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# Treatment of experimental infections of mice with bacteriophages

J. S. SOOTHILL*

*Department of Infection, The Medical School, Edgbaston, Birmingham B15 2TJ*

**Summary.** Bacteriophages for *Acinetobacter baumanii, Pseudomonas aeruginosa* and *Staphylococcus aureus* were tested in experimental infections of mice to investigate their potential for the treatment of infections of man. As few as $10^2$ particles of an acinetobacter phage protected mice against 5 LD50 ($1 \times 10^8$) of a virulent strain of *A. baumanii*, and phage was demonstrated to have multiplied in the mice. A pseudomonas phage protected mice against 5 LD50 of a virulent strain of *P. aeruginosa*, with a PD50 of $1{\cdot}2 \times 10^7$ particles. A staphylococcal phage failed to protect mice infected with a strain of *S. aureus*. These studies support the view that bacteriophages could be useful in the treatment of human infections caused by antibiotic-resistant strains of bacteria.

## Introduction

Bacteriophages were discovered in 1915 by Twort,[1] and d'Herelle.[2] There were early claims of their successful use in the treatment of infection,[3] but well conducted trials[4] failed to substantiate the early claims, and, hastened by the introduction of antibiotics, investigations into their role in therapy were largely abandoned. Uncontrolled studies of phage therapy by Slopek[5] and Abdul-Hassan[6] have included burn patients infected by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. In Slopek's studies the most frequently used phage was $\phi$–131, a staphylococcal phage with a broad host range. Smith[7–9] demonstrated great efficacy of phage in the treatment and prophylaxis of experimental *Escherichia coli* infections of mice and calves. In view of these findings the effect of phage therapy was investigated against three species of bacteria for which antibiotic resistance is frequently a problem, and that commonly colonise burn patients—*P. aeruginosa*, *S. aureus*, and *Acinetobacter baumanii*.

## Materials and methods

### *Bacterial strains*

The *A. baumanii* strain AC54, known to be highly virulent for mice,[10] was obtained from Dr Obana of Kyoto Pharmaceutical University, Japan. *P. aeruginosa* strain 3719 was a clinical isolate. Two *S. aureus* strains were used, 6409 and a more virulent strain M60, obtained from Dr A. J. Bramley, Institute for Animal Health, Compton, England.

### *Phage strains*

Phages active against pseudomonas (BS24) and acinetobacter (BS46) were isolated from sewage by a scaled-up version of the enrichment method of Adams,[11] from 15-L batches of sewage. The staphylococcal phage strain $\phi$-131, and its propagating strain of *S. aureus* 6409, were obtained from Mrs B. Weber-Daçbrowska, Polish Academy of Sciences, Wroclaw. The in-vitro activity of the phages was assessed in shaken broth cultures by a method modified from that of Smith.[8] Flat 100-ml bottles containing 5 ml of broth were pre-warmed to 37°C and then inoculated with $1 \times 10^5$ bacteria and varying doses of phage. A control bottle containing the same inoculum of bacteria but no phage was also set up. The bottles were then incubated in a shaking water bath at 37°C and their turbidity was assessed every 30 min. The minimum dose of phage that caused complete lysis was determined. The activity of BS24 against *P. aeruginosa* 3719 has also been assessed in an in-vitro pig skin model.[12]

Bacteria were counted by spreading 0·1-ml volumes of serial 10-fold dilutions on to plates of Nutrient Agar (Oxoid).

Phage counts were done by the agar-overlay technique described by Adams[11] on plates of nutrient agar with calcium chloride 0·1 %.

### *Preparation of bacteriophage suspensions*

To grow bacteriophage, 10 ml of an overnight broth culture of the appropriate bacterial species (*A. baumanii* AC54, *P. aeruginosa* 3719 or *S. aureus* 6409) and

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* Correspondence should be sent to Dr J. S. Soothill, The Burns Research Group, Birmingham Accident Hospital, Bath Row, Birmingham B15 1NA.

20 ml of a phage-lysed broth culture were added to 5-L flasks each containing 2·5 L of Tryptone Soya Broth (TSB; Oxoid) at 37°C. The cultures were incubated with shaking at 37°C and the absorption at 470 nm was checked at the start of culture and then every 30 min. A control flask to which bacteria but no phage had been added was also incubated. The turbidity of the phage-containing flasks rose initially, but then fell. When the turbidity ceased to fall the flasks of phage-lysed bacterial culture (crude phage suspension) were removed and placed in the refrigerator overnight. The crude phage suspension was filtered with a "tangential flow" 0·2-$\mu$m filter (Sartocon Mini; Sartorius Ltd, Epsom). This removed any remaining bacteria and larger bacterial fragments. The filtrate was then filtered with a custom-made 0·07 $\mu$m filter (in the Sartocon Mini system) and the retentate (which contained the bulk of the phage particles) was washed twice with saline. The retentate was then diluted with 1 L of Dulbecco's modification of Eagle's Medium (DMEM) and reduced to 100 ml of retentate. This was diluted with 1 L of DMEM and then reduced to 100 ml. The purpose of the 0·07 $\mu$m filtration-redilution stage was to wash away toxins, small bacterial fragments and TSB etc.

To prepare a phage-free control, similar cultures were made, but no phage was added and they were incubated for a shorter period—the same time that it had taken the turbidity of the corresponding phage culture to start to fall. After culture the bacterial suspensions were centrifuged, the supernate was removed and the bacteria, for *P. aeruginosa* and *A. baumanii*, were lysed by passing them through a French press. *S. aureus* is not lysed in the French press, and was therefore incubated with lysostaphin (Sigma) 0·1 mg/ml for 1 h. The lysed bacteria were added to the supernate and the resulting mixture was processed in the same way as the phage suspension to give 100 ml of control suspension.

### *Preparation of bacterial inocula*

Stocks of bacteria were grown at 37°C for 5 h in shaken 5-L flasks containing 2·5 L of TSB. After growth, the bacterial suspension was centrifuged and the precipitate was then resuspended in saline. This was repeated once after the precipitate was resuspended in saline containing glycerol 15%. The resulting suspension was mixed, divided into aliquots and stored at $-70°$ until required.

Adult CBA outbred mice weighing 20–23 g were used, males for the pseudomonas and acinetobacter studies, and because of differences in availability, females for staphylococcal studies. Bacteria, phage and control suspensions (0·25 ml) were injected into the peritoneal cavity. Simultaneous injection of bacteria and phage was done with a specially made holder which held two syringes. During LD50 estimations, the mice were housed two or three/cage; all animals in each cage received the same inoculum. During the phage protection studies, mice were caged singly. All cages were coded.

Doses of bacteria to be used in the protection studies were established by LD50 measurements. Mice were given injections of four inocula of bacteria, with five mice/inoculum level. Mice were killed when it was considered that they were terminally ill (reduced mobility, partially-closed eyes, abnormal posture and an altered breathing pattern).

The toxicity of phage and control suspensions for mice was investigated by injecting 0·25 ml of the suspension into each of a group of three mice. Three uninjected mice were retained as normal controls. The mice were observed for signs of illness, and temperatures were taken hourly during the first 5 h after injection and then daily during the next 4 days.

The protective effect of each strain of phage was assessed in a group of mice, each of which had been given the same lethal dose of bacteria into the peritoneal cavity. To minimise the suffering of mice in survival studies, an earlier endpoint than that used previously during the LD50 studies was established, based on a fall in colonic temperature.[13] A fall below 34°C was found to predict terminal illness accurately, and was used as an endpoint in protection studies.

In the acinetobacter phage protection study, five groups of five mice were given 5 LD50 of *A. baumanii* AC54 by injection; four of the groups also received 0·25 ml of phage BS46 in three-fold dilution steps (the highest containing $10^8$ pfu); the remaining group received 0·25 ml of control suspension. As the efficacy of this phage was unknown, pilot studies to determine the doses of phage were needed, before doing the definitive study. In the first study, six groups of two mice received 8 LD50 of strain AC54, five of the groups also receiving 0·25 ml of phage, the doses decreasing in five-fold dilution steps, the highest dose being $8{\cdot}3 \times 10^6$ pfu; the remaining group received 0·25 ml of control suspension. In the second pilot study, five mice were used; each received 3 LD50 of bacteria, four of the mice also receiving 0·25 ml of phage, the doses decreasing in 10-fold steps (one mouse/dose), the highest being 1200 pfu; the remaining mouse received 0·25 ml of control suspension.

Two pseudomonas phage mouse-protection studies were done. In the first study, 10 LD50 ($1{\cdot}5 \times 10^8$ cfu) of *P. aeruginosa* 3719 were injected into 20 mice, with 10-fold dose steps of phage for each of the four groups of five mice, the highest being $10^8$ pfu. In the second study, the lower dose of *P. aeruginosa* 3719 of $8 \times 10^7$ ($5 \times$ LD50) and four three-fold dose steps of phage from $2 \times 10^7$ pfu were used.

Staphylococcal protection studies were done with phage $\phi$-131 and two strains of *S. aureus* (strains 6409 and M60). In the study with strain 6409, two groups of five mice were used. Each mouse received $1{\cdot}7 \times 10^{10}$ cfu (6 LD50) of strain 6409 in 0·25 ml injected into the peritoneal cavity, and either $3 \times 10^8$ pfu of phage $\phi$-131 (the maximum available dose) in 0·25 ml, or 0·25 ml of control suspension. In the study with strain M60, 14

mice were used, each receiving an intraperitoneal dose of 9 LD50 ($4{\cdot}3 \times 10^9$ cfu) of strain M60. Seven of these mice also received the maximal available dose of $3{\cdot}7 \times 10^8$ pfu in 0·25 ml DMEM; the remaining seven (controls) received 0·25 ml of control suspension.

For counts of phage and bacteria in the tissues, each mouse was dissected, the bones and skin were discarded, and the remaining tissues were homogenised in an Ultra-Turrax T25 homogeniser (Northern Media, Nottingham).

Estimation of LD50 and PD50 was done by fitting the experimental data to a probit curve.[14] Significance testing was by Fisher's exact test (two-tailed).

## Results

Only 60 pfu of phage BS24 and 340 pfu of phage BS46 were required to cause complete clearing of the standard broth cultures, demonstrating the high activity of these phages *in vitro*. Phage $\phi$-131 was less active, $10^6$ pfu being required to cause complete clearing of the strain M60 broth culture, but $10^5$ pfu caused almost complete clearing.

The LD50s for CBA mice of the four strains of bacteria were, $1{\cdot}9 \times 10^7$ cfu for *A. baumanii* strain AC54, and $1{\cdot}5 \times 10^7$ cfu for *P. aeruginosa* strain G17, $2{\cdot}6 \times 10^9$ and $4{\cdot}8 \times 10^8$ cfu for *S. aureus* strains 6409 and M60 respectively. No toxicity of the phage and control suspensions was detected, i.e., there were no signs of illness and the temperatures of the mice did not differ significantly from those of controls.

Both pilot studies of phage BS46 in *A. baumanii*-infected CBA mice showed clear protection (table I) and indicated an effective dose of *c.* one phage particle for $10^6$ bacteria. In the definitive study this dose (the highest dose used) protected the mice significantly as compared with the untreated controls ($p = 0{\cdot}046$). Since the lower doses did not confer protection, the PD50 (i.e., the dose which protects 50% of the animals) cannot be calculated precisely from these data, but it is very low, within the range 36–108 pfu. The much higher maximum doses of pseudomonas phage BS24 (table II) significantly protected mice against 10 LD50 ($p = 0{\cdot}0079$) and 5 LD50 ($p = 0{\cdot}046$) of *P. aeruginosa* (when survival in each of the groups receiving maximum doses are compared with that in the equivalent groups receiving lowest doses). For the 5 LD50 dose the PD50 of BS24 was calculable; it was $1{\cdot}2 \times 10^7$ pfu.

In contrast, phage $\phi$-131, which is poorly lytic *in vitro*, failed to protect mice from the lethal effects of two *S. aureus* strains (M60 and 6409) at the maximum doses available.

In all groups, the visual assessment of the well-being of the mice precisely paralleled temperature measurement.

One hundred thousand times as many phages for *A. baumanii* were grown from the tissues of the animals which survived, than were injected into them (table III), supporting the view that the phage was infecting the bacteria *in vivo*, and providing a basis for the remarkable effectiveness of the very small dose given. In the three mice in which intestinal tract and other tissues were studied separately, there were similar amounts in the two compartments. In all mice the plaques produced on lawns of AC54 had the characteristic "halo" of phage BS46 (figure). No *A. baumanii* were isolated from any of the surviving mice.

**Table I.** Numbers of survivors in three studies of groups of mice given three doses of *A. baumanii* and varying doses of phage

| Study I, 8 LD50 (Two mice/dose) | | Study II, 3 LD50 (One mouse/dose) | | Study III, 5 LD50 (Five mice/dose) | |
|---|---|---|---|---|---|
| Phage dose (pfu) | Number of survivors | Phage dose (pfu) | Number of survivors | Phage dose (pfu) | Number of survivors |
| $8{\cdot}3 \times 10^6$ | 2 | 1200 | 1 | 108 | 4 |
| $1{\cdot}7 \times 10^6$ | 2 | 120 | 1 | 36 | 0 |
| $3{\cdot}3 \times 10^5$ | 2 | 12 | 1 | 12 | 0 |
| $6{\cdot}6 \times 10^4$ | 2 | 1 | 0 | 4 | 0 |
| $1{\cdot}3 \times 10^4$ | 2 | None | 0 | None | 0 |
| None | 0 | ... | ... | ... | ... |

3 LD50 = $5{\cdot}6 \times 10^7$ cfu; 5 LD50 = $9{\cdot}5 \times 10^7$ cfu; 8 LD50 = $1{\cdot}5 \times 10^8$ cfu.

**Table II.** Numbers of survivors in two studies of groups of five mice given two doses of *P. aeruginosa* and varying doses of phage

| Study I, 10 LD50 | | Study II, 5 LD50 | |
|---|---|---|---|
| Phage dose (pfu) | Number of survivors | Phage dose (pfu) | Number of survivors |
| $2{\cdot}9 \times 10^8$ | 5 | $1{\cdot}8 \times 10^7$ | 4 |
| $2{\cdot}9 \times 10^7$ | 1 | $6{\cdot}0 \times 10^6$ | 0 |
| $5{\cdot}8 \times 10^6$ | 0 | $2{\cdot}0 \times 10^6$ | 1 |
| $2{\cdot}9 \times 10^5$ | 0 | $6{\cdot}7 \times 10^5$ | 0 |
| PD50 | $> 2{\cdot}9 \times 10^7$ $< 2{\cdot}9 \times 10^8$ | PD50 $1{\cdot}2 \times 10^7$ | |

5 LD50 = $8 \times 10^7$ cfu; 10 LD50 = $1{\cdot}5\ 10^8$ cfu.

**Table III.** Total tissue counts of phage in mice surviving 7 days after being given $9{\cdot}7 \times 10^7$ cfu of *A. baumanii* AC54 and $10^8$ pfu of phage BS46

| Mouse no. | Total count (pfu) in | | |
|---|---|---|---|
| | Tissues* | GI Tract | Total |
| 1 | ND | ND | $5{\cdot}5 \times 10^5$ |
| 2 | $7{\cdot}2 \times 10^7$ | $2{\cdot}8 \times 10^7$ | $1{\cdot}0 \times 10^8$ |
| 3 | $3{\cdot}2 \times 10^5$ | $4{\cdot}3 \times 10^5$ | $7{\cdot}5 \times 10^5$ |
| 4 | $2{\cdot}4 \times 10^5$ | $8{\cdot}2 \times 10^4$ | $3{\cdot}2 \times 10^5$ |
| Mean | ... | ... | $2{\cdot}5 \times 10^7$ |

* Excluding gastrointestinal tract.
ND, not done.

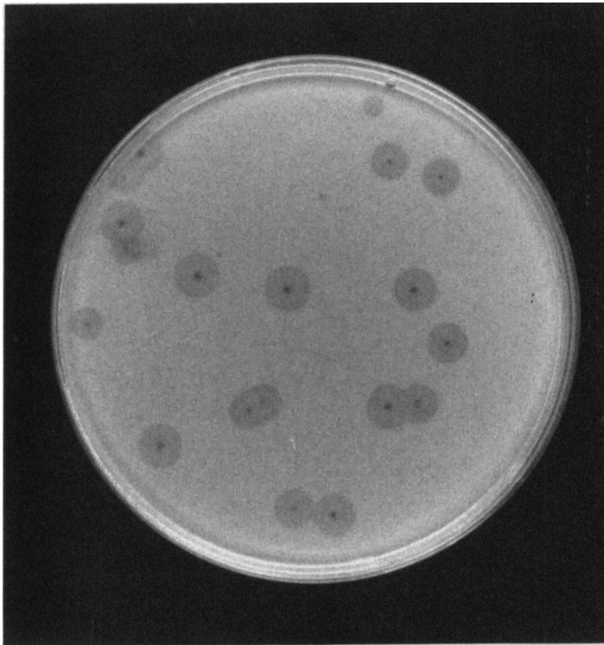

**Figure.** Plaques of phage BS46 grown on *A. baumanii* AC54.

## Discussion

Highly lytic bacteriophages protect mice against susceptible strains of two bacterial species, *A. baumanii* and *P. aeruginosa*, given in doses which are fatal to them. The acinetobacter phage, which was very active *in vitro*, was protective *in vivo* at the remarkably low dose ratio of 1 pfu to $10^6$ bacterial cfu. The numbers of phages detected in the tissues of the surviving animals, far in excess of those given, indicate replication *in vivo* during the bacterial killing process.

Early studies of such protection[15] have shown only small effects with very large doses, but Smith[7–9] showed similarly effective protection against *E. coli* in mice, and also protection in calves, piglets and lambs. Replication of phages *in vivo* could be invaluable in the treatment of infections where the blood supply is poor and in infections of orthopaedic implants, since the concentration of phage could rise rapidly at the site of infection. Failure to demonstrate protection against staphylococci in this study, despite reported clinical success with this phage,[5] may have been due to the poor lytic properties of the phage, selected because it exhibited a broad host (it kills 70% of Birmingham *S. aureus* isolates). The LD50s for both strains of *S. aureus* were high, making protection by any antibacterial agent less likely because the dose of toxin present in the inoculum might have been fatal *per se*, and the highest phage: bacteria ratio obtainable was 1:12; the pseudomonas phage was effective only when given in a ratio of *c*. 1:4. A model with a lower infecting dose of bacteria would be preferable for studying staphylococcal infection.

Provided that mixtures of highly lytic phages covering the required range of bacterial strains can be produced, phage may well be useful for the treatment of clinical infections, especially those caused by antibiotic-resistant organisms.

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