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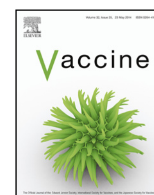

Staphylococcus aureus avirulent mutant vaccine induces humoral and cellular immune responses on pregnant heifers

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A B S T R A C T

Bovine mastitis produces economic losses, attributable to the decrease in milk production, reduced milk quality, costs of treatment and replacement of animals. A successful prophylactic vaccine against *Staphylococcus aureus* should elicit both humoral and cellular immune responses. In a previous report we evaluated the effectiveness of a live vaccine to protect heifers against challenge with a virulent strain. In the present study the immunological response of heifers after combined immunization schedule was investigated. In a first experimental trial, heifers were vaccinated with 3 subcutaneous doses of avirulent mutant *S. aureus* RC122 before calving and one intramammary dose (IMD) after calving. Antibodies concentration in blood, bactericidal effect of serum from vaccinated animals and lymphocyte proliferation was determined. The levels of total IgG, IgG1 and IgG2 in colostrum and the lymphocyte proliferation index were significantly higher in vaccinated respect to non-vaccinated group throughout the experiment. The second trial, where animals were inoculated with different vaccination schedules, was carried out to determine the effect of the IMD on the level of antibodies in blood and milk, cytokines (IL-13 and IFN-γ) concentration and milk's SCC and bacteriology. The bacterial growth of the *S. aureus* strains was totally inhibited at $1–3 \times 10^6$ and $1–3 \times 10^3$ cfu/ml, when the strains were mixed with pooled serum diluted 1/40. The results shown that IMD has not a significant effect on the features determinate. In conclusion, a vaccination schedule involving three SC doses before calving would be enough to stimulate antibodies production in milk without an IMD. Furthermore, the results showed a bactericidal effect of serum from vaccinated animals and this provides further evidence about serum functionality. Immune responses, humoral (antigen-specific antibodies and Th2 type cytokines) and cellular (T-lymphocyte proliferation responses and Th1 type cytokines), were augmented by administration of the avirulent mutant which represent an antigenic pool.

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1. Introduction

Q5 Bovine mastitis occurs when pathogenic bacteria are able to enter to the udder, establish an infection there and produce inflammation of udder secretory tissue [1,2]. This disease produces economic losses that are attributable to the decrease in milk production, reduced milk quality, costs of treatment and replacement of animals [3].

In Argentina, as in many other countries, *Staphylococcus aureus* is still one of the major causes of clinical and subclinical mastitis, especially due to resistance to antibiotic treatment and its ability to persist in a herd in an undetected form [4–6].

Dry cow therapy is one of the recommended methods to cure existing intramammary infections and to prevent new ones at the peripartum period where, animals are most susceptible to new infections [7,8]. These treatments are only partly successful because of the intracellular biological face characteristic of *S. aureus* and because the maximum efficacy of antibiotic therapy is only 50% [9,10]. Moreover, there is a global pressure to limit antibiotic therapy in dairy cattle with the aim of reducing the incidence of drug residues in milk to make safe food [11–13].

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Numerous strategies have been developed in order to increase the resistance of herd to *S. aureus* mastitis and to reduce the clinical and economic consequences of this disease. Although vaccination is a common practice for the control of many infectious diseases, it is not yet a major prophylactic measure against bovine mastitis [14,15]. A successful prophylactic vaccine should elicit both humoral and cellular immune responses in immunized individuals. The potential of immunization to induce an effective immune response is directly related to the titters of antigen-specific antibodies and T-lymphocyte proliferation [8,16]. Specific and innate immune factors associated with mammary gland tissues and secretion also play a vital role in protecting the gland from infectious disease [7]. During mastitis, CD4+ T lymphocytes prevail, are activated in response to recognition of an antigen and activate macrophages through the production of cytokines. Depending on the repertoire of cytokines produced, the T helper cells were divided in type Th1 or type Th2 [17,18]. Th1 clones were characterized by the production of IFN-γ and IL-2, whereas Th2 clones produced IL-4 [19,20], IL-5, IL-6, IL-10 and IL-13 [21]. Th1 cytokines activate macrophages, which are responsible for cell mediated immunity and phagocyte dependent protective responses such as the production of opsonizing b-complement-fixing antibodies [22]. In contrast, Th2 cytokines are responsible for strong antibody production and eosinophil activation as well as inhibition of several macrophage functions, thus resulting in phagocyte-independent protective responses [22–24].

The development of vaccines for protection against *S. aureus* is of considerable interest in the milk production industry. While some formulations have demonstrated promise in ameliorating the disease, few *S. aureus* vaccines have adequately prevented new infections [2,9,15,25,26]. The use of an attenuated vaccine could enhance both cellular and humoral protective responses by simulating natural infection without causing the disease. Attenuated vaccines may contain native antigens (proteins, polysaccharides, lipids, nucleic acids, etc.) that are expressed for extended periods of time [27].

In a previous report [27], we investigated the response of heifers after combined immunization schedule (three subcutaneous doses before calving and one intramammary dose (IMD) after calving) with the avirulent mutant *S. aureus* vaccine. The results of this study demonstrated that immunization of dairy heifers with the strain *S. aureus* RC122 was able to elicit a specific and significant opsonic antibody response in blood and milk and to provide protection by a significant reduction in post challenge milk bacterial shedding. These results could be due to activation and proliferation of blood memory cells previously stimulated. In order to demonstrate this, we investigate the blood memory cell proliferation and immunoglobulin isotype concentration in colostrum. The effect of IMD was determined by evaluation of antibodies in blood, bactericidal effect of serum from vaccinated animals and by the type of lymphocytes that proliferate through the quantification of IL-13 and IFN-γ in heifers immunized with different schemes.

2. Materials and methods

2.1. Experimental trial I

2.1.1. Animals, vaccine and immunization

Eleven clinically healthy Holtein heifers, free of antibodies for *S. aureus* after specific serological testing, were randomly divided into two groups. One group, the vaccinated group (VG) ($n = 8$), was inoculated subcutaneously with three doses of vaccine preparation (subcutaneous dose, SCD) containing 5×10^8 cfu/ml of *S. aureus* RC122, lyophilized and homogenized in phosphate-buffered saline (PBS) pH 7.2 at the original concentration, as described by Pellegrino et al. [25]. The first vaccine dose was administered to 14–16-month-old heifers immediately after they arrived to dairy farm. Heifers were inseminated artificially approximately 30 days after the first dose. The second dose was administrated 30 days after pregnancy was diagnosed. Ten days before calving, a third dose was administrated. Twenty days after calving, all quarters of the vaccinated group were inoculated intramammarily with 1 ml of 10^9 cfu of formol-killed avirulent mutant. The other group of animals, non-vaccinated group (NVG) ($n = 3$), was used as control.

Table 1
Immunization schedule of heifers during trials I and II.

Treatment (T)/no. of cows	Subcutaneous dose (SCD)	Intramammary dose (IMD)
T1/8[a]	Yes	Yes
T2/2	Yes	No
T3/2	No	Yes
T4/3[a]	No	No

[a] Animals from experimental trial I.

2.2. Experimental trial II

2.2.1. Animals and immunization

The level of antibodies on blood and milk after the intramammary dose (IMD) was determined. Four additional clinically healthy Holstein heifers were included to animal trial I and inoculated subcutaneously or intramammarily as described in Table 1.

2.3. Blood sampling

For ELISA assay, blood samples were collected from heifers immediately before the administration of each vaccine dose and 20, 21 and 26 days after calving. Approximately 30 ml of blood were obtained from the tail vein and placed into sterile tubes. Bactericidal assay was performed using 21 day pool serum. Samples were maintained at room temperature, centrifuged at $1200 \times g$ for 10 min and blood sera was collected and stored at −20 °C.

For lymphocyte proliferation assay, heparinized whole blood samples were obtained from the jugular vein (approximately 30 ml) immediately before the administration of each subcutaneous dose of vaccine and 27 days after calving. To avoid blood coagulation, syringes were rotated slowly and immediately processed in the laboratory for the isolation of lymphocytes. An additional sample was taken at day 27, after calving, for cytokines quantification.

2.4. Milk sampling and analysis

Heifers were milked daily at 12-h intervals and individual milk samples collected aseptically 20 (immediately before the administration of IMD), 21 and 26 days after calving. Colostrum samples were collected 24–48 h after calving. Samples were collected according to the National Mastitis Council procedure [28]. For ELISA assay, the serum obtained after centrifugation of whole milk or colostrum at $3700 \times g$ for 15 min was stored at −20 °C.

Bacteriological assays were performed according to the criteria of the National Mastitis Council [28]. The presence of bacterial strains of the genus *Staphylococcus* was determined using 10 μl of milk plated onto blood-agar (Tryptic Soy agar with 5% of sheep blood) and incubated at 37 °C for 24 h. The Gram-positive cocci were characterized by standard biochemical tests [29].

The somatic cell count (SCC) was performed with a Somacount 300 (Bentley, USA, 1997), according to the revised protocol of the 148A method C, fluoro-opto-electronic, International Dairy Federation Laboratory (1995).

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