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DETERMINATION OF HOST RANGE AND BACTERIAL GROWTH REDUCTION POTENTIAL OF BACTERIOPHAGE AGAINST *E. COLI*

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Abstract

A gram-negative bacillus called Escherichia coli (E. coli) is considered to be a normal component of intestinal flora, but it may also cause intestinal and extraintestinal illnesses in people. The emergence of antibiotic resistance has made treating E. Coli infections with antibiotics more difficult. Therefore, a novel alternative approach to treating these infections is needed. With an estimated 1031 particles, bacteriophages are the most common biological entity on Earth. They invade bacterial cells as required intracellular parasites. They can multiply and lyse bacterial cells, and they have a high host specificity. Two new native bacteriophages that fight E. coli were discovered, identified, and given the names EP1 and EP2 during this study. Both these phages were found to have a specific host range for E. coli. EP1 was able to lyse 7 out of 10 different E. coli clinical isolates and EP2 was able to lyse 5 out of 10 isolates but could not infect bacteria from other species. The distinct phages were viable at temperatures as high as 50°C and pH values between 3 and 9. For eighteen hours, EP1 prevented the development of germs. However, by blocking and lowering the initial bacterial inoculum count until 24 hours of observation, EP2 demonstrated exceptional bacterial reduction abilities. It is proposed that both of these bacteriophages (EP1 and EP2) may be viable phage treatment options in the future following the required animal models and clinical studies, given their superior bacterial growth reduction, phage titer, pH and temperature stability, and host range.

INTRODUCTION

The Enterobacteriaceae family of facultative anaerobic Gram-negative bacteria, *E. coli*, colonizes the gastrointestinal tract of warm-blooded animals soon after birth and persists in colonizing adults for the rest of their lives. This species interacts with the host in a mutualistic way while remaining a harmless commensal in the mucous layer. The predominant facultative anaerobe in the intestinal flora of warm-blooded animals and humans is *E. coli*, which is often non-pathogenic (Blount, 2015). The majority of the *E. Coli* strains that have been identified since 1895 belong to the commensal microbial population of the gut; they are generally the most common microbe in coprocultures of all mammals and are

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usually found in feces in amounts between 10^7 and 10^9 CFU/g. However, certain strains have evolved pathogenetic processes that can cause a variety of diseases, including serious ones in individuals as well as livestock (Nguyen & Sperandio, 2012).

Nonpathogenic intestinal *E. coli* can eventually cause or contribute to disease in weakened hosts, however other strains of the bacteria have pathogenic qualities that can cause illness. Escherichia coli has been identified as the primary cause of a large number of foodborne disease cases. Food is often preserved via a physical or chemical process to avoid foodborne illness. These techniques, however, can degrade the food's organoleptic qualities and quality. Globally, *E. Coli* O157:H7 poses a serious cost and health burden, with low-income nations and vulnerable groups such as various age groups facing particular challenges (Paitan, 2018). This pathogen is a major foodborne threat, causing a substantial number of infections, hospitalizations, and deaths each year (Hayat *et al.,* 2018). Within the bounds of the United States alone, *E. coli* O157:H7 is responsible for many cases of disease each year, leading to hospitalizations and deaths. The infections are severe and potentially fatal due to the pathogen's low infectious threshold and increased virulence, which especially affects small children, the elderly, and those with weakened immune systems (DeFilipp *et al.,* 2019).

Since *E. Coli* are Gram-negative bacteria, they are resistant to a wide range of antibiotics, and multiresistant high-risk *E. Coli* clones are becoming more prevalent. In fact, the World Health Organization views strains of bacteria that produce carbapenem-resistant extended-spectrum beta-lactamase (ESBL) as a crucial priority for the development of new medicines (Manohar *et al.,* 2019). However, because antibiotics have a significant impact on the human microbiota, innovative approaches including immunization, phage therapy, antiadhesives, and very-narrow-spectrum medications are encouraged. Because it can result in the development of bacteria resistant to antibiotics, the use of chemical agents such as antibiotics remains a worry. Bacteriophages are viruses that may precisely target bacteria without causing damage to human, plant, or animal cells, making them a natural alternative means of food preservation. Because of their specificity, capacity for self-replication, and abundance in food, bacteriophages have been used more often as biocontrol agents in recent years. In order to ensure safety, the bacteriophage must be lytic and non-transducing (Cieplak *et al.,* 2018). A phage's effectiveness for phage treatment is impacted by host range in a number of ways. Because it keeps the remainder of the host's microbiome intact and stops the phage from killing other species, a host range restricted to a single species is preferable. Therefore, the phage should have a smaller host range in terms of bacterial species (Wang *et al.,* 2018).

But within that bacterial species, a phage that infects most, if not all, strains are helpful because it means that many bacterial infections by that species can be treated somewhat empirically (i.e., presumptively), meaning that the sensitivity of the infecting strain for susceptibility is not required to be investigated (de Jonge *et al.,* 2019). This is comparable to the usage of broad-spectrum antibiotics prior to pathogen identification or antibiotic sensitivity testing (antibiotics that impact bacteria across genera, families, orders, or even greater taxonomic ranges). Using phages with a wider host range for phage therapy might reduce treatment failures caused by mismatched host and phage combinations. Therefore, a wider host range is preferred in terms of strains within target species. (However, it is unquestionably preferable to aim for a suitable host range width earlier rather than later during the preclinical development of phages for phage treatment, as highlighted here (Yehl *et al.,* 2019).

Materials and method

Study Area

The current study was carried out at Abbottabad University of Science and Technology's Microbiology Lab. On the other hand, bacteriophage samples were gathered from the Butt Pull Sewer Drain in Mansehram and DHQ Peshawar.

Bacterial Isolates

E. Coli samples were streaked on a MacConkey agar plate and left to incubate for the whole night. The bacterium was identified using microscopy and Gram staining. Before every experiment, bacterial

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samples were subcultured, and cultures that were 6–8 hours' old were utilized. Through routine sub-culturing, the culture's viability and purity were preserved.

Isolation of Bacteriophages from Sewage

To isolate bacteriophages, the gathered samples were taken to Abbottabad University of Science and Technology's Microbiology Laboratory. First, the water samples were shaken for two minutes. To get rid of the germs, big particles, and silt, they were centrifuged for ten minutes at 10,000 rpm. Additionally, 40ml of clear sewage supernatant was added to 10ml of sterile 5x nutritional broth in a conical flask. 200ul of an overnight culture of E. coli was added to the flask as an inoculant. Overnight, the inoculated flask was shaken (at 120 rpm) at 37°C. Following incubation, the flask's contents were centrifuged for five minutes at 10,000 rpm. The clear supernatant was collected in a fresh, clean falcon tube and stored at 4°C for later use after being filtered with a 0.22 ul syringe filter. By using a spot test, the presence of bacteriophages in the filtrate was identified (Asif *et al.*, 2018).

Lytic Spectrum Determination by Spot Test

The spot test method was used to assess the phages' capacity to induce lysis in various bacterial strains. The nutrient agar plate was covered with 100ul of an overnight-grown culture of E. coli for the spot test. After applying 5 ul of the filtrate, the plates were allowed to dry for nearly ten minutes. The plates were then incubated for the whole night at 37°C. After that, the plates were examined for a distinct lysis zone produced by bacteriophages. Any bacterial lawn showing a lysis zone at the end of the incubation time is considered vulnerable to the phages (Asif *et al.*, 2018).

Quantification and Purification of Bacteriophages using Double Layer Agar Assay

Bacteriophages from the lysate were quantified and purified using the double layer agar overlay technique. The first step included serially diluting the lysate (1:9). Log phage bacteria (100µl) were introduced to each dilution. After pouring the liquid onto an LB plate, 3-5 milliliters of LB semisolid agar were added, and the mixture was stirred to ensure adequate mixing. Overnight, the plates were incubated at 37°C. Following incubation, the plaque morphology was examined and tallied. Plaque forming units per milliliter (pfu/ml) were used to count the plaques that developed on the plate. After the phage was purified, their pfu was ascertained. Plates with unique plaque were chosen for phage purification. Carefully, a sterile micropippete tip was used to tap the plaque surface. For phage propagation, the tip was placed in a test tube with 10 ml of nutrient broth and 1 ml of fresh *E. coli* culture. It was then cultured for 24 hours at 37 °C, after which plaque was visible and purified. Up to ten repetitions of the purification stage were carried out. The lysate titer was then determined (Alvi *et al.*, 2020).

Characterization of Bacteriophages

Determination of Host Range

Using a standard methodology, bacterial cultures from several strains of *E. coli* and other species were used to analyze host range. Phage lysate (5μl) was used to detect a bacterial lawn that had been created on a plate. After the plates were incubated for 24 hours at 37°C, bacterial lysis was found (Alvi *et al.*, 2020).

Determination of Thermal Stability of Bacteriophages

The aliquots of the known titers of *E. coli* phages were placed at 4°C, 25°C, 37°C, 50°C, and 60°C for one hour in order to assess the thermal stability of the isolated bacteriophages. 1011, 109, 1011, 109, and 101 were the titers. The double layer agar overlay technique was used to measure the bacteriophage titer following incubation. The initial titer and the titer reduction were compared (Du *et al.*, 2009).

Determination of pH Stability of Bacteriophages

HCl and NaOH were used to change the lysate's pH. A pH paper was used to measure the pH. Aliquots of the known titers of *E. coli* phages were placed at pH 2 10 for one hour in order to assess the pH stability of the isolated bacteriophages. The two-layer agar overlay technique was used to measure the bacteriophage titer following incubation. The initial

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titer and the titer reduction were compared (Du *et al.*, 2009).

Long-term Storage Stability.

For three months, bacteriophages were maintained in LB broth at 4°C, 25°C, and 37°C. The bacteriophages *E. coli* had titers of 6×109 pfu/ml and 8×1010 pfu/ml prior to storage. The two-layer agar method was used to measure each bacteriophage's titer both before and after storage (Alvi *et al.,* 2018).

Bacterial Growth Reduction

The reduction test was used to assess bacteriophages' capacity to inhibit bacterial growth. To put it briefly, an 8-hour-old culture of the *E. coli* phages strain (3×108 CFU/ml, 1ml) was added to two flasks that held 50ml of broth each. *E. Coli* bacteriophages (3×107pfu/ml, 1ml) were introduced to the second flask (MOI=0.1), while the first flask served as a control. By measuring optical density at a wavelength of 600 nm (O. D600), bacterial proliferation was quantified. The growth decline was shown on a graph and contrasted with the control. Three separate runs of the experiment were conducted (Ullah et al., 2023).

Results

Bacterial Growth

E. coli is a straight, rod shape bacterium. On MacConkey agar media, the colonies of *E. coli* were pink in color.

Figure 1: Bacterial growth on MacConkey media

Spot Test for the Detection of *E. Coli* Specific Bacteriophages

The presence of bacteriophages against the *E. coli* strain was detected in two of the four sewage samples. The KTH-obtained bacteriophage was designated EP1, whereas the Butt pull canal Manshera-obtained bacteriophage was designated EP2.

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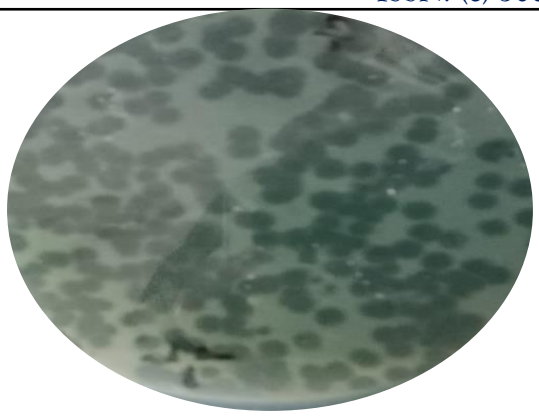

Figure 2: Detection of bacteriophage through spot test

Isolated Bacteriophages Produced Clear Transparent Plaques

The isolated phages created circular transparent plaques against *E. coli* on the double layer agar plate. The plaques had a hazy layer around them.

Isolated Phages Had High Titer

The isolated phages' lytic activity and potential as therapeutic agents were demonstrated by the translucent, clear plaques. After being propagated in liquid culture for

24 hours, the titer of EP1 and EP2 phages was determined to be 5×10^{9} pfu/ml and 3×10^{10} pfu/ml, respectively.

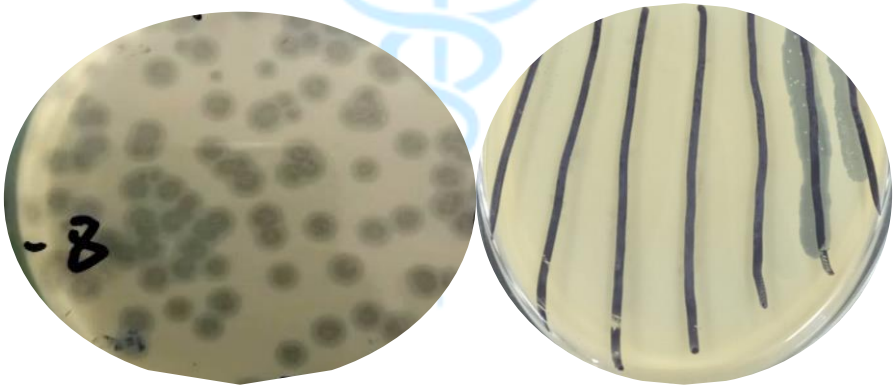

Figure 3: Titer of isolated bacteriophages

Isolated bacteriophages were found to have narrow spectrum

It was discovered that the isolated *E. coli* EP1 and EP2 phages were strain-specific. While no infectivity was seen for other genera, EP1 and EP2 bacteriophages were able to infect and create a lytic zone against isolates of *E. coli*. According to host range specificity test results, EP1 could infect five out of ten *E. coli* strains, whereas EP2 could infect seven out of ten. However, it did not infect bacteria belonging to other genera, such as *S. aureus, P. aeruginosa*, Enterobacter, and *S. typhi.*

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Table: 1 Host range spectrum of EP1 and EP2 bacteriophages against various bacterial cultures

S.No	Bacterial culture	Spot test EP1	Spot test EP2
1	*E. coli 1*	+	+
2	*E. coli 2*	-	+
3	*E. coli 3*	+	+
4	*E. coli 4*	-	+
5	*E. coli 5*	+	-
6	*E. coli 6*	+	+
7	*E. coli 7*	+	+
8	*E. coli 8*	-	-
9	*E. coli 9*	-	+
10	*E. coli 10*	-	-

Isolated Bacteriophages Were Thermally Stable

The stability of bacteriophages is significantly impacted by temperature. It impacts adhesion, penetration, and proliferation in all aspects of phage replication. It was discovered that the EP1 and EP2 phages remained stable at 50°C without experiencing any titer changes. At 4°C there was no drop in phage titer for EP2, but at 50°C, there was a three-fold reduction at 25 fourfold reduce and 37 one fold reduce. The EP1 titer decreased by two, one, and three at 25°C, 37°C, 50°C, respectively, whereas there was no change at 4°C.

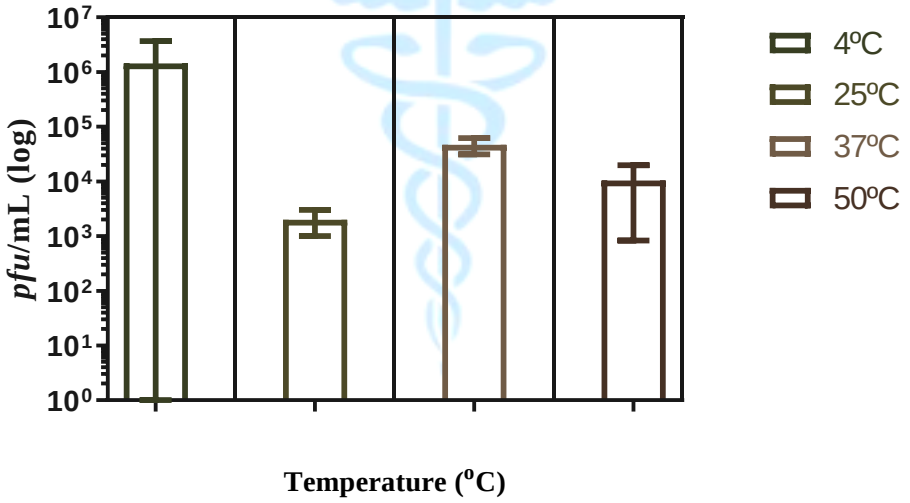

Figure 4: Thermal stability of EP1 bacteriophage at different temperatures

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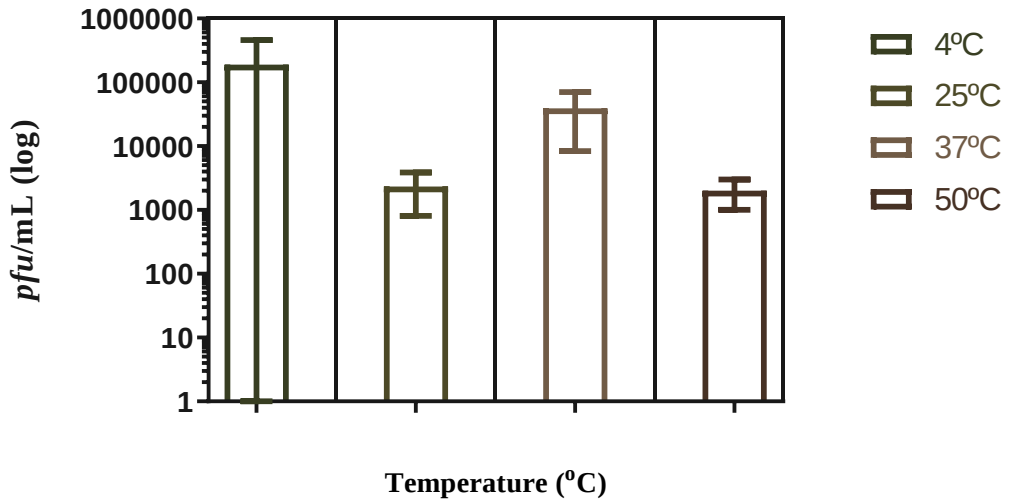

Figure 5: Thermal stability of EP2 bacteriophage at different temperatures

PH Stability of Bacteriophages

One important aspect influencing phage stability is the environment's acidity and alkalinity. After an hour, it was discovered that the EP1 and EP2 phages were resistant to the pH range of 3.0 to 9.0. It was discovered that the ideal pH range for phage EP1 was 3–9. Additionally, at pH 9, 5, and 4, a drop of 4, 3, and 2 log was seen, respectively. The ideal pH for phage EP2 was discovered to be 5-7. Additionally, a 1 log drop was noted at pH 6, Ph5. Nevertheless, a 2 log and 7 log drop was noted at pH 8 and pH 9, respectively. At pH 2 no viability was observed.

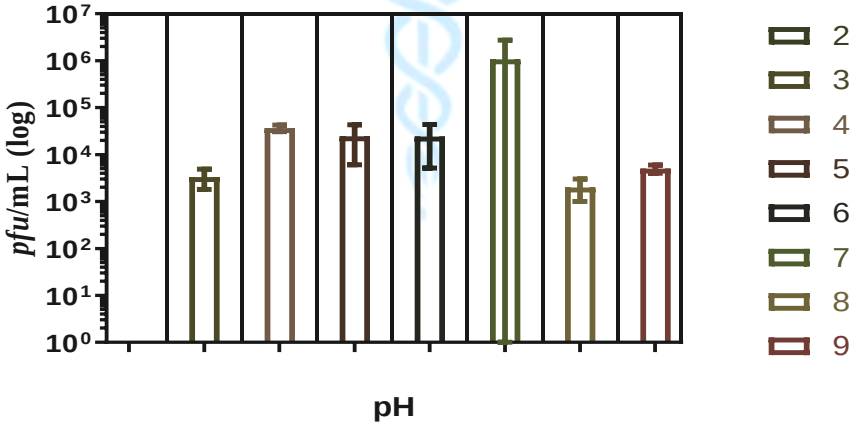

Figure 6: Effect of various pH on the viability of EP1 bacteriophage

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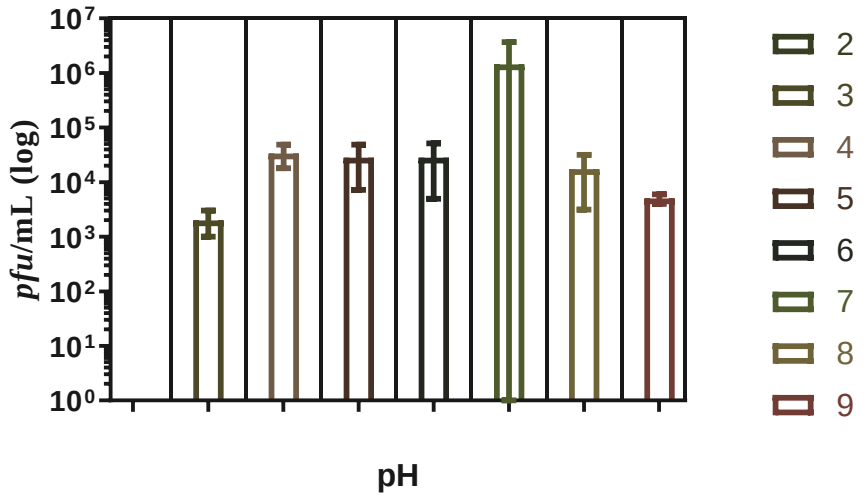

Figure 7: Effect of various pH on the viability of EP2 bacteriophage

Best Temperature for Storage

After three months of storage, the titer of phages EP1 and EP2 was determined to remain steady. At 4°C, there was no discernible drop in titer for phage EP1. However, at 25°C and 37°C, respectively, a drop of 1 and 2 log was noted (Table 2). At 4°C and 25°C, there was no discernible drop in titer for phage EP2. However, during three months of storage, a 1 log drop in phage titer was noted at 37°C.

Table 2: Storage stability of bacteriophage at different temperature

Phage	Titer Before Storage	Titer After Storage		
		4°C	25°C	37°C
EP1	8×10^{8} *pfu*/Ml	5×10^{8}	7×10^{8}	4×10^{6}
EP2	7×10^{9} *pfu*/Ml	5×10^{9}	7×10^{8}	4×10^{9}

EP1 and EP2 Reduced *E. coli* growth

The bacterial growth reduction test was used to evaluate the antibacterial activity of EP1 and EP2. Using a spectrophotometer to measure OD600 every two hours for the following twenty-four hours, the capacity of both phages to inhibit bacterial growth was tracked and contrasted with the growth of the control. No increase in bacterial growth was seen throughout the 18 hours when EP2 suppressed bacterial growth. After 18 hours, there was a little rise in the number of bacteria, nevertheless. The emergence of phage-resistant bacterial mutants may be the cause of this rise in the number of bacteria. It has previously been observed that phages that can limit the growth of *E. coli* can do so for 16 hours. By suppressing and lowering the initial bacterial inoculum count throughout the course of a 24-hour observation period, EP1 also demonstrated exceptional bacterial reduction capabilities. These phages are extremely uncommon, and the absence of resistant mutants during EP1 infection raises the possibility that the phage may connect to several bacterial receptors. Bacteriophages in cocktails may block or reduce bacterial growth, whereas phages in isolation are said to have less effective bacterial reduction capabilities. Following the required clinical and animal model research, it is hypothesized that EP1 and EP2 might be viable phage treatment candidates based on the decrease of bacterial growth.

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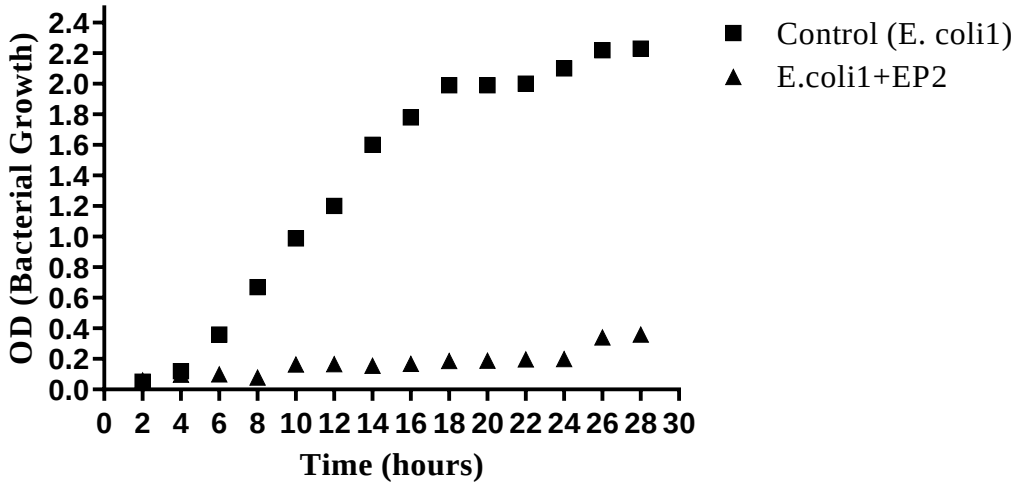

Figure 8. Bacterial growth reduction potential of EP2 bacteriophage

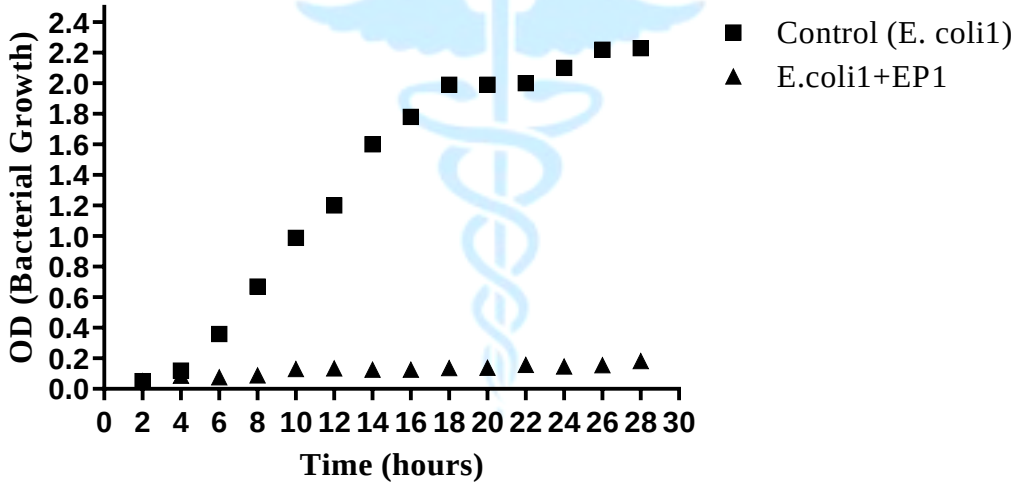

Figure 9: Bacterial growth reduction potential of EP1 bacteriophage

Discussion

In a spot test, two of the four water samples produced infectious bacteriophages of *E. coli*, whereas the other three samples did not generate any lytic zone. This is due to the fact that bacteriophages are obligatory parasites that rely on their host bacteria for growth. Variations in the amount of host bacteria at sample collection locations may be one cause of this disease (Alvi *et al.,* 2018). Since phage particles gradually stick to the sediment gathered at the water body's bottom, the lack of phage identification in the three water samples may have resulted from collecting the water without sediments. The plaque's surrounding halo demonstrated that the phages EP1 and EP2 generated soluble enzymes, namely depolymerase, that broke down the bacterial host cell. The halo indicated that the bacterial capsular polysaccharide (CPS) was broken up into distinct oligosaccharide components by the depolymerase enzyme as it permeated the agar layer. According to earlier studies, certain *E. coli* bacteriophages produced the enzyme from the infected bacteria, depolymerase, during phage replication, and utilized it to target other bacteria's

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CPS. *E. Coli* phage (108 pfu/ml) has been reported to have a phage titer in this range before (Alvi et al., 2018). Only a minimal amount of the phage lysate is needed to cure bacterial infections, and the titer of the separated phages is rather reasonable. The findings suggested that within the genus *E. coli*, the EP1 and EP2 phages exhibit host specificity.

In a different investigation, the EClpp1 bacteriophage used a spot test to infect and create lytic zones against different bacteria. In fact, it failed to form a lytic zone on four of the eleven *E. coli* isolates, although it was able to infect seven of them. *E. coli* species were lysed by the EClpp1 bacteriophage, but no additional bacteria were infected. The separated phages' range of infectivity was unique to *E. coli*. Since these phages would not harm normal microflora, this property can be useful in treating *E. coli* infections. Additionally, at 25°C and 37°C, the EP1 and EP2 phages were stable. If these phages were to be used as therapeutic agents, they might be easily employed because the average human body temperature is 37°C. Furthermore, no specific heating conditions are needed for the transmission of these phages. It is quite uncommon to find phages that can withstand such a wide pH range. Phage EP1 can be used orally because EP1/EP2 demonstrated survivability at pH 3 and the stomach pH is between 1.5 and 2.5 (Alvi *et al.,* 2018) . In contrast, the phage EP2 may be used by encapsulating it and ingesting it orally. However, it is safe to utilize these phages topically, intraperitoneally, intravenously, and intranasally. Because EP1 and EP2 are stable across a broad pH range, they may be used to treat UTIs brought on by *E. coli*. Additionally, they might be used to impregnate urinary catheters, as suggested by Verma and Harjai (2015), to stop the production of bacterial biofilms. The optimal storage temperature for tailed phages, without the use of preservatives, was determined to be 4°C by Ackermann and Tremblay and Alvi and Asif (2015) his indicates that there is no titer loss when both of these phages are kept at 4°C.

Conclusion

Infections and the effects of diseases caused by multidrug-resistant (MDR) bacterial pathogens today pose a serious threat to human health. Curing common infections becomes difficult. Bacteriophages remain a potent alternative to conventional medicinal approaches, despite the existence of various alternatives. They are capable of being isolated from the bacterial environment. Because of their in vitro efficacy in inhibiting bacterial growth, causing stability across a broad pH and temperature range, Host range and promoting long-term storage stability without the need for preservatives, bacteriophages EP1 and EP1 seem to be promising candidates for phage therapy after the necessary animal modeling and clinical trials.

Conflict of Interest:

No potential conflict of interest relevant to this article was reported.

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