Case Report

Bacteriophage therapy for refractory *Pseudomonas aeruginosa* urinary tract infection

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We describe the success of adjunctive bacteriophage therapy for refractory *Pseudomonas aeruginosa* urinary tract infection in the context of bilateral ureteric stents and bladder ulceration, after repeated failure of antibiotics alone. No bacteriophage-resistant bacteria arose, and the kinetics of bacteriophage and bacteria in urine suggest self-sustaining and self-limiting infection.

Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen associated with biofilm infections refractory to antibiotic therapy, particularly in cystic fibrosis and burn wounds and in other immunocompromised hosts (Azeredo & Sutherland, 2008; Fothergill *et al.*, 2010). Lytic bacteriophage has long been known to have the potential to deliver effective targeted therapy (Himmelweit, 1945), including for *P. aeruginosa*, and increasing antibiotic resistance has sparked renewed interest in this approach (Azeredo & Sutherland, 2008; Kutter *et al.*, 2010; Harper & Enright, 2011).

Case report

A 67-year-old woman underwent extensive intra-abdominal resections and pelvic irradiation for adenocarcinoma followed by bilateral ureteric stent placement to relieve obstruction. This was complicated by symptomatic *P. aeruginosa* infection, for which she received multiple courses of gentamicin, ceftazidime, ciprofloxacin and meropenem over a 2 year period, and two stent replacements. Symptomatic relapse with *P. aeruginosa* susceptible to ciprofloxacin (MIC 0.25 µg ml⁻¹), gentamicin (2 µg ml⁻¹), ceftazidime (4 µg ml⁻¹) and meropenem (MIC 2 µg ml⁻¹; determined by Etest performed according to

manufacturer's instructions; Etest, AB bioMérieux) occurred usually within a week after ceasing antibiotics, always at >10⁵ c.f.u. ml⁻¹ and in association with pyuria (>100 white blood cells ml⁻¹) and haematuria.

Several isolates of the infecting *P. aeruginosa* strain were screened against existing bacteriophage libraries at the Eliava Institute in Tbilisi (Democratic Republic of Georgia, DPR) which have been used extensively to treat patients (Kutter *et al.*, 2010; Stone, 2002). Six lytic bacteriophages, each at a titre of 10⁶ p.f.u. ml⁻¹ against the target *P. aeruginosa*, were combined into a filter-sterilized bacteriophage product (Pyophage #051007) licensed under DPR Ministry of Health guidelines (Stone, 2002; Sulakvelidze *et al.*, 2001). Routine bacterial culture on chocolate agar (Difco) at 37 °C of a sample vial was again negative in the Centre for Infectious Diseases and Microbiology Laboratory Services laboratory.

Ethical approval was obtained from the Western Sydney Human Research Ethics Committee on a compassionate use basis, and informed consent was obtained from the patient. The patient was hospitalized and 20 ml (~2 × 10⁷ p.f.u.) Pyophage #051007 was instilled directly into the bladder every 12 h for 10 days, with the catheter clamped for 30 min after each instillation. Urine was collected before treatment and at 4 h intervals after the morning dose for the first 2 days, and then each morning prior to bacteriophage and antibiotic treatment. The catheter was changed on days 3 and 10. Meropenem (1 g twice daily) and colistin (polymixin E, 100 mg twice daily) commenced

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Abbreviation: MT-PCR, multiplexed tandem PCR.

on day six. Colistin was used for a total of 5 days (days 6–10) and the patient was discharged home on intravenous meropenem alone after day 10, to complete a total meropenem course of 30 days.

Both stents were removed for a third time and the right ureteric stent was reinserted while still on meropenem (day 20). Cystoscopy at that time revealed an inflamed bladder with bilateral lower ureteric strictures and an ulcerated bladder base. Midstream urine sampled every 8 weeks remained sterile for 6 months after completion of antibacterial treatment, during which time the patient remained asymptomatic. Intermittent haematuria continued but no further antibiotic therapy was used. Urine cultures were negative when the patient was admitted with recurrent intra-abdominal malignancy a year later.

Microbiological studies

For quantifying bacteriophage in urine, samples were diluted 1:1 in 0.9% (w/v) PBS, filtered through a 0.2 µm filter and evaluated in an overlay assay with the relevant *P. aeruginosa* strain. All assays were performed in triplicate. Serial dilutions of urine were also incubated aerobically on blood agar (Oxoid) at 37 °C for bacterial colony counts. All *P. aeruginosa* and *Enterococcus faecalis* isolates were tested against the bacteriophage cocktail: 100 µl ~10⁶ c.f.u. exponential phase organisms ml⁻¹ in 3 ml 0.4% nutrient agar (NA; Difco) at

50 °C was overlaid onto 0.8% NA and allowed to solidify at 25 °C for 20 min; 5 µl bacteriophage serially diluted in modified Tris buffer [5 ml 2% (w/v) gelatin in 50 ml 1.0 M Tris/HCl (pH 7.0) with 5.8 g NaCl l⁻¹, 2.0 g MgSO₄·7H₂O l⁻¹] was adsorbed into the overlay. Plaques were counted after 18 h aerobic incubation at 37 °C. To establish the frequency of resistance to bacteriophages after the therapeutic intervention, the bacteriophage preparation was applied as above in 10-fold dilutions (neat to 1:10⁴) onto bacterial lawns prepared from phage-treated samples days 1, 3 and 7. Bacteriophage was enumerated in urine by dilution as above. All assays were performed in triplicate.

Two hundred microlitre aliquots of urine were also extracted (GenElute Mammalian DNA Extraction kit; Sigma-Aldrich) for bacterial DNA load estimation, as were control strains (suspended at ~10³ c.f.u. ml⁻¹ in sterile 0.9% (w/v, NaCl). The 16S rDNA of *Enterobacteriaceae*, *Pseudomonas* species, *Enterococcus* species, *Streptococcus* species and *Staphylococcus* species was quantified by multiplexed tandem PCR (MT-PCR) (Bacterial Count; AusDiagnostics), essentially as previously described (Szewczuk *et al.*, 2010), in a RotorGene RG6000 cyclor (Corbett Research). Limits of detection were determined as the means of three different experiments using sterile urine spiked with *P. aeruginosa* ATCC 27853 (183 c.f.u. ml⁻¹; 1.5 c.f.u. per reaction), *Escherichia coli* ATCC 25922 (3733 c.f.u. ml⁻¹; 30 c.f.u. per reaction), *E. faecalis* ATCC 29212 (1719 c.f.u. ml⁻¹; 14 c.f.u. per reaction),

Table 1. *P. aeruginosa* viable count (c.f.u. ml⁻¹, by serial dilution and culture), DNA load of *Enterococcus* and *Pseudomonas* species [by MT-PCR, both expressed in c.f.u. (ml urine)⁻¹] and bacteriophage titre against original *P. aeruginosa* isolate [approx. p.f.u. (ml urine)⁻¹]

Antibiotics were present from day 6 after pre-dose sampling (bold).

	Bacterial viable count (c.f.u. ml ⁻¹)*	Bacterial DNA load (c.f.u. ml ⁻¹)		Phage titre (p.f.u. ml ⁻¹)
		<i>Enterococcus</i> c.f.u. (mean ± sd)	<i>Pseudomonas</i> c.f.u. (mean ± sd)	
Day 1 (start)	3.00 × 10 ⁶	3.10 × 10 ⁷ ± 1.45 × 10 ⁷	4.27 × 10 ⁵ ± 1.42 × 10 ⁵	0
Day 1 (+4 h)	1.60 × 10 ⁵	5.41 × 10 ⁶ ± 9.18 × 10 ⁵	4.92 × 10 ⁴ ± 1.78 × 10 ⁴	3.09 × 10 ³
Day 1 (+8 h)	2.40 × 10 ⁵	8.59 × 10 ⁶ ± 7.87 × 10 ⁵	5.70 × 10 ⁴ ± 1.03 × 10 ⁴	4.30 × 10 ³
Day 1 (+12 h)	(–)	8.59 × 10 ⁵ ± 1.07 × 10 ⁵	1.53 × 10 ⁴ ± 2.27 × 10 ³	5.30 × 10 ⁴
Day 2 (pre-)	3.00 × 10 ⁵	3.85 × 10 ⁶ ± 4.44 × 10 ⁴	8.37 × 10 ⁴ ± 2.20 × 10 ⁴	1.60 × 10 ⁶
Day 2 (+4 h)	1.12 × 10 ⁵	9.43 × 10 ⁶ ± 9.54 × 10 ⁶	3.58 × 10 ⁵ ± 1.68 × 10 ⁵	1.40 × 10 ⁶
Day 2 (+8 h)	3.00 × 10 ⁵	2.91 × 10 ⁶ ± 1.61 × 10 ³	3.03 × 10 ⁴ ± 4.43 × 10 ⁴	9.30 × 10 ⁵
Day 2 (+12 h)	3.00 × 10 ⁵	1.31 × 10 ⁶ ± 3.99 × 10 ⁵	7.55 × 10 ⁴ ± 5.81 × 10 ³	9.30 × 10 ⁵
Day 3 (pre-)	3.00 × 10 ⁵	3.77 × 10 ⁶ ± 1.57 × 10 ⁶	3.87 × 10 ⁴ ± 6.17 × 10 ⁴	2.30 × 10 ⁴
Day 4 (pre-)	(–)	2.82 × 10 ⁶ ± 7.55 × 10 ⁵	2.66 × 10 ⁴ ± 8.14 × 10 ²	3.70 × 10 ⁵
Day 5 (pre-)	3.00 × 10 ⁵	1.38 × 10 ⁷ ± 3.12 × 10 ⁵	2.25 × 10 ⁵ ± 3.02 × 10 ⁴	9.30 × 10 ⁵
Day 6 (pre-)	6.40 × 10 ⁴	2.69 × 10 ⁶ ± 2.85 × 10 ⁵	1.94 × 10 ⁵ ± 1.48 × 10 ⁵	3.70 × 10 ⁵
Day 7 (pre-)	6.30 × 10 ³	1.70 × 10 ⁷ ± 1.48 × 10 ⁶	6.75 × 10 ⁴ ± 3.35 × 10 ⁴	4.00 × 10 ⁵
Day 8 (pre-)	0	1.93 × 10 ⁷ ± 3.12 × 10 ⁶	8.39 × 10 ⁴ ± 4.29 × 10 ⁴	2.60 × 10 ⁴
Day 9 (pre-)	0	1.58 × 10 ⁷ ± 1.08 × 10 ⁶	2.57 × 10 ³ ± 6.73 × 10 ²	1.00 × 10 ³
Day 10 (pre-)	0	9.81 × 10 ⁶ ± 8.57 × 10 ⁵	1.43 × 10 ³ ± 5.64 × 10 ²	3
Day 21 (pre-)	0	6.66 × 10 ³ ± 1.74 × 10 ³	≤ 10	0

* (–), Not tested.

Streptococcus pneumoniae ATCC 49615 ($2022 \text{ c.f.u. ml}^{-1}$; 16 c.f.u. per reaction) and *Staphylococcus aureus* ATCC 25923 ($2718 \text{ c.f.u. ml}^{-1}$; 22 c.f.u. per reaction). Melt temperatures were consistent in triplicate runs and reproducibility was high (standard curves for all targets r^2 0.9922–0.9997; not shown).

Bacteriophage counts remained high until after disappearance of the target organism and then diminished sharply (Table 1). Quantitative PCR data indicated that bacterial DNA persisted beyond the point of culture-positivity, perhaps reflecting the release of dead organisms from biofilms, although experiments were not done to formally evaluate this. Initial viable *P. aeruginosa* counts were $>3 \times 10^6 \text{ c.f.u. ml}^{-1}$ (Table 1). Viable bacterial counts declined before antibiotics commenced on day 6 (logarithmic graph, Fig. 1). DNA fingerprinting by enterobacterial repetitive intergenic consensus-PCR (Versalovic *et al.*, 1991) showed that all *P. aeruginosa* isolates were identical, and all were susceptible to colistin and meropenem and to the bacteriophage cocktail in the same titre. Bacteriophage counts in urine rose from $3 \times 10^3 \text{ p.f.u. ml}^{-1}$, 4 h after the initial dose, to $\sim 5 \times 10^4 \text{ p.f.u. ml}^{-1}$ after 12 h and to $>10^6 \text{ p.f.u. ml}^{-1}$ 20 h later before the first dose on day 2. Bacteriophage levels remained at at least $\sim 10^5 \text{ p.f.u. ml}^{-1}$ for a week but then fell sharply. Small amounts of bacteriophage were still detectable ($\sim 10^3 \text{ p.f.u. ml}^{-1}$) immediately prior to bacteriophage administration on day 9, and none thereafter (Table 1). Cultures and MT-PCR detected both *Pseudomonas* and *Enterococcus* species in urine. *E. faecalis*, susceptible to ampicillin but not to bacteriophage, was cultured and *Enterococcus* species were enumerated by MT-PCR at $\sim 10^{6-7}$ from admission until day 9, and then fell to $\sim 10^3 \text{ c.f.u. ml}^{-1}$ on discharge from hospital. *P. aeruginosa* was culturable at $>10^5 \text{ c.f.u. ml}^{-1}$ to day 6 and was quantified by MT-PCR at $1.9 \times 10^5 \text{ c.f.u. ml}^{-1}$ (Table 1), and then in reduced numbers up to day 7 ($6.75 \times 10^4 \text{ viable c.f.u. ml}^{-1}$; 10-fold higher DNA load equivalent), but no antibiotic- or bacteriophage-resistant *P. aeruginosa* was recovered at any stage. *P. aeruginosa* DNA could still be detected by MT-PCR up to day 10 (DNA equivalent to $\sim 10^3 \text{ c.f.u. ml}^{-1}$), but not thereafter (Table 1).

Discussion

Combined therapy was well-tolerated, apparently resulting in symptomatic relief and microbiological cure where repeated courses of antibiotics combined with stent removal had failed. Previous reports indicate that bacteriophage therapy is a safe and potentially effective antipseudomonal treatment (Marza *et al.*, 2006; Rhoads *et al.*, 2009; Wright *et al.*, 2009), although data about the kinetics of bacteriophage infection are scant. Bacteriophage did not persist nor was any antibiotic- or bacteriophage-resistant *P. aeruginosa* identified. The additive effort of combined phage-antibiotic treatment has been known since the beginning of the antibiotic era (Himmelweit,

Fig. 1. Logarithmic plot of early morning urine viable *P. aeruginosa* (c.f.u. ml⁻¹) and bacteriophage (p.f.u. ml⁻¹) counts. Antibiotic administration and catheter change and removal (days 3 and 10, respectively; boxed) are indicated.

1945), but optimal dosing and duration of therapy remain unknowns. The nearly 2 log fall in *P. aeruginosa* concentration in the urine which appeared to precede the administration of antibiotics strongly suggests an action *in vivo* that mimics that observed *in vitro*. The data indicate that bacteriophage infection was self-sustaining and self-limiting, with the phage decreasing in number along with the viable target organisms in which they replicated.

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