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Characterization of a Multidrug-resistant *Escherichia Coli* Lytic Bacteriophage Isolate as a Safe Alternative to Synthetic Antibiotics

By Najwa Alharbi[*] & Reham Yahya[±]

The control of pathogenic bacteria depends mainly on the use of antibiotics; however, several problems can arise due to the overuse of broad-spectrum antibiotics that destroy not only pathogens, but also natural beneficial microbes in the gut micro-flora. Furthermore, the development of antibiotic resistance poses a major challenge in clinical medicine. Therefore, there is an urgent therapeutic need to develop safe alternative bactericidal or bacteriostatic approaches that will selectively kill only pathogens without disrupting the micro-flora. One such approach is the use of bacteriophages to suppress the growth of bacterial pathogens in humans and other animals. Sewage water can be a rich source of pathogenic bacteria and their lytic phages; thus, we sampled sewage water in this study and successfully isolated an Escherichia coli strain and its lytic phage. We demonstrated that the phage could repress bacterial growth of Salmonella enterica (ATCC 14028), Klebsiella oxytoca (ATCC49131), Shigella sonnei (ATCC 25931), and E. coli (ATCC25922). The isolated phage was stable at room temperature, but survival declined with an increase in temperature to 70 °C, and complete inhibition was observed at temperatures ≥80 °C. In addition, the phage was stable over a pH range of 6–8, and was completely inactivated at pH 14.

Keywords: *bacteriophage, Escherichia coli strain M00057, EC phage, multidrug-resistance*

Abbreviations: *EC phage, Escherichia coli lytic bacteriophage; EHEC, enteric hemorrhagic E.coli; PFU, plaque forming units; STEC, Shiga toxin-producing E. coli; Stxs, Shiga toxins; TEM, transmission electron microscopy*

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Introduction

Antibiotics are widely used to control pathogenic bacteria; however, excessive use poses several problems. In particular, broad-spectrum antibiotics may destroy the pathogen of concern, but they may also destroy beneficial microbes in the gut micro-flora. Furthermore, excessive antibiotic use can lead to the development of antibiotic resistance, which is a major challenge facing clinical medicine today (Cieplak et al. 2018). Safe alternative bactericidal or bacteriostatic approaches need to be developed that will selectively kill only the pathogen, and not lead to resistance. One of these approaches is the use of bacteriophages to repress the growth of bacterial pathogens in humans and other animals. One of the unique features of bacteriophages is their high host specificity; bacteriophages usually attack only specific bacterial strains and they do not infect the cells of any human organs, unlike broad-spectrum antibiotics (Woolston et al. 2013). Bacteriophages also play an important role in Figurehting some pathogenic viruses; therefore, they may contribute to the formulation of vaccines as well as antiviral compounds (Wdowiak et al. 2022).

Several bacterial species commonly cause diseases in humans and other animals; however, some of these bacteria are also commensal within the gut. For example, *Escherichia coli* lives within the digestive tract of humans and warm-blooded animals, but it also causes the highest frequency of infections in healthcare settings (Kaper et al. 2004, Nguyen et al. 2012). Furthermore, *E. coli* is involved in the progress of diseases caused by other types of bacteria; for instance in the urinary tract, bloodstream, and intra-abdominal infections, in addition to enteritis and neonatal meningitis. In contrast, several research studies have documented that the majority of *E. coli* strains are harmless to humans.

Severe foodborne diseases are associated with particular types of *E. coli*, e.g., Shiga toxin-producing *E. coli* (STEC). STEC is passed to humans mainly through eating contaminated foods, such as undercooked ground meat, raw milk or milk products, and uncooked vegetables or their products (Kaper et al. 2004, Nguyen et al. 2012). *E. coli* was first described by Theodor Escherich in 1885 as an anaerobic, rod-shaped bacterium. *E. coli* pathogenicity comes from the acquisition of virulence factors from phages, such as plasmids and transposons.

Based on clinical symptoms, serogroups, virulence factors, and mechanisms of pathogenicity, *E. coli* can be classified as enteric hemorrhagic or invasive *E. coli* (EHEC) (Kaper et al. 2004, Nguyen et al. 2012). EHEC as a group was recognized previously to include pathogenic strains of *E. coli* that secrete Shiga toxins (Stxs). These toxins lead to certain illnesses, such as hemorrhagic colitis. Moreover, infection by hemolytic *E. coli* can be life-threatening disease.

Phages, also known as bacteriophages, were first discovered by Frederick Twort and Félix d'Hérelle in 1915 and 1917 respectively. These virus particles consist of genetic material enclosed in a protein capsid or head, often accompanied by a protein tail. The term "bacteriophage" literally translates to "something that eats bacteria." Biologists consider phages as natural parasites or enemies of bacteria, playing a crucial role in maintaining microbial balance on Earth (Wittebole et al. 2014). Tailed phages, belonging to the Cudoviricetes class, encompass various types such as podoviruses, myoviruses, and siphoviruses (Liu et al. 2021), along with the polyhedral

Microviridae family. These phages are commonly associated with applications in phage therapy (Harper and Enright 2011, Lin et al. 2017). Scientists attribute the antibacterial effectiveness of phages to a well-established life cycle, which can be either lytic or lysogenic. In certain situations, phages have the ability to adapt and employ both lysogenic and lytic strategies, known as pseudolysogenicity, as alternative infection pathways in response to different host strains, host physiology, and changes in the environment (Mäntynen et al. 2021). Given their widespread presence, it is not surprising that phages can be found and isolated from various sampling sites, including feces, seawater, sewage, soil, sludge, and any environment where bacteria can thrive (Khawaja et al. 2016, Bhetwal et al. 2017). Bacteriophages (phages) were discovered by Frederick Twort and Félix d'Hérelle in 1915 and 1917, respectively. Phages are virus particles consisting of genetic material wrapped in a capsid or head, most often with a protein tail. The term "bacteriophage" literally means "something that eats bacteria." Biologists describe them as natural parasites (or enemies) of bacteria and associate them with maintaining microbial balance on Earth (Wittebole et al. 2014). Tailed phages of the class Cudoviricetes, which includes podoviruses, myoviruses, siphoviruses (Liu et al. 2021), and the polyhedral Microviridae family are commonly associated with applications in phage therapy (Harper and Enright 2011, Lin et al. 2017).

Scientists attribute the antibacterial activity of phages to an established life cycle (lytic or lysogenic). In some cases, phages adapt both lysogenic and lytic strategies (i.e., pseudolysogenicity) as alternative infection steps in response to different host strains, host physiology, and environmental changes (Mäntynen et al. 2021). Because phages are ubiquitous, it is not surprising that phages can be isolated from a variety of sampling sites such as feces, seawater, sewage, soil, sludge, and anywhere bacteria can grow (Khawaja et al. 2016, Bhetwal et al. 2017). A number of studies have focused on phages that control *E. coli*; these phages usually present in sewage, polluted river water, hospital waste water, and the feces of humans and other animals (Dalmasso et al. 2016). Our aim in this study was to isolate a safe alternative agent, such as a bacteriophage, in sewage water that could be exploited to specifically repress the development of pathogenic bacteria.

Materials and Methods

Phage Host Bacteria Isolation and Identification

Sample Collection

This study was carried out between October 2020 and December 2020. A grand total of 30 samples of sewage were gathered from the waste disposal site in Jeddah, with the gracious authorization of the sewage plant management. These samples were meticulously collected in hermetically sealed zip-lock plastic bags, ensuring the preservation of aseptic conditions. Each sample contained a volume of 50 ml and was promptly stored at a temperature of 4 °C, diligently labeled to denote their origin and specific location. To ensure their integrity, the collected samples were then transported to the esteemed King Fahd Medical Research Center (KFMRC)

microbiology laboratory in an ice box, where they were carefully maintained at refrigerator temperature until they could be processed.

Isolation of Bacteria from Sewage Sample

The sample underwent thorough agitation to achieve a uniform mixture, and it was subsequently filtered using a syringe filter with a pore size of 0.22 μm before a portion was extracted for culturing. All bacteriological analyses followed standard protocols. Wastewater samples were subjected to serial 10-fold dilutions in sterile distilled water. This dilution process was based on the principle that obtaining a reduced number of bacterial colonies in the presence of water samples would yield purer colonies. Each sample, measuring one milliliter, was mixed with nine milliliters of sterile distilled water and subsequently subjected to serial dilution, starting from one tube containing nine milliliters of sterile distilled water and progressing to the next tube in a 10-fold dilution until an 8-fold dilution was achieved. Subsequently, 0.5 milliliters of the desired serial dilutions of bacterial suspensions were plated for bacterial cultivation. Duplicate samples were plated on various differential media, including MacConkey (MAC) agar, Eosin methylene blue agar (EMB), Blood agar, S.S agar, and nutrient agar. The plates were then incubated at a temperature of 37 ºC for a period of 24-48 hours. Identification of the most significant pathogenic bacteria present in the sewage water was carried out based on colony appearance, gram staining, growth on selective media, and biochemical testing, as per the standard methods outlined in the examination of water and wastewater by the American Public Health Association (1999).

Pure colonies were obtained by subculturing on MacConkey (MAC) agar, Eosin methylene blue agar (EMB), Blood agar, S.S agar, and nutrient agar. Once pure colonies were obtained and their important characteristics were recorded, the isolated organisms were further identified to the species level using biochemical methods following standard procedures outlined by Vandepitte (1996). Upon successful growth of the microorganisms, the pure cultures of bacteria were sub-cultured in slants, nutrient broth, and glycerol stock. These cultures were then incubated at a temperature of 37 °C to promote vigorous growth and subsequently preserved in 20% glycerol vials at -80 °C, as described by Williams (1971). The sample was thoroughly shaken to get a homogenous mixture, and filtrated by syringe filter (0.22 μm) before a portion was taken for culture. Standard procedures were followed in all bacteriological analyses. Serial 10-fold dilutions of wastewater samples were prepared in sterile distilled water. Serial dilution was done based on the principle that when water samples along with bacterial colonies are taken, the result obtained in the form of reduced bacterial colonies would be more appropriate to get pure colonies. One ml of each sample was poured into nine ml of sterile distilled water and serially diluted from one tube (containing 9 ml sterile distilled water) to the next in a 10-fold dilution until an 8-fold dilution was reached. The bacteria were cultivated by plating 0.5 ml each of the desired serial dilutions of the bacterial suspensions. Duplicate samples were plated onto differential media, such as MacConkey (MAC) agar, Eosin methylene blue agar (EMB), Blood agar, S.S agar and nutrient agar, then incubated at 37 ºC for 24-48 hours. Identification of the most important pathogenic bacteria found in sewage water was done based on their

colony appearance, gram staining, growth on selective media and biochemical test according to standard methods for examination of water and wastewater (American Public Health Association 1999).

Pure colonies were prepared by subculturing on MacConkey (MAC) agar, Eosin methylene blue agar (EMB), Blood agar, S.S agar and nutrient agar. After obtaining pure colonies and recording important features, isolated organisms were further identified to the species level biochemically, following standard methods (Vandepitte 1996). After successful growth of microorganisms, the pure cultures of bacteria were sub-cultured in slants, nutrient broth and glycerol stock; incubated at 37 °C to achieve vigorous growth and then preserved in 20% glycerol vials at -80 °C (Williams 1971).

Molecular Identification of the Bacterial Isolate Through 16S rRNA Gene Sequencing

The isolated bacteria were cultivated in nutrient broth medium for 24 h. The cells were then harvested using centrifugation and subjected to genomic DNA extraction using a QlAamp® DNA Mini Kit (Venlo, Limburg, Netherlands) according to the manufacturer's guidelines, with minor changes as previously recommended (Atashpaz et al., 2010). The extracted genomic DNA was used to amplify the 16S rRNA gene, employing an MJ Research Peltier Thermal Cycler (Watertown, MA. USA). The following primers were used: 5′-AGAGTTTGATCMTGGCTCAG-3′ (forward) and 1492R 5′-TACGGYTACCTTGTTACGACTT-3′ (reverse). The amplification products of the PCR were cleaned up utilizing a Millipore kit (Sigma-Aldrich, Burlington, MA, USA) according to the manufacture instruction, and then sent to Macrogen (Seoul, South Korea) for sequencing.

Isolation of the Bacteriophage

Collection and Transport of Samples

Phages were isolated from the thirty previously-described samples. The selection of wastewater sample collection sites was based on the amount of pollution in the water sample, in addition to the distance between the collection site and the laboratory analysis site. In each case, the samples were collected under aseptic conditions and placed in screw cap 50 ml bottles. All samples were then transported in an icebox to the KFMRC and stored until further processing within 2 h.

Spot Assay of Bacteriophage Lytic Activity

A spot assay was used to assess bacteriophage lytic activity against host strains according to Pereira et al. (2011). Following incubation of the plates at suitable conditions (37 °C for 24 h), they were scanned for the existence of any plaques associated with the added lysate. If a clear plaque was detected from the tested phages, they were identified as virulent phages.

Bacteriophage Purification and Titration Assay

Identified phage were purified using a top agar (soft agar) overlay protocol according to Gencay et al. (2017). To confirm the purification of a phage, the protocol

was repeated three times, until the appearance of single plaques characterized by the same morphological features. The filtrate containing the purified phage was stored at 4 °C. A double-layered protocol was applied to assay the phage titers, as well as to determine the concentration of each bacteriophage according the following formula (https://barricklab.org/twiki/ bin/view/Lab/ProtocolsPhageTiters (https://barricklab.org)):

$$\text{Titration of phage} = \frac{\text{PFU}}{mL} = \frac{\text{Plaque } n\text{umber}}{\text{D} \times \text{V}}$$

where PFU stands for plaque forming units, D is the quantity of phage (10^8), and V is the purified phage volume.

Effect of Physical Conditions on Phage Stability

Assay of Phage Thermal Stability

The isolated phage were subjected to thermal stability testing using the procedures of Jurczak-Kurek et al. (2016). Briefly, 100 µL (108 PFU/mL) of phage lysate were collected in an Eppendorf tube under sterile conditions and then incubated for 120 min at temperatures ranging from 30–90 °C in a water bath**.** At each temperature, the bacteriophage titer was determined using the double-layer agar protocol. The stability percentage was calculated based on the ratio of titers of live phage remaining after treatment, to that prior to treatment (×100). The thermal stability data were plotted using Excel 2010 software (mean of triplicate experiments vs. time).

Assay of Phage pH Stability

The pH stability of the phage was assessed by making a slight modification to the procedure outlined by Verma et al. (2009). To create a pH range of 2–14, 1 M HCl or 1 N NaOH, prepared in nutrient broth, was added drop by drop. The pH values were measured using a pH meter (JENWAY, Bibby Scientific Ltd, Stone, UK). Following this, 1 mL of phage suspension was added to 9 mL of growth medium that had been adjusted to a specific pH and then incubated at 37 ºC for 3 hours. The phage titer at each pH was subsequently determined using the double-layer agar technique, and the percentage of surviving phage was calculated. To ensure accuracy, triplicate experiments were conducted to calculate the mean survival percentages. These data were then plotted as a pH stability curve using Excel 2010 software.

Determination of Phage Host Range

The range of hosts that the phage isolated in this study can infect was evaluated using different strains of bacteria, specifically six bacterial species. The evaluation method used was based on the approach described by Jurczak-Kurek et al. in 2016. To carry out the evaluation, a lawn of each bacterial species was prepared by mixing 1 mL of a bacterial culture suspension that had been incubated for 12 hours with liquefied soft agar containing 7% agar. The molten agar mixture was then poured onto the surface of nutrient agar plates. Next, 5 mL of a purified lytic phage lysate, with a concentration of 108 plaque-forming units (PFU) per milliliter, was spotted

onto the bacterial lawn. The plates were then incubated for 12 hours at a temperature of 37 ºC. The lytic potential of the phage lysate was assessed by examining the appearance of plaques that formed on the bacterial lawn. The results were recorded as follows: no plaques (-), turbid plaques (+), or clear plaques (++).

The host range of the isolated phage was tested against different strains of bacteria (six bacterial species) based on the method of Jurczak-Kurek et al. (2016)**.** Briefly, each bacterial species was prepared as a lawn by mixing 1 mL of 12 h-incubated bacterial culture suspension with liquefied soft agar (7% agar). The prepared molten agar was then poured over the surface of the nutrient agar in the plates. Subsequently, 5 µL of purified lytic phage lysate (10^8 PFU/mL) was spotted onto the lawn of bacteria and the plates were incubated for 12 h at 37 ºC. The lytic potential of the phage lysate was determined based on the clearness of the produced plaques and recorded as no plaques (-), turbid plaques (+), or clear plaques (++).

Examination of Phage Using Transmission Electron Microscopy (TEM)

The phage isolates were analyzed using transmission electron microscopy (TEM), specifically the Hitachi H-7000 instrument in Tokyo, Japan. The technique employed was based on the method described by van Regenmortel et al. (2000), with some minor modifications. In brief, a volume of 20–30 µL of the purified phage lysate (at a concentration of 108 plaque-forming units per milliliter) was applied onto a 400-mesh carbon-coated copper grid and allowed to incubate for 2 minutes. Subsequently, the samples were treated with a solution of phosphotungstic acid (2% weight/volume, pH 7.0) for staining purposes. Excess stain was eliminated by gently blotting the side of the grid with soft paper, followed by air-drying of the samples. The examination of the prepared samples was conducted at magnifications ranging from 15,000 to 25,000 times, using an accelerating voltage of 80 kilovolts. Based on the TEM images acquired, the classification of the phage isolate was determined by referencing the available data on the International Committee on Taxonomy of Viruses website (https://talk.ictvonline.org/).

Results

Isolation of Bacteria from Urban Sewage

This research was conducted using three samples of urban wastewater collected in Jeddah, Saudi Arabia. A total of 30 bacteria found in the wastewater were isolated. These bacteria were divided into three groups: ten isolates from sample I (coded as MB1, MB2, MB3, MB4, MB5, MB6, MB7, MB8, MB9, and MB10), ten isolates from sample II (coded as MN1, MN2, MN3, MN4, MN5, MN6, MN7, MN8, MN9, and MN10), and ten isolates from sample III (coded as MB11, MB12, MB13, MB14, MB15, MB16, MB17, MB18, MB19, and MB20). Additionally, two pathogenic isolates were obtained from King Abdelaziz hospital.

After analysing the bacteria on the plates, we selected six strains of E.coli for

further identification, which accounted for 31.57% of the total isolates. Table 3.1 provides a summary of the micromorphology characteristics of the bacterial strain MB1. As the colonies of the isolated bacteria developed on nutrient agar plates, they displayed various morphological features (Figures 1-2).

Bacterial Identification

The tables labelled 1 and 2 showcase the biochemical and sugar fermentation test outcomes for the bacterial strain MB1 that has been isolated. In Table 3.1, the identified bacteria are presented after undergoing phenotypic characterization.

The biochemical and sugar fermentation test results of the isolated bacterial strain MB1 are represented in Tables 1 and 2. The bacteria as identified, following phenotypic characterization, are depicted in Table 1.

Table 1. *Colony and Cell Characteristics of the isolated Bacterial Strains from Urban Sewage*

	Bacterial Isolation	**Colony features**		**Cell feature - Name of Bacteria**		
		Color of colony	**Type of Media**	**Gram stain**	**Cell morphology**	**Bacterial identity by 16s RNA**
1	MB1	Metallic green	EMB	Negative	Rod	*Escherichia coli* strain M00057

Morphology and Biochemical Tests of Bacterial Isolate

Several biochemical tests were used to document the properties of the bacterial isolates found. The results of most of the biochemical tests were negative; however, tests for D-mannitol, D-trehalose, lysine decarboxylase, D-glucose, D-mannose, coumarate, D-sorbitol, β-galactosidase, D-sorbitol, and β-glucoronidase were positive (Table 2).

Figure 1. *Results for Gram Stain of Bacteria Isolated from Wastewater Samples*

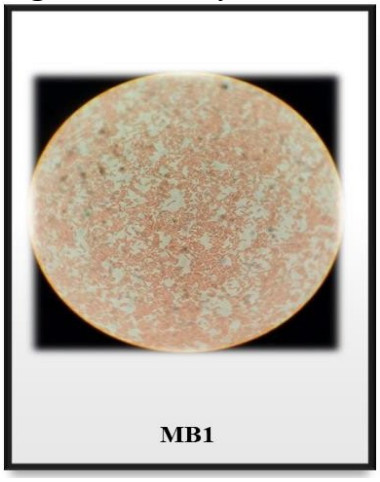


Figure 2. *Colony Characteristics of Bacteria Isolated from Wastewater Samples*

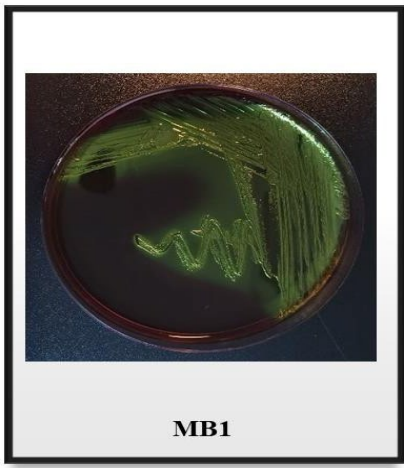


Table 2. *Biochemical Test Results for Bacteria Isolated from Wastewater Samples and a Hospital Clinic*

Biochemical test	**Results**
β-Glucoronidase	+ve
D-Mannitol	
D-Trehalose	
Coumarate	
β-Galactosidase	
D-Sorbitol	
Lysine decarboxylase	
D-Glucose	
D-Mannose	
Production of H_2S	-ve
β-Glucosidase	
L-Proline arylamidase	
Saccharose/Sucralose	
L-Lactate alkalinization	
Glycine arylamidase	
O/129 Resistance	
Adonitol	
β-N-acetyl-glucosaminidase	
D-Maltose	
Lipase	
D-Tagatose	
α-Glucosidase	
Ornithine decarboxylase	
Glu-Gly-Arg-arylamidase	
L-Pyrrolydonyl arilamidase	
Glutamyl arylamidase pNA	
Palatinose	
Succinate alkalinization	
L-Malate assimilation	
L-Arabitol	
Tyrosine arylamidase	
Citrate (sodium)	

β-N-acetyl-galactosaminidase	
L-Histidine assimilation	
Ellman	
D-Cellobiose	
γ-Glutamyl-transferase	
β-Xylosidase	
Urease	
Malonate	
Alpha-galactosidase	
L-Lactate assimilation	
Fermentation-glucose	
β-Alaninearylamidase pNA	
5-Keto-D-gluconate	
Phosphatase	
α-Phe-Pro-arylamidase	

Sensitivity of *E. coli* Strain to Different Antibiotics

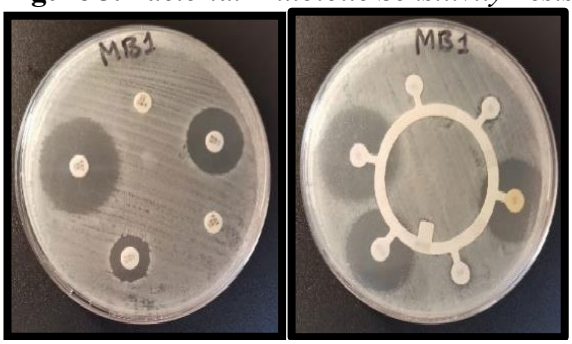
The isolated *E. coli* strain MB1 was susceptible to a number of antibiotics, including gentamicin (aminoglycoside), norfloxacin (fluoroquinolone), and colistin sulfate (polymycin), with inhibition zones of 20 mm, 24 mm, and 15 mm, respectively (Table 3, Figure 3). In contrast, MB1 was resistant to ampicillin, cefoxitin, cephalothin, nalidixic acid, and amoxycillin.

Table 3. *Sensitivity of Isolated E. coli Strain to Different Antibiotics*

	Antibiotic	**Result**	**MIC**
ViTEK2 2 technique	Ampicillin	R	-
	Tazobactam/Piperacillin	S	≤ 4
	Imipenem	-	-
	Amikacin	-	-
	Cefepime	-	-
	Ceftazidime	-	-
	Meropenem	-	-
	Gentamicin	-	-
	Tigecycline	-	-
	Ciprofloxacin	-	-

	Antibiotic	**Result**	**MIC**
Agar disc diffusion technique	Nitrofurantoin	S	22 mm
	Norfloxacin	S	24 mm
	Cotrimoxazole	S	27 mm
	Nalidixic acid	R	-
	Cephalothin	R	-
	Sulphamethoxazole	S	29 mm
	Gentamicin	S	20 mm
	Colistin sulphate	S	15 mm
	Amoxycillin	R	-
	Cefoxitin	R	-

Figure 3. *Bacterial Antibiotic Sensitivity Tests by the Disc Diffusion Method*



Phylogenetic Analyses

Sequences were analyzed for similarity against existing databases using the BLAST (Basic Local Alignment Search Tool) network service provided by the National Center for Biotechnology Information (NCBI). The BLAST tool, developed by Altschul et al. in 1997, allows for the comparison of sequences and identification of potential matches. Additionally, partial sequences of the 16S rRNA gene were compiled using the AlignIR 2.0 Fragment Assembly and Contig Editor software.

Figure 4. *16S rRNA Gene of Bacteria (1100bp)*

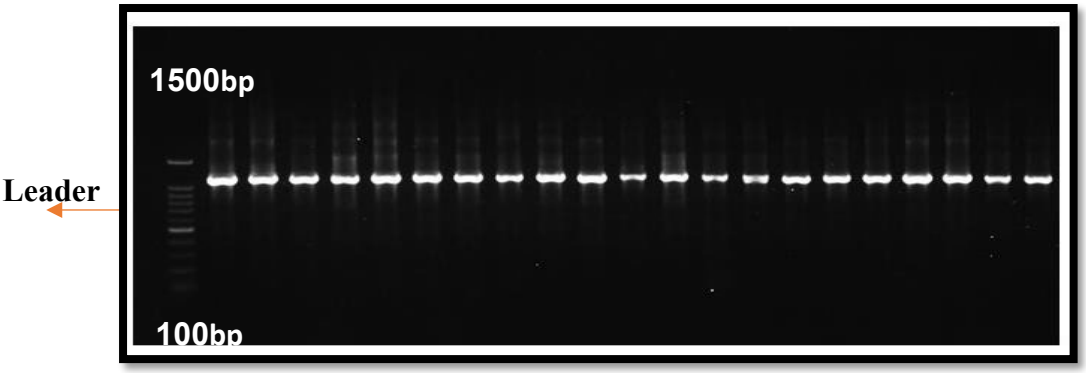
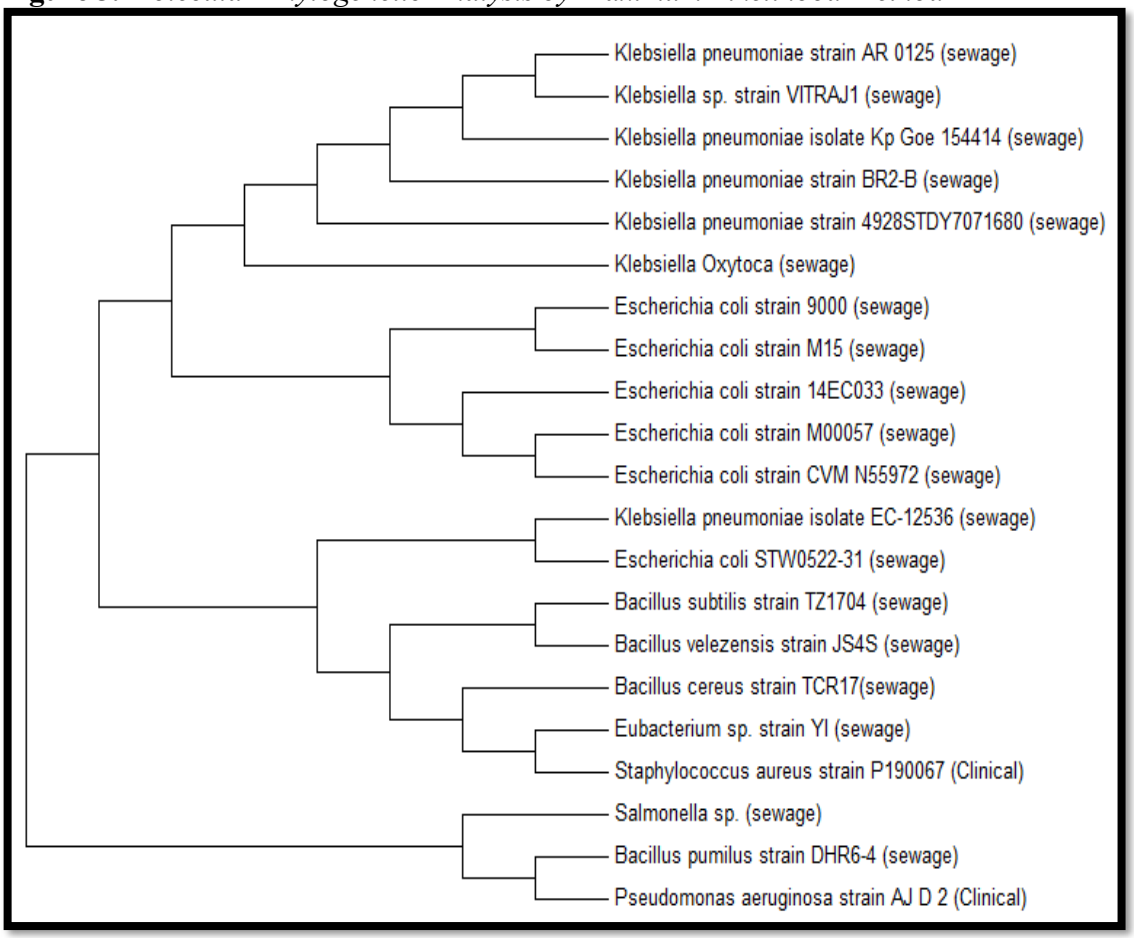


Figure 3. *Molecular Phylogenetic Analysis by Maximum Likelihood Method*



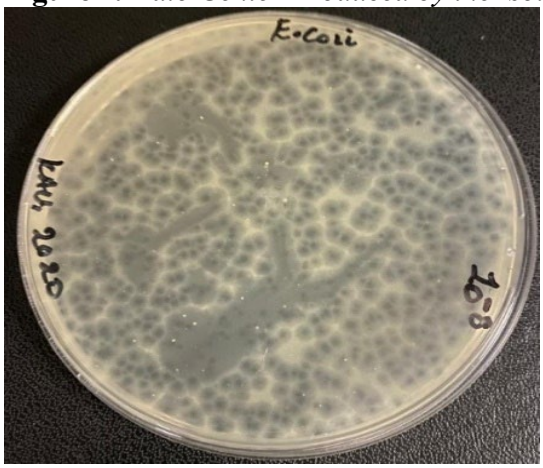
Isolation and Morphological Characteristics of the Bacteriophage

From these results, and using *E. coli* as a host, one lytic bacteriophage was detected in the sewage water ("EC phage"). After incubation of the EC phage with *E. coli* at 37 ºC for 48 h, the bacteriophage titration was calculated to be 10^9 PFU/mL. According to this assay, 131 PFU/mL was effective for *E. coli* inhibition (Table 4). In this context, the formation of clear plaques by the bacteriophage was observed with a 1 mm halo center, and well-defined boundaries (Figure 2).

Table 4. *Bacteriophage Titration at 10^{-9}*

Phage type	Equation used	Total PFUs
EC titer	$\frac{131\ \text{PFU/mL}}{0.1 \times 10^{-8}}$	$1.31 \times 10^9\ \text{PFU/mL}$

Figure 2. *Halo Center Produced by the Isolated Bacteriophage*



Bacteriophage–Host Range Interaction

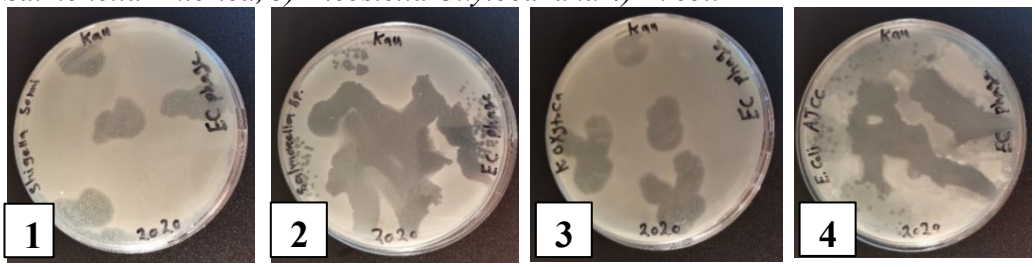
Analysis of the host range of the EC phage showed that a number of bacterial hosts were sensitive to its lytic potential, including *Salmonella enterica* (ATCC 14028), *Klebsiella oxytoca* (ATCC49131), *Shigella sonnei* (ATCC 25931) (Table 5). On the other hand, no lytic activity was observed against *Enterococcus faecalis* (ATCC 29212) and *Proteus vulgaris* (ATTC49132). The lytic activity of PaMAsh phage was then tested using a spot assay against four out of the six bacteria, *Shigella sonnei* (ATCC 25931), *Salmonella enterica* (ATCC 14028), *Klebsiella oxytoca* (ATCC49131), and *E. coli* (ATCC25922) (Figure 3).

Table 5. *Host Range of Isolated Phage*

	Enterococcus faecalis	*Shigella sonnei*	*Klebsiella oxytoca*	*Proteus vulgaris*	*Escherichia coli*	*Salmonella enterica*
	-	+	+	-	+	+

(+) lysed, (-) not lysed.

Figure 3. *Host Range of EC Phage Using Spot Test Against (1) Shigella Sonni, 2) Salmonella Enterica, 3) Klebsiella Oxytoca and 4) E. coli*



Bacteriophage Stability Testing

The isolated bacteriophage was 100% stable at room temperature (Figure 4). EC phage incubated for 1 h exhibited excellent activity, as reflected in their survival

percentages, at temperatures of 37 and 65 °C; however, activity declined with incremental increases in incubation temperature. At 70 °C, the phage showed minimal activity, and at ≥80 °C, complete inhibition was observed. The influence of pH on survival of the EC phage showed that it was more susceptible to acidic pH than alkaline pH (Figure 5). The graph of phage survival over a pH range of 2–14 indicated an increase in survival with an increase in pH from 2 to 7, where survival peaked. There was no survival at pH values ≥11.

Figure 4. *EC Phage Thermal Stability*

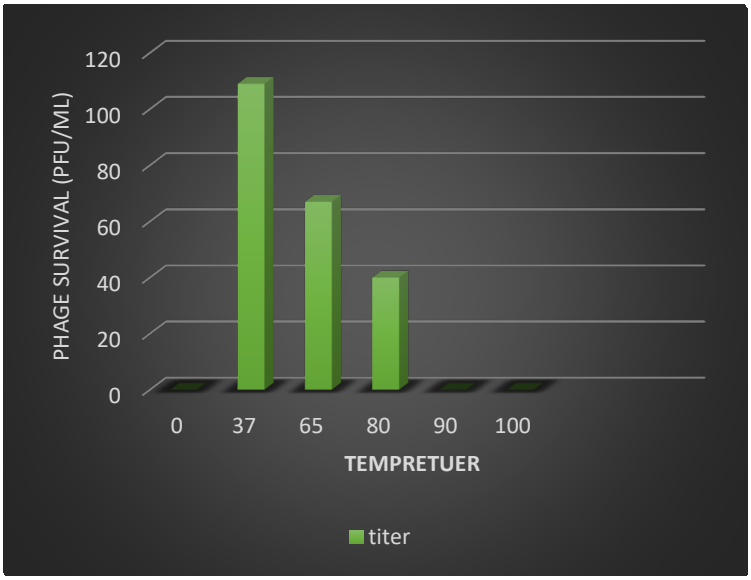
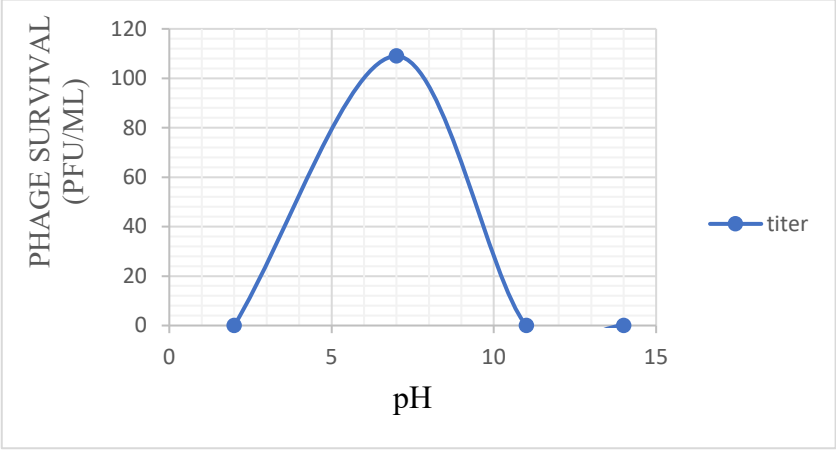


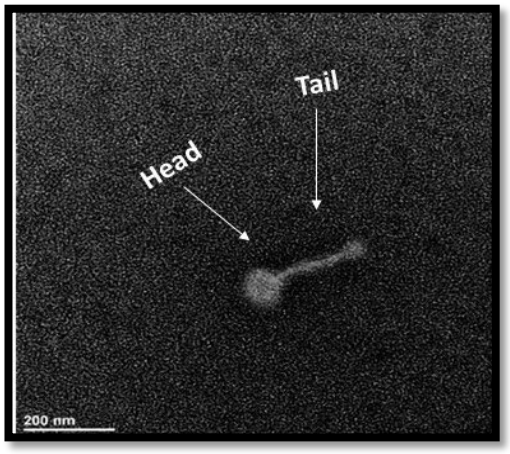
Figure 5. *EC Phage Stability at Different pH*



Morphological Characteristics of the Isolated Phage

The morphological features of the EC phage were examined using TEM, revealing the presence of a non-enveloped, head-tail structure. The head is about 60 nm in diameter (Figure 6), indicating its relationship to members of the family Siphoviridea.

Figure 6. *EC Phage Image via Transmission Electron Microscopy*



Discussion

The current study focused on wastewater as a source of bacteria and bacteriophages, owing to contamination of the water with fecal material exhibiting a great diversity of coliform species that can cause several diseases. The *E. coli* isolated from the wastewater coliform bacteria population (MB1) showed resistance to five antibiotics, classifying it as one of the MDR bacteria. Multi-drug resistant *E.coli* strains have been isolated previously from various sources, including drinking water and waste water, by Odonkor and his colleagues in 2018. Wastewater, in general, contains a large diversity of coliforms due to fecal contamination. Therefore, wastewater is a tank of intestinal pathogens, as well as their lytic phages, proposed in this study as a possible substitute for antibiotics.

The greatest recognizable plaque characteristic of the EC phage recovered from the wastewater sample in the present study was a distinctive halo zone, whose width incrementally increased over time, and which was encircled by a plaque that remained the same diameter. This is explained by Cornelissen et al. (2011), who demonstrated that bacteriophages produce a diverse array of hydrolytic enzymes that are responsible for the degradation of polysaccharides in the bacterial cell wall, as well as other constituents. Thus, the halo zones observed with our EC phage are presumed to be associated with these enzymes. The phage titer of 1.31×10^9 PFU/mL in our investigation is similar to the 7×10^8 PFU/mL titer reported by Swati et al. (2019) for an EC phage also isolated from wastewater.

The morphology of phages is one of the most important criteria for their characterization (Shukla & Hirpurkar 2011). In our study, the variation in the morphology of plaques may correspond to the difference in gel strength, phage strain and addition of cations (calcium chloride) (Ghasemian et al. 2017). However, Jothikumar et al. (2000) reported that the morphology of phage plaques was not affected by cationic supplements. Pedrosa & Martins (Pedroso & Martins 1995) also could not find any association of specific plaque morphology among the coliphage family.

Host range is an essential factor to consider when choosing phages for phage therapy (Duc et al. 2018). One of the fundamental biological properties of a bacteriophage is its host range (Kutter 2009). The EC phage isolated in this study exhibited successful and efficient hydrolytic activity against *K. oxytoca, Shigella sonnei, E. coli*, and *Salmonella enterica* (Figure 3, Table 4); however, the current results are not in agreement with the findings of (Kafshgari et al. 2019). The phage they isolated was effective, but its host range was limited to five strains of *E. coli*; other bacterial species, such as *Staphylococcus aureus* and *Salmonella enterica,* were not affected. A similar result was found by Dalmasso et al. (2015) who isolated three narrow host range phages from human feces in wastewater against nine strains of *E.coli*, so he proposed that using a cocktail of mixed phages would be more successful as a biocontrol than using a single phage.

Temperature is known to be a significant factor in the stability of phages under various conditions, but in particular during storage and/or transportation (Kering et al. 2020). The EC phage isolated in this study exhibited a high thermal stability over a wide range of temperature (37–65 ºC), but it was unstable when exposed for 2 h to high temperatures ≥80 ºC. In addition, pH influences phage stability; the isolated phage was not viable at a very low (pH 2) or very high (pH 14) pH, but remained viable over a pH range of 7–10 Earlier studies by Tiwari et al. (2010) indicated that *E. coli* phage remained viable at 70 °C for 2 min, but increasing the exposure time to 3 min resulted in death of the phage. They also found that phage propagation at a low (pH 3) or high (pH 11) pH was completely inactivated, whereas it remained active within the pH range of 5–9.

Conclusions & Limitations

In this study we successfully isolated a lytic phage active against *E. coli* strain M00057*,* which can cause stomach cramps, fever, and occasionally diarrhea. The ability of the isolated phage to completely lyse the cell makes it a good candidate to treat disease caused by this bacterium. However, more *in vivo* studies are needed to expand this research, and more phages against *E.coli* need to be isolated to cover related bacterial strains.

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