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Detection of *Staphylococcus aureus* adhesion and biofilm-producing genes and their expression during internalization in bovine mammary epithelial cells

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ABSTRACT

Staphylococcus aureus is one of the most prevalent pathogens isolated from bovine mastitis, causing chronic intramammary infections (IMI) that limit profitable dairying. The course of infection is often associated with factors both related to the host and the bacterium. Aims of this study were to select *S. aureus* isolates from bovine IMI with different genotypic profiles harboring genes involved in adherence and biofilm production, to determine the behavior of these strains in contact with bovine mammary epithelial cells (MAC-T) and the expression of those genes during bacterial-cell early interactions. The genetic diversity of 20 *S. aureus* strains that were isolated from milk samples taken from cows with persistent-P and non-persistent-NP IMI was high, discriminated into 13 fingerprint groups. The occurrence of genes coding for *S. aureus* surface proteins (*clfA*,*clfB*, *fnbA*, *fnbB*, *fib*, *cna*) and biofilm formation (*icaA*, *icaD*, *icaC*, *bap*) and *in vitro* biofilm-forming ability was not related to strain clinical origin (NP or P). Internalization of *S. aureus* into MAC-T cells was strain-dependent and internalized bacteria overexpressed adherence and biofilm-forming genes compared with those that remained in the supernatant of co-cultures; particularly those genes encoding FnBPs and IcaD. Strains yielding highest invasion percentages were those able to overexpress *fnBP*, irrespectively of the presence of other evaluated genes. Strains from NP IMI showed a greater multiplication capacity *in vitro* compared with strains from P IMI. These results provide new insights about *S. aureus* differential gene expression of adhesion-internalization factors during early interaction with mammary epithelial cells.

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1. Introduction

Staphylococcus aureus is one of the most prevalent major pathogens causing intramammary infections (IMI) in cattle (Zecconi et al., 2006). Commonly, an acute episode of mild to moderate clinical mastitis occurs, but in many cases the infection is not successfully eliminated, resulting in development of chronic subclinical mastitis. Persistence of *S. aureus* in the mammary gland and poor response of the pathogen to antibiotic therapy makes *S. aureus* IMI a common cause of culling (Hebert et al., 2000; Zecconi et al., 2006). Both early interactions between *S. aureus* and host cells, as well as the events that lead to establishment of chronic mastitis are not fully understood, but persistent infections are often associated with an impairment of the immune response due to factors related both to the bacterium and the host. *S. aureus* has a variety of virulence factors, which favor bacterial survival and multiplication in the mammary gland (Zecconi and Scali, 2013).

Previous studies found associations between *S. aureus* genotypes and severity of mastitis clinical signs (Haveri et al., 2007). More recent studies showed that genotypes of *S. aureus* isolated

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from bovine IMI were highly related to their virulence gene pattern (Graber et al., 2009; Piccinini et al., 2012). Furthermore, some subtypes were highly associated with selected epidemiological features like within-herd prevalence and persistence; however, the relationship to pathogenic properties was less evident (Graber et al., 2009). In this regard, in a study comparing subclinical and clinical isolates from *S. aureus* ovine mastitis, Le Maréchal et al. (2011) found no genotypic, but proteomic and transcriptomic differences. While the strain from ewe subclinical mastitis showed overexpression of genes encoding surface molecules, the strain causing lethal gangrenous mastitis mainly overexpressed genes encoding exoproteins (Le Maréchal et al., 2011). Studies characterizing gene expression of *S. aureus* from bovine IMI in the presence of host cells have not yet been carried out. Information about potential differential gene expression during bacterial-cell interactions can contribute to increase the knowledge of both bacteria and cell mechanisms that lead to establishment of infection.

In vitro and *in vivo* studies have demonstrated that *S. aureus* binds to cells and extracellular matrix components and invades bovine mammary epithelial cells, as well as other cells from mammary tissue (Almeida et al., 1996; Hebert et al., 2000) displaying several virulence factors that mediate adhesion to the host cells. The main mechanism for host cell adhesion is mediated by fibronectin binding proteins (FnBP) A and B, that allow bacterial-cell interaction *via* a fibronectin bridge with fibronectin receptors of mammal cells ($\alpha5\beta1$ integrins) (Sinha et al., 1999). Another pathogen adhesion mechanism is through clumping factors (Clf) A and B; these are fibrinogen binding proteins that contribute to initiate infection (Zecconi and Scali, 2013). This attachment also prevents pathogen destruction via opsonophagocytosis, promoting cleavage and inactivation of complement components that mediate opsonization (Hair et al., 2010). Furthermore, biofilm formation, a highly organized multicellular complex, is associated not only with epithelial adhesion but also with evasion of host immune defense (Melchior et al., 2009). Biofilm production requires the presence of the gene cluster *icaADBC* (intracellular adhesion locus). Both the high prevalence of *S. aureus* isolated from bovine IMI harboring the *ica* locus (Vasudevan et al., 2003) and the proportion of biofilm producers among isolates belonging to pulsotypes associated with milk rather than to bovine extramammary sites (Fox et al., 2005), suggest its potential role as a virulence factor in the pathogenesis of mastitis.

The multiplicity of virulence factors and the evidence that potential key elements are not equally expressed *in vivo* by different isolates (Klein et al., 2012) has to be taken into account for the development of effective vaccines against *S. aureus*. An ideal immunotherapeutic agent should prevent bacterial adhesion, promote phagocytosis-induced bacterial elimination and neutralize toxic exoproteins. Therefore, among strategies for vaccine development, antigen selection to generate a protective immune response is probably the most critical and challenging step. Antigen candidates must be conserved and expressed by most strains and, theoretically, adhesion factors should be prime targets for vaccine development (Zecconi and Scali, 2013). Nevertheless, immunization attempts using individual antigens have shown only partial protection against intramammary staphylococcal infections in murine models (Tuchscherr et al., 2008). This suggests that formulations comprising more than one target antigen should increase defensive functions against IMI. Therefore, achievement of more effective anti-staphylococcal vaccines requires a better understanding of the mechanisms by which *S. aureus* can colonize and persist inside its host. Aims of this study were to select *S. aureus* isolates from bovine IMI with different genotypic profiles harboring genes involved in adherence and biofilm production, to determine the behavior of these strains in contact with mammary epithelial cells and the expression of those genes during bacterial-cell early interactions.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Staphylococcus aureus ($n = 20$) were isolated from milk samples taken from Holstein cows with clinical and subclinical mastitis belonging to different dairy farms located in Santa Fe, Córdoba and Buenos Aires provinces (Argentina) collected between 2000 and 2014. Bacteria were identified as *S. aureus* according to standard phenotypic methodology and stored at −80 °C in trypticase soy broth (TSB) (Britania, Buenos Aires, Argentina) with 15% glycerol until use. Fifteen *S. aureus* isolates were from clinical mastitis and 5 from subclinical mastitis. Mastitis were characterized as subclinical (milk somatic cell count of $>250 \times 10^3$/mL, no macroscopic changes) or clinical (visual abnormalities in milk and/or swelling or tenderness in the udder). Isolates from clinical mastitis belonged to 15 different unrelated dairy farms, 9 from Santa Fe province, 2 from Buenos Aires province and 4 from Córdoba province. Samples from clinical cases were obtained by the veterinary practitioners that acted as advisor of the dairy farm ($n = 11$) or by trained personnel during a visit that included sampling of every lactating cow at the farm ($n = 4$). Cows with clinical IMI were treated with standard treatments based of beta lactam or macrolide antibiotics for 3 days. Strains from clinical IMI isolated only once from a mammary quarter and not re-isolated in two consecutive milk samplings after antibiotic therapy, were considered with low adaptation to bovine mammary gland and designated as nonpersistent-NP. Strains from subclinical IMI belonged to two different dairy farms located in Santa Fe province. Two isolates were obtained each from the same mammary quarter before and 15 and 21 days following a standard treatment with beta lactam antibiotics for 3 days (Farm A), while the remainder three isolates were obtained from the same mammary quarter from three different cows in three or more consecutive monthly milk samplings over a period of six months (Farm B). These isolates were tested by pulsed field gel electrophoresis (PFGE) showing the same pattern and were considered highly adapted to bovine mammary gland and designated as persistent-P.

2.2. DNA isolation and PCR

Bacteria were activated from frozen stocks by overnight culture at 37 °C on trypticase soy agar (TSA) (Britania) under aerobic conditions. Bacterial suspensions were grown overnight in TSB at 37 °C, harvested by centrifugation, and incubated for 2 h at 37 °C in 10 mM Tris–HCl pH 8 and 2.5 mg/mL lysozyme (Genbiotech SRL, BA, Argentina). Lysis was achieved by incubating with lysis buffer (50 mM Tris, 100 mM EDTA, 1% SDS, pH 8) and 1 mg/mL of proteinase-K (Genbiotech SRL) at 50 °C for 1 h. After phenol: chloroform extraction and ethanol precipitation, DNA was resuspended in Milli-Q sterile water and quantified spectrophotometrically using an UV/vis Lambda 20 (PerkinElmer) spectrophotometer.

For *S. aureus* characterization, species-specific oligonucleotide primers were used to amplify a 108 bp-segment of a glutamate synthetase family protein gene previously described by Martineau et al. (1998) (Table 1). PCR amplification was performed using 20 μL of reaction mixture containing 1X *Taq* buffer, 250 μM of each deoxynucleotide, 1 μM of each primer (Invitrogen, Life technology, CA, USA), 2.4 mM $MgCl_2$, 1.5 U of *Taq* DNA polymerase (Promega, WI, USA), and 100 ng of template DNA. The PCR reaction was carried out in a thermocycler (Ivema T-18, Ivema Desarrollos SRL) using the following program: an initial step at 96 °C for 3 min and

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