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Isolation and Characterization of Lytic Bacteriophages from various sources in Addis Ababa against Antimicrobial Resistant Diarrheagenic *E. coli* Strains and Evaluate their Therapeutic Potentials

M.Sc. Thesis

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A Thesis Submitted to the Institute of Biotechnology in Partial Fulfillment of the Requirements for the Master of Science Degree in Biotechnology

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We certify that Tamirat Salile's M.Sc. thesis entitled **"**Isolation and Characterization of Lytic Bacteriophages from various sources in Addis Ababa against Antimicrobial Resistant Diarrheagenic *E. coli* Strains and Evaluate their Therapeutic Potentials" is the final version. Therefore; we, as the examining board, approved it as the final document to be accepted as fulfilling the requirement for the degree of Master of Science in Biotechnology.

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Abbreviations and Acronyms

E. coli	*E. coli*
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
IBD	Inflammatory bowel disease
PFU	Plaque forming unit
SM	Salt of magnesium
STEC	Shiga toxin-producing *E. coli*
TSA	Tryptone soya agar
TSB	Tryptone soya broth
UPEC	Uropathogenic *E. coli*
UTI	Urinary tract infection
AMR	Antimicrobial resistance
DEC	Diarrheagenic *E. coli*
CF	Colonization factor
LT	Heat-labile
ST	Heat-stable
SEM	Scanning electron microscope
EOP	Efficiency of plating
MOI	Multiplicity of infection
HUS	Hemolytic uremic syndrome
DAEC	Diffusely adherent *E. coli*
WHO	World health organization
CDS	Coding sequence
MCP	Major capsid protein
BWA	Burrow-wheeler alignment
BAM	Binary alignment map
BBMap	Big blue mutation analysis page

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Abstract

Escherichia coli is a common fecal coliform, facultative aerobic, gram-negative bacterium. Pathogenic strains of such microbe have evolved to cause diarrhea, urinary tract infection and septicemias. The emergences of antibiotic-resistance urged to find an alternative strategy. The use of lytic bacteriophages against the control of pathogenic *E. coli* in the clinics and different environmental setups (waste and drink water management) become an alternative therapy to antibiotic therapy. Thus, this study aimed to isolate and characterize the lytic bacteriophage from various sources in Addis Ababa and tested against antimicrobial resistant diahrrgenic *E. coli* strains and evaluates their therapeutic potentials under *invitro* conditions. A total of 14 samples were processed against six different diahrrgenic *E. coli* strains. Conventional culture and plaque analysis agar overlay method was conducted to recover lytic bacteriophage isolates. The phage isolates were characterized to determine their lytic effect, growth characteristics, host range activity and stability under different temperature and pH conditions. Phage isolates were identified by Scanning Electron Microscope (SEM), molecular techniques (PCR), and whole genome sequencing. Totally, 17 phages were recovered from 84 tested plates. Totally, 17 phages were recovered from 84 tested plates. Of the 17 phage isolates, 11(65%) were *Myoviridae*-like phages, 6 (35%) phage isolates were *Podoviridae* and *Siphoviridae* by morphology and PCR identification. Bacteriophage genome sequencing revealed that each bacteriophage has a linear double-stranded DNA genome. The GC content of phage genomes ranged from 43 to 54%, while their sizes ranged from 40,427 to 143,710 bp. The whole genome sequence analysis of 7 potent coliphages showed that phage isolates were taxonomically classified as 4 (57%) of *Myoviridae* phages and 3 (43%) of *Siphoviridae* phages. Based on the host range test, growth characteristics and stability test 7 potent phages were selected. These phages demonstrated better growth characteristics, including short latent periods, highest burst sizes, and wider host ranges, as well as thermal stability and the ability to survive in a wide range of pH levels. These phages' promising effect against AMR pathogens has raised the possibility of their use in biological control of bacterial infections.

Key words: Lytic bacteriophages**,** Diahrrgenic *E. coli*, *Myoviridae*, *Siphoviridae*, *Podoviridae*

1: Introduction

1.1. Background of the study

E. coli is a prominent fecal coliform facultative aerobic gram-negative rod-shaped bacterium. Pathogenic strains have evolved to cause human and animal diseases leading to human and animal morbidity and mortality all over the world. As a result, a study on pathogenic *E. coli* in humans, animals, food, and the environment is extensive. Both developed and developing nations frequently experience outbreaks, which can occasionally have fatal consequences (Croxen *et al*., 2013).

Despite the fact that the majorities of *E. coli* are thought to be relatively harmless and are part of the normal flora of the intestine, certain strains have evolved pathogenicity mechanisms, and can cause diseases in human and animals. Intestinal (diarrhea) and extraintestinal (urinary tract infection (UTI), septicemia, pneumonia, and meningitis) disorders are examples (Cabal *et al*., 2016; Millar *et al*., 2016).

There are seven diahrrgenic *E. coli* serotypes, these are: Enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC/O157), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), diffusely adherent *E. coli* (DAEC) and Shiga-toxin generating *E. coli* (Non-O157 STEC) serovar which is mostly related with EHEC, but different pathogenic characteristics. These have been identified as *E. coli* pathotypes based on pathogenicity profiles virulence factors, clinical disease, and phylogenetic profile. The adherent invasive *E. coli* (AIEC), which is typically linked to inflammatory bowel disease (IBD) is recently evolved (Mora *et al*., 2011; Agus e*t al*., 2014; Conte *et al*., 2014).

The treatment of those pathogenic *E. coli* is threated by their antimicrobial resistance (AMR). There are growing reviews of *E. coli* AMR all around the globe. Because these organisms are naturally found in high concentrations in human and animal feces, when the feces are disposed of and reach drainage systems, where already overused or misused antibiotics released from clinical aspects and agricultural run-offs predominate, coliforms are under pressure and AMR strains emerge (Akhwale *et al*., 2019; Bhetwal, *et al*., 2017).

The persistence of AMR microbes not only puts selective pressure on nearby exposed bacteria, but it also increases opportunities for resistance genes to be transferred to nearby susceptible bacteria (via horizontal gene transfer using plasmids, transposons, or integrons) and eventually

into the human food chain (Ahn and Biswas, 2014). This allows them to thrive in the absence of inhibition or toxicity. As a result, more emphasis is needed on developing antimicrobials that can combat such circumstances.

The spread of multidrug resistance among bacterial strains has posed a substantial threat to public health in the treatment of infectious diseases on a global scale (Kutateladze and Adamia, 2010; Yu, 2013). Even though there is a high global rise in antibiotic-resistant bacteria, the search for new antibiotics has slowed in recent decades (Haque *et al.*, 2020). As a result, continuous efforts to discover promising alternative therapies for treatment of infectious diseases and proscribing the emergence and unfold of antibiotic resistance amongst microorganisms are required (Ahn and Biswas, 2014).

E. coli is one of the 12 most hazardous superbugs that pose a health risk, according to the World Health Organization (Puvača and de Llanos, 2021). Despite the fact that various researchers around the world are striving to combat it by developing new medications and other options, they have failed to solve the problem. In the current situation, alternative antimicrobials to substitute antibiotics for treating a wide range of bacterial infections are urgently needed. Scientists' interest in phages as an alternative medicine was rekindled as a result of this. The use of lytic bacteriophage as a means of combating pathogenic bacterial contamination is an alternate technique (Amarillas *et al.*, 2017).

Phage therapy is the therapeutic application of bacteriophages (natural bacteria predators) to treat bacterial illnesses (De vos and Pirnay, 2015). Phage therapy has regained popularity as an alternative to antibiotics. Natural phage communities are presumed to be the reservoirs of significant uncharacterized genetic variation on Earth (Hambly and Suttle, 2005). Complete phage genomes make it easier to research phage evolution, relationships, biodiversity, biogeography, and the discovery of new phage taxa (Akhwale *et al.*, 2019). Understanding phage biology can lead to a wide range of applications, including innovative nanotechnologies, bacterial detection techniques, and industrial-scale biological control of harmful bacteria (Monka, 2010).

Individual phages or a cocktail of phages are often used in therapeutic techniques to specifically infect and kill target microorganisms. Bacteriophages have the ability to influence pathogenic bacteria without inflicting collateral damage to commensal microbiota due to their limited

species-specific host range (Niu *et al.*, 2014; Tomat *et al.*, 2013; Khan Mirzaei and Nilsson, 2015).

Furthermore, phages are the most "safe" and "green" creatures that can be used in clinical settings (Liu *et al*., 2021, Podlacha *et al.*, 2021). Apart from their ability to self-replicate and destroy antibiotic-resistant bacteria, they are abundant in nature and have excellent selectivity, causing minimal damage to regular flora (Koskella and Meaden, 2013). Unlike antibiotics, which lose potency over time after administration, phages continue to reproduce and infect the target bacteria (Kutateladze and Adamia, 2010). When compared to antibiotics, phage resistance is not transferable. In addition, as compared to the development of a new antibiotic, the isolation of a new phage is comparatively quick and inexpensive. The use of bacteriophages as antimicrobial agents necessitates a thorough understanding of phage biology in order to assess their potential as an alternative effective technique for the control of pathogenic bacteria (Sillankorva *et al*., 2010; Ateba and Akindolire, 2019).

Over 95 % of the lytic phages reported in the scientific literature belong to the order *Caudovirales* (tailed phages) (Guttman *et al*., 2005; Bebeacua *et al*., 2013) that have double stranded linear DNA. This order mainly includes *myoviridae* (T4-like phages), *podoviridae* (T7-like phages) and *siphoviridae* (T5-like phages) family of bacteriophages that are actively involved in the lysis and destruction of bacteria. However, lytic phages are not limited to the order C*audovirales*, and there are many bacteriophages exist in the earth like single stranded linear RNA MS2 phages as well as many others that are mostly temperate or lysogenic (Bolocan *et al.*, 2016).

Phage therapy or use of bacteriophages as therapeutic agents for eradicating bacterial infections was first introduced by preliminary study of Twort and d'Herelle in the beginning of the 20th century (Twort, 1915; d'Herelle, 1917). Keeping this in view, present investigation was carried out to isolate pure phage strain against diahrrgenic *E. coli* strains from various sources.

1.2. Statement of the problem

Pathogenic *E. coli* strains are a major cause of sickness and mortality around the world, and their prevalence is on the rise. These bacteria are becoming increasingly common in many nations and are posing a severe public health threat (Croxen *et al*., 2013). Furthermore, the globalization of food marketing and distribution exacerbates the risk of sickness connected with this pathogenic

E. coli. Antibiotic treatments for *E. coli* frequently result in the spread of multidrug resistance (MDR) in the clinics. Antibiotics are becoming less and less efficient as pathogenic *E. coli* develop resistance to them as a result of widespread clinical, veterinary, and agricultural use (Puvača and Frutos, 2021).

AMR among pathogenic *E. coli* strains is steadily increasing in Ethiopia (Belete *et al*., 2022; Adugna and Sivalingam, 2022). Despite the growing threat of antimicrobial resistance in our country, little attention is paid to its management, and novel alternatives have yet to be explored. Furthermore, as a biological resource-rich country with a diverse ranges of environmental elements, investigating lytic phages in our capital city environment could be a potential solution to combating antibiotic resistance. However, there is a few study on the individual bacteriophages that infect *E. coli* strains has documented the extraction of phages from various environmental samples in Ethiopia and Addis Ababa in particular.

Thus, identifying efficient bio-control medicines against pathogenic *E. coli* requires the isolation and description of novel phages. Phages can be found in any environment where bacteria exist. As a result, they're found in a variety of samples, including contaminated rivers, animal faces, hospital fluid wastes, food reservoirs, and human faces. Therapeutic phages have advantages over antibiotics. Phages, for example, minimize damage to normal flora because to their limited host range to non-pathogenic strains; phage resistance is neither worldwide nor transferable, unlike antibiotic resistance; and the isolation of a new phage is comparatively quick and inexpensive compared to the discovery of novel antibiotics (Hassan *et al*., 2021; Gordillo Altamirano and Barr, 2019).

1.3. Objectives

1.3.1. General objective

The general objective of this study was to isolate and characterize lytic bacteriophages against AMR diarrheagenic *E. coli* strains from various sources

1.3.2. Specific objectives

- To isolate and characterize lytic bacteriophages specific to multidrug resistant diarrheagenic *E. coli* stains from environmental samples.
- To examine the therapeutic potential of the isolated phages against multidrug resistant diarrheagenic *E. coli* strains.

- To characterize the phages in to morphological, molecular, family, genus level and to develop phylogenetic trees.
- To determine some phage essential genes from selected phages sequence data.

2: Literature review

2.1. Pathogenic *E. coli*

E. coli is a gram-negative, rod-formed bacterium which is usually determined within the lower gut of warm-blooded organisms (endotherms). According to Vogt and Dippold (2005), the majority of *E. coli* strains are not harmful, but some can lead to severe food poisoning, septic shock, meningitis, or urinary tract infections in humans. The pathogenic strains of *E. coli*, in contrast to the normal flora, create toxins and other virulence factors that allow them to live in areas of the body where *E. coli* would not ordinarily reside and cause harm to host cells. Only the pathogens have the virulence genes that encode these harmful features (Mobley *et al*., 2004).

Oral and fecal pathways are common routes for the spread of pathogenic *E. coli*. Unsanitary food preparation, agricultural contamination from manure fertilizer, crop irrigation using contaminated grey water or raw sewage, feral pigs on cropland, and direct ingestion of sewage-contaminated water are common modes of transmission (Jin *et al*., 2013). The main sources of *E. coli* O157:H7 are dairy and beef cattle, which can harbor the infection asymptomatically and excrete it in their feces (Ferens and Hovde, 2011). Cucumber, raw ground beef, raw spinach or seed sprouts, raw milk, unpasteurized juice, unpasteurized cheese, and foods contaminated by infected food workers through the fecal–oral pathway are food products linked to *E. coli* outbreaks.

According to Eckburg *et al*. (2005), *E. coli* and similar bacteria make up approximately 0.1% of the gut flora. The main route via which pathogenic strains of the bacterium cause illness is fecal-oral. Because they can only exist outside of the body for a brief period of time, cells are perfect indicator organisms to check environmental samples for the presence of feces (Thompson and Andrea, 2007). The bacterium has been the subject of extensive research for more than 60 years and is also easily and affordably produced in a laboratory setting. Vibrant strains of *E. coli* can cause a variety of illnesses in both humans and domestic animals. Neonatal meningitis, urinary tract infections, and gastroenteritis among these illnesses. Rarely, virulent strains can also cause

extraintestinal diseases such as gram-negative pneumonia, peritonitis, mastitis, septicemia, and hemolytic-uremic syndrome (Todar, 2007).

2.1.1. Diarrheagenic Gastroenteritis

Normally, *E. coli* stays within the intestinal lumen without causing any harm. However, in individuals who are immunocompromised or have weakened gastrointestinal barriers, even the non-pathogenic strains of *E. coli* can lead to infections. Moreover, a number of highly adapted E. Coli clones that have evolved to cause a wide range of diseases in humans and animals can infect even the healthiest people. Pathogenic E. Coli infections can spread throughout the body or just affect mucosal surfaces. The three primary clinical syndromes resulting from infections produced by intrinsically harmful strains of Escherichia coli include urinary tract infections, sepsis/meningitis, and enteric/diarrheal disorders.

In children and diary calves, diarrheagenic *E. coli* (DEC) is a major cause of acute gastroenteritis. According to Liu *et al*. (2016), acute gastroenteritis ranks fourth in the world for children under the age of five years old in terms of mortality and is a frequent cause of morbidity in both developing and developed nations during childhood. *E. coli* pathotypes that are diarrheagenic (DEC) are distinguished from non-pathogenic and extraintestinal pathogenic (ExPEC) based on virulence factors found in their genomes and phenotypic traits. According to Kaper *et al*. (2004), the three types of ExPEC are neonatal meningitis-associated *E. coli* (NMEC), sepsis-inflicting *E. coli* (SEPEC), and uropathogenic *E. coli* (UPEC).

The DEC group has been reexamined as seven distinct pathotypes by pathogenomics and phenotypic classification. These pathotypes are defined by their essential virulence genes and differential features, which include enteropathogenic *E. coli* (EPEC), Shiga toxin producing *E. coli* (Non-O157/STEC), enterohemorrhagic *E. coli* (EHEC/O157), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and *E. coli* that adheres diffusely (DAEC) (Croxen *et al.*, 2013).

i) Enteropathogenic E. coli (EPEC)

EPEC was the first pathotype of *E. coli* to be described. For many years, O:H serotyping was the only way to identify this pathotype and the mechanisms underlying EPEC-induced diarrhea remained a mystery. However, since 1979, numerous advances in understanding of the

pathogenesis of EPEC diarrhea have been made, such that EPEC is now among the best understood of all the pathogenic *E. coli*.

EPEC infections are linked to a distinct intestinal histopathology. Known as "attaching and effacing" (A/E) infections, these bacteria form close attachments to intestinal epithelial cells and induce dramatic cytoskeletal alterations, such as the accumulation of polymerized actin directly beneath the adherent bacteria. Microvilli effacement and close adherence between the bacteria and the epithelial cell membrane characterize this remarkable phenotype. A whole family of enteric pathogens that cause A/E lesions on epithelial cells has its origins in EPEC strains.

ii) E. coli that is enterohemorrhagic (EHEC/O157)

An *E. coli* strain EHEC is responsible for producing the Shiga toxin. The intestinal wall's lining is harmed by the toxin. EHEC was identified as the source of bloody diarrhea in 1982, which occurred when a person consumed raw or undercooked hamburger meat tainted with the bacteria. Since then, unpasteurized milk, unsalted apple juice or cider, salami, spinach, lettuce, sprouts, well water, and surface water areas that animals frequently visit have all been connected to EHEC outbreaks. According to Gomes *et al*. (2016), outbreaks have also been linked to animals at petting zoos and daycare facilities.

Hemorrhagic colitis, hemolytic-uremic syndrome (HUS), diarrhea, and hemorrhagic diarrhea are all caused by the enterohemorrhagic bacterial strain *E. coli* O157: H7, which is also a significant food source. Three to eight days following infection are when EHEC symptoms start to appear. They include diarrhea that can turn into bloody diarrhea (hemorrhagic colitis) and abdominal pain. Fever and vomiting are possible side effects (Welinder-Olsson & Kaijser, 2005). The primary means of transmission for EHEC pathogens is eating contaminated food. Raw or undercooked meat, raw (unpasteurized) dairy products, and occasionally raw vegetable products are the main food products affected, as the digestive tract of cattle serves as the primary natural reservoir of EHEC. Such animals may also become contaminated when they are milked or killed. Another possible source of contamination is ruminant feces in the ground, in manure, or in water (ponds and streams). Although uncommon, EHEC can also spread from person to person. Most often, it is seen in a community or family setting (Miko *et al*., 2009).

iii) E. coli that is enterotoxigenic (ETEC)

The bacterium known as enterotoxigenic *E. coli* (ETEC) is a pathogenic version or pathotype of *E. coli* that produces heat-labile (LT) and heat-stable (ST) enterotoxins that cause diarrhea. Nearly fifty years have passed since these bacteria were first linked to cholera-like watery diarrhea (Sack, 2011). Despite this, the bacteria continue to pose a serious threat to global health, especially to young children living in low-resource areas of the world. Here, it is estimated that more than a billion cases of diarrheal illness occur in children under five each year, with hundreds of millions of episodes of diarrhea linked to ETEC alone (Khalil *et al*., 2018). Watery diarrhea is caused by ETEC and can vary in severity from a mild self-limiting illness to a severe purging illness. Symptoms of an ETEC infection can include headaches, cramping in the stomach, vomiting, and, in rare instances, a low-grade fever. According to some research, ETEC infection may have some side effects, including an increased risk of childhood stunting from malnourishment and immunological deficiencies, an increased risk of getting other infectious diseases, and even an impact on cognitive development (Troeger *et al*., 2018). Moreover, there is a connection between post-infectious irritable bowel syndrome and traveler's diarrhea (Steffe *et al.*, 2014). Food or water tainted with human or animal excrement is how it spreads. Hand washing with soap on a regular basis and avoiding or properly preparing foods and beverages that may be contaminated with the bacteria are two ways to prevent infection.

IV. Enter invasive E.coli (EIEC)

EIEC, which shares a close kinship with Shigella, is believed to induce watery diarrhea by invading the colon's epithelial cells. They attach to and penetrate intestinal cells using adhesin proteins, making them extremely invasive. Although they don't produce any toxins, they mechanically destroy intestinal wall cells, causing severe damage. A few minor biochemical tests separate EIEC from Shigella, but these pathotypes share important factors that contribute to their virulence (Van den Beld & Reubsaet, 2012). It is believed that EIEC infection is an example of inflammatory colitis, even though many patients appear to have small bowel syndrome with secretory symptoms. Abdominal cramps, malaise, tenesmus, and occasionally fever are among the symptoms. Dysentery or bloody diarrhea is an unusual consequence (Schuetz, 2019).

v) E. coli that is enteroaggregative

A pathotype of E. coli known as enteroaggregative E. coli (EAEC) causes both acute and chronic diarrhea in both developed and developing nations. Additionally, they might result in UTIs. According to Jensen *et al*. (2014), EAEC are identified by their "stacked-brick" pattern of adhesion to the HEp-2 human laryngeal epithelial cell line. It is now accepted that EAEC is a newly discovered enteric pathogen. Specifically, EAEC are known to be a common cause of diarrhea in pediatric populations and the second most common cause of traveler's diarrhea, behind enterotoxigenic E. coli. Additionally, it has been linked to long-term infections in the latter group as well as in immunocompromised hosts, including those with HIV (Huang *et al*., 2006). Intestinal infections are brought on by EAEC; these infections can cause fever, diarrhea, and stomach pain. The majority of severe cases may result in kidney failure, dehydration, or bloody diarrhea (Jensen *et al*., 2014).

iv) Shiga toxin generating E. coli (Non-O157 STEC)

The most well-known serotype of Shiga toxin-producing *E. coli* (STEC) is probably *E. coli* O157:H7 (EHEC), but non-O157 STEC refers to the at least 150 other serotypes of STEC that can infect humans and animals. For a long while, *E. coli* O157:H7 was linked to the majority of STEC outbreaks that were known to occur. That was mostly due to the ease with which *E. coli* O157 could be found in stool cultures ordered by medical professionals and carried out in clinical laboratories. Though the virulence of non-O157 STEC is highly variable, some strains can undoubtedly be just as dangerous as O157, even having the capacity to cause hemolytic uremic syndrome (HUS) and even death. All non-O157 STEC pathogenic strains have the potential to result in bloody diarrhea and hospitalization. But only strains carrying Stx2 (as opposed to only strains carrying Stx1) usually cause HUS. The sources and risk factors of non-O157 STEC outbreaks are often comparable to those of *E. coli* O157:H7. The main means of transmission are foodborne, although it can also spread through contact with animals, water, and other people.

Vii) E. coli that is diffusely adherent (DAEC)

One group of *E. coli* that has been linked to diarrhea is diffusely adherent *E. coli* (DEC). Their diffuse adherence pattern on HeLa or HEp-2 cultured epithelial cells is what distinguishes them (Servin, 2005). This adherence phenotype is caused by adhesins from the Afa/Dr family, which

are present in about 75% of DAEC. Much attention has been given to DAEC strains possessing Afa/Dr adhesions, but only those that were positive to daaC probe, which recognizes a conserved region from Afa/Dr adhesins operons, were found in higher frequency in diarrhoeic patients than asymptomatic controls (Mansan-Almeida *et al*., 2013).

Table-1: Major Pathotypes of *E. coli* that cause diarrhea (Cabrera-Sosa and Ochoa, 2020)

No	**Strains**	**Diarrhea pattern**	**Antecedent condition**
1	Enteropathogenic *E. coli* (EPEC)	Watery	Can cause diarrhea outbreaks in newborn nurseries
2	Enterotoxigenic *E. coli* (ETEC)	Watery	Produces a toxin that acts on the intestinal lining, and is the most common cause of traveler's diarrhea
3	Enterohemorrhagic *E. coli* (O157/EHEC)	Bloody/non-bloody	A type of EHEC on which Bloody diarrhea and hemolytic uremic syndrome (anemia and kidney failure) can be brought on by *E. coli* O157:H7.
4	Entero invasive *E. coli* (EIEC)	Bloody/non-bloody	Invades (passes into) the intestinal wall to produce severe diarrhea.
5	Enteroaggregative *E. coli* (EAEC)	Watery	Can cause acute and chronic (long-lasting) diarrhea in children.
6	Shiga toxin producing (non-O157 STEC)	Bloody/non-bloody	Causes of acute diarrhea, dysentery, and HUS.
7	Diffusely adherent *E. coli* (DAEC)	Watery diarrhea	Leads to diarrhea, stomach pain and cramps and low-grade fever

2.1.2. Non-Diarrheal pathogenic *E. coli*

Extraintestinal pathogenic *E. coli* encompass several well-described pathogens, i.e., uropathogenic *E. coli* (UPEC) that causes sepsis and urinary tract infections, and neonatal meningitis *E. coli* (NMEC) that causes sepsis and brain infections. These subspecies are important pathogens and implicated in the global spread of antibiotic resistance genes.

i) Uropathogenic E. coli (UPEC)

Roughly 90% of urinary tract infections (UTI) in people with normal anatomy are caused by uropathogenic E. coli (UPEC) (Todar, 2007). Fecal bacteria colonize the urethra and travel up the urinary tract to the bladder, kidneys (causing pyelonephritis), or, in the case of males, the prostate in ascending infections. Women are 14 times more likely than men to experience an ascending UTI due to their shorter urethras (Nicolle, 2008). P fimbriae, or pyelonephritis-associated pili, are used by uropathogenic *E. coli* to bind urinary tract urothelial cells and colonize the bladder. This receptor is absent in about 1% of the human population, and its presence determines whether a person is susceptible to urinary tract infections caused by Escherichia coli or not. Alpha- and beta-hemolysins produced by uropathogenic *E. coli* that cause lysis of urinary tract cells (Todar, 2007).

The Dr family of adhesins, which is especially linked to cystitis and pregnancy-associated pyelonephritis, is another virulence factor frequently found in UPEC. The Dr blood group antigen (Dra), which is found on the decay accelerating factor (DAF) on erythrocytes and other cell types, is bound by the Dr adhesins. According to Justice et al. (2006), the Dr adhesins cause the development of lengthy cellular extensions that encircle the bacteria, activating multiple signal transduction cascades along the way, including PI-3 kinase. By infiltrating superficial umbrella cells, UPEC can circumvent the body's innate immune defenses, such as the complement system, and create intracellular bacterial communities (IBCs). Additionally, they are capable of forming capsular polysaccharides, which aid in the formation of biofilms and the K antigen. Biofilm-generating *E. coli* is frequently the cause of persistent urinary tract infections because it is resistant to immune factors and antibiotic treatment. K antigen synthesising upper urinary tract infections caused by *E. coli* are frequent (Ehrlich *et al*., 2005).

ii) Meningitis/sepsis-associated E. coli (MNEC)

Gram-negative neonatal meningitis is most frequently caused by this *E. coli* pathotype, which has a 15–40% case fatality rate and causes severe neurological defects in many survivors1. While infections by gram-positive organisms seem to be declining, the incidence of infants with early onset sepsis caused by *E. coli* infections appears to be increasing. Meningitis-causing *E. coli* strains are primarily composed of K1 capsule strains, accounting for 80% of the strains, and are only represented by a small number of O serogroups, similar to *E. coli* pathotypes with well-established genetic bases for virulence. An intriguing distinction between MNEC and *E. coli*

strains that cause urinary tract or intestinal infections is that, while the latter strains are easily spread through urine or feces, infection of the central nervous system does not seem to provide a clear advantage for the selection and spread of highly pathogenic MNEC strains. (Stoll *et al*., 2002).

These strains, found in the mother's vagina, colonize the newborn's intestines and cause bacteremia, which eventually results in meningitis. Additionally, the lack of maternal IgM antibodies (which only transfer IgG across the placenta because FcRn only mediates the transfer of IgG) combined with the body's recognition of the K1 antigen as self due to its similarity to cerebral glycopeptides causes severe meningitis in newborns. These strains, found in the mother's vagina, colonize the newborn's intestines and cause bacteremia, which eventually results in meningitis. Additionally, the lack of maternal IgM antibodies (which only transfer IgG across the placenta because FcRn only mediates the transfer of IgG) combined with the body's recognition of the K1 antigen as self due to its similarity to cerebral glycopeptides causes severe meningitis in newborns (Croxen and Finlay, 2010) .

2.2. *E. coli* infection diagnosis

Stool cultures are used to diagnose infectious pathogenic *E. coli* and identify antimicrobial resistance, with antibiotic sensitivity testing coming next. The bacteria may be cultured in order to confirm the diagnosis and identify specific toxins, such as those produced by *E. coli* O157:H7. To culture gastrointestinal pathogens, two days is the minimum and several weeks is the maximum. Although some human pathogens cannot be cultured, stool culture has varying rates of sensitivity (true positive) and specificity (true negative). Antimicrobial resistance testing takes an extra 12 to 24 hours to complete for culture-positive samples (Gould, 2010).

Point of concern right now molecular diagnostic tests are much quicker at identifying *E. coli* and antimicrobial resistance in the strains that are identified than culture and sensitivity testing (Zhang *et al*., 2021). Microarray-based platforms with high sensitivity and specificity can identify particular pathogenic strains of Escherichia coli and AMR genes unique to that strain in two hours or less; the size of the test panel, which includes all pathogens and antimicrobial resistance genes, is limited. Newer platforms for the identification and diagnostics of pathogenic *E. coli* based on PCR and sequencing are currently being developed to overcome the limitations of available molecular diagnostic technologies and culture (Maurer, 2011).

2.3. Antibiotic therapy and resistance

Bacterial infections are typically handled with antibiotics. However, there are significant differences in the antibiotic sensitivity of various *E. coli* strains. Since *E. coli* are gram-negative bacteria, they are immune to many antibiotics that work well against gram-positive bacteria. Amoxicillin and other semisynthetic penicillins, numerous cephalosporins, carbapenems, aztreonam, trimethoprim-sulfamethoxazole, ciprofloxacin, nitrofurantoin, and aminoglycosides are among the antibiotics that can be used to treat an *E. coli* infection. The issue of antibiotic resistance is getting worse. A portion of this can be attributed to human antibiotic overuse, but a portion is most likely caused by the use of antibiotics in animal feed as growth promoters (Johnson *et al*., 2006). "On the order of 10−5 per genome per generation, which is 1,000 times as high as previous estimates," according to a study, is the rate of adaptive mutations in *E. coli*. This finding may be important for the management and study of bacterial antibiotic resistance (Perfeito *et al*., 2007).

resistant to antibiotics Moreover, *E. coli* may use a process known as horizontal gene transfer to transfer the antibiotic resistance genes to other bacterial species, including Staphylococcus aureus. *E. coli* frequently carries several drug resistance plasmids, which it can easily spread to other species when under stress. Plasmids from and to other bacteria can be accepted and transferred by *E. coli* due to species mixing in the intestines. Consequently, *E. coli* and other enterobacteria are significant sources of antibiotic resistance that can be transferred. (Salyers *et al*., 2004). Since the prevalence of bacterial strains that produce extended-spectrum beta-lactamases has increased in recent decades, resistance to beta-lactam antibiotics has become a particular issue. These beta-lactamase make many, if not all, of the penicillins and cephalosporins ineffective as therapy. Extended-spectrum beta-lactamase–producing *E. coli* (ESBL *E. coli*) are highly resistant to an array of antibiotics, and infections by these strains are difficult to treat. In many instances, only two oral antibiotics and a very limited group of intravenous antibiotics remain effective (Paterson and Bonomo, 2005).

2.4. Phage therapy

2.4.1. Fundamentals of phage biology

Phages are non-living biological entities that are simple yet highly diverse. They are made of protein capsids containing either DNA or RNA (**Figure 1**). Phages are naturally occurring bacterial parasites that are dependent on their bacterial host for survival because they are unable to reproduce on their own and are therefore non-living.. Generally speaking, phages attach themselves to particular receptors on the surface of the bacterial cell, inject their genetic material into the host cell, and either integrate this material into the bacterial genome (temperate phages reproduce vertically from mother to daughter cell) or use the bacterial replication machinery to produce the next generation of phage progeny (lytic phages), which lyse the destination cell. When the number of phage progeny reaches a critical mass, which varies based on the environment and can range from a few to over 1000 viral particles, the lytic proteins activate and hydrolyze the peptidoglycan cell wall, releasing new phage to restart the lytic cycle (Weinbauer, 2004).

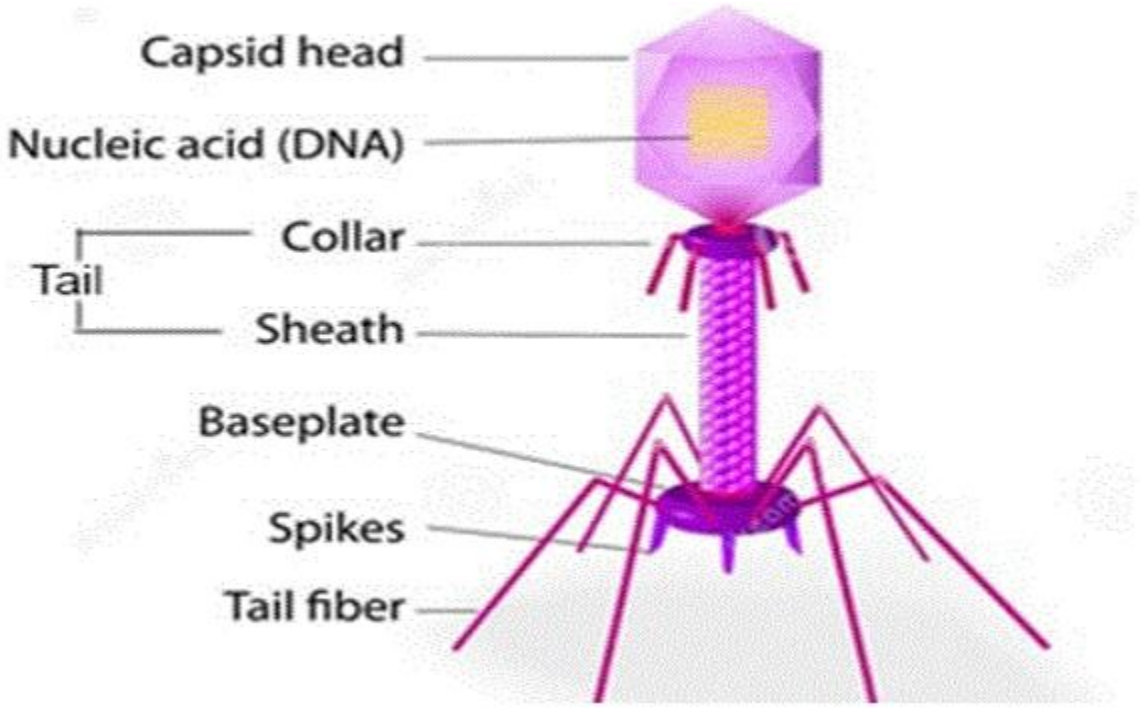


Figure 1: Typical bacteriophage structure (Mansour, 2017)

The majority of phages exhibit infectious properties solely to bacteria harboring their corresponding receptor, thereby effectively defining the host range of lytic phages (*Rakhuba et*

al., 2010). Phages differ in their host specificity; some are strain-specific, while others have shown the ability to infect a variety of bacterial strains and even genera (Motlagh *et al*., 2016). Bacteria have developed a multitude of defense mechanisms against lytic phage infection, and phages possess an equally remarkable array of defense mechanisms against this resistance. The integration of phage DNA into the clustered regularly interspaced palindromic repeats/CRISPR associated system (CRISPR/Cas) system and the alteration or loss of receptors in bacteria are examples of this (Labrie et al., 2010). For phages, this can include the recognition of new or altered receptors and anti-CRISPR genes. The two orders of lytic phages that are most frequently linked to human pathogens and the gut microbiota are *Microviridae*, which are tailless single-stranded DNA viruses, and Caudovirales, also referred to as "tailed phages" because they have double-stranded DNA genomes(Minot *et al*., 2011).

Lysogenic phages incorporate their genetic material into the bacterial chromosome as an endogenous prophage, as opposed to lytic phages (**Figure 2**). The bacterial lysogen then multiplies the prophage with each cell division. The lytic cycle and the release of phage progeny into the environment can be initiated by environmental stressors acting on the bacterial host, which can also induce the lysogenic phage from the latent prophage form. Prophage-encoded genes become accessible for transcription by the host upon integration of their genetic material into the bacterial genome (Czajkowski, 2019). Conventional phage therapy employs only lytic phages, which are inherently fatal to their bacterial host. Lithic phages are used in "phage cocktails," which are preparations made up of several phages that have been shown to be

effective in vitro against the pathogen of interest.

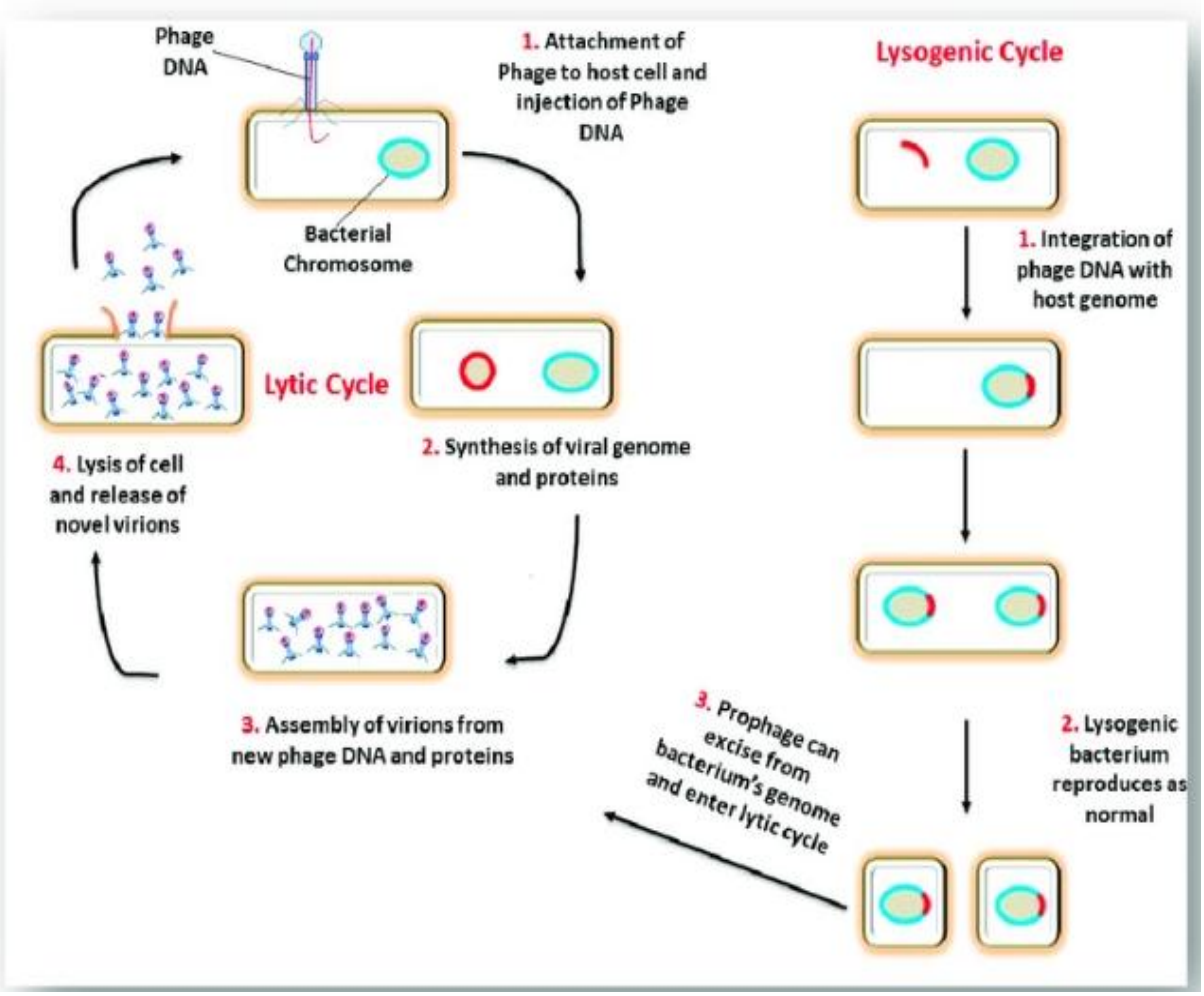


Figure 2: Bacteriophage lytic and lysogenic cycles (Stone *et al.*, 2019)

Lytic bacteriophages undergo the lytic cycle, in which the host is lysed and offspring bacteriophages are released into the surroundings. Bacteriophages specifically attach to the bacterial host on a receptor present on the surface of the bacteria and inject their genetic material into the cell. The host cell supplies the necessary molecular building blocks and enzymes to replicate the bacteriophage's genetic material and produce offspring bacteriophages. During the release of new viruses, bacteriophage enzymes participate in disrupting the structures of the host cell - cell lysis (Leprince *et al*., 2022). Bacteriophage-encoded proteins such as endolysin and holin lyse the host cell internally. Holins are small proteins that accumulate in the cytoplasmic membrane of the host and allow endolysin to degrade peptidoglycan, enabling the offspring

bacteriophages to escape. Subsequently, in the external environment, lytic bacteriophages can infect and destroy all nearby bacteria. The production of large numbers of offspring by lytic bacteriophages is an advantage when they are used in bacteriophage therapy.

2.4.2. Phage classifications

Bacteriophages are classified on the basis of their morphological structure and the genetic materials. The majorities of phages are tailed phages with dsDNA and are members of the *Caudovirales* order. The DNA translocase molecular motors that pack the chromosomes of tailed phages into procapsid are identical, but the DNA replication technique and the resulting genome end are different (Casjens and Gilcrease, 2009). The type and unique features of the receptor on the surface of the host cell determine how the phages are absorbed. Phages are primarily divided into virulent and temperate phages based on their life cycles. The lytic life cycle is followed by virulent phages. However, under certain circumstances, temperate phages can occasionally switch from the lysogenic to the lytic cycle. The two main proteins employed by lytic phages to kill their host cells are holin and lysine. The International Committee on Taxonomy of Viruses (ICTV) categorizes phages based on their appearance and nucleic acid (**Table 2**). The Caudovirales order, which contains the Myoviridae family with a contractile tail, the Podoviridae family with a short tail, and the Siphoviridae family with a non-contractile long tail, makes up about 96% of the documented bacteriophages. The same order includes filamentous, cubic, and polymorphic phages, which are divided into 10 distinct families and account for around 3.6% of all known bacteriophages (Kaliniene *et al*., 2017).

Table-2: Bacteriophage classification (Giri, 2021)

Family	**Morphology**	**Nucleic acid**	**Examples**
Myoviridae	Contractile tail, Non-enveloped	Linear dsDNA	*T4 virus, P1, P2, FO1, Jilinvirus, Vequintavirus*
Siphoviridae	Long on-contractile tail, Non-enveloped	Linear dsDNA	*Lambda, T5, N15, Kagunavirus, Dhillonvirus*
Podoviridae	Short non-contractile tail, Non-enveloped	Linear dsDNA	*T7 virus, P22, T3, SP6*
Tectiviridae	Isometric, Non-enveloped	Linear dsDNA	*PRD1*
Corticoviridae	Isometric, Non-enveloped	Circular dsDNA	*PM2*

Lipothrixviridae	Rod-shaped, Enveloped	Linear dsDNA	*Acidianus filamentous virus*
Plasmaviridae	Pleomorphic, Enveloped	Circular dsDNA	*Acholeplasma laidlawii virus L2*
Rudiviriade	Isometric, non-enveloped	Linear dsDNA	*SIRV1*
Fuselloviridae	Lemon-shaped, None-enveloped	Circular dsDNA	*SSV-1*
Inoviridae	Flamentous, Non-enveloped	Circular ssDNA	*M13*
Microviridae	Isometric, Non-enveloped	Circular ssDNA	*ΦX174*
Leviviridae	Isometric, Non-enveloped	Linear ssDNA	
Cystoviridae	Spherical, Enveloped	Segmented dsDNA	*Φ6*

2.4.3. Therapeutic application of phages

i) History of phage therapy

Bacteriophages were separately discovered in 1915 by Frederick William Twort and in 1917 by Felix d'Hérelle. Twort reported on a possible "ultra-microscopic virus" that he recovered from vaccinia virus cultures using "white micrococcus" cultures. Looking back, it appears that the lytic phages that were identified were actually bacteriophages that targeted *Staphylococcus* species that were present in a vaccinia virus culture.

On the other hand, Felix d'Hérelle isolated bacteriophage active against *Shigella bacillus* from the stools of patients recuperating from bacillary dysentery. According to Abedon *et al*. (2011), there were indications of bacteriophages' presence even prior to their discovery, a time frame known as bacteriophage prehistory. Felix d'Hérelle used bacteriophages in medicine for the first time in 1919 (Summers, 1999).

Worldwide, the use of bacteriophages to treat infectious disorders increased between the early 1920s and the late 1930s (d'Herelle, 1931). This phase of inflated expectations was succeeded by a period of waning excitement for phage therapy throughout much of the western world, which was followed by antibiotics replacing its usage following World War II and a shift in emphasis toward the use of phages as model genetic systems. Phage therapy was difficult to administer since, at the time of its discovery, relatively little was understood about phages. In fact, until they

were seen in the 1940s with the development of electron microscopy, their very existence was a matter of debate. Even though phage research did not cease in the former USSR, with the establishment of the Eliava Institute in Tbilissi, Georgia, and other nations like Poland (including its well-known Hirsfeld Institute in Wroclaw), phage therapy for animals was rediscovered in the English literature in the 1980s (Smith and Huggins, 1983).

Phages have been used therapeutically for a very long time in Eastern Europe and the former Soviet Union (Sulakvelidze and Morris, 2001). It was proposed that bacteriophages could be used to prevent and/or treat bacterial infections before the discovery and widespread use of antibiotics (Sulakvelidze *et al*., 2001). After antibiotics were discovered, phage therapy was widely abandoned due to a number of logistical and technical challenges. The English literature rediscovered phage therapy in animals in the 1980s, despite the fact that phage research was never abandoned in the former USSR. This was due to the establishment of the Eliava Institute in Tbilissi, Georgia, as well as other countries like Poland, which included the well-known Hirsfeld Institute in Wroclaw.

Eastern Europe and the former Soviet Union have long employed phages as medicinal agents (Sulakvelidze and Morris, 2001). It was proposed that bacteriophages had been used to prevent and/or treat bacterial infections before the discovery and widespread use of antibiotics (Sulakvelidze *et al*., 2001). Phage therapy was largely dropped after antibiotics were discovered because of numerous logistical and technological difficulties. But in a world before antibiotics, when the standard of care for treating bacterial illnesses was incredibly ineffective, phages, with their innate antibacterial qualities, might give much-needed hope. Poor use documentation and inconsistent results were major contributing factors to the phage therapy issue (Lin *et al*., 2017). However, there is still a lot of data supporting their clinical application at current time, and several historical innovations have been linked to significant phage therapy-influencing events (**Figure 3**).

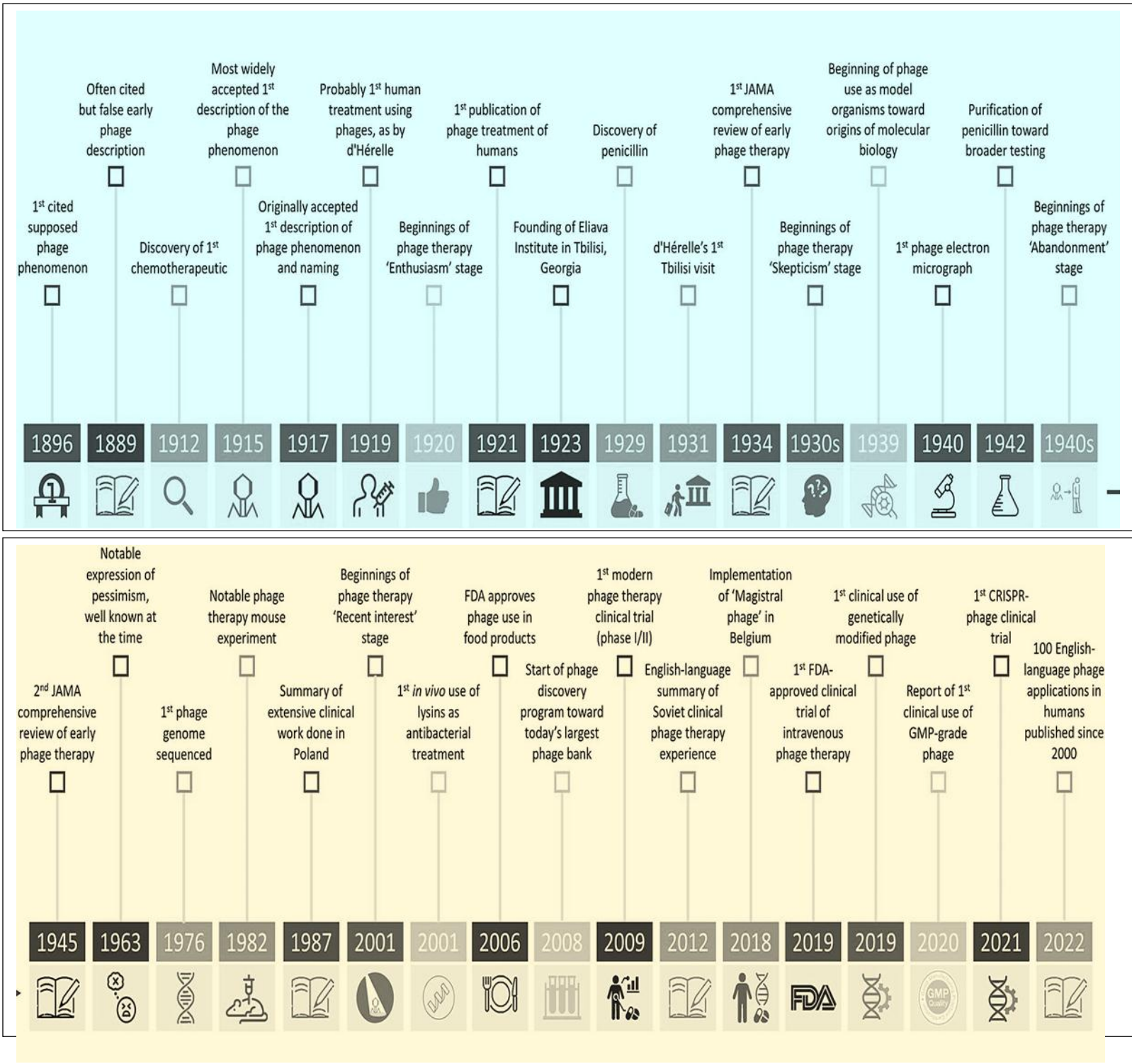


Figure 3: Major breakthroughs in the fields of phage therapy and science: JAMA, Journal of the American Medical Association; IV, intravenous; GMP, good manufacturing practice (Petrovic Fabijan *et al*., 2023).

ii) Phage therapy principles

The keys to antibacterial therapy success is building a library of different bacteriophages. According to Cui *et al*. (2017), bacteriophages exhibit stringent specificity and can range in host range from very narrow to broad. A range of virulent bacteriophages that can kill the same strain as well as different lytic bacteriophages that can kill different species should be kept in the library. The establishment of stringent enrollment criteria for bacteriophages intended for clinical use is crucial.

For bacteriophage therapy to be used in clinical settings, standard operating procedures for bacteriophage preparations, storage, and transportation must be developed. Several crucial steps for their application were included in the documented clinical trials of bacteriophage therapy: bacteriophage isolation, characterization, susceptibility testing, endotoxin removal, and production of relevant products. Monitoring of bacteria resistant to bacteriophages and assessment of bacteriophage pharmacokinetics during therapy were also aspects of a documented successful case of bacteriophage therapy (Chooley *et al*., 2017). Nevertheless, bacteriophage pharmacokinetics, endotoxin removal, and monitoring of bacteriophage-resistant bacteria were not part of a double-blind phase 1/2 trial. In the event of a bacterial infection in the gastrointestinal tract, it is important to prevent the phage from being neutralized by stomach acid when administering it orally. Additionally, other methods of treatment that have the potential to deactivate phages, such as antiseptic agents, should not be utilized in conjunction with phage preparations (Dedrick *et al*., 2019).

Before lytic phages can be used therapeutically in the West, more study is required to gather reliable pharmacological data about them, including thorough toxicological studies. Therapeutic phages were believed to kill their target bacteria by multiplying inside and lysing the host cell through a lytic cycle as part of their bactericidal function. However, later research showed that lytic and lysogenic phages have significantly different replication cycles and that not all phages replicate in the same way (**Figure-2**).

A lytic phage's lysis of host bacteria is a complex process involving a cascade of events involving several structural and regulatory genes, as demonstrated by the recent delineation of the full sequence of the T4 phage and years of elegant studies of the mechanism of T4 phage replication. Since the T4 phage is a typical lytic phage, it is conceivable that many therapeutic

phages work in a manner similar to this; however, it is also conceivable that some therapeutic phages possess particular, as yet undiscovered genes or mechanisms, which enable them to successfully lyse their target bacteria (Lin *et al*., 2017).

Phages have been given to humans orally, in tablet or liquid formulations (10^5 to 10^{11} PFU/dose); rectally; locally (skin, eye, ear, nasal mucosa, etc.), in tampons, rinses, and creams; as compared to the first four methods, there have been almost no reports of serious complications related to the use of aerosols or intrapleural injections, and intravenously (Golkar *et al*., 2013).

Two different phage therapy methods had been created at the time of the early 2000s phage therapy renaissance (Pirnay *et al*., 2011). These are one size fits all strategy and personalized phage therapy approaches. Broad-spectrum defined phage cocktails, which were intended to target the majority of bacteria thought to be responsible for several infectious disorders, were used in what might be considered the one-size-fits-all method (Adesanya, *et al*., 2020). These predetermined broad-spectrum phage mixtures were created, manufactured, and evaluated using the pharmaco-economic models that are now in use and were created to support "static" medications like antibiotics (Azeredo *et al*., 2021). However, true broad-spectrum phage cocktails that were effective against the majority of gram-positive and/or gram-negative bacteria frequently found in infectious disorders required a significant number of phages and proved to be extremely challenging to create. It was possible to create phage cocktails with a narrower spectrum that were only effective against one or a small number of bacterial species, to be utilized in specific situations and with the knowledge of the bacterial species that would be infected beforehand. Phages with very extensive host ranges have been isolated and characterized for various bacterial species, including *E. coli* (Vandersteegen *et al*., 2011).

In case of personalized one or more phages were chosen for the phage therapy concepts from phage banks or the environment, and they may have been modified (in vitro selection of phage mutants exhibiting increased infectivity) to more effectively infect the bacteria isolated from the patient's infection site. Large therapeutic phage banks were set up and maintained by several phage therapy facilities. These banks were frequently updated with new phages, expanding and adapting the bank's host range to the constantly shifting bacterial populations. As only the infecting bacterium is targeted, there is less selection pressure toward the development of bacterial phage resistance, making personalized phage therapy approaches potentially more

sustainable (Friman *et al*., 2016). They shipped bacterial strains and corresponding phages all over the world, which made them more intricate and logistically challenging than one-size-fits-all methods.

2.4.4. Phage therapy against *E. coli*

Phage treatment for *E.coli* involves identifying and isolating specific phages that can infect and kill the target *E. coli* strain. These phages are then purified and prepared for treatment. *E. coli* phages are commonly isolated from marine environments including fresh and salt water, sewage, hospital waste, human and animal faces, various food sources (such as vegetables, fruits, dairy, and fish), soil, plants, and other environmental sources (Kutateladze and Adamia, 2010). Additionally, phages are frequently found in human skin, vagina, mouth, and other parts of the gastrointestinal tract, where their population is thought to be approximately 1 × 1015 (Dalmasso *et al*., 2016).

Pathogenic *E. coli* bacteria are divided into two groups mainly according to the location of the disease: extraintestinal pathogenic *E. coli* (ExPEC) and enteric pathogenic *E. coli* (InPEC). Enteric pathogenic *E. coli* are generally divided into those causing diarrhea by expressing heat-labile or heat-stable toxin or Shiga toxin. Diarrheal diseases are one of the leading causes of death in children under the age of five, and the enteric pathogen One of the main worldwide causative groups of these infections is *E. coli.* Enteropathogenic *E. coli* (EPEC) and enterotoxigenic (ETEC) *E. coli* pathogens are endemic primarily in developing nations, and ETEC strains are the primary cause of diarrhea in visitors to these regions. Conversely, the enterohemohergic *E. coli* (EHEC) is the origin of major epidemics in the world, mainly affecting industrialized countries, responsible not only for diarrhea but also for serious clinical complications such as hemorrhagic colitis and hemolytic-uremic syndrome. Overall, the emergence of antibiotic-resistant strains, the annual increase in healthcare costs, the high incidence of travelers' diarrhea, and the increase in the number of episodes of the hemolytic-uremic syndrome have increased the need for effective treatments. The bacteriophage may be an alternative to antibiotics and have potential therapeutic capacities in treating disease *E. coil* (**Table 3**).

A mouse study by Chibani-Chennoufi *et al*. (2004) showed that broad host range T4-like coliphages for diarrhea-associated *E. coli* serotypes were isolated from stool samples from

patients with diarrhea infants and from ambient water samples. All of these isolated phages showed very efficient passage through the digestive tract of adult mice when added to drinking water. Viable phages were recovered from the feces in a dose-dependent manner. Just 10^3 PFU of phage per milliliter of drinking water was the lowest oral dose required for sustained fecal recovery. In conventional mice, orally administered phage remained confined to the gut lumen and, as expected for non-invasive phage, no histopathological changes were observed in the gut mucosa of phage-exposed animals. *E. coli* strains introduced into the gut of conventional mice and monitored for ampicillin-resistant colonies were successfully lysed in vivo by phage added to the drinking water (Chibani-Chennoufi *et al*., 2004).

According to Dissanayake *et al*. (2019), phage treatment reduced viable *E. coli* O157:H7 in infected mice with efficacy comparable to ampicillin therapy. But compared to ampicillin, the bacteriophage preparation had less of an impact on the gut microbiota. With no negative effects on the normal, and frequently beneficial, gut flora, lytic bacteriophage preparations can be used prophylactically or therapeutically to prevent or treat bacterial infections of the gastrointestinal tract, including those brought on by eating food contaminated with important foodborne bacterial pathogens like *L*. monocytogenes, *Salmonella* spp., and enterohemorrhagic *E. coli* (e.g., *E. coli* O157:H7) (Sulakvelidze and Kutter, 2004).

The reports from Dalmasso *et al*. (2016) demonstrate that three human intestinal phages showed promise as potential phage therapy. According to them, the three-phage cocktail completely inhibited the growth of *E. coli*. The phage cocktail also reduced biofilm formation and prevented the emergence of phage-resistant mutants that appeared in a single phage. Phage combined with ciprofloxacin alone or in cocktails inhibited the growth of *E. coli* and interrupted the emergence of resistant mutants. These new phage isolates are promising agents for the biological control of *E. coli* infections.The human gut is a natural reservoir of many phages with promising antibacterial properties (Dalmasso, 2014).

Bruttin and Brussow. (2005) used *E. coli* T4 phage to treat acute infectious bacterial diarrhea in adults and children. Fifteen healthy adult volunteers received a lower dose of *E. coli* T4 phage (103 PFU/ml), a higher dose of phage (105 PFU/ml), and a placebo via the drinking water. Fecal coliphage was detected in a dose-dependent manner in volunteers who were orally exposed to the phage. All volunteers receiving the highest dose of phage showed fecal phage 1 day after the

challenge; this rate was only± 50% in subjects receiving the lowest dose of phage. One week after a 2-day oral phage application, no fecal phage was detectable. Oral administration of the phage did not cause a reduction in the total number of *E. coli* in the stool. In addition, in the commensal population of *E. coli*. No side effects associated with the use of phage have been reported. They found that while the *E. coli* T4 phage is safe, its therapeutic efficacy is still controversial (Bruttin and Brussow, 2005).

However, a report by Sarker *et al*. (2015) indicated that oral administration of phage to hospitalized children with acute diarrhea did not improve diarrhea scores, possibly due to phage's insufficient ability to fight a broad spectrum of diarrhea or genetic variability of *E. coli*. In addition, it was not clear whether *E. coli* was actually responsible for diarrhea since the fecal samples were largely dominated by streptococci. Reduced efficiency of the phage titers after passing through the gastric acid was identified as another possible reason for the failure of the assay. Beside this, possible differences between the fecal and intestinal physiological status of *E. coli* and a low titer of the fecal pathogen could have prevented *E. coli* phage replication. These results confirm that much more knowledge of phage-bacteria interactions in vivo is required if we are to develop effective phage therapy assays. On a positive note, the coliphages administered during the study passed the gut safely, which helped demonstrate the safety aspects of phage therapy (Sarker *et al*., 2015).

Table-3: Some reports of phage therapy in pathogenic *E. coli*

Diseases	Phage/s applied	Effectiveness	Reference
Urinary tract infection	Single-phage/T4	Bacterial inoculum rendered untreated mice 100% fatal; however, phage (MOI 60) saved all mice.	Nishikawa *et al*., 2008
Gastroenteritis	Single-phage/unspecified	Dysbiosis-related weight loss and behavioral abnormalities were found in rats given antibiotics alone or in combination, despite the absence of bacterial contamination in these groups.	Vahedi *et al*., 2018
Urinary tract infection	Single-phage/KEP10	Bacterial inoculum rendered infected, untreated mice 100% fatal; however, phage (MOI 60) saved 90% of the mice.	Nishikawa *et al*., 2008
Gastroenteritis	Single-phage/T4	Rats treated with phage outlived untreated rats by 83% to 0%.	Rastogi *et al.*, 2017

Lung infection	Single phage/536-P1	The phage saved 100% of the animals from death compared to 25% survival in infected, untreated controls; Reduction of mortality from 80% to 25% by adapted phages	Dufour *et al*., 2015
Systemic infection	Single-phage/K1 phages	Following the lowest treatment dose, K1 capsule-dependent phages produced a 6 log10 reduction (specimen unspecified) in comparison to K1 capsule-independent phages.	Bull *et al*., 2012
Gastroenteritis	Cocktail-phages/ EcD7, V18, SE40, SI3, CH1, Lm1, ST11	Mice not given any treatment had 104 cfu of bacteria per gram of stool, while mice given the phage had none.	Aleshkin *et al*., 2015
Gastroenteritis	Cocktail/ CLB_P1, CLB_P2, CLB_P3	Bacterial colonization in ileum-treated mice was 88% lower than in control mice, but by day 7 post-treatment, bacterial density had rebounded to levels similar in both groups.	Maura *et al*., 2012
Systemic infection	Single-phage/ $EC200^{PP}$	7-hour post-infection treatment results in 100% rescue and bacterial elimination in blood; 24-hour post-infection treatment results in 50% rescue.	Pouillot et al., 2012
Meningitis	Single-phage/ $EC200^{PP}$	100 of the 100 meningitis-induced death rats were saved after receiving treatment with 108 pfu 1 or 7 hours	Pouillot *et al.*, 2012

2.4.5. Challenges regarding phage therapies

Bacteriophages typically affect specific types of bacteria, and some only affect a few species, and therefore cannot attack all pathogenic strains of the same bacterial species (Hyman and Abedon, 2010). Although single-bacterium diseases can be effectively treated with bacteriophages, many infections reported in case studies involve multiple pathogenic bacteria. As a result, certain bacteriophages frequently struggle to produce the intended therapeutic outcome. The lysogenic phenomenon is caused by certain lysogenic phages that, once integrated with the host bacterium, are unable to lyse the host bacterium and prevent other phages from acting lytically on the host bacterium. When a virus is lysogenic, it replicates its genome from

the host DNA, either before or after joining the bacterial chromosome. In addition, there is a major concern that bacteriophages in the lysogenic state can also transmit toxins and antibiotic-resistance genes to bacteria (Carascal *et al*., 2022). The limits of phage therapy, therefore, lie in the stability of the phages in the preparation, the evolutionary resistance of the bacteria, the limited effect of the phages, and the difficulty of the screening methods.

i) Phage preparation stability

A promising phage therapies candidate really should have a long-life span; it should be stored in a preparation that provides activity without a significant decrease in phage titer during treatment and long-term storage, as such a decrease may adversely affect treatment outcome (Jault *et al*., 2019). However, the stability of phages in different preparations (e.g. liquids, gels, powders) is very variable, especially between different phage types. An alternative strategy to improve the durability of phages is to encapsulate them on various matrices such as liposomes, alginate, cellulose or other polymers. In vitro and in vivo studies demonstrated the ability of encapsulated phage to persist for a long time at low pH, improving the efficacy of oral administration in animal models (Vinner *et al*., 2019). Another issue with phage stability is the occurrence of spontaneous mutations in phage stocks stored for long periods or accumulated during phage production and production, which can affect viral fitness (Botka *et al*., 2019). Although difficult, it would be useful to predict the evolution of phages during production in order to establish a production process that minimizes the mutation rate in phage genomes (García *et al*., 2019).

ii) Evolving of bacterial phage resistance

One of the main problems with phage therapy is the possible emergence of bacteriophage-insensitive mutants (BIMs), which could impede the success of this therapy. In recent years, several studies have addressed the issue of bacterial phage resistance, showing that the emergence of phage-resistant mutants is common and almost inevitable (Oechslin., 2018). In most of these studies, bacterial phage resistance was caused by mutations in genes encoding phage receptors, which include lipopolysaccharides, outer membrane proteins, envelopes, flagella, pili, and others. Several animal models, human pilot studies, and case reports have all seen the emergence of phage-resistant variants in action. Bacterial phage resistance can be circumvented by different approaches (McCallin *et al*., 2019). The most typical is the phage combination, which preferentially targets various receptors and has complementary host ranges,

into a single preparation, commonly known as a phage cocktail. Such cocktails not only show greater coverage against a specific bacterial species but can also prevent BIM from occurring. Finally, the combination of phage with antibiotics or other antibacterial agents can also be used to avoid the development of bacterial resistance and improve therapeutic efficacy (Tagliaferri *et al*., 2019).

iii) Problems of phage screening techniques

Because of the increased selectivity of phage action, finding a phage that is preys a particular strain often requires screening large collections of phage. The most traditional method for detecting phage activity against a strain is the bilayer agar (DLA) method, in which multiple phages are plated on a bacterial carpet of interest (Kakasis and Panitsa, 2019). Depending on the growth rate of the specific target strain, it can take up to 48 hours to obtain results and therefore the DLA method is impractical in a therapeutic setting where rapid diagnosis is essential. High throughput and rapid screening methods are desirable to quickly identify phages capable of successfully infecting target strains. Many methods for the detection and quantification of phages by direct or indirect measurement have been developed, but few appear to be applicable in the clinical setting. Real-time PCR (qPCR) (Ly-Chatain *et al*., 2011), flow cytometry, surface plasmon resonance capacity (SPR), cellular respiration, and optical density kinetics have been developed for rapid and sensitive detection analysis and identification of infections, e.g. by detecting elevated concentrations of Phage. If phage therapy is to be widely used as a treatment option in the future, a simple and fast, high-throughput method should be developed and implemented in clinical and banking settings. The strict host effect and non-unique pharmaceutical properties of phage are also major limitations of phage therapies.

3: Materials and Methods

3.1. Description of study area and period

The study was conducted at selected areas of Addis Ababa namely, Kebena River, Akaki River, dairy farm sewages of Gulele, Shiromeda and Ferensay area as well as Tikur Anbesa Hospital fluid waste disposal. Samples were processed at health biotechnology laboratory, Institute of Biotechnology, Addis Ababa University from April 2022 to May 2023. Ethiopia's largest city and capital is Addis Ababa. Depending on elevation and predominant wind patterns, the city has a complex mix of highland climate zones with temperature variations of up to 10°C (50° F). Degrees, min, and seconds (DMS) latitude longitude coordinates for Addis Ababa are: 9° 0' 19.4436" N, 38° 45' 48.9996" E (**Figure 3**). The high elevation moderates' temperatures year-round and the city's position near the equator means that temperatures are very constant from month to month. The temperature in January ranges from 20°C (68°F) to 12°C (53°F). The area of the city increased from 85.73 square miles (222.04 square kilometers) in 1984 to 204.7 square miles (530.21 square kilometers) in 1994 to 2023. Addis Ababa had a population of 5,461,000 and a 4.46 percent annual growth rate in 2023 according to Macrotrends, Metro area population.

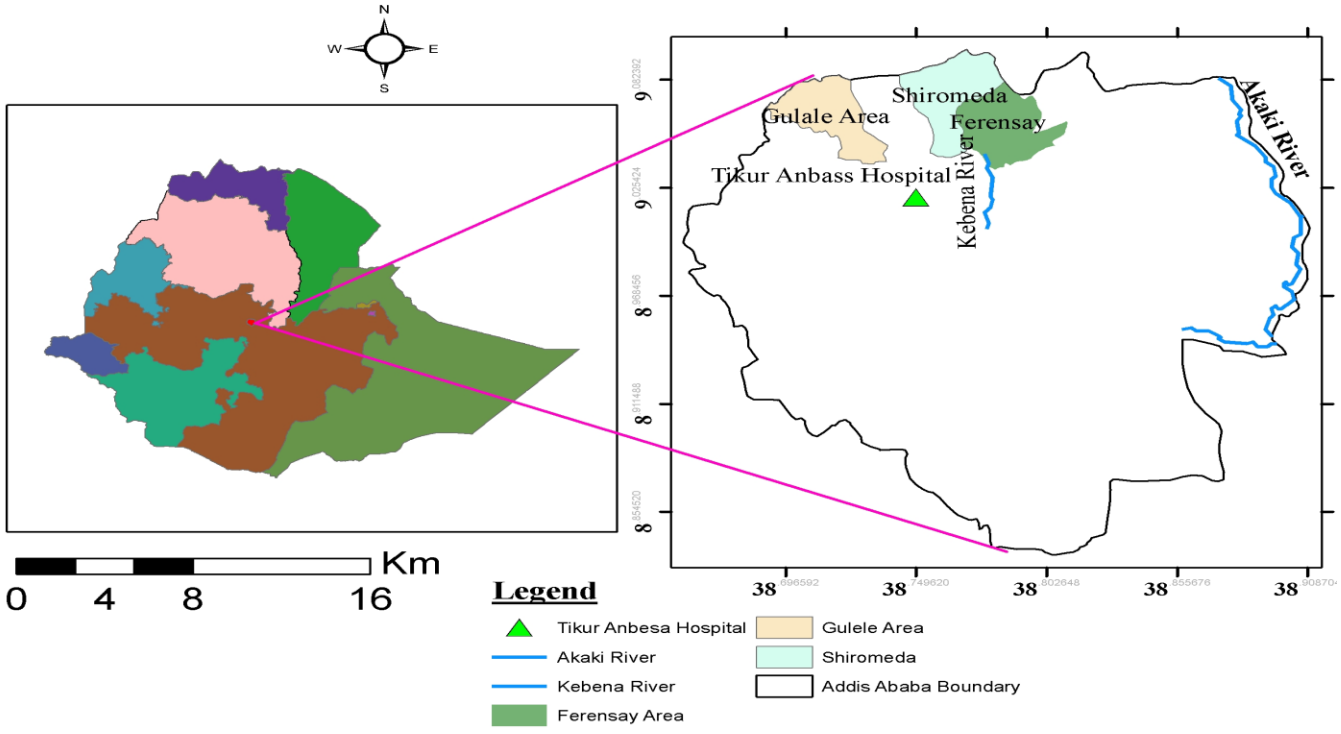


Figure 4: Study area geographic map

3.2. Study Design and sampling method

A descriptive study design was employed for isolation and characterization of potent *E. coli* lytic phages present in sampling sites mentioned above at the Institute of Biotechnology, Addis Ababa University. A convenient non-probability purposive sampling was used for the selection of sampling site and sample size determinations. Thus, a total of 14 samples including 3 from Kebena river (Top, Middle, and Bottom surface of collection pond), 3 from Akaki river (Top, Middle, and Bottom surface of collection pond), 6 from dairy sewages of Gulele, Shiromeda and Ferensay area (Superficial and stirred sediment) as well as 2 from Tikur Anbessa hospital waste samples (Superficial and stirred sediment) were tested against the six *E. coli* strains.

3.3. Sample collection and processing

Fifty milliliter of samples were collected from stagnant surface of river water from 3 regions (top (5cm), middle (25cm) and bottom (50cm)) while in case of hospital effluent and dairy farm effluent samples were collected from superficial and stirred sediment of selected site in a 50 ml sterile screw capped glass bottle. Totally fourteen samples were collected from selected sites; water (3 from Kebena and 3 from Akaki river effluents, 2 from Gulele dairy farms, 2 from Shiromeda dairy farms, 2 from Ferensay dairy farms and 2 from Tikur Anbessa hospital waste) samples.

After collection the sample containing bottles were then brought to the lab in ice box and stored overnight at room temperature to settle large debris and insoluble waste. The remaining particulates were removed by centrifugation at 3000 rpm for 10 min using 15 ml falcon tubes, and the supernatant was slowly filtered through a syringe filter with a pore size of 0.22 μm to a sterile screw capped tube.

3.4. Bacterial host strains and culture conditions

The bacterial host cells used in this study include a total of six clinically isolated pathogenic *E. coli* strains, shown in **Table 4,** that are found as culture collections at the laboratory of Institute of Biotechnology, Addis Ababa University. The bacterial isolates were stored as glycerol stock at -20°C. The *E. coli* isolates were grown at 37°C in Eosin methylene blue, tryptone soya broth and soft agar overlays (0.75% agar).

Table 4: *E. coli* strains used as a host and host range test

No	Isolate code	Strains	Antibiotics profile	Source
1	EHEC-52A	Enterohemorrhagic *E. coli* (EHEC)	Resistant to CMP, GM, AMX	Children, diarrheic
2	EPEC-46A	Enteropathogenic *E. coli (*EPEC)	Resistant to AMP, AMX, NOR, CS3	Childern, diarrheic
3	STEC-29A	Shiga-toxin producing *E. coli* (STEC)	Resistant to TMT, CS3, AMP, CMP, AMX	Calf, diarrheic
4	ETEC-41A	Enterotoxigenic *E. coli* (ETEC)	Resistant to TMT, GM, AMX	Calf, diarrheic
5	EAEC-02A	Enteroaggregative *E. coli* (EAEC)	Resistant to CIP, AMP	Children, diarrheic
6	EIEC-24	Enteroinvasive *E. coli* (EIEC)	Resistant to CIP, TC, AMX, GC, AMP	Calf, diarrheic

E. coli strains with resistant to three or more antibiotics were considered as MDR

3.5. Enrichment of the samples

After the samples were purified through preliminary filtration using centrifugation and 0.22 μm syringe filter and *E. coli* host strains prepared in broth as overnight culture, 5 ml of tryptone soya broth culture for each sample was prepared that was supplemented with 2mM calcium chloride. Five ml of tryptone soya broth, 3 ml of each of the 6 *E. coli* strains broth culture and 3ml of each sample was aseptically added to an appropriately labeled sterile falcon tube and incubated at 37^{o}C for 24 hours at 150 rpm shaking condition (Dubey and Maheswori 2012). Following incubation, phage infected cultures were poured into several 15 ml centrifuge tubes and centrifuged at 3000 rpm for 20 min to remove residue. The supernatant was decanted into a sterile tube and filtered through syringe filter (0.22μm) to remove bacterial cells.

3.6. Bacteriophage isolation

Double layer plaque technique was performed for bacteriophage isolation. Each of the 14 sample filtrates were individually tested in each of the 6 *E. coli* strains. Thus, 14*6= 84 plates were

tested for lytic phages. One ml of purified phage filtrate was transferred into a sterile test tube for each one of the samples. Then, 2 ml of the respective *E. coli* tryptone soy broth grown culture at log phase was added and mixed well. The mixture was left for 10 min at ambient temperature for allowing phages to adsorb to the host. After 10 min, 5 mL tryptone soy agar (0.7% agar) was added, mixed well, and poured over the surface of tryptone agar plate. It was allowed to set at room temperature and incubated at 37° C for 24 hours. Plates were observed and scored positive if there is a presence of clear zone (plaque formation) over the surface of the agar plate. Plaques were counted from all positive samples and recorded as a plaque forming unit (pfu/ml) (Vitaliano *et al.*, 2007).

3.6.1. Purification of Phages

For purification of the phages, clear plaques were selected and plugged off from the agar surface using sterile pipette tips and then mixed in 1 ml salt of magnesium phage buffer (100 mM Nacl, 8 mM MgSO4, and 50 mM Tris-HCl (pH 7.5) with agitation in vortex mixer. The agar and cell residues were removed by centrifugation at 3000 rpm for 10 min, followed by filtration of the supernatant through a 0.22 μm pore sized syringe filter. Resulting filtrate (phage lysate) was used to test the lytic efficacy of bacteriophages in vitro and preserved at 4° C until processing. This step was repeated three times in order to get pure single plaque from each isolate.

3.6.2. Quantitative assay of bacteriophages

Bacteriophage concentration was measured by counting the number of plaques formed in tryptone soy agar media plates as plaque forming units/mL (PFU/mL). Each lytic bacteriophage stock was diluted 10-fold serially in sterile phage buffer solution; then 100 μL from each dilution of the bacteriophage isolate was transferred to 100 μL of the *E. coli* culture which was incubated for 24 hours on tryptone soya broth medium. The suspension was incubated for 30 min at 37° C and a total of 5 mL of molten tryptone agar was mixed. After that, each one of the suspensions was poured onto TSA plate and incubated at 37° C for 24 hours. After incubation, the plates were observed for the plaque formation and expressed as PFU/mL which was determined by the following formula: PFU/ml= (Plaques per plate) X (dilution factor)/ (Volume of phage plated in ml). The substantial plaque-forming sample material was chosen for the following procedures.

3.6.3. Morphological imaging of phages

The scanning electron microscope (SEM) analysis was performed at Adama science and Technology University, Department of Biology. The liquid phage samples were lyophilized for SEM analysis using lyophilizer machine (ALPHA 2-4 LD Plus, Christ) at the Microbial, Cellular and Molecular Biology department, CNCS, Addis Ababa University. Gelatin and glucose were used as stabilizers of phage sample during lyophilization in order to protect phage degradation. For imaging SEM was operated between 5 and 15 kV accelerating voltage and focal depth between 5 and 50 μm with magnifications power ranging from 1000X to 5000X.

3.7. Characterization of selected phages

3.7.1. Determination of host range

Host range spectrum determination was done using spot method to check the ability of phages to lyse other bacteria besides its own host bacteria. The lytic spectra of a phage, which is a significant biological characteristic, refers to the range of bacteria genera, species, and strains that a phage can kil. The isolated bacteriophages were screened for their ability to infect bacterial cells in order to determine their infectivity range or lysis efficiency. This assay was based on the ability of a phage to either produce a clear plaque, turbid plaque or no lysis against 6 *E. coli* strains and other bacterial species including *Shigella flexneri, Klebsiella pneumoniae* and *Salmonella sp*. Bacterial lawns of all the strains were prepared on tryptone soy agar and 10 µl droplets of phages lysates was spotted on these lawns using sterile pipette tips. The plates were incubated at 37 ºC for 24 hours and checked for the presence of plaques. The most efficient phages based on the lysis profiles, plaque clarity and sizes thus displaying zones of lysis against most of the isolates were selected for further studies.

3.7.2. Efficiency of plating (EOP)

The efficiency of plating (EOP) was tested to measure of the ability of bacteriophages (phages) to form plaques on a bacterial lawn. It represents the ratio of the number of plaques formed by a particular phage on a host bacterial lawn to the number of phage particles applied to the lawn. The EOP analysis was performed on five bacterial strains including *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Shigella flexineri*, and STEC that were susceptible to phages on the spot test.

3.7.3. Optimal Multiplicity of Infection (MOI)

The optimal MOI was determined as the ratio of the number of phages to that of host bacteria present in a defined space that is best for phage proliferation to obtain maximum titers. It represents the number of phage particles added per bacterial cell in a culture. *E. coli* cultures of the exponential growth phase were infected with different amounts of phages with a set of serial dilutions at 10 (10/1), 1 (1/1), 0.1 (1/10), 0.01(1/100), 0.001(1/1000), and 0.0001(1/10,000). Once the phage was incubated for two hours, the titers were measured.

3.7.4. One-step growth curve of phages

The one-step growth curve was drawn using MOI of phages with respect to their isolation host. Phage isolates growth curve was plotted at 15 min to 60 min incubation time range and titer was calculated at each 15 min time interval using double layer agar assay. Then, latent period, relative burst size and burst period were obtained from each graph for each phage isolates.

3.7.5. Thermal and pH stability assay

The stability of isolated pure bacteriophage at different conditions of temperature and pH was determined. To ascertain the stability of bacteriophages at different temperatures, $1x10^9$ PFU/ml titer of phage lysate was assayed after storage at 25°C, 37°C, 70°C and 90°C for 24 hours by the double agar layer method. Similarly, phage stability at different pH was evaluated by incubating the phages in phage buffer of different pH, 3, 5, 7, 9 and 11 following double- layer agar plate methods.

3.8. Molecular identification of phages

3.8.1. DNA extraction

Organic DNA extraction method with subsequent ethanol precipitation was employed to retrieve DNA from bacteriophages. Bacteriophage DNA isolation was performed by adding 0.5 µL of DNaseI and 1 µL of RNase A to 1 mL of purified bacteriophage and incubating at 37 °C for 30 min in order to digest contaminating bacterial DNA and RNA. Phage nucleic acid was extracted by adding 40 µL of EDTA 0.5 M, 50 µL of 10% sodium dodecyl sulfate (SDS) and 5 µL of proteinase K (10 mg/ mL). The mixture was incubated at 37 °C for 1h. After incubation, 700 µL of phenol–chloroform–isoamyl alcohol solution (25:24:1) was added to remove unwanted materials, and the solution was centrifuged at 13000 RPM for 5 min. Isopropyl alcohol was added to precipitate the DNA. Approximately 700 µL of 70% ethanol was added to the pellet,

and the mixture was centrifuged again at 12,749×g for 10 min. The supernatant was removed and the pellet was dried. Fifty microliters of nuclease-free water (NFW) solution was added to the pellet for DNA storage at 4 °C (Dewanggana *et al.*, 2022). In addition, to check the quality of phage DNA a comparative DNA extraction was performed using DNase, RNase, SDS and Proteinase K treatment at the different levels. That was phage filtrate with treatment of DNase and RNase and without treatment of DNase and RNase; Phage-host mix with DNase, RNase, SDS, proteinase K treatment and without treatment. The comparative results were observed after 1% agarose gel electrophoresis.

3.8.2. Assessments of the purified phage DNA

Quantification and purity of the DNA extracted was estimated using a Nanodrop spectrophotometer. By measuring the absorbance of the DNA, the concentration and quality of extracted DNA was measured. In the case of DNA, the maximal absorbance is at 260nm. Protein maximally absorbs at 280nm and the ratio of nucleic acid to protein (260/280) was generally used as an indicator of the purity of DNA samples. The quality with respect to the host DNA contamination was assessed using gel electrophoresis. The quantified and assessed DNA extract was prepared and used for PCR.

3.8.3. Polymerase Chain Reaction (PCR)

The determination of lytic phage type was performed by conventional PCR using isolated phage DNA as template and primers specific for phage family and genus. Polymerase chain reaction was employed for rapid and definite identification of bacteriophages using family-specific primers targeting major capsid protein gene g23 for T4-like *myoviridae,* major capsid protein for T7-like *podoviridae*, and major coat protein gene for T5-like *sphirovidae* that are mostly conserved among those phage families (**Table 5**). The amplification process was done individually for each family specific primer for all targeted isolates to confirm the type of lytic phage at which family of *Caudovirales* they are classified. PCR amplification reactions were performed in 20 µl reaction volumes consisting of 2.5 mM MgCl2, 2.5 PCR buffer, 0.2 mM each deoxynucleotide triphosphate, 1 U of Taq DNA polymerase, 1.5 µl of each primer, 3 µl of template DNA and remaining amount of nuclease free water. The amplification was done in 35 cycles with an initial denaturation at 95 for 2 min followed by a denaturation step of 95 for 30 sec, primer annealing at 55 and primer extension at 72 with the final extension at 72.

Table-5: PCR primers used in phage identifications

Gene	Family	Sub-family	Genus	Sequence (5'→3')	Reference
MCP	*Myoviridae*	*Tevenvirinae*	*T4-like*	T4-fw: CCC TGC TGT TCC AGA TCG ANA ARG ARG C	Born *et al*., 2019
				T4-rev: CTG CCT GGC GTA CTG GTC DAT RWA NAC	
		Ounavirinae	*FO1-like*	FO1-fw: CGC CAT TGA AGA ACT GCG TRW RCA YAT GGA	
				FO1-rev: GGC ATC ATA TAG GAA TGC GCY TCR AAR TC	
MCP	*Podoviridae*	*Autographivirinae*	*T7-like*	T7-fw: GAC AAG CGG AAG GAC ATC AAN CAY ACN GAR A	
				T7-rev: CGC GTA GTT GGC GGC RTT NGG CAT NA	
McoP	*Siphoviridae*	-	*T5-like*	MCF-2F: GCGTGATGGTTGGGATGGTA	Alanazi *et al*., 2022
				MCF-2R: GACGCTCAATCTGACGACCA	

3.8.4. Agarose gel electrophoresis

The amplified DNA products from phages family specific-PCR were further analyzed using agarose gel electrophoresis in order to detect the presence of targeted phage DNA. The DNA samples were simultaneously checked on 1% agarose gel along with a DNA ladder with 0.3 µL of 10 × loading dye, undder 1× TAE buffer. The gel products were visualized on ultraviolet illuminator and imaged with gel documentation system (BIO-RAD).

3.9. Sequencing and bioinformatics analysis

3.9.1. Phage genome sequencing

Based on the host range, stability test and one step growth characteristics seven best-selected phage genomes were sequenced for further identification and analysis of lytic phage isolates. Phage lysate DNA samples were sent to Armauer Hansen Research Institute (AHRI), Addis Ababa, Ethiopia for whole phage genome sequencing using an Illumina NextSeq 500/550 sequencer with 2x150 bp read length. Ten (10µl) of the DNA was used for each seven samples for the whole genome library preparation with a modified Illumina COVIDseq RUO kit. In short, the DNA was enzymatically fragmented and tagmented simultaneously. The tagmented DNA was purified and amplified with a limited PCR cycle for the addition of indexes and amplification. The concentration of the libraries was measured using Qubit HS assay kit. The concentrations are listed in **Table 6**. The libraries were finally loaded on the sequencer targeting 80x depth coverage. The resulting paired-end reads were obtained in FASTQ format.

Table 6: The concentration of phage DNA library prepared for whole genome sequencing

No	Sample id	Concentration(ng/µl)
1	S41-EH-B-A (A1)	0.106
2	S42-EH-SD-TH	0.152
3	S43-EP-M-A	1.95
4	S44-EP-B-K (E2)	3.20
5	S45-EI-SP-GF	2.9
6	S46-ET-SD-TH	0.152
7	S47-ST-T-K	0.206

3.9.2. Sequence assembly and consensus generation

In this study, a comprehensive analysis workflow was used for generating a high-quality consensus phage genome from Illumina sequencing data. The workflow involved several crucial steps designed to ensure accurate assembly and error detection/correction. A widely adopted bioinformatics tools such as FastQC, BBMap package, SPAdes, BWA, Sam tools, and Pilon were utilized to perform the necessary data processing and analysis. Initially performed quality control checks on the raw reads using FastQC and used the bbduk.sh script from the BBMap package to perform adapter trimming and quality filtering, followed by genome assembly using SPAdes. From the resulting assembly, the longest contig was selected as the representative genome sequence. To prepare the longest contig for read alignment, it was indexed using the Burrows-Wheeler Aligner (BWA). Paired-end reads were then aligned to the longest contig using BWA's mem algorithm, and the resulting alignment was stored in a Sequence Alignment/Map (SAM) file. To facilitate downstream analysis, the SAM file was sorted by genomic coordinates using Sam tools, generating a sorted Binary Alignment/Map (BAM) file. Subsequently, an index file (.bai) was created for the sorted BAM file using Sam tools. The sorted and indexed BAM file was then subjected to error detection and correction using Pilon. Pilon utilizes the alignment information to identify and correct errors, including SNPs, indels, and gaps, resulting in an improved consensus phage genome. The corrected genome assembly was stored in a FASTA file and used for the downstream analysis (Shen and Millard, 2021; Petrillo *et al*., 2022). The variant calling and consensus sequence was generated using ivar tools.

3.9.3. Phage genome sequence analysis

The entire genome sequences were queried against the viruses (taxid:10239) nucleotide collection (nr/nt) using NCBI blastn and default settings (http://www.ncbi.nlm.nih.gov/genome). In addition, Genome detective web-based software was used to determine genome size and to classify phage genomes using sequence-derived taxonomic features whether phages belong to the lytic phage families of *Myoviridae*, *Podoviridae,* or *Siphoviridae.* Genome detective web-based and GeneMarkS software were used for the identification of CDSs and initial annotation of the phage genomes including identification of the phage terminase large subunit, major capsid proteins and phage lytic enzymes. The GeneMarkS and GC content calculator was used to determine the G+C content of the phages. Utilizing web-based software called tRNAscan-SE 2.0, the amount of tRNAs were anticipated. To ascertain the diversity of phage genomes and the evolutionary connections between phages, multiple sequence alignment was carried out using ClustalW, and a phylogenetic tree was built using the neighbor-joining and MEGA11 software methods. Major capsid protein and the conserved gene were utilized as phylogenetic phylomarkers for the variety and evolutionary relationship of each phage isolates. Reference sequences used in the analysis were obtained from the GenBank database. Phylogenetic trees were supported statistically by bootstrapping with 1000 replicates. Homologues were identified with NCBI GENE database using the nucleotides as queries. The accession numbers of the viruses used in the alignments and phylogenetic analyses are listed on the trees.

3.10. Data management and statistical analysis

The collected data was computed by using R studio 4.1 and excel for appropriate statistical analysis. Statistical analysis was performed to describe different variables and parameters in the study, and to describe relationship with each other as well. The isolation percentages of phages with related to host strains from different sample sources was represented by pie chart. The number of phage isolates and sample site analysis as well as pH and temperature effect on isolates was indicated in bar graphs. Descriptive statistics was used to derive percentage, standard deviation, and to tabulate tables and graphs as well to describe the lytic bacteriophages in relation to their lysis profile and their exposure to pH and temperature. The MEGA11 bootstrap statistical tool as 1000 replicates was used in case of phylogenetic tree construction in order to confirm inferred relationships in taxa. Effects were reported as statistically significant as *P* value of less than or equal to 0.05.

4: Results

4.1. Recovery of bacteriophages

Totally fourteen samples of river water, dairy farm sewage and hospital sewages were screened within each of the six *E. coli* strains for the presence of phages. Using 6 different *E. coli* host strains as host organisms, 17 phages were isolated from eighty-four culture plates tested by the double agar overlay method. All the sample sites yield phages against *E. coli* host strains. These phages produced clear and centered plaques of different sizes (**Figure 5**). All positive results were denoted as lytic bacteriophages due to the clear zone plaques on the agar. Isolated phages were named according to the *E. coli* host and sample site (e.g., phage ET-SD-TH stands for host organism ETEC and sample site sediment of Tikur Anbessa Hospital from where it was isolated). The spectrum of effective phages, their isolation site, and the hosts from which they are isolated are illustrated in **Table 7** and **Table 8**.

Table 7: Over all samples processed with respective phages isolated

Sample No	Sample name	Presence of lytic phages for *E. coli*	Pathogenic *E. coli* strain (s) tested
1	EA-T-K	Absent	EAEC
2	EH-T-K	Absent	EHEC
3	EI-T-K	Absent	EIEC
4	EP-T-K	Absent	EPEC
5	ET-T-K	Absent	ETEC
6	ST-T-K	Present	STEC
7	EA-M-K	Absent	EAEC
8	EH-M-K	Absent	EHEC
9	EI-M-K	Absent	EIEC
10	EP-M-K	Present	EPEC
11	ET-M-K	Absent	ETEC
12	ST-M-K	Present	STEC
13	EA-B-K	Absent	EAEC
14	EH-B-K	Absent	EHEC

15	EI-B-K	Absent	EIEC
16	EP-B-K (	Present/ 2 phages/ EP-B-K (B) & EP-B-K (E2)	EPEC
17	ET-B-K	Absent	ETEC
18	ST-B-K	Absent	STEC
19	EA-T-A	Present	EAEC
20	EH-T-A	Absent	EHEC
21	EI-T-A	Absent	EIEC
22	EP-T-A	Absent	EPEC
23	ET-T-A	Absent	ETEC
24	ST-T-A	Absent	STEC
25	EA-M-A	Present	EAEC
26	EH-M-A	Absent	EHEC
27	EI-M-A	Absent	EIEC
28	EP-M-A	Present	EPEC
29	EH-M-A	Absent	EHEC
30	ST-M-A	Present	STEC
31	EA-B-A	Absent	EAEC
32	EH-B-A (A1 & A2)	Present/2 phages/ EH-B-A (A1) & EH-B-A (A2)	EHEC
33	EI-B-A	Absent	EIEC
34	EP-B-A	Absent	EPEC
35	ET-B-A	Absent	ETEC
36	ST-B-A	Absent	STEC
37	EA-SP-GF	Absent	EAEC
38	EH-SP-GF	Absent	EHEC
39	EI-SP-GF	Present	EIEC
40	EP-SP-GF	Absent	EPEC
41	ET-SP-GF	Absent	ETEC

42	ST-SP-GF	Absent	STEC
43	EA-SD-GF	Absent	EAEC
44	EH-SD-GF	Absent	EHEC
45	EI-SD-GF	Absent	EIEC
46	EP-SD-GF	Absent	EPEC
47	ET-SD-GF	Absent	ETEC
48	ST-SD-GF	Absent	STEC
49	EA-SP-TH	Absent	EAEC
50	EH-SP-TH	Present	EHEC
51	EI-SP-TH	Absent	EIEC
52	EP-SP-TH	Absent	EPEC
53	ET-SP-TH	Absent	ETEC
54	ST-SP-TH	Absent	STEC
55	EA-SD-TH	Absent	EAEC
56	EH-SD-TH	Present	EHEC
57	EI-SD-TH	Absent	EIEC
58	EP-SD-TH	Absent	EPEC
59	ET-SD-TH	Present	ETEC
60	ST-SD-TH	Absent	STEC
61	EA-SP-SMA	Present	EAEC
62	EH-SP-SMA	Absent	EHEC
63	EI-SP-SMA	Absent	EIEC
64	EP-SP-SMA	Absent	EPEC
65	ET-SP-SMA	Absent	ETEC
66	ST-SP-SMA	Absent	STEC
67	EA-SD-SMA	Absent	EAEC
68	EH-SD-SMA	Absent	EHEC
69	EI-SD-SMA	Absent	EIEC
70	EP-SD-SMA	Absent	EPEC

71	ET-SD-SMA	Absent	ETEC
72	ST-SD-SMA	Absent	STEC
73	EA-SP-FA	Absent	EAEC
74	EH-SP-FA	Absent	EHEC
75	EI-SP-FA	Absent	EIEC
76	EP-SP-FA	Absent	EPEC
77	ET-SP-FA	Absent	ETEC
78	ST-SP-FA	Absent	STEC
79	EA-SD-FA	Present	EAEC
80	EH-SD-FA	Absent	EHEC
81	EI-SD-FA	Absent	EIEC
82	EP-SD-FA	Absent	EPEC
83	ET-SD-FA	Absent	ETEC
84	ST-SD-FA	Absent	STEC

Table 8: Phages recovered with respective hosts and sites

No	Phage	Host	Sample site and location
1	ET-SD-TH	ETEC	Tikur Anbessa Hospital (Sediment)
2	ST-M-K	STEC	Kebena river (Medium)
3	EI-SP-GF	EIEC	Gullele farm (Superficial)
4	EA-T-A	EAEC	Akaki river (Top)
5	EP-B-K (B)	EPEC	Kebena river (Bottom)
6	EH-SD-TH	EHEC	Tikur Anbessa Hospital (Sediment)
7	EP-B-K (E2)	EPEC	Kebena river (Bottom)
8	ST-T-K	STEC	Kebena river (Top)
9	EH-SP-TH	EHEC	Tikur Anbessa Hospital (Superficial)
10	EH-B-A (A1)	EHEC	Akaki river (Bottom)
11	EH-B-A (A2)	EHEC	Akaki river (Bottom)
12	ST-M-A	STEC	Akaki river (Medium)
13	EA-M-A	EAEC	Akaki river (Medium)
14	EP-M-K	EPEC	Kebena river (Medium)
15	EP-M-A	EPEC	Akaki river (Medium)
16	EA-SP-SMA	EAEC	Shiromed area farm (Superficial)
17	EA-SD-FA	EAEC	Ferensay area farm (Sediment)

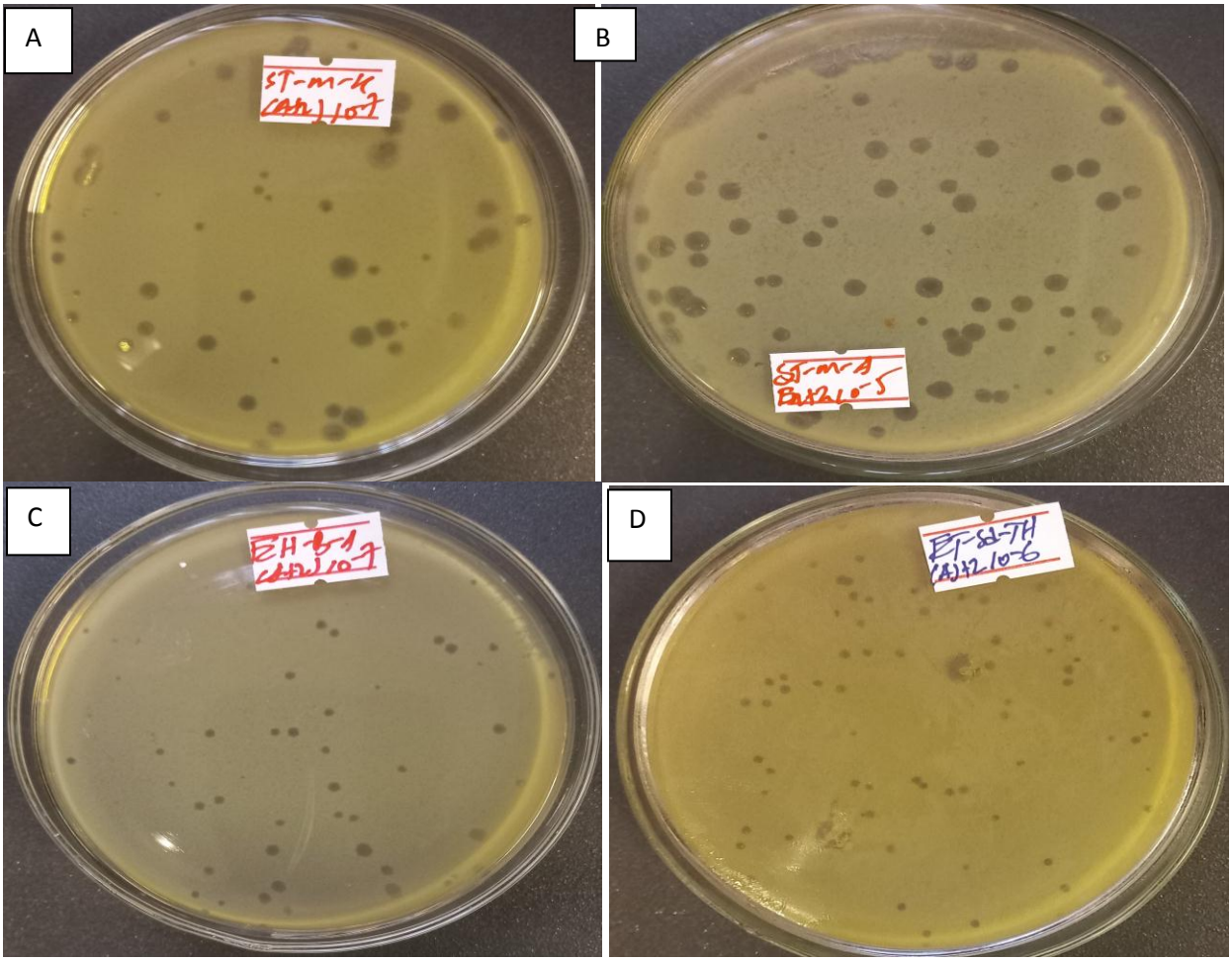


Figure 5: Representative bacteriophage plaque images during isolation and purification depicting different morphologies: A) Clear plaque with elevated halos B) Large size clear plaques C) Small sized clear plaques D) Small pinned clear plaques.

The number of phage strains recovered were 3 (17.7 %) against STEC, 1 (5.9 %) against both ETEC and EIEC, and 4 (23.5 %) against EPEC, EHEC, and EAEC (**Figure 6**). Recovery of phages was slightly higher in river water samples as compared to dairy farm waste and hospital waste, and the concentration of phage was greater in the bottom and middle layers than the superficial and/or top layers of the collection tank (**Figure 7**; **Table 7**).

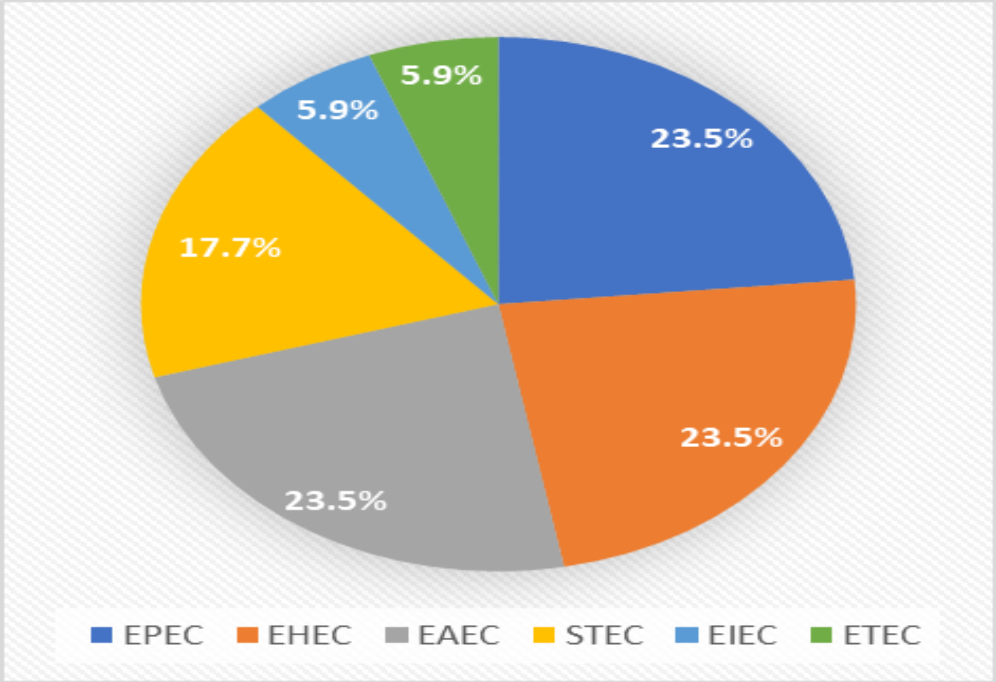


Figure 6: Frequency of lytic phages isolated with respect to the six pathogenic *E. coli* strains

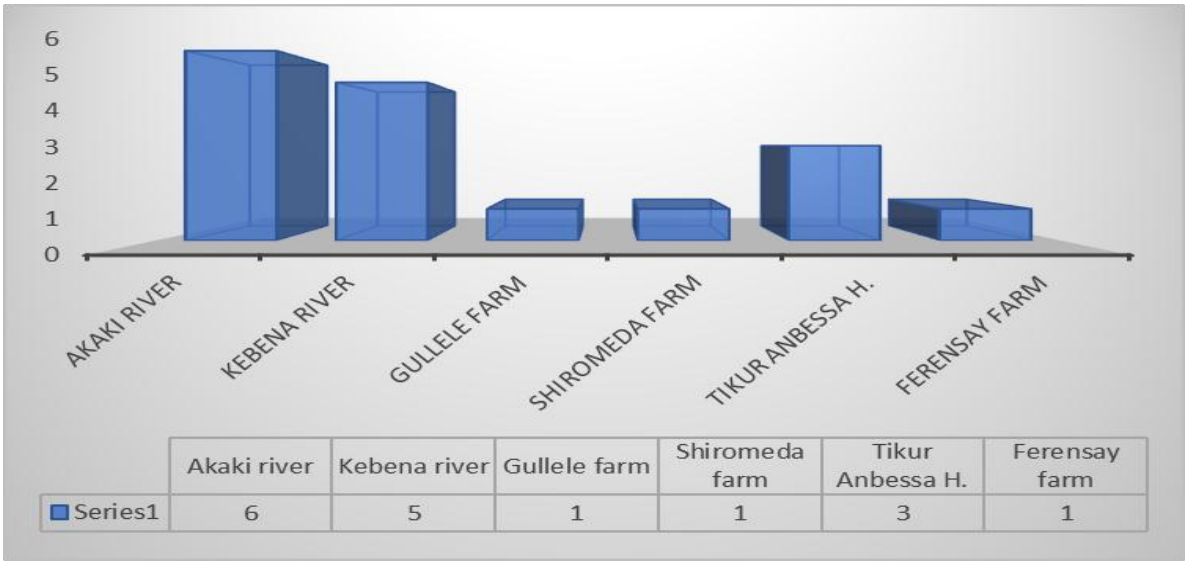


Figure 7: Frequency of lytic phages recovered with respect to the sample site

4.2. Bacteriophage titer determination

Bacteriophage titers were observed between 2.8 x 10^{7} and 3.12 x 10^{10} PFU mL − 1 (**Table 9**). The highest titer of bacteriophage was isolated from EP-M-K bacteriophage with a titer of 3.12 × 10^{10} PFU mL − 1. The titer of phages from the medium and bottom of the river and sediment

surface of the dairy farm, as well as hospital waste, was highly concentrated than the top surface of the river and superficial surface of farm and hospital fluid waste.

Table 9: Phage titer and plaque morphology

No	Phages	Titer (PFU/ml)	Plaque morphology
1	EP-M-K	3.12×10^{10}	Clear plaque with small halos
2	EP-B-K (B)	6.0×10^{9}	Haloed clear plaques
3	EP-B-K (EN2)	1.7×10^{9}	Clear plaque with halos
4	EP-M-A	2×10^{10}	Clear plaque with diffused surrounding
5	EA-SP-SMA	3.0×10^{10}	Elevated-type haloed plaque
6	EA-T-A	1.4×10^{9}	Haloed clear plaques
7	EA-SD-FA	3.1×10^{10}	Clear plaque with halo surrounded
8	EA-M-A	12.0×10^{9}	Small clear plaques
9	EH-B-A (A1)	5.1×10^{7}	Small-sized clear plaques
10	EH-B-A (A2)	12.0×10^{9}	Clear plaque with small pin headed
11	EH-SD-TH	7.5×10^{7}	Medium size clear plaques
12	EH-SP-TH	9.8×10^{9}	Large size clear plaques
13	EI-SP-GF	9.0×10^{7}	Small size clear plaques
14	ET-SD-TH	2.8×10^{7}	Small clear plaques
15	ST-T-K	17.5×10^{8}	Large-sized diffused plaques
16	ST-M-A	8.4×10^{9}	Large-sized clear plaques
17	ST-M-K	16.5×10^{8}	Clear-centered plaque with halos

4.3. Morphology of phages analyzed by SEM

Seventeen phage isolates were subjected to Scanning Electron Microscopy analysis to determine their morphotype. Scanning electron micrograph images of the phages and structural dimensions are shown in **Figure 8**. Phage isolates were classified as per the International Committee on Taxonomy of Virus (ICTV) classification based on the three-dimensional structure observed. Only one phage isolates (EP-M-K) clearly and three relative phages (EA-SD-FA, ST-M-A & EH-SP-TH) were identified by using SEM imaging of the isolates. Identified phage isolates were in the order *Caudovirales* of three families such as *Myoviridae*, *Siphoviridae and Podoviridae*.

Among 4 phages, 2 phage isolates were classified as *Myoviridae*-like phages while 2 phage isolates were classified as *Siphoviridae*-like and *Podoviridae*-like phages. *Myoviridae* phages have relatively large icosahedral heads and a long, thick, complex, contractile tail, consisting of a central tube surrounded by a contractile sheath and ancillary structures. *Siphoviridae* consists of a long non-contractile thin flexible tail with short, kinked, terminal fibers (**Figure 8, B**). *Podoviridae* phages have non-enveloped icosahedral heads with short, non-contractile tails (**Figure 8, C**).

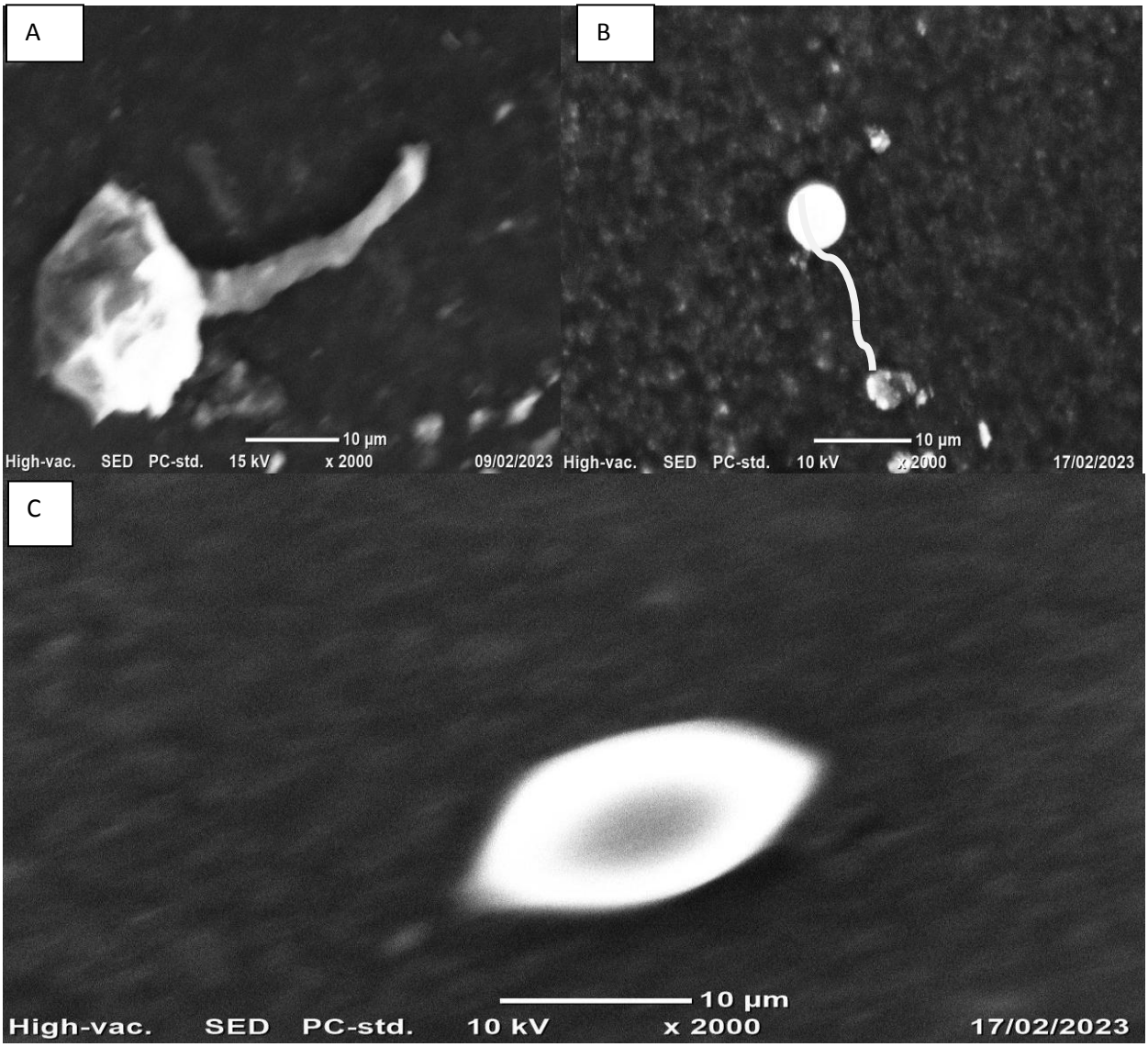


Figure 8: Scanning electron microscopy representative images of phage families: A) *Myoviridae* like phage B) *Siphoviridae* like phage C) *Podoviridae* like phage morphology

4.4. Delineation of bacteriophage isolates

4.4.1. Host range test

Phages were selected on the basis of the size and clarity of plaques they produced for screening their host range to determine the lytic profile (**Figure 9**). Thus, the infectivity of seventeen phages was analyzed against 3 other bacterial pathogenic species including *Salmonella Typhimurium, Shigella flexineri, Klebsiella pneumoniae,* and other *E. coli* isolate apart from host strains. The host range of these phages was investigated by spot method which revealed that most of the phages were able to lyse pathogenic strains.

The results in **Table 10** showed that a large proportion, 11(65%) of the phages were effective against available bacterial species since clear plaques were formed when phages were spotted on the bacterial lawn. However, 6(35%) of the phages demonstrated no lysis and were not able to produce areas of clear zones on a bacterial lawn. Among 17 phages, EP-M-A was the most effective phage with 62.5% lytic ability killing 5 different bacterial strains followed by the phage EI-SP-GF which lysed 4 different strains. The intensity of EP-M-A phage was effective in *Klebsiella pneumoniae, Salmonella Typhimurium, Shigella flexineri, EAEC, and STEC* whereas EI-SP-TH was effective in *Salmonella Typhimurium, Shigella flexineri, EHC and STEC.* Phages EP-B-K (B), EH-SD-TH, ET-SD-TH, EH-B-A (A1), and EH-B-A (A2) were each able to lyse three bacterial hosts while phages ST-M-A and EA-M-A were each able to lyse two *E. coli* strains. Phages EP-M-K and ST-M-K only lyse one bacterial host when compared to other phages. Contrary to the low levels of virulence displayed by these phages, seven phages (EP-M-A, EI-SP-GF, EP-B-K (E2), EH-SD-TH, ET-SD-TH, EH-B-A (A1), and EH-B-A (A2)) demonstrated the broadest spectrum of activity since they could be infected between 3 and 5 bacterial host strains. These seven super-phages were selected for further characterization.

Table 10: Host range test of phages

Phages	Test organisms of bacterial species and other *E. coli* strains								
	Sal. Typhimurium	*Sh. Flexinari*	*K. Pneumoniae*	*EPEC*	*ETEC*	*EHEC*	*EIEC*	*EAEC*	*STEC*
EP-M-A	+	+	+	H	-	-	-	+	+
EP-M-K	-	-	-	H	-	-	-	-	+
EP-B-K (B)	-	-	-	H	-	-	-	-	-
EP-B-K	+	-	+	H	-	-	-	-	+

(E2)									
EH-SD-TH	+	-	-	-	+	H	-	-	+
ET-SD-TH	+	+	-	-	H	+	-	-	-
EI-SP-GF	+	+	-	-	-	+	H	-	+
EH-B-A (A1)	+	+	-	-	-	H	-	-	+
EH-SP-TH	-	-	-	-	-	H	-	-	-
EH-B-A (A2)	+	+	-	-	-	H	-	-	+
ST-T-K	-	-	-	-	-	-	-	-	H
ST-M-A	-	-	-	+	-	-	-	+	H
EA-SP-SMA	-	-	-	-	-	-	-	H	-
EA-SD-FA	-	-	-	-	-	-	-	H	-
ST-M-K	-	-	-	+	-	-	-	-	H
EA-M-A	-	-	-	+	-	-	-	H	+
EA-T-A	-	-	-	-	-	-	-	H	-

+= lysis; -= no plaques; H= host isolated

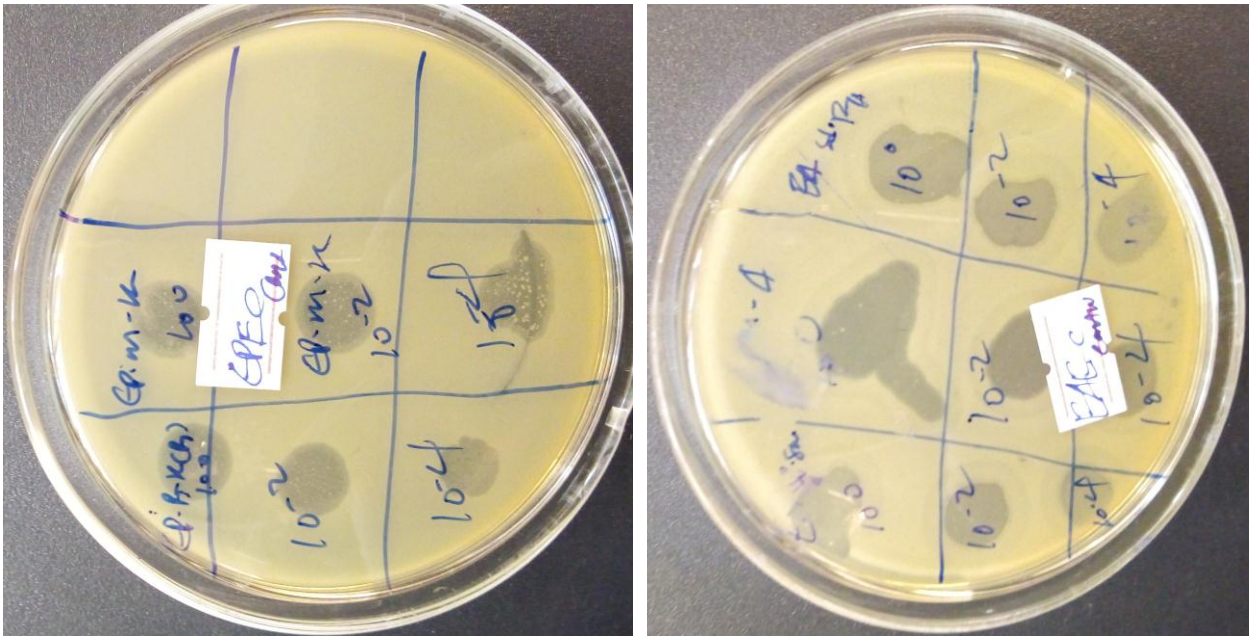


Figure 9: Analysis of the separated phages' host range representative image: The spot lysed by different phages that depicting clear lysis zone on TSA agar plate.

4.4.2. Efficiency of plating (EOP) in seven phages

The EOP analysis was performed on four bacterial strains including *Klebsiella pneumoniae*, *Salmonella Typhimurium, Shigella flexineri*, and *STEC* that were susceptible to phages on the

spot test. The EOP was calculated as the ratio between the average number of plaques of target host bacteria (PFUs) and the average number of plaques of reference host bacteria (PFUs). The EOP was classified as high (EOP≥0.5), moderate (EOP > 0.1< 0.5), and low ((EOP ≤ 0.1) based on the reproducible infection on the target bacteria. Although spot test results revealed clear plaques on reference hosts (**Figure 10**), EOP results exhibited various lytic patterns of the phages. Even though EOP analysis revealed high (EOP≥0.5) productive infection on reference bacterial hosts, moderate and low infections were observed (**Table 11**). Four phages (EH-SD-TH, EI-SP-GF, EP-M-A, EH-B-A (A1)) revealed high EOP values (0.7–1.4) on reference hosts. On the other hand, EOP analysis exhibited moderate and low productive infections on the hosts' EOP values ranging from 0.1 to 0.5 and less than 0.1 respectively.

Table 11: Efficiency of plating for spot test effective phages

Phages	Reference host	Titer in isolate host	Titer in EOP test sp.	EOP
EP-M-A	*Shigella flexineri*	2×10^{10}	2.8×10^{9}	0.7
ET-SD-TH	*Salmonella typi.*	2.8×10^{7}	1.1×10^{8}	0.2
EH-SD-TH	*STEC*	7.5×10^{7}	5.2×10^{7}	1.4
EH-B-A (A1)	*STEC*	5.1×10^{7}	4.4×10^{8}	1.2
EI-SP-GF	*Shigella flexineri*	9.0×10^{7}	3.2 x 108	1.38
EH-B-A (A2)	*Shigella flexineri*	12.0×10^{9}	3.0×10^{10}	0.4
EP-B-K (E2)	*Klebsiella pneumoniae*	1.7×10^{9}	1.8×10^{9}	0.09

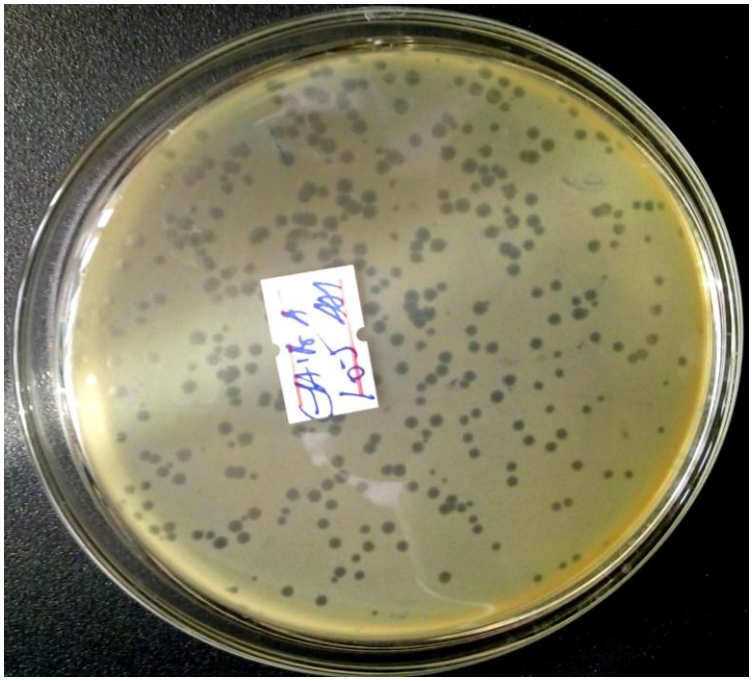


Figure 10: Representative image of phage plaques in EOP reference host

4.4.3. Optimal Multiplicity of Infection (MOI)

The optimal MOI was determined as the ratio of the number of phages to that of host bacteria present in a defined space that is best for phage proliferation to obtain maximum titers. *E. coli* strain cultures of the exponential growth phase were infected with different amounts of phages with a set of serial dilutions at 10 (10/1), 1 (1/1), 0.1 (1/10), 0.01(1/100), 0.001(1/1000), and 0.0001(1/10,000). The phage titers were measured after incubation for 2 h. The results indicated that the optimal MOIs of phage isolates EP-M-A, EI-SP-GF, EP-B-K (B), EH-SD-TH, ET-SD-TH, EH-B-A (A1), and EH-B-A (A2) were 1, 0.1, 0.1, 0.01, 0.1, 0.01 and 1, respectively, which gave the highest production of phage progeny (**Table 12**). Phage EP-M-A had highest MOI (1) which was also comparable with host range test result, but EI-SP-GF had broad host range with slight difference in MOI. In general, phage with broader host range had highest MOI and vice versa.

Table 12: The multiplicity of infection in phages isolates

E. coli strain (CFU/500 ul)		MOI	Phage isolates titer (PFU/ml) after infection						
			EP-M-A	EP-B-K	EH-SD-TH	ET-SD-TH	EH-B-A (A1)	EH-B-A, (A2)	EI-SP-GF
EPEC	1.8×10^{10}	10	1.3×10^{8}	2.7×10^{8}	5.7×10^{7}	6.1×10^{5}	2.7×10^{7}	8.4×10^{8}	9.1×10^{5}
ETEC	3.4×10^{8}	1	**2×10^{10}**	3.9×10^{7}	3.9×10^{7}	1.6×10^{7}	3.3×10^{7}	**12.0×10^{9}**	8.6×10^{7}
EHEC	8.4×10^{8}	0.1	1.2×10^{9}	**1.7×10^{9}**	1.5×10^{6}	**2.8×10^{7}**	2.4×10^{6}	2.6×10^{8}	**9.0×10^{7}**
EIEC	5.2×10^{7}	0.01	3.7×10^{8}	1.5×10^{8}	**7.5×10^{7}**	2.7×10^{7}	**5.1×10^{7}**	7.3×10^{9}	7.7×10^{7}
EAEC	3.4×10^{5}	0.001	7.2×10^{5}	1.7×10^{8}	8.1×10^{5}	3.9×10^{6}	7.1×10^{5}	5.4×10^{8}	8.3×10^{7}
STEC	1.2×10^{6}	0.0001	6.7×10^{7}	3.5×10^{6}	4.3×10^{7}	1.4×10^{6}	4.6×10^{7}	6.2×10^{7}	9.4×10^{6}

4.4.4. One-step growth curve of phages

The latent duration and relative burst size per infected bacterial cell were determined using a one-step growth curve analysis for the seven phage isolates using optimal MOI. Triphasic curves were created using the data generated after analysis (**Figure 11**). All phages had a latent duration of between 10 and 15 min followed by a burst period of between 15 and 30 min. The relative burst size, defined as the mean phage titer value at the plateau phase divided by that of the latent phase, was approximately between 87 and 364 virions per infected cell. Phages EH-B-A (A1) and EP-B-K (E2) exhibited the biggest burst sizes (364 and 268 PFUs, respectively) per infected cell; whereas phage ET-SD-TH had the smallest burst size (87 PFUs). The mean burst size of phages EH-B-A (A2), EI-SP-GF, EH-SD-TH, and EP-M-A was (200, 193, 105, and 129 PFUs respectively).

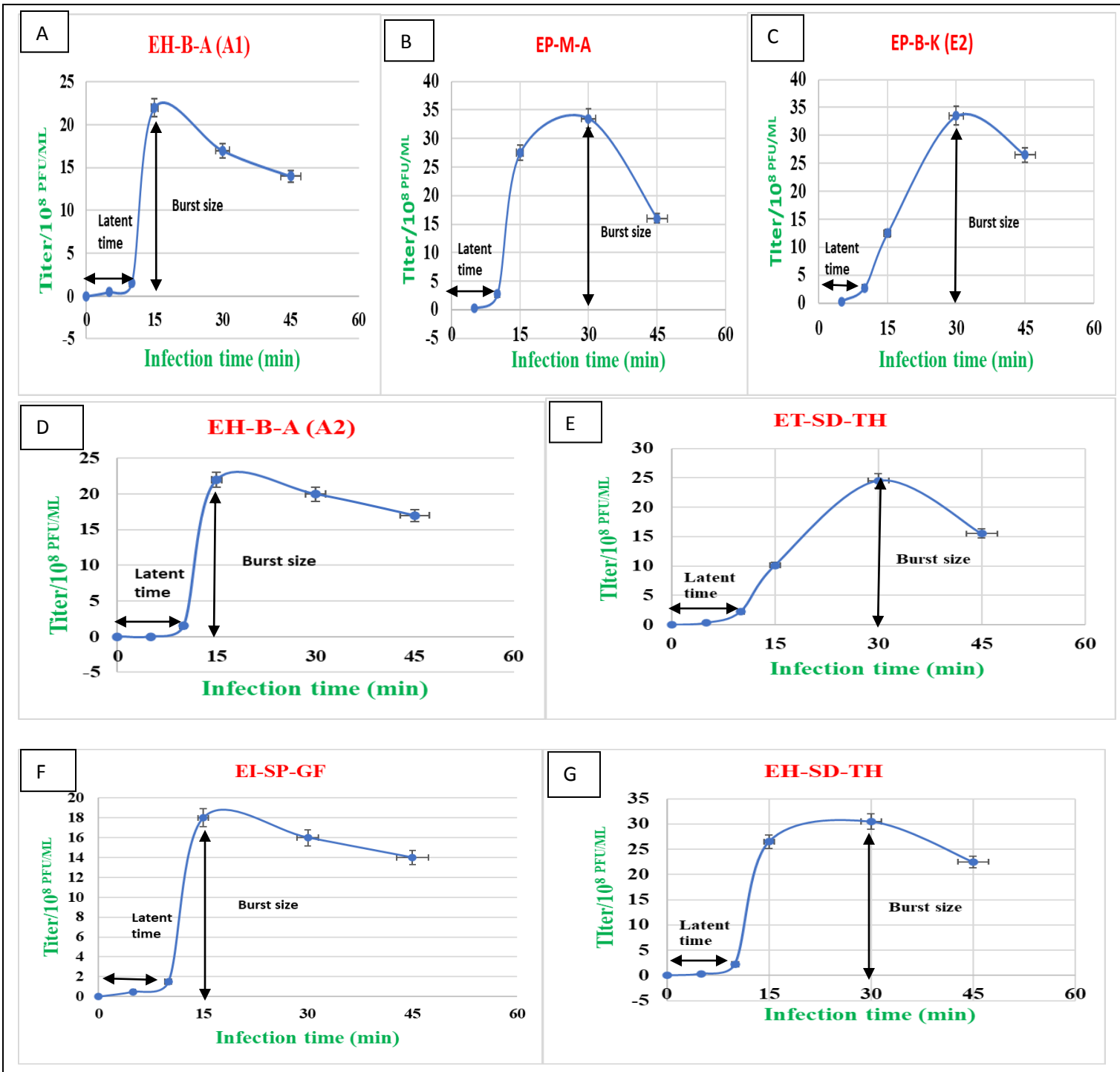


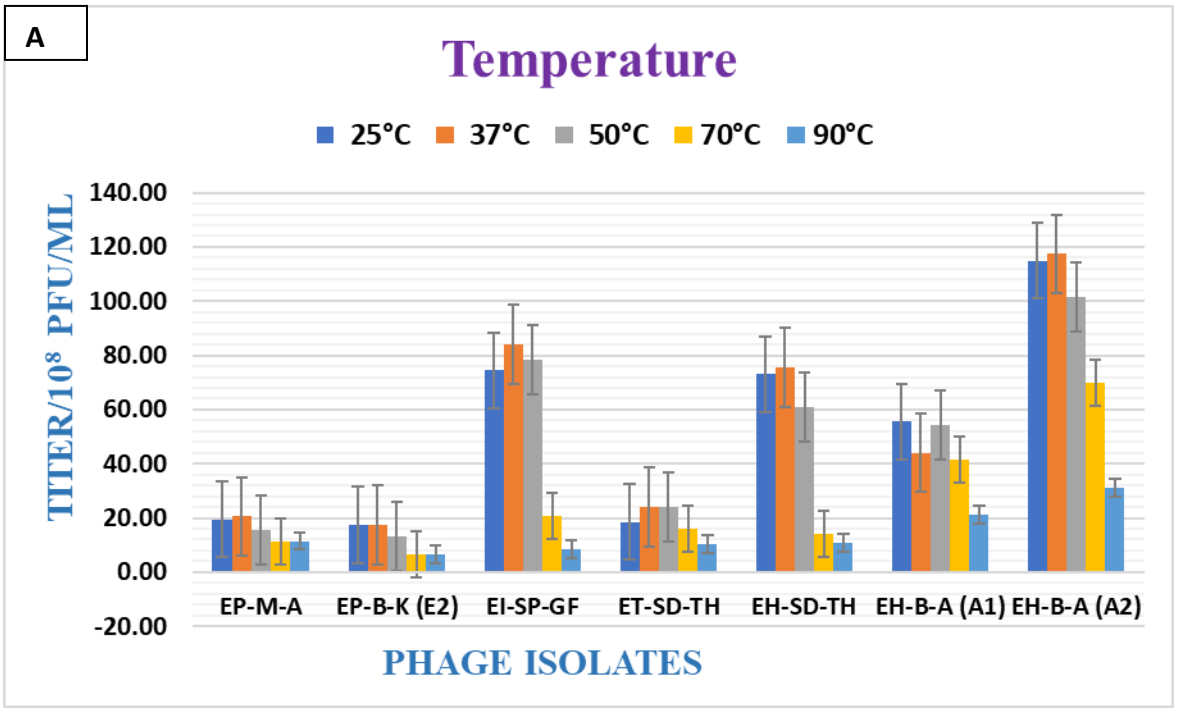
Figure 11: One-step growth curves in seven potent phages: The double-layer agar method was used to calculate the phage titers. Latent periods and burst sizes were estimated from the graph in which an arrow line indicated for each phage. **A)** EH-B-A (A1) phage growth curve; **B)** EP-M-A growth curve; **C**) EP-B-K (E2); **D**) EH-B-A (A2); **E**) ET-SD-TH; **F**) EI-SP-GF; **G**) EH-SD-TH phage growth curve. The average of two separate trials is shown by each data point. Standard deviations between the duplicate samples are displayed by error bars.

4.4.5. Temperature and pH stability

Plaque-forming units (PFU) were used to measure changes in survival in order to evaluate the pH and thermal stabilities of phages. After incubating the phage at temperatures ranging from 25 to 90°C for 6 hours, the thermal stability of the phage isolates was assessed using a phage titration experiment with 37°C as isolation temperature point. In the case of pH test, phages were also incubated at different pH values of phage buffer ranging from 3 to 11 at 37°C for 6 hours in relation to pH of the isolation, pH 7.5 as medium.

According to the results of thermal stability tests, all phages were stable at temperatures between 25^{o}C and 70^{o}C and did not become less viable after being incubated for 6 hours at the appropriate temperatures except EP-B-K (E2) and EH-SD-TH which showed high loss in titer at 70^{o}C. The infectivity of the phage was entirely seen to decrease after incubation at 90°C for 6 hours in all isolates. When temperature increase the viability of phage was significantly decrease. All phage have optimum or good infectivity at 37°C (**Figure 112, A**).

The results of pH stability tests showed that phage viability was largely unaltered following incubation in buffer at pH values ranging from 5 to 9 with slight loss at pH 9 particularly EI-SP-GF and ET-SD-TH, while decreases of titer were roughly recorded at pH 3 and pH 11 in all phage isolates (**Figure 12, B**). EH-B-A (A2) was the most stable phage at pH 3 out of all of them. After 6 hours of incubation, all the phages survived well at pH 7 and pH 9 with no appreciable drop in titer. A pH 7 and temperature 37°C were best optimum conditions for phages. These findings demonstrate the wide temperature and pH tolerance of the phage isolate.



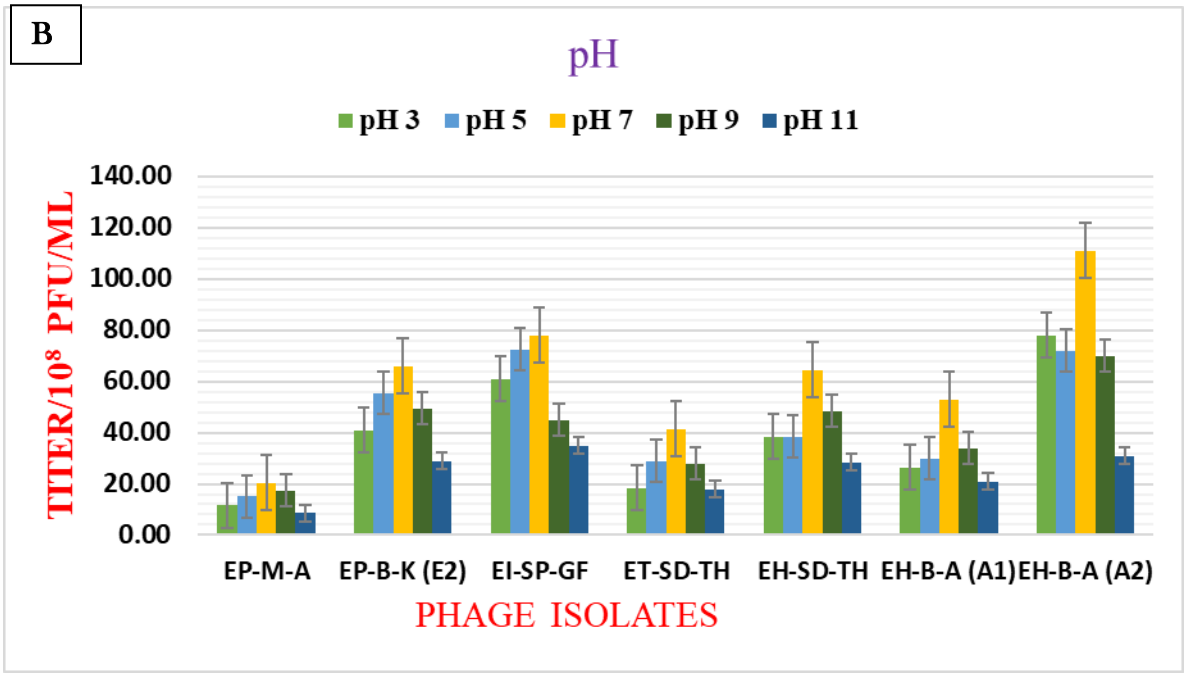


Figure 12: Temperature and pH stability test: (A) Phages were incubated at various temperatures between 25 and 90°C. (B) pH sensitivity test at different pH ranges between 3 and 11. The average of two separate experiments was used to draw the graph. Lines that are vertical represent standard deviations.

4.5. Molecular characterization of phages

4.5.1. DNA extraction and analysis

DNA was successfully extracted from 17 phages that are active against Diahrrgenic *E. coli strains*. Nanodrop was used to evaluate the DNA concentration and purity. The range of the DNA concentrations was 58.6-1241.9 ng/L. The amount of DNA that was extracted and the initial phage concentration were not linearly correlated. For example, the highest titer of bacteriophage was isolated from EP-M-K phage with a titer of 3.12×10^{10} PFU mL – 1 while in the case of DNA, the highest DNA concentration was extracted from EP-B-K (E2) which is 1241.9 ng/L.

The measurement of DNA revealed that twelve out of seventeen DNA samples were high quality and others were relatively acceptable. An intact band indicates that the DNA has not been damaged or tainted with host RNA and DNA. The genomic DNA extracted from phage isolates were indicated below in **Figure 13**. The result of possible contaminant check revealed that phage sample treated with DNase and RNase had some clear gel image whereas those samples that did not treated with DNase and RNase showed cloudy or indistinct gel image (**Figure 14**).

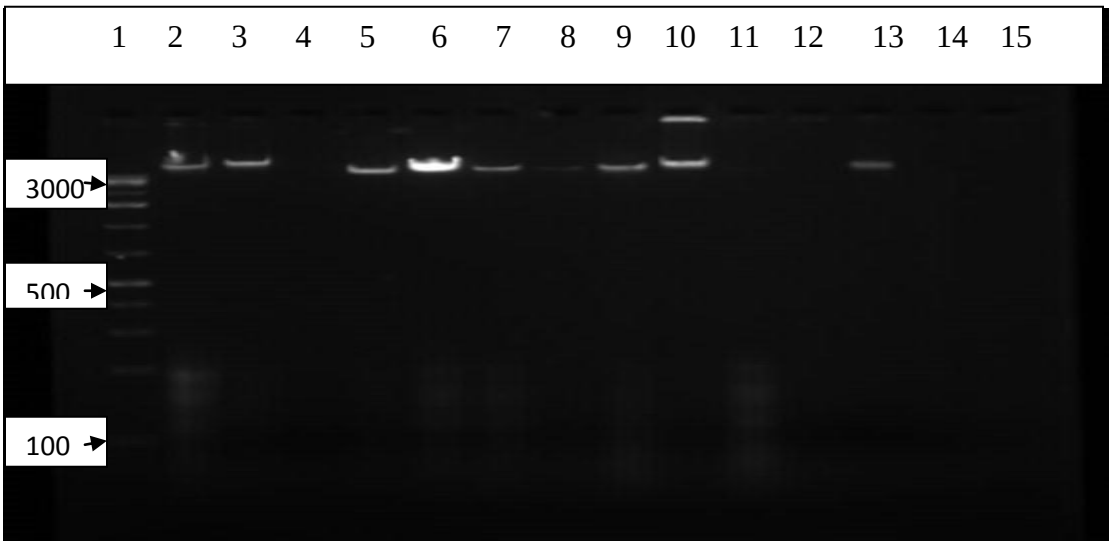


Figure 13: Phage genomic DNA on 1.2 % agarose gel electrophoresis: Lane1=100bp ladder; Lane2=EP-M-A; Lane3=EP-K-K B&E2; Lane4=EH-SD-TH; Lane5=EH-B-A, A1&A2; Lane6=ST-M-A; Lane7=ST-T-K; Lane8=EI-SP-GF; Lane9=EA-T-A; Lane10=EA-SP-SMA; Lane11=EA-SD-FA; Lane12=EH-SP-TH; Lane13=ET-SD-TH; Lane14=ST-M-K and Lane15=EP-M-K

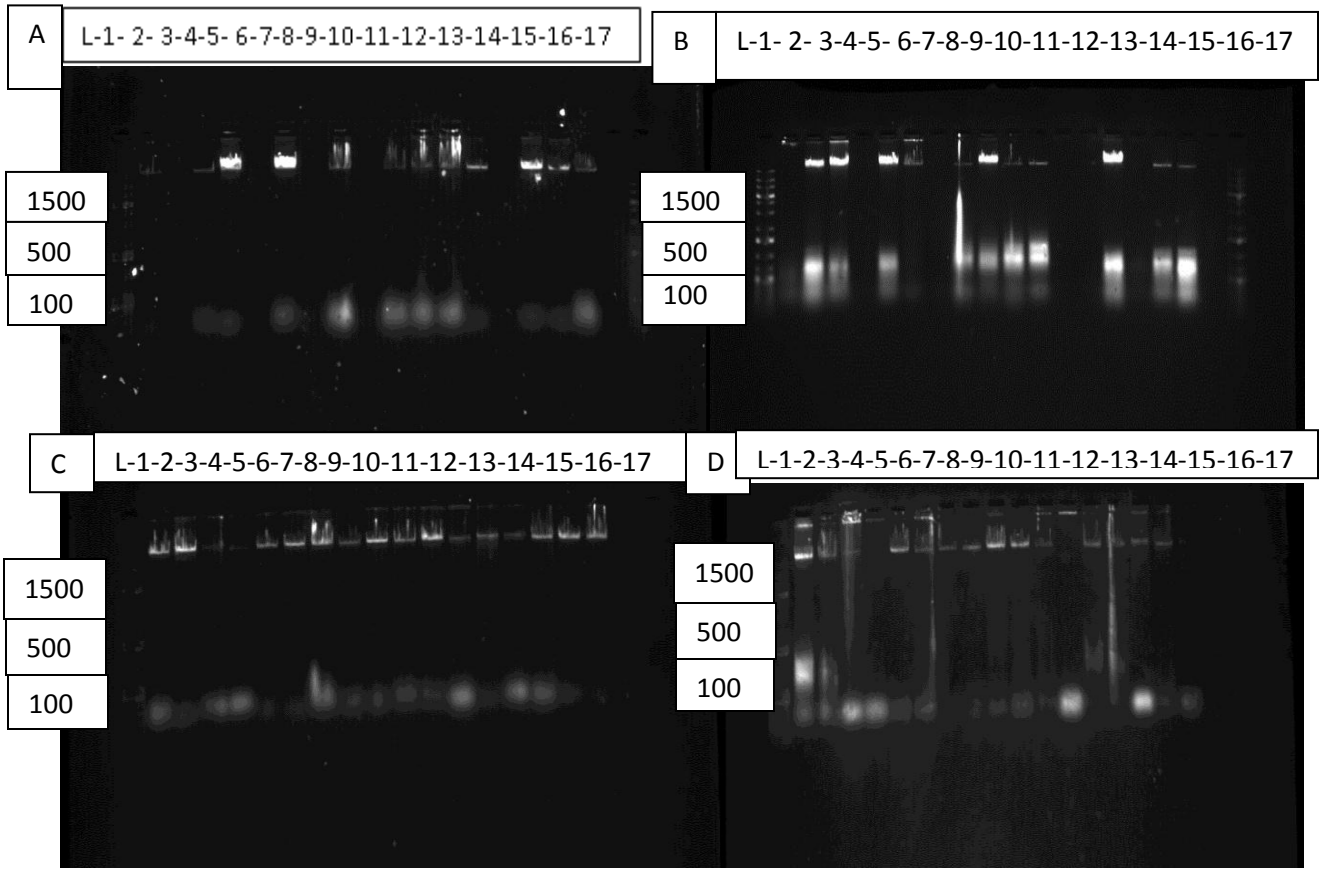
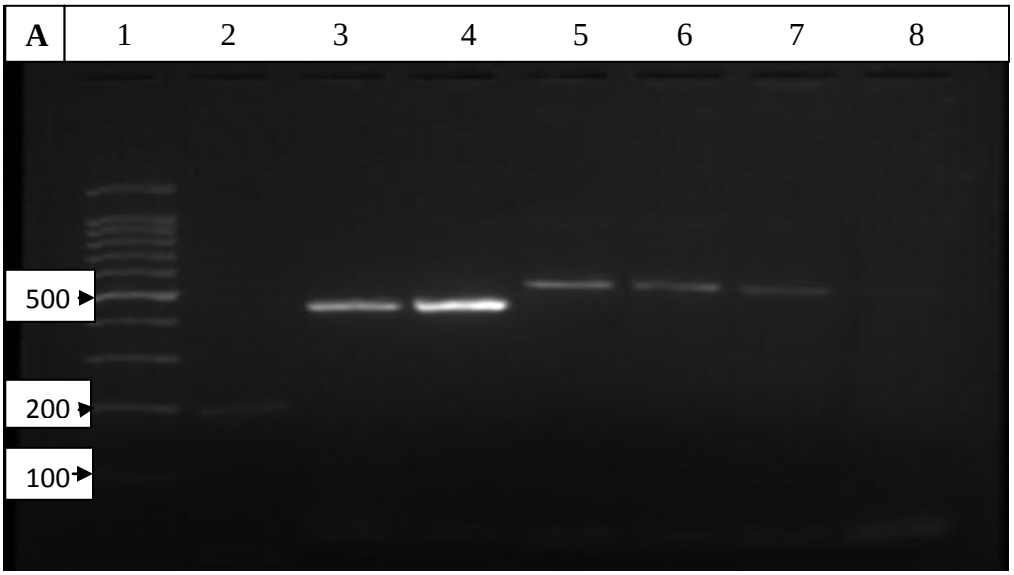


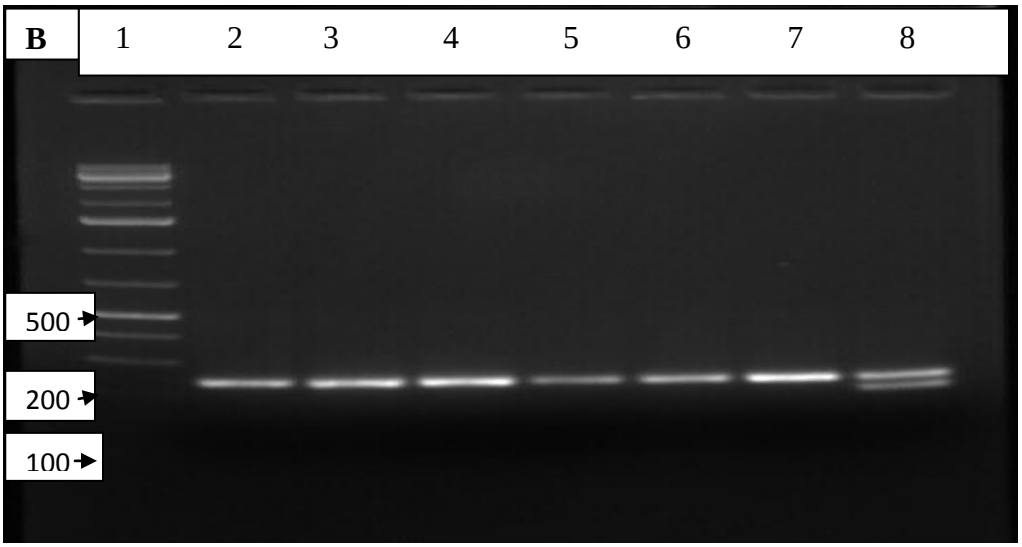
Figure 14: The comparative phage DNA extraction of 17 phage isolates to check quality of DNA: A) Phage filtrate with DNase and RNase treated; B) Filtrate with no DNase and RNase treatment; C) Phage-*E. coli* mix treated by DNase, RNase, SDS and Proteinase K; D) Phage-*E. coli* mix not treated by DNase, RNase, SDS and Proteinase K.

4.5.2. Polymerase chain reaction (PCR) based identification of phages

PCR was used to verify phages at the family and genus levels using gene specific primers for each of the families. Out of 17 phage isolates 15 phages were identified by PCR targeting the major capsid protein of *myoviridae* and *podoviridae* phages as well as the major coat protein of *siphoviridae* phages. *Siphoviridae* and *Podoviridae* families were detected with amplicon sizes of 200 bp and 461 bp, respectively. Moreover, *Myoviridae* was detected with different amplicon sizes 240 bp of T4 virus and 519 bp of Felixo 1 virus (**Figure 15, A & B**). Of 15 identified phage isolates two phages (EI-SP-GF and EA-T-A) were in the family *Podoviridae* and the other two phages (EA-SP-SMA and ST-M-A) were in the family *Siphoviridae*. The remaining all phages were in the family *Myoviridae* with four phages (EP-M-K, EP-M-A, EP-B-K (B), and EP-B-K (E2)) identified as Felixo 1 virus and seven phages (EH-SD-TH, ET-SD-TH, EH-B-A (A1), EH-

B-A (A2), EA-M-A, EA-SD-FA, and ST-M-K) were identified as T4 virus. Similarly, among seven potent phage isolates six were *myoviridae* phages with four of them being T4 viruses and 2 of them being FO1 phage viruses (**Figure 15, C**). PCR unidentified phage isolates were ST-T-K and EH-SP-TH.





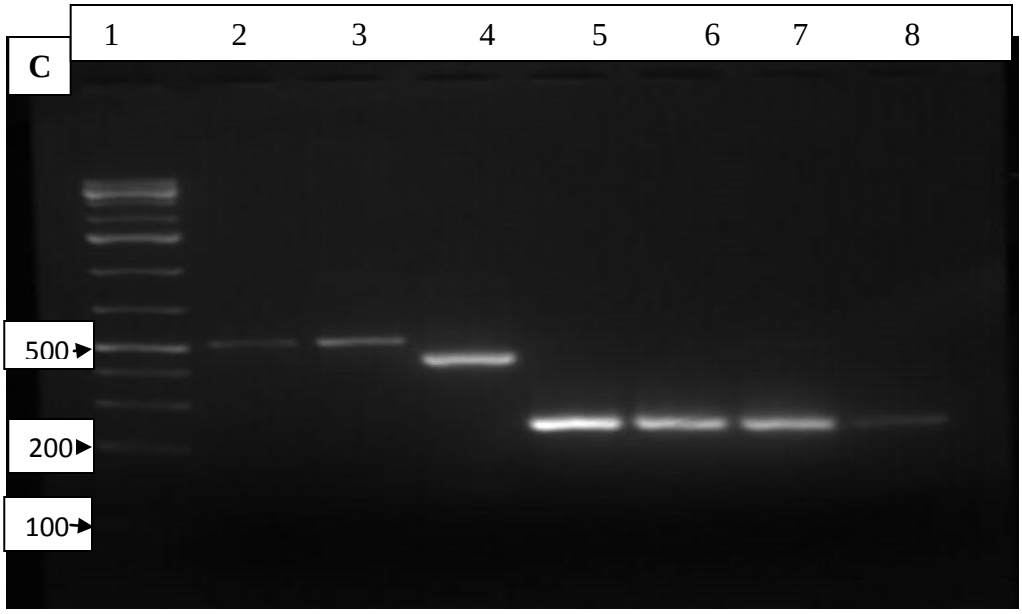


Figure 15: Gel images of PCR identified phage isolates: **A**) Lane-1, 1kb plus ladder; Lane-2, T5; Lane-3 & 4, T7 and Lane-5 to 8, FO1 phages with 200 bp, 461 bp, and 519 bp respectively **B**) Lane-1, 1kb plus ladder; lane-2 to 8 all phages were T4 phages with 240 bp size **C**) Seven potent phages selected in separate gel; Lane-1, 1kb plus ladder; Lane -2 & 3, 519 bp sized FO1 phages of EP-M-A & EP-B-K (E2), Lane-4, 461 bp sized T7 phage EI-SP-GF and Lane- 5 to 8, 240 bp sized phages of EH-SD-TH, EH-B-A (A1), EH-B-A (A2), ET-SD-TH.

According to the PCR result, about 73 % of phage isolates were *myoviridae*-like phages with 47 % being T4 phages as well as 26 % of phages were FO1. *Podoviridae*-like and *Siphoviridae*-like phages were 27 % with 13.5 % each identified from the PCR (**Table 13**). Therefore, *Myoviridae* phages were the most dominant coliphages isolated from different environmental samples. EI-SP-GF *Podoviridae* phage was the only isolated among seven potent phages.

Table 13: PCR-identified phage isolates with their family and genus

Phages	Family	Genus	Amplicon size
EP-M-A	*Myoviridae*	Felixo 1 virus	519 bp
EP-B-K/B	*Myoviridae*	Felixo 1 virus	519 bp
EP-B-K/EN2	*Myoviridae*	Felixo 1 virus	519 bp
ET-SD-TH	*Myoviridae*	T4 virus	240 bp
EH-SD-TH	*Myoviridae*	T4 virus	240 bp
EH-B-A/A1	*Myoviridae*	T4 virus	240 bp

EH-B-A/A2	*Myoviridae*	T4 virus	240 bp
ST-M-A	*Siphoviridae*	T5 virus	200 bp
ST-M-K	*Myoviridae*	T4 virus	240 bp
EI-SP-GF	*Podoviridae*	T7 virus	461 bp
EA-T-A	*Podoviridae*	T7 virus	461 bp
EA-SD-FA	*Myoviridae*	T4 virus	240 bp
EA-SP-SM	*Siphoviridae*	T5 virus	200 bp
EA-M-A	*Myoviridae*	T4 virus	240 bp
EP-M-K	*Myoviridae*	Felixo 1 virus	519 bp

4.5.3. Whole Genome Sequence Analysis

To examine the genomic structures and the potential diversity in the genomes, the 7 potent newly isolated bacteriophage genome sequences were determined and some genomic comparison was conducted. The raw sequence data was submitted to NCBI data base as sequence read archive (SRA) with PRJNA1006193 bioproject identifier and SAMN37015700 to SAMN37015706 biosample identifiers as well as accession numbers from SRR25691062 to SRR25691068. Good quality of raw sequence data for all phages was ensured according to FastQC parameters. Therefore, phage isolate EH-B-A,A1 and EH-SD-TH sequences were not further analyzed due to low quality FastQC result for consensus sequence generation. Genome sequencing of the bacteriophages showed that all the bacteriophages have a linear double-stranded DNA genome. Phage genomes ranged in size from 40,427 to 143,710 bp, with a GC content of 43 to 54% (**Tables 14 and 15**). Aligned in NCBI database, BLASTn analysis showed that phage isolate EP-M-A had the highest genome similarity with Escherichia phage ZCEC5, (GenBank: NC-073321.1), with a nucleotide similarity of 91.81% and a genome coverage of 76%. EP-B-K, E2, EI-SP-GF, ET-SD-TH and ST-T-K had the highest genome similarity with Escherichia phage K1G, (GenBank: NC-027993.1), Escherichia phage slur16 (GenBank: NC-028248.1), Escherichia phage vB_EcoM_ECO1230-10 (GenBank: NC-027995.1) and Escherichia phage vB_EcoS-101114BS4 (GenBank: NC-073061.1) respectively.

Taxonomic classification of the 7 isolated potent coliphages was performed using multiple WGS genome comparisons (http://www.ncbi.nlm.nih.gov/genome/viruses/) and phage DB. These coliphages included 4 (57%) of *Myoviridae E. coli* phages and 3 (43%) of *Siphoviridae E. coli*

phages. According to ICTV guidelines, phage family, subfamily, and genus were predicted based on genome similarity. The results are shown in Table 13. EH-B-A (A1), EH-SD-TH, EI-SP-GF and ET-SD-TH phages belong to the *Myoviridae* family. These phages were thought to belong to the *Tevenvirinae* subfamily, which includes the *T4 virus*, also known as *Tequatrovirus*, except for phage ET-SD-TH, which probably belongs to the Jilinvirus family. EP-M-A, EP-B-K (E2), and ST-T-K phages belonged to the *Siphoviridae* family with subfamily *Guernseyvirinae* with genus *Kagunavirus*; Except for ST-T-K which was in the genus *Dhillonvirus*.

Table 14: The whole genome analysis of phage DNA sequence

Sample	Specimen	No raw reads	No trimmed reads	Genome size (bp)	Family	Subfamily	Genus
S41	EH-B-A (A1)	1095	398	131660	*Myoviridae*	*Tevenvirinae*	*Tequatrovirus T4*
S42	EH-SD-TH	4329	1880	143710	*Myoviridae*	*Tevenvirinae*	*Tequatrovirus T4*
S43	EP-M-A	534845	276179	42850	*Siphoviridae*	*Guernseyvirinae*	*Kagunavirus*
S44	EP-B-K (E2)	803828	377929	42228	*Siphoviridae*	*Guernseyvirinae*	*Kagunavirus*
S45	EI-SP-GF	624628	325380	136820	*Myoviridae*	*Vequintavirinae*	*Vequintavirus*
S46	ET-SD-TH	10079	5024	40427	*Myoviridae*	-	*Jilinvirus*
S47	ST-T-K	53297	22236	45249	*Siphoviridae*	-	*Dhillonvirus*

As indicated in **Table 15**, 52-268 putative CDSs were discovered for each *E. coli* phage using both automatic and manual annotation. All 7 coliphages genome sequences had CDSs that encoded the phage terminase small subunit, DNA polymerase, phage terminase big subunit, the phage lysis enzyme, and the phage capsid and tail proteins. In addition, tiny terminase component and phage DNA polymerase were found in the majority of the phage genomes. One to three CDSs for the tail and capsid proteins of each phage were discovered. None of the 7 phage genomes contained any known acquired resistance or virulence genes. The updated tRNAscan-SE based predictions of tRNAs indicated that EI-SP-GF had 5 tRNAs and EH-SD-TH had 9 tRNAs. The remaining 5 phage isolates have no tRNA.

Table 15: Genome detective and GeneMarkS-2 wed-based annotations of phage DNA

Phages	Alignment score	No stop codon	No CDS	No proteins	GC content (%)	NT (%) Identity	AA (%) Identity	Reference phage
EH-B-A (A1)	25200	13	266	45	45	98.6	96.4	Escherichia phage ECML-134 (taxon:1204522)
EH-SD-TH	121469	59	268	65	43	97.8	98.5	Escherichia phage ime09 (taxon:1054834)
EP-M-A	119175	56	72	64	52	91.8	96.1	Escherichia phage ZCEC5 (taxon:2530021)
EP-B-K (E2)	108109	36	52	49	51	91.35	96.23	Escherichia phage K1G (taxon:698486)
EI-SP-GF	194829	190	206	204	44	94.3	97.2	Escherichia phage slur16 (taxon:1720495)
ET-SD-TH	76790	2	51	8	53	88.1	88.4	Escherichia phage vB_EcoM-ep3 (taxon:1541883)
ST-T-K	153955	58	71	64	54	94.4	96.9	Escherichia phage vB_EcoS-101114BS4: (taxon:2865793)

4.5.4. Phylogenetic Analysis

In phylogenetic trees of relation among phage isolates EP-M-A clearly clustered together and in the same clade with phage EP-B-K, E2 (**Figure 16**) with 100% bootstrap values as both phages were in the same family of *Siphoviridae* and genus *Kagunavirus*. These two phage isolates again showed 100% evolutionarily relationship with phage isolate ET-SD-TH. Phages EI-SP-GF and

ST-T-K were out grouped from the relationship, but within cluster having distant evolutionary relation.

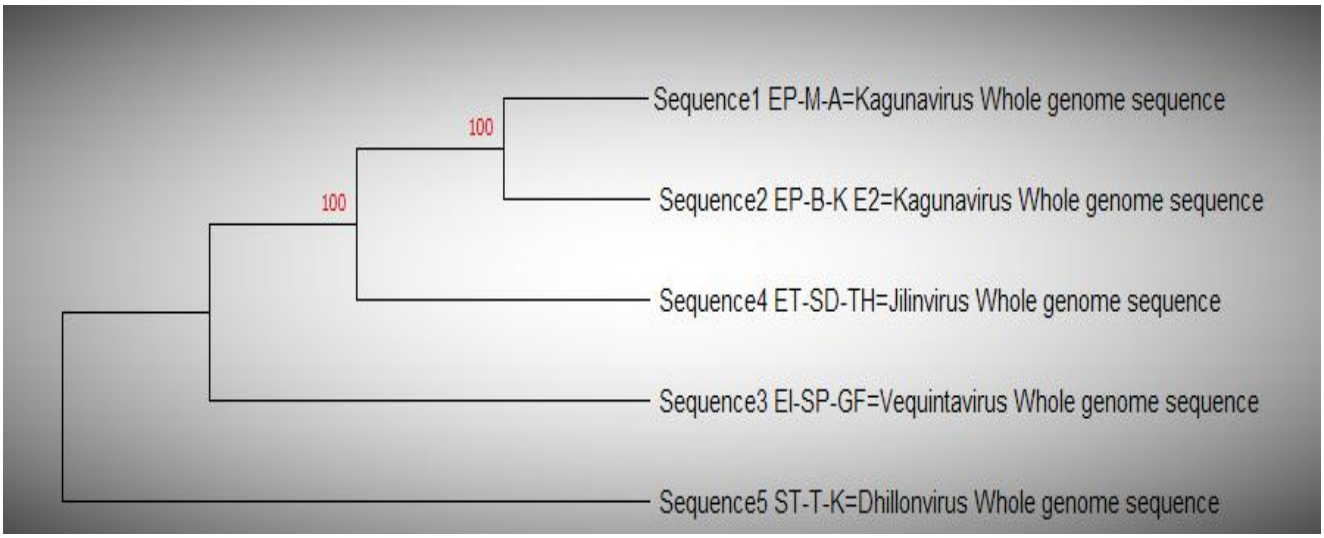


Figure 16: Phylogenetic tree among phage isolates constructed by using the sequence alignment of whole genome: A bootstrap value 100 in the branch of tree indicates level of support in branch that implying 100% inferred relationship to be accurate.

In order to analyze the evolutionary relationship between phage isolates and other *Caudoviricetes* phages, a phylogenetic tree was constructed based on the nucleotide sequences of the relatively conserved phage major capsid protein (MCP) phylomarker gene using the Neighbor-joining (NJ) method. Constructing a major capsid protein-based phylogenetic tree of phages involves analyzing the genetic sequences of the major capsid protein gene from database of different strains.

In case of phage isolates EP-M-A and EP-B-K, E2 major capsid protein (MCP) gene search was limited to the genus *Kagunavirus.* The MCP gene sequence search for phage isolates EI-SP-GF, ET-SD-TH and ST-T-K was limited to *Vequintavirinae*, *Jilinvirus* and *Dhillonvirus* respectively. Therefore, NCBI MCP gene sequences were retrieved from database and MCP gene was cut out from each phage isolate mapping with reference sequences and multiple sequences was performed in MEGA11 software for tree construction. There were 10 NCBI search hit sequences obtained related to *Kagunavirus;* the accession numbers are indicated in the tree. The phylogenetic tree of phage isolate EP-M-A showed that phage EP-M-A and Escherichia phage vB EcoSfFiEco02 clustered onto a single branch with 73% bootstrap value which supports strongly the inferred relations (**Figure 17**). It was related by four phages by 39 bootstrap values

which means that the branch in question was supported in approximately 39% of the resampled trees.

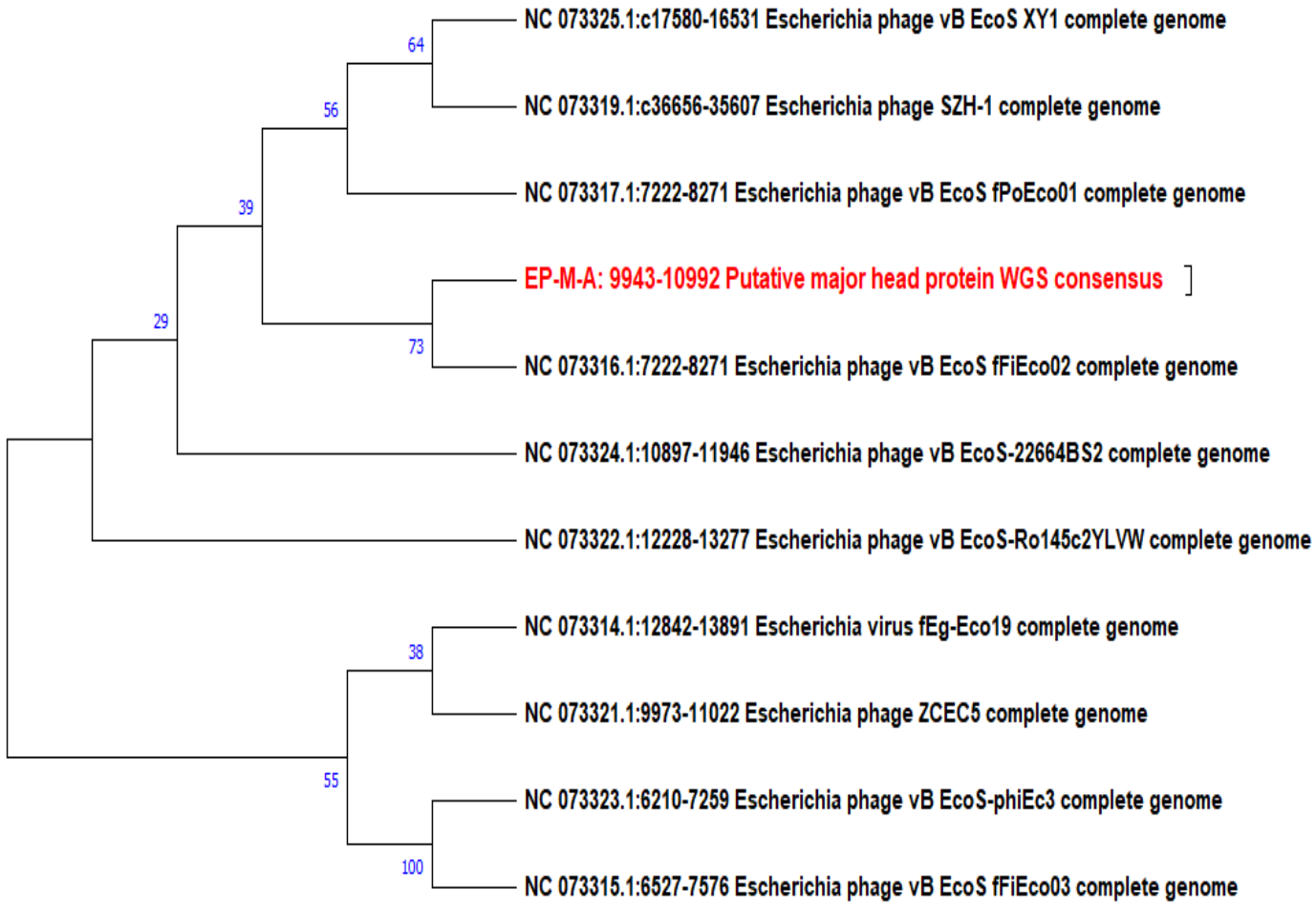


Figure 17: Phylogenetic tree of EP-M-A phage based on major capsid protein gene: The phylogenetic tree was constructed in relation with 10 different phages under genus *Kagunavirus.* The evolutionary relationship of phages EP-B-K (E2) showed that it was related to Escherichia phage vB EcoSfFiEco02 phage from the database with 81% support of the grouping of taxa by bootstrap and 61% support of the evolutionary relationship by two phages (**Figure 18**).

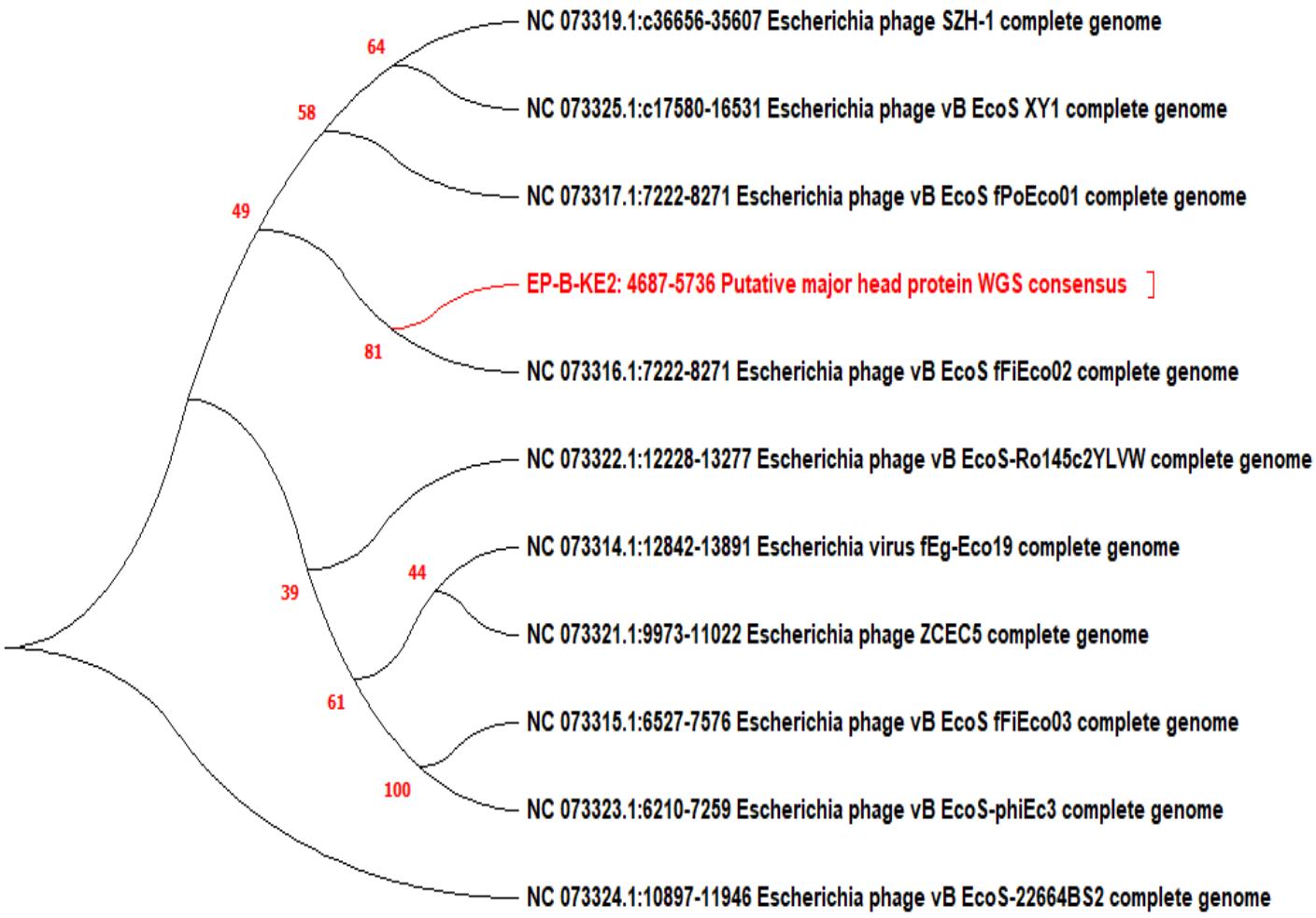


Figure 18: Phylogenetic tree of EP-B-K (E2) phage based on major capsid protein gene: The phylogenetic tree was constructed in relation with 10 different phages under genus *Kagunavirus.*

The phage isolate EI-SP-GF was related 100% with four phages including *Salmonella* and *Klebsiella* phages (**Figure 19**).

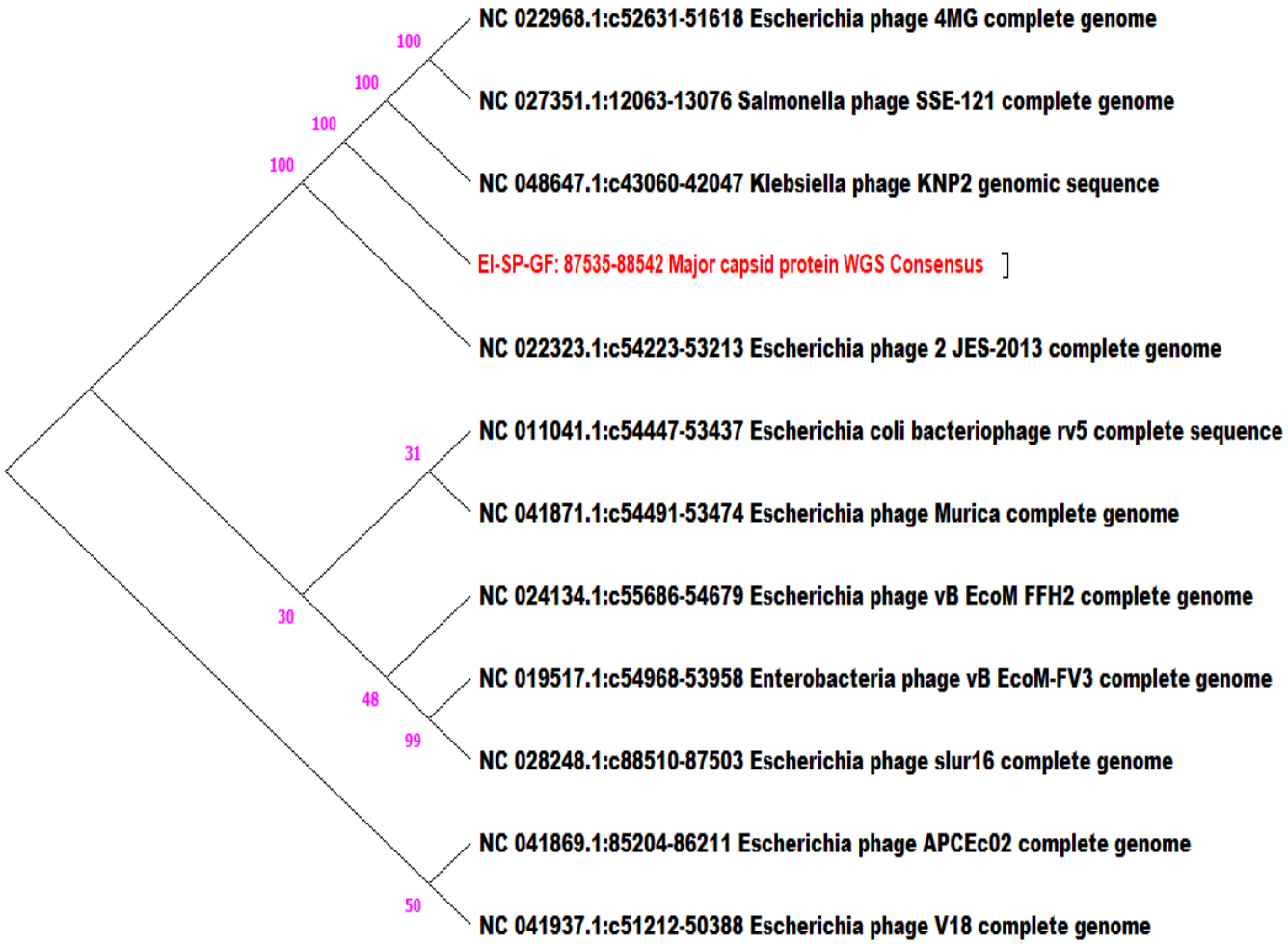


Figure 19: Phylogenetic tree of EI-SP-GF phage based on major capsid protein gene: The phylogenetic tree was constructed in relation with 11 different phages under Subfamily *Vequintavirinae*.

ET-SD-TH and ST-T-K were related 100% with *Enterobacter* phage Arya and 94% with two *E. coli* phages respectively (**Figure 20 & Figure 21**).

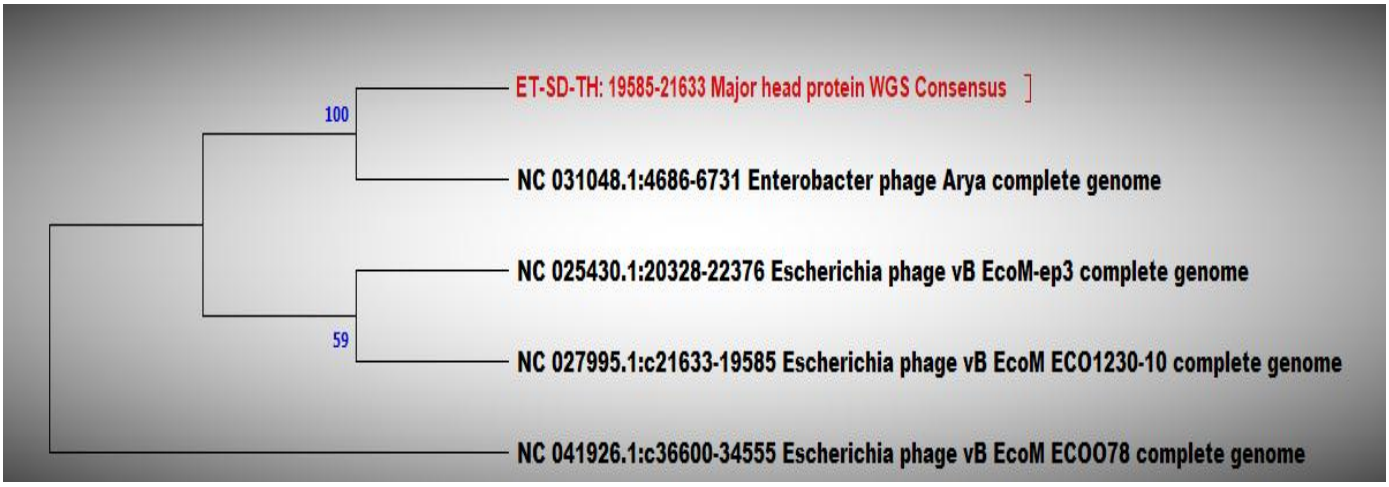


Figure 20: Phylogenetic tree of ET-SD-TH phage based on major capsid protein gene: The phylogenetic tree was constructed in relation with 4 different phages under genus *Jilinvirus*

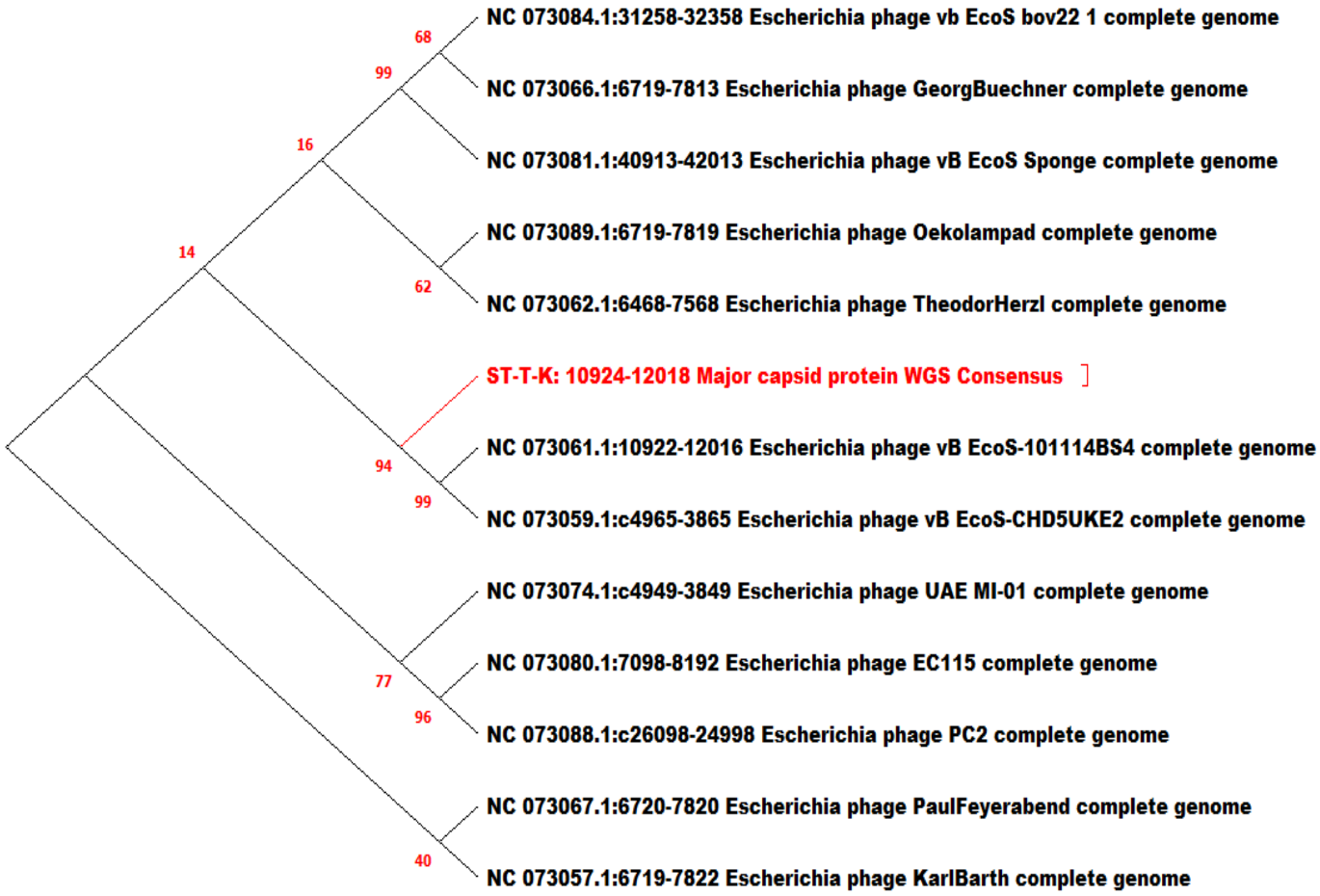


Figure 21: Phylogenetic tree of ST-T-K phage based on the major capsid protein gene: The phylogenetic tree was constructed in relation with 12 different phages under genus *Dhillonvirus.*

5: Discussion

Pathogenic *E. coli* is known to cause diarrhea and related diseases in both animals and humans. The emergence of antibiotic resistance has led to renewed interest in exploring lytic bacteriophages as a natural and eco-friendly biocontrol strategy. These phages have the ability to lyse multidrug-resistant pathogens, making them an effective tool in eliminating pathogenic bacteria (Lin *et al*., 2017). Consequently, there is a growing interest in searching and utilizing lytic bacteriophages for the treatment of these harmful pathogens (Yang *et al.*, 2010). This study focused on the isolation and characterization of lytic phages against multidrug resistant *E. coli* strains from various sources and evaluates their therapeutic potentials. Bacteriophages are widely distributed in the environment, including rivers, soil, sewage, animal feces, water ponds, and seawater where their hosts reside (Mulani *et al.*, 2015). In Ethiopia, river water and sewage are heavily contaminated with fecal and waste matter, resulting in a high diversity of enteric organisms. This study aimed to isolate phages from relevant sources, such as river water, dairy sewage, and hospital liquid waste samples. Other studies have also successfully isolated phages from freshwater ponds, animal waste, and soil. For instance, Shukla *et al*. (2014) isolated phages from animal waste collected from different livestock farms, while Alonso *et al*. (2002) isolated 26 phages from water samples of Alboran Sea, Western Mediterranean. Leta *et al*., (2017) and Betemaryam, 2020 also successfully isolated lytic phages from sewage water collected in Jimma town, Jimma, and National Veterinary Institute, Bishoftu respectively in Ethiopia against *E. coli*. These findings suggest that phages can be isolated from a wide range of sources.

Before successfully isolating suitable lytic bacteriophages as antimicrobial agents, it is necessary to isolate, identify, and fully characterize the bacterial host (Moye *et al*., 2018). In this particular investigation, multidrug-resistant pathogenic *E. coli* strains were determined as resistant to most available antibiotics and available as glycerol stocks at the Institute of Biotechnology, Addis Ababa University, Health Biotechnology laboratory. As a potential alternative and effective treatment for diseases caused by these resistant *E. coli* strains, phages were employed. Thus, 17 lytic phages were successfully isolated from various sources including river water, hospital fluid waste, and dairy farm sewages. Multidrug-resistant diahearrgenic *E. coli* strains were used as hosts. The selected phages were those that formed clear zones of lysis or plaques on the bacterial lawn, indicating their virulence and suitability for biocontrol applications. Different sizes of clear

and discrete plaques were observed, ranging from small to large with phage titers ranging from 2.8×10^7 to 3.12×10^{10} PFU/ml. Interestingly, 65% of phage isolates produced large and clear plaques on their preferred hosts, similar to *E. coli* O157, *Listeria*, *Pseudomonas* and, *Salmonella*-specific phages (Perera *et al.*, 2015 and Zhang *et al*., 2018). All the phage isolates were unique respect to their host *E. coli* strain, but from some sample site two or more phages were detected from different partitions like middle and top surface of Kebena river. The main reason for this is phages similar receptor bindings with different *E. coli* strains (Fischer *et al*., 2004). To determine their therapeutic value, phage properties such as host range, stability, growth kinetics, and viral yield must be characterized (Jacquinot *et al*., 2018).

The selection of phages for biocontrol of AMR pathogens is primarily based on the host range, which is considered a crucial factor (Duc *et al*., 2018). The lytic spectra of a phage, which is a significant biological characteristic, refers to the range of bacteria genera, species, and strains that a phage can kill. In biocontrol applications, it is essential to select virulent phage candidates with broad lytic spectra instead of temperate and narrow lytic ones (Hagens and Loessner, 2010) because temperate phages have the ability to transfer virulence or antibiotic resistance genes (Haaber *et al*., 2016), and narrow lytic ones cannot cover many bacterial strains.

The diversity among phages was evident in this study as their lytic profiles varied when different host strains of *E. coli* and other gram-negative Enterobacteria were used. Seventeen phages underwent spot tests to determine their host range, based on lytic profiles, plaque clarity, and phage size. The phages were capable of infecting different *E. coli* strains and gram-negative Enterobacteria. Interestingly, seven phages (7/17) exhibited clear plaques on different hosts, suggesting that they were polyvalent and had broader host ranges. EOP analysis revealed that four (4/7) phages had high efficiency (EOP $\geq$ 0.5) on the reference host strains. Although all seven phages formed clear plaques on *E. coli* hosts and gram-negative Enterobacteria species during the spot test, three phages exhibited medium to low EOP (<0.5) on the reference host, indicating that they were highly specific to the isolation host strain. The infectivity variation might be due to non-specific binding receptors on the host cell wall or the presence of phage-resistant strains. Host specificity is considered a desirable characteristic for selecting therapeutic phage application, particularly in live animals, to ensure that they have little or no impact on the beneficial gut microflora (Akhtar *et al.*, 2017).

The phages were further characterized by means of a one-step growth experiment to determine their infection characteristics, including latent period and burst size. A one-step growth curve was a graphical representation of the various stages of a phage infection cycle within a host cell population over time. It provides insights into the dynamics of viral replication and the progression of infection. Typically, a one-step growth curve consists of a series of data points plotted against time. The x-axis represents the time elapsed since the start of the infection, while the y-axis represents the number of infectious viral particles or viral progeny produced during each time point represented in PFU/ml. Those parameters are important in assessing a phage's efficacy in infecting and developing within a specific host.

The average latent period of the phages was found to be between 10 and 15 min, with burst sizes ranging from 87 to 364 particles per cell. The observed burst sizes were considered large compared to other *E. coli* phages, where average burst sizes as small as 33 and 51 pfu/cell have been reported (Lee and Park, 2015). However, a burst size of 9000 pfu/cell has been reported for a Podovirus-phage phiAxp-3 (Ma *et al.*, 2016). Differences in the latent period and burst size of phages can be attributed to host cells, growth medium, pH, and temperature of incubation (Guttman *et al*., 2005). The short latent period and large burst size of the seven potent phages suggest that they have a competitive advantage over other phages, as they can produce enough virions to lyse host bacteria in a short amount of time. Therefore, these phages possess favorable characteristics that make them attractive candidates for a biocontrol treatment program.

Apart from biological properties, newly isolated phages should be evaluated for their stability and persistence in different environmental conditions to confirm their biocontrol potential (Hagens and Loessner, 2010). Therefore, the study aimed to investigate the response of the seven potent phages to physicochemical stress factors that might be encountered during phage production or biocontrol application. The seven phages showed similar behavior and stability at different incubation temperatures and pH. They were stable between 25°C and 70°C for six hours, but temperatures above 70°C resulted in a reduction of titers, possibly due to the effect of high temperatures on phage proteins (Ackermann *et al*., 2004). Similar observations have been reported in studies investigating the effect of temperature on phage stability (Litt and Jaroni, 2017). The thermal stability of the phages to high temperatures (50°C-70°C) in this study suggests that they could be suitable for biocontrol applications against pathogenic *E. coli* strains.

The infectivity of *E. coli* phages is affected by acidic environments, which can lead to denaturation of phage proteins and subsequent loss of viability (Hazem, 2002). Previous studies have shown that most tailed phages remain stable at pH levels between 5.0 and 9.0 (Smolarska *et al*., 2018), which is nearly similar with the results of this study. While all of the isolated phages showed high resistance to acidic and alkaline conditions (pH 5.0 to pH 9.0) after 6 hours of exposure, some phages experienced a loss of titer at pH levels of 3.0 and 9.0. However, phages EP-M-A and EP-B-K exhibited resistance to higher alkaline environments (pH 9.0), similar to previous findings on the preference of *Podoviruses* for alkaline conditions and their sensitivity to acidic conditions (Jończyk *et al*., 2011). This alkaline stability could expand the potential applications of these phages.

SEM analysis is a rapid and simple method of characterizing phages, aiding in the identification of novel phages and attribution to families (Aprea *et al*., 2015). SEM observation indicated that 4 out of 17 phages, isolated from different samples, belonged to the order *Caudovirales*, with 2 of the isolates being part of the *Myoviridae* family. This family is one of the three main families of *Caudovirales,* also known as tailed phages. Although previous research has shown that tailed phages (*Caudovirales*) represent the most diverse, numerous, and widespread of all bacterial viruses, the families *Siphoviridae* and *Myoviridae* are the most prevalent, accounting for 86% of the order, while the *Podoviridae* family is the least represented, accounting for approximately 14% (Ackermann, 2011). Similarly, studies on the morphology of environmental *E. coli* O157:H7 bacteriophages have shown the dominance of the *Myoviridae* and *Siphoviridae* families (Lee and Park, 2015). The remaining 13 phages were not identified by SEM analysis due to various factors such as phage degradation during lyophilization processes, SEM imaging errors, and sample handling. It provides detailed structural information about the phage, such as its size, shape, and surface characteristics. SEM can quickly identify the presence of phages in a sample and provide visual evidence of their morphological features. However, SEM alone cannot provide information about the specific genetic makeup or identity of the phage (Abdelsattar *et al*., 2022).

The PCR assays presented in this report offers the benefit of detecting the presence of *E. coli* phages and their family and genus directly from phage lysate samples in a single reaction. In this investigation, the major capsid protein and major coat protein were utilized as molecular markers

to promptly classify new phages into a certain group, thereby providing a preliminary identification of their family and genus which is similar to Hopkins *et al*., 2014 and Born *et al*., 2019 who target MCP gene for the PCR identification of their phage isolates. The sequences of virulent phages and their genus-specificity were the basis for the selection of all primers used in this investigation. The specificity of each primer set is at a specific taxonomic level based on the current taxonomy of these phages (Barylski *et al*., 2020).

Fifteen out of seventeen phage isolates were identified by PCR amplification using specific primers. Among these fifteen phages, eleven phages were in the *Myoviridae* family. This suggests that *Myoviridae* phages are the most common type of phage in river water, hospital, and diary sewages, which is consistent with the findings of Alanazi *et al.*, (2022) who used direct concentration of phages from environment by PEG precipitation and Born *et al.*, (2019), who used *E. coli* and other Enterobacteria as a host, then identified *Myoviridae* as the dominant phages in sewage water. In contrast, Jurczak-Kurek *et al*., (2016), found a greater abundance of *Siphoviridae* compared to *Myoviridae* and reported the least abundance of *Podoviridae* in coliphages from sewage by using *E.coli* and different bacterial hosts. Additionally, a study of viral communities in Lake Baikal indicated the prevalence of various families, including *Myoviridae*, *Siphoviridae*, and *Podoviridae*, supporting this study (Potapov *et al*., 2019). Phage isolates ST-T-K and EH-SP-TH were not identified by PCR which might be the absence of target gene in the phages as well as they are different phages from *Caudovirales*.

Sequencing reads were aligned to a reference genome, calling variants using the ivar tool, and consensus sequence was generated using the samtools. The phage isolates EH-B-A, A1 and EH-SD-TH had low quality reads and very short contiguous sequences were assembled from the reads. Therefore, it was unreliable to generate consensus sequence from these phage isolates read. The main factor for low quality reads and inability to generate consensus might be initial library preparation problems as well as the quality of DNA used for sequencing (Russell, 2018; Shen and Millard, 2021). Genome sequencing of the *E. coli* phages EP-M-A, EP-B-K, E2, EI-SP-GF, ET-SD-TH ST-TK and the scaffold sequences of EH-B-A, A1 and EH-SD-TH revealed that these phages have linear double-stranded DNA (dsDNA) which is similar to previous reports (Xiao *et al*., 2023; Li *et al*., 2023) who extracted double-stranded DNA genome from animals and sewage of hospital. Genome map of phage isolates EH-B-A, A1, EH-SD-TH, ET-SD-TH

and EI-SP-GF belonged to the family *Myoviridae* and phages EP-M-A, EP-B-K,E2 and ST-T-K belonged to the family *Siphoviridae* which is comparable with previous reports (Wójcicki *et al*., 2021; Cepko *et al*., 2020; Grami *et al*., 2023). The genome based comparative phage taxonomy of isolates was different from the PCR identification of phages for the isolates EP-M-A, EP-B-K,E2 and EI-SP-GF that could be due to PCR primer non-specific binding as well as primer designing problem.

PCR is commonly used method for phage identification, has limitations in capturing the complete genomic diversity of phages (Ács *et al*., 2020). On the other hand, genome-based comparative phage taxonomy analyzes whole phage genomes to determine relationships and classify them accurately. When comparing the results of PCR identification with genome-based taxonomy, differences can arise. These differences may be due to limitations of PCR primers or designing problem, due to degeneracy of primers and horizontal gene transfer events (Clokie, 2009; Clermont *et al*., 2015).

Genome-based taxonomy offers advantages by providing a comprehensive understanding of phage diversity. It enables the detection of novel phage groups, identification of evolutionary relationships, and insights into phage-host interactions. It also allows for the identification of functional genes and potential applications in fields like phage therapy. Integrating both PCR-based and genome-based methods is crucial for a better understanding of phage diversity. This approach will contribute to advancing our knowledge of phages and their potential applications in various fields. In general, SEM is a visual technique that provides structural information, PCR enables targeted detection of specific phage DNA or genes, and sequencing offers a comprehensive analysis of the phage's genetic material.

In phage isolates EH-B-A, A1, EH-SD-TH and ET-SD-TH only 20% of open reading frames (ORFs) code for putative and hypothetical proteins whereas in case of EP-M-A, EP-B-K, E2, EI-SP-GF, and ST-T-K more than 90% of ORFs code for proteins. The lower percentage of ORFs (20%) coding for proteins in the EH-B-A, A1, EH-SD-TH and ET-SD-TH isolates might be attributed to sequence low read numbers. This suggests that the sequencing depth or coverage for these isolates might be insufficient, resulting in a smaller fraction of identified ORFs. The higher percentage of ORFs coding for proteins in the EP-M-A, EP-B-K, E2, EI-SP-GF, and ST-T-K isolates (more than 90%) indicates a greater level of genome completeness. These isolates likely

have higher quality and depth of sequencing, which enables the identification of a larger proportion of functional protein-coding genes.

The tRNAscan-SE v. 2.0 analysis indicated that phage isolates EH-SD-TH and EI-SP-GF had tRNAs in their genome, but the isolates EP-M-A, EP-B-K, E2, ET-SD-TH, EH-B-A, A1 and ST-T-K had no tRNAs. The absence of tRNA sequences in the phage genome implies that the phage is more reliant on the host cell's resources for translation and may have evolved to exploit the host's existing translational machinery. The presence of tRNAs in the phage genome suggests that the phage has adapted to replicate efficiently within the host cell by utilizing its own translation machinery. In this study, the genome detective web-based and GeneMarkS-2 analysis showed that the genomes of all phage isolates do not contain sequences of genes encoding integrase, recombinase, repressors, or excisionase, which are the main markers of lysogenic viruses (Necel, *et al*., 2020). Therefore, the results indicated that these phages should be considered as strictly lytic (virulent) phages.

To obtain a more global phylogenetic overview of the relationships between the different *E. coli* phage isolates, whole genome-based alignment was employed for tree construction against each other. The major capsid protein gene database sequences of the same genus as well as subfamily particularly isolate EI-SP-GF was obtained for determination of evolutionary relationship of each phage isolates with available database sequences. Bootstrapping, a resampling statistical technique was used to assess the robustness of the inferred phylogenetic relationships. The resulting trees were compared to calculate the frequency at which a particular branch appears in the replicate trees. This frequency was expressed as a bootstrap value, which represents the statistical support for that branch. Higher bootstrap values (typically ranging from 70 to 100) indicate greater support for the branch (Wiens *et al*., 2008).

They are constructed based on similarities and differences in genetic sequences, typically using techniques multiple sequence alignment and evolutionary models. The phages EP-M-A and EP-B-, E2 were clustered together having 100% supports by bootstrap. In phylogenetic analysis, bootstrap support is a measure of the statistical confidence or robustness of a particular branch or grouping in the tree. It is often represented as a percentage and indicates how often a particular grouping appears in replicate analyses of the data. A bootstrap value of 100% suggests that in multiple iterations of the analysis, the sequences EP-M-A and EP-B-E2 consistently clustered

together as a distinct group. This high bootstrap support indicates a strong statistical confidence in the grouping or relationship between these two sequences. Therefore, based on the available information, it can be concluded that EP-M-A and EP-B-E2 are closely related and form a cluster in the phage phylogenetic tree, supported by a bootstrap value of 100% which is comparable with study by Al-Shayeb *et al*., (2020) who constructed phylogenetic tree using major capsid protein and many of the sequences from their phage genomes cluster together with high bootstrap support that defining clades.

The major capsid protein (MCP) gene was used for phylogenetic tree construction for each phage isolate in order to observe evolutionary relationships with existing data base sequences. The MCP gene was the gold standard for classification of the lytic phage family. An essential function of the major capsid protein (MCP) gene is to maintain the structure and function of bacteriophages, which are viruses that specifically infect and replicate within bacteria. The MCP gene encodes the major structural protein that forms the outer capsid of the phage, encompassing the viral genome and protecting it during infection. The MCP gene is highly conserved within a specific phage family, meaning that it displays a relatively low rate of mutation across different phage isolates within the same family. This conservation leads to utilize the MCP gene for phylogenetic tree construction, which helps to elucidate the evolutionary relationships between different phage isolates (Lee *et al.,* 2022; Dion *et al*., 2020).

The MCP gene database search was specified to the genus *Kagunavirus* for isolates EP-M-A and EP-B-K, E2 whereas in case of EI-SP-GF specified to the family *Vequintirinae, Jilinvirus* for ET-SD-TH and *Dhillonvirus* for ST-TK to retrieve sequence from database. Isolates EP-M-A and EP-B-K were classified within the genus *Kagunavirus*. This suggests that these isolates share significant similarities in their MCP gene sequences, indicating a close evolutionary relationship that is similar to the report by Grose and Casjens, (2014). The isolate EI-SP-GF was classified within the family *Vequintirinae*, a taxonomic family that encompasses a group of viruses showing similarities in their MCP gene sequences. The isolate ET-SD-TH was classified within the genus *Jilinvirus*, while the isolate ST-TK was classified within the genus *Dhillonvirus*. These genera represent distinct groups of viruses with shared MCP gene characteristics.

6: Conclusion

The occurrence and proliferation of antibiotic-resistant bacterial pathogens causes a severe challenge in both clinical and dairy settings due to the dearth of available treatment alternatives. Of particular concern is the rise of multidrug-resistant strains of pathogenic *E. coli*, which has become a significant issue for both medical and veterinary professionals. This study's major discovery was the isolation, morphological, PCR identification and whole genome-based analysis of potential lytic phages against various multidrug resistant diahrrgenic *E. coli* strains commonly involved in human and animal infections. Seventeen phages that were lytic against multidrug-resistant pathogenic *E. coli* strains were discovered. The phages belonged to the order *Caudovirales*, with the family *Myoviridae*, *Siphoviridae*, and *Podoviridae*, and the genus T4 virus, Vequintavirus, Dhillonvirus, and Kagunavirus with a rapid growth rate. Genome sequencing of the seven bacteriophage isolates showed that all the phages have a linear double-stranded DNA genome. This study provides a better understanding of the infection kinetics of seven potent phages that target *E. coli* strains, as well as their survivability under various stress conditions and growth characteristics. The phages demonstrated better growth characteristics, including short latent periods, highest burst sizes, and wider host ranges, as well as thermal stability and the ability to survive in a wide range of pH levels. This information will enable researchers to determine the potential application of phage-based interventions. These phages' promising effect against MDR pathogens has raised the possibility of their use in biological control of bacterial infections. Further characterization of specific phages is necessary to explore their potential clinical applications.

Recommendations

- This study only examined some environmental waste and one hospital sample. Dairy farm effluents are also limited. Therefore, there is a need for more research on other environmental, hospital and agricultural/dairy farm samples.
- On the other hand, they need to be further characterized for their *in vivo* activity in mouse models and assess their performance.
- Genomic factors such as virulence and drug resistance genes and the survival of these bacteriophages under different physical and chemical conditions need to be investigated.

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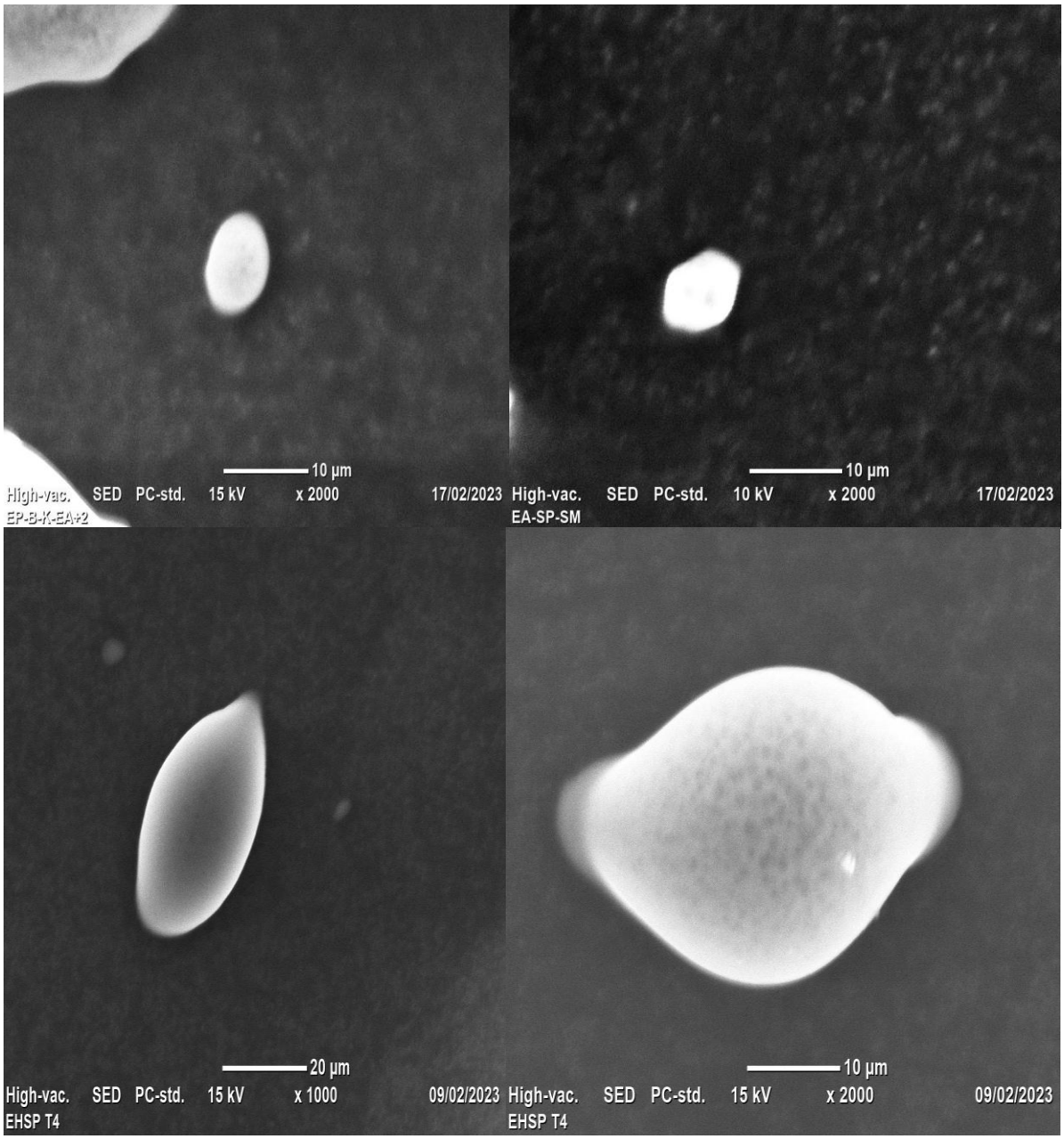
Appendixes

1. DNA quality and quantity measured by Nanodrop

Phages	Concentration	Unit	A260 (Abs)	A280 (Abs)	260/280	Factor
EP-M-A	110.2	ng/µl	2.205	1.252	1.76	50
EP-B-K/B	347.6	ng/µl	6.953	3.61	1.93	50
EP-B-K/EN2	1241.9	ng/µl	24.838	15.754	1.72	50
ET-SD-TH	387	ng/µl	0.779	0.511	1.67	50
EH-SD-TH	91.6	ng/µl	1.833	1.016	1.8	50
EH-B-A/A1	129.1	ng/µl	2.583	1.459	1.77	50
EH-B-A/A2	152.2	ng/µl	1.625	0.921	1.76	50
ST-T-K	751.5	ng/µl	15.029	7.394	2.03	50
ST-M-A	164.9	ng/µl	3.298	1.744	1.89	50
ST-M-K	161.9	ng/µl	3.239	1.932	1.73	50
EI-SP-GF	70.6	ng/µl	1.412	0.821	1.72	50
EA-T-A	138.3	ng/µl	2.765	1.528	1.81	50
EA-SD-FA	103.6	ng/µl	0.071	0.04	1.75	50
EA-SP-SM	164.2	ng/µl	3.284	1.852	1.77	50
EA-M-A	164	ng/µl	3.28	1.84	1.78	50
EP-M-K	190.8	ng/µl	1.841	1.032	1.78	50
EH-SP-TH	58.6	ng/µl	0.289	0.17	1.71	50

2. Scanning electron microscopy images of other phages tested that don’t showed relative phage structure



3. NCBI submitted biosample attributes and SRA metadata

Sequence accession	**Sample accession**	**Sample**	**Organism**	**Strain**	**Isolate source**	**Propagation**	**Sequence type**
SRR25691068	SAMN37015700	EH-B-A (A1)	Bacteriophage sp.	Caudovirales	Akaki river water	Lytic	WGS
SRR25691067	SAMN37015701	EH-SD-TH	Bacteriophage sp.	Caudovirales	Hospital waste water	Lytic	WGS
SRR25691066	SAMN37015702	EP-M-A	Bacteriophage sp.	Caudovirales	Akaki river water	Lytic	WGS
SRR25691065	SAMN37015703	EP-B-K (E2)	Bacteriophage sp.	Caudovirales	Kebena river water	Lytic	WGS
SRR25691064	SAMN37015704	EI-SP-GF	Bacteriophage sp.	Caudovirales	Diary farm waste sludge	Lytic	WGS
SRR25691063	SAMN37015705	ET-SD-TH	Bacteriophage sp.	Caudovirales	Hospital waste water	Lytic	WGS
SRR25691062	SAMN37015706	ST-T-K	Bacteriophage sp.	Caudovirales	Kebena river water	Lytic	WGS