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Immuno-enhancement of Taishan *Pinus massoniana* pollen polysaccharides on recombinant *Bordetella avium* ompA expressed in *Pichia pastoris*

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ABSTRACT

Bordetellosis, caused by *Bordetella avium*, continues to be an economic problem in the poultry industry of China. Vaccines with good protective ability are lacking. Thus, developing a novel vaccine against the *B. avium* infection is crucial. Here, we constructed a recombinant *Pichia pastoris* transformant capable of expressing the outer membrane protein A (ompA) of *B. avium* to prepare the recombinant ompA subunit vaccine and then evaluated its immune effects. To further investigate the immunomodulation effects of Taishan *Pinus massoniana* pollen polysaccharides (TPPPS) on this subunit vaccine, three concentrations (20, 40, and 60 mg/mL) of TPPPS were used as the adjuvants of the ompA subunit vaccine respectively. The conventional Freund's incomplete adjuvant served as the control of TPPPS. Chickens in different groups were separately vaccinated with these vaccines thrice. During the monitoring period, serum antibody titers, concentrations of serum IL-4, percentages of $CD4^+$ and $CD8^+$ T-lymphocytes in the peripheral blood, lymphocyte transformation rate, and protection rate were detected. Results showed that the pure ompA vaccine induced the production of anti-ompA antibody, the secretion of IL-4, the increase of $CD4^+$ T-lymphocytes counts and lymphocyte transformation rate in the peripheral blood. Moreover, the pure ompA vaccine provided a protection rate of 71.67% after the *B. avium* challenge. Notably, TPPPS adjuvant vaccines induced higher levels of immune responses than the pure ompA vaccine, and 60 mg/mL TPPPS adjuvant vaccine showed optimal immune effects and had a 91.67% protection rate. Our findings indicated that this recombinant *B. avium* ompA subunit vaccine combined with TPPPS had high immunostimulatory potential. Results provided a new perspective for *B. avium* subunit vaccine research.

1. Introduction

Bordetella avium is the etiological agent of bordetellosis, which is clinically characterized by labored breathing, sneezing, and oculonasal discharges [1,2]. This pathogen has been widespread among avian species, such as Muscovy ducks, domesticated geese, partridges, turkeys, chickens, ostriches, cockatoos, conures, macaws, parrot finches, and cockatiels [3,4]. In addition, it was also described in previous studies as an opportunistic human pathogen [5]. Because of its increasing secondary infection prevalence and slow growth rates, *B. avium* has brought significant economic losses in commercial flocks. Currently, the use of antibiotics in controlling diseases has not been recommended as they possess health hazards to consumers. Moreover, the live-attenuated vaccines and bacterin used to prevent bordetellosis cannot limit the infection and spread of *B. avium*, although they offer protection against severe diseases [6]. Therefore, developing a novel vaccine against *B. avium* infection is crucial.

OmpA, which is an important component of Gram-negative bacteria, has many active biological functions [7]. In our laboratory, the previous studies have shown that the ompA of *B. avium* expressed in *Escherichia coli* (*E. coli*) has certain immunogenicity [8]. Herein, to explore a novel subunit vaccine with better effects against *B. avium*, we intended to express the ompA using *Pichia pastoris* GS115 eukaryotic expression system. This system has been widely used in the expression of recombinant proteins because of its various advantages, such as easy cultivation, high yield, and

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capability to perform post-translational modifications [9]. Moreover, this expression system secretes few native proteins into the medium [10]. In the development of bacterial vaccines, the yeast-expressed antigen had been investigated in several animals and was proven to have good effects [11–13]. Therefore, the ompA expressed in *P. pastoris* GS115 may be a potential candidate in the development of a subunit vaccine against *B. avium*.

The application of adjuvant in vaccination is indispensable because adjuvant enhances the effects of vaccines. Adjuvant is believed to establish high and long-lasting immune responses by activating the innate immune system, thereby enhancing the adaptive immune response to an administered antigen [14,15]. Currently, plant-derived polysaccharides have attracted an increasing attention in the field of immune modulator [16]. As effective biological response modifiers, polysaccharides had been proven with excellent immuno-enhancement effects both *in vivo* and *in vitro* [17–19]. In our previous studies, we explored the polysaccharides from *Aloe vera*, Taishan *Robinia pseudoacacia*, and *Firmiana simplex* flower, and found that these polysaccharides possess immune enhancement function with different levels [20–22]. Notably, TPPPS, which we have been studying since 2003, has been proven as an effective adjuvant for the inactivated and subunit vaccines [23,24]. However, little is known about the effects of TPPPS on the recombinant ompA based on eukaryotic expression system.

Therefore, to develop a novel *B. avium* vaccine, we constructed a recombinant *P. pastoris* transformant expressing *B. avium* ompA. Furthermore, to improve the effects of the prepared subunit vaccine, we used TPPPS as adjuvant. We evaluated the antibody production, cytokine secretion, T lymphocyte counts, lymphocyte transformation rate, and protection rate to assess the immune modulatory functions of TPPPS in chickens.

2. Materials and methods

2.1. Ethics statement

The animal procedures were approved by the Animal Care and Use Committee of Shandong Agricultural University (Permit number: 20010510), and performed in accordance with the "*Guidelines for Experimental Animals*" of the Ministry of Science and Technology (Beijing, China).

2.2. Bacterial strains and plasmids

B. avium strain LL was isolated from the sick chickens in 2008 (Shandong, China) and then preserved by our laboratory. The genetic homology of 16 S rRNA between this *B. avium* strain LL and the reference strain S5 was 100%. The *B. avium* strain was grown and maintained on lysogeny broth (LB) agar and broth at 37 °C. *P. pastoris* GS115 and plasmid pPIC9 were purchased from Invitrogen (Carlsbad, CA, USA). The media were prepared according to the manuals of *Pichia* expression.

2.3. Construction of recombinant transformant

Based on the ompA gene sequence of *B. avium* (GeneBank accession number: M96550.1), a pair of primers (F: 5′-CCG*GAATTC*ATGAACAAACCCTCCAAAAT- 3′, the underlined bases encode an *Eco*R I restriction site, R: 5′-TT*GCGGCCGC*TTA ATGATGATGATGATGATGCTTGCGGCTACCGACGATT-3′, the underlined bases encode a *Not*I restriction site) were designed to produce a 636 bp fragment. The PCR product, which was confirmed by sequencing, was cloned into plasmid pPIC9, named pPIC9-ompA. This plasmid was transformed into a competent *P. pastoris* GS115 to obtain a transformant *P. pastoris* pPIC9-ompA in accordance with the manufacturer's instructions (Invitrogen). Blank plasmid pPIC9 was transformed into *P. pastoris* as a negative control.

2.4. Expression, purification, and identification of B. avium ompA

The methanol-induced expression of ompA in *P. pastoris* GS115 was performed following the manufacturer's instructions (Invitrogen). At 24, 48, 72, and 96 h post methanol induction, the culture supernatants of the *P. pastoris* transformed with the recombinant pPIC9-ompA plasmid and the *P. pastoris* transformed with blank pPIC9 plasmid were harvested through centrifugation, respectively. SDS-PAGE and Western blot analysis were carried out to identify the ompA as described in previous study [25]. The mouse anti-omp polyclonal antibody used in western blot analysis was prepared by ourselves according to the previous method [26]. The recombinant protein ompA was purified using ProteinIso™ Ni-NTA Resin kit (TRANS, Beijing, China). Protein concentration was determined by Easy II Protein Quantitative Kit (BCA) (TRANS, Beijing, China).

2.5. Preparation of vaccines

TPPPS was provided by our laboratory and was prepared via hot water extraction and ethanol precipitation [24]. The purified recombinant ompA was mixed with TPPPS and Freund's incomplete adjuvant (Solarbio, Beijing) at a ratio of 1:1 respectively, reaching three final concentrations of 50, 100, and 150 μg/mL. The concentration of TPPPS was set at three doses of 20, 40, and 60 mg/mL in three separate TPPPS adjuvant vaccines. The recombinant ompA mixed with TPPPS and Freund's incomplete adjuvant, were separately prepared to the corresponding adjuvant subunit vaccines.

2.6. Animal experiment

A total of 180 one-day-old SPF white leghorn chickens (male; Spirax Ferrer Poultry Co., Ltd, Jinan) were randomly divided into six sterilized isolators (groups I to VI), with 30 chickens in each. The ambient conditions were set at 20 °C–25 °C and 30%–40% relative humidity, and air entering the isolators was filtered. The chickens were allowed to acclimatize for three days before the start of the experiments.

Each chicken in groups I–VI was subcutaneously inoculated with 0.2 mL of the 20, 40, and 60 mg/mL TPPPS adjuvant recombinant ompA vaccines, the Freund's incomplete adjuvant recombinant ompA vaccine, the pure recombinant ompA vaccine, and PBS respectively at 0, 7, and 14 days post the first vaccination (dpv). Groups I–VI were named OmpA-TPPPS (20), OmpA-TPPPS (40), OmpA-TPPPS (60), OmpA-Freund, OmpA, and PBS respectively. The final concentration of ompA in groups I–V reached 50, 100, and 150 μg/mL at the first, second, and third vaccination respectively. At 3, 7, 14, 21, 28, 35, 42, and 49 dpv, three chickens from each group were selected randomly to determine the serum antibody titers, serum IL-4 concentrations, $CD4^+$ and $CD8^+$ T lymphocytes counts in the peripheral blood, and T-lymphocyte transformation rate. The chickens were not fed for 12 h before sampling.

One week after the third vaccination (at 21 dpv), 20 chickens from each group (except from the control group) were placed into a new isolator and challenged intranasally with 10 LD_{50} *B. avium* LL strain. Clinical manifestation and survival status of the chickens were recorded for seven days after challenge. Clinical symptoms, including labored breathing, sneezing, and oculonasal discharges were monitored [1,2]. The mortality, morbidity, and protection rate in each group were calculated according to the following formulas: