Research Note: The immune enhancement ability of inulin on *ptfA* gene DNA vaccine of avian *Pasteurella multocida*

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ABSTRACT To evaluate the ability of inulin to enhance the immune response of a *ptfA* gene DNA vaccine for avian *Pasteurella multocida*, inulin was added as an adjuvant to the *ptfA*-DNA vaccine, obtaining an inulin-adjuvant DNA vaccine. The DNA vaccine was administered to chickens; a fimbria protein vaccine and an attenuated live vaccine were used as positive controls. The levels of the serum antibody and concentrations of interferon-γ (**IFN-γ**), interleukin-2 (**IL-2**), and interleukin-4 (**IL-4**) were determined, and a lymphocyte proliferation assay was performed. After being challenged with virulent *P. multocida*, the protective efficacy was evaluated. The results showed that the serum antibodies induced by the *ptfA*-DNA vaccine were not enhanced by inulin. The stimulation index values and the concentrations of IL-2 and IFN-γ in chickens vaccinated with inulin-adjuvant DNA vaccine were significantly higher than those in chickens vaccinated with the DNA vaccine, those with the fimbria protein vaccine, and the chickens gavaged with inulin. The concentrations of IL-4 in the inulin-adjuvant DNA vaccine group and the fimbria protein vaccine group were higher than those in the DNA vaccine group and the inulin-gavage group. The protective efficacy rates of the attenuated live vaccine group, the fimbria protein vaccine group, the DNA vaccine group, the inulin-adjuvant DNA vaccine group, and the inulin-gavage group were 90, 70, 55, 65, and 55%, respectively.

Key words: avian *Pasteurella multocida*, *ptfA* gene, inulin, adjuvant, DNA vaccine

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INTRODUCTION

Pasteurella multocida (*P. multocida*) is a very important pathogen in many types of animals. It can cause lower respiratory tract infections (Aida et al., 2019). The major method used to control this disease is drug treatment, particularly treatment with antibiotics. Unfortunately, the disease easily relapses after drug withdrawal, and the pathogen is susceptible to drug resistance after long-term medication. In addition, the laying rate of the layers may decrease significantly, and there is drug residue in the broilers. Thus, it is necessary to seek a more effective strategy to prevent and control this disease. Vaccination is an effective strategy. The currently available vaccines for fowl cholera include attenuated vaccines and inactivated vaccines. However, the protective efficacy of commercial vaccines is not ideal (Ahmad et al., 2014). Therefore, it is necessary to develop more effective vaccines to prevent fowl cholera.

Currently, DNA vaccines have become a forerunner in the field of vaccine research because of their advantages, which include ease of preparation and low costs (Golshani et al., 2015). There are several reports of DNA vaccines for *P. multocida* (Register et al., 2007; Okay et al., 2012; Gong et al., 2013). However, the protective effects of most DNA vaccines are not superior to those of traditional vaccines. In previous studies, we prepared a nanoparticle DNA vaccine based on the *ptfA* gene of avian *P. multocida* with chitosan as the adjuvant, and we prepared a recombinant subunit vaccine of *ptfA* gene. We also determined the immunogenicity and protective efficacy of these vaccines. Although both vaccines were able to provide a certain level of protection in the experimental animals, the level of protection did not exceed that of the attenuated live vaccine (Gong et al., 2016, 2018a). Therefore, it is necessary to select a new adjuvant to improve the immune efficacy of *ptfA* vaccines. In this study, we constructed a DNA vaccine

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based on the *ptfA* gene of avian *P. multocida*, and the natural plant polysaccharide inulin was used as the adjuvant. The immune response and protective efficacy in chickens vaccinated with the DNA vaccine were examined. The goal of this study was to lay a foundation for the development of a DNA vaccine against fowl cholera and a foundation for the research of a novel adjuvant for DNA vaccines.

MATERIALS AND METHODS

Bacterial Strains and Chickens

A commercially available avian *P. multocida* strain (CVCC474, serotype A:1) was used for this study (China Institute of Veterinary Drug Control). Healthy 1-day-old broilers were purchased from the Animal Center Laboratory of Henan province, China. They were kept and handled using procedures consistent with the regulations for experimental animals in China. The study protocol was approved by the Animal Monitoring Committee of Henan University of Science and Technology (permit number: 2018-0066; 12 June 2018).

Extraction and Vaccine Preparation of Fimbria Protein of P. multocida

Avian *P. multocida* was grown in tryptone soy broth medium for 48 h, and then the culture was centrifuged at 4,000 *g* for 10 min. The pellet was then washed twice and resuspended in phosphate buffered saline (pH 7.2). The bacteria were incubated at 60°C for 30 min and then stirred violently for 50 min. Then, the sample was centrifuged for 20 min at 10,000 g at 4°C. The supernatant was removed, and an equal volume of saturated ammonium sulfate solution was added. The mixture was incubated at 4°C for 12 h. Then, the sample was centrifuged for 20 min at 10,000 g at 4°C. The pellet, namely the fimbria protein of avian *P. multocida*, was obtained, and the concentration was determined using the Bradford method. Tween-80 (6% of the total volume) was added to the fimbria protein liquid and thoroughly mixed to an aqueous phase. The oil phase consisted of 94% white oil, 6% span-80, and 2% aluminum stearate. The aqueous phase and the oil phase were mixed in a ratio of 1:2 to yield the oil-emulsion fimbria protein vaccine. The concentration of the antigen in the oil-emulsion fimbria protein vaccine was 1 μg/μL.

Animal Vaccination

The *ptfA*-DNA vaccine was constructed and prepared in large scale as previously described (Gong et al., 2018a). Before vaccination, healthy 1-day-old chickens ($n = 140$) were reared in a purpose-built animal house with a controlled environment for light, temperature, and humidity. All chickens had *ad libitum* access to water and nonmedicated feed. Chickens were assigned to 7 groups ($n = 20$ chickens/group) after a period of adaptation to the new feeding environment. Vaccination was performed at 4 wk of age by intramuscular injection. Chickens in the DNA vaccine group and the pCDNA3.1 (+) group were injected with 200 μL of *ptfA*-DNA vaccine and empty vector pcDNA3.1 (+) solution, respectively. Each of these solutions contained 200 μg of DNA. Chickens in the inulin-adjuvant DNA vaccine group were administered with 200 μL of the inulin-adjuvant DNA vaccine, which contains inulin at a final concentration of 20% and 200 μg of DNA. Chickens in the inulin-gavage group were gavaged with 200 μL of inulin liquid every day, which contains 400 μg of inulin. This was followed by vaccination with 200 μL of the DNA vaccine (containing 200 μg of DNA) after 2 wk of continuous gavage. Chickens in the fimbria protein vaccine and the negative control groups were vaccinated with 200 μL of the oil-emulsion fimbria protein vaccine and sterile normal saline, respectively. Chickens in each of the aforementioned groups were immunized 3 times at 2-wk intervals. In the positive control group, chickens were inoculated with 0.5 mL of the attenuated live vaccine of avian *P. multocida* at the time of the initial vaccination. After each gavage and vaccination, chickens were closely observed for any adverse reactions. Any chickens that presented as depressed, seen as lacking appetite, or showed other clinical signs of illness were isolated to a quiet feeding environment and fed more palatable feed until they recovered.

Detection of Serum Antibody

After the first vaccination, 5 chickens were randomly selected from each group, and blood samples were collected from the wing veins weekly for 6 wk before challenge. Then, serum antibodies were detected using an indirect ELISA, according to the previously published method (Gong et al., 2018a). The fimbria protein and suspension of avian *P. multocida* was used as a coating antigen, and rabbit antichicken IgG-horseradish peroxidase (Sigma-Aldrich, St. Louis, MO) was used as the secondary antibody.

Lymphocyte Proliferation Assay and Cytokine Secretion Test

Two weeks after each vaccination, blood samples were collected from 5 vaccinated chickens in each group. Then, the peripheral blood lymphocytes (**PBLs**) were separated, and the concentration was adjusted to 2×10^7 cells/mL. The 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide method was performed to measure the proliferation of PBL and the interferon-γ (**IFN-γ**), interleukin-2 (**IL-2**), and interleukin-4 (**IL-4**) concentrations secreted by the PBL of the chickens (Gong et al., 2018b).

Challenge Experiment

Two weeks after the third vaccination, all chickens were challenged with the virulent avian *P. multocida*

strain CVCC474 (5LD50/chicken) by intramuscular injection. After the challenge, chickens were reared for a further 15 D. Chickens were closely observed for clinical signs of illness every day after challenge. Chickens showing signs of depression or inappetence were isolated and kept under further observation. Chickens that were anorexic or dyspneic, and those with hemorrhagic diarrhea or other abnormal gastrointestinal signs, were withdrawn from the experiment and euthanized by intravenous injection of pentobarbital sodium. The survival time and survival number of each group were calculated.

Statistical Analysis

Statistical analyses were conducted using SAS software (version 9.4; SAS Institute, Cary, NC). ANOVA was used to determine significant differences between the means of the experimental groups. Differences with $P < 0.05$ were considered significant, and differences with $P < 0.01$ were considered extremely significant. Differences with $P > 0.05$ were considered not significant.

RESULTS AND DISCUSSION

Results of Serum Antibody Detection

The humoral immune response is an important factor in the protection against avian *P. multocida* infection. In this study, we used the fimbria protein and avian *P. multocida* suspension as a coating antigen to detect the levels of antibodies in vaccinated chickens. The results showed that the antibodies in the DNA vaccine group, the inulin-adjuvant DNA vaccine group, and the

Table 1. Levels of serum antibody, SI value, and concentrations of IFN∼γ, IL-2 and IL-4 from vaccinated chickens.

	Serum antibodies (A492) coating antigen: fimbria protein					
Groups	1 wk	2 wk	3 wk	4 wk	5 wk	6 wk
Attenuated live vaccine group	0.249 ± 0.019	0.389 ± 0.045[b]	0.483 ± 0.047[b]	0.599 ± 0.051[b]	1.178 ± 0.036[b]	1.341 ± 0.055[b]
Fimbria protein vaccine group	0.288 ± 0.012	0.457 ± 0.033[b]	0.771 ± 0.019[d]	1.373 ± 0.048[d]	1.769 ± 0.039[d]	1.947 ± 0.049[d]
DNA vaccine group	0.253 ± 0.025	0.426 ± 0.037[b]	0.647 ± 0.055[c]	0.851 ± 0.037[c]	1.373 ± 0.075[c]	1.622 ± 0.052[c]
Inulin-adjuvant DNA vaccine group	0.272 ± 0.019	0.434 ± 0.044[b]	0.654 ± 0.042[c]	0.894 ± 0.056[c]	1.518 ± 0.062[c]	1.681 ± 0.078[c]
Inulin-gavage group	0.26 ± 0.014	0.419 ± 0.053[b]	0.625 ± 0.038[c]	0.794 ± 0.074[c]	1.459 ± 0.088[c]	1.547 ± 0.036[c]
pCDNA3.1 (+) group	0.254 ± 0.018	0.198 ± 0.022[a]	0.277 ± 0.031[a]	0.274 ± 0.035[a]	0.266 ± 0.019[a]	0.202 ± 0.027[a]
Normal saline group	0.231 ± 0.027	0.226 ± 0.021[a]	0.199 ± 0.027[a]	0.213 ± 0.017[a]	0.187 ± 0.038[a]	0.294 ± 0.026[a]

	Serum antibodies (A492) coating antigen: suspension of avian *P. multocida*					
Groups	1 wk	2 wk	3 wk	4 wk	5 wk	6 wk
Attenuated live vaccine group	0.301 ± 0.025[b]	0.455 ± 0.056[b]	0.869 ± 0.037[c]	1.452 ± 0.091[d]	1.866 ± 0.077[d]	2.047 ± 0.062[d]
Fimbria protein vaccine group	0.274 ± 0.033[b]	0.399 ± 0.042[b]	0.613 ± 0.057[b]	1.127 ± 0.072[c]	1.354 ± 0.045[c]	1.719 ± 0.066[c]
DNA vaccine group	0.246 ± 0.031[b]	0.342 ± 0.039[b]	0.556 ± 0.057[b]	0.793 ± 0.041[b]	0.979 ± 0.083[b]	1.349 ± 0.061[b]
Inulin-adjuvant DNA vaccine group	0.263 ± 0.022[b]	0.387 ± 0.047[b]	0.492 ± 0.068[b]	0.838 ± 0.053[b]	0.925 ± 0.071[b]	1.465 ± 0.054[b]
Inulin-gavage group	0.251 ± 0.027[b]	0.371 ± 0.055[b]	0.538 ± 0.056[b]	0.726 ± 0.086[b]	1.084 ± 0.049[b]	1.402 ± 0.071[b]
pCDNA3.1 (+) group	0.191 ± 0.019[a]	0.254 ± 0.035[a]	0.187 ± 0.022[a]	0.204 ± 0.047[a]	0.263 ± 0.045[a]	0.272 ± 0.038[a]
Normal saline group	0.186 ± 0.015[a]	0.207 ± 0.034[a]	0.239 ± 0.041[a]	0.271 ± 0.026[a]	0.199 ± 0.033[a]	0.234 ± 0.027[a]

	SI value			Concentrations of IFN-γ (pg/mL)		
Groups	First vaccination	Second vaccination	Third vaccination	First vaccination	Second vaccination	Third vaccination
Attenuated live vaccine group	1.32 ± 0.037[b]	1.78 ± 0.066[d]	2.05 ± 0.083[d]	305.3 ± 15.37[b]	560.9 ± 20.06[d]	906.7 ± 25.31[d]
Fimbria protein vaccine group	1.22 ± 0.029[b]	1.46 ± 0.057[b]	1.62 ± 0.043[b]	287.9 ± 11.25[b]	400.5 ± 13.62[b]	606.5 ± 18.74[b]
DNA vaccine group	1.22 ± 0.033[b]	1.39 ± 0.028[b]	1.55 ± 0.051[b]	279.6 ± 13.92[b]	388.4 ± 13.81[b]	586.3 ± 16.25[b]
Inulin-adjuvant DNA vaccine group	1.28 ± 0.026[b]	1.61 ± 0.035[c]	1.82 ± 0.081[c]	290.5 ± 8.71[b]	471.1 ± 15.14[c]	712.1 ± 15.97[c]
Inulin-gavage group	1.25 ± 0.035[b]	1.42 ± 0.036[b]	1.58 ± 0.039[b]	280.2 ± 10.56[b]	391.2 ± 10.09[b]	597.7 ± 16.83[b]
pCDNA3.1 (+) group	1.03 ± 0.021[a]	1.06 ± 0.019[a]	1.11 ± 0.022[a]	93.4 ± 5.44[a]	105.6 ± 8.13[a]	113.5 ± 9.35[a]
Normal saline group	1.06 ± 0.025[a]	1.03 ± 0.027[a]	1.09 ± 0.018[a]	87.7 ± 7.21[a]	99.3 ± 6.65[a]	109.6 ± 11.16[a]

	Concentrations of IL-2 (pg/mL)			Concentrations of IL-4 (pg/mL)		
Groups	First vaccination	Second vaccination	Third vaccination	First vaccination	Second vaccination	Third vaccination
Attenuated live vaccine group	202.3 ± 5.42[b]	432.5 ± 12.65[d]	705.4 ± 21.39[d]	191.6 ± 15.37[b]	408.9 ± 20.06[c]	672.3 ± 25.31[d]
Fimbria protein vaccine group	193.7 ± 6.37[b]	334.4 ± 7.38[b]	560.7 ± 15.63[b]	185.5 ± 11.25[b]	389.5 ± 13.62[c]	571.8 ± 18.74[c]
DNA vaccine group	201.5 ± 6.66[b]	327.6 ± 5.67[b]	537.7 ± 11.86[b]	174.9 ± 13.92[b]	297.4 ± 13.81[b]	462.8 ± 16.25[b]
Inulin-adjuvant DNA vaccine group	199.2 ± 4.32[b]	385.1 ± 8.25[c]	632.1 ± 16.63[c]	180.5 ± 8.71[b]	314.1 ± 15.14[b]	562.4 ± 15.97[c]
Inulin-gavage group	189.5 ± 5.78[b]	320.9 ± 7.71[b]	553.3 ± 18.21[b]	177.3 ± 10.56[b]	306.2 ± 10.09[b]	477.3 ± 16.83[b]
pCDNA3.1 (+) group	55.7 ± 3.19[a]	66.9 ± 4.73[a]	78.8 ± 5.11[a]	62.2 ± 5.44[a]	65.4 ± 8.13[a]	74.7 ± 9.35[a]
Normal saline group	52.1 ± 4.24[a]	61.3 ± 4.26[a]	72.1 ± 6.28[a]	58.9 ± 7.21[a]	71.5 ± 6.65[a]	68.2 ± 11.16[a]

[a-d]Different letters in the same column represent significant differences.

Abbreviations: IFN-γ, interferon-γ; SI, stimulation index.

inulin-gavage group were not statistically significant different. They were all lower than those in the fimbria protein vaccine group and the attenuated live vaccine group when the coating antigen was fimbria protein and avian *P. multocida* suspension, respectively (Table 1). These results indicate that the antibody response induced by the *ptfA*-DNA vaccine could not be enhanced regardless of if inulin was used as an adjuvant or if the chickens were gavaged with it before vaccination with the DNA vaccine.

PBL Proliferation, IFN-γ, IL-2, and Il-4 Assay Results

In addition to the antibody response, the cellular immune response also plays an important role during the process of antiinfective immunity. Two common indexes that are used to evaluate cellular immune function are the ability of lymphocytes to proliferate and the levels of cytokine secretion. Thus, in this study, we detected the ability of lymphocytes to proliferate and measured cytokine levels after the vaccination (Table 1). After the first vaccination, the stimulation index values and the concentrations of IFN-γ, IL-2, and IL-4 demonstrated no significant differences among all the vaccine groups. After the second and third vaccinations, the stimulation index values and the concentrations of IFN-γ and IL-2 in the attenuated live vaccine group were significantly higher than those in other groups ($P < 0.05$). In addition, the 3 aforementioned index scores were higher in the inulin-adjuvant DNA vaccine group than those in the DNA vaccine group, the inulin-gavage group, and the fimbria protein vaccine group ($P < 0.05$) after the second and third vaccination. No significant differences were detected among the 3 latter groups, which indicates that the inulin-adjuvant DNA vaccine could induce a better Th1 response than the DNA vaccine. After the second vaccination, the concentrations of IL-4 in the DNA vaccine group, the inulin-adjuvant DNA vaccine group, and the inulin-gavage group were not significantly different, but they were all significantly lower than those in the attenuated live vaccine group and the fimbria protein vaccine group ($P < 0.05$). After the third vaccination, the concentrations of IL-4 in the inulin-adjuvant DNA vaccine group were equivalent to those in the fimbria protein vaccine group. It was significantly higher than those in the DNA vaccine group and the inulin-gavage group ($P < 0.05$), and it was lower than that in the attenuated live vaccine group ($P < 0.05$). Th2 cytokines can reflect the humoral immune response to some degree. The levels of serum antibodies in the inulin-adjuvant DNA vaccine group were not higher than those in the DNA vaccine group. However, the concentrations of the Th2 cytokine IL-4 in chickens vaccinated with the inulin-adjuvant DNA vaccine were higher than those in chickens vaccinated with the DNA vaccine after the third vaccination. The reasons need to be further studied.

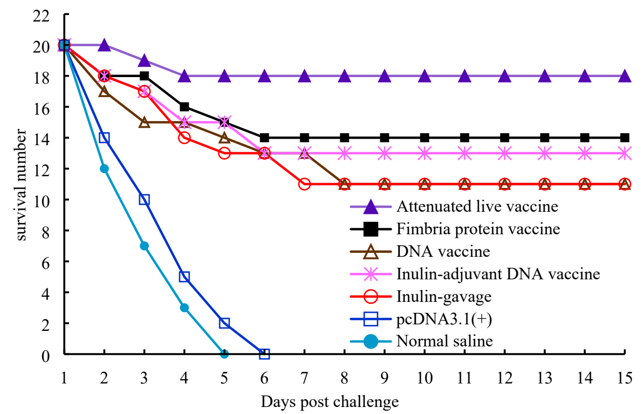

Figure 1. Survival curve of chickens after challenge with avian *P. multocida*. The chickens ($n = 20$) were observed over a period of 15 D after challenge.

Results of the Challenge Study

Two weeks after the last vaccination, the groups of chickens were challenged with live virulent avian *P. multocida*. The number of surviving chickens was counted every day until 15 D after challenge (Figure 1). After the challenge, none of the chickens in the pcDNA3.1 (+) group and normal saline group survived more than 6 D. Chickens in the attenuated live vaccine group began to die on the third day, and the number of surviving chickens remained unchanged from the fourth. The protective rate of the attenuated live vaccine was 90%. Chickens in the fimbria protein vaccine group, the DNA vaccine group, the inulin-adjuvant DNA vaccine group, and the inulin-gavage group began to die on the second day. In the fimbria protein vaccine group and the inulin-adjuvant DNA vaccine group, death occurred between days 2 and 6, and from then on, the number of surviving chickens did not change further. The number of surviving chickens in the DNA vaccine group and the inulin-gavage group remained unchanged from the eighth and seventh day, respectively. Till 15 D after challenge, the survival numbers in the fimbria protein vaccine group, the DNA vaccine group, the inulin-adjuvant DNA vaccine group, and the inulin-gavage group were 14, 11, 13, and 11, respectively, and the protective rates were 70, 55, 65, and 55%, respectively. These results suggest that the protective efficiency induced by the *ptfA*-DNA vaccine could be enhanced by inulin, when it was adopted as an adjuvant. However, the protective efficiency provided by the inulin-adjuvant DNA vaccine was inferior to that provided by the attenuated live vaccine and the fimbria protein vaccine. Therefore, further measures should be taken to improve the immune efficacy of an inulin-adjuvant DNA vaccine.

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