HS-06

Bacteriophage, Isolated from Mouse Feces, Attenuates *Pseudomonas aeruginosa* in Pneumonia Mouse Model

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Abstract

Pseudomonas aeruginosa is one of the most antibiotic-resistant nosocomial infections. It commonly causes respiratory infections (pneumonia). The emergence of MDR *P. aeruginosa* has made it increasingly difficult to treat with current antibiotics, highlighting the necessity for novel treatments. Phage therapy is the therapeutic use of bacteriophages (bacterial viruses) for the treatment of pathogenic bacterial infections. The use of phages in killing MDR bacteria offers a possible alternative to conventional antibiotic treatments for MDR bacterial infections. In this study, phages were isolated from mouse feces using a *P. aeruginosa* clinical isolate as the host and were used to treat a *Pseudomonas*-associated pneumonia mouse model. The results showed that intratracheal administration of phages significantly improved survival rates in pneumonia models ($p<0.01$). The number of bacteria in the bronchoalveolar lavage fluid (BALF) and blood was significantly decreased ($p<0.0001$) in phage-treated mice compared to untreated mice. In contrast, the presence of phages in BALF and blood increased ($p<0.05$). Pathological examination showed that the phage-treated group had significantly reduced hemorrhage, inflammatory cell infiltration, and lung injury score ($p<0.01$). The levels of inflammatory cytokines in the serum, including IL-6, TNFα, IL-1β, and IL-10, were significantly lower ($p<0.0001$) than in the PA group. The study results suggest that phage therapy can potentially be used to treat pneumonia caused by *P. aeruginosa*.

Keywords: Pseudomonas aeruginosa, Pneumonia, Phage, Phage therapy

Introduction

Pneumonia is a common acute respiratory infection that results to the alveoli and distal bronchial tree of the lungs. Aspiration pneumonia is widely categorized into community-acquired pneumonia (CAP) and hospital-acquired pneumonia (HAP, including ventilation-associated pneumonia (VAP)) (Torres et al., 2021). According to the difference of the causative microorganisms for CAP and HAP, the most common causal microorganisms in CAP containing *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Mycoplasma pneumoniae*, and *Legionella pneumophila*, as well as respiratory viruses. Conversely, the most recurrent pathogens in HAP are both methicillin-susceptible and resistant *Staphylococcus aureus*, *Acinetobacter* spp., and non-fermenting Gram-negative *Pseudomonas aeruginosa*.

P. aeruginosa is a gram-negative opportunistic nosocomial pathogen in human that *can* causes various types of healthcare-associated infections in bloodstream, lungs (pneumonia), urinary tract, and other parts of the body after surgery, especially the patients in the intensive care unit (ICU). Interestingly, most patients with *Pseudomonas* caused pneumonia were detected colonization and biofilm formation within 12 h of intubation and remained more 96 h (Feldman et al., 1999), indicating that treatments of *P. aeruginosa* infection are extremely difficult because of its adaptation to gain resistance to antibiotics and biofilm formation. Therefore, the crisis of *Pseudomonas* caused pneumonia was concerned about the exploration of the alternative treatment against *pseudomonas* infection. Because the effectiveness of currently used antibiotics was limited, therapy with bacteriophage has gained increased attention.

Bacteriophages or phages are viruses that specifically target bacteria to treat or prevent infectious diseases. Bacteriophage therapy is one of the interesting alternative treatments because they are capable of disrupting the target bacterial pathogens without affecting the host or host commensal microbiota (Abdelsattar et al., 2021), and there are the specific target site that different from broad-spectrum antibiotics (Hasan & Ahn, 2022), which means that a lower therapeutic dose has more effective than antibiotic therapy (Śliwka, Ochocka, & Skaradziński, 2022). In addition, bacteriophages can be easily isolated from various environments using simple and inexpensive methods (Domingo-Calap, Georgel, & Bahram, 2016). Bacteriophages can enter mammalial cells through cell-surface receptors and phagocytosis by attaching pathogens during infection or via bacterial lysogens (Domingo-Calap et al., 2016). In pathogen-related pneumonia, neutrophils are the first leukocytes which recruited from the circulation to the lungs and remove pathogens by various mechanisms including phagocytosis, release of reactive oxygen species (ROS), neutrophil extracellular traps (NETs) formation (NETosis), and release of antimicrobial products such as α-defensins, azurocidin, and lactoferrin (Teng, Ji, Ji, & Li, 2017). Subsequently, bacteriophage therapy can induce bacterial phagocytosis of immune cells by attachment on the surface of bacteria, which is recognized by innate immune cells especially on neutrophils (Kaur, Harjai, &

Chhibber, 2014), indicating that bacteriophages not only interact with bacteria but also the responsiveness of host. In addition, several reports revealed that host innate immunity is necessary for bacteriophages to be effective in treating respiratory bacterial infections (Leung & Weitz, 2017; Pincus et al., 2015; Roach et al., 2017; Tiwari, Kim, Rahman, & Kim, 2011)

Phage therapy has also demonstrated up to success in pre-clinical animal experiments for treatment of infections caused by multidrug-resistant pathogens. This study will be evaluated bacteriophage specific to *P. aeruginosa* isolated from mouse feces that can attenuate *P. aeruginosa in vivo* pneumonia mouse model.

Purposes

1. To isolate bacteriophages against *P. aeruginosa* from mouse feces
2. To elucidate phage therapy in *Pseudomonas* associated pneumonia mouse model

Research Methodology

Bacteriophage isolation

The feces from the cecum and colon of mice were mixed with *P. aeruginosa* in trypticase soy broth (TSB) and incubated at 37°C and 200 rpm for 18 hours. After incubation, the overnight culture was centrifuged at 5,000 xg and 4°C for 30 minutes, and the supernatant was collected and filtered using a 0.22 µm filter. The presence of *Pseudomonas* phage was confirmed by plaque assay. Briefly, an overnight culture of P. aeruginosa was mixed with filtered phage supernatant and transferred to soft agar (TSB + 0.7% agar; top) and overlaid on trypticase soy agar (TSA; bottom). The plates were then incubated at 37°C for 16 hours to allow plaques to form, indicating the presence of phages.

Host range determination

Seventeen clinical *P. aeruginosa* isolates were used to assess the host range of bacteriophage by spot test. Three milliliters of 0.7% agar TSB were mixed with 1 mL of log-phase bacterial culture and poured on TSA. After solidification of top agar, 10 µL of bacteriophage was spotted on the plate and incubated for 18 h at 37°C to detect the presence of bacterial lysis.

Thermal and pH sensitivity

The thermal stability was determined in 1X SM buffer in temperature ranging of 20°C to 80°C for 1 h to characterize the thermotolerant property. pH stability was determined by incubating bacteriophage in 1X SM buffer at pH ranging from 1 to 14 for 24 h at 37°C. Then, both thermal and pH stabilities of bacteriophage detected by plaque assay as described above.

Bactericidal activity

Overnight culture of *P. aeruginosa* was adjusted to a turbidity of 0.5 McFarland (approximately 1×10^8 CFU/mL), and then 100-fold serial diluted to 1×10^6 CFU/mL. Subsequently, the bacteria were added into 96-well plates (200 µL/well) with or without (growth control) phage suspension at multiplicity of infection (MOI) of 0.1, 1, and 10, and incubated at 37°C. The bacterial turbidity was measured by spectrophotometry at OD_{600nm} every 1 h intervals for 6 h.

One step growth curve

Bacteria was mixed with phage at MOI 0.1 and incubated to adsorb for 10 min at 37°C with shaking 200 rpm. Then the mixture was centrifuged 12000 rpm for 2 min. The pellet was suspended with 5 ml TSB and incubated at 37°C 200 rpm in incubator shaker. 200 µl of sample was take every 20 min intervals between 0 and 160 min. The phage was estimate by plaque assay.

Pneumonia mouse model

Male 12-week-old C57BL/6 mice was intratracheally administrated with 0.2 mL of 10^8 CFU/mL of *P. aeruginosa*. After 2 h of infection, *Pseudomonas* related pneumonia mice was intratracheally administrated by 0.2 mL of 10^9 PFU/mL of bacteriophage. After treatment, mice were sacrificed by cardiac puncture under isoflurane anesthesia before sample collection (blood and internal organs) for bacterial and phage enumerations by colony count and plaque assay, cytokine measurement by ELISA, and histological analysis.

Histological analysis

The lung tissues of Pseudomonas-associated pneumonia were fixed with 10% neutral buffered formalin and embedded in paraffin. Samples were sliced at a thickness of 5 µM and stained using hematoxylin and eosin (H&E), which is the standard method. Each sample was visualized using an inverted microscope (Olympus, Inc., Japan) at 200x magnification in 10 randomly selected fields. The lung injury score was determined based on neutrophils in the alveolar space, neutrophils in the interstitial space, proteinaceous debris filling the air spaces, and alveolar septal thickening, using a modified lung injury score (Matute-Bello et al., 2011) as follows.

Data analysis

Statistical differences will be examined using an unpaired student's t-test or one-way analysis of variance (ANOVA) with Tukey's to analyze the two or multiple groups, respectively, and presented as mean ± standard error (SE), with $p < 0.05$ considered significant. All experiments will be repeated three times before being statistically analyzed using GraphPad software version 7.

Results

Isolation and characterization of bacteriophage

Isolation of bacteriophage specific to *P. aeruginosa* from mouse feces.

The mouse feces from healthy mice were collected to enrich *Pseudomonas* phage, and the presence of the phage was observed by plaque using a double agar assay. Four phages, including PaP_AL1, PaP_AL2, PaP_AL3, and PaP_AL4, were isolated from mouse feces, each with different plaque morphology and size, as shown in Fig. 1. PaP_AL1 showed a clear plaque with a diameter of approximately 2 millimeters (Fig. 1A). PaP_AL2 and PaP_AL3 had a similar plaque morphology with a clear point (Fig. 1B, C). PaP_AL4 showed two circular layers with an inner circle of approximately 1 millimeter in width and a completely clear center surrounded by a 0.5-millimeter-wide turbid outer circle (Fig. 1D). The phages were collected and enriched for host range determination.

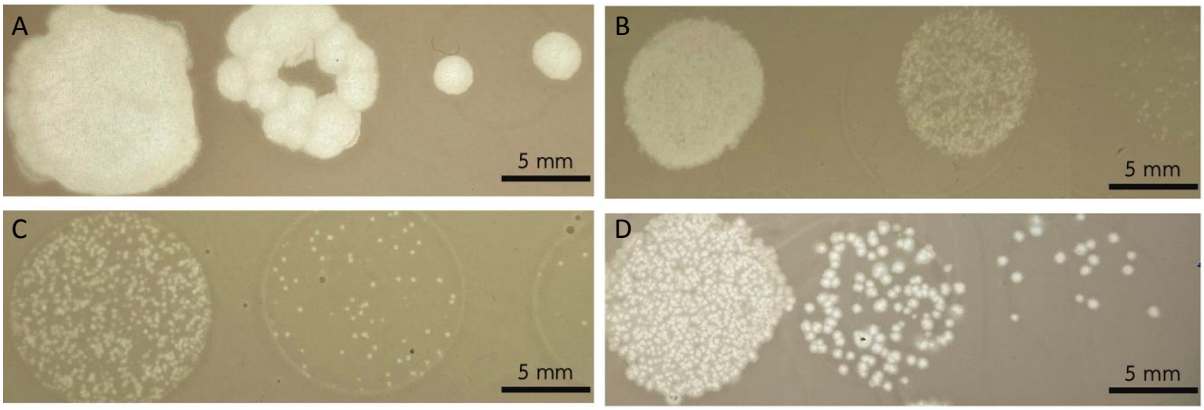

Figure 1 Plaque morphology of PaP_AL1(A), PaP_AL2 (B), PaP_AL3 (C), and PaP_AL4 (D) in double agar layer plate with different concentration

Host range determination

The ability of each phage to infect different bacteria was tested using spot test analysis. The different phages showed a varying number of hosts that they could infect, as shown in Table 1. PaP_AL1 was found to be the most effective, being able to infect 10 out of 17 P. aeruginosa isolates. PaP_AL1 was then collected, purified, and propagated for further study.

Table 1 host range of different phages against *P. aeruginosa* isolates. Host bacteria isolates are indicated on the left, and phage isolates are indicated at the top. The lysis of host is indicated by black color.

Hosts	PaP_AL1	PaP_AL2	PaP_AL3	PaP_AL4
PA1				
PA2	■			
PA3	■	■		■
PA4	■			
PA5	■	■		■
PA6	■			
PA7	■			■
PA8				
PA9	■			■
PA10				
PA11				
PA12				
PA13	■		■	
PA14				
PA15	■			
PACl	■	■	■	■
PAO1				
% Killing	58.82	11.76	5.88	29.41

Characterization of bacteriophage specific to *P. aeruginosa*

Bactericidal activity

To identify the *in vitro* bactericidal activity of PaP_AL1 phage, *P. aeruginosa* was cultured and then infected with PaP_AL1 phage at MOI of 0.1, 1, and 10 at 37°C. As shown in Fig. 2, the results show that PaP_AL1 has bactericidal activity at MOI of 0.1, 1, and 10.

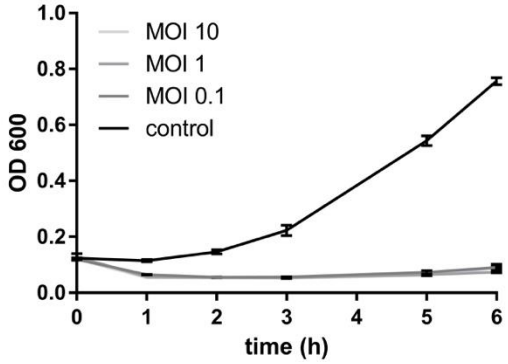

Figure 2 Bactericidal activity of PaP_AL1 was monitored for 6 h. Biological three independent experiments were conducted, and the mean and SEM are shown.

One step growth curve

One-step growth curve experiments were performed to determine the phage growth parameters, namely, the latent period and burst size. The phage latent period is defined as the time between phage infection and host cell lysis, while the burst size refers to the expected number of virions produced by one infected cell. PaP_AL1 was found to have a latent period of 80 minutes, and the burst size was approximately 188 PFU/infected cell, as shown in Fig. 3.

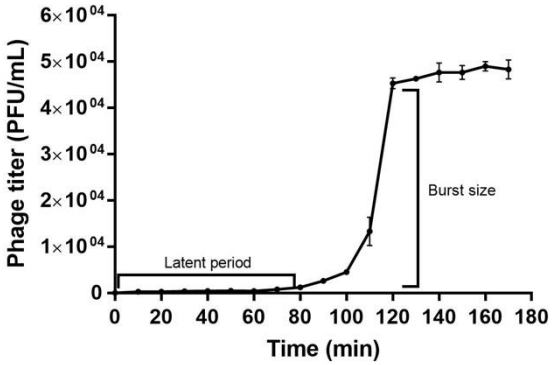

Figure 3 One-step growth curves of PaP_AL1. Experiments were conducted independently in triplicated, and the mean and SEM are represented.

Thermal and pH sensitivity

The pH stability of PaP_AL1 was tested at different pH values ranging from pH 1 to pH 12. After 60 minutes of incubation, the phage showed stable infectivity at a wide range of pH values from pH 3 to pH 13. The phage's thermal tolerance was also tested, showing stable infection after incubation at 4°C, 20°C, and 40°C for 60 minutes. However, the phage's infectivity decreased at 60°C, and at 80°C, the phage particles lost their infectivity.

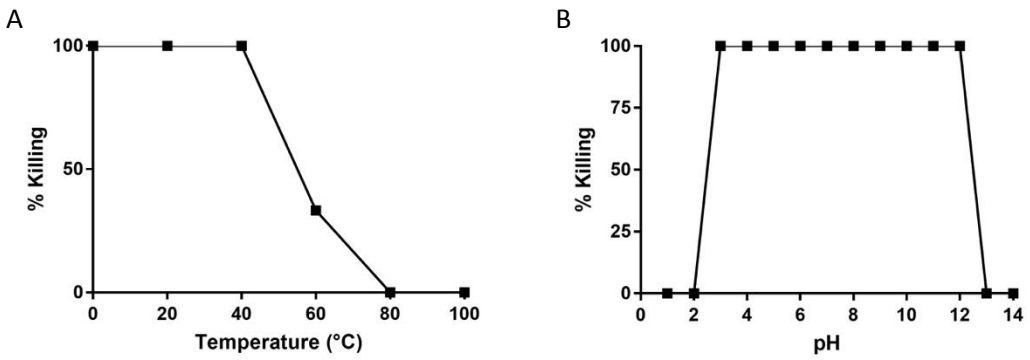

Figure 4 Thermal (A) and pH (B) sensitivity of PaP_AL1. The % killing of phages were shown as mean ± SEM from the triplicate experiments.

Phage therapy in pneumonia mouse model

The survival rates of the treatment group and the PA group were compared to determine whether PaP_AL1 has a protective effect against pneumonia caused by *P. aeruginosa*. The survival rate of mice in the treatment group was significantly higher than that in the PA group (75% vs. 12%; $p < 0.01$). This indicates that PaP_AL1 has a protective effect against pneumonia caused by *P. aeruginosa*.

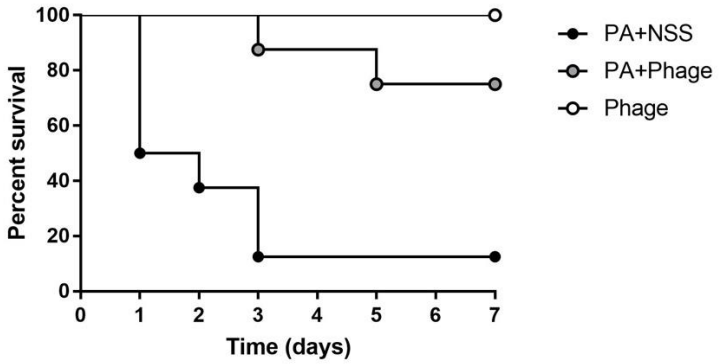

Figure 5 Survival rate in pneumonia mice. Mice were inhaled 200 μL of *P. aeruginosa* (1×10^8 CFU/ml) and inhaled with 200 μL of phage PaP_AL1 (1×10^9 PFU/ml) at 2 h post bacterial inoculation.

Number of Bacteria and Phage in BALF and Serum

The ability of PaP_AL1 to clear bacterial pneumonia was measured by colony count in the BALF and serum. The number of viable bacteria in the BALF of the treatment group was significantly lower than the PA group (treatment group $1.33\text{x}10^3$ CFU/mL vs. PA group $1.43\text{x}10^7$ CFU/mL; $p < 0.0001$). Similarly, in the serum, the number of viable bacteria in the treatment group was significantly lower than the control group (treatment group $3.66\text{x}10^3$ CFU/mL vs. control group $2.27\text{x}10^4$ CFU/mL; $p < 0.0001$). This indicates that PaP_AL1 can clear bacteria from the lungs and blood of mice at 2 hours post-treatment. On the other hand, the presence of PaP_AL1 in BALF and serum was measured by plaque assay. The phage titer in the BALF of the treatment group was significantly higher than in the control group (treatment group 1.77x107 PFU/mL vs. control group 1.31x106 PFU/mL; $p < 0.01$). Similarly, in the serum, the number of phages in the treatment group was significantly higher than in the control group (treatment group $1.62\text{x}10^4$ PFU/mL vs. control group 0 PFU/mL; $p < 0.0001$).

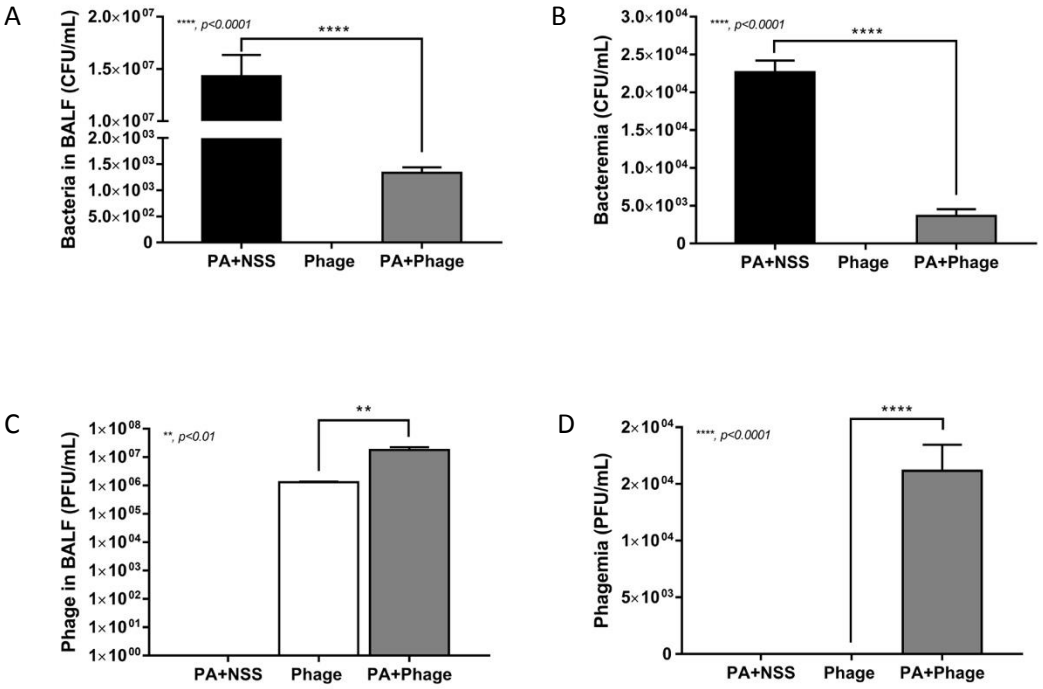

Figure 6 The number of bacteria and phage in the BALF and blood of mice 24 h after infection. The number of viable bacteria in the BALF (A), the number of viable bacteria in the blood (B), the number of viable phages in the BALF (C), and the number of viable phages in the blood (D). Data are presented as mean ± SEM.

Cytokine levels in Serum

The serum cytokines including TNF-α, IL-6, IL-1β, and IL-10 were measured by ELISA. Levels of TNF-α, IL-6, IL-1β, and IL-10 in the phage-treatment group were significantly lower than those in the PA group (TNFα in treatment group: 6.27 pg/mL vs. PA group: 37.84 pg/mL; $p < 0.0001$; IL-6 in treatment group: 128.13 pg/mL vs. PA group: 1373.37 pg/mL; $p < 0.0001$, IL-1β in treatment group: 21.14 pg/mL vs. PA group: 269.08 pg/mL; $p < 0. 0.0001$; IL-10 in treatment group: 374.09 pg/mL vs. PA group: 1080.16 pg/mL; $p < 0.0001$).

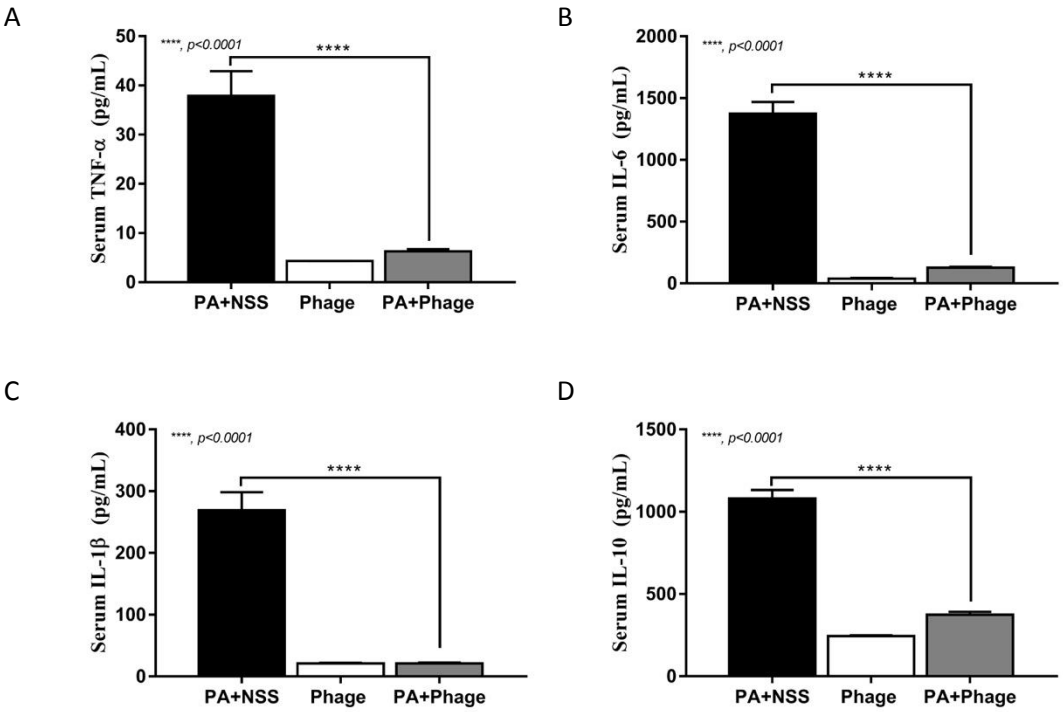

Figure 7 The levels of inflammatory cytokines in serum including TNF-α (A), IL-6 (B), IL-1β (C), and IL-10 (D). Data are presented as mean ± SEM.

Pathological Examination

Histopathology of lungs harvested at 24 h post-infection demonstrated that the PA group had infiltrates of neutrophils and macrophages within alveolar and perivascular spaces. The alveoli were filled with mucus, along with areas of hemorrhage. In contrast, in the treated mice, the lungs were observed to be cleared, and the observed hemorrhage was reduced. The alveoli were filled with fewer neutrophils and macrophages. The lung injury score in the treatment group was lower than the PA group (treatment group: 51.5 vs. PA group: 89.2; $p < 0.001$). This indicates that phage therapy effectively eliminated *P. aeruginosa* in the lungs.

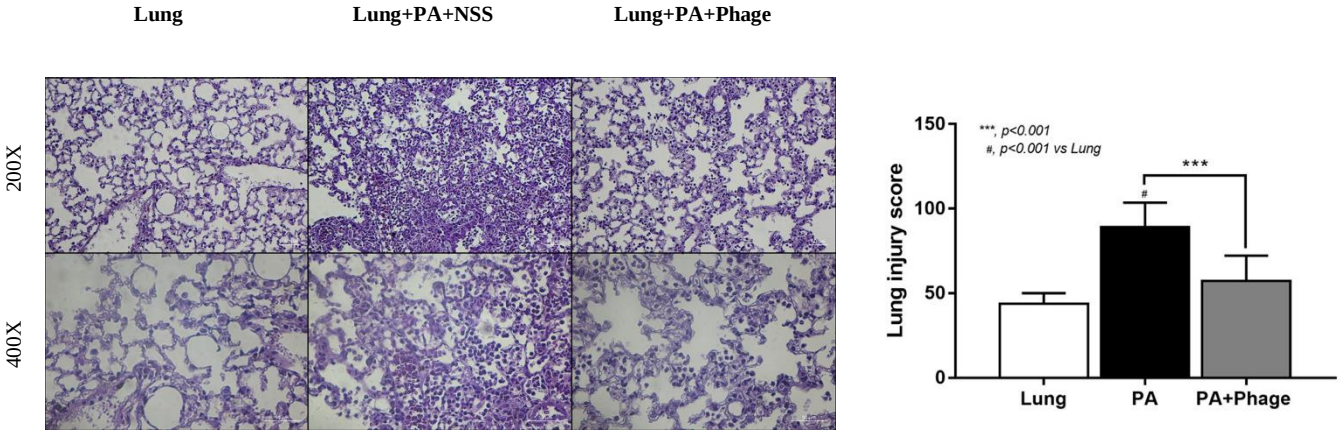

Figure 8 Characteristics of lung injury in mice of normal lung, pneumonic lung, and phage treated lung including pathological examination of mouse lungs (A) and lung injury score (B).

Discussion

Pneumonia is an inflammation of the tissue in one or both lungs usually caused by a wide spectrum of pathogens including bacteria virus and fungi. Hospital-acquired pneumonia is a one type of pneumonia, it is a respiratory infection that develops more than 48 h after hospital admission, occurs at a rate of 5–10 cases per 1000 hospitalizations (Zaragoza et al., 2020), and is the second most common nosocomial infection in the USA (Kumar, Yassin, Bhowmick, & Dixit, 2017). The common pathogen cause hospital-acquired pneumonia is *P. aeruginosa* (Restrepo et al., 2018). The ability to form biofilms of *P. aeruginosa* can protect it from various type of antibiotic, escape them from host immune response allowing them to persist in host tissues and on medical device surfaces (Tuon, Dantas, Suss, & Tasca Ribeiro, 2022). Therefore, it is difficult to treat the patient that *P. aeruginosa* infected and challenge to find the new therapeutic agents. Phage-based therapeutic strategies have been used to prevent bacterial infection sine emergence of antibiotic resistant bacteria (Lin, Koskella, & Lin, 2017). In this study, phages were isolated from mouse feces and treat in *P. aeruginosa* pneumonia mouse model.

We isolated four phages from mouse feces, including PaP_AL1, PaP_AL2, PaP_AL3, and PaP_AL4, that could effectively control clinical isolates of *P. aeruginosa*. We chose to use PaP_AL1 in the experiment because it was the most effective at killing *P. aeruginosa*. In the pneumonia mouse model, we observed that most of the pneumonic mice that didn't receive phage therapy died within three days of infection, but in the phage

treatment group, more than 70% of mice survived. Inhalation of phage reduced the number of bacteria in the BALF in the treatment group, and the viral load increased. This finding supports that phage can replicate and lyse the infected bacteria within several hours (Payne & Jansen, 2001). Inhalation of bacteria can induce bacteremia in pneumonic mice, but in phage-treated mice, the number of bacteria in serum was lower than in pneumonic mice. This observation was also consistent with previous work (Yang, Haque, Matsuzaki, Matsumoto, & Nakamura, 2021). Although we did not detect phages in the blood after inhalation in pneumonic mouse, but in treatment mice, we found phages in the treatment group's blood because the phage-treated mice had *P. aeruginosa* hosts. Phages can infect and lyse the host to produce progeny. This indicates that inhalation of phages is not only beneficial for protecting against pulmonary infections but also for reducing septicemia triggered by pulmonary infections. Respiratory infection with *P. aeruginosa* is associated with increased pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β, as well as anti-inflammatory cytokine (IL-10) (Phuengmaung et al., 2022). In the phage treatment group, the levels of inflammatory cytokines including TNF- α, IL-6, IL-1β, and IL-10 were reduced when compared with pneumonic mice. As a result, the mice treated with phages exhibited a significant reduction in both pro-inflammatory and anti-inflammatory cytokines, which may also have been attributed to the decreased number of pathogenic bacteria in the body following therapeutic application of the phages.

The results presented here indicate that intratracheal PaP_AL1 can increase the survival rate in pneumonia mice, reduce the bacterial load in both BALF and serum, reduce inflammatory cytokines, and improve pathological changes in the lung. The use of phage therapy in the treatment of *P. aeruginosa* infections in the lungs could prove beneficial. However, additional research is necessary to determine the safety of phage therapy and its potential as an alternative treatment option in clinical settings.

Conclusions

The effectiveness of phage therapy was previously believed to be due to a reduction in the number of bacteria causing infections. However, a recent study showed that PaP_AL1 improved Pseudomonas-associated pneumonia in mice by reducing the bacterial burden through its ability to break down bacteria and change the nature of bacterial infections. This also led to a decrease in inflammation by reducing levels of both pro-inflammatory and anti-inflammatory cytokines. The results of the study suggest that phage therapy could be used to treat lung infections caused by *P. aeruginosa*.

Recommendations

Additional preclinical and clinical investigations are necessary to fully understand the mechanisms and limitations of phage therapy. This will enable phage to reach its full potential as a disruptive therapy that can effectively combat the escalating threat of MDR bacterial infections.

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