Evaluation of immunogenicity and protective efficacy of recombinant *ptf*A of avian *Pasteurella multocida*

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Summary

Avian *Pasteurella multocida* is the causative agent of fowl cholera, a disease much affecting the poultry industry. In order to study the efficacy of the recombinant subunit vaccine constructed with *ptf*A gene of avian *P. multocida*, the *ptf*A gene fragment amplified by PCR from avian *P. multocida* was cloned into the prokaryotic expression vector pET32a and the recombinant plasmid pET32a-ptfA was obtained. The pET32a-ptfA was expressed in *Escherichia coli* BL21(DE3) and the target protein rPtfA was purified. The purified protein was then mixed with Freund's adjuvant and the recombinant subunit vaccine was obtained. Three groups of chickens labeled as rPtfA, attenuated live vaccine and PBS were vaccinated with the recombinant subunit vaccine, attenuated live vaccine and PBS, respectively. Serum antibodies, peripheral blood lymphocyte proliferation (PBLP) and interferon-γ (IFN-γ) level secreted by peripheral blood lymphocyte were tested. The immunized chickens were finally challenged with virulent avian *P. multocida* and the protection rate was counted. Indirect ELISA showed the levels of antibodies in rPtfA and attenuated vaccine groups were most significantly higher than the other groups ($P<0.01$), and the former was slightly lower than the latter. Peripheral blood lymphocyte proliferation experiments and IFN-γ experiments indicated that SI value and the levels of IFN-γ induced by ConA in the two vaccine groups were significantly higher than those of the PBS groups ($P<0.01$), and that the attenuated vaccine group was higher than the rPtfA group. The protection rates of rPtfA and attenuated live vaccines were 45% and 75%, respectively. The results indicated that the PtfA recombinant subunit vaccine was capable of improving the immunity level and inducing a protective effect for the vaccinated chickens, but it was barely satisfactory.

Key words: Avain *Pasteurella multocida*, Immune efficacy, Recombinant subunit vaccine, rPtfA

Introduction

Pasteurella multocida is the pathogenic bacteria of hemorrhagic Septicemia, an acute, fatal and septicemic disease of many kinds of animals. Fowl cholera, caused by avian *P. multocida*, is a multiple disease for poultry and fowl and is widely distributed in many countries. This disease affects the healthy development of poultry and causes great economic losses. Antibiotics are used in many countries and regions to control and treat this disease; however, such treatment might create drug residues and cause laying rate decreases in layers. Additionally, the therapeutic effect will be reduced because the pathogen becomes susceptible to drug resistance after long-term medication.

Vaccine immunity is one of the effective prevention measures against infectious diseases. Commercial vaccines against fowl cholera currently include attenuated live vaccines and inactivated vaccines. The protective efficacy of these vaccines, however, is not ideal. Attenuated live vaccines have considerable side effects, can cause excretion virion, and are difficult to store. On the other hand, inactivated vaccines can cause poor immunogenicity and short term immunoprotection. Nevertheless, novel vaccines such as genetically engineered subunit vaccines and DNA vaccines have represented a promising approach towards the prevention of hemorrhagic septicemia caused by *P. multocida* (Gong *et al*., 2013). Recombinant subunit vaccine, which was developed after the 1980s, has many advantages including better safety and low manufacturing costs, and has become one of hotspots in the field of vaccine research. Up to now, several recombinant subunit vaccines such as those for infectious bursal disease and chicken chlamydia have been studied in clinical cases. However, while previous research has been conducted on the fowl cholera recombinant subunit vaccine, no vaccine has been applied to clinical immunization (Lee *et al*., 2007; Dabo *et al*., 2008; Sthitmatee *et al*., 2008).

In the present study, we used the fimbria protein gene *ptf*A of avian *P. multocida* as the basis for constructing the recombinant subunit vaccine. Host chickens immunized by this recombinant subunit vaccine were adopted and challenged with the avian *P. multocida* virulent strain. Immune protection efficacy was evaluated based on the detection of immunological indicators and protection rates. The aim was to lay a foundation for the development of recombinant subunit vaccines against fowl cholera.

Materials and Methods

Bacterial strains, vaccine, animals

Avian *P. multocida*-CVCC474 (serotype A:1) strain was purchased from the Chinese Institute of Veterinary Drug Control (IVDC). Attenuated live vaccine was purchased from Qilu Animal Health Products Co., Ltd. Competent cell BL21(DE3) was conserved in the laboratory of He Nan University of Science and Technology, China. One-day-old chickens were purchased from the Animal Center Laboratory of Henan province, China.

Construction of recombinant plasmid

Genomic DNA of the avian *P. multocida*-CVCC474 strain was extracted according to the conventional method. Primers were designed according to the nucleotide sequences of the *ptf*A gene of *P. multocida* strain Pm-17 (GenBank accession number DQ417897.1). The forward primer contained *BamH* I site and the reverse primer contained *Hind III* site. Using PCR technology, the *ptf*A gene fragment was amplified using the genomic DNA as a template. This amplified product was purified using a gel extraction mini kit and digested with restricition enzyme *BamH* I and *Hind III*. It was then ligated into the prokaryotic expression vector pET32a (Invitrogen, San Diego, CA, USA), dealing in advance with *BamH* I and *Hind III*, resulting in the recombinant plasmid labeled as pET32a-ptfA.

Expression and purification of recombinant protein

Competent *Escherichia coli* BL21(DE3) was transformed with the recombinant plasmid pET32a-ptfA. Ampicillin-resistant colonies were grown in Luria-Bertani (LB) medium containing 50 μg/ml ampicillin and cultured overnight with shaking at 200 r/min at 37°C. The seed culture was then transferred into fresh LB medium supplemented with 50 μg/ml of a 1:100 proportion of ampicillin and cultured until OD600 reached 0.8. Isopropyl-beta-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 mM, and incubation was continued for 5 h. Cells were harvested by centrifugation at 3000 g for 5 min at 4°C. The protein expression was then visualized on 12% (w/v) polyacrylamide gel according to the SDS-PAGE method. The expression form was authenticated with ultrasound pyrolysis and SDS-PAGE after the successful expression. The results showed the recombinant protein expressed in soluble form. The target protein was purified by His-Tag fusion protein purification kit according to the instructions and the concentration of purified recombinant protein was measured with coomassie brilliant blue. The protein was then named rPtfA and stored at -80°C.

Immunization of chickens

1-day-old nonimmune chickens (n=60) were reared to 4 weeks of age and assigned randomly to three groups, an rPtfA group, an attenuated live vaccine group and a PBS group. In the first immunization, purified rPtfA protein was blended with the same volume of Freund's complete adjuvant and injected to the subcutaneous tissue of the chickens in the rPtfA group with a 0.5 ml dose. In the second and third immunizations, chickens in the rPtfA group were subcutaneously injected with 0.5 ml vaccines which were composed of equal volumes of purified rPtfA protein and Freud's incomplete adjuvant. Chickens in the attenuated live vaccine group were immunized with 0.5 ml attenuated live vaccine of avian *P. multocida* by intramuscular injection. Chickens in the PBS group were injected with 0.5 ml PBS (0.01 M, pH = 7.2) by subcutaneous injection. The chickens in each of the above groups were immunized 3 times at 2-week intervals.

Detection of serum antibody levels

About 100 μL blood samples were collected from the chickens each week after the first vaccination, until week six, and serum specimens were isolated. Serum antibody levels were tested using indirect enzyme-linked immunosorbent assay (ELISA). Briefly, ELISA micro-titer plates (eBioscience, San Digeo, CA, USA) were coated with 10^9 CFU/ml avian *P. multocida* suspension, followed by blocking nonspecific binding using 100 μL 5% bovine serum albumin (BSA, Sigma, St. Louis, USA) for 2 h. This was followed by the addition of 50 μL serum (1:100 dilution), and the samples were incubated in a moist chamber at 37°C for 1.5 h. The plates were washed 3 times with PBST (0.01 M PBS-0.05% Tween-80, pH = 7.2), and a rabbit anti-chicken IgG-Horseradish peroxidase (HRP) (Sigma-Aldrich, St. Louis, MO, USA) was added and incubated at 37°C for 1.5 h. Plates were washed 3 times with PBST, then 50 μL Ortho-phenylene diamine (OPD, Sigma) was added and incubated for 10 min. Enzyme activity was stopped by adding an equal volume of 2 M H_2SO_4, and the absorption was measured at 490 nm.

Peripheral blood lymphocyte proliferation assay of the immunized chickens

Two weeks after each immunization, blood was collected from the chickens of each group. Peripheral blood lymphocytes (PBL) were separated by density gradient centrifugation. After collecting the cells under sterile conditions, they were resuspended to a concentration of 1×10^7 cells/ml. A volume of 50 μL of cell suspension was added to a 96-well plate (Greiner Bio-One, Longwood, German). Each experiment was repeated 3 times. Each well was pulsed with 50 μL of 5 μg/ml rPtfA protein (experimental well) or 50 μL RPM1640 medium (Gibco, Grand Island, USA) (as negative control), and the plates were kept at a temperature of 37°C under 5% CO_2 for 60 h. Following this, 10 μL of 5 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich) was added to each culture well and incubated for 3 h. After being centrifuged for 10 min, the supernatant was discarded and 150 μL dimethyl sulfoxide (DMSO) was

added to the pellet and incubated for 10 min until crystals were dissolved. The optical density (OD) value of each well was measured using a microculture plate reader (Elx-800, bio-Tek instruments, Inc., American) with a test wavelength of 570 nm. The stimulation index (SI) was determined from the formula:

SI = OD (experimental well)/OD (negative control well) (Avantika *et al*., 2010; Magdalena *et al*., 2014)

IFN-γ assays

At 2, 4 and 6 weeks after the first immunization, PBLs were separated and adjusted to a concentration of 1×10^7 cells/ml. Later on, rPtfA protein was added to the PBLs according to the above mentioned method. The cells were incubated at 37°C under 5% CO_2 for 72 h, when the supernatants were harvested and stored at -20°C. The levels of IFN-γ in the supernatant were detected using a commercial ELISA kit (Yuan Ye Biotech Co., Shanghai, China) according to the manufacturer's instructions.

Challenge experiment

Two weeks after the 3rd immunization, every experimental chicken was challenged with the virulent avian *P. multocida* strain CVCC474 (5LD50/one chicken) by intramuscular injection. After the challenge, the chickens were reared for two weeks, and the survival number and protection rate provided by rPtfA subunit vaccine and attenuated live vaccine were counted.

Results

Purification of recombinant protein

The recombinant plasmid pET32a-ptfA was transformed into *E. coli* BL21(DE3) and expressed. The target protein was analyzed by SDS-PAGE (Fig. 1) and purified using a protein purification kit. The result showed the molecular mass of rPtfA protein to be about 33 ku, which was consistent with our expectations.

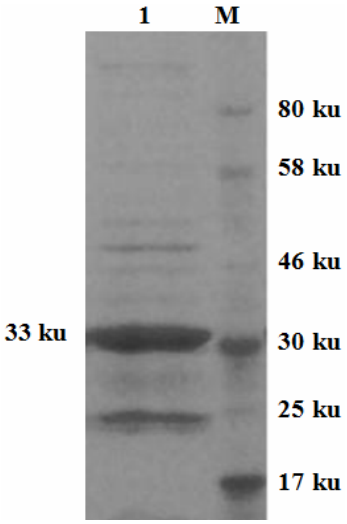

Fig. 1: SDS-PAGE of recombinant protein rPtfA. Lane 1: Recombinant protein rPtfA, Lane M: Protein molecular weight Maker

Serum antibody levels

Serum antibody levels were detected using indirect ELISA. As shown in Fig. 2, serum antibody levels of the rPtfA group and the attenuated live vaccine group exhibited an increasing trend after immunization and were significantly higher than those of the PBS group (P<0.01). In addition, there were no differences in the antibody levels between the attenuated live vaccine group and rPtfA group (P>0.05), although the former was slightly higher than the latter.

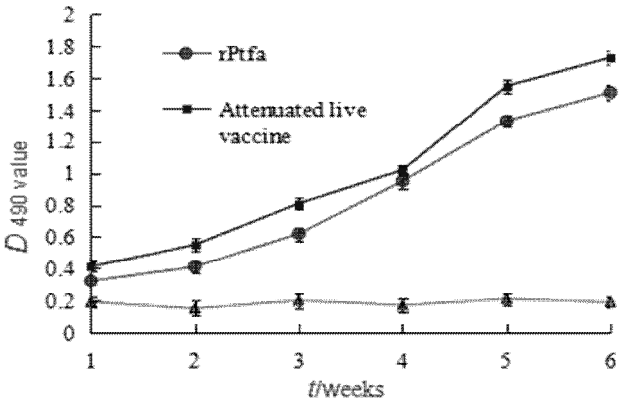

Fig. 2: Dynamic changes in the serum antibody levels of immunized chickens. The serum antibody levels were measured by indirect ELISA weekly, until 6 weeks. Chickens were immunized with rPtfA group (●), attenuated live vaccine group (■), and PBS group (▲)

MTT assay

The results of the MTT assay are shown in Fig. 3. In each experiment, the SI values for the rPtfA and attenuated live vaccine groups were consistently higher than those for the negative control groups. After second and third immunizations, the SI values in the two vaccine groups were significantly higher than those of the PBS group (P<0.01). There were also no differences between the two vaccine groups (P>0.05), although the attenuated live vaccine group was slightly higher than the rPtfA group.

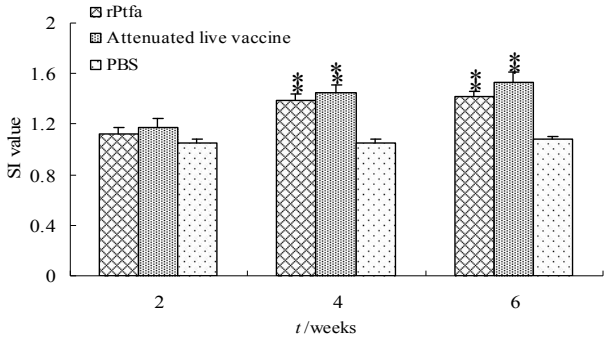

Fig. 3: Proliferation of lymphocytes from chickens immunized with rPtfA vaccine. ConA was administered to stimulate peripheral blood lymphocytes 2 weeks after each immunization. ** P<0.01

IFN-γ secretion

As shown in Fig. 4, after rPtfA protein stimulation, the levels of IFN-γ secreted by the peripheral blood lymphocytes increased in the chickens vaccinated with the rPtfA and attenuated live vaccines. After the first immunization, no significant differences were detected between the two vaccine groups (P>0.05). After the second and third immunization, the levels of IFN-γ were similar in the rPtfA and attenuated live vaccine groups; however, the rPtfA group was slightly lower than the attenuated live vaccine group, and significantly higher than the PBS group (P<0.01).

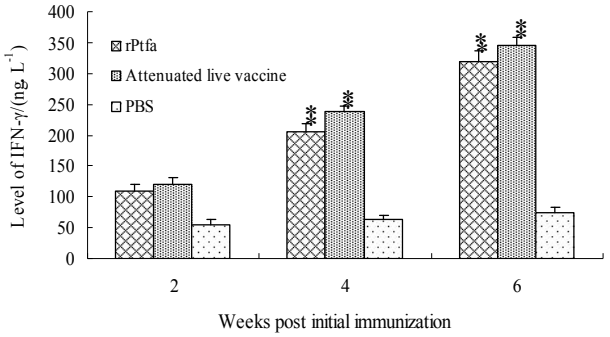

Fig. 4: The level of IFN~γ secreted by peripheral blood lymphocytes from immunized chickens. ConA was added to stimulate peripheral blood lymphocytes 2, 4 and 6 weeks after the first immunization. ** P<0.01

Protection study

After the challenge, the survival number and protection rate were counted (Table 1). All chickens injected with PBS died. On the contrary, rPtfA vaccine and attenuated live vaccine showed some degree of protection for the vaccinated chickens. Nevertheless, for the attenuated live vaccine group, the protection rate was higher (75%) than that of the rPtfA group (45%).

Table 1: Protection of immunized chickens against lethal challenge with avian *Pasteurella multocida*

Groups	Survival number/total	Protection rate (%)
rPtfA	9/20	45
Attenuated live vaccine	15/20	75
PBS	0/20	0

Discussion

Fimbria is a special construction of pathogenic bacterium which can participate in the invasion, settlement and proliferation of pathogenic micro-organisms. Studies have showed that the fimbria protein encoded by the *ptf*A gene of *P. multocida* plays an important role in the adhesion of pathogenic bacteria onto the cell (Craig *et al*., 2004; Sellyei *et al*., 2010; Varga *et al*., 2013). Therefore, the fimbria protein is one of the major pathogenic factors of *P. multocida*. Intensive research on the *ptf*A gene will undoubtedly help find the pathogenic molecular mechanism of *P. multocida* and provide the potential means for the development of new drugs and the design of clinical diagnosis and vaccines.

Fimbria protein seems to have a certain immunogenicity besides its relationship with the pathogenicity of pathogenic bacterium. When extracted from pathogenic bacterium, this protein can be used to study subunit vaccines such as *Moraxella bovis* and *Dichelobacter nodosus* (Hatfaludi *et al*., 2010). In addition, a number of subunit vaccines such as K88 and K99 subunit vaccines of pathogenic *E. coli*, which are based on the fimbria protein, have been applied to clinical cases. Previous works have studied the fimbria protein of *P. multocida*. Results of Hatfaludi *et al*. (2010), for instance, showed that the molecular weight of fimbria protein encoded by *ptf*A gene of *P. multocida* is 14.9 ku and that this protein contains 144 amino acids, including a signal peptide, which is made up of 12 amino acids. Shivachandra *et al*. (2012) expressed and purified the recombinant type IV pili protein of bovis *P. multocida* in *E. coli*, investigated its structure and carried Western-blotting analysis. The results of their study showed that this recombinant protein has some degrees of immunogenicity. The research carried out by Harper *et al*. (2006) also indicated that the antibody against fimbria protein of *P. multocida* could block the adhesion of *P. multocida* to epithelial cells. Other research has shown that the inactivated recombinant vaccine expressing the fimbrial protein of *P. multocida* B:2 could provide protection against challenges and enhance the stimulation of local and systemic immunities in goats (Yasin *et al*., 2011; Ahmad *et al*., 2014). The fimbrial protein of *P. multocida* is thus expected to be the potential candidate antigen of subunit vaccines. In this study, the prokaryotic expression vector including the *ptf*A gene of avian *P. multocida* was constructed and transformed into *E. coli* competent cells for expression. The expressed recombinant protein was then purified. In order to study the immunogenicity of this recombinant protein, we prepared the oil emulsion vaccine using this recombinant protein and carried out animal immunization and challenge experiments. The levels of humoral and cellular immune responses induced by this vaccine and the protective efficacy it offered were then evaluated.

Antibody response is an important factor when seeking protection against avian *P. multocida* infection. In this study, we detected the level of antibodies induced by the rPtfA subunit vaccine and found that it had the ability to stimulate humoral immunoresponse, but was lower than that of the attenuated live vaccine. The antibodies produced by the animals were a mixture of antibodies against various kinds of antigens due to the fact that the ingredient of attenuated live vaccine was complicated. Although the composition of the rPtfA subunit vaccine was relatively simple, the antibody induced by the rPtfA subunit vaccine was mainly against this recombinant protein. In this study, we chose avian *P. multocida* whole cells as the coating antigen, which contain a similar composition as the attenuated live vaccine. This may be one of the reasons why the antibody level induced by the attenuated live vaccine was higher than that of the rPtfA subunit vaccine. In

addition, we detected the levels of lymphocyte proliferation and IFN-γ secretion stimulated by the two vaccines. The results showed that the rPtfA subunit vaccine induced some degree of cellular immune responses; however, the ability was lower than that of the attenuated live vaccine.

Challenge experiments are one of the important indices used to evaluate the protective efficacy of vaccines. In the present study, the results of the challenge experiment showed that the rPtfA subunit vaccine provided a certain protection for the immunized animals and exhibited promising application prospects. Nevertheless, its protective efficacy was obviously inferior to that of the attenuated live vaccine. The reason for this may be either that the PtfA recombinant protein contains relatively simple antigens or that the immunogenicity of fimbrial protein of avian *P. multocida* is not as good, and so cannot provide enough protection for the experiment animals against the attack of avian *P. multocida*.

The main goal of this study was to provide a reference for genetic engineering subunit vaccine research against fowl cholera. We have tried, therefore, to construct the multigenic fusion subunit vaccine based on ptfA and other genes of avian *P. multocida* to enhance immune efficacy.

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