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Assessment of Immunogenicity of CP8, CLFA-FnBPB and CP8-CLFA-FnBPB Antigens

[1,2]Xiuli Liu, [1]Yongqing Hao, [1]Shuyi Wang, [1]Airong Zhang, [3]Yuxi Wang, [1]Yu Guo and [1]Chunguang Xu

[1]Faculty of Veterinary Medicine, Inner Mongolia Agricultural University, Hohhot, 010020, China
[2]Veterinary Research Institute, Inner Mongolia Academy of Agricultural & Animal Husbandy Sciences, Hohhot, 010031, China
[3]Lethbridge Research Centre, Agriculture and Agri-Food Canada, P.O. Box 3000, Lethbridge, AB, T1J 4B1, Canada

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ABSTRACT

To assess the effects of conjugating Capsular Polysaccharide (CP) with Clumping factor A (ClfA) and Fibronectin-Binding Proteins B (FnBPB) on the immunogenicity of the antigen, CP was isolated and purified from serotype 8 (CP8) *Staphylococcus aureus* from bovine mastitis dairy herds in Inner Mongolia and ClfA-FnBPB was produced with Escherichia coli encoding a fusion protein ClfA-FnBPB gene. The CP8-ClfA-FnBPB complete antigen was constructed by first attaching Alcohol Dehydrogenase (ADH) to CP8 which was then combined with ClfA-FnBPB at the presence of EDC (1-ethyl-3, (3-di-methylaminopropyl carbodiimide). The immunogenicity of CP8, ClfA-FnBPB and CP8- ClfA-FnBPB was determined by intraperitoneal injecting 40 8-wk old of Balb/c mice four groups with 0.2 ml/mouse of 0.9% NaCl (Control) or CP8, ClfA-FnBPB and CP8-ClfA-FnBPB solution. The mice were group-fed with same pellet diet in an environmentally controlled room with a constant temperature of 22°C and 12h/24h light. The concentration of CP8, ClfA-FnBPB or CP-ClfA-FnBPB in respective solutions was 50, 25 or 75 μg mL^{-1}. Two injections were given, on d7 and d21 of the experiment. Blood samples were taken on d0, d14, d21 and d28 and measured for the antibodies titer. On d28, all mice were injected with CP8 type of *S. aureus* and the mortality of the mice determined for the following 14 days (challenge test). The results showed that ClfA-FnBPB was successfully expressed by *E coli* and the isolated and purified ClfA-FnBPB was successfully conjugated with CP8 to yield a CP8-ClfA-FnBPB complete antigen. Mice immunized with CP8-ClfA-FnBPB had higher antibody titer than that of CP8 ($p<0.01$) and ClfA-FnBPB ($p<0.05$) groups. Antibody titer in mice immunized with ClfA-FnBPB antigen was also higher ($p<0.05$) than that of CP8 and 0.9% NaCl groups. Survival rate of the mice at the end of 2-wk challenge test for Control, CP8, ClfA-FnBPB and CP8-ClfA-FnBPB groups were 20 30, 60 and 80% respectively. These results demonstrated that conjugating CP8 with ClfA-FnBPB to form CP8-ClfA-FnBPB complete antigen significantly increased immunogenicity of the antigen and markedly enhanced the survival rate of the mice infected with the bacteria. The information suggests that vaccine based on the CP8-ClfA-FnBPB antigen would be more effective in controlling bovine mastitis than those based on CP8 and ClfA-FnBPB alone, but further animal study is needed.

Keywords: *S. Aureus*, Capsular Polysaccharide, CP8-ClfA-FnBPB, Immunogenicity Mice

1. INTRODUCTION

Mastitis caused by the infection of *Staphylococcus aureus* occurs in dairy herds globally and is a serious disease threatening dairy industry in Inner Mongolia, a major dairy production region of China (Siqinmenghe, 2012). It has been regarded that the primary virulent factor in *S. aureus* infection is extracellular Capsule Polysaccharide (CP) serotype (Wu and Huang, 2003). Eleven serotypes of CP (CP1 to CP11) have been

Corresponding Author: Yongqing Hao, Faculty of Veterinary Medicine, Inner Mongolia Agricultural University, Hohhot, 010020, China

defined so far (Zhang and Wang, 2003), which exist in more than 90% of *S.aureus* strains. Among them, CP8 is the most prevalent serotype globally (Creuzenet and Joseph, 2001; Lin *et al*., 2005; Zhang *et al*., 2004). Our previous study showed that CP8 strains accounted 52.6% of high pathogenic *S.aureus* strains isolated from bovine mastitis in Inner Mongolia of China and the rest (47.4%) were other types and/or nontyped strains (Guo *et al*., 2011). The other factors determining the virulence of *S. aureus* are adhesins such as Staphylococcal Protein A (SPA), Fibronectin-Binding Proteins A (FnBPA) and B (FnBPB) and Clumping factor A (ClfA) and B (ClfB) proteins (Ni Eidhin *et al*., 1998; Visai *et al*., 2006; Wann *et al*., 2000) that play a crucial role in bacterial adhension and colonization to breast tissue (Stutz *et al*., 2011). Both CP and adhensins have individually been considered as potential antigen candidates for developing vaccine against *S. aureus* (Daum and Spellberg, 2012). However, the traditional vaccine available for mastitis (Chang *et al*., 2008; Middleton, 2008) is rather ineffective due to the fact that animal is lack of immune response to new infections (Reid and Szymanski, 2010). It has been suggested that vaccine produced from the combinations of antigens including CPs and adhensins would be an effective way against bovine mastitis caused by *S. aureus* (Lorena *et al*., 2008; Josefsson *et al*., 2001), but little information is available in this area.

The objectives of this study were to assess the immunogenicity of CP8, ClfA-FnBPB and CP8-ClfA-FnBPB antigens and to determine the efficacy of the antibody produced from these antigens against *S. aureus.*

2. MATERIALS AND METHODS

Monoclonal antibody of CP8 was produced from *S.aureus* anti-CP8 MAb 8E8 (Lot#237.118) by Dr. Tessie McNeely of Merck & Co., Inc; Rahway, NJ. US. Alcohol Dehydrogenase (ADH), 1-ethyl-3, (3-di-methylaminopropyl carbodiimide, EDC), N-Succinimidyl N-methylcarbamate (sulfo-NHS), DNase I, RNase A, Lysostaphin and Proteinase K were obtained from Sigma (St Louse, MS, US) and Sephacryls-300 was obtained from Duly Shanghai (Shanghai, China).

Isopropyl-β-d-thiogalactoside (IPTG) was obtained from TaKaRa (Dalian, China).

2.1. Construction of CP8-ClfA-FnBPB Complete Antigen

2.1.1. Extraction and Purification of CP8

The isolation and identification of CP8 serotype of *S. aureus* strains were reported by Guo *et al*. (2011). The extraction of CP8 was carried out using a method described by Fournier *et al*. (1984) and James *et al*. (2009) with some modifications. Briefly, the identified CP8 strain of *S. aureus* as described in Guo *et al*. (2011) was first cultured at 37°C on 200 Columbia blood agar plates for 24 h. Colonies of the bacteria in each plate were then suspended into 5 mL of phosphate buffer (PB, 10 mM; pH = 7.4) with scratching and suspensions from all plates were combined which was subsequently autoclaved at 121°C for 2h. The autoclaved cell suspension was centrifuged (3,500 × g, 30 min) and the supernatant (~ 400 mL) was added DNase (0.5 mg mL^{-1}), RNase A (25 μg mL^{-1}), lysostaphin (0.25 mg mL^{-1}) and proteinase K (1.25 mg mL^{-1}). The mixture was stored at room temperature for 24 h, filtered through a 0.22-μm filter membrane (PES, φ40, Haining Ltd., Zhejiang, China) thereafter and the filtrate was freeze-dried. The dried powder of CP8 crude extract was then dissolved into PB solution and purified by sephacryls-300 with method described by James *et al*. (2009), lyophilized and the dried powder was stored in sealed container at -20°C. The CP8 content of the purified extract was determined by phenol-sulfuric acid method Wang (2008) and the contents of protein, dsDNA, ssDNA and RNA were determined using a Nucleic Acid Protein Instrument (AG 22331, Eppendorf Hamburg).

2.2. Induction, Expression and Purification of ClfA-FnBPB

The induction and expression of ClfA-FnBPB was conducted with *Escherichia coli* encoding a fusion protein ClfA-FnBPB gene that was constructed in this laboratory (Fan, 2011). *E. coil* (BL21) was firstly pre-cultured in Liquid Broth (LB) medium for three generations. The overnight-grown third generation of *E. coli* suspension was inoculated to fresh LB medium at the ratio of 1:100 (v:v) and the culture was incubated at 37°C with horizontal shaking (200 rpm) for 16h. The culture was then added IPTG at the concentration of 1.0 mmol, incubated for a further 5.0 h and the bacteria cells were harvested by centrifugation (15,000×g, 4°C, 10 min). Subsample (500 mg) of the isolated bacterial pellet was then suspended into 5.0 ml of PB and lysozyme (Sigma, 10 mg mL^{-1}) was added to the final concentration of 1.0 mg mL^{-1}. The mixture was placed on ice for 2.0 h, followed by mixing with TritonX-100 in the ratio of 1:9 (V:V) and subsequently centrifuged (15,000× *g*, 4°C, 5 min). The resultant supernatant was analyzed by SDS-PAGE with a Bay Gene BG-Power 600 electrophoresis apparatus (Crystal Biological

Technology Co., Ltd, Beijing, China) and the image taken by MultiMate (Universal Hood Ⅱ,BIO-RAD,US). The molecular weight of the target ClfA-FnBPB protein was 51KD, which was purified using Ni^+ column using procedure described by Fan (2011). The purified ClfA - FnBPB was determined for protein content as described above and was divided into two portions and stored at -80°C prior to use.

2.3. Construction of Complete Antigen

The CP8 (78 mg) obtained from a strain as described above was firstly dissolved into 15 mL of PB, followed by addition of ADH to the final concentration of 0.5 mol/l. The solution, after adjusting pH to 6.5-7.2 with concentrate HCl was dialyzed (MW cutoff 8,000~12,000 Dalton; Shanghai Yuanye Biological Technology Co., Ltd) against 1,000 ml of 0.2 M NaCl solution for 2 d, with the dialyzing solution being changed every 12 h. At the end of the dialysis, the content in the dialysis bag that contained ADH modified CP8 was mixed with one portion of ClfA-FnBPB prepared above in the ratio (w:w) of 1:1, 2:1 or 3:1 (CP8:ClfA-FnBPB) and the EDC and sulfo-NHS were added to the final concentrations of 0.1 M and 8.0 mM respectively. The solution after adjusted to pH of 6.5-7.2 was dialyzed again against 0.2 M NaCl solution at 4°C overnight. The CP8-protein conjugates (conjugation of CP8-ClfA-FnBPB) in the dialyzed solution was then purified using method described by Yang (2009). The purified CP8- ClfA-FnBPB product was stored at -80°C before use.

2.4. Determination of Immunologenicity of CP8, ClfA-FnBPB and CP8-ClfA-FnBPB Antigens

Forty Balb/c mice (SPF, 8-wk old) were randomly divided into 4 groups, which were then randomly allocated to one of the following treatments: 0.9 %NaCl (Control), CP8, ClfA-FnBPB and CP- ClfA-FnBPB. The mice were group-fed in an environmentally controlled room with a constant temperature of 22°C and 12h /24h light. All mice were fed same pellet diet that was formulated to meet or exceed the nutrient requirements of the mice for *ad libitum* intake and had free access to water. After 7-d adaption, the mice in relevant group received the first immunization through intraperitoneal injection of 0.2 ml of freshly prepared 0.9% NaCl, CP8, ClfA-FnBPB or CP-ClfA-FnBPB solution. The CP8 was prepared by dissolving CP8 into 0.9% NaCl solution to designated concentration and ClfA-FnBPB and CP-ClfA-FnBPB solutions were prepared by diluted with 0.9% NaCl solution which was then mixed with equal volume of Freund's Complete Adjuvant solution. The final concentrations of CP8, ClfA-FnBPB and CP- ClfA-FnBPB in respective solutions were 50, 25 and 75 μg mL^{-1}. The second immunization was done on d14 (d 21 of the experiment) after the first immunization using the same procedure with Freund's Inomplete Adjuvant solution as that for the first immunization. Blood samples were collected into tubes sterilized from each mouse on d0, d14, d21 and d28 of the experiment through amputation of tail. The blood samples were kept at room temperature for 20 min to obtain serum and the antibodies to the CP, ClfA-FnBPB and CP-ClfA-FnBPB in the serum of relevant treatment was determined by indirect enzyme-linked immunosorbent assay (ELISA) with spectrophotometer (TU-1800PC, PERSEE, Beijing) using procedure described by Yang (2009).

2.5. In Vivo Challenge Testing

At d28 of the experiment (7 days after 2nd immunization), the 40 mice were intraperitoneally injected (0.2 mL per mouse) with a *S.aureus* ($CP8^+$) cell suspension using procedure described by Wang and Wu (2010). The homologous *S.aureus* ($CP8^+$) was first aerobically grown in LB broth medium for two subcultures 18~24h each and harvested through centrifugation (6,000×g, 15 min). Bacteria cell pellet was subsequently washed with 5.0 mL of PB once and the bacterial injection solution was prepared by suspending the bacterial pellet into PB to the cell density of about 1×10^9 colony forming unit. Immediately after injection, the mice were observed for mortality daily over the next 14 days. The dead mouse was removed from the treatment and *S.aureus* were isolated and identified using method of Guo *et al*. (2011). The live mice were killed by spinal dislocation on d14 after bacteria injection and the bacteria were isolated and identified as described above. The mortality of the mice was calculated as the percentage of dead mice in the total injected mice for each treatment over the 2-wk period.

All protocols of this study, treatment and disposal of the experimental animal were reviewed and approved by the Department of Veterinary science, Inner Mongolia Agricultural University.

2.6. Data Analysis

The data of immunologenicity assay and challenge test were statistically analyzed using IBM SPSS Statistics 19. Data obtained from two days of sampling in immunologenicity assay were analyzed separately using analysis of variance with antigens as main factors and

individual animal as statistical unit and data for survivability of mice in challenge test were analyzed by Chi-Square test Difference among treatment was determined by least square mean and declared at $p<0.05$.

3. RESULTS

3.1. Construction of CP8-ClfA-FnBPB Complete Antigen

The content and purity of CP8 in the CP crude extract were 34.43 and 79.68% respectively. The information for ClfA-FnBPB protein was shown in **Fig. 1**. A clear target band shown in **Fig. 1a** (column 2; crude extract) was corresponding to the 51KD sized marker protein (column M in figure). This showed that target ClfA-FnBPB protein was expressed successfully and it was soluble protein because this band of protein was not observed in precipitant (Column 1 in **Fig. 1a**). A major single band corresponding to the marker protein appeared after the extract being purified by Ni^+ column (column 1 in **Fig. 1b**). This indicated that purification by Ni^+ column increased the purity of target ClfA-FnBPB. The content of ClfA-FnBPB in the final product was 0.78 mg mL^{-1} with purity of 98%.

The CP8-ClfA-FnBPB complete antigen was obtained in this study by EDC with a bridge ADH. A major absorption peak in spectrophotometry was found at 210 nm for the constructed CP8-ClfA-FnBPB complete antigen whist this peak was observed at 206 and 217 nm for CP8 and ClfA-FnBPB respectively (**Fig. 2**). This indicated that CP8 was successfully conjugated with ClfA-FnBPB to form the CP8-ClfA-FnBPB complete antigen. The CP8 in the product was also confirmed to be conjugated to ClfA-FnBPB by phenol-sulfuric acid assay (data not shown). The contents of CP8 and ClfA-FnBPB for the CP8-ClfA-FnBPB conjugate were 499±10.4 and 262±4.8 μg mL^{-1} (mean±SD) respectively, resulting in a CP8:ClfA-FnBPB being 1.89:1 for the purified CP8-ClfA-FnBPB complete antigen.

3.2. Immunologenicity of CP8, ClfA-FnBPB and CP8- ClfA-FnBPB Antigens

No antibody titer was found from all mice at d 0 and d14 after the first immunization (data not shown). Antibody titer at d21 and d28 (7 and 14 d after the 2nd immunization) were significant different (**Fig. 3a and b**). Mice immunized with CP8 had only numerical higher antibody titer than that of immunized with 0.9% of NaCl at both d21 and d28. In contrast, mice immunized with CP8-ClfA-FnBPB had the highest antibody titer at d21, which was higher ($p<0.01$) than that of CP8 and ClfA-FnBPB groups. Antibody titer in mice immunized with ClfA-FnBPB antigen was also higher ($p<0.05$) than that of CP8 and 0.9%NaCl groups at d21. The same trend was also observed at d28. The numerical higher antibody titer in CP8 group than in Control indicated that CP8 alone had very weak immunogenicity although it plays key role in infecting animal by bacteria.

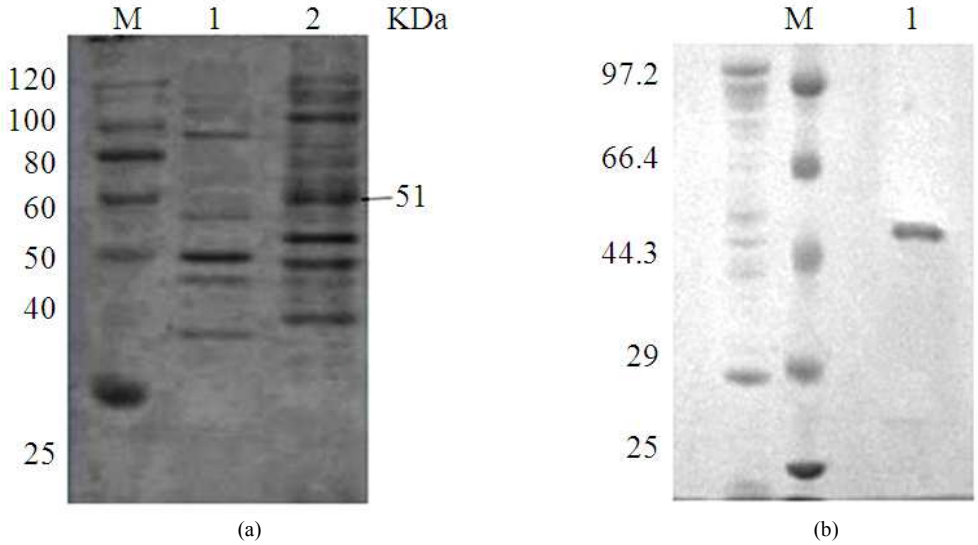

Fig. 1. Bacteria lysis analysis of pET-FnBPB-CLFA (a) and result of FnBPB-CLFA purification (b) M: Protein marker (low); A 1: pET-FnBPB-CLFA precipitate; 2: pET-FnBPB-CLFA supernatant. B 1: eluant of FnBPB-CLFA purified

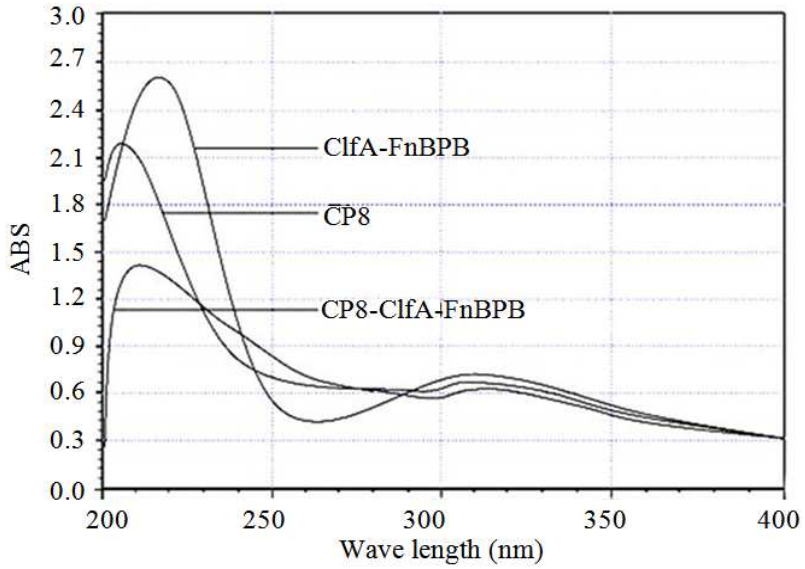

Fig. 2. Comparision of ultraviolet spectra of CP8, AlfA-FnBPB and CP8-ClfA-FnBPB

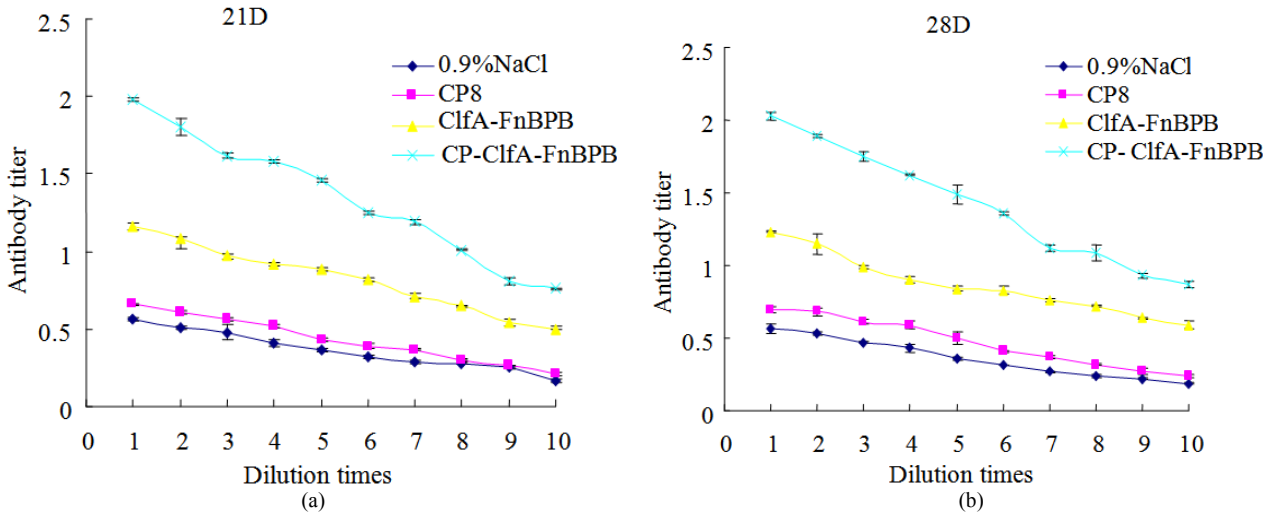

Fig. 3. Antibody titer in serum of mice immunized with 0.9%NaCl, CP8, ClfA-FnBPB or CP-ClfA-FnBPB on d 7 and d 14 of 2nd immunization (d 21 and d 28 of the 1st immunization). Dilution times were: 1, 1:50; 2, 1:100; 3, 1:200; 4, 1:400; 5, 1:800; 6, 1:1600; 7, 1:3200; 8, 1:6400; 9, 1:12800; 10, 1:25600

Table 1. Survival rate of mice that had been immunized with 0.9%NaCl, CP8, ClfA-FnBPB or CP- ClfA-FnBPB for 28 d followed by intraperitoneally injection of CP8 serotype of *S.aureus* cell suspension . The number was the sum of survived mice over the two week period after bacterial infection

Groups	Total infected	Survived	Survival rate (%)	Chi-square[a]	P value
0.9%Nacl	10	2 (8)*	20	13.438	0.004
CP8	10	3 (7)	30		
ClfA-FnBPB	10	6 (4)	60		
CP8-ClfA-FnBPB	10	8 (2)	80		

3.3. Challenge Test

Survival rate of the mice at the end of 2-wk challenge test differed ($p<0.05$) among the four treatment groups (**Table 1**). Compared with 20% for Control (immunized with 0.9% NaCl), survival rate for the groups immunized with CP8, ClfA-FnBPB or CP8-ClfA-FnBPB was increased to 30, 60 ($p<0.05$) and 80% ($p<0.01$) respectively. The survival rate of the mice immunized with CP8-ClfA-FnBPB was also higher ($P< 0.05$) than that of CP8 group, higher ($p<0.01$) than 0.9% NaCl, higher ($p<0.05$) than ClfA-FnBPB. *S. aureus* were isolated from all dead mice and were confirmed by both positive mannitol test and positive coagulase test. In contrast, no *S. aureus* were isolated from any survived mice slaughtered at the end of challenge test.

The survival rate of the *S. aureus* infected mice was consistent with antibody titer of each group as described above. Therefore, the highest survival rate of mice in CP8- ClfA- FnBPB immunized group was due to the greatest immunogenicity of the CP8- ClfA-FnBPB that stimulated highest antibody titer. This demonstrated that immunization animal with CP8-ClfA-FnBPB complete antigen is much more effective than with CP8 or ClfA-FnBPB alone in prevent animal from *S. aureus* infection.

4. DISCUSSION

Both physical and chemical methods have been used to couple CP with protein (Middleton, 2008). This study used chemical method with ADH as a bridge to couple CP with protein. The principle is that some functional groups existing in carrier can connect with half of an antigen material and ADH is needed as bridge to revitalize CP prior to making a coupling. Research has shown that coupling antigen with ADH produced increased immunogenicity (Liu *et al*., 2005; Pan, 2007) and the finding of the present research is in agreement with this. It has been shown that coupling CP with protein yielded greater antigenicity than CP alone (Woodland, 2004). Fetal bovine serum albumin, tetanus toxoid, *Pseudomonas aeruginosa* outside A poison, diphtheria toxin have been used as carrier protein with varying successes (Fattom *et al*., 2004; Mei *et al*., 2006; Pan, 2007; Liu *et al*., 2006). However, there is little information on-using ClfA-FnBPB as a carrier. This study showed that ClfA-FnBPB was successfully coagulated with CP8 forming complete antigen, indicating ClfA-FnBPB could be potentially effective protein carrier.

This is due to the fact that CP8 belongs to small molecular weight substances that usually possess weak or no immunogenicity (Katherine and Lee, 2004; Wu, 2011). Similarly, the higher antibody titer observed in ClfA-FnBPB group than that in Control and in CP8 is also likely due to the increased molecule size of the ClfA-FnBPB. The markedly higher antibody titer in CP8- ClfA-FnBPB group than in other groups indicated that coupling CP8 with ClfA-FnBPB significantly increased immunogenicity of ClfA-FnBPB. Because CP belonged to T-Independent Antigen (TI-Ag), connecting it conjugately with protein, would transfer it into T-Dependent Antigen (TD-Ag) and yield enhanced immunologenicity. It has also been suggested that CP would become effective antigen when it is connected with carrier protein into conjugates (Wu, 2011). Other researches have showed that CP itself had weaker antigenicity that was unable to stimulate animal's body to produce antibody. John *et al*. (2011) also suggested that immunogenicity of conjugation of CPs and bacteria protein would be considerably higher than that of CPs alone.

Reasearchers (Lorena *et al*., 2008; Gaudreau *et al*., 2007; Broullette *et al*., 2002) also reported that immunization with protein conjugation vaccines were used efficently. CPs-ClfA conjugation was more effective than CPs and ClfA separately in preventing staphylococcal infection in a mouse model of mastitis. Further research is need to evaluate the immunogenicity of the CP8- ClfA- FnBPB in large animals.

5. CONCLUSION

CP8 and ClfA-FnBPB were successfully conjugated to produce CP8-ClfA-FnBPB complete antigen. Conjugation of ClfA-FnBPB with CP8 markedly increased level of antibody titre, immunogenicity of the antigen and survival rate of the infected animal as compared to CP8 or ClfA-FnBPB alone. Therefore, developing vaccine based on CP8-ClfA-FnBPB complete antigen is a potential strategy to battle with bovine mastitis.

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