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Immunology

Immune Efficacy of different immunization doses of divalent combination DNA vaccine pOPRL+pOPRF of *Pseudomonas aeruginosa*

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ABSTRACT. At present, there is no vaccine available against *Pseudomonas aeruginosa*, a common zoonotic pathogenic bacterium. In a previous study, the authors prepared a divalent combination DNA vaccine, pOPRL+pOPRF, which exhibited good protective efficacy. To explore the optimal immunization dose of this divalent combination DNA vaccine, in the present study, chickens were vaccinated with 25, 50, 100, and 200 µg doses. The levels of serum antibody, interferon-γ (IFN-γ), and interleukin-2 (IL-2) were determined, and lymphocyte proliferation assays were performed. After challenge with virulent *P. aeruginosa*, the protective efficacy was evaluated. Following vaccination, the serum antibodies, stimulation index values, and concentrations of IFN-γ and IL-2 were significantly higher in chickens vaccinated with 100 and 200 µg vaccines than in those vaccinated with 25 and 50 µg doses ($P<0.05$). IFN-γ and IL-2 concentrations in chickens immunized with 100 µg vaccine were slightly higher than those in chickens immunized with 200 µg vaccine, although the difference was not statistically significant. The protective rates were 55%, 65%, 85%, and 85% with 25, 50, 100, and 200 µg of the pOPRL+pOPRF DNA vaccine, respectively. Thus, the immune efficacy of the pOPRL+pOPRF DNA vaccine increased with an increase in immunization dose, but this does not imply that a higher dose necessarily achieves a better outcome. The optimal immunization dose of pOPRL+pOPRF DNA vaccine in chickens was 100 µg.

KEY WORDS: DNA vaccine, Immune efficacy, Immunization dose, pOPRL+pOPRF, *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a zoonotic pathogenic bacterium that can cause diseases in humans and animals, and is widely distributed in many countries. In humans, it is a causative agent of pneumonia, urinary tract infections, meningitis, and other diseases [1, 16]. In particular, pulmonary infection caused by this pathogen is associated with high mortality rates, and has become a considerable challenge in clinical settings. In addition, it can cause hemorrhagic pneumonia and septicemia among other diseases [7]. The incidence of *P. aeruginosa* infections has significantly increased in recent years [10]. In addition to humans, other animals can also be infected. In countries such as China, the rapid development in intensive animal farming has led to a significant rise in the incidence of *P. aeruginosa* infection. This pathogen can infect chickens, turkeys, pigeons, cockroaches, ducks, birds, and other poultry. In chickens, *P. aeruginosa* is often a secondary infection of other diseases and can present as a mixed infection with other pathogens such as *Escherichia coli* and *Salmonella*. Drug treatment, particularly with antibiotics, is a major control measure for this disease. However, the pathogen is susceptible to drugs after long-term treatment. *P. aeruginosa* has become resistant to multiple classes of antibacterial agents (e.g., carbapenems), and the rate of resistance has rapidly risen [8, 14, 17]. Therefore, it is necessary to develop a new strategy for the prevention and control of this pathogen-borne diseases.

Vaccine immunization is currently one of the most effective ways to prevent and control infectious diseases. Although reports on *P. aeruginosa* vaccines have been published, there are no commercialized vaccines that can be used in clinical settings [12]. Therefore, there is an urgent need to develop *P. aeruginosa* vaccines. New generation vaccines against infectious diseases include gene engineering subunit vaccines, synthetic peptide vaccines, and DNA vaccines. Since the first successful preparation of a DNA vaccine by Wollf in the 1990s, which offers the advantages of convenient preparation, low cost, and simple preservation, this type of vaccine is currently the subject of intense investigation in the field of vaccine research. Immunogen genes currently explored for research on novel vaccines include the outer membrane protein gene, flagellin gene, and toxin gene. The outer membrane protein, encoded by the *oprL*, *oprF*, and *oprD* genes, is one of the major protective antigens of *P. aeruginosa*. Several groups have studied recombinant subunit vaccines and DNA vaccines based on *oprF* and *oprI* genes [3, 4, 13, 18]. However, research on a DNA vaccine based on the *oprL* gene of *P. aeruginosa* has been minimal. In a previous study, the authors constructed monovalent,

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divalent, and two-gene fusion DNA vaccines based on the *oprL* and *oprF* genes of *P. aeruginosa* that exhibited an immune response and protective efficacy [5]. The levels of immune response and protective efficacy induced by divalent combination DNA vaccines were superior to those by others.

In the present study, different immunization doses of the divalent combination DNA vaccine were evaluated for their immune response and protective efficacy. The goal of this study was to explore the optimal immunization dose of the divalent combination DNA vaccine of the *oprL* and *oprF* genes of *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strain and experimental animals

P. aeruginosa was purchased from the Chinese Institute of Veterinary Drug Control. Healthy 1-day old chickens were obtained from the Animal Center Laboratory of the College of Medical Technology and Engineering of Henan University of Science and Technology, China. The study protocol was approved by the Animal Monitoring Committee of Henan University of Science and Technology (Permit Number 2019-0025; July 23, 2019).

Construction of DNA vaccines

DNA vaccines, pOPRL and pOPRF, were constructed according to previous methods [5]. In brief, primers were designed according to the nucleotide sequences of the *oprL* and *oprF* genes of *P. aeruginosa* (GenBank accession number AE004091.2). The primer sequences used are as follows:

F-*oprL*: 5′-ATCGGGATCCATGGAAATGCTGAAATTC-3′,
L-*oprL*: 5′-CAGAATTCTTACTTCTTCAGCTCGACGCGAC-3′,
F-*oprF*: 5′-GTGGATCCATGAAACTGAAGAACACC-3′,
L-*oprF*: 5′-ATGGAATTCTTACTTGGCTTCAGCT-3′.

Genomic DNA of the *P. aeruginosa* CAU0792 strain was extracted using the cetyltrimethylammonium bromide (CTAB) method. The *oprL* and *oprF* gene fragments were amplified using genomic DNA as a template. The polymerase chain reaction (PCR) mixture comprised 1 µl template DNA, 1 µl of each primer (final concentration 0.25 µM), 12.5 µl of 2× Taq Plus Master Mix, and 9.5 µl of sterile deionized water. PCR amplification was carried out with pre-denaturation for 5 min at 94°C, followed by 30 cycles of denaturation for 45 sec at 94°C, annealing for 30 sec at 60°C, extension for 45 sec at 72°C, and a final extension step for 10 min at 72°C. Amplified products were purified using a gel extraction mini kit (Shanghai Watson Biological Engineering Co., Shanghai, China), followed by sequencing. The products were digested with *Bam*HI/*Eco*RI, ligated into the eukaryotic expression vector pcDNA3.1 (+), and transformed into *E. coli* DH5α competent cells. The plasmids were extracted and identified using the restriction enzymes *Bam*HI and *Eco*RI. The positive plasmids (i.e., DNA vaccines) were named pOPRL and pOPRF [5]. The two DNA vaccines were prepared on a large scale and adjusted to 1 µg/µl concentration using phosphate-buffered saline (PBS, 0.01 M, pH 7.2) for animal experiments.

Immunization of chickens

Healthy 1-day old chickens (n=120) were reared in a purpose-built animal house with controlled environmental light, temperature, and humidity. The chickens had *ad libitum* access to water and non-medicated feed. General health monitoring was performed on all chickens from the day of arrival until the completion of the experiment. After adaptation to the new environment, chickens (1-week old) were randomly assigned to six groups, namely, 25, 50, 100, and 200 µg, PBS, and empty vector groups, and were injected accordingly. The pOPRL and pOPRF were mixed in equal proportions as the divalent combination DNA vaccine (pOPRL+pOPRF). The chickens from 25, 50, 100, and 200 µg groups were intramuscularly injected with 25 µg divalent combination DNA vaccine (containing 12.5 µg pOPRL and 12.5 µg pOPRF), 50 µg divalent combination DNA vaccine (containing 25 µg pOPRL and 25 µg pOPRF), 100 µg divalent combination DNA vaccine (containing 50 µg pOPRL and 50 µg pOPRF), and 200 µg divalent combination DNA vaccine (containing 100 µg pOPRL and 100 µg pOPRF), respectively. Chickens from the PBS and empty vector groups were injected with 200 µl PBS (0.01 M, pH 7.2) and 200 µl (1 µg/µl) empty vector pcDNA3.1 (+) solution, respectively. The chickens from all groups were immunized thrice at 2-week intervals. After each vaccination, chickens were closely observed for adverse reactions. Any chickens that presented with depression, lack of appetite, or other clinical signs of illness were isolated to a quiet feeding environment where they had access to more palatable feed until they recovered.

Detection of antibody levels

Following vaccination, blood samples were weekly collected for 6 weeks prior to the challenge. Serum antibody levels were detected using indirect enzyme-linked immunosorbent assays (ELISAs) with the outer membrane protein of *P. aeruginosa* as a coating antigen and horseradish peroxidase (HRP)-labeled rabbit anti-chicken IgG (Sigma-Aldrich, St. Louis, MO, USA) as the secondary antibody. Briefly, ELISA microtiter plates (eBioscience, San Diego, CA, USA) were coated with 50 µl of the outer membrane protein (20 µg/ml) of *P. aeruginosa*, and nonspecific binding was blocked with 5% bovine serum albumin (Sigma-Aldrich). Subsequently, 50 µl of serum samples (1:100 diluted) were added to the plates and incubated at 37°C for 1.5 hr. The plates were washed thrice with PBST (0.01 M PBS-0.05% Tween-80, pH 7.2), and probed with rabbit anti-chicken IgG. The plates were then incubated at 37°C for 1.5 hr. After three washes, ortho-phenylene diamine (OPD) was added and the reaction was allowed to proceed for 10 min. The enzyme activity was terminated by adding 2 M sulfuric acid (H_2SO_4) and the absorbance value was measured at 492 nm wavelength.

Peripheral blood lymphocyte (PBL) proliferation assay

Two weeks after each vaccination, a PBL proliferation assay was performed according to a previously published method [6]. Blood samples were collected from vaccinated chickens, and PBLs were separated using a commercial chicken PBL separation kit (Solarbio, Beijing, China). In brief, blood samples were mixed with a lymphocyte separation solution and centrifuged at 1,000 × *g* for 20 min. The lymphocytes between the plasma layer and the separation solution layer were carefully removed. After washing twice with 1× PBS (pH 7.2), PBLs were obtained. The concentration of PBL was adjusted to 2×10^7 cells/ml. The cell suspension (50 µl) was seeded into a 96-well culture plate (Greiner Bio-One, Longwood, Germany), and treated with 50 µl of 20 µg/ml outer membrane protein of *P. aeruginosa* (experimental well) or 50 µl of Roswell Park Memorial Institute (RPMI)-1,640 medium (negative control). The plates were then incubated at 37°C under 5% CO_2 for 72 hr. Subsequently, each well was treated with 10 µl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and the plates were incubated for 3 hr. After centrifugation, the supernatant was discarded and 150 µl of dimethyl sulfoxide (DMSO) was added. The plates were then incubated for 10 min, and the absorbance value of each well was measured at 570 nm. The stimulation index (SI) was measured using the following equation: SI=A (experimental well)/A (negative control well).

Cytokine assay

Two weeks after each vaccination, PBL suspensions of chickens were prepared and stimulated with the outer membrane protein of *P. aeruginosa*. The cells were then cultured at 37°C under 5% CO_2 for 72 hr. Interferon (IFN)-γ and interleukin (IL)-2 concentrations in the supernatants were assayed using a commercial ELISA kit (Yuan Ye Biotech Co.) according to the manufacturer's instructions.

Challenge experiment

Several different challenge models are available, such as intranasal, subcutaneous, intraperitoneal, and intramuscular. In this study, chickens were challenged with the virulent *P. aeruginosa* strain CAU0792 (5 LD50 per chicken) by intramuscular injection 2 weeks after the third DNA immunization. The injection site was prepared with 70% alcohol, and 0.5 ml *P. aeruginosa* suspension was administered using sterile 1 ml syringes. Following this challenge, chickens were reared for 15 days and closely observed every day for clinical signs of illness. Chickens showing signs of depression and/or a lack of appetite were isolated and placed under close observation. Chickens that were anorexic or dyspneic and those with hemorrhagic diarrhea or other abnormal signs were withdrawn from the experiment and euthanized by intravenous injection of pentobarbital sodium. At the end of the experiment, survival and protection rates were calculated for each group.

Statistical analysis

Statistical analysis was conducted using SAS software (Version 9.4; SAS Institute). Analysis of variance was used to determine significant differences in the means between the experimental groups. Differences were considered significant at $P<0.05$.

RESULTS

Identification of pOPRL and pOPRF

The *oprL* and *oprF* genes were ligated with pcDNA3.1 (+) to construct the recombinant plasmids pOPRL and pOPRF, respectively. The plasmids were extracted and digested with restriction enzymes *Bam*HI and *Eco*RI, and the products were identified by agarose gel electrophoresis (Fig. 1). DNA fragments of 517 and 1,063 bps were obtained, indicating the successful construction of the recombinant plasmids pOPRL and pOPRF.

Serum antibody levels

Serum antibody levels were detected using indirect ELISA. As shown in Fig. 2, serum antibodies increased following vaccination in the groups vaccinated with different doses of pOPRL+pOPRF DNA and significantly exceeded the values reported in the PBS and empty vector groups ($P<0.01$). Four weeks after the first vaccination, antibodies detected in the 100 and 200 µg groups were higher than those detected in the 25 and 50 µg groups ($P<0.05$). However, no differences were observed between the 100 and 200 µg groups ($P>0.05$).

Lymphocyte proliferation assay

To evaluate the cellular immune response induced by different dosages of the pOPRL+pOPRF DNA vaccine, we performed the MTT assay to assess the proliferation of PBLs at three time points following vaccination. The results are shown in Fig. 3. After each vaccination, the SI values in all DNA vaccine groups were consistently higher than the values obtained for the PBS and empty vector groups ($P<0.05$). In addition, the SI values of the 100 and 200 µg groups were higher than those of the 25 and 50 µg groups after second and third vaccinations ($P<0.05$). There were no differences between the groups treated with 100 and 200 µg vaccine ($P>0.05$).

Concentrations of IFN-γ and IL-2

After vaccination, the concentrations of IFN-γ and IL-2 secreted by the PBLs of immunized chickens were determined (Fig. 4). After each vaccination, the concentrations of the two cytokines in each of the DNA vaccine groups were higher than those reported

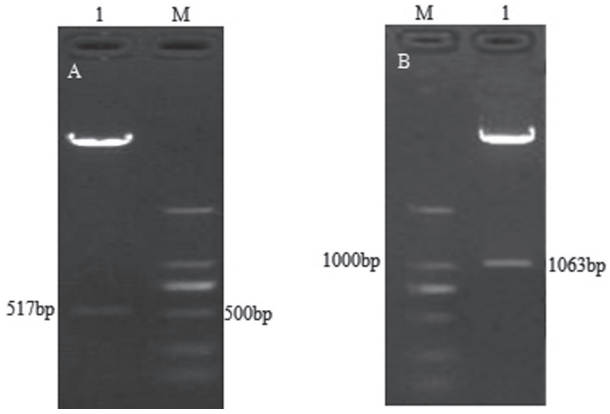

Fig. 1. Agarose gel electrophoresis analysis of *Bam*HI/*Eco*RI digestion of recombinant plasmid pOPRL **(A)** and pOPRF **(B)**. Lane M: DL2000 DNA marker; Lane 1: pOPRL (A) and pOPRF (B) digested with restriction enzymes.

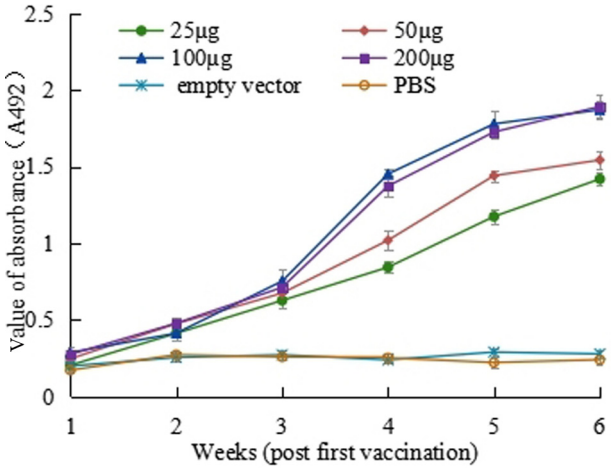

Fig. 2. Dynamic changes in serum antibody concentrations in vaccinated chickens. Following the first vaccination, serum antibody concentrations were determined by indirect ELISA weekly for 6 weeks. Chickens were vaccinated with the pOPRL+pOPRF DNA vaccine at different doses as follows: 25 μg (●), 50 μg (◆), 100 μg (▲), 200 μg (■), empty vector (*), and PBS (○).

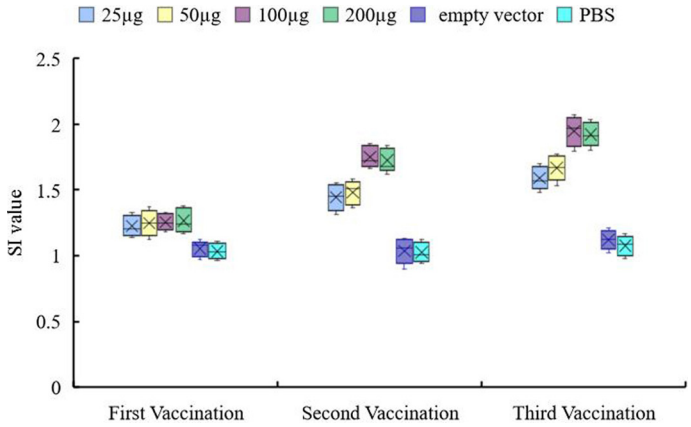

Fig. 3. Lymphocyte proliferation assays from chickens vaccinated with various dosages of pOPRL+pOPRF DNA vaccine. *Pseudomonas aeruginosa* outer membrane protein was administered to stimulate peripheral blood lymphocytes 2 weeks after each vaccination.

in the PBS and empty vector groups ($P<0.05$). After the first vaccination, no significant differences were detected in IFN-γ and IL-2 levels among the four dosage groups ($P>0.05$). However, following the second and third vaccinations, the concentrations of the two cytokines in 100 and 200 μg groups were significantly higher than those in 25 and 50 μg treatment groups ($P<0.05$). IFN-γ and IL-2 levels did not statistically differ between 100 and 200 groups, although the former was slightly higher than the latter.

Results of challenge

Two weeks after the third vaccination, the chickens were challenged with live virulent *P. aeruginosa.* We analyzed the survival of chickens and evaluated the survival curves (Fig. 5). After challenge, the chickens from PBS and empty vector groups died within 4 days. Chickens from 25 and 50 μg groups began to die on the second day and continued to do so until 15 days after the challenge. The survival numbers in these two groups were 11 and 13, respectively. Chickens from 100 and 200 μg groups began to die on the third day, and the numbers of surviving chickens remained unchanged from day 5 and 6, respectively. After 15 days of challenge, the survival numbers in the 100 and 200 μg groups were 17. The protective rates were 55%, 65%, 85%, and 85% for 25, 50, 100, and 200 μg of the pOPRL+pOPRF DNA vaccine, respectively.

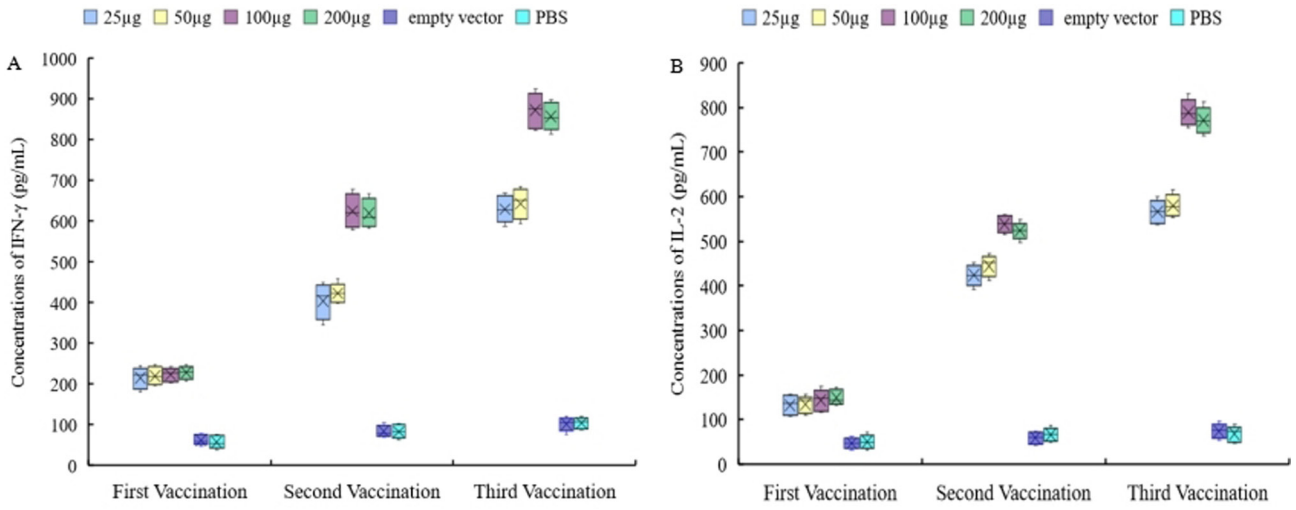

Fig. 4. Concentrations of interferon-γ (IFN-γ) **(A)** and interleukin-2 (IL-2) **(B)** from spleen lymphocytes of vaccinated chickens. Outer membrane protein of *Pseudomonas aeruginosa* was added to stimulate peripheral blood lymphocytes 2 weeks after each vaccination.

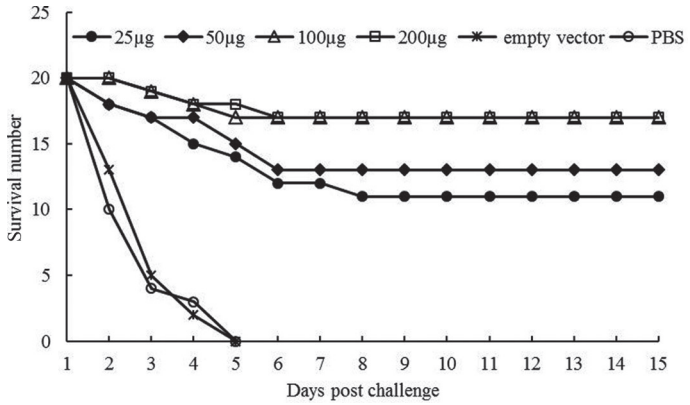

Fig. 5. Survival of chickens after challenge with *Pseudomonas aeruginosa.*

DISCUSSION

P. aeruginosa is a common pathogenic bacterium and a causative agent of several diseases in both humans and animals. The effects of clinical treatment have been unsatisfactory because of increasingly serious drug resistance. Therefore, it is imperative to develop effective vaccines for the prevention of these diseases. At present, a few studies have described *P. aeruginosa* vaccines such as attenuated live vaccines, inactivated vaccines, DNA vaccines, recombinant subunit vaccines, and conjugate vaccines [2, 9, 11, 15, 19]. In the present study, various DNA vaccines of *P. aeruginosa* were prepared using *oprL* and *oprF* genes in the early stages. The results of animal experiments showed that the divalent combination DNA vaccine has good prospects for application in the prevention of *P. aeruginosa* infection. In the present study, the optimal immunization dose was determined to further improve the immune efficacy of the divalent combination DNA vaccine.

Serum-specific antibodies play an important role during the immune response. In this study, the levels of antibodies induced by different dosages of the divalent combination DNA vaccine were assessed. The results showed that the ability to stimulate humoral immune response at high dosages of this DNA vaccine was superior to that at low dosages. Apart from the antibody response, the levels of cellular immune responses induced by these DNA vaccines were also detected. The ability of lymphocyte proliferation and levels of cytokine secreted can reflect cellular immune functions to a certain extent. The results of this study indicated that high dosages of divalent combination DNA vaccine could induce more effective lymphocyte proliferation as compared with low dosages. A previous study demonstrated that a divalent combination DNA vaccine could induce a predominantly Th1 cellular immune response instead of a Th2 cellular immune response [5]. Based on this, in the present study, the Th1-type cytokines IFN-γ and IL-2 secreted by PBLs of vaccinated chickens were assessed. The results indicated that 100 and 200 μg of the divalent combination DNA vaccine induced higher concentrations of IFN-γ and IL-2 than the other dosages. Challenge experiment is an important index used to evaluate the protective efficacy of vaccines. The challenge experiment in the present study demonstrated

that the protective efficiencies achieved by 100 and 200 µg of the divalent combination DNA vaccine pOPRL+pOPRF were superior to those achieved by 25 and 50 µg doses. In conclusion, 100 and 200 µg of the divalent combination DNA vaccine pOPRL+pOPRF could induce an effective immune response and protection in vaccinated chickens. Considering animal welfare and costs, the optimum recommended dose for immunization of chickens is 100 µg.

CONFLICT OF INTEREST. No authors have any financial or personal relationships that could inappropriately influence or bias the content of this paper.

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