https://doi.org/10.17221/86/2021-VETMED

# Immune responses and protective efficacy of a trivalent combination DNA vaccine based on *oprL*, *oprF* and *flgE* genes of *Pseudomonas aeruginosa*

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**Citation:** Gong Q, Li Y, Zhai W, Niu M (2022): Immune responses and protective efficacy of a trivalent combination DNA vaccine based on *oprL*, *oprF* and *flgE* genes of *Pseudomonas aeruginosa*. Vet Med-Czech 67, 611–619.

**Abstract:** *Pseudomonas aeruginosa* is an infectious pathogenic bacteria infecting many different species of animals. Currently, it lacks a commercial vaccine. In this study, three monovalent DNA vaccines (poprL, poprF, and pflgE), three bivalent combination DNA vaccines (poprL+poprF, poprL+pflgE, poprF+pflgE), and a trivalent DNA vaccine (poprL+poprF+pflgE) were constructed. Consequently, we immunised chickens with these DNA vaccines and used inactivated vaccines as the positive controls. Then, the immune efficacy was evaluated through serum antibody detection, a lymphocyte proliferation assay, and cytokine concentration determination. Lastly, we assessed the protection rate through a challenge experiment. Following vaccination, the serum antibody levels induced using these DNA vaccines were different due to the different coating antigens. In the trivalent combination DNA vaccine group, we established that the lymphocyte proliferation (SI values), IFN-γ, IL-2, and IL-4 levels were significantly higher than those of the other six DNA vaccine groups and the inactivated vaccine group. However, the protection provided was slightly lower than that of the inactivated vaccine and higher than those of other DNA vaccines. The protection rate of poprL, poprF, pflgE, poprL+poprF, poprL+pflgE, poprF+pflgE, poprL+poprF+pflgE, and the inactivated vaccine were 50, 45, 60, 75, 80, 80, 90, and 95%, respectively. The results of this study indicated the trivalent DNA vaccine based on *oprL*, *oprF* and *flgE* genes represents a promising approach for the prevention of *Pseudomonas aeruginosa* infections.

**Keywords:** cytokine concentration; poprL+poprF+pflgE; protection rate; serum antibody; SI value

*Pseudomonas aeruginosa*, a gram-negative pathogen, exists in different environments like the air, soil, and water. Moreover, it adheres to the body surface and respiratory tract of healthy people. It is one of the usual conditional pathogens in hospitals, mostly causing infections in immunocompromised persons and intensive care unit (ICU) patients (Pachori et al. 2019). Additionally, this bacterium is a food-borne pathogen, potentially causing meningitis, pneumonia, or septicaemia (Garvey et al. 2017; Xu et al. 2019). Apart from humans, other animals, including pigs, cattle, poultry, mink, and even fish, could be infected with this bacterium.

In human and veterinary medicine, the primary control measure of this disease-causing pathogen is treatment with antibiotics, but it has a certain adverse effect. Despite the large and broad application of antibiotics, *P. aeruginosa* has developed resistance

Supported by a grant from the Natural Science Foundation of Henan province, P.R. China (No. 162300410068).

https://doi.org/10.17221/86/2021-VETMED

to many types of antibiotics (Shariati et al. 2017; Fernandez-Esgueva et al. 2020; Hashemizadeh et al. 2020). Presently, the pathogen has developed severe resistance to meropenem, imipenem, and carbapenem antibiotics (Judd et al. 2016; Rao et al. 2018; Fournier et al. 2020). The World Health Organization (WHO) has listed carbapenem-resistant *P. aeruginosa* as one of the three major pathogens that urgently needs new drug development to control (Tacconelli et al. 2018). Therefore, it is indispensable to investigate more effective strategies to control this pathogenic bacterium.

Immunisation is now the most practical and effective way to prevent and control infectious diseases. However, no vaccine of *P. aeruginosa* can be clinically applied so far (Rashid et al. 2017). Previously, we constructed monovalent, a bivalent combination, and two genes fusion DNA vaccines using the *oprL* and *oprF* genes of *P. aeruginosa*, and evaluated their immune response and protective efficacy (Gong et al. 2018a). Here, the immune response levels and protective efficacy induced by the bivalent combination DNA vaccines were superior to the other DNA vaccines. The optimal immunisation dose of the bivalent combination DNA vaccine in chickens was 100 μg (Gong et al. 2021). However, its protective rate was inferior to that of the inactivated vaccine. Thus, further measures are needed to enhance the protective rate of the bivalent combination DNA vaccine.

This study prepared a trivalent DNA vaccine using the *oprL*, *oprF*, and *flgE* genes of *P. aeruginosa* and evaluated the immune efficacy induced by them. Taken together, this research aims to provide a reference for the study of a new multivalent DNA vaccine of *P. aeruginosa* in chickens.

## MATERIAL AND METHODS

### Bacterial strain and experimental animals

The *P. aeruginosa* was purchased from the Chinese Institute of Veterinary Drug Control (IVDC). The laying hens were procured from the Animal Center Laboratory of the College of Medical Technology and Engineering of Henan University of Science and Technology, P.R. China. Our study protocol was approved by the Animal Monitoring Committee of Henan University of Science and Technology (Permit No. 2020-0023; 2020 July 7).

### Preparation of the DNA vaccines and inactivated vaccine

Primers were designed according to the nucleotide sequences of the *oprL*, *oprF*, and *flgE* genes of *P. aeruginosa*. The genomic DNA of the *P. aeruginosa* CAU0792 strain was extracted. The target gene fragments were amplified using the genomic DNA as a template. Then, they were ligated into a eukaryotic expression vector pcDNA3.1(+), resulting in three recombinant plasmids. Competent *E. coli* DH5α was transformed with these recombinant plasmids. Ampicillin resistant colonies were then grown in a Luria-Bertani medium containing 50 μg/ml ampicillin at 37 °C with shaking. The plasmids were extracted and identified using restriction enzymes. The positive plasmids were named poprL, poprF, and pflgE, namely the DNA vaccines of the *oprL*, *oprF*, and *flgE* genes. Next, we prepared the three DNA vaccines on a large scale and adjusted them to 1 μg/μl with phosphate-buffered saline (PBS) for the animal experiments. Concurrently, the concentration of the *P. aeruginosa* suspension was adjusted to $2 \times 10^{10}$ cfu/ml with sterile saline and inactivated with formaldehyde. Tween-80 (6% of the total volume) was added to the inactivated bacteria liquid and thoroughly mixed to an aqueous phase. The oil phase consisted of 92% white oil, 6% span-80, and 2% aluminium stearate. The aqueous phase and the oil phase were mixed in a ratio of 1 : 2 to yield the *P. aeruginosa* inactivated vaccine (Gong et al. 2018a).

### Immunisation of the chickens

Healthy one-day-old chickens ($n$ = 180) were reared in an animal house with controlled environmental light, humidity, and temperature. Notably, they were given drinking water, non-medicated feed, and routine health monitoring was carried out daily throughout the experiment. After the chickens adapted to the new environment, they were categorised into nine groups ($n$ = 20 chickens/group). When they were two weeks old, the vaccines were administered through a leg intramuscular injection. The chickens in the three monovalent DNA vaccine (poprL, poprF, and pflgE) groups were vaccinated with 200 μg of recombinant plasmid poprL, poprF, and pflgE, respectively. The chickens in the three bivalent combination DNA

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vaccine groups (poprL+poprF, poprL+pflgE, and poprF+pflgE) were vaccinated using 200 µg of the mixed DNA solution containing equal proportions of poprL and poprF, or poprL and pflgE, or poprF and pflgE. The chickens in the trivalent DNA vaccine group (poprL+poprF+pflgE) were vaccinated using 200 µg of the mixed DNA solution containing equal proportions of poprL, poprF, and pflgE. The inactivated vaccine group received 200 µl of the inactivated vaccine, whereas those in the PBS group received 200 µl of PBS (0.01 M, pH 7.2). Of note, the chickens in all the groups were vaccinated three times at two-week intervals.

### Detection of the serum antibody

Following the first vaccination, serum specimens were isolated from the chickens every week until the sixth week. Subsequently, the serum antibody levels were determined using an enzyme-linked immunoassay (ELISA) according to the previous methods, which had $10^9$ cfu/ml of *P. aeruginosa* suspension or 20 µg/ml of the outer membrane protein of *P. aeruginosa* or 20 µg/ml of the fimbriae protein of *P. aeruginosa* as the coating antigens (Gong et al. 2018a).

### Lymphocyte proliferation assay

Two weeks after each immunisation, we collected blood samples from the vaccinated chickens and separated the peripheral blood lymphocytes (PBLs). Two millilitres (2 ml) of a Hanks' solution and 2 ml of a chicken lymphocyte separation solution (Solarbio, Beijing, P.R. China) were added to 2 ml of the blood sample, and were subsequently well mixed. Then, the mixed solution was centrifuged at 3 500 *g* for 20 minutes. After centrifuging the solution, the white lymphocyte layer in the middle was carefully drawn and washed twice with the Hanks' solution at 3 100 *g* for 10 minutes. The supernatant was discarded and an RPMI-1640 solution was added to the pellet to resuspend it. After counting with an inverted microscope, the cell concentration was adjusted to $2 \times 10^7$ cells/ml with the RPMI-1640 solution. Then, their proliferation ability was detected after they were activated using concanavalin A (Gong et al. 2018b). Then, we estimated the stimulation index (SI) using the formula:

$$SI = \frac{A \text{ (experimental well)}}{A \text{ (negative control well)}} \quad (1)$$

### Cytokine assays

Two weeks after each vaccination, the PBLs induced by concanavalin A were prepared. Then, the cells were cultured at 37 °C under 5% $CO_2$ for 72 h, and the supernatants were harvested. Next, we measured the concentrations of interferon-γ (IFN-γ), interleukin-2 (IL-2), and interleukin-4 (IL-4) using a commercial ELISA kit (Yuan Ye Biotech Company, Shanghai, P.R. China) per the instructions from the manufacturer.

### Challenge experiment

Two weeks after the third vaccination, we intraperitoneally injected the virulent *P. aeruginosa* strain CAU0792 (5LD50/one chicken) into the chickens for a challenge experiment. Then, the chickens were reared for a further 15 days, and the mortality rate in each group was recorded. After the challenge experiment, the survival curve was drawn, and the protection rate of each group was calculated.

## RESULTS

### Construction of the DNA vaccines

The recombinant plasmids of the *oprL*, *oprF* and *flgE* gene were digested with restriction enzymes and 525 bp, 1 063 bp, and 1 400 bp DNA fragments were obtained. As indicated in Figure 1, we successfully constructed the following DNA vaccines: poprL, poprF, and pflgE.

### The serum antibody levels

As demonstrated in Figure 2A, when the *P. aeruginosa* suspension was adopted as the coating antigen, we found that the antibody levels in the poprL+poprF+pflgE group were significantly higher than other DNA vaccine groups ($P < 0.05$). However, there were no significant differences between the inactivated and the trivalent combination DNA vaccine groups. Afterwards, the antibody

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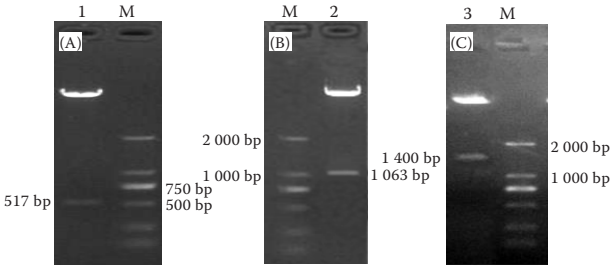


Figure 1. Identification of poprL (A), poprF (B) and pflgE (C) by restriction digestion

Lane M: DL2000 DNA marker; Lane 1–3: poprL, poprF and pflgE enzymes with *Bam*HI and *Eco*RI

levels in the three monovalent DNA vaccine groups were lower than those in the three bivalent combination DNA vaccine groups ($P < 0.05$). The antibodies in the poprL+pflgE and poprF+pflgE groups were slightly higher than those in the poprL+poprF group, however, there were no significant differences among them ($P > 0.05$). Besides, there were no differences among the three monovalent DNA vaccine groups.

After the first vaccination, no differences among all the vaccination groups were observed except for the PBS and the monovalent DNA vaccine pflgE groups, when the coating antigen was the outer membrane protein. After the second and third vaccinations, we observed that the antibodies detected in the inactivated vaccine group and the poprL+poprF group were higher than the other DNA vaccine groups ($P < 0.05$). Furthermore, the antibody levels in the poprL+poprF+pflgE group, poprL group and poprF group were significantly higher than those in the poprL+pflgE and poprF+pflgE groups ($P < 0.05$). Following the three vaccinations, the serum antibody levels in the trivalent combination DNA vaccine group were higher than those in the poprL and poprF groups ($P < 0.05$). However, we observed no significant differences in the antibody levels between the inactivated vaccine and the poprL+poprF groups (Figure 2B).

When the fimbriae protein was adopted as the coating antigen, the antibody levels in the poprL, poprF, and poprL+poprF groups remained low and was equivalent to those in the PBS group. From the third week, the antibodies in the inactivated vaccine group were higher than the other groups ($P < 0.05$). Additionally, we observed that the antibody levels in the pflgE group from the fourth week were higher than those in the two bivalent combinations DNA

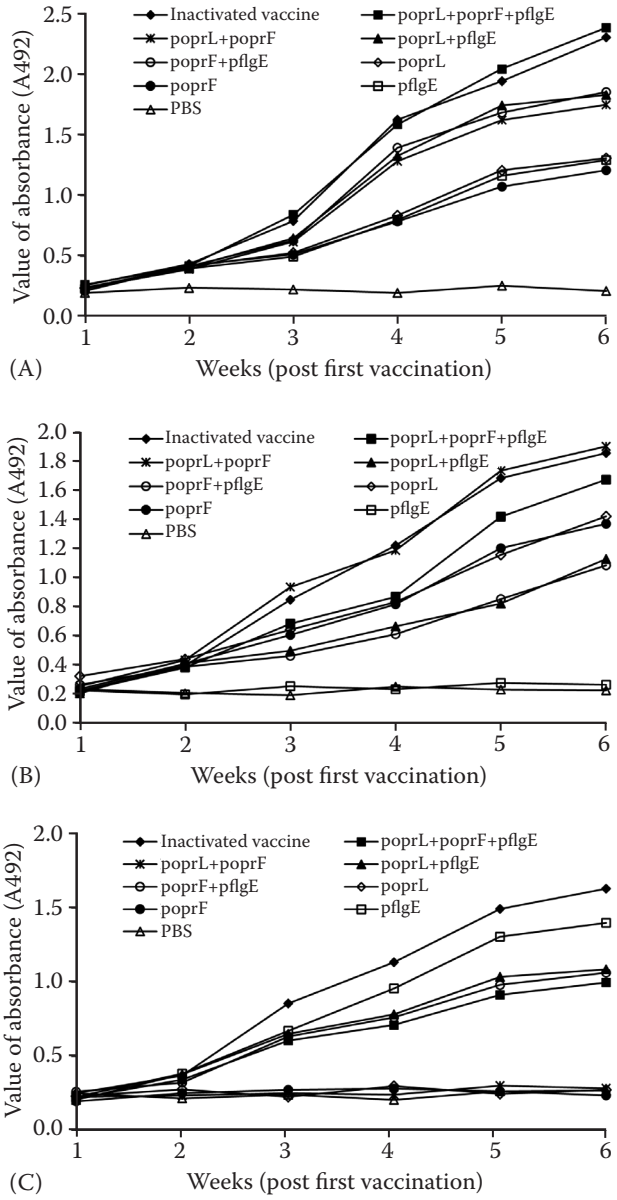


Figure 2. Dynamic changes in the serum antibodies in the vaccinated chickens

Indirect ELISAs were performed with the *P. aeruginosa* suspension (A) or outer membrane protein (B) or fimbriae protein (C) as the coating antigen. Chickens were vaccinated with inactivated vaccine (◆), poprL+poprF+pflgE (■), poprL+poprF (*), poprL+pflgE (▲), poprF+pflgE (○), poprL (◇), poprF (●), pflgE (□), or PBS (△)

vaccine (poprL+pflgE and poprF+pflgE) groups and the trivalent combination DNA vaccine groups. There were no significant differences among the trivalent combination DNA vaccine, poprL+pflgE, and poprF+pflgE groups, though the former was slightly lower than the other two aforementioned groups (Figure 2C).

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### Lymphocyte proliferation assay

After vaccination, we assessed the proliferation of the PBLs of the vaccinated chickens (Figure 3). The SI values of the PBS group were always significantly lower than those in the other groups ($P < 0.05$) throughout the experiment. However, after the first vaccination, there were no significant differences in the SI values among all the vaccine groups. After the latter two vaccinations, the SI values in the inactivated vaccine group, trivalent combination DNA vaccine group, and the three bivalent DNA vaccine groups were higher than those in the three monovalent DNA vaccine groups ($P < 0.05$). Moreover, following the third vaccination, the SI values in the poprL+poprF+pflgE group were significantly higher than the inactivated vaccine group and the three bivalent combination DNA vaccine groups ($P < 0.05$).

### IFN-γ, IL-2, and IL-4 secretion

Two weeks after each vaccination, we detected the IFN-γ, IL-2, and IL-4 concentrations that the PBLs of the vaccinated chickens secreted (Figure 4). No significant differences were detected in the three cytokines concentrations among all the vaccine groups after the first vaccination. After the latter two vaccinations, we observed that the IFN-γ and IL-2 concentrations in the poprL+poprF+pflgE group and the three bivalent combination DNA vaccine groups were significantly higher than

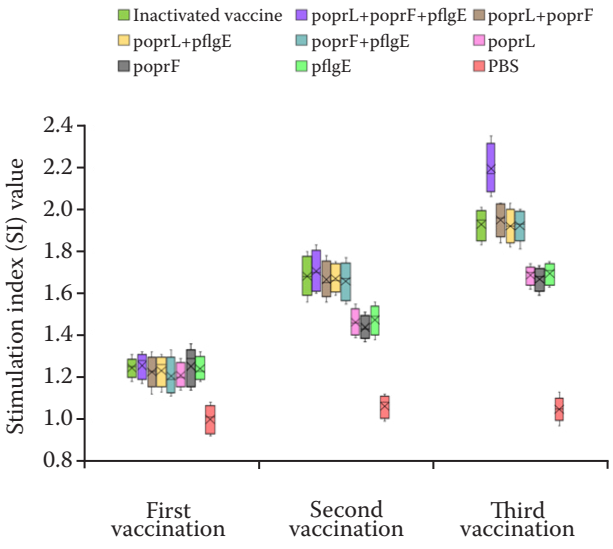


Figure 3. Results of the lymphocyte proliferation assays

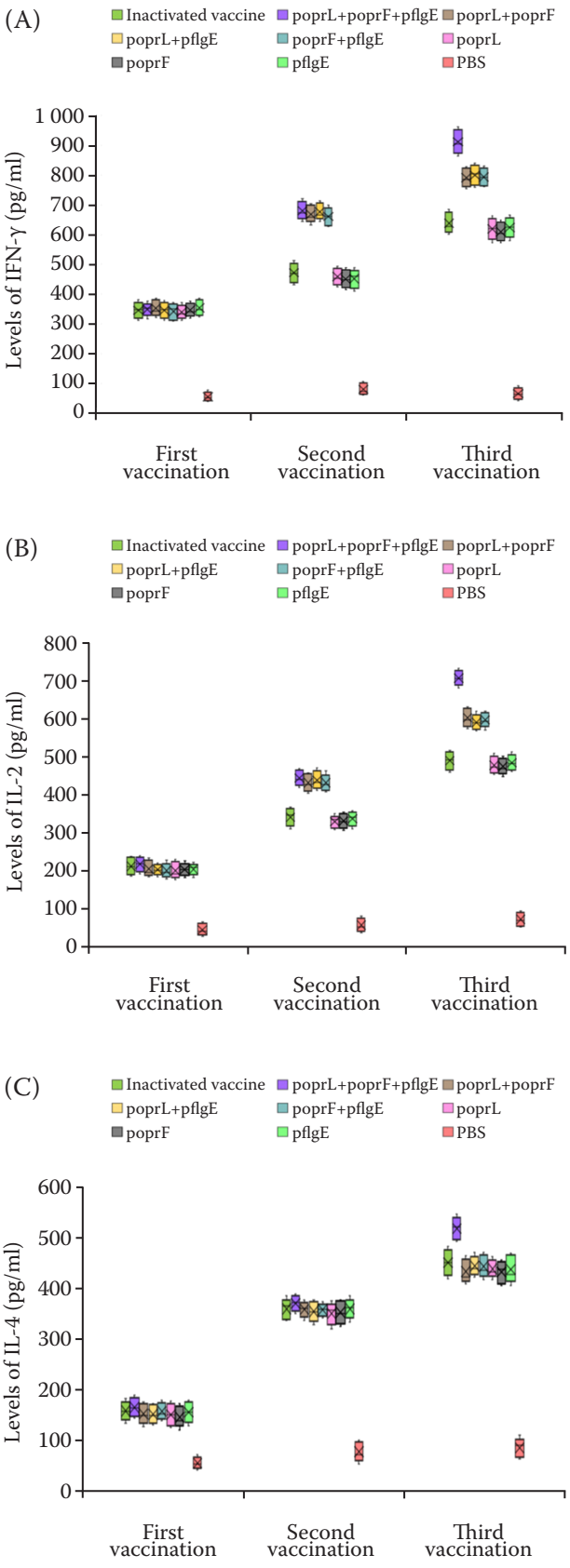


Figure 4. Concentrations of IFN-γ (A), IL-2 (B) and IL-4 (C) from the PBLs of the vaccinated chickens

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those in the inactivated vaccine group and the three monovalent DNA vaccine groups ($P < 0.05$). Besides, after the last vaccination, the IFN-γ and IL-2 concentrations in the trivalent combination DNA vaccine group were higher than the three bivalent combination DNA vaccine groups ($P < 0.05$) (Figure 4A,B). Following the second vaccination, no differences were detected in the IL-4 concentrations among all the vaccine groups. However, after the third vaccination, we observed that the IL-4 concentrations in the trivalent combination DNA vaccine group were higher than other vaccine groups ($P < 0.05$) (Figure 4C).

### The challenge experiment

After the challenge experiment, the chickens in the PBS group died rapidly, and none of the remaining chickens survived more than five days. The chickens in the trivalent combination DNA vaccine group only died on the 4th day. The chickens in the three bivalent combination DNA vaccine groups died from the 3rd day, and the survival numbers in the poprL+poprF, poprL+pflgE, and poprF+pflgE groups remained unchanged from the 7th, 6th, and 7th day, respectively. The chickens in the three monovalent DNA vaccine groups died from on the day of the challenge until 9 days later, the number of surviving chickens in the poprL, pflgE, and poprF groups were 10, 12, and 9, respectively. Following the challenge, only one chicken died in the inactivated vaccine group. The protection rates of poprL, poprF, pflgE, poprL+poprF, poprL+pflgE, poprF+pflgE, poprL+poprF+pflgE, and the inactivated vaccine were 50, 45, 60, 75, 80, 80, 90, and 95%, respectively (Figure 5).

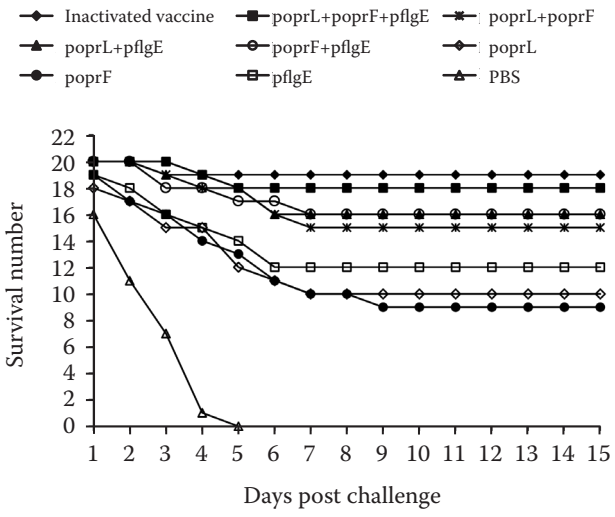


Figure 5. Survival curve of the chickens after the challenge

## DISCUSSION

*P. aeruginosa* is a conditional pathogen that globally causes various diseases in humans and animals. Since drug resistance is an increasingly serious issue, the clinical treatment effect is barely satisfactory. Therefore, vaccines would be a new strategy to prevent this disease. Currently, there are some reports on vaccines of *P. aeruginosa*, like the DNA vaccine, conjugate vaccine, live vaccine, recombinant subunit vaccine, and inactivated vaccine (Staczek et al. 2003; Zuercher et al. 2006; Doring and Pier 2008; Bumann et al. 2010; Mousavi et al. 2016; Meynet et al. 2018).

As a new generation vaccine, the DNA vaccine has become an interesting alternative in the field of vaccine research. The protective antigen genes currently available for *P. aeruginosa* vaccines research include *oprI*, *oprF*, *pcrV*, and *pilA* (Hassan et al. 2018; Ranjbar et al. 2019; Bahey-El-Din et al. 2020; Hashemi et al. 2020). We prepared a bivalent combination DNA vaccine of *P. aeruginosa* using the *oprL* and *oprF* genes in the early stage (Gong et al. 2018a). The animal experiments results show that though it could induce a good immune response, its protective efficacy was lower than that of the inactivated vaccine. The immunogen genes currently available for research on novel vaccines include the outer membrane protein gene, flagellin gene, and toxin gene, etc. The outer membrane protein, encoded by the *oprL* gene, *oprF* gene, and so on, is one of the major protective antigens of *P. aeruginosa*. A study by Gomi et al. (2017) showed the *oprF* gene DNA vaccine could be used to treat respiratory infections caused by *P. aeruginosa*. Yu et al. (2016) prepared a fusion DNA vaccine with the *oprF* gene of *P. aeruginosa* and the *VP22* gene of herpes simplex virus and evaluated the immune effect. The study of Gao et al. (2017) showed a recombinant subunit vaccine construct based on the *oprL* gene could effectively induce a Th17 response in vaccinated animals. Apart for the outer membrane protein, the fimbriae protein of *P. aeruginosa* encoded by the *flgE* gene is also a protective antigen gene of *P. aeruginosa* (Weimer et al. 2009). The study of Wan et al. (2021) showed

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that mice vaccinated with recombinant FlgE could induce a Th2-predominant immune response and reduced bacterial load and inflammation. Based on this, in this study, we chose the *oprL*, *oprF*, and *flgE* genes of *P. aeruginosa* to construct a trivalent combination DNA vaccine and detected the immune response and protective efficacy.

To evaluate the humoral immune response levels induced by the trivalent combination DNA vaccine, we performed an indirect ELISA using a *P. aeruginosa* suspension, a fimbriae protein, and an outer membrane protein as the coating antigens. The results indicated that the antibodies induced through the trivalent combination DNA vaccine were higher than those of three bivalent combination DNA vaccines and three monovalent DNA vaccines when the coating antigen was the *P. aeruginosa* suspension. This is equivalent to the inactivated vaccine. However, the antibodies detected in the poprL+poprF+pflgE group were lower than those detected in the poprL+poprF group and pflgE group when the coating antigens were the outer membrane protein and the fimbriae protein, respectively. This could be caused by the antigen composition and vaccine concentrations. After vaccination using the trivalent combination DNA vaccine, the chickens could produce antibodies against three antigens. The chickens vaccinated with bivalent combination DNA vaccines or with monovalent DNA vaccines could only produce antibodies against the corresponding antigen. Besides, the concentrations of poprL and poprF in the trivalent combination DNA vaccine are only two-thirds of the bivalent combination poprL+poprF DNA vaccine. Hence, the antibody concentrations against poprL and poprF in the chickens vaccinated with the trivalent combination DNA vaccine were lower than the chickens vaccinated with the poprL+poprF DNA vaccine. Similarly, the antibody concentrations against pflgE in the poprL+poprF+pflgE group were lower than those in the pflgE group. This might explain why the antibody concentrations in the poprL+poprF, poprL and poprF groups were higher than those in the poprL+pflgE, poprF+pflgE and pflgE groups, when the coating antigen was the outer membrane protein, and vice versa.

As is known DNA vaccines can induce strong cellular immune responses, the lymphocyte proliferation and cytokine levels can reflect the cellular immune function (Shebannavar et al. 2010). This study detects the PBL proliferation and the secretion of IFN-γ, IL-2, and IL-4 in vaccinated chickens. All the DNA vaccines and the inactivated vaccine could induce the proliferation of PBL, and as the number of boosters increases, the proliferation level of the PBL also increases gradually. Particularly, the levels of lymphocyte proliferation induced by the poprL+poprF+pflgE vaccine were higher than that of the other DNA vaccines and the inactivated vaccine. The cytokine assay results showed bivalent combination DNA vaccines could induce higher concentrations of the Th1 cytokines IL-2 and IFN-γ than the monovalent DNA vaccines and inactivated vaccines. The trivalent combination DNA vaccine could induce a higher IL-2 and IFN-γ response than the bivalent combination DNA vaccines. The concentrations of Th2 cytokine IL-4 induced by the three bivalent combination DNA vaccines, three monovalent DNA vaccines, and the inactivated vaccine were not statistically different. However, the trivalent combination DNA vaccine induced higher concentrations of IL-4 than the other vaccines.

Thus, we observed that the trivalent combination DNA vaccine in this study could induce both a stronger Th1 and Th2 response than the other vaccines.

Challenge experiments are important indices used to evaluate the protective efficacy of vaccines. Here, the challenge experiment demonstrated that the inactivated vaccine, trivalent combination DNA vaccine, and the three bivalent combination DNA vaccines could provide good protective efficacy for the vaccinated chickens. However, the protection rate of the trivalent combination DNA vaccine was higher than that of the other DNA vaccines. Though the trivalent combination DNA vaccine could induce good humoral and cellular immune responses, its protection rate was slightly lower than that of the inactivated vaccine. Therefore, further measures, such as optimising the vaccination dose, selecting more protective antigen genes and applying effective DNA vaccine adjuvants, need consideration to improve the protective efficacy of the trivalent combination poprL+poprF+pflgE DNA vaccine.

## Conflict of interest

The authors declare no conflict of interest.

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Received: June 24, 2021
Accepted: September 29, 2022
Published online: November 10, 2022