Scholars Journal of Engineering and Technology

OPEN ACCESS

Abbreviated Key Title: Sch J Eng Tech
ISSN 2347-9523 (Print) | ISSN 2321-435X (Online)
Journal homepage: https://saspublishers.com/journal/sjet/home

Expression and Immunogenicity of ClfB/Lbp Chimeric Protein in *Staphylococcus Aureus*

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DOI: 10.36347/sjet.2019.v07i12.003 | **Received:** 20.12.2019 | **Accepted:** 27.12.2019 | **Published:** 30.12.2019

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Abstract **Original Research Article**

Staphylococcus aureus (*S. aureus*) is an important pathogen causing the mastitis of dairy cows, which often causes great economic losses. In order to prevent the mastitis of dairy cows caused by *S. aureus*, a successful recombinant plasmid pET-32a-ClfB (ClfB, Clumping factor B), pET-32a-Lbp (lbp, lamin binding protein), and pET- 32 a-ClfB'/ Lbp were transformed into Rosetta (DE3), induced expression and purified. The immunogen was prepared by mixing purified protein and adjuvant to immunize mice and challenge the virus, and the protective difference between single protein immunization and chimerism protein immunization was compared. The results showed that we successfully expressed and purified the protein. After immunizing mice, chimeric proteins were immunized with a single protein, which greatly improved the protective efficiency. This study will lay a foundation for further study of effective vaccines to prevent *S.aureus* infection.

Keywords: *Staphylococcus aureus*, Chimeric protein, Immunogenicity.

INTRODUCTION

Staphylococcus aureus (*S.aureus*) is a kind of opportunistic pathogenic bacteria, which often lives on the surface of skin and mucous membrane, invades the body under certain conditions, grows and proliferates in tissues and blood, and can cause many kinds of diseases. *S. aureus* can form biofilm in mammary gland tissue. It often recurred after the treatment of mastitis with antibiotics, resulting in the alternation of recessive mastitis and clinical mastitis in dairy cows, leading to the delay of the disease. With the emergence of a variety of *S. aureus* resistant strains, the treatment of cow mastitis caused by *S. aureus* is facing great difficulties [1]. Therefore, how to protect dairy cows from mastitis caused by *S. aureus* is an urgent problem.

S. aureus expresses a series of secretory proteins or cell surface-related proteins, which are important virulence factors of *S. aureus*, such as proteins that promote adhesion to damaged tissues or host cells, surface proteins that can bind to proteins in blood to escape immune response and surface proteins that promote iron absorption. Most strains express polysaccharide capsule. In the stage of host infection and the initiation of immune response, the invading microorganisms and their products were swallowed into the lymph nodes by macrophages and other antigen-presenting cells and transported to the lymph nodes. B cells differentiated, secreted antibodies to fix toxins, and promoted the phagocytosis of bacterial cells more effectively [2]. However, this immune response can not effectively defend against *S. aureus*. Almost every individual animal has antibodies against the antigen and increases with the dose of infection, but those antibodies and immune memory do not effectively prevent the next infection. Therefore, many researchers have been studying a protein with good immunogenicity and high protection rate for many years. However, it was found that the combined immunization of single protein or several proteins could not achieve a good immune effect because of the high virulence factors of *S.aureus*. It has been reported that the immunogenicity and protective rate of chimeric expressed proteins are significantly higher than those of single protein or several proteins [3]. For the above reasons, according to the frequency of *S.aureus* virulence factors stored in the laboratory and the related literature reports, the coagulation factor B (Clumping f was selected. Actor B, ClfB and laminin binding protein (lbp)-related genes are used as the target gene for protein expression, and the immunogen is prepared to prevent the mastitis of dairy cows caused by *S. aureus*. The study will provide a good foundation for further

research on the effective prevention of *S. aureus* infection.

MATERIALS AND METHODS

MATERIALS

Bacteria and Plasmids

The recombinant plasmid pET-32a-ClfB, pET-32a-Lbp, pET-32a-ClfB and pET-32a-ClfB, pET-32a-ClfB and pET-32a-ClfB-/ Lbp of the *S. aureus* standard strain, wod46, BL21, and Rosetta (DE3), were stored in the cell biology laboratory of the Life Science and Technology College of the University of Agricultural Science and Technology of Heilongjiang Province. Kunming-series clean-grade mice were purchased from Changchun biological products, China.

Main Reagents and Antibodies

Protein Ruler I was purchased from Beijing All-type Gold Biotech Co., Ltd., the product of IPTG, kanamycin, and trypsin Inhbitor Agar is the product of the company of Sigma, MagneHisTM Ni-Particles was purchased from Promega, tryptone and yeast extract for bacterial culture were OXOID products, and TSB medium for bacterial culture was DB products. Topure plasmid kit and glue were purchased from GeneTech Ltd. HRP labeled sheep anti-mouse IgG monoclonal antibody was purchased from Zhongshan Jinqiao Company. Anti his tag monoclonal antibody was purchased from New England Biolabs.

METHOD

Expression and Purification of Protein

In the previous work, PCR primers were designed according to the published *S. aureus* ClfB, Lbp gene sequence of Genbank, and the ClfB, Lbp gene was amplified from *S. aureus* standard strain wood46, and the ClfB`/ Lbp gene sequence was obtained by overlap PCR. Then the three genes were constructed into the prokaryotic expression vector of pET-32a.

In this experiment, the recombinant plasmid pET-32a-ClfB,pET-32a-Lbp,pET-32a- ClfB`/ Lbp containing the correct sequence was transformed into the transformed colony of the expressing strain BL21, and inoculated into 5 mL LB medium containing ampicillin ($\mu g \cdot mL^{-1}$). The strain was cultured at 37 ℃ for 10 h. 1: 100 was inoculated in 500 mL LB medium containing ampicillin (100 $\mu g \cdot mL^{-1}$), cultured at 37 ℃ for 2 h, A600 was 0.3 ~ 0.4, and the final concentration of IPTG was 0.25 $mmol \cdot L^{-1}$. After 3 hours, the induced and uninduced bacteria were taken for 12% SDS-PAGE electrophoresis and the percentage of the expressed target protein to the total bacterial protein was determined [4]. The bacteria were centrifuged by ultrasonic fragmentation and centrifuged by HisTrapTM FF crude colum affinity chromatography. The proteins were eluted with miscellaneous protein eluent (40 mmol $\cdot L^{-1}$ imidazole, 300 mmol $\cdot L^{-1}$ NaCl, 50 mmol $\cdot L^{-1}$ Na_3PO_4, pH 8.0). The eluted proteins were eluted with eluent (500 mmol ·L-1 NaCl, 50 mmol ·L-1 Na_3PO_4, pH 8.0. The eluting protein was eluted with the eluent solution (500 mmol $\cdot L^{-1}$ imidazole, 300 mmol $\cdot L^{-1}$ NaCl, 50 mmol $\cdot L^{-1}$ Na_3PO_4, pH 8.0. The eluting protein was eluted with the eluent peak。

Preparation of Immunogens

The purified protein was diluted with PBS to a concentration of 1 $mg \cdot mL^{-1}$ and then emulsified in two syringes in a 1:1 manner with Freund's complete adjuvant for the preparation of the first immunoprotein. The second immune recombinant protein subunit vaccine is prepared by mixing the immunogen with the same concentration and proportion with the Freund's incomplete adjuvant.

Animal Immunization

Eighty healthy mice were randomly divided into 4 groups with 20 mice in each group. The results were as follows: ClfB immunization group, LBP immunization group, ClfB'/Lbp` immunization group and adjuvant immunization control group. The mice were immunized subcutaneously with the dose of 100 μg/ mice, for the second week after the first immunization, and the mice were challenged for two weeks after the second immunization. The death of the mice within one week was recorded [5]. The IgG content of specific antigen in serum samples isolated three weeks after immunization and two weeks after immunization was detected by indirect ELISA. The specific steps are as follows: after the antigen is diluted by 5$\mu g \cdot mL^{-1}$, a 96-well enzyme-labeled plate (0.5μg/well) is coated per well of 100 μL per well, overnight at 4℃, washed with 5% PBST for 3 times, each time is 5 min, 100 μL PBST-diluted skim milk powder blocking solution is added per well, incubated for 1 hour at 37℃, and washed with PBST for 3 times, 5 min each, add 100 μL of PBS-diluted to-be-tested immune serum per well, incubate at 37℃ for 1 h; then wash with PBST for 3 times, 5 min each time, 100 μL of PBS dilution per well Goat anti-Mouse IgG-Peroxidase II, incubated at 37℃ for 1 h, washed 3 times with PBST, 5 min each time,100 μL of TMB solution per well, and protected from light for 15 min at room temperature, 50 μL of 2 mol $\cdot L^{-1}$ sulfuric acid termination solution was added to each well, and the absorbance at 450 nm was measured with a microplate reader.

Challenge Test for Mice

Taking the Wood46 strain liquid stored at -80℃ in a TSB solid plate for streaking, culturing for 24 h, picking up a single colony, inoculating and culturing in a 5 mL TSB culture medium overnight to forming a strain, inoculating to the non-resistant TSB liquid culture medium according to the amount of 1% of the inoculation amount, and culturing for 8 hours at 37℃, the number of bacteria was determined by plate counting, and the bacteria in the logarithmic growth phase were washed, centrifuged and diluted with PBS. The challenge test [6] was conducted two weeks after

the second immunization as determined by the absolute lethal dose of the mouse. The immune efficacy of the recombinant protein, ClfB, LBP, ClfB`/Lbp`, was determined daily on the death of the mice in one week.

RESULTS AND ANALYSIS

Expression, purification and identification of protein

The BL21 strain containing recombinant plasmid pET-32a-eno was inoculated and induced by IPTG for 3 hours. The protein electrophoresis band was located at the molecular weight of about 52kDa. The collected bacteria were broken and centrifuged by ultrasound, and the culture fluid was desalinated by AKTA purifier 100. The proteins were collected and analyzed by electrophoresis. Because the expressed protein can be detected in the upper serum, it can be seen that the protein is soluble expression, and the expression of the protein is consistent with the expected size.

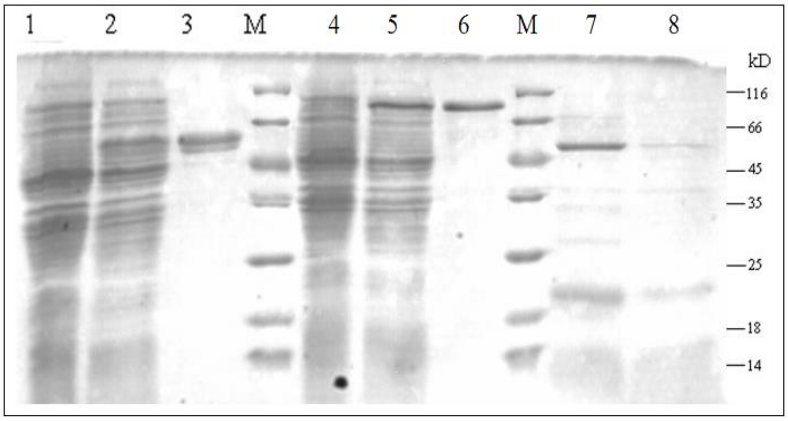

Fig-1: SDS-PAGE result of purified recombinant protein ClfB, LBP and ClfB`/Lbp`

Note, 1-Expressed protein ClfB after purified, 2-Expressed protein LBP after purified, 3-Expressed protein ClfB`/Lbp` after purified, M-Protein marker

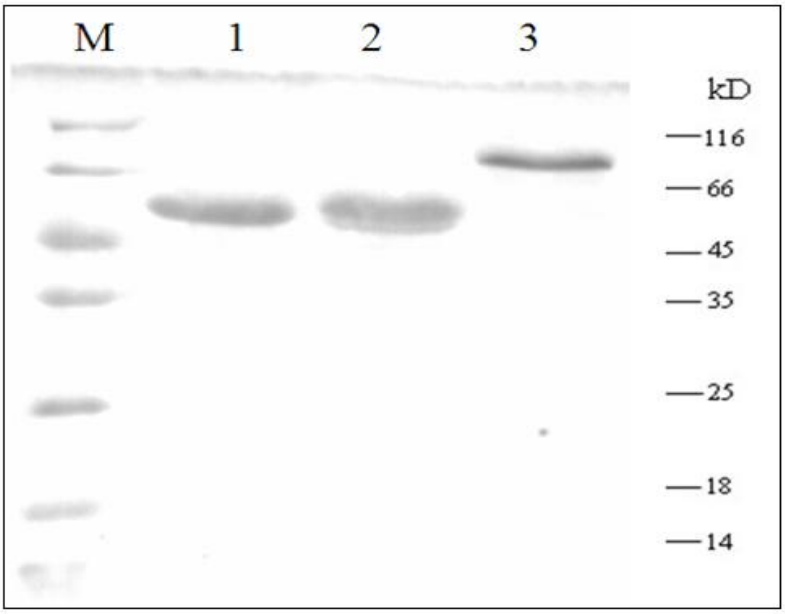

Fig-2: SDS-PAGE result of purified recombinant protein ClfB, LBP and ClfB`/Lbp`

Note, 1-Expressed protein ClfB after purified, 2-Expressed protein LBP after purified, 3-Expressed protein ClfB`/Lbp` after purified, M-Protein marker

Determination of Antibody Potency

The serum of the first immunized mice was diluted 1 000 times, then diluted twice (serum of three mice in each group), that is, 2 000, 4 000, 8 000, 16 000, 32 000, 64 000 times diluted, and the serum of the second immunized mice was diluted with 4 000, 8 000, 16 000, 32 000, 64 000, 128 000, 256 000, 512 000 times, and then indirect ELISA was detected with HRP labeled sheep anti-mouse IgG monoclonal antibody as the second antibody. The average value plus 3 times standard deviation was positive. The titer of ELISA antibody in serum is shown in Table-1. The results showed that the titer of IgG could be as high as 1:32000 to 1:128000 two weeks later.

Table-1: Antibody IgG titres of serums from immunized mouse

Days	ClfB`/Lbp`			LBP			ClfB		
	#1	#2	#3	#1	#2	#3	#1	#2	#3
7	1:<1000	1:2000	1:1000	1:1000	1:1000	<1:1000	1:1000	<1:1000	1:1000
14	1:2000	1:4000	1:4000	1:2000	1:1000	1:1000	1:4000	1:2000	1:4000
21	1:8000	1:8000	1:16000	1:16000	1:8000	1:4000	1:8000	1:8000	1:16000
28	1:64000	1:64000	1:128000	1:64000	1:32000	1:32000	1:32000	1:32000	1:64000
35	1:64000	1:64000	1:128000	1:32000	1:32000	1:32000	1:32000	1:64000	1:64000

Determination of antibody growth and decline level

In order to compare the antibody level of clfb`/LBP` protein immune group with that of each single control protein immune group and non immune group, the serum of one week, two weeks, three weeks, were diluted by 1:100 times, and the antibody of sheep anti mouse IgG mono croton labeled with HRP was used as the second antibody for indirect ELISA, and its OD450 value was measured [7]. The results showed that the titer increased rapidly after immunization and became stable one week later. The results are shown in Table-2 and Figure-3.

Table-2: Recombinant protein antibody IgG titres of serums from immunized mouse

Days	7	14	21	28	35
Control	0.129±0.011	0.122±0.014	0.098±0.007	0.092±0.006	0.091±0.001
Mock	0.094±0.013	0.109±0.001	0.102±0.005	0.081±0.002	0.085±0.005
Lbp	0.199±0.148	0.278±0.024	0.709±0.036	0.998±0.061	0.714±0.070
ClfB`/Lbp`	0.212±0.148	0.716±0.024	0.735±0.036	1.184±0.061	0.926±0.070
ClfB	0.211±0.148	0.283±0.024	0.715±0.036	1.034±0.061	0.725±0.070

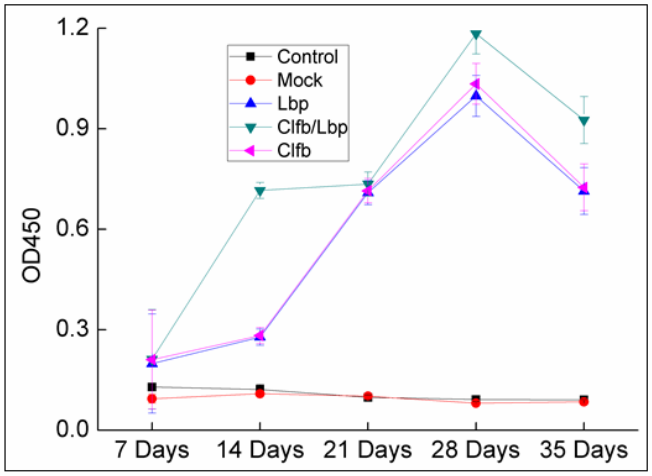

Fig-3: Recombinant protein antibody IgG titres of serums from immunized mouse

Immune Protection Test Results

Two weeks after the second immunization, the experimental animals in each protein immune group and control group were challenged with *S. aureus* wood46 strain respectively, with an absolute lethal dose of 6×10^8 CFU. The death of experimental animals was observed for 7 days after the attack. The results are shown in Table-3 and Figure-4.

Table-3: Mice were challenged with *S. aureus* Wood46

Day	0	1	2	3	4	5	6	7
Negative Control	12	0	0	0	0	0	0	0
Mock	12	1	0	0	0	0	0	0
LBP	12	6	5	5	4	4	4	4
ClfB	12	6	5	5	5	4	4	4
ClfB`/Lbp`	12	8	7	7	7	7	7	7

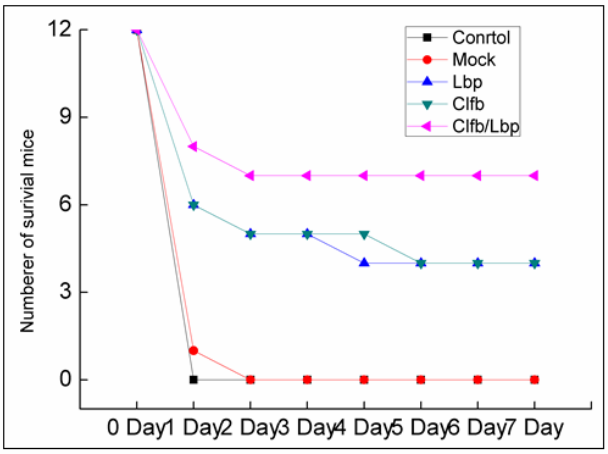

Fig-4: Mice were challenged with *S. aureus* Wood46

DISCUSSION AND CONCLUSION

Since the discovery of *S. aureus*, people have taken the defense against invasive *S. aureus* infection as a target. In 1965, Rogers could not produce a good immune protective response with whole cell live vaccine or inactivated vaccine [8]. Type 5 and type 8 capsular polysaccharide conjugate vaccines are in the third phase of clinical practice, and the protection rate is good. The mice were immunized with multin-acetylglucosamine encoded by icaadb operon, which had a protective effect on *S. aureus*-induced infection. The immune response of subunit vaccine composed of single surface protein, such as cohesion factor A (CLFA), cohesion factor B (clfb), ISDB, FnBPs and so on, provided partial protection for *S. aureus* to attack the experimental animals.

In 1998, eidhin discovered a new *S. aureus* fibrinogen binding adhesin, which was named as cohesion factor B (clfb). Clfb is a bacterial surface protein, which is expressed in the early stage of bacterial logarithmic growth and can bind to fibrinogen and 1-type cytokeratin molecule K10. CLFA and clfb have similar composition and a lot of sequence similarity [9]. However, only 26% of their fibrinogen binding domains (a domains) are identical. CLFA interacts with the γ chain of fibrinogen, clfb interacts with the α-chain and β-chain of fibrinogen. In 2006, Adam et al. found that clfb was related to *S. aureus* colonization in mouse model. Immunization with rclfb can reduce the colonization of *S. aureus* in the nasal cavity.

The LBP protein exists on the cell surface, with a molecular size of 52 kDa, and binds to laminin. Laminin is the most abundant extracellular matrix protein in host cells. LBP binds to laminin and is related to its invasive phenotype. The binding ability to fibroin is related to the pathogenicity of *S. aureus*. So it is important to use LBP as the target gene to produce vaccine [10].

In 2006, Moisan analyzed virulence factors by transcriptome and found that clfb and LBP were differentially expressed. The differential expression of clfb and LBP was also found in the proteomic analysis of biofilm formation and plankton cells [11]. In 2008, Baba Moussa *et al*., tested the virulence factors of *S. aureus* clinical isolates, and found that only clfb and LBP genes existed in all strains, and had nothing to do with the source of the strain [12]. This is consistent with other people's research.

This project is to use gene engineering method to chime and express *S. aureus* virulence factor clfb and LBP, enhance the immunogenicity of the protein, improve the humoral immune response and cellular immune response of the body, so as to achieve the purpose of preventing *S. aureus* infection and avoiding the occurrence of mastitis in dairy cows. It can be seen from the above experiments that we have successfully expressed and purified three kinds of foreign proteins. Through immunoassay, it is found that the immunoprotective effect of recombinant chimeric protein clfb`/LBP` is better than that of clfb and LBP respectively. This study will lay a good foundation for further research on the effective vaccine against *S. aureus* infection.

ACKNOWLEDGEMENTS

This work was supported by Innovation and Entrepreneurship Program for College Students in Heilongjiang Province (201910223085).

REFERENCE

1. Herron-Olson L, Fitzgerald JR, Musser JM, Kapur V. Molecular correlates of host specialization in *Staphylococcus aureus*. PLoS One. 2007 Oct 31; 2(10):e1120.
2. Tawaf R. Physical and technical feasibility procedure slaughter at the Government Abattoir in West Java, Seminar Nasional Peternakan Berkelanjutan ke 5 Fapet Unpad, 2013.
3. Hisatsune J, Hagiya H, Shiota S, Sugai M. Complete genome sequence of systemically disseminated sequence type 8 staphylococcal cassette chromosome mec type IVl community-acquired methicillin-resistant *Staphylococcus aureus*. Genome Announc. 2017 Aug 31;5(35):e00852-17.
4. Directorate General of Livestock and Animal Health, Realizing the Indonesia Slaughtering Management that has the Principle of Animal Welfare, Te seminar of indolivestock held by PB ISPI-PDHI, Jakarta, Indonesia, 2012.
5. Soepranianondo K, Wardhana DK. Analysis of bacterial contamination and antibiotic residue of beef meat from city slaughterhouses in East Java Province, Indonesia. Veterinary World. 2019;12(2):243-248.
6. Prawesthirini S, Siswanto HP, Estoepangestie ATS. Analysis of Meat, Milk, and Egg Quality,

Surabaya, Indonesia, 8th edition, 2016.
7. Petersen TN, Brunak S, Von HG, Nielsen H. Signal P 4.0: discriminating signal peptides from transmem-brane regions. Nat Methods. 2011 Sep 29; 8(10):785-786.
8. Pele J, Becu JM, Abdi H, Chabbert M. Bios2mds: an R package for comparing orthologous protein fami-lies by metric multidimensional scaling. BMC Bioinformatics. 2012 Jun 15;13:133.
9. Nowak A, Rygala A, Oltuszak-Walczak EP, Walczak P. Te prevalence and some metabolic traits of Brochothrixthermosphacta in meat and meat products packaged in different ways. Journal of the Science of Food and Agriculture, 2012;92(6):1304-1310.
10. Capra E, Cremonesi P, Pietrelli A, Puccio S, Luini M, Stella A, Castiglioni B. Genomic and transcriptomic comparison between Staphylococcus aureus strains associated with high and low within herd prevalence of intra-mammary infection. BMC microbiology. 2017 Dec;17(1):21.
11. Parkhill J, Wren BW. Bacterial epidemiology and biology—lessons from genome sequencing. Genome Biol. 2011; 12(10):230.
12. Bakthtiary F, Sayevandi HR, Remely M, Hippe B, Hosseini H, Halsberger AG. Evaluation of bacterial contamination sources in meat production line. Journal of food quality, 2016;39(6):750-756.