Original Article

Immune efficacy of *oprH* chitosan nanoparticle DNA vaccine against *Pseudomonas aeruginosa*

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Abstract

Background: *Pseudomonas aeruginosa* is a zoonotic pathogen that poses a threat to human and animal health. However, no vaccine exists for controlling this bacterium. **Aims:** This study aimed to evaluate the immune efficacy of a chitosan nanoparticle DNA vaccine of the *oprH* gene from *P. aeruginosa*. **Methods:** The naked DNA vaccine based on the *oprH* gene of *P. aeruginosa* was constructed. Then, the chitosan nanoparticle DNA vaccine of the *oprH* gene was prepared and the shape, size, encapsulation efficiency, stability, and ability of anti-DNA enzyme degradation were detected. Chickens were divided into five groups, namely the naked DNA vaccine group (poprH group), chitosan nanoparticle DNA vaccine group (CpoprH group), outer membrane protein vaccine group (OMP group), inactive vaccine group, and PBS group. After being vaccinated with corresponding vaccines, the levels of serum antibodies, lymphocyte proliferation assays, interferon-γ (IFN-γ), interleukin-2 (IL-2), and interleukin-4 (IL-4) concentrations were detected. Groups of chickens were challenged with live virulent *P. aeruginosa* 2 weeks after the last vaccination and the survival numbers were counted until day 15 post challenge. Then, the protective rates were calculated. **Results:** The particle size of the chitosan nanoparticle DNA vaccine was approximately 200 nm and close to spherical; the encapsulation efficiency was 95.88%, and it could effectively resist degradation by DNase. Following vaccination, serum antibodies, stimulation index (SI) value, and concentrations of IFN-γ, IL-2, and IL-4 in chickens immunized with the chitosan nanoparticle DNA vaccine were significantly higher than those that were vaccinated with the naked DNA vaccine ($P<0.05$). The protective rates of poprH, CoprH, OMP vaccine, and inactive vaccine groups were 55%, 75%, 75%, and 90%, respectively. **Conclusion:** Chitosan could significantly enhance the immune response and protection provided by the naked DNA vaccine of the *oprH* gene.

Key words: Chitosan nanoparticle DNA vaccine, Immune efficacy, Naked DNA vaccine, *oprH* gene, *Pseudomonas aeruginosa*

Introduction

Pseudomonas aeruginosa is a Gram-negative bacteria, widely distributed in nature. It is a classic zoonotic pathogen. It can cause infections of the urinary tract, wounds, bones, and joints, pneumonia, and even bacteremia, sepsis, and other diseases (Parra-Millán *et al*., 2022; Sathe *et al*., 2023; Yang *et al*., 2024). In addition, it can infect pigs, cattle, birds, mink, and even fish. Accordingly, *P. aeruginosa* is a common pathogen of zoonosis (Xiang *et al*., 2020; Bai *et al*., 2022; Mo *et al*., 2023).

Antibiotics are now commonly used to control infections caused by this pathogen. However, with the extensive use of antibiotics, the bacteria gradually developed drug resistance, and drug resistance is becoming increasingly serious. *P. aeruginosa* is now severely resistant to a variety of antibiotics, including β-lactam, aminoglycosides, quinolones, carbapenems, and so on (Khatami *et al*., 2022; Santos *et al*., 2022). This has led to increasingly difficult treatment. Therefore, it is necessary to seek more effective prevention and control measures. In addition to clinical drug therapy, immune prevention is also one of the most important measures to prevent and cure the infection caused by pathogenic microorganisms. However, up to now, there is no commercialized *P. aeruginosa* vaccine available in the clinic, so it is imperative to develop an effective *P. aeruginosa* vaccine. In this study, a naked DNA vaccine was constructed from the *oprH* gene encoding outer membrane protein H of *P. aeruginosa* using the eukaryotic expression vector pcaggs-HA. At the same time, a nanoparticle DNA vaccine was prepared with chitosan as an adjuvant. Then, the immune response and protective effect induced by two DNA vaccines in chickens were tested. The study aimed to provide some

theoretical basis for the study of a novel vaccine for *P. aeruginosa*.

Materials and Methods

Major reagents, bacterial strain, and experimental animals

Chitosan (deacetylation degree 90%) was provided by Zhejiang Golden Shell Pharmaceutical Co., Ltd. (Taizhou, China). DNase I, T4 DNA ligase, restricted endonuclease *Kpn*I, and *Xho*I were purchased from Takara Biotechnology Co., Ltd. (Beijing, China). Mouse IFN-γ, IL-2, and IL-4 test kits were products of Sangon Biotech Co., Ltd. (Shanghai, China). The *P. aeruginosa* CAU0792 strain was stored in the microbial genetic engineering laboratory of Henan University of Science and Technology, China. Healthy 1-day-old chickens were kept in the laboratory animal house of Henan University of Science and Technology, China.

Construction of naked DNA vaccine of the *oprH* gene

The naked DNA vaccine of the *oprH* gene was constructed according to previous methods (Gong *et al*., 2022). In brief, PCR methods were used to amplify the *oprH* gene from *P. aeruginosa* genomic DNA. Then, the *oprH* gene was ligated with the pMD™ 19-T vector. Subsequently, the recombinant plasmid was digested with *Kpn*I/*Xho*I and subcloned into the eukaryotic expression vector pcaggs-HA. The positive plasmid (i.e., naked DNA vaccine) was named poprH.

Preparation and detection of *oprH* gene chitosan nanoparticle DNA vaccine

The recombinant plasmid poprH was mixed with 30 mM Na_2SO_4 and preheated to 55°C. At the same time, 0.075% chitosan solution was prepared and was rapidly mixed with the above plasmid solution in equal volume after being preheated to 55°C. Subsequently, the mixture is swirled for 30 s and left at room temperature for 30 min to obtain a chitosan nanoparticle DNA vaccine of the *oprH* gene, named CpoprH. Then, the agarose gel electrophoresis was performed with naked DNA vaccine poprH as a control.

Electron microscopic observation of nanoparticle DNA vaccine

3 μL of the chitosan nanoparticle DNA vaccine solution was dropped onto the clean copper net supporting membrane and was left at room temperature for 2 min. After chitosan nano-DNA particles were fully contacted with the carbon membrane, the excess water was absorbed and dried at room temperature, and the morphology and size of nanoparticles were observed under the transmission electron microscope (TEM).

Determination of encapsulation efficiency of nanoparticle DNA vaccine

The encapsulation efficiency of nanoparticle DNA vaccine was analyzed by spectrophotometry. Firstly, the absorbance of recombinant plasmid poprH at 260 nm (A_{260}) was determined. Then, the nano-DNA vaccine was prepared according to the above method, and the prepared nanoparticle DNA vaccine was centrifuged for 20 min at 10,000 g. The supernatant was collected following centrifugation, and the A value at 260 nm (A'260) was calculated. The following formula was used to determine the effectiveness of encapsulation:

$$\text{Encapsulation efficiency} = (A260\text{-}A'260)/A260 \times 100\%$$

Anti-DNase I degradation assay

1 μg chitosan nanoparticle DNA vaccine and naked DNA vaccine were added into two centrifuge tubes, and then 2 μL of DNase I (2 U/μL) was added to each of them. Then, the two centrifuge tubes were placed for 2 h in a 37°C water bath. After incubation, the agarose gel electrophoresis was performed to determine the anti-degradation capability of the vaccine.

Stability detection of nanoparticle DNA vaccine

The prepared chitosan nanoparticle DNA vaccine and naked DNA vaccine containing the same amount of DNA were placed in a 37°C incubator for 1, 3, and 5 days. Agarose gel electrophoresis and Image Lab™ software were subsequently used to analyze the brightness of the bands to detect the stability of the chitosan nanoparticle DNA vaccine.

Animal vaccination

The outer membrane protein vaccine (OMP vaccine), which contains 1 μg/μL of *P. aeruginosa* outer membrane proteins, and the inactivated vaccine, which contains 2×10^{10} CFU/ml of *P. aeruginosa*, were made using the previously described procedure (Gong *et al*., 2018). Using phosphate-buffered saline (PBS, 0.01 M, pH 7.2), the naked DNA vaccine and the chitosan nanoparticle DNA vaccine were made in large quantities and adjusted to a concentration of 1 μg/μL DNA. Healthy one-day-old chickens (n=100) were raised in a specially designed animal home with regulated humidity, temperature, and light levels before vaccination. After a week of environmental acclimatization, the chickens were randomly divided into five groups, namely, the inactivated vaccine group, the OMP vaccine group, the naked DNA vaccine (poprH) group, the chitosan nanoparticle DNA vaccine (CpoprH) group, and PBS groups. The chickens from poprH, CpoprH, and PBS groups were intramuscularly injected with 200 μL poprH, CpoprH, and PBS (0.01 M, pH 7.2). Next, 200 μL of the *P. aeruginosa* inactivated vaccine and OMP vaccine were subcutaneously injected into the chickens in the inactivated and OMP vaccination groups, respectively. Chickens in the inactivated vaccine and OMP vaccine groups were subcutaneously injected with 200 μL of the inactivated vaccine and OMP vaccine, respectively. Chickens in all groups were vaccinated three times at 2-week intervals. Chickens were observed closely for adverse events after each vaccination. Any

chickens with depression, anorexia, or other clinical symptoms were housed in a calm feeding area where they could eat more appetizing food until they were healed.

Detection of serum-specific antibodies

Blood samples were taken every week for six weeks prior to the challenge after vaccination. Indirect enzyme-linked immunosorbent assays (ELISAs) were used to evaluate serum antibodies using coated antigens of *P. aeruginosa* OMPs (20 µg/ml) and suspension of *P. aeruginosa* (2×10^9 CFU/ml). Following vaccination, blood samples were collected weekly for 6 weeks before the challenge. Serum antibodies were measured using indirect enzyme-linked immunosorbent assays (ELISAs) tests with 2×10^9 CFU/ml of *P. aeruginosa* suspension and 20 µg/ml OMPs of *P. aeruginosa* as coating antigens. In brief, 50 µL of the OMP or suspension of *P. aeruginosa* was applied to the 96 well microliter plates, and 5% BSA was added to the microliter plate to block the nonspecific binding. Following three PBST (0.01 M PBS-0.05% Tween-80, pH 7.2) washes, 50 µL of blood samples (1:100 dilution) were added to the plates and incubated for 1.5 h at 37°C. After that, 50 µL of secondary antibody (rabbit anti-chicken IgG-horseradish peroxidase, HRP) was added, and it was incubated for 1.5 h at 37°C. Next, 50 µL of ortho-phenylene diamine (OPD) was added after washing, and the reaction lasted for 10 min. Ultimately, 50 µL of 2 M sulfuric acid (H_2SO_4) was used to terminate the reaction, and the absorbance at 492 nm was recorded.

Lymphocyte proliferation test

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method was used to assay the proliferation of peripheral blood lymphocytes (PBL) in immunized chickens (Gong *et al*., 2021). Blood samples were obtained from vaccinated chickens two weeks following each vaccine, and a kit was used to separate PBLs. PBL's concentration was brought down to 2×10^7 cells/ml. A 96-well culture plate was seeded with 50 µL of cell suspension. Then, each well was added with 20 µg/ml OMP of *P. aeruginosa* (test well) or cell culture medium (negative control) at 50 µL. The plates were then incubated at 37°C for 72 h at 5% CO_2. After culture, each well was added with 50 µL of 5 mg/ml MTT for 3 h. Following centrifugation, 150 µL of dimethyl sulfoxide (DMSO) was added after the supernatant was extracted. The absorbance of each well was then measured at 570 nm after the plates had been incubated for 10 min. The formula used to calculate the stimulation index (SI) was:

SI = A (test well)/A (negative control well)

Determination of serum cytokine concentration

Blood samples were taken two weeks following each vaccination, and sera were separated. Then, the concentrations of interferon (IFN)-γ, interleukin (IL)-2, and IL-4 in sera were assayed using a commercial ELISA kit according to the manufacturer's instructions (Sangon Biotech Co., Ltd.).

Chicken challenge experiment

All of the chickens received an intramuscular injection of the virulent *P. aeruginosa* strain CAU0792 (5 LD50 per chicken) two weeks following the third immunization. Firstly, the injection site was disinfected with 70% alcohol. Then, using sterile 1 ml syringes, 0.5 ml of *P. aeruginosa* suspension was injected into the chickens. All of the chickens were housed for 15 days following the challenge, during which time they were closely watched for any indications of the disease. Any chickens exhibiting signs of depression and/or appetite loss were segregated and kept under strict observation. The survival and protection rates for each group were determined 15 days following the test.

Analysis of statistics

For statistical analysis, SAS software (ver. 9.4; SAS Institute) was used. To identify significant differences in the mean value between the experimental groups, the Fisher's least significant difference (LSD) test and one-way analysis of variance (ANOVA) were employed. A p-value less than 0.05 was considered to be statistically significant.

Results

Identification of naked DNA vaccine poprH

The *oprH* gene of *P. aeruginosa* was ligated with the eukaryotic expression vector pcaggs-HA and the recombinant plasmid poprH was obtained. Agarose gel electrophoresis was used to identify the product after the plasmids were isolated and digested using the restriction enzymes *Kp*nI and *Xho*I (Fig. 1). A DNA fragment of 610 bp was obtained, indicating the successful construction of the naked DNA vaccine poprH.

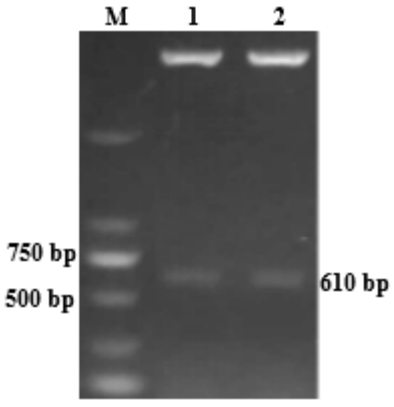

Fig. 1: Identification of recombinant plasmid poprH by restriction digestion. Lane M: DNA marker DL2000. Lanes 1, 2: poprH enzymed with *Kp*nI and *Xho*I

Gel retardation assay of the nanoparticle DNA vaccine

After preparation, the chitosan nanoparticle DNA

vaccine of the *oprH* gene was detected by agarose gel electrophoresis (Fig. 2). The naked DNA vaccine could migrate in the electric field. The chitosan nanoparticle DNA vaccine was confined near the gel pore because the negative charge of DNA is neutralized by positively charged chitosan molecules. The result showed that DNA was closely bound to chitosan molecules.

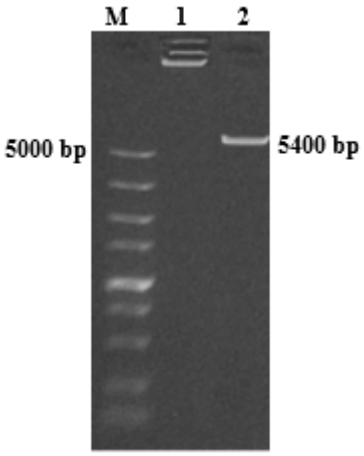

Fig. 2: Results of gel retardation assay of *oprH* chitosan nanoparticle DNA vaccine. Lane M: DNA marker DL5000. Lane 1: Chitosan nanoparticle DNA vaccine, and Lane 2: Naked DNA vaccine poprH

Results of electron microscopic observation and encapsulation efficacy

The shape of the chitosan nanoparticle DNA vaccine of the *oprH* gene was observed under TEM. The type of nanoparticle appeared regular spherical shape, and the particle size was about 200 nm (Fig. 3).

After centrifuging, the supernatant of the nanoparticle DNA vaccine had a value of 0.087, and the naked DNA vaccine had a value of 2.112 at 260 nm. Using the above formula, the encapsulation efficacy of the nanoparticle DNA vaccine was 95.88%.

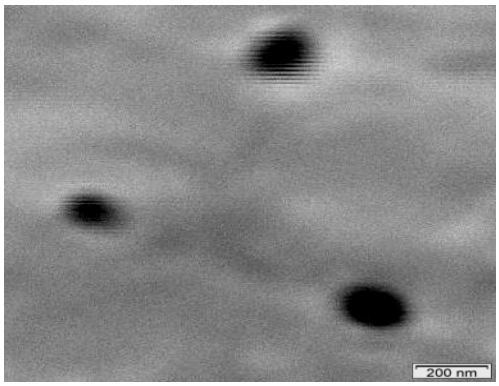

Fig. 3: TEM image of *oprH* chitosan nanoparticle DNA vaccine (×50,000)

Result of DNaseI degradation

To determine the protective effect of chitosan on DNA, DNase I was put into both the naked DNA vaccine and the nanoparticle DNA vaccination. Prior to and following DNase digestion, the chitosan nanoparticle DNA vaccine's brightness stayed essentially constant. However, DNase I totally degraded the naked DNA vaccine (Fig. 4). The result showed that the chitosan nanoparticle DNA vaccine of the *oprH* gene has a good ability against DNase I degradation.

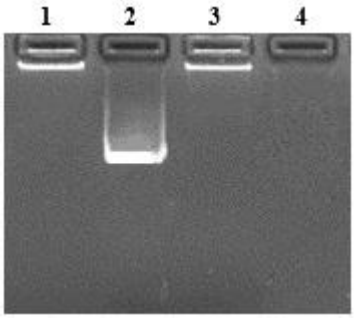

Fig. 4: Analysis to determine protection from DNase degradation of *oprH* chitosan nanoparticle DNA vaccine. Lanes 1, 3: Chitosan nanoparticle DNA vaccine before and after degradation, and Lanes 2, 4: Naked DNA vaccine before and after degradation

Result of the stability assay

Agarose gel electrophoresis was performed after the chitosan nanoparticle DNA vaccine and naked DNA vaccine were placed for 1, 3, and 5 days at 37°C. The results are shown in Fig. 5. Image Lab™ software analysis showed that the brightness of the electrophoretic bands of the naked DNA vaccine decreased gradually with the time of extension, while the brightness of the nanoparticle DNA vaccine did not change significantly from beginning to end. The result indicated that the chitosan nanoparticle DNA vaccine of the *oprH* gene had good stability.

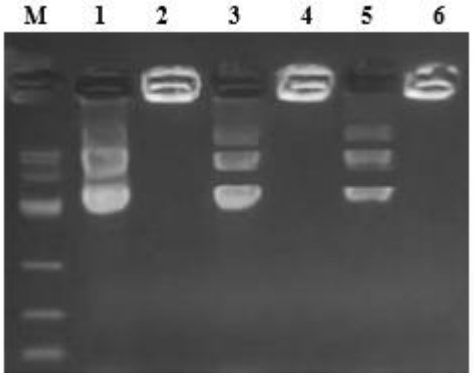

Fig. 5: Stability test of chitosan nanoparticle DNA vaccine. Lane M: DNA marker DL 15000. Lanes 1, 3, 5: Electrophoresis of naked DNA vaccines after 1, 3, and 5 days at 37°C, and Lanes 2, 4, 6: Electrophoresis of chitosan nanoparticle DNA vaccine after 1, 3, and 5 days at 37°C

Results of serum-specific antibody detection

Indirect ELISA was used to measure the serum antibody levels of immunized chickens, and the results are shown in Figs. 6A and B. There were no differences in antibody levels among the inactivated vaccine group,

OMP vaccine group, poprH group, and CpoprH group after the initial vaccination (P>0.05). From the third week, the antibody levels in the OMP vaccine group and CpoprH group were significantly higher than those in the poprH group (P<0.05), and the antibodies found in the inactivated vaccine group were higher than those found in the other three vaccine groups (P<0.05), when the *P. aeruginosa* suspension was the coating antigen (Fig. 6A). Following the second and third vaccinations, antibodies detected in the OMP vaccine group were higher than those detected in the inactivated vaccine group and CpoprH group, and the antibody levels in the latter two groups were significantly higher than those in the poprH group (P<0.05), when OMP of *P. aeruginosa* was the coating antigen (Fig. 6B).

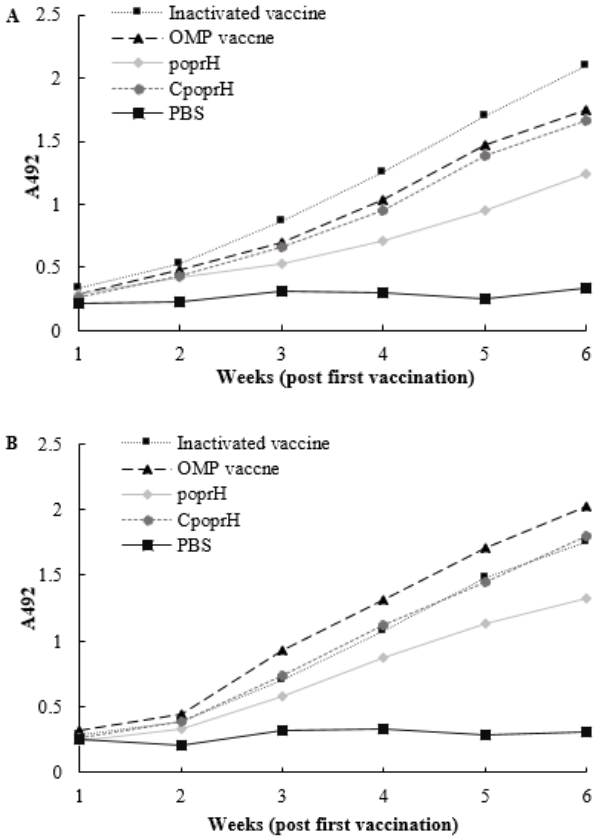

Fig. 6: Dynamic changes in serum antibody concentrations in vaccinated chickens. Following the first vaccination, serum antibody concentrations were determined by indirect ELISA weekly for 6 weeks with *P. aeruginosa* suspension (**A**) or OMPs of *P. aeruginosa* (**B**) as coating antigen. Chickens were vaccinated with inactivated vaccine (■), OMP vaccine (▲), naked DNA vaccine poprH (◇), chitosan nanoparticle DNA vaccine CpoprH (O), and PBS (*)

Lymphocyte proliferation assay

At three time points after vaccination, the proliferation of PBLs was assessed using an MTT test (Fig. 7). Following each vaccination, SI values of the four vaccine groups were higher than that in the PBS group (P<0.05). There was no difference between the four vaccine groups after the first vaccination (P>0.05). The SI values of the inactivated vaccine group and OMP vaccine group were significantly higher than those in the poprH and CpoprH groups after the second and third vaccinations (P<0.05). The SI value in the CpoprH group was higher than that in the poprH group after the third immunization (P<0.05).

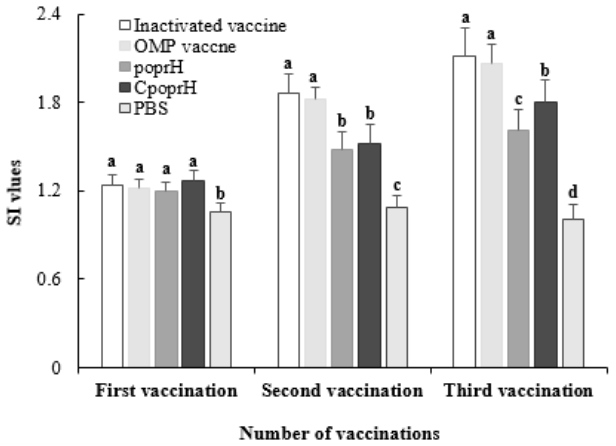

Fig. 7: Lymphocyte proliferation assays from chickens vaccinated with DNA vaccines. *P. aeruginosa* OMPs were administered to stimulate peripheral blood lymphocytes 2 weeks after each vaccination

Results of cytokine assay

Following vaccination, IFN-γ, IL-2, and IL-4 concentrations were determined in the sera (Figs. 8A-C). Following each vaccination, the concentrations of the three cytokines in the four vaccine groups were higher than that in the PBS group (P<0.05). The concentrations of the three cytokines in the four vaccine groups showed no differences after the first vaccination (P>0.05). Following the second vaccination, the inactivated vaccine group and OMP vaccine group had significantly higher levels of IFN-γ and IL-2 than the poprH and CpoprH groups (P<0.05), and the CpoprH group had higher levels of the two cytokines than the poprH group (P<0.05). Following the third vaccination, IFN-γ and IL-2 concentrations in the inactivated vaccine group were higher than those of the other three vaccine groups (P<0.05), while the concentrations in the OMP vaccine and CpoprH groups were higher than those of the poprH group (P<0.05). Nevertheless, following the third immunization, there were no differences in the concentrations of IL-2 and IFN-γ between the CpoprH and OMP vaccine groups (P>0.05) (Figs. 8A and B). The inactivated vaccine group had higher levels of IL-4 than the other groups after the second and third vaccinations (P<0.05). After the second vaccination, no significant differences were detected in the concentrations of IL-4 among the OMP vaccine, poprH, and CpoprH groups (P>0.05). After the third vaccination, the concentrations of IL-4 in the OMP vaccine group and CpoprH group were higher than those in the poprH group (P<0.05), and no differences were detected between the OMP vaccine group and CpoprH group (P>0.05).

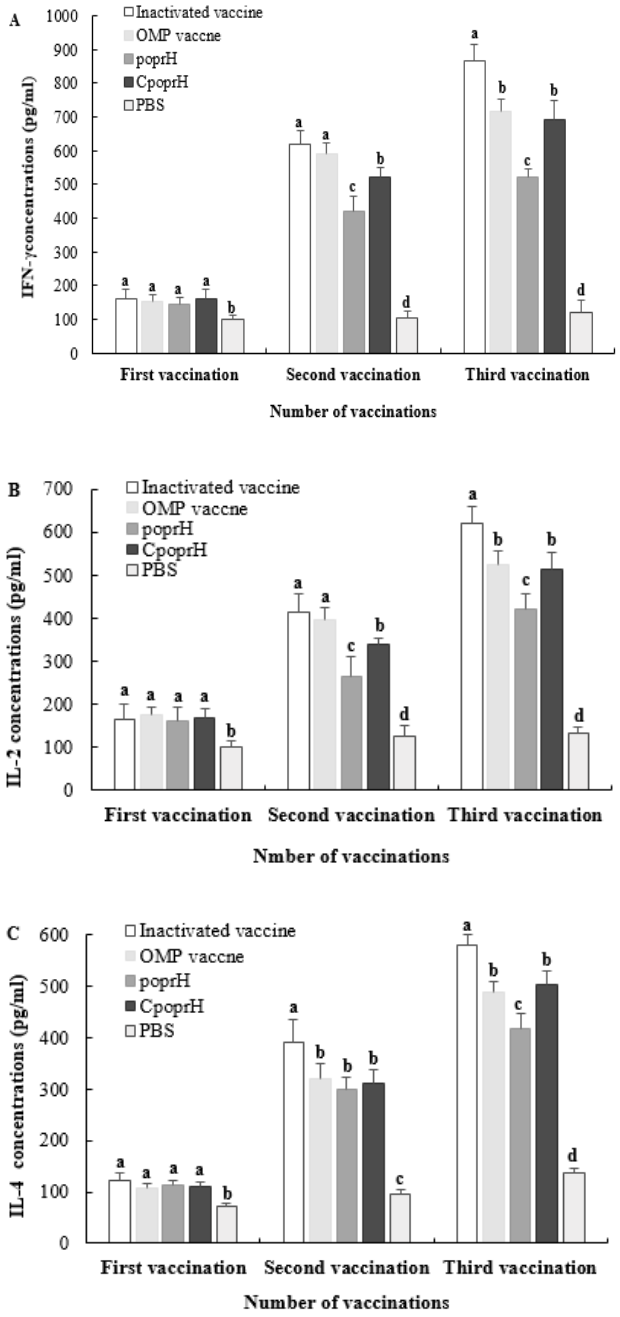

Fig. 8: Concentration of cytokines in the serum of immunized chickens. Concentrations of IFN~γ (**A**), IL-2 (**B**), and IL-4 (**C**) from sera of vaccinated chickens 2 weeks after each vaccination

Results of the challenge study

Two weeks following the third immunization, the chickens were challenged with live, virulent *P. aeruginosa*. The survival curve was drawn by counting the number of chickens that survived (Fig. 9). The chickens of the PBS group all died within 4 days after the challenge. After the challenge, chickens from poprH, CpoprH, and OMP vaccine groups began to die on the first day, second day, and second day, respectively. The survival numbers for the poprH, CpoprH, and OMP vaccination groups were 11, 15, and 15 following a 15-day challenge. Following the challenge, just two chickens in the inactivated vaccination group perished. The results showed that the protection rates of poprH, CpoprH, OMP vaccine, and inactivated vaccine were 55%, 75%, 75%, and 90%.

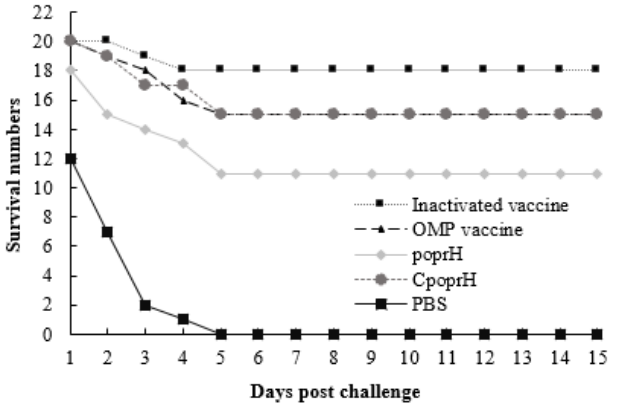

Fig. 9: Survival of chickens after challenge with avian *P. aeruginosa*. The chickens (n=20) were observed for 15 days post-challenge

Discussion

As a common conditioned pathogen of zoonosis, *P. aeruginosa* threatens human health and the development of animal husbandry to some extent. Since the pathogen has developed serious resistance to a variety of antibiotics in recent years, there are fewer and fewer effective drugs for the clinical treatment of this pathogen, and the difficulty of the treatment is increasing. In addition, there is no effective vaccine for the prevention of the pathogen. Therefore, to effectively control this pathogen, it is necessary to strengthen the research and development of therapeutic drugs and vaccines against *P. aeruginosa*. At present, there have been some related research reports on the *P. aeruginosa* vaccine, including multi-epitope vaccine, chimeric vaccine, inactivated vaccine, and DNA vaccine (Beg *et al*., 2021; Jiang *et al*., 2023; Korpi *et al*., 2023; Zhang *et al*., 2024). Among them, DNA vaccines have attracted much attention because of their advantages of low cost, simple process, and easy preservation (Eusébio *et al*., 2021; Yu *et al*., 2022). Although DNA vaccines against a variety of important human and animal infectious diseases have related research, there are few DNA vaccines available for clinical use. The main reason is that the protective effect of most DNA vaccines is inferior to the traditional vaccines. Effective measures should therefore be implemented to increase the immunological efficacy of DNA vaccines.

Given this, researchers have taken a variety of measures to explore, such as the application of ubiquitin to enhance the release of antigen to the proteasome, optimization of the inoculation method, the use of new adjuvants, etc. At present, the new adjuvants used in vaccine research include cytokine adjuvant, CpG-ODN adjuvant, ISCOM adjuvant, polysaccharide adjuvant, nanoparticle adjuvant, etc. Among them, nanoparticle

adjuvant is a kind of vaccine adjuvant that has attracted more attention in recent years. This kind of adjuvant has many advantages, such as the ability to adsorb, concentrate, and protect nucleotides from degradation by nuclease, and the susceptibility to be internalized and presented by antigen-presenting cells (Mao *et al*., 2021; Shi *et al*., 2022). Some studies have shown that poly lactic-co-glycolic acids (PLGA), silica, chitosan, etc. can be used as carriers and adjuvants for vaccine research (Abianeh *et al*., 2023; Barbey *et al*., 2023; Huo *et al*., 2023). Chitosan is a widely used nanoparticle adjuvant. As the only alkaline polysaccharide with a positive charge in nature, chitosan can adsorb negatively charged DNA molecules to form nanoparticles. After the DNA nanoparticles enter the animal body, the adsorption of chitosan can play a role in slow and controlled release so that DNA molecules can be slowly released, thereby prolonging the immune duration and improving the immune effect. Accordingly, we constructed a chitosan nano-DNA vaccine of the *P. aeruginosa oprH* gene, and tested its immune efficacy by animal experiments. The results showed that chitosan could closely combine with the naked DNA vaccine of the *P. aeruginosa oprH* gene to form relatively regular nanoparticles, and could delay the natural degradation of the naked DNA vaccine and improve its resistance to DNA enzymes.

To evaluate the ability of the naked DNA vaccine and chitosan nanoparticle DNA vaccine to induce humoral immune responses in experimental animals, the serum-specific antibody levels of chickens in each group were determined by indirect ELISA using *P. aeruginosa* suspension and OMP as coating antigens. The results indicated that the chitosan nanoparticle DNA vaccine caused a more intense humoral immune response since the antibodies induced by CpoprH were higher than poprH regardless of which antigen was coated.

The ability of lymphocytes to proliferate and levels of cytokines secreted are common indices that are used to evaluate cellular immune function. In this experiment, the proliferation of PBLs and concentrations of three cytokines in immunized chickens were assessed. With the increase in immunization times, IFN-γ, IL-2, and IL-4 concentrations as well as SI values steadily rose in each vaccination group. Although the chitosan nanoparticle DNA vaccine was less effective at inducing lymphocyte proliferation than the inactivated and OMP vaccines, it is superior to the naked DNA vaccine. IFN-γ, IL-2, and IL-4 concentrations from sera of chickens vaccinated with the chitosan nanoparticle DNA vaccine were similar to those of the OMP vaccine, and superior to those of the naked DNA vaccine. These results showed that the *oprH* chitosan nanoparticle DNA vaccine was more effective than the naked DNA vaccination at inducing Th 1 and Th 2 immune responses.

Challenge experiment is the most important and intuitive indicator to assess the vaccine's protective effect. The result of the challenge experiment showed that the protective effectiveness of the chitosan nanoparticle DNA vaccine was comparable to that of the OMP vaccine, better than that of the naked DNA vaccine, but still less effective than that of the inactivated vaccine. The reason may be that although the use of chitosan as an adjuvant enhanced the immunogenicity of the *oprH* gene DNA vaccine to some extent, it is still a monovalent vaccine containing only one immunogen gene, and the *oprH* gene is only one of the many outer membrane protein-coding genes of *P. aeruginosa*. Due to the large genome and many protective antigen genes of *P. aeruginosa*, the immune response induced by a single antigen is insufficient to provide adequate protection for experimental animals.

In conclusion, the chitosan nanoparticle DNA vaccine induced a better immune response and protective efficacy than the naked DNA vaccine. This implies that the *oprH* gene chitosan nanoparticle DNA vaccine is a viable strategy for researching new DNA vaccines against *P. aeruginosa*.

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Conflict of interest

None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

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