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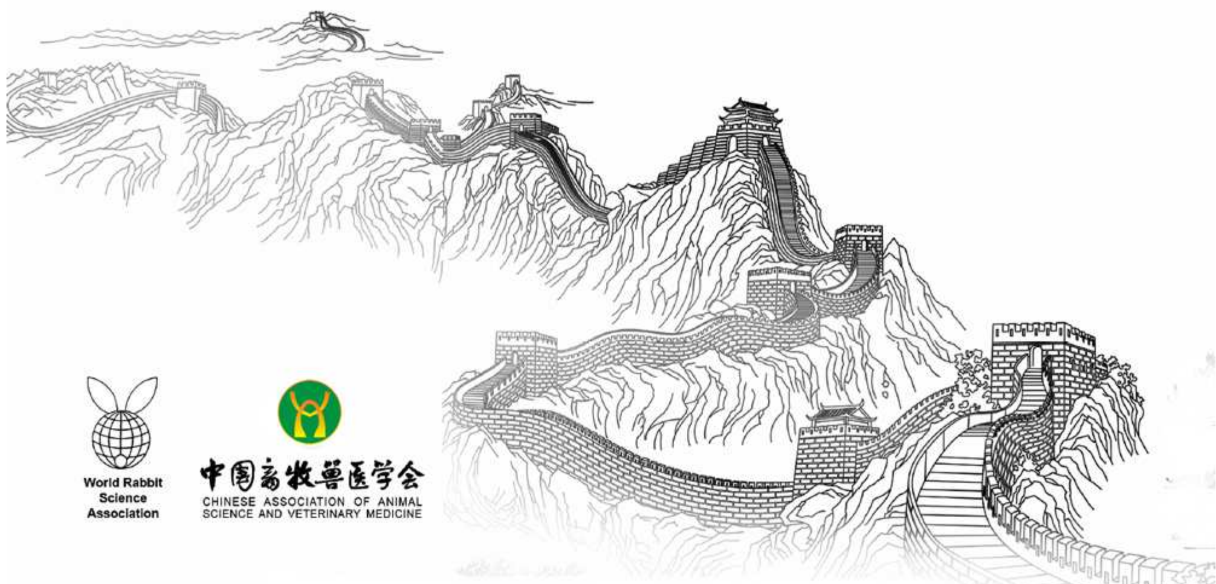

IDENTIFICATION OF TWO NEW IMMUN-PROTECTIVE CANDIDATES PROTEINS FOR THE DEVELOPMENT OF *BORDETELLA BRONCHISEPTICA* SUBUNIT VACCINE

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ABSTRACT

The Gram-negative pathogen *Bordetella bronchiseptica* causes acute and chronic respiratory infection in a variety of animals. To identify useful candidate antigens for such a vaccine, immunoproteomic analysis was adopted to analyse outer membrane proteins of *B. bronchiseptica*. The outer membrane proteins extracted from *B. bronchiseptica* were separated by two-dimensional gel electrophoresis and analyzed by Western blotting for their reactivity with the convalescent serum against two strains. Immunogenic proteins were identified by Matrix-Assisted Laser Desorption/Ionization Time of Flight-Mass Spectrometry (MALDI-TOF-MS). A total of 14 proteins were common immunoreactive proteins, of which 1 was a known antigen and 13 were novel immunogenic proteins for *B. bronchiseptica*. We selected five of these newly discovered immunogenic proteins including amino acid ATP-binding cassette transporter substrate-binding protein (ABC), lipoprotein (PL), outer membrane porin protein (PPP), leu/ile/val-binding protein (BPP), and conserved hypothetical protein (CHP) as targets for recombinant prokaryotic expression.We tested the recombinant proteins for immunogenicity and protection against B. bronchiseptica in mice to find novel immune-protective antigens. The immune responses of mice to vaccination with individual recombinant proteins were measured. Each of the tested recombinant proteins induced high antibody titer, and PPP and PL showed protective indices against challenges with B. bronchiseptica. The protection ratios were 62.5% and 50%, respectively, compared with 12.5% for control vaccinations. The protection ratios of ABC, BPP, and CHP were not significantly different from the controls.

Keywords: *Bordetella bronchiseptica*, immunoproteomic analysis, immune-protective protein, subunit vaccine.

INTRODUCTION

Bordetella bronchiseptica is an important bacterial pathogen that causes a number of respiratory diseases in livestock and poultry. They include atrophic rhinitis in swine, kennel cough in dogs, and snuffles in rabbits (Mattoo *et al*., 2005). *B. bronchiseptica* infections are often endemic in commercial rabbitries, and they are difficult to control due to the rapid spread and persistence of the infection (Long *et al*., 2010). Although many available *B. bronchiseptica* vaccines induce high titers of serum antibodies and offer protection against severe diseases (Glass *et al*., 1989), *B. bronchiseptica* is often isolated from animals in vaccinated populations, suggesting that the vaccines are not satisfactory in terms of efficacy and safety (Yevsa *et al*., 2012; Gopinathan *et al*., 2007). Development of a new vaccine is critical to prevent and control *B. bronchiseptica* infection.

In this study, we performed immunoproteomic analyses to analyze the outer membrane proteins of *B. bronchiseptica* and we selected five newly discovered immunogenic proteins as targets for recombinant prokaryotic expression. We tested the recombinant proteins for immunogenicity and protection against *B. bronchiseptica* in mice to find novel immun-protective antigens.

MATERIALS AND METHODS

Immunoproteomic analysis of *Bordetella bronchiseptica* outer membrane proteins

B. bronchiseptica strain HB originally isolated from a rabbit with infectious rhinitis and RB50 (ATCC) were grown routinely in trypticase soy broth (TSB) containing 5% (v/v) sheep blood at 37°C for 16 h. Protein extraction was performed as previously reported by Molly et al. (2000) . Isoelectric focusing (IEF) was performed with the IPGphor II TM system (GE Healthcare, USA) and the Immobiline DryStripTM IPG strips of 13 cm (pH 4～7). The prepared OMPs sample (750 µg/strip) was mixed with rehydration buffer and focused for 410 000 VHrs. After equilibration, the proteins were separated on a 12% SDS polyacrylamide gel using Hoefer SE600 Ruby (GE Healthcare, USA) and analyzed by Western blotting for their reactivity with the convalescent serum against two strains. Immunogenic proteins were identified by Matrix-Assisted Laser Desorption/Ionization Time of Flight-Mass Spectrometry (MALDI-TOF-MS).

Immune efficacy of five recombinant *Bordetella bronchiseptica* proteins

Five proteins including ABC , PL, PPP, BPP and CHP were selected as targets on the basis of immunoproteomic analysis. Target genes encoding the mature, full-length proteins without signal peptide sequences were amplified by PCR, and ligated to a pET32a+ vector, whose cloning site is available for producing fusion proteins also containing a His Tag.The inserts were confirmed by DNA sequencing and the resulting plasmids were used to transform competent Escherichia coli BL21 (DE3) cells. The recombinant proteins were produced as soluble proteins, the expression products were purified by passing the Ni-affinity chromatograph column using the recombinant proteins with the tag of 6× His and then analyzed by Western blotting.

Table 1: The primer sequences used to amplify the selected proteins

Primer name	Sequence（5′-3′）	Target gene (reference Gene ID)	PCR amplicon size
ABC-F	GCGCCATGGGCAAGAAAATCACCGCTGTA	ABC(ID:2660093)	789bp
ABC-R	TATCTCGAGCTTGATGATGTTGGCGCCGAA		
BPP-F	GCGCCATGGGCAACAAGGCATTTCGTTTC	BPP (ID: 2663902)	1125bp
BPP-R	GCGCTCGAGTTACTTGTACGACGTCTTGCT		
CHP-F	TATCCATGGGCTATCCCAACCGCCGGCTGTAC	CHP(ID: 2660968)	573bp
CHP-R	TATCTCGAGCTACTTGCCGCGCCCGCCTTG		
PL-F	GCGCCATGGGCCGTATGAACAAACGTCAT	PL(ID: 2662792)	1125bp
PL-R	GCGCTCGAGTCAGACCATCTTGTCCTTCAG		
PPP-F	GCGCCATGGGCAAAAAGACTCTGCTCGCT	PPP(ID: 2660513)	1164bp
PPP-R	GCGCTCGAGTTAGAAGCGGTGACGGATACC		

Animals and experimental design

Female closed line of ICR white mouse (18–22 g) were purchased from the Zhejiang Experimental Animal Center (China) and maintained under standard conditions. A total of 48 ICR mice were divided into six groups. Five groups were inoculated with the recombinant proteins (50 µg/dose), respectively, in a 200 µL volume mixed with Freund's complete adjuvant. A control group was inoculated with 200 µL PBS. Each group was inoculated twice at a 2-week interval. Two weeks after the second inoculation, the mice were challenged by intraperitoneally injecting 1.74×10^7 CFU of *B. bronchiseptica* HB (LD_{50}= 2.42×10^6 CFU, a pre-experiment was carried out to define LD_{50}). Prior to challenge, serum was collected from each mice via tail bleeding. Antibody titers were measured using indirect enzyme-linked immunosorbent assay (ELISA). Mortality was recorded 10 days post challenge, protection ratio (survival quantity/total

quantity) was calculated. All surgeries were performed in accordance with the recommendations proposed by the European Commission (1997), and efforts were made to minimize animal suffering.

Statistical Analysis

All data were reported as the mean ± standard error. To determine the significance of the observations, Student's t-tests were carried out using the SPSS 17.0 software (SPSS Inc., Chicago, US). P values ≤ 0.05 were considered significant.

RESULTS AND DISCUSSION

A total of 14 proteins are common immunoreactive proteins by immunoproteomic analysis (Fig.1) , of which 1 was known antigen and 13 were novel immunogenic proteins for *Bordetella bronchiseptica.* Five of those proteins were expressed, Western blot analysis indicated that all five recombinant proteins were immunogenic in mice(Fig.2). There was a significant increase (P < 0.005) of the antibodies against the recombinant proteins on day 17 before *B. bronchiseptica* challenge for groups 1, 2, 3, 4, and 5 compared with the control (group 6) (Fig.3). Up to 10 days after challenge with *B. bronchiseptica* HB, rPPP and rPL provided better protection (protection ratio of 62.5% (5/8) and 50% (4/8), respectively compared with PBS alone (protection ratio of 12.5%)Fig. 4. No statistically significant differences were observed among the protection ratios of rABC (1/8), rBPP (2/8), or rCHP (1/8), and the PBS control (1/8). Viable *B. bronchiseptica* was recovered from all the mice that died.

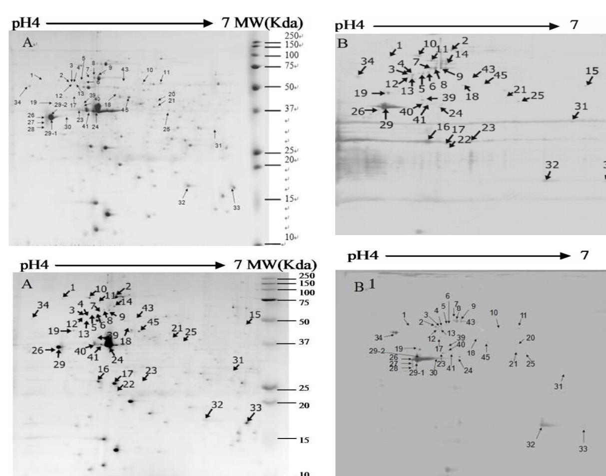

Figure 1: Two-dimensional maps (pH 4-7) and Western immunoblot profile of *B. bronchiseptica* outer membrane proteins, with the immunoreactive proteins indicated. A, A1, preparative 2-D gels of HB and RB50 outer membrane proteins stained with Coomassie brilliant blue. B, B1, Western blot reacted with pooled convalescent sera against HB and RB50 strains. (all sera were diluted 1:1000). Spots identified by MALDI-TOF-MS or MALDI-TOF-TOF-MS are labeled.

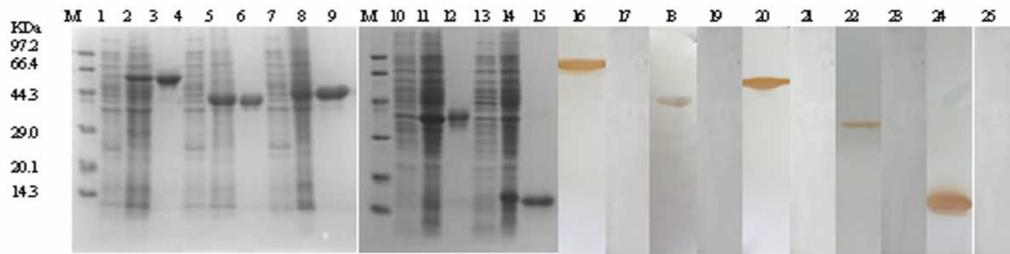

Figure 2: Expression and western blot analysis of recombinant target proteins. Lanes are as follows: Lane M, protein molecular weight marker. Lanes 1–2, 4–5, 7–8, 10–11, 13–14: CBB staining of the recombinant *E. coli* whole-cell lysate before and after induction with IPTG for recombinant ABC, BPP, PPP, PL, and CHP, respectively. Lanes 3, 6, 9, 12, 15: purified recombinant ABC, BPP, PPP, PL, and CHP, respectively. Lanes 16–17, 18–19, 20–21, 22–23, 24–25: western blot analysis of the purified recombinant ABC, BPP, PPP, PL, and CHP with convalescent sera of rabbits before and after infection with *B. bronchiseptica* HB, respectively.

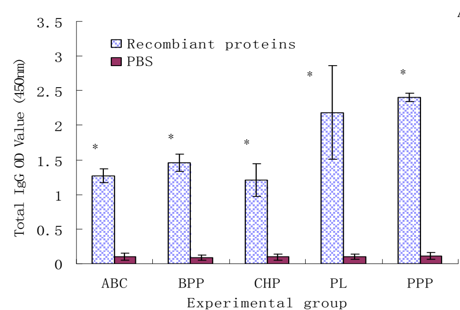

Figure 3: Antibody response elicited after immunization with recombinant proteins or mock-vaccinated (PBS control) before challenge. Histograms indicate the recombinant protein specific serum antibody titers (total IgG) of sera collected from all mice 28 days after first immunization. Data are presented as the mean optical density obtained from ELISA analysis of individual serum samples (n = 8 per treatment) at a 1:200 dilution. The curve of antibody response was drawn where the ordinate axis represents the absorbance at 450 nm ± standard deviation. * indicates highly significant differences compared with the mice immunized with PBS (P < 0.001).

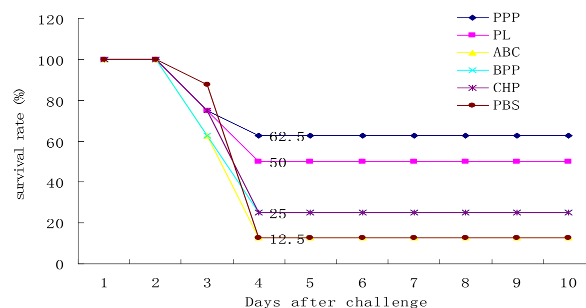

Figure 4: Protective efficacy of recombinant proteins in the mouse model
The percentage survival curve showing survival pattern of immunized and control mice (8 mice/group) following challenge with 1.74×10^{7} cfu/mL *B. bronchiseptica* HB, respectively.

The most direct way to evaluate a vaccine candidate is to measure the survival rate of vaccinated mice challenged with the lethal pathogen. In this study, an effective degree of protection was observed when mice were immunized with rPPP and rPL compared with controls, suggesting that rPPP and rPL are effective vaccine candidates .

CONCLUSIONS

In conclusion, this study shows that rPPP and rPL can induce protective immune responses against *B. bronchiseptica*. These data suggest that rPPP and rPL are potential immunomodulators or vaccine candidates if different vaccination approaches are followed.

ACKNOWLEDGEMENTS

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REFERENCES

Glass L.S., Beasley J.N., 1989. Infection with and antibody response to *Pasteurella multocida* and *Bordetella bronchiseptica* in immature rabbits. *Lab. Anim. Sci., 39, 406- 410.*

Gopinathan L., Kirimanjeswara G.S., Wolfe D.N., et al., 2007. Different mechanisms of vaccine-induced and infection-induced immunity to *Bordetella bronchiseptica*. *Micrbe. Infect*., *9, 442- 448.*

Long G.H., Sinha D., Read A.F., et al., 2010. Identifying the age cohort responsible for transmission in a natural outbreak of *Bordetella bronchiseptica*. *PLoS Pathog. 6, e1001224.*

Mattoo S., Cherry J.D, 2005. Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other *Bordetella* subspecies. *Clin. Microbiol. Rev., 18, 326- 382.*

Toobak H., Rasooli I., Talei D., et al., 2013. Immune response variations to *Salmonella enterica serovar Typhi r*ecombinant porin proteins in mice. *Biologicals., 41, 224- 230.*

Yevsa T, Ebensen T, Fuchs B, et al., 2012. Development and characterization of attenuated metabolic mutants of *Bordetella bronchiseptica* for applications in vaccinology. *Environ. Microbiol., 15, 64- 76.*

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