People seldom see the halting and painful steps by which the most insignificant success is achieved.

- Anne Sullivan

University of Alberta

Aerosol phage therapy for *in vivo* *Burkholderia cepacia* complex respiratory infections

by

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Abstract

In recent times, increased attention has been given to evaluating the efficacy of phage therapy, especially in scenarios where the bacterial infectious agent of interest is highly antibiotic resistant. In this regard, phage therapy is especially applicable to infections caused by the *Burkholderia cepacia* complex (BCC) since members of the BCC are antibiotic pan-resistant. Aerosol drug delivery is a common therapeutic delivery method for respiratory ailments and yet aerosol phage therapy has not previously been demonstrated to be effective for BCC infections.

This study outlines the development of an *in vivo* model for evaluating aerosol delivery of phage therapeutics using a Nose-Only Inhalation Device (NOID). Immunocompromised mice infected with *Burkholderia cenocepacia* demonstrated a significant reduction in bacterial concentration when treated 24 h post-infection provided that the phage therapeutic was delivered at a sufficient concentration. This method shows promise for future evaluation of aerosolised respiratory phage therapeutics.

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List of Symbols and Abbreviations

ASMDM – Artificial Sputum Medium

BCC – *Burkholderia cepacia* complex

°C – degrees Celsius

CF – Cystic Fibrosis

CFU – Colony Forming Units

CGD - Chronic Granulomatous Disease

CPA - Cyclophosphamide

CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats

EOP – Efficiency of Plating

gHBSS – Hank's Balanced Salt Solution with 1% gelatin

h - hour

IP - intraperitoneal

LB – Luria Bertani

LPS - lipopolysaccharide

MOI – Multiplicity of Infection

min - minute

mL – millilitre

NOID – Nose-Only Inhalation Device

OD_{600} – Optical Density measured at a wavelength of 600 nm

PFU – Plaque Forming Units

RFLP – Restriction Fragment Length Polymorphism

RPM – Revolutions per Minute

s - second

µm – micrometre or micron

Introduction

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The *Burkholderia cepacia* complex (BCC) is a group of 17 genetically diverse, but phenotypically similar Gram-negative rod-shaped bacteria (59). First described as the cause of soft onion rot (5), these opportunistic pathogens are both environmentally and clinically significant. Environmentally, the BCC are not only phytopathogens but are also found as plant commensals found both in the rhizosphere as well as within the roots of some plants. They can also be used in biocontrol applications, such as preventing damping-off in seedlings, likely due to the production of antifungal agents (reviewed by Parke and Gurian-Sherman (60)). Additionally, members of the BCC may be useful as bioremediation agents as they are able to degrade chemical pollutants such as trichloroethylene and toluene (reviewed by Lessie *et al*. (37)).

In contrast to the many environmental benefits the BCC may confer, clinically the BCC can cause respiratory infections in individuals who are immunocompromised or have been diagnosed with cystic fibrosis (CF) or chronic granulomatous disease (CGD) (29, 31). BCC infections can spread rapidly between at-risk individuals (41) and significantly reduce the life expectancy of CF patients. In severe cases infection can lead to "cepacia syndrome", which is characterised by rapid deterioration of pulmonary function, septicemia and reduced life expectancy (29, 71). BCC infections are difficult to treat because they are highly antibiotic resistant (40, 64, 76), making alternative treatments such as phage therapy attractive. Phage therapy utilises bacteriophages

(phages), viruses that specifically target particular species of bacteria, to combat the infection. This introduction outlines the origins of phage therapy as well as highlights current research applicable to phage therapy for combating BCC infections.

Discovery of phages and beginnings of phage therapy

Bacteriophages were discovered independently by Twort (87) at Browns Veterinary Hospital in London and by d'Herelle (14) at the Institut Pasteur in Paris. Phages are viruses that exclusively attack and lyse bacteria (often of a particular species or strain), which prompted d'Herelle to develop the term "bacteriophage", meaning "bacteria-eater" (3, 14). Shortly after the discovery of phages, d'Herelle proposed using them as a possible method of combating infection and used phages to treat patients with dysentery, bubonic plague and cholera, with some success. Although there were reports of successful phage treatment, not all phage therapy testing was effective. These variable results were most likely due to a lack of knowledge in the area of phage biology, a lack of understanding regarding phage specificity, the inappropriate use of phage to treat conditions not caused by a bacterial infection, improper phage preparation leading to phage inactivation, and an inability to produce high volumes of purified phage (reviewed by Sulakvelidze and Morris (83) and Bradbury (3)). The discovery of chemical antibiotics, combined with a perceived inconsistency in

phage therapy performance, led to the discontinuation of phage therapy in the West by the 1940s (53).

Mechanisms of phage infection and phage therapy

The main principle upon which phage therapy rests is that after replication within a bacterium, phages lyse and kill their host cell. The best candidates for phage therapy are lytic phages (77). The basic steps of lytic phage infection for phages of the order *Caudovirales* (composed of three families of tailed phages: *Siphoviridae*, *Myoviridae*, and *Podoviridae*) are as follows (Figure 1): First, a phage binds to a receptor on the surface of a bacterium. On a Gram-negative bacterium, such as the BCC, phage receptors can include lipopolysaccharide (both smooth and rough types) and outer membrane components such as porins, transport proteins, enzymes and structural proteins (67). Phage interaction with a surface receptor may be enhanced through the production of a phage-associated enzyme capable of degrading exopolysaccharide or biofilm components. Once bound to the receptor, the phage injects its nucleic acid into the bacterial host. The phage early genes are expressed, disrupting bacterial systems and inducing replication of the phage genome. Late phage genes are then expressed, allowing production of proteins required for phage assembly and host lysis. Finally, mature phage particles are assembled and the host is lysed, releasing the newly assembled phages (77).

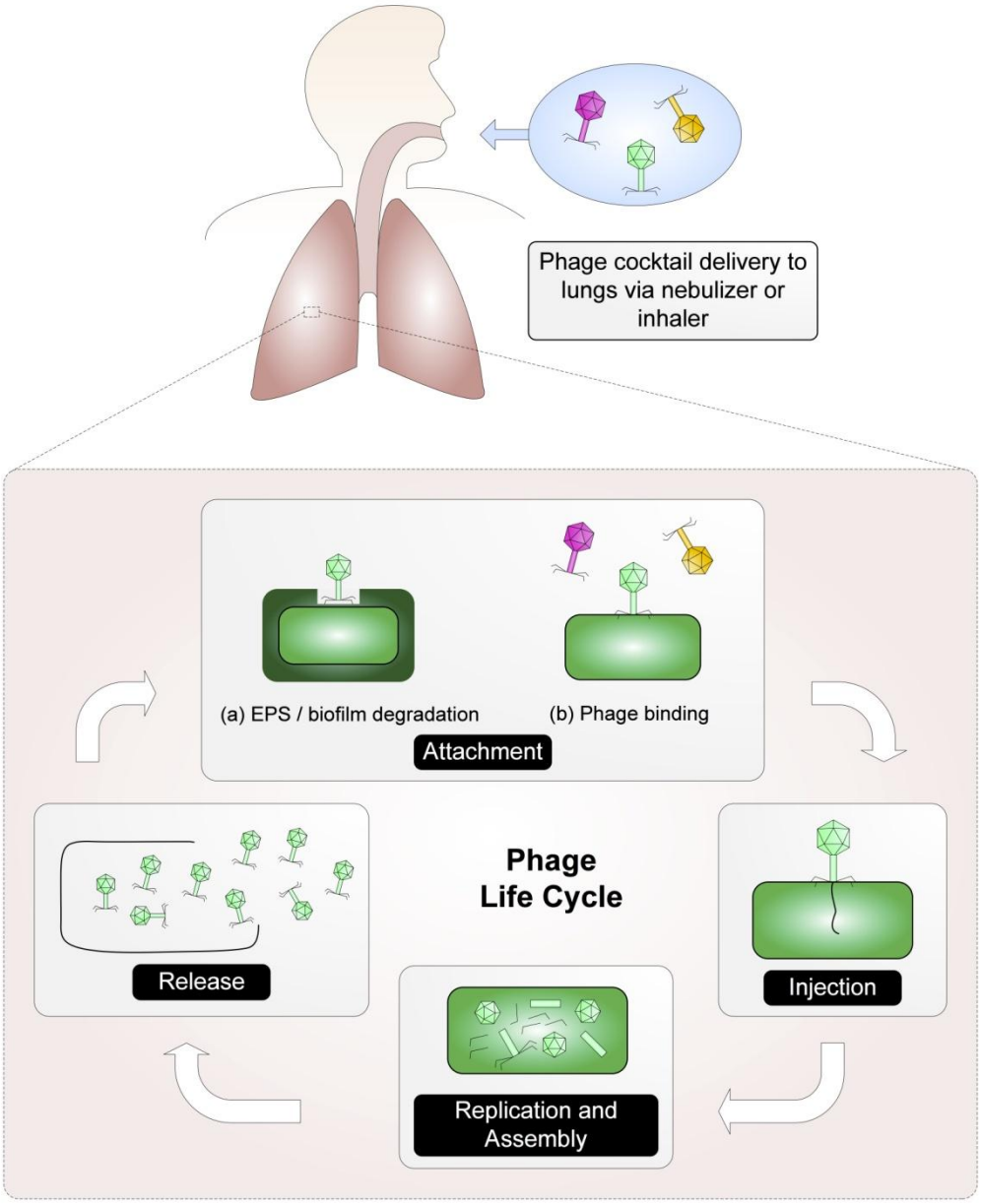


Figure 1. The phage life cycle. A phage cocktail is delivered via an aerosol to the site of the infection where the phage life cycle can be established. First, phage attach to receptors on the host. (Phage may need to degrade exopolysaccharide [EPS] in order to reach the phage receptor). After binding, the phage are able to transfer their nucleic acid into the host. Host systems are disrupted and the replication of the phage genome begins. Once mature phage particles have been assembled the host is lysed and the phages are released.

Some phages are capable of a temperate (lysogenic) lifestyle in which their genome integrates into the host genome or exists as a plasmid and remains dormant, simply being replicated along with normal bacterial cell division. Such phages will remain in this state until conditions trigger the phage to enter a lytic lifecycle. This dormancy can be problematic when using phages in a phage therapy strategy because additional new phages are not being released (33). Temperate phages are also not optimal candidates for phage therapy because they do not cause the rapid host death required for effective phage therapy treatment (77). Additional concerns include lysogenic conversion, superinfection immunity and transduction. Lysogenic conversion is the utilisation of prophage genes by the host which may render the host more virulent than prior to infection. Superinfection immunity may develop in situations where the host cell becomes resistant to subsequent infection by a similar phage. Finally, transduction occurs when bacterial DNA (possibly containing virulence factor genes) is packaged into the phage capsid and transferred to a new host via the phage (reviewed by Lynch *et al*. (43, 44)). Although lytic phages remain the optimal choice for phage therapy, in situations where the use of lytic phages is not possible, the efficacy of phage therapy using temperate (or putatively temperate) virions has also been demonstrated (6, 7, 43, 61, 75).

There are three basic steps in treating a clinical infection with phage therapy. First, the cause of the infection must be isolated and characterised in order to

assess strain sensitivity. Second, a phage preparation must be chosen that is active against the appropriate host. Preferably, the preparation will be a high titre cocktail of phages with different host receptors, thereby increasing the activity of the preparation. Finally, the treatment must be appropriately administered to the patient according to the location of the infection (3). Many different methods of phage administration have been described in the literature including oral, rectal, topical, intravenous and inhalation (83).

The pharmacokinetics of phage therapy are quite different from any drug pharmacokinetics currently employed. With a chemical drug, the concentration decreases after administration. The opposite is true of phages. After the originally administered phage replicate, they provide an increasing phage dose that will continue until the infection is cleared. This phenomenon is known as active phage therapy (62). Passive phage therapy is also possible if the initial phage dose is high enough to clear the infection without phage replication (62). Although the concept of active phage therapy suggests that a low phage titre is all that is required for successful treatment, this is not necessarily the case. Using a mathematical model simulating the pharmacokinetics of phage therapy, Payne *et al*. (62) demonstrated that selecting the correct phage concentration required for successful treatment should be based on the bacterial density of the infection. A sufficiently dense bacterial community will allow for active phage therapy. However, a less dense population will not be able to support active

phage therapy because the phage will not be able to reproduce quickly enough to yield and maintain the high *in vivo* concentrations required to sustain a high bacterial kill rate. In situations such as this, multiple high titre phage treatments, administered with a similar schedule to that of antibiotic therapy, would be required in order to completely eliminate the infection (38).

In addition to titre, timing in phage administration is critical. Using mathematical modeling, Payne *et al*. (62) demonstrated that a delay in treatment would prove detrimental to combating an infection. These modeled data have also been demonstrated in a mouse model (8). Interestingly, Payne *et al*. (62) also demonstrated that a treatment delivered too early may be detrimental to clearing of the infection. This detrimental effect is related to low bacterial density. Sparse bacterial densities, in which the probability of a phage encountering a bacterial host is low, do not lend themselves to sustainable active phage therapy. If treatment is delayed until active phage therapy is possible, it will likely be more effective.

Advantages of phage therapy

Although there are some concerns regarding phage therapy (see below), there are a large number of potentially positive aspects to phage therapy as well. Phages are active against bacterial cells using completely different mechanisms than classical antibiotics, allowing them to be active against antibiotic resistant

bacteria (3). Phage activity against antibiotic resistant bacterial strains is likely the most promising feature of phage therapy, as phages can be used in instances where antibiotic treatment is no longer possible. Phages possess many positive qualities as potential antimicrobials, as shown in Table 1. In direct contrast to antibiotics, which are often broad-spectrum, phages show more host specificity, and in most cases show specificity to a single species or strain of bacteria. Using phages would allow for the treatment of an infection without harming the natural microflora of a patient (49). Phages do not cause negative side effects to patients, as demonstrated in a study in which human volunteers drank water containing the *E. coli* phage T4 (4). In this study there were no reported adverse effects caused by the phage, as well as no T4-specific immune response in the volunteers. These three key features demonstrate phage therapy's potential as an effective tool in combating bacterial infections.

Table 1. A comparison of some of the main advantages and disadvantages of antibiotics and phage therapy.

	Antibiotics	Phage Therapy
Specificity	Broad spectrum, affecting more than the targeted organism	Generally species- or strain-specific
Side Effects	Many, including allergies and intestinal disorders	No side effects (4, 52, 69, 89)
Resistance	Occurs and can also be transmitted to non-target bacteria	Occurs, but can be linked to host virulence attenuation (90). Also, phage can co-evolve with host.
Development	Time-consuming and expensive	Rapid

Challenges of phage therapy

The specificity of phages, which is certainly an advantage, can also provide one of the challenges that must be overcome when using phage therapy as a treatment method. Before an infection can be treated, the causative agent must be characterised or typed in order to ensure that the correct phage is being used for its treatment. Strain characterisation can be time-consuming and is especially troublesome in cases that must be treated rapidly. In order to overcome such problems, phage cocktails can be developed that are active against a broad range of strains or even different bacterial species (38).

A potentially serious complication of phage therapy that must be taken into consideration is that bacterial strains can develop phage resistance or semi-resistance. Phage resistance (or semi-resistance) may pose significant problems,

especially if a phage must undergo rigorous and time-consuming testing before it is approved for clinical use (49). Although phages can still infect semi-resistant bacteria, their infection rate is far lower than in sensitive bacteria, rendering phage therapy less effective (38). Semi-resistance is most often seen in mucoid colonies, as the mucoid barrier provides an additional obstacle to phage contacting the appropriate receptor. Phage resistance generally occurs by a mutation in the receptor to which the phage binds. Unlike semi-resistant bacteria, receptor mutations prevent phages from binding to the cell, thereby preventing the initial step in phage infection. Phage resistance and semi-resistance can be minimised by treating infections with a phage cocktail containing a mixture of phages that will target different binding receptors on the bacterial cell. This allows the cocktail to be effective, even if a receptor is altered. Interestingly, receptor mutation leading to phage resistance may not always be a bad thing. In some instances, bacteria that develop phage resistance are also less competitive and less virulent, or even avirulent. This phenomenon is most noticeable in phage-resistant bacteria where the phage receptor lost is a capsule or other virulence factor (38), as was demonstrated by Zahid *et al*. (90) who found that *Vibrio cholerae* serotype 01 cultured with phage in a nutrient medium quickly became phage resistant, but concurrently lost their LPS O1 side-chain antigen. Another form of resistance that must be considered is restriction endonuclease resistance. In this case, the bacterium encodes restriction endonucleases active against the phage genome, causing destruction of the

phage's genetic material and inhibiting phage replication. Although restriction endonucleases must be taken into consideration, it may not be of significant importance as many phages are able to evade restriction (38).

Phage therapy critics have suggested that the presence of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) in 40% of sequenced bacterial genomes is a detriment to phage therapy. Briefly, CRISPRs are genomic sequences of alternating spacers and palindromic repeats. The spacers are sequences of phage or plasmid DNA that the host had previously obtained. Identical matches between the spacers and foreign DNA entering the cell alert the bacterium to the presence of a potential predator, which triggers the degradation of the foreign DNA. CRISPRs have been reviewed in detail by Horvath and Barrangou (27). It should be noted, however, that if a phage has even a single point mutation in the sequence to be matched to the CRISPR sequence, the phage will be able to evade the CRISPR system and proceed with host infection. Additionally, CRISPRs are not a pressing concern in regards to BCC phage therapy as only one strain of the analyzed BCC species (*Burkholderia ambifaria* AMMD) contains a confirmed CRISPR locus (19).

Clinical uses and current evaluation of phage therapy

Current evaluation of phage therapy is often performed using animal models, as phage therapy must be proven with controlled studies before its efficacy in a

clinical setting can be evaluated. One of the most commonly used animal models is the mouse. Mice are an attractive animal model for many reasons. From a practical standpoint, their small size and low maintenance costs allow for comprehensive studies with many subjects (56). Mice have been well characterised and their immune systems have similarities to the human immune system (56).

Examples of the application of phage therapy to animal models are numerous and many of these have been and are currently being evaluated, including the treatment of thermal injury infection (34, 51) and systemic infections (6, 8), including bacterial pathogens that are antibiotic resistant (2). The application of phage therapy in veterinary medicine is also being assessed. There is an increased interest in finding alternatives to antibiotics for use in animal husbandry as more restrictive regulations against antibiotic use in livestock and fowl are put in place (30). Such research has included the evaluation of treating enterotoxigenic *Escherichia coli* infections in pigs, calves and lambs (78) and airsaculitis in chickens (28).

In Eastern Europe, particularly Georgia, phage therapy research and treatment has continued at the Eliava Institute. During the Soviet Era, the Eliava Institute was responsible for producing phage preparations for both prophylactic uses as well as for the treatment of infections in patients across the Soviet Union. The

Eliava Institute has reported many successes, treating patients with a wide range of infections including skin wounds, and eye, respiratory and gastrointestinal infections (83). Unfortunately, these reports are anecdotal in nature and have rarely been published in scientific literature, especially in the English language. With the advent of antibiotic resistant infections and an improved knowledge of phage biology and bacterial identification, phage therapy is now being revisited by researchers in the West.

Phage therapy clinical studies

Recently, three human safety studies and one safety and efficacy clinical study have evaluated either the safety or efficacy of phage therapy. The first of the recent phage administration human safety trials in English literature was performed by Bruttin and Brüssow (4). Fifteen volunteers were given either a placebo, a low phage titre preparation (10^3 PFU/mL) or a high phage titre preparation (10^5 PFU/mL) orally on two consecutive days followed by five days without phage administration. This was repeated for the following two weeks with each patient receiving a different phage preparation or placebo each week. Throughout the study five adverse effects were reported, however, there was no difference in the number of adverse effects in the placebo, low phage titre and high phage titre groups, and the effects were deemed to be unrelated to the study. Phage was never recovered from stool samples prior to phage administration or when the placebo was administered. However, phage was

recovered from stool samples in a dose-dependent manner after phage administration, demonstrating that the phage was able to survive transit through the gastrointestinal system. Interestingly, the presence of phage T4 did not correlate with a decrease in natural *Escherichia coli* counts in the stool samples, demonstrating that the phage did not affect the natural microflora of the volunteers. Throughout the study there was no change in serum alanine aminotransferase and aspartate aminotransferase levels, which are liver enzymes that would have indicated an increase in liver toxicity. There was also no detected immune response to the phage, demonstrating the safety of phage administration.

A phase I safety trial was performed by Rhoads *et al*. (69) to ensure that the application of WPP-201, a phage cocktail containing eight lytic phages targeting *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *E. coli*, would not pose any health risks to patients with venous leg ulcers. Thirty-nine patients completed the 24 week trial, with every patient receiving the same compression therapy and wound dressings with the exception that approximately half received WPP-201 applied topically, while the controls received only a sterile saline application. Phage application occurred weekly for 12 weeks, while dressings were changed three times weekly for the same duration. Patients were monitored weekly for the first 12 weeks of the study and data collected included wound size and severity, vital signs, and adverse events which may or may not have been related

to the phage application (adverse events included a variety of infections, injuries, medical procedures, pain, and cardiovascular issues). Wound bacterial counts, blood chemistry, and blood cell counts were performed every other week as well. Two additional assessments were made on weeks 16 and 24. There were no statistically significant differences between the test and control groups in either the number of adverse events experienced or the frequency with which wounds healed. This study did not set out to evaluate phage efficacy, but instead appraised the safety of phage application. With this objective successfully completed, the authors were able to proceed to Phase II studies, evaluating the efficacy of WPP-201.

In the final phage safety trial Merabishvili *et al*. (52) developed and began evaluating BFC-1, a phage cocktail consisting of three phages that targeted *P. aeruginosa* and *S. aureus* strains isolated from burn wounds of patients in the Burn Centre of the Queen Astrid Military in Belgium. The cocktail was sprayed onto the burn wounds of eight patients using a micromister. None of these patients were reported to experience any adverse events resulting from the phage application.

Chronic otitis caused by *P. aeruginosa* can be problematic to treat, as *P. aeruginosa* is highly antibiotic resistant. Wright *et al.* (89) conducted phase I/II trials to evaluate the treatment of this infection using Biophage-PA, a cocktail of

six phages specific for *P. aeruginosa*. The 24 volunteers in this study (each with a history of chronic otitis lasting two to 58 years) were found to have *P. aeruginosa* infections sensitive to Biophage-PA prior to participation. A single dose of either Biophage-PA or placebo was applied directly into the ear of each participant. The median bacterial counts significantly decreased in phage-treated volunteers on days 21 and 42 (swabs were taken on days zero, seven, 21 and 42), whereas there was no significant decrease in placebo treated volunteers. This study demonstrated the advantage of administering a self-replicating treatment, as the mean phage count isolated on days seven, 21 and 42 was $1.27x10^8$ PFU while the initial phage count administered was $6x10^5$ PFU. The phage persisted at the site of the infection for an average of 23.1 days and the phage were cleared in cases where the infection was successfully treated.

Each case was also scored on a visual analogue scale (VAS) by the patient and the physician. Patients scored the infection on discomfort, itchiness, wetness and odour while the physician scored the infection on inflammation, ulceration/granulation/polyps, discharge quantity, discharge type, and odour. When the patient VAS scores from days seven, 21 and 42 were averaged, there was a significant decrease from day zero for discomfort, itchiness, and wetness, as well as overall patient VAS scores, while placebo treated volunteers did not show any significant reductions. When the physician VAS scores from days seven, 21 and 42 were averaged, there was a significant decrease in

inflammation, ulceration/granulation/polyps, discharge type, and odour, as well as overall physician VAS scores, while the placebo group did not show any significant reductions. As with the other clinical trials reviewed, none of the adverse events reported during the course of the trial were deemed by the trial physician to be related to phage treatment. This study provided sufficient evidence of phage therapy efficacy to warrant the design of a phase III clinical trial.

The studies reviewed above (4, 52, 69, 89) demonstrate that regardless of the phage delivery strategy used, phages are safe for use in phage therapy applications.

Phage therapy in the BCC

Aerosol phage delivery

Although aerosol phage therapy has not been frequently reported in the literature, it remains a popular option for treating respiratory infections and many of the techniques used for aerosol drug delivery are being adapted to phage therapy. Aerosol drug delivery has been successfully employed for many pharmaceuticals including bronchodilators, antibiotics, mucolytics and anesthetics (24), as it enables a drug to reach the diseased site rapidly and requires lower doses of medication than other delivery methods (86). As phages require direct contact with their bacterial hosts in order to be active, aerosol

phage delivery appears to be a logical method of delivering phages rapidly to the site of a pulmonary infection. There are many aspects of aerosol phage therapy to be evaluated in order to ensure that it can be used as a reliable treatment method. These research areas include phage preparation, storage and delivery methods, as well as demonstration of activity *in vitro* and effective treatment of an infection *in vivo*.

Clinical treatment using inhalation phage therapy

There are three case studies that mention the use of inhalation phage therapy as a clinical treatment method. Unfortunately, one of these papers (16) is written in Russian and therefore is difficult to review. The other two papers (25, 35) are case studies describing the treatment and recovery rates of patients treated by inhalation phage therapy. Although these studies do not directly address the treatment of BCC infections, they are still of interest because they outline situations in which aerosol phage therapy has been employed in the past. The first clinical study (25) evaluated the recovery of 29 respiratory infection patients treated by an aerosolised phage cocktail. The patients were chosen because they could not be treated by conventional methods such as expectorants or secretolytics (medications used to clear mucus from the respiratory system) or antibiotic therapies. Approximately two-thirds of the patients had *Streptococcus* infections, while the remaining patients had *Staphylococcus* infections. The phage cocktail used was Diriphagen, a commercially available cocktail containing

180 to 200 phages specific for different bacterial species. In addition to phages, Diriphagen also contained an "aimed antimicrobic", which targeted bacterial strains that became phage resistant during the course of the treatment. No mention was made as to the identity or mechanism of action of the aimed antimicrobiotic. This particular phage cocktail was chosen because of the large diversity of phages it contained. During the course of the study, the patients received daily aerosolised phage treatments. The treatments lasted 10 - 15 min. The average number of treatments per patient was 11; some patients received as few as three treatments and one patient received as many as 40 treatments. After treatment, the symptoms of the 29 patients were classified into three groups: 55% were reported to have made a complete recovery, 35% showed considerable improvements and 10% showed no change. When the bacterial load in the sputum cultures was determined, it yielded contradictory results. All patients had detectable levels of either *Staphylococcus* or *Streptococcus*, with 30% of the patients showing considerable improvement, 55% showing some improvement and 10% showing no improvement. Interestingly, at least one patient showed no change in symptoms even though her bacterial counts decreased substantially. Unfortunately, there were many aspects of this study that were not properly described or quantified. There was no information given as to what exact infection each of the patients had (as it was not uniform throughout the group), making it difficult to determine if the phage cocktail was more effective against certain species or strains. No mention was made as to

what criteria were used to classify the patients' symptoms as "considerably improved" and neither were quantitative values associated with a "considerable decrease" or "some decrease" in bacterial load in the sputum. This study provides some anecdotal evidence that phage therapy may be an effective method of treating respiratory infections, but it cannot provide any conclusive results.

A more recent case study (35) described the treatment of a five-year-old cystic fibrosis patient at the Eliava Institute in Tbilisi, Georgia. This patient had both *S. aureus* and *P. aeruginosa* respiratory infections that were resistant to antibiotic treatment. These strains were sensitive to pyophage, a commercially available phage cocktail active against *S. aureus*, *P. aeruginosa*, *Streptococcus*, *Proteus* and *E. coli*. The patient completed a treatment regimen of three multi-day treatments administered at one month intervals. During the first treatment period, pyophage was administered by nebuliser daily over a six-day period. In addition to the pyophage treatment, the patient received vitamins and an antimucosal treatment. After this treatment, the patient's condition was described as improved; this characterisation was based on weight gain (the patient had gained no weight in the previous year and gained one kilogram after the treatment), facilitation of expectoration and lack of sputum. No mention was made to indicate if the bacterial load in the lungs had changed after this treatment. A second ten-day treatment was performed a month after the initial

treatment. The concentration of *S. aureus* increased while the *P. aeruginosa* concentration did not change during this treatment period. The second treatment was not effective and based on the increase in *S. aureus* concentration, it was assumed that the *S. aureus* had become pyophage resistant. The third and final treatment administered in this case study was a combination of pyophage and tetracycline. After this treatment *S. aureus* and *P. aeruginosa* could not be detected in the patient. At the time of publication the patient was still receiving phage therapy on occasion as respiratory infections were identified. It is unfortunate that this particular case study was presented as an example of successful inhalation phage therapy as the evidence is anecdotal and not particularly convincing. A quantitative change in respiratory bacterial load before and after the treatments was not reported. Instead, the efficacy of the treatment was generally evaluated based on the change in patient symptoms. The *S. aureus* and *P. aeruginosa* strains appeared to become phage resistant during the second treatment and did not decrease to undetectable levels until the phage treatment was combined with antibiotics, suggesting that the antibiotic treatment or the combination therapy at best, but not the phage therapy alone, cleared the infection. In order to demonstrate that phage therapy is an effective medical treatment, full clinical studies with control groups will be required and this case study emphasises this point.

Simulated aerosol phage delivery to humans

Methods of aerosol drug delivery are continually evolving and so it is important to assess these methods with respect to the efficacy of aerosol phage delivery, ensuring that the phages are delivered to the location of the infection and also that they are delivered intact and able to infect and lyse their bacterial host. In a respiratory infection one must ensure that the phage aerosol has the correct characteristics required for delivery to the area of the lung where the infection resides. Phage liquids can be delivered using a nebuliser. Two common types of nebulisers in use in a clinical setting are the jet nebuliser, such as the Collison or the LC Star, and the ultrasonic nebuliser, such as the eFLOW (17). The key feature of a nebuliser is that it must generate particles of the appropriate size to impact the correct location within the pulmonary system. The typical particle size generated by a nebuliser is generally 1 – 5 μm, which allows the aerosol to reach the smaller bronchioles (17).

One study has been performed to assess the feasibility of aerosolizing phage using a nebuliser that is commonly used for delivering drugs via an inhalation route (17). This *in vitro* method employed a computer controlled breath simulator to mimic the natural breathing patterns of an adult. A BCC phage, KS4-M, able to infect *Burkholderia cenocepacia* strain K56-2 was aerosolised using one of two commonly used nebulisers: the LC star (a jet nebuliser) and the eFLOW (an ultrasonic nebuliser). The aerosol size distribution of the particles

was measured as the aerosol exited the nebuliser using phase Doppler anemometry. A filter placed in line with the breath simulator collected the aerosolised phage, allowing the quantification of phage exiting the nebuliser, which was classified in the study as the inhaled phage. The collected phage were quantified by plating using a soft agar overlay method. As this method only quantified viable phage, it only took into account the number of phage that were able to survive the aerosolisation process (and would therefore theoretically be active within a host). In addition to enumerating the number of phage able to survive aerosolisation, these data were also employed in mathematical models to determine the regional lung deposition of the phage aerosol based on a numerical lung deposition model. Using this model, calculations were performed to compare the PFU deposition in the extrathoracic, tracheobronchial and alveolar regions of the lung with both of the nebulisers. The results for both nebulisers were quite similar suggesting that either class of nebuliser would be effective in generating phage aerosols. The LC star and eFLOW nebulisers produced aerosols with similar properties with mass median diameters of 4.98 µm and 5.83 µm, respectively and geometric standard deviations (the measure of particle monodisperity) of 1.48 µm and 1.44 µm, respectively. The inhaled phage concentrations produced by both of the nebulisers was also quite comparable with the LC star aerosolizing 1.06×10^8 PFU and the eFLOW aerosolizing 1.15×10^8 PFU. There was an order-of-magnitude decrease from the inhaled phage concentration (approximately 10^8 PFU) to the predicted

concentrations deposited in each of the three lung areas (approximately 10^7 PFU) for both of the nebulisers tested, however this is still a significant phage concentration being delivered to the lungs and the particle deposition in each region of the lung was similar in both cases. The results of this study add credibility to aerosol phage delivery as a potential treatment method for respiratory infection. While mathematical modeling provides an indication as to the outcome of an experiment, it is not a replacement for biological experimental data. This study serves as an excellent starting point for experimentally determining phage deposition within the lungs during aerosol phage therapy.

Phage powders

Freeze-drying

A number of recent studies have been performed to investigate phage preparation methods for use in phage aerosols, including freeze-drying (lyophilisation) and spray drying (18, 48, 66). Freeze-drying and spray drying allow the phage stock to be converted to a dry powder, which is delivered to a patient via an inhaler. Delivering phage as a powder would be a rather simple method of treating patients. A dry powder would also be simpler to handle and easier to transport than the liquid phage suspension required for nebulisation. There are a number of advantages to dry powder phage preparations including easier transport and longer-term storage capabilities. Treatments can be

delivered using a dry powder inhaler in a single breath, making it a faster delivery method than nebulisation. A recent study (66) outlines a method for freeze-drying and encapsulating phage in biodegradable poly(DL-lactic-co-glycolic acid) microspheres. The microspheres can be delivered directly to the lungs via a dry powder inhaler and have been previously approved for use in humans (42). This proof of concept was performed to determine if phages specific to *S. aureus* and *P. aeruginosa* could be freeze-dried and disseminated as well as to determine the shelf-life of the phages after freeze-drying and encapsulation. The microcapsules were developed for use in a dry-powder inhaler. The encapsulation efficiency for *S. aureus* phage was 18%, while the efficiency for the *P. aeruginosa* phage was 27%. In dispersion tests, 55% of the total released contents of the *S. aureus* phage microcapsules was released within 30 min and release of the phage continued for 6 h. Similarly, within 30 min 63% of the total released *P. aeruginosa* phage were dispersed.

Both of the phages tested could survive freeze-drying and remain at high titres for at least three days, at 4°C as well as at 22°C, which would also allow for easier handling and transport. However, after seven days the phages were no longer viable. Unfortunately, the phage titres dropped quickly enough that counts of "too numerous to count" could not be replated for accurate counts and had to be reported in a semi-quantitative fashion, so only trends could be reported. This study demonstrated that freeze-drying followed by encapsulation into

microspheres shows promise, especially as a dispersal technique, however the phages would need to be further stabilised for increased survivability.

An additional study (18) took a different approach to developing phage aerosol powders, formulating powders for both KS4-M, a BCC phage, and ΦKZ, a *P. aeruginosa* phage. Instead of encapsulating the phage powder in a microsphere, the endotoxin-removed phage stocks were lyophilised in a mixture of carriers that help to stabilise the phages as well as enhance the dispersibility of the powder. The latter is an important consideration for the future use of phage powders as a therapeutic agent because dispersibility affects the efficacy with which the phages will reach the site of infection. Golshahi *et al*. (18) demonstrated that a combination of lactose/lactoferrin 60 : 40 w/w was an effective carrier for both phage stability and dispersibility. Lactose is an excipient (or carrier) that is currently used in dry powder inhalers in the United States (85) while lactoferrin was chosen for its effect on particle size as well as for its antimicrobial properties. The combination of lactoferrin and phages has also been previously demonstrated to be more effective in treating infection in mice than either treatment separately (91). The freeze-drying process caused a reduction in phage titre, with a reduction of two orders of magnitude for KS4-M and one order of magnitude for ΦKZ. This reduction associated with freeze-drying should not pose a problem because both phages can be grown to a high initial titre. Additionally, in contrast to freeze dried, encapsulated phages (66),

both the freeze dried KS4-M and ΦKZ phages were able to maintain viability for long periods of time. Both phage titres remained essentially constant at the same order of magnitude (approximately 10^8 PFU/100mg) for three months, regardless of storage temperature (both 4°C and 22°C were tested). After a year of storage at 4°C, ΦKZ remained at 10^8 PFU/100mg while KS4-M dropped to 10^7 PFU/100mg. Even at 22°C ΦKZ fared well with the titre remaining at 10^7 PFU/100mg, while KS4-M dropped to 10^2 PFU/100mg (unpublished results). This freeze-drying method shows great promise for long-term phage storage. In addition to testing phage stability, the phage powders were tested in a commercially available Aerolizer® dry powder inhaler. When attached to a mouth-throat replica known as the "Alberta Idealized Geometry", the efficacy with which the freeze dried phages could be delivered to the site of a pulmonary infection was evaluated. This study also provided promising results with less than an order of magnitude drop from the phage load in the inhaler to deposition within the lungs. This study demonstrated that phage freeze drying is a viable method of preserving phages for long-term storage. The powdered phages also show a great deal of promise as a therapeutic delivery method in aerosol phage therapy and should be evaluated further.

Spray drying phages

Using a different approach, Matinkhoo *et al*. (48) evaluated spray drying rather than freeze-drying as a method of developing phage powders. Spray drying is

another method of producing pharmaceutical powders which has the added advantage of allowing for a greater selection in particle characteristics (88). As with Golshahi *et al*. (18), KS4-M and ΦKZ were used in the study, but an additional BCC phage, KS14 was also tested. The spray drying process was less damaging to the phages than freeze-drying with one-half to one order of magnitude drop in titre for each of the phages, depending on the excipients used. These findings show an improvement over the two orders of magnitude drop in titre after freeze drying. Although the long-term viability of the spray dried phages was not determined in this study and therefore cannot be compared to freeze dried phages, the phage powders were tested in the same mouth-throat apparatus as the freeze dried phages in the previously described study (18). The spray dried phages outperformed the freeze dried phages with respect to lung deposition with two times more spray dried phages delivered to the lungs in comparison to the freeze dried phages. Also, more than 50% of the total inhaler dose was delivered to the lungs. In comparison, a survey of 12 commercially available inhalers showed a range of delivery efficiencies from 6% to 41% (57). These data demonstrate the plausibility of using spray drying as a method of developing phage therapeutics, provided that the spray dried phages can remain stable for long periods of time.

Phage therapy evaluation *in vivo*

To date, there are two major *in vivo* models that have been used for evaluating phage therapy against BCC infections: the *Galleria mellonella* (Greater wax moth) larvae model (43, 75) and the mouse lung infection model (7). The *G. mellonella* larvae infection model is used to determine the efficacy of phages to rescue infected larvae from death. This model is relatively simple to work with: the larvae are injected first with bacteria, then phage (either immediately after infection or after a specified duration) and the percentage of deaths is then calculated 48 h post-infection. Prior to the use of *G. mellonella* as a phage therapy model, the pathogenicity of 23 BCC strains in *G. mellonella* was compared to pathogenicity in alfalfa seedlings, in rats and in *Caenorhabditis elegans*. It was demonstrated that *G. mellonella* results are comparable to those in other *in vivo* models (74). Seed and Dennis (75) initially evaluated this model for BCC infections using two strains of *B. cenocepacia*, K56-2 and C6433, given at lethal concentrations. The survival rate of the larvae increased with increased phage multiplicity of infection (MOI) for KS12. Also, a decrease in duration between infection and treatment also rescued more larvae. Using the same model, Lynch *et al*. (43) were able to compare the efficacy of a genetically modified lytic phage and the wildtype phage in rescuing infected *G. mellonella* larvae. Gene *41* of KS9, a temperate phage isolated from *B. pyrrocinia* LMG 21824, was identified to putatively encode the phage repressor. By disrupting the gene, KS9 became a lytic variant (named KS9c). KS9c was tested in the *G.*

mellonella model to determine if the conversion from a temperate phage to a lytic phage would allow it to be a more effective phage therapeutic. Unexpectedly, although the KS9c variant did not stably lysogenise the bacterial host and was active *in vivo*, it performed similarly to the wildtype phage KS9. These results demonstrate the value in having a simple animal model for testing hypotheses such as the one described. Both of these papers demonstrate the usefulness of *G. mellonella* as an initial test for evaluating the efficacy of a phage against the BCC *in vivo*.

The mouse lung infection model involves delivering first bacteria and then phage to the lungs of mice. The bacterial or phage suspensions are often delivered intranasally by placing small volumes of liquid on the nares of anesthetised mice, allowing the mice to inhale the liquid. Intranasal sample delivery is used to mimic aerosol delivery to a human via a nebuliser. This method was used for evaluating the use of aerosol phage therapy to treat a BCC infection (7). In this study mice were infected with *B. cenocepacia* via tracheotomy and 24 h post-infection were treated with phage BcepIL02 delivered by either intranasal inhalation or intraperitoneal injection. Forty-eight hours after treatment the mice were euthanised and the lungs assayed for bacterial and phage titre. Interestingly, the mice that received the intranasal phage treatment had approximately a one order of magnitude decrease in BCC bacterial load while the mice that received the intraperitoneal phage treatment had a two log drop in

BCC bacterial load. Although neither of the described phage delivery methods produced substantial therapeutic results, the authors suggest that phages delivered indirectly are more effective than phages applied directly to the infection site.

It should be noted that although intranasal delivery of liquids to the lung is meant to mimic aerosol delivery via a nebuliser, it is not an optimal replacement for actual aerosol delivery in mice. Intranasal delivery in mice has been demonstrated to be far more variable and less reproducible than aerosol delivery. Halperin *et al*. (20) demonstrated that mice given *Bordetella pertussis* respiratory infections via two methods, either intranasal inhalation or a whole body exposure chamber, had very different bacterial lung counts. The mice receiving intranasal installation showed a far higher degree of variability in bacterial load in comparison to those receiving aerosolised bacteria (a 1000-fold variability as compared to a five-fold variability, respectively). The mice receiving intranasal installation also showed a far greater variability in bacterial distribution within the lungs (right lung 43 – 84%, left lung 16 – 57%) in comparison to those receiving aerosols via the whole body exposure chamber (right lung 60 – 68%, left lung 32 – 40%). This may also translate into higher variability when treating mice in phage therapy studies and it would be of interest to see additional studies performed using a whole body exposure chamber or nose-only inhalation chamber in order to observe the effect of

aerosol phage delivery method on treatment efficacy. These data suggest that a study with similar methodologies to that of Carmody *et al*. (7) should be performed using an aerosol delivery method and the results compared to determine if there is a difference between intranasal and aerosol phage delivery in mice.

Although these studies are not directly comparable due to different host-phage interactions, it is interesting to contrast the findings of Carmody *et al*. (7) with the results found by Debarbieux *et al*. (13), Morello *et al*. (55) and Alemayehu *et al*. (1) when treating *P. aeruginosa* respiratory infections in mice. All three studies infected and treated the mice via intranasal inhalation. Debarbieux *et al*. (13) treated the mice 2 h, 4 h or 6 h post-infection and tracked mortality for 72 h. At the endpoint, 100% of mice treated 2 h post-infection, 75% of mice treated 4 h post-infection and 25% of mice treated 6 h post-infection survived. The mice treated 2 h post-infection had a bacterial load six orders of magnitude lower than untreated mice 24 h after treatment. Similarly, Morello *et al*. (55) demonstrated that mice infected with a strain of *P. aeruginosa* isolated from a cystic fibrosis patient and treated with a sufficiently high MOI 2 h post-infection and euthanised 20 h post-infection had a reduction in bacterial load of two orders of magnitude. Interestingly, they were also able to demonstrate, using immunohistochemistry, that the remaining bacteria in the treated mice were mainly in the macrophages in the lungs while the untreated mice had bacteria

throughout the macrophages, alveolae and extracellular spaces within the lungs. Additionally, Alemayehu *et al*. (1) demonstrated that a phage cocktail of ΦNH-4 and ΦMR299-2 delivered 2 h after infection was able to clear *P. aeruginosa* respiratory infections in mice. Two separate bioluminescent strains of *P. aeruginosa* (MR299 and NH57388A) were delivered intranasally and the bioluminescence within the lungs measured at 2 h intervals up to 8 h after infection. The luminescence within the lungs (which is proportional to bacterial concentration) showed a significant decrease in phage-treated mice while untreated mice showed a three-fold increase in luminescence intensity.

These findings indicate that phage therapy was able to work with the immune system in order to effectively clear the *P. aeruginosa* infection. These conflicting results between Carmody *et al*. (7) and the results obtained by Debarbieux *et al*. (13), Morello *et al*. (55) and Alemayehu (1) demonstrate the necessity for further evaluation of aerosol phage therapy.

In two of the *P. aeruginosa* studies, the effect of phage as a prophylactic was also evaluated. Debarbieux *et al*. (13) demonstrated that phage administered 24 h prior to infection allowed for 100% survival, while all mice in the control group died within two days. Morello *et al*. (55) reported a similar trend with a pretreatment occurring four days prior to infection. Although generally phage therapy is thought of as an infection treatment method, these results suggest

that aerosol phage therapy may also be a useful method for preventing BCC infection.

Conclusion

Aerosol phage therapy and phage therapy against the BCC are two fields of study that have not been investigated in great detail until recently. Through the use of two different *in vivo* models, the *G. mellonella* model and the mouse lung infection model, phages have recently been demonstrated to be effective against the BCC. Using a mechanical lung model it was demonstrated that BCC phages could successfully be delivered to the human lung, remaining viable after deposition within the lung. Additionally, dry powder aerosol phage delivery appears to be a promising alternative to liquid phage nebulisation. BCC phages have been successfully freeze dried and spray dried and can be successfully stored and aerosolised after processing. With advancing research, BCC phage therapy continues to show promise as an alternative antimicrobial therapy.

Aerosol phage therapy in BCC

Introduction

Patients diagnosed with cystic fibrosis (CF) are predisposed to acquiring a wide range of respiratory infections including *S. aureus*, *Haemophilus influenzae*, *P. aeruginosa* and BCC organisms (23). Although a minority of CF patients acquire BCC infections, these infections are problematic due to their ability to spread rapidly between patients (41), and in up to 20% of patients the infection will progress to cepacia syndrome which is characterised by acute respiratory failure, septicemia, and reduced life expectancy (29, 71). Of the 17 species that comprise the BCC, *B. cenocepacia* (the species under investigation in this study) has been of special interest to the CF community as it has been the most common BCC isolate in Canadian (83% of BCC infections) (81) and American (46%) (68) patients. Treatment of BCC infections is complicated by the innate multi-antibiotic resistance of the BCC (40, 64, 76).

Phage therapy provides an intriguing alternative to antibiotic treatment by employing phages to reduce or eliminate an infection. Phages penetrate and lyse a bacterial host with specificity (often targeting a single species or strain), allowing for targeted treatment of an infection without disruption of natural host microflora. Previous research using animal models has shown that phage therapy can be effective against a wide range of infections including, but not limited to, burn wounds (34, 51), systemic (8) and respiratory (1, 7, 13, 55). A number of clinical trials have also been successful. These include phase I clinical

studies of phage application to venous leg ulcers (69) and burn wounds (52) and a phase I/II clinical study of phage therapy for chronic otitis caused by *P. aeruginosa* (89).

Aerosol drug delivery is routinely employed as a mode of delivering a wide range of therapeutics (24) directly to the lungs of a patient and requires lower drug doses than would be required for other routes of delivery (86). However, until recently there has been very little written about aerosol phage therapy in the scientific literature other than case studies (25, 35). Contemporary studies using mice have begun to investigate phage therapy as treatment for *P. aeruginosa* (1, 13, 55) and BCC (7) respiratory infections. Although previous studies have investigated phage therapy for treating respiratory infections in a mouse model, they have always done so using intranasal instillation as the delivery method (1, 7, 13, 55). Since prior research has demonstrated that phages can be successfully aerosolised without being damaged (17), this study aimed to demonstrate that aerosol phage therapy is effective when phages are delivered as a nebulised aerosol. Aerosolizing the therapeutic agent would be the most ideal method of phage delivery when treating a patient with a respiratory infection, as it is already an established mode of drug delivery.

This study set to develop a mouse model for evaluating aerosol phage therapy. A Nose-Only Inhalation Device (NOID) was used both to deliver *B. cenocepacia* to

the lungs of immunocompromised mice as well as to deliver the phage therapeutic one day after infection. Five BCC phages were tested to compare the efficacy of the aerosolised phages to treat acute bacterial lung infections *in vivo*. Of these five phages used in this study, KS4-M and KS12 have been previously shown to cure *B. cenocepacia* strain K56-2 infections in the *G. mellonella* (greater wax moth) model and KS14 has been shown to treat *B. cenocepacia* C6433 in *G. mellonella* (75). Also, BcepIL02, a phage closely related to DC1, has been shown to be effective against *B. cenocepacia* AU0728 in mice (7, 46). Additionally, the efficacy of aerosol phage therapy was compared to intraperitoneal (IP) phage delivery in order to determine which phage delivery method was more effective.

Materials and Methods

Bacterial preparation

The strains used, *B. cenocepacia* K56-2 and C6433, are both respiratory isolates from Canadian cystic fibrosis patients (12, 47). When cultured for mouse infection, the hosts were grown aerobically in half-strength Luria Bertani broth (½ LB; 5 g/L bactotryptone, 2.5 g/L yeast extract, 2.5 g/L NaCl) at 30°C for 16 hr with shaking to an optical density of approximately two. After growth the cells were centrifuged at 3200 x g for 10 min using a Centrifuge 5810 R (Eppendorf) and resuspended in Hank's Balanced Salt Solution with 1% gelatin (gHBSS) (Sigma

Aldrich) to the starting volume. The final host concentration delivered to the mice was generally 2 - 6x10^9 CFU/mL.

Phage preparation

All phages used in this study (see Table 2) were initially grown either in liquid media or using an agar overlay method. In order to grow phage in liquid media, 200 mL of ½ LB broth was inoculated with 2 mL of host pre-grown overnight and incubated at 30°C with shaking. After 2 h, 20 mL of previously propagated phage (with a concentration of 10^8 – 10^9 PFU/mL) was added and the flask was incubated under the same conditions for an additional 4 h. The final phage stock was filter sterilised using a Rapid-Flow sterile bottle top filter with 0.45 µm pore size (Nalgene).

When grown on solid medium, 300 µL of previously propagated phage (at a concentration of 10^8 – 10^9 PFU/mL) and 300 µL of host pre-grown overnight were incubated at room temperature for 20 min in a 16x125 mm glass culture tube. After incubation 10 mL of 55°C ½ LB top agar was added and the mixture was poured onto a 150 mm ½ LB agar plate. (Standard ½ LB agar contains 15 g/L select agar while top agar contains a reduced concentration of 7 g/L select agar). A total of 25 agar plates were inoculated. After at least 16 hr incubation at 30°C, the plates were overlaid with 10 mL of sterile milliQ water and rocked for a minimum of 2 h. The liquid was removed from the agar plates and filter

sterilised using a Rapid-Flow sterile bottle top filter with 0.45 µm pore size (Nalgene).

The resulting 200 mL of phage lysate (obtained from either method) was ultracentrifuged in an Optima LE-80k ultracentrifuge (Beckmann) with a Type 70 Ti rotor for 70 min at 371 000 x g (60 000 rpm), the pellet resuspended in 6 - 8 mL sterile milliQ water and filter sterilised using a sterile 0.45 µm mixed cellulose ester Millex syringe filter unit (Millipore). Endotoxin was removed from the final phage preparation using either a Detoxi-Gel Endotoxin Removing Column (Thermo Scientific) or Pierce High Capacity Endotoxin Removal Spin Column (Thermo Scientific).

Preparation of heat inactivated phage followed the protocol outlined above. After endotoxin removal the KS12 stock was incubated at 80°C for 15 min. The phage inactivation was confirmed by a plaque assay.

Table 2. A summary of phages used in this study.

Phage	Host Used in this Study	Temperate/ Lytic	Family	Reference(s)
DC1	*B. cenocepacia* C6433	Temperate	Podoviridae	(46)
KS4-M	*B. cenocepacia* K56-2	Temperate	Myoviridae	(75)
KS5	*B. cenocepacia* K56-2	Temperate	Myoviridae	(73)
KS12	*B. cenocepacia* K56-2	Likely lytic	Myoviridae	(45, 75)
KS14	*B. cenocepacia* C6433	Temperate	Myoviridae	(75)

Mouse model

This mouse model used six to eight week old female BALB/c mice (Charles River or Jackson Labs). Prior to infection the mice were immunocompromised by intraperitoneal (IP) cyclophosphamide (CPA) injections in order to allow the infection to persist for the three-day period from infection to euthanisation. CPA acts by causing leukopenia (10). The CPA was administered twice, three days apart, at a concentration of 150 μg/g of mouse weight.

Infections were performed either one or two days after the final CPA injection (after a reduction of approximately 5% from the average starting mouse weight). All mice were either infected with *B. cenocepacia* (either K56-2 or C6433 depending on the phage tested) or mock-infected with gHBSS using the NOID (Figure 2). NOID functional details and efficiencies are described in detail by Nadithe *et al*. (56). Briefly, the mice were anesthetised by a ketamine (0.85 mg/g of mouse weight; Ketaline, Bimeda MTC) xylazine (0.1 mg/g of mouse weight; Rompun, Bayer HealthCare) mixture administered by an IP injection, their eyes coated with Refresh Lacri-lube (Allergan) to prevent drying and inserted into the

NOID. The *B. cenocepacia* or gHBSS was disseminated using an LC Star nebuliser (Pari International) attached to a Proneb Ultra air compressor (Pari International). The aerosolised solution was drawn through the NOID and delivered directly to the nose of the mice and inhaled into the lungs (Figure 2). All excess and exhaled aerosol was exhausted through Respirgard II filters (Vital Signs, Inc.), which are suitable for filtering both bacteria and viruses. Exposure lasted 10 min and used approximately 2.5 mL of liquid. Prior to further use the NOID was decontaminated by Virkon (DuPont Chemical Solutions Enterprise) aerosolisation, followed by sterile milliQ water aerosolisation.

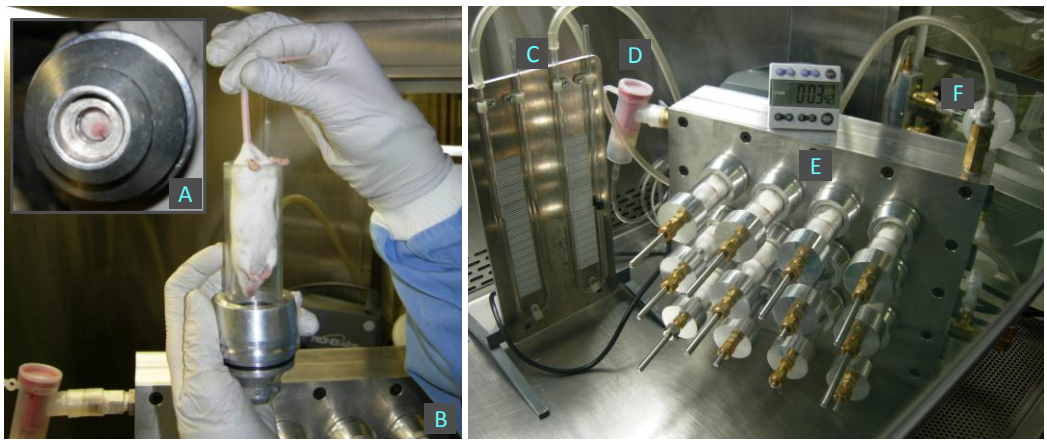


Figure 2. Functional operation of the Nose-Only Inhalation Device (NOID). The anesthetised mice are inserted into plexiglas tubes (B) containing an adaptor that allows only the nose of the mouse to be exposed to aerosol (A) once attached to the NOID (E). The solution to be aerosolised is disseminated by a Pari LC Star nebuliser (D). The aerosol is drawn through the NOID by a vacuum pump (not shown) and exhausted through filters (F), thereby preventing aerosol release from the apparatus. The flow through the NOID is monitored by a manometer (C). The complete NOID system is contained within a biosafety cabinet.

One day after infection mice were either treated with phage or mock-treated with sterile milliQ water. The treatment was either delivered by a 200 µL IP injection or by the NOID, using the same procedure as for infection. Heat-killed KS12 was also delivered by the NOID.

Food and water was provided *ad libitum* throughout the test period. At the trial endpoints the mice were euthanised by CO_2 asphyxiation, the lungs harvested, rinsed in sterile gHBSS and placed in 3 mL gHBSS. All procedures involving mice were approved by the University of Alberta Animal Care and Use Committee.

Sample processing and data analysis

The lungs were weighed and mechanically homogenised with a Brinkmann Polytron Homogeniser PCU-11 (Kinematica AG) for 2 min and plated by spread plate method (for host colony counts) or by agar overlay method (for phage plaque counts) on ½ LB agar containing 300 mg/L Ampicillin sodium salt (Sigma-Aldrich).

Randomly chosen colonies from the lungs of 20 mice treated with KS12 delivered by NOID and 20 mice treated with KS12 delivered by IP were tested to determine if the passaged K56-2 had developed phage resistance using a plaque assay. In no case was resistance observed.

Data were plotted as a box and whisker plot (63) and statistical significance was determined by a one-tailed Mann-Whitney U test.

Luminescent plasposon library screening

A previously created bioluminescent plasposon mutagenesis library of *B. cenocepacia* K56-2 containing pTn*Modlux*OTp' (70) was screened both for luminescence intensity and duration using a Victor X3 Multilabel Plate Reader (PerkinElmer). The library was initially grown in 96-well plates with 150 µL ½ LB broth and 30 µL host at 30°C with shaking for 72 h. Plates were incubated for 72 h with OD_{600} and luminescence readings taken at 0 h, 24 h, 48 h and 72 h. The

normalised luminescence (calculated as luminescence divided by OD_{600}) was calculated for each mutant (data not shown). Samples demonstrating a consistent high luminescence were further tested under three different growth conditions: at room temperature with no shaking, at 30°C with shaking and 30°C for 6 h with shaking followed by incubation at room temperature.

The final mutant chosen was 9H08, which has an insertion at position 2 136 597, which is a putative phage integrase pseudogene BcenGI13 (26).

Imaging

The luminescent K56-2 strain 9H08 was assayed for luminescence intensity using the previously described *G. mellonella* waxworm infection model (75). Initial screens were performed to determine what concentration of K56-2 9H08 was required to be detected by the IVIS Spectrum (Caliper Lifesciences) imaging system. The larvae were injected with a range of bacterial concentrations serially diluted from $1.7x10^7$ CFU to $1.7x10^1$ CFU in sextuplet and imaged within 15 min of infection (exposure time 1 s).

For phage clearance tests in *G. mellonella*, larvae were injected with approximately $2x10^7$ CFU/larva of K56-2 9H08, imaged and treated with approximately $6x10^7$ PFU/larva within 1 h of infection. Larvae were imaged regularly over a 6 h period with either a 1 s or 3 s exposure time.

For phage clearance tests in mice, the same infection model as described above was used with minor additions. Mice were imaged lying on their backs immediately after infection, prior to and immediately after treatment, 2 h, 4 h, 6 h, 24 h and 48 h after treatment using an exposure time of 15 min. With the exception of immediately after infection and treatment, mice were anesthetised using isoflurorane prior to imaging.

Results and Discussion

Aerosol drug delivery is a popular and effective method of delivering therapeutics directly to the diseased site in a patient with respiratory illness. However, to date murine respiratory phage therapy studies have not evaluated this method of phage delivery. Previous studies have established experimental lung infections using either intranasal instillation or tracheotomy and delivered phage via intranasal instillation or IP injection (1, 7, 13, 55). Placing a phage preparation on a mouse's nares and having the mouse aspirate the liquid often results in a great deal of variability in particle deposition within the lung. Conversely, the NOID uses a jet nebuliser designed for medical applications to generate aerosolised phage particles that are better suited for alveolar deposition (20). This study demonstrates that aerosol phage therapy is not only possible, but an effective method of delivering phages directly to the site of an

infection. Under the correct conditions, infected mice receiving phage treatment via aerosol demonstrated a significant decrease in bacterial load within the lung.

Determining the necessity of CPA and infection delivery schedule

As previous studies have shown (10, 11), a significant complication with the *B. cenocepacia* respiratory infection mouse model is maintaining the infection. Mice that are not immunocompromised show a dramatic decrease in bacterial titre even 24 h post-infection (10), while immunocompromising the mice will delay the dramatic clearing by a few days (11). However, previous studies had not investigated the effect of multiple infections on bacterial clearance. The bacterial concentration in the lungs of mice after infections on three consecutive days or six days of infection delivery (three consecutive days, one day break followed by another three consecutive days of infection) was compared to mice given two CPA treatments (one and four days before infection) followed by a single infection (Figure 3). One day after the final infection the mice in each group showed quite different levels of bacterial clearance. The mice receiving three infections maintained approximately 10^3 CFU/g of lung one or two days after the final infection. Mice receiving six infections were able to maintain a bacterial load (10^6 CFU/g of lung) far closer to those receiving two CPA injections (10^7 - 10^8 CFU/g of lung). Infecting the mice six times showed some promise, however mice develop a resistance to anesthetic, making it difficult use this infection model while maintaining a consistent level of anesthesia throughout

the course of the test. The use of an immunocompromising agent was chosen as it was a much simpler method of maintaining the bacterial load within the lung than multiple infections.

CPA delivery schedules of either two or three injections were compared for efficacy. In at least one instance the use of three CPA injections led to significant weight loss and two mice needed to be euthanised prior to infection. This led to the choice to use two CPA injections. Generally mice received CPA four and one days prior to infection. However, in rare instances there appeared to be very little or no weight loss after this treatment and an extra day was given to allow the CPA to have an effect before the mice were infected.

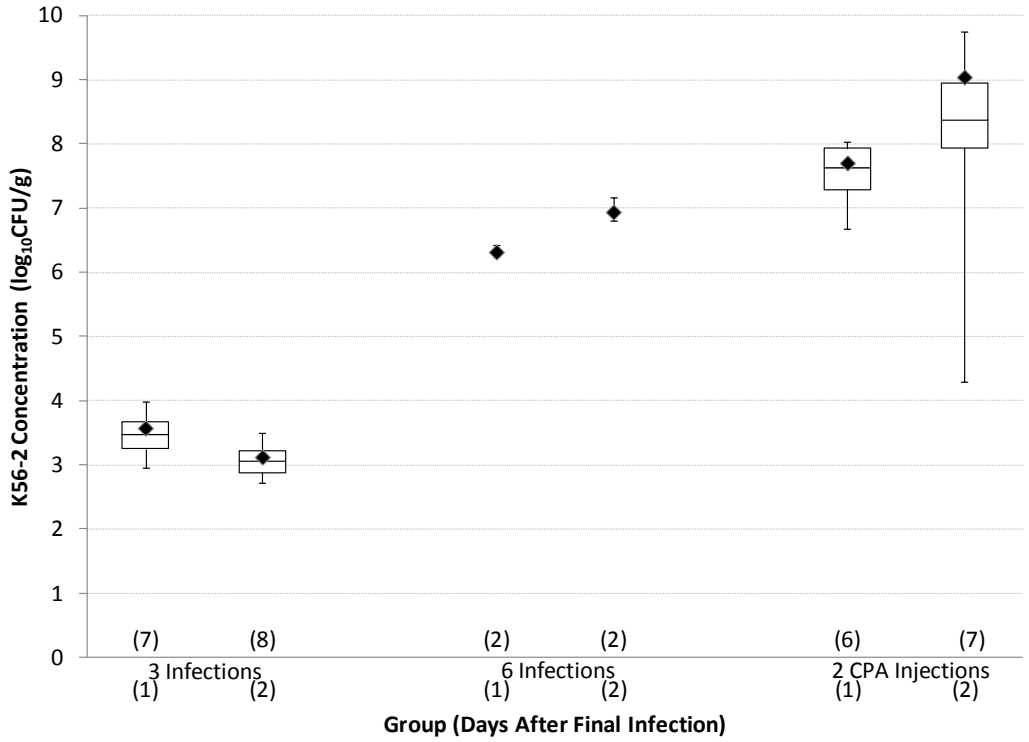


Figure 3. A comparison of *B. cenocepacia* K56-2 lung concentration with non-immunocompromised mice given multiple infections or immunocompromised mice given a single infection. Bacterial concentrations were determined one and two days after the final infection. The boxes indicate the first quartile, median and third quartile while the whiskers indicate the maximum and minimum values. The diamond marks the mean value. This trial was performed once. The number of mice in each group is indicated above the x-axis.

Determining the best sampling days

In order to determine the optimal duration from treatment to euthanisation, the bacterial load within the lungs was followed over a four-day period post-infection. Although one day after treatment was not sufficient for some phages to demonstrate efficacy (Figure 4), it was sufficient for other phages, provided that the phage concentration administered was high enough (Figure 5). (Optimal phage concentration is discussed further below.) By three days after treatment,

mice that did not receive phage were clearing the infection to the same degree as those that did receive phage (Figure 4). Two days after treatment was the optimal sampling duration for evaluating the efficacy of a phage treatment. Phage that appeared to be effective one day after treatment still showed efficacy after two days and yet this was not a long enough duration for mock-treated control mice to clear their infections.

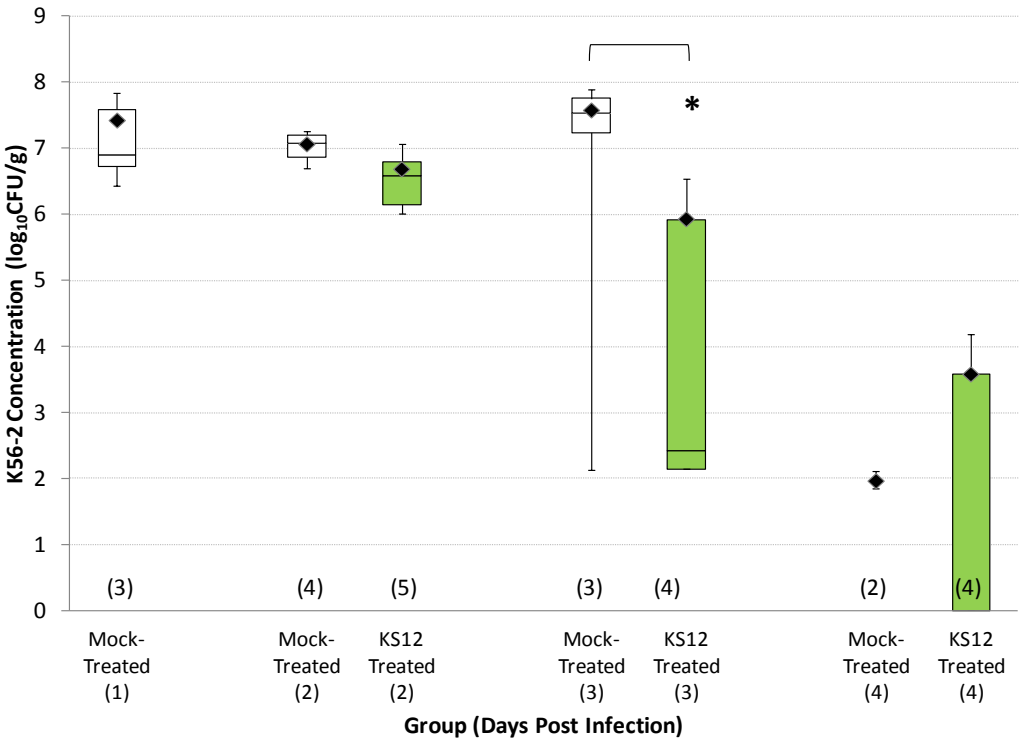


Figure 4. The bacterial clearance in mock-treated (white boxes) and KS12 treated (green boxes) mice over a four-day period. Bacterial concentrations were determined on the day of treatment as well as the following three days. The boxes indicate the first quartile, median and third quartile while the whiskers indicate the maximum and minimum values. The diamond marks the mean value. This trial was performed once. The number of mice in each group is indicated above the x-axis. *P<0.05 (Mann-Whitney U test)

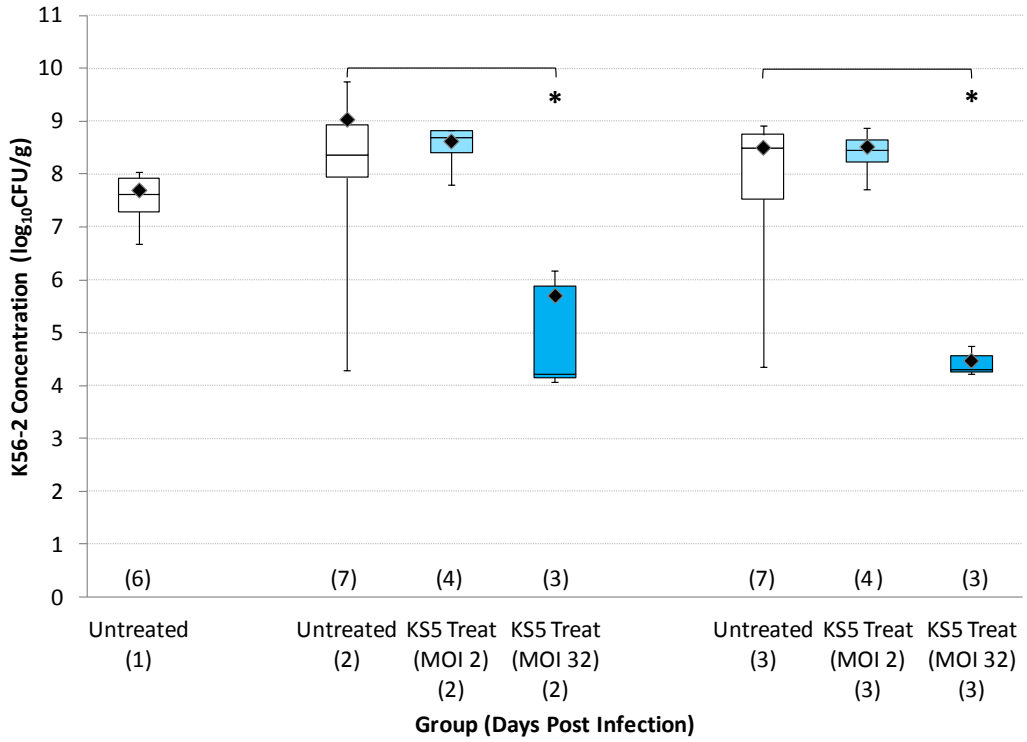


Figure 5. The bacterial clearance in mock-treated (white boxes) and mice treated with KS5 at a low MOI (2; marked with light blue boxes) or a high MOI (131; marked with dark blue boxes) over a three-day period. Bacterial concentrations were determined on the day of treatment as well as the following two days. The boxes indicate the first quartile, median and third quartile while the whiskers indicate the maximum and minimum values. The diamond marks the mean value. This trial was performed once. The number of mice in each group is indicated above the x-axis. *P<0.05 (Mann-Whitney U test)

Evaluating the effect of *P. aeruginosa* on mouse health

The BALB/c mice used in this experiment appeared to be healthy at the beginning of each experiment, however, they were not germ-free. In a number of experiments, certain mice would appear to lose more weight than others in the group and although they would appear to recover normally after infection, they would either be found dead the next morning or lose > 20% of their body weight and could not continue in the trial due to a rapid decline in health. When

the lungs of these mice were plated, they would yield not only *B. cenocepacia* colonies, but also greenish colonies characteristic of *P. aeruginosa*. These colonies were confirmed to be *P. aeruginosa* by 16S RNA sequencing. The University of Alberta Biosciences Animal Services Veterinarian indicated that the presence of *P. aeruginosa* should not be affecting the overall health of the mice, but instead, once the mouse had died the *P. aeruginosa* would have rapidly grown within the mouse (Dr. Craig Wilkinson, personal communication). Yet, the BCC and *P. aeruginosa* co-colonize the CF lung and are able to communicate through quorum sensing (9, 22, 39, 50, 72). The current CF infection model within the CF lung suggests that *P. aeruginosa* is able to promote BCC attachment to epithelial cells and upregulate the production of BCC virulence factors (9, 22, 39, 50, 72). This suggests that it is plausible for *P. aeruginosa* to promote further decline in mouse health. Regardless, once the mouse supplier was changed to one in which the mice tested negative for *P. aeruginosa*, mouse health improved dramatically throughout a test period. Mice were no longer found dead the morning after infection (with the exception of those unable to recover from anesthesia, see below) and *P. aeruginosa* was no longer isolated from the lung.

Optimising ketamine concentration

There has been some conflicting information in recent literature regarding considerations one when choosing an anesthetic for murine respiratory studies.

One study found that intranasal instillation was more effective when mice were anesthetised by an inhaled anesthetic rather than a parenteral (or injected) anesthetic (54), while an earlier study found no difference between the two forms on anesthetic (80). In this model the use of a parenteral anesthetic (ketamine/xylazine mixture) was necessary in order to maintain anesthesia throughout the NOID exposure period.

One of the complications with using a ketamine/xylazine mixture is that the drugs must be metabolised before the mouse is able to recover (in contrast to the reversal of an inhaled anesthetic such as isoflurane which is quickly nulled after mice are removed from the anesthetic). This makes overdosing a greater threat. Mice that receive too high a level of anesthetic become hypotensive and respiration rates are seriously impeded. In severe cases the mice will not recover from anesthesia (in an extreme case over 10% of mice in a trial were lost to anesthetic overdosing). In an effort to reduce or eliminate anesthesia overdosing a range of ketamine concentrations was tested. With a dose of 50% of the original ketamine concentration (0.57 mg/g) the mice were not sufficiently sedated, however, a dose of 75% (0.85 mg/g) of the original ketamine concentration was sufficient to lightly anesthetise the mice while maintaining proper respiration rates and allowing for rapid recovery (10 - 15 min as opposed to 30 - 45 min).

Prior to reducing the ketamine dose the bacterial concentration delivered to the mouse lungs was often quite varied. When assayed immediately after bacterial exposure and anesthetic recovery, the group of mice would show a wide range of bacterial concentrations, sometimes spanning more than two orders of magnitude (Figure 6). However, after the reduction in ketamine dose, none of the groups spanned more than two orders of magnitude and only one of the trials had a span of greater than one order of magnitude. This highlights the necessity of determining the appropriate level of anesthetic in an aerosol delivery study. When the mice are able to respire easily and naturally this enhances the repeatability of particle deposition within the group.

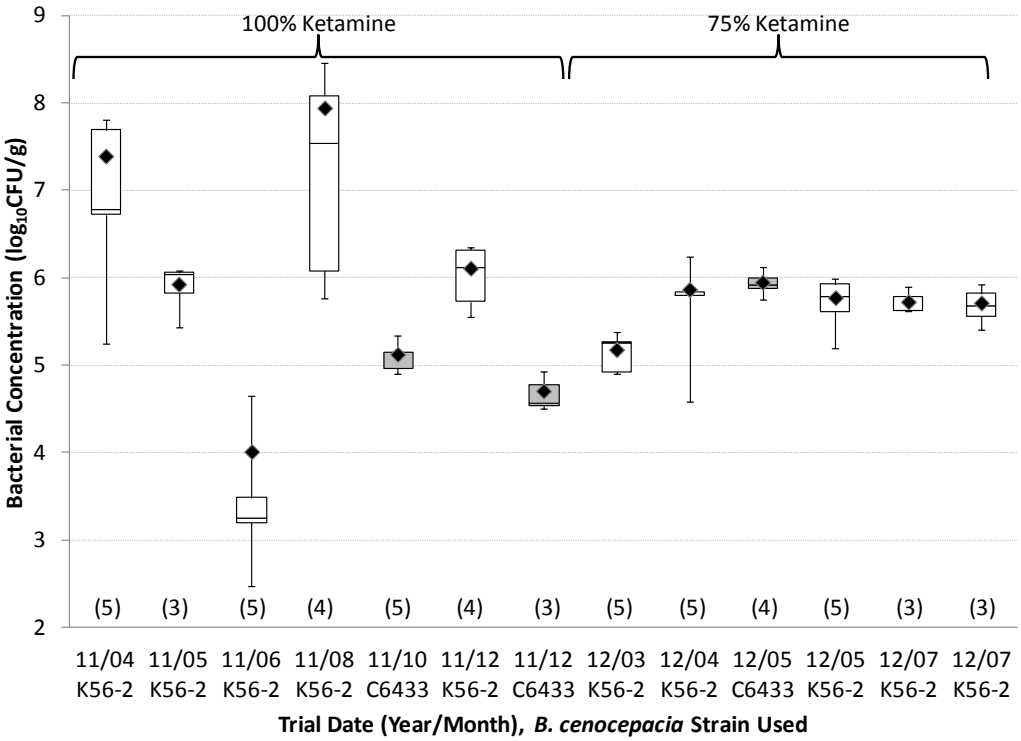


Figure 6. The bacterial lung concentration the same day as infection in mice given the original ketamine concentration (100%) or a reduced ketamine concentration (75%). Two different *B. cenocepacia* strains were used in this study: K56-2 (white boxes) and C6433 (grey boxes). The boxes indicate the first quartile, median and third quartile while the whiskers indicate the maximum and minimum values. The diamond marks the mean value. The number of mice in each group is indicated above the x-axis.

Optimising bacterial and phage starting titres

One of the challenges in developing a respiratory BCC infection model in mice is rapid bacterial clearance. Bacterial clearance can be delayed by immunocompromisation and can also be optimised by delivering the correct bacterial concentration to mice. The starting titre used in this study was chosen to optimise the *B. cenocepacia* residence time, MOI and mouse health.

If the starting titre in the lungs of the mice was too low, the infection was cleared too rapidly, even in immunocompromised mice. As shown in Figure 7, if the mice began the test with a low bacterial lung concentration such as 10^3 CFU/g, the bacterial concentration would remain low and continue to drop over the course of the next three days of the trial. This made it difficult to distinguish between natural clearance and clearance due to phage activity. Conversely, if the starting titre was over 10^6 CFU/g of lung (Figure 8) the phage did not appear to have any bacterial clearing effect and some mice became too ill to complete the study.

One of the limiting factors in treating mice with high bacterial lung concentrations may have been the highest phage titre that could be delivered to the mice. The highest titre the phages used in this study could be repeatably grown to ranged from about 10^8 to 10^9 PFU/mL. After concentration by ultracentrifugation, the titre was increased approximately five to ten times, generally giving a final phage titre of 10^9 to 10^{10} PFU/mL. The bacteria delivered to the lungs were at a concentration of $2 – 6x10^9$ CFU/mL, generally giving a final MOI between one and ten. (In this study MOI was calculated as the concentration of phage in the aerosol solution divided by the concentration of bacterial host in the aerosol solution.) Although KS12 showed some activity when delivered at these MOIs, others (especially KS5 and KS4-M) required higher MOIs (which could not be reliably obtained) in order to be active. Although

other concentration methods (polyethylene glycol precipitation and centrifugal filter units) were tested, they were not effective (data not shown). Were a more effective concentration method available, it may have been possible both to start with a higher bacterial titre as well as to reliably demonstrate positive phage activity with more phages.

Determining the correct balance between starting bacterial and phage titres highlights one of the complications with phage therapy pharmacokinetics. Theoretically speaking, active phage therapy (administering a single phage treatment and using phage replication as a means of further phage dosing) should be effective even with low starting phage concentrations (62), however, this may not be the case in practice, especially when an infection is widespread or at a very high concentration. In these situations delivering multiple phage treatments throughout the treatment period may be a more feasible alternative. In this study, administering a second phage treatment prior to the end of the trial may have yielded further reduction in the bacterial lung concentration.

The pharmacokinetics of phage therapy are completely different from a traditional antibiotic. Whereas an antibiotic will be degraded shortly after being administered, phages are able to replicate once infecting a host. This should hypothetically render even a low phage concentration effective as the phage concentration will increase until the host is eliminated. This theory was tested by administering a low number of phages to the mice. As shown in Figure 5, low

phage titres were not effective in treating the infection. The figure shown is representative of all phages tested at an MOI of two or lower (KS4-M, KS5, KS12, DC1, KS14).

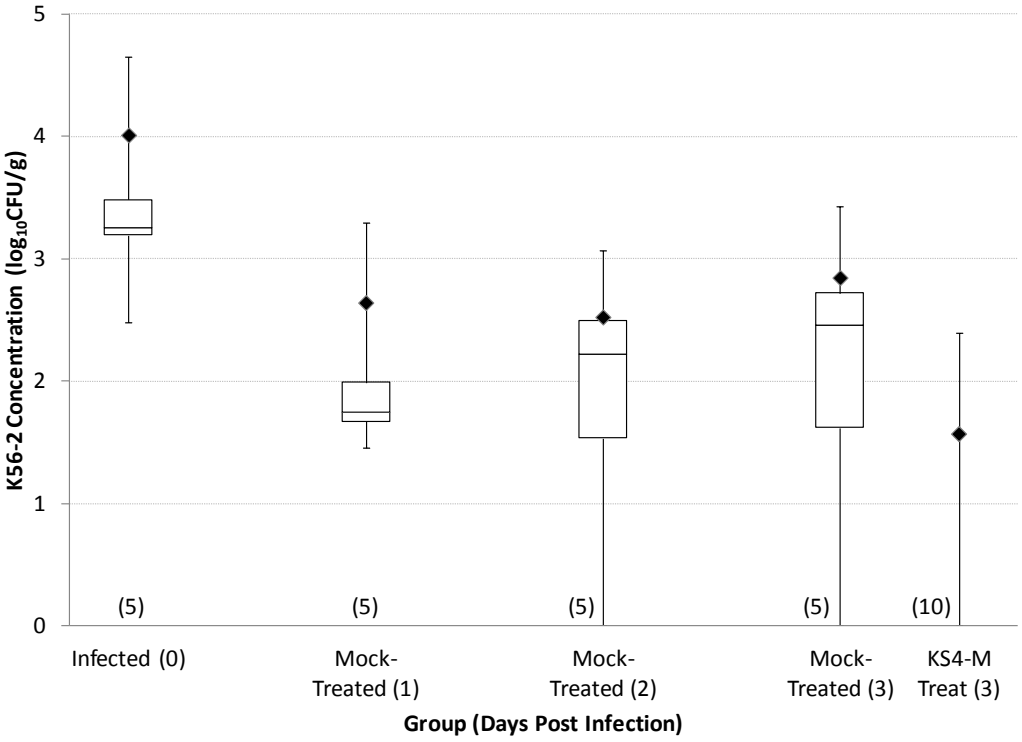


Figure 7. The bacterial clearance in mock-treated (white boxes) mice and mice treated with KS4-M over a four-day period when mice begin the trial with a low starting bacterial concentration ($1.8x10^3$ CFU/g of lung). Bacterial concentrations were determined on the day of infection, on the day of treatment as well as the following two days. The boxes indicate the first quartile, median and third quartile while the whiskers indicate the maximum and minimum values. The diamond marks the mean value. This trial was performed once. The number of mice in each group is indicated above the x-axis.

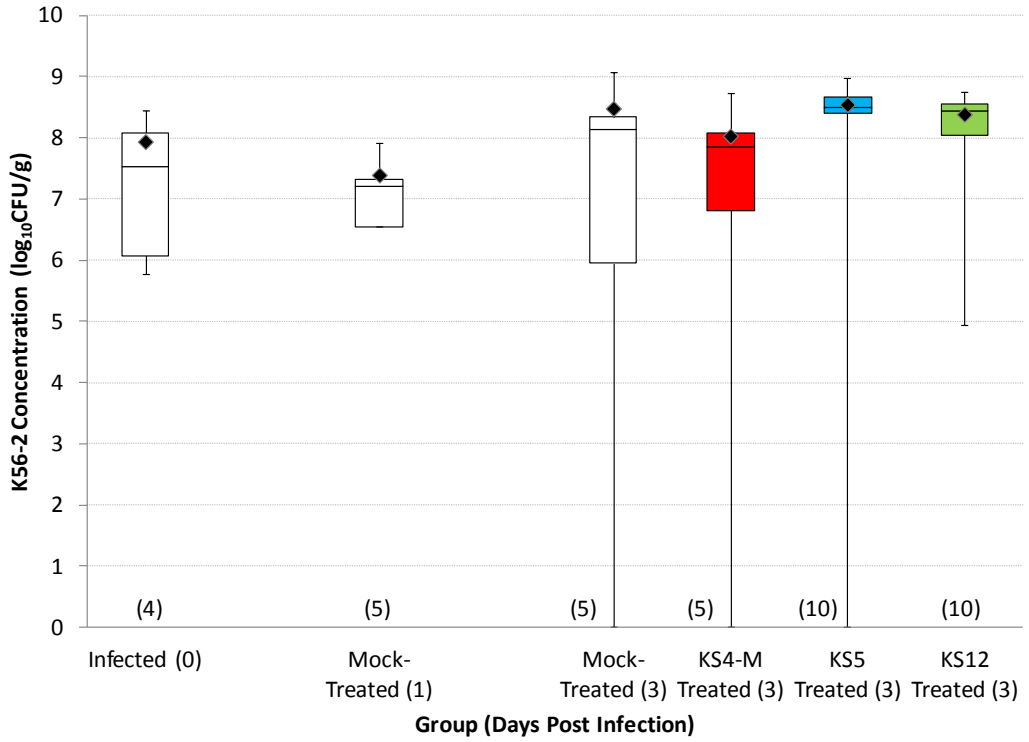


Figure 8. The bacterial clearance in mock-treated (white boxes) mice and mice treated with KS4-M (red box), KS5 (blue box) and KS12 (green box) over a four-day period when mice begin with a high starting bacterial concentration ($3.4x10^7$ CFU/g of lung). Bacterial concentrations were determined on the day of infection, on the day of treatment (one day after infection) as well three days after infection. The boxes indicate the first quartile, median and third quartile while the whiskers indicate the maximum and minimum values. The diamond marks the mean value. This trial was performed once. The number of mice in each group is indicated above the x-axis.

Comparing phage efficacy

Initial tests were performed with five different phages all able to infect *B. cenocepacia* (KS4-M, KS5 and KS12 infected strain K56-2, DC1 and KS14 infected strain C6433). Some of these phages had previously been shown to be active against *B. cenocepacia* in the *G. mellonella* infection model (75). In this study phages were evaluated for their ability to be repeatably propagated to high titres (at least 10^9 PFU/mL) in addition to their activity in the mouse model. KS12 was

found to be the best candidate for phage therapy in the mouse lung infection model as it could be reliably grown to a titre that was effective in the mouse model (Figure 14). The reduction in median bacterial concentration with KS12 was dramatic (five orders of magnitude) when phages were administered at an MOI of 131 (Figure 4), however, a reduction of over one order of magnitude could be achieved with an MOI as low as three. Additionally, there appears to be a correlation between the KS12 MOI delivered to the mice and efficacy of the phage (Figure 11) suggesting that phage efficacy will increase as delivered phage titre is increased.

KS5 (Figure 5) initially showed a great deal of promise as an effective phage therapeutic as one of the initial tests showed a dramatic decrease in bacterial titre after treatment. However, it could rarely be grown to a titre of 10^{10} PFU/mL and the phage stock could not be concentrated by a full order of magnitude to 10^{11} PFU/mL, which was the concentration required to achieve an MOI of greater than 10. KS5 could not be further evaluated as a therapeutic because it could not be concentrated to a high enough titre. Similarly, consistently obtaining a higher phage titre might have also demonstrated the efficacy of KS4-M (Figure 9), because it showed activity when delivered at an MOI of 11. Again, this MOI could not be achieved consistently because KS4-M could not be grown to a high enough titre regularly.

DC1 (Figure 10) appears to be the least effective phage therapeutic as, even when administered at an MOI of 122, it did not show a reduction in median bacterial concentration in the lungs. KS14 (Figure 10) did not show a reduction median bacterial concentration in the lungs either, however it was administered at an MOI of 5, so again, this might have been effective if it could have been grown to a higher titre.

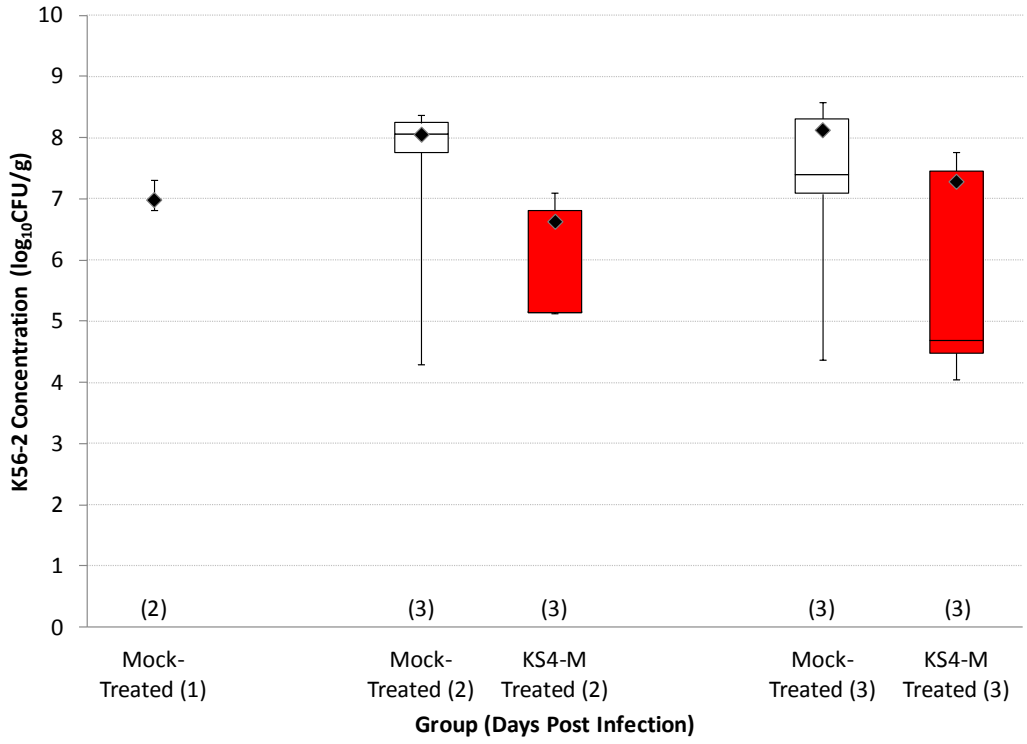


Figure 9. The bacterial clearance in mock-treated (white boxes) and KS4-M treated (red boxes) mice over a three-day period. Bacterial concentrations were determined on the day of treatment as well as the following two days. The MOI of the treatment was 11. The boxes indicate the first quartile, median and third quartile while the whiskers indicate the maximum and minimum values. The diamond marks the mean value. This trial was performed once. The number of mice in each group is indicated above the x-axis.

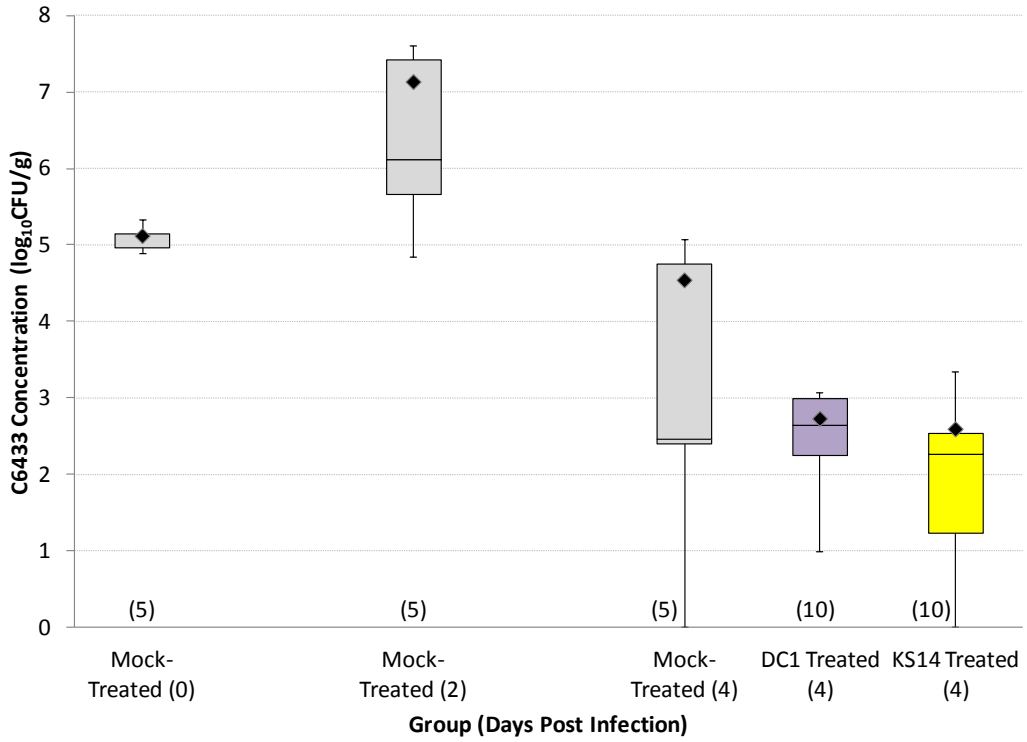


Figure 10. The bacterial clearance in mock-treated (grey boxes), DC1 treated (purple box) and KS14 treated (yellow box) mice over a four-day period. Bacterial concentrations were determined on the day of infection, the day of treatment as well as two days after treatment. The MOI of the DC1 treatment was 122 and the MOI of the KS14 treatment was five. The boxes indicate the first quartile, median and third quartile while the whiskers indicate the maximum and minimum values. The diamond marks the mean value. This trial was performed once. The number of mice in each group is indicated above the x-axis.

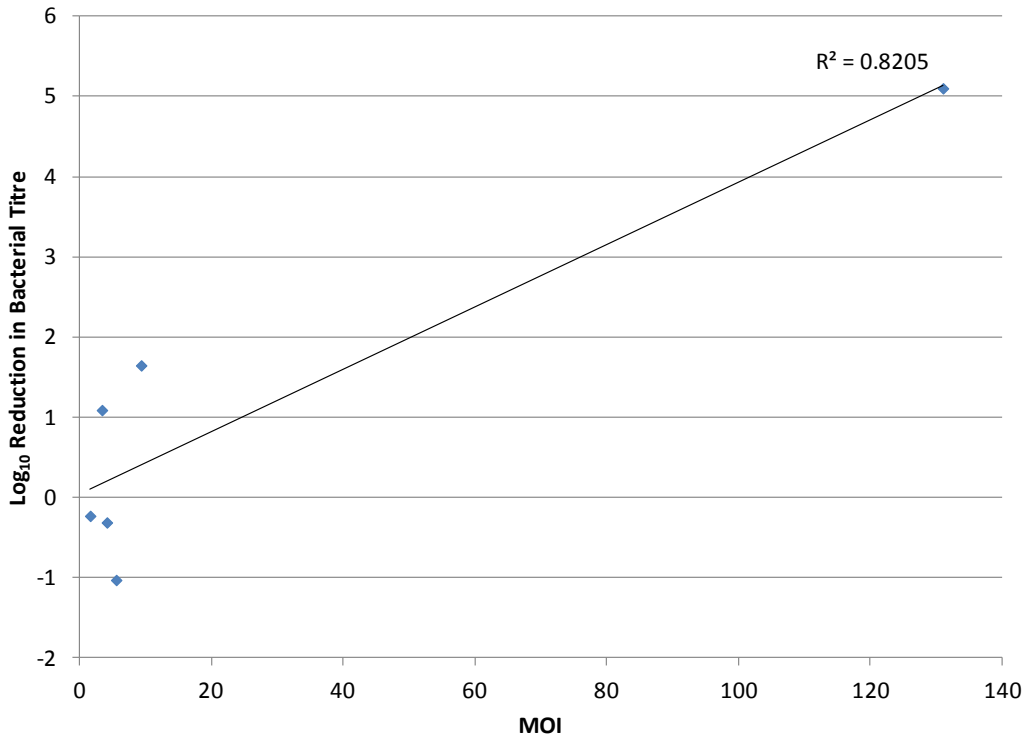


Figure 11. The correlation between KS12 MOI and the reduction in bacterial lung titre. Each data point notes the bacterial reduction from mock-treated to KS12 treated mice in a single trial.

In order to demonstrate that phage activity is required for effective phage therapy, the efficacy of heat-killed phage was also tested. Infected mice treated with aerosolised heat-killed KS12 showed no bacterial clearance when compared to mice mock-treated with sterile milliQ water. This is in contrast to the significant decrease in bacterial titre found in mice treated with active KS12 (Figure 12). This demonstrates that phage activity is required for effective phage therapy and bacterial clearance is not a result of upregulation of the immune system due to the introduction of foreign proteins.

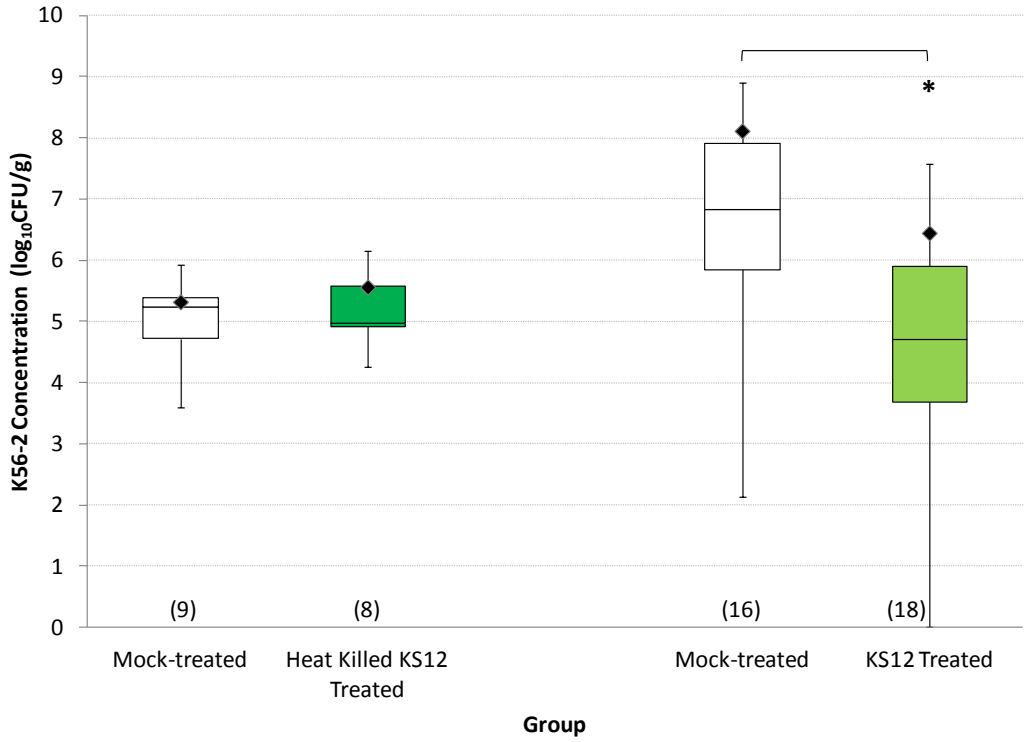


Figure 12. The bacterial clearance in mock-treated (white boxes) and heat-killed KS12 (dark green box) or KS12 (light green box) two days after treatment. The boxes indicate the first quartile, median and third quartile while the whiskers indicate the maximum and minimum values. The diamond marks the mean value. This trial was performed in triplicate. The number of mice in each group is indicated above the x-axis. *P<0.05 (Mann-Whitney U test)

Comparison of IP and NOID phage delivery

A previous study (7) found IP delivery of phage therapeutics to be a more effective method of phage delivery than intranasal instillation in treating a *B. cenocepacia* respiratory infection in a mouse model. In order to evaluate whether IP phage delivery was also more effective than aerosol phage delivery two different treatment delivery methods were compared for KS12: NOID and IP injection.

After treatment and emergence from anesthesia (approximately 1 – 2 h), phage could be isolated from the lungs, regardless of phage delivery method (Figure 13). This demonstrates that the phage are able to move from the peritoneum to the lung. Interestingly, mice receiving phage via IP injection had far higher phage concentrations within the lung than those that received phage via the NOID. Phage remained within the lung, regardless of the presence of bacterial host, but phage delivered via IP injection appeared to decrease at a more rapid rate than phage delivered by the NOID.

As predicted, infected mice receiving phage treatment via aerosol demonstrated a significant decrease in bacterial load within the lung (Figure 14). However, even though IP delivered phages were able to reach the lungs, mice receiving treatment via IP injection did not show any decrease in bacterial load (Figure 14). There may be some explanation for the discrepancy in these findings as opposed to those found by Carmody *et al*. (7) related to the delivery method of the phage. As mentioned above, aerosolisation is an optimal method of particle delivery and has previously been shown to be a more effective method of delivering particles to the mouse lung than intranasal instillation. Aerosolisation provides wider-spread and uniform particle deposition than intranasal instillation (20). It may be that while IP phage delivery is more effective than intranasal instillation, it is not as effective as aerosolisation. Also, although the IP-delivered phage may be able to reach the lungs, they may not be able to co-localise with the bacterial host.

Using immunofluorescence Carmody *et al*. (7) found that 48 h after treatment intranasally delivered phage were localised to the alveolar macrophages whereas IP-delivered phage localised to the perivascular areas and the alveolar septa. By 24 h post-infection the bacterial host (delivered by tracheotomy) was localised mainly to the lung parenchyma, especially the peribronchiolar and perivascular areas and the host remained in the lung parenchyma throughout the course of the study. Depending on where the bacteria localise during infection, the phage may not be able to access their bacterial target. If a NOID-delivered bacterial host does not localise to the perivascular areas, the phage concentration in the lung will not matter as the phage may not be able to reach their target host.

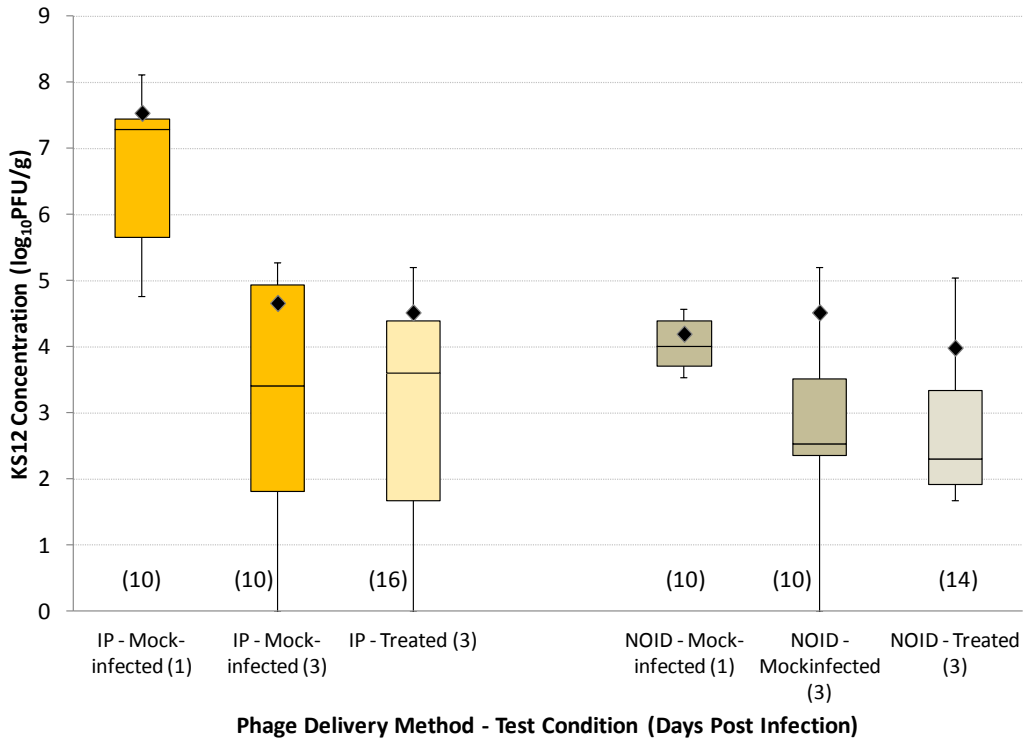


Figure 13. The KS12 phage titre in mock-infected mice treated with a KS12 IP injection (dark orange boxes) or NOID-delivered KS12 (dark grey boxes) and K56-2 infected mice treated by KS12 IP injection (pale orange box box) or NOID-delivered KS12 (pale grey box). Phage concentrations were determined on the day of treatment as well as two days after treatment. The boxes indicate the first quartile, median and third quartile while the whiskers indicate the maximum and minimum values. The diamond marks the mean value. This trial was performed in duplicate. The number of mice in each group is indicated above the x-axis.

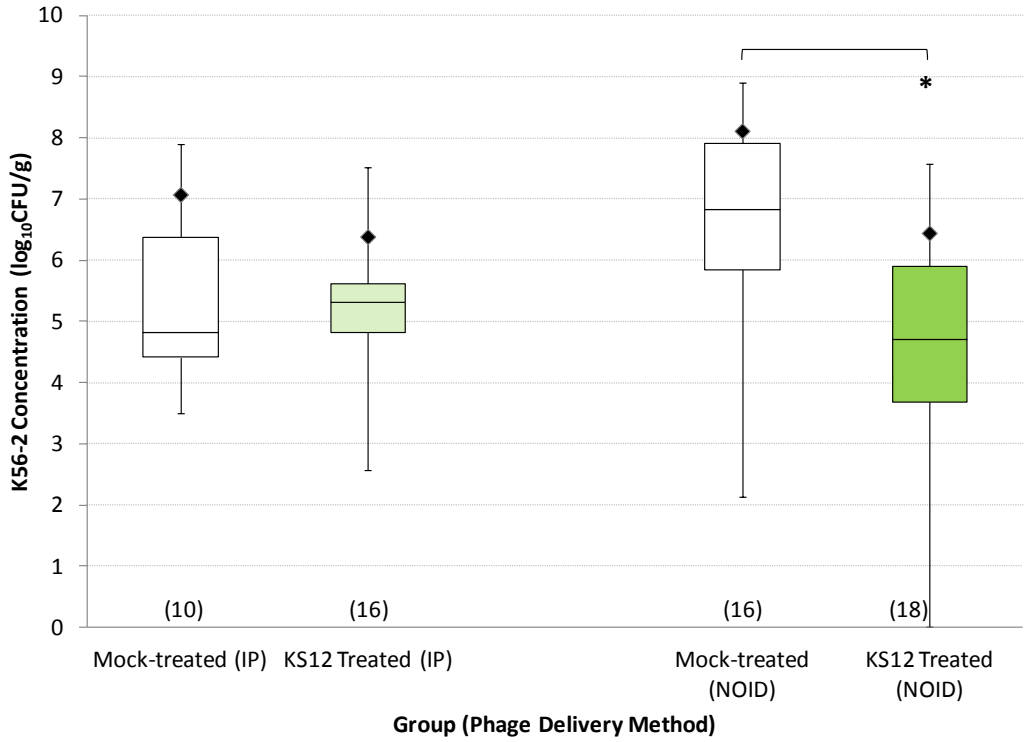


Figure 14. The bacterial clearance in mock-treated (white boxes) mice and KS12 treated mice receiving an IP injection (pale green box) or NOID treatment (dark green box) two days after treatment. The boxes indicate the first quartile, median and third quartile while the whiskers indicate the maximum and minimum values. The diamond marks the mean value. The trial for IP treated mice was performed in duplicate and the trial for NOID treated mice was performed in triplicate. The number of mice in each group is indicated above the x-axis. *P<0.05 (Mann-Whitney U test)

Demonstrating phage activity by host luminescence imaging

Some phages (especially KS4-M and KS5) appeared to be effective after 24 h *in vivo*, however, this does not give a clear picture of how quickly phages are able to act *in vivo*. Previous studies with *P. aeruginosa* (1, 13) have shown that the effect of phage activity on bacterial concentration in the respiratory system can be visualised *in vivo* non-invasively with the use of a luminescent host.

Initial screening of the plasposon mutagenesis library demonstrated that not all mutants were equally luminescent and some did not maintain luminescence once entering stationary phage. A representative screen of a range of mutants (Figure 15) demonstrates the variation in luminescence over time. The mutant used in this study, K56-2 9H08, was chosen because it maintains relatively consistent luminescence over a 72 h period.

Initially the *G. mellonella* larvae model was used to ensure that K56-2 9H08 could be detected using the IVIS spectrum. A relatively high concentration of host was required to detect luminescence (Figure 16) with a short camera exposure time (1 -3 s). The short exposure time was required because the larvae were active during imaging, producing a "smear effect" during long exposures. Using the *G. mellonella* larvae infection model, rapid activity of KS12 was demonstrated. Over the course of a 6-h period, untreated larvae showed a rapid increase in *B. cenocepacia* K56-2 concentration while larvae treated within 1 h of infection decreased in bacterial titre (see Figure 17 and Figure 18).

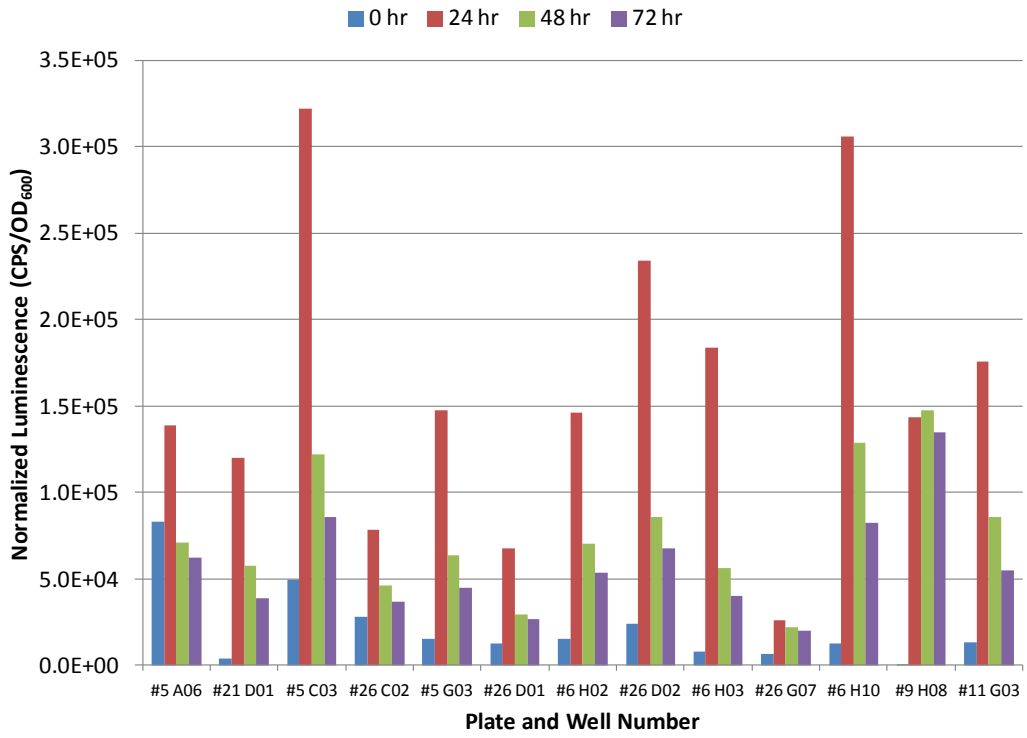


Figure 15. A screen of normalised luminescence emitted by plasposon mutagenised K56-2 shown 0 h (blue bars), 24 h (red bars), 48 h (green bars) and 72 h (purple bars). Cells were grown at 30°C for 6 h, then remained at room temperature for the remainder of the test. This test was performed once.

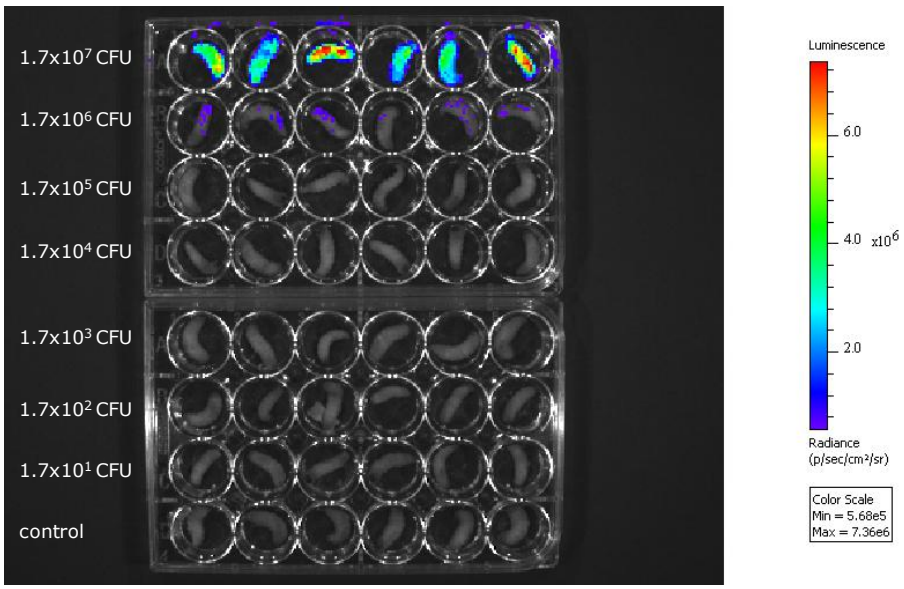


Figure 16. The luminescence intensity of *G. mellonella* injected with K56-2 9H09 at a concentration of $1.7x10^1$ CFU/larva to $1.7x10^7$ CFU/larva within 15 minutes of infection.

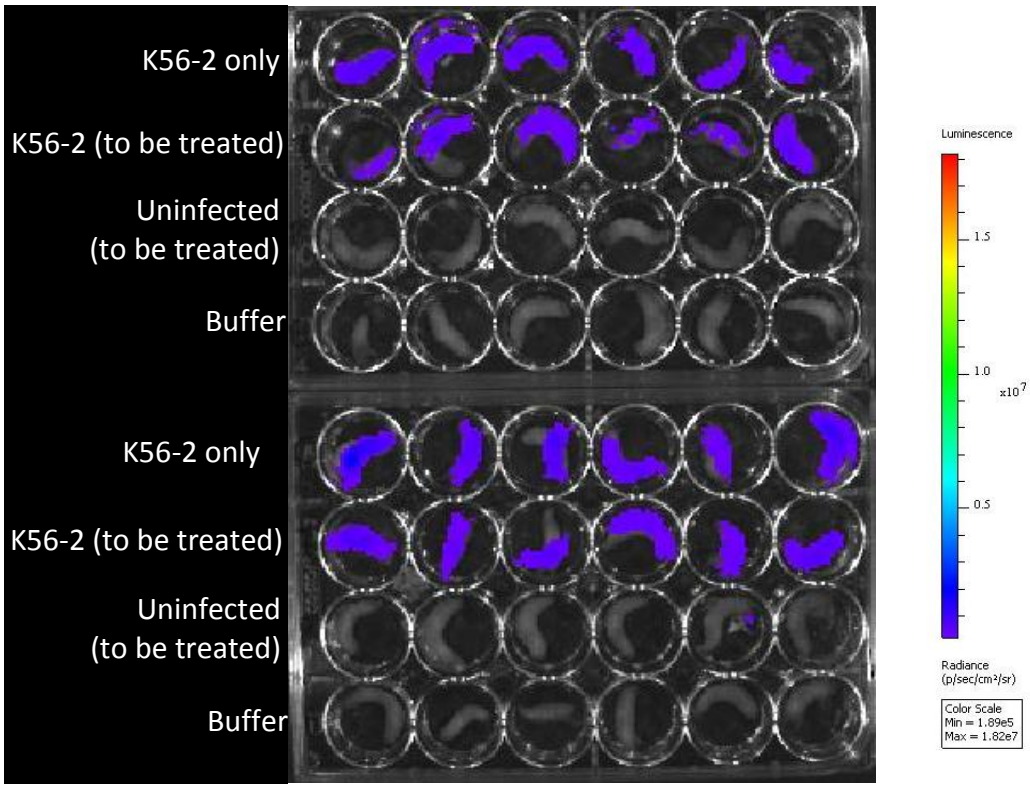
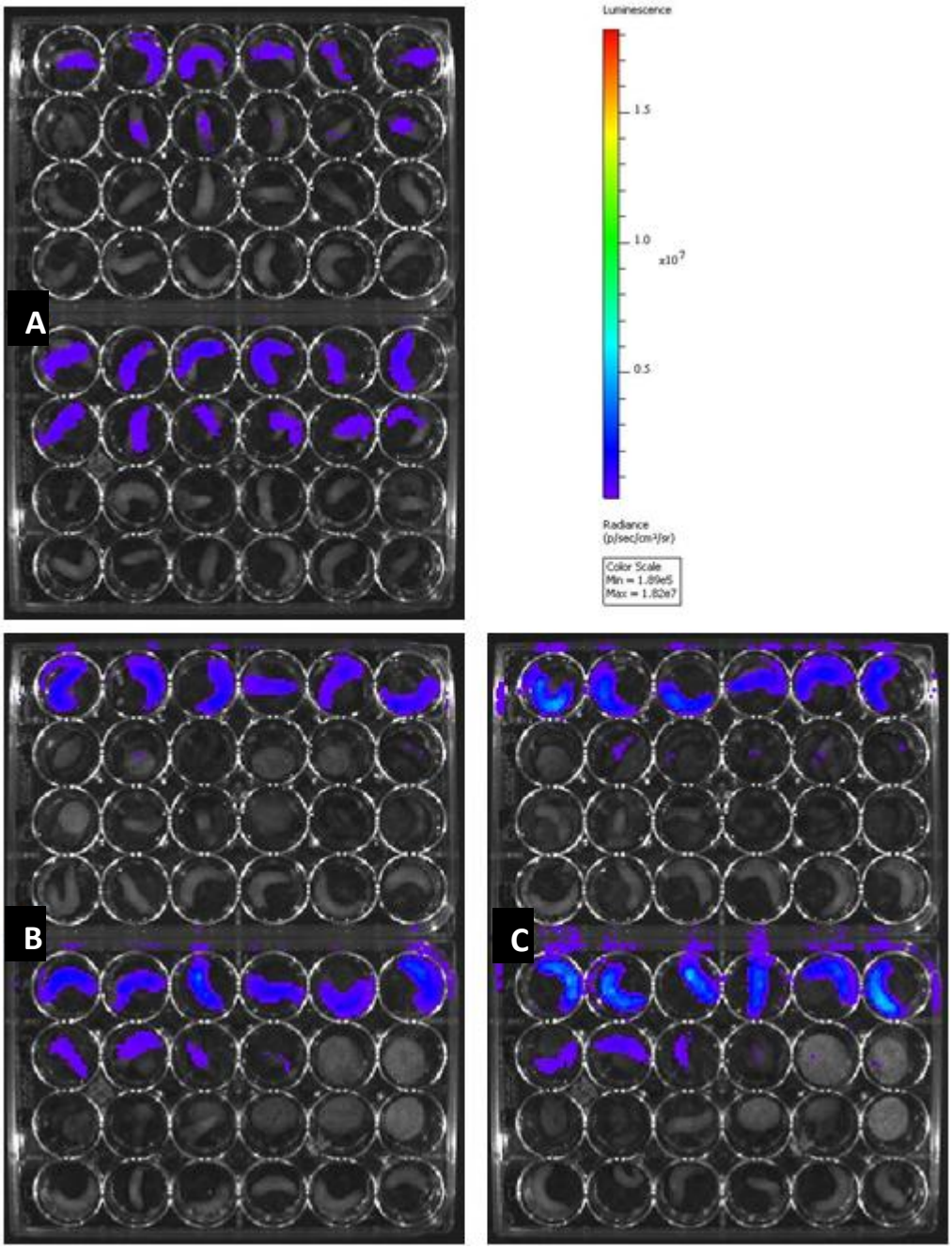


Figure 17. The luminescence intensity of *G. mellonella* injected with *B. cenocepacia* K56-2 9H08 (with a concentration of $3x10^7$ CFU/larva) prior to treatment. This image was taken 45 min after infection. This test was performed in triplicate, however images from the third trial are not shown as these two trials are representative of the dataset.


A
B
C
Luminescence
1.5
1.0
x10^7
0.5
Radiance
(p/sec/cm²/sr)
Color Scale
Min = 1.89e5
Max = 1.82e7

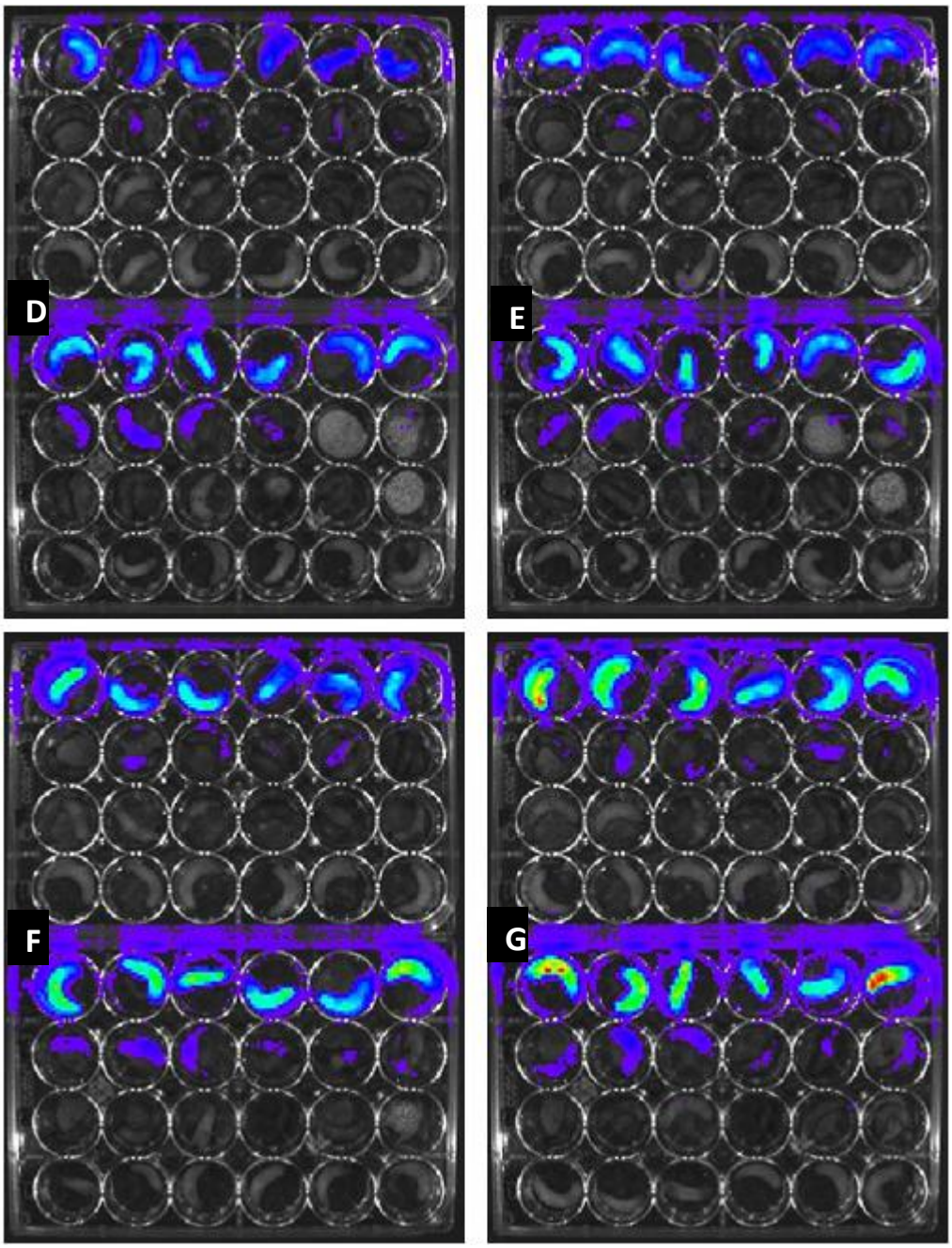


Figure 18. The luminescence intensity of *G. mellonella* injected with $6x10^7$ PFU/larva (A) immediately, (B) 1 h, (C) 2 h, (D) 3 h, (E) 4 h, (F) 5 h and (G) 6 h after treatment with KS12. The larvae were treated 45 min after infection with an MOI of three. This test was performed in triplicate, images from the third trial are not shown as these two trials are representative of the dataset. The test groups for the top half of each image from top to bottom are *B. cenocepacia* K56-2 9H08 only, *B. cenocepacia* K56-2 9H08 infected and KS12 treated, KS12 only and buffer only and then repeated for the bottom half of the image.

Bacterial clearance was not demonstrated in the mouse model using luminescence imaging. Interestingly, the development of the infection within approximately 2 h of infection was demonstrated with luminescence starting at the nose and travelling down the trachea toward the lung (see Figure 19, Figure 20 and Figure 21) as well as throughout the body. Particle delivery outside the lung (especially in the gastrointestinal tract) is likely due to high initial particle deposition in the nose and throat, which is then swallowed (56). However, even with a very long exposure time (15 min), luminescence within the lung could not be distinguished from general luminescence throughout the body (see Figure 22).

As with previous tests, mice were treated 24 h after infection. The mice were divided into three groups: untreated, gHBSS mock-treated and KS12 treated. The untreated mice showed a decrease in bacterial luminescence when compared to the mock-treated and KS12-treated mice (see Figure 23 and Figure 24), with minimal luminescence detected 24 h after the treatment day (48 h after infection). This suggests that the untreated mice, which had not been subjected to a second anesthetic dose and NOID exposure, were recovering quicker than the mock-treated or KS12 treated mice that had been subjected to the stresses of a second NOID exposure. There was, however, no distinguishable difference between mock-treated and KS12 treated mice (see Figure 24).

There may be a number of reasons why luminescence could not be detected in the lungs including a bacterial host that is not bright enough to be imaged from the depth of the lung (which is obscured by not only fur and skin, but also the heart) or a bacterial concentration that is too low to be detected. In the larval infection model the starting bacterial concentration is 10^7 CFU/worm, however, in the mice the starting lung concentration is commonly 10^6 CFU/lung. After euthanisation (48 h post treatment), the lungs of the mice were removed and imaged. Even without the fur, skin or heart obscuring of the lungs, no luminescence could be detected from the lungs (Figure 25).

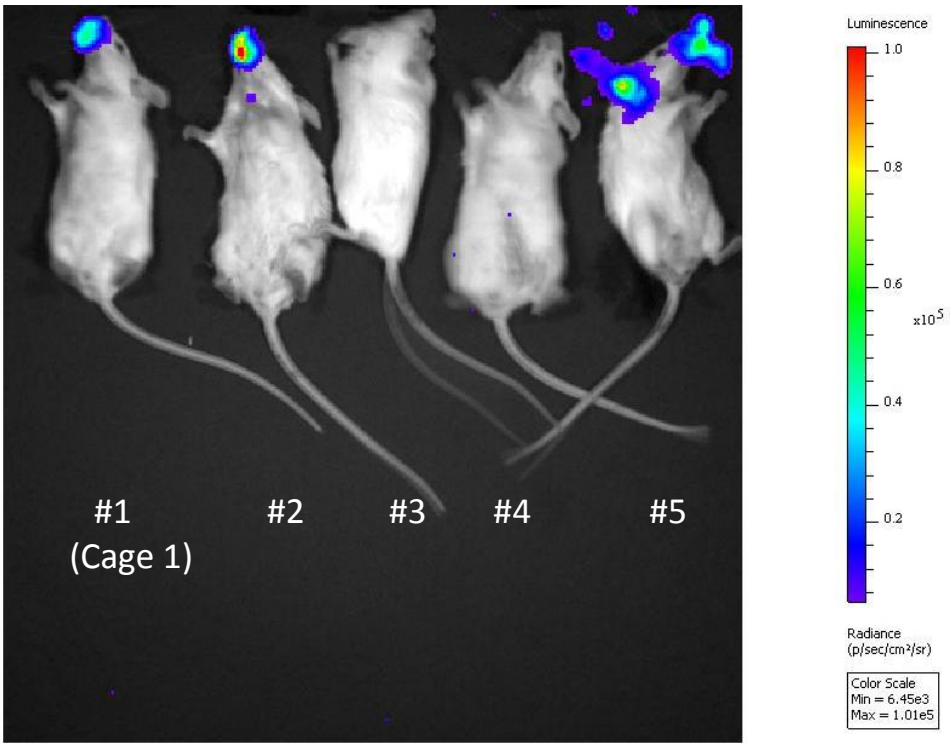


Figure 19. The luminescence intensity of *B. cenocepacia* K56-2 9H08 infected mice immediately after infection. Note that the images of mice # 3 – 5 should be disregarded. These mice emerged from anesthesia during imaging and were moving during the luminescence exposure time, so the images of these mice can not be correlated to the luminescence images.

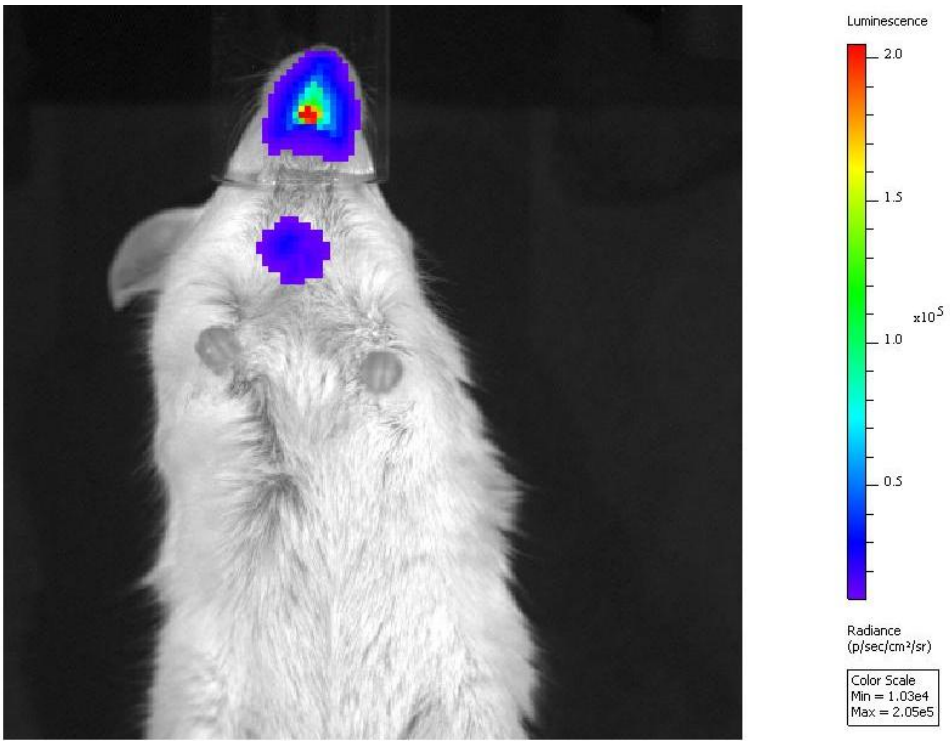


Figure 20. The close-up image of luminescence intensity of *B. cenocepacia* K56-2 9H08 infected mouse #2 (cage 1). This image was taken 40 min after infection.

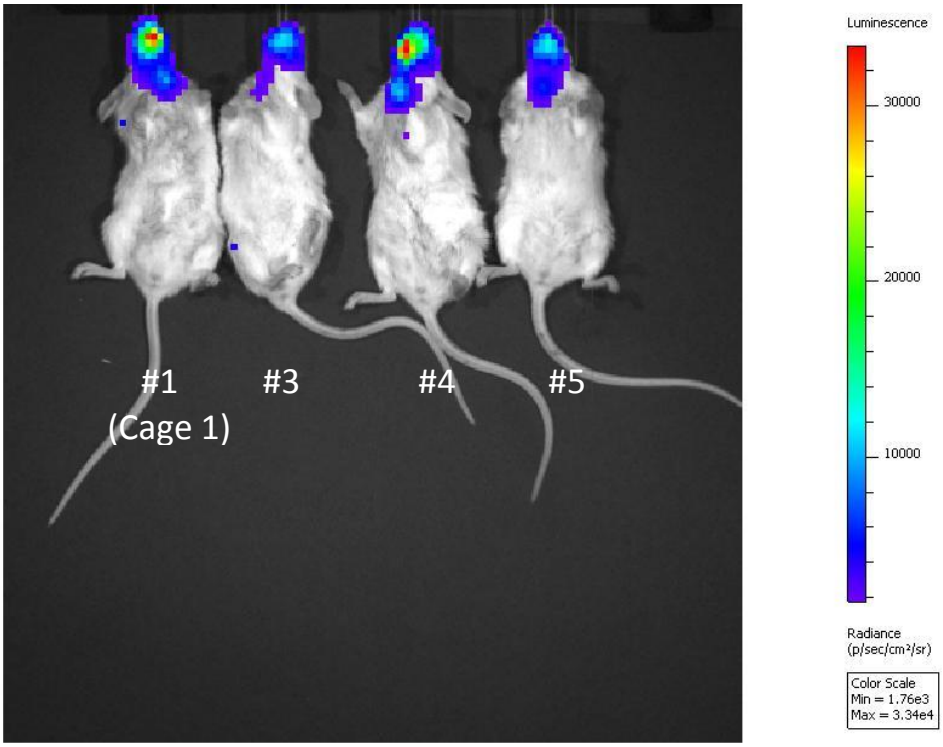


Figure 21. The luminescence intensity of *B. cenocepacia* K56-2 9H08 infected mice 1 h after infection.

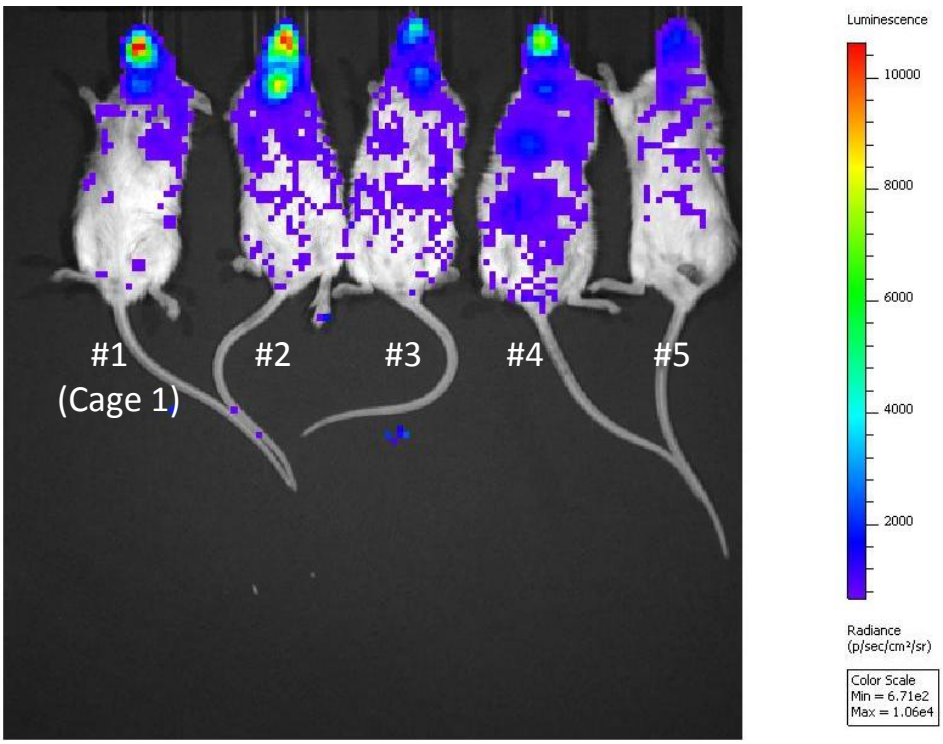


Figure 22. The luminescence intensity of *B. cenocepacia* K56-2 9H08 infected mice 2 h after infection.

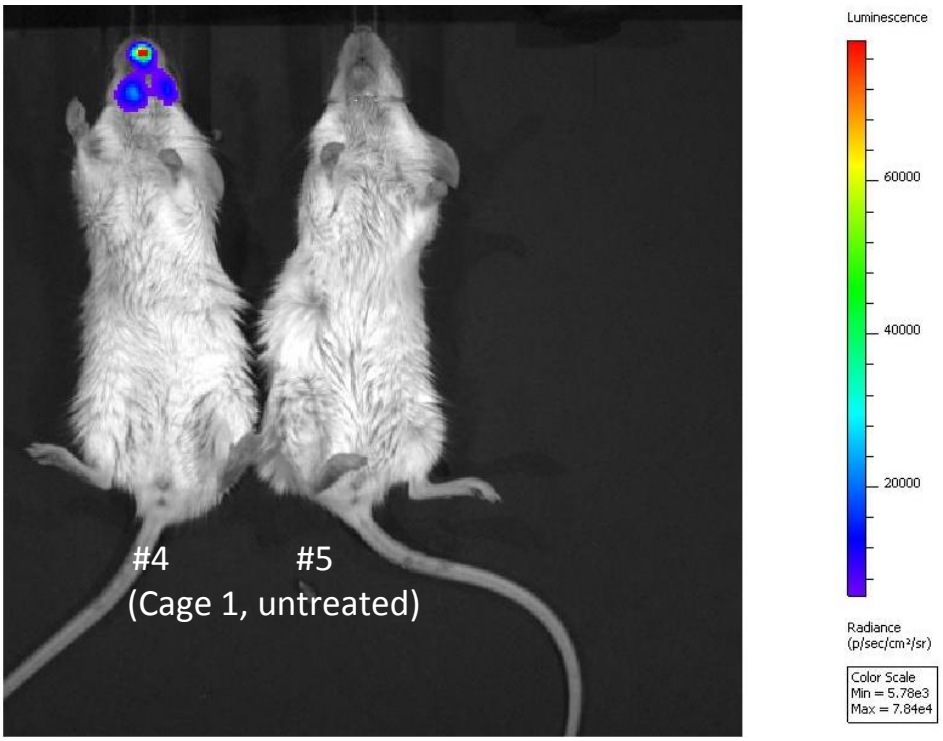


Figure 23. The luminescence intensity of *B. cenocepacia* K56-2 9H08 infected mice having received no treatment or mock-treatment, 24 h after treatment day.

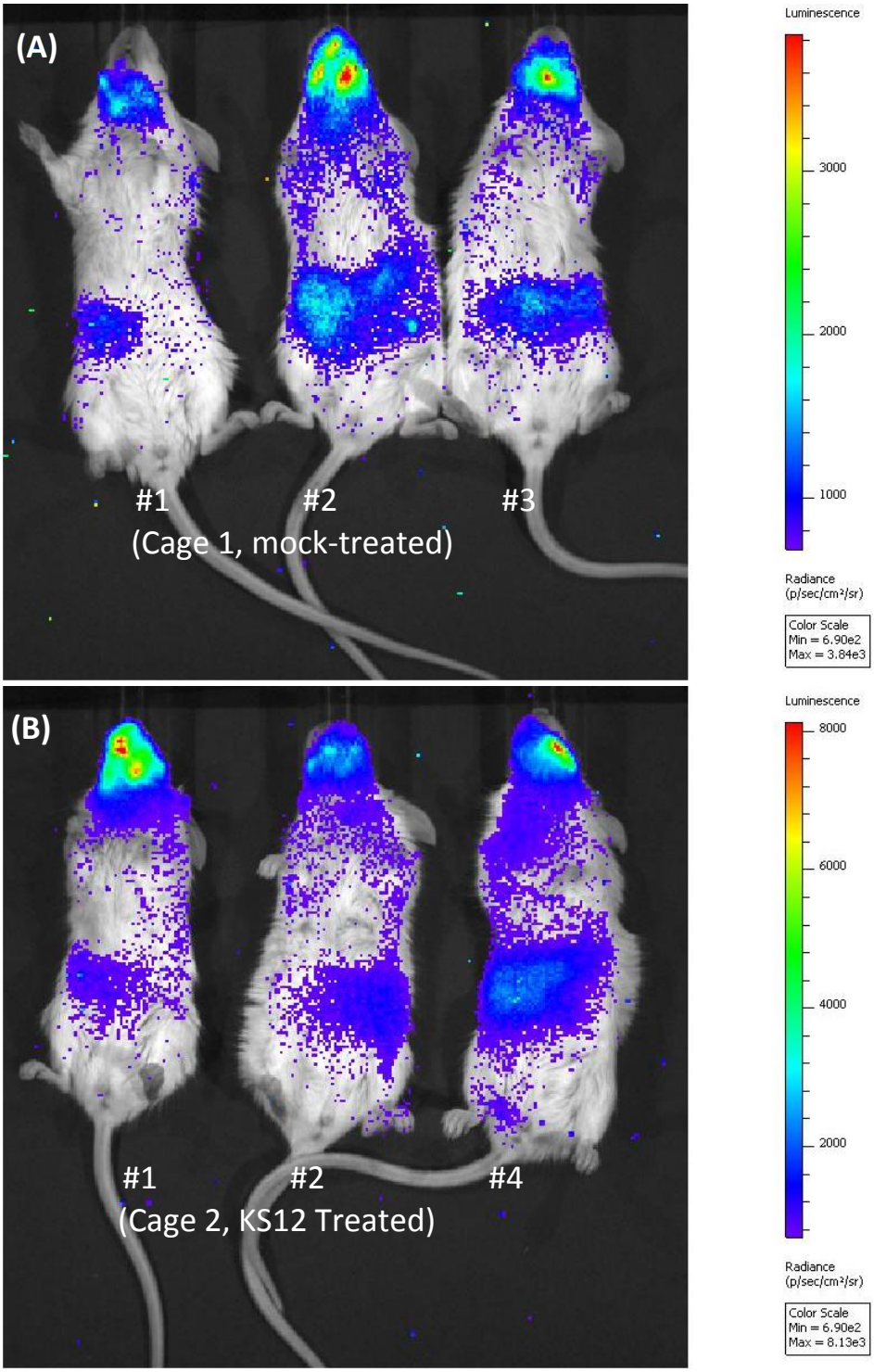


Figure 24. The luminescence intensity of *B. cenocepacia* K56-2 9H08 infected mice, 24 h after (A) gHBSS mock-treatment or (B) KS12 treatment.

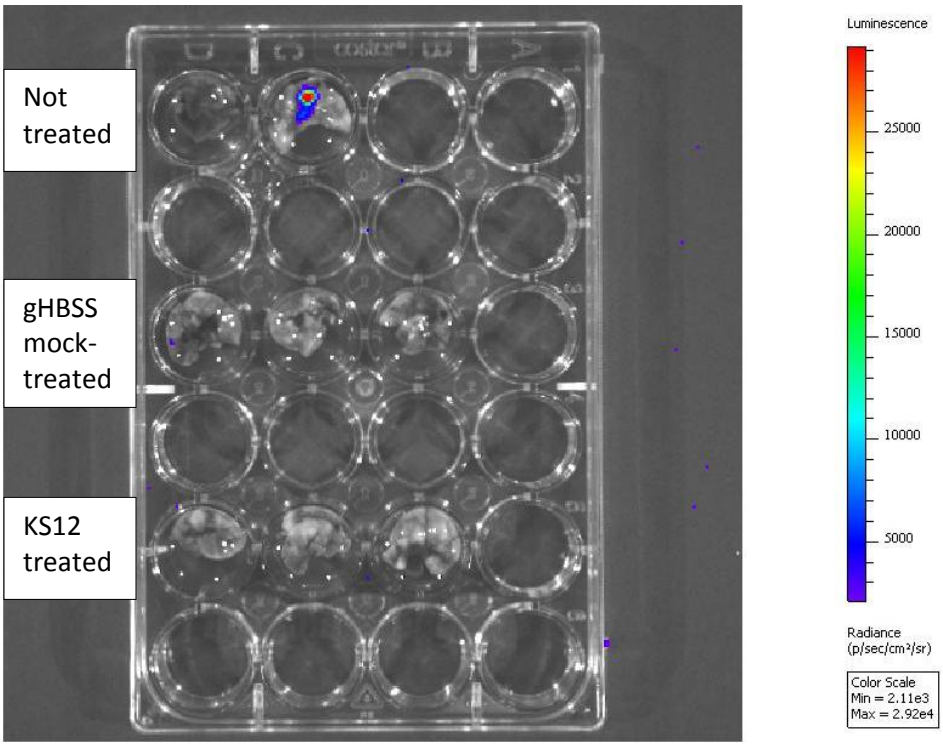


Figure 25. The luminescence intensity of lungs removed from *B. cenocepacia* K56-2 infected mice 72 h after infection (48 h post treatment).

Conclusions

Future Directions

Through the use of a bacterial culturing method, this study demonstrated that aerosol phage therapy can be effective in reducing pulmonary BCC concentrations in a mouse model. However, some adjustments would likely make this model more robust as well as more adaptable.

One of the biggest difficulties in this study was obtaining a phage titre that would be effective in the mice. Although ultracentrifugation could increase the phage concentration to a certain degree, it was not sufficient to increase the titres of some of the most promising phages used in the study (KS4-M, KS5) to a

concentration at which they could be properly evaluated. Finding an alternate method that could be used to increase a phage titre by two orders of magnitude would dramatically expand the scope of this study. One phage concentration method that could be attempted is the use of a Convective Interaction Media Monolithic Column (BIA Separations). This concentration method purifies and concentrates phages using anion exchange chromatography. Although this would be both costly and time-consuming to optimise, after the initial setup it is both rapid and relatively efficient. A 30 mL T4 phage sample containing $8x10^8$ PFU/mL can be concentrated to a 3 mL sample containing $5.7x10^9$ PFU/mL in 10 – 40 min, depending on the flow rate used (79). With such a rapid concentration time it would be possible to concentrate 300 mL of phage sample down to 3 mL, thereby increasing the phage titre by approximately two orders of magnitude. This would provide an effective phage titre for any of the phages used in this study.

Even if phage titres could not be increased to the extent required to treat mice with a single dose, multiple treatments may prove to be an effective treatment method. There has been some evidence that multiple phage treatments are effective. Two case studies (25, 35) found multiple aerosol phage treatments to be effective in a clinical setting. Although the current model does not allow for the extension of the trial past three days post-infection, a second treatment

could still be given a day after the initial treatment giving a full day of activity for the second treatment.

A commonly proposed phage treatment method is the use of phage cocktails and they have been used in published case studies and clinical trials (25, 35, 52, 69, 89). Provided multiple phages effective for a single host are available (which there were for this study), a phage cocktail can be prepared. Phage cocktails can target multiple host receptors and reduce the chances of phage resistance developing over time. The efficacy of a BCC phage cocktail could easily be evaluated using the aerosol phage therapy mouse infection model developed for this study.

Two different solutions could be attempted to image the effect of phage on the bacterial host in mice. First, the starting titre of the bacterial host could be increased in order to be able to detect the luminescence in the lung. This would, however, also require that the starting phage titre administered is also increased. Second, a new brighter bioluminescent host could be engineered, perhaps by creating a high copy constitutively expressed plasmid containing *luxCDABE*.

Significance

Prior to this study the only study to evaluate phage therapy for BCC *in vivo* infections did not successfully demonstrate the efficacy of aerosol phage therapy (7). In direct contrast, this study demonstrates not only that aerosol phage therapy can be effective, but also that a NOID is a relatively simple and effective method of both developing a respiratory infection in mice and delivering aerosolised phage treatment *in vivo*. Furthermore, this study has laid the foundation for further aerosol phage therapy research. The basic model can be adapted to different respiratory infection models. Additionally, it can be used to test the efficacy of newly discovered BCC phages or phage cocktails *in vivo*. In conclusion, this study supports the use of aerosolised phages as a treatment method for respiratory infections and more specifically, for *B. cenocepacia* infections.

Acknowledgements

Thank you to Dr. C. Wilkinson for providing advice and assistance in developing our mouse anesthetising protocol as well as the University of Alberta Biosciences Animal Services for providing animal care. We would also like to thank Dr. D. Coltman for offering assistance with statistical analysis.

Appendix A. Determining the genetic source of variation between phage KS4 and its liquid clearing variant, KS4-M

Introduction

A previously isolated prophage of *B. cenocepacia* J2315, KS4 (or BcepMu) (73, 84) was reported to have a liquid clearing variant, KS4-M (75). KS4-M was observed to lyse cultures when grown in a liquid medium, causing a reduction in turbidity (or clearing) of the medium, while this was not observed for KS4. Both KS4 and KS4-M were previously sequenced and comparisons of the two genomes led to the hypothesis that this variant was able to clear liquid due to a point mutation at position 31518 in a putative base plate assembly gene (gene 50). Although there were some other sequence differences between KS4 and KS4-M, an A to G change in a base plate assembly gene, causing an amino acid change from threonine to alanine, appeared to be the most likely cause of this phenotypic change (21). The purpose of this study was to determine if the putative base plate assembly gene affected ability of KS4-M to propagate in a liquid medium.

Materials and Methods

A set of KS4 mutants containing gene 50 from KS4-M were previously created in order to determine if the liquid clearing ability of KS4-M came from the point mutation in gene 50 or from a mutation in another gene (21). Briefly, these mutants were created by development of a plasmid containing the alternate base plate assembly gene and electroporated into J2315. Using an antibiotic resistance screen, nine mutant strains were found to contain the gene of interest. Phage stocks were created by growth of the J2315 strains in ½ LB broth

at 30°C with shaking for at least 16 h followed by centrifugation and filter sterilisation of the phage-containing supernatant. Of these nine mutants, three (NM7, NM8 and NM9) were chosen at random for further experimentation.

High titre stocks of KS4, KS4-M, NM7, NM8 and NM9 were made using an agar overlay plating method with 100 µL phage, 100 µL *B. cenocepacia* K56-2 and 3 mL ½ LB broth. After incubation at 30°C for 16 h the plates were overlaid with 3 mL sterile milliQ water and rocked for a minimum of 2 h. The liquid was removed and filter-sterilised.

The genomic region of interest in the five high-titre phage stocks was sequenced using previously designed primers (Table 3) to ensure that KS4 had maintained the original sequence and that the other phage stocks had not reverted to the original sequence. Sequencing was performed using an ABI 3730 DNA Analyzer (Applied Biosystems) by the University of Alberta Department of Biology Molecular Biology Service Unit. The phage stocks were also tested to ensure that they were not contaminated with the other phages used in the lab that are also able to infect K56-2 (KS5 and KS12). Since primers are available for the identification of KS5, they were used to ensure that the phage stocks were free of KS5. The phage stocks were tested for the presence of KS12 by growth on *B. cenocepacia* C6433 or *B. multivorans* C5274, hosts that are susceptible to KS12 but not KS4/KS4-M.

Table 3. The primers used for sequencing the portion of KS4/KS4-M containing the point mutation in gene 50. The final product is 449 base pairs in length.

Primer	Primer Position	Direction	Sequence
Nigel Forward 3	31292 – 31310	Forward	5' CCTGGTCGACATCATTGCC 3'
Nigel Reverse 2	31721 – 31740	Reverse	5' GATCGTCCGTTTCCTCCTCG 3'

The liquid clearing ability of the five phages was monitored over a 6 h period by measuring the optical density (OD_{600}) of the cultures in 96-well plates. In total, 12 conditions were tested in each plate: growth media only, phage (KS4, KS4-M, NM7, NM8 or NM9) in growth media only, host in growth media only and phage and host in growth media. The titre of the phage stocks was determined prior to each test and the stocks diluted as necessary to ensure that the same starting titre was used for each phage stock. (The starting phage titre ranged from $7.7x10^7$ PFU/mL to $5.9x10^8$ PFU/mL.) The K56-2 stock used for the experiment was grown for 16 h in ½ LB at 30°C with shaking. The 96-well plates were contained 150 µL ½ LB broth and were inoculated with 50 µL phage and 10 µL K56-2 as necessary. The OD_{600} was measured at four time points: 0, 2, 4 and 6 h. The complete test was repeated three times, twice with each condition tested eight times and once with each condition tested in triplicate.

In order to ensure that the point mutation was stable, the region in question was sequenced for KS4, KS4-M, NM7, NM8 and NM9 after the 6 h growth period was

completed and also after 16 h incubation. The phages sequenced were chosen from a random well. The point mutation was stable for each phage.

Results and Discussion

Unexpectedly, under controlled conditions KS4 consistently cleared liquid to the same extent as the phages with the base plate assembly gene point mutation (Figure 26). For the first 4 h of the trial the OD_{600} rose similarly for the host grown without phage as for the host combined with phage. However, by the 6 h mark there was a significant difference in OD_{600} between the host grown with or without phages.

Although previous tests indicated that there was a difference in the ability of KS4 and KS4-M to clear liquid, this study indicates that there is no difference in the ability of the two phage to propagate in liquid. Previous tests did not account for variables such as starting phage titres and once this test was performed under controlled conditions both KS4 and KS4-M cleared liquids at the same rate.

The amino acid change from KS4 to KS4-M of threonine to alanine appears to have no effect on the liquid clearing abilities of the phages. The three KS4 mutants (NM7, NM8 and NM9) containing the KS4-M base pair change were

equally effective as KS4-M at clearing liquid, demonstrating that any of the other genetic differences between KS4 and KS4-M also had no effect on liquid clearing.

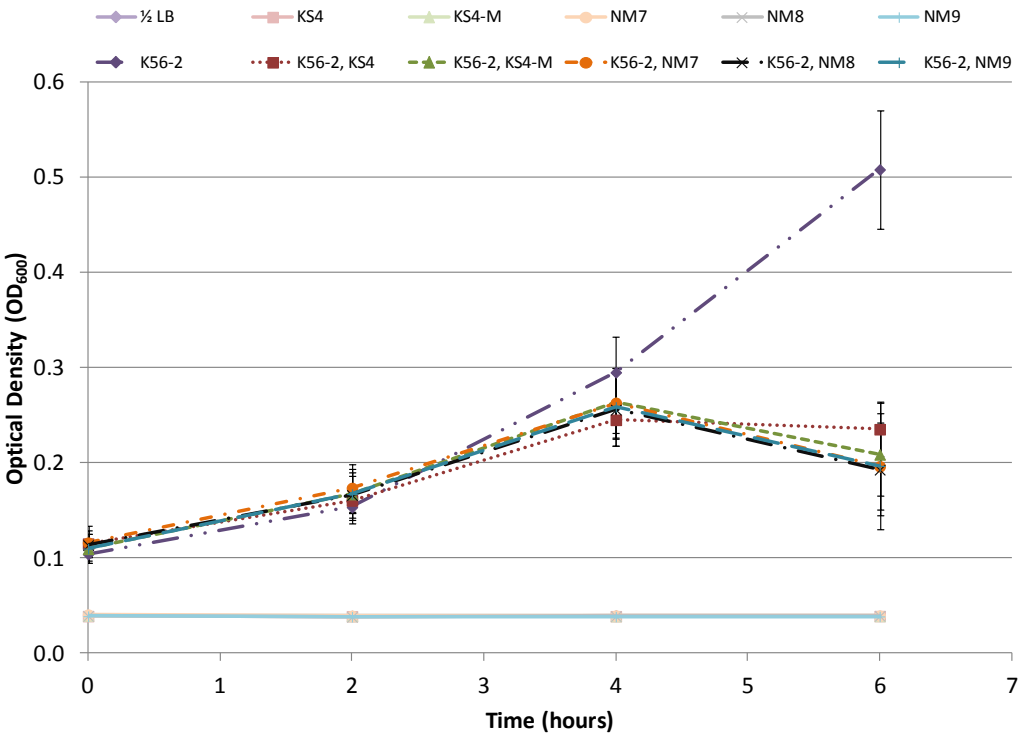


Figure 26. The optical density (OD_{600}) of cultures inoculated with phage only (KS4, KS4-M, NM7, NM9 or NM9), host only (*B. cenocepacia* K56-2) or phage and host. Each data point is an average of three independent trials performed once in triplicate and twice in octuplet.

Appendix B. Testing for dual host phages

Introduction

Phages infect hosts with a great deal of specificity, often infecting a single species or even strain. However, there has been scant mention in the literature of phage that are able to infect hosts of different species, or even genera. A report of phage able to infect both *P. aeruginosa* and members of the BCC (58) found two phages, NS1 and NS2 that, in addition to infecting strains of four BCC species, were able to infect strains of *P. aeruginosa*. Additionally, *P. aeruginosa* was found to be susceptible to the BCC phage JB3 (36). Further research demonstrated that NS2 was also active against *Burkholderia pseudomallei* and *Burkholderia gladioli*, which are not members of the BCC. A number of other BCC phages, DK1, RL1c, RL1t and JB5, are also able to infect *B. gladioli* (36).

In addition to screening BCC phages on *P. aeruginosa* strains, Nzula *et al*. (58) also evaluated the growth of four *P. aeruginosa* phages, E79, B3, F116L and G101, on BCC hosts and found that all four phages were able to infect three BCC species: *B. cepacia*, *B. cenocepacia* and *B. vietnamiensis*.

Understanding the host range of a phage beyond the host on which it was isolated may be important for a few reasons. First, when working towards a phage therapy or biocontrol application, it is beneficial to understand the full potential of a phage. For instance, a phage that is able to lyse both BCC and *P. aeruginosa* hosts may be an excellent addition to a phage cocktail for use in

treating a CF patient. Alternatively, a phage able to infect both BCC strains and *B. pseudomallei* may be useful in both a phage therapy and biocontrol applications. Second, determining the broader host range of a phage may give further insights into the host receptors a single phage is able to bind.

In an effort to further characterise a collection of BCC and *P. aeruginosa* phages, a set of *P. aeruginosa* strains was tested for susceptibility to BCC phages (and vice versa).

Materials and Methods

Phage propagation

Phages assayed for the presence of plaques were grown by incubating 200 µL of phage and 100 µL of host grown overnight in liquid at room temperature for 20 min in a glass culture tube. After incubation 3 mL of 55°C ½ LB top agar was added and the mixture was poured onto a 100 mm ½ LB agar plate. After at least 16 hr incubation at 30°C, the phages were collected either by liquid overlay or picking the plaques. A liquid overlay was performed by adding 3 mL of sterile milliQ water to the plate and rocking it for a minimum of 2 h. The liquid was removed from the agar plates and filter sterilised using 0.45 µm filters. Plaques were picked by removing the plug of agar containing the plaque with a Pasteur pipette and resuspending it in sterile milliQ water for a minimum of 1 h.

A phage spot test was performed as described above with the exception that the host-top agar mixture is poured on the plate without the phage. After the top agar dried for a minimum of 10 min, 100 µL of phage was spotted onto the plate and allowed to dry prior to incubation upside down.

High titre phage stocks were prepared by repeatedly propagating phage using the agar overlay method described above until plates had confluent lysis.

Restriction fragment length polymorphism (RFLP) analysis

The DNA extraction was performed as previously described (65). RFLP analysis was performed by incubating 600 µg DNA with EcoR1 (Invitrogen) for 1 h.

Results and Discussion

Initial phage screen

After the initial screen of 13 BCC phages on 14 *P. aeruginosa* strains and four *P. aeruginosa* phages on 26 BCC strains all potential plaques (plaque definition was not always clear on a new host) were retested on the original host as well as the host from which the phage was isolated. Phages able to form plaques on both the original propagation host and the alternate host from which it was isolated (see Table 4 and Table 5) were brought to high titre and tested for efficiency of plating (EOP).

Once the same phage stock was tested for growth on both the original propagation host and the alternate isolation host, very large discrepancies were found between the EOP of the phage on the two hosts (Figure 27 and Figure 28). In fact, five of the phage lysates were entirely unable to grow on both hosts once brought to high titre.

However, once the phage stocks used for testing the efficiency of plating were tested for purity, they were found to be contaminated. When the *P. aeruginosa* phages (44 and E79) used for this test were tested for the presence of BCC phages by PCR amplification, they both tested positive for DC1. DC1 is able to grow on *B. cenocepacia* C6433. This explains why these "*P. aeruginosa*" phages were able to grow on C6433. Although there were no PCR primers available to test for the identity of the *P. aeruginosa* phages, the KS4, KS4-M and KL3 phage stocks propagated on a *P. aeruginosa* host were all found to test negative when PCR tested for the presence of their respective phage. This would indicate that all these phage stocks were contaminated with a *P. aeruginosa* phage. Although no PCR primers are available to test for the presence of KS12, when a completely different KS12 stock was used to attempt to replicate the ability of KS12 to grow on HER1006, this finding could not be replicated.

Two additional BCC phages (J6068 and ϕH111) were tested for the ability to grow on the *P. aeruginosa* hosts outlined in Table 5 using a spot test. J6068 was

found to be able to grow on *P. aeruginosa* HER1006 and 14715. HER1006, however, releases a prophage, making it difficult to distinguish between the plaques of the prophage and the plaques of the phage being grown on the host. The J6068 isolated from HER1006 was transferred to 14715 in order to produce high titre phage stocks of this phage. In order to determine if the phage that was able to be propagated on a *P. aeruginosa* host was actually J6068, an RFLP analysis was performed. Three phage stocks were tested: J6068 isolated from HER1006 and then repropagated on 14715 five times (performed in duplicate) and J6068 isolated from HER1006 and then repropagated on *B. cenocepacia* K56-2 five times. The RFLP analysis revealed that the J6068 that had been repropagated on K56-2 was likely J6068, however the J6068 repropagated on 14715 had an entirely different profile (Figure 29). This indicates that there was likely some remaining J6068 in the original sample isolated from HER1006, which was repropagated once the stock was plated again on K56-2. The phage propagated on 14715, however, was likely a *P. aeruginosa* phage. This may be a novel phage or a prophage of 14715, which could be determined by genomic sequencing.

This study indicates that none of the phages tested are able to grow on both a *P. aeruginosa* and a BCC host. It also demonstrates the importance of testing for phage stock purity in such studies.

Table 4. Four *P. aeruginosa* phages were tested for the ability to grow on 26 BCC strains. An 'X' indicates the ability of the phage to plaque on a host after being picked and replated.

		P. aeruginosa phages			
		24	44	E79	ΦKZ
B.cepacia	LMG18821				
	ATCC17759				
B. multivorans	C5393				
	C3430				
	C5274				
B. cenocepacia	715J				
	J2315				
	K56-2				
	C6433		X	X	
	C1257				
	C5425				
	C4455				
	PC184				
	Cep 511				
B. stabilis	LMG14294				
	LMG18870				
B. vietnamiensis	LMG10929				
	LMG18835				
	DB01				
B. dolosa	LMG18943				
	LMG21443				
B. ambifaria	LMG17828				
	LMG19182				
	LMG19467				
B. anthine	LMG16670				
B. pyrocinia	LMG14191				

Table 5. Thirteen BCC phages were tested for the ability to grow on 14 *P. aeruginosa* strains. An 'X' indicates the ability of the phage to plaque on a host after being picked and replated.

		BCC phages												
		DC1	KS4	KS4-M	KS5	KS9	KS10	KS12	KS14	KL1	KL3	KL4	AH2	SR1
P. aeruginosa strains	PAO1			X							X			
	PAK													
	HER1004													
	HER1006							X						
	HER1012													
	PA103		X											
	Utah4													
	Utah3													
	ATCC27853													
	PA14													
	14655													
	14715													
	6106													
	14679													

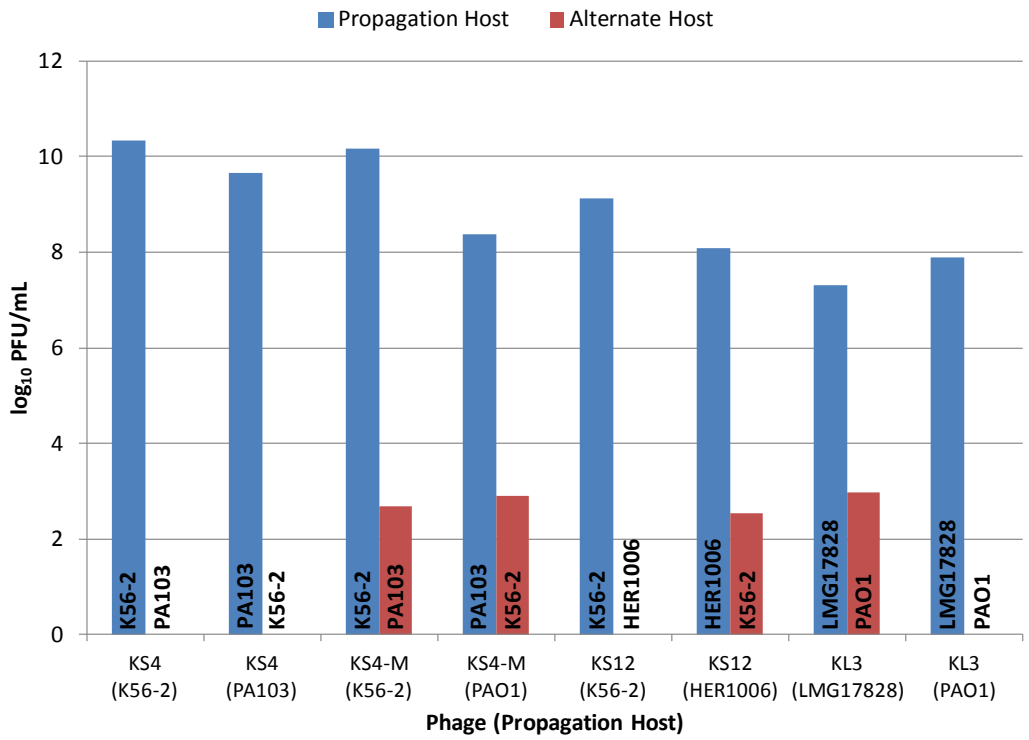


Figure 27. A comparison of calculated phage concentrations of BCC phages on two different hosts. Each phage was brought to a high titre stock on either a BCC host or a *P. aeruginosa* host and then plated on both hosts.

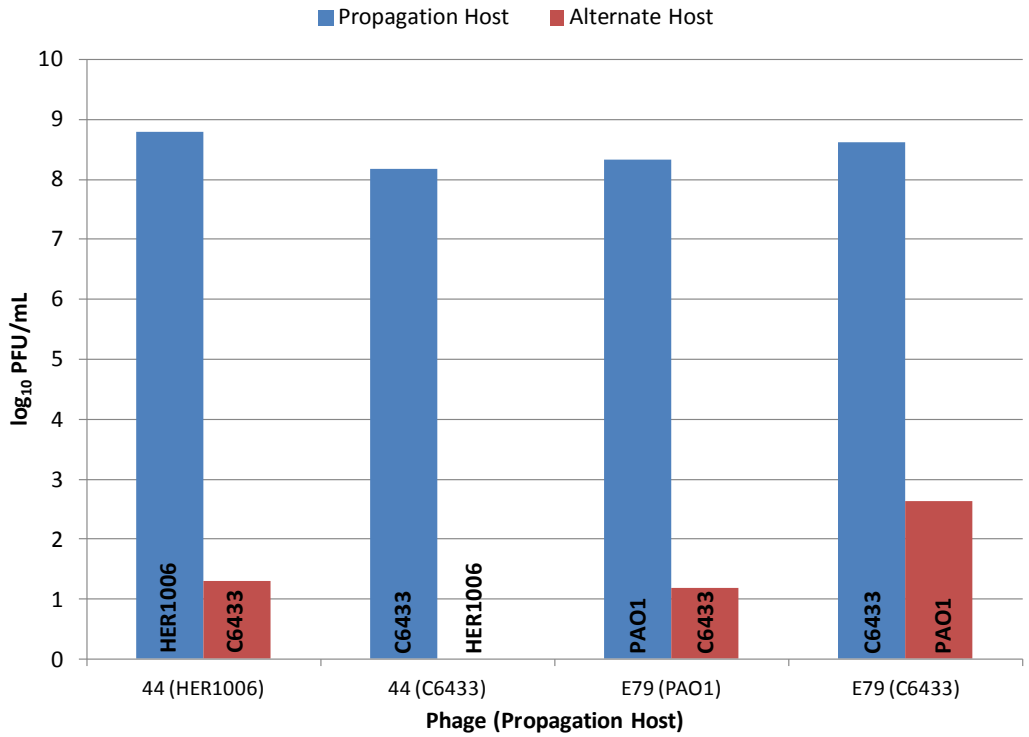


Figure 28. A comparison of calculated phage concentrations of *P. aeruginosa* phages on two different hosts. Each phage was brought to a high titre stock on either a BCC host or a *P. aeruginosa* host and then plated on both hosts.

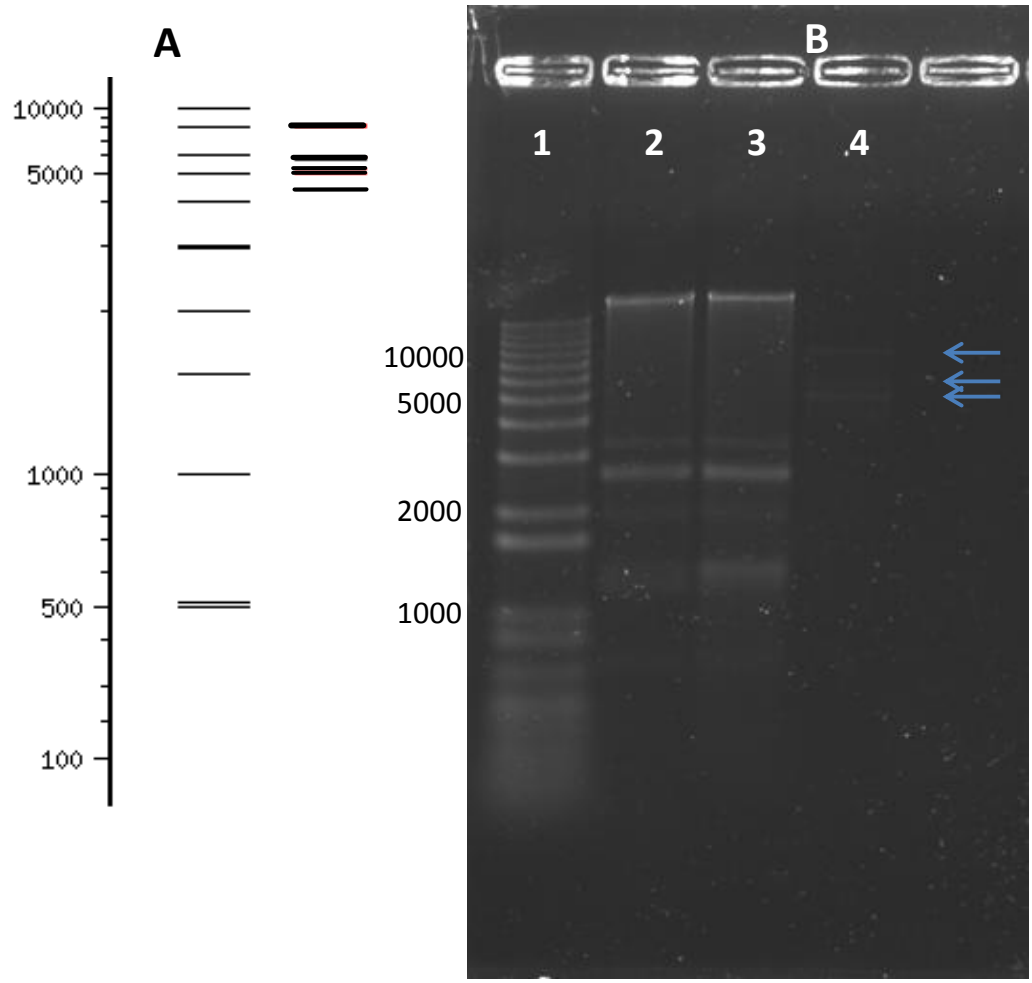


Figure 29. (A) A schematic of the expected bands obtained from RFLP analysis of J6068 cut with EcoR1. (B) A 1 Kb Plus Ladder (Invitrogen) marker and the RFLP analysis of J6068 isolated from *P. aeruginosa* HER1006 and repropagated on 14715 five times, performed in duplicate (Lanes 2 and 3) and J6068 isolated from *P. aeruginosa* HER1006 and repropagated on *B. cenocepacia* K56-2 five times (Lane 4). Arrows indicate the bands in lane 4. In all cases 600 ng of genomic DNA was digested with EcoR1 for 1 h and then separated on a 0.8% agarose gel.

Appendix C. Time course comparing the maintenance of bacterial load in the mouse lung for *B. cencocepacia* K56-2 grown in ½ LB broth and artificial sputum medium broth

Introduction

Bacteria grown in a laboratory environment with optimal conditions will not behave in a similar fashion to those surviving in the harsher environmental conditions from which they were initially isolated. This is certainly true of pathogens of the CF lung. Grown in a rich medium, cells are able to replicate easily, however organisms adapted to living within the CF lung must upregulate genes that enable survival in this environment. The difference in gene expression between laboratory grown and environmentally isolated cells can have a dramatic effect on how the cells will respond under experimental conditions such as testing for antibiotic resistance (32). Artificial sputum media have been previously developed that attempt to mimic the nutrient conditions of CF sputum (15, 32, 82). These media generally contain ingredients such as DNA, mucin and amino acids, which play a role in biofilm formation (15).

A modified artificial sputum medium (ASMDM) has been shown to induce different gene expression in *P. aeruginosa* than when grown in a rich medium, simulating the growth conditions in the CF lung. This includes upregulation of genes for type III secretion systems, quorum sensing and anaerobic respiration during exponential phase while downregulating type III secretion systems and quorum sensing and upregulating iron acquisition genes and microcolony formation during stationary phase growth (15).

One of the major drawbacks to the use of a BCC mouse lung infection model is rapid bacterial clearing. A preliminary test was performed to determine if a BCC strain grown on an artificial sputum medium could be maintained within the lung for a longer duration (because it has already upregulated the genes required for survival within the lung) than the same strain grown on a non-specific laboratory medium. This was a time-course study, comparing the K56-2 concentration within the mouse lungs when grown in either ½ LB broth or ASMDM broth.

Materials and Methods

Bacterial preparation

The strain used, *B. cenocepacia* K56-2, is the same strain used that was used in the mouse model described earlier. When cultured for mouse infection, K56-2 was grown aerobically in ½ LB broth at 30°C for 16 h with shaking to an optical density of approximately 2.0. After growth the cells were centrifuged at 5000 rpm for 10 min and resuspended in gHBSS (Sigma Aldrich) to the starting volume. The final concentration of K56-2 grown in ½ LB delivered to the mice was approximately $7.8x10^8$ CFU/mL.

ASMDM was prepared as described by Fung *et al*. (15) with one modification. The antibiotics added to the original preparation were omitted because broth contamination was not a concern. When cultured for mouse infection, K56-2 was grown aerobically in ASMDM broth at 30°C for 36 h with shaking to an

optical density of approximately 2.0. After growth the cells were prepared the same way as the ½ LB grown cells. The final concentration delivered to the mice was approximately 5.99×10^9 CFU/mL.

Mouse Model

The mouse model used in this study was the same as described previously, with two modifications. First, phage were not delivered to the mice, as this study was performed to determine the bacterial persistence within the lung. Second, a fourth endpoint was added at seven days post-infection to determine if the K56-2 was able to persist within the lungs for twice as long as the usual test duration.

Results and Discussion

The bacterial lung concentration appeared to differ between mice that were infected with K56-2 grown in ½ LB broth and ASMDM broth (Figure 30). The ASMDM-grown K56-2 appeared to persist at a higher concentration within the lungs for the first three days of the test, while the ½ LB-grown K56-2 concentration decreased more rapidly. Regardless, within one week post-infection the bacterial titres were similar for all mice.

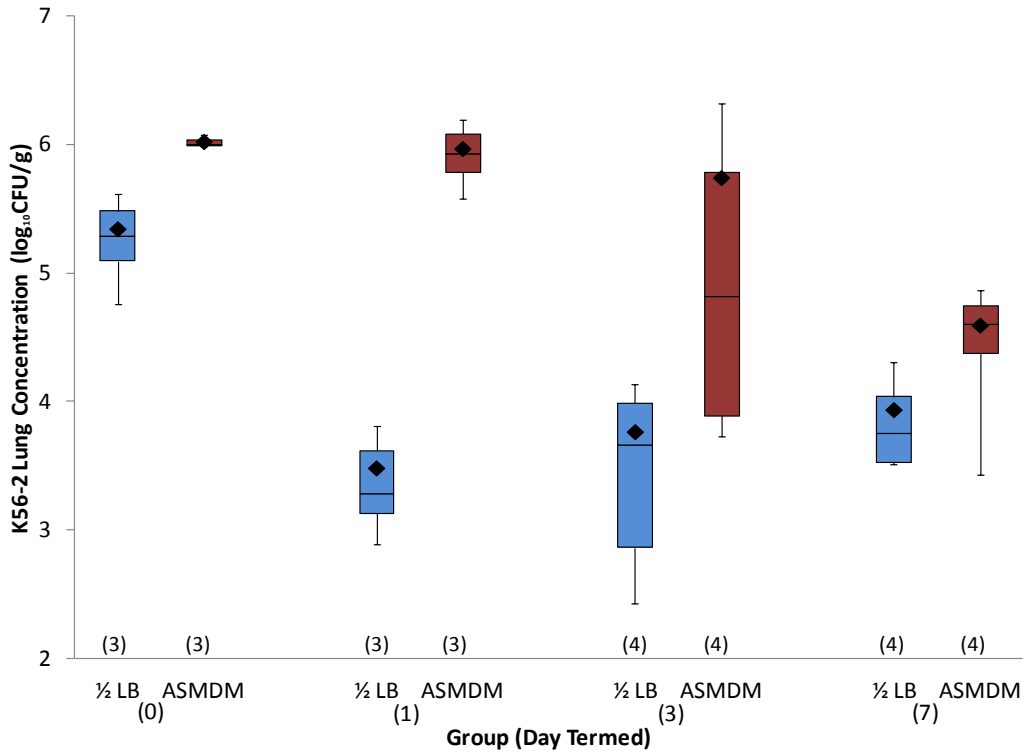


Figure 30. A box and whisker plot depicting the bacterial concentration in mouse lungs after aerosol exposure to K56-2 grown in either ½ LB (blue boxes) or ASMDM (red boxes). The outer box lines indicate the first and third quartile of the data set, the centre box line indicates the median and the whiskers mark the maximum and minimum values of the data set. The mean is indicated by a black diamond. Samples were obtained the same day as exposure as well as one, three and seven days after exposure. The number of mice in each group is noted across the bottom of the x-axis.

One of the difficulties with this test was accurately comparing the two test conditions. The exposure titre for the two groups could not be determined until two days after the bacterial exposure was completed because colonies require 48 h incubation before they can be counted. Therefore, the starting bacterial titre could not be normalised to be the same for both test groups outside of maintaining a similar OD_{600} for both cultures. In an effort to compare the two

exposure groups more accurately the bacterial lung concentration throughout the test duration was compared as a percentage of the original bacterial lung concentration (Figure 31). When the decrease in bacterial lung concentration calculated as a percent decrease of the original concentration, the K56-2 grown in ASMDM appears to persist at a higher concentration within the mice in comparison to K56-2 grown in ½ LB until three days post-infection. By one week after infection only minimal K56-2 concentrations are found in either mouse group.

Although growth in ASMDM does not appear to enable the K56-2 to persist within the mouse lung for longer than K56-2 grown in a generic medium, it still appears that this may be useful for maintaining a higher bacterial load within the lungs. One of the difficulties with the current aerosol phage therapy mouse model is that the bacteria are rapidly cleared from the lung, even if the mice are immunocompromised. However, the model only extends for three days post-infection and this may be a useful method of maintaining a higher bacterial load throughout a test, thereby better highlighting the significant difference in bacterial load between mock- and phage-treated mice.

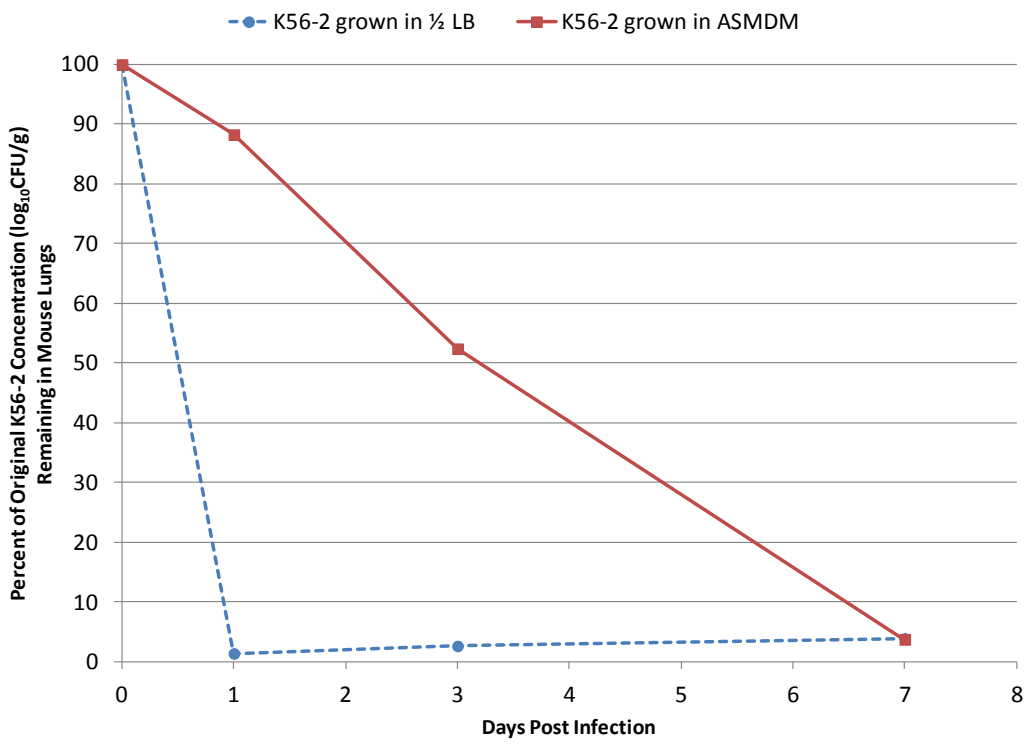


Figure 31. The percentage of the original bacterial concentration remaining in mouse lungs after aerosol exposure to K56-2 grown in either ½ LB (blue dashed line) or ASMDM (red solid line).

Although this test appears promising, it has only been performed once. In the future this study should be repeated in order to determine if there is any statistical validity to the data. Provided that this trend is repeatable, the growth of a BCC host on ASMDM should be incorporated into the aerosol phage therapy mouse model in order to determine if it allows higher bacterial concentrations to be maintained in the lungs of mock-treated mice while still allowing the reduction in bacterial load of phage-treated mice.

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