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Immunizing mice using different combination antigens of the PI-2a fimbria subunit of *Streptococcus agalactiae*

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Abstract

Background: *Streptococcus agalactiae* is the main causal pathogen of bovine mastitis (BM), causing considerable economic loss to the dairy industry worldwide. Vaccines against *S. agalactiae* play an important role in preventing disease. **Aims:** The aim of this study was to evaluate the immunoprotection of *S. agalactiae* pilus island fusion proteins, ancillary protein 1-ancillary protein 2 (AP1-AP2), ancillary protein 1-bone protein (AP1-BP), bone protein-ancillary protein 2 (BP-AP2), and ancillary protein 1-bone protein-ancillary protein 2 (AP1-BP-AP2) in Balb/c mice. **Methods:** Four kinds of fusion antigens and the same volume of Freund's complete adjuvant were mixed vigorously to prepare fusion antigen immuno-samples. The mice were immunized 4 times (on the 0th, 7th, 14th, and 28th days) with these samples with an immunizing dose of 50 μg/mouse. After the 4th immunization, serology tests were used to evaluate the antibody. The antibody titre produced by AP1-BP-AP2 fusion antigen was the highest, at up to 1:25600. The mice were then injected with 0.5 ml of 2×10^4 CFU/ml clinically isolated *S. agalactiae* at day 50 and observed daily for the following 7 days. **Results:** Statistical analyses showed that these 4 kinds of fusion antigens had good protective immunity. Among them, AP1-BP-AP2 fusion antigen had the best protective immunity in Balb/c mice, with an immune protection index (PI) of 80%. **Conclusion:** This research provides a reliable theoretical basis for screening candidate antigens of the subunit vaccine and detecting antigen preparations of *S. agalactiae*.

Key words: Fusion antigen, Immunoprotection, Pilus island

Introduction

Bovine mastitis (BM) is a common threat to mammary gland health in dairy cattle. It not only causes a decline in the milk yield of dairy cows, but also causes a significant reduction in milk quality. The usual method of treating clinical BM is the use of antibiotics. However, overusing antibiotics has caused an increase in bacterial resistance. Many clinical *Streptococcus* isolates have been found to obtain broad-spectrum resistance (Hong-Sheng *et al*., 2012; Lin *et al*., 2016). The produced milk in such cases contains different levels of antibiotic residues after treatment, which may cause various allergies and other adverse effects in sensitive individuals after drinking the milk.

Streptococcus agalactiae (group B *Streptococcus agalactiae*, GBS) is an important pathogenic bacterium that causes BM. It is hazardous to infants, causes serious financial loss to the dairy industry, and threatens human and livestock health (Weihong *et al*., 2014; Huanhuan *et al*., 2016). Traditional mastitis vaccines are either inactivated whole bacteria vaccines or live-attenuated vaccines. Inactivated whole bacteria vaccines contain large amounts of non-immunogenic substances, which have greater toxicity and often require multiple immunizations to work. Mastitis live-attenuated vaccines can be propagated *in vivo* and can even stimulate cellular immunity after injection into dairy cattle. However, live-attenuated vaccines are prone to recovery of virulence and causes disease, meaning that its security could not be guaranteed. Compared with traditional vaccines, genetic subunit vaccines have the advantages of good security, high purity, and being obtained easily (Herrera Ramírez *et al*., 2017; Mukherjee *et al*., 2017). Therefore, the recombinant subunit vaccine for most mastitis pathogens has broad application prospects. *Streptococcus agalactiae* has a pilus-like structure similar to *Escherichia coli*, bone protein (BP), ancillary protein 1 (AP1) and ancillary protein 2 (AP2), which are the effective conserved antigen components of the pilus island with good antigenicity and certain immunoprotection. Based on previous studies, the different combinations of pilus island fusion proteins AP1-AP2, AP1-BP, BP-AP2, and AP1-BP-AP2 were used to perform immunoprotection experiments in mice to screen and determine optimal antigen combinations. These findings lay a solid foundation for the research of effective genetic vaccines for mastitis.

Materials and Methods

Strains, reagents and laboratory animals

Clinical isolations of type Ia pathogenic *S. agalactiae* (ID_{50}: 10^4 CFU/ml); AP1-AP2, AP1-BP, BP-AP2, and AP1-BP-AP2 fusion antigens were prepared by the Inner Mongolia Autonomous Region Engineering Technology Research Centre of Prevention and Control. The pathogenic bacteria in milk, Complete Freund's Adjuvant, and horseradish peroxidase (HRP)-labelled goat anti-mouse immunoglobulin G (IgG) antibody were purchased from Ruitaibio (China). Additionally, two hundred 6- to 8-week-old female BALB/c mice were provided by Shandong University of Traditional Chinese Medicine Experimental Animal Room.

Preparation of different fusion antigens

AP1-AP2, AP1-BP, BP-AP2, and AP1-BP-AP2 fusion antigen immuno-samples were prepared according to the "The Regulations of Animal Usage Biological Products of people's republic of China". These purified fusion antigens were quantified accurately to 2.0 mg/ml and emulsified with the same volume of Freund's complete adjuvant. Twenty mice were randomly divided into 4 groups (n=5/group), and each group was subcutaneously injected with 0.5 ml of a mixture of fusion protein and adjuvant, and the activity, food and water intake of the mice were observed daily for two weeks. After confirming that there was no abnormal reaction, the mixture of fusion protein and adjuvant was stored at 4°C for subsequent experimental studies.

Mouse grouping and immunization

From the thirty mice, six were injected subcutaneously with 50 µL of AP1-AP2 fusion antigen (50 µg antigen per mouse) mixed with an equal amount of Freund's complete adjuvant on day 0 (the same day), 7, 14, and 28 of the experiment. Three mice were randomly selected from each group for blood collection before each immunization and on the 35th, 42th, and 49th days after the first immunization. The AP1-BP, BP-AP2, and AP1-BP-AP2 fusion antigen preparations were identical to the AP1-AP2 immunizations. In the control group, physiological saline was used instead of the fusion antigen. Each mouse was injected with 50 µL physiological saline mixed with equal amount of Freund's complete adjuvant. The specific operation was the same as that of the experimental group.

Detecting antibodies in mouse immune serum

The purified AP1-AP2, AP1-BP, BP-AP2, and AP1-BP-AP2 fusion antigens were used as coating antigens. Optimal working conditions for each antigen were determined by titration, and an indirect enzyme-linked immunosorbent assay (ELISA) method was established for the detection of immune antibody levels. Briefly, polystyrene microplates were coated with 100 µL of the fusion protein (2 µg/ml) in 0.05 M carbonate buffer (pH = 9.6) overnight at 4°C and washed twice with 200 µL of phosphate buffered saline (PBS) with 0.05% Tween-20. The plates were blocked with 200 µL of 2% dry milk in 0.01 M PBS at 37°C for 2 h. The wells were then washed three times. The washing step was followed after incubation with primary and secondary antibodies as well. Following this step, 100 µL of serum samples with a 1:100 dilution were dispensed into microplates coated with fusion protein at 37°C for 1 h. Then, 100 µL of goat anti-mouse IgG peroxidase conjugate (at 1:4000 dilution) was added to each well, and plates were incubated for 1 h at 37°C. To develop color, 100 µL of Ultra 3,3´,5,5´-tetramethylbenzidine (TMB) was added to each well, and the plates were incubated for 15 min at room temperature followed by the addition of 50 µL of stopping solution (1 N HCl). The optical density (OD) was determined at 450 nm using a plate reader.

Twenty-four negative sera collected from each group of mice before the immunization were used for threshold determination. The level of immune antibody and the final titre were determined while the serum collected on the 0th, 7th, 14th, 28th, 35th, 42th, and 49th days was used as the first antibody, and the HRP-labelled goat anti-mouse IgG antibody was used as the second antibody. The regulation of the increase and decrease of antibodies were clarified, and the fusion antigen that produced the highest antibody titre after immunization was determined.

Immunoprotection experiment

Mice were randomly divided into 5 groups (n=30 /group). On day 50 after immunization, 20 mice were taken separately from the four experimental groups and the control group. Each mouse was intraperitoneally injected with 2×10^4 CFU/ml of clinically isolated type Ia pathogenic *S. agalactiae*. The injection volume was 0.5 ml/mouse. The mice were observed daily for 7 days after injection. The morbidity and protection index (PI) of each group were then calculated as follows

PI = (morbidity of control group - morbidity of fusion antigen immunization group)/morbidity of control group × 100%

During immunization, the mice were observed for mental status and were fed carefully.

Results

Detecting immunized mouse serum antibody levels

The optimized working concentration of each antigen was 5.0 µg/ml, and optimal working conditions were 12 h incubation at 4°C. The best sealing time at 37°C was 2 h with 10% calf serum as the sealing reagent. The optimal dilution ratio of the sample serum was 1:100 and the optimal reaction time was 1 h at 37°C. The optimal dilution ratio of the enzyme-labelled antibody was 1:4000 and the optimal reaction time at 37°C was 1 h. The optimal development time at room temperature was 15 min with TMB as the chromogenic reagent.

The negative sera of 24 mice was detected with an AP1-AP2-coated plate under the optimal conditions. The

mean of OD_{450} of the negative serum was calculated as $\overline{X}$ = 0.2320, the standard deviation (SD) was 0.03426, and the critical value was $OD_{450} \geq \overline{X}$ + 3SD, which means that the sample was found to be positive when $OD_{450} \geq 0.3348$. The serum antibody levels collected at 0d, 7d, 14d, 28d, 35d, 42d, and 49d of the AP1-AP2 antigen-immunized group were detected in the same way. After similar calculations, the critical values with ELISA detection methods for AP1-BP, BP-AP2, and AP1-BP-AP2 fusion antigens were found to be ≥0.3445, ≥0.3389, and ≥0.3317, respectively.

All mice secreted more antibodies after immunization with AP1-AP2, AP1-BP, BP-AP2, and AP1-BP-AP2 fusion antigens (Fig. 1), antibody levels measured by ELISA showed significant differences between AP1-BP-AB2 and AP1-AP2 ($P<0.01$), AP1-BP ($P<0.01$), BP-AP2 ($P<0.01$). This shows that the AP1-BP-AP2 fusion antigen was more likely to stimulate the organism to produce antibodies.

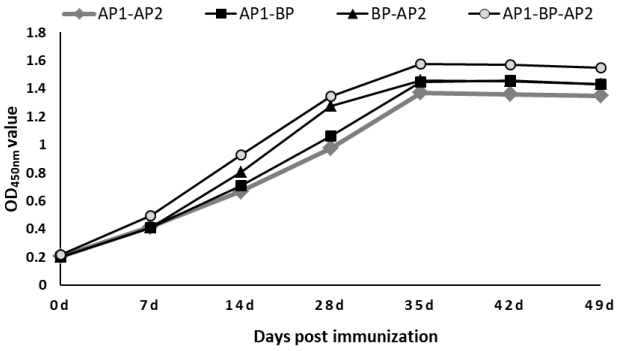

Fig. 1: Changes of antibody level in the serum samples of the mice in different groups after immunization with 4 kinds (AP1-AP2, AP1-BP, BP-AP2, and AP1-BP-AP2) of fusion antigens

The antibody titres stimulated by AP1-AP2, AP1-BP, and BP-AP2 fusion antigens on the 35th and 49th days after four immunizations were 1:12800, while the AP1-BP-AP2 fusion antigen was 1:25600 (Figs. 2-5). This indicates that the AP1-BP-AP2 fusion antigen-stimulated mice to produce antibodies with higher titres than AP1-AP2, AP1-BP, and BP-AP2 fusion antigens under the same immunization conditions.

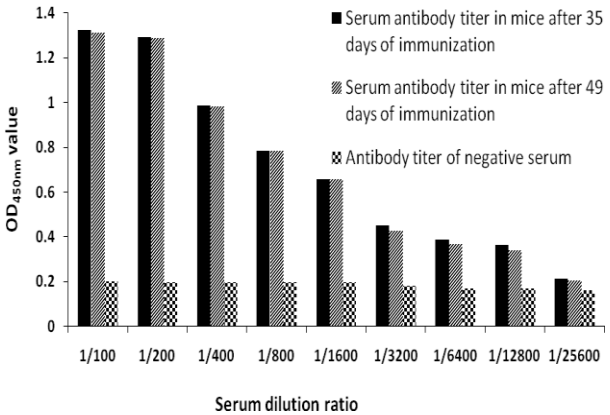

Fig. 2: The antibody titers stimulated by AP1-AP2 fusion antigens on the 35th and 49th days

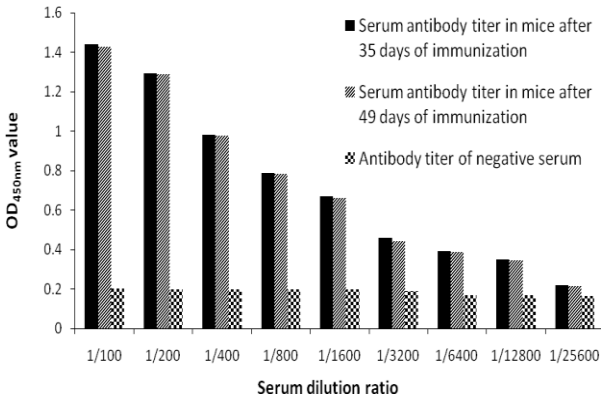

Fig. 3: The antibody titers stimulated by AP1-BP fusion antigens on the 35th and 49th days

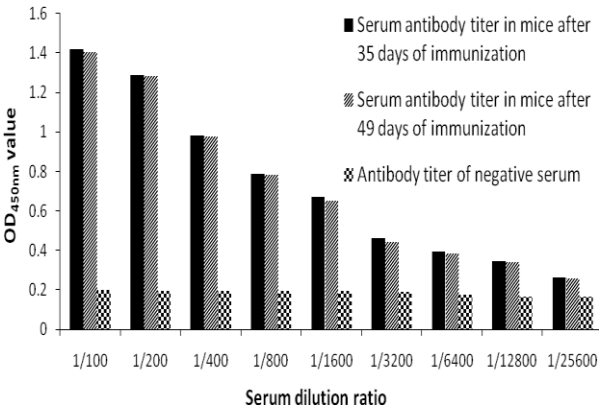

Fig. 4: The antibody titers stimulated by BP-AP2 fusion antigens on the 35th and 49th days

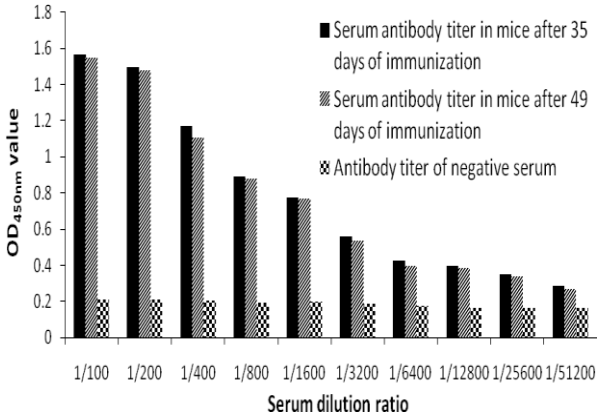

Fig. 5: The antibody titres stimulated by AP1-BP-AP2 fusion antigens on the 35th and 49th days

Immunoprotection experiment

Thirty mice were collected after immunization for 50 days and the mental status of the mice was observed continuously for 7 days after intraperitoneal injection of 0.5 ml clinically isolated type Ia pathogenic *S. agalactiae*. The morbidity and immune PI were calculated according to formula. The mental state of the mice showed that the number of mice suffering from mental insufficiency in each group peaked 48 h after injection. In addition, the number of mice with poor mental status in the antigen-

Table 1: The mental state and immune PI of mice

Experimental group	Observation time (h)								Incidence	PI
	0 h	24 h	48 h	72 h	96 h	120 h	144 h	168 h		
AP1-AP2	0	4	5	4	0	0	0	0	0.25	50%
AP1-BP	0	3	4	4	0	0	0	0	0.2	60%
BP-AP2	0	3	4	3	0	0	0	0	0.2	60%
AP1-BP-AP2	0	2	2	1	0	0	0	0	0.1	80%
Control group	0	8	10	8	3	1	1	1	0.5	--

The number of mice in each group of poor mental condition (20 mice in each group). Note: After 48 h, there was one death in the control group. Death rate was counted as the number of mentally depressed mice. PI: Protection index, AP1-AP2: Ancillary protein 1-ancillary protein 2, AP1-BP: Ancillary protein 1-bone protein, BP-AP2: Bone protein-ancillary protein 2, and AP1-BP-AP2: Ancillary protein 1-bone protein-ancillary protein 2

immunized group was significantly lower than that of the control group. The PI value of each group of mice showed that four kinds of recombinant antigens had a certain degree of immunoprotection in mice, among which the AP1-BP-AP2 recombinant antigen had the best immune protection effect, when the PI was up to 80% (Table 1). This indicates that the AP1-BP-AP2 recombinant antigen can be used as a candidate antigen for the subunit vaccine of mastitis *S. agalactiae*.

Discussion

Bacterial pilus (pili) is an appendage of many Gram-negative bacteria such as *E. coli*, and a few Gram-positive bacteria such as *Corynebacterium bovis* (Xicohtencatl-Cortés *et al*., 2006; Brochet *et al*., 2008; Mandlik *et al*., 2008). It is a pathogenic factor related to the adhesion and invasiveness of the bacteria and has good antigenicity (Brodeur *et al*., 2000; Yang and Li, 2016; Khara and Narayana, 2017). Since the discovery of the pilus-like structure of *E. coli* with *S. agalactiae*, scholars have paid special attention to the research and development of *S. agalactiae* pilus (Khodaei *et al*., 2018). Rosini *et al*. (2006) performed an in-depth study of the genomic structure of the pilin islands of the assembled pilus proteins and pointed out that pilus-like structures were important virulence factors and potential vaccine candidates of *S. agalactiae*. Konto-Ghiorghi *et al*. (2009) confirmed that the pilus of *S. agalactiae* played an important role in the adhesion of tissue epithelial cells and the formation of *S. agalactiae* biofilms. Khare *et al*. (2011) studied the regulation of the expression signal of the pilus genes and confirmed that *S. agalactiae* needed a sortase transpeptidase during assembly. This enzyme has two Srt A and Srt C forms; the former participates in the assembly of the pili, while the latter participates in anchoring the pili with cell wall polymers (Khare *et al*., 2011). Sharma *et al*. (2013) reported that specific sera with pilin proteins can inhibit more than 88% of lung epithelial macrophages infected with *S. agalactiae* VII. Their results provide theoretical support for the study of pilus subunit vaccines of *S. agalactiae*. Another study shows that at least one pilus structural gene existed in the isolates of *S. agalactiae*. These strains were confirmed to have two kinds of islands, PI-1 and PI-2. The latter has two types, namely, PI-2a and PI-2b. PI-2a was found to have a high proportion of 79%, followed by PI-1 pili islands accounting for 70% (MAatins *et al*., 2013).

In this study, we immunized mice with the fusion antigens AP1-AP2, AP1-BP, BP-AP2, and AP1-BP-AP2 and detected different levels of antibodies, among which ap1-bp-ap2 fusion antigen stimulated mice to produce antibodies with the highest titre, reaching 1:25600 on the 49th day after immunization. The immune PI is 80%, indicating a good immunoprotective effect. Therefore, the AP1-BP-AP2 fusion antigen constructed based on the pilus island gene can be used as a candidate antigen for the pilus subunit vaccine of *Streptococcus agalactiae*. This can lay a solid research foundation for the development of an effective mastitis vaccine.

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Conflict of interest

There is no conflict of interest.

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