

Full Length Research Paper

Efficacy of immunization with the recombinant collagen adhesin A region against *Staphylococcus aureus*–induced mouse mastitis

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***Staphylococcus aureus* (*S. aureus*) is a major cause of bovine mastitis. In this present study, we assessed the efficacy of the recombinant A region of collagen adhesin (rCna-A) as a mastitis vaccine in a mouse model. Three groups of mice were immunized with either rCna-A, inactivated *S. aureus*, or phosphate-buffered saline (PBS) as a control. IgG and IgG subtype titers of mice in the rCna-A vaccine group were significantly higher than those in the killed vaccine group ($P<0.01$). Immunized lactating mice were challenged with *S. aureus* via the intramammary route. Significantly, fewer bacteria were recovered from mice in the rCna-A group than from those in the killed vaccine group ($P<0.001$). Histopathology indicated that the mammary structure showed greater integrity and a milder inflammatory response in the rCna-A group as compared with both the inactivated vaccine and PBS control groups. These results suggested that the rCna-A protein may be an attractive target for a vaccine against *S. aureus*-induced mastitis.**

Key words: *Staphylococcus aureus*, collagen adhesion, inactivated vaccine, mastitis.

INTRODUCTION

Mastitis is recognized as one of the most costly diseases affecting the dairy industry (Miller et al., 1993; Xu et al., 2011). *Staphylococcus aureus* (*S. aureus*) is a major bovine mastitis pathogen, and causes nearly 50% of bovine mastitis (Hynes, 1992; Haas and Plow, 1994). Because of the dramatic increase in antibiotic multi-resistant staphylococci and the slow development of new antibacterial agents, resolution of *S. aureus*-induced mastitis remains as a major problem to control. Vaccination is a logical approach to the control of infectious diseases in food production animals (Nilsson et al., 1998; Leitner et al., 2003). Over the past several decades live, heat-killed, and formalin-inactivated preparations of *S. aureus* cells or toxins have been tested as vaccines to

to prevent staphylococcal infections (Gong et al., 2010). However, these attempts to vaccinate against *S. aureus* infections have most often been unsuccessful (Flock, 1999; Hu et al., 2010).

Microbial adhesion to host tissue is the initial critical event in the pathogenesis of *S. aureus* infections (Patti et al., 1994a), and immunotherapies designed to inhibit the adhesion of pathogenic microorganisms appear to be an effective way to prevent or minimize the severity of an infection (Talbot and Lacasse, 2005). Collagen adhesin (Cna) has been reported to be the major adhesin responsible for high affinity collagen binding and is a major virulence factor in *S. aureus* infection (Holderbaum et al., 1986; Gillaspay et al., 1998). Cna was shown to be involved in the pathogenesis of *S. aureus* infection of the cornea in rabbit (Rhem et al., 2000), osteomyelitis (Elasri et al., 2002) and septic arthritis in mice, respectively (Patti et al., 1994b). Moreover, passive immunization with antibodies against Cna also showed promising protection

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in a mouse model of sepsis (Flock, 1999).

S. aureus Cna consists of an N-terminal signal peptide, a non-repetitive A region, and one to four repeating units, followed by a cell wall anchor region, a transmembrane segment, and a short, positively-charged cytoplasmic tail. The A region of Cna was found to be fully responsible for the collagen binding activity of *S. aureus* (Rich et al., 1998). Nilsson et al. (1998) reported that mice vaccinated with the collagen binding portion of Cna were partially protected from septic death. Molecular characterization of the Cna gene has shown that the A region is conserved among clinical *S. aureus* isolates. It is thus an attractive target for development of Cna-A vaccines against *S. aureus* mastitis. However, there is at present no reports on the efficacy of Cna-A as a vaccine against *S. aureus* mastitis. In this present study, rCna-A was expressed and subunit vaccines were prepared and evaluated in a mouse mastitis model. The mechanism of protection against *S. aureus* was further investigated.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

Escherichia coli strain DH5 α , grown in Luria-Bertani broth or agar (Difco), was used for cloning plasmids, and pET-28a (+) was used as an expression vector. *E. coli* BL21-CodonPlus (DE3) was used as a host for protein production. *S. aureus* strain J9 was isolated from bovine mastitis in China, and grown in either Tryptic Soy Broth or Agar (BD Difco, Sparks, MD, USA).

Cloning of Cna-A

Genomic DNA was extracted from *S. aureus* strain J9 using an extraction kit (Clontech, Palo Alto, CA, USA). Extracted DNA was then used as the template for polymerase chain reaction (PCR). The gene encoding Cna-A was amplified using the forward primer Can-A-Fwd (5'-TATGGATCCGTAGCTGCAGATGCACC-3'), which includes a *Bam*HI restriction site (underlined), and the reverse primer Cna-A-Rev (5'-CGCCTCGAGCTCTGGAATTGGTTCAATTTC-3'), which includes a *Xho*I restriction site. PCR was conducted under the following conditions: an initial 5 min denaturation at 94°C followed by 30 cycles of 30 s at 94°C, 45 s at 64°C, and 2 min at 72°C with a final extension at 72°C for 10 min. PCR product was purified with a Qiagen PCR Product Purification kit (Qiagen, Chatsworth, CA) as described by the manufacturer, and the rCna-A was subsequently cloned into pET-28a (+) digested with *Bam*HI and *Xho*I to generate the recombinant plasmid pET-28a-Cna-A. Restriction digestion, ligation, and transformation of DNA into competent *E. coli* cells were performed using standard methods (Sambrook and Russell, 2001) or following the manufacturer's instructions.

Expression and purification of rCna-A

Recombinant Can-A protein expression was induced by the addition of 1 mM isopropyl- β -D-thiogalactoside (IPTG), and the cells were harvested 4 h post-induction. The cells were then collected by centrifugation at 10,800 \times g for 10 min at 4°C, washed in 0.1 M PBS (pH 7.2), and disrupted by sonication. rCna-A was purified by affinity

chromatography using nickel-nitrilo triacetic acid resin (Ni-NTA, Qiagen) according to the manufacturer's specifications.

Western blot analysis

The protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto Immobilon-P polyvinylidene difluoride membranes and blocked with 3% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at 25°C. The membranes were washed with PBS-Tween 20 (PBST) three times and further incubated for 4 h with rabbit anti-*S. aureus* antibody prepared in our laboratory. After extensive washing, bound antibody was detected using horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Southern Biotech, Birmingham, AL, USA). The membranes were washed with PBST, followed by washing with 0.1 M sodium acetate for 5 min. Finally, the reaction on the membranes was developed with 3, 3'-diaminobenzidine in 0.1 M sodium acetate buffer containing hydrogen peroxide.

Vaccine preparation

In order to prepare the protein vaccines, 40 μ g of purified rCna-A was dissolved with 100 μ l of PBS and emulsified with an oil adjuvant. *S. aureus* strain J9 was cultured and adjusted to 1×10^6 CFU/ml, and then inactivated at 37°C for 24 h by the addition of formaldehyde. After confirmation that no bacteria had survived by culture assay, the bacteria were emulsified at a 1:1 ratio with oil adjuvant to prepare the bacterial vaccine.

Mouse immunization

Forty-eight specific pathogen free (SPF) BALB/c mice (12 male, 36 female, age 7 to 9 weeks) were maintained and handled according to the Regulations for the Administration of Affairs Concerning Experimental Animals. The female mice were randomly divided into three groups of twelve, one of which was injected intraperitoneally with rCna-A protein vaccine. Another group was immunized with inactivated *S. aureus*. Aliquots of 100 μ l of each vaccine were injected intraperitoneally into mice. Booster injections were performed two weeks later. The control group was injected with PBS following the same protocol.

IgG detection

Enzyme-linked immunosorbent assays (ELISAs) were used to determine the IgG titers against rCna-A according to the method of Gong et al. (2010). Polystyrene Maxisorp 96-well plates (Nalgen Nunc International Corp., Rochester, NY, USA) were coated overnight at 4°C with 0.1 μ g rCna-A per well. Following saturation of the plates with a skim milk solution (5% w/v) overnight at 4°C, the serum samples were added to the plate and incubated for 45 min at 37°C. HRP-conjugated goat anti-mouse IgG antibodies were then added and incubated for 30 min at 37°C. Chromogenic substrate solution (100 μ l; 42 mM tetramethylbenzidine and 0.01% hydrogen peroxide) was added for 10 min and the enzymatic reaction was stopped by the addition of 50 μ l hydrofluoric acid (HF). Three washes with PBS-0.05% Tween-20 were performed between each step. The optical density (OD) was read on a plate reader (BioTek Instruments, Winooski, VT, USA) at 630 nm. Endpoint titers were calculated as the reciprocal of the last serum dilution that gave a value twofold higher than PBS control group.

The method used to determine antibodies titers against *S. aureus* was the same as that described above for rCna-A except that the

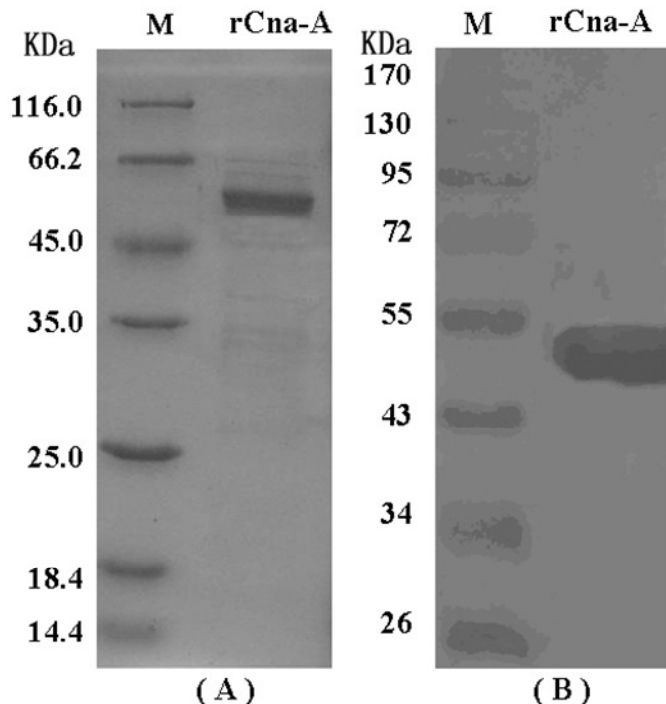


Figure 1. SDS-PAGE analysis of rCna-A expressed in *E. coli* (A). Lanes rCna-A, rCna-A purified by His affinity chromatography; Lanes M, reference proteins with the molecular weight labeled on the left. Western blot analysis of rCna-A (B). Lanes rCna-A, rCna-A protein; Lanes M, pre-stained reference proteins with the molecular weight labeled on the left.

coated antigen was replaced with killed *S. aureus*.

Detection of IgG1 and IgG2a subtypes

ELISAs were performed as described above except that the secondary antibody was either mouse anti-IgG1-HRP (Southern) or mouse anti-IgG2a-HRP (Southern). The dilution was 1:100 in the inactivated *S. aureus* group and 1:102,400 in the rCna-A group.

Challenge of lactating mice and enumeration of bacteria

Female and male mice were placed in a 3:1 ratio in the same cage so that the female mice could become pregnant. The mice were anaesthetized and mammary glands were inoculated under a binocular stereomicroscope. According to the methods of Hu et al. (2010), mice were infected in the R4 (fourth on the right) and L4 (fourth on the left) mammary abdominal glands by injecting 50 μ l of a bacterial suspension containing approximately 5×10^6 CFU in PBS into the mammary ducts of each mouse. After 24 h, the mice were killed and their mammary glands were aseptically removed. The glands were then placed in sterile PBS (1:10, w/v) and homogenized. Appropriate dilutions were seeded in tryptic soy agar (TSA) to determine the numbers of CFU per gland.

Histological examination of mammary glands

Mammary glands at 24 h after inoculation were fixed in 4% formaldehyde in PBS for 24 h then embedded in paraffin wax. Fixed

mammary glands were dehydrated through a graded alcohol series, immersed in xylene, and embedded in paraffin. The slices were then cut and stained with hematoxylin and eosin (H&E). Light microscopy was performed at a magnification $\times 400$ and histological micrographs were obtained. Morphological characteristics of the test and control groups were compared.

Statistical analysis

The arithmetic mean and standard error of the mean were calculated for each treatment group. The data were analyzed using an analysis of variance (ANOVA) with the SAS software package (SAS Institute, Cary, NC, USA). P values < 0.05 were considered statistically significant.

RESULTS

Expression of the recombinant plasmids

Recombinant plasmids containing the Cna-A protein were constructed in the backbone of a pET-28a (+) vector for expression of the recombinant protein. The rCna-A protein before and after purification was analyzed by SDS-PAGE, and the bands with the expected size (50 kDa) were observed (Figure 1A). Western blot assay confirmed that rCna-A was immunogenic and reacted with rabbit antiserum to *S. aureus*, as shown by the band present at the expected size on the blotted membrane (Figure 1B).

Antibody response detected by ELISA

Both inactivated *S. aureus* and rCna-A induced an immune response in mice. Two weeks after the first immunization, antibody titers reached over 1:200 in both groups. Furthermore, mice primed with rCna-A produced significantly higher antibody titers as compared to mice vaccinated with inactivated bacteria. The antibody levels continued to rise and reached a peak after the third immunization (1:526,628 in the rCna-A group and 1:3,200 in the inactivated *S. aureus* group, $P < 0.001$), when sample collection was stopped (Figure 2A). However, antibody production rates in the subunit vaccine group were faster than in the inactivated vaccine group for each immunization. IgG1 and IgG2a isotype levels were evaluated in each group (Figure 2B). Only the groups vaccinated with rCna-A demonstrated a higher proportion of IgG1 than IgG2a, with 2000-fold higher levels induced by the inactivated bacterial vaccine.

Bacterial loads of infected mammary glands

Immunized mice were then challenged with an injection of *S. aureus* (50 μ l of a bacterial suspension containing approximately 5×10^6 CFU in PBS) in the R4 and L4 glands. Twenty-four hours after infection, the numbers of

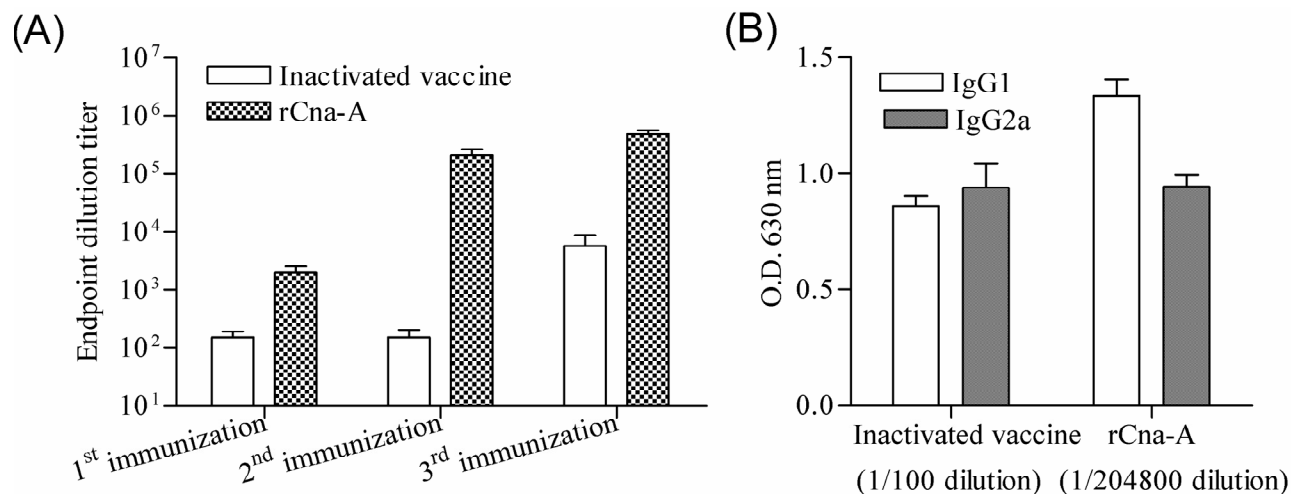


Figure 2. Serum total IgG (A) and IgG1 and IgG2a (B) titers as determined by ELISA. Mice were immunized three times, and sera were obtained after each vaccination. Results are shown as mean \pm SD.

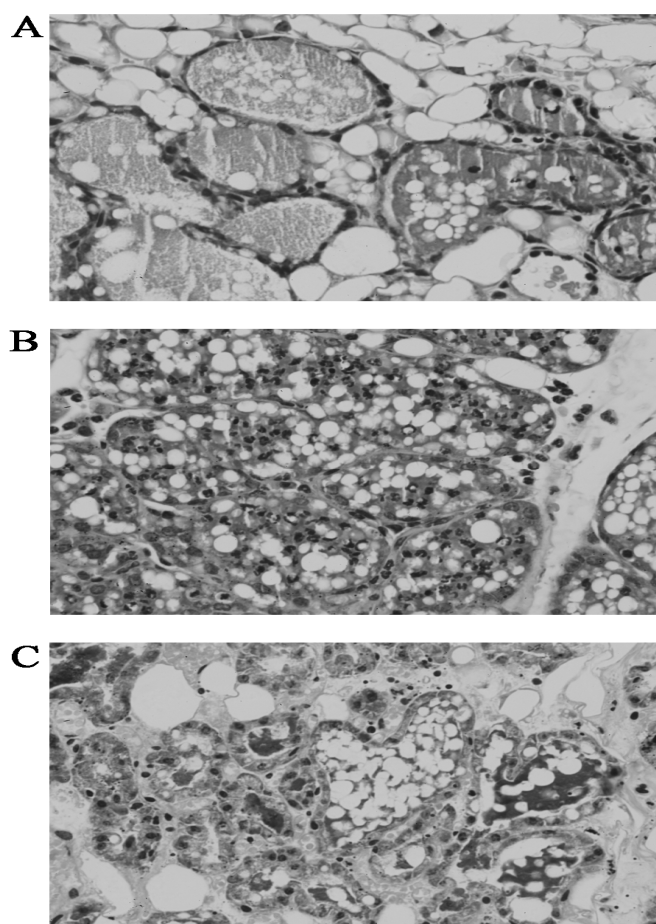


Figure 3. Microscopic images of mammary glands in the rCna-A group (A), inactivated *S. aureus* group (B), and PBS control group (C). A = The structure of breast tissue intact with only few PMNs visible. B = The mammary acinar structure partially destroyed with greater PMN infiltration. C = Most mammary glands damaged and necrotic. Magnification: 400.

CFU in the mammary glands of the rCna-A group ($6.40 \times 10^2 \pm 4.73 \times 10^2$ CFU/gland) were significant lower than those in both the inactivated vaccine ($3.24 \times 10^8 \pm 8.73 \times 10^7$ CFU/gland, $P < 0.001$) and PBS control ($5.16 \times 10^9 \pm 1.23 \times 10^8$ CFU/gland, $P < 0.001$) groups.

Histology

Mammary gland sections were stained with H&E and examined (Figure 3). In the rCna-A group, breast tissue structures were intact and some PMN infiltration had occurred in a portion of the mammary gland. In the inactivated vaccine group, many mammary glands inflammatory showed cell infiltration, and some proliferation of mammary stroma were observed. In the PBS control group, most mammary glands were damaged, mammary epithelial cells were swollen, milk was coagulated, and a large number of epithelial cells were necrotic and lost.

DISCUSSION

Although infection by *S. aureus* is a complex process, adhesins play the most important role in *S. aureus* infection and invasion (Joh et al., 1999; Miller and Cho, 2011). Thus, blocking bacterial adhesion to cells and colonization of the mucosal surface may be the most effective strategy for preventing *S. aureus* infection (Nour El-Din et al., 2006), and vaccines that target adhesins have shown promise in terms of preventing *S. aureus*-induced mastitis (Gong et al., 2010). Many adhesins have proved to be attractive for inclusion in vaccines against *S. aureus* infection. Cna, one of the most important adhesins, proved to be an ideal candidate component for inclusion in a vaccine against *S. aureus* infection (Flock,

1999). In this present study, the gene Can-A encoding the A region of Cna was cloned and rCna-A was successfully expressed. Western blot analysis showed that rCna-A possessed good immunogenicity and reactogenicity, which provided a basis for further evaluation of the efficacy of the mastitis vaccine against *S. aureus* infection.

In this study, mice were immunized with rCna-A, inactivated *S. aureus*, and PBS, respectively. The rCna-A group was superior to the inactivated vaccine group in terms of both the antibody production rate and the final titer. Higher antibody titers are thought to induce better protection against intramammary infection (Otto, 2008). In the rCna-A immunized group levels of the IgG1 subtype were 2000 times higher than in the inactivated vaccine group, showing that IgG1 is the dominant subclass. Further, the IgG1 subtype is the most effective inducer of opsonophagocytosis (Schlageter and Kozel, 1990; Gong et al., 2010).

In order to evaluate the potential effects of these vaccines on *S. aureus* mastitis, a mouse mastitis model was used as described by Gong et al. (2010). Only a small number of *S. aureus* (500 to 5000 CFU) are required for induction of mouse mastitis symptoms, and both the appearance and histology of infected mammary glands in mice are similar to those in naturally-infected bovine mammary glands (Kim et al., 1999; Akers and Nickerson, 2011). In this study, the numbers of bacteria in the mammary glands of the rCna-A immunized group were significantly lower than in either the inactivated *S. aureus* or PBS groups. These results indicate that rCna-A could prevent *S. aureus* invasion more effectively than inactivated bacteria. Histological examination lent support to this view.

The mammary glands of mice in the rCna-A group showed greater structural integrity and a milder inflammatory response as compared to the inactivated vaccine and PBS control groups. These observations indicate that vaccination with rCna-A protected mice against *S. aureus* infection.

In summary, the rCna-A subunit vaccine developed in this study provided significantly better protection against *S. aureus*-induced mastitis compared to that produced either by the inactivated *S. aureus* vaccine or the mock immunization in mouse model. The rCna-A protein may thus be a potential candidate of a vaccine against *S. aureus*-induced mastitis. The precise mechanism by which rCna-A provides protection against *S. aureus* requires further elucidated.

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