Benefits of aerosolized phages for the treatment of pneumonia due to methicillin-resistant *Staphylococcus aureus* (MRSA): an experimental study in rats

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Main point: Nebulized bacteriophages (aerophages) showed promise for the treatment of pneumonia due to MRSA; aerophages reduced bacterial loads and lung damage, and when combined with systemically applied phages, rescued >90% of animals from lethal infection.

Abstract

Background The optimal method for delivering phages in the context of ventilator-associated pneumonia (VAP) is unknown. In the current study, we assessed the utility of aerosolized phages (aerophages) for experimental MRSA pneumonia.

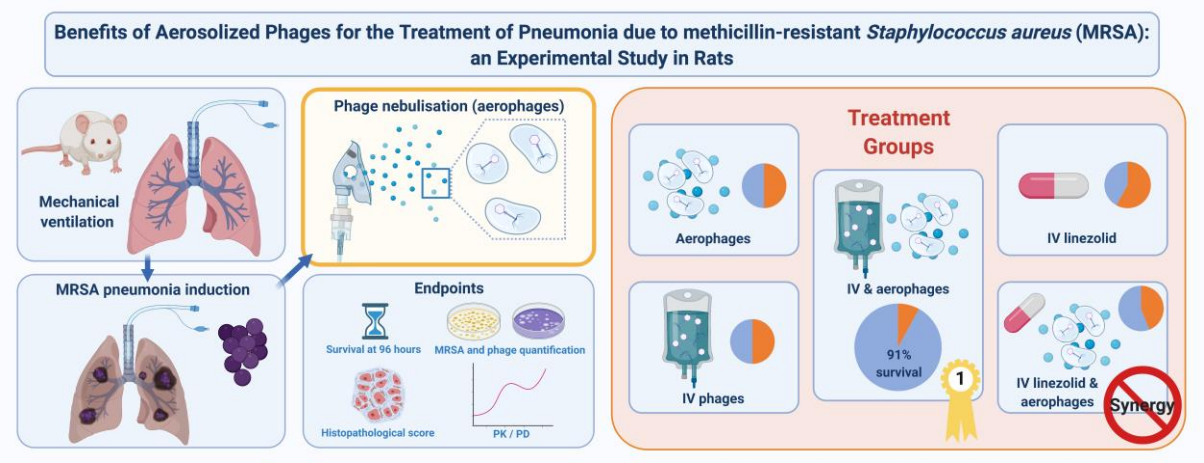
Methods: Rats were ventilated for 4h before induction of pneumonia. Animals received either: 1) aerophages; 2) intravenous (IV) phages; 3) a combination of IV and aerophages; 4) IV linezolid; and 5) a combination of IV linezolid and aerophages. Phages were administered at 2, 12, 24, 48 and 72h, and linezolid at 2, 12, 24, 36, 48, 60 and 72h. The primary outcome was survival at 96h. Secondary outcomes were bacterial and phage counts in tissues, and histopathological scoring of the lungs.

Results: Aerophages (1) and IV phages (2) each rescued 50% of animals from severe MRSA pneumonia (P<0.01 compared to placebo controls). The combination of aerophages and IV phages rescued 91% of animals, which was higher than either monotherapy (P<0.05) (3). Standard-of-care antibiotic linezolid (4) rescued 38% of animals. Linezolid and aerophages (5), however did not synergise in this setting (55% survival).

Conclusions: Aerosolized phage therapy showed potential for the treatment of MRSA pneumonia in an experimental animal model and warrant further investigation for application in humans.

Key words: antibiotic resistance; phage therapy; inhalative; nosocomial infections; ventilator associated pneumonia

Visual_Abstract



INTRODUCTION

Mechanically ventilated patients are at risk of contracting ventilator-associated pneumonia (VAP) caused by multidrug resistant (MDR) bacterial pathogens [1]. *Staphylococcus aureus* remains a frequent contributor to infection in this setting and despite active programs focused on eliminating methicillin-resistant *S. aureus* (MRSA), it still accounts for 5-15% of all cases of VAP [1-4].

There is a well-recognised need to develop new strategies to combat MDR infections. One such approach is the application of bacterial viruses known as phage therapy [5]. We have shown previously that intravenous (IV) phage therapy was as effective as standard-of-care antibiotics for the treatment of experimental VAP due to MRSA [6]. The findings were promising, however phages did not rescue all of the animals nor did it completely eradicate MRSA from the lungs. Additionally, phages and antibiotics did not synergise to improve outcomes in this setting [6].

To maximise the potential for phage therapy in the context of lung infections, alternative administration strategies warrant investigation. Inhalative therapy using nebulized antibiotics has gained clinical acceptance for infections caused by gram-negative bacteria, and current guidelines recommend adjunctive inhalative treatments in cases of bacterial persistence and antibiotic resistance [7, 8]. Nebulized therapeutics have been shown to concentrate at the lung infection site, effectively reduce bacterial loads, and limit systemic exposure and the emergence of antibiotic resistance [9, 10]. Proof-of-concept experimental studies investigating aerosolized phage therapy revealed efficacy for the treatment of respiratory tract infections caused by gram-negative bacteria [11, 12], and a case report detailing the treatment of a patient with complex *Pseudomonas aeruginosa* VAP treated with IV and aerosolized phages revealed positive outcomes [13]. Additionally, prophylactic application of nebulized phages improved survival of rats in an experimental MRSA VAP model [14]. However, no study assessing the utility of nebulized phages for the treatment of establish

MRSA pneumonia has been performed.

Here, the efficacy of aerosolized phages ('aerophage') for the treatment of experimental MRSA pneumonia was investigated. Aerophages alone, and in combination with IV administered therapies (phages and standard-of-care antibiotics) were assessed. Additional experiments were performed to improve our understanding of phage pharmacokinetics and pharmacodynamics (PK/PD) in the context of lung infection.

MATERIAL AND METHODS

MRSA pneumonia model. All animal experiments were approved by the Cantonal Committee on Animal Experiments of the State of Bern, Switzerland (approval BE 83/17) and according to ARRIVE Guidelines. The model has been described previously [6]. Briefly, Wistar rats [Crl:WI(Han), male, 9-10 weeks old, Charles River, Germany] were intubated, then ventilated for 4h (10mL/kg tidal volume, 5cmH_2O of positive end-expiratory pressure, 50 breaths/min with FiO_2 0.35). This strategy produced mild signs of inflammation and pulmonary oedema commonly associated with mechanical ventilation in critical care patients [6]. MRSA clinical isolate AW7 was used to establish pneumonia [15]. Following ventilation, rats were inoculated via the endotracheal tube with ~1x10^{10}CFU then extubated. Animals were monitored for signs of illness using a score system described previously [6, 14], and were euthanized with pentobarbital (150mg/kg) as a humane endpoint. Survival at 96h was the primary outcome. The secondary outcomes were bacterial and phage loads in the lungs and spleen, and histopathological scoring of pneumonia [16]. All secondary outcomes were assessed as described previously [6, 14].

Aerophage procedure. The phage cocktail consisted of equal titers of four genetically unique phages called 2003, 2002, 3A and phage K (1.5x10^{10} plaque forming units [PFU]/ml) [6, 14]. The combination of these phages was shown to be effective against 92% of *S. aureus* isolates tested [14], and each phage was capable of infecting MRSA strain AW7. Aerophages were delivered using a modified vibrating mesh aerosol drug delivery system

used in humans (Aerogen Solo technology, USA, average particle size 3.1µm) **(Fig. 1A)**. Animals were put into an adapted induction chamber and sedated with Sevoflurane (1–3%). To achieve optimal drug delivery, sedated spontaneously breathing animals were connected to the nebulizer via a full-face mask (**Fig. 1A**). Each treatment (2ml volume) lasted ~10 minutes. Three animals were administered aerophages and were immediately euthanized to assess phage distribution (PFU) in lung tissue.

For 'sham' animals, MRSA was replaced by 0.15ml 0.9%NaCl following ventilation (0h). At 2h and 12h, animals received either aerophages (n=3), IV phages (n=4), or a combination of both (n=4). Animals were euthanized at 13h and PFU determinations were performed for the blood, lung tissue, bronchoalveolar lavage (BAL) fluid, spleen, liver, and kidney. A second set of uninfected animals received either placebo (n=5), aerophages (n=6), or IV phages (n=5) at 2, 12, 24, 48 and 72h, and were then sacrificed at 96h. Blood was taken for IL-1β quantification, as described previously [6]. The placebo consisted of a filtered bacterial supernatant that did not contain phages.

Treatment protocol. In the first round of experiments MRSA infected animals were randomized into three groups following inoculation. All animals received both an inhalative treatment and an IV treatment each consisting of either phages or placebo. The therapy was administered in an investigator/operator blinded manner. Treatment groups were as follows: aerophages (n=10); IV phages (0.3ml per treatment, n=10); a combination of both (n=11). Each treatment was further applied at 12, 24, 48 and 72h. We knew from previous studies that IV phage therapy alone would result in 50% survival [6], and we hypothesized that aerophage treatments would increase survival to 99-100%. These estimates (alpha=0.05, power 1-β=0.8) required n=11 per group (SigmaPlot 12.0). Two animals were not fit enough to be randomized following surgery. Eight animals were included as untreated controls.

A second round of experiments was performed to assess the additive effects of aerophages with IV linezolid. Animals received either IV linezolid (10mg/kg) and inhaled placebo (n=8),

or linezolid and aerophages (n=9). Inhalative therapy was administered at 2, 24, 48 and 72h after infection and linezolid according to manufacturer's recommendation twice daily (at 2, 12, 24, 36, 48, 60 and 72h after infection). Sample sizes were reduced following an interim analysis of survival that revealed no synergistic effects.

Phage resistance determination. The possible emergence of phage-resistant MRSA clones following phage treatment was assessed using phage cross streak assays as described previously [6].

In vitro phage-linezolid assessment. An overnight culture of AW7 was diluted in tryptic soy broth containing 2mM $CaCl_2$ to reach ~1x10^{6}CFU/ml. Cultures were supplemented with either phages (multiplicity of infection [MOI] of 0.01) or phages and linezolid (10μg/ml), then incubated at 37° with shaking for 24h. PFU were quantified before (0h) and after treatment (24h). The experiment was performed twice in biological triplicates.

Statistics. Survival of animals was assessed using Kaplan Meier curves and log-rank tests. One- or two-way ANOVA (normal distribution), or Kruskal-Wallis tests (non-normal) were used. Multiple comparisons were corrected for using Dunnett's method. Correlations were determined using Pearson two-way correlation tests. All analyses were performed using GraphPad Prism (v7). Data were considered significant when P<0.05.

RESULTS

Aerosolised phages remain active in vitro and in vivo. Phage titres were quantified before and after nebulization. On average 93% of phages (SD=1.52%, n=3) were recoverable following nebulization. Aerophages were then applied to the lungs of uninfected rats to determine bioavailability. On average, 1.4x10^{6}PFU/g of phages were recovered. No difference in phage titres were detected between cranial and caudal sections, suggesting a uniform distribution of aerophages within the lung tissue (**Fig. 1B**).

Aerophages reduce mortality for animals with established MRSA pneumonia. MRSA pneumonia was lethal for untreated controls (**Fig. 2A**). Treatment with aerophages significantly improved survival (50%, *P*< 0.01, **Fig. 2A**). Survival was associated with reduced bacterial loads in the lungs (**Fig. 2B, Fig. S1A**), and lower histopathological scoring for lung tissue (**Fig. 2C**), when compared to non-surviving animals.

Failure to clear MRSA from the lungs was not due to phage resistance. Aerophages did not eradicate MRSA from the lungs of animals with pneumonia (**Fig. 2B**). To determine if bacterial persistence in the lung was attributable to phage resistance, the phage susceptibility of 100 bacterial colonies taken from the lungs of 10 phage-treated animals was tested. Each isolate remained susceptible to the phage cocktail suggesting that the failure to eradicate the bacteria was not due to the selection of phage-resistant clones.

Aerophages localise in the lung, and do not spread to other organs. To explain the limited therapeutic efficacy of the aerophages, we performed a series of experiments addressing phage PK/PD in uninfected animals. First, we assessed phage distribution in various tissues following two rounds of phage treatment (2h, 12h). Aerophages revealed a local distribution, highly abundant in lung tissue and BAL fluid, and less abundant in the blood, spleen and liver (**Fig. 3A**). The distribution of aerophages was then compared with that of animals treated with an IV bolus of phages using the same treatment regimen. IV phages were abundant in the blood, liver and spleen, but less concentrated in the BAL and lung tissue when compared to aerophage treatment (~4,000 and ~22–times fewer, respectively *P*<0.0001, **Fig. 3A**). Combining the two routes resulted in high local and systemic distribution of phages (**Fig. 3A**).

Aerophages do not induce an inflammatory response. We showed previously that repeated IV administration of phages resulted in an elevated inflammatory response, as determined by increased levels of the proinflammatory cytokine IL-1β in blood [6]. The limited distribution of aerophages in the blood, liver and kidney led to the hypothesis that

phages localised primarily in the lung will produce a dampened systemic inflammatory response. In support of this, repeated administration of phages IV (2h, 12h, 24h, 48h, 72h) produced an IL-1β response in the blood at 96h that was 11-fold higher than that produced by aerophages (P< 0.0001, **Fig. 3B**).

Aerophages adjunct to IV phages is an effective treatment for experimental MRSA pneumonia. In the context of MRSA pneumonia, while IV phage therapy alone resulted in 50% survival, the combination of aerophages and IV phages significantly improved survival when compared to each monotherapy (91% survival, p<0.05 for each comparison, **Fig. 3C**).

There was a significant difference in MRSA counts in the lungs between treatment groups (*P*=0.008, one-way ANOVA, **Fig. S1B**). When performing pairwise comparisons, the average MRSA load in the lungs for animals receiving aerophages or IV monotherapies was ~100 times lower when compared to placebo controls; however this was not statistically significant (*P*=0.226 and 0.152 respectively, **Fig. S1B**), likely owing to the large difference between non-surviving and surviving animals in each group (**Fig. S1A**). In contrast, the combination aerophages and IV treatment group which had the best survival animals, had ~1,000-times less MRSA in the lungs compared to untreated controls, and this difference was significant (*P*=0.005, **Fig. S1B**). When compared with aerophages alone, reduced lung bacterial densities associated with combination IV phages and aerophages were not statistically different (*P*=0.340). No statistical differences in MRSA counts in the spleen were detected between treatments (*P*=0.512, Kruskal-Wallis test, **Fig. S1C**).

Aerophages adjunct to IV linezolid did not improve survival for rats with experimental MRSA pneumonia. Given the efficacy of combined systemic and local phage therapy (**Fig. 3C**), we next evaluated the aerophages/linezolid IV combination. While IV linezolid alone rescued 37.5% of animals from lethal pneumonia, the combination with aerophages did not synergise in vivo. No improved survival (**Fig. 4A**), nor improved bacterial clearance (**Fig. S1B**), was observed compared to either monotherapy. There was a positive correlation

between phage and bacterial loads in the lungs for animals treated with aerophages alone (**Fig. 4B**). In contrast, no correlation was observed for animals treated with the combination (**Fig. 4B**). Additionally, phages were detected in the spleen for half of the animals treated with aerophages, but not detected in any of those that received the combination (P=0.033, Fischer's exact test, **Fig. S1E**), suggesting linezolid may have a detrimental effect on phage amplification. To lend support to these in vivo associations, MRSA was exposed to phages, linezolid, or a combination of both, in vitro. In the absence of linezolid, phages increased by ~10^6 PFU/ml fold after 24h. In contrast, the addition of linezolid abolished phage amplification at the tested MOI of 0.01 (**Fig. 4C**).

DISCUSSION

We systematically evaluated the utility of nebulized phages for the treatment of experimental MRSA pneumonia in rats. The key findings of this study, presented in Supp. Tab. 1, are as follows: (i) aerophages administered directly to the lungs retained their activity, and remained localised at high concentration within lung tissue following application; (ii) aerophages alone improved survival for animals with MRSA pneumonia, and when combined with IV phages rescued almost all of the animal subjects; and (iii) aerophages adjunct to IV linezolid did not synergise in this experimental setting.

IV phage therapy improved survival for animals with MRSA pneumonia, supporting previous findings [6]. IV therapy, however, did not rescue all animals, or eradicate MRSA from the lungs. We postulated that IV therapy failed in non-survivors due to poor PK/PD, including limited penetration into the lung, and failure of the phages to overcome the clearing effects of the blood [17]. Inhalative therapy has shown potential to overcome the caveats of IV therapy in the context of pneumonia; at least for antibiotics, high concentrations can be achieved at the site of infection that are not tolerable using systemic application, and this is associated with reduced systemic side effects such as toxicity and antibiotic resistance development at non-respiratory sites (10, 20). Similar benefits were associated with aerophage therapy;

compared with IV delivery, aerophages were more concentrated in the lungs, as demonstrated by high titres in the BAL, and they were not associated with systemic side effects such as a heightened proinflammatory response or the emergence of phage resistance.

Aerophages, however, did not rescue all of the animals from MRSA pneumonia. It is possible that aerophage treatment failure may have occurred due to insufficient penetration of phages to poorly aerated areas of lung parenchyma; a hypothesis that is worthy of further investigation. It is also possible that, given the localised distribution of aerophages in the lung, mortality for non-surviving animals was due to infection metastasis. In humans, bacteraemia appears to be a major predictor of mortality for patients with VAP [18, 19]. Indeed, in the experimental model of pneumonia used in this study, mortality was significantly associated with the presence of MRSA in the spleen, which we use as a proxy for systemic spread (**Fig. S1A**)**.** Combining a localized therapy (aerophages) with systemic therapy (IV phages) improved survival compared to either therapy alone, and was associated with the best microbiological outcomes in the lungs and spleen.

Glycopeptides and linezolid are the first line treatments for MRSA pneumonia [20]. We chose linezolid for assessing aerophage-antibiotic combination therapy because unlike for glycopeptides (teicoplanin), it synergised with phages in vitro [6]. Combined linezolid and aerophages therapy in the pneumonia model did not result in improved outcomes compared to either therapy alone. It is apparent that phage amplification (termed ‘autodosing’ [21]), is important for successful treatment of MRSA pneumonia. For aerophages alone, there was a positive correlation between bacteria and phage counts in the lung, suggesting bacterial host-dependent phage amplification, which was not observed for animals receiving adjunct linezolid. Linezolid is a bacteriostatic agent that inhibits protein synthesis [22]. Phages rely on bacterial machinery to replicate, and the action of linezolid impaired phage amplification.

When tested in vitro, some phage-antibiotic combinations reveal either synergisms or

antagonisms, depending on the concentration of each agent [23]. Previously, we showed synergy between linezolid and phages using a checkerboard assay [6]. In contrast, when simulating the PK parameters of the pneumonia model in vitro (Linezolid 10 µg/ml, based on a C*max* of ~15µg/ml [24], and phage MOI of 0.01), linezolid drastically impaired phage amplification, which may explain the poor treatment outcomes for the aerophage-linezolid combination therapy. Future studies are required to further understand the interaction between phages and antibiotics, in order to exploit synergies, and avoid antagonisms.

The model used in this study has important limitations. It is rapidly lethal, and animals require treatment shortly after inoculation (2h), which does not accurately reflect the clinical scenario. The model also relies on a high dose of bacteria to establish a reproducible infection ($1x10^{10}$CFU). In order to achieve a reasonable MOI for therapy, high phage doses were administered ($3x10^{10}$PFU per treatment). In contrast, in the two published human case studies using nebulized phages, each patient was administered $1.5x10^{10}$PFU per treatment [13, 25], which is considerably lower when the size of the subject is accounted for. Thus, it is difficult to extrapolate optimal phage dosing as it pertains to humans using the current rodent model. Further studies are warranted addressing aerophage dosing and lung distribution using larger experimental animals such as pigs [26].

In summary, aerophage therapy has shown potential for the treatment of pneumonia due to MRSA, and when combined with systemic phage therapy, improved animal survival and reduced MRSA burdens in tissues. Results from this translational study pave the way for future placebo-controlled trials assessing the safety and efficacy of adjunct aerophages for the treatment of VAP due to MRSA in humans.

Footnote Page:

Conflict of interest: All authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf and declare no conflicts of interest.

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Authors Contribution: JP, MH, DRC, YAQ conceived the project; JP, LV, MI, LF, DRC, performed experiments; SS performed histopathology; DG performed the cytokine analysis; DRC, JP, LV, MI, DG, SJ, GR, SLL, MH, and YAQ analysed the data; DRC, JP, YAQ wrote the manuscript; all authors reviewed and edited the manuscript, and approve of the final submission**.**

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FIGURES

Figure 1. Nebulized delivery of phages to the lungs of rats. (A) Administration of phages aerosolized via an Aerogen® Solo vibrating mesh nebulizer used in humans and generating particles of 3.1µm average size. **(B)** Phage titres in cranial and caudal sections of lungs in uninfected animals immediately following one aerophage treatment. PFU, plaque forming units.

Figure 2. Aerosolized phage treatment of rats with MRSA pneumonia. (A) Kaplan-Meier survival curves for rats with untreated MRSA pneumonia (n=8), and those treated with aerophages (n=10). Significance was determined by log-rank test. **(B)** MRSA bacterial loads in the lungs of rats with MRSA pneumonia. CFU; colony forming units. **(C)** Histopathological score was determined for the lung of rats with MRSA pneumonia. For each panel, 'AERO' (blue) represents aerophage treatment. Secondary endpoints (CFU, histology) were assessed immediately after an animal succumbed to infection (represented by crosses), or at the end of the 96 hour trial (represented by closed circles). For B and C, significance was assessed using one-way ANOVA with corrections for multiple comparisons using Tukey's method. ** $P<0.01$; **** $P<0.0001$.

Figure 3. PK/PD and treatment efficacy comparisons for uninfected animals treated with aerophages, IV phages, or the combination of both. (A) Animals received two doses of phages (2h, and 12h after ventilation) and were sacrificed at 13h. Animals received either aerophages (AERO, n=3), IV phages (IV, n=4), or a combination of each (AERO+IV, n=4) Phage loads were quantified from the blood, bronchoalveolar lavage (BAL) fluid, and various organs. Statistical differences were determined using two-way ANOVA with corrections for multiple comparisons using Tukey's method, **** $P<0.0001$. PFU, plaque forming units. **(B)** Additional uninfected animals received repeated doses (12, 24, 48 and 72h) of either aerophages (n=6) or IV phages (n=5). Blood was taken at 96h

and IL-1β was compared to baseline (0h) for each animal. Untreated animals (n=5) were included as controls. Statistical significance was determined using one-way ANOVA with multiple comparison correction using Tukey method, **** $P<0.0001$. **(C)** Kaplan-Meier survival curves for rats with MRSA pneumonia treated with aerophages (n=10), IV phages (n=11), or a combination of each (n=11). Significance was determined by log-rank test, * $P<0.05$.

Figure 4. Combination linezolid and aerophage therapy for the treatment of MRSA pneumonia. (A) Kaplan-Meier survival curves for rats with MRSA pneumonia treated with aerophages ('AERO', n=10), IV linezolid ('LZD_IV', n=8), or a combination of each ('AERO+LZD_IV', n=9). Significance was determined by log-rank test; no differences were significant. **(B)** Correlation analysis of bacteria and phage loads in the lung of animals treated with either aerophages or aerophages and linezolid. CFU, colony forming units (CFU) and plaque forming units (PFU) were assessed immediately after an animal succumbed to infection (represented by crosses), or at the end of the 96 hour trial (represented by closed circles). Pearson two-way correlation tests were used to determine statistical significance. **(C)** MRSA strain AW7 was exposed to phages in vitro (multiplicity of infection 0.01), with, or without linezolid (10µg/ml). Phages were quantified before (0h) and after treatment (24h).

Figure 1

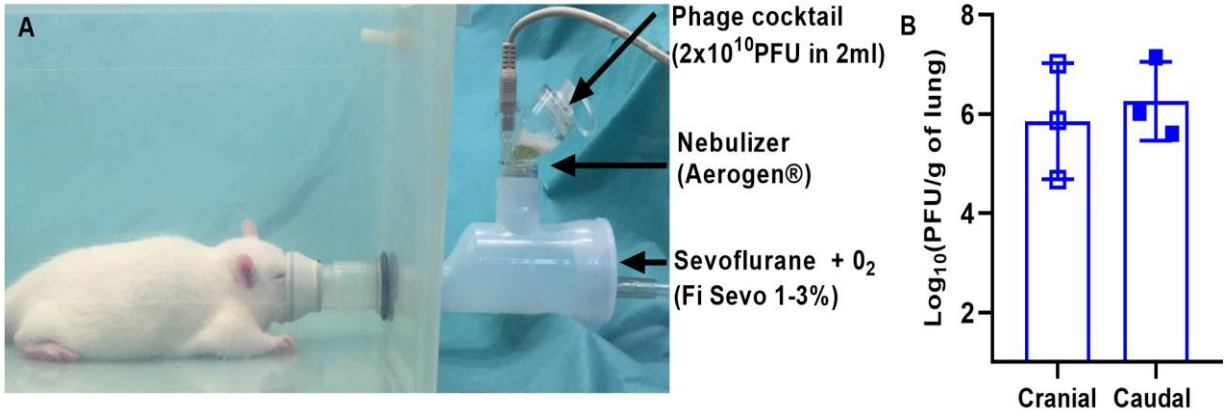

Figure 2

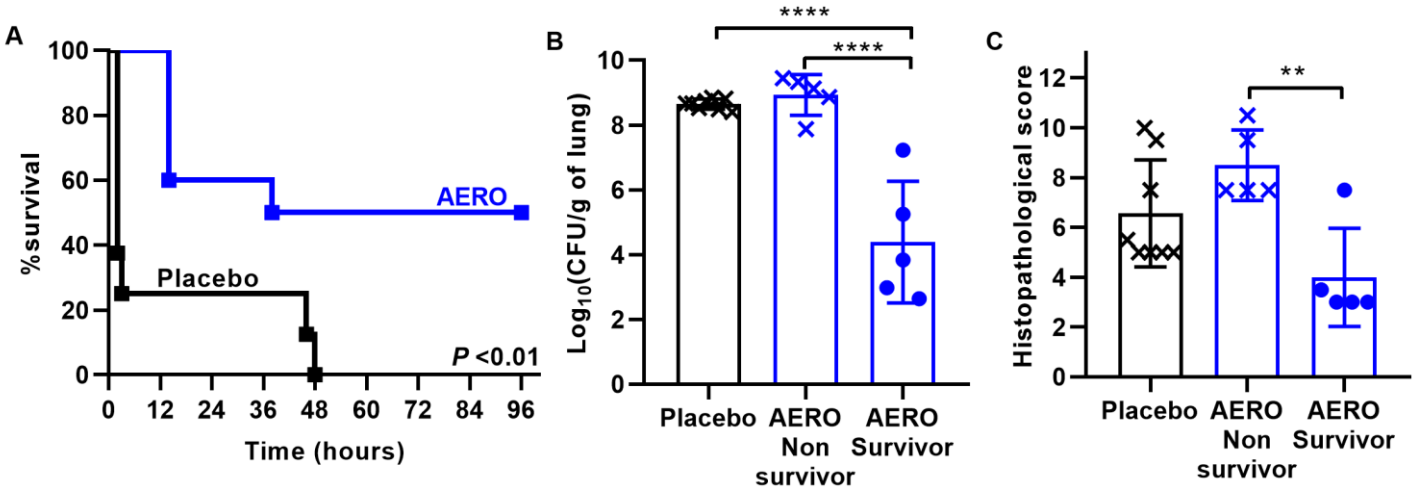

Figure 3

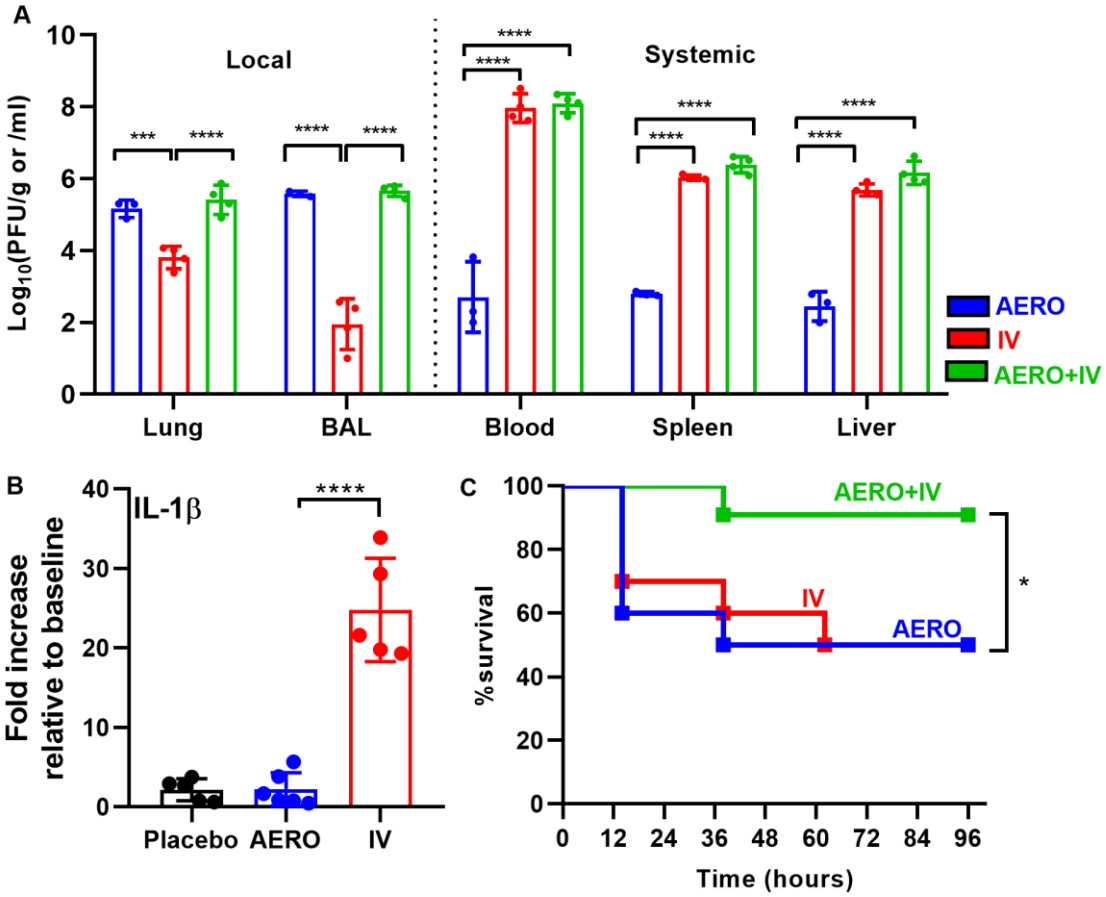

Figure 4