Phage cocktail therapy against pathogenic *Escherichia coli*

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English summary

The worldwide emergence of multidrug-resistant Gram-negative bacteria poses a growing hazard to human health. Enterohemorrhagic *E. coli* (EHEC) is classified as Shiga toxin-producing *E. coli*, which causes diarrhea, hemorrhagic colitis (HC), and hemolytic uremic syndrome (HUS) in humans, and has been associated with several foodborne outbreaks in developed nations. The prevalence of *E. coli* O157 exhibiting multidrug resistance has significantly escalated recently.

Therefore, the formulation of novel treatment techniques for managing *E. coli* O157 infection is essential, and the application of bacteriophages presents a promising alternative to antibiotics for bacterial infection management.

This study aimed to isolate, characterize, and apply specific bacteriophages as biocontrol agents against MDR *E. coli* O157.

The study was carried out as follows:

A total of 80 clinical samples (Urine & stool) were collected from gastrointestinal patients admitted to Fayoum General Hospital and Fayoum University Hospital, Fayoum, Egypt for isolation of *E. coli* O157. Sorbitol MacConkey agar medium was used for the isolation and purification of *E. coli* O157.

Thirty-seven isolates were positive for *E. coli* O157 from the eighty clinical samples and confirmed morphologically by their growth on (SMAC) agar, investigated microscopically by Gram staining, and some biochemical characteristics.

The pathogenic *E. coli* isolates were tested for their susceptibility to seventeen antibiotics using the disk diffusion method. All 37 *E. coli* exhibit multidrug resistance patterns, exhibiting resistance from five to eight different antibiotic classes. The results demonstrate that 75.6% (29/37) of *E. coli* isolates showed resistance to cefoperazone, 81% (30/37) demonstrated resistance to

amoxicillin/clavulanic acid, and 91% (34/37) were resistant to both ampicillin/sulbactam and azithromycin. 86.4% (32/37) of *E. coli* isolates displayed resistance to amoxicillin, 72.9% (27/37) exhibited resistance to gentamicin, 89.1% (33/37) were resistant to amikacin, 56.7% (21/37) demonstrated resistance to cefoperazone/sulbactam, 70.2% (26/37) were resistant to both tetracycline and cefotaxime, and 75% (28/37) showed resistance to tigecycline. Complete resistance has been documented against erythromycin, ampicillin, ceftazidime, clindamycin, and vancomycin. The results demonstrate that 94.2% (35/37) of *Escherichia coli* isolates showed susceptibility to ciprofloxacin. The results demonstrated that the isolate (ES13) showed the greatest resistance to all evaluated antibiotics, except for ciprofloxacin. Bacterial isolate (ES13) was genetically identified using the 16S rRNA gene by PCR technique and exhibited a high degree of similarity to *Escherichia coli* O157, with a percentage of 99.3%, and submitted to GenBank under accession number (PP838722.1).

Four lytic coliphages have been isolated and identified for *E. coli* O157. These phages were designated as Ø1EC0157, Ø2EC0157, Ø3EC0157, and Ø4EC0157. From Qahafa Sewage Station, Fayoum, Egypt, Ø1EC0157 and Ø2EC0157 were isolated. From Sinouris Sewage Station, Fayoum, Egypt, Ø3EC0157 was isolated, and Ø4EC0157 was isolated from Atsa Sewage Station, Fayoum, Egypt.

The morphological plaques of Ø1EC0157 were irregular, clear plaques ranging from 1 to 2mm in diameter. In contrast, Ø2EC0157 yielded clear and circular plaques, measuring 1mm in diameter. Ø3EC0157 generated clear, circular plaques with a diameter of 2mm. Lastly, Ø4EC0157 produced circular plaques with a clear center encircled by a turbid halo zone, measuring 4 to 5mm in diameter.

According to TEM micrographs, three coliphages had both a head and a tail, while one exhibited only a head. The findings indicated that Ø1EC0157 is

classified within the *Siphoviridae* family, characterized by a hexagonal head measuring 64.2 nm in diameter and a non-contractile long tail extending 157.3 nm. In contrast, Ø2EC0157 had an icosahedral head with a diameter of 35 nm and a contractile tail measuring 132.2 nm in length. Ø3EC0157 had a cubic head measuring 61 nm in diameter and an elongated contractile tail extending 139 nm in length. Ø2EC0157 and Ø3EC0157 are classified within the *Myoviridae* family, while Ø4EC0157 is categorized under the *Podoviridae*.

A phage cocktail was prepared by mixing an equal quantity of each purified coliphage solution at 10^{10} PFU/mL.

Four isolated coliphages and phage cocktail were exposed to different pH values (3-12) .it was found that Ø1EC0157 and Ø4EC0157, Ø3EC0157 activity was completely inhibited at extremely acidic (pH= 3) and alkaline (pH= 11 and12) conditions. Maximum activity was observed at a pH equal to 7. As detected other coliphages, Similar results were observed for Ø2EC0157 except at pH = 4 there was no detectable activity. The phage cocktail exhibited the highest overall stability and activity across pH levels. It achieved maximum activity at pH= 7 followed closely by pH= 8 and pH =6. Activity remained robust at pH =9 and pH =10, indicating that the cocktail is more resilient to pH fluctuations compared to individual phages. However, like the individual phage, it was inactive at pH =3, pH =11, and pH =12.

The isolated coliphages and their cocktail are extremely thermotolerant, however, Ø2EC0157 and Ø3EC0157 lost their infectivity at 70°C. Whereas Ø1EC0157 and Ø4EC0157 lost their infectivity at 80°C. The phage cocktail demonstrated the highest stability and adaptability among the tested treatments; it lost infectivity at 90°C.

The isolated coliphages and their cocktail had a high level of stability across all tested concentrations of NaCl (0.5%,5%, 10%, and 15%). For Ø1EC0157, Ø2EC0157, and Ø4EC0157. But Ø3EC0157 showed complete inhibition at 15%, demonstrating that the individual phages and phage cocktail,

except for Ø3EC0157, have extraordinary resistance to higher NaCl levels.

The coliphages and phage cocktail host range was tested against 37 *E. coli* O157 isolates and other bacterial species, including three strains of *S. aureus*, two *Salmonella* strains, and one *P. aeruginosa* strain. The Ø1EC0157 was able to infect ten *E. coli* O157 isolates and one *Salmonella* strain. On the other hand, Ø2EC0157 infected eleven *E. coli* O157 isolates and one *Salmonella* strain, while Ø3EC0157 infected fifteen *E. coli* O157 isolates. Ø4EC0157 demonstrated the capacity to infect the greatest number of *E. coli* O157 isolates among the four coliphages, reaching 23 isolates. The phage cocktail infected all tested *E. coli* isolates and two *Salmonella* strains, thus demonstrating a wide host range.

The latent periods and burst sizes for coliphages were determined. Ø1EC0157 and Ø2EC0157 have lower burst sizes (180 and 191 PFU/cell) with latent periods of 53 and 79 min, respectively. Ø3EC0157and Ø4EC0157 phages showed the highest burst sizes 285 and 375 PFU/cell with latent periods of 49 and 27 min, respectively.

The accuracy and amount of purity for four DNA coliphages were validated by Nanodrop's A260/280 absorbance ratio and gel electrophoresis. Analysis with *EcoRI, HindIII*, and *BamHI* restriction enzymes revealed diversity in fragment numbers and densities among the gel patterns of four coliphage DNA genomes. The approach found 138 DNA polymorphisms and three genetic marker segments, spanning from 2 to 25 Kbp.

The *E. coli* O157 strain was treated with Ø1EC0157, Ø2EC0157, Ø3EC0157, and Ø4EC0157 at different MOIs (0.01,0.1,1) and the bacterial turbidity was measured at OD_{600} over 12 hours. There was a significant decrease in viability of the *E. coli* O157 strain infected with coliphages at different MOIs when compared with the control bacterial culture without phage treatment.

The phage cocktail was preserved at three different temperature degrees: 4°C, room temperature (RT), and -20°C. There were significant differences in

the preservation efficacy of the phage cocktail across different temperatures. While -20°C provided moderate stability during the initial weeks, phage activity declined substantially after day 28. Storage at RT was the least effective, with rapid titer loss observed as early as day 14. Conversely, 4°C proved to be the optimal storage condition, maintaining the highest phage viability over more than two months.

The phage cocktail was mixed with three different carriers: royal jelly, sorbitol, and trehalose, with a ratio of 1:1 V: W. The three formulas were packed in HMPC capsules at 4°C and checked weekly to determine the viability using the DLA method. Trehalose provided the best protection, followed by sorbitol and royal jelly. This suggests trehalose is the most effective stabilizer for formulated phages, making it the preferred choice for applications requiring long-term stability.

An applied experiment was designed to use a coliphage cocktail with (10^{10} CFU/mL)as a biocontrol agent against *E. coli* O157 infection in animal rat model. Thirty rats were allocated into five groups as follows:

Healthy group: functioned as a negative control, receiving only 1 mL of PBS orally.

Illness group: acted as the positive control, receiving 1 mL of *E. coli* O157 bacterial solution orally via a metallic probe straight into the stomach of the rats.

Cip. group: each rat was administered 640 µL of ciprofloxacin daily.

Ph. cocktail group: each rat was administered 1 mL of phage mixture.

Cip+ Ph. cocktail group: each rat was administered a combination of phage and ciprofloxacin.

The rat experiment findings revealed substantial alterations in the CFU/mL values of *E. coli* O157 across several treatments during the observation period. The illness group exhibited a rise in bacterial count from the

1st day (7.30) to the 3rd day (10.29 log CFU/mL), then stabilizing at (9.12 log CFU/mL) on the 5th day and 7th day. The Cip. group demonstrated a consistent reduction in bacterial count from the 1st day (7.36 log CFU/mL) to the 7th day (2.74 log CFU/mL), signifying its efficacy in diminishing bacterial levels. The Ph. Cocktail group also exhibited a very similar significant decrease in bacterial count like ciprofloxacin, declining from (7.19 log CFU/mL) on the 1st day to (2.86 log CFU/mL) on the 7th. The synergistic administration of ciprofloxacin and the phage cocktail demonstrated superior efficacy, with CFU/mL levels markedly declining from (7.30) on the 1st day to (1.84) on the 7th day. The previous results indicate that both ciprofloxacin and the phage mixture efficiently reduced bacterial counts throughout the study duration.

Hematological and serological analyses for rat blood samples were assessed. The parameters examined were RBCs and TLC and the sera samples were subjected to the following examinations interleukin -6, SGOT, and SGPT. There were differential impacts of the treatments on blood parameters in *E. coli*-infected rats. The phage cocktail alone or in combination with Ciprofloxacin exhibited potential anti-inflammatory and hepatoprotective effects, as evidenced by reduced IL-6, SGOT, and SGPT levels compared to the illness group.