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Original Article

Evaluation of the humoral immune response to a multicomponent recombinant vaccine against *S. aureus* in healthy pregnant heifers

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ABSTRACT

Staphylococcus aureus is a worldwide pathogen that causes mastitis in dairy herds. Shortcomings in control programs have encouraged the development of vaccines against this pathogen. This study evaluated the vaccine candidate VacR, which included recombinant *S. aureus* protein clumping factor A (rClf), fibronectin binding protein A (rFnBP) and hemolysin beta (rBt), formulated with a novel immune-stimulating complex. Comparisons were made between healthy pregnant heifers that received either VacR ($n = 8$; VacR group) or phosphate buffered saline (PBS) plus adjuvant (control group) SC in the supramammary lymph node area on days 45 and 15 before the expected calving date. Blood and foremilk samples were collected from 7 to 60 days post-calving.

After calving, heifers in the VacR group produced higher total IgG (IgG_{total}) titers against each component, in both serum (rBt, 3.4×10^5; rClf, 3.1×10^5; rFnBP, 2.3×10^5) and milk (rBt, 2.6×10^4; rClf, 1.3×10^4; rFnBP, 1.1×10^4), than control heifers ($P < 0.0001$). There were increased concentrations of IgG_1 and IgG_2 in VacR group ($P < 0.05$), in both serum and milk. Humoral responses remained high throughout the period most susceptible to intramammary infections ($P < 0.01$). Antibodies produced against *S. aureus* rClf and rFnBP reduced bacterial adherence to fibronectin and fibrinogen by 73% and 67%, respectively ($P < 0.001$). Milk antibodies against these adhesins inhibited *S. aureus* invasion of a mammary epithelial cell line (MAC-T), resulting in 15.7% of bacteria internalized ($P < 0.0001$). There was an approximately 6-fold reduction in the hemolysis titer for the native hemolysin in the VacR group compared to the control group ($P < 0.0001$) and a significantly increase in the proportion of positive neutrophils (VacR, 29.7%; PBS, 13.1%) and the mean fluorescent index (VacR, 217.4; PBS, 152.6; $P < 0.01$) in the VacR group. The results suggest that VacR is a valuable vaccine candidate against *S. aureus* infections, and merits further field trials and experimental challenges.

Introduction

Staphylococcus aureus is one of the most prevalent mastitis pathogens in dairy herds worldwide (Zecconi et al., 2006; Persson et al., 2011). Classical control programs based on antibiotic therapy and milking-time hygiene (Dodd and Jackson, 1971) have failed to eliminate bacterial infections; therefore, research efforts have been focused on vaccine development as a complementary control measure (Barkema et al., 2006). There is strong evidence that *S. aureus* vaccines using recombinant DNA technology yield the best results (Middleton, 2008; Anderson et al., 2012; Pozzi et al., 2012); however, only few studies have evaluated these vaccines in cattle (Pereira et al., 2011). In a previous study, our group evaluated a formulation which included some recombinant antigens in combination with a *S. aureus* lysate (Lys + Rec group; Camussone et al., 2014a). Incorporation of the recombinant molecules contributed to a more robust immune response than lysate alone (Lys group); however, the protection given by recombinant proteins alone was not assessed. The aim of this study was to evaluate a recombinant vaccine that included only recombinant antigens and an appropriate adjuvant for this type of formulation. It is understood that subunit vaccines are poor immunogens when

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administered alone; hence, the adjuvant is a key component in the formulation (Nascimento and Leite, 2012; Mohan et al., 2013). Ideal adjuvants for subunit vaccines upgrade antigen immunogenicity and serve as delivery systems to elicit optimal immune responses that cannot be achieved with traditional adjuvants (Singh et al., 2006; Mohan et al., 2013). Some recently developed immune-stimulating complexes combine antigens and adjuvant in the same nanoparticle, triggering balanced humoral/cellular immune responses in many different animal models (Morein et al., 2004; Pearse and Drane, 2005; Sun et al., 2009). Previous work by our group evaluated the performance of a new generation adjuvant (ISCOMATRIX, Isconova) as part of a project to develop a staphylococcal vaccine. When combined with different antigens from *S. aureus*, the adjuvant was effective in stimulating robust immune responses in the blood and milk of heifers (Camussone et al., 2013, 2014a,b).

In this controlled study, a recombinant multicomponent vaccine composed of three crucial virulence factors of *S. aureus* and formulated with the same novel adjuvant was prepared. Clumping factor A (rClf) and fibronectin binding protein A (rFnBP) were selected because of their roles in mammary gland invasion, whereas beta hemolysin (rBt) was used because it causes injury to the host when bacteria colonize tissues (Camussone and Calvinho, 2013; Scali et al., 2015). The potential of this new vaccine candidate to induce a robust humoral immune response was assessed.

Materials and methods

Vaccine formulation

E. coli BL21 (DE3) clones expressing *S. aureus* proteins rBt, rClf or rFnBP had been used for a previous study by our research group (Camussone et al., 2014a) and were available in our laboratory. Data about the cloning process, protein sequences and antigen purification are reported in Camussone et al. (2014a).

The multicomponent vaccine was composed of 200 μg/dose of each of three recombinant proteins (sterilized by filtration) and formulated with 2 mg/dose of adjuvant (ISCOMATRIX, Isconova). The candidate immunogen was named VacR. Heifers sham-inoculated with phosphate buffered saline (PBS) and adjuvant were used as control group (PBS). Vaccine sterility was tested by plating 100 μL of the formulation on blood agar plates in duplicate, and incubating at 37 °C for 48 h.

Animals and sampling

Sixteen healthy pregnant Holstein heifers were selected from the dairy herd at the Instituto Nacional de Tecnología Agropecuaria (INTA) Rafaela Experiment Station and randomly divided by draw in two groups of eight heifers each. Two doses of either VacR (VacR group; $n = 8$) or PBS plus adjuvant (control group; $n = 8$) were administered to heifers SC in the supramammary lymph node area on days 45 and 15 before the expected calving date (days −5 and −15, respectively). On day −15, udders were clinically examined by palpation; samples of pre-partum mammary secretion were collected using standard procedures (Oliver et al., 2004) and cultured to test for the presence of *S. aureus*. Foremilk samples were collected weekly between days 7 and 30, and on day 60 after calving. Samples were acidified by adding acetic acid (1 drop/mL) and then neutralized before centrifugation ($300 \times g$ for 15 min), as described in previous experiments (Camussone et al., 2013, 2014a). Whey samples were stored at −20 °C until used.

Blood samples were collected from the coccygeal vein before each vaccination or PBS plus adjuvant administration and on days 7, 14, 21, 30 and 60 after calving (+7, +14, +21, +30 and +60). All the procedures followed the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1999) and were approved by the Committee of Animal Ethics and Security of the Facultad de Ciencias Veterinarias, UNL (Protocol No. 85/11, 30th May 2011).

Antibody determination

Indirect ELISA was used to measure specific antibodies against rBt, rClf and rFnBP in serum and whey samples from each heifer. To characterize the kinetics of total IgG (IgG_{total}) production, antibody concentrations (expressed as optical densities; ODs), were determined at days −45, −15, +7, +15, +30 and +60. Briefly, flat-bottomed 96-well microtiter plates (Greiner Bio-One International) were coated overnight with rBt, rClf or rFnBP (0.5 μg/well) mixed with carbonate buffer (pH = 9.6) at 4 °C. Serum samples were titrated to a 1:8000 concentration while whey samples were titrated to a 1:2000 concentration, following optimization in preliminary experiments. Mouse anti-bovine IgG_{total}/horseradish peroxidase (HRP; Sigma–Aldrich) was used as a secondary antibody. The assay was visualized by adding 3,3′,5,5′-tetramethylbenzidine as a substrate; after 10 min, H_2SO_4 (0.5N; Invitrogen) was added to stop the colorimetric reaction. The OD was measured at 450 nm on a plate reader (BioTek, ELx808).

IgG_{total} titers were determined following the procedure described above, but using 2-fold serially diluted serum or whey samples, taken on day +7. Titers were determined by a previously described linear regression method (Crowther, 2008). IgG_1 and IgG_2 subclasses were also measured by ELISA in serum and whey samples extracted on day +7. Mouse anti-bovine IgG_1/HRP (Sigma–Aldrich) or a mouse anti-bovine IgG_2 (Sigma–Aldrich) were applied as secondary antibodies, as appropriate, followed by a rabbit anti-mouse IgG/HRP as marker reagent (Jackson Immunoresearch).

Inhibition of the hemolytic activity of the native beta toxin (nBt)

The nBt protein was partially purified from a culture supernatant of a *S. aureus* strain isolated from a bovine mastitis case and characterized to produce only nBt in vitro (Calvinho and Dodd, 1994). Hemolytic activity against sheep erythgrocytes was assessed by pre-incubating the nBt with 2-fold serially diluted serum samples from vaccinated heifers. The hemolytic titer was the last serum dilution which caused lysis of erythrocytes. In this experiment, hemolytic titers were defined as the highest dilution that caused complete hemolysis.

Blocking assays

Fibronectin (Fn) protein was acquired commercially (Invitrogen) and fibrinogen (Fg) was purified from bovine plasma (Ismail, 2012). Blocking assays were conducted as previously described (Camussone et al., 2014a). A suspension of 1×10^9 CFU/mL of *S. aureus* Reynolds strain was pre-incubated with either anti-rClf or anti-rFnBP antibodies and purified by affinity from serum samples; these samples were collected from vaccinated heifers 7 days post-calving. Mouse sera with specificity for *S. aureus* Reynolds and a rabbit anti-mouse IgG/HRP conjugate (Sigma–Aldrich) were used in this assay. Results presented here are expressed as binding percentage, with 100% binding indicating bacteria that were not pre-incubated with serum samples.

Internalization assay

The established bovine mammary epithelial cell line (MAC-T; Huynh et al., 1991) was used. MAC-T cells were grown in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL), insulin (5 μg/mL), hydrocortisone (1 μg/mL), penicillin (100 U/mL) and streptomycin sulfate (100 μg/mL; Sigma–Aldrich). The bacterial internalization assay was performed as previously described (Camussone et al., 2014a). *S. aureus* cells (Reynolds strain) were opsonized with whey samples diluted 1/10, obtained from each heifer on day 7 post-calving. Each assay was run in triplicate. Data are expressed as percentage of internalization compared to the control group (100%).

Opsonophagocytic assays

Bovine polymorphonuclear cells (PMN) were obtained from healthy cattle, as previously described (Siemsen et al., 2007). For the opsonophagocytosis assays, fluorescein-labeled cells from *S. aureus* Reynolds (1×10^8 CFU/mL in Hanks balanced salt solution, HBSS) were incubated with heat inactivated sera obtained from each heifer on day 7 post-calving, as described in detail previously (Camussone et al., 2014a). Fluorescence intensity was read by flow cytometry (FACSCanto II, BD Biosciences) and data was analyzed using WinMDI software. Results are expressed as the proportion of PMN containing ingested bacteria (% positive neutrophils) and mean fluorescence intensity (MFI; Zetterlund et al., 1998).

Statistical analysis

Statistical analyses were performed using GraphPad Instat 4.0 software (GraphPad). Differences beteween the vaccine and control groups were analyzed using the non-parametric Mann–Whitney test. The Kruskall–Wallis non-parametric test was used when several groups compared, followed by pairwise comparison using Mann–Whitney U-tests.

Results

Adverse reactions were not detected in either group and all calves were born healthy. IgG_{total} kinetics are represented in Fig. 1A and the curves for rBt, rClf and rFnBP were similar in shape. Each dose of VacR increased IgG_{total} and maximum concentrations were achieved on day 7 post-calving. IgG_{total} was significantly higher in the VacR group than the control group until the end of the experiment ($P < 0.05$). Serum IgG_{total} titers obtained on day +7 in the VacR group were statistically higher than those in the control