Original Article

# Active immunization with *Pseudomonas aeruginosa* vaccine protects mice from secondary *Pseudomonas aeruginosa* challenge post-influenza virus infection

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**Background:** Influenza virus infection complicated by secondary bacterial pneumonia contributes significantly to death during seasonal or pandemic influenza. Secondary infection of *Pseudomonas aeruginosa* (*P. aeruginosa*) in influenza virus-infected patients contributes to morbidity and mortality.

**Methods:** Mice were first infected with PR8 influenza virus, followed by a secondary infection of *P. aeruginosa*. Body weights and survival rate of mice was monitored daily over 20 days. Bronchoalveolar lavage fluids (BALFs) and lung homogenates were harvested for measuring bacterial titers. Lung tissue section slides were stained with hematoxylin and eosin for microscopic observation. After vaccination with inactivated *P. aeruginosa* cells or recombinant PcrV protein, the mice were subjected to PR8 influenza virus infection followed by a secondary infection of a *P. aeruginosa*. The inhibition against *P. aeruginosa* of serum was evaluated by detecting the growth of *P. aeruginosa* in broth containing diluted sera.

**Results:** The prior influenza infection greatly enhanced the susceptibility to secondary infection of *P. aeruginosa* and increased morbidity and mortality in mice. Active immunization with inactivated *P. aeruginosa* cells could protect mice from secondary *P. aeruginosa* challenge in influenza virus infected mice.

**Conclusions:** To develop an effective *P. aeruginosa* vaccine might be a promising strategy to decrease the threat of secondary *P. aeruginosa* infection in influenza patients.

**Keywords:** Influenza virus; *Pseudomonas aeruginosa* (*P. aeruginosa*); secondary infection; active immunization

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## Introduction

Pneumonia is the leading cause of infectious death worldwide (1), and acute lower respiratory infection is the leading cause of death in children under 5 years old in developing countries (2). Influenza virus is one of the predominant causative agents of seasonal flu and infectious mortality worldwide. Most influenza-related mortality is not due to the viral infection alone (3,4). Recent studies suggested that bacterial pneumonia secondary to influenza virus infection was identified as the major cause of increased hospitalizations and deaths during influenza virus epidemics (5,6).

Earlier studies about influenza-related secondary infections were concentrated on the gram-positive bacterium. Several gram-positive bacterial pathogens have been isolated from patients who suffered from secondary infections during influenza pandemics, such as *Streptococcus pneumoniae* (*S. pneumoniae*), *Staphylococcus aureus* (*S. aureus*), *Streptococcus pyogenes* (*S. pyogenes*) and methicillin resistant *S. aureus* (MRSA) (7-9). Recently, a gram-negative bacterium such as *Pseudomonas aeruginosa* (*P. aeruginosa*), *Haemophilus influenzae* (*H. influenzae*), *Klebsiella pneumoniae* (*K. pneumoniae*) and anaerobes were also isolated from institutionalized elderly influenza patients (10-12). During avian influenza A virus H7N9 epidemic, H7N9 patients older than 60 were more likely to suffer from secondary infections of *P. aeruginosa*, *K. pneumoniae* or *Acinetobacter baumannii* (*A. baumannii*) (13,14). Secondary infection with gram-negative bacteria caused more serious disease and higher mortality than gram-positive bacteria (15,16).

Active immunization with a vaccine is a common strategy to protect humans from microbial infections. Vaccination with attenuated bacterial or inactivated bacterial cells were often used to induce a protective immunity response. In the 1990's, a candidate inactivated vaccine was tested in acute *P. aeruginosa* pneumonia model (17). It showed that vaccination not only decreased the *P. aeruginosa* titers in the lungs but also improved the survival rate.

In this study, we prepared candidate vaccines of inactivated whole *P. aeruginosa* cells and recombinant type III secretion system PcrV protein, and established a secondary infection mouse model with a prior infection of influenza virus and secondary infection of *P. aeruginosa*. Furthermore, the protection of candidate vaccines were evaluated against *P. aeruginosa* with the secondary infectious model. We present the following article in accordance with the ARRIVE reporting checklist (available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-1012/rc).

### Highlight box

**Key findings**

- The prior influenza infection greatly enhanced the susceptibility to secondary infection of *Pseudomonas aeruginosa* and increased morbidity and mortality in mice.

**What is known and what is new?**

- Active immunization with inactivated *Pseudomonas aeruginosa* cells could protect mice from secondary *Pseudomonas aeruginosa* challenge in influenza virus infected mice.

**What is the implication, and what should change now?**

- To develop an effective *Pseudomonas aeruginosa* vaccine might be a promising strategy to decrease the threat of secondary *Pseudomonas aeruginosa* infection in influenza patients.

## Methods

### *Mice*

Six to eight weeks old female BALB/c mice were housed with 24-hour access to food and water. The study protocol was approved by the Guangzhou Institutes of Biomedicine and Health (GIBH) Institutional Animal Care and Use Committee (ID: 2013028). All animal experiments were performed under the guidelines established by the Association for the Assessment and Accreditation of Laboratory Animal Care.

### *Influenza virus and bacterial strain*

The mouse-adapted influenza A virus (PR8) was diluted in sterile phosphate-buffered saline (PBS) and with a mouse $LD_{50}$ ($MLD_{50}$) of 100 PFU. The *P. aeruginosa* strain (ATCC27853) was purchased from China General Microbiological Culture Collection Center (CGMCC). The bacterial strain had a $MLD_{50}$ of $3.2\times10^7$ colony-forming units (CFU).

### *Influenza virus—P. aeruginosa secondary infection model (Figure 1)*

For infection, mice were anesthetized with isoflurane and held upright. Then, the mice were intranasally (i.n.) inoculated with PR8 virus and/or *P. aeruginosa* strain respectively. PR8 virus was administered at a dose of 0.4 $MLD_{50}$ in 40 μL PBS per mouse, and the *P. aeruginosa* was administered at a dose of 0.0625 $MLD_{50}$ ($2\times10^6$ CFU). Secondary infection of *P. aeruginosa* was administered at day 2, 7 or 14 post PR8 virus infection (18). A group of

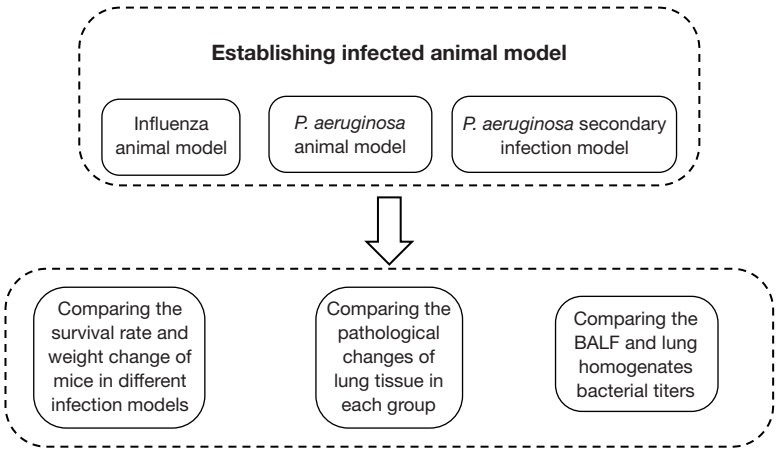


**Figure 1** Flowchart of testing and verifying secondary *P. aeruginosa* infection increased lethality after influenza virus infection in BALB/c mice. *P. aeruginosa*, *Pseudomonas aeruginosa*; BALF, bronchoalveolar lavage fluid.

mice inoculated with PBS served as controls. Once PR8 virus was inoculated, body weights and survival rate of mice were monitored daily for 20 days. Mice were euthanized for further experiments when their body weights fell below 70% of the initial body weights.

### *Bronchoalveolar lavage fluid (BALF) and lung homogenates bacterial titers*

The mice were euthanized 3, 6, 8, or 24 hours post *P. aeruginosa* infection. The bronchoalveolar lavage was performed with three aliquots of 0.6 mL sterile PBS, and about 1.6 mL in a total of lavage fluid was retrieved for each mouse. Lungs were collected and homogenized in PBS. Ten-fold serial dilutions of BALFs and lung homogenates were inoculated onto Luria-Bertani (LB) agar plates, and bacterial CFU were counted after incubation at 37 ℃ overnight.

### *Histological analysis of lungs*

Lungs were removed from euthanized mice and fixed immediately with 10% buffered formalin phosphate for 24 hours. After paraffin embedding, representative lateral and medial lung areas were cut into 7 μm thick sections. After deparaffinization, the sections were stained with hematoxylin and eosin, and then observed under microscopy (19).

### *Preparation of inactivated P. aeruginosa*

The *P. aeruginosa* was cultured in broth to an OD600 of 1.0. The bacterial cells were washed 3 times with PBS and then inactivated with 10% formalin for 1 hour. The complete inactivation efficiency of the bacteria was confirmed by the index of no viable bacteria growing on blood agar. The complete inactivated *P. aeruginosa* was purified by centrifugation after being shaken in cold PBS for 1 hour, and the purification was repeated 3 times. The vaccine was prepared in PBS with a concentration of $3\times10^{10}$ cells/mL and immunized after being mixed with an equal volume of aluminum phosphate adjuvant (18).

### *Preparation of recombinant type III secretion system PcrV protein*

PcrV expression plasmid (pET22b-PcrV) was made by inserting PcrV gene into the pET-22b vector with C-terminal His-Tag. The E. coli BL21 were transformed with pET22b-PcrV, then cultured in LB containing ampicillin at 37 ℃ and shaken at 250 rpm/min. When OD600 of culture solution arrived at 0.5, the culture was induced with 1 mmol/L IPTG and then shaken for another four hours. The bacterial cells were harvested by centrifugation, and the protein expression was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). PcrV protein was purified by Nickel-Nitrilotriacetic Acid (Ni-NTA) affinity chromatography and identified by Western-blotting using rat anti-His-Tag antibody and horseradish peroxidase (HRP)-labeled goat anti-rat antibody. 1 mg/mL Purified PcrV protein in PBS was mixed with an equal volume of aluminum phosphate adjuvant for immunization.

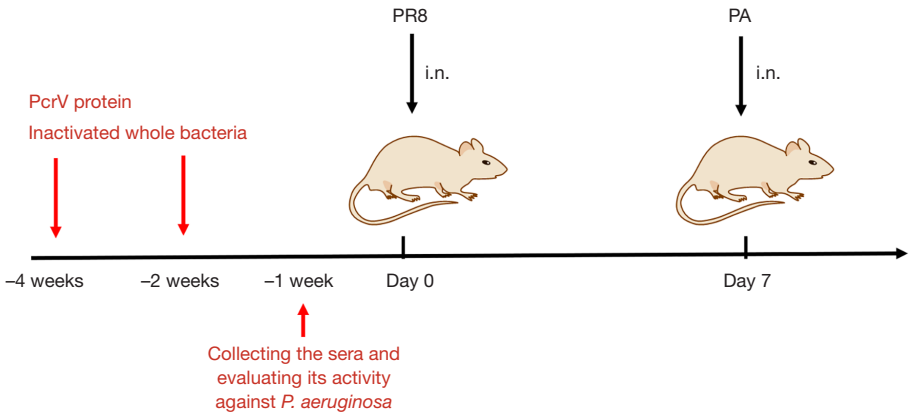


**Figure 2** Flowchart of protection of candidate vaccines against *P. aeruginosa* were evaluated in a secondary infectious model. *P. aeruginosa*, *Pseudomonas aeruginosa*; PA, *Pseudomonas aeruginosa*.

### *Active immunization studies (Figure 2)*

The 6-week-old female BALB/c mice were immunized by intramuscular quadriceps injection of 100 µL of the inactivated *P. aeruginosa* ($1\times10^8$ inactivated bacterial cells), PcrV protein (0.5 mg/mL) or PBS, respectively. The booster were performed on the 14th day after the first immunization. On day 21 after immunization, the sera were collected, and its activity against *P. aeruginosa* was evaluated by detecting the growth of *P. aeruginosa* in broth containing diluted serum (1:4, 1:8, 1:16, and 1:32). The immunized mice were infected by PR8 virus on day 28 and followed by a secondary infection of *P. aeruginosa* on day 35 post-immunization.

### *Statistical analysis*

The data was analyzed by the GraphPad Prism 6.01 software package (GraphPad Software) and statistical significance was determined by unpaired Student's *t*-test with two-tailed analysis. Logrank trend test was used to compare the survival rate of the experimental group and the mock control. A P value less than 0.05 was statistically significant.

## Results

### *Secondary P. aeruginosa challenge increased lethality after influenza virus infection in BALB/c mice*

To evaluate the effect of prior influenza virus infection on secondary *P. aeruginosa* infection, we monitored the body weight loss and mortality after the challenge of *P. aeruginosa* (0.0625 $MLD_{50}$, $2\times10^6$ CFU) in PR8 influenza virus-infected mice (0.4 $MLD_{50}$). The body weight showed a loss of less than 20% within 9 days post PR8 influenza virus sole infection and then gradually recovered (*Figure 3*). *P. aeruginosa* sole infection caused no apparent loss of body weight. No mouse died in the groups with sole infection of PR8 virus or *P. aeruginosa*. However, significant body weight loss and mortality were observed in the mice with secondary infection with *P. aeruginosa*. More importantly, secondary *P. aeruginosa* on day 2 or day 7 post-PR8 infection caused more significant body weight loss and higher mortality. Furthermore, secondary infection with an even lower dose of *P. aeruginosa* ($2\times10^5$ CFU) on day 2 post PR8-infection (0.00625 $MLD_{50}$) could also induce significant weight loss and death (Figure S1). The results indicated that mice become more susceptible to *P. aeruginosa* after PR8 virus infection, and secondary *P. aeruginosa* infection might cause severe outcomes.

### *Secondary infection recruited inflammatory cells and induced severe lung injury*

We next examined the histopathology of lungs in mice infected with PR8 virus and *P. aeruginosa* (*Figure 4*). The mice were sacrificed at the indicated time after PR8 virus infection or 24 hours after *P. aeruginosa* infection. In this study, many more inflammatory cells could be observed in the alveolar space on day 8 than on day 3 after PR8 infection, indicating that PR8 virus infection induced progressive inflammatory cell infiltration (*Figure 4C*,*4E*). However, *P. aeruginosa* could induce severe inflammatory

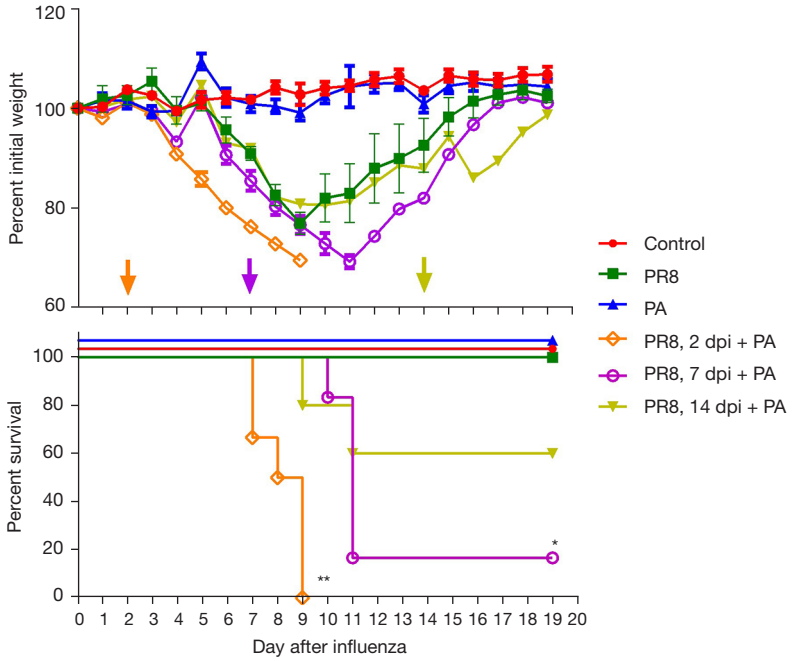


**Figure 3** Morbidity and mortality of mice after influenza: *P. aeruginosa* secondary infection. Mice were divided into 6 groups and inoculated intranasally (i.n.) with either PBS + PBS (Control, n=5), PR8 virus + PBS (PR8, n=5), PBS + *P. aeruginosa* (PA, n=5), PR8 virus + *P. aeruginosa* (2 dpi) (PR8, 2 dpi + PA, n=6), PR8 virus + *P. aeruginosa* (7 dpi) (PR8, 7 dpi + PA, n=6) or PR8 virus + *P. aeruginosa* (14 dpi) (PR8, 14 dpi + PA, n=5). After infection, the weight loss (morbidity) and survival (mortality) were monitored daily. **, indicates a significant difference (P<0.01); *, indicates a significant difference (P<0.05) compared to non-secondary infection groups, using a logrank test for trend. PA, *Pseudomonas aeruginosa*; *P. aeruginosa*, *Pseudomonas aeruginosa*; PBS, phosphate-buffered saline.

cell infiltration within 24 hours (*Figure 4B*) compared to control mice without PR8 or *P. aeruginosa* instillation (*Figure 4A*). In comparison, more severe inflammatory cell infiltration and more severe lung injury were observed in the lung tissue with secondary infection (*Figure 4D,4F*) than that with sole infection of PR8 virus or *P. aeruginosa* (*Figure 4B,4C,4E*). Severe lung injury in mice with the secondary infection of *P. aeruginosa* was consistent with body weight loss and mortality. These findings indicated that it tended to develop severe bacterial pneumonia when a secondary *P. aeruginosa* happened after influenza virus infection.

### *Prior infection of influenza binders the clearance of P. aeruginosa*

The secondary infection mice were prepared by inoculating with *P. aeruginosa* on day 2 post influenza infection. BALFs were collected from the mice at 3, 6, 8, and 24 hours after *P. aeruginosa* inoculation (*Figure 5A*). The viable *P. aeruginosa* in BALF was detected by clone formation in an agarose medium. The number of viable *P. aeruginosa* in BALF gradually decreased after infection. There were almost no viable bacteria detected in the BALF from mice with sole disease of *P. aeruginosa* for 24 hours. In the BALF collected at 3 hours and 6 hours after *P. aeruginosa* infection, there was no significant difference in the number of viable bacteria between the *P. aeruginosa* sole infection group and the secondary infection group. However, in the BALF collected at 8 and 24 hours after *P. aeruginosa* infection, the number of viable bacterial from the secondary infection group was higher than that from sole *P. aeruginosa* infection group. Likewise, viable bacterial titers in the whole lung homogenates (collected 24 hours after *P. aeruginosa* infection) from the secondary infection group were about 100-folds higher than that from the sole *P. aeruginosa* infection group (*Figure 5B*). The consistent results of viable bacteria titer in BALF and lung homogenate suggest that prior infection of influenza virus attenuates the clearance of bacteria, leading to severe secondary bacterial pneumonia.

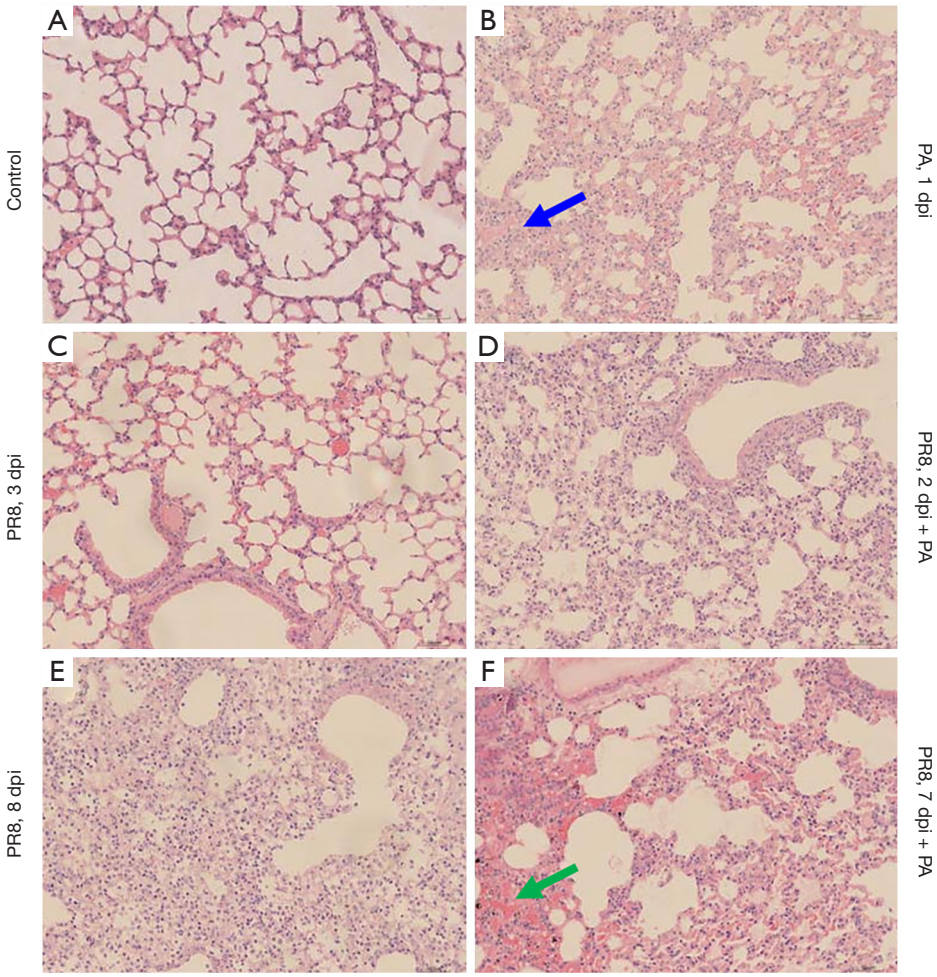


**Figure 4** Histological assessment (hematoxylin-eosin staining) of mouse lung tissue. Mice were divided into 6 groups. (A) Tissue from control mouse without PR8 or P. aeruginosa instillation. (B) Tissues of PA infected mice were collected 24 hours after inoculation of PA (Numerous inflammatory cells exude indicated as the blue arrow). (C) Tissues of PR8 infected mice were collected 3 days after injection of PR8. (D) Tissues of PR8, 2 dpi + PA secondary-infected mice were collected 3 days after inoculation of PR8. (E) Tissues of PR8-infected mice were collected 8 days after inoculation of PR8. (F) Tissues of PR8, 7 dpi + PA secondary-infected mouse were collected 8 days after inoculation of PR8 (Necrosis and hemorrhage indicated as the green arrow, and the alveolar structure was damaged). Original magnification, ×100. PA, *Pseudomonas aeruginosa*; *P. aeruginosa*, *Pseudomonas aeruginosa*.

### *Whole bacteria vaccine protects mice from P. aeruginosa secondary challenge after influenza infection*

Currently, there are several vaccine candidates against *P. aeruginosa* are under development, including protein vaccines, inactivated bacterial and attenuated bacterial (17). Since IC43, OprI, OprF, and PcrV are expressed in most of the pathogenic strains of PA and play an essential role for *P. aeruginosa*, these proteins have been selected as vaccine candidates (20). Based on the results that prior influenza infection hinders the clearance of *P. aeruginosa*, we investigated whether a vaccine could protect against *P. aeruginosa* secondary infection. PcrV protein is an essential component of the type III secretion system (T3SS), which is a trans-membrane syringe-like structure and related to the secretion of Virulence factors. PcrV protein was proven to be with immunogenicity (20,21). Antibodies induced by PcrV protein have been reported to block the cytotoxicity mediated by T3SS and relieve the acute lung injury caused by *P. aeruginosa* (22). We expressed recombinant

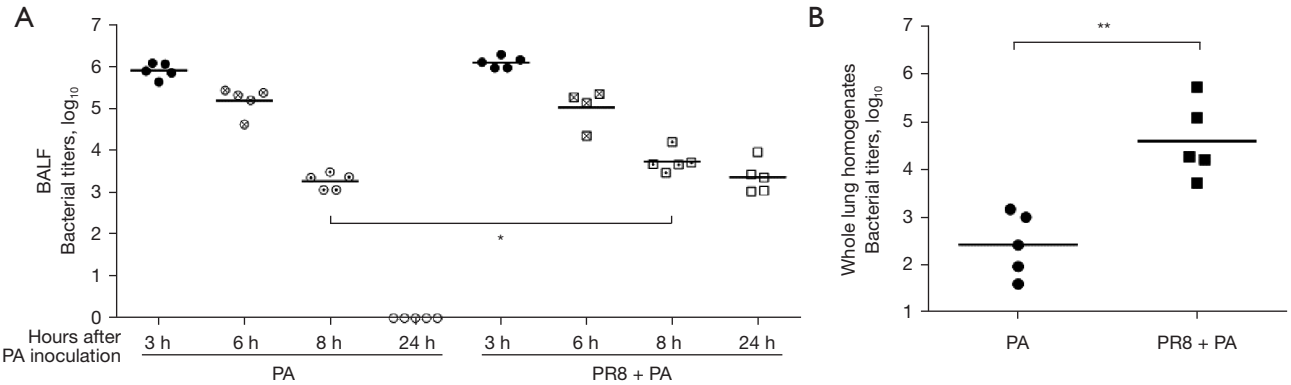


**Figure 5** Bacterial titers in BALF and lung homogenates from sole *P. aeruginosa* infected mice and secondary-infected mice: (A) mice were divided into 8 groups (n=5). At 3, 6, 8 and 24 hours after *P. aeruginosa* inoculation, viable bacteria was detected in BALF from separate groups of mice. Bacterial titers were quantitated and analyzed by unpaired *t* test. *, indicates a significant difference (P<0.05). (B) At 24 hours after *P. aeruginosa* inoculation, viable bacteria were detected in lung homogenates from separate groups of mice. Bacterial titers were quantitated and analyzed by unpaired *t*-test. **, indicates a significant difference (P<0.01). BALF, bronchoalveolar lavage fluid; PA, *Pseudomonas aeruginosa*; *P. aeruginosa*, *Pseudomonas aeruginosa*.

PcrV protein (Figure S2) and prepared inactivated *P. aeruginosa* cells as two candidate vaccines. Mice received intramuscular injection of either inactivated *P. aeruginosa* ($1\times10^8$ inactivated bacterial cells) or recombinant PcrV protein (50 μg) with Alum adjuvant. The booster was vaccinated on the 14th day after the first immunization. On day 7 after booster vaccination, the sera were collected and evaluated for bacteriostatic activity against *P. aeruginosa* in vitro. On days 21 and 28 after booster vaccination, the mice were challenged with sublethal dose of PR8 virus and *P. aeruginosa* (0.0625 $MLD_{50}$, $2\times10^6$ CFU), respectively. The mice vaccinated with inactivated *P. aeruginosa* cells had 80% survival and less body weight loss, while mice vaccinated with recombinant PcrV protein only had 20% survival (*Figure 6*). However, all unvaccinated mice died on day 5 after *P. aeruginosa* challenge.

We next detected the bacteriostatic activity of immune sera against *P. aeruginosa* in vitro. The growth of *P. aeruginosa* was inhibited in the medium with immune sera. The sera from mice vaccinated with inactivated vaccine completely inhibited *P. aeruginosa* at 4-fold dilution and showed significant bacteriostatic activity at 32-fold dilution. However, the growth of *P. aeruginosa* was only partially inhibited by sera from mice vaccinated with recombinant PcrV protein at 4-fold dilution and was not inhibited by sera from unvaccinated mice (*Figure* 7).

These results suggested that the vaccination with inactivated *P. aeruginosa* cells could provide adequate protection against secondary *P. aeruginosa* infection post influenza virus infection, whereas recombinant PcrV protein could only provide weak protection.

## Discussion

*P. aeruginosa* is one of the six pathogens that are the major causes of nosocomial infections. Secondary infection of *P. aeruginosa* always results in severe pneumonia and even death in influenza patients. Treatment of *P. aeruginosa* infections is extremely challenging due to multi-drug-resistant (MDR) *P. aeruginosa* strains in the hospital environment, which is becoming increasingly resistant to all available antibiotics. Vaccination against *P. aeruginosa* would provide protection and overcome difficulties associated with antibiotic resistance. It is important to develop a *P. aeruginosa* secondary infection model and to explore the possibility of creating a candidate vaccine.

In this study, we successfully established an influenza-*P. aeruginosa* secondary infection model by sequential intranasal inoculation with influenza virus and *P. aeruginosa* strain. Gram-positive bacterial streptococcus pneumonia could be completely cleared within three hours after sole infection (23). However, we found that viable *P. aeruginosa* could still be detected in BALF until 24 hours after *P. aeruginosa* sole infection. Further study showed that the influenza virus prior to infection decreases the clearance of *P. aeruginosa* and increases the susceptibility to secondary

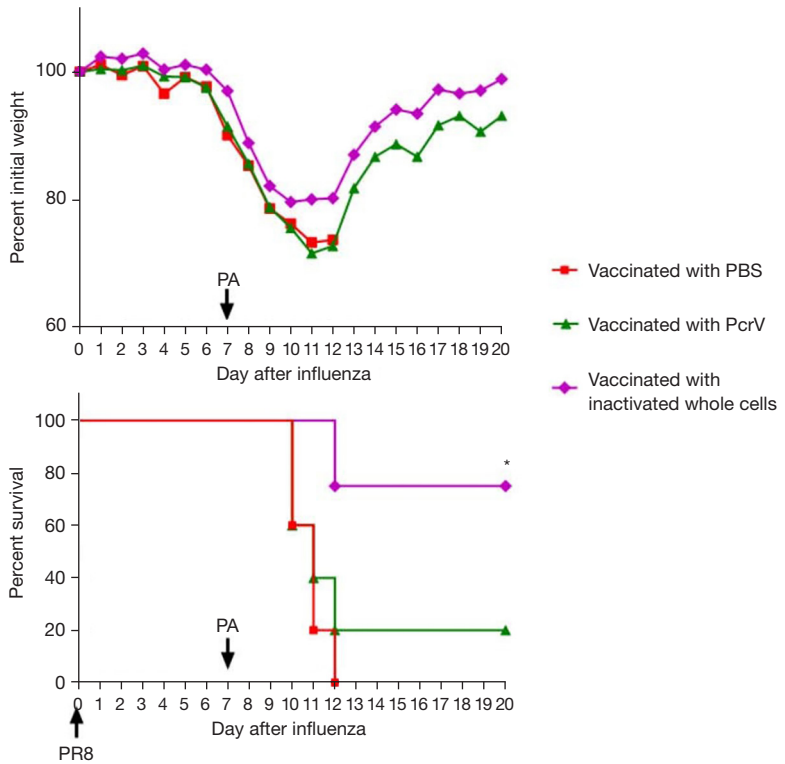


**Figure 6** Protection of inactivated whole cells or PcrV protein vaccine. Mice were divided into 3 groups (n=5). *, indicates a significant difference (P<0.05) compared to PBS group, using a logrank test. PA, *Pseudomonas aeruginosa*. PBS, phosphate-buffered saline.

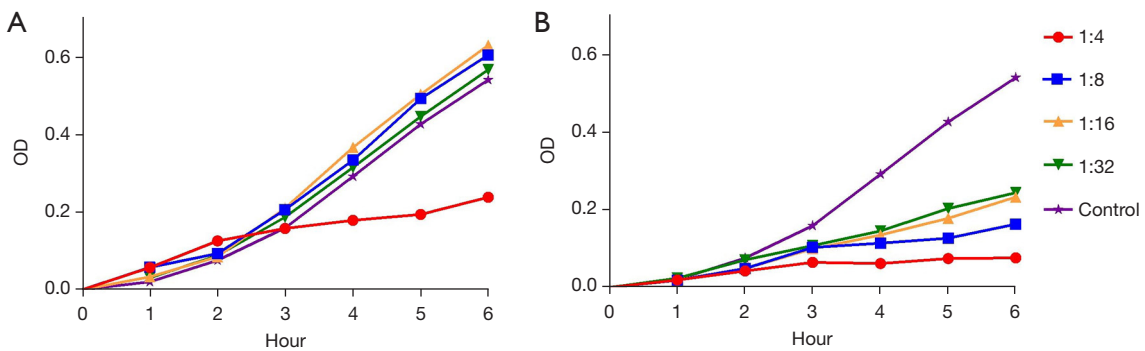


**Figure 7** Bacteriostatic activity of immune serum (time-$OD_{600}$ curve): (A) growth curve of bacteria incubated with serum from mice vaccinated with PcrV protein. (B) Growth curve of bacteria incubated with serum from mice vaccinated with inactivated whole cells. 100 µL serum samples were diluted at 1:4, 1:8, 1:16, and 1:32 and added to micro broth. The control is the serum at 1:4 dilution from unvaccinated mice. OD, optical density.

*P. aeruginosa* infection. The viable bacterial titer in the lung tissues from secondary infection at 24 hours after *P. aeruginosa* infection was almost 100 folds higher than that from *P. aeruginosa* sole infection. Our study demonstrated that prior influenza virus infection damaged the ability of the host to remove bacteria, which made it possible for bacteria to invade into the lung tissue and replicate. Therefore, the *P. aeruginosa* secondary infection caused

much more severe morbidity and mortality than sole infection with influenza or *P. aeruginosa*.

What's more, challenges with *P. aeruginosa* at different times after influenza viral infection could all aggravate the disease, even at 14 days after influenza viral infection. This result indicates that *P. aeruginosa* is a severe threat to patients during the progression or recovery of influenza. Therefore, to minimize *P. aeruginosa* associated morbidity and mortality after influenza infection, the development of an effective *P. aeruginosa* vaccine is highly desirable.

Vaccines against *P. aeruginosa* have been explored in the past, so far there are no licensed vaccines (17). Currently, several vaccine candidates against *P. aeruginosa* are under clinical trials, including protein vaccines, inactivated bacterial and attenuated bacterial. Since the protein IC43, OprI, OprF, and PcrV are expressed in most pathogenic *P. aeruginosa* strains and are associated with *P. aeruginosa* reproduction or pathogenicity (20), these proteins are selected as promising vaccine candidates. It was reported that infection of *P. aeruginosa* with type-3 secretion system (T3SS) proteins caused 6-fold higher mortality in patients than without T3SS proteins (21). T3SS proteins expression was correlated with increasing incidence of bacteremia and organ failure induced by *P. aeruginosa* (19,24). PcrV protein is one of the important components of T3SS and has strong immunogenicity (25-28), which functions as an essential virulence factor to cause acute necrotic cell death and significant lung injury (29). Antibodies against PcrV protein can block the cytotoxicity mediated by T3SS and relieve the acute lung injury caused by *P. aeruginosa*. Mab166, a monoclonal antibody against PcrV protein from mice, was reported to protect mice from *P. aeruginosa* infection. In this study, serum against PcrV could inhibit *P. aeruginosa* growth in vitro. Still, PcrV protein vaccine showed less protection in *P. aeruginosa* secondary infection mice, indicating that one recombinant protein alone might not be enough to induce sufficient protection. Combination of multiple *P. aeruginosa* candidate proteins might be a better strategy for developing a candidate vaccine.

Attenuated bacterial and inactivated bacterial cells have been used as a common strategy to develop a vaccine, which may induce a broader immune response to multiple antigenic targets on *P. aeruginosa*. It was reported that immunization with inactivated *P. aeruginosa* significantly enhanced bacterial clearance and improved the survival rate in an acute *P. aeruginosa* pneumonia rat model (17). Our study demonstrated that vaccination with inactivated *P. aeruginosa* could provide protection and reduce mortality caused by *P. aeruginosa* secondary infection in mice. Further analysis showed that serum induced by the inactivated vaccine could inhibit *P. aeruginosa* growth in vitro. In addition to PcrV protein, delineating and evaluating the protective effects of other components from the inactivated *P. aeruginosa* cells might be helpful for the development of an effective vaccine.

## Conclusions

In summary, prior infection with non-lethal influenza virus decreases the capacity of clearing *P. aeruginosa* and increases the susceptibility to *P. aeruginosa* secondary infection. *P. aeruginosa* secondary infection is a severe threat to influenza patients. Vaccination against *P. aeruginosa* could provide protection and should be a strategy to overcome high morbidity caused by *P. aeruginosa* secondary infection in influenza patients.

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## Footnote

*Reporting Checklist:* The authors have completed the ARRIVE reporting checklist. Available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-1012/rc

*Data Sharing Statement:* Available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-1012/dss

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-1012/coif). NZ serves as Editor-in-Chief of *Journal of Thoracic Disease*. All authors report funding from National Natural Science Foundation of China (No. 91442102) and National Science and Technology Major Project of China (No. 2018YFC1200100). The authors have no other conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related

to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study protocol was approved by the Guangzhou Institutes of Biomedicine and Health Institutional Animal Care and Use Committee (ID: 2013028). All animal experiments were performed under the guidelines established by the Association for the Assessment and Accreditation of Laboratory Animal Care.



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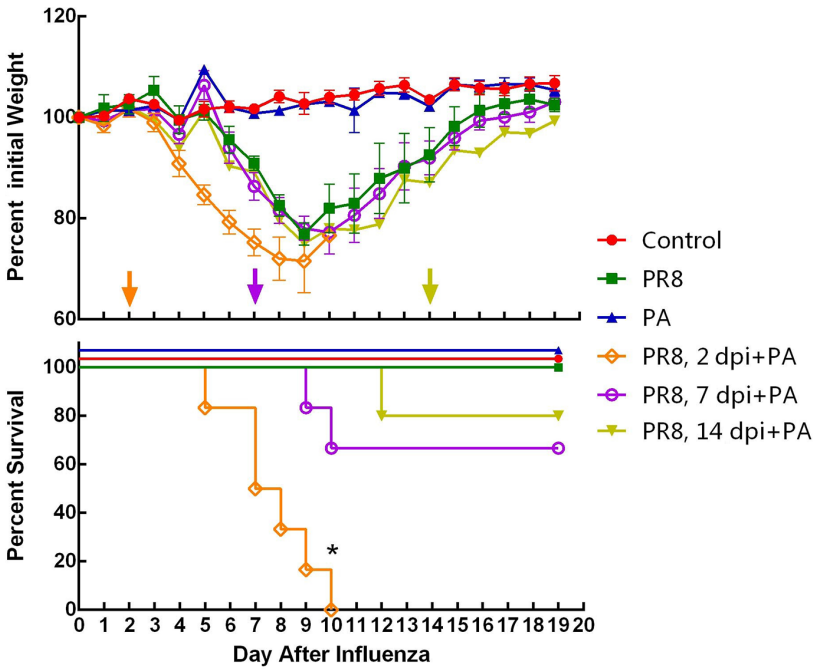


**Figure S1** Morbidity and mortality of mice after influenza: *P. aeruginosa* (lower dose, 0.00625 MLD50) secondary infection. Mice were also divided into 6 groups and inoculated intranasally with either PBS + PBS (control, n=5), PR8 virus + PBS (PR8, n=5), PBS + *P. aeruginosa* (PA, n=5), PR8 virus + *P. aeruginosa* (2 dpi) (PR8, 2 dpi + PA, n=6), PR8 virus + *P. aeruginosa* (7 dpi) (PR8, 7 dpi + PA, n=6) or PR8 virus + *P. aeruginosa* (14 dpi) (PR8, 14 dpi + PA, n=5). After infection, the weight loss (morbidity) and survival (mortality) were monitored daily. *Indicates a significant difference (P<0.01) compared to non-secondary infection groups, using a logrank test for trend. PA, *Pseudomonas aeruginosa*; *P. aeruginosa*, *Pseudomonas aeruginosa*; PBS, phosphate-buffered saline.

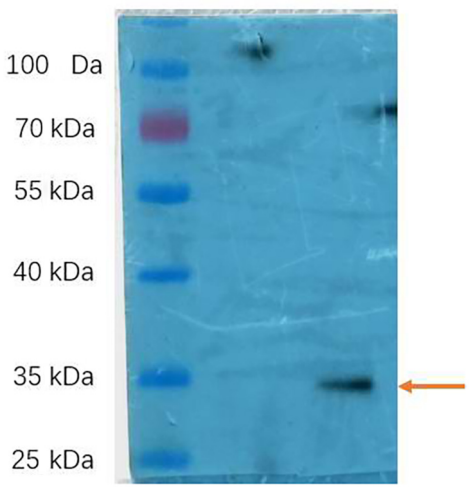


**Figure S2** PcrV protein (molecular weight 32 kDa) was detected by western blotting using rat anti-His-Tag antibody and HRP-labeled goat anti-rat antibody. HRP, horseradish peroxidase.


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