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Aerosol Phage Therapy Efficacy in *Burkholderia cepacia* Complex Respiratory Infections

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Phage therapy has been suggested as a potential treatment for highly antibiotic-resistant bacteria, such as the species of the *Burkholderia cepacia* complex (BCC). To address this hypothesis, experimental *B. cenocepacia* respiratory infections were established in mice using a nebulizer and a nose-only inhalation device. Following infection, the mice were treated with one of five *B. cenocepacia*-specific phages delivered as either an aerosol or intraperitoneal injection. The bacterial and phage titers within the lungs were assayed 2 days after treatment, and mice that received the aerosolized phage therapy demonstrated significant decreases in bacterial loads. Differences in phage activity were observed *in vivo*. Mice that received phage treatment by intraperitoneal injection did not demonstrate significantly reduced bacterial loads, although phage particles were isolated from their lung tissue. Based on these data, aerosol phage therapy appears to be an effective method for treating highly antibiotic-resistant bacterial respiratory infections, including those caused by BCC bacteria.

Patients diagnosed with cystic fibrosis (CF) are predisposed to acquiring a wide range of respiratory infections, including those caused by *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia* complex (BCC) organisms (1). The BCC is a group of Gram-negative opportunistic bacterial pathogens that can infect a variety of immunocompromised individuals, including those suffering from the genetic diseases cystic fibrosis and chronic granulomatous disease (CGD) (2). BCC infection is the second leading cause of death among CGD sufferers (3). Among CF patients, the BCC infection rate is 5% in Canada, with a mortality rate following BCC acquisition of as high as 20% (4). Of the 18 species that comprise the BCC (5), *B. cenocepacia* is an especially important major bacterial pathogen, as it has been the most common isolate from Canadian (83% of BCC infections) (6) and American (46%) BCC-infected patients (7). More recently, *Burkholderia multivorans* has become the most dominant BCC species isolated in many clinics (8), even though *B. multivorans* strains are generally less virulent than *B. cenocepacia* strains. *B. cenocepacia*-colonized patients undergoing lung transplant have a poorer prognosis than do patients colonized with other members of the BCC (9). Although a minority of CF patients acquire BCC infections, BCC infections are problematic due to their ability to spread rapidly between patients (10) and their progression to cepacia syndrome, an acute infection phase resulting in septicemia, respiratory failure, and reduced life expectancy (11, 12). Therapeutic treatment of BCC infections in CF patients is confounded by the extreme antibiotic resistance demonstrated by the BCC (13–15).

Phage therapy provides an intriguing alternative to antibiotic treatment by employing bacterial viruses, or phages, to reduce or eliminate infection (16). Phages can lyse a bacterial cell with acute specificity (often infecting only a few strains of a species), allowing for targeted treatment of a bacterial infection without disruption of the natural host microflora (17–25). Previous research using animal models has shown that phage therapy can be effective against a wide range of infections, including those involving burn (17, 18), systemic (19), gut (20, 21), and respiratory infections (22–25). A number of phage therapy clinical trials have also been successful (24–26). These include phase I clinical studies of phage application to venous leg ulcers (26) and burn wounds (27), as well as a phase I/II clinical study of phage therapy for chronic otitis caused by *P. aeruginosa* (28).

Aerosol drug delivery is routinely employed as a mode for delivering a wide range of therapeutics (29) directly to the lungs of a patient, and it requires lower drug doses than would be required for other routes of delivery (30). However, until recently, there has been very little written about aerosol phage therapy in the scientific literature other than case studies (29, 31). Contemporary studies using mice have begun to investigate phage therapy as a treatment for *P. aeruginosa* (23–25) and BCC (22) respiratory infections. Although these studies have investigated phage therapy in different mouse infection models, they have done so using intranasal instillation as the delivery method (22–25), which is inefficient and produces significant phage loss through ingestion. We have previously demonstrated that phages can be successfully aerosolized without damage (32, 33), and utilizing this knowledge, we demonstrate here that aerosol phage therapy in mice is effective with phages delivered as a nebulized aerosol. Direct aerosolization of a therapeutic agent is the ideal method of delivery when treating a patient with a respiratory infection, and this method is an established mode of chemical drug delivery (34).

Using a jet nebulizer attached to a nose-only inhalation device (NOID), we first determined that *B. cenocepacia* can be efficiently delivered to the lungs of neutropenic mice to establish infection. Subsequently, we compared the deliveries of five different BCC-specific phages via aerosols and demonstrated efficacy in the treatment of an acute BCC lung infection. Our prior findings in an

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TABLE 1 Relevant characteristics of bacterial strains and bacteriophages used in this study

Bacterium or bacteriophage	Relevant genotype or phenotype (reference)[a]	LD_{50} in *L. minor* (CFU/ml) ± SE (45)[b]	*G. mellonella*		
			LD_{50} (CFU/ml) (54)	PT efficacy (35)[c]	MOI (35)
Burkholderia cenocepacia strains					
K56-2	CF-e, ET12 (Canada) (37)	$1.2 \times 10^1 \pm 7.0 \times 10^0$	9.0×10^2		
C6433	CF-e, RAPD type 4 (Canada) (37)	$2.8 \times 10^4 \pm 2.2 \times 10^4$	3.0×10^4		
Bacteriophage strains					
KS4-M	*Myoviridae*, Mu-like (41)			~60%	1–10
KS5	*Myoviridae*, P2-like (41, 55)			ND	ND
KS12	*Myoviridae* (41, 45)			~60%	5×10^2 to 5×10^3
KS14	*Myoviridae*, P2-like (41, 55)			~50%	0.1
DC1	*Podoviridae*, BHR (56)			ND	ND

[a] CF, cystic fibrosis; CF-e, strain that has spread epidemically among CF patients; BHR, broad host range.
[b] LD_{50}, 50% lethal dose.
[c] PT, phage therapy; ND, not determined.

invertebrate infection model (35) strongly suggested that a therapeutic effect would also be observed in a mouse infection model. Finally, the activity of phages delivered as a respiratory aerosol was compared to that of phages delivered via an intraperitoneal route, and significant differences were discovered.

MATERIALS AND METHODS

Bacterial preparation. The BCC strains used, *B. cenocepacia* K56-2 and C6433, are both respiratory isolates from Canadian cystic fibrosis patients (Table 1) (36, 37). Both strains can spread epidemically and were originally classified as genomovar III-A. Both strains are highly antibiotic resistant, completely resistant to cefepime, imipenem, ciprofloxacin, amikacin, ticarcillin, ampicillin-sulbactam, aztreonam, and rifampin, and have MICs to the frontline drugs ceftazidime, 128 μg/ml; chloramphenicol, >64 μg/ml; doxycycline, >32 μg/ml; meropenem, 32 μg/ml; minocycline, 32 μg/ml; piperacillin, 256 μg/ml; and trimethoprim, >16 μg/ml (38). When cultured for mouse infection, the bacteria were grown aerobically in half-strength Luria-Bertani broth (½LB) (5 g/liter Bacto tryptone, 2.5 g/liter yeast extract, 2.5 g/liter NaCl) at 30°C for 16 h, with shaking, to an optical density of approximately 2.0. After growth, the cells were centrifuged at 3,200 × *g* for 10 min using a centrifuge 5810 R (Eppendorf) and resuspended in Hanks' balanced salt solution with 1% gelatin (gHBSS) (Sigma-Aldrich) to the starting volume. The final bacterial concentrations delivered to the mice, as calculated by serial dilutions of harvested lung tissue samples, were 2×10^9 CFU/ml (for K56-2) and 6×10^9 CFU/ml (for C6433). To determine whether the passaged bacterial cells were developing phage resistance, randomly chosen colonies plated from the harvested lungs of 20 mice treated with phage KS12 delivered by NOID and 20 mice treated with KS12 delivered intraperitoneally (i.p.) were isolated 3 days postinfection. The colonies were plated, purified from single cells, and then tested in plaque assays with purified phage KS12. The plaques were inspected visually, and in all instances, phage sensitivity was observed.

Phage propagation and preparation. All phages used in this study (Table 1) were initially grown either in liquid medium or using an agar overlay method. In liquid medium, 200 ml of ½LB broth was inoculated with 2 ml of bacteria pregrown overnight and incubated at 30°C, with shaking. After 2 h, 20 ml of phage (with a concentration of 10^8 to 10^9 PFU/ml) was added and incubated for an additional 4 h. The final phage stock was filter sterilized using a Rapid-Flow sterile bottle top filter (Nalgene). On solid medium, 300 μl of previously propagated phage (at a concentration of 10^8 to 10^9 PFU/ml) and 300 μl of bacteria pregrown overnight were incubated at room temperature for 20 min in a 16 by 125-mm glass culture tube. After incubation, 10 ml of 55°C ½LB top agar was added, and the mixture was poured onto a 150-mm ½LB agar plate. After ≥16 h of incubation at 30°C, the 25 agar plates were overlaid with 10 ml of sterile Milli-Q water and rocked for 2 h. The liquid was filter sterilized using a Rapid-Flow sterile bottle top filter with 0.45-μm pore size (Nalgene). The resulting 200 ml of phage lysate was centrifuged in an Optima LE-80k ultracentrifuge (Beckman) for 70 min at 371,000 × *g*, and the pellet was resuspended in 7 ml sterile Milli-Q water and filter sterilized 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 a Pierce high-capacity endotoxin removal spin column (Thermo Scientific) (39, 40). The heat-inactivated phage preparation was similar, but after endotoxin removal, the phage stock was incubated at 80°C for 15 min. Phage inactivation was confirmed by a loss of activity in a plaque assay.

Phage choice and concentration. Initial tests were performed with a number of phages previously shown to be active against *B. cenocepacia* strains K56-2 and C6433 (Table 1) (35, 41). The phages were evaluated for their ability to be repeatedly propagated to high titers and for their activity in the mouse model. Additionally, the most effective phage-to-bacteria titer ratios were determined (data not shown). Therapeutic treatment was generally found to be ineffective, or less effective, if the multiplicity of infection (MOI) (the relative number of phage particles to bacterial cells) was <10. Although we were only able to back-calculate the phage numbers actually delivered to the lung tissue, we attempted to test the effects of phages applied at MOIs of approximately 20 (mid-range) and 120 (maximum). To determine the optimal duration from treatment to termination, the bacterial load within the lungs was followed over a 4-day period postinfection. Three days after treatment was found to be the optimal endpoint, as it was before immune suppression began to wane and yet long enough to observe a reduction in bacterial numbers. After 4 days, mouse-to-mouse variability increased, as some control mice began to clear the respiratory infection independently of any treatment. Figure 1 illustrates a typical mouse infection and phage therapy experiment time course.

Immunocompromised mouse infection model. The mouse infection model employed 6- to 8-week-old female BALB/c mice (Charles River Laboratories or the Jackson Laboratory). Prior to infection, the mice were immunocompromised by intraperitoneal (i.p.) cyclophosphamide (CPA) injections causing leukopenia (42, 43). The CPA was administered twice, 3 days apart, at a concentration of 150 μg/g of mouse weight. The infections were performed 1 day after the final CPA injection (after a reduction of approximately 5% from the average starting mouse weight). The mice were infected with either *B. cenocepacia* (K56-2 or C6433) or mock in-

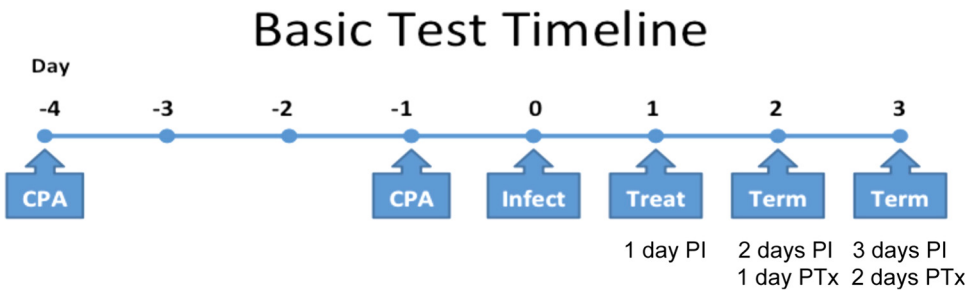

FIG 1 Basic time course of the BCC phage therapy experiments. Cyclophosphamide (CPA) was administered by intraperitoneal delivery to mice on days −4 and −1 relative to the day-0 bacterial infection (Infect) with nebulized bacteria delivered via the NOID. One day postinfection (PI), the mice were again sedated and treated with NOID-delivered nebulized phages (Treat). On day 2 (2 days postinfection/1 day posttreatment [PTx]), the mice were terminated (Term), lung tissue was harvested, and cell and phage counts were obtained. On day 3 (3 days postinfection/2 days posttreatment), similarly, the mice were terminated, lung tissue was harvested, and cell and phage counts were obtained. For one experiment (see Fig. 5), the mice were also terminated on day 4 (4 days postinfection/3 days posttreatment). For several control experiments, the mice were terminated on day 5 or 6. The controls included mock-infected mice (no bacteria delivered, treated with phage), mock-treated mice (bacteria delivered, treated with no phage), and heat-killed phage-treated mice (bacteria delivered, treated with heat-killed phage).

fected with gHBSS using the NOID, as described previously (44). The mice were anesthetized with a ketamine (0.85 mg/g of body weight; Bimeda MTC) and xylazine (0.1 mg/g of body weight; Rompun; Bayer HealthCare) mixture administered by i.p. injection, their eyes were coated with Refresh Lacri-Lube (Allergan) to prevent drying, and mice were inserted into the NOID. Aerosolized *B. cenocepacia* or gHBSS was disseminated using an LC Star nebulizer (Pari International) attached to a Proneb Ultra air compressor (Pari International). All excess and exhaled aerosols were exhausted through RespirGard II filters (Vital Signs, Inc.). The exposure lasted 10 min and used approximately 2.5 ml of liquid. The NOID was decontaminated between runs by aerosolized Virkon (DuPont Chemical) and sterile Milli-Q water. One day after infection, the mice were treated with phages or mock treated with sterile Milli-Q water (Fig. 1). The treatment was delivered by either a 200-μl i.p. injection or by NOID (exposure for 10 min, approximately 2.5 ml liquid). Food and water were provided *ad libitum* throughout the test period. At the trial endpoints, the mice were euthanized by CO_2 asphyxiation and the lungs were harvested, rinsed in sterile gHBSS, and placed in 3 ml gHBSS. The lungs were weighed and mechanically homogenized with a sterilized Brinkmann Polytron homogenizer (Kinematica AG) for 2 min and plated (for bacterial colony counts) or by agar overlay method (for phage plaque counts) on ½LB agar containing 300 mg/liter ampicillin (Sigma-Aldrich). The data were plotted as a box-and-whisker plot (Peltier Tech), and statistical significance was determined by a one-tailed Mann-Whitney U test. All procedures involving the mice were approved by the University of Alberta Animal Care and Use Committee-Biological Sciences (ACUC-BS), in compliance with the Canadian Council on Animal Care (CCAC).

RESULTS

Phage therapy efficacy. Initial tests were performed with five different phages, each able to kill *B. cenocepacia* (phages KS4-M, KS5, and KS12 lyse *B. cenocepacia* K56-2, and phages DC1 and KS14 lyse *B. cenocepacia* C6433). Some of these phages had been shown to be active against *B. cenocepacia* in *Galleria mellonella* and *Lemna minor* infection models (Table 1) (35, 45). In the current study, the phages were evaluated for their ability to be repeatedly propagated to high titers of $\geq 10^9$ PFU/ml and to retain lytic activity in the mouse model. Phage KS12 tested against *B. cenocepacia* strain K56-2 was found to be the best candidate for phage therapy in this infection model, as it was reliably grown to a high titer and the phage persisted in the lungs for ≥3 days (Fig. 2). Although fewer KS12 phages were detected in the lungs approximately 2 h after aerosol NOID-delivered instillation versus i.p. instillation (1 day postinfection), similar amounts of KS12 were found in the lungs 2 days after phage instillation (3 days postinfection), regardless of the route of delivery. A comparison of the median values for either i.p. or aerosol delivery suggests that active phage replication occurs, as more phages are present in the lungs of the mice containing bacteria (i.p., 5×10^3; NOID, 3×10^3) than in the mock-infected mice (i.p., 1×10^2; NOID, 5×10^2) (Fig. 2).

The reduction in the bacterial numbers of *B. cenocepacia* K56-2 by phage KS12 at 3 days postinfection (2 days after phage treatment) was significant and dramatic (a median difference of five orders of magnitude) when the phages were administered at an estimated MOI of 150 (actual MOI, 131, due to off-target loss during NOID delivery) (Fig. 3). The mean change was a 2.5-log difference at 3 days postinfection. However, even nonsignificant reductions in bacterial numbers of over one order of magnitude were still achieved with phage KS12 MOIs as low as 3 (data not shown). Similarly, KS4-M demonstrated a therapeutic effect against *B. cenocepacia* strain K56-2 compared to controls (Fig. 3). At both 2 and 3 days postinfection (1 and 2 days after phage treatment, respectively), at an MOI of 11, the median decrease in bacterial numbers was ≥2.5 orders of magnitude.

Similarly, aerosol phage therapy efficacy was observed with all other BCC phages tested. Phage KS5 tested against *B. cenocepacia* K56-2 also showed a great deal of promise as an effective aerosol phage therapeutic, as initial tests with NOID delivery indicated a significant 4-log decrease in the median bacterial titer 1 day after treatment (2 days postinfection) and a 5-log decrease in the median bacterial titer 2 days after treatment (3 days postinfection) (MOI, 32; Fig. 4). At a lesser MOI of 2, phage KS5 was ineffective at reducing the numbers of *B. cenocepacia* K56-2 cells from the lungs of the infected mice at either time point. However, phage KS5 could not be grown to a titer of $>10^{10}$ PFU/ml, and thus the phage stock to be aerosolized could not be effectively concentrated to 10^{11} PFU/ml, the concentration required to achieve an MOI of >10 in the NOID.

Aerosol phage therapy efficacy was also demonstrated by phage KS14 against a different BCC strain, *B. cenocepacia* C6433. Phage KS14 showed a 2-log mean reduction in strain C6433 concentration in the lungs compared to phage-free controls 4 days postinfection (3 days after treatment) when delivered by nebulization at an MOI of 5 (Fig. 5). In contrast, although still exhibiting therapeutic efficacy, phage DC1 was one of the least effective phages we

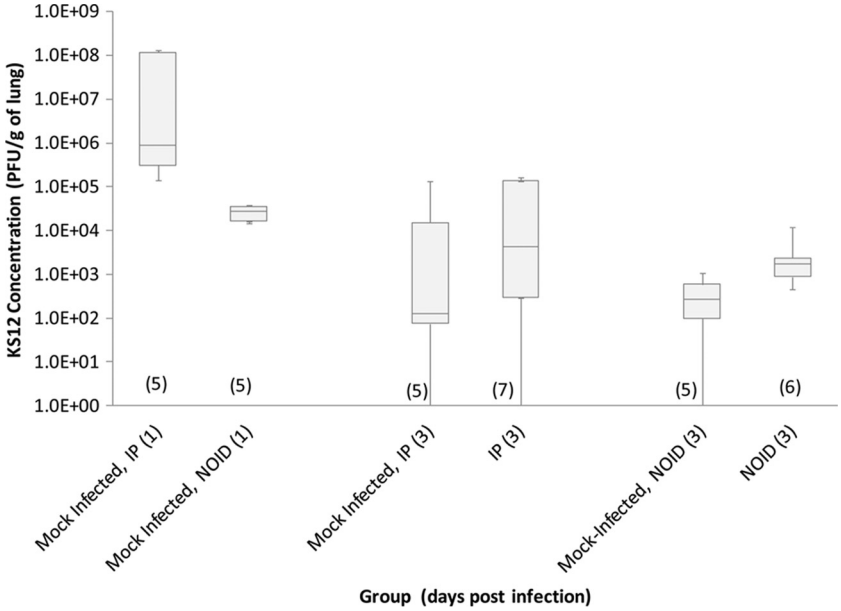

FIG 2 Recovery of BCC phage KS12 from lungs 24 h (1 day) and 72 (3 days) after phage instillation. The phage concentration was determined in mice infected with *B. cenocepacia* K56-2 and in mock-infected mice (no bacteria). Phages were delivered to the mice by intraperitoneal (IP) injection or by NOID aerosolization, as noted. The boxes indicate the first quartile, median, and third quartile values, and the whiskers indicate the maximum and minimum values. The phages were collected either 1 or 3 days postdelivery, as noted below the *x* axis. The number of mice tested in each group is noted above the *x* axis.

tested *in vivo*. Phage DC1 also showed a 2-log reduction in the mean bacterial numbers of *B. cenocepacia* strain C6433 compared to the controls, but only when administered by NOID at a high calculated MOI of 122. In comparison, phage KS14 administered at an MOI of 5 was approximately as effective as phage DC1 administered at an MOI of 122 (Fig. 5), indicating that phage KS14 is more efficient at killing bacteria *in vivo*. Also shown in Fig. 5, the mock-treated controls indicate that *B. cenocepacia* C6433 numbers increased for the first 2 days (mean population increased from 10^5 to 10^7), subsequently decreasing over the next 2 days (10^7

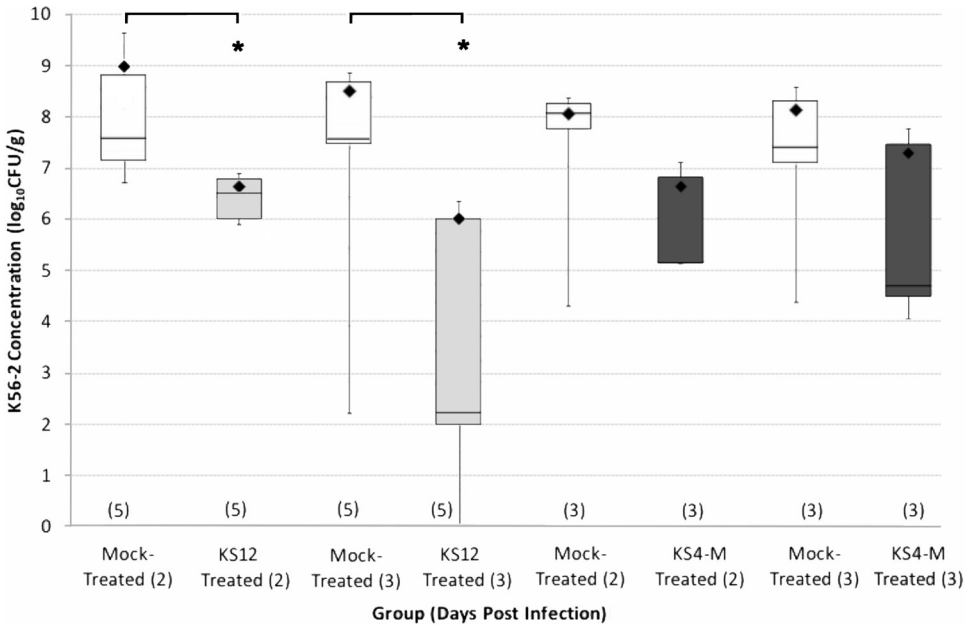

FIG 3 Phage treatment is effective at reducing *B. cenocepacia* cell numbers *in vivo*. The bacterial clearance of *B. cenocepacia* strain K56-2 in mock-treated (white boxes), KS12-treated (light shaded boxes), and KS4-M-treated (dark shaded boxes) mice over a 3-day period is shown. Phages were delivered by NOID 24 h postinfection, and the bacterial concentrations were determined 2 and 3 days (48 and 72 h) postinfection. The KS12 MOI was calculated to be 131, and the KS4-M MOI was calculated to be 11. The data are representative of three independent trials. The boxes indicate the first quartile, median, and third quartile values, and the whiskers indicate the maximum and minimum values. The diamonds indicate mean values. The number of mice in each group is indicated above the *x* axis. *, $P < 0.05$ (Mann-Whitney U test).

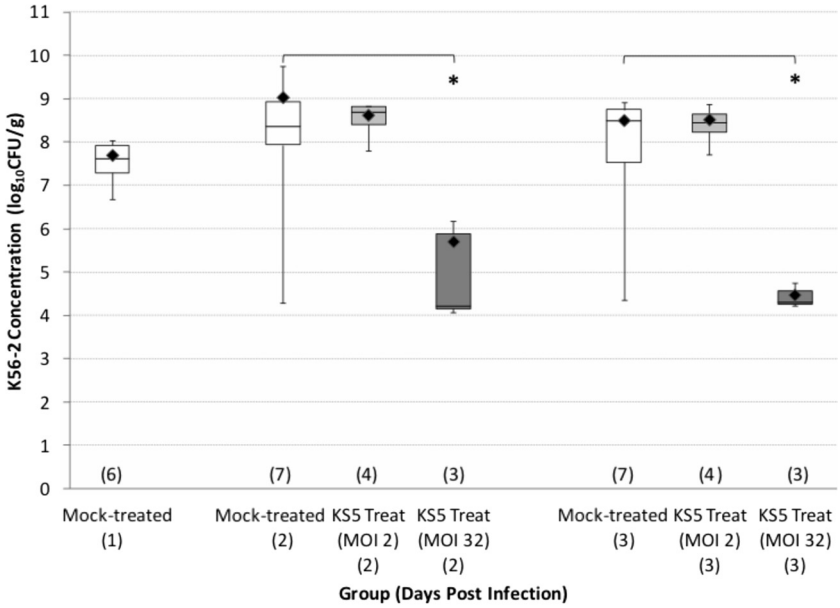

FIG 4 Phage KS5 therapy efficacy is significant at high but not low MOIs. The bacterial clearance of *B. cenocepacia* strain K56-2 in mock-treated mice (white boxes) and mice treated with KS5 at a low MOI of 2 (shaded boxes) or a high MOI of 32 (dark shaded boxes) over a 3-day period is shown. Phages were delivered 24 h postinfection by NOID, and bacterial concentrations were determined on the day of treatment as well as 2 and 3 days (48 and 72 h) postinfection. The boxes indicate the first quartile, median, and third quartile values, and the whiskers indicate the maximum and minimum values. The diamonds indicate mean values. The number of mice in each group is indicated above the *x* axis. *, $P < 0.05$ (Mann-Whitney U test).

to $10^{4.5}$). However, the reduction in bacterial numbers 4 days postinfection was greater when either phage DC1 or KS14 was administered.

To demonstrate that phage activity is required for effective phage therapy, the efficacy of heat-killed phages delivered by NOID was tested *in vivo*. *B. cenocepacia* K56-2-infected mice treated with aerosolized heat-killed KS12 phage (3×10^{10} PFU/ml) showed no bacterial clearance, which was similar to the results obtained when mice were mock treated with aerosolized sterile Milli-Q water (Fig. 6). This is in contrast to the significant decrease

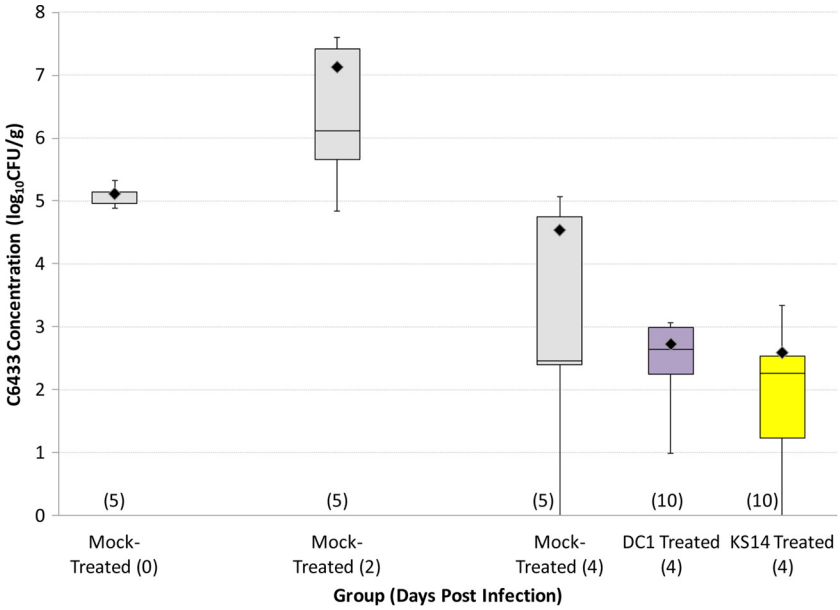

FIG 5 Four days postinfection (3 days after treatment), DC1 and KS14 phage-treated mice exhibit increased bacterial clearance of *B. cenocepacia* strain C6433 compared to controls. Bacterial numbers from mock-treated mice (gray boxes), BCC phage DC1-treated mice (purple box), and phage KS14-treated mice (yellow box) over a 4-day period are shown. Phages were delivered 24 h postinfection by NOID, and bacterial concentrations were determined on the day of infection as well as 2 and 4 days (48 and 96 h) postinfection. The MOI of the DC1 treatment was 122 and the MOI of the KS14 treatment was 5. The boxes indicate the first quartile, median, and third quartile values, and the whiskers indicate the maximum and minimum values. The diamonds indicate mean values. The number of mice in each group is indicated above the *x* axis.

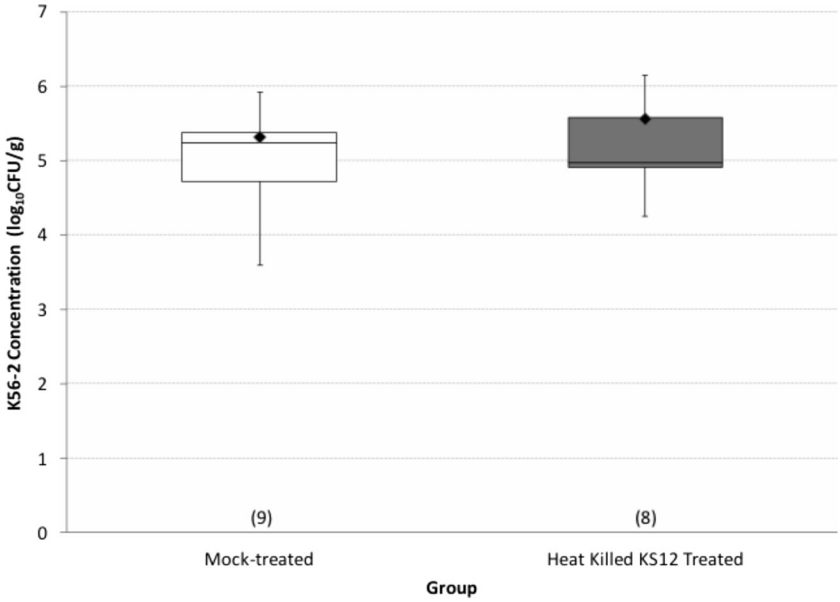

FIG 6 No difference in bacterial clearance is observed in mice treated with heat-killed KS12 phages compared to mock-treated controls. The bacterial clearance levels in NOID-delivered mock-treated mice (sterile water) (white box) and heat-killed KS12-treated mice (initial concentration, 3×10^{10} PFU/ml) (shaded box) 2 days posttreatment (3 days postinfection) are shown. The boxes indicate the first quartile, median, and third quartile values, and the whiskers indicate the maximum and minimum values. The diamonds indicate mean values. This trial was performed in triplicate. The number of mice in each group is indicated above the *x* axis.

in bacterial titer observed in mice treated with the same initial stock of NOID-delivered KS12 at a calculated MOI of 120 (mean, 1×10^4 CFU/g; median, 5×10^2 CFU/g). This demonstrates that active phages are required for effective phage therapy and suggests that bacterial clearance is not merely a result of immune system activation due to the introduction of foreign phage proteins.

Comparison of phage KS12 NOID and i.p. delivery. A previous study found i.p. delivery of phage therapeutics to be a more effective method of phage delivery than intranasal instillation for treating a *B. cenocepacia* respiratory infection in a mouse model (22). To evaluate whether i.p. phage delivery was more effective than aerosol phage delivery, two different treatment delivery methods were compared: KS12 aerosol inhalation via NOID versus i.p. injection. After treatment and emergence from anesthesia (approximately 2 h), phages were isolated from the lungs of mice from each phage delivery group, demonstrating that i.p.-injected KS12 phages are able to move from the peritoneum to the lung. Initially, there was an average of 5×10^7 KS12 phage particles per g of lung tissue recovered from the i.p. mock-infected mice ($n = 10$), as opposed to 2×10^4 phage particles per g of lung tissue from the NOID mock-infected mice ($n = 10$). Two days after treatment, there was an average of 7×10^4 phage particles per g of lung tissue from the i.p. mock-infected mice ($n = 10$) versus 5×10^4 phage particles per g of lung tissue from the NOID mock-infected mice ($n = 10$). Similarly, 2 days after treatment, there were 5×10^4 phage particles per g of lung tissue from i.p.-infected and -treated mice ($n = 16$) and 1×10^4 phage particles per g of lung tissue from the NOID-treated mice ($n = 14$). However, 2 days after treatment, the *B. cenocepacia* K56-2 bacterial numbers remained relatively constant between i.p.-delivered mock-treated (no phage) mice and i.p.-delivered KS12-treated mice (mean values, 1×10^7 versus 4×10^6, respectively), suggesting that i.p.-delivered phages have little effect on the bacteria inside the lungs (Fig. 7). In contrast, K56-2-infected mice receiving KS12 phage treatment via NOID aerosol demonstrated a significant decrease in the mean bacterial load within the lungs compared to NOID mock-treated (no phage) mice (Fig. 7) (4×10^6 versus 1×10^8, respectively). A similar 2-log decrease in the median bacterial numbers was observed in the NOID-delivered KS12-treated mice (7×10^4) compared to the NOID-delivered mock-treated mice (8×10^6).

DISCUSSION

In a previous study, we demonstrated that phage therapy can be used to significantly reduce the number of BCC bacteria in a *G. mellonella* infection model (35). This initial study showed that treatment efficacy was influenced by both the phage MOI and the postinfection timing of treatment delivery. Moreover, individual BCC phages were observed to possess differences in their *in vivo* activity often unrelated to their *in vitro* activity, a trend that has recently been studied in more detail (46, 47). Although our previous article described the first use of phages to eliminate or reduce BCC infections in a higher organism, it was performed in an alternative infection model with limited physiological relevance to human BCC lung infections. Therefore, in this current study, the aim was to determine whether our previous BCC phage therapy success could be reproduced in a murine model of lung infection using aerosolized phages.

Aerosolized drug delivery is an effective method for delivering therapeutics directly to diseased pulmonary sites in patients with respiratory illness (34). However, to date, respiratory phage therapy studies have not evaluated the aerosol method of phage delivery. Previous studies have utilized experimental lung infections using either intranasal instillation or tracheotomy and subsequent phage delivery using intranasal instillation or i.p. injection (22–25). In intranasal phage delivery, the phage preparation is placed on the nares, and the anesthetized animal aspirates the liquid,

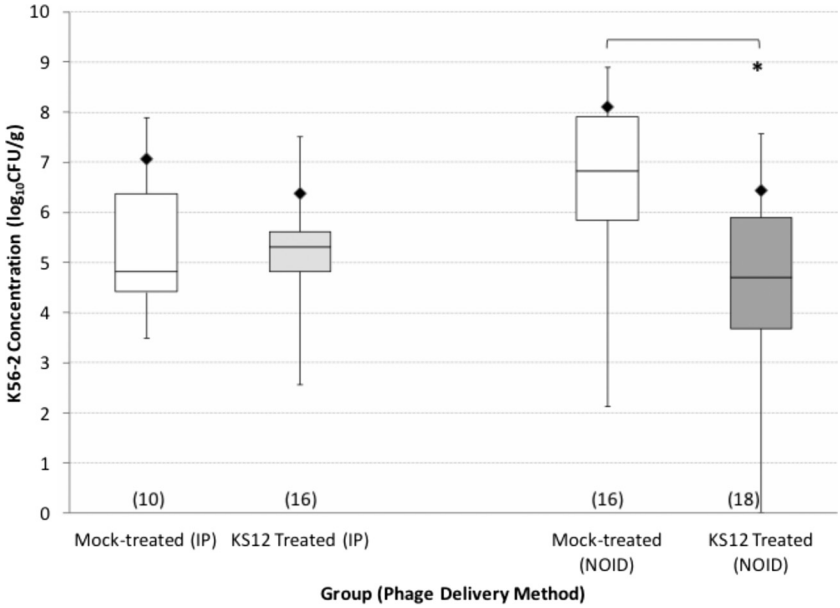

FIG 7 i.p.-delivered phage KS12 produces no more bacterial clearance than that in i.p. mock-treated mice, whereas NOID-delivered KS12 produces more bacterial clearance than that in NOID mock-treated mice. The bacterial clearance levels in mock-treated (white boxes) mice and KS12-treated mice receiving an i.p. injection (shaded box) or NOID treatment (dark shaded box) 2 days after treatment are shown. The boxes indicate the first quartile, median, and third quartile values, and the whiskers indicate the maximum and minimum values. The diamonds indicate mean values. The trial for i.p.-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).

which often results in a high percentage of phage particles being deposited into the gastrointestinal tract. In contrast, phage particle aerosols generated by a nebulizer and delivered via a NOID constitute a better method for alveolar phage deposition (32). Several recent studies suggested that aerosol phage therapy is not only possible but that it can be an effective method for delivering phages directly to the site of a pulmonary bacterial infection (32, 33, 48). In this study, immunocompromised and BCC-infected mice receiving aerosolized phage treatments exhibited significant decreases in bacterial loads within the lungs. For example, after 2 days posttreatment, phage KS12 at an MOI of 131 produced a 2.5-log mean reduction (a median 5-log reduction) in *B. cenocepacia* K56-2 (Fig. 2). Similarly, phage KS5 at an MOI of 32 produced a 3-log mean reduction in *B. cenocepacia* K56-2 in the lungs 1 day posttreatment and a 4-log mean reduction in bacterial numbers 3 days posttreatment; both changes were statistically significant (Fig. 3). A therapeutic effect was observed to some extent across all five different phages and across the two different strains of *B. cenocepacia* tested. For example, in *B. cenocepacia* C6433, phages DC1 (MOI, 122) and KS14 (MOI, 5) both produced approximately a 2-log mean reduction in bacterial numbers 3 days posttreatment, despite the fact that at this time point, some mock-treated control mice were beginning to show some clearance of the bacterial infection (Fig. 5). Decreases in the bacterial numbers in the lungs due to aerosol phage therapy ranged from approximately 1.5- to 5-log difference from the mock-treated controls, depending upon both the activity of the phage *in vivo* and its ability to be concentrated *in vitro* to achieve an effective MOI following NOID delivery. Both of these variables appear to be inherent characteristics of each specific phage that differ irrespective of phage morphology, size, or host range.

The observed reduction in bacterial numbers was in some cases (Fig. 2 and 3) similar to the best therapeutic effect that can be achieved using chemical antibiotics (49, 50). However, the pharmacokinetics of phage therapy is different from that of traditional antibiotics (51). While a chemical antibiotic will typically be degraded shortly after being administered, phages are potentially able to replicate once reaching their bacterial host (active phage therapy). As can be seen in Fig. 1, although the overall phage KS12 numbers dropped from day one to day three, active phage replication was evident, as more phages were present in mice containing BCC bacteria than in the mock-infected control mice. Hypothetically, the ability to replicate should render even a low phage concentration efficacious, as the phage concentration will increase autonomously until the bacterium is eliminated. Our results for each individual phage varied somewhat, primarily due to differences in MOI (generally, the higher the MOI, the better the therapeutic effect), which was directly dependent upon our ability to produce high-titer phage stocks. Our results suggest that an optimum quantity of phages (generally producing an MOI of >10) was better at reducing bacterial numbers than was a lower number of phages. The number of phages obtained during high-titer stock production is dependent upon a number of biological parameters (that are different for each phage), including phage-bacterium interactions, phage burst size, and generation time. The number of phages that are deposited in the lungs of mice may also vary depending upon the ability of phage particles to form aerosols and travel through the airspace to find and adhere to their specific bacterial hosts, in contrast to, for example, phages suspended in the gut environment, in which chance bacterial cell encounters may be more frequent. Going forward, the ability to achieve a specific reproducible dosage of phages will be an important factor in developing an aerosol phage therapy strategy.

During the phage preparation, we used ultracentrifugation to

increase phage concentrations; however, this approach was not sufficient to adequately increase the titers of some BCC phages (KS4-M and KS5) for optimal NOID delivery. As increasing phage titers of the delivered phage stock generally improved the therapeutic effect, a promising alternative preparative method that might be used in the future to purify and further concentrate phages is anion-exchange chromatography, such as the Convective Interaction Media monolithic column (BIA Separations). A recent report indicates that a 30-ml T4 phage sample containing 8×10^8 PFU/ml can be concentrated to a 3-ml sample containing 5.7×10^9 PFU/ml in 10 to 40 min (52). A similar concentration outcome would provide an effective phage titer for aerosol delivery using any of the BCC phages tested in this study. Even if phage titers cannot be increased to the extent required to treat mice with a single dose, multiple doses may also prove to be an effective treatment method. Two case studies found multiple aerosol phage treatments to be effective in a clinical setting (29, 31). In our study, the administration of a second phage treatment prior to the end of the trial may have yielded a further reduction in the bacterial concentration in the lungs; however, it would have also required additional CPA administration, greatly increasing the complexity of the experimental design.

A previous study found i.p. 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 (22). Interestingly, we found that infected mice receiving aerosol phage therapy demonstrated a significant decrease in bacterial load within the lung, while mice receiving treatment via i.p. injection did not show any significant decrease in bacterial numbers. A potential explanation for the discrepancy in these findings relates to the delivery method of the phages. Aerosolization is an optimal method of particle delivery and has previously been shown to be a more effective method for delivering particles to the lung than intranasal instillation, with aerosolization providing more widespread and uniform particle deposition than intranasal instillation (53). Similarly, although i.p. phage delivery may be more effective than intranasal phage instillation, it is not as effective as phage aerosolization. Also, although the i.p.-delivered phages may be able to reach the lungs, they may be restricted to certain areas within the lung tissue and unable to colocalize with the bacteria in the lung lumen. Using immunofluorescence, Carmody et al. (22) found that 48 h after treatment, intranasally delivered phages localized to the alveolar macrophages, whereas i.p.-delivered phages localized to the perivascular areas and the alveolar septa. Conversely, by 24 h postinfection, the bacteria delivered by tracheotomy were localized mainly to the lung parenchyma, especially the peribronchiolar and perivascular areas, and they remained in the lung parenchyma throughout the course of the study. Thus, depending upon where bacteria are localized during pulmonary infection, i.p.-delivered phages may not be able to access the bacterial targets.

Prior to this study, the only previous study to evaluate phage therapy for BCC infections in mouse lungs did not demonstrate phage therapy efficacy using noninvasive phage delivery (22). In direct contrast, our results demonstrate that aerosol phage therapy for the BCC is efficient and effective. This study lays the foundation for further aerosol phage therapy research, as this experimental methodology can be adapted to different pathogenic bacteria and respiratory infection models. In conclusion, this report supports the use of aerosol phage therapy as a viable treatment method for bacterial infections and, specifically, for pulmonary infections caused by bacteria of the BCC.

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