

Evaluation of the humoral immune response in BALB/c mice immunized with a naked DNA vaccine anti-methicillin-resistant *Staphylococcus aureus*

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ABSTRACT. Methicillin-resistant *Staphylococcus aureus* (MRSA) is the major pathogen involved in nosocomial infections, leading to high rates of morbidity and mortality in hospitals worldwide. The methicillin resistance occurs due to the presence of an additional penicillin-binding protein, PBP2a, which has low affinity for β -lactam antibiotics. In the past few years, vancomycin has been the only antibiotic option for treatment of infections caused by multiresistant MRSA; however, reports of vancomycin-resistant strains have generated great concerns regarding the treatment to overcome these infections. In the present study, we report preliminary results regarding the humoral immune response generated in BALB/c mice by two different doses of naked DNA vaccine containing an internal region, comprising the serine-protease domain, of the PBP2a of MRSA. The immunization procedure consisted of four immunizations given intramuscularly within 15-day intervals. Blood was

collect weekly and anti-PBP2a-specific antibodies were screened by ELISA. BALB/c mice immunized with DNA vaccine anti-PBP2a have shown higher antibody titers mainly after the fourth immunization, and intriguingly, no correlation between the humoral immune response and DNA dose was observed. Our results suggest that the DNA vaccine anti-PBP2a induced an immune response by production of specific antibodies anti-MRSA in a non-dose-dependent manner, and it could represent a new and valuable approach to produce specific antibodies for passive immunization to overcome MRSA infections.

Key words: Methicillin-resistant *Staphylococcus aureus*, Antibodies, DNA vaccine, PBP2a

INTRODUCTION

Antibiotic resistance has emerged as a major public health threat due to drug overuse (Swartz, 1994), and one of the major concerns, regarding multiresistant pathogens within the medical community is the methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA was first described in 1961 by Jevons (Jevons, 1961), and since then it has spread worldwide and become endemic in many hospital environments (Hiramatsu et al., 2001). MRSA has an extra penicillin-binding protein (PBP2a) with low affinity for β -lactam antibiotics (Fontana, 1985; Reynolds and Fullers, 1986), which is encoded by the *mecA* gene, located in a chromosomal cassette of a foreign DNA region integrated into the bacterial chromosome (Beck et al., 1986). PBP2a is classified as a multimodular class B penicillin-binding protein, comprising a no PBP domain, whose function is not known, and the transpeptidase domain, which is responsible by the transpeptidase reactions of the bacterial cell wall (Goffin and Ghuysen, 1998). In presence of β -lactam antibiotics, normal PBPs are blocked, in contrast the PBP2a is able to proceed the transpeptidation reactions alone, allowing the cell wall synthesis.

Until recently MRSA has exhibited sensitivity to vancomycin, which is the final option to overcome infections caused by strains that are resistant to other antibiotics. However, the first case of new “super bug” bacterium that is completely resistant to vancomycin was reported (Pearson, 2002). Therefore, alternative methods to prevent and treat multiresistant bacterial infections, such as vaccines and passive immunization, are being extensively evaluated, but none have yet been fully successful (Lee et al., 1988; Greenberg et al., 1989; Mamo et al., 1994; McKenney et al., 1999).

Genetic immunization is an attractive advance in vaccine development (Manickan et al., 1997) that has been used to elicit protective antibodies and cell-mediated immune responses in a variety of animal models of viral and bacterial diseases (Donnelly et al., 1999; Tuteja, 1999). One of the advantages of this method is that the antigen is produced *in vivo* and subsequently presented either by MHC class I or class II molecules, eliciting an efficient immune response (Condon et al., 1996).

It is well described that MRSA morbidity is dependent on the host immunity status, especially humoral immunity, which is believed to play a significant role against staphylococcal

infections (Cohen, 1986). However, patients infected by MRSA are in most of the cases immunosuppressed, and probably unable to produce antibodies after vaccination, being passive immunization of specific antibodies against MRSA a more attractive approach to fight multiresistant pathogens. Our study evaluates the anti-PBP2a antibody production generated by two different doses of a naked DNA vaccine that codifies the serine-protease domain of the PBP2a in a murine model (BALB/c). Our results suggest that the DNA vaccine anti-PBP2a induced an immune response by production of specific antibodies anti-MRSA in a non-dose-dependent manner and this strategy can be used in large scale animals for antibody production and passive immunization to overcome MRSA infections.

MATERIAL AND METHODS

Plasmid constructions

DNA vaccine - pCI-Neo-mecA

A 249-bp fragment of the *mecA* gene referent to the active site of the codified enzyme (PBP2a) was amplified by PCR from an MRSA clinical isolate from the Hospital de Pronto Socorro, Porto Alegre, RS, Brazil. The synthetic oligonucleotide primers (5'-**GCTAGCCAAGGAGGTCCAGCCATGAGTAACGAAGAA**-3' and 5'-**TACGAATTCATATCTTGTAAC**-3') were used for PCR amplification of the *mecA* gene. The amplification conditions were as follows: 1 cycle of 5 min at 94°C, 20 cycles of 1 min at 94°C, 1 min at 52°C, 1 min at 72°C, and a final cycle of 5 min at 72°C. The PCR product was digested with *NheI* and *EcoRI* (in bold) and cloned into pCI-Neo Mammalian Expression Vector (Promega) to perform the immunization experiments.

Recombinant peptide

A 228-bp *mecA* fragment was amplified by PCR using the recombinant plasmid pCI-Neo-*mecA* as template. The forward primer, containing a restriction site for *NdeI* (5'-GGAGGTC CACATATGAGTAACGAAG-3') and the reverse primer containing a restriction site for *HindIII* (5'-CAAGCTTTCATACCACTTCATATCTTG-3') were used for nucleic acid amplification with DNA polymerase of *Pyrococcus furiosus* (*Pfu* DNA polymerase; Stratagene). The PCR conditions consisted of 1 cycle of 3 min at 98°C, followed by 35 cycles of 45 s at 98°C, 45 s at 60°C, 1 min at 72°C, and a final cycle of 3 min at 72°C. The PCR product was cloned into pCR-Blunt plasmid (Invitrogen) and subcloned into *NdeI* and *HindIII* sites into the expression plasmid pET23a(+) (Novagen).

Protein expression

In preliminary experiments, *E. coli* BL21(DE3) cells transformed with pET23a(+)-*mecA* were grown at 37°C in Luria-Bertani medium (LB) containing carbenicillin (50 mg/mL) until exponential phase ($OD_{600\text{ nm}} = 0.6$), followed by induction with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). Samples were collected every 3 h for 24 h and analyzed by SDS-PAGE to follow the best time point of protein expression. After several trials varying the

growth temperature and IPTG concentration, the best protein expression levels was obtained when the cells were grown at 30°C and incubated for 6 h after induction with 1 mM IPTG.

Purification of recombinant peptide

Cells expressing the peptide were harvested, resuspended in phosphate-buffered saline (PBS), pH 7.4, containing protease inhibitors, and submitted to sonication. After centrifugation, the recombinant peptide (approximately 8.0 kDa) was purified from the supernatant by ultrafiltration, using a 30-kDa size exclusion membrane filter and subsequently, a 3-kDa size exclusion membrane filter to concentrate the peptide. Fractions were analyzed by SDS-PAGE and quantified using the kit Protein Assay (Bio-Rad).

Immunization procedure

The DNA vaccine pCI-Neo-*mecA* was purified using Plasmid Giga Kit (Qiagen) and diluted in PBS, pH 7.4, to immunize the mice. Five-week-old female BALB/c mice purchased from CEMIB (Unicamp, São Paulo, SP, Brazil) were divided into four different groups, as shown in Table 1. Two groups were immunized with different doses of the naked DNA vaccine (pCI-Neo-*mecA*). As negative control, one group received only the empty recombinant plasmid and the other group received the vaccine vehicle (only PBS). Three days before the first immunization, 2.5 µL/g per animal weight 0.5% bupivacain chloridrate was injected in the left quadriceps muscle to promote cell damage and increase the efficiency of DNA entry into the cells. The immunization procedure consisted of three doses of DNA and one booster given intramuscularly, at the same site as used to inject the bupivacain chloridrate. The vaccine was injected within 15-day intervals and blood was collected before immunizations (pre-immune serum) and every week to analyze the humoral immune response (Figure 1).

Table 1. Immunization schedule of experimental groups and comparisons between titers of specific anti-PBP2a antibodies on pre-immune serum and serum after the fourth immunization.

Groups	Number of mice	Vaccine/dose	Serum pre-immune ^a	Serum after the 4th immunization ^a	P
I	5	10 µg pCI-Neo- <i>mecA</i>	0.10 ± 0.026	0.30 ± 0.08	<0.001*
II	5	100 µg pCI-Neo- <i>mecA</i>	0.11 ± 0.038	0.31 ± 0.11	0.008**
III	3	10 µg pCI-Neo	0.13 ± 0.06	0.18 ± 0.07	0.426*
IV	3	PBS	0.18 ± 0.10	0.18 ± 0.07	1.0*

^aAverage titer ± SD; *Student *t*-test; **Mann-Whitney test. PBS = phosphate-buffered saline.

Analysis of the immune response

The enzyme-linked immunosorbent assay (ELISA) was used to determine the presence of specific antibodies anti-PBP2a in the serum of immunized mice. Plates were coated with 10 µg/mL recombinant peptide, diluted in 0.1 M carbonate/bicarbonate coating buffer, pH

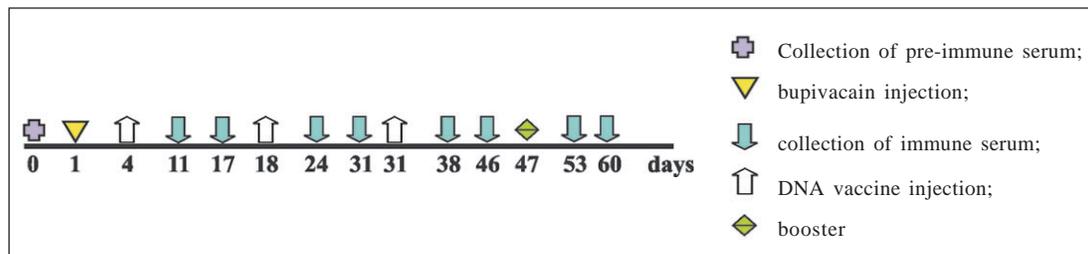


Figure 1. Diagram showing the immunization schedule used in this study.

9.6, and incubated overnight at 4°C. After blocking (PBS containing 5% milk, for 2 h at 37°C) the plates were incubated for further 2 h at 37°C with mouse sera diluted 1:10 in blocking buffer. Then, the plates were washed (PBS 0.05% Tween 20) and incubated at 37°C for 2 h with the secondary antibody (anti-mouse polyvalent peroxidase-conjugated; Dako) diluted 1:500. To develop the reaction, plates were washed as previously and incubated for 15 min at room temperature with a chromogenic substrate solution (3 mg o-phenylenediamine hydrochloride - OPD, 4 µL hydrogen peroxide diluted in 10 mL 0.2 M citrate/phosphate buffer, pH 5.0). To stop the reaction 50 µL/well 2 M H₂SO₄ was added and the optical density (OD) was read on a microplate reader Benchmark (BioRad) at 490 nm. All samples were tested in duplicate and a horse sera anti-PLP2a was used as positive control.

Statistical analysis

Student *t*-test and Mann-Whitney test were applied to determine the P values between vaccinated and control groups.

RESULTS

Protein expression and purification

Induction and expression of the PBP2a fragment containing the serine-protease domain in *E. coli* yielded a peptide with a molecular mass of approximately 8.0 kDa. However, the peptide expressed by *E. coli* BL21(DE3) cells transformed with the recombinant plasmid pET23a(+)-*mecA* was in inclusion body fractions (data not shown). Changes in temperature, IPTG concentration and incubation time had positive effect on solubility and ideal conditions were obtained after 6 h induction at 30°C with 1 mM IPTG. *E. coli* BL21(DE3) cells carrying the empty plasmid pET23a(+) were also included as negative control. Figure 2 shows the protein fractions present in the cell pellet and supernatant of *E. coli* BL21(DE3) containing the pET23a(+)-*mecA* plasmid and the empty plasmid pET23a(+). The peptide was present in the soluble fraction after 6 h of induction using 1 mM IPTG at 30°C. The recombinant peptide was successfully purified and concentrated by ultrafiltration and used for serum screening by ELISA. Figure 3 shows the samples obtained during the purification procedure, and the peptide samples obtained before and after concentration, relatively pure to use as antigen in ELISA experiments.

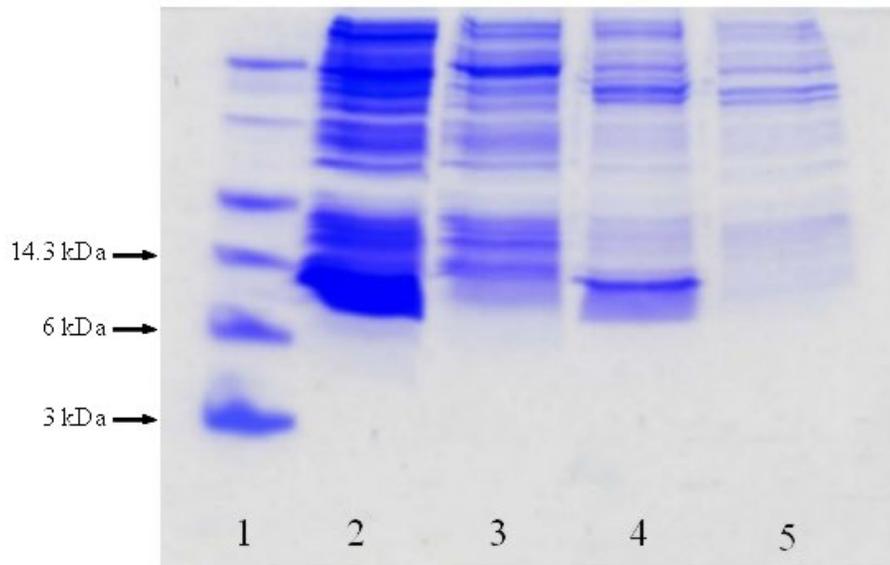


Figure 2. SDS-PAGE of protein fractions from *Escherichia coli* BL21(DE3) strain transformed with the recombinant plasmid pET23a(+)-*mecA* and pET23a(+) as negative control, showing the peptide *mec* being expressed mostly in the supernatant (soluble fraction) after sonication. Both bacterial cultures (cells transformed with recombinant plasmid or the negative control) were induced with 1 mM IPTG for 6 h at 30°C. *Lane 1.* 3-43-kDa Protein Molecular Weight Standards - Low Range (Life Technologies). *Lanes 2 and 4.* Soluble and insoluble (pellet) fractions of cells expressing the peptide *mec*, respectively. *Lanes 3 and 5.* Soluble and insoluble fractions of the negative control cells, respectively.

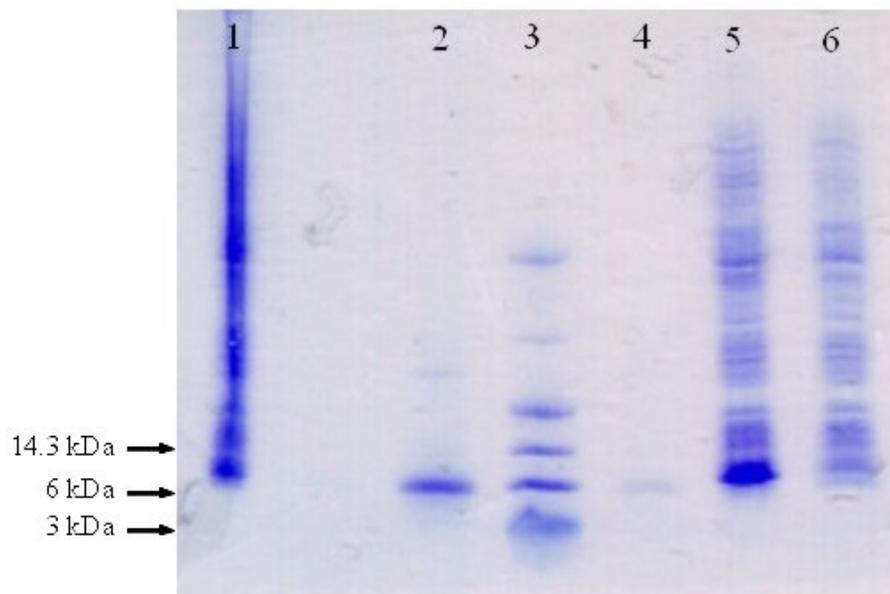


Figure 3. SDS polyacrylamide gel gradient showing recombinant peptide expressed by *Escherichia coli* BL21(DE3) and purified by ultrafiltration. *Lane 1.* Proteins retained by 30-kDa size exclusion membrane filter. *Lane 2.* Purified and concentrated *mecA* peptide-fraction obtained after ultrafiltration and concentrated by the 3-kDa size exclusion membrane filter. *Lane 3.* 3-43-kDa Protein Molecular Weight Standards - Low Range (Life Technologies). *Lane 4.* Peptide obtained after ultrafiltration before concentration. *Lanes 5 and 6.* Protein fraction from the *E. coli* BL21(DE3) cell lysates, transformed with pET23a(+)-*mecA* plasmid or pET23a(+) alone, respectively.

Animal immunization and humoral immune response evaluation

There were no deaths or signs of inflammation at the site of injection among the animals used in this study. We evaluated the presence of specific antibodies anti-PBP2a raised in BALB/c mice after DNA vaccine immunization by ELISA. All animals from the two different groups immunized with DNA vaccine (pCI-Neo-*mecA*) were able to produce specific antibodies anti-PBP2a, and higher antibody titers were observed after the fourth immunization (booster). Surprisingly, no difference between the immune response from the mice immunized with different vaccine doses (10 and 100 µg of DNA) was observed. Similar titer of specific antibodies was obtained from mice immunized with 10 or 100 µg of naked DNA vaccine, indicating that maybe DNA doses were not crucial to elicit humoral immune responses, being lower doses sufficient to stimulate antibody production. In contrast, as expected, the control groups failed in produce specific antibodies anti-PBP2a (Table 1), indicating that the DNA vaccine elicited a specific humoral immune response *in vivo* (Figure 4).

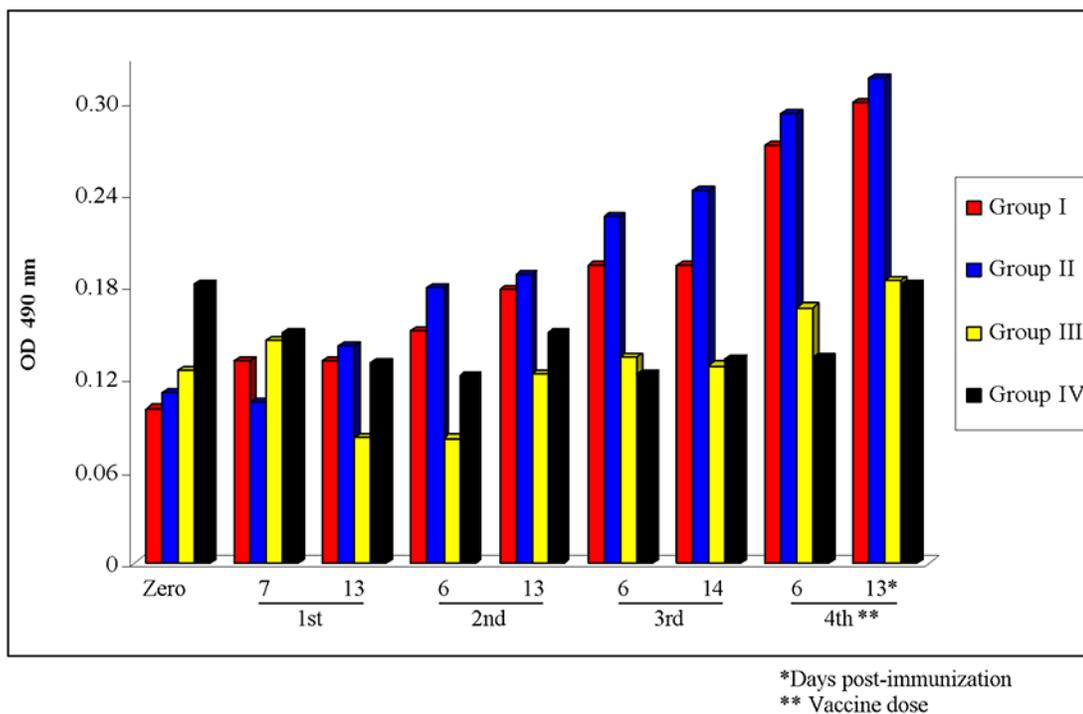


Figure 4. Presence of specific antibodies anti-PBP2a in the serum of mice immunized with two different doses of the DNA vaccine and the negative controls. Group I: 10 µg pCI-Neo-*mecA*/injection. Group II: 100 µg pCI-Neo-*mecA*/injection. Group III: 10 µg pCI-Neo plasmid. Group IV: PBS (100 µL/injection).

DISCUSSION

Staphylococcus aureus is an important community-acquired and nosocomial pathogen (Sheagren, 1984). Staphylococcal resistance to first-line drugs, such as synthetic penicillin and

clinically useful antibiotics, resulted in a major problem to treat MRSA infections, which are increasingly common, especially in hospitalized patients (McKenney et al., 1999). As a result, vancomycin is often the only remaining effective antibiotic. However, it has been reported in the past few years some cases of multiresistant bacteria, and no treatment is available to overcome these pathogens at all. Thus, alternative methods for prevention and treatment of multiresistant bacterial infections are eagerly sought.

Other methods, such as vaccination, have been evaluated as an alternative, but none has been currently in use to treat infections caused by MRSA multiresistant strains. In our study, we evaluated the antibody production in murine model to better understand the humoral immune response elicited by DNA vaccination to produce antibodies in large scale for passive immunization. We employed a naked DNA vaccine containing a fragment of *mecA* gene which codifies the active site of the PBP2a of MRSA. Firstly, this protein was chosen as antigen since the *mecA* sequence is a unique genetic marker for MRSA. Secondly, the PBP2a is located on the outer surface of the bacterial cytoplasmic membrane where it may be easily recognized by antibodies raised by the host immune system (Navarre and Schneewind, 1999).

Nowadays, several protocols employing DNA vaccine have been described. Priming-boost with recombinant proteins (Estcourt et al., 2004), DNA vaccine delivered by auxotrophic bacteria (Grillot-Courvalin et al., 1999), DNA vaccine with adjuvants (Lima et al., 2004), and associated with chemotherapy (Silva et al., 2005) are new strategies to increase the efficiency of DNA vaccines. However, most of them are time consuming and more expensive than the traditional protocols employing just the naked DNA to elicit immune response. The protocol employed in our study showed to be robust and easy to perform, allowing us to compare different doses of DNA vaccine and in the future it can be used in large scale for antibody production.

In a previous study, we report that our DNA vaccine anti-PBP2a is able to protect mice against MRSA infection (Senna et al., 2003). However, although DNA vaccines have been shown to be safer than some traditional vaccines like live vaccines and viral vectors, none of them are in current use for any infectious diseases in humans (Herrmann, in press), and unfortunately, there is still a number of questions that need to be elucidated before DNA vaccine commercialization, such as risk of integration into host cell genome and immune response against the DNA. More importantly, when MRSA is the infection disease in the context, a humoral immune response is needed to eliminate the infection (Cohen, 1986), but most of the MRSA-infected patients are immunosuppressed and probable unable to produce antibodies after immunization. Thus, a more attractive approach in these cases might be the direct use of antibodies by passive immunization to treat infected patients. We have shown here that our naked DNA vaccine anti-MRSA can elicit a humoral immune response stimulating the production of specific antibodies in mice. All animals vaccinated with DNA vaccine (pCI-Neo-*mecA*) produced specific antibodies anti-PBP2a of MRSA and higher titers were obtained after the fourth immunization (booster). Interestingly, we have demonstrated that the DNA dose, at least under conditions used in our study, was not crucial for the antibody production, being 10 µg per dose within four doses sufficient to elicit a humoral immune response *in vivo*. This is an important point to be established since high doses of DNA could stimulate tolerance. According to Liu et al., 2001, different doses of DNA can differ in eliciting antibody production. They demonstrate that 50 µg elicits weak responses, strong and long-lasting humoral responses were obtained with 100 µg, and 200 µg induced fast and high immune response as soon as after the first injection, but the titers decrease quickly from 3 to 5 weeks of the primary immunization. However, they em-

ployed different vaccination profile with only 2 doses of vaccine, whereas our study employed 4 doses. Therefore, maybe lower doses, as 10 µg of DNA, are efficient as higher doses when more immunizations are performed in the DNA vaccination regime. In conclusion, our naked DNA vaccine was efficient to elicit a humoral immune response in mice and can represent in the near future a valuable approach to produce specific antibodies against MRSA in large scale for passive immunization.

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