Assessing phage therapy against *Pseudomonas aeruginosa* using a *Galleria mellonella* infection model

Beeton M. L.[1]*, Alves D. R.[2]*, Enright M. C[2]. and Jenkins A. T. A.[2]

[1]Cardiff Metropolitan University, Western Avenue, Cardiff CF5 2YB, United Kingdom.

[2]Department of Chemistry, University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom.

*both authors contributed equally

Corresponding author. E-mail. mbeeton@cardiffmet.ac.uk

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Synopsis

Objectives

To develop a *Galleria mellonella* infection model to assess the *in vivo* efficacy of phage therapy against laboratory and clinical strains of *Pseudomonas aeruginosa*.

Methods

Galleria were infected with *P. aeruginosa* PA01 and treated with varying multiplicity of infection of phage either two hours post-infection (treatment) or two hours pre-infection (prevention) via injection into the haemolymph. To address the kinetics of infection larvae were bled over a period of

24 hours for quantification of bacteria and phage. Finally, clinical strains from acute and chronic cystic fibrosis infections were used in the prevention model to further validate the model.

Results

Survival rates at 24 hours when infected with 10 cells/larvae were greater with the prevention model versus treatment model (47 vs 40% MOI 10, 47 vs 20 % MOI 1 and 33 vs 7% MOI 0.1). This pattern also held true when infected with 100 cells/larvae (87% vs 20% MOI 10; 53% vs 13 % MOI 1; 67% vs 7% MOI 0.1). By 24 hours post infection phage had kept bacterial numbers in the haemolymph to 1000-fold lower than the non-treated group. Phage increased survival of *Galleria* when infected with both the acute (0% vs 85%) and chronic (80% vs 100%) clinical *P. aeruginosa* strains.

Conclusions

Here we present data for the use of *G. mellonella* as a simple, robust and cost-effective model for the initial *in vivo* examination *P. aeruginosa* targeted phage therapy which may be applied to other pathogens with similarly low infective doses.

Introduction

Multi-drug resistant bacterial pathogens pose an ever increasing threat to human health. This problem is in part due to a lack of novel antibiotics approved for use over the last few decades resulting in an urgent need to identify new avenues for treating bacterial infections, especially those caused by gram-negative pathogens[1] *Pseudomonas aeruginosa* is an opportunistic pathogen that is a leading cause of infection among burns victim and patients with cystic fibrosis. It is also responsible for a large number of health-care associated infections. To make matters worse, *P. aeruginosa* is associated with hypermutability and due to high antibiotic selective pressure has given rise to the emergence of multi-drug resistant strains in the population and concerns about available effective treatments are growing.[2, 3] In the UK resistance to two or more antibiotics among *P. aeruginosa* isolated from the

lungs of cystic fibrosis (CF) patients has risen to 40%.[4] This is a worrying statistic as colonisation of the CF lung with *P. aeruginosa* is a predictor of poor prognosis and associated with a 2-3 fold increased chance of death over an eight year period.[5] For this reason novel anti-invectives are needed.

Facing such a scenario interest in phage therapy in Western society has experienced resurgence after research into this area fell out of favour following the discovery of antibiotics. Bacteriophages (or phages) are viral particles able to infect bacterial cells with high specificity, taking over cellular function to replicate its genome. Upon maturation is complete the bacterial cell wall is lysed to release viral progeny.

Phage therapy can be broadly subdivided in to four main categories.[6] (1) Conventional phage therapy principally uses lytic phage to lyse target bacterial species. (2) Modified phage therapy utilizes genetically altered phage with favourable properties such as non-lytic replication to avoid the possibility of endotoxin shock, when bacterial cells are lysed. (3) Treatment with enzymes derived from phage such as administration of endolysins to selectively degrade the bacterial peptidoglycan cell wall. (4) Finally is the concept of combination therapy with phage and antibiotics where phage exhibit properties to degrade polysaccharide components of biofilms therefore allowing antibiotics to penetrate and elicit an action.[7]

Although *in vitro* systems allow for a reductionist approach to examining phage interactions with target bacteria, it does not take into account a more complex *in vivo* system. Mammalian models are an excellent means of testing phage therapy, but require ethical approval, significant infrastructure and funds. The *Galleria mellonella* model fills the void between these two systems providing a cheap, reliable and ethics free system for testing novel antimicrobials.[8] Here we describe the first use of the *G. mellonella* model to examine the treatment of *P. aeruginosa* infection with phage therapy.

Materials and Methods

Bacterial strains and preparation of inoculum

Phage therapy was assessed using *P. aeruginosa* PAO1 and two clinical isolates, PA45291 and BC00907, isolated from cystic fibrosis patients with acute and chronic infections, respectively. Bacteria were grown to mid-log phase in LB and washed once in PBS. Cells were resuspended in PBS to give a final cell number of $1x10^8$ cfu ml^{-1} and diluted accordingly in PBS to give the required inoculum size for each experiment.

Phage cocktail preparation and titration

Six distinct phages infecting all the *P. aeruginosa* strains mentioned above were combined to establish a cocktail suspension. Phage solutions were propagated on PAO1. Briefly, 100 µl of phage lysate and 100 µl of host growing culture were mixed and left for 5 min at room temperature. Following incubation, 3 ml of LB soft-agar containing 0.65% bacteriological agar was added and poured onto agar plates. After an overnight incubation at 37°C, plates displaying confluent lysis were selected and 3 ml of SM buffer (5 M NaCl, 1M MgSO4, 1 M Tris–HCl [pH 7.5], 0.01% w/v gelatine) and 2% (vol/vol) chloroform were added before incubation at 37°C for 4h. High-titre phage solution was removed from the plates, centrifuged (8,000 x g, 10 min) to remove cell debris, and then filter sterilized (pore size, 0.22 µm). A PEG purification step was further added to remove any possible bacterial remainings from the suspensions and the final solutions were stored at 4°C. All the necessary dilutions were performed in SM buffer. For the titration of the bacteriophage content in the haemolymph, a similar methodology to the propagation was followed. The several dilutions were mixed with the host bacterial cells and 3 ml of soft agar was added and poured onto agar plates. After an overnight incubation plaques were counted to determine phage titre.

***G. mellonella* phage therapy assay**

Larvae of *G. mellonella* were obtained from Livefood UK Ltd (Somerset, UK). Larvae were stored at 4 ^{o}C and used within 1 week of receipt. A modified methodology developed by Peleg was used to infect each *Galleria*, but in brief.[8] *Galleria* were surface sterilized with a FASTAID pre-injection swab containing 70 % ethanol. Using a pair of tweezers each *Galleria* was restrained and with a 26 gauge Terumo syringe 10 µl of inoculum containing either 100 or 10 cells of *P. aeruginosa* was delivered into the larval haemolymph behind the last proleg. For the treatment model phage suspension was delivered behind the last proleg on the opposite side to the infection two hours post-infection and for the prevention experiment phage suspension was given two hours pre-infection. All experiments used 15 larvae per treatment. A positive control group, where the larvae were infected and treated with PBS solution, and two negative control groups were also included: one group injected with PBS only, assessing the impact of any negative effect from the injection process, and one group injected with phage suspension. Larvae were placed into petri dishes and incubated at 37 °C for 48 hours, being examined regularly and recorded as dead when they did not move in response to touch.

Bleeding larvae haemolymph

The prevention model was used to follow the kinetics of bacteria and phage interactions within the larval haemolymph over time. The phage cocktail, or PBS, was administered two hours prior to infection with phage initially quantified within the haemolymph at time of infection (time zero). *Galleria* were infected with 100 cells of *P. aeruginosa* PA01 and at time points of eight and 24 hours three *Galleria* were sacrificed and bled following incision made with forceps to quantify phage and *P. aeruginosa* in both phage and PBS treated Galleria. Titrations of haemolymph were made in SM buffer for phage counts and for PAO1 counts titrations were made in 10 mM of ferrous ammonium sulfate (FAS) (Sigma Aldrich, UK) for inactivation of extracellular phage prior to viable bacterial counts.

Statistical analysis

Kaplan-Meier survival curves were plotted using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Treatment of infection

In this study two models of phage and infection interactions were examined. The first was a treatment whereby *Galleria* were infected with either 10 or 100 cells of *P. aeruginosa* PA01 and left to allow an infection to establish for two hours. Varying MOIs of phage were then administered and death was observed over 48 hours. No death was seen in the PBS controls and *Galleria* which were treated with PBS died quicker when infected with 100 cells versus 10 cells. Administration of phage prolonged the survival of the *Galleria* in a dose dependent manner, but 0% survival was eventually seen in all groups by 30 hours [Figure 1a]. At 24 hours there was 100% mortality in the infected and untreated *Galleria*, but 40% survival for those infected with 10 cells and treated with an MOI of 10 compared with 20% survival with those infected with 100 cells at the same MOI [Figure 1b].

Prevention of infection

The second model examined the effect of prevention of infection whereby *Galleria* were given a prophylactic dose of phage two hours prior to infection with *P. aeruginosa* PA01. Similarly to the treatment experiment, *Galleria* infected with 100 cells died quicker than those infected with 10 cells when given PBS two hours before infection. [Figure 2a]. At 24 hours survival ranged from 80% in *Galleria* given an MOI of 100 to 35% in those given an MOI of 0.1 [Figre 2b]. Compared with 10 cells there was greater survival at 24 hours for all comparable MOIs when infection was established with 100 cells. Survival ranged from 90% to 60% in *Galleria* given MOIs of 100 and 1, respectively.

Kinetics of *P. aeruginosa* infection and effect of phage treatment

To understand the kinetics of a *Pseudomonas* infection within *Galleria*, larvae were infected with 100 cells using the prevention model of infection. Bacteria and phage were quantified at set time points by bleeding the haemolymph. No endogenous *Pseudomonas* or phage with lytic activity against *P. aeruginosa* PA01 were detected in the uninfected controls. For the *Galleria* which were given *P. aeruginosa* PA01 only the numbers of cells isolated from the heamolymph increased over the duration of the experiment. By 24 hours all *Galleria* were dead and numbers of *P. aeruginosa* were in the order of 10^8 c.f.u/ml. The second group of *Galleria* were given a prophylactic dose of phage 2 hours prior to infection and phage and bacteria were then quantified over the course of infection. Numbers of *P. aeruginosa* PA01 were comparable to that of the non-treated Galleria after 8 hours infection, but were three orders of magnitude less cells at 24 hours compared with the non-treated *Galleria*. These *Galleria* were alive at 24 hours. Numbers of phage increased over the duration of the infection reaching a peak titre at 24 hours of 10^8 p.f.u/ml.

Clinical isolates of *P. aeruginosa*

To validate the model of phage therapy with *P. aeruginosa* we sought to test the model with clinical strains isolated from cystic fibrosis patients experiencing acute and chronic episodes of *P. aeruginosa* infections. With the PA45291 acute strain all infected *Galleria* were dead by 24 hours whereas there was 60 % survival at 28 hours in the group which were treated with phage at an MOI 10. When *Galleria* were infected with the BC09007 chronic strain there was little death at 24 hours (90%) when given PBS as treatment, but 100 % survival in the phage treated group. By 40 hours all *Galleria* were then dead.

Discussion

To avoid a scenario whereby society is plunged back into a pre-antibiotic era we need to urgently identify novel anti-bacterial agents. Phage therapy offers a novel non-antibiotic approach to help in this battle. The benefits of phage therapy include no cross resistance from pre-existing antibiotic resistant organisms, high selectivity therefore not wiping out the host microbiota unlike antibiotics as well as being deemed as safe in trials.[9-11]

The *G. mellonella* infection model provides a system that can bridge the gap between *in vitro* studies and more advanced mammalian studies giving initial proof of principle data. Mammalian models are crucial for testing the efficacy of phage prior to human trials, but drawbacks include the need for sufficient infrastructure, substantial costs, as well as the need for ethical approval. *Galleria* larvae have been used to examine numerous host-pathogen interactions ranging from studies of pathogenicity to antimicrobial activity with a small number of these examining the potential for phage therapy.[12-14]

The strain of *P. aeruginosa* PA01 proved to be highly virulent with only 10 cells per *Galleria* required to result in death at 24 hours. This is a very low infective dose in this model with organism such as *S. aureus* requiring 10^5 -10^6 cells/*Galleria* for death, *A. baumannii* requires greater than 10^4 and for *Helicobacter pylori* 10^6-10^7 cells are required for establishment of infection.[15-17] This low infectious dose is of particular interest as it reduces the chances of endotoxin shock due to rapid lysis of high numbers of Gram negative cells.

Two models of therapy were examined. The first was a treatment methodology whereby an acute 2 hour infection was allowed to establish prior to administration of phage. At all MOIs of phage there was prolonged survival of the *Galleria* regardless whether 10 or 100 bacterial cells were used as the inoculum. Although there was increased survival compared with the control there was a difference in

survival depending on the number of cells in the inoculum. Presumably the 10-fold higher inoculum of 100 cells vs 10 cells had meant that the infection had become more established within the two hour time frame therefore reducing the efficacy of the phage to prolong survival.

The second model examined the ability to prevent infection using a prophylactic administration of phage two hours prior to infection. When compared with the treatment model, prophylactic administration of phage resulted in greater survival after 24 hours at all comparable MOI values. Presumably this increased efficacy was the result of phage being able to distribute throughout the haemolymph over the two hour period prior to infection, where as in the treatment model the bacteria will have had opportunity to establish and begin to express toxins. Interesting was the observation of greater survival among *Galleria* which received the higher inoculum of 100 cells, compared with 10 cells. This may have been due to the higher number of bacterial cells resulting in an increased chance of bacteria and phage interaction resulting in a more rapid amplification of the phage.

In both models phage treated *Galleria* eventually succumbed to the infection resulting in death by 30 hours post infection. For this reason we explored the kinetics of both the *P. aeruginosa* infection as well as effect phage had on bacterial numbers *in vivo*. The most striking observation was the comparison between numbers of *P. aeruginosa* in the phage treated and untreated *Galleria*. At 24 hours the phage had kept the number of *P. aeruginosa* to 1000-fold less than the non-treated *Galleria*, but even in the presence of high titres of phage there had still been active growth, and therefore infection, from the *P. aeruginosa* over the duration of the experiment. We had previously hypothesised that the reason for eventual death was the lack of available phage for clearance. From Figure 3 it is clear that this is not the case due to the high titre of phage within the haemolymph, although the MOI had shifted from 100 to less than 1 by 24 hours. This hypothesis was also ruled out by an experiment where *Galleria* were given a second dose of phage four hours after an initial dosing, but there was no difference when compared with the single dose control (data not shown). One

possibility for the continual survival of PA01 in the presence of a high titre of phage was the evolution of phage resistance within the *Galleria*. This was ruled out after observation of no bacterial growth when co-cultivating *P. aeruginosa* single colonies, recovered at 24 hours after phage treatment, and a suspension of phage cocktail (data not shown). The final explanation for the survival would be the intracellular localisation of *Pseudomonas*. In these experiments we only examined bacterial numbers within extracted haemolymph. *Pseudomonas aeruginosa* is known to have the ability to invade epithelial cells which would protect from attack from the phage.[18] This highlights one of the limitations of phage therapy on pathogens which are able to exist and replicate in an intracellular environment. Perhaps combination therapy with antibiotics which can enter host cells such as a fluoroquinolone or tetracycline would have aided in clearance, but this was beyond the scope of this study. This potential intracellular survival strategy would also explain why the prevention model showed improved survival compared with the treatment model where the *P. aeruginosa* will have had time to establish within cells before the *Galleria* received a dose of the phage. Although we hypothesise the lack of *P. aeruginosa* clearance was due to intracellular localisation, there must have been a degree of extracellular replication of cells within the haemolymph to allow for the observed propagation of the phage over time.

Finally we looked to demonstrate the effectiveness of the phage model on clinical isolates of *P. aeruginosa*. To do this the prevention model was repeated with clinical isolates from an acute and a chronic CF infection. Here, the acute isolate resulted in rapid death of the *Galleria* within 24 hours, with 85 % survival when given phage at an MOI of 10. Interestingly the chronic isolate was less virulent at 24 hours compared with the acute and PA01 strains, but 100 % mortality was then seen by 40 hours. In conclusion we present data for the use of the *G. mellonella* as a simple, robust and cost-effective model for initial examination *P. aeruginosa* targeted phage therapy.

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The authors declare that they have no conflicts of interest.

Transparency declarations

None to declare

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Figure legends

Figure 1. Kaplan-Meier survival curves of *G. mellonella* infected with (A) 100 cells or (B) 10 cells of *P. aeruginosa* PA01 and treated with phage at varying multiplicities of infection two hours post-infection. C. Percentage of *G. mellonella* survival at 24 hours.

Figure 2. Kaplan-Meier survival curves of *G. mellonella* infected with (A) 100 cells or (B) 10 cells of *P. aeruginosa* PA01 and pre-treated with phage at varying multiplicities of infection two hours pre-infection. C. Percentage of *G. mellonella* survival at 24 hours.

Figure 3. *In vivo* kinetics of *P. aeruginosa* infection within *G. mellonella* with and without phage treatment.

Figure 4. Kaplan-Meier survival curves of *G. mellonella* infected with 10 cells of (A) acute *P. aeruginosa* PA45291 or (B) chronic *P. aeruginosa* BC09007 and pre-treated with phage at an MOI 10 two hours pre-infection.