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Stability and purity of a bacteriophage cocktail preparation for nebuliser delivery

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Running heading: preparation of bacteriophage product

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Significance and Impact of Study: With increasing reports of bacterial resistance to antibiotics and the lack of new antibiotics being produced, bacteriophage therapy is becoming an attractive alternative. There has been no published report on the quality assurance of bacteriophage product to date. This is the first study on the quality assurance of a *Ps. aeruginosa* phage cocktail following pharmacopoeial requirements. The presence of bacterial endotoxin was found to be the key stumbling block for meeting regulatory criteria.

Abstract

The aim of this study was to determine the stability and purity of a phage cocktail to be delivered by nebulisation. A cocktail of three phages active against *Pseudomonas aeruginosa* isolates from cystic fibrosis patients was developed for a potential nebulised formulation. The individual phages were examined for their retention of activity over time, while the phage cocktail was analysed for bacterial contaminant and endotoxin level according to regulatory requirements for nebulised products. The phage cocktail was nebulised using a Porta-neb nebuliser connected to an Anderson cascade impactor. The three phages retained activity over a period of 180 days storage at room temperature and at 4°C. Nebulised phages were recovered in the lower stages of the cascade impactor indicative of potential delivery deep into the lungs. The phage cocktail met bacterial limits but the endotoxin levels measured with the Limulus amoebocyte lysate (LAL) test remained considerably in excess of acceptable levels even following purification. These findings suggest that nebulisation of

phage is a viable delivery option; although there is a need for appropriate depyrogenation strategies to remove bacterial endotoxins from phage based preparations in order to meet regulatory requirements.

Introduction

With renewed interest in the use of phage to treat bacterial infection, attention is drawn to the production of phage formulations for clinical use and the regulatory requirements that such a product must satisfy. These requirements vary depending on the intended application and route of administration with topical applications requiring less stringent microbiological controls than, for example, intravenous injections (Anon., 2013a,b). In this regard, many of the commercially produced phage products currently undergoing trials are of a topical nature (Wright *et al.*, 2009). There is currently a paucity of information on the quality of phage products for clinical applications. This paper aims to investigate the alignment of a prototype nebulised phage formulation with selected pharmacopoeial requirements.

Results and Discussion

The phage cocktail described in this study met the British Pharmacopoeial bacterial limit test for non-sterile manufactured products (Anon., 2013c). All phage cocktails showed an absence of microbial contamination according to pharmacopoeial standards (data not shown). In addition, there was no significant

difference in viable phage at both room temperature and 4°C ($P \geq 0.05$) for all 3 phages (Fig. 1). There was no statistically significant difference in phage viability due to storage conditions ($P \geq 0.05$) with the exception of LP-M 10 at 180 days storage ($P \leq 0.003$). In general phage LP-M 10 was most susceptible to loss of viability following storage (approx. $1 \log_{10}$ reduction in phage concentration at 4°C; Figure 1c) and GL-1 least susceptible to loss following storage (approx. $0.5 \log_{10}$ at 4°C reduction in phage titre; Figure 1a) over a period of six months. While the current crude preparation demonstrated stability over a period of six months, final commercially available preparations may require the addition of stabilizing agents. These formulations would subsequently require BP or EP based preservative efficacy testing in order to demonstrate long term stability. Due to the enhanced nature of pharmacopeial compliance for some products, many of the phage preparations currently undergoing clinical trials (Wright et al., 2009) or currently licensed for use (e.g. ListShield™, Intralytix, USA) are for topical use or for applications for the food industry. For approval for the use in the food industry, the Listshield™ product has demonstrated that the phage cocktail used is at little or no risk of causing adverse side effects and that no potentially pathogenic sequences for *Listeria* reside within the phage genome (Food and Drug Administration, 2006; <http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm154675.htm>, accessed 07/05/2013). The current investigation demonstrated the ease with which phage preparations could meet the basic microbiological requirements for non-sterile topical applications, however for more complex

applications, further work including the sequencing of the phage genome would need to be undertaken.

This study demonstrated that a phage cocktail can be successfully nebulised with very little loss of infectivity. There was no significant difference ($P=0.848$) between the total number of phage at the start of the nebulisation process and the total number of phage recovered after nebulisation. In addition, the deposition pattern of the phage throughout the 7 stage cascade impactor indicated that most of the phages would reach the throat and upper airways (corresponding to stages 0-3 of the cascade impactor), while a quarter would reach secondary bronchi and alveoli (represented by deposition between stages 4 and 7). Indeed 99.2% ($\pm 4.2\%$) of phages were recovered by stage 7 of the cascade impactor; 75% of phages were deposited between stages 0 and 3, and the remaining phages between stage 4 and 7 (results not shown).

The production of endotoxin from the lysis of the *Ps. aeruginosa* strains used here represents a major hurdle for the further development of non-topical phage products and notably for nebulised applications. When endotoxin levels were assessed using the Endosafe PTS LAL kinetic assay, the phage cocktail showed high levels of endotoxin ($>5 \text{ Log}_{10} \text{ EU ml}^{-1}$; Table 1). Following purification via passage through the Endotrap system, there was a small drop in endotoxin levels (approximately $0.2 \text{ Log}_{10} \text{ EU ml}^{-1}$) but this was not significant when compared to the “unpurified” phage cocktails ($P=0.327$). This level of endotoxin far exceeds

the pharmacopeial required levels ($<0.2 \text{ EU ml}^{-1}$) and as such would fail quality assurance. To note, because of the level of endotoxin present in the phage cocktail, the LAL assay used here required the dilution of samples that were above the detection threshold of the cartridge (1 EU ml^{-1}), requiring the extrapolation of endotoxin levels from a positive result. Furthermore, the activity assessment of both “purified” and “unpurified” phage preparations on the Bioscreen C analyser shows a small but not significant decrease ($P \geq 0.05$) in the level of activity of the phages at 8 and 20 h (Table 1). This is most probably caused by the small but non-significant reduction ($P=0.124$) in the phage number following passage through the Endotrap system. Although the use of an overlay based propagation method has been used in the current investigation this would not be appropriate for large scale manufacture, due to the relatively low throughput.

The determination of endotoxin levels in phage preparations have previously only been measured with the rabbit pyrogenicity assay (Merabishvili *et al.*, 2009). In our study, the pharmacopeial “gold standard” LAL assay (Anon, 2013d,e) has been used to quantify endotoxin levels in both “non-purified” and “purified” phage cocktails demonstrating unacceptably high endotoxin levels. The LAL assay not only provides quantitative data with regards to the total endotoxin levels within samples, but also is more sensitive than animal models (Ong *et al.*, 2006). The purification of phage preparations with the Endotrap Blue system has previously been demonstrated to be effective at reducing endotoxin levels in phage preparations (Merabishvili *et al.*, 2009). In the current investigation endotoxin

levels were not significantly altered by passage through the Endotrap Blue system. The saturation of the column with the high level of endotoxin present in our sample could explain the overall low reduction in the endotoxin level, although this possibility was not tested at the time of the experiment. For laboratory scale preparation, caesium chloride gradients and PEG precipitations have been demonstrated to purify phages (Gill and Hyman, 2010) although this may be impractical for manufacturing processes due to associated costs with large scale centrifugation and the requirement to know buoyant densities of phages particularly for caesium chloride gradients.

This investigation illustrated the range of microbial quality control matters that should be addressed in the formulation of phage based products, in particular the endotoxin levels within phage preparations. Although the nebulised phage cocktail was stable over a 6-month period showing no bacterial contamination, the level of endotoxin remained high. Reducing the endotoxin level is the limiting factor in this study, (practical and financial), to produce an appropriate nebulised phage product.

Materials and Methods

Preparation of bacterial and bacteriophage inocula

Ps. aeruginosa strain PAO1 (Cooper *et al.*, 2011) was routinely cultured for 24 h in 10 ml of tryptone soya broth (TSB, Oxoid, UK) at 37°C prior to use and

standardised to contain between $1\text{--}5 \times 10^8$ CFU ml⁻¹ by reference to optical density measurements at 600nm.

Phage cocktail preparation

Phage GL-1, GL-1_{2.5} and LP-M₁₀ described by Cooper *et al.* (2011) were used.

Individual phage stock was prepared by using *Ps. aeruginosa* PAO1 as host using the classical agar overlay method (Adams, 1959). In brief, 10µl of a bacterial suspension (approx. 10^8 CFU ml⁻¹) was mixed with 10 µl phage stock suspension and added to 5 ml of overlay agar (26 g/l TSA) containing 50 µmol l⁻¹ CaCl₂. Phages were diluted 1:10 in PBS and enumerated using the overlay agar method as described above. Phages were harvested as described previously by Cooper *et al.* (2011). A cocktail containing equal amounts of phage GL-1, GL-1_{2.5} and LP-M₁₀ (approx. 10^{11} PFU ml⁻¹) was prepared in 0.9% (w/v) saline containing 50 µmol l⁻¹ CaCl₂.

Removal of endotoxins from the phage cocktail

Endotoxins were removed from the phage cocktail preparation using the Endotrap Blue Chromatography system (Hyglos, Germany) as follows: the prepacked column was drained and regenerated using the regeneration buffer supplied by the manufacturer. Following regeneration the column was washed twice in 0.9% (w/v) saline containing 50 µmol l⁻¹ CaCl₂ (equilibration buffer). The phage cocktail was added and flushed through with the equilibration buffer. The column was then washed and regenerated prior to the addition of the next

sample. When not in use the column was stored in 0.2% (w/v) sodium azide at 4°C. Samples of purified phage cocktail were assessed for their endotoxin content within 1 h. The activity of bacteriophage cocktails was assessed by a Bioscreen C based assay as previously described by Cooper *et al.* (2011) for lytic activity compared to untreated growth at 8 and 20h post phage addition as well as potential regrowth from bacterial survivors.

Nebulisation

Ten ml of the phage cocktail was transferred to the Ventstream drug chamber of a Porta-neb nebuliser (Respironics Respiratory Drug Delivery Ltd., Chichester, UK). The nebuliser was connected to an Anderson cascade impactor (Pandey *et al.*, 2003) and air was drawn through the impactor at a rate of approximately 28 L min⁻¹. The phage suspension was then nebulised for 10 min after which the remaining phages in the Ventstream drug chamber were recovered and enumerated by the agar overlay method (Cooper *et al.*, 2011) using *Ps. aeruginosa* PAO1 as a host.

Steel sampling plates from each impactor stage were removed aseptically from the cascade impactor and transferred to sterile petri dishes. Ten ml sterile PBS was added to each petri dish, and the dishes shaken at 150 RPM for 15 min. The PBS wash fluid was then pipetted into a sterile centrifuge tube, vortexed and the phage enumerated by the agar overlay method (Cooper *et al.*, 2011) using *Ps. aeruginosa* PAO1 as a host.

Phage stability

Ps. aeruginosa phage suspensions of GL-1, GL-1_{2.5} and LP-M₁₀ were prepared in phosphate buffered saline (PBS) using the agar overlay method (Cooper *et al.*, 2011). Following an initial quantification of phage viability, triplicate samples were stored at $4 \pm 1^\circ\text{C}$ or room temperature ($21 \pm 1^\circ\text{C}$). Phage viability was then measured by the agar overlay method using *Ps. aeruginosa* PAO1 as a host after 7, 30, 60, 90 and 180 days storage.

Endotoxin content

All glassware and containers were depyrogenated by dry heat sterilization at 200°C for 1 h (Anon., 2013f). Buffers and reagents were prepared in pyrogen-free cell culture grade water (Sigma Aldrich, Dorset, UK).

The endotoxin level in the phage cocktail was determined with a Limulus amoebocyte lysate (LAL) kinetic chromogenic method using the Endosafe®-PTS (Charles River Laboratories International Inc., MA, USA) according to manufacturer's instructions (Charles River Laboratories International Inc., MA, USA) with a PTS testing cartridge (0.05 endotoxin units per millilitre (EU ml⁻¹); Charles River Laboratories International Inc., MA, USA). Measurements were performed in triplicate. A negative control consisted of 0.9% (w/v) saline prepared in pyrogen-free cell culture grade water. All samples were diluted in LAL reagent grade water (Charles River Laboratories International Inc., MA, USA).

Determination of the bacterial content of the phage product

The level of microbial contamination in the phage cocktails before and after passaging through the Endotrap Blue Chromatography system was assessed in accordance with Pharmacopoeial requirements for non-sterile respiratory products (Anon. 2013c,g) as follows:

Total aerobic viable count: one ml of the phage cocktail was used to inoculate 20 ml of molten tryptone soya agar (TSA, Oxoid, UK) or 20 ml of Sabouraud dextrose agar (SAB; Oxoid, UK). This was repeated for triplicate plates. TSA plates were incubated at 37°C for 5 days and SAB plates were incubated at 30°C for 5 days. Plates were checked for growth every 24 h.

Absence of *Ps. aeruginosa*: one ml of phage cocktail was added to 100 ml TSB and incubated at 37°C for 48 h. After incubation, a sample was streaked onto Cetrimide agar (Oxoid, UK) and incubated at 41-43°C for 24 h. *Ps. aeruginosa*, if present, appeared as blue/green colonies.

Absence of *Staphylococcus aureus*: one ml phage cocktail was added to 100 mL TSB and incubated at 37°C for 48 h. After incubation, a sample was streaked onto Baird-Parker Agar (Oxoid, UK) and incubated at 37°C for 72 h. *S. aureus*, if present, appeared as black colonies with a clear halo.

Conflict of interest

None to declare

References

- Adams, M.H. (1959). *Bacteriophage*. New York: Interscience Publishers Inc, p. 7.
- Anonymous (2013a). *Parenteral preparation*. The British Pharmacopoeia; Volume III Formulated Preparations: General Monographs. Norwich: The Stationary Office.
- Anonymous (2013b). *Test for Sterility*. The British Pharmacopoeia; Volume V Appendix XVI A. Norwich: The Stationary Office.
- Anonymous (2013c). *Microbiological Examinations of Non-Sterile Products*. The British Pharmacopoeia; Volume V Appendix XVI B. Norwich: The Stationary Office.

Anonymous (2013d). *LAL Test*. The British Pharmacopoeia; Volume V Appendix XIV C. Test for Bacterial Endotoxins. Norwich: The Stationary Office.

Anonymous (2013e). *Bacterial Endotoxin Testing*. The British Pharmacopoeia; Volume V Supplementary Chapters: SC I C. Norwich: The Stationary Office.

Anonymous (2013f). *Methods of Preparation of Sterile Products* The British Pharmacopoeia; Volume V Appendix XVIII Methods of Sterilisation. Norwich: The Stationary Office.

Anonymous (2013g). *Microbiological Quality of Non-Sterile Pharmaceutical Preparations and Substances for Pharmaceutical Use*. The British Pharmacopoeia; Volume V Appendix XVI D. Norwich: The Stationary Office.

Cooper, C.J., Denyer, S.P. and Maillard, J.-Y. (2011) Rapid and quantitative automated measurement of bacteriophage activity against cystic fibrosis Isolates of *Pseudomonas aeruginosa*. *J Appl Microbiol* **110**, 631-640

Gill, J.J. and Hyman, P. (2010) Phage Choice, Isolation, and Preparation for Phage Therapy. *Curr Pharm Biotechnol* **11**, 2-14

Merabishvili, M., Pirnay, J-P., Verbeken, G., Chanishvili, N., Tediashvili, M., Lashkhi, N., Glonti, T., Krylov, V., Mast, J., Van Parys, L., Lavigne, R., Volckaert, G., Mattheus, W., Verween, G., De Corte, P., Rose, T., Jennes, S., ZIZI, M., De Vos, D. and Vaneechoutte, M. (2009) Quality-Controlled Small-Scale Production of a Well-Defined Bacteriophage Cocktail for Use in Human Clinical Trials. *PLoS ONE*, **4**, e4944.

Ong K.G., Leland J.M., Zeng K., Barrett G., Zourob M. and Grimes C.A. (2006) A rapid highly sensitive endotoxin detection system. *Biosens Bioelectron* **21**, 2270-2274.

Pandey, R., Sharma, A., Zahoor, A., Sharma, S., Kuhlner, G.K. and Prasad, B. (2003) Poly (DL-lactide-co-glycolide) nanoparticle-based inhalable sustained drug delivery system for experimental tuberculosis. *J Antimicrob Chemother* **52**, 981-986

Wright, A., Hawkins, C.H., Änggård, E.E. and Harper, D.R. (2009) A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant *Pseudomonas aeruginosa*; a preliminary report of efficacy. *Clin Otolaryngol* **34**, 349-357.

Figure 1: Stability of phage cocktail components over a period of 180 days.

a, GL-1; b, GL-1 2.5 and c, LP-M 10. ■, Room temperature, ■, 4°C. Data shown are the mean of 3 replicates \pm SD.

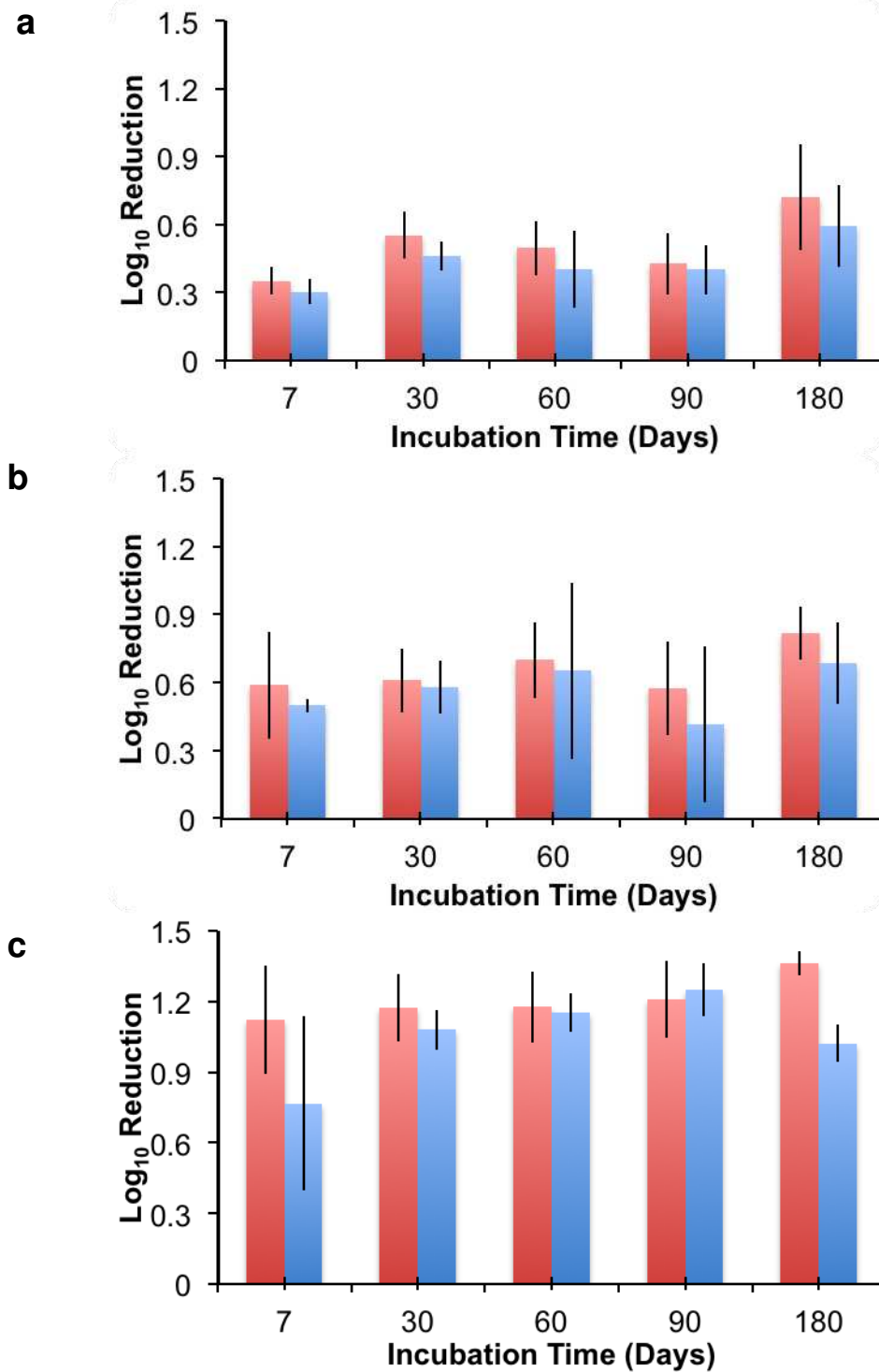


Table 1: Activity of *Ps. aeruginosa* cocktails following purification on the Endotrap Blue system against *Ps. aeruginosa* strain PAO1. Data shown are the mean of 3 replicates (\pm SD). ¹: Time taken to increase OD_{420-580nm} by 0.1 above the original OD_{420-580nm}.

Recovery - Log ₁₀ PFU/ml (SD)	Bioscreen C Assay		Time ¹ (min)	Endotoxin Levels -Log ₁₀ EU/ml (SD)
	Log ₁₀ Reduction (SD)			
	8 h	20 h		
“unpurified” Preparation				
11.02 (0.07)	1.85 (0.11)	1.84 (0.19)	>1200	6.05(0.06)
“Purified” Preparation				
10.81 (0.18)	1.78 (0.12)	1.77 (0.29)	>1200	5.81(0.37)