Veterinary Immunology and Immunopathology 214 (2019) 109890

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Immunological responses and evaluation of the protection in dairy cows vaccinated with staphylococcal surface proteins

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ARTICLE INFO

Keywords:
Mastitis
Immune response
Vaccine
Surface protein
Staphylococcus aureus
Staphylococcus chromogenes
Protective effect

ABSTRACT

Bovine mastitis is a significant cause of economic losses in the dairy industry. *Staphylococcus aureus* is one of the most common contagious mastitis pathogens, whereas *Staphylococcus chromogenes* increasingly became a significant cause of subclinical mastitis in dairy cows. Current mastitis control measures are not effective on all mastitis pathogens. There is no effective vaccine to control Staphylococcal mastitis in dairy cows. The objective of this study was to evaluate the immune responses and protection in dairy cows vaccinated with *S. aureus* surface proteins (SASP) or *S. chromogenes* surface proteins (SCSP). We divided eighteen Holstein dairy cows randomly into three groups of 6 animals each. We vaccinated group 1 and 2 animals with SASP and SCSP with Emulsigen-D adjuvant, respectively. We injected control (group 3) animals with PBS (pH 7.2) in Emulsigen®-D. We vaccinated animals three times at 28 and 14 days before drying off, and at dry off. Two weeks after the third vaccination, we challenged each animal by dipping all teats in *S. aureus* culture suspension once daily for 14 consecutive days. We evaluated milk or mammary secretion and serum antibody titers during vaccination and challenge periods. We evaluated milk samples for the number of bacteria shedding and somatic cell counts (SCC). Out of six cows vaccinated with SASP, one cow was removed from the study due to injury, two were infected clinically, another two were infected subclinically, and the remaining cow was not infected. No SCSP vaccinated cows developed clinical or subclinical mastitis. Out of six control cows, two developed clinical mastitis whereas four were infected subclinically. The SCSP vaccine cross-protected against *S. aureus* mastitis and reduced number of *S. aureus* shedding in milk. We concluded that the SCSP is a promising vaccine to control Staphylococcal mastitis in dairy cows.

1. Introduction

Bovine mastitis is the major cause of economic losses in the dairy industry worldwide (De Vliegher et al., 2012). Economic losses due to bovine mastitis are estimated to be $2 billion in the United States (NMC, 2005), $400 million in Canada (Carson et al., 2017) and $130 million in Australia (Ismail, 2017) per year. Intramammary infection (IMI) also has a significant effect on public health since some of the causative pathogens and/or their toxins may enter the food supply resulting in foodborne disease (Fitzgerald, 2012a, 2012b; Graveland et al., 2011; Holmes and Zadoks, 2011; Pantosti, 2012; Srinivasan et al., 2006). Bovine mastitis is usually caused by bacteria. *Staphylococcus aureus* is a frequent causative agent of mastitis in dairy cows, which usually results in chronic IMI. *Staphylococcus aureus* is one of the most common contagious mastitis pathogens, whereas *S. chromogenes* increasingly became a major causative agent of subclinical mastitis in dairy cows (Bradley, 2002; De Vliegher et al., 2012; Pyorala and Taponen, 2009; Vanderhaeghen et al., 2014). *In vitro* studies showed that non-coagulase positive *Staphylococcus* species (CNS) from bovine mammary glands (Carson et al., 2017) and some *S. chromogenes* isolate from teat apices of dairy heifers (De Vliegher et al., 2004) produce bacteriocin which prevented the growth of *S. aureus* and other bacterial mastitis pathogens. *In vivo* study in humans showed that nasal colonization by *Staphylococcus lugdunensis* strain, which produce lugdunin, an

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https://doi.org/10.1016/j.vetimm.2019.109890
Received 1 March 2019; Received in revised form 19 June 2019; Accepted 3 July 2019

antibacterial agent, reduced *S. aureus* nasal carriage (Zipperer et al., 2016). We also found that prior colonization of mammary glands of a dairy cow with our vaccine strain of *S. chromogenes,* which does not produce bacteriocin prevent subsequent colonization by *S. aureus*. This was observed only in one particular animal in one experiment with repeated experimental challenges over 14 days (None published data). It is unknown whether this *in vivo* preventive effect of *S. chromogenes* is related to previously reported *in vitro* antibacterial effect of bacteriocin from *S. chromogenes* (De Vliegher et al., 2004) or related to inducible innate or acquired immune responses. Even though this *in vivo* preventive effect against *S. aureus* may be related to the bacteriocidal or bacteriostatic product from *S. chromogenes*, other studies showed that bacteriocidal/static proteins induced strong protective immune response characterized by increased production of interferon gamma and IgA in mice (Wang et al., 2017). We selected *S. chromogenes* strain for SCSP vaccine preparation because of its ability to prevent the mammary gland colonization by *S. aureus* under *in vivo* condition.

Dairy cows are more susceptible to IMI during early dry and transition (periparturient) periods. The incidence of IMI is high during the early dry period because of an absence of hygienic milking practices (teat washing and drying, as well as pre- and post-milking teat dipping in antiseptic solutions) that are known to reduce teat end colonization and infection. The high incidence of mastitis during transition (periparturient) period is due to combined effects of parturition hormones which cause immunosuppression, negative energy balance at early lactation due to the mobilization of body energy reserve to meet high energy demand for milk production and parturition related physical stress (Esposito et al., 2014).

Current *S. aureus* mastitis control measures are good milking routines, use of properly functioning milking machines, segregation, and culling of persistently infected animals, dry cow antibiotic therapy; and prompt identification and treatment of cows with mastitis (Barkema et al., 2006). These control measures reduced the incidence of contagious mastitis pathogens when fully adopted and applied. However, staphylococcal mastitis remains to be a significant problem in dairy farms since most farms not fully adopted current control measures.

Dairy farmers in the United States and many other parts of the globe rely on the prophylactic intramammary infusion of long-acting antimicrobials at drying off to prevent new infections during dry period or to treat existing infections (USDA APHIS, 2008; Oliver et al., 2011). The intramammary administration of antimicrobials at drying off is a growing issue since this practice exposes a large number of healthy animals to antimicrobials. This exposure puts pressure on commensal bacteria (*e.g.*, Gram-negative bacteria in the gastrointestinal tract) in the body of dairy cows, which are serious human pathogens and other opportunistic bacteria to develop antimicrobial resistance. *Staphylococcus aureus* is one of the pathogens with a known ability to develop antimicrobial resistance and established *S. aureus* infections are persistent and difficult to clear. Other factors associated with the difficulty of curing *S. aureus* intramammary infection includes biofilm formation (Donlan and Costerton, 2002; Melchior et al., 2006; Stewart and Costerton, 2001), formation of small colony variant (Alkasir et al., 2013; Atalla et al., 2008; Tubby et al., 2013; Zhu et al., 2016) and survival of the bacterium within the epithelial cells and macrophages (Almeida et al., 1996). All these factors enable *S. aureus* to resist clearance by immune defense (*i.e.*, phagocytosis) or antibiotic treatment (Almeida et al., 1996; Hébert et al., 2000; Hensen et al., 2000). The failure to control these infections leads to the presence of reservoirs in the dairy herd, which, ultimately leads to the spread of the infection and the culling of the chronically infected cows (Barkema et al., 2006; McDougall et al., 2009).

Effective vaccine against staphylococcal mastitis is a sustainable control measure than use of antimicrobials for treatment and control of mastitis in dairy cows.

There are two commercial inactivated whole cell (Bacterin) vaccines for the control of Staphylococcal mastitis in dairy cows. These are the Lysigin® (Boehringer Ingelheim Vetmedica, Inc, St. Joseph, MO) in the US and the Startvac® (Hipra, Girona, Spain) in Europe and some other countries (Freick et al., 2016). Some field and controlled experimental efficacy studies on Lysigin® and Startvac® showed that these vaccines reduced the incidence, severity, and duration of mastitis in vaccinated cows compared with non-vaccinated control cows (Bradley et al., 2015; Piepers et al., 2017; Schukken et al., 2014). Contrary to these observations in other studies, these vaccines did not improve udder health or showed no difference between vaccinated and non-vaccinated control cows (Freick et al., 2016; Landin et al., 2015). None of these Bacterin-based vaccines prevents new *S. aureus* IMI (Bradley et al., 2015; Middleton et al., 2009, 2006; Schukken et al., 2014). Therefore, developing an effective vaccine that overcomes staphylococcal immune evasion mechanisms is a sustainable alternative approach to control staphylococcal mastitis than prophylactic use of antimicrobials. We hypothesize that the strategic vaccination of dairy cows with immune-reactive staphylococcal surface proteins during the early dry period will enhance intramammary immune responses during the transition period, thus conferring protection against *S. aureus* IMI. The objective was to evaluate the efficacies of *S. aureus* surface proteins (SASP) and *S. chromogenes* surface proteins (SCSP) as vaccine antigens to control *S. aureus* mastitis in dairy cows.

2. Materials and methods

2.1. Study animals

We divided eighteen pregnant Holstein dairy cows randomly into three groups of six cows each. Group 1 and Group 2 cows were vaccinated with *S. aureus* surface proteins (SASP) and *S. chromogenes* surface proteins (SCSP) with Emulsigen®-D adjuvant (Phibro Animal Health Corporation, Omaha, NE), respectively. We injected Group three cows with PBS (pH7.2) mixed with Emulsigen®-D adjuvant at an equal proportion (1.5 ml each) and used as a control. Before enrolment in the study all cows had somatic cell counts of less than 250, 000 SCC/ml of milk except two cows which had more than 250,000 SCC/ml of milk in all quarters and four cows that had more than 250,000 SCC/ml of milk in one of their four quarters. However, there was no bacterial growth from those quarters with SCC more than 250,000 cells/ml. The remaining quarters of all cows had less than 250,000 SCC/ml of milk before enrolment in the study. On average, all animals had a background serum, and milk LS mean log titers of about 3 to 4. This indicated that these animals had some titer against vaccine antigens before vaccination which we have seen in almost all previous studies we conducted. This background titer is from previous exposure to Staphylococcus or similar bacteria in life. This study was approved by the University of Tennessee, Institutional Animal Care and Use Committee (IACUC No. 2394-1115). Experimental and control cows were under the same herd management throughout the study and housed at the East Tennessee Research and Education Center-Little River Animal and Environmental Unit (ETREC-LAEU, Walland, TN).

2.2. Vaccine strain selection, antigens preparation, and vaccine formulation

S. aureus surface proteins (SASP) and *S. chromogenes* surface proteins (SCSP) were extracted using 1% cholic acid (Sigma-Aldrich, St. Luis, MO). We select a *S. aureus* vaccine strain based on analysis of pulsed-field gel electrophoresis (PFGE) results of 111 *S. aureus* isolates from cases of bovine mastitis from 11 dairy farms in Eastern Tennessee collected during 2005–2012 (none published data). We found 16 different PFGE types. Of 16 PFGE types, three were predominantly distributed among 11 farms included in the study. The criteria for selection was dominance among strains or the most frequent isolates among our *S. aureus* collection as determined by PFGE typing. We selected *S. aureus* strain 38 (SAUT1), one of the three most prevalent isolates for SASP vaccine preparation. Similarly, the challenge strain, *S. aureus*

strain 60 (SAUT2) was in the different PFGE type but most frequently isolated from different farms like vaccine strain. We used *S. chromogenes* isolate that prevented mammary gland colonization by our challenge strain of *S. aureus* under *in vivo* condition.

For antigen preparation, selected strains were streaked on blood agar plates and incubated at 37 °C overnight (16–18 h). After incubation, we suspended three isolated colonies in 450 ml of tryptic soy broth (TSB) and grown to mid-log phase with shaking at 125 rpm at 37 °C in 5% CO2: 95% air balanced incubator. We centrifuged the culture at 500X g for 10 min at 4 °C and the pellet was resuspended in 1% cholic acid (Sigma-Aldrich) and incubated at room temperature for 2 h with shaking (125 rpm). The bacterial suspension was centrifuged at 10,000X g for 30 min at 4 °C and proteins in the supernatant were concentrated using Centriprep Ultracel-10 K YM concentrators with ten kDa cut off (EMD Millipore Corporation, Billerica, MA). We measure protein concentration using a BCA protein assay kit (Thermofisher Scientific, Waltham, MA). The vaccine was prepared, using either SASP or SCSP (1.2 mg in 1.5 ml of PBS [pH 7.2]) mixed with 1.5 ml of Emulsigen®-D (Phibro Animal Health Corporation), resulting in a final total volume of 3 ml. Similarly, we injected control cows with a total volume of 3 ml (1.5 ml of Emulsigen®-D mixed with 1.5 ml of PBS).

2.3. Vaccination schedule

All cows were given three series of vaccinations at about 14 days interval subcutaneously (SQ) on alternate sides of the neck area, approximately midway between the base of the ear and the point of the shoulder at 28 days before drying off (D-28), 14 days before drying off (D-14) and at drying off (D0).

2.4. Vaccine safety

To monitor any possible adverse reactions against vaccine, we monitored rectal temperature and injection site reaction and recorded. We measured rectal temperatures 24 h before vaccination, immediately before vaccination, and for three consecutive days following vaccination. We monitored injection site reactions at the same time points by measuring the length (cranial/caudal), width (dorsal/ventral), and height (thickness) in millimeters (mm). We monitored all vaccinated cows closely for any adverse reaction to the vaccine, loss of appetite or any other complications at 24 h, daily for 1–3 days and at days 7 and 14 after each vaccination.

2.5. Evaluation of vaccine-induced immune response by enzyme-linked immunosorbent assay (ELISA)

We analyzed serum- and milk/mammary secretion-anti-SASP and -SCSP IgG, IgG1, IgG2, and IgA antibody titers using indirect enzyme-linked immunosorbent assay (ELISA) as described elsewhere (Kerro Dego et al., 2006). Briefly, we coated 96 well Polystyrene plates (Immulon® 2 HB) (ThermoScientific, Rochester, NY) with 1 μg/ml of SASP or SCSP in a sodium bicarbonate (NaHCO3) coating buffer [0.015 molar (M) Sodium Carbonate (Na_2CO_3) and 0.034 M Sodium Bicarbonate ($NaHCO_3$) solution of pH 9.6] and incubated overnight (16–18 h) at 4 °C. We removed coating buffer and plates were washed 5X using automated 405 touch screen (TS) microplate washer (Biotek instrument Inc., Winooski, VT) with PBS containing 0.05% Tween ®20 (v/v) (PBS-T) (Thermofisher Scientific) and blocked with PBS-T containing 1% gelatin (W/V) (PBS-TG) (Thermofisher Scientific) for 2 h. The Plates were washed 5X with PBS-T and serum and skimmed samples were serially diluted four folds starting from 1:100 dilution for serum and from 1:10 dilution for skim horizontally across 12 wells of 96 well plates and incubated at room temperature for 1 h. After incubation plates were washed 5X with PBS-T and 100 μl of 1: 10,000 diluted (in PBS-TG) horseradish peroxidase-conjugated polyclonal sheep anti-bovine IgG or IgA (heavy + Light Chains), or monoclonal sheep anti-bovine IgG1 or IgG2 (Bethyl Laboratories, Inc. Montgomery, TX) were added and incubated for 1 h at room temperature. After incubation, plates were washed 5X with PBS-T, and 100 μl of ABTS™ horseradish peroxidase substrate (SeraCare Life Sciences, Milford, MA) were added and incubated for 20 min at room temperature. We read the absorbance at a wavelength of 405 nm using a Synergy H1 Microplate reader (Biotek instrument Inc). We exported the data to Excel (Microsoft Corporation), and the average + 2 standard deviations (avg + 2stdev) of the blank row (A1– A12) was used to determine the cutoff point for titer calculation. We calculated serum or skim titers by the intersection of least-square regression of A405 *versus* the logarithm of dilution.

2.6. Experimental challenge (infection) and evaluation of protection

S. aureus strain 60 (SAUT2), which is different from our vaccine strain 38 (SAUT1), was used as our heterologous challenge strain. This strain was originally isolated from a dairy cow with mastitis. It was another dominant strain in our *S. aureus* collection from different farms in Eastern Tennessee. An aliquot from a frozen vial of *S. aureus* strain UT60 (SAUT2) stored at −80 °C in 50% tryptic soy broth/glycerol was inoculated to a blood agar plate and grown overnight (16 to 18 h) at 37 °C. After incubation, five colonies were inoculated into 2 liters of tryptic soy broth (TSB) and grown for 3.5 h at 37 °C to create our challenge culture. Before the challenge, teats were cleaned thoroughly with mild non-bactericidal dish detergent and dried with an individual paper towel. Two weeks after the third vaccination, we challenged each teat by dipping in an *S. aureus* culture suspension using a single cup per cow, containing approximately 100 ml of *S. aureus* suspension at 1×10^7 CFU/ml of culture media for approximately 15 s once daily for 14 consecutive days. The number of *S. aureus* in the challenge suspension was determined using a viable plate count before and after the challenge. We challenged each cow once a day for 14 consecutive days, or until removed from the study when a cow developed clinical mastitis. After the challenge, we allowed teats to air dry for about 10 min before releasing cows from the parlor, and the floor of the parlor was disinfected by hypochlorite (Bleach or Clorox) (Thermo Fisher Scientific) at 1:10 dilution.

2.7. Clinical examination of challenged cows

During the challenge period, we examined rectal temperature, conducted a clinical assessment of inflammatory changes in the mammary gland tissue, and mammary secretion and recorded the findings. The following scoring system was used to determine inflammatory changes in the milk or mammary secretion: 0 = Normal, 1 = Flakes, 2 = Clots, 3 = Stringy/watery/bloody. We scored inflammatory changes in the mammary gland tissue as follows: 0 = Normal; the udder is pliable, no detection of heat, pain, redness, and/or swelling, 1 = Slight swelling; the udder is less pliable, some firmness detected, heat, pain, redness, and/or swelling not necessarily detected, 2 = Moderate swelling; the udder is firm, redness and heat detected, discomfort detected, 3 = Severe swelling; the udder is very hard, red and hot, noticeable difference compared to other quarters and the cow exhibits signs of irritation.

We measured rectal temperature of each cow daily during challenge period to monitor for potential systemic reactions to challenge with *S. aureus*. We declared that a cow developed clinical mastitis when inflammatory changes of both mammary secretion and mammary gland tissue were scored 2 or when either inflammatory changes in the mammary secretion or in the mammary gland tissue was scored 3 for three consecutive days. Before challenge, in this study, a lactating cow is declared subclinically infected when somatic cell count (SCC) of composite milk is > 200,000 cells/ml of milk with positive isolation of the mastitis pathogen from milk for three consecutive days without manifestation of clinical signs of mastitis. A quarter of a lactating cow is declared subclinically infected when SCC is > 100,000 cells/ml of milk

without manifestation of clinical signs of mastitis. For a dry cow, these scores are higher because of the increase in SCC due to a decrease in mammary secretion volume. So in this study, a cow was declared subclinically infected when composite mammary secretion SCC was > 250,000 cells/ml of mammary secretion, with positive isolation of the challenge strain from mammary secretion for three consecutive days without manifestation of clinical signs of mastitis. A quarter of a cow was declared subclinically infected when SCC was > 150,000 cells/ml of mammary secretion with positive isolation of challenge strain for three consecutive days without clinical manifestation of mastitis. A quarter with *S. aureus* count in the mammary secretion but did not develop clinical or subclinical mastitis is categorized as no mastitis but shedding *S. aureus*. Clinically infected cows were removed from the challenge and treated with an antibiotic (Ceftiofur hydrochloride/ Spectramast® DC) (Zoetis Inc., Kalamazoo, MI) following manufacturer instructions for dry cow treatment. We conducted the antibiotic sensitivity test on the challenge *S. aureus* strain 60 (SAUT2) before the beginning of the challenge study during challenge model development, and the selected challenge strain was sensitive to Ceftiofur. At the end of challenge period, all cows were treated with Ceftiofur and the clearance of infection and bacterial shedding through milk was checked by culturing milk samples collected on the day of calving (C) and three days after calving (C + 3).

2.8. Mammary gland secretion and blood samples collection

Milk or mammary gland secretion samples were collected one week before the beginning of the study, at 28 and 14 days before drying off (D-28 and D-14) and at drying off (D0), and daily during challenge period at days 0–7 (Ch0 – Ch + 7) and at days 10 and 14 (Ch + 10 and Ch + 14). We collected samples aseptically by washing all teats, dried each teat with individual paper towel and then disinfect each teat opening by scrubbing with 70% alcohol starting from teat far away from person collecting sample to the closest teat to prevent contamination from contact with hands of the person after alcohol application. We squirt 1–3 ml of mammary secretion from each teat into a sterile 15 ml tube, starting from a teat closest to a person collecting sample to a teat far away from a person collecting sample to avoid contamination. We placed tubes on ice and transported to the laboratory. We prepared the skim of mammary secretion samples by centrifugation at 20,000X g for 30 min to remove fat and cellular debris. We stored skim samples at -20 °C until antibody titers analyzed by ELISA.

We collected blood samples 1 week before the beginning of the study and immediately before each vaccination at 28 days before drying off (D-28), 14 days before drying off (D-14), and at drying off (D0), and then during challenge period of days 0–7 (Ch0 – Ch + 7) and on days 10 and 14 of challenge. Immediately after collection, we centrifuged samples at 2500 rpm for 20 min at 4 °C and serum was separated out and stored at -20 °C until antibody titers evaluated using ELISA.

2.9. Bacteriological analysis of milk and mammary secretion samples

We measured somatic cell count (SCC) at the Dairy Herd Improvement Association Laboratory (Knoxville, TN). We conducted bacteriological analysis following the National Mastitis Council guidelines as described elsewhere (Oliver et al., 2004). Briefly, 100 µl of milk or mammary secretion was spread on tryptic soy agar (TSA) with 5% sheep blood (blood agar plates) (Becton, Dickinson and Company, Franklin Lakes, NJ) and incubated at 37 °C for 24 h to 48 h until colony growth detected. We recorded colony characteristics such as morphology, color, and hemolysis on blood agar plates. We further tested those suspected to be *Staphylococcus spp*. by gram staining and catalase test to differentiate them from *Streptococcus* spp. We used the coagulase tube test using rabbit plasma to differentiate *S. aureus* from coagulase-negative *Staphylococcus species* (CNS). We identified those, which were catalase positive and coagulase positive as *S. aureus*.

2.10. Statistical analysis

To assess the effect of SASP and SCSP vaccines on antibody responses, antibody impacts on experimental intramammary infection and measures of infection post-challenge, we used a mixed model ANOVA (SAS 9.4). We assessed continuous measures using a mixed model ANOVA evaluating the fixed effects of SASP and SCSP vaccines (treatments) at specific time points post vaccination (*e.g.*, D-28, D-14, D0, Ch0, Ch + 1 - Ch + 3, Ch + 7, & Ch + 14), and the interaction of the treatment and day. A significant effect was declared when $P \leq 0.05$. We evaluated multiple comparisons among treatment means with Fisher's least significant difference (LSD). The linear regression models were conducted within treatment groups to evaluate combinations of serum and milk or mammary secretion anti-SCSP and -SASP IgG, IgG1, IgG2, and IgA antibody titers, somatic cell count (SCC), and *S. aureus* count from mammary secretion. Titers and CFU were log transformed before statistical analysis. Regression models were conducted within treatment groups to evaluate the relationship between milk and serum antibody titers, and 2017 calendar year, milk production patterns (7–47 days in milk (DIM), 47–87 DIM, 87–127 DIM, milk per day, and peak milk) with 2016 milk production added as a covariate.

3. Results

3.1. Vaccine safety

Out of six SASP vaccinated cows, one cow was removed from the study and euthanized due to physical injury that resulted in permanent lameness. Only five cows completed the study in the SASP vaccinated group. There were no signs of systemic reactions to the vaccine throughout the vaccination period. All vaccinated cows developed local reactions at injection sites characterized by slight to moderate swelling. There was no significant difference in regards to size (mm) of injection site reaction among the SASP (39.12 ± 2.20), SCSP (42.46 ± 2.10), and the control (40.24 ± 2.10) groups (Data not shown). All vaccinated cows had normal rectal body temperature at 24 h before vaccination, immediately before vaccination and for 3 consecutive days (1–3 days) after each vaccination and at 7 and 14 days after each vaccination (data not shown). Rectal body temperatures were also monitored each day throughout the 14 days of the challenging period. There was no significant difference in regards to the mean rectal temperatures (°F) among the SASP (100.96 ± 0.12), SCSP (100.97 ± 0.12), and the control (101.2 ± 0.12) groups (Data not shown).

3.2. Immune responses to vaccines

Serum anti-SASP IgG2 titers were significantly higher in SASP vaccinated group compared to the control group at D-14, Ch0, and Ch + 7 (Fig. 1, panel A). There was no significant difference in serum anti-SASP IgG2 titers at D-28, D0, or Ch + 14. Serum anti-SCSP IgG1 titers were significantly higher in vaccinated group compared to control group at D-14, Ch0, Ch + 7 and Ch + 14 (Fig. 1, panel B). There was no significant difference in serum anti-SCSP IgG1 titers at D-28 or D0. There was an overall significant difference in serum anti-SASP IgG1 titers in the vaccinated cows compared to the control cows. There were no significant differences in serum anti-SASP IgG and IgA. There were no significant differences in serum anti-SCSP IgG, IgG2, and IgA. There was no significant difference in milk anti-SASP IgG, IgG1, IgG2, or IgA antibody titers in the vaccinated cows compared to the control cows. Similarly, there was no significant difference in milk anti-SCSP IgG, IgG1, IgG2, and IgA antibody titers in the vaccinated cows compared to the control cows.

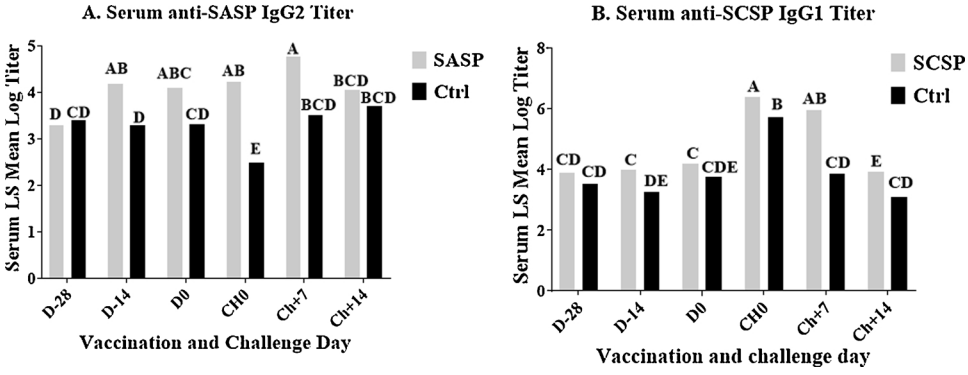

Fig. 1. The serum anti-SASP and -SCSP antibody titers. SASP = *Staphylococcus aureus* surface proteins, SCSP = *Staphylococcus chromogenes* surface proteins, D-28 = 28 days before drying off, D-14 = 14 days before drying off, D0 = at drying off, Ch = Challenge, Ch0 = right before challenge, Ch + 7 = on day 7 of challenge, Ch + 14 = on day 14 of challenge, Ctrl = Control. The different letters represent statistically different titers ($P < 0.05$).

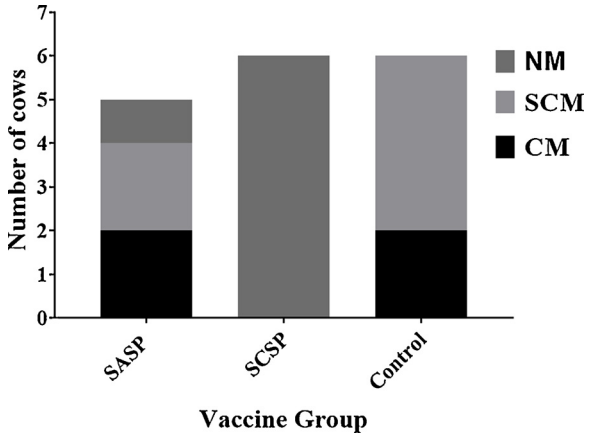

Fig. 2. The response of cows to *S. aureus* challenge throughout 14 days. SASP = *Staphylococcus aureus* surface proteins (SASP) vaccinated group, SCSP = *Staphylococcus chromogenes* surface proteins (SCSP) vaccinated group. NM = No mastitis, SCM = Subclinical mastitis, CM = Clinical Mastitis.

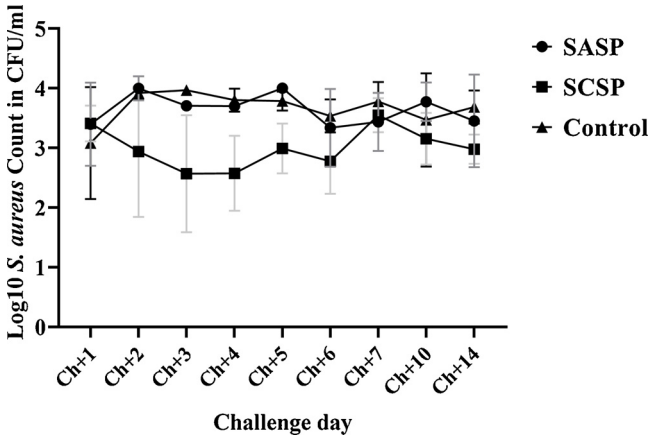

Fig. 3. The number of *S. aureus* shedding through milk over 14 days of challenge period. Ch = Challenge (infection), SASP = *Staphylococcus aureus* surface proteins vaccinated group (●), SCSP = *Staphylococcus chromogenes* surface proteins vaccinated group (▬), control group (▲) Ch + 1 – Ch + 7 = Day 1 to Day 7 of challenge and at days 10 and 14 (Ch + 10 & Ch + 14) of challenge, each symbol on the curve showed mean of Log10 *S. aureus counts* in CFU/ml of milk per group per day, the symbols showed the mean and the bars showed standard deviation of the mean, and overall, there was no significant difference in bacterial shedding in the vaccinated cows compared to the control cows over the 14 days of challenge period ($P > 0.05$).

3.3. Protective effects of SASP and SCSP vaccines

Of the five cows in the SASP vaccine group, two were infected clinically and another two were infected subclinically, and the remaining cow was neither infected clinically nor subclinically but shed a low number of *S. aureus* through mammary secretion on Ch + 1 (Figs. 2 and 3). Of six control cows, two were infected clinically, and the remaining four cows were infected subclinically (Figs. 2 and 3). Out of six SCSP vaccinated cows, all cows were neither clinically nor subclinically infected, but they were shedding a relatively low number of *S. aureus* through mammary secretion during challenge time of 14 days (Figs. 2 and 3). Vaccination of dairy cows during late lactation period with SASP or SCSP proteins resulted in a significantly increased serum antibody titers and a relatively lowest *S. aureus* counts in CFU/ml of mammary secretion in SCSP vaccinated group followed by lower *S. aureus* counts in SASP vaccinated group compared to the control group that had highest counts during experimental challenge period of 14 days. However, statistical analysis results showed that there was no significant difference in the clinical frequency among the SCSP (0%), SASP (33.33%), and control (33.33%) groups (Figs. 3).

The somatic cell count from the dry cow secretion is higher than SCC from the milk of a cow in lactation because of the decrease in volume of fluid in the mammary gland during the dry period. Therefore, SCC is not good criteria for evaluation of intramammary infection in dry cows, but infected cows still have relatively higher count compared to non-infected cows (Data not shown).

Evaluation of the relationship between the number of *S. aureus* shedding through mammary secretion and serum antibody titers showed a significant relationship between serum anti-SCSP IgG antibody titer and an average number of *S. aureus* shedding in SCSP vaccinated cows compared with the control cows. There were no significant (data not shown) relationship among mammary secretion anti-SCSP IgG or serum or mammary secretion anti-SCSP IgG1, IgG2 and IgA antibody titers of SCSP vaccinated cows and *S. aureus* count from their mammary secretion during the challenge period. Similarly, there were no significant (data not shown) relationship among serum anti-SASP IgA or serum or mammary secretion anti-SASP IgG, IgG1, IgG2, or IgA antibody titers of SASP vaccinated cows and *S. aureus* count from their mammary secretion during the challenge period.

A significant positive relationship was found between serum anti-SASP IgG1 titer at Ch0 and milk production during 7–47 DIM in the SASP vaccinated group compared with the control group. There was no significant (data not shown) relationship between the milk or serum anti-SASP and anti-SCSP antibody titers at D-28 and milk production status.

4. Discussion

The SASP and SCSP vaccines both induced an increased immune response in the vaccinated cows compared with the control cows. Results of subsequent experimental challenge (infection) with a heterologous strain of *S. aureus* showed that the SCSP vaccine cross-protected vaccinated cows from *S. aureus* mastitis. The reduction of *S. aureus* shedding through mammary secretion was highest in SCSP

vaccinated group followed by SASP compared with the control group. The SASP vaccine did not induce strong protection since two of the five animals were clinically infected, and another two were subclinically infected. However, the remaining animal was shedding a low number of *S. aureus* in the mammary secretion but was not infected clinically or subclinically. It is not clearly defined which specific immune response, cell mediated (Th1) or antibody (humoral) mediated (Th2) or balanced combination of both cell mediated and antibody mediated responses that clears staphylococcal intramammary infections.

We don't know what kind of immune response was predominantly induced in our two vaccines. Further study with an increased number of animals and detailed evaluation of cytokine expression patterns is required to determine predominant immune response induced by SASP and SCSP vaccines. The type of protective response induced by commercial vaccines are also not clearly known. Similar to our observation, Piepers et al. (2017), evaluated the efficacy of Startvac® through vaccination and subsequent challenge with a heterologous killed *S. aureus* strain and found that the inflammatory response in the vaccinated cows was less severe compared to the control cows. However, there were methodological differences between our study and their study. These authors (Piepers et al., 2017) suggested that Startvac® elicited a strong Th2 immune response against *S. aureus* in vaccinated animals and was more effective at clearing bacteria compared to the control animals. The challenge was with killed *S. aureus* which cannot replicate in the host and unable to produce known virulence factors that enable *S. aureus* to evade host immune system. So this observation alone cannot be considered as clearance of *S. aureus* by Th2 immune response.

Similar to Piepers et al. (2017) suggestion, in our study there was a significant increase in the serum anti-SCSP and anti-SASP IgG1 titers (Th2 response) in the vaccinated cows compared with the control cows. The anti-SASP IgG2 titers (Th1 response) in vaccinated cows was significantly higher than that of the control cows. However, upon challenge better protection was achieved by SCSP vaccine compared to SASP vaccine. Without further detailed evaluation of cytokine expression patterns in conjunction with analysis of antibody isotypes (IgG1, IgG2) in vaccinated and control cows, it is not possible to conclude which immune response (Th1 or Th2 or both or Th17 or other) induced observed protection. In the SCSP vaccine group, there were no clinical or subclinical infections. With the significant increase in IgG1, the protection may be by IgG1 and IgG2 mediated opsonophagocytic removal of *S. aureus* from the mammary gland. We observed high background titers at the beginning of the study in all cows. The increased background titer could be due to prior exposure since the cows in this study were not free from exposure to *S. aureus* or other similar bacterial pathogens earlier in life before this study. However, there were increases in titers from baseline titers after vaccination in all vaccinated cows compared with control cows. The low number of *S. aureus* shedding throughout the challenging period, without an increase in somatic cell count may be due to daily challenge with *S. aureus*. These bacteria could have been unable to colonize glands and stay in secretion rather than establishing infection, in which *S. aureus* is growing and multiplying inside the mammary gland inducing high SCC. Monitoring cows over a more extended period after completion of challenge period before treating them with antibiotics would clarify these findings. This study was conducted during the dry period, which resulted in a relatively increased SCC due to decreased volume of dry cow secretion. However, there was lower SCC in the SASP, and SCSP vaccinated group compared to the control group. A meta-analysis showed that SCC during *S. aureus* infections was roughly 357,000 cells/ml whereas during CNS infections was roughly 138,000 cells/ml (Djabri et al., 2002). Results from Djabri et al. (2002) observation showed that overall, *S. aureus* infection caused a more significant increase in SCC compared to CNS infections. Contrary to this observation, Sharma et al. (2011), found that both *S. aureus* and *S. chromogenes* had a relatively similar increase in SCC compared to other bacterial species. Due to the high SCC during the dry period, the use of SCC as an indicator of inflammation may not be accurate for a dry cow.

Similarly, Prenafeta et al. (2010) evaluated the efficacy of the vaccine that contains a low or high amount of slime associated antigenic complex (SAAC), which is a major antigenic component of Startvac. These authors (Prenafeta et al., 2010) found no difference in the occurrence of mastitis in high or low or none SAAC vaccinated groups upon subsequent experimental challenge despite higher antibody production against high SAAC than low SAAC content in the vaccine.

The Lysigin® (Boehringer Ingelheim Vetmediaca, Inc.) is another Bacterin vaccine commercially available in the US for the control of staphylococcal mastitis in dairy cows. Different investigators evaluated the efficacy of Lysigin® in different studies and similar to Startvac® there were discrepancies and variation on its effect as a vaccine to control staphylococcal mastitis in dairy cows. Nickerson and others showed that the Lysigin® vaccinated heifers had a 45% reduction in both new *S. aureus* IMI during pregnancy and new *S. aureus* IMI at calving relative to controls when first Lysigin® vaccination was given at 6 months of age followed by a booster dose 2 weeks later and subsequent vaccinations every 6 months until calving (Nickerson et al., 1999). These authors (Nickerson et al., 1999) also showed that vaccinated heifers had reduced new coagulase-negative *Staphylococcus species* (CNS) IMI at calving relative to controls, thus indicating Lysigin® may be of use in reducing staphylococcal mastitis in periparturient heifers. Contrary to this observation, Middleton et al. (2006) showed that vaccination of heifers with Lysigin® in late gestation with a subsequent experimental challenge with a heterologous strain of *S. aureus* during early lactation showed no protection.

Similarly, other studies (Luby et al., 2007; Middleton et al., 2006) showed that there was no difference in *S. aureus* clearance rates and milk IgG1, IgG2, and IgM antibody titers in Lysigin® vaccinates and control animals. Based on their data these authors (Luby et al., 2007; Middleton et al., 2006) suggested the presence of insufficient vaccine-induced opsonizing antibody in milk to facilitate clearance of *S. aureus* from the mammary gland. Even though our study was on dry cows, we also noticed that anti-SCSP and –SASP antibody titers in mammary secretion was less than that of serum or colostrum. This suggest that optimization of intramammary immune response through antigen dose titration, site and route of immunization and antigen-adjuvant formulation is required to enhance protective effect.

Almost all studies showed that vaccination with Startvac® or Lysigin® induced increased antibody titers in vaccinated animals and both Startvac® and Lysigin® did not protect new intramammary infection (Bradley et al., 2015; Middleton et al., 2009, 2006; Schukken et al., 2014). However, there were discrepancies and variations in the protective effects of Startvac® and Lysigin® as vaccines to control staphylococcal mastitis in dairy cows. One of the major causes of variations is a methodological difference in mastitis vaccines efficacy testing. In the controlled experimental vaccination and challenge studies, it is critical to have a uniform and good (infection model that mimic natural infection) challenge infection model. Most of the controlled experimental vaccine efficacy studies used intramammary infusion challenge model. Intramammary infusion is good in causing infection, but it is not a good model for vaccine efficacy evaluation because bacteria were infused directly into the intramammary area bypassing inducible innate and acquired immune responses as well as natural defense mechanisms at teat orifice and in the teat canal. These potentially vaccine induced as well as natural defense mechanisms at teat orifice and in the teat canal could have protected or reduced severity of infection if not bypassed during a challenge. Therefore, infection model that mimic natural infection is required for the evaluation of the efficacy of mastitis vaccines. In this regard, we used teats dipping in *S. aureus* bacterial suspension at a mid-log phase of growth to induce infection (teats dipping based infection model) that is closely similar to the natural infection. The growth phase of bacteria used to cause experimental challenge (infection) is also important because if most bacterial cells in challenge dose

are not viable or not actively growing the ability to cause infection is dramatically reduced.

We observed better protection with SCSP vaccine from *S. chromogenes* that prevent colonization of mammary glands by *S. aureus*. *In vitro* studies showed that non-coagulase positive Staphylococcus species (CNS) from bovine mammary glands (Carson et al., 2017) and some *S. chromogenes* isolate from teat apices of dairy heifers (De Vliegher et al., 2004) produce bacteriocin which prevented the growth of *S. aureus* and other bacterial mastitis pathogens. *In vivo* study in humans showed that nasal colonization by *Staphylococcus lugdunensis* strain, which produce lugdunin, an antibacterial agent, reduced *S. aureus* nasal carriage (Zipperer et al., 2016). The *in vivo* prevention of *S. aureus* infection by prior colonization of mammary glands with some *S. chromonges* species showed potential use of *S. chromogenes* as a vectored vaccine to control *S. aureus* mastitis in dairy cows. However, *S. chromogenes* is not microflora of mammary gland but increasingly shown to be an important causative agent of subclinical intramammary infection characterized by high somatic cell count in dairy cows. However, removal of virulence factor/s of *S. chromogenes* strain that induces increased somatic cell count may render this strain suitable for a vectored vaccine to control mastitis in dairy cows.

Almost all commercial as well as experimental mastitis vaccines are dead whole bacterial cells (Bacterin) which makes it difficult to know and improve which antigenic component of whole bacterial cell induced some protective effect. Our vaccines are purified *S. aureus* surface proteins (SASP) and *S. chromogenes* surface proteins (SCSP) that can be improved by further evaluation and identification of the most immunogenic proteins in the vaccine using a highly resolving 2-D SDS-PAGE and Western blotting followed by sequencing of proteins in the spots. The most immunogenic proteins can be cloned and expressed individually and further tested in different formulations to optimize protective effects.

5. Conclusion

In conclusion, we observed that three series of vaccinations of dairy cows at 28 and 14 days before drying off, and at drying off with SASP and SCSP induced a significant increase in immune responses in vaccinated cows compared to control cows. The subsequent experimental challenge of vaccinated cows with the heterologous strain of *S. aureus* resulted in reduced number of bacterial shedding in milk in vaccinated cows compared to control cows. More interestingly, SCSP vaccine cross-protected vaccinated cows from *S. aureus* mastitis indicating that SCSP seems to perform better than SASP as a vaccine to control *S. aureus* mastitis in dairy cows. Further detailed studies including identification of the most immunogenic proteins in the SCSP vaccine and combining the most immunogenic proteins at different doses with different adjuvants and routes of vaccination may optimize the efficacy of SCSP vaccine to control Staphylococcal mastitis in dairy cows.

Funding sources

This study was funded by the University of Tennessee, College of Veterinary Medicine, Center of Excellence in Livestock Diseases and Human Health (UTCVM COE).

Acknowledgments

We thank the University of Tennessee, College of Veterinary Medicine, Center of Excellence in Livestock Diseases and Human Health (UTCVM COE) for funding this study.

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