

Isolation, purification and identification of *E. coli* O157 phage for medical purposes

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Abstract

Phages are the most important natural antimicrobial agents. They have some important advantages in comparison with other chemical antibiotics. *Escherichia coli* as a gram-negative bacterium belong to the *Enterobacteriaceae* family. *E. coli* O157 as the most well-known member of this family is the cause of some diseases and food poisonings. Symptoms of infection with these bacteria are mild to severe bloody diarrhea and even death. Researches in recent years have proven the existence of multidrug resistant strains of bacteria. Phages can be alternative biological agents against resistant bacteria that can cause serious problems. The most difficult step in the development of phage drugs for the purpose of treatment is isolation and purification of strong phages. In this article we tried to isolate coliphages from environmental samples like waste water and different types of soils. Phage isolation and purification steps of environmental samples and wastewater were performed according to plaque and spot assay methods. Eventually we isolated a phage belonged to the siphoviridae family that specifically destroys *E. coli* O157. It can be used as an agent for treatment of *E. coli* O157 infections with nearly no side effects.

Keywords: Antibiotic resistance, Diarrhea, *E. coli* O157, Isolation, Phage therapy.

1. Introduction

Phages are the most important natural antimicrobial agents (1). In addition to their antimicrobial properties, phages have other advantages compared to antibiotics or chemical agents (2). Briefly, fewer resistance exists to phages

compared to chemical agents (Phage resistance genes in bacteria are fewer than the widespread resistance genes against chemical agents, for example there is a few genes like BREX (3) that could make resistant bacteria against phages). In contrast, there are many genes for resistance to other anti-bacterial agents. Also phages have the ability to destroy targeted particular strains of bacteria without harming the normal flora (4). "Auto-

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mated Dosing” is another ability which means that phages have the potential to increase their dose in treatment of infections. Moreover the phages can be easily isolated from environmental samples. They can be produced at low costs and in high volumes (2). Also, phages have the potential to be effective in various pharmaceutical forms (5).

Escherichia coli as a gram-negative bacterium belong to the Enterobacteriaceae family. Most serotypes of *E. coli* that live in the intestines are avirulent and flora, but some of them can cause severe food poisonings (6). Also, in the case of infected wounds, the bacteria can act as pathogens on the wound (7). Shiga toxin-producing *E. coli* (STEC) are a group of *E. coli* species that can be potential pathogens by producing this toxin. *E. coli* O104: H4 and *E. coli* O157: H7 (or *E. coli* O157) in this category are better known (8). *E. coli* O157 is the most well-known member in this group. The rest of serotypes of STEC except O157, are named “non-O157 STEC” (9). Symptoms of infection with these bacteria have a wide range from mild to severe bloody diarrhea that even can be lead to death (8). Researches in recent years prove the existence of multidrug resistance (MDR) strains of bacteria in STEC category, and especially *E. coli* O157 (10). Existence and emergence of antibiotic resistant strains in these dangerous pathogens could be an alarm for global health. According to previous experiences in the field of phage therapy on various bacteria and their acceptable results, this issue can also be a promising solution to the problem of antibiotic resistant *E. coli* O157 (11).

Along with the first phage based antimicrobial agent, approved in 2006 by the Food and Drug Administration (FDA), efforts to build different biological drugs based on phages grew. “Safe and usable in humans and human consumption products” is the messages that were established with the approval of the patent (12).

The most difficult step toward the development of phage drugs for the purpose of treatment is isolation and purification of strong and selective phages. In this study, we attempted to separate the strongest possible phages from samples of waste water and soils of Fasa city for treatment of *E. coli* O157 infections.

2. Materials and methods

2.1. Chemicals

Suspension medium (SM) solution, 5.8 g NaCl, 2.0 g MgSO₄ · 7H₂O and 0.1 M CaCl₂ was purchased from Merck (Germany). 5 ml of 2% gelatin in 1 liter, Luria Bertani broth medium, Luria Bertani agar medium, PEG 6000 Solution (207 g PEG 6000 and 49.9 g NaCl, in 350 ml distilled water) was prepared from Sigma-Aldrich (USA).

2.2. Bacteria and phage isolation

E. coli O157 was purchased from Pasteur Institute of Iran (ATCC 35150). Phage isolation and purification steps of environmental samples and wastewater were performed according to plaque and spot assay methods.

Soil samples were dissolved in saline (From here on, the steps for water and sewage samples are similar). Samples were centrifuged at 5,000 rpm for 5 minutes. The supernatant was passed through 0.45 and then 0.22 µm syringe filters (Orange, UK). Then filtered fluid is a concentrated phage solution. A colony of bacteria (*E. coli* O157) was inoculated in a 10 ml Luria Bertani broth culture medium. After about three hours of incubation at 37 °C, relative turbidity can be seen. 10 ml of filtered samples was mixed with 10 ml of bacteria (in the exponential growth phase). This solution was inoculated with 100 ml Luria Bertani broth supplemented with 0.1 M calcium chloride and 0.1 M magnesium sulphate. This mixture was placed in a shaker incubator (Chest-Type GYROMAX 777, Amerex Instruments, Inc.) for 16 hours at 37 °C and 120 rpm. Samples were centrifuged (5000 rpm for 7 minutes) and the pellet was discarded. The supernatant was passed through 0.45 and then 0.22 µm filters. The serial dilution of filtered samples was performed to achieve appropriate concentration of phages (up to 10⁻⁶).

2.3. Double layer plaque assay

In this method, 200 µl of each serial concentration was mixed with 500 µl of fresh bacterial culture (3 hours in Luria Bertani broth) in separate sterile tubes. Tubes were incubated 20 minutes at 37 °C. Then it was combined with 3

ml of soft agar (0.7% agar Luria Bertani supplemented with 0.1 M magnesium sulfate) and poured on solid agar plates (1.5% agar Luria Bertani). After 24 hours of incubation at 37 °C, plaques were visible on the plates.

2.4. Spot assay

In spot assay, a combination of 3 ml of soft agar and 500 µl of fresh bacterial culture were poured on the plate. The plates were left at room temperature about 30 minutes to be firmed. Then three drops of phage solution (10 µl), were placed on three different sections of plates. After 24 hours of incubation at 37 °C, clear zones were appeared.

All the steps above were done separately for samples of animal husbandry, agricultural soils and wastewaters. The best results and the highest diversity of specific phages were obtained from samples of animal husbandry soil and sewage respectively.

2.5. Phage Purification

After plaque assay, the plaques were formed on the plate surface. An isolated plaque was selected and removed by a Pasteur pipette (superficial and deep layers of agar). Removed plaque (about 10^5 to 10^6 phages) was solved in 1.5 ml of SM solution. Then 200 µl of chloroform was added to eliminate bacterial contaminations. With this solution, plaque assay was performed in triplicate.

2.6. Phage amplification

2.6.1. Small lysate method

A mixture of 500 µl of bacterial solution in the exponential growth phase, 200 µl of purified phage solution and 3 ml of soft agar was poured on the LB medium with 1.5% agar. After 4 hours of incubation at 37 °C, about 3 ml of SM solution was poured on the plate surface and placed for 16 hours at 4 °C. If SM solution was absorbed by the underneath agar, another 3 ml of SM solution should be added on the plate surface and kept for another 4 hours. Then the entire SM solution which was full of selective phages, was collected by a fine syringe. Achieved phages had a concentration of about 10^9 Pfu/ml. This solution could be concentrated and precipitated

by PEG 6000.

2.6.2. Large lysate method

A combination of 10 ml of bacterial suspension, 1 ml of purified phage solution and 500 ml of LB broth was placed in a shaker incubator for 18 hours at 37 °C. Then the mixture was centrifuged at 5000 rpm for 7 minutes. The supernatant was passed through 0.45 and 0.22 µm filters. For achieving higher phage concentrations, this solution was precipitated by PEG 6000.

2.7. Precipitation of phages

PEG (5X) was mixed with the phage solution in a ratio of 1:4 (in the sterile 50 ml Falcon tube with a gentle shake). After leaving on ice for 1 hour, samples were centrifuged at 9000 rpm for 15 min at 4 °C. The supernatant was discarded and the Falcons were put on absorbent paper upside down. After drying, pellets were dissolved in 10 ml of SM solution (if it is not solved in the solution, 1 M KCl could be added).

2.8. Transmission electron microscopy (TEM)

Phage lysate with high titer (10 µl) was placed on carbon grids and stained with 1% uranyl acetate. The negatively stained grids were seen by TEM device model CM-10 construction by Philips.

3. Results and discussion

Phage samples were soil, waste water and soil enriched with manure. Phage samples were collected from all environments. According to variety and abundance of plaques, the most variety in types and potency of phages were in soil enriched with manure.

The differences between plaque forming patterns of phages from the initial stages of isolation and the phages after complete purification can be seen in Figure 1. Plaques that were formed from initial stages of isolation were very diverse in size and shape, but after purification of a selected type, all the plaques were the same in shape and size (Figure 1).

The most clear and larger plaque, was isolated, purified and amplified.

TEM image that was taken from purified

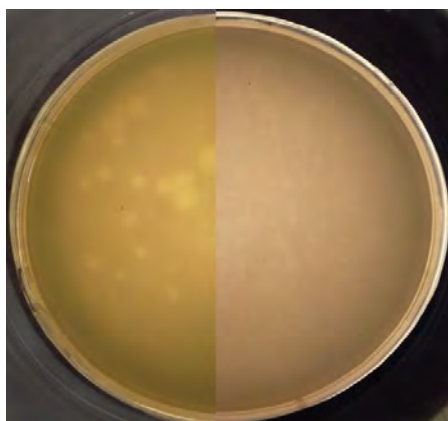


Figure 1. Left: plaques of phages in initial stages of the isolation. Right: plaques of phages that was formed after purification.

E. coli O157 phage indicates that the zone with greater diameter belongs to the order of caudovirals and family of siphoviridae (Figure 2).

The specificity of purified phage was tested on other bacteria such as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. All test results were negative and no plaques were formed. In the next step of specificity examination, isolated phages were tested on non-O157 *E. coli*. The result of this test was also negative. The highest concentration of achieved phages was 1.2×10^{10} Pfu/ml.

The isolated phage that infected *E. coli* O157 was belonging to the siphoviridae family that completely destroys the host and can be used as an agent for phage therapy (Figure 2). In other articles lytic phages were isolated and showed powerful effects when used in treatment of infections. Their results showed that phages were very powerful agents. In some comparisons among

phages, antibiotics and chemical agents such as silver nitrate, phages response to treatment was better than the other factors (13-14). Phages can be used in various pharmaceutical dosage forms such as topical, oral, gel, injection and patches without losing their potency, activity and effectiveness (13, 15-16).

In another study by Hudson *et al.*, they used *E. coli* selective phages to remove the meat from *E. coli* contaminations (17). Tomat *et al.*, used *E. coli* phages to eliminate contamination of milk (16). These articles show that we can use phages as an agent of preventing of disease and food corruption (18).

4. Conclusion

The selectivity of phage effects on bacteria ensures that there are not any harmful effects on human body's normal flora and even non-

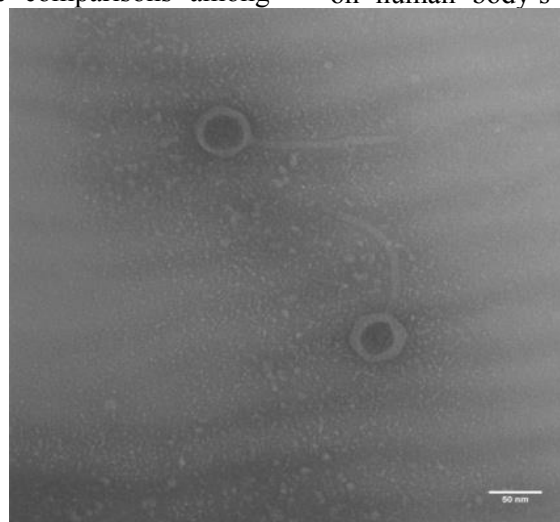


Figure 2. TEM image of phages isolated from soil enriched with manure that infected *E. coli* O157.

pathogenic *E. coli* strains.

In recent years, prevention of bacterial infections has become a major challenge. Using phages can be an appropriate and efficient alternative antibiotic in the fight against bacteria (19).

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Conflict of Interest

None declared.

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