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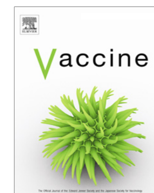

Evaluation of carbopol as an adjuvant on the effectiveness of progressive atrophic rhinitis vaccine

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ABSTRACT

The Gram-negative pathogen toxigenic *P. multocida* causes progressive atrophic rhinitis (PAR) in swine throughout the world. Although some vaccines are being developed against PAR, their efficacy has not been evaluated using carbopol. In our study, a mixture of killed *B. bronchiseptica* and *P. multocida* bacteria, combined with recombinant proteins containing the C- and N-termini of PMT, was emulsified using two different adjuvants (ISA-15A and carbopol 971). The efficacy of these two vaccines was evaluated in a mouse model. Balb/C mice were immunized twice at a 14-day interval. Two weeks after the secondary immunization, blood samples were collected and the mice were challenged with toxigenic *P. multocida*. Thirty-five days later, the mice were euthanized, blood and tissue samples were collected. Compared with mice inoculated with vaccine emulsified with ISA-15A, higher titers of SN (1:64) and significantly increased levels of TNF-α, IL-6 and IL-17A were observed in mice inoculated with vaccine emulsified with the carbopol 971P. Especially, mice immunized with vaccine emulsified with the carbopol 971P had no detectable pathological changes in snouts or organs after challenge. The results demonstrated that carbopol adjuvanted vaccine provides good protection against PAR and *P. multocida* infection which can induce robust humoral and cell-mediated responses. We conclude that the carbopol adjuvanted vaccine is a good candidate for PAR prevention.

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1. Introduction

Toxigenic *Pasteurella multocida* (*P. multocida*), a rod-shaped Gram-negative bacterium in the family *Pasteurellaceae*, is a widespread pathogen responsible for progressive atrophic rhinitis (PAR) in swine [1]. Though PAR can be experimentally induced by toxigenic *P. multocida* infection alone, infection by *B. bronchiseptica* can facilitate *P. multocida* colonization in respiratory tract [2]. The main clinical signs of PAR are shortening or twisting of the snout and atrophy of the nasal turbinate. Pathological effects on the nasal turbinate vary, from slight atrophy of the inferior scroll of one ventral turbinate, to complete bilateral atrophy of the dorsal and ventral turbinate [3]. The extent of turbinate atrophy has been used as a measure of disease severity, and several scoring systems are available [4]. Currently, vaccination is considered to be the most effective approach for preventing and controlling this nonfatal and economically damaging infectious disease.

P. multocida toxin (PMT), also known as dermonecrotic toxin, osteolytic toxin, or turbinate atrophy toxin, has an apparent molecular mass of 143 kDa (kDa) and is a primary virulence factor in PAR [5]. The C-terminal portion of the toxin contains the catalytically active moiety, and the N-terminal region is responsible for cell binding [6–8]. PMT has good immunogenicity, and formalin-inactivated PMT can induce specific antibodies against PAR [9–11]. Unfortunately, native PMT comprises less than 0.6% of *P. multocida* protein by weight, and large-scale purification and inactivation of full-length recombinant PMT expressed in *E. coli* is difficult. However, because the C-terminus of PMT is essential for maximal immunogenicity [12], and the N-terminal 487 amino acids contribute to protective immunity [13], it has been possible to develop efficacious subunit vaccines.

Adjuvants are used to increase the immunogenicity of antigens and thus increasing vaccine efficacy [14,15]. Traditional adjuvants such as complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA) [16], and aluminum hydroxyl gel, have been used in PAR vaccines [17], but few novel adjuvants were used in this vaccine development. Compared to aqueous vaccines with equivalent antigenic content, the novel adjuvant carbopol (Code name PD – Solvay Dupha) elicits robust humoral immunity and T-cell responses to some subunit vaccines in mice [18].

Cytokines play crucial roles in fighting infections and in immune responses [19,20]. The two types of T helper cells (Th1 and Th2) were classified which differ in cytokine secretion patterns

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and other functions [21]. Th1 cells produce IL-1, IL-2, IL-12, TNF-α, and IFN-γ, which are classified as pro-inflammatory cytokines. In contrast, the anti-inflammatory cytokines IL-4, IL-5, IL-6, and IL-10 are produced by Th2 cells. The recently identified IL-17 has also been classified as a pro-inflammatory cytokine, and IL-17A, a significant factor in the host defense against extracellular bacterial and fungal pathogens, such as Pseudomonas aeruginosa, *K. pneumoniae* [22–24].

In this study, a mixture consisting of formalin-inactivated *B. bronchiseptica* and *P. multocida*, plus recombinant proteins containing the N-terminal and C-terminal regions of PMT, was prepared and then emulsified with either carbopol or ISA-15A. The two vaccines were evaluated in mice by assessing the level of PMT-specific antibody, the serum neutralization (SN) titers, and the concentration of cytokines in the serum. Snout sections and selected organs were also examined for pathological variations.

2. Materials and methods

2.1. Bacterial strains, plasmids, and enzymes

P. multocida ZXT^+Pm is a type D toxigenic strain obtained from a pig farm affected by PAR in Jiangsu, China. *B. bronchiseptica* AHBb was obtained from a porcine necropsy in Anhui, China. ZXT^+Pm and AHBb were cultured in Brain Heart Infusion (BHI) Agar (Oxoid Ltd, Hants, UK). The expression vector pET-32aM and the *E. coli* Rosseta were maintained as stocks in our laboratory. Restriction enzymes and T4 DNA ligase were purchased from Takara (Takara Biotechnology (Dalian) Co., Ltd.).

2.2. Growth curves for ZXT^+Pm and AHBb

The ZXT^+Pm and AHBb were cultured as the recommendation in provious study [25]. Briefly, ZXT^+Pm and AHBb were continuously inoculated into TSB cultures for more than three generations until stable growth was achieved. Then, 1 mL of the two bacterial cultures (1×10^6 CFU/ml) was inoculate into 100 mL fresh TSB cultures and incubated on a shaker at 200 rpm at 37 °C. Growth curves were determined by measuring the optical density at 600 nm (OD_{600}) of the cultures every 1 h and 2 h respectively with Eppendorf BioPhotometer (Eppendorf AG, Germany), and the CFU count per milliliter was determined at the same time.

2.3. Protein expression and identification

The complete *toxA* gene encoding PMT, a 1459 bp fragment encoding amino acids 1–487 at the N-terminus of PMT (*tox*N), and a 891 bp fragment encoding amino acids 986–1282 at the C-terminus of PMT (*tox*C), were amplified using primers containing BamH I and Sac I restriction enzyme recognition sites (Table 1).

Table 1
Primer sequences used to amplify selected regions of *toxA*.

Primer name	Sequence (5′-3′)	PCR amplicon size
toxA-F	CGC<u>GGATCC</u>ACTAACATAGAGGTTAT	3858 bp
toxA-R	CTC<u>GAGCTC</u>TTATAGTGCTCTTGTTAAGC	
toxN-F	GG<u>GGATCC</u>ATGAAAACAAAAC	1461 bp
toxN-R	GT<u>GAGCTC</u>TTATTGAGCTAAAGC	
toxC-F	GT<u>GGATCC</u>CTGCAATCAGCAAAAGATAAT	903 bp
toxC-R	AT<u>GAGCTC</u>TTATAGTGCTCTTGTTAAGCGAGGC	

Restriction enzyme recognition sites are underlined.

The PCR products were digested with restriction enzymes and then cloned into the pET-32aM vector to construct recombinant plasmids (pET-toxA, pET-toxN and pET-toxC) that encoded His-tagged proteins. Recombinant plasmids were transformed into *E. coli* Rosseta by the $CaCl_2$ method [26] and cultured to mid-logarithmic phase in LB medium at 37 °C. Protein expression was induced by addition of isopropyl-b-D-thiogalactopyranoside (IPTG) (Biosharp, Anhui, China) to a final concentration of 1 mM. After incubation at 37 °C for 4 h, cells were harvested by centrifugation and disrupted by sonication. His-tagged proteins were purified under native conditions using Ni-NTA Agarose (GE Healthcare Bio-Sciences, Uppsala, Sweden) as recommended by the manufacturer. Purified proteins were identified by western blot using anti-6X His tag antibody (Abcam, Shanghai, China). Endotoxin was removed following methods described previously [27] and detected using the Chromogenic End-point TAL Kit (Chinese Horseshoe Crab Reagent Manufactory Co., Ltd., Xiamen, China). Protein concentration was determined using the BCA Protein Assay Kit (Cwbiotech, Nanjing, China) and stored at −80 °C.

2.4. Immunogen preparation

Whole-cell bacterins were produced by culturing ZXT^+Pm and AHBb in BHI medium at 37 °C to mid-logarithmic phase. Cells were harvested by centrifugation, re-suspended in PBS with 0.3% (v/v) formalin (Fisher), and incubated at 37 °C for 24 h with constant gentle shaking. Two vaccine preparations were made as follows: the vaccine mixture was composed of two recombinant proteins containing the N-terminal and C-terminal region of PMT (N-PMT and C-PMT) at 40 μg/dose, inactivated ZXT^+Pm (1×10^9 CFU/dose), and inactivated AHBb (1×10^9 CFU/dose). The mixtures were emulsified with either equal volume 0.25% carbopol or 1/10 volume ISA-15A (SEPPIC, Paris, France) to produce the two vaccines.

2.5. Animal experiments

Forty female specific pathogen free (SPF) BALB/c mice (3 weeks old) were purchased from the Laboratory Animal Centre of Yangzhou University (Yangzhou, China) and housed in a biosafety level 2 animal facility. Animal experiments were approved by the Institutional Animal Care and Ethics Committee of Nanjing Agricultural University (Approval No. IACECNAU20100902). Mice were randomly divided into four groups of 10 mice each and raised separately with independent ventilation sources. After acclimating for 3 days, mice in the two groups were injected intramuscularly with 0.2 mL of vaccine emulsified with carbopol and ISA-15A, respectively, and the other two groups (PBS and control) were injected with 0.2 mL of PBS. All mice were boosted 3 weeks later. Two weeks after boosting, blood samples were collected from five mice (anesthetized by ether inhalation) in each group, and the remaining mice were challenged with ZXT^+Pm, using a modified protocol described by Jordan [28]. Briefly, all mice were pre-treated intranasally by dropping 10 μL of 1% acetic acid onto the external nares. This procedure was repeated 24 h later. Sixteen hours after the secondary acetic acid treatment, all mice (except in the control group) were challenged intranasally with 1×10^{10} CFU of ZXT^+Pm. After 35 days, all mice were euthanized by exsanguination under terminal anesthesia and blood samples were collected. All blood samples were treated and stored as described previously [29].

2.6. Pathological examination of snout sections and organs

Immediately after euthanasia, the heads, livers, and lungs from all mice were collected and fixed for 14 days in 10% neutral

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