THERAPEUTIC POTENTIAL OF BACTERIOPHAGE ISOLATED FROM SEWAGE TO RESCUE *ESCHERICHIA COLI* INFECTION IN SWISS MICE (*Mus musculus*)

By

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ABSTRACT

E. coli is a gram negative enteric bacillus which is the causes of enteritis, urinary tract infection, septicemia and other clinical infections including neonatal meningitis. Moreover, it is also associated with diarrhoea in pet and farm animals. The prevalence of multidrug-resistant *E. coli* strains is increasing worldwide principally due to the spread of mobile genetic elements, such as plasmids. Therefore, the spread of antibiotic resistance in *E. coli* is an increasing public health concern in the world. This work has come to study the therapeutic effect of bacteriophages isolated from our environments for promising alternative therapy against *E. coli* bacterium. Bacteriophages are bacterial viruses ubiquitous in our world in various places including the oceans, soil, deep sea vents, the water we drink, and wastewater and food we eat. Phages have been proposed as natural antimicrobial agents to fight bacterial infections in humans, in animals or in crops of agricultural importance. This study is currently developed to isolate potential phages from sewage samples and use it as a candidate for therapeutic purpose against lethal dose (10^9) of *Escherichia coli* infection in Swiss mice. Lytic phage was isolated from wastewater collected from Jimma town following standard enrichment method against the bacterium. Intraperitoneal injections (cells) of *E. coli* caused death in mice within 5 days. In contrast, subsequent intraperitoneal administration of purified bacteriophages (ϕJS3) suppressed *E. coli* induced lethality. Inoculation mice with high-dose of ϕJS3 alone produced no adverse effects attributable to the phage. These results uphold the efficacy of phage therapy against *E. coli* infections in mice and suggest that ϕJS3 phage may be a potential therapeutic alternative to antibiotics in human.

Keywords: Phage therapy, **Pathogenic, Survivability of mice**

INTRODUCTION

Common illnesses such as pneumonia, strep throat, urinary tract infections, and gastrointestinal infections are the result of bacterial infections in our body. To treat the infection, medical doctors frequently prescribe antibiotics. Some antibiotics inhibit bacterial growth by interfering with the production of components needed for bacterial multiplication to form new bacterial cells. For example, penicillin inhibits the biosynthesis of peptidoglycan, an essential component of bacterial cell walls and also inhibits their growth. Vancomycin also impedes proper synthesis of the bacterial cell wall .Other antibiotics, such as tetracycline, bind to ribosomes and impair protein manufacture (Biswas *et al*., 2002).

Since the 1940's, antibiotics have become increasingly available. However, as a result of over-prescription of broad-spectrum antibiotics, wide-spread use in animal feeds and unregulated accessibility in many developing countries, bacteria have evolved to become increasingly resistant to their actions. It has been recently found that there are bacteria that are resistant to all known antibiotics .These include *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Mycobacterium tuberculosis* (Wills *et al*., 2005). There is also concerns that increasing antimicrobial resistance of pathogenic *Escherichia coli* in mammals (Fairbrother *et al.*, 2005) may compromise the therapeutic use of antimicrobials threaten human health through transfer of drug resistance genes to zoonotic pathogens. There is therefore a need for safe and practical alternatives to antimicrobials for prophylaxis and therapy.

The increasing rate of emerging antibiotic-resistant bacteria has become so alarming that alternative treatments to bacterial infection are being examined. One alternative method to antibiotic treatment is phage therapy, a procedure that employs bacterial viruses, often called "bacteriophage" or "phage," to combat bacterial infections. Phage therapy was first employed in the early 1900's by many countries including the United States (Twort, 1915).

The first clinical studies of phage therapy began in 1919 when d'Herelle used a phage preparation to treat a twelve-year old boy suffering from dysentery (d'Herelle, 1922). The success was rapidly evident when the boy began to improve within 24 hours after one dose of the phage, and completely recovered within a few days. Subsequently, three more patients

suffering from dysentery were treated and began to improve within 24 hours after administration of one dose of phages (Sulakvelidze *et al*., 2001).

Interest in phage therapy began to resurface in the early 1980s. In 1982 and 1983, H. W. Smith and M. B. Huggins investigated the use of phage to control systemic *E. coli* infections in mice (Smith and Huggins, 1983). They injected mice with a pathogenic strain *of E. coli* intramuscularly and found that they all died. However, if they injected an *E. coli* phage which could infect this pathogen (i.e., a phage specific for the Kl capsule *of E. coli)* simultaneously with the *E. coli* pathogen, the mice lived. Furthermore, they were able to demonstrate that phage therapy was more effective than treatment with tetracycline, streptomycin, ampicillin, or trimethoprim/sulfafurazole (Smith and Huggins 1982). In 1994, Barrow and Soothill (Soothill *et al*., 1997) carried out skin-graft investigations using guinea pigs. Since skin grafts are rejected due to *P. aeruginosa* colonization, they treated the guinea pigs with *Pseudomonas aeruginosa {P. a.)* phage, they showed that skin-graft rejection could be prevented by prior treatment with *P. a.* phage.

The advantage of this treatment over the use of antibiotics lies in its infective process: one lytic phage that infects a bacterium makes hundreds of new phage, multiplying as the infection proceeds until all of the bacteria targeted have been destroyed. Theoretically, this would require only a few phages to rid a patient of its bacterial pathogen. Each type of phage will usually infect only a specific type of bacteria, as determined, in part, by protein receptors on the surface of the bacteria.

Bacteriophage is highly abundant and chiefly concentrated in, the niches of almost all natural environments on this planet (Bruttin and Brussow, 2005). In nature they have been observed in open and coastal waters, marine sediments and particularly in terrestrial ecosystems such as soil. About up to 2.5 x 10^8 plaques forming units (PFU)/ml concentration of phages was determined in natural unpolluted water (Bergh *et al.*, 1989). They are also commonly found in association with diverse higher organisms extending from insects to humans. This association is not only superficial; surface mediated but extends within their bodies particularly the gastrointestinal tracts of humans, animals, and insects. Bacteriophage and the related viruses infect bacteria and

thus obligate intracellular parasites that must multiply inside the prokaryotes by making use of some or all biosynthetic machinery of the host (Fairbrother *et al*., 2005).

Bacteriophage can be targeted to specific bacterial strains (Wagner and Waldor, 2002) and thus can targeted against pathogens or non-beneficial microbes .Unlike the spread of antibiotic resistance from one strain to another, the high host-specificity of bacteriophage would alleviate this pattern of transmission. Bacteriophages are self-replicating as well as self-limiting with decrease in number of the specific bacteria concomitantly leading to their decrease and eventual elimination of both from the body without any harm. Since bacteriophages are capable of exponential growth, they can concomitantly accumulate and replicate at the site of infection. As the resistance of pathogenic organisms with antibiotics is increasing world widely and phage therapy may be an alternative to alleviate this risk. Unlike bacterial resistance to antibiotics, phages can mutate in step with evolving bacteria and if the bacteria become resistant to one phage, there is a natural abundance of phage species which can be targeted by other phages having a similar target range (Sulakvelidze *et al.,* 2001).

Vernment agencies in the West have for several years been going to Georgia and the former Soviet Union for help of phages to counteract bioweapons and toxins, such as anthrax and botulism (Schuch *et al*., 2002). Other uses of phages include spray application in horticulture for protecting plants and vegetables from the spread of bacterial diseases and Applications of phages in hospitals as preventative treatment for catheters and medical devices prior to use in clinical settings. The technology for phages to be applied to dry surfaces, e.g., uniforms, curtains, or even sutures for surgery now exists. Clinical trial reports show success in veterinary treatment of pet dogs with otitis (Wright *et al.,* 2009).

Another major advantage of the bacteriophages as a therapeutic option for patients with antibiotic allergies, since there is no allergies reported against phage therapies (Chan and Abedon, 2012). Since phages are found throughout nature, it is relatively easy to find a new phage if a bacterium becomes resistant to it and involves a rapid and cheap process as compared to the approval and the costly manufacture of new antibiotics. Examples of many successful

phage therapies in diverse experimental animal model systems were reviewed (Ackermann, 2005).

In general, bacteriophages have several characteristics that make them potentially attractive therapeutic agents (Summers, 2001). They are (i) highly specific and very effective in lysing targeted pathogenic bacteria, (ii) safe, as underscored by their extensive clinical use in Eastern Europe and the former Soviet Union and the commercial sale of phages in the 1940s in the United States, and (iii) rapidly modifiable to combat the emergence of newly arising bacterial threats. Many of these studies do not meet the current rigorous standards for clinical trials and there still remain many important questions that must be addressed before lytic phages can be widely endorsed for therapeutic use (Gill and Hyman, 2010).

To this effect, this study was designed to address isolation of bacteriophages from environmental wastewater sewages and to evaluate their potential for treatment of diseases caused by pathogenic *E.coli* on mice.

2. OBJECTIVES

2.1. General Objectives

The general objectives of this current study was to isolate bacteriophages from sewage sample and to evaluate their therapeutic efficacy in treating pathogenic bacterial isolate infection in animal model

2.2. Specific Objectives

The specific of objectives of these current studies were:

- To isolate lytic bacteriophage from sewage samples collected from Jimma town area
- To characterize the located bacteriophage against selected biophysical properties.
- To develop *E. coli* infection model in mice to define lethal dose in mice experiment
- To evaluate the efficacy of bacteriophage against infection in a mice model.

3. REVIEW OF LITERATURE

3.1. Escherichia coli

Escherichia coli commonly abbreviated *E. coli* is a gram-negative, facultative anaerobe, rod shaped bacterium of the genus *Escherichia* that is commonly found in the lower intestines of warm-blooded organisms. Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in their hosts. The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K, and preventing colonization of the intestine with pathogenic bacteria (Reid *et al.,* 2001).

The World Health Organization estimates that 5 million children die each year as a consequence of acute diarrhea (WHO, 2014). *Escherichia coli* is the cause of a third of cases of childhood diarrhea in developing countries and is also the most prominent cause of diarrhea in travelers in developing countries. *E. coli* is also prominently associated with diarrhea in pet and farm animals. Due to its malleable genetic character, *E. coli* has one of the widest spectra of disease of any bacterial species. The recent emergence of *E. coli* $O_{157:}H_7$ as a major food pathogen is a lively reminder of its dynamic character. According to O'Flynn *et al,* (2004), infections with *E. coli* O157:H7 may be caused by as few as 10 bacterial cells. If the infection is severe enough, it causes damage to the kidneys and leads to hemolytic-uremic syndrome (HUS), which is most common in young children, and may result in death. Furthermore, *Shigella* species, the cause of dysentery, taxonomically constitute a subspecies of *E. coli* (Donnenberg, 2002). *Escherichia coli* are also responsible for extra intestinal infections, including urinary tract infections, bacteremia, and meningitis. Neonatal meningitis is one of the most severe infections due to the bacteria killing up to one-quarter of those infected (Gaschignard *et al*., 2011).

The future prognosis of this disease may be worsened by the increasing incidence of multidrug-resistant strains, especially those producing extended-spectrum beta-lactamases (ESBL) enzymes. *E. coli* is among the most important human pathogens accounting for the majority of bacterial strains isolated from clinical patient samples in Jimma University Specialized Hospital

(Mulualem *et al.,* 2012).The results showed that *E. coli* was isolated from 67 (18.66%) clinical specimens, of which 24 (36%) isolates were ESBL producers. The resistance pattern to the tested antibiotics: Penicillin , Amoxacillin and Ampicillin , Tetracycline , Amoxacillin-clavulanate , Co-trimoxazole , chloramphenicol , Ciprofloxacin , Norfloxacine , Cefotaxime , Ceftazidime , Gentamicin was observed. All the isolates tested showed resistance to two or more drugs, and were considered to be multi-drug resistant. Over all a higher rate (46%) of ESBL production and multi-drug resistance was seen among isolates from inpatients as in Jimma University Specialized Hospital.

The clinical manifestations of infections with *E coli* and the other enteric bacteria depend on the site of the infection and cannot be differentiated by symptoms or signs from processes caused by other bacteria. *E coli* is the most common cause of urinary tract infection and accounts for approximately 90% of first urinary tract infections in young women (Kropinski,2006) .The symptoms and signs include frequent urination, dysuria, hematuria, and pyuria. Flank pain is associated with upper urinary tract infection. None of these symptoms or signs is specific for *E coli* infection. Urinary tract infection can result in bacteremia with clinical signs of sepsis. *E coli* that cause diarrhea are also extremely common worldwide.

E. coli O157:H7 can cause a range of symptoms - from asymptomatic infection or mild diarrhea, to bloody diarrhoea (hemorrhagic colitis) and hemolytic uremic syndrome (HUS) .Shiga-toxin-producing *Escherichia coli* and hemolytic uremic syndrome. Goshen (2005) states possible clinical scenarios are: no symptoms or mild diarrhoea, painful diarrhoea or hemorrhagic colitis: starts as diarrhoea and abdominal cramps. In most cases, the diarrhoea becomes bloody after 1-3 days, patients usually have no fever by the time they see a doctor. Compared to other forms of bacterial gastroenteritis, the abdominal pain is generally more severe, abdominal tenderness on examination is common and defecation tends to be painful. hemolytic uremic syndrome (a triad of acute renal failure , hemolytic anemia usually diagnosed 5-13 days after the onset of diarrhoea and thrombocytopenia mainly occurs in young children, which is the first feature and similar to HUS, but with less renal involvement and more prominent neurological features (Mead and Griffin ,1998)

E. coli is classified by the characteristics of its virulence properties and each group causes disease by a different mechanism. Most of the infections are caused by *E coli* of a small number of O antigen types. K antigen appears to be important in the pathogenesis of upper urinary tract infection (Donnenberg, 2002).

The most common known pathogenic *E.coli* in the intestine are listed below (Bopp *et al.,* 2003). i,enteropathogenic *E coli* (EPEC) is an important cause of diarrhea in infants, especially in developing countries. EPEC previously was associated with outbreaks of diarrhea in nurseries in developed countries. The bacteria adhere to the mucosal cells of the small bowel. ii,enterotoxigenic *E coli* (ETEC) is a common cause of "traveler's diarrhea" and a very important cause of diarrhea in infants in developing countries. ETEC colonization factors specific for humans promote adherence of ETEC to epithelial cells of the small bowel and produce a more severe diarrhea. iii, Enterohemorrhagic *E coli* (EHEC) produces verotoxin, named for its cytotoxic effect on Vero cells. Of the *E coli* serotypes that produce verotoxin, O157:H7 is the most common and is the one that can be identified in clinical specimens.iv, Enteroinvasive *E coli* (EIEC) produces a disease very similar to shigellosis. The disease occurs most commonly in children in developing countries and in travelers to these countries. v, Enteroaggregative *E coli* (EAEC) causes acute and chronic diarrhea (> 14 days in duration) in persons in developing countries. This organism is also the cause of food-borne illnesses in industrialized countries. It is characterized by its characteristic pattern of adherence to human cells.

Water and sanitation programmes could improve the quality of drinking water but are prohibitively expensive for many developing countries. In addition, effective treatment and prevention measures are lacking for *E. coli* diarrhea. Antibiotic use is of doubtful value since resistance is widespread in *E. coli*, and vaccines are still in the early development phase (Savarino *et al*., 2002). *E. coli* is frequently used as a model organism in microbiology studies. Cultivated strains (e.g. *E. coli* K12) is well-adapted to the laboratory environment, and, unlike wild type strains, has lost its ability to thrive in the intestine. These features protect wild type strains from antibiotic and other chemical attacks but require a large expenditure of energy and

material resources (Fux *et al.,* 2005). Thus scientists have been finding the alternative method of controlling and treating the E.coli infection disease by using bacteriophages

3.2. Bacteriophages

Bacteriophages are ubiquitous on earth, with an estimated 10^{32} particles, and are approximately 10 times more abundant than bacteria and archaea (Brussow, 2005). As they are obligate bacterial parasites and are found everywhere, including various terrestrial ecosystems and even in extreme conditions such as the deep sea, solar salterns acidic hot springs (> 80°C) alkaline lakes (pH = 10) (Jensen, 2006), and Antarctic lakes, where bacteria and archaea are present .Their densities have been estimated to be up to $2.5x10^{8}$ particles per milliliter and $1.5x10^{7}$ particles per gram in aquatic and soil environments respectively. In theory, all bacteria are susceptible to viral infection, often by several types of phages.

3.2.1. Types of Phages and Phage Biology

More than 6000 different bacteriophages have been discovered and described morphologically including 6196 bacterial and 88 archeal viruses (Jenson, 2006) .The vast majority of these viruses are tailed while a small proportion are polyhedral, filamentous or pleomorphic. They may be classified according to their morphology, their genetic content (DNA vs. RNA), their specific host, the place where they live (marine virus vs. other habitats), and their life cycle .Evolving classification formats have been proposed over time and abbreviations for these viruses were proposed by Fauquet and Pringle (Ackermann, 2007).

As obligatory intracellular parasite of a bacterial cell, phages display different life cycles within the bacterial host: lytic, lysogenic, pseudo-lysogenic, and chronic infection. The most common life cycles are lytic and lysogenic. For phage therapy, the main interest has focused upon lytic phages, mainly represented in 3 families of the *Caudovirales* order: the *Myoviridae*, the *Siphoviridae* and the *Podoviridae*. There are also some reports on cubic phages and filamentous phages applications. General description of those phages may be summarized as follows: the genetic material is contained in a protein shell or capsid which has a form of an icosahedrons;

this head is connected through a collar or neckline to the tail which may be contractile or not and whose distal extremity is in contact with tail fibers with tips that recognize (Kuhl and Mazure, 2011).

3.2.2. Strictures of bacteriophages

Bacteriophage, or more commonly called phages, was discovered nearly 100 years ago by Frederick Twort and Felix d'Herelle (Boyd and Brussow 2002). These small viral entities that specifically infect bacteria exist as nucleic acids (single or double stranded DNA/RNA, circular or linear) packaged within a protein capsid, which protects the nucleic acids from the environment (Fig .1). Understanding the family to which a phage belongs can provide valuable information regarding shape, genetic material, and similar phages. A commonly researched phage is T4 of the *Myoviridae* family; this phage infects *E. coli* bacterium. T4 phage contains more than 200 genes and its DNA genome is approximately 10μm in length (Snyder and Champness, 2003). The most sophisticated and complex structured Myoviridae member is shown in Figure 1 (Lavigne *et al*., 2009).

The anatomical features of a typical phage comprise of a head filled with genetic material, a syringe shaped tail and several fibres for the attachment to specific receptors on the surface of host bacteria. Phages bore into their relevance host bacteria and inject their genetic material in the form of either single stranded or double stranded nucleic acid genome which is enclosed in a protein or lipoprotein coat. For their replication in the host cells, phages contain yet unidentified lipolytic enzymes to facilitate the opening of the bacterial cell wall barrier and subsequently inject their DNA or RNA into the cytoplasm. The simplest phages code on average for 3-5 gene products, while the more complex phages can code for over 100 gene products (Kutter and Sulakvelidze, 2005).

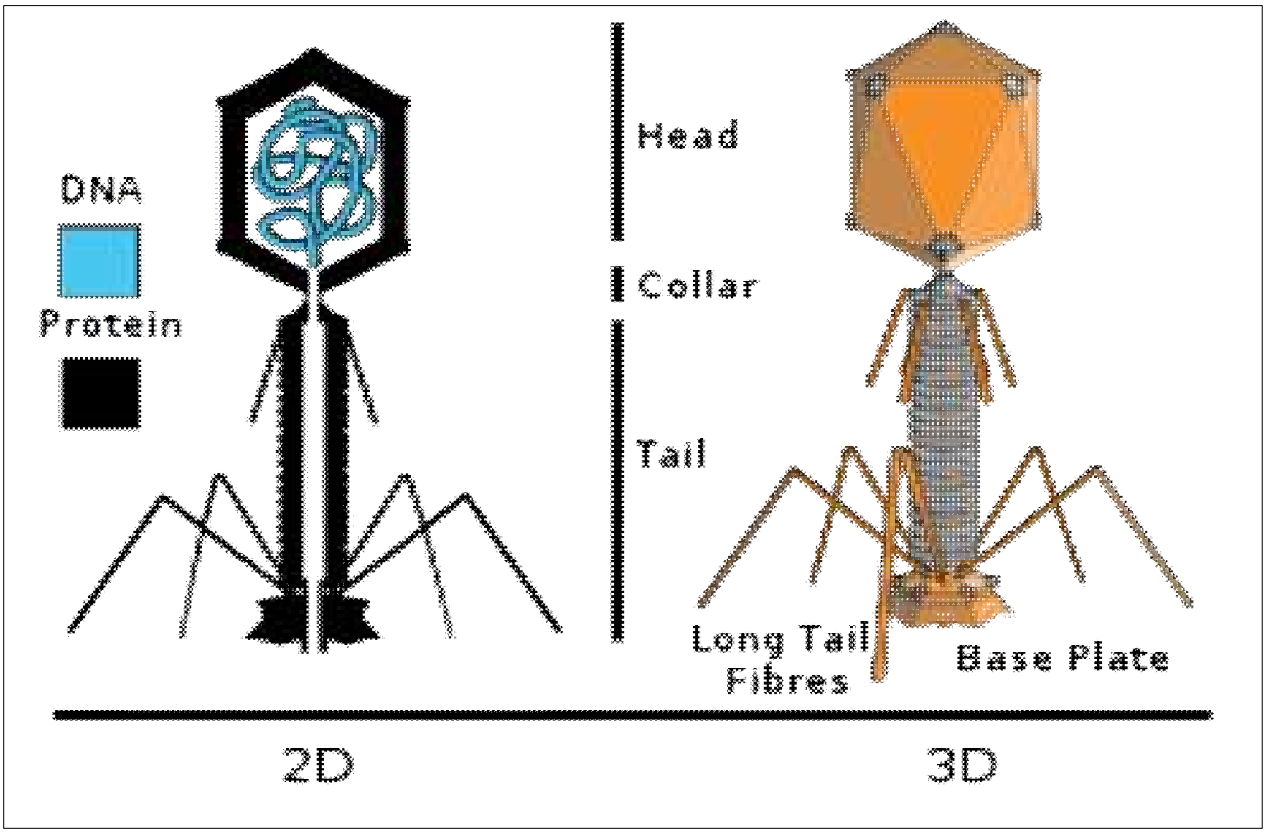

Fig.1. Diagram of typical tailed Myovirus bacteriophages

3.3. Phage virulence factors

A number of important human bacterial pathogens owe their virulence factors to prophage integrated into the bacterial genome (Brussow *et al*., 2004). This is also true for coliphages: in the sequenced *E. coli* O_{157}:H_7 strains prophage encodes the major virulence factor, the Shiga-like toxin. Depending on their nature, after injection of their nucleic acids inside the bacteria, phages induce: a) lysis of the bacterial host with the release of newly formed viral particles (lytic phages); b) release of the progeny viruses by extrusion or budding without lysis of the host cell over several generations (filamentous phages); c) reside as a stable element called prophage inside the host cell as a free plasmid molecule or integrated into the host chromosome (temperate phages).

Many other genera of coliphages can establish lysogeny, but only few have actually been shown to contain established virulence genes. Nevertheless, to be on the safe side, temperate phages should not be selected for phage therapy. A priori, candidates for phage therapy should come from the group of 'professional virulents'lytic phages. They degrade and recycle the bacterial host genome for their own DNA synthesis and thus lack the molecular basis for coexistence with the host .This property also reduces the likelihood of *in situ* DNA transformation resulting from phage lysis (Miller *et al*., 2003).

Soon after their discovery, bacteriophages were utilized as natural antimicrobial drugs to control bacterial infections. They are still used in Eastern Europe, contrary to Western Europe where antibiotics rapidly overtook the use of bacteriophage as antibacterial agents. Due to the excessive use of antibiotics, the increasing prevalence of antibiotic-resistant bacteria has become a worldwide issue, and the development of alternatives to antibiotics is now one of the highest priorities in the field. Since phages have already been proven to be a good natural antimicrobial treatment, the use of phages as alternative bacterial therapeutics may have a strong promise (Fiorentin *et al*., 2005)

In addition, phage-based techniques (phage-display or phage typing) have become routine procedures in molecular biology laboratories and have been adapted to various further applications. For example, Bacteriophages are now used as delivery vehicles for protein and DNA vaccines as potential gene therapy vectors and in nanotechnology techniques (Wagner and Waldor, 2002).

Pathogenicity of phages generally thought to be harmless to humans, phages can actually carry virulence factors that may be transferred to bacterial populations and cause human diseases. Indeed, some examples of well-known bacteria, such as *Vibrio cholera, Streptococcus pyogenes,* or *Escherichia coli* O_{157}:H_7, have gained their Pathogenicity thanks to the acquisition of phages in their genomes (Sulakvelidze *et al*., 2001).

Cured of phages, the pathogenic strains become non-pathogenic again, providing evidence that phages can sometimes be the actual vector that spreads disease among humans. Besides coding for toxins, phages can also encode virulence factors that can affect all stages of the bacterial

infections process such as bacterial adhesion (*Streptococcus mitis*) or bacterial invasion of human tissues (*Salmonella enterica*). Phages can also enhance bacterial resistance to serum and phagocytes (*E. coli* or *Pseudomonas aeruginosa)* or alter bacterial susceptibility to antibiotics (*Staphylococcus aureus* or *S. pyogenes*) by transferring resistance genes (Brussow, 2005).

3.4. Life cycle of the Bacteriophages

Bacteriophages (phages) are non-hazardous self-replicating agents that increase their numbers as they destroy target bacteria (Huff *et al*., 2005). Phages are viruses that infect bacteria and use the bacterial cell's genetic apparatus to produce more phages. In the process, they kill their host. By harnessing phages' natural ability to destroy bacteria, infections can be cleared (Abedon *et al*., 2011).

The various proteins function in both the infection process and act as a coat to protect their nucleic acid from nucleases in the environment. For entry into their host, phages have to negotiate, in the case of Gram negative bacteria, two layers of lipid membranes typically separated by a peptidoglycan layer. The penetration may require specific entry enzymes, presently poorly characterized (Kutter and Sulakvelidze, 2005).

As bacteriophages infect and kill bacteria, in the case of lytic phages, interferes unidirectional with the normal bacterial metabolism, meaning that the bacteria enter a lytic cycle. The most exact definition refers to a group of bacteriophage-associated enzymes that are produced actively during the lytic cycle. These enzymes are able to degrade the peptidoglycan layer of the bacterial cell wall. When this degradation has been carried out, new mature particles of bacteriophages can be released from bacterial cell (Fig. 2). The cycle include- 1: Bacteriophage attaches to a specific host bacterium. 2: it injects its DNA. 3: Bacteriophage uses bacterial DNA and protein synthesis machinery to make the different bacteriophage parts. 4: Assembly of new bacteriophage. 5: The new bacteriophages are released after cell lysis so that new cycles can begin again (Tunail, 2009).

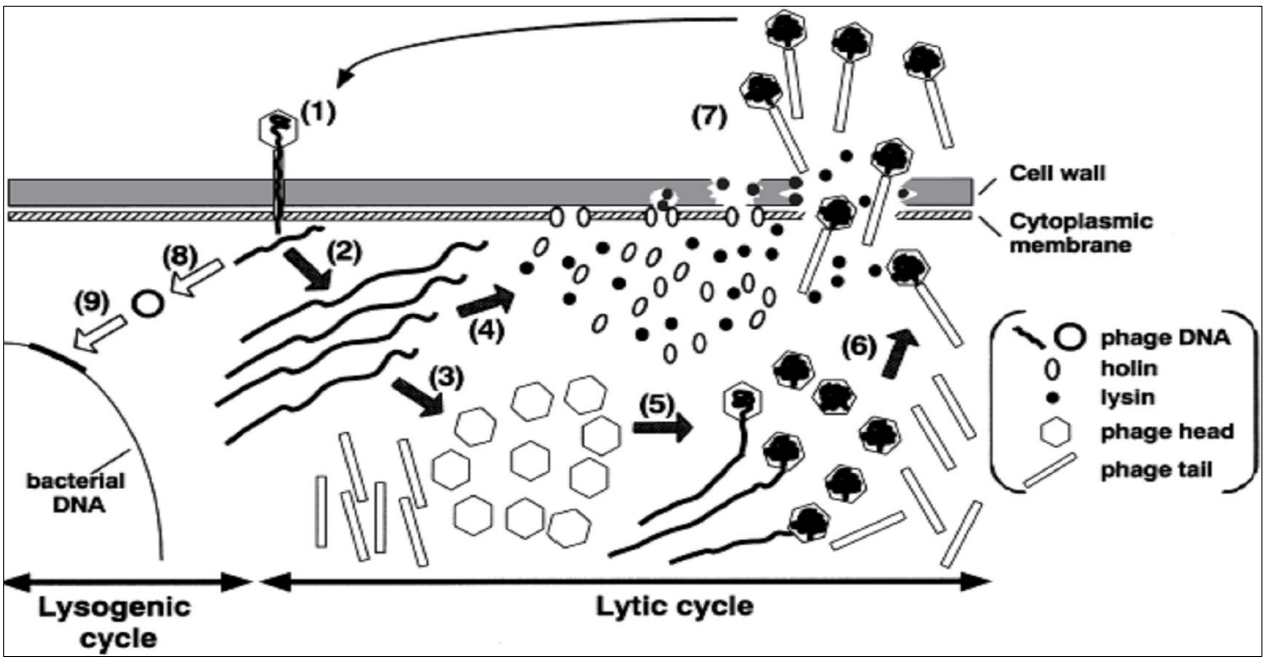

Figure 2. Lytic and lysogenic life pathways of bacteriophages (1) adsorption and DNA injection; (2) DNA replication; (3) head and tail production; (4) synthesis of holing and lysin; (5) DNA packaging; (6) maturation ;(7) disruption of the cell wall and release of the progeny; (8) circularization of phage DNA; (9) integration of the phage DNA into the host genome .

For their release from the producing cell, most bacteriophages synthesize two types of enzymes: holins and lysins. Holins are small membrane proteins which are believed to accumulate in the cytoplasmic membrane as oligomers. They are responsible for the collapse of the membrane potential and the forming of non-specific membrane lesions, allowing lysins to access the peptidoglycan layer. They are the "regulators" of the lytic cycle (Young, 1992).

3.5. Host specificity

Antibiotics kill bacteria rather unspecifically and can therefore lead to numerous side effects. In contrast, species specificity is the rule for phages and is commonly quoted as one of the major assets of phage therapy (Morita *et al*., 2002) .They kill only targeted bacterium but others.

Because of their high specificity, cell wall binding domain (CBD's) fused with indicator proteins have significant potential as tools for rapid detection of bacterial pathogens.

Phages are inexpensive and quick to produce .New phages can sometimes be selected in days or weeks. In contrast, the process of discovering and testing a new antibiotic can take decades. Like with antibiotics, bacteria can also develop resistance to phages. But unlike antibiotics, phages are dynamic and can evolve alongside bacteria in a mutually escalating arms race (Inal, 2003). Because of concerns about resistance; phages are usually used in cycle, with multiple phages directed against a specific pathogen. Even if the bacteria being targeted have evolved resistance to one phage, there remains a high likelihood that they will be killed by the other phage, minimizing both the risk of treatment failure and the possibility that resistance will be passed on to new generations of bacteria. As antibiotic resistance mechanisms do not affect phages, phage therapy provides an ideal way to treat highly antibiotic resistant microorganisms, such as the *Acinetobacter* species found to infect soldiers returning from Iraq (Donlan, 2009).

3.6. Safety Profile and Limitations of bacteriophages

Bacteriophages were considered to be safe during the long therapeutic history in Eastern Europe, former Soviet Union. Before antibiotic era so far, no major side effects have been reported with the exception of liberation of endotoxins from bacteria lyses by the bacteriophage therapy (Schuch *et al*., 2002). During World War II the German and Soviet armies used phages against dysentery and the US army conducted classified research on it. After the war the Eliava Phage Institute in Tbilisi, Georgia, conducted a well-designed field study in the 1960s that came close to the standards of a placebo-controlled clinical trial. More recently, British scientists reported on the successful veterinary application of *E. coli* phages in the 1980s, and excellent studies on phage therapy were carried out by Smith and colleagues, using *E. coli* infection in mice and farm animals. Treatment of infected mice with phages was recorded as more effective than treatment with antibiotics, and phages were effective when they were administered before or after infection (Merril *et al*., 2003).

Phages are similar to antibiotics as they have remarkable antibacterial activity. All phages are specific to their own host (bacteria); they react to only their targeted bacterial host but not to human or other eukaryotic cells. For example, phages specific to *V. cholerae,* always lyse *V. cholerae* and will not lyse *Shigella, Salmonella* or *E. coli* bacteria. This is a clear contrast to antibiotics which target both pathogenic microorganisms and normal micro flora. As a result, the microbial balances in the patient are disturbed and may lead to serious secondary infections (Soothill, 1992).

Several reports about the use of phages in clinical settings have come from many countries especially the former USSR and Eastern Europe. Virtually, all of them supported favorably the prophylactic and therapeutic use of phages. In all cases, phage therapy appeared to be safe and there have been virtually no reports of serious complications associated with the use of lytic phages in humans (Sulakvelidze *et al.,* 2001). However, despite favorable reports, the phages are not commonly used prophylactically or therapeutically throughout the world and their efficacy is still a matter of controversy. One limitation is the high specificity of phages against targeted bacterial species. Phage susceptibility is necessary before administered and polyvalent phage cocktails lyse the majority of strains of the etiological agents. Today, interest in this subject has regained and phages as therapeutic agents seem to have the effect of diminishing the chances of selecting multi drug resistant bacteria in clinical trials of phage therapy.

One particular advantage of phage therapy is the apparent lack of serious side effects (Sulakvelidze and Kutter 2005). So far, phages have been well tolerated, while antibiotics have a range of side effects. There are concerns about possibly harmful molecules released when bacterial cells burst because of phage activity-specifically endotoxins released from the cell walls of bacteria killed by phages into a patient's bloodstream. The same side effects may occur after antibiotic therapy, and various approaches (corticosteroid therapy, for example) used to reduce the problem during antibiotic therapy may also be used during phage therapy (Donlan 2009; Sulakvelidze and Kutter, 2005). These issues remained unresolved, however, because there has been little experience with them. The comparisons of phage and antibiotics therapy were listed in the table below (Table 1).

Table1. Comparison of Bacteriophages and antibiotics treatment.

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

Source: taken from Sulakvelidze, 2001

3.7. Other Applications

Fighting and destroying bacterial infections (both in humans and animals) are the primary applications of phage therapy, but it can also be employed for other uses. It can be the key to fighting the NDM-1, a gene that can be included in the DNA of bacteria, enabling them to resist antibiotics. Waste water from sewage systems are not really considered waste because it is a rich source of phage strains for various kinds of bacteria that lead to the most up-to-date medicines. Skin grafting for extensive wounds, trauma, burns, and skin cancer can also be improved by using phage therapy to lessen the *Pseudomonas aeruginosa* infection (Wright *et al*., 2009). Some experiments for cells in tissue culture have also discovered antitumor agents in phages. Bacteria cause food to spoil faster, and phages have been studied for their potential to increase the freshness of food and decrease the incidents of food spoilage.

Besides direct treatment of infected patients, creative scientists may find a host of uses for phages, including reducing bacterial levels in places such as hospitals and food processing plants. The U.S. Environmental Protection Agency recently registered Intralytix's LMP-102 phage preparation for various environmental applications targeted at eradicating or reducing contamination with L. monocytogenes (as in food processing plants) (Chibani-Chennoufi *et al* 2004).

4. MATERIALS AND METHODS

4.1. Host Bacterial strains

The bacterium strain used in this study was *Escherichia coli* clinically isolated from urine sample obtained from Jimma University Specialized Hospital (JUSH). The bacterium was biochemically identified with IMViC series of tests performed on *E. coli*. Cultures of Escherichia coli were grown for 24 to 48 hours at 37°C and the respective tests were performed and pure culture was grown from single colony on Nutrient agar (NA) and then in Nutrient broth (both from Oxoid, Milan, Italy) at 37^{O}C (approximately 10^{10} CFU⁄ ml) using McFarland standard. Culture was centrifuged (10,000 x g for 10 min) and the pellet was suspended in Phosphate buffer solution to a concentration of 10^{10}CFU⁄mL. Appropriate ten-fold dilutions from this preparation were made in Normal saline solution and used for *in vivo* and *in vitro* experiments. The host bacterium was grown aerobically in Nutrient broth at 37ºC and used in logarithmic phase in all the experiments. It was stored fridge of 0 ºC -4 ºC

4.2. Bacteriophage Isolation:

Isolation of Bacteriophages specific against *E. coli* was carried out from sewage sample .A culture of clinically isolated pathogenic *E. coli* from urinary tract infected patient at Jimma University Specialized Hospital was used for isolation of lytic phages by the enrichment method of (Waddell *et al* .,2009) and used in all subsequent tests.

Sewage samples of waste water were collected randomly in sterile 200 ml containers from five different areas; Jimma University Hospital (1 area), College of Natural Sciences (2 areas) and 'Jimma town Kochi' (2 areas) and among which three lytic bacteriophages were isolated. The samples collected were processed by the enrichment method in Medical Microbiology Laboratory of Jimma University main campus. Briefly, 50 mL of each sample were centrifuged at 10,000 g for 10 minutes to remove particulate materials. The supernatants were filter sterilized by passing through a 0.45-micrometer membrane filter (MERK, EUROLAB [PTFE], U.S.A). The filtrate (50 ml) along with log phase grown (four to six hours) *E. coli* strain of the sample was then mixed with equal volume of sterile double strength Nutrient broth in a 250 ml

Erlenmeyer flask. The flask was incubated with 5 ml of the indicator strain prepared as described in the above section overnight at 37 ^{O}C in static incubator shaking in between every 2 to 4 hours. Next day, the mixture was centrifuged at 10,000xg at 4^{O}C for 15 minutes. Then the supernatant containing phage was passed through a 0.45 micrometer pore membrane filter under aseptic condition. The pellet was discarded.

4.3. Bacteriophage enrichments and Propagation

A 50 ml volume of Nutrient broth in a 100 ml conical flask was inoculated with aliquots of broth culture to contain approximately 10^{10} CFU/ml and a phage (ϕJS3) preparation to contain 10^{10} PFU/ml. The culture was then centrifuged at 10,000 rpm for 15 min at 4°C, and the supernatant was filtered (0.45-mm pore size) as shown below (Fig.3).

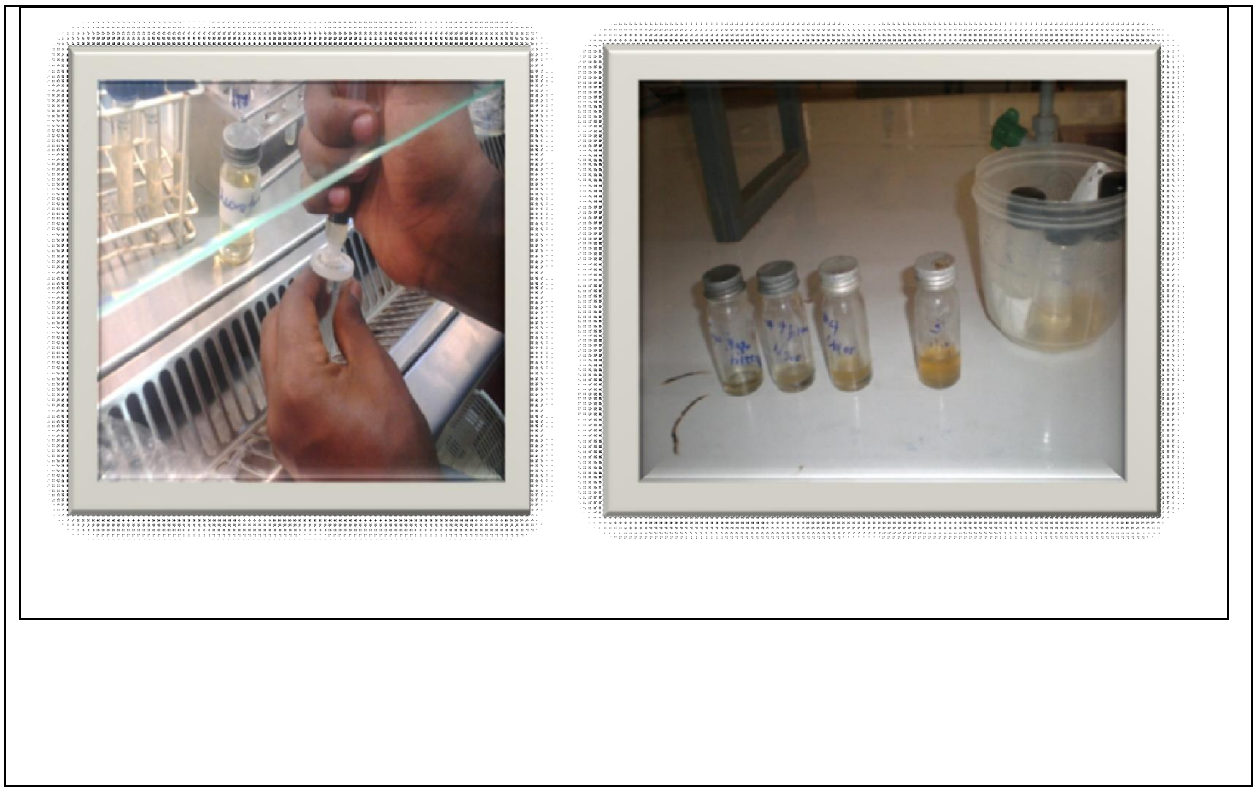

Fig. 3. Bacteriophage flteration processing

4.4. Amplifying (concentrating) of Bacteriophage

Amplification step was run in the filtrate from the last step by mixing 50 ml of the filtrate with equal volume of double strength nutrient broth containing 2 mM $MgSO_4$ and incubated with 5 ml of the indicator strain (clinically isolated pathogenic *E. coli*).The mixture was incubated at 37^{O}C in the incubator shaking every two to four hours overnight. The next day, the mixture was centrifuged at 10,000 x g at 4^{o}C for 15 minutes. The supernatant considered to contain phages filter sterilized through a 0.45 micrometer pore membrane filter under aseptic condition. The amplified filtrate obtained was tested for phage activity by the method of spot assay.

4.5. Detection of Bacteriophages Activity

Bacteriophage activity against the host bacterium, *E. coli*, was checked using spot assay as described by Cerveny *et al,* (2002) and Kumari *et al,* (2010). The test cells (0.1 ml) were added to sterile molten soft agar (0.75%) prepared and maintained at 45^{O}C in a water bath and quickly mixed. The contents were poured on previously prepared nutrient agar plate.

Around 12-15 micrometer (two drops) of amplified /concentrated filtrate was spotted on each plate at 2 different places using micropipette. The plates were allowed to dry at room temperature and then incubated overnight at 37^{O}C. The plates were examined the next day for clearance or plaque formation at the spotted area.

4.6. Phage Purification

All the isolated phages were purified by successive single plaque isolation until homogenous plaque was obtained following standard procedure described by Schuch *et al*, (2012). To purify a single strain from a heterogeneous stock, a single plaque was picked aseptically and transferred into tube containing 5 ml broth of fresh log phase grown (about 4-6hrs) test strain. Another tube containing the pathogen strain left as a control. Both tubes were incubated at 37^{O}C under shaking condition until complete lysis occurred in the test preparation. Phage host mixture and control preparation were centrifuged at 10,000xg for 15 minutes at 4^{O}C. The supernatant was filter sterilized by passing through 0.45 micrometer pore membrane to remove any bacterial

contaminants. The filtrate was tenfold serially diluted in phosphate buffer solution and assayed for plaques in order to quantify the number of plaques. The procedure was repeated for three times to ascertain the purity of isolated phage. The plaque recorded and scored as: confluent lysis with some halo formation. Purified ϕJS3 was stored for the next purpose at 0 - 4ºC for routine use after sterilized.

4.7. Quantitative Assay of Bacteriophages (Titration)

Titre of the phage preparation [plaque forming units per milliliter (PFU/ml)] was estimated by the soft agar overlay method as described by Adams (1959). High titer was prepared by adding phages to early log phase (about 4-6 hrs) host culture at an MOI (multiplicity of infection) of 1 and incubating at 37ºC, until complete clearance was obtained. The large plaque forming sample content was selected for the next processes.

Serial dilution (10^{-1}-$10^{-10)}$ of the bacteriophage sample was made in sterile physiological saline solution. 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 8 ml of molten soft agar held at 45^{o}C.The mixture without producing air bubbles thoroughly mixed and quickly poured over previously moisture dried nutrient agar plate. The pellets were swirled gently to ensure even distribution of the mixture. The overlays allowed to solidify upright for 30 minutes at room temperature and then incubated in inverted position at 37^{O}C for overnight. Next day plates with 30-300 plaques were selected and counted. Original phage count (titer) was determined by using the following formula for calculation of phage number.

$$\text{Number of plaques} = \frac{\text{Plaques counted x Reciprocal of the dilution}}{\text{Amount of plated in milliliter}}$$

= PFU/ml (plaque forming units per millimeter)

Note: A plaque formed is a result of successive infection of bacterial cells by phages originating from a single phage (PFU) at a point.

4.8. Phage (ϕJS3) characterization

E. coli stain in exponential growth phase was incubated with purified phage. The adsorption rate, plaque size, sensitivity to chloroform and its specificity to *E. coli* was determined according to the method of Adams (1959). Sensitivity of the ϕJS3 phage to chloroform/ether solvents was determined according to the method of Deutsch and Wassermann (1965) to purify. One ml of bacteriophage suspension having a known titre of ≈1× 10^8 PFU/ml was taken in tubes labeled as 'Test 1' for chloroform, 'Test 2' for diethyl ether, and 'Control'. One ml of chloroform, diethyl ether, and sterile saline (as 'Control') were added to the respective tubes. Tubes were shaken vigorously for 10 seconds at room temperature and then centrifuged at 4000×*g* for 10 min. A 100 µl of the uppermost aqueous layer was carefully transferred to an empty sterile tube after an hour. Phage titration of both the test and control was then performed by agar overlay method. Plates were incubated at 37^0C overnight. Next day, the phage titre of tests and control was compared. A drop in titre of more than 10 times compared to the control was considered as sensitivity of phage to the chloroform/ether.

4.9. Toxicity testing of phage in mice.

Female Swiss mice, 6–10 weeks old and average weight of 30 g, were used in this study. Mice injected with 0.1 ml phage suspension in sterile normal saline (10^{11}PFU/ ml) by the intraperitoneal (i.p.) route in three groups of three mice each were followed for any signs of illness and scored accordingly. Three normal saline injected mice were retained as controls. The mice were observed for signs of illness, and reduction of weight were taken hourly during the first 5 h after injection and then daily for the next 5 days. The toxicity of phage suspension was investigated in mice according to the method of Soothill (1992). Points were given as follows: Point (pt) 5=normal, unremarkable condition, Pt 4=slight illness, lethargy and ruffled fur, Pt

3=Moderate illness, severe lethargy, ruffled fur and humbled back, Pt 2=severe illness above signs plus exudative accumulation around closed eye, Pt 1=Moribund state and Pt 0=Death.

4.10. Mice infection model development using the Lethal Dose (LD100) *E. coli*

An experiment was carried out on female Swiss mice (8-10 weeks old) of average weight of 25-30g at Jimma University College of Agriculture and Veterinary medicine in one of veterinary medicine laboratory. All mice were fed an antibiotics free diet and given food and water. For the animal handling in this experiment, ethical clearance was approved by Jimma University and Agricultural College and Veterinary medicine, School of Veterinary Medicine.

E. coli cells were grown in 5 mL nutrient broth medium at 37°C and were centrifuged at 10,000 *g* for 10 min at the late logarithm phase. The cell pellet was washed with sterile normal saline, centrifuged again under the same conditions, and finally resuspended in 1ml sterile normal saline. After appropriate dilution, and determining bacterial cell number, as the same method with number of plaque forming units, different concentration of *E. coli* dose was injected in three mice groups. One of the three mice groups was given with sterile normal saline as a control. Mice were challenged with bacterial doses ranging from 10^6 to 10^{10} CFU/ml bacterial cells suspended in 0.1 mL sterile normal saline. The test animals were observed over 5 days for any type of signs of illness and death. To record this, a score system was used in determining the signs of illness observed in mice in 3-5 days, with the following points given:

4.11. Bacterial counts in faeces of the mice

To evaluate the disease causing bacteria in mice, one day old faeces from three group of mice (1= only lethal dose *E.coli* infected in three mice, 2=only bacteriophage injected in three mice, and 3= only sterile saline injected in three mice) were collected 3 days after inoculation and homogenized in PBS (1:100, w/v). Ten-fold serial dilutions were prepared and 0.1ml aliquots from each dilution factor were plated onto MacConkey agar. The Petri dishes were incubated aerobically at 37°C overnight and colonies were counted after 24 h.

In all the groups of mice used, *E.coli* isolated from the faeces of mice was tested for their susceptibility against the bacteriophage (ϕJS3) using the spot assay method. Results were reported as CFU/ml of *E.coli* from the three groups obtained and their susceptibility to ϕJS3.

4.12. Measuring the efficacy of phage to rescue mice from *E. coli* infection in vivo

The first group of 6 mice was inoculated with *E. coli* only (10^9 CFU/mouse). The second group of 6 mice was inoculated with *E. coli* (10^9 CFU/mouse) and immediately challenged with ϕJS3 (10^9 PFU/mouse). The diluents used in *E. coli* and phages were normal saline solution given to the mice by intraperitoneal injection. The third group 6 mice were inoculated with LD100 *E. coli* and then immediately treated with antibiotic Ciprofloxacin. The mortality rate between mice treated with phage and Ciprofloxacin and those not treated was compared.

4.13. Statistical analysis

To investigate the effectiveness of bacteriophage to treat *E.coli*, three replicate experiments were conducted, with phages evaluated per replicate. The Microsoft Excel software was used to determine the mean, Standard deviation and survival rate percentages of the mice .The result was represented using tables and graph which represents the phages potential to rescue the mice from death.

For the Animal handling in this experiment, ethical clearance was approved by responsible body in Jimma University, College of Agricultural and veterinary medicine, School of Veterinary medicine.

5. RESULTS

5.1. Isolation of *E. coli* – specific phages (ϕJS3)

To evaluate lytic spectra of all isolated phages, they were tested on host cell and the plaque size they formed was measured .On the basis of this, among the samples processed for phage isolation, sample three obtained from around Jiren secondary school was selected on the basis of big size plaque formation that indicate it is more effective in lysing bacteria and used in subsequent study, indicating that this phage can adsorb efficiently to the indicator host and kills it (Fig.4). It was named as ϕJS3 to represent Jiren School Sample 3 .The supernatants was harvested and then subjected to the spot test (plaque formation).

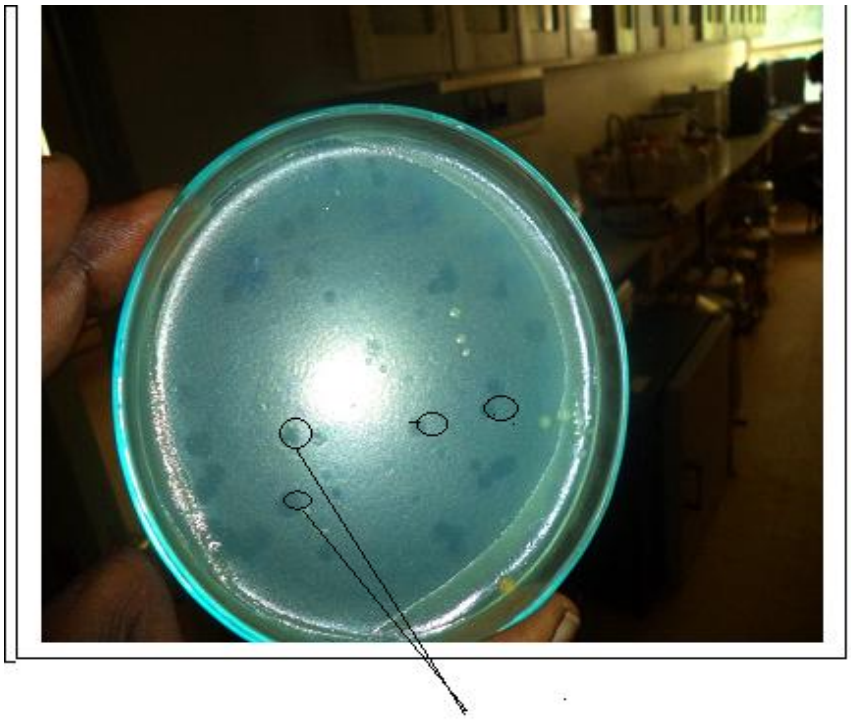

Fig. 4. Plaques formatted by isolated Bacteriophages

5.2. Phage characterization

E. coli strain in exponential growth phase was incubated with purified phage. The lysis rate, plaque size, sensitivity to chloroform and ether and its specificity to *E. coli* was measured. The *in vitro* characteristic of lysis rate (plaque formation) was observed in 4 hours after inoculation on the host on the plate and a burst plaque size of 5 mm. The phage specificity was checked by inoculating the phages on *Salmonella* spp*, Pseudomonas* spp, *Niesserria* spp and *E.coli* ATCC 25922 forming no plaques, except on the specific host *E.coli*. The phage is resistant to chloroform and ether which indicates they are not enveloped viruses.

All enriched samples produced zones of clearing on host bacterial lawns (E. *coli*) (Fig.3.B).

5.3. Lethal dose of *E. coli* in infected mice

Mice challenged with bacterial doses ranging from 10^6 to 10^8 CFU/ml were not died.

The number of bacteria when increased to 10^9 to 10^{10}CFU/ml for intraperitoneal administration, all the mice died within 3 to 5 days. Those mice inoculated with 10^9 CFU/ml died within 5 days (Pt=0). All infected mice receiving normal saline treatment only (control) did not show any signs of bacteraemia or slight illness over the period of 5 days.

Mice inoculated with 10^9 CFU/ml died within 5 days. This dose was recorded as minimum lethal dose (LD100) of bacterial cells and was used in all subsequent experiments for the induction of infection and death. Then determine change of signs on mice in 3 days

Table 2.Determination of Lethal Dose (LD100) *E. coli*

Dose	Amount	No of mice	Result
10^{10}	100microlter or 0.1ml	3	Pt 0
10^{9}	100microlter or 0.1ml	3	Pt 0
10^{8}	100microlter or 0.1ml	3	Pt 1
10^{7}	100microlter or 0.1ml	3	Pt 2
10^{6}	100microlter or 0.1ml	3	Pt 2
Normal saline (as control)	100microlter or 0.1ml	3	Pt 2

Key; Pt 2=normal unremarkable health, Pt 1=slight illness lethargy and abnormal and Pt 0=death.

Regarding Phage toxicity test a 0.1ml of 10^{10} or (10^{11}) phage concentration was injected intraperitoneally to 3 mice and 3 mice with normal saline as a control signs observed on mice in 3 days were determined. Points were given as**: Pt** 2=normal unremarkable health, Pt 1=slight illness lethargy and abnormal and pt 0=death

From the result obtained, there were no observable changes found due to administration of the bacteriophages (Pt 2). Thus the phages were found safe to be used for the subsequent activities.

5.4. Infected Mice model treatment using specific phage (ϕJS3)

On the evidence cited above, the isolated phage was considered to be a suitable candidate for the therapeutic phage, or its prototype, for the treatment of *E. coli* infections. Therefore, the following experiments were undertaken using these phages in a mice model.

Of 10^9 –10^{10} cells lowered the survival rate in a dose-dependent manner. Because the injection of 10^9 bacterial cells was fatal in >60% of mice within 3 days and in 100% within 5 days of post injection, this level of challenge was considered to be optimal for observing the (ϕJS3) effect on bacterial lethality (Fig.5.B). A more precise time-based analysis showed that intraperitoneal injection of 10^9 *E. coli* cells killed all mice between 3-5 days after injection, with associated preceding bacteremia (Fig.)

A B C

Fig.5. Mice treatment groups: A= mice injected only with *E. coli;* B=mice injected with *E. coli* and then challenged with bacteriophag*es,* C= mice injected with *E. coli* and then with Ciprofloxacin.

From the three experimental groups and their percentage of survival are shown in Tables 3, the mice in first group had an induced pathogenic *E. coli* infection, the second group induced with

E. coli and immediately with ϕJS3 and the third group was induced *E. coli* immediately with antibiotic Ciprofloxacin were injected with Phage. The following results were obtained.

In each group, 6 mice were treated. Review of the data revealed explicit time intervals for reporting mouse survival in days post infection injected with *E. coli* alone. As seen in table 3, the group of mice was observed daily consecutively for 5 days. At day 2 of post infection, all the mice were still alive. At third day of post infection, there were distinct differences in survival and morbidity. One of the mice was died .On the fifth day of post infection, all mice were died, but the ϕJS3 and Ciprofloxacin treated groups were 100% set free the mice from the lethal infection. The survival rates percentage of the experimental mouse injected with *E.coli* alone showed declining during the study and eventually at the fifth day all the mice were died out.However the other treatment group *(E.coli*+ phages and *E.coli*+ Ciprofloxacin)

Table 3 .Average change of weight survival rates of mice in each study groups

Mouse	**Average Weight and Survival rates during each study day**					
	Date 0 □± SD(%)	**Date 1** □±SD(%)	**Date 2** □± SD(%)	**Date 3** □± SD(%)	**Date 4** □± SD(%)	**Date 5** □±SD(%)
E.coli aolone	23.67±3.14 (100)	24.67±2.67 (100)	22.17±2.56 (100)	1.83±4.96 (100)	7.33±11.43 (100)	0
E.coli+ Phage	24.33±4.22 (100)	24.67±3.89(00)	24.83±3.93(100)	25.50±3.83 (100)	26.33±3.56(100)	27.33±4.3(100)
E.coli +Ciprofloxacin	22.33±1.86 (100)	23.0±1.67 (100)	23±1.26(10 0)	24.0±1.26(100)	24.67±1.37(100)	25.67±1.37 (100)

Key: Date 0=before treatment :□ =Mean weight :SD (%) =Standard deviation and percentage of survival rates respectively

Data represent percentage the change of weight and survival rates of mice that were subjected to *E. coli* infection, LD100 *E. coli* infection and then immediately administered with phage (ϕJS3) and LD100 *E. coli* infection and then immediately administered with Ciprofloxacin (antibiotic).

In case mice taken only LD100 E.coli injection, their average weights were decreased and one of the mice died on third day after injection with the LD100 *E. coli* (Table 3). At fifth day all the challenged mice were dead. On the other hand the weight of mice injected with *E.coli* and the treated with bacteriophages and Ciprofloxacin were increased and all the mice were survived death. The above results (Table 3) was summarized as the figure below (Fig.6)

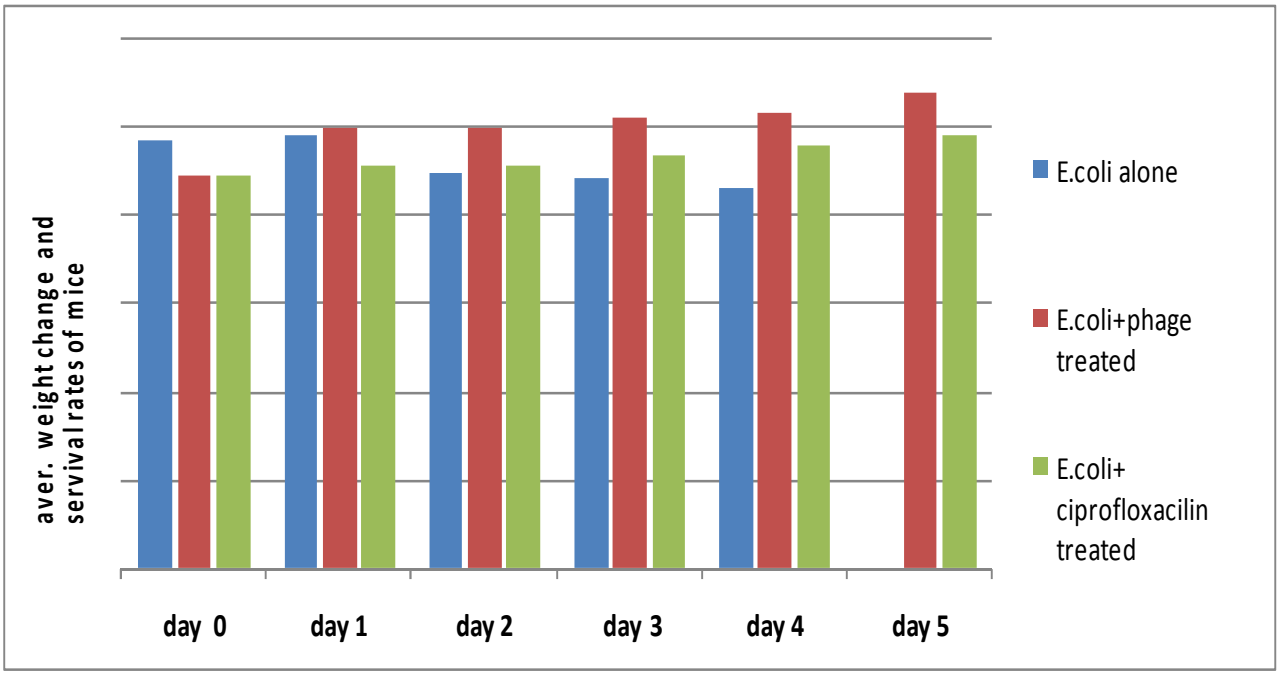

Fig.6. Average change of weight survival rates of mice in each study groups

5.7. Bacterium enumeration from animal faeces

From this study, the number of colonies counted were averagely 80, and the dilution factor was 10^{-8} of a 0.1ml suspension (amount of plated). Accordingly, the number of original cell concentration is $=80x10^{8}/0.1 = 80x10^{9} = 8x\ 10^{10.}$ The fore the number of *E.coli* present in 1gram of faeces of infected mice was $8x10^{10}$.

To check whether the bacteria colony was the pathogenic one the susceptibility test to phages ϕJS3 was applied on it. The formation of plaque was observed which indicates that, those bacteria are specific to ϕJS3, the strain that led the mice to death.

On the other study groups of mice although the bacteria were seen on the culture media they didn't show any plaque formations when the same phages (ϕJS3) was applied on them which implies that they were not the experimentally used pathogenic bacteria.

6. DISCUSSION

Using bacteriophages as potential chemotherapy-independent schemes can helps to address clinical problem and drug-resistant bacteria by using microbial interference. In the present study, using a newly isolated phage, it was experimentally evaluated the therapeutic potential of phages in mice infected with clinically isolated pathogenic *E. coli*

The successes reported in the present study were similar to those reported previously for neonatal pigs (Smith and Huggins, 1983; Smith *et al*., 1987).Because both the challenge ETEC and the phages may be susceptible to low pH in the stomach and upper small intestine, the model of experimental ETEC diarrhea was modified by the pre-challenge oral administration of sodium bicarbonate. Administration of the phages shortly after feeding also is another strategy for protecting them from exposure to low pH in the stomach (Brussow, 2005).

Since phages are obligate parasites, a host must be provided in order to enumerate them from any environment samples. Providing a host and counting the number of phages is most easily accomplished by using an agar overlay technique of (Adams, 1959). 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 and the same guidelines for CFU's apply to plaque forming units (PFU's) (Adams, 1959).

6.1. The lethal dose of *E. coli*

The minimum lethal dose of the bacteria less than10^9 cells hold no effect on mice but both 10^9 and 10^{10} killed the mice after three days of post infection and 10^9 were taken as the minimum lethal dose (MLD). This is to develop a phage therapy system that avoids the necessity of continually administering multiple doses of phage.

6.2. Effects of Phages treatment and associative Safety issues

By administration of purified phages immediately injected after the infections of mice with *E. coli* and effective results were observed. ΦJS3 was injected into the mouse intra peritoneally phages at MOI of 1:1 protected mice from *E. coli* induced death (Fig.5.B). On the other hand, administration of a large amount (up to1.0×10^{11}PFU) of phages alone to did not affect their physical condition or survival during 5 days observation period after injection. These findings indicate that the phages themselves, at least when inoculated into the peritoneal cavity, does not give rise to any detectable adverse effects.

Another important factor that can modify the effectiveness of phage treatment is single dose versus multiple doses. Several studies have shown that multiple doses are better than a single dose**.** However, in our study doses MIO of 1:1 was successful because phages can multiply rapidly in suitable conditions. Huff *et al*, (2002) found that the application of bacteriophage was most useful very soon after the chickens had been exposed to the bacteria and that, if treated early, multiple doses were better than a single dose. Interestingly, if treatment starts later, there is no difference between single and multiple doses, but treatment is still very helpful. A performed experiment by Biswas *et al.,* (2002) using a mouse model of vancomycin- resistant *Enterococcus faecium* infection was showed that a phage administered intraperitoneally 45 min post-infection was able to liberate mice from *E. faecium* and that the rescue or set free was associated with a significant decrease in bacterial numbers in the blood. They also demonstrated that phage administration up to 5 h post-infection still fully rescued the mice while treatment delayed beyond 5 h save only some of the mice. In our study, the ϕJS3 were immediately injected after the infections of mice with *E. coli* and effective results were observed.

The present study has shown that the selected phage (ϕJS3) was effective in treating *E. coli* infections in an animal model. The result of this study is in consistent in this context with Soothill's findings (Soothill, 1992) who indicated that a multiple of infection (MOI) of 1 is the minimum required for □MR11 to yield a fully protective effect, at least in the mouse model. Taken together, these data imply that determination of the appropriate dose of phage is a prerequisite for successful phage therapy.

This study investigated whether the direct bactericidal activity of phage was actually responsible for its protective effects in *Invitro* and *Invivo* environment. An intraperitoneal injection of mechanical lysate, an analogue of phage lysate, prepared from 10^9 PFU/ml cells, was followed by the injection of an equivalent number of living *E, coli* cells. After 3 days, all mice treated with phages were still alive, whereas 100% of mice not treated with phages were died within 3-5 days. The therapeutic efficacy of phage was even predictable in mice treated two days after injection with bacteria, when all the control mice injected with *E. coli* only already exhibited signs of physical deterioration, such as reduced activity and ruffled hair mice.

Another study showed that phages virions themselves stimulate an antibacterial immune response (Huff *et al*., 2005). If the net life saving effect is mediated, not through phage-induced bacteriolysis but via a virion-stimulated immune response (e.g., production of cytokines), the administration of phage should be effective against the host bacterial infections. These results support the conclusion that direct bactericidal activity exerted by phage is the prime and perhaps the sole determinant of the protective effect observed in the present study (Barrow *et al.,* 1998).

With the expanded knowledge of phage molecular biology and interactions with mammalian immune systems it is possible to genetically engineer phage that might be more efficacious than the wild types found in nature. This potential was shown in the development of long circulating phage strains that could stay in the circulatory system longer than the laboratory strains from which they were derived. There have been a number of successful demonstrations of the effectiveness of phage in animal models of bacterial infectious diseases like success in the treatment of rats from sepsis and meningitis caused *Staphylococcus* and a fatal neonatal meningitis *E. coli* strain (Biswas *et al.,* 2002).

Another concern regarding the therapeutic use of lytic phages is that the development of phage resistance may hamper their effectiveness develop .But this has no much effect since the rate of developing resistance to phages is approximately 10-fold lower than that to antibiotics ( ). The rate of developing resistance against phages can be partially circumvented by using several phages in one preparation (much like using two or more antibiotics simultaneously). Most importantly, when resistance against a given phage occurs, it should be possible to select rapidly

(in a few days or weeks) a new phage active against the phage-resistant bacteria (Pouillot *et al.,* 2012).

All mice injected only with *E. coli* died within 3-5 days, whereas phage and antibiotic treated mice were all saved. These results illustrate the *in vivo* kinetics; intraperitoneal injected phage was amplified perhaps resulting in the systemic propagation of the phages. Under these circumstances, the circulating phage was sustained at a significant level until the target cells were eradicated, which must have counteracted the progression of bacteremia (Nakai *et al.,* 1999). The beneficial *in vivo* kinetics of phage in curing the *E. coli* infection also may be applicable in general to phages given therapeutically to counteract other bacterial infections.

The protective effect induced by phage is attributable to direct bacteriolysis by the phage or associated with vaccine like immune activation by dispersed bacterial components (Krylov et *al*., 1992). The irrelevancy of immune mediation of toxins and antigens released from bacteria due to lysis in phage treatment is also supported by the fact that bacterial antigens were completely removed from the phage preparations during repeated centrifugation and purification processes. In this study, the phages (ϕJS3) were purified for three times to ensure this .Furthermore, any activation by phage particles themselves of an antibacterial immune response also was negligible, because phage had no therapeutic effect against infections with the phage-lysogenic bacterial strain (Soothill *et al*., 2004).

After a single dose, phages get rapidly into the bloodstream of laboratory animals within two to four hours and into internal organs within approximately ten hours which implies rapid systemic distribution of the phages (Dubos *et al*., 1943). As other study stated, infectious phage were subsequently detected in various tissues and organs which suggests an extension of the medical application of phage to systemic infections (Chan and Abedon, 2012). Merril *et al*., (2003), also observed similar results of *in vivo* translocation of *E. coli* λ phage and *Salmonella* phage P22 administered in a mouse system. As an unfavorable consequence, however, these phages were rapidly eliminated from the blood, making the capture of circulating phages by the splenic reticuloendothelial system an anticipated problem of phage therapy.

To establish more efficient phage-therapy system, generally applicable against human bacterial induced diseases, several other obstacles must be overcome in the future. The incidence of bacteria that resist phage attack is very low than the incidence of bacteria insensitive to an antibiotic (Kumari *et al*.,2008).It will be necessary to create advanced therapeutic phages to circumvent other inevitable problems, at least at present, such as the lysogenicity of therapeutic phages and the restriction-modification systems of bacteria. Phages are a suitable candidate for a prototype with which to pursue these objectives, because its genome size is relatively small and allows for easy genetic manipulation. It also lacks known toxin and drug-resistance genes (Westwater *et al.,* 2003).

6.3. Safety of the therapeutic phage preparation

During the long history of using phages as therapeutic agents through Eastern Europe and the former Soviet Union, there has been no report of serious complications associated with their use (Sulakvelidze & Morris, 2001). Phages are extremely common in every environment and regularly consumed in foods (Bergh *et al.* 1989).They have been commonly found in human gastrointestinal tract, skin and mouth, where they are harboured in saliva and dental plaques and they have been shown to be unintentional contents of some vaccines and sera commercially available in United States (Merril *et al.,* 2003*).*

Phages have high specificity for specific bacterial strains, a characteristic which requires careful targeting host (Merril *et al.,* 2003). Therefore, phage therapy can be used to lyse specific pathogens without disturbing normal bacterial flora and phages pose no risk to anything other than their specific bacterial host (Sulakvelidze *et al.,* 2001). From a clinical standpoint, phage therapy appears to be very safe. Efficacy of natural phages against antibiotic-resistant *Streptococci, Escherichia, Pseudomonas, Proteus, Salmonella, Shigella, Serratia, Klebsiella* (Kumari *et al.,* 2010), *Enterobacter, Campylobacter, Yersinia, Acinetobacter* and *Brucella* were being evaluated by researchers (Matsuzaki *et al.,* 2005).Our study also proved that the ϕJS3 were specific to the selected *E.coli* that cause death to mice.

Phages can be modified to be an excellent therapeutic agent by directed mutation of the phage genome or recombination of phage. These new modified phages have been shown to

successfully overcome challenges to earlier phage therapy such as countering bacterial infections, endotoxins (lipopolysaccharide) released by the gram negative bacteria as a component of outer membrane and cause fever, (Morita *et al*.,2002) .Toxic shock can be tackled by recombinant phage derived by genetic modifications (Levin *et al.,* 2004). This phage had the benefit of minimizing the release of membrane associated endotoxins during phage therapy (Parisien *et al.,* 2008). In order not to compromise on the issue of the safe use of therapeutic phage preparation, rigorous characterizations of each phage to be used therapeutically should be done (Payne & Jensen, 2000).

7. CONCLUSION

- ✓ The results of this study suggest that phage therapy has a potential to check the growth of pathogenic *E. coli* in the intraperitoneal system of mice if initiated at appropriate time.
- ✓ The ability of lytic phages to rapidly kill and lyse infected bacteria, the specificity of phages to particular bacteria, the ability of phages to increase in number (self dosing) in the presence of the target bacteria and no multiplication in the absence of host make phages excellent potential therapeutic agents for fighting bacterial disease.
- ✓ Since phages are ubiquitous and adsorb to different receptor molecules on specific bacteria, it can be readily isolated from the environment using simple, low-cost techniques compared to developing a new antibiotic.
- ✓ This study has opened a broad research horizon in Jimma and further in Ethiopia that will enable future researchers to alleviate the use of bacteriophage as an alternative therapeutic agents of diseases caused by bacteria including multidrug resistant once.
- ✓ It is easy to isolate bacteriophages from sewage sample, and evaluate their therapeutic efficacy to treat pathogenic clinical isolate *Escherichia coli* infection in mice model by comparing the study results that all the mice treated with bacteriophages were saved from the lethal dose of *E.coli*, while the untreated groups were died.
- ✓ Bacteriophages are effective to treat *E. coli* in the mouse system.
- ✓ They cannot replicate in non targeted organisms and eukaryotic cells thus have no harmful effects on eukaryotic cells.

8. RECOMMENDATION

Appropriately selected phages well-characterized and purified phages will be desirable for therapeutic use and can easily be used to help prevent bacterial diseases in animals, with potential for alternative applications and special interest for developing countries, because currently, many pathogenic bacteria have acquired multiple drug resistance, which is a serious clinical problem.

- In order to achieve successful therapeutic bacteriophages preparation to be used as a clinical product, standardization and specific protocols should be considered.
- Effectiveness and biological activity of bacteriophages preparations, stability and storage conditions of phage preparations and the methods employed for the evaluation of the bacteriophages preparations should be considered
- Appropriate procedures need to reduce the chance of impure product or contamination with bacteriophages carrying genes encoding toxins or factors that enhance bacterial pathogen city in order to assure therapeutic safety (repeated purification is needed).
- Going forward, industry and the regulatory bodies should work closely to bridge the gaps and find mutually acceptable solutions to overcome the barriers.
- The negative public perception of viruses should be overcome by increasing the awareness among people about the benefits of phage therapy.
- Well-conducted studies are required to define the role and safety of phage therapy in daily clinical practice to treat patients with various infections.
- There is also a need for in-depth characterization of the bacteriophage to understand more about the biology of the virus.
- Furthermore, better understanding of the interactions between phage and their bacterial host can help identify novel targets for phage-based drug development.

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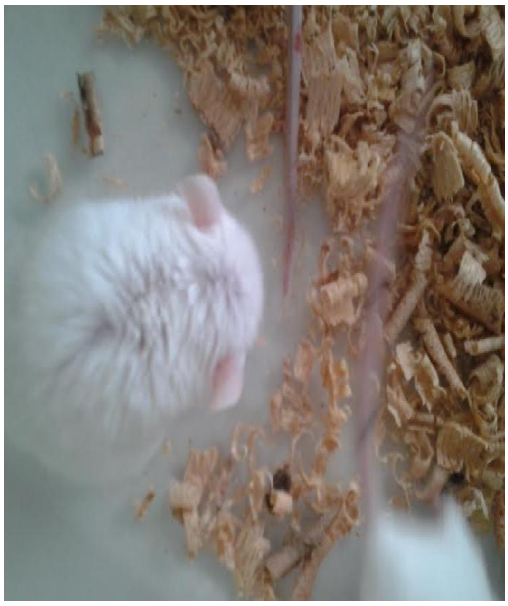
Appendixes

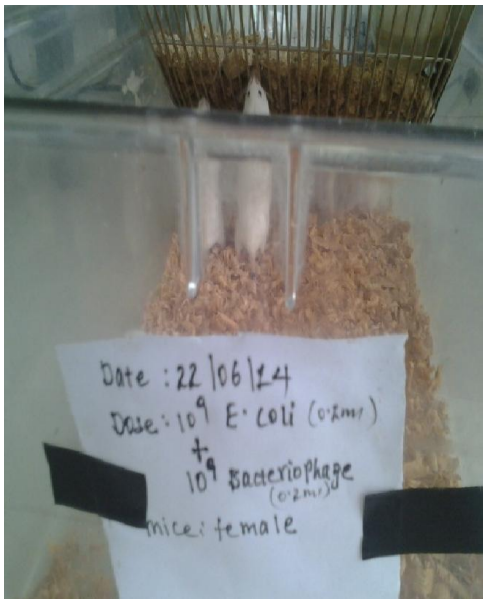
A Collected sewage **B Phage processing** **C. Plaques formed by phages**

Sewage sample and bacteriophages isolations processes and plaque formations

Researchers' photograph



Mouse untreated by phages



Mouse treated by phages

The three Experimental groups of mice

Average change of weight survival rates of mice in each study groups

Weight (gram)																	
Mouse	**Date 0**			**Date 1**			**Date 2**			**Date 3**			**Date 4**			**Date 5**		
	E.	**E +P**	**E+C**	**E**	**E+P**	**E+C**	**E**	**E+P**	**E+C**	**E**	**E+P**	**E+C**	**E**	**E+P**	**E+C**	**E**	**E+P**	**E+C**
M1	19	27	24	21	28	25	20	28	25	19	28	26	d	29	27	-	30	28
M2	22	26	22	23	25	22	20	26	22	22	27	23	20	28	24	d	29	25
M3	26	24	20	27	24	21	26	24	22	26	24	24	24	25	25	d	26	26
M4	27	19	25	27	20	25	24	20	24	24	21	25	d	23	25	-	23	26
M5	22	30	21	23	30	22	20	30	23	20	31	23	d	31	23	-	33	24
M6	26	20	22	27	21	23	23	21	22	d	22	23	-	22	24	-	23	25

Key: E=*E.coli*; P= Phage; C=Ciprofloxacin

M1= one black color mark on mice tail M1= one red color mark on mice tail

M2=two black color marks on mice tail M2= two red color marks on mice tail

M3= three black color marks on mice tail M3=three red color marks on mice tail

d=died mice