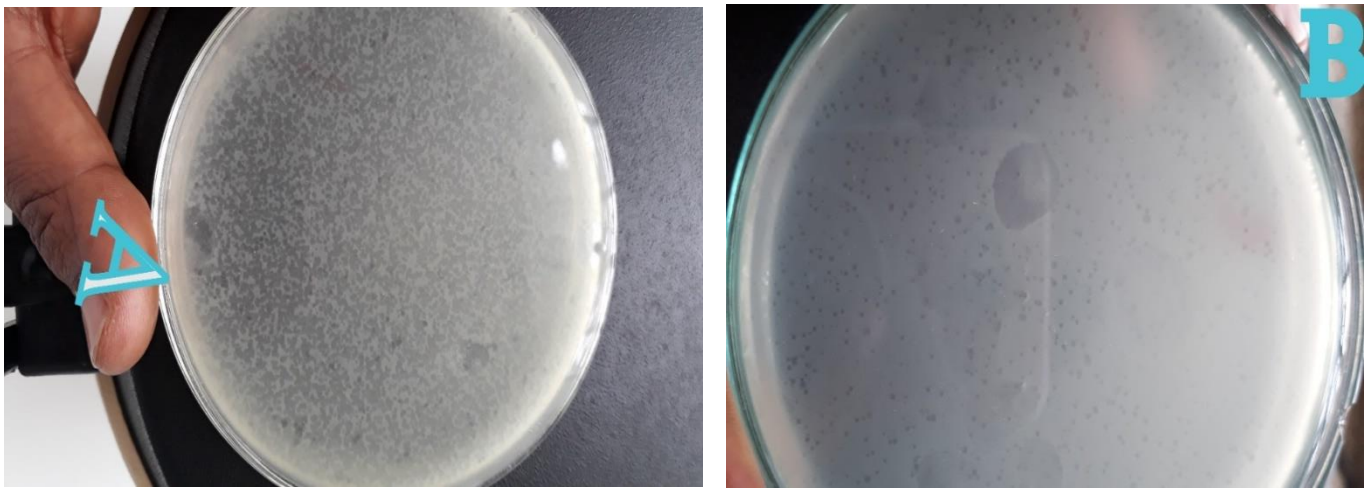

Figure 9: Effect of chloroform on phage activity
Key: Plate **A=** phage contain chloroform, plate **B=** phage do not contain chloroform

4.6 *In vitro* phage therapy (tube lysis test)

The isolated phage was further tested against MDR-*E.coli* 7 isolate from sewage using a bacterial reduction assay in liquid medium to ascertain the lytic function. *In vitro* lysis of MDR-*E.coli* 7 by the corresponding bacteriophage was monitored for 24 hr. during incubation at 37^{o}C. The bacterial reductions assay by phages were compared with the corresponding controls. Phage infections produced a drastic decrease in MDR-*E. coli* 7 density compared to the corresponding controls which showed normal bacterial growth kinetics (Fig 10).

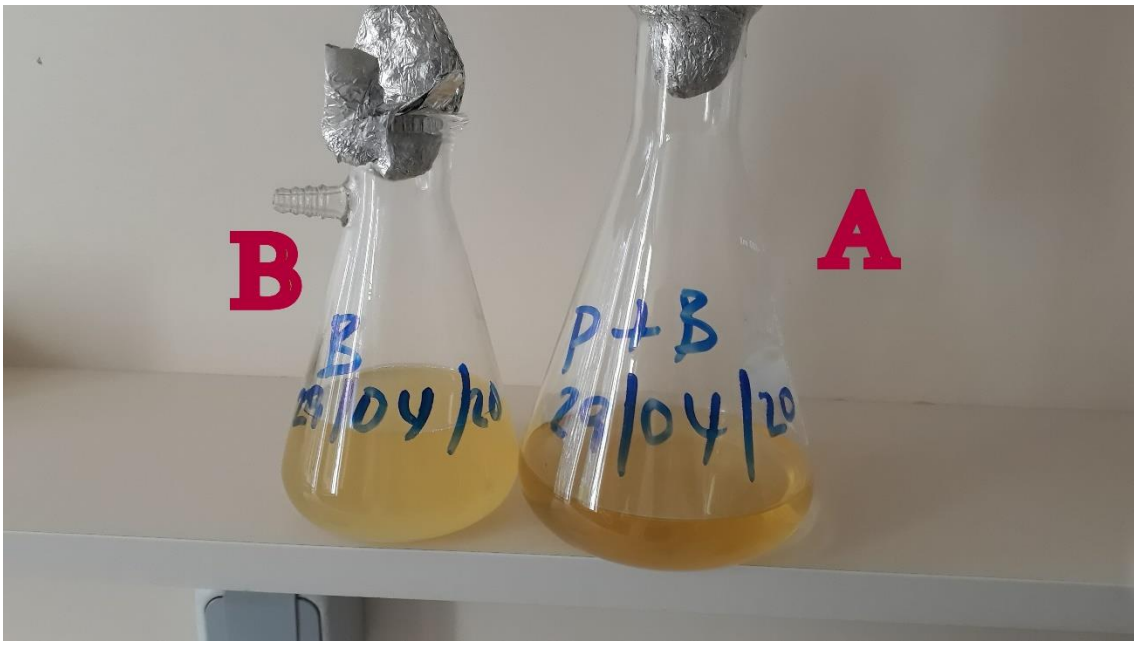

Figure 10: Lytic efficacy of Phage on MDR *E.Coli* 7 and conltrol

Key: flask **A=** contain phage and MDR *E. coli* 7, flask **B=** contain only bacteria

4.7. *In vivo* phage activity

To investigate whether phage could protect mice from *E.coli 7*, three groups of mice (seven mice per group) were injected intrapreoteinaly with a dose of *E. coli* 7 (3.1×10^8 CFU/mouse). One group served as an untreated control, and the remaining two groups were given the phage (3.2×10^7 PFU/mouse) intraperitoneally immediately after infection and the result have been monitored in different time frame.

Generally After three days of in vivo experiment on mice 71.4% (5 out of 7) mice have survived *E.coli* **7** infection in experimental group receiving phage therapy immediately after bacterial infection and 100% (7 out of 7) of the mice have survived in the group that gets phage and PBS. However 85.7% (6 out of 7) of the mice were died in the group that *E. coli* **7** and PBS administered. Therefore this result showed that, there was a significant difference in survival of mice between phage and bacterial challenged and bacteria and PBS challenged group (figure 11).

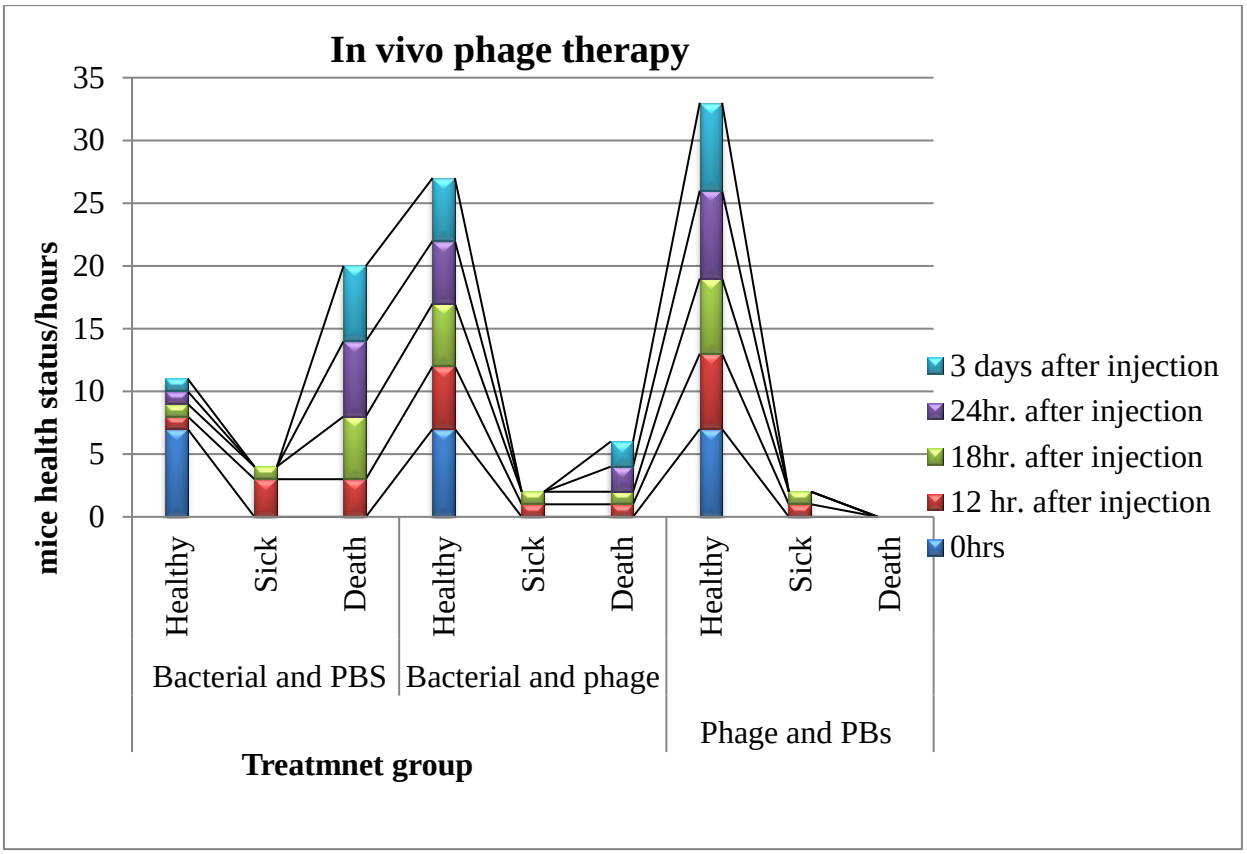

Figure 11: ***In vivo*** **phage therapy in different time frame**

5. DISCUSSION

Resistant pathogens are ever increasing and it is anticipated that those pathogens would emerge as a substantial global problem. These emerging MDR pathogens and the unavailability of newer antibiotics have reintroduced the use of phages cited to its specificity and novel mode of action. Hence, the treatment of these nasty pathogens with lytic bacteriophage and researches on it is gaining the spotlight in this era (Yang *et al*., 2010). Bacteriophages are abundant in the environment where their host resides such as rivers, soil, sewage, poultry or animal feces, water ponds, and seawater (Mulani *et al*., 2015). In general, sewage contains a large diversity of enteric organisms due to different sources. The present study attempted to isolate phages from sewage and drainage samples as they are the most relevant sources for its isolation. Moreover, in other studies, isolation of phages from freshwater ponds, animal wastes, and soil was successful too. Shukla *et al*. (2014) isolated phages in animal waste collected from different livestock farming. Likewise, the study carried out by Li and Zhang (2014) isolated phage specific against Staphylococcus aureus by processing fresh milk samples collected from the local dairy farm. Similarly, Alonso *et al*. (2002) isolated 26 phages from water samples of the Alboran Sea. Seaman and Day (2007) and Yordpratum *et al*. (2011) isolated phages from soil samples. This indicates that phages can be isolated from a wide variety of sources.

Using virulent bacteriophages as potential chemotherapy-independent schemes, drug-resistant bacteria can be destructed using microbial interference. virulent phages were isolated against MDR *E. coli* 7, and it was tested for its killing ability to the host (multidrug resistance *E.coli*). Since phages are obligate parasites, a host must be provided to isolate and enumerate them from any environment samples. In samples where phages were present, they multiplied and lyse the bacteria, causing a zone of clearing (a plaque) on the plate. Theoretically, each plaque is formed by one virus and the number of plaques multiplied by the dilution factor is equal to the total number of viruses in a test suspension. This is analogous to bacterial cell enumeration guidelines to apply to plaque-forming units (PFUs) (Adams, 1959). The selection of best phages was done according to the results of the lytic spectrum.

Bacteriophage was propagated and tested for lysis rate, sensitivity to chloroform, and its stability against different pH-values and temperatures was determined. One of the mechanism to identify

the phage weather it is enveloped or not is via exposing the phag to organic solvent like chloroform. In the present investigation, phage was resistant to chloroform exposure which implies that it is a non enveloped bacteriophage because as a rule enveloped virus as suscptable for organic solvent. The stability study of the phage held at different pH for 1 hr. and kept at room temperature showed that the phage lost its infectivity completely at pH 3 and 12. Maximum phage stability was observed between pH 5 to 9. However, the isolated phage was very unstable below pH 4. This is because phages are sensitive to protein denaturation in acidic conditions (Ackermann, 2006). While on the alkaline side, the isolated phage was unable to survive above pH 9. The maximum stability seemed to be at pH 7 followed by a decline in infectivity below pH 5 and greater than pH 9. This result is consistent with the previous study that survival of most phages is within pH 5 to 9 that may maintain the native virion structure and stability (Jamaluddin *et al*., 2008).

Temperature is one of the most important environmental factors that strongly affects many aspects of biological systems. One of the important characteristics of the temperature, as an environmental factor, is its fluctuation over a wide range of spatial and temporal scales that make possible as well as limit the existence of life in different niches. The influence of temperature upon the biological system is very vivid and it has been observed that the evolution of phenotypic traits, species distributions, and extinctions in many cases can be traced to changes in temperature regimes (Vale *et al*., 2008). The result of the present study in the stability of phage against varying temperature ranging from 4°C to 60°C indicated that, the phage was stable in the temperature range within 15°C to 42°C. However, it became less stable following exposure to tempraure range between 50°C and 60°C as the numbers of phage count were reduced to zero.The region of optimum stability was close to human body temperature (37°C) as the phage appeared to survive in this range. The isolated phage was unable to grow well in temperature that is higher than body temperature although the loss was at 42°C and most phages could not survive above this temperature. Thus, it was seen that the was rapidly inactivated at 50°C temperature. This result could show that extreme conditions might affect the phage structure and cellular functions (Kutter and Sulakvelidze, 2005).

The present study results ware in agreement with the others findings as observed that yield of was highly temperature-dependent. The was unable to develop and perform lysis on *E.coli* at 4°C, while on 15°C, 25°Cand 37°C, the activity was carried out but in a little bit delayed manner. These

findings of the study are in line with those of Taj *et al*, (2014) who observed that lysis by phage was delayed if the incubation temperature was below 37°C. Similarly, this study showed that at thermophilic temperature 42°C developed and performed lysis on its host bacteria and support the results of Taj *et al.* (2014), who reported that bacteriophage developed at 42°C. While temperature regimes above 42°C proved as a limiting factor and caused the actual inactivation of the (Table 9). Study results regarding the inactivation of phage were in agreement with those observed by Basdew and Laing (2014) who reported that an increase in temperature decreases virus survival and activity. In the same way, findings by Popp *et al*. (2004) indicated that an increase in bacteriophage yield till 30°C and 39°C which support the findings of the present study that indicated 37°C was the ideal temperature for the bacteriolytic activity of phage against *E. coli* **7**

Phages have high specificity for specific bacterial strains, a characteristic that requires careful targeting (Merril, *et al*., 2003). From a clinical standpoint, phage therapy appears to be very safe if further preclinical and clinical tests can be ruled out. Efficacy of natural phages against antibiotic-resistant *E. coli* was being evaluated by researchers (Kumari *et al*., 2010). The current study also proved that the was specific to the selected *E. coli* 7 that produced strong drug resistance but not even to the other *E. coli* strains isolated from other sewage and cattle. It has been found/concluded that lysis of the bacterial host is the final event in the infection cycle of a lytic bacteriophage (Wang *et al*., 2000). The result showed that did lysis from 10^{-1} to 10^{-7} dilutions while from 10^{-8} to 10^{-10} dilutions had no lysis activity as shown in table 6. It means that at the highest dilutions, fails to do lysis. Lysis can be produced by phage dilution up to a certain point; as Worley-Morse *et al*. (2014) reported that bacteriophage concentration is very important for lysis activity.The storage of the isolated bacteriophages was tested at three different temperatures; the bacteriophages remained stable at 4°C and -20°C for a month, while none survived at 37°C. The best storage temperature for the phage was -20°C, as reported by previous authors (Mishra *et al*., 2012).

In this study, using newly isolated lytic phages we developed an animal model to determine the potential use of the phage to cure MDR *E.coli*. A single dose of phage was administered immediately after the challenge with the *E.coli*. By 24 hrs. a dose effect on the state of health of the infected animals was visible. All of the mice that were alive and healthy at 24 hrs. remained so for an additional 3 days, at which point the experiment was terminated. Purified phage was

administered intraperitoneally to mice after *E. coli* **7** injection. After 24 hrs. most mice treated with phages were still alive, whereas 6 out of 7 (85.7%) of mice not treated with phages were dead.

The results obtained in the present experiments were encouraging, after Intraperitoneal (i.p.) injection of $3.1x10^8$ CFU dosage of MDR *E. coli* 7 strains from one side of the abdomen, i.p. injection of the phages at 3.2×10^7 PFU from the other side rescued 71.7% of bacteremia mice. These experiments demonstrate a powerful curative effect of phages on MDR *E.coli* 7 bacteremia in our mouse model. Healthy animals did not display apparent reactions to these factors, as evidenced by the lack of any adverse effects in the control groups inoculated with phage preparation.

Mortality is certainly an important criterion to judge the efficacy of treatment, yet the present study also supported this idea because the one which take bacteria and PBs only one mouse was recovered from seven mice. Phage was well tolerated by the animals, it drastically reduced the mortality of mice because from seven mice five mice were recovered. Phage treated mice remained healthy for 3 days after infection when the experiment was ended. The above results become more convincing when examined in the context of numerous reports documenting phage efficacy *in vivo* against several bacterial species (Biswas *et al*., 2002, Matsuzaki *et al*., 2003, Qazi *et al*., 2004, Wills *et al*., 2005). We also attempted to test the efficacy of phage when bacterial infection was already established. To demonstrate that phage can be effective several days after experimental infection, we shifted the criterion for measuring phage efficacy from the survival rate for animals treated with a lethal bacterial dose to the sterilization rate for animals infected with a low bacterial dose.

6. CONCLUSION AND RECOMMENDATION

Bacteriophages have bactericidal activity against the pathogenic bacteria responsible for diseases, and they may quickly reduce bacterial loads. Majority of *E. coli* isolate were resistant to for more than five out of eight drugs. From seven trials of phage isolation one lytic bacteriophage namly was successfully isolated against MDR *E. coli*. The phage was naked virion because formed plaque when exposed to chloroform and also having stability at wider pH ranges between 5 and 9 and temperatures between 4 and 42°C. After the candidate phage has adequate lytic spectrum with maximum adsorption capability it used for *in vitro* and *in vivo* therapeutic purposes. Since phages are abundant, it can be readily isolated from the environment using simple, low-cost techniques than that of developing a new antibiotic. The results of this study indicated that phage has a potential to treat MDR *E.coli*. Phages are alternative therapeutics against multidrug-resistant bacteria, but there are still questions about their practical use, especially in systemic infections. Besides the immunological aspects, a good understanding of phage-bacteria interactions and phage survival in the body is required. Our results demonstrate that, by previous findings, phages can not only combat bacteria already present in the one system but also survive in the different system afterward. Therefore isolation, characterization and therapeutic application of potential phages lytic against *E.coli* bacteria commonly involved in infections is a major finding of this study. Notably, we found phage lytic against multidrug-resistant *E.coli* 7. This promising effect against MDR pathogens has raised the probable utility of these phages for biological control of bacterial infection. Based on the above conculussion the following recomedations are forwarded:

- Future work must be dedicated to those factors that influence this and possibly enhance the therapeutic potential of phages.
- Further characterization of specific phages is needed to explore the potential use of these phages for their clinical application.

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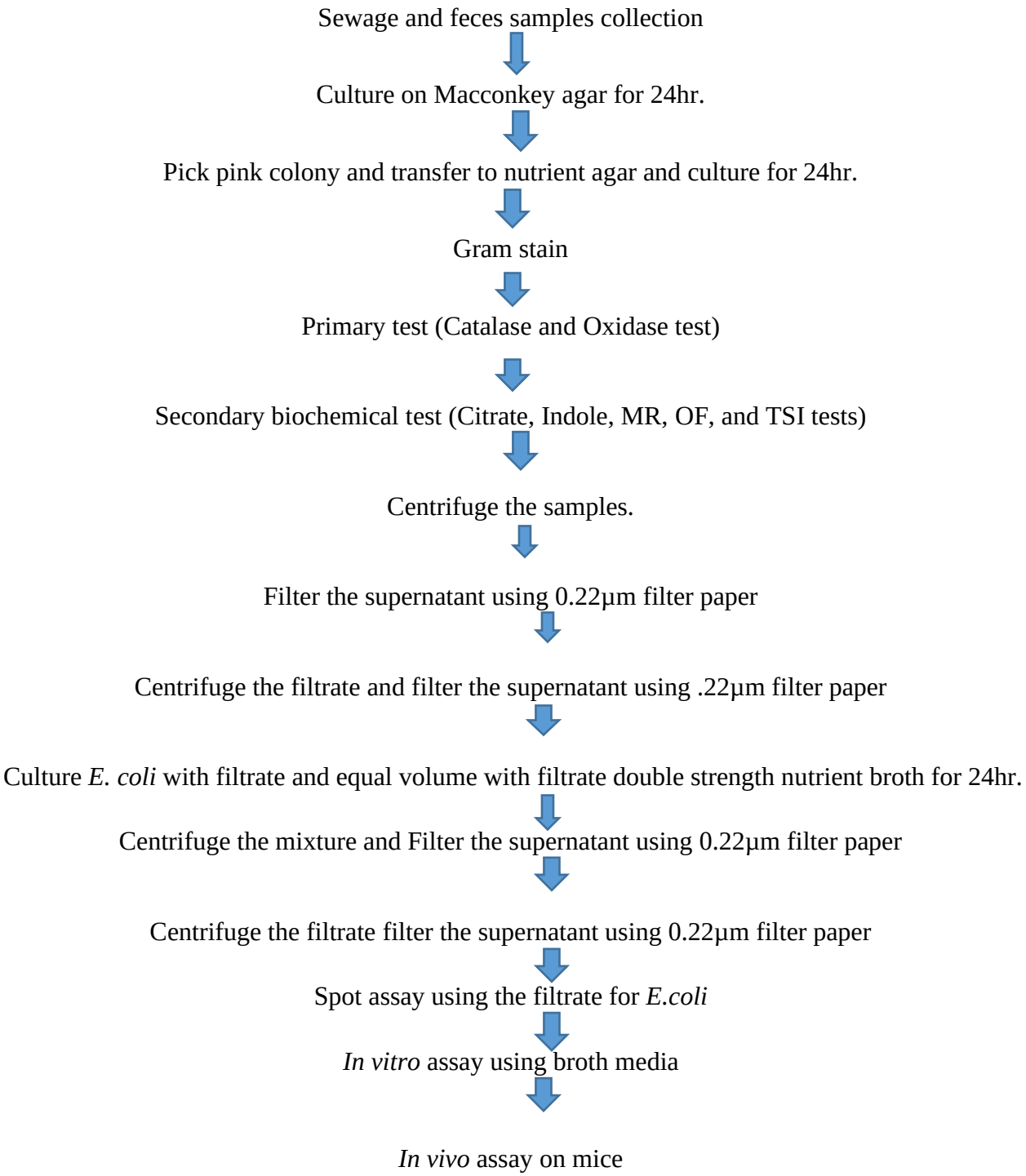
8. ANNEXES

Annex 1: Experimental procedure from sample collection up to in vivo therapy on mice



Annex2: Biochemical test (composition, preparation), Reagents and solutions and Test procedures

- **Biochemical tests (composition, preparation)**

MacConkey agar (CMO115, Oxoid)

Composition (g/l): Peptone 20.0, Lactose 10.0, Bile salts No.3 1.5, Sodium chloride 5.0, Neutral red 0.03, Crystal violet 0.001, Agar 15.0, PH 7.1 (Approximately)

Preparation: 51.5 grams of the powder were suspended in a litre of distilled water brought to boil to dissolve completely and dissolved completely and sterilised at (1210C for 15 minutes).The molten agar was cooled to 500c and approximately 20ml poured into a Petri dish (90mm in diameter) and allowed to cool and solidify at room temperature.

Methyl Red-Vogues Proskauer Medium (Buffered Glucose Broth) (HIMEDIA M070-500G)

Composition (g/l): Peptone mixture 7.00 (Peptic digest of animal tissue 5.00); Dextrose 5.00; Dipotassium Phosphate 5.00. Final pH: 7.5 ± 0.2 at 25ºC, Distilled water 1 liter

Preparation: Suspend 15 grams of the medium in one liter of distilled water. Heat to dissolve. Distribute into tubes in 1oml amount and sterilize by autoclaving at 121 Dispense in tubes in 10ml amounts and sterilize at 121ºC (15 lbs sp) for 15 minutes.

Simmons Citrate Agar (HIMEDIA M099 500G)

Composition (g/l): Ammonium Dihydrogen Phosphate 1.00; Dipotassium Phosphate 1.00; Sodium Chloride 5.00; Sodium Citrate 2.00; Magnesium Sulphate 0.20; Bacteriological Agar 15.00; Bromthymol Blue 0.08. Final PH: 6.8 ± 0.2 at 25ºC, Distilled water 1 liter

Preparation: Suspend 24.28 grams of the medium in one liter of distilled water. Heat to boiling to dissolve the medium completely. Dispense in tubes and sterilize in the autoclave at 121ºC for 15 minutes. Cool the tubes in a slanted position so that the base is short (1-1.5 cm. deep).

Triple Sugar Iron Agar (OXOID CM0277 500G)

Composition (g/l): Peptone Mixture 20.00; Lactose 10.00; Sucrose 10.00; Sodium Chloride 5.00; Beef Extract 3.00; Yeast Extract 3.00; Glucose 1.00; Ferrous Ammonium Citrate 0.30; Sodium

Thiosulphate 0.30; Phenol Red 0.024; Bacteriological Agar 12.00. Final pH: 7.4 ± 0.2 at 25ºC, Distilled water 1 liter

Preparation: Suspend 65 grams of the medium in one liter of distilled water. Bring to the boil to dissolve completely. Mix well and distribute in tubes. Sterilize by autoclaving at 121º C for 15 minutes and cool in a slanted position, as to obtain butts of 1.5 – 2 cm depth.

Mueller Hinton agar (OXOID CM0337 500G)

Composition (g/l): Beef Infusion 300.00; Casein hydrolysate 17.50; Starch 1.50; Bacteriological Agar 17.00.Final PH: 7.4 ± 0.2 at 25ºC, Distilled water 1 liter

Preparation: Suspend 38 grams of medium in one liter of distilled water. Bring to boil to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes.

- **Reagents and solutions**

Kovac's reagent (Indole test reagent)

Composition:4- dimethylaminobenzaldehyde 5gm; ethanol alcohol 75ml; hydrochloric acid 25ml.
Preparation: Mix the components with constant stirring. The final reagent should be stored in brown bottle.

Methyl Red alcoholic indicator solution (UNI-CHEM M76653-8G 100ml)

Dissolve 0.1g of methyl red powder in 300ml 95% ethanol and 200ml distilled water

1% solution of 2, 3, 5-Triphenyl Tetrazolium Chloride (TTC, FD057)

Dissolve 500mg of triphenyl Tetrazolium chloride in dehydrated alcohol to make100ml

McFarland turbidity standard preparation

A 0.5 McFarland turbidity standard is used to make the inoculum for disk susceptibility testing.

Reagents: Anhydrous barium chloride $BaCl_2$ 1% w/v

Cold pure sulphuric acid H_2SO_4 1% v/v

Preparation: Add 0.5 ml of 1% BaCl2 to 99.5 ml of 1% H2SO4. Stir to mix the suspension evenly. Distribute 5 ml into clear glass tubes with the same diameter, or the same tubes in which the

inoculum will be prepared. Store tubes at room temperature in the dark. The turbidity is equivalent to a density of 1.5 x 10^8 cells.

- **Test procedures**

Gram's staining

Principle: Bacteria also differ from one another chemically and physically and may react differently to a given staining procedure. This is the principle of differential staining. Differential staining can distinguish between types of bacteria based on the thickness of the cell wall. It divides bacteria into two groups: Gram negative and gram positive.

Procedure: Fix the smear, Stain with the primary stain – crystal violet - for 30 seconds, Wash crystal violet off with water, Add iodine for 10 seconds (mordant) (the mordant can also be added without washing the crystal violet), Wash iodine off with water, Decolorize with ethyl alcohol for 10 – 20 seconds (decolorizing agent), Wash the alcohol off with water, Counter stain with safranin for 30 seconds, Wash the safranin off with water, Blot the smear dry, Observe under oil immersion.

Catalase test

*Principle***:** Catalase is an enzyme, which is produced by microorganisms that live in oxygenated environments to neutralize toxic forms of oxygen metabolites; H_2O_2. A positive result is the rapid evolution of O2 as evidenced by bubbling and a negative result is no bubbles or only a few scattered bubbles.

Procedure: Pick a colony from an 24 hours culture and place it on a clean glass slide. Then put one drop of 3% H2O2 over the organism on the slide. Observe for immediate bubbling (gas liberation) and record the result.

Oxidase test

Principle: The oxidase test is used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. When present, the cytochrome c oxidase oxidizes the reagent (tetra methyl-p-phenylene-diamine) to (indophenols) purple color end product. When the enzyme is not present, the reagent remains reduced and is colorless.

Procedure: First we dilute the substrate tetra methyl-p-phenylene-diamine di-hydrochloride by distilled water. Moisten the paper with a diluted solution. Pick the colony to be tested with wooden and smear in the filter paper. Observe inoculated area of paper for a color change to deep blue or purple within 10 seconds.

Oxidation-Fermentation (O.F.) test

Principle: Oxidation-Fermentation test is used to determine the oxidative or fermentative metabolism of a carbohydrate or its non-utilization. Fermentation is a anaerobic process and bacterial fermenters of carbohydrates are usually facultative anaerobes. Oxidation is a aerobic process and bacterial oxidizers are usually strict aerobes.

Procedures: The method described, sometimes referred to as the Hugh and Leifson test employs a semi-solid medium in tubes containing the carbohydrate under test (usually glucose) and a pH indicator. Two tubes are inoculated and one is immediately sealed with paraffin oil to produce anaerobic conditions.

Indole test

Principle: Organisms those possess the enzyme tryptophanase can break down the amino acid tryptophan to indole. When indole reacts with para-dimethylaminobenzaldehye (Kovac's reagent) a pink -colored complex is produced. Tryptophan is plentiful in most media, but growth on blood agar or chocolate agar produces the best effects.

Procedure: Take loopful of inoculum by touching the 3-5 representative colonies with inoculating loop from pure colonies and inoculate Tryptone soya broth tube. Incubate the tube at 37°C for 24 hours and cap left loosen to aerate the tube. After incubation, add 5-10 drops (0.5ml) of Kovac's reagent to the culture broth and agitate gently. Then observe the tube for color change within 5 minutes.

Citrate utilization test

Principle: Citrate contains carbon. If an organism can use citrate as its only source of carbon the citrate in the media will be metabolized. Bromthymol blue is incorporated into the media as an indicator. Under alkaline conditions this indicator turns from green to blue. The utilization of citrate

in the media releases alkaline bicarbonate ions that cause the media pH to increase above 7.4 causes the media blue.

*Procedure***:** Take loopful of inoculum by touching the center of 3-5 representative colonies with inoculating loop and streak it onto the surface of a Citrate slant. Incubate the tube aerobically at 35°C with cap left loosen for 22 hours. After 22 hrs incubation observe the tube for growth and color change.

Triple sugar iron test

Principle: Bacteria that ferment any of the three sugars in the medium will produce byproducts which will change the color of the red pH-sensitive dye (phenol red). A bacterium that is a non-lactose fermenter and ferments glucose, initially causes a yellow slant/yellow bottom (acid/acid reaction) after 8 hours, but then converts to a red slant/yellow bottom after 24 hours (alkali/acid reaction). Where as if it ferments both lactose and glucose, it results in a yellow/yellow tube and remains that way due to the large amount of acid produced in the reaction.

Procedure: By sterile inoculating loop touching the center of colony from isolated pure colony take loop full of inoculum. Streak the inoculum back and forth on Triple Sugar Iron agar in tube along the surface of the slant. Incubate the tube with the cap loosened at 35 ºC for 22 hours.

Methyl red test

Principle: Some organisms produce acid from the metabolism of glucose in a sufficient quantity to produce a pH of 4.4 in the media. These acids are not further metabolized and are said to be stable acids. At a pH of 4.4 or less the pH indicator methyl red is a bright cherry red. While also some organisms initially produce acid from glucose metabolism but further metabolize the acid produced to neutral end products, such as acetoin, and 2, 3-butanediol. Initially the pH may drop to 4.4 but the neutral end products raise the pH so the methyl red test will be negative.

Procedure: Take loopful of inoculum by touching the center of 3-5 representative colonies with inoculating loop from the pure isolated colonies and inoculate MR-VP broth with inoculum, incubated 37 ºC for 48 hours. Aseptically from incubated broth after 48 hrs transfer aliquot to two clean test tubes each with two ml of broth culture with sterile pipette. Add 5 drops of methyl red to one tube. The result read immediately. The tube didn’t mix.

Antimicrobial susceptibility test

Principle: The pathogenic organism is grown on Mueller-Hinton agar in the presence of various antimicrobial impregnated filter paper disks. The presence or absence of growth around the disks is an indirect measure of the ability of that compound to inhibit that organism.

Procedure: Using a sterile inoculating loop or needle, touch four or five isolated colonies of the organism to be tested. Suspend the organism in 5 ml of sterile saline. Vortex the saline tube to create a smooth suspension. Adjust the turbidity of this suspension to a 0.5 McFarland standard by adding more organism if the suspension is too light or diluting with sterile saline if the suspension is too heavy. Use this suspension within 15 minutes of preparation.

Annex 3: Phage Isolation Protocol, Phage enrichment from water

Sewage treatment plants are ideal sources from which to isolate bacteriophages since it contains high numbers of diverse bacteria as well as different ecosystems including raw input sewage, as well as aerobic and anaerobic digestion tanks. Alternative sources include pond, lake or ocean waters.

- Collect sewage from various points in the sewage treatment plan using caution not to come into direct contact with the liquids since they will most definitely contain human pathogens.

Notes:

- ✓ It is recommended that one obtain water samples at least several days after a heavy rain since the latter will dilute the sewage.
- ✓ While it may initially seem illogical the container should be sterile, since you don't want to contaminate the newly acquired phage source with desiccation-resistant phages from your laboratory.
- Centrifuge the sewage suspensions at 10,000 x g for 10 min. to remove particulates.

Notes:

- ✓ This material can be used directly in enrichments involving rapidly dividing bacterial cultures (enterics, Pseudomonas, Bacillus); alternatively, it can be filter sterilized by passage through 0.2 µm low protein-binding membrane filters: Millipore Express (PES) or Dura pore (PVDF), Pall-Gel man Sup or membrane (PES), What man in organic An o pore membranes. Membrane chemistries

with reported low protein binding are poly tetra fluoro ethylene, polypropylene, polycarbonate, polysulfide, and cellulose acetate.

- ✓ If you do not use filtration bacterial spores which may be present in the clarified sewage will germinate and after 48h incubation the smell will elicit very negative remarks from your coworkers.
- ➢ For aerobic hosts, pipette 10 ml of sterile double strength broth containing2 mM CaCl2 into a 125 ml Erlenmeyer flask, and add 10 ml of clarified (and filtered) sewage.

Notes:

- ✓ For the bacteria listed above Difco Luria broth or Tryptic Soy Broth work well.
- ✓ For other bacteria not mentioned above we would suggest initially using the medium recommended for the propagating the host bacterium (see for example the American Type Culture Collection (ATCC at http://www.atcc.org/Home.cfm) or the DeutscheSammlung von Microorganism und Zellkulturen (DSMZ athttp://www.dsmz.de/).
- ✓ Many phages require 1-10 mM divalent ions such as Ca for attachment or intracellular growth (11, 13, and 14). It is a good idea when working with phage mixtures or uncharacterized phages to include 1-2 mM Ca^{2+} in the all media.

- ➢ Inoculate the flask with 0.1 ml of an overnight broth culture of the desired host bacterium and incubate at the appropriate growth temperature with gentle mixing (50 rpm).

- ➢ After 24-48h incubation, centrifuge the contents of the flask at 10,000 x g for 10 min. Decant the supernatant into a small screw-capped bottle or a series of capped test-tubes.

Notes:

- ✓ As a general protocol add approximately 0.5 ml of chloroform to the clarified crude lysate, shake and store at 43^{o}C.
- ✓ Please note that lipid-containing phages (members of the Cystoviridae [φ6], Corticoviridae [PM2], Plasmaviridae [L2], and Tectiviridae [PRD1]) will be inactivated by this treatment. In addition, certain members of the Inoviridae, while lacking lipids, are also solvent sensitive.
- ✓ In the latter cases the primary lysate could be filter sterilized.
- ➢ At this stage of the enrichment it is recommended that you spot-test your lysate on the host cells to see whether it contains any phage active on your host bacterium.

- Spread lapful's of host bacteria down the surface of agar plates prepared with the same medium supplemented with Ca^{2+}
- Allow the fluid to dry and then place 5 µL of the clarified enrichments on the streaks, incubate overnight and observe for zones of cell killing.

Underlay procedure for phage purification

- Resuspended phage particles from a well-isolated plaque in 1 ml of sterile broth.
- Streak a nutrient agar plate as though one were attempting to obtain single colony isolates from a bacterial culture. Allow the plate to dry at room temperature.
- Prepare an overlay containing host cells it over the surface of the plate starting from the region containing the most dilute phage particles and allowing it to diffuse towards the primary inoculation area.
- After the overlay sets (15 min) incubate the plate. Individual well-separated plaques will be visible after overnight incubation.

Annex 4: MDR E.coli isolation and characterization

Fig: Pink colony on MacConkey agar

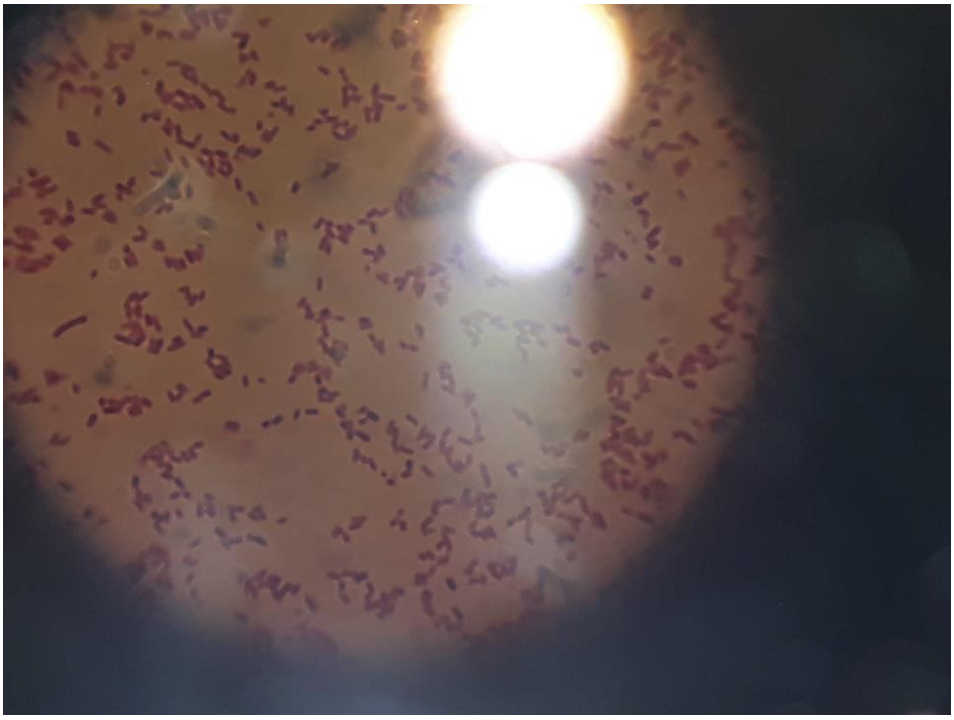


Fig: Gram stain pink road shaped bacteria

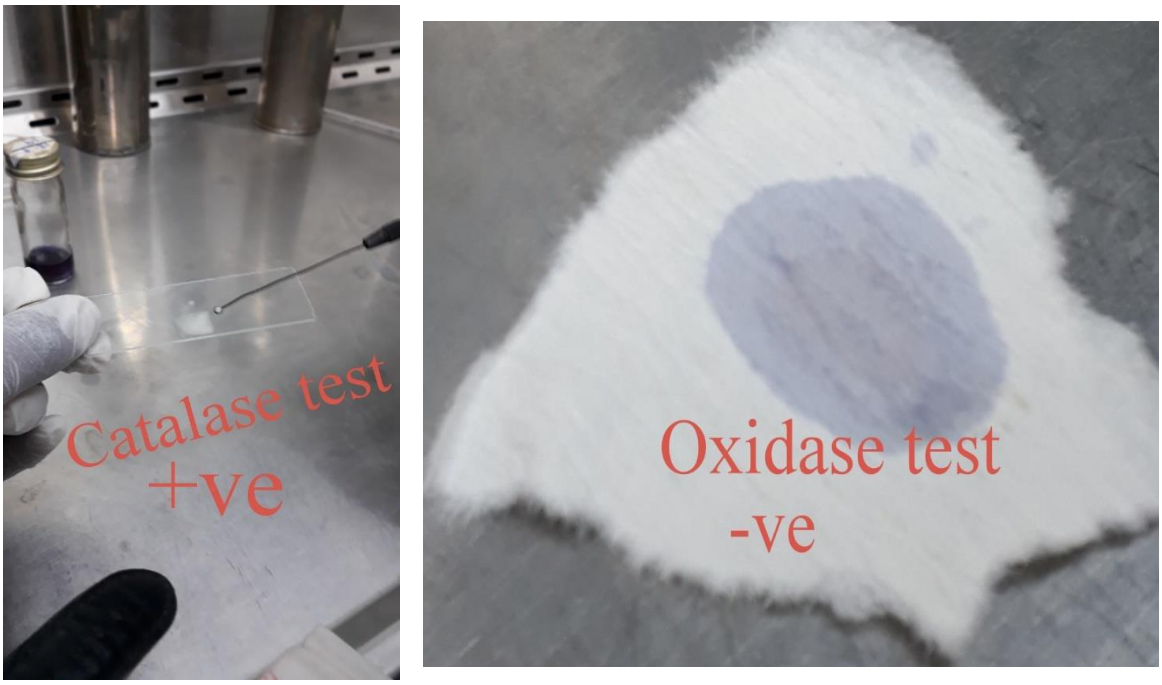


Fig: catalase and oxidase test

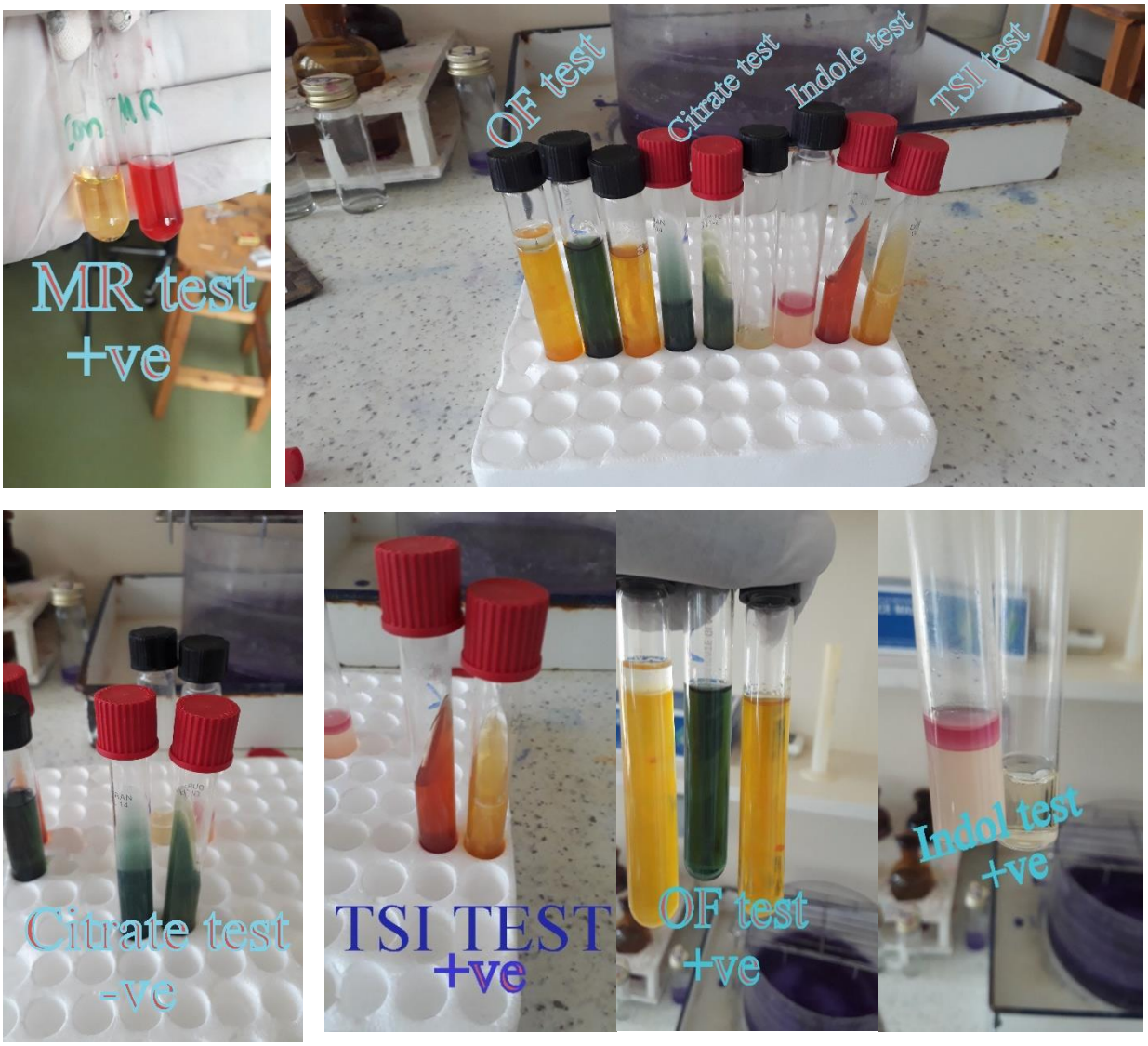


Fig: Secondary Biochemical test result for *E.coli*

Annex 5: Antibiotic susceptibility test for MDR *E.coli*

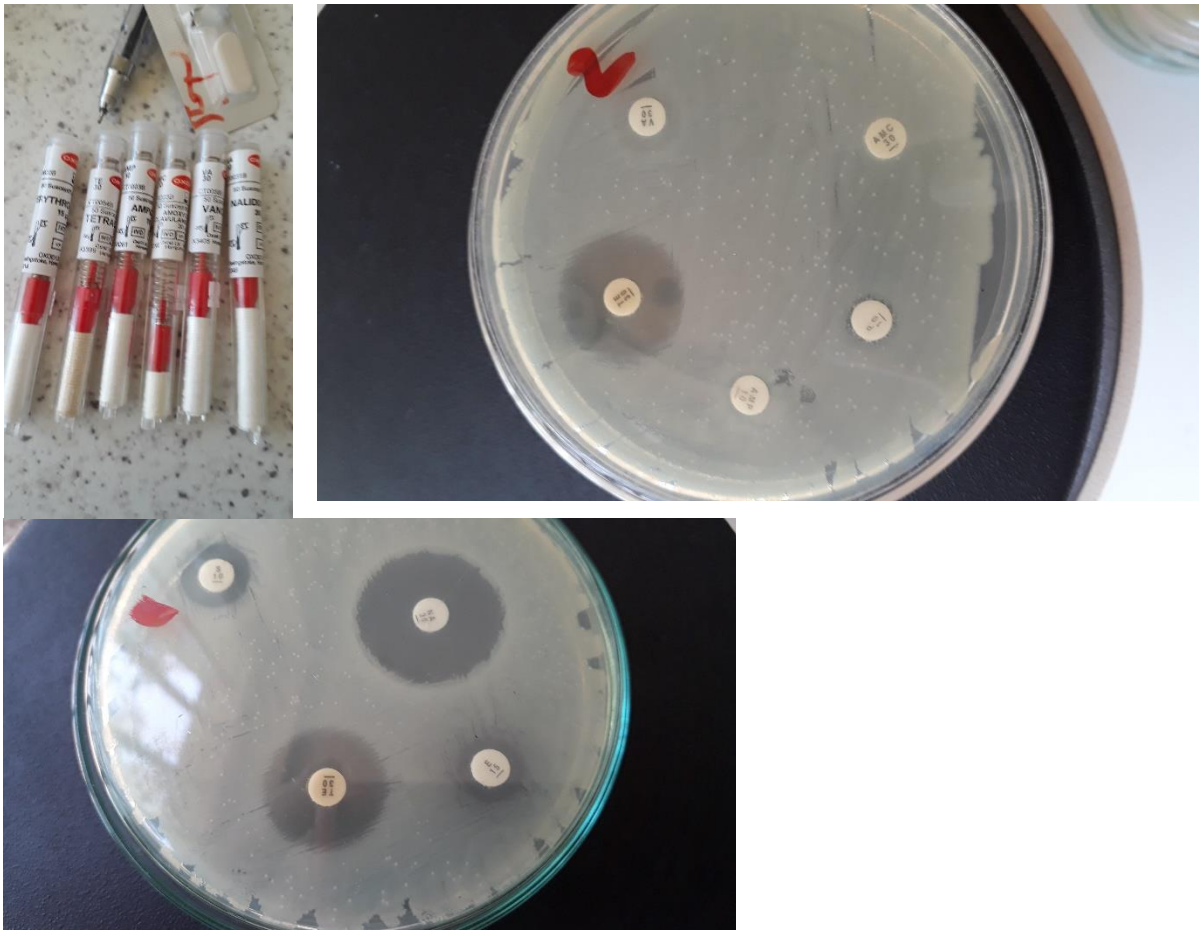


Fig: Antibiotic susceptibility test for MDR *E.coli*

Annex 6: Phage isolation procedure and isoleted trials

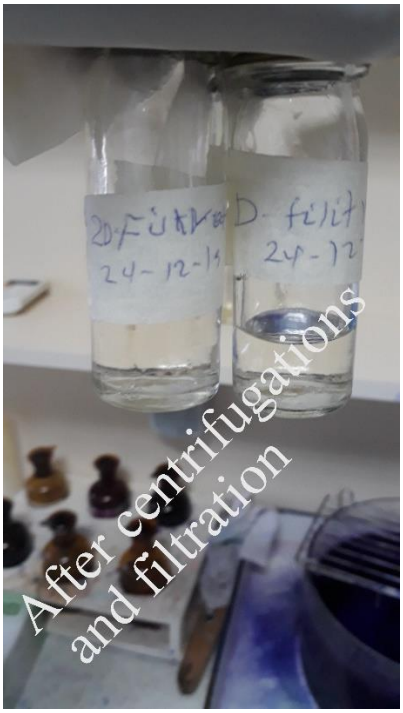
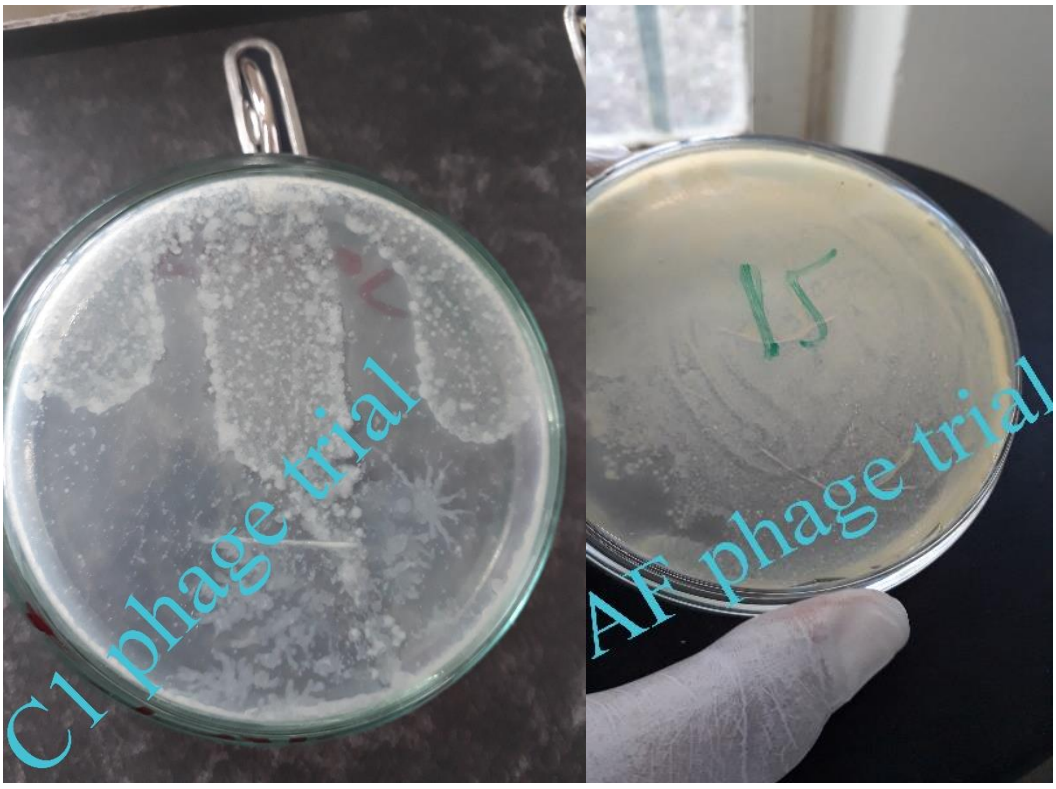
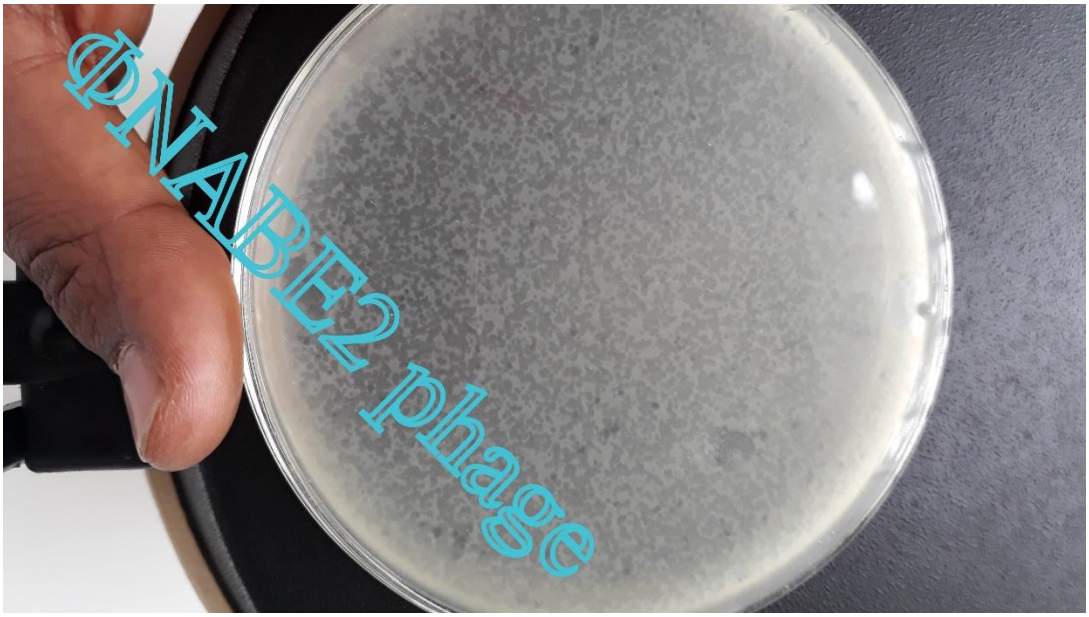


Fig: some phage Isolation steps


C1 phage trial
15
AF phage trial


ΦNABE2 phage

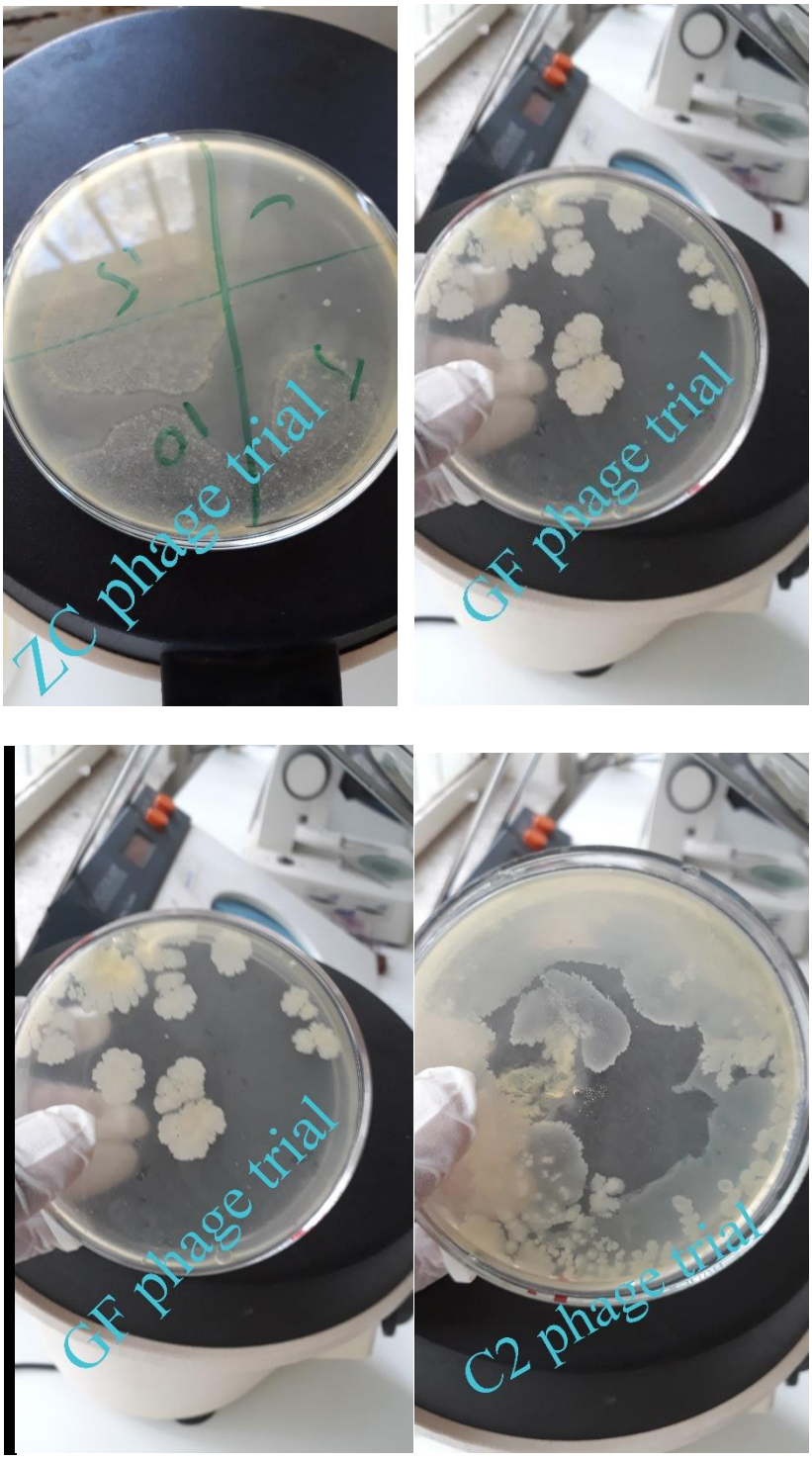


Fig: six phage trial and one successful phage isolate against MDR *E. coli*

Annex 7: *In vitro* phage therapy

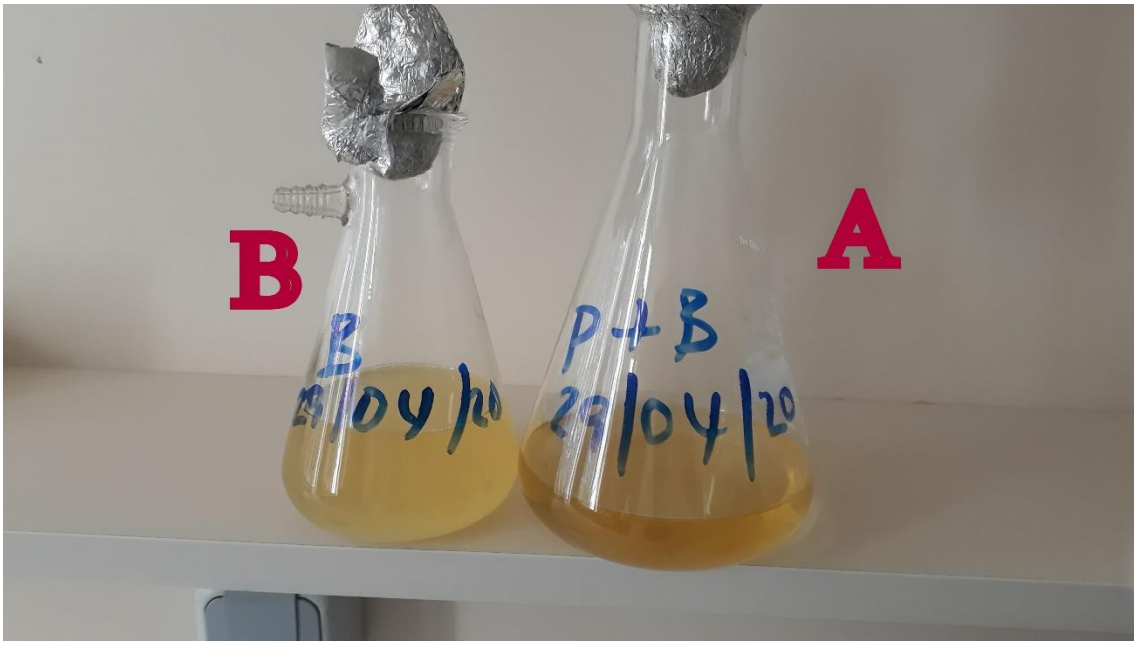


Fig: flask A contain both bacteria and phage while flask B containing only bacteria

Annex 8: *In vivo* phage therapy on mice

Fig: before mice challenge test

Fig: **during mice challenge test**

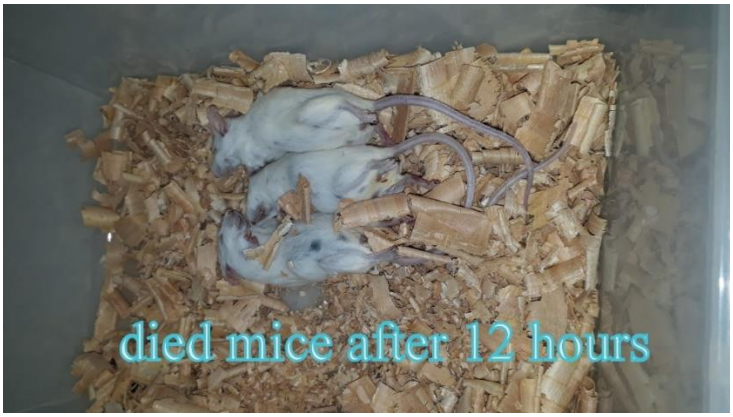


Fig**:** After mice challenge test

Annex: 9 Ethical clearance certificate

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu/Debre Zeit

Animal Research Ethics Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/08/01/12/2020

Name of Applicant: Betemaryam Getaneh (DVM, MVSc fellow)

Address: College of Veterinary Medicine and Agriculture (Addis Ababa University)

Title of the project: *Isolation and characterization of bacteriophage from sewage and its therapeutic potential for multidrug resistant Escherchia coli.*

Date of application:	**06/11/2019**
Nature of the project:	**invasive (mice infection)**
Target animal species:	**cattle and mice**
Number of animals involved:	**20 cattle and 6 mice**
Study area:	**Bishoftu, Ethiopia**

Minutes No. and date of review: **VM/ERC/01/12/020, 03/01/2020**

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is ethically sound to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected, minor comments are corrected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee when this is deemed necessary

Dr Getachew Terefe
Chairman

Signature

The Federal Democratic Republic of Ethiopia
Addis Ababa University
College of Veterinary Medicine and Agriculture

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Please quote Our Ref. No. When replying

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