Isolation and identification bacteriophage of E coli O157: H7 Acute Diarrhea children patients

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ABSTRACT

Bacteriophages are viral particles present everywhere bacteria can be found. Current estimates suggest that over 10^31 bacteriophages exist in the world, which is greater than the total of all living organisms and even bacteria combined. This study gathered a total of5out of 100samples comprising stool from children patient with acute diarrhea in order to investigate if a highly effective phage alternative therapy could be developed. Four different antibiotics were tested using Kirby- Bauer Method for sensitivity tests the results indicated isolation showed high resistance (100%) for Ceftazidime, Levofloxacin, Ciprofloxacin ceftriaxone, Antibiotics. while showed Low resistance with (0%) for Meropenem. Phage antibacterial efficacy was evaluated against four strains of Escherichia coli O157:H7 expressing multidrug-resistance isolated from children suffering from diarrhea feverish illness. All tested isolates were responsive (40%) to the action of bacteriophage lysis.. Final results indicated that 60% of our study was highly active against phages in vivo, whereas 50% was sensitive for them in vitro E.coli O157:H7. This indicates that indeed bacteriophage applied as an anti E.coli O157:H7 therapy works remarkably well on the targeted strain of application gastrointestinal tract.

1. Introduction

A long-standing survival mechanism utilized by bacteria to compete against other microbes that produce antibiotics is antibiotic resistance [4.]. Despite this phenomenon is a natural biological process, it becomes concerning when it arises in clinical bacterial infections. Globally, human health is seriously threatened by antibiotic resistance. with its prevalence and severity on the rise, making it one of the most urgent challenges of the 21st century [6].

Bacteriophage therapy was introduced into medical practice well before the advent of sulphonamides and antibiotics, but its application as an alternative treatment has been limited. Nevertheless, the lytic activity of bacteriophages in laboratory settings has allowed researchers to utilize specific bacteriophages for distinguishing between various bacterial species (McGrath and Sinderen, 2007). Currently, phages are being explored for their potential applications.

2. Subjects and Sampling

A total of 5outof 100 stool samples were collected from patients at the emergency units of Baghdad Teaching Hospital, Child Protection Hospitals, and a private central public health laboratory. The samples were isolated on Eosin-methylene blue (EMB) after being grown on blood agar and MacConkey agar and the ethics committees of the Iraqi University, Faculty of Medicine approved the study protocol (No.: FM.SA.98, dated: 5/3/2025).

2.1. Antibiotic Susceptibility Testing

We utilized Kirby-Bauer disk diffusion method to test ten antibiotics on E. coli isolates to evaluate the sensitivity they were to them. (Perilla et al., 2010). antimicrobial susceptibility of each isolate was determined on Mueller-Hinton agar (CM0337) (Oxoid Ltd., Basingstoke, Hampshire, England). The antibiotics tested included meropenem (10 µg), levofloxacin (30 µg), ciprofloxacin (5 µg), ceftazidime (30 µg), and ceftriaxone (30 µg) (Remel, U.S.A). The inoculated plates were incubated at 37°C for 24 hours. The inhibitory zones (measured in millimeters) were utilized to record the results, and they were interpreted in accordance with laboratory and clinical standards [1].

2.2. Preparation of Viral Suspension

Sewage sludge was prepared from viral suspension. A 25 ml centrifuge tube that is sterile moved 10 ml of sludge, which was then centrifuged for five minutes at 2000 rpm. Pellet was not disturbed during the aseptic transfer of the supernatant to a sterile 15 ml tube after centrifugation. Supernatant was aseptically filtered through a 0.8 mm pore-sized cellulose filter

to eliminate particles and then filtered through a 0.45 mm pore-sized filter to exclude bacterial cells and cellular debris to prepare a viral suspension [2,3].

For phage enrichment, 200 milliliters of fresh sewage samples were mixed with 20 milliliters of bacteriophage broth, 20 milliliters of a mixture containing seven multidrug-resistant E. coli O157:H7, and 20 milliliters of brain heart infusion broth in a sterile 1-liter flask. For 48 hours, this mixture was shaken at 120 rpm while being incubated at 37°C. After mixture was incubated solid debris was removed by centrifuging it at 10,000 × g for 10 minutes. Supernatant was then filtered using a Millipore filter with a pore size of 0.2 μm [4].

2.3. Enumeration of Bacteriophage Particles Isolated

To promote plaque formation, the double-layer agar plate technique also referred to as the overlay method was used. All phage filtrate solutions were first serially diluted (10^8 Plaque-Forming Unit / milliliter) in 50 milliliters of SM buffer, which is made up of 1 M Tris-HCl at pH 7.5, 5.8 g of NaCl, and 2 g of MgSO4·6H2O in 1 litter of distilled water. Subsequently, 0.1 milliliter from all dilution was combined with 0.1 milliliter of fresh bacterial culture (10^8 Colony Forming Units / milliliter). This mixture was then added to 5 milliliters of melted Brain Heart Infusion medium containing 0.7% agar, which was subsequently layered onto surface of Brain Heart Infusion with 1.5% agar. At 37 °C, prepared culture was incubated for 24 hours. Observations and counts were performed of resultant plaques after incubation. After incubation, Plaques were viewed and counted as a result [5].

2.4. Phage Plaque Purification and Bacteriophage Serial Dilution

Overlay agar plates were prepared utilizing supernatants diluted from 10^-4 to 10^-8 for the purpose of purifying specified phages. Utilizing a sterile scalpel, each plaque that grew on the plate surface was carefully removed and placed into sterile microtubes with 1 milliliter of Systemic Mastocytosis buffer, where they were mixed thoroughly for 30 seconds. The mixtures were subsequently centrifuged for five minutes at 4 °C at 8000 g. After centrifugation, 0.9 milliliters of SM buffer were mixed with 0.1 milliliters of each supernatant. Each solution's plaque formation was evaluated using the overlay method, and following a 24-hour incubation period at 37 °C, the number of plaques was counted [6,7].

2.5. Determining the Host Range of Bacteriophages

The host range of the isolated phage was assessed using a spot test with various bacteria. phage was identified by marking the plate accordingly. A sterile cotton swab, moistened with broth culture, was used to create a lawn culture on the surface of a nutrient agar plate (HiMedia Laboratories, India) for each bacterial strain. Five microliters (5 µl) of each phage lysate were then spotted onto the designated areas of the bacterial lawn. After allowing the lysates to dry, the

the plates were incubated at 37°C for 24 hours. Following incubation, the plates were examined for lytic zones in the spotted areas, which indicated the effectiveness of each individual phage [8].

2.6. Use of Mice in the Laboratory

I obtained 30 male albino Swiss mice aged 6-12 weeks and weighing 25-30 grams from the Iraqi Center for Genetics and Cancer Research at Mustansiriyah University. These mice were used to study the therapeutic effects of phages on E. coliO157:H7 infections. The mice were kept in plastic cages and maintained at room temperature of 25°C, with free access to water and proper nutrition. They were separated into four experimental groups as follows:

1. Control Group: Ten healthy mice kept and well-maintained to serve as a positive control baseline for the remaining groups.

2. Infection-Only Group: This group consisted of ten mice who were injured with 50 µL of nutrient broth containing *E. coli* O157:H7.

3. Treatment Group: Ten infected mice were injured as above, then treated topically and orally with phage solution at a dose of 50 µL.

The goal of these conditions was to assess the effectiveness of phage therapy as compared to a healthy control group and untreated infections.

2.7. Transmission Electron Microscopy (TEM)

Filtrate of bacteriophages was centrifuged for one hour at 25,000 xg. After that, A phage filtrate drop (10^9) was put on a copper grid coated with carbon and negatively staining with 2% uranyl acetate. Transmission electron microscopy was subsequently utilized for analyzing the morphology of the purified phages. The morphology of the purified phages was subsequently analyzed using transmission electron microscopy [9,10].

3. Results

Isolation and Characterization of Bacteriophages via Plaque Assay samples were utilized to screen and isolate various bacteriophages. These were subsequently tested for their effectiveness against multiple antibiotic-resistant (MAR) E. coli O157 : H7 isolates using plaque assay and spot techniques, as illustrated in Figure 1.

Figure1. Formation of large and small plaques by bacteriophages derived from stool tested against E. coli O157 : H7 isolates derived from diarrhea patients.

3.1. Identification of Bacteriophages Using Electron Microscopy

Electron microscopy (EM) revealed the presence of phages in samples obtained from a mixture of urine and stool. The identified phage belong to thefamily, characterized by a short tail measuring approximately 100 nm, which was isolated from the stool sample (see Figure 2)

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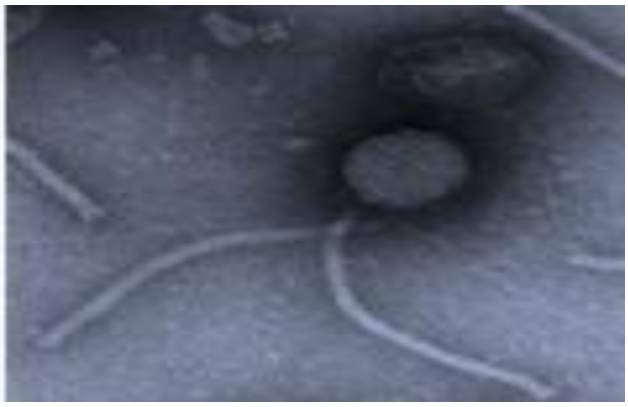

Figure 2. Electron micrographs images of the three types of bacteriopahges isolated from local E coli O157 : H7 isolates which appeared to be belong to Siphoviridae viruse.

This study involved determining phage titer through 10-fold serial dilutions (10-1, 10-2, 10-3, 10-4, 10-5, 10-6, 10-7, 10-8, 10-9, and 10-10) and counting the plaque-forming units (PFU). The dilutions of 10-1, 10-2, and 10^-3 resulted in complete bacterial lysis and could not be quantified. The remaining dilutions produced lysis counts of3.65 × 104, 1.9 × 104, 1. 56 × 104, 9.5× 103, 4× 103, 3× 103, and 0, respectively. These findings indicate that the 10-6 dilution yielded the most countable number of plaques. This dilution factor was subsequently used for all other experiments in this section of the study, the results suggested that bacteriophages were the most effective in achieving bacterial lysis, as shown in Table 1.

Table1: lysis activity of phage derived from sewage Specificity of Bacteriophages Tested Against E. coli O157 : H7 Isolates

Plate no	Factor of dilution	Plaque per plate	Dilution titer
1	10-1	Clear	Clear
2	10-2	Clear	Clear
3	10-3	Clear	Clear
4	10-4	3.65	3.65 x 104
5	10-5	190	1.9 x 104
6	10-6	156	1.5 6 x 104
7	10-7	95	9.5 x 103
8	10-8	50	4x 103
9	10-9	30	3x 103
10	10-10	0	0

From various bacteriophages were evaluated, yielding similar results. Consequently, the findings for phage A will be presented here. This phage was chosen to **assess** host specificity and demonstrated no activity against the tested bacterial species, including Shigella, Staphylococcus haemolyticus, Acinetobacter baumannii complex, Streptococcus pyogenes, and Micrococcus. These bacteriophages were effective solely in lysing their bacterial host, E. coli O157 : H7, as shown in Table 2.

Table 2: Specificity of bacteriophage lysis against a number of bacterial isolates

Bacteriophage lysis ability	` Specificity
E.coliO157:H7	+
Shigela Spp	–
Salmonella Typhi	–
Pantoea Spp	–
Enterococcus cloacac	–
Proteus mirabilis	–
Klebsella pneumonae	–
Proteus mirabilis	–
Acinetobacter baumannii complex	–
Streptococcus pyogen	–
Micrococcus Spp	–

Table (3): Percentages and Observed Frequencies for E. Coli O157:H7 Sensitivity.

Sensitivity level	Isolations no	Percentage
Sensitive S	2	40 %
Resistance R	3	60 %
Total	5	100 %

The in vivo testing for E.coliO157:H7

phage treatment was done in a study involving 40 albino Swiss mice as described in table (4). This study included 40 albino Swiss mice which were split into four groups of 10 mice each.

Table (4): Prior to test day and three days after test, the frequency and percentages of mice in each of the three states (live, dead, and healed from infection) were recorded.

	After 3 days form test						
Mice groups (No. =30)	1st numbers before test	Lived mice		Dead mice		healed mice from infection	
		No.	%	No.	%	No.	%
1st group: Normal rats	10	10	100	0	0	0	0
2nd group : Negative Control group	10	0	0	10	100	0	0
4th group : treated with phages in mouth	1o	6	60	4	40	6	60

4. Discussion

Bacteriophages have effectively sustained their populations by replicating through new bacterial infections. Research indicates that bacteriophages tend to persist longer than their host bacteria, allowing them to remain viable [11]. This suggests that the distribution of bacteriophages in humans may primarily be influenced by infection rates and opportunities for phage shedding and transmission [12,13].

A major concern regarding antibiotic resistance is its potential to compromise antibiotic treatment for patients with infectious diseases. Pathogens resistant to antibiotics present a significant risk to individuals undergoing such therapies[14,15]. The use of antibiotics to treat E. coli O157:H7 infections has sparked considerable debate, with one argument suggests that these antibiotics are not producing effective results [16].Although bacteria can produce β-lactamases, this study did not identify any E. Coli isolates that have extended-spectrum β-lactamases (ESBL)[17].

indicate that antimicrobials can lead to the emergence of resistant mutant strains through the transfer of resistance from other bacterial species [18].The main causes of gram-negative bacilli's resistance to quinolones involve alterations in outer membrane proteins or efflux pumps, as well as gene mutations that influencequinolone targets. This finding is supported by research from [19,20].

Studies discovered meropenem resistance is a growing public health concern, as it is an antibiotic as a last line of defense against hospital infections.[21,22,23] Discovered that genes encoding carbapenemases on plasmids are often linked to this resistance. E. Coli isolates from

control carcasses that exhibited meropenem resistance were identified to have the class C beta-lactamase gene. [24].

Cross contamination with bacteria that carry the resistance gene during the slaughtering process is most likely the cause of this resistance. Integrons may contribute to the development of antibiotic resistance [25,26].

E. coli is specifically target of bacteriophages isolated from urine and feces, according to host range analysis of these phages. Adsorption of phages to host cells is influenced by molecular interactions between the phage tail fibers and the binding sites on the cell surface [26].

Reduced adsorption to appropriate bacterial receptors may be associated with differences in the host range of bacteriophages. Both Salmonella and E. coli serotypes exhibited evasion mechanisms [28,29,30] which may be related to changes in the polysaccharides that make up the receptors. The test revealed that only E. coli 40% of isolates were susceptible to the phage PAPFH. This result is consistent with a number of previous studies.[30] found 50% of Escherichia coli bacteria were sensitive to Coliphage while [31] reported that P. aeruginosa was sensitive to the SL1 phage 41.2% of the time. Likewise, found 28.6% of Acinetobacter isolates sensitive to phages. Mice were grouped for special purposes to conduct the study. The first group which we called the positive control group included the healthy mice which were cared for and treated. It was noted that all the mice in this group survived till the third day. On the other hand, the second group formed the negative control where we soutured the mice and infected them with approximately 50 μm of *E. coli O157:H7*. Result were in such a way that all the mice in the negative control group died by third dayThe third group included the mice which were treated with bacteriophage prior to the *E. coli O157:H7* infection soutured the way the negative control group was treated. Survival analysis showed that the 60% of mice in this treatment group survived to the third day. The fourth group was similar to the third group where they were given bacteriophages orally prior to being infected with E. coli O157:H7. The survival rates for this group was similar to the third group. These results are consistent with results that were previously published by Watanabe (2007) where he recorded approximately 41.7% survival rate of phage treated mice infected Also, the survival rate drastically increased to 92.3% when the mice infected with Pseudomonas aeruginosa* were treated with bacteriophage.

5. Conclusion

This study provided a detailed characterization of bacteriophages isolated from sewage . The findings revealed that phages obtained from these sources exhibited greater lytic activity against multi-antibiotic resistant. E.coliO157:H7.Consequently, these phages could serve as a more effective alternative antibacterial therapy for treating multidrug resistant E.coliO157:H7 in patients with diarrhea.

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Conflicts of interest

The authors declare no conflict of interest

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عزل وتشخيص العاثيات (البكتيريوفاج) الخاصة ببكتيريا الإيشيريكية القولونية O157:H7 من الأطفال المصابين بالإسهال الحاد

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المستخلص

تم تحضير وتشخيص خمسة مركبات من نوع قاعدة شيف ذات الطور البلوري السائل, حيث تم استبدال طرف هذه المركبات بأنواع مختلفة من المجموعات القطبية ($-OCH_3$ ، $-COCH_3$ ، $-Br$ ، $-COOC_2H_5$ ، $-CF_3$) , لقد تم تشخيص التراكيب الكيميائية لجميع المركبات بواسطة التحليل الطيفي(FTIR) ، بينما لوحظت انتقالات الطور والتراكيب النسيجيه للعينات بواسطة المجهر ذو الضوء المستقطب ، وتم تحديد درجات حرارة الانتقال للمركبات ومدى درجة الحرارة للاطوار البلورية السائلة باستخدام جهاز المسح المسعري التفاضلي. أظهرت جميع المركبات ميلًا أعلى لإظهار أطوار وسطيه من النوع النيماتي مع مديات درجات حرارة مختلفة. تم قياس التوصيليه الكهربائية وثابت العازل وعلاقتهما مع درجة الحراره عند التصفيف الموازي والعمودي . إن استخدام مجموعات قطبية مختلفة في المركبات البلورية السائلة لوحظ بانه مصحوبًا بتغيرات في الاستقرار الحراري للاطوار البلوريه السائله بالإضافة إلى الخصائص الفيزيائية وجميع العينات اظهرت تباينا في العزل الكهربائي من النوع الموجب.

الكلمات المفتاحية : العلاج بالعاثيات (البكتيريوفاج) ، المضادات الحيوية .