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**Original Research Article**

# Construction and evaluation of a novel triple cell epitope-based polypeptide vaccine against cow mastitis induced by Staphylococcus aureus, Escherichia coli and Streptococcus

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## Abstract

***Purpose:*** *To construct a novel triple cell epitope-based polypeptide vaccine against cow mastitis induced by Staphylococcus aureus, Escherichia coli and Streptococcus and to reduce the use of antibiotics.*
***Methods:*** *Based on bioinformatics approach, a novel triple epitope-based polypeptide (CM-TEP) was designed and subjected to Ni-NTA flow resin purification. Purified CM-TEP was immunized into mice to prepare a polyclonal antibody. Pull-down assays and enzyme-linked immunosorbent assay (ELISA) were used to detect the interaction between CM-TEP antibodies and S. aureus, E. coli and Streptococcus. Active immunity mice and challenge of bacterial pathogens were used to detect immune protection of CM-TEP. Additionally, the optimal expressing conditions of CM-TEP strain were analyzed using orthogonal test design.*
***Results:*** *A novel cow mastitis triple cell epitope-based polypeptide (CM-TEP) with a MW of 36 kDa was designed, purified and used to immunize mice to prepare a polyclonal antibody. Pull-down assays and ELISA data showed that CM-TEP antibodies directly interacted with S. aureus, E. coli and Streptococcus. CM-TEP displayed a significant immune protective effect against infection by S. aureus (50 %, $p < 0.05$) and E. coli (54.54 %, $p < 0.05$) and provided some immune protective effect (30.78 %, $p > 0.05$) against Streptococcus. The optimum expressing conditions of CM-TEP were as follows: IPTG concentration of 0.3 mmol/L, strain OD600 value of 1, inducing temperature of 37 $^{o}$C, and inducing time of 8 h.*
***Conclusion:*** *The findings suggest that epitope-based vaccine of CM-TEP may be a useful strategy for treating cow mastitis induced by S. aureus, E. coli and Streptococcus.*

***Keywords:*** *Cow mastitis, Epitope vaccine, Immunogenicity, Immune protective*

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## INTRODUCTION

Cow mastitis is the most frequent and costly disease affecting dairy products worldwide [1], and its main pathogens include *Staphylococcus aureus* (*S. aureus*), *Streptococcus* and *Escherichia coli* (*E. coli*). At present, using antibiotics to treat mastitis can cause drug residues to be present in dairy products and can lead to the development of antibiotic resistance [2]. However, a single-dose vaccine that is effective against all three major bacterial

pathogens of cow mastitis has not been developed.

Epitope vaccines are a new type of vaccines that have been developed in recent years. Compared with traditional vaccines, epitope vaccines can easily be recognized by major histocompatibility complex (MHC) molecules with different genetic backgrounds, and they have been studied for use against viruses and bacteria and in cancer immunotherapy [3,4]. To date, research has shown that many proteins are good candidates for mastitis vaccine, including clumping factor A (ClfA) and elastin binding protein (EbpS) of *S. aureus* [5,6], outer membrane protein C (OmpC) and outer membrane protein A (OmpA) of *E. coli* [7,8], and surface immunogenic protein (SIP) and phosphoglycerate kinase (PGK) of *Streptococcus* [9,10]. This study sought to construct and evaluate a novel triple-cell epitope-based vaccine for cow mastitis (CM-TEP), using these six proteins.

## EXPERIMENTAL

### Chemicals and reagents

(HRP)-conjugated secondary antibodies, PBS and TMB were purchased from Sigma. Freund's Complete Adjuvant and Freund's Incomplete Adjuvant was purchased from Shanghai Sangon Biotech. Corp (Shanghai, China). CM-TEP nucleic acid sequences were synthesized and cloned in *E. coli* BL21 by the Shanghai Xuguan Biotech Development Corp (Shanghai, China).

### Animals

SPF Kunming mice (6 weeks old) were purchased from Xian Jiaotong University College of Medicine (Xian, China). All the animal experimental protocols were performed in accordance with the guidelines prescribed in Guide for the Care and Use of Laboratory Animals [11] and were approved by the Animal Ethical Committee (ref. no. 20150208), Shaanxi University of Technology, China.

### Protein materials

According to the amino acid sequence deposited in NCBI GenBank, the accession numbers of ClfA and EbpS proteins of *S. aureus* are EFM05894.1 and EFM06457.1, respectively; OmpC and OmpA of *E. coli* are AIL15476.1 and ELV73478.1, respectively; and SIP and PGK of *Streptococcus* are AEL21614.1 and AKU04271.1, respectively.

### Cell epitope analysis

ABCpred (http://www.imtech.res.in/raghava/abcpred/ABC_submission.html) and the BepiPred 1.0b (http://www.cbs.dtu.dk/services/BepiPred/) [12] software programs were used to predict the B cell epitopes of the proteins. nHLAPred (http://www.imtech.res.in/raghava/nhlapred/comp.html) [3] and ProPred (http://www.imtech.res.in/raghava/propred/) [4] were used to predict the cell epitope peptide segments of helper T cells (Th) and cytotoxic T lymphocytes (CTL), respectively. Four glycine residues (GGGG) were inserted into each epitope, and DNASTAR software was used to optimize the arrangement of the cell epitopes. The optimal antigenic combination was chosen as the final triple epitope polypeptide vaccine for cow mastitis (CM-TEP). The corresponding nucleic acid sequences were obtained by translation of the CM-TEP sequence.

### Prokaryotic expression, purification and antiserum preparation of CM-TEP

CM-TEP nucleic acid sequences were synthesized and cloned in *E. coli* BL21 by the Shanghai Xuguan Biotech Development Corp., China. Prokaryotic expression and purification were performed as described previously [13]. Five-week-old Kunming mice were administered purified protein (100 μg per mouse) emulsified with Freund's Complete Adjuvant (Shanghai Sangon Biotech. Corp., China), then given two injections of Freund's Incomplete Adjuvant (Shanghai Sangon Biotech. Corp., China) at intervals of 14 days. Serum samples were collected from the mice and stored at −80 °C.

### Pull-down assays and ELISA to detect the interaction between CM-TEP antiserum and bacteria

The bacterial pull-down assay was implemented as described previously [13]. Briefly, Bacterial cultures at $OD_{600}$ 1.0 were harvested by centrifugation, and the bacteria were washed with physiological saline (0.85 % NaCl). After the addition of 1 % oxymethylene (W/V) for 90 min at 80 °C, the bacterial pellet was resuspended in physiological saline, and the final concentration was adjusted to 0.2 OD at 600 nm. One milliliter of bacterial suspension containing approximately $10^8$ CFU/mL bacterial cells was transferred into each 1.5-mL tube. After centrifugation, 100 μL of CM-TEP antiserum at various dilutions was added to the tubes and incubated for 1 h at 37 °C. Additionally, 2 μg/μL BSA was used as the negative control. After being washed, the bacteria were combined with rabbit anti-mouse

secondary antibodies at a dilution of 1:3000 for 1 h at 37 °C and washed again with PBS. After suspension of the bacteria in 20 µL of PBS, the samples were transferred to enzyme-labeled plates, and coloration liquid (50 µL of $H_2O_2$ and 50 µL of TMB) was added to each well. Plates were maintained in the dark at 37 °C to allow the color reaction to develop. After 10 min, 50 µL of stop solution (2 M $H_2SO_4$) was added to each well, and the absorbance was read at 450 nm using a microplate reader (Bio-Rad, USA).

### Active immunity and challenge

Mice were randomly divided into groups, and purified CM-TEP protein was intraperitoneally injected two times at an interval of 10 days, to deliver the primary and booster doses. Freund's Complete Adjuvant and Freund's Incomplete Adjuvant emulsified with CM-TEP protein (100 µg per mouse) was utilized in the primary and booster immunizations, respectively. The control groups were immunized with Freund's adjuvant. Immunized mice were intraperitoneally challenged with *S. aureus*, *E. coli* and *Streptococcus* at one week after the second immunization. Mice were observed for 15 days to measure their relative percentage survival (RPS). Protection rates were expressed as in Eq 1.

$$RPS = \left[1 - \frac{Nt}{Nc}\right] 100 \quad \text{..... (1)}$$

where *Nt* represents the vaccinated mortality in test group (triple epitope polypeptide vaccine of cow mastitis, CM-TEP), and *Nc* the non-vaccinated mortality for control group. Statistical significance between groups was tested using SPSS software.

### Optimization of expression condition of CM-TEP

In order to optimize the expression conditions of CM-TEP protein, we have chosen orthogonal experiment design $L_9(3^4)$ [14], which is a four factors and three-level orthogonal design, and the factors are as follows: strain $OD_{600}$ value (A), IPTG concentration (B), inducing time (C), and inducing temperature (D). In brief, according to $L_9(3^4)$ matrix mode, once the concentration of bacterial culture was reached, different concentrations of IPTG was added to the culture to induce protein production with appropriate temperature. Bacterial cells were harvested at the corresponding time based $L_9(3^4)$ matrix, heated for 5 min in boiling water and the proteins were electrophoresed by SDS-PAGE. The protein bands were visualized by staining with G-250. The optical density of CM-TEP protein bands was analyzed by Phoretix 1D software. Statistical analysis of the dataset was conducted using Statistical Package for the Social Science (SPSS) software. The experiment was repeated three times.

## RESULTS

### *In silico* prediction of B cell epitopes

B cell epitopes of proteins were predicted by using the BepiPred 1.0b (Table 1) and ABCpred (Table 2) programs. On the basis of the common epitope sequence predicted by the two programs, the B cell epitope segment of *S. aureus* ClfA protein was at residues 114 – 129 and 326 – 330, and that of the Ebps protein was at residues 87 – 102 and 132 – 147. The B cell epitope segment of *E. coli* OmpA protein was at residues 44 – 50 and 126 – 135, and that of the OmpC protein was at residues 19 – 31 and 129 – 144. The B cell epitope segment of *Streptococcus* SIP protein was at residues 224 – 238 and 346 – 358, and that of the PGK protein was at residues 64 – 79 and 324 – 329.

### *In silico* prediction of CTL cell epitopes

On the basis of the nHLAPred method, the CTL cell epitope segment of Ebps and ClfA protein was at residues 318 – 326 and 895 – 902, respectively; the OmpA and OmpC of *E. coli* were at residues 237 – 245 and 295 – 303, respectively; and the *Streptococcus* PGK protein was at resides 73 – 81, but the SIP protein did not display a CTL cell epitope (Table 3).

**Table 1:** Identification of B cell epitopes for proteins by using the BepiPred 1.0b method

| Protein name | Peptide segment position |
|---|---|
| Ebps | 6-60, 69-121, 127-318, 346-446, 457-467, 473-481 |
| ClfA | 34-203, 205-211, 223-234, 242-254, 264-272, 288-299, 323-330, 338-347, 355-362, 385-394, 399-407, 415-428, 464-484, |
| OmpA | 19-30, 44-59, 61-67, 83-95, 126-145, 165-181 |
| OmpC | 19-31, 46-55, 64-93, 129-144, 170-212, 221-252, 264-270, 277-283, 315-321 |
| SIP | 23-35, 45-56, 97-104, 107-125, 144-154, 160-169, 179-216, 218-238, 242-249, 253-288, 290-325, 334-342, 346-358, 375-384, 402-423 |
| PGK | 64-76, 120-136, 188-194, 269-277, 280-290, 324-334, 344-359 |

**Table 2:** *Identified B cell epitopes for proteins based on* ABCpred method

| Protein name | Peptide segment position |
|---|---|
| Ebps | 87-102, 132-147 |
| ClfA | 114-129, 326-341, 395-410, 533-548, 848-863 |
| OmpA | 35-50, 87-102, 120-135, 153-168, 214-229, 298-313 |
| OmpC | 16-31, 129-144, 283-298 |
| SIP | 106-121, 224-239, 346-361 |
| PGK | 61-76, 135-150, 221-236, 314-329 |

### *In silico* prediction of Th cell epitopes

Using the ProPred method, we predicted the Th epitopes of the six proteins (Table 4). Th cell epitopes segments of *S. aureus* Ebps and ClfA protein were at residues 479 – 487 and 201 – 209, respectively; the OmpA and OmpC protein of *E. coli* were at residues 284 – 292 and 6 – 12, respectively; and the PGK and SIP protein of *Streptococcus* were at residues 317 – 325 and 240 – 248, respectively.

### Reorganization of series epitopes

Different possible arrangement and combinations of epitopes were optimized by using DNAStar software (Figure 1), and the final amino acid sequence was as follows:

KDDVKGGGGIRNGQQIVIGGGGVVNQAVNTS
GGGGGMAKVLLPLGGGGPEPIEDNDKHDTIKN
AGGGGAGTIDDRQVESSHSTEGGGGTGEATT
TTTNQANTPAGGGGLLASIGSLGGGGANAAEV
YNKDGNKGGGGALDQLYSQLGGGGDTKSNVY
GKNGGGGLSLLVPAGGGGFINNNGPGGGGPE
FGGDTYGSDNFMQQGGGGGLRPSVAYLGGG
GYLISKGIPAGGGGGVFENPGGGGVRTVAAPR
VGGGGTYRAGDPGDHGKGGGGGSLAPVAAD
LGGGGKEEADKEGKSLAPVAAGGGGVVWNG
PMGVGGGGASVAAETPAPVAKVA.

**Table 3:** *Identified* CTL epitopes for proteins

| Protein name | Rank | Type | Peptide segment position |
|---|---|---|---|
| EbpS | 1 | HLA-A2 | 119-127, 318-330, 337-345 |
| | 2 | HLA-A*0201 | 318-330 |
| | 3 | HLA-A*0202 | 318-330 |
| | 4 | HLA-A*0203 | 318-326 |
| | 5 | HLA-A*0205 | 269-277, 318-331, 332-340 |
| ClfA | 1 | HLA-A2 | 504-512, 894-902 |
| | 2 | HLA-A*0201 | 895-903 |
| | 3 | HLA-A*0202 | 895-903 |
| | 4 | HLA-A*0203 | 121-129, 290-296, 297-305, 425-433, 895-903 |
| | 5 | HLA-A*0205 | 220-228, 353-361, 406-141, 538-546, 891-899 |
| OmpA | 1 | HLA-A2 | 117-125, 181-189, 237-245 |
| | 2 | HLA-A*0201 | 237-248 |
| | 3 | HLA-A*0202 | 237-245 |
| | 4 | HLA-A*0203 | 11-19, 100-108 |
| | 5 | HLA-A*0205 | 11-18, 19-27, 55-63, 92-99, 100-108, 110-116, 117-125, 152-160, 209-217, 222-230, 237-245 |
| OmpC | 1 | HLA-A2 | 7-15, 73-81, 113-121, 258-266, 295-303 |
| | 2 | HLA-A*0201 | 7-15, 295-303 |
| | 3 | HLA-A*0202 | 7-15, 197-205, 295-303 |
| | 4 | HLA-A*0203 | 258-266, 288-296 |
| | 5 | HLA-A*0205 | 33-41, 97-105, 295-303, 322-330, 353-361 |
| SIP | 1 | HLA-A2 | ------ |
| | 2 | HLA-A*0201 | 12-20, 67-75 |
| | 3 | HLA-A*0202 | 226-234 |
| | 4 | HLA-A*0203 | 2-10, 147-155, 254-262 |
| | 5 | HLA-A*0205 | 9-17, 53-61, 67-75, 85-93, 132-140, 151-159, 160-168, 184-192, 229-237, 269-277, 298-306, 316-324, 356-364 |
| PGK | 1 | HLA-A2 | 55-63 |
| | 2 | HLA-A*0201 | 16-24, 73-81, 99-105, 106-114, 134-142 |
| | 3 | HLA-A*0202 | 16-24, 55-63, 73-81, 99-107, 161-169, 260-268, 310-318 |
| | 4 | HLA-A*0203 | 73-81 |
| | 5 | HLA-A*0205 | 33-39, 40-48, 73-85, 134-142, 151-159, 310-318, 334-342, 344-352, 387-395 |

**Table 4:** Identified Th epitopes for proteins

| Protein name | Rank | Type | Peptide segment position |
|---|---|---|---|
| EbpS | 1 | DRB1-0101 | 324-329, 330-335, 336-344, 450-458, 468-476, 479-487 |
| | 2 | DRB1-0102 | 324-329, 336-344, 468-476, 479-487 |
| | 3 | DRB1-0301 | 331-339, 449-157, 479-487 |
| ClfA | 1 | DRB1-0101 | 23-28, 29-37, 141-149, 201-209, 261-269, 275-283, 372-379, 893-901 |
| | 2 | DRB1-0102 | 29-37, 141-149, 201-209, 257-265, 275-283, 350-358, 896-904 |
| | 3 | DRB1-0301 | 21-29, 201-209, 221-229, 241-249, 303-311, 376-384 |
| OmpA | 1 | DRB1-0101 | 44-52, 189-197, 284-292 |
| | 2 | DRB1-0102 | 6-13, 284-292 |
| | 3 | DRB1-0301 | 74-82, 98-106, 123-131, 148-155, 185-193, 217-225, 256-264, 290-298, 324-331 |
| OmpC | 1 | DRB1-0101 | 6-12, 14-22, 111-119, 141-149, 152-157, 158-166, 170-178, 216-224, 302-310, 335-343, 355-363 |
| | 2 | DRB1-0102 | 6-12, 14-22, 158-166, 216-224, 296-304, 355-363 |
| | 3 | DRB1-0301 | 5-17, 43-51, 57-65, 101-109, 117-124, 250-258, 366-375 |
| SIP | 1 | DRB1-0101 | 1-9, 17-25, 84-92, 240-248, 363-371 |
| | 2 | DRB1-0102 | 1-9, 17-25, 84-92, 107-115, 240-248 |
| | 3 | DRB1-0301 | 45-53, 55-63, 68-76, 95-102, 132-140, 155-163, 240-248, 359-367, 391-399 |
| PGK | 1 | DRB1-0101 | 46-54, 146-154, 197-205, 232-240, 317-325, 342-350, 381-389 |
| | 2 | DRB1-0102 | 146-154, 197-205, 232-240, 317-325, 342-350, 381-389 |
| | 3 | DRB1-0301 | 18-26, 31-39, 55-63, 89-97, 222-230, 252-260, 295-303, 317-325 |

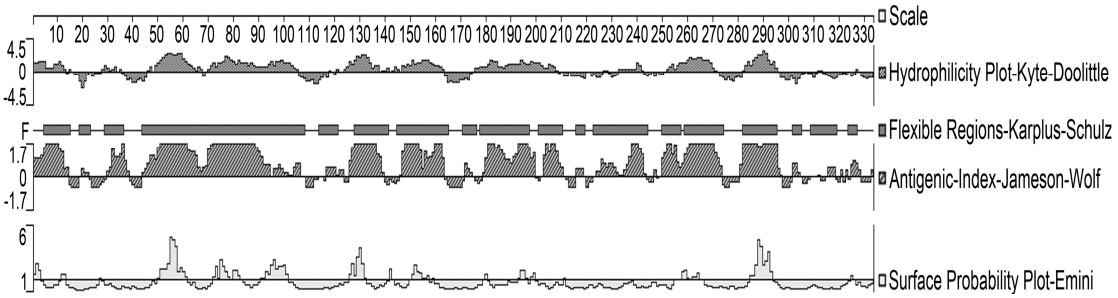


**Figure 1:** Antigenic index of the CM-TEP. The predicted epitopes were connected by GGGG flexible regions, thus making them independent of each other

## Prokaryotic expression, purification and immunogenicity analysis of CM-TEP

Heterologous expression of CM-TEP in *E. coli* and induction with IPTG resulted in the production of a protein of approximately 56 kDa in size, which corresponded to a fusion protein of 20.4 kDa and CM-TEP of 36 kDa (Figure 2). This was the expected size of the recombinant protein. A reasonable quantity of CM-TEP was purified by Ni-NTA super flow resin (Figure 2 and Figure 3). Purified CM-TEP was used to immunize mice to prepare a polyclonal antibody. Pull-down and ELISA results showed that the $OD_{450nm}$ value decreased as the CM-TEP antiserum dilution increased and was near to zero in the control serum (Figure 3). These data indicated a direct interaction between CM-TEP antiserum and *S. aureus*, *E. coli* and *Streptococcus*.

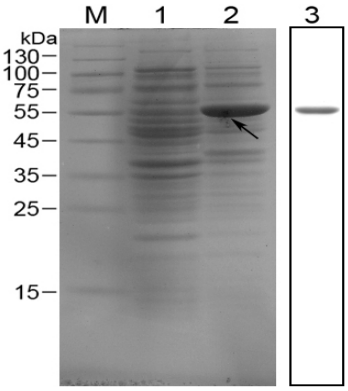


**Figure 2:** Expression and purification of CM-TEP. M, protein marker. 1, non-induced strain. 2, IPTG-induced strain. 3, purified CM-TEP. Heterologous expression of CM-TEP in *E. coli* and induction with IPTG resulted in the production of a protein of approximately 56 kDa in size, including a fusion protein of 20.4 kDa and CM-TEP of 36 kDa. This was the expected size of the recombinant protein. A reasonable quantity of CM-TEP was purified using Ni-NTA super flow resin and produced only one band

**CM-TEP immune protective**

An active immunization approach was used to investigate the ability of CM-TEP to protect mice against infection. After infection with *S. aureus*, *E. coli* and *Streptococcus*, the mice showed severe symptoms. Many of the mice died, but those that survived were able to gradually resume activities after 4 days. Compared with the control group of Freund's immunized adjuvant, the CM-TEP group displayed a significant immune protective effect against infection by *S. aureus* (50 %, $p < 0.05$) and *E. coli* (54.54 %, $P < 0.05$) and provided some immune protection against *Streptococcus* (30.78 %, $p > 0.05$) (Table 5).

**Optimization of expression condition of CM-TEP expression strain**

Based on orthogonal design and SDS-PAGE electrophoresis, the expression of CM-TEP was shown in Figure 4. Optical density value of CM-TEP bands was analyzed by Phoretix 1D software (Table 5). By comparing the K1, K2 and K3 in Table 5, the optimal expression condition of CM-TEP was A3, B2, C2 and D3, which meant strain $OD_{600}$ value of 1, IPTG concentration of 0.3 mmol/L, inducing time of 8 h and inducing temperature of 37 $^{o}$C, respectively. Range analysis showed that the influence degree of each factors on the expression of CM-TEP was C > B > D > A (Table 6). For CM-TEP expressing, notable statistical significance of factors were IPTG concentration, inducing time and inducing temperature (Table 7).

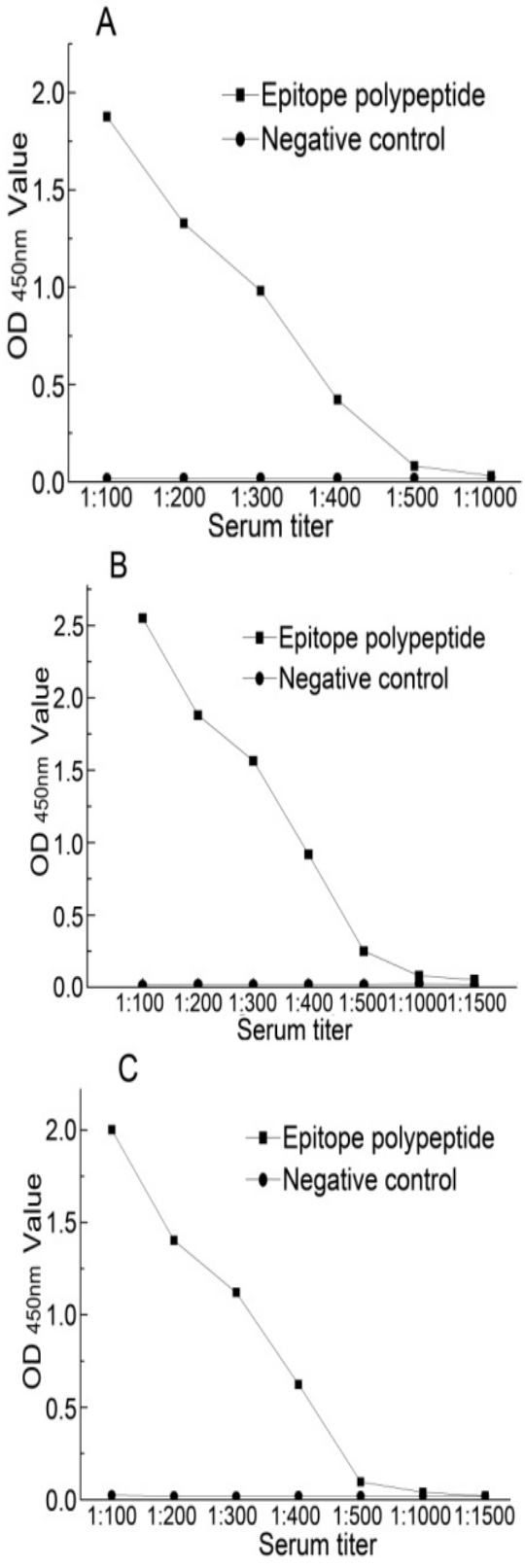


**Figure 3:** The interaction between CM-TEP antiserum and the three bacterial pathogens, as determined by ELISA. A, B and C were CM-TEP antiserum interaction with *S. aureus*, *E. coli* and *Streptococcus*, respectively. The $OD_{450}$ value decreased as the serum dilution increased and was almost zero in control serum

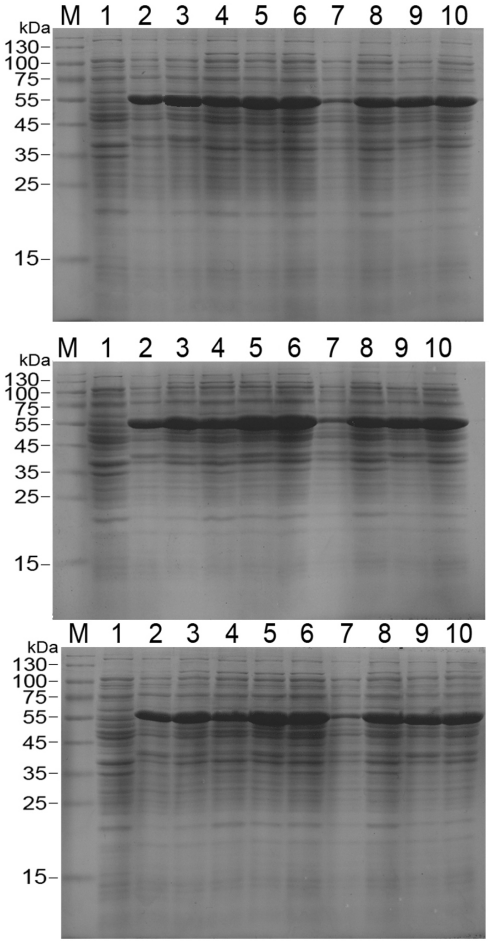


**Figure 4:** Inducing expressing of CM-TEP expression strain. M: Protein marker. 1, non-induced strain. 2 – 4: strain $OD_{600}$ value of 0.5, IPTG concentration of 0.1, 0.3 and 0.5 mmol/L, inducing time was 3, 8 and 12 h, and inducing temperature: 28, 32 and 37 ℃. 5 – 7: strain $OD_{600}$ value of 0.8, IPTG concentration of 0.1, 0.3 and 0.5 mmol/L, inducing time: 8, 12 and 3 h, and inducing temperature: 32, 28 and 37 $^{o}$C. 8 – 10: strain $OD_{600}$ value of 1, IPTG concentration of 0.1, 0.3 and 0.5 mmol/L, inducing time: 12, 3 and 8 h, and inducing temperature: 32, 37 and 28 $^{o}$C

## DISCUSSION

There are many methods available for predicting B cell epitopes, including BepiPred, ABCpred and BPAP [15]. Through BepiPred and ABCpred, the B cell epitopes were predicted for membrane-associated proteins of *C. jejuni* [12] and Gly m Bd 28K of soybean allergens [16]. This study used a combination of the BepiPred and ABCpred methods to improve the prediction accuracy of the B cell epitope for the six proteins.

CTL cell epitopes play an integral role in the adaptive immune response by recognizing peptides combine to major histocompatibility complex class I (MHC-I) molecules; the prediction methods include nHLAPred, IEDB, SYFPEITHI and IMTECH [17]. The nHLAPred method has been used to predict CTL cell epitopes for *Leishmania* major-related candidate antigens and HIV Gag protein [3]. We used the same method to identify a novel critical epitope, which may play a major role in activating $CD8^{+}$T cells [3], thereby increasing the development of immunogenicity.

The ProPred method was found to be efficient for the Th epitope prediction of bacteria [4] and viral proteins [18]. In this article, we used the ProPred method to obtain protein Th epitopes, which could be combined with MHC-II molecules to enhance the immune response.

The flexible portions are underlined. Epitope joints can be divided into flexible and rigid, and frequently used amino acid connection joints sequences are GGGG, AAY, KK and GGGGS [19]. DNAStar software can be used to recombine epitopes for optimal antigenicity [20]. We used the GGGG amino acid joint and used DNAStar software to optimize different epitopes to obtain a polypeptide (CM-TEP) with optimal antigenicity.

The cow mastitis researchers have found that recombinant epitope (Sip-ClfA) antiserum capacities for opsonizing, adhesion and phagocytosis are significantly greater than those of the killed bacteria after immunization, and that these recombinant epitopes also exhibit good immunogenicity [21]. This study indicated a direct interaction between CM-TEP antiserum and *S. aureus*, *E. coli* and *Streptococcus*. Thus, CM-TEP was expected to have good immunogenicity. Additionally, several studies have found that immunization with the B cell epitope (Sip-ClfA) provides better protection against both *S. agalactiae* and *S. aureus* challenges [21]. In this study, CM-TEP immunization developed an immune protection against all three major bacterial pathogens of cow mastitis. Thus, CM-TEP is expected to be an approach to the development of a vaccine against cow mastitis.

In order to apply to industrial production, the optimum expression conditions were studied to obtain abundant and high quality of CM-TEP, which were strain $OD_{600}$ value of 1, IPTG concentration of 0.3 mmol/L, inducing time of 8 h and inducing temperature of 37 ℃, respectively. IPTG is toxic [22] and should be induced by low concentration in practical production, and the same to our research. In addition, the suitable induction time is favorable to the synthesis of protein [23], and the production cost can be

**Table 5:** Active immune protection achieved by CM-TEP

| Immune | *S. aureus* ADR(%)/RPS(%)/Sig(*P*) | *E. coli* ADR(%)/RPS(%)/Sig(*P*) | *Streptococcus* ADR(%)/RPS(%)/Sig(*P*) |
|---|---|---|---|
| CM-TEP | 40 / 50* / 0.025 | 33.33 / 54.54* / 0.028 | 60 / 30.78 / 0.099 |
| Control (Freund's adjuvant) | 80 / − / − | 73.33 / − / − | 86.67 / − / − |

ADR, accumulating death rates. RPS (%) = 1 − (% vaccinated mortality/% non-vaccinated mortality) × 100. Sig., significance. * $p < 0.05$ (compared with control). CM-TEP displayed a significant immune protective effect against the infection of *S. aureus* (50 %, $p < 0.05$) and *E. coli* (54.54 %, $p < 0.05$), and displayed some immune protection against *Streptococcus* (30.78 %, $p > 0.05$)

**Table 6:** Inducing expression condition of CM-TEP strain

| Number | A | B (mmol/L) | C (h) | D (℃) | Optical density/SD (×10$^5$) |
|---|---|---|---|---|---|
| 1 (A1B1C1D1) | 0.5 | 0.1 | 3 | 28 | 1.552 ± 0.026 |
| 2 (A1B2C2D2) | 0.5 | 0.3 | 8 | 32 | 2.325 ± 0.144 |
| 3 (A1B3C3D3) | 0.5 | 0.5 | 12 | 37 | 2.102 ± 0.184 |
| 4 (A2B1C2D3) | 0.8 | 0.1 | 8 | 37 | 2.777 ± 0.283 |
| 5 (A2B2C3D1) | 0.8 | 0.3 | 12 | 28 | 2.840 ± 0.082 |
| 6 (A2B3C1D2) | 0.8 | 0.5 | 3 | 32 | 0.601 ± 0.173 |
| 7 (A3B1C3D2) | 1.0 | 0.1 | 12 | 32 | 2.218 ± 0.194 |
| 8 (A3B2C1D3) | 1.0 | 0.3 | 3 | 37 | 2.052 ± 0.100 |
| 9 (A3B3C2D1) | 1.0 | 0.5 | 8 | 28 | 2.338 ± 0.271 |
| K1 (Mean value 1) | 1.993 | 2.182 | 1.401 | 2.243 | |
| K2 (Mean value 2) | 2.073 | ***2.406*** | ***2.480*** | 1.715 | |
| K3 (Mean value 3) | ***2.203*** | 1.681 | 2.387 | ***2.310*** | |
| Range analysis | 0.210 | 0.725 | 1.079 | 0.595 | |

*The factor of A, B, C, and D means strain $OD_{600}$ value, IPTG concentration, inducing time and inducing temperature, respectively. By comparing the data size of K1, K2 and K3, the optimal expression condition of CM-TEP was A3, B2, C2 and D3. Range analysis showed that the influence degree of each factors on the expression of CM-TEP was C > B > D > A*

**Table 7:** Variance results for CM-TEP expressing

| Factor | Optical density value analysis | | |
|---|---|---|---|
| | *Mean square* | *F value* | *P value* |
| A | 0.101 | 3.096 | 0.070 |
| B | 1.241 | 38.109 | 0.000 |
| C | 3.215 | 98.738 | 0.000 |
| D | 0.957 | 29.406 | 0.000 |

The factor of A, B, C, and D means strain $OD_{600}$ value, IPTG concentration, inducing time and inducing temperature, respectively. $P < 0.05$ means the difference with control group is significant

saved compared with the blind setting time. Thus, appropriate induction time should be used.

## CONCLUSION

A novel triple-cell epitope-based polypeptide (CM-TEP) has been designed, optimized expression. It displays a significant immune protective effect against the infection of *S. aureus* and *E. coli* while providing some immune protection against *Streptococcus*. Thus, a new member has been added to the group of triple epitope-based vaccines against cow mastitis.

## DECLARATIONS

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### *Conflict of Interest*

No conflict of interest associated with this work.

### *Contribution of Authors*

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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