ORIGINAL ARTICLE

ASSESSING THE EFFICACY OF PHAGE-COCKTAIL AND PHAGE-ANTIBIOTIC COMBINATIONS AGAINST ENTEROTOXIGENIC ESCHERICHIA COLI USING A GALLERIA MELLONELLA INFECTION MODEL

Prasanth Manohar[1,2], Loganathan Archana[3], Belinda Loh[1] , Sebastian Leptihn[1,4,5], Nachimuthu Ramesh*[3]

[1]Zhejiang University-University of Edinburgh (ZJU-UoE) Institute, Zhejiang University, Haining, Zhejiang 314400, P.R. China
[2]The Second Affiliated Hospital Zhejiang University (SAHZU), School of Medicine, Hangzhou, P.R. China.
[3]Antibiotic Resistance and Phage Therapy Laboratory, School of Bio-Sciences and Technology, Vellore Institute of Technology (VIT), Vellore, Tamil Nadu 632014, India
[4]Department of Infectious Diseases, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, P.R. China
[5]University of Edinburgh Medical School, Biomedical Sciences, College of Medicine & Veterinary Medicine, The University of Edinburgh, 1 George Square, Edinburgh, EH8 9JZ, United Kingdom.

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Address for Corresponding : *Dr. Nachimuthu Ramesh,* *Assistant Professor, School of Bio-Sciences and Technology, Vellore Institute of Technology (VIT), Vellore, Tamil Nadu, INDIA. Mobile: 9842660673. Email: drpnramesh@gmail.com*

ABSTRACT

Context: Phage therapy has become a revolutionary medical approach to control bacterial infections in the post-antibiotic era.
Objective: This study aims to evaluate the efficacy of phage cocktails and combination of phages together with antibiotics in treating *E. coli* infections. Methods: In our work, pathogenic Enterotoxigenic *E. coli* (ETEC) and the previously characterized Escherichia phages EP1 and EP2 were used.
Results: *In-vivo* studies using the *Galleria mellonella* infection model showed that a single dose of EP1 was able to clear the infection while phage EP2 was unable to eliminate the bacterial pathogen. A phage cocktail consisting of EP1 together with EP2 protected the larvae from bacterial infection with a 100% larval survival rate. To study phage-antibiotic synergy, a combination of phage with cefotaxime was investigated. Here, we observed a 100% survival rate within 72 hrs, while no significant survival was observed in the control group.
Conclusion: This study highlights the importance of phage cocktails and phage-antibiotic combinations as a strategy in the preparation of phages for therapy.

Keywords: Phage therapy; *Escherichia* phage; Phage cocktail; Phage-antibiotic; *Galleria mellonella*

INTRODUCTION

With the increasing globally occurring crisis of antimicrobial resistance in bacteria, phage therapy -as a non-antibiotic therapy is considered one of the most promising alternatives. Recent studies and clinical data demonstrate that the use of bacteriophages is a powerful approach to treat infections.[1,2,3] A crucial problem, however, is the evolution of bacteria to develop resistance towards phages. Phage-bacterial coevolution is a natural phenomenon, important for microbial diversity.[4] The development of phage-resistant bacterial strains is almost unavoidable. In *in-vitro* studies and clinical trials alike, the emergence of phage-resistant bacterial strains is frequently reported.[5-8]

Thus, phage therapy requires approaches that are able to constrain the emergence of phage-resistant bacterial strains. This can be achieved by deploying so-called phage cocktails (consisting of two or more therapeutic phages) or by using phages in combination with antibiotics.[9-11] The microbial viruses in phage cocktails ideally target different bacterial surface receptors, making the emergence of double or triple mutations affecting all receptors simultaneously unlikely. For example, when a phage cocktail (containing six phages) was used to treat *P. aeruginosa* infection in mice, no phage-resistant bacterial mutants were observed, even though phage-resistant mutants were identified during *in vitro* experiments.[12]

During the *in vivo* test, the occurrence of a small number of mutants might be cleared by the immune system. In the case of combining phages together with antibiotics, sub-lethal concentrations of some antibiotics have been shown to increase phage replication.[13] *G. mellonella* infection model is found to be a more predictive model system to study the therapeutic effects of phages against bacterial infections.[12,14-16] There is a significant correlation observed between the results obtained from *G. mellonella* model and a mouse infection model.[17] Most of the studies have noted that survival was largely dependent on phage dose and time.[17,18]

Phage resistance was also observed in the case of *Escherichia coli* strains, however, there is very limited knowledge about the elimination of the resistant bacterial *in vivo*.[19,20] Previous studies have reported that the phage-resistant mutants are less often observed in animal models or during clinical trials, but there are several exceptions.[5,6,12] In this study, we focused on the evaluation of the efficacy of phage cocktails (a combination of two phages) and phage-antibiotics (synergistic activity) in eliminating the occurrence of phage-resistant bacterial strains using *G. mellonella* (wax worms) as a model organism.

MATERIALS AND METHODS:

Bacterial strains and bacteriophages:

The two clinical *E. coli* isolates used in this study were collected from a diagnostic center in Chennai. The ETEC isolates (ETEC01 and ETEC02) were identified based on molecular analysis as previously described.[21] MIC was used to study the susceptibility of ETEC isolates towards cefotaxime and recorded as per CLSI guidelines.[22,23] The two bacteriophages, *Escherichia* phage EP1 and *Escherichia* phage EP2 were isolated from the sewage water sample collected from Karur and the Ganges river water sample collected from Varanasi, respectively. Both the bacteriophages were previously characterized and based on the genomic analysis the phages were found to be virulent / lytic.[14,15,24] For this study, purified phages were used as described in our previous study.[21] Phage cocktail was prepared mixing the equal concentrations (10^4 PFU/mL) of *Escherichia* phage EP1 and *Escherichia* phage EP2 at 1:1 ratio. Phage-antibiotic combinations were prepared using 10^4 PFU/mL of *Escherichia* phage plus 1 mg/L of cefotaxime.

In vivo experiments:

For the *in-vivo* studies the *G. mellonella* was used as a model organism. The late larval stage (app. 2-2.5 cm, creamy white color) worms were used to evaluate the therapeutic potential of bacteriophages as explained elsewhere.15 Briefly, the *E. coli* isolates were grown exponentially in LB broth to reach OD_{600} =0.65, and 20 µL of bacteria was used to infect the larvae. The larvae used for the experiment was starved for 24 hrs and the injection was made on the last left pro-leg. The experimental set-up consists of four controls and in each set 8 larvae were used. Control 1: Larvae were injected with saline (20 µL); control 2: Larvae were infected with *E. coli* (20 µL, 108 CFU/mL); control 3: Larvae were injected with phage/phage cocktail (20 µL, 104 PFU/mL); control 4: Larvae were injected with cefotaxime (20 µL, 1.0 mg/L).
In the treatment group 1, larvae were infected with bacteria and treated with phages/phage cocktails within 1 hr of infection. The test group consists of; A) larvae infected with ETEC01 and treated with EP1, B) larvae infected with ETEC02 and treated with EP2, C) larvae infected with ETEC02 and treated with phage cocktail, D) larvae infected with both ETEC01 and ETEC02 and treated with phage cocktail.

In the treatment group 2, the larvae were infected with bacteria and treated with phage-cefotaxime within 1 hr of infection. The test group consists of; A) larvae infected with ETEC01 and treated with EP1 plus cefotaxime, B) larvae infected with ETEC02 and treated with EP2 plus cefotaxime, C) larvae infected with ETEC01 and ETEC02 and treated with phage cocktail plus cefotaxime.

The treated larvae were evaluated for their survival at every 12 hrs up to 96 hrs. After the conclusion of the experiment, *G. mellonella* health index scoring was performed using the larvae survival as categorized by either alive or dead. The larvae were considered to be dead only when there was no movement or minimal movement upon stimulation or larva appearing black (melanization) or >3 black spots on brown larvae, and considered alive only when there was movement without stimulation and no melanization. All the experiments were performed in duplicates and analysed using GraphPad Prism 7.0.

Statistical analysis:

All the experiments were repeated twice and the results represent the standard error of mean (SEM). The survival curves were plotted using Kaplan-Meier method and log-rank test was used to calculate the difference in survival rates using GraphPad Prism software 7.0 (GraphPad Software, Inc., La Jolla, USA). $p<0.05$ was considered as statistically significant.

Results and Discussion:

To study the efficiency and efficacy of therapeutic phages in preparations composed of either multiple phages or in combination with antibiotics, we performed *in vivo* studies using the *Galleria mellonella* larvae (wax moth larvae) model. Here, the phages EP1, EP2, as a phage cocktail (EP1 plus EP2) or as a phage-antibiotic (cefotaxime) combination, were investigated in the model. Recent studies have demonstrated that the use of *G. mellonella* larvae as models of higher organisms is highly appropriate to study the efficacy of phages *in vivo*.[17,25,26] Based on our previous studies, bacteria at 10^8 CFU/mL in a 20 µL volume were found to be lethal for a single *G. mellonella* larva.[15] In the control groups, in which saline, phage lysate (or phage cocktail) (20 µL), or antibiotic (20 µL) were injected, 100% larval survival was observed (Fig.1). This also confirms the absence of toxins in the phage products, which often contain endo- or exotoxins that are released from the bacteria. Prior to medical applications of phages, it is necessary to prepare the phage products free of endotoxins or any other bacterial components that may complicate the infection and sometimes lead to life-threatening conditions, such as septic shock.[15] In the case of the infection group, where bacteria where injected without antibiotics or phages, all the larvae were dead within 48 hrs, confirming the virulence of the *E. coli* isolates (ETEC).

When the larvae were infected with ETEC01 and treated with EP1, 100% larval survival was observed (Fig.2), indicating that EP1 can efficiently inactivate the pathogen, allowing the phage to prevent bacterial infection. In contrast, when the larvae were infected with ETEC02 and then treated with EP2 phage, we observed survival rates between 80% at 36 hrs and 0% at 96 hrs (Fig. 2). This finding illustrates that phage EP2 is not highly effective in inactivating the pathogen. In most of the earlier studies, a single dose containing a single type of phage increased the survival of infected *G. mellonella* larvae.[14,15,17] A dose dependent increase in survival rates was also reported in earlier studies.[27,28] In our study, a single dose of *Escherichia* phage EP2 was unable to mediate clearance of the pathogens to allow recovery of the infected larvae, although the phage showed efficient killing of the bacterium *in vitro*.

The *in vivo* results might be due to the emergence of phage-resistant mutant or the release of endotoxins during bacterial cell lysis. In our earlier study, we observed that phage cocktails can eliminate the infections caused by multiple bacterial species in the *G. mellonella* model.[15] In order to confirm whether the phage cocktail can increase larval survival, the ETEC02 infected larvae were treated with a phage cocktail composed of EP1 and EP2. Here, the infection was fully cleared and 100% survival rate was observed (Fig.2). Interestingly, when the larvae infected with multiple bacteria i.e. ETEC01 and ETEC02 were treated with phage cocktail the larval survivability was 100%. While this experiment cannot conclude that the use of a phage cocktail eliminated phage resistant mutants developed during monophage therapy, it highlights the efficacy of a phage cocktail for therapy. These results are in agreement with earlier studies have also noted the efficacy of phage cocktails in treating *P. aeruginosa* infections using *G. mellonella* larvae.[12,16]

During the phage-antibiotic combination therapy, all the infected groups (ETEC01, ETEC02) recovered from the bacterial infection and 100% larval survival was observed (Fig.3). This is one of the very few studies to evaluate the efficacy of phage-antibiotic combinations using the *G. mellonella* model. Earlier Wang *et al*., reported *in vivo* antibacterial activity of φWL-3 and fosfomycin against *E. coli* strains using the *G. mellonella* model.[29] In another study, *G. mellonella* larvae were infected with *A. baumannii* and treated with a combination of phage, vB_AbaP_AGC01 and ciprofloxacin or meropenem to increase larval survival from 35% to 77%.[30] Similarly, our result suggests that phages and antibiotics can act synergistically to eliminate the bacterial infection when used as combination therapy.

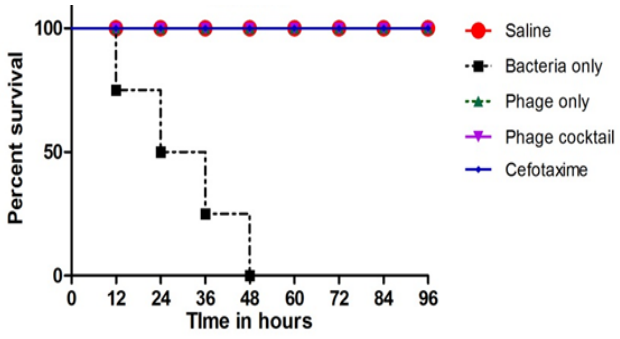

Figure 1: Control groups [Saline, bacteria only, phage only, phage cocktail and cefotaxime] used for the *in vivo* studies. The larvae (*G. mellonella*) were injected with 20 µL of saline, infected with 10^8 CFU/mL of bacteria, a single dose of phage EP1/EP2 (10^4 PFU/mL), phage cocktail (EP1 plus EP2, 10^4 PFU/mL), phage-antibiotic (EP1/EP2 at 10^4 PFU/mL plus cefotaxime at 1 mg/L). The survival rates were plotted using the Kaplan-Meier method and log-rank test was used to analyze the difference in survival rates in GraphPad Prism 7.0.

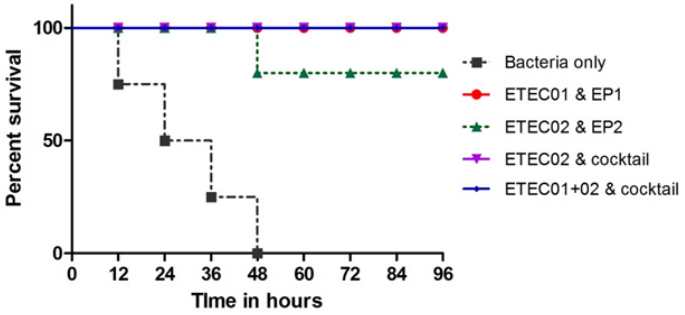

Figure 2: Efficacy of phages, EP1 & EP2, and phage cocktail in the treatment of *E. coli* (ETEC) infection and survival rates of *G. mellonella* larvae. A single dose of phage EP1/EP2 (10^4 PFU/mL) or phage cocktail (EP1 plus EP2, 10^4 PFU/mL) was injected after the larvae was pre-infected with 10^8 CFU/mL of bacteria. Survival rates of the larvae infected with ETEC01, ETEC02, and ETEC01+ETEC02 that were treated with phage EP1 or EP2 or phage cocktail (p=0.0001). The survival rates were plotted using the Kaplan-Meier method and log-rank test was used to analyze the difference in survival rates in GraphPad Prism 7.0. All phage treatment results were compared with the control. A statistically significant difference (p<0.05) was observed using the 8 worms per group of phage treatment.

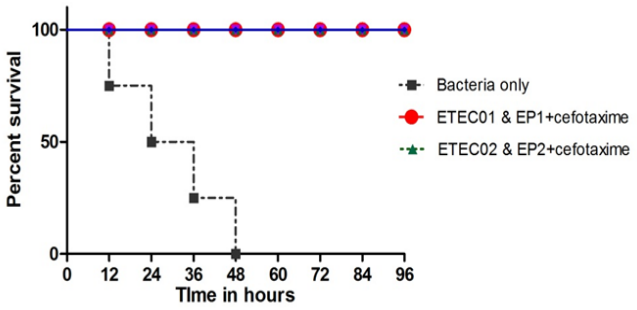

Figure 3: Efficacy of phage-antibiotic combination in the treatment of *E. coli* (ETEC) infection and survival rates of *G. mellonella* larvae. A combination of (single dose) phage EP1/EP2 (10^4 PFU/mL) and cefotaxime (1 mg/L) was injected after the larvae was pre-infected with 10^8 CFU/mL of bacteria (ETEC01 and ETEC02). Survival rates of the larvae infected with bacteria that were treated with phage EP1/EP2 plus cefotaxime (p=0.0001). The survival rates were plotted using the Kaplan-Meier method and log-rank test was used to analyze the difference in survival rates in GraphPad Prism 7.0. All phage treatment results were compared with the control. A statistically significant difference (p<0.05) was observed using the 8 worms per group of phage treatment.

Conclusion:

The antibiotic resistance crisis has stimulated a renewed interest in the application of bacteriophages to treat bacterial infections. In our study, we evaluated the therapeutic effects of phage cocktails and phage-antibiotic combinations against ETEC clinical strains in the *G. mellonella* model. A significant reduction in mortality was observed in the larval model when phages were administered to eliminate the bacterial pathogens; moreover, the use of a 2-phage cocktail and a phage-antibiotic combination, increased the efficacy in treating the ETEC infection. Our study shows that the *G. mellonella* model is ideal to study therapeutic phages, their combination with more than one phage, or together with antibiotics. Our work also demonstrates the importance of using phages as therapeutic agents for treating ETEC infections, in particular to control MDR strains of the bacterium.

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Conflicts of interest:

The authors declare that there is no conflict of interest.

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