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Beeton, ML, Alves, DR, Enright, Mark and Jenkins, ATA (2015) Assessing phage therapy against *Pseudomonas aeruginosa* using a *Galleria mellonella* infection model. *International Journal of Antimicrobial Agents*, 46 (2). pp. 196-200. ISSN 0924-8579

DOI: <https://doi.org/10.1016/j.ijantimicag.2015.04.005>

Publisher: Elsevier

Version: Accepted Version

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

2

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10

11 Keywords. *Pseudomonas aeruginosa*, phage therapy, *Galleria mellonella*, infection model

12

13 Synopsis

14 Objectives

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

17 Methods

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

21 24 hours for quantification of bacteria and phage. Finally, clinical strains from acute and chronic cystic
22 fibrosis infections were used in the prevention model to further validate the model.

23 **Results**

24 Survival rates at 24 hours when infected with 10 cells/larvae were greater with the prevention model
25 versus treatment model (47 vs 40% MOI 10, 47 vs 20 % MOI 1 and 33 vs 7% MOI 0.1). This pattern
26 also held true when infected with 100 cells/larvae (87% vs 20% MOI 10; 53% vs 13 % MOI 1; 67% vs
27 7% MOI 0.1). By 24 hours post infection phage had kept bacterial numbers in the haemolymph to
28 1000-fold lower than the non-treated group. Phage increased survival of *Galleria* when infected with
29 both the acute (0% vs 85%) and chronic (80% vs 100%) clinical *P. aeruginosa* strains.

30 **Conclusions**

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

34

35 **Introduction**

36 Multi-drug resistant bacterial pathogens pose an ever increasing threat to human health. This
37 problem is in part due to a lack of novel antibiotics approved for use over the last few decades resulting
38 in an urgent need to identify new avenues for treating bacterial infections, especially those caused by
39 gram-negative pathogens¹ *Pseudomonas aeruginosa* is an opportunistic pathogen that is a leading
40 cause of infection among burns victim and patients with cystic fibrosis. It is also responsible for a large
41 number of health-care associated infections. To make matters worse, *P. aeruginosa* is associated with
42 hypermutability and due to high antibiotic selective pressure has given rise to the emergence of multi-
43 drug resistant strains in the population and concerns about available effective treatments are
44 growing.^{2, 3} In the UK resistance to two or more antibiotics among *P. aeruginosa* isolated from the

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

48

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

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

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

68

69 **Materials and Methods**

70 **Bacterial strains and preparation of inoculum**

71 Phage therapy was assessed using *P. aeruginosa* PAO1 and two clinical isolates, PA45291 and
72 BC00907, isolated from cystic fibrosis patients with acute and chronic infections, respectively. Bacteria
73 were grown to mid-log phase in LB and washed once in PBS. Cells were resuspended in PBS to give a
74 final cell number of 1×10^8 cfu ml⁻¹ and diluted accordingly in PBS to give the required inoculum size for
75 each experiment.

76

77 **Phage cocktail preparation and titration**

78 Six distinct phages infecting all the *P. aeruginosa* strains mentioned above were combined to establish
79 a cocktail suspension. Phage solutions were propagated on PAO1. Briefly, 100 µl of phage lysate and
80 100 µl of host growing culture were mixed and left for 5 min at room temperature. Following
81 incubation, 3 ml of LB soft-agar containing 0.65% bacteriological agar was added and poured onto
82 agar plates. After an overnight incubation at 37°C, plates displaying confluent lysis were selected and
83 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)
84 chloroform were added before incubation at 37°C for 4h. High-titre phage solution was removed from
85 the plates, centrifuged (8,000 x g, 10 min) to remove cell debris, and then filter sterilized (pore size,
86 0.22 µm). A PEG purification step was further added to remove any possible bacterial remainings from
87 the suspensions and the final solutions were stored at 4°C. All the necessary dilutions were performed
88 in SM buffer. For the titration of the bacteriophage content in the haemolymph, a similar methodology
89 to the propagation was followed. The several dilutions were mixed with the host bacterial cells and 3
90 ml of soft agar was added and poured onto agar plates. After an overnight incubation plaques were
91 counted to determine phage titre.

92

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

94 Larvae of *G. mellonella* were obtained from Livefood UK Ltd (Somerset, UK). Larvae were stored at 4
95 °C and used within 1 week of receipt. A modified methodology developed by Peleg was used to infect
96 each *Galleria*, but in brief.⁸ *Galleria* were surface sterilized with a FASTAID pre-injection swab
97 containing 70 % ethanol. Using a pair of tweezers each *Galleria* was restrained and with a 26 gauge
98 Terumo syringe 10 µl of inoculum containing either 100 or 10 cells of *P. aeruginosa* was delivered into
99 the larval haemolymph behind the last proleg. For the treatment model phage suspension was
100 delivered behind the last proleg on the opposite side to the infection two hours post-infection and for
101 the prevention experiment phage suspension was given two hours pre-infection. All experiments used
102 15 larvae per treatment. A positive control group, where the larvae were infected and treated with
103 PBS solution, and two negative control groups were also included: one group injected with PBS only,
104 assessing the impact of any negative effect from the injection process, and one group injected with
105 phage suspension. Larvae were placed into petri dishes and incubated at 37 °C for 48 hours, being
106 examined regularly and recorded as dead when they did not move in response to touch.

107

108 **Bleeding larvae haemolymph**

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

117 **Statistical analysis**

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

120

121 **Results**

122 **Treatment of infection**

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

132

133 **Prevention of infection**

134 The second model examined the effect of prevention of infection whereby *Galleria* were given a
135 prophylactic dose of phage two hours prior to infection with *P. aeruginosa* PA01. Similarly to the
136 treatment experiment, *Galleria* infected with 100 cells died quicker than those infected with 10 cells
137 when given PBS two hours before infection. [Figure 2a]. At 24 hours survival ranged from 80% in
138 *Galleria* given an MOI of 100 to 35% in those given an MOI of 0.1 [Figure 2b]. Compared with 10 cells
139 there was greater survival at 24 hours for all comparable MOIs when infection was established with
140 100 cells. Survival ranged from 90% to 60% in *Galleria* given MOIs of 100 and 1, respectively.

141

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

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

155

156 Clinical isolates of *P. aeruginosa*

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

164 Discussion

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

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

177

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

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

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

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

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

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

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

236

237 **Acknowledgements**

238 We thank the Engineering and Physical Sciences Research Council (EPSRC) Healthcare Partnership fund
239 for their funding and support.

240 The authors declare that they have no conflicts of interest.

241 **Transparency declarations**

242 None to declare

243

244

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291 **Figure legends**

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

295

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

299

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

302

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

306