



Research paper

Immune response and functional role of antibodies raised in heifers against a *Staphylococcus aureus* CP5 lysate and recombinant antigens vaccine formulated with Iscom Matrix adjuvant



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ABSTRACT

Staphylococcus aureus is the most frequently isolated pathogen from bovine intramammary infections worldwide. Commercially available vaccines for mastitis control are composed either of *S. aureus* lysates or inactivated whole-cells formulated with traditional adjuvants. We recently showed the ability of a *S. aureus* CP5 lysate vaccine adjuvanted with Iscom Matrix to generate a longer lasting specific antibody response in blood and milk, with improved opsonic capacity, compared with a *S. aureus* CP5 whole-cell formulation. The aim of the present study was to obtain an experimental immunogen composed of lysed cells of a CP5 *S. aureus* strain supplemented with recombinant clumping factor A, fibronectin binding protein A and β -toxin formulated with Iscom Matrix, characterize the immune response generated when immunizing pregnant heifers and assess the functional role of antibodies raised against this immunogen in experimental models. Both a lysate vaccine and a lysate + recombinant antigens vaccine elicited antibodies that promoted neutrophil phagocytosis and inhibited internalization into mammary epithelial cells, *in vitro*. Incorporation of defined antigenic molecules to the lysate formulation elicited a strong specific humoral immune response against both lysate and recombinant antigens and was associated with higher expression of regulatory and pro-inflammatory cytokines. In addition, antibodies were efficient for blocking *S. aureus* binding to bovine fibrinogen and fibronectin, and neutralizing β -toxin effect *in vitro*, placing these antigens as candidates to be included in a formulation directed to prevent staphylococcal bovine mastitis.

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1. Introduction

Staphylococcus aureus is the most frequently isolated pathogen from bovine intramammary infections (IMI) worldwide (Zecconi et al., 2006). Lack of effectiveness of traditional control measures based on milking time

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hygiene and antibiotic therapy against this organism (Zecconi et al., 2006), has led to the development of complementary alternatives directed to prevent the disease. Among them, manipulation of the host immune mechanisms through vaccination has been explored. During the last two decades several experimental immunogens for *S. aureus* mastitis control have been evaluated (Pereira et al., 2011). However, only two vaccines, composed of lysates of different *S. aureus* strains (Lysigin™, Boehringer Ingelheim Vetmedica Inc., St Joseph, MO, USA) and inactivated *S. aureus* expressing slime associated antigenic complex (Startvac® Laboratorios Hipra, Spain), are currently commercially available worldwide. However, field trials or experimental challenge carried out with Lysigin™ in adult cows showed only some effectiveness in reducing infection duration and severity (Middleton et al., 2006, 2009), whereas Startvac® efficacy was estimated in two herds based on infection transmission and duration parameters, resulting in a relatively low reduction of new IMI and a moderate efficacy for cure (Schukken et al., 2014).

S. aureus has multiple virulence factors that interact with the host at different stages of an IMI; hence, a successful *S. aureus* vaccine will probably need to include a variety of antigens to stimulate antibody production against key pathogen factors involved in IMI (Middleton, 2008). In addition, increasing evidence of the importance of cell-mediated immunity in protection against *S. aureus* infections indicates that a vaccine should be capable of T-cell stimulation for inducing a superior protective efficacy (Gómez et al., 2002; Middleton, 2008; Proctor, 2012). Several molecules that contribute to pathogenesis and also stimulate mammary gland defences were identified and proposed as vaccine candidates. The ability of the organism to internalize in epithelial and phagocytic cells is considered one of the key steps in staphylococcal pathogenesis, and this mechanism is mainly attributed to the presence of fibronectin (Fn) binding protein (FnBP) A and B (Dziewanowska et al., 1999; Lammers et al., 1999; Fowler et al., 2000). The *S. aureus* clumping factors A and B (Clf) allow interaction between bacteria and plasmatic fibrinogen (Fb) leading to an instantaneous clumping of bacterial cells (Hawiger et al., 1982). It was also demonstrated that these factors have anti-phagocytic activity *in vitro* (Higgins et al., 2006). Capsular polysaccharides (CP) 5 and 8 are considered important components for vaccine development since antibodies against them opsonize *S. aureus*, enhancing polymorphonuclear neutrophil (PMN) phagocytosis (Guidry et al., 1991, 1994). Beta toxin (β -toxin) is a sphingomyelinase secreted by most *S. aureus* strains (Aarestrup et al., 1999). This toxin lyses red blood cells (RBCs), PMN (Marshall et al., 2000) and proliferating T-cells (Huseby et al., 2007), and it may be involved in endosomal membrane lysis resulting in the escape of bacteria into the cytoplasm (Shompole et al., 2003).

The combination of *S. aureus* whole or lysed cells with inactivated toxoids and capsular or extracellular products has been a widely used strategy for vaccine formulation (Pereira et al., 2011). However, most of these components were used as crude extracts and therefore not completely characterized (Pereira et al., 2011). In addition, there is scarce information about the use of multicomponent

vaccines formulated with defined antigens against *S. aureus* IMI and only one of them has been evaluated in a bovine model (Shkreta et al., 2004). In previous studies, we have shown the ability of experimental immunogens composed by *S. aureus* whole or lysed cells formulated with Iscom Matrix to stimulate strong humoral immune responses in blood and milk, with production of opsonic antibodies and expression of regulatory and pro-inflammatory cytokines (Camussone et al., 2013, 2014). We hypothesized that inclusion of key antigenic components involved in different steps of staphylococcal IMI in vaccine formulations would extend the immune response reached by our previous immunogen (Camussone et al., 2014). The aim of this study was to obtain an experimental immunogen composed of lysed cells of a CP5 *S. aureus* strain supplemented with recombinant β -toxin, FnBPA and ClfA, formulated with Iscom Matrix, characterize the immune response generated when immunizing pregnant heifers and assess the functional role of antibodies raised against this immunogen in experimental models.

2. Materials and methods

2.1. Vaccine components

Construction of expression plasmids: The nucleotide sequence of *S. aureus* β -toxin, ClfA and FnBPA genes were obtained from the GenBank database (Accession numbers X13404, Z18852 and AJ629121.1, respectively). Genomic DNA was extracted using a commercial kit (Genomics DNA extraction kit, Real Biotech Corporation), from either a *S. aureus* isolated from a mastitis case for β -toxin coding sequence or from Reynolds reference strain for ClfA and FnBPA coding sequences. PCR was performed in a total volume of 50 μ l containing: 1 $\times$ PCR buffer, 2 mM MgCl₂, 1 U/ μ l *Thermus aquaticus* DNA polymerase (Fermentas, Germany), 0.2 mM dNTPs (Genbiotech, Argentina), 0.3 μ M primers, and 50 ng of genomic DNA. Specific primers for full length β -toxin protein were: BTfw (5'-ggattcAAAGGAGTGATAATGATG-3') and BTrv (5'-gtcgacCTATTACTATAGGCTT-3'). ClfA primers were designed to obtain the Region A containing the Fb binding domain being: ClfAfw (5'-gaattcGAAAATAGTGTTACGCAATCT-3') and ClfArv (5'-gtcgacCTCTGGAATTGGTTCAATTTC-3'). FnBPA primers were designed to obtain the Fn binding domains D1D2D3 being: FnBPAfw (5'-gaattcGGTGGCCAAAATAGCGGTA-3') and FnBPArv (5'-gtcgacTTGGTGGCACGATTGGAG-3'). Lower case sequences correspond to restriction enzymes sites used for cloning. Primers were purchased from PB-L (Argentina). Amplification was performed in GeneAmp PCR System (Applied Biosystems, USA) using the following program: an initial 3 min denaturation step at 95 °C, followed by 10 cycles of 30 s of denaturation at 95 °C, 30 s of annealing at 50 °C for β -toxin or 51 °C for ClfA or FnBPA, and 1 min of extension at 72 °C, and 20 cycles of 30 s of denaturation at 95 °C, 30 s of annealing at 58 °C for β -toxin or 65 °C for ClfA or FnBPA, and 1 min of extension at 72 °C; with a final extension step at 72 °C for 5 min. PCR products were analyzed by electrophoresis on GelRed (Biotium, USA)-stained 1% agarose gels (Biodynamics, Argentina), and sequenced

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