

Cloning, Expression and Purification of Penicillin Binding Protein2a (PBP2a) from Methicillin Resistant *Staphylococcus aureus*: A Study on Immunoreactivity in Balb/C Mouse

Setareh Haghighat^{1*}, Seyed Davar Siadat², Seyed Mehdi Rezayat Sorkhabadi³,
Abbas Akhavan Sepahi⁴, and Mehdi Mahdavi⁵

1. Department of Biology, Faculty of Basic Sciences, Science and Research branch, Islamic Azad University, Tehran, Iran

2. Department of Microbiology, Pasteur Institute of Iran, Tehran, Iran

3. Department of Pharmacology, School of Advanced Sciences and Technology in Medicine, Tehran University of Medical Sciences, Tehran, Iran

4. Department of Microbiology, Faculty of Basic Sciences, North Tehran Branch, Islamic Azad University, Tehran, Iran

5. Department of Virology, Pasteur Institute of Iran, Tehran, Iran

Abstract

Background: *Staphylococcus aureus* (*S. aureus*) is a major nosocomial pathogen and the infection with this organism in human is increasing due to the spread of antibiotic resistant strains. One of the resistance mechanisms of *S. aureus* comprises modification in binding proteins to penicillin. Vaccine strategy may be useful in controlling the infections induced by this organism. This study aimed at developing and producing the recombinant protein PBP2a as a vaccine candidate and evaluating the related humoral immune response in a murine model.

Methods: A 242 bp fragment of *mecA* gene was amplified by PCR from *S. aureus* COL strain and then cloned into prokaryotic expression vector pET-24a. For expression of recombinant protein, pET24a-*mec* plasmid was transformed into competent *E. coli* BL21 (DE3) cells. Recombinant protein was over expressed with 1 mM isopropylthio- β -D-galactoside (IPTG) and purified using Ni-NTA agarose. SDS-PAGE and western blotting were carried out to confirm protein expression. For immunization of experimental groups, Balb/c mice were injected subcutaneously with 20 μ g of recombinant PBP2a three times with three weeks intervals. The sera of experimental groups were collected three weeks after the last immunization and then specific antibodies were evaluated by ELISA method.

Results: Successful cloning of *mecA* was confirmed by colony-PCR, enzymatic digestion, and sequencing. SDS-PAGE and western blot analysis showed that recombinant protein with molecular weight of 13 kDa is over expressed. In addition, high titer of specific antibody against PBP2a in vaccinated mice was developed as compared with the control group and confirmed the immunogenicity of the vaccine candidate.

Conclusion: Results suggest that PBP2a recombinant induced specific antibodies and can be used as Staphylococcal vaccine candidate after further studies.

* Corresponding author:

Setareh Haghighat, Ph.D.,
Department of Biology,
Faculty of Basic Sciences,
Science and Research branch,
Islamic Azad University,
Hesarak, Tehran, Iran

Tel: +98 21 47911

E-mail:

setareh_haghighat@yahoo.com

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Introduction

S. aureus is a gram positive bacterium, identified as one of the major nosocomial agents responsible for several hospital-acquired infections, including septic shock,

skin infections, and bacteremia¹⁻³. Hospital-acquired infection is a critical problem especially because Methicillin-resistant *Staphylococcus aureus* (MRSA) becomes increasingly

prevalent^{4,5}. MRSA is resistant to all β -lactam antibiotics, due to the presence of an extra penicillin-binding protein (PBP2a) with low affinity to β -lactam antibiotics⁶.

PBP2a is encoded by *mecA* gene, which is located in a chromosomal cassette of a foreign DNA region integrated into the bacterial chromosome⁶⁻⁸. PBP2a is classified by Goffin and Ghuysen as a multimodular class B penicillin-binding protein harboring transpeptidase domains⁹. While in the presence of β -lactam antibiotics, normal PBPs are blocked, PBP2a precedes the transpeptidation reactions, thereby results in normal cell wall synthesis^{8,10}.

Given the inherent and acquired antibiotic resistance of *S. aureus*, antibiotic therapy for MRSA infections has a limited effectiveness. While vancomycin is the only remaining effective antibiotic¹¹ against *S. aureus*, instances of multiresistant bacteria have been frequently reported which won't be affected by any conventional antibiotics^{6,11,12}. In addition, the morbidity rate due to MRSA infection can be dependent on status of host immunity, in particular humoral immunity, which is believed to play a significant role against staphylococcal infections^{6,13}. These ever-increasing obstacles related to conventional antibiotic treatments, point to the need for adopting an alternative approach for effective prevention and treatment of multiresistant bacterial infections¹⁴⁻¹⁶. Vaccine strategy is proven to be useful in controlling such infections⁶⁻⁸.

Several vaccine strategies against *S. aureus* have been proposed using bacterial structures¹⁷⁻¹⁹ such as surface polysaccharide^{8,13,20} whole cells^{8,13} or surface proteins¹ such as clumping factor A²¹ and fibronectin-binding protein²² as target, but none of them revealed protectivity in animal studies and human trials⁸. *mecA* sequence alignments demonstrate a high homology among all MRSA, which substantiates the intention to use this antigen as a vaccine candidate²³. Comparison of the nucleotide and amino acid sequences of *mecA* among different species has indicated that whereas the N and C-terminal of these se-

quences are highly conserved, the central hyper variable region is not equally conserved²³. These previous works suggest *mecA* as an antigen candidate for designing an anti-MRSA vaccine; furthermore, prokaryotic expression system provides a facile method for producing recombinant proteins and may also be useful for the production of PBP2a and other bacterial outer membrane proteins for vaccine studies. The main purpose of the present study was to construct a prokaryotic high level expression system for producing recombinant PBP2a which can be used for vaccine development in future.

Materials and Methods

Bacterial strains and vector

S. aureus COL strain (methicillin-resistant *S. aureus*) was kindly obtained from Dr. Mohammad Emameini (Tehran University of Medical Science). *Escherichia coli* (*E. coli*) strains DH5 α (Invitrogen, California, USA) and *E. coli* strains BL21 (DE3) (Novagen, Wisconsin, USA) were used for cloning and expression of recombinant protein, respectively. *E. coli* cells harboring recombinant plasmids were grown aerobically at 37°C in Luria-Bertani broth (Merck, Darmstadt, Germany) with or without 50 μ g/ml kanamycin (Sigma, Saint Louis, MO, USA). Plasmid pET-24a (Novagen, Wisconsin, USA) was used as an expression vector in prokaryotic system.

PCR of *mecA* gene segment

S. aureus COL strains (methicillin-resistant *S. aureus*) were cultured on Luria-Bertani (LB) broth and bacteria were collected with centrifugation and then genomic DNA was extracted with mi-Bacterial Genomic DNA isolation kit (Me-tabion, South Korea) according to the manufacturer's instructions. A 242 bp (amino acids 370-451) fragment of *mecA* gene with trans-peptidase activity was amplified from genomic DNA as the template. Primer pair used for *mecA* amplification had the nucleotide sequence as follows: forward primer, containing a restriction site

for *HindIII* (5'- GGTAAGCTTTTATGTAT GCATGAGTAACGTAAG -3') and reverse primer with *XhoI* restriction site (5'- GCCT CGAGACCATTTCACCTTCATATCT TG -3'). *Pfu* DNA polymerase (Fermentase) was used in the reaction. The PCR conditions consisted of 1 cycle of 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 40 s at 57°C, 1 min at 72°C, and a final cycle of 10 min at 72°C. The PCR products were recovered from the gel and purified by using PCR purification kit (Roche, Germany). The purified *mecA* fragment was digested with restriction enzymes *HindIII* and *XhoI* and ligated into the digested pET-24a vector, which provides six His residues at the C-terminus of the expressed protein. Recombinant vector pET24a-*mecA* was transformed into the competent *E. coli* DH5a cells. The integrity of the recovered plasmid was confirmed by colony PCR, restriction endonuclease digestion and sequencing⁶⁻⁸.

Protein expression

For expression of recombinant protein, pET24a-*mecA* plasmid was transformed into competent *E. coli* BL21 (DE3). Transformed cells were grown at 37°C in LB medium containing kanamycin (50 µg/ml) until exponential phase (OD_{600 nm}=0.6), followed by induction with 1 mM IPTG (Fermentase). Samples were collected every 3 hr for 24 hr and analyzed by SDS-PAGE to follow the best time point of protein expression. SDS-PAGE and western blot analysis were carried out to confirm protein expression^{1,6-8,26}.

Western blot analysis

For Western blot analysis, the separated proteins by SDS-PAGE gel were transferred to a nitrocellulose membrane (Schleicher & Schuell). The membrane was then blocked in Tris-Buffered Saline (TBS) containing 5% Bovine Serum Albumin (BSA) overnight at 4°C and washed three times with TBS containing 0.05% Tween 20 (TBST).

Afterwards, the nitrocellulose membrane was incubated for 2 hr at room temperature with mouse anti-histag antibody (Qiagen, USA) diluted 1:10000 in TBS-T. Then the

membrane was washed with TBS-T and then incubated with Rabbit anti-mouse immunoglobulin G (heavy and light chain) Horseradish Peroxidase (HRP) conjugate antibody (diluted 1:5000 in TBS-T) for 2 hr at room temperature. After three times of washing, the membrane was treated using DAB solution (Sigma, Saint Louis, MO, USA) and placed in darkness until the appearance of the protein band²⁶.

Purification of recombinant protein

E. coli BL21 (DE3) containing pET24a-*mecA* plasmid was grown in large scale and the pellets of bacterial cells expressing protein were harvested and resuspended in lysis buffer (8 M urea, 0.1 M NaH₂PO₄, and 0.01 M Tris, pH=8.0) containing protease inhibitors. Cell suspension was sonicated and centrifuged for 20 min at 10000 rpm. After centrifugation, the recombinant protein (approximately 13 kDa) was purified from supernatant under denaturing conditions via its His-tag using Ni-nitrilotriacetic acid (Ni-NTA) affinity chromatography (Ni-NTA Agarose; Qiagen) according to the manufacture's instruction^{1,7,23,27}. The output fractions were analyzed by SDS-PAGE and the quantity of protein was determined with Bradford protein assay and Nanodrop analyzer. The PBP2a protein solution was dialyzed against 0.1 M phosphate buffered saline (PBS, pH=7.4) for 72 hr to remove urea, filtered (0.22 µm, Sartorius, Germany) and then stored at -70°C until use^{1,7,21,23}.

Experimental groups and immunization procedures

Six-to-eight week old female BALB/c mice were purchased from Pasteur Institute of Iran and kept in standard condition. The mice were assigned into two groups (n=14) and immunized subcutaneously with 20 µg of recombinant PBP2a or PBS (the control group) in complete Freund's adjuvant (Sigma, Saint Louis, MO, USA). Booster doses were also given in incomplete Freund's adjuvant with three and six week intervals. Three weeks after the last immunization, the mice were bled and serum samples were collected and

stored at -20°C until use^{1,6-8,21,23}.

Performing ELISA for specific antibody

The enzyme-linked immunosorbent assay (ELISA) was used to determine the presence of anti-PBP2a antibodies in the sera of immunized mice. Ninety-six well microtiter plates (Extragate, USA) were coated with $100\ \mu\text{l}$ of $10\ \mu\text{g/ml}$ of recombinant protein ($1\ \mu\text{g/well}$) diluted in PBS, and incubated overnight at 4°C . Each plate was washed three times with PBS-T and blocked with PBS containing 5% BSA (blocking buffer) for 2 hr at 37°C . Following blocking and washing, mouse sera were diluted in blocking buffer (1:200 to 1/402240). The plates were incubated for 2 hr at 37°C . Then, the plates were washed three times and incubated with HRP-conjugated anti-mouse IgG (Sigma, USA) diluted 1:7000 (as secondary antibody) at 37°C for 2 hr. To develop the reaction, plates were washed and incubated with TMB (tetramethylbenzidine) as the substrate for 15 min at room temperature in dark condition. The reaction was stopped with $100\ \mu\text{l}$ of 2N H_2SO_4 , and the results were read at optical density of 450 nm (OD_{450}) by ELISA reader^{1,6-8}.

Statistical analysis

Data were summarized using descriptive statistical methods. Student's independent t-test (Statview) and one-way analysis of variance (ANOVA) were used to compare the mean values. A p-value less than 0.05 represented a significant difference. All data were analyzed using SPSS Software Version 20.0^{1,6-8}.

Results

Amplification of *mecA* and construction of pET24a-*mecA*

Specific primers were designed to amplify *mecA* from the *S. aureus* COL strain. The expected size of *mecA* PCR product, approximately 242 bp, is shown in figure 1. The integrity of the recombinant vector pET24a-*mecA* was confirmed by double digestion using *Hind*III and *Xho*I restriction enzymes (Figure 2) and colony-PCR with specific pri-

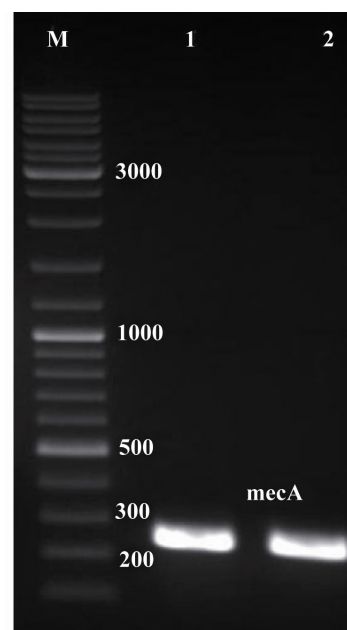


Figure 1. Electrophoresis of PCR products on agarose gel (1% w/v). Lanes 1 and 2, single expected band of *mecA* (approximately 242 bp); lane M, 1 kb DNA size marker

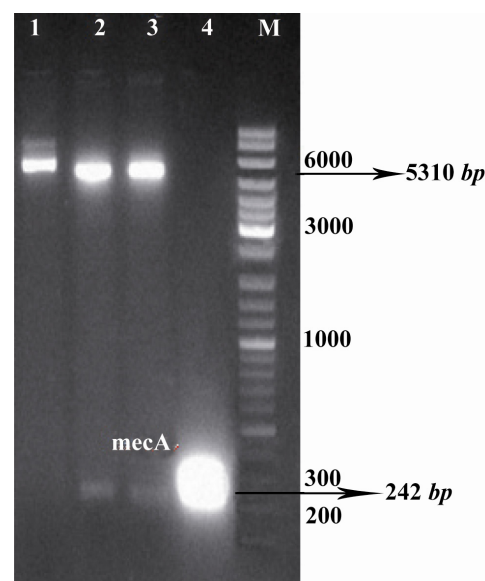


Figure 2. Confirmation of recombinant vector by restriction enzyme digestion. The plasmids were extracted and digested with appropriate restriction enzymes. Lane 1, undigested recombinant vector, pET24a-*mecA*; lanes 2, 3, recombinant vector, pET24a-*mecA*, digested with *Xho*I and *Hind*III; lane 4, PCR product of *mecA* gene (approximately 242 bp); lane M, 1 kb DNA size marker. Products were electrophoresed on 1% w/v agarose gel

mers. Identity and orientation of *mecA* in the construct were confirmed by sequencing the recombinant vector. Cloned *mecA* gene sequence showed 99.9% homology with reference sequences.

SDS page analysis

Cells harboring pET24a-mecA plasmid were cultured at 37°C in the presence of IPTG. The whole-cell lysates were analyzed by 12% SDS-PAGE. One major band appeared approximately at the 13 *kDa* position in the case of IPTG induction, which was the expected position of PBP2a (Figure 3). Induction of the cells at 37°C for 6 *hr* and IPTG with dosage of 1.0 *mmol/L* was found to be optimal to achieve the highest-level of PBP2a expression. Both supernatant and the pellet of cell lysates were tested for the presence of recombinant proteins. The majority of the expressed protein was detected in inclusion bodies. Therefore, recombinant protein was carefully purified with Ni-NTA affinity chromatography under denaturing condition (Figure 3). This purification yielded 100 *mg* of highly purified recombinant PBP2a protein from one of induced culture.

Western blot analysis

Western blot analysis was performed to de-

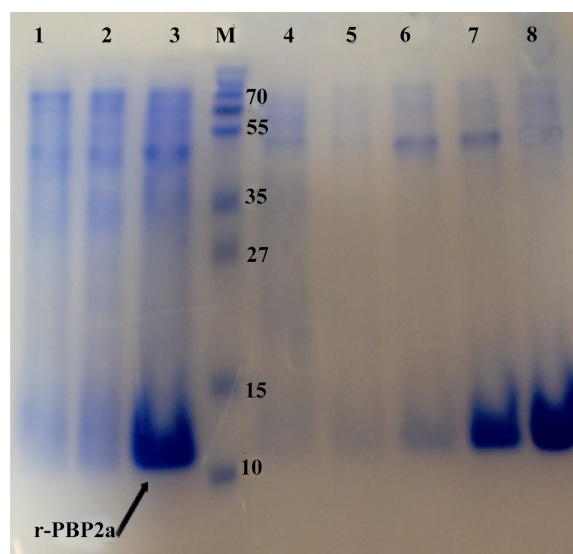


Figure 3. Detection of expressed and purified recombinant PBP2a in SDS-PAGE (12% w/v). The gel was stained with Coomassie blue G-250. Samples were resuspended directly in SDS loading buffer and boiled for 5 *min*. Amount of proteins loaded in each well was about 50 μ g. Lane 1, Negative control cells (BL21 with pET24a+); lane 2, pellet of un-induced bacteria; lane 3, pellet of IPTG induced bacteria; lane 4, flow through material, lanes 5 and 6 inclusion bodies after washing and solubilizing; lane 7, purified r-PBP2a from Ni-NTA Agarose column lane 8, PBP2a protein after dialysis; lane M, standard protein size marker (*kDa*). The r-PBP2a proteins have been shown by the arrow

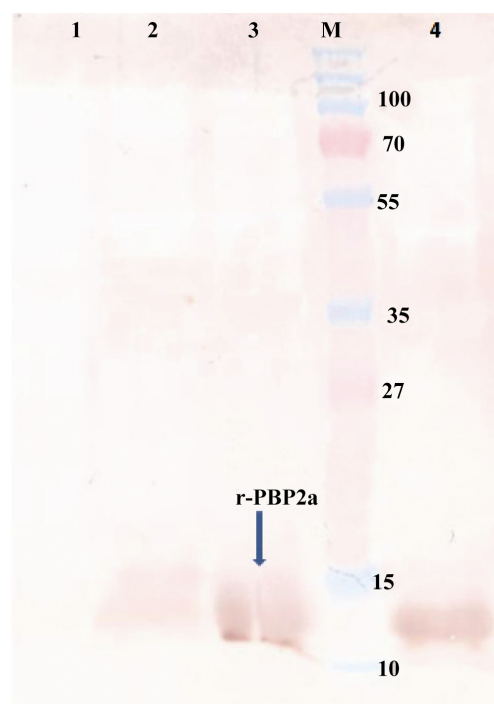


Figure 4. Western blot of recombinant autolysin protein probed by anti-His (1:10,000). Lane 1, control negative (pET24a+ without mecA fragment); lane 2, pellet of un-induced bacteria; lane 3, pellet of IPTG induced bacteria; lane 4, purified r-PBP2a; lane M, pre-stained protein size marker (*kDa*). HRP-conjugated anti-rabbit IgG (1:7000) and DAB were used

tect the expression of desired protein. The major band observed in SDS-PAGE (13 *kDa*) (Figure 4) was confirmed as PBP2a protein by western blot analysis with mouse serum anti-his-tag, which indicates the apparent molecular mass of 13 *kDa*.

Humoral immune response

The specific antibody production was measured by an optimized ELISA method with sera obtained before (pre-immune serum) and after each immunization course with recombinant vaccine. All animals immunized with recombinant vaccine (mecA) were able to produce specific antibodies anti-mecA and the highest antibody titers were observed after the third immunization (second booster). The vaccinated group (Group I) produced higher anti-PBP2a specific antibody after each course of immunization as compared with the control group (after first immunization $p=0.023$, after second immunization $p=0.017$, after third immunization $p=0.0001$) (Figure

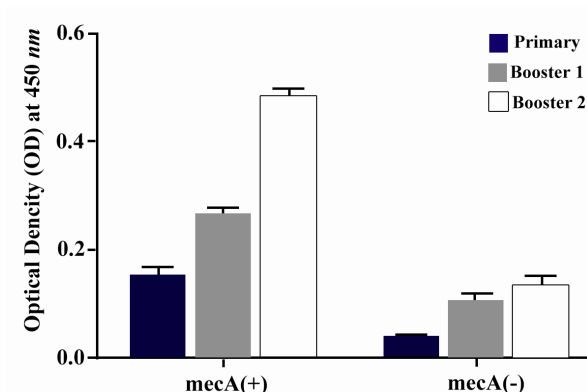


Figure 5. Presence of specific antibodies anti-PBP2a in the sera of mice immunized with the recombinant protein vaccine and the negative controls after each course of immunization. Data presented as mean \pm S.D of experimental group (n=14)

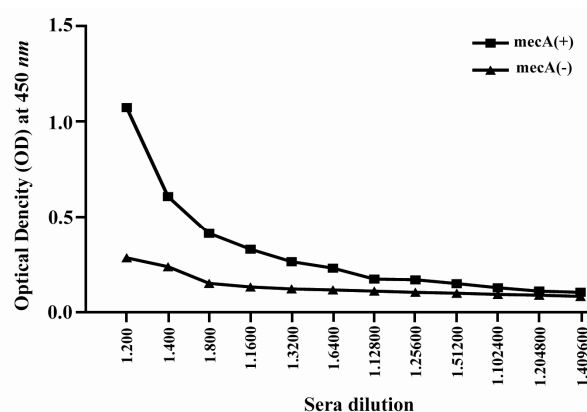


Figure 6. Titration of anti-PBP2a antibody in experimental group with ELISA method. Sera of experimental groups were diluted and ELISA was carried out. Values are presented as mean \pm S.D of 14 mice in each group

5). Result of titration in the experimental group after the third immunization showed that immunization with *mecA* significantly increased total antibody at the dilution of 1/200 to 1/25600 as compared with the control group ($p < 0.03$) (Figure 6).

Discussion

Staphylococcal resistance to first-line drugs, including synthetic penicillin and other conventional antibiotics, is a major problem in treating MRSA infection, which is increasingly common, especially in hospitalized patients^{7,24}. While vancomycin is often used as the only remaining effective antibiotic against MRSA, there are reports that vancomycin re-

sistant species of this infection has emerged^{7,11}. As there is no effective antibiotic treatment for multiresistant infections, there is a need to develop alternative methods for prevention and treatment of these diseases^{6,7,11}. In this study, we reported that PBP2 expressed in *E. coli* BL21 (DE3) can be a potential vaccine candidate.

Being responsible for resistance to all beta-lactam antibiotics, PBP2a was selected as the target protein in developing our potential vaccine candidate⁶⁻¹⁰. This enzyme consists of a spanning region which is responsible for its attachment to cytoplasmic membrane, a non-penicillin binding domain of unknown function, and a transpeptidase domain^{8,9}.

PBP2a is located on the outer surface of the bacterial cell wall, where it can be easily recognized by antibodies raised by host immune system⁶⁻⁹. In the present study, recombinant DNA technology was used to obtain a high PBP2a production yield. In a similar study by Senna *et al*⁸ a virtually identical fragment of *mecA* was cloned and expressed using pCI-Neo mammalian vector, in order to achieve a DNA vaccine candidate. Other experiments in which whole *mecA* gene was used as antigen were reported previously^{6,25}. While in other studies pET23a and 32a vectors belonging to pET family were used to express *mecA* gene,^{8,25} we used pET24a vector, because of its better expression in comparison with the above mentioned vectors. In addition, pET24 carries six histidine residues in C-terminal that facilitates purification of recombinant protein by Ni NTA agarose^{26,27}. Since inclusion bodies can be unfolded and require denaturing condition for their solubilization²⁷, the desired protein was purified in denatured condition effectively and then refolded by dialysis in PBS.

In our study, ELISA results indicated that anti-PBP2a specific antibody was induced. In addition, in this study the titer of antibody was higher than that produced by Roth *et al*⁷, though they used a naked DNA vaccine for immunization.

Humoral immune response is a major factor in the removal of extracellular pathogens such

as *S. aureus*²⁸. In the presence of this factor, antibody could bind to the surface antigen of the pathogen, activating complement system, and increasing the phagocytosis of the pathogen through opsonization process. PBP2a is able to induce humoral immune response and could eliminate the pathogen with complement activation and induction of opsonization²⁹.

Conclusion

In conclusion, our results show that PBP2a can be expressed in *E. coli* BL21 (DE3) at a high level and stimulate humoral immune response in a murine model. Investigating other aspects of PBP2a as a potential vaccine against MRSA infection, including protective effect against bacterial challenge and survival rate is a ground for future studies.

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