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The varying effects of a range of preservatives on *Myoviridae* and *Siphoviridae* bacteriophages formulated in a semi-solid cream preparation.

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SIGNIFICANCE AND IMPACT OF THE STUDY

Bacteriophages are being increasingly investigated as alternatives to antibiotics. While bacteriophages can be formulated in diverse ways for therapeutic delivery, there has been scant work on how excipients and preservatives in these formulations affect stability of different bacteriophages. We demonstrate that the nature of preservatives in formulations will affect

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bacteriophage stability, and that in these formulations, viability of bacteriophage differs according to their morphology. Our work highlights the need for individual testing of specific bacteriophages in pharmaceutical formulations, as efficacy when exposed to preservatives and excipients in these delivery forms may vary.

ABSTRACT

Bacteriophages may be formulated into semi-solid bases for therapeutic delivery. This work investigated the effects of a range of preservatives on the viability of *Myoviridae* and *Siphoviridae* bacteriophages when these were formulated into a standard semi-solid cream base. The six preservatives tested included: benzoic acid (0.1%), chlorocresol (0.1%), combination hydroxybenzoates (propyl 4-hydroxybenzoates with methyl 4-hydroxybenzoates) (0.1%), methyl 4-hydroxybenzoate (0.08%), 2-phenoxyethanol (1%), and propyl 4-hydroxybenzoate (0.02%). These were each formulated into cetomacrogol cream aqueous to generate six individual semi-solid bases into which *Myoviridae* and *Siphoviridae* bacteriophages were added and tested for stability. Optimal bacteriophage stability was seen when the preservative chlorocresol was used. Bacteriophage in the acidic benzoic acid were the least stable, resulting in complete loss of viability after four to five weeks. Of the bacteriophages tested, the *Myoviridae* KOX1 was significantly more stable than the *Siphoviridae* PAC1 after 91 days in formulations with each of the preservatives. Our results suggest the need for individual testing of specific bacteriophages in pharmaceutical formulations, as their efficacy when exposed to preservatives and excipients in these delivery forms may vary.

Key words

Bacteriophage, cetomacrogol cream aqueous, preservatives, stability, *Myoviridae*, *Siphoviridae*

INTRODUCTION

Bacteriophages are uniquely specific viruses capable of killing pathogenic bacteria. Their potential for use in clinical applications has been assessed in a variety of disease models (Rhoads *et al.*, 2009, Wright *et al.*, 2009, Bodier-Montagutelli *et al.*, 2017, Fish *et al.*, 2018, Nir-Paz *et al.*, 2019, Jault *et al.*, 2018, Dedrick *et al.*, 2019). In topical applications, they can be tested against a range of bacterial infections of the epidermis and epithelia. To achieve this, the bacteriophages are formulated in aqueous solutions such as lotions, drops and sprays (Chen *et al.*, 2013, Rose *et al.*, 2014); in viscous preparations such as hydrophilic gels (Curtin and Donlan, 2006, Chen *et al.*, 2013); or semi-solid preparations such as creams, ointments and pastes (O'Flaherty *et al.*, 2005, Brown *et al.*, 2017b). The efficacy of formulation, stability and release of bacteriophages from semi-solid preparations has been shown (Brown *et al.*, 2017b). Thermostability and photodegradation are important issues, as optimal stability is seen when

bacteriophage formulations are stored at four degrees Celsius in light protected containers (Brown *et al.*, 2016, Brown *et al.*, 2017b).

The ionic nature of semi-solid bases will affect release of medicaments from formulations. Bacteriophages appear to be similarly affected. It has been suggested that the overall charge of bacteriophages is negative from acidic (pH 3.6) to neutral (pH 7.6) ranges (Todd, 1927), and that phage capsids possess a negative charge while tail fibres are positively charged (Serwer and Pichler, 1978, Serwer and Hayes, 1982). Ionic polymers within some cream bases have the potential to interact with bacteriophages through electrostatic forces, and in experimental conditions, differential release of bacteriophages from creams of varying ionic nature was seen, with optimal release from non-ionic formulations (Brown *et al.*, 2017b). Therefore, in clinical applications, the ionic nature of the cream may be important in allowing “release” of the bacteriophages to access the bacteria in underlying tissue upon which it is spread. Semi-solid formulations which are thicker than creams, such as ointments and pastes, may not be as optimal for the delivery of bacteriophages for therapy. Some components of pastes, such as starch, appear to inhibit release of bacteriophage, possibly because of the thickness it imparts, and the potential for starch macromolecules to entangle structures such as bacteriophage tails (Brown *et al.*, 2017b). Another issue is that some ointments, for instance, emulsifying ointment, contain sodium lauryl sulphate, a surfactant capable of denaturing protein and which has been shown to act as a virucide (Piret *et al.*, 2002).

Yet despite these analyses, there has been little research surrounding how other components of these semi-solid formulations, for instance, preservatives, may inhibit or allow bacteriophage release. In one study, commercial burn wound care products were combined with *Myoviridae* and *Podoviridae* bacteriophages (lytic for *A. baumannii*, *P. aeruginosa* and *S. aureus*), which were then assessed for stability and viability following 24 hours storage at 37 degrees Celsius (Merabishvili *et al.*, 2017). These commercial products included creams, gels, suspensions and ointments, and assays involved the dilution of these products 1:1 with bacteriophage suspensions. Therefore, the study did not investigate the capacity of intact semi-solid preparations to deliver viable bacteriophages, but did demonstrate that some of the antibacterial agents, in particular, acidic compounds, had an adverse effect on bacteriophages (Merabishvili *et al.*, 2017). In another study, when the anti-bacterial agent sodium benzoate was added to *Escherichia coli* bacteriophages in suspension, it inhibited activity at concentrations similar to those at which it is employed as a preservative in a range of semi-solid formulations (Subils *et al.*, 2012).

In this study, we aimed to investigate the functional effects of a range of preservatives commonly used in semi-solid formulations on the viability of *Myoviridae* and *Siphoviridae* bacteriophages. To negate the potential effects of ionic and physical interaction with the bacteriophage, we used

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as a semi-solid base the hydrophilic non-ionic cetomacrogol cream aqueous, which we have previously shown to be efficacious in releasing bacteriophages (Brown *et al.*, 2016). The morphologically distinct bacteriophages employed were the *Siphoviridae* *Cutibacterium* (formerly *Propionibacterium*) *acnes* bacteriophage PAC1 (Brown *et al.*, 2016) and the *Myoviridae* *Klebsiella oxytoca* bacteriophage KOX1 (Brown *et al.*, 2017a). The effects of the preservatives on these bacteriophages were assessed regularly over a period of 91 days.

RESULTS & DISCUSSION

PAC1 *Siphoviridae* bacteriophage and KOX1 *Myoviridae* bacteriophage were each tested in six cetomacrogol cream aqueous formulations, which differed according to the preservative added: benzoic acid (0.1%), chlorocresol (0.1%), combination hydroxybenzoates (propyl 4-hydroxybenzoates with methyl 4-hydroxybenzoates) (0.1%), methyl 4-hydroxybenzoate (0.08%), 2-phenoxyethanol (1%), and propyl 4-hydroxybenzoate (0.02%). The stability of the bacteriophage in these formulations was tested weekly by determining viable numbers [Plaque Forming Units per gram (PFU g⁻¹)]. PAC1 was formulated at an initial concentration of 1.5 x 10⁸ PFU g⁻¹ and KOX1 formulated at 5.5 x 10⁸ PFU g⁻¹. Both KOX1 and PAC1 bacteriophages showed monotonic decreases over time as shown in Figures 1 and 2, where each data point reflects the standard error of the mean bacteriophage concentration for the three samples tested at that time. For KOX1 bacteriophage, unit root was detected in all preservatives except benzoic acid; whereas for PAC1 bacteriophage it was detected in all except benzoic acid and combination hydroxybenzoates. In all preservatives where time series was apparent, the Mackinnon approximate p value was > 0.05. The pairwise comparisons of percent change, comparing baseline with day 91 by preservative used for each of the bacteriophages, are presented in Table 1. Statistical differences between the preservatives were more prominently observed for KOX1 than PAC1 (Table 1). Comparing baseline with day 91, survival was significantly higher for KOX1 than for PAC1, and this was observed across all preservatives except benzoic acid in which survival was 0% in both (Table 2).

While there have been assessments of the viability of bacteriophages in suspensions of commercial topical applications, the variation in the composition and additives in these preparations makes it difficult to specify which component may be causing bacteriophage inactivation (Merabishvili *et al.*, 2017). The study we present here is the first to specifically define the effects of different anti-bacterial agents, or preservatives, on the viability and stability of *Myoviridae* and *Siphoviridae* bacteriophages in a standard semi-solid formulation. Results show that bacteriophages were most severely affected by benzoic acid, with no viable numbers retrieved after 28 days for the *Siphoviridae* PAC1, and 35 days for the *Myoviridae* KOX1.

Exposure to chlorocresol resulted in the least decrease in viability after 91 days for KOX1. For

PAC1, there was no significant difference in loss of viability when formulated in creams where either methyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate or chlorocresol was the preservative. Chlorocresol proved to be significantly better than 2-phenoxyethanol, combination hydroxybenzoates, or benzoic acid for PAC1 survival. While the presence of acidic compounds in suspensions (Merabishvili *et al.*, 2017), solutions (Subils *et al.*, 2012) or the environment (Jonczyk *et al.*, 2011) have been previously shown to adversely affect bacteriophages, our study is the first to report detrimental effects of the acidic benzoic acid when it is used as a preservative in a semi-solid base into which bacteriophages have been formulated. After four to five weeks in these formulations, the bacteriophages were no longer capable of killing their hosts.

The effects of the benzoic acid on both PAC1 and KOX1 are dramatic. This may have been facilitated by the nature of the semi-solid base chosen in these experiments. For instance, cetomacrogol cream aqueous is an emulsified oil-in-water system prepared by adding water to a liquid paraffin. The oil emulsion formed is stabilised by the non-ionic surfactant cetomacrogol (polyethylene glycol hexadecyl ether). The hydrophobic region of the surfactant sorbs to the surface of the oil droplets, effectively stabilising the formulation. The presence of these hydrophobic droplets in the cream system, however, also provides a hydrophobic environment where other low molecular weight molecules may partition to. If we consider the preservatives used in this study, it is the more hydrophobic molecules (e.g. chlorocresol) that have lower impact on bacteriophage viability. The most polar molecule, benzoic acid, has a dramatic effect, which may be facilitated by its dispersal in higher concentrations throughout the cream matrix so that it can interact with bacteriophage in sufficient concentrations to inhibit their activity.

The survival of the *Myoviridae* KOX1 at day 91 was significantly better than that of the *Siphoviridae* PAC1 when exposed to creams with each of the preservatives used in this study. The robustness of Myobacteriophages in comparison to bacteriophages belonging to other morphological groups has been noted before (Merabishvili *et al.*, 2017). While other reports suggest that *Siphoviridae* bacteriophages may be more resistant to adverse conditions (Lasobras *et al.*, 1997), it is important to note that these studies focussed on direct environmental influences on the virus, whereas our work reflected on the stability of bacteriophages in formulation. As such it may provide a more accurate assessment of pharmaceutical stability considerations when delivering formulated bacteriophages to the epithelia.

This work shows that different types of bacteriophages have different stability profiles depending on the nature of the preservative used in a formulation. The more acidic preservative was more detrimental to bacteriophage viability. We show that the Myovirus we used was more robust than the Siphovirus under our conditions, and as such, our work highlights the need for individual testing of specific bacteriophages in pharmaceutical formulations, as their efficacy when exposed to preservatives and excipients in these delivery forms may vary. Finally, it is

important to note that while we show a significant decrease over time in bacteriophage viability in some semi-solid formulations, this may not correlate exactly with the potential for bacterial control when applied therapeutically, as the latter is dependent on the bacterial numbers present, and the consequent multiplicity of infection.

MATERIALS AND METHODS

Bacterial strains and bacteriophages

Cutibacterium acnes bacteria, which had been identified and used in previous studies, was used as a host for the *Siphoviridae* bacteriophage PAC1 (Brown *et al.*, 2016). The culture was grown on reinforced clostridial media containing 1% peptone (Oxoid, Adelaide, Australia), 1% Lab Lemco powder (Oxoid), 0.5% glucose (Sigma, Sydney, Australia), 0.5% sodium chloride (Sigma), 0.3% yeast extract (Oxoid), 0.3% anhydrous sodium acetate (Chem-Supply, Gillman, Australia), 0.05% soluble starch (Difco, Detroit, USA) and 1.5% bacteriological agar (Oxoid). The plates were incubated anaerobically using the Anaerogen system (Oxoid) at 37°C. The *Klebsiella oxytoca* strain previously characterised and reported by us (Brown *et al.*, 2017a) was used as a host for the *Myoviridae* bacteriophage KOX1. This strain was cultured on Tryptone Soya Agar (Oxoid) under anaerobic conditions at 37°C. All chemicals were purchased from Sigma (Sydney, Australia) unless stated otherwise.

Phage cream formulation

Preparation of bacteriophage stocks was as follows: environmental samples containing the bacteriophages were filtered through 0.2 µm cellulose acetate filters (Advantec, Melbourne, Australia). 10 µL of the filtrate was then placed onto a lawn of the *K. oxytoca* or *C. acnes* bacteria. The presence of bacteriophage was identified by plaque formation on the bacterial lawn. Individual plaques were excised along with the underlying agar, placed into 600 µL of broth and vortexed to allow suspension of the bacteriophage into the broth. After centrifugation (15,000 g for 2 min) the supernatant was serially diluted (1:10) so that decreasing concentrations were obtained, to a concentration of 10⁻⁸. 10 µL of each dilution was spotted onto a lawn of *K. oxytoca* or *C. acnes* to obtain single plaques. The purification process was repeated four times to ensure that each plaque was the result of one virion infection. In these experiments, each bacteriophage was always propagated on the host it was obtained from. A bacteriophage stock was generated by suspending an individual plaque (as described in the purification process above) in 50 mL of broth inoculated with 1 mL of an exponential culture of *K. oxytoca* or *C. acnes* using a Multiplicity of Infection of 0.1 PFU per bacterium, for 24 hours. Following

incubation, the culture was centrifuged (800 g for 5 min) and the supernatant filtered through a 0.2 μ M cellulose acetate filter (Advantec). To assess the concentration of this phage stock (PFU mL⁻¹), serial dilutions were performed as described above.

Bacteriophage stocks ($>10^9$ PFU mL⁻¹) in phosphate buffered saline (137 mmol L⁻¹ NaCl, 2.7 mmol L⁻¹ KCl, 10 mmol L⁻¹ Na₂HPO₄ and 1 mmol L⁻¹ KH₂PO₄) were used for formulation. Cetomacrogol cream aqueous (cetomacrogol 1000 3%w/w, cetostearyl alcohol 12%w/w, liquid paraffin 10%w/w, white soft paraffin 10%w/w, propylene glycol 5.2%w/w; deionised water to 100g) was manufactured according to the published formulary (Sansom, 2018), however, a range of preservatives were used to observe their effects on *Siphoviridae* and *Myoviridae* bacteriophage viability. Cetomacrogol cream aqueous was used as the base for formulation in this study because we had previously shown that formulated PAC1 *Siphoviridae* bacteriophage and KOX1 *Myoviridae* bacteriophage were able to be released from this cream and retained their capacity to lyse their bacterial hosts after extended periods of storage (Brown et al. 2016; Brown et al. 2017). Six preservatives commonly used in semi-solid preparations were tested: benzoic acid (0.1%), chlorocresol (0.1%) (the standard preservative used in cetomacrogol cream aqueous), combination hydroxybenzoates (propyl 4-hydroxybenzoates with methyl 4-hydroxybenzoates) (0.1%), methyl 4-hydroxybenzoate (0.08%), 2-phenoxyethanol (1%), and propyl 4-hydroxybenzoate (0.02%). Therefore, in these experiments, the PAC1 *Siphoviridae* bacteriophage and KOX1 *Myoviridae* bacteriophage were each tested in six cetomacrogol cream aqueous formulations, which differed according to the preservative added.

Bacteriophages were added to the creams by geometric dilution as described previously (Brown *et al.*, 2017b). Briefly, the bacteriophage stock was thoroughly mixed into 2 grams of cream before another 2 gram portion of cream was added and thoroughly mixed. This process was repeated until the phage was dispersed evenly throughout the cream. Bacteriophages PAC1 and KOX1 were formulated at final concentrations of 1.5×10^8 PFU g⁻¹ and 5.5×10^8 PFU g⁻¹ respectively.

Phage stability testing

Bacteriophage creams with the various preservatives were stored at 4°C in light protected glass containers for the duration of this study and portions of cream were removed at weekly intervals for testing. To test the viability, 0.05 g of the bacteriophage cream was mixed with PBS to a total volume of 1 mL, and a 10-fold serial dilution was completed. Each dilution was spotted onto an agar lawn of the bacterial host and the viable bacteriophage concentration determined (PFU g⁻¹). This was performed weekly in triplicate for a duration of 91 days.

STATISTICAL ANALYSIS

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Non-stationarity or presence of unit root in the time series of PAC1 *Siphoviridae* bacteriophage and KOX1 *Myoviridae* bacteriophage by type of preservative were each tested using the Augmented Dickey-Fuller test (ADF). Relative percent changes in bacteriophage numbers (baseline versus day 91) were compared between the six preservatives for each bacteriophage and between PAC1 bacteriophage and KOX1 bacteriophage within each preservative using Pearson chi-square tests that tested the null hypothesis that the percent changes were the same. Statistical significance was set at a p value of < 0.05 (two-sided). The analyses were performed using Stata SE version 15 (Stata Corp, College Station, TX).

Conflict of Interest: The authors declare no conflict of interest

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Table 1. Pairwise comparisons of percent change (baseline versus day 91) in PAC1 and KOX1 viable numbers in different preservatives

Preservative	PAC1 P-value	KOX1 P-value
Chlorocresol vs 2-phenoxyethanol	0.017	0.031
Chlorocresol vs Benzoic acid	< 0.001	< 0.001
Chlorocresol vs Propyl 4-hydroxybenzoate	0.196	< 0.001
Chlorocresol vs Methyl 4-hydroxybenzoate	0.150	< 0.001
Chlorocresol vs Combination hydroxybenzoates	0.009	< 0.001
2-phenoxyethanol vs Benzoic acid	< 0.001	< 0.001
2-phenoxyethanol vs Propyl 4-hydroxybenzoate	0.249	< 0.001
2-phenoxyethanol vs Methyl 4-hydroxybenzoate	0.315	< 0.001
2-phenoxyethanol vs Combination hydroxybenzoates	0.795	< 0.001
Benzoic acid vs Propyl 4-hydroxybenzoate	< 0.001	< 0.001
Benzoic acid vs Methyl 4-hydroxybenzoate	< 0.001	< 0.001
Benzoic acid vs Combination hydroxybenzoates	< 0.001	< 0.001
Propyl 4-hydroxybenzoate vs Methyl 4-hydroxybenzoate	0.881	0.398
Propyl 4-hydroxybenzoate vs Combination hydroxybenzoates	0.161	0.025
Methyl 4-hydroxybenzoate vs Combination hydroxybenzoates	0.208	0.002

Table 2. Comparison of percentage survival between PAC1 and KOX1 at day 91 compared to numbers in initial formulations.

Preservative used in cream	PAC1	KOX1	p-value
Chlorocresol	17.8%	72.7%	< 0.001
2-phenoxyethanol	6.7%	58.2%	< 0.001
Benzoic acid	0%	0%	1.0
Propyl 4-hydroxybenzoate	11.3%	26.7%	< 0.001
Methyl 4-hydroxybenzoate	10.7%	32.2%	< 0.001
Combination hydroxybenzoates	5.8%	13.9%	< 0.001

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