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Caracterización de factores genotípicos y fenotípicos de *Staphylococcus aureus* asociados a la formación de biopelículas en mastitis bovina

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Tabla de contenido

Lista de tablas

Artículo 1

Artículo 2

Artículo 3

Artículo 4

Artículo 5

- **Table 1.** Cows included in the study infected with a pathogen different to *S. aureus.*
- **Table 2**. Genotypes identified in *S. aureus* isolates.
- **Table 3**. IgG levels against the proteins evaluated by sample type and cows status.
- **Table 4.** Results obtained in the ROC curves analysis for each protein and sample type.

Lista de figuras

- **Figure 3**. Total IgG response against the proteins identified in milk serum from infected and uninfected cows.
- **Figure 4**. Total IgG titers in blood serum versus genotype isolated.
- **Figure 5.** ROC curve.

Lista de abreviaturas

- AA: amino acids
- ALT: ácido lipoteicoico
- ATCC: American type culture collection
- Bap: Biofilm-associated protein
- CC: Clonal complex
- CDS: coding sequences
- CID: collision-induced dissociation
- Clf: Clumping factors
- CWA: Cell Wall Anchored
- DMEM: Dulbecco`s modified eagle`s medium
- EDTA: ácido etilendiaminotetraacético
- ELISA: Enzyme-linked immunosorbent assay
- FnBPs: Fibronectin-Binding Proteins
- IgG: immunoglobulin G
- Isd: Iron-regulated Surface determinants
- LC–MS/MS: liquid chromatography-tandem mass spectrometry
- LPS: lipopolisacarido
- M: molar
- MAMP: Microorganisms-Associated Molecular Pattern
- MEC: mammary epithelial cells
- MLST: Multilocus sequence typing
- mL: mililitro
- MOI: multiplicity of infection
- MRSA: methicillin-resistant *S. aureus*
- MSCRAMM: Microbial Surface Components Recognizing Adhesive Matrix Molecule
- NET: neutrophil extracellular trap

- ng: nano gramo
- nm: nanometros
- OD: optical densities
- PIA: Polysaccharide Intercellular Adhesion
- PBS: phosphate-buffered saline
- PCR: Polymerase Chain Reaction
- PFGE: Pulsed-field gel electrophoresis
- PRR: Pattern Recognition Receptors
- RP: recombinant proteins
- SpA: Staphylococcal protein A
- Sdr: Serine-aspartate repeat family proteins
- SEM: scanning electron microscopy
- SCC: somatic cell count
- SCCmec: Staphylococcal Cassette Chromosome
- SCV: Small Colony Variants
- t: spa tipo
- TBS-T: tris-buffered saline and tween
- TLR: Toll-Like Receptors
- TSA: Trypticase soy agar
- TSB: Trypticase soy broth
- µM: micro molar
- µL: micro litro
- WGS: whole genome sequence

Resumen general

Staphylococcus aureus es una de las principales bacterias causantes de infecciones intramamarias en bovinos, las cuales se caracterizan por su limitada respuesta a los antibióticos y persistencia en glándula mamaria. Uno de los factores de virulencia que más se ha asociado con estas características es la formación de biopelícula, debido a que le confiere al patógeno capacidad para evadir la respuesta inmune y la acción de los antibióticos. En Colombia, donde *S. aureus* todavía es uno de los patogenos más prevalentes, se desconocía sobre las características genotípicas y fenotípicas relacionadas con la capacidad de formación de biopelícula de las cepas circulantes. Por lo tanto, el objetivo fue caracterizar factores genotípicos y fenotípicos de *S. aureus* asociados a la formación de biopelícula en mastitis bovina.

En total, 277 cepas de *S. aureus* fueron caracterizadas genotípica y fenotípicamente en términos de formación de biopelículas y linajes mediante técnicas microbiológicas y genómicas. Además, se identificaron y evaluaron potenciales marcadores (proteínas) de infección empleando un análisis proteómico y un inmunoensayo.

Los resultados mostraron tres diferentes genotipos en cuanto a formación de biopelícula. El genotipo 1 (*ica* positivo y *bap* negativo) fue el más prevalente (78,2%), seguido del genotipo 2 (*ica* y *bap* positivos) (12,7%) y el genotipo 0 (*ica* y *bap* negativos) (9,2%). El 81,3% de las cepas evaluadas fueron formadoras de biopelícula, observandose que el 100% de las cepas del genotipo 2 fueron formadoras y presentaron las densidades ópticas más altas (>2,4), demostrando su alta capacidad para generar biopelícula. En cuanto a los linajes, se identificaron 38 spa tipos diferentes, los cuales fueron agrupados en 16 complejos clonales. Los spa tipos más frecuentes fueron el t267 y el t521, ambos pertenecientes al complejo clonal CC97. Además, 51 cepas tipificadas se clasificaron como genotipos nuevos (no reportadas

previamente). El 68% de los genotipos identificados también han sido encontrados causando infecciones en humanos.

Por otra parte, el determinante de superficie regulador del hierro A (IsdA), el factor de agregación A (ClfA) y la proteína con repeticiones de aspartato-serina D (SdrD) fueron las proteínas identificadas por métodos proteómicos, que mostraron potencial para ser usadas como marcadores de infección, pues fueron expresadas en la fase inicial de la infección, se han asociado con formación de biopelícula y tienen la capacidad de desencadenar una respuesta inmune.

El inmunoensayo realizado mostró diferencias significativas en la respuesta de IgG contra las proteínas evaluadas, resaltando el potencial antigénico de IsdA. Sin embargo, fue la mezcla de las proteínas la que presentó la mayor capacidad para diferenciar entre animales infectados y controles empleando muestras de sangre. Aunque, es importante resaltar que cuando esta mezcla fue evaluada con muestras de leche, se evidenció una alta especificidad (94%).

En conclusión, la mayoría (81,26%) de las cepas de *S. aureus* circulantes en Antioquia son formadoras de biopéliculas, resaltando la alta capacidad de las cepas *bap* positivas. Igualmente, se encontró que estas cepas fueron ampliamente diversas en cuanto a los genotipos, evidenciándose un alto porcentaje (68%) de genotipos causantes también de infecciones en humanos. Además, se identificaron proteínas implicadas en la formación de biopelículas que podrian ser usadas en inmunoensayos como marcadores de infección.

General abstract

Staphylococcus aureus is one of the most common pathogens causing bovine intramammary infections, which are characterized by the presence of persistent microorganisms along with a poor response to antibiotic therapy. The ability of *S. aureus* to persist in the mammary gland has been associated with multiple virulence factors. Among these, biofilm formation is one of the most important because biofilms confer protection against antibiotics and the host immune response. In Colombia however, where this pathogen is one of the major causes of IMI in dairy cows, biofilm formation ability remains unknown. Therefore, the aim of this study was to characterizer genotypic and phenotypic factors from *S. aureus* associated with biofilm formation in bovine mastitis.

In total, 277 *S. aureus* strains were characterized in terms of biofilm formation and genetic lineages using microbiological and genomics techniques. In addition, potential serologic markers were identified and evaluated by proteomic analysis and an immunoassay.

Three different genotypes grouped into three separate clusters were identified from *in vitro* assays. Genotype 1 (*ica* positive and *bap* negative) was the most prevalent (78.17%), followed by genotype 2 (*ica* and *bap* positive) (12.66%) and genotype 0 (*ica* and *bap* negative) (9.17%). Biofilm formation was observed in 81.26% of the strains from which 100% of genotype 2 isolates showed biofilm formation. The biofilms formed by genotype 2 isolates were also found to have the highest optical density (>2.4). Regarding the lineages found, 38 different spa types were identified, which were grouped into 16 clonal complexes. The most frequent spa types were t267 and t521. Furthermore, it was determined that 51 of the genotypes described had been unreported so far (new) and 68% had also been previously reported in humans.

On the other hands, IsdA, ClfA and SdrD were the identified proteins that could be used as markers of infection, since are involved in the early stage of the infection, in the biofilm formation and have the ability of elicit an immune response.

The result showed significant differences in the IgG response against markers evaluated, highlighting the antigenic potential of IsdA. According to ROC curves analysis, the protein mixture was the one that showed the greatest capacity to differentiate between infected and uninfected cows when the blood samples were used. In addition, this mixture also showed the highest specificity (94%) using milk samples, suggesting its possible use as a diagnostic complement to somatic cell count, which is highly sensitive but not so specific.

In conclusion, most (81,26%) *S. aureus* strains evaluated were biofilm former, highlighting the high ability of the *bap* positive strains to produce biofilm. We also identified that the *S. aureus* strains included were very diverse in terms of lineages, evidencing a high percentage (68%) of genotypes that can also cause infections in humans. Furthermore, three proteins involved in the biofilm formation process were identified, which could be used as potential markers of infection.

Introducción general

Staphylococcus aureus es uno de los patógenos más aislado de infecciones intramamarias (IIM) bovinas tanto en Colombia como en diferentes partes del mundo (Keefe, 2012; Vidal et al., 2016). Estas infecciones se caracterizan por la persistencia de la bacteria en glándula mamaria y limitada respuesta a los antibióticos (Veh et al., 2015a). Los animales con infecciones crónicas se convierten en importantes reservorios del patógeno y mantienen su ocurrencia dentro del hato, lo cual desencadena pérdidas económicas importantes en las granjas lecheras (Halasa et al., 2007; Hernández-Castellano et al., 2017a; Schukken et al., 2011; Veh et al., 2015a).

La capacidad de persistencia de *S. aureus* en la glándula mamaria bovina se ha asociado con múltiples factores de virulencia, entre estos, la formación de biopelícula, dado que esta conformación le confiere al patógeno protección frente a antibióticos y la respuesta inmune del huesped, ambos elementos claves para la eliminación de la infección (Gomes et al., 2016). Estudios previos han demostrado que el proceso de formación de biopelículas por *S. aureus* es diverso y redundante (Zapotoczna et al., 2016). Este patógeno puede formar biopelículas, principalmente, mediante dos mecanismos. El más común y estudiado a la fecha es la biopelícula mediada por el polisacárido de adhesión intercelular (PIA por sus siglas en inglés), el cual es codificado por el operón *ica* y conocido como dependiente de PIA (Cucarella et al., 2004; McCarthy et al., 2015). Se ha evidenciado que la mayoría de los aislados que causan infecciones tanto en humanos como animales son portadoras del operón *ica* (Cucarella et al., 2004). El otro mecanismo descrito es denominado independiente de PIA y se ha relacionado con la proteína asociada a biopelícula (Bap por sus siglas en inglés) y la meticilino resistencia (McCarthy et al., 2015). La proteína Bap es codificada por el gen *bap* y se ha localizado en un transposón contenido en la isla de patogenicidad SaPIbov2, uno de los elementos genéticos moviles de *S. aureus* (Cucarella et al., 2004). Algunos reportes han indicado que las cepas portadoras del gen *bap* son

generalmente fuertes formadoras de biopelículas, incluso, en ausencia del locus *ica*, y pueden generar infecciones más persistentes que las cepas *bap* negativas (Cucarella et al., 2004; Torres et al., 2019). Sin embargo, algunos estudios también han descrito otros elementos que parecen jugar un papel importante en el proceso, como es el ADN extracelular (eADN), la aglomeración dependiente de fibrina y los agregados de amiloide (Zapotoczna et al., 2016).

La fase inicial en el proceso de formación de biopelícula es la adhesión de la bacteria a la superficie biótica o abiótica (Moormeier & Bayles, 2017). No se formará una biopelícula madura sin una unión adecuada y fuerte de *S. aureus* a la superficie, debido a que las bacterias no ancladas serán removidas del huésped (Gong et al., 2010). *S. aureus* puede unirse a las superficies mediante diferentes proteínas pertenecientes al grupo conocido como componentes de la superficie microbiana que reconocen moléculas adhesivas de la matriz (MSCRAMMs por sus siglas en inglés), las cuales hacen parte de las proteínas de anclaje a la pared (CWA por sus siglas en inglés) (Foster et al., 2014). Algunas de las proteínas de este grupo que han sido implicadas en la adherencia inicial y posterior formación de biopelícula son las proteínas de unión a la fibronectina (FnBPA y FnBPB), los factores de agregación (ClfA y ClfB), proteínas con repeticiones de aspartato-serina (SdrC, SdrD y SdrE), determinantes de superficie reguladores del hierro (IsdA, IsdB, IsdC y IsdH) y Bap (Moormeier & Bayles, 2017).

Diferentes enfoques proteómicos y herramientas bioinformáticas se han usado para estudiar la compleja interacción entre *S. aureus* y el huesped (Huang et al., 2014). La mayoría de estudios proteómicos acerca de biopelículas han usado cultivos bacterianos sobre superficies abióticas, los cuales probablemente no reflejan la total realidad *in vivo* (Lei et al., 2017). Estas discrepancias han sido demostradas en algunos trabajos, en los cuales se han observado diferencias importantes entre lo encontrado usando superficies inertes y modelos vivos (Brady et al., 2006; den Reijer et al., 2016). Lo

anterior, demuestra la importancia de emplear modelos (celulares o animales) que permitan acercar la bacteria a las condiciones más reales.

La detección temprana y precisa de *S. aureus* es una de las herramientas esenciales en el adecuado control de las IIM causadas por este patógeno, pues permite optimizar los tratamientos antibióticos y separar oportunamente los animales infectados, por consiguiente, interrumpir la cadena de transmisión (Hernández-Castellano et al., 2017b). Usualmente, el diagnóstico de IIM se realiza mediante el recuento de células somáticas (RCS) y el cultivo bacteriológico (Rainard et al., 2018). Sin embargo, un estudio reciente encontró que el RCS tenía una sensibilidad y especificidad (con un umbral de detección de 200.000 células/mL) para detectar infecciones causadas por *S. aureus* de 84,2% y 52,8%, respectivamente (Petzer et al., 2017). Estos hallazgos no resultan totalmente convenientes para el control de este patógeno, pues al no ser tan específico conlleva a que se apliquen tratamientos que probablemente no serán efectivos. Al final, se hará un uso inadecuado de antibióticos, los animales seguirán infectados con *S. aureus* y propagarán la bacteria dentro del hato, lo cual perpetuará el ciclo. En el caso del cultivo bacteriólogico, este resulta complejo de realizar en campo, consume tiempo y su sensibilidad frente al patógeno es variable (Rainard et al., 2018). De ahí, la importancia de encontrar alternativas diagnósticas eficientes que permitan mejorar la detección de *S. aureus* o complementar las técnicas convencionales.

Existe evidencia que ha demostrado el potencial inmunogénico de algunas proteínas expresadas por *S. aureus* durante la fase inicial de la formación de biopelícula, las cuales podrían ser usadas a nivel diagnóstico, pues estudios han demostrado que individuos infectados con la bacteria generaron títulos de anticuerpos más altos que los controles sanos (den Reijer et al., 2017; Joost et al., 2011). Por lo tanto, estos resultados conllevan a seguir buscando y evaluando marcadores que permitan discriminar eficientemente los animales con IIM de los sanos, lo cual permitirá

implementar medidas de manejo que impacten oportunamente la prevalencia de este patógeno dentro del hato.

Objetivos

Objetivo general

Caracterizar factores genotípicos y fenotípicos de *Staphylococcus aureus* asociados a la formación de biopelícula en mastitis bovina.

Objetivos específicos

- Caracterizar genotípica y fenotípicamente cepas de *S. aureus* y evaluar diferencias en susceptibilidad a meticilina y capacidad formadora de biopelículas.
- Caracterizar y comparar los proteomas generados *In vitro* durante la formación de biopelícula por cepas de *S. aureus* aisladas de mastitis.
- Determinar anticuerpos en muestras de leche y sangre frente a las proteínas recombinantes de interés.

Marco teórico

Mastitis bovina

La mastitis se define como la inflamación de la glándula mamaria de la vaca, la cual puede ser causada por factores como traumatismos o lesiones, irritaciones químicas e infecciones; aunque, son estas últimas las más comunes. Las infecciones intramamarias (IIM) pueden ser causadas por diferentes microorganismos. Sin embargo, son las bacterias las principales causantes de esta patología en bovinos (Philpot & Nickerson, 2000).

La IIM se presenta luego de que los microorganismos penetran por el canal del pezón, se establecen en el tejido, evaden la respuesta inmune, se multiplican y producen el daño. La interacción entre el tejido mamario y el microorganismo en condiciones normales desencadena una respuesta inflamatoria en la gándula (Philpot & Nickerson, 2000). La reacción inflamatoria es un mecanismo de protección que busca eliminar los microorganismos, neutralizar sus toxinas y ayuda a reparar los tejidos comprometidos. Esta respuesta está mediada principalmente por leucocitos (células somáticas), especialmente neutrófilos, los cuales migran desde la sangre hacia el tejido afectado con el objetivo de eliminar la infección (Wellnitz & Bruckmaier, 2012).

Dependiendo de los signos y síntomas que se presenten en la glándula mamaria y la leche, la mastitis se puede clasificar en subclínica o clínica. La mastitis subclínica no presenta síntomas ni signos evidentes y la apariencia de la leche es normal. No obstante, su calidad y cantidad se ve afectada. Su diagnóstico solo se puede realizar mediante pruebas de laboratorio que permiten detectar el microorganismo responsable de la infección o el aumento del recuento de células somáticas (RCS). Este tipo de mastitis es la más prevalente e importante, pues generalmente pasa desapercibida para el productor durante mucho tiempo y le genera pérdidas económicas significativas. Un estudio realizado en Antioquia por Ramírez et al. (2014) encontró una prevalencia de

mastitis subclínica alrededor del 37%. Por el contrario, la mastitis clínica se caracteriza por las anormalidades visibles en la ubre o en la leche, lo que facilita su diagnóstico, y la severidad puede variar durante el curso de la enfermedad (Philpot & Nickerson, 2000).

La mastitis es considerada la enfermedad que más pérdidas económicas le genera a los productores de leche debido a la reducción en la calidad y cantidad de leche producida, el descarte temprano de animales y de leche, así como, por el aumento de costos veterinarios (Shim et al., 2004). La industria lactea también se ve impactada por esta enfermedad, pues se requiere más litros de leche para obtener un kilo de producto final (por ejemplo, de queso o leche en polvo) y por acortarse la vida útil del producto en el mercado (Keefe, 2010). Se estima que las pérdidas económicas anualmente en Estados Unidos por mastitis pueden alcanzar los dos billones de dólares y en el Reino Unido £300 millones (Viguier et al., 2009). En Canadá, los costos por un caso de mastitis están alrededor de US$290 y en Holanda promedia los €150 por vaca al año (Keefe, 2010; Viguier et al., 2009). En Colombia, la cifra puede acercarse al millón de pesos por animal (Bedoya & Ponce de León, 2008).

Infecciones intramamarias bovinas causadas por *S. aureus*

Se han descrito alrededor de 150 microorganismos causantes de IIM, pero son las bacterias, especialmente los cocos Gram positivos de los géneros *Staphylococcus* y *Streptococcus,* los responsables de la mayoría de casos (Fu et al., 2013). *S. aureus* es uno de los patógenos más aislado de las IIM a nivel mundial y es reconocido por generar infecciones crónicas difíciles de eliminar. Diferentes artículos han evidenciado que la patogenicidad de *S. aureus* es un proceso complejo, en el cual se involucran diferentes factores de virulencia y condiciones del huésped. A la fecha, se han descrito diferentes factores de virulencia del patógeno involucrados con la adhesión, invasión celular, daño a tejido, formación de biopelícula y evasión de la respuesta inmune. Estos elementos en conjunto le confieren a la bacteria un amplio repertorio de mecanismos

para infectar y persistir dentro de la glándula, con pocas probabilidades de eliminación por el sistema inmune y los antibióticos (Zecconi & Scali, 2013).

S. aureus puede llegar a la piel del pezón a través de las manos del ordeñador, pezoneras, leche o cualquier elemento contaminado con el que tenga contacto. Para ingresar al pezón, la bacteria primero debe sobrepasar las barreras naturales generadas por el esfínter y el tapón de queratina, mediante sus propios mecanismos de adhesión y multiplicación o a través de elementos físicos que la pueden arrastrar hacia el interior como las cánulas de aplicación de antibióticos y las diferencias de presión ocasionadas por las fluctuaciones de vacío durante el ordeño mecánico. Una vez la bacteria alcanza el interior del pezón, también debe superar la acción expulsiva del ordeño frecuente, por lo que en este punto, su capacidad de adherencia al tejido influirá sobre la permanencia dentro del cuarto infectado, evento decisivo en la fase temprana de la patogénesis (Philpot & Nickerson, 2000).

La capacidad de adherencia de *S. aureus*, uno de los factores de virulencia más relevantes, se le atribuye a una familia de proteínas conocidas como componentes de la superficie microbiana que reconocen moléculas adhesivas de la matriz (*Microbial Surface Components Recognizing Adhesive Matrix Molecule* - MSCRAMM), las cuales hacen parte del grupo de proteínas de anclaje a la pared celular (*Cell Wall Anchored* - CWA) (Foster et al., 2014). En *S. aureus* se han identificado alrededor de 28 diferentes CWA y su expresión puede variar entre cepas, dependiendo del genotipo, condiciones ambientales o necesidades bacterianas. Las MSCRAMM son proteínas de superficie que median el ataque a componentes extracelulares tales como fibrinógeno, fibronectina, laminina y colágeno, entre otras; incluso, algunas presentan funciones adicionales a la adhesión (Foster et al., 2014).

Algunas de las MSCRAMM más estudiadas en *S. aureus* aislado de bovinos, debido a su alta capacidad de adhesión y posterior participación en formación de biopelículas, son las proteínas de unión a fibronectina A y B (*Fibronectin-Binding Proteins A and B* –

FnBPA *and* FnBPB), los factores de agregación A y B (*Clumping factors A and B* – ClfA and ClfB), los determinantes de superficie reguladores de hierro (*Iron-regulated Surface determinants* – IsdA, IsdB, IsdC, IsdH), proteínas con repeticiones de aspartato-serina (*Serine-aspartate repeat family proteins* – SdrC, SdrD, SdrE) y la proteína asociada a la biopelícula (*Biofilm-associated protein* – Bap). La mayoría de estas proteínas comparten el mismo motivo (LPXTG); sin embargo, estas tienen diferentes blancos de unión sobre la matriz extracelular del huésped (Moormeier & Bayles, 2017).

Las FnBPs, además de mediar la unión a fibronectina, también están involucradas en el proceso de invasión celular, otro de los factores de virulencia importantes de la bacteria (Brouillette et al., 2003; Budd et al., 2016). La fibronectina es una glicoproteína presente en la matriz extracelular, compuesta por tres módulos estructurales (Tipo 1, Tipo 2 y Tipo 3), donde las FnBPs de *S. aureus* presentan alta afinidad por el módulo tipo 1, mientras que, el módulo tipo 3 es reconocido por la integrina α5β1, un receptor expresado en la superficie de las células epiteliales mamarias (CEM) (Foster et al., 2014; Schwarz-Linek et al., 2003). En este caso, la fibronectina actúa como un puente entre la bacteria y la célula (Fowler et al., 2000). Un estudio demostró que la expresión de las FnBPs es cepa dependiente y su ausencia en la cepa evaluada redujo la adhesión a las CEM hasta en un 40% y la invasión en un 95%, comparado con la cepa control (Budd et al., 2016).

La proteína IsdA juega un papel importante en el proceso de adhesión y en la formación de biopelícula bajo condiciones limitadas de hierro. El hierro es un elemento esencial para el crecimiento de la bacteria y la formación de biopelícula, pero su disponibilidad en el huésped generalmente es restringido, pues más del 90% del hierro disponible está localizado dentro de las células (Hammer & Skaar, 2011). Uno de los mecanismos que tiene *S. aureus* para captar hierro del ambiente es mediante IsdA. Algunos estudios han demostrado que hay un aumento en la expresión de la proteína cuando la disponibilidad de hierro se ha limitado (Clarke et al., 2004; Lin et al., 2012). No obstante, IsdA también

ha sido implicada en el proceso de adhesión a la fibronectina y al fibrinógeno durante la etapa inicial de la colonización (Clarke et al., 2004). Estudios que han bloqueado esta proteína han observado una reducción en la capacidad de colonizar el tejido del huésped (Bennett et al., 2019; Cheung et al., 2009; Hammer & Skaar, 2011). Adicionalmente, la capacidad antigénica de IsdA también ha sido demostrada por Stranger-Jones et al. (2006), quienes lograron una inmunidad protectora en murinos previamente sensibilizados con una vacuna compuesta de cuatro antígenos, entre estos IsdA y SdrD, y retados con cepas patógenas aisladas de humanos.

ClfA es una importante adhesina, usualmente anclada covalentemente a la pared bacteriana (Gong et al., 2010). Aunque, ClfA generalmente ha sido relacionada con la unión al fibrinógeno plasmático, un estudio mostró que esta molécula también tiene un mecanismo directo de anclaje a la célula en ausencia de fibrinógeno. Esta proteína puede interactuar con la AnnexinaA2, una molécula encontrada en la superficie de las CEM (Ashraf et al., 2017). En cuanto al papel de ClfA en la formación de biopelícula, Zapotoczna et al. (2015) informó sobre un fenotipo de biopelícula mediado por esta proteína, dado que tiene la capacidad de unirse a la fibrina formada por la enzima coagulasa o al fibrinógeno disponible, conllevando a una agregación bacteriana. Diferentes estudios han propuesto que ClfA es un buen candidato para vacuna, porque es un importante factor de virulencia, la mayoría de las cepas son portadoras del gen *clfA* y es antigénica (Camussone et al., 2014; Nour El-Din et al., 2006).

Sdr es otra de las proteínas importantes en el proceso de colonización y formación de biopelícula por *S. aureus*, aunque, no todas las cepas son portadoras de los genes que conforman este grupo (Josefsson et al., 1998). Ma et al. (2012) y Vaishampayan et al. (2018) observaron en sus trabajos una reducción en la expresión del gen *sdrD* y en la capacidad de formación de biopelícula de las cepas evaluadas después de ser enfrentadas a productos que buscaban impactar la producción de biopelícula. Otro estudio, demostró que la proteína SdrD contribuía a la supervivencia de *S. aureus* en

sangre debido a que participaba en la evasión de la respuesta inmune (Askarian et al., 2017). Como fue mencionado anteriormente, SdrD también fue evaluada como parte de la vacuna compuesta desarrollada por Stranger-Jones et al. (2006), donde quedó demostrado su potencial antigénico.

En cuanto a Bap, esta proteína tiene un tamaño de 2.276 aminoácidos, permite la unión a las superficies, así como, la adhesión intercelular y es codificada por el gen *bap*, el cual hace parte de un transposón insertado en la isla de patogenicidad SaPIbov2 (Cucarella et al., 2004). La evidencia sugiere que el gen *bap* se puede transmitir entre cepas mediante transferencia horizontal de genes (Tormo et al., 2005). El gen *bap* se han encontrado en cepas aisladas de bovinos y cerdos, pero su frecuencia entre cepas puede variar por regiones (Cucarella et al., 2004; Feltrin et al., 2016; Torres et al., 2019). Estudios han demostrado que las cepas portadoras de este gen son generalmente fuertes formadoras de biopelículas y generan infecciones más persistentes en bovinos (Cucarella et al., 2004; Torres et al., 2019). Otro estudio demostró que las cepas con este genotipo son menos invasivas, debido a que Bap bloquea la interacción de las FnBPs con su ligando (fibronectina), un proceso fundamental para que la bacteria pueda ingresar a la CEM (Cucarella et al., 2002; Valle et al., 2012).

Una vez establecida la infección, las bacterias recubren la cisterna del pezón, así como, la glandular, y luego migran al sistema de conductos donde se multiplican activamente hasta formar agregados bacterianos. En la fase inicial de la infección el daño puede ser mínimo y su eliminación mediante los tratamientos antibióticos puede tener mejores resultados. Durante una infección crónica con *S. aureus*, el daño puede ser variable y la zona afectada será total o parcialmente improductiva, pues las células secretoras se degeneran y junto con los leucocitos obstruyen los conductos galactóforos, lo que conlleva a que se genere tejido cicatricial, y en algunos casos, dependiendo de la extensión del daño, a que se atrofie el alveolo. (Philpot & Nickerson, 2000).

Respuesta inmune contra *S. aureus*

Inmunidad innata

El contacto de *S. aureus* con las células epiteliales del tejido y con las células somáticas durante su ingreso desencadena la respuesta inicial contra el patógeno. La importancia de las células epiteliales mamarias en la respuesta inmune temprana ha sido reconocida en numerosas investigaciones; primero, porque son las células con las que inicialmente entra en contacto la bacteria al ingresar; segundo, por la capacidad de producir mediadores inflamatorios que desencadenan la activación y migración de los leucocitos, principalmente de neutrófilos, la célula somática predominante durante la fase aguda de la respuesta (Bougarn et al., 2010; Yang et al., 2008).

Las células epiteliales como las somáticas pueden reconocer a este patógeno mediante los receptores de reconocimiento de patrones (*Pattern Recognition Receptors* – PRR) ubicados en la superficie o a nivel intracelular. Estos receptores se unen a los patrones moleculares asociados a los microorganismos (*Microorganisms-Associated Molecular Pattern* – MAMPs), estructuras conservadas y características entre grupos de microorganismos. Es el caso de los lipopolisacáridos (LPS) en las bacterias Gram negativas y los ácidos lipoteicoicos (ALT) en las Gram positivas. Los receptores tipo Toll (*Toll-Like Receptors* – TLR) son una clase de PRRs que reconocen un amplio rango de MAMPs (Abbas et al., 2014; Bougarn et al., 2010). Generalmente los TLRs son específicos y cada uno tiene la capacidad de unirse a un MAMP. En el caso de *S. aureus*, bacteria Gram positiva, el TLR2 es el que se ha asociado a su reconocimiento, debido a que pueden unirse a ALT y al peptidoglicano presente en la pared bacteriana; aunque, se ha descrito que este TLR no es tan específico y también puede reconocer LPS de algunas bacterias Gram negativas (Smith et al., 2003). Debido a que *S. aureus* invade eficientemente las células como mecanismo de evasión del sistema inmune, también puede ser reconocido por los receptores tipo NOD, un PRR ubicado en el citosol de la célula hospedera y que interacciona con el peptidoglicano (Jintaek et al.,

2014). En un estudio donde se inoculó *S. aureus* en la glándula mamaria de ratas, se evidenció un incremento en la expresión del RNAm para NOD después de la infección, lo que demostró su actividad durante la invasión intracelular del patógeno (Wang et al., 2015).

Una vez es reconocida la bacteria, se activan los mecanismos moleculares que promueven la expresión de genes que codifican para las principales citoquinas pro-inflamatorias, el factor de necrosis tumoral alfa (*Tumor Necrosis Factor Alpha* - TNF- α), la Interleuquina 1β (*Interleukin* 1β - IL-1β) y la IL-6. La importancia de estas citoquinas dentro del proceso inflamatorio está dada por la capacidad que tienen de preparar a las células endoteliales para que permitan la migración de los leucocitos al sitio de la agresión (TNF- α y la IL-1β), promover la fagocitosis en neutrófilos (TNF- α) y estimular la producción de reactantes de fase aguda (IL-1β e IL-6) (Fu et al., 2013). Los TLR2 en condiciones normales desencadenan la expresión de estos genes por la vía dependiente de la proteína adaptadora MyD88 y del factor nuclear κB (*Nuclear Factor κappa B* - NF-κB). Se ha observado en diferentes estudios que *S. aureus* tiene la capacidad de bloquear o inhibir la activación del NF-κB, por consiguiente, alterar la magnitud y tiempo de respuesta de estas citoquinas; contrario a lo que sucede con otros patógenos como *Escherichia coli,* donde se ha descrito una fuerte y rápida respuesta (Lara, 2011; Yang et al., 2008). No obstante, algunos autores, observaron expresión de estas citoquinas en ausencia del factor, sugiriendo la participación de vías independientes, tales como la proteína de activación 1 (*Activating Protein 1* - AP-1) y el elemento de respuesta al AMP cíclico (*Cyclic AMP-Responsive Element* - CRE). Incluso, en otros casos lo que se obtuvo fue transcripción de los genes después del reto pero no hubo traducción, pues no se detectaron las citoquinas (Bougarn et al., 2010; Kim et al., 2011). La variabilidad en los resultados se les ha atribuido a los modelos de ensayo (animal o celular), a las técnicas de medición y al tipo de inmunógeno (bacterias vivas, inactivas o componentes de la pared) empleados.

Los neutrófilos son los primeros fagocitos en migrar al sitio de la agresión y pueden reconocer la bacteria directamente mediante los PRRs, o indirectamente, por receptores que se unen a opsoninas (fracción C3a y C5a del complemento o IgG) presentes en la superficie del patógeno. Posteriormente, la célula ingresa la bacteria dentro una vacuola fagocítica (fagosoma) para eliminarla mediante los diferentes mecanismos microbicidas que dispone, entre estos: a) el estallido respiratorio, donde se generan las especies reactivas del oxígeno, moléculas altamente oxidantes; b) la producción de especies reactivas del nitrógeno, especialmente de óxido nítrico; c) la descarga de enzimas proteolíticas dentro del fagosoma. Estos mecanismos en conjunto y bajo condiciones normales lesionan la bacteria hasta su destrucción (Abbas et al., 2014). La rápida y efectiva respuesta de los neutrófilos es clave para la resolución de la infección; sin embargo, en algunos trabajos se ha cuestionado su función fagocítica y bactericida en leche, debido a los efectos inhibitorios que pueden ejercer sobre el proceso la caseína y los glóbulos de grasa cuando son fagocitados. Además, porque *S. aureus* posee varios mecanismos que le permiten escapar de su acción; por ejemplo, cuando invaden las célula epiteliales, forman biopelículas o bloquean la migración (Mehrzad et al., 2005; Thammavongsa et al., 2015). Aunque este proceso puede verse alterado, no es la única función que emplean los neutrófilos para eliminar los microorganismos. Las trampas extracelulares de neutrófilos (NET) es uno de los mecanismos alternos, en el cual la célula libera todo su material nuclear (DNA y proteínas) y el de sus gránulos sobre la bacteria para causarle daño. La presencia en leche de componentes relacionados con la formación de NETs ha sido la evidencia de que los neutrófilos también recurren a este mecanismo para enfrentar al patógeno (Swain et al., 2014).

Inmunidad adaptativa

Los anticuerpos son los principales mediadores de esta respuesta y sus funciones son neutralizar, activar el complemento y opsonizar; pero como el *S. aureus* puede invadir eficientemente las células, así como, formar biopelículas y cápsula, se ha sugerido que

este tipo de respuesta no es suficiente ni efectiva (Atalla et al., 2010; Grönlund et al., 2006). Una de las inmunoglobulinas que participa en la respuesta inmune frente a patógenos como *S. aureus* es la IgG, especialmente los subtipos IgG1 e IgG2 (Atalla et al., 2010). En condiciones normales, la IgG1 es la inmunoglobulina predominante en la secreción mamaria, mientras que, la IgG2 se encuentra en bajas concentraciones, pero ambas pueden aumentar significativamente en los procesos inflamatorios. La IgG2 se reconoce como una potente opsonina para los *S. aureus* (Atalla et al., 2010). Un estudio donde se vacunó conejos con una cepa de *S. aureus* fuertemente productora de biopelícula PIA dependiente aislada de mastitis bovina, reportó títulos de anticuerpos en sangre más altos en los animales vacunados que en los no vacunados (Raza et al., 2015). Prenafeta et al. (2010) demostró que las vacas a las que se les aplicó una bacterina que tenía alto contenido de biopelícula, generaron títulos de anticuerpos en leche y sangre superiores a los animales vacunados con la bacterina que tenía bajo contenido de biopelícula y los no vacunados. Por otra parte, Cucarella et al. (2004) detectaron anticuerpos IgG contra Bap en sangre de animales infectados con *S. aureus bap* positivos.

Con respecto a las células que intervienen en esta respuesta, se ha observado, que además de los linfocitos B, también se pueden encontrar linfocitos T (LT) CD4 y CD8. Sin embargo, son los LT CD8 los que tienden a predominar en la fase crónica de la mastitis (Grönlund et al., 2006).

Detección de *S. aureus* en leche de bovinos con infecciones intramamarias

La detección de *S. aureus* se realiza mediante técnicas microbiológicas convencionales como el cultivo o moleculares como la Reacción en Cadena de la Polimerasa (*Polymerase Chain Reaction* – PCR). Su eliminación cíclica en leche, la cual puede estar asociada con la invasión celular y con la formación de biopelícula y de abscesos, dificulta su diagnóstico e impacta la sensibilidad de las técnicas empleadas (Keefe, 2012). La sensibilidad reportada del cultivo estándar (siembra de 0,01 ml) para una sola

muestra de leche está entre el 75% a 90%, la cual mejora con cultivos seriados. La sensibilidad de la PCR puede ser superior que la del cultivo; sin embargo, su estandarización debido al tipo de muestra (leche) y su nivel de complejidad dificultan su implementación dentro de la rutina diagnóstica (Dohoo et al., 2011; Greg Keefe, 2012; Sears et al., 1990).

Otra de las estrategias disponibles para diagnosticar IIM causadas por *S. aureus* es el RCS, la cual presenta ventajas con respecto a las dos mencionadas anteriormente en términos de costos y practicidad (Petzer et al., 2017). Aunque, la sensibilidad reportada para esta técnica es buena, 84,2%, su especificidad es baja, 52,8% (Petzer et al., 2017). Un estudio reciente encontró que el 30,8% de las vacas con IIM generadas por este patógeno pueden no ser detectadas cuando el umbral es de >150.000 células/ml en leche compuesta, dado que se pueden presentar infección con bajos recuentos (Petzer et al., 2017). El problema de las técnicas sensibles, pero no tan específicas, es que también presentan un número importante de falsos positivos. Lo anterior, junto con el hecho de que esta técnica no permita identificar los animales infectados específicamente con *S. aureus*, podría conllevar a prácticas inadecuadas durante su control, debido a que se tratarán infecciones que probablemente no van a responder al tratamiento y facilitarán su propagación al interior del hato.

Por otra parte, varios inmunoensayos también han sido descritos como alternativa diagnóstica de IIM causadas por *S. aureus* (Rainard et al., 2018). Sin embargo, este tipo de técnicas aún representan un reto por resolver, debido a que los animales sin IIM, pero colonizados naturalmente, pueden presentar títulos de anticuerpos similares a los infectados (Boerhout et al., 2016). Por lo tanto, el principal reto de este tipo de prueba es encontrar marcadores serológicos que permitan diferenciar entre animales con infecciones activas y sanos. Fabres-Klein et al. (2014) en su revisión, presenta los resultados de algunas de las pruebas realizadas para diagnosticar mastitis empleando

muestras de leche. Las sensibilidades y especificidades reportadas variaron entre 75% - 89% y 70% - 90%, respectivamente.

Tratamiento antibiótico contra *S. aureus*

El objetivo de cualquier tratamiento antibiótico contra *S. aureus* es reducir el grupo de animales infectados dentro del hato. Sin embargo, las tasas de cura de *S. aureus* son muy bajas comparadas con otras bacterias, lo que dificulta su control y erradicación (Keefe, 2012). Diferentes estudios han demostrado que la probabilidad de curación depende de factores asociados con el animal, el patógeno y el tratamiento (Barkema et al., 2006). Barkema et al. (2006) calcularon que la tasa de curación esperada para animales jóvenes es de aproximadamente del 60%; mientras que, para animales viejos con altos RCS y múltiples cuartos infectados podría ser inferior al 1%. Factores del patógeno como la capacidad de invasión celular, el fenotipo de variantes de colonia pequeña (*Small Colony Variants* – SCV), la resistencia a los antibióticos, la formación de abscesos y de biopelícula dificultan el tratamiento (Barkema et al., 2006). Algunos estudios han demostrado que el uso de terapias extendidas (>5 días) y mezcla de antibióticos generan mejores resultados que los tratamientos de corta duración (2- 3 días). La decisión sobre emplearlas o no para tratar las infecciones con *S. aureus*, es un tema de discusión, pues dependerá del análisis costo-beneficio que se le realice a la opción de mantener el animal dentro del hato (Pinzón-Sánchez et al., 2011; Roy & Keefe, 2012; Steeneveld et al., 2011).

Resistencia de *S. aureus* a los beta-lactámicos

Uno de los problemas que han tenido que afrontar los profesionales responsables de la salud en humanos como en animales, es el aumento de cepas resistentes a los antimicrobianos disponibles y la falta de nuevas moléculas con actividad antibiótica (Oldfield & Feng, 2014). Durante los últimos años la Organización Mundial de Sanidad Animal (OIE) junto con la Organización Mundial de la Salud (OMS) vienen desarrollando

estrategias y políticas para evitar el uso inadecuado de los antimicrobianos y minimizar los riesgos que lo anterior conlleva sobre la salud pública y el bienestar animal (*AMR_ES: OIE - World Organisation for Animal Health*, 2016). Se ha reportado que, alrededor del 80% de los antibióticos empleados en Estados Unidos en un año, fueron destinados al manejo del ganado (Chang et al., 2015; Hollis & Ahmed, 2013) y que el uso rutinario de antibióticos a nivel agropecuario ha conllevado a la generación de cepas bacterianas resistentes que impactan la salud pública (Chang et al., 2015); un verdadero problema si se tiene en cuenta que aproximadamente el 60% de los patógenos humanos provienen de animales (Organización Mundial de Sanidad Animal, 2015).

La resistencia a la meticilina desarrollada por *S. aureus* actualmente es reconocida como la resistencia a los antibióticos ß-lactámicos. Estos antibióticos tienen la capacidad de unirse a las proteínas de unión a la penicilina (*Penicillin-Binding Protein* – PBP), esenciales para la síntesis de la pared celular e inhibir la formación del peptidoglicano (Paterson et al., 2014). La meticilino resistencia es conferida por un elemento genético móvil conocido como cassette cromosómico estafilocóccico (*Staphylococcal Cassette Chromosome* – SCC*mec*), donde se ubica el gen *mecA* o *mecC* (Paterson et al., 2014). Estos genes codifican para unas PBP alteradas (PBP2a/PBP2´) que tiene poca afinidad por los antibióticos ß-lactámicos, lo cual evita que se afecte la síntesis de la pared celular, por consiguiente, la viabilidad de la bacteria (Paterson et al., 2014). Una de las cepas más reconocidas de *S. aureus* meticilino resistente asociada a animales (cerdos, bovinos, caballos, aves de corral, perros) y con potencial zoonótico es la ST398 (*sequence type* 398); sin embargo, las cepas más comunes entre los bovinos son la ST97, ST126, ST133, ST151, ST479 y ST771 (Holmes & Zadoks, 2011).

La correcta y oportuna identificación de cepas de *S. aureus* meticilino resistentes se ha convertido en una necesidad y obligación para todos los profesionales de la salud, pues

de esta forma se puede minimizar el uso indiscriminado e irresponsable de antibióticos y evitar la diseminación de estas cepas; dos acciones que al final van a impactar positivamente sobre la salud pública y el bienestar animal.

La meticilino resistencia y el fenotipo de biopelícula

La relación entre la susceptibilidad a los antibióticos ß-lactámicos y el fenotipo de la biopelícula fue descrito inicialmente en *Staphylococcus epidermidis*, donde se identificó que diferentes niveles en la producción de PIA fue asociado significativamente con diferentes niveles de susceptibilidad a los ß-lactámicos (McCarthy et al., 2015). En *S. aureus* se ha encontrado que la producción de PIA fue esencial para la formación de biopelícula en cepas meticilino sensibles, pero no en las meticilino resistentes (O'Neill et al., 2007). Incluso, la delección del locus *ica* en cepas de *S. aureus* meticilino sensibles suprimió la formación de biopelículas (O'Neill et al., 2007). En cuanto al fenotipo formado por las cepas meticilino resistentes, se ha encontrado que el locus *ica* está presente, pero no parece producirse PIA (O'Neill et al., 2007). Además, se observó en un estudio que la expresión del locus *ica* fue reprimida hasta 300 veces en una cepa meticilino resistente, comparado con la meticilino sensible, lo cual conllevó a que se formara una biopelícula de tipo proteico, es decir, independiente de PIA (Pozzi et al., 2012).

Factores de virulencia de *S. aureus* asociados con la evasión de la respuesta inmune

Las mastitis por *S. aureus* generalmente evolucionan a infecciones crónicas difíciles de erradicar; hecho que se le ha atribuido a la capacidad que posee el patógeno para evadir y manipular la respuesta inmune a través de los múltiples factores de virulencia descritos. Los mecanismos involucrados en este proceso se pueden dividir en dos categorías; primero, los que le permiten al microorganismo pasar desapercibido ante los

elementos de alarma; segundo, aquellos que le ayudan a escapar o manipular la respuesta inmune cuando es reconocido y atacado.

En la primera categoría, fundamentales para establecer la infección, se pueden incluir los mecanismos de invasión celular, la formación de cápsula y la producción de una proteína tipo superantígeno de los *Staphylococcus* (*Staphylococcal Superantigen-Like* - SSLs). *S. aureus* siempre se ha considerado un microorganismo extracelular, sin embargo, su habilidad de invadir y sobrevivir dentro de varios tipos de células ha sido evidenciada en numerosos estudios (Finlay & Cossart, 1997; Kim et al., 2011). La importancia de este factor de virulencia es que lo deja fuera del alcance de la fagocitosis y los anticuerpos, dos de los principales mecanismos efectores de la respuesta inmune. Varios elementos se han relacionado con la capacidad de invasión, entre estos, la expresión de receptores e integrinas, el bloqueo o inactivación de NF-κB, el fenotipo conocido como variantes de colonia pequeña (*Small Colony Variants* - SCV), la presencia de polisacáridos capsulares y la formación de biopelícula. Algunos estudios demostraron que el bloqueo de la integrina α5β1 y de elementos relacionados con la activación de NF-κB reducen significativamente la invasión intracelular, al igual que la expresión de Bap en la superficie de la bacteria y la presencia de polisacáridos capsulares (Bardiau et al., 2014; Valle et al., 2012). Por el contrario, se ha observado que cuando la bacteria presenta el fenotipo de SCV es más eficiente para ingresar que el fenotipo normal (Atalla et al., 2008). Con respecto a la cápsula, se han descrito alrededor de 11 polisacáridos capsulares, siendo el CP5 y el CP8 los más frecuentes tanto en humanos como en bovinos. Esta estructura le confiere a la célula bacteriana protección frente al reconocimiento y la fagocitosis, debido a que no permite la unión de los PRRs y opsoninas a sus moléculas blanco (Barrio et al., 2003; Camussone et al., 2014). Las SSLs pueden variar entre cepas; sin embargo, la evidencia indica que los genes *ssl1*, *ssl2, ssl3, ssl11, ssl12, ssl13, ssl14* son frecuentes entre todos los aislados. Una de las más importantes es la SSL3, pues se puede unir a los TLR2 expresados en

las células epiteliales e inmunes y bloquear el reconocimiento de la bacteria (Thammavongsa et al., 2015; Yokoyama et al., 2012).

En cuanto a los mecanismos que componen la segunda categoría, claves para mantener la infección después de reconocida, se pueden dividir en cinco grupos: a) los factores que intervienen con la quimoatracción y migración de neutrófilos; b) los factores que inhiben el accionar de los neutrófilos y del complemento; c) los factores de destrucción celular; d) los factores con los que se aísla del entorno; e) los factores que alteran la inmunidad adaptativa.

a) *Factores que intervienen con la quimoatracción y migración de neutrófilos*

S. aureus puede afectar estos procesos, fundamentales en el control de la infección, a través de la secreción de algunas SSLs o por proteínas inhibitorias de la quimioatracción (CHIPS). El patógeno puede alterar la generación de las moléculas C3a y C5a del complemento, importantes quimiotácticos de células fagocíticas, al no permitir que se active el sistema. Por ejemplo, la SSL7 tiene la capacidad de secuestrar inmunoglobulinas mediante la unión a la fracción Fc para que no puedan activar el complemento por la vía clásica o puede bloquear las tres vías al inactivar directamente los factores C3 y C5 (Thammavongsa et al., 2015). En caso que se activen las vías del complemento y se produzca el quimioatrayente C5a, la bacteria puede bloquear su función mediante las CHIPS, pues compiten y tienen alta afinidad por los receptores de esta fracción (Postma et al., 2004). Además, la adhesión al endotelio y migración de los neutrófilos puede ser alterada por la SSL5 y la SSL11, quienes se adhieren a la glicoproteína PSGL-1, presente en las membranas de los fagocitos y no permiten su unión con la P-selectina, una molécula expresada en el endotelio del huésped, la cual es clave para la adhesión y posterior migración de los leucocitos al sitio de la infección (Bestebroer et al., 2007).

b) *Factores que inhiben el accionar de los neutrófilos y del complemento*

La sola fagocitosis de *S. aureus* no garantiza su eliminación, pues la bacteria posee varias estrategias con las que puede alterar los mecanismos efectores de este proceso. Frente a las especies reactivas del oxígeno (ROS), como el peróxido de hidrógeno, emplea elementos antioxidantes, tales como el pigmento estafiloxantina y la catalasa, mientras que, contra el superóxido utiliza las superóxido dismutasas (*Superoxide dismutase* - SodA and SodM) (Cosgrove et al., 2007; Karavolos et al., 2003; Liu et al., 2005). Como respuesta al óxido nítrico la bacteria produce flavohemoglobina y Lactato deshidrogenasa. Incluso, puede inhibir la desgranulación al incrementar la concentración extracelular de Adenosina mediante la enzima catalítica de superficie Adenosin sintetasa A (*Adenosine sinthase A* - AdsA). La Adenosina producida se une a su receptor en la membrana del fagocito y desencadena una cascada de señalización anti-inflamatoria que bloquea la liberación de los gránulos dentro del fagosoma (Thammavongsa et al., 2009). Otra de las estrategias, es modificar químicamente el peptidoglicano (acetilación) para generar resistencia frente a la lisozima y péptidos antimicrobianos (Bera et al., 2005). Con respecto a las NET, esta bacteria las evade mediante DNasas, enzimas que le permiten degradar este material antes de que le cause daño (Thammavongsa et al., 2013).

Además de la SSL7 y el SSL10, descritas anteriormente, *S. aureus* presenta otros mecanismos con los que también puede afectar el sistema del complemento. La Aureolisina (Aur) es una metaloproteasa dependiente de Zinc, secretada por la bacteria para degradar el factor C3, por lo que no se van a generar las fracciones encargadas de la quimioatracción (C3a y C5a), la opsonización (C3b y C5b) y el complejo de ataque a la membrana, objetivo final de este sistema (Laarman et al., 2011). Otra manera es inhibiendo la C3 convertasa por medio del inhibidor del complemento (*Staphylococcal Complement Inhibitor* - SCIN), que en condiciones normales es la responsable de fragmentar C3 en C3a y C3b, dos productos claves

en el proceso; pero al igual que con la Aur, se bloquea todo lo que dependa de la activación y correcto funcionamiento de estas moléculas (Rooijakkers et al., 2005).

c) *Factores que le permiten destruir las células con quien tiene contacto*

La destrucción de diferentes tipos de células inmunes esta mediada básicamente por toxinas, especialmente, por las β-toxinas formadoras de poro (β-PFTs). Las más representativas de este grupo son la α-Hemolisina (Hla) y las leucocidinas. Todas las cepas pueden producir al menos tres leucocidinas (Luk): γ-Hemolisina AB (HlgAB), HLgCB y la Leucocidina AB (LukAB), mientras que, la producción de la LukED es cepa dependiente. La Hla forma un anillo heptamérico y las Luk uno octamérico, que contienen un poro central, con el cual debilitan la membrana de la células blanco hasta causarles lisis osmótica (Heuck et al., 2001; Miles et al., 2002).

d) *Factores que la aíslan y protegen del entorno*

La formación de biopelícula y de abscesos son los principales representantes de este grupo. Las biopelículas son un conglomerado de células inmersas en una matriz que pueden adherirse a una superficie viva o inerte, la cual le confiere protección frente al sistema inmune y antibióticos, pues quedan fuera de su alcance y acción (Gomes et al., 2016). La formación de biopelícula por *S. aureus* será tratada más adelante.

Los abscesos son comunidades bacterianas que se protegen dentro de una pseudocápsula formada a base de depósitos de fibrina y de células inmunes muertas. Esta estructura evita la entrada de fagocitos y promueve la supervivencia dentro de la lesión. Su formación esta mediada por la Deoxiadenosina generada durante la degradación de los NET a través de nucleasas secretadas por la bacteria, la cual desencadena la apoptosis de los macrófagos que la rodean y que terminan aislándola de su entorno (Thammavongsa et al., 2013).

e) *Factores que alteran la inmunidad adaptativa*

S. aureus también puede interferir con los mecanismos efectores de la inmunidad adaptativa. En este proceso participan básicamente superantígenos, sobre todo la Proteína A (*Staphylococcal protein A* – SpA), la cual es expresada y secretada por todas las cepas de interés clínico. SpA tiene la capacidad de unirse tanto a la fracción Fc como a la fracción Fab de las inmunoglobulinas. De acuerdo a la evidencia, cuando se une a la fracción Fab de la IgM de superficie de los Linfocitos B desencadena apoptosis; mientras que, cuando sucede en los plasmoblastos, el resultado es la producción de anticuerpos no específicos debido a que altera la hipermutación somática (Goodyear & Silverman, 2004; Thammavongsa et al., 2015). Por otra parte, la respuesta de los Linfocitos T (LT) también se ve afectada por superantígenos, aunque diferentes a la SpA. En este caso, los superantígenos interfieren entre las células presentadoras de antígenos y los LT ayudadores, pues se unen a los receptores involucrados en esta comunicación, el complejo mayor de histocompatibilidad (MHC II) y los receptores de las células T (TCR), lo que desencadena una respuesta no específica para el patógeno (Jardetzky et al., 1994; Thammavongsa et al., 2015).

Formación de biopelícula en glándula mamaria por *S. aureus*

La formación de biopelícula es considerada un factor de virulencia que le confiere ventajas a los patógenos causantes de mastitis debido a que les facilita su persistencia en glándula mamaria. De hecho, diferentes autores en los últimos años se han interesado en la formación de biopelícula, pues esta estructura le permite a la bacteria evadir la respuesta inmune e incrementar la tolerancia frente a los diferentes agentes antimicrobianos, por lo que estas infecciones serán más difíciles de eliminar (Gomes et al., 2016). Se ha descrito que alrededor del 40% de las mastitis corresponden a casos que no resolvieron con los tratamientos antibióticos aplicados (Hillerton & Kliem, 2002). Las bacterias cuando forman biopelícula pueden tolerar 10 veces o más la

concentración de antibióticos requerida para eliminarla en condiciones normales y tampoco son accesibles a la opsonización y fagocitosis, procesos que son fundamentales para la eliminación de las infecciones mamarias (Donlan & Costerton, 2002; Gomes et al., 2016).

Las biopelículas son ampliamente definidas como un grupo de células inmersas en una matriz que estas mismas forman. Recientemente Moormeier & Bayles, (2017) describieron que S. aureus forma biopelículas en cinco etapas: 1) adhesión, 2) multiplicación, 3) éxodo, 4) maduración, y 5) dispersión. La formación de biopelícula inicia con la adhesión de la bacteria a la superficie viva, que en este caso serían las CEM, mediante las diferentes proteínas de superficie descritas. Posteriormente, las bacterias se multiplican y forman varias capas de células. Al tiempo en que éstas se multiplican, se van generando los componentes que conforman la matriz en la cual quedarán inmersas las células bacterianas y demás material proveniente del medio ambiente. Además, durante la formación de la biopelícula se crean en su interior canales por donde van a fluir elementos indispensables para la supervivencia de las bacterias, tales como el oxígeno, metabolitos y nutrientes. Pero antes de madurar la biopelícula, S. aureus forma microcolonias y desprende algunas células con el propósito de restructurar la biopelícula; este proceso es conocido como éxodo. Una vez madura la biopelícula, las bacterias allí contenidas generan una ruptura de la estructura mediante enzimas líticas para liberarse y diseminarse, así como, para renovar la estructura de la ya establecida. Mediante este mecanismo la bacteria coloniza otras áreas y se expande la infección (Gomes et al., 2016; McCarthy et al., 2015; Moormeier & Bayles, 2017).

S. aureus es uno de los patógenos más aislado de las infecciones intramamarias a nivel mundial y una de las bacterias con mayor capacidad de formar biopelícula sobre superficies vivas e inertes (Gomes et al., 2016; Greg Keefe, 2012). Una particularidad de las mastitis causadas por *S. aureus* es que tienden a evolucionar a infecciones

crónicas difíciles de eliminar; hecho que se ha asociado en diferentes estudios a su capacidad de formación de biopelícula (Carme Cucarella et al., 2004; Gomes et al., 2016).

S. aureus puede formar dos tipos de biopelículas, una donde la matriz tiene como base el polisacárido de adhesión intercelular (*Polysaccharide Intercellular Adhesion* - PIA) y la otra es a base de proteínas; la primera es conocida como dependiente de PIA y codificada por el operón *ica*; la segunda es conocida como independiente de PIA y codificada por gen *bap* y asociada a la meticilino resistencia (McCarthy et al., 2015). Además, otros genes como el gen regulador accesorio (*accesory gene regulator – agr*) y el regulador accesorio estafilococcico (*staphylococcal accesory regulator – sar*) también se han involucrado en el proceso de formación de biopelícula, al igual que factores como el pH, algunos componentes de la leche, el Cloruro de Sodio (NaCl) y la glucosa (Gomes et al., 2016; McCarthy et al., 2015).

Entre los mecanismos de formación de biopelícula, el más estudiado ha sido el dependiente de PIA. En este se produce básicamente el polisacárido poli-N-acetilglucosamina (PNAG), también conocido como PIA, el cual es codificado por el operón *ica*. El operón está compuesto por cinco genes; cuatro (A, D, B, C) codifican para las proteínas involucradas en la biosíntesis y exportación de PIA a la superficie de la pared bacteriana; el quinto (R) codifica para un regulador transcripcional. Las proteínas codificadas por los genes *icaA, icaD* y el *icaC* corresponden a proteínas transmembrana. La proteína producto del gen *icaA* tiene actividad glicosíltransferasa y es la responsable de la síntesis del polisacárido; en este proceso también interviene el producto del gen *icaD*. Inmediatamente el polisacárido alcanza un tamaño máximo de 20 residuos, el producto derivado del gen *icaC* lo transloca a la superficie celular; sin embargo, para que el polisacárido formado se desprenda de la bacteria y quede totalmente sobre su superficie, este debe ser deacetilado por el producto del gen *icaB* (O'Gara, 2007). El PIA formado queda con una carga neta positiva que promueve la

agregación intercelular y la unión de la bacteria a la superficie inerte (Rohde et al., 2010). La mayoría de las cepas de importancia clínica son portadoras del operón *ica* (Cucarella et al., 2004).

Uno de los primeros reportes de formación de biopelícula independiente de PIA en *S. aureus*, fue realizado precisamente en un aislado de mastitis bovina, en el cual se identificó la proteína asociada a la biopelícula (*Biofilm-Associated Protein* – Bap) (Cucarella et al., 2001). Bap tiene un tamaño de 2.276 aminoácidos y permite la unión a las superficies y la adhesión intercelular; es codificada por el gen *bap*, el cual hace parte de un transposón que se inserta en la isla de patogenicidad SaPIbov2 (Cucarella et al., 2004). La evidencia sugiere que el gen *bap* se puede transmitir entre cepas mediante transferencia horizontal de genes (Tormo et al., 2005). Otro de los mecanismos que se ha relacionado con la formación de biopelícula independiente de PIA es la meticilino resistencia en *S. aureus* (McCarthy et al., 2015). Algunos estudios han mostrado que las cepas resistentes a la meticilina expresan el fenotipo proteico; diferente a lo evidenciado con las cepas sensibles quienes presentan el fenotipo PIA dependiente (McCarthy et al., 2015). Se ha demostrado que, en cepas de *S. aureus* meticilino resistentes, el operón *ica* puede estar presente y expresado pero el PIA no se produce (O'Neill et al., 2007). El operón *ica* en estos casos puede estar reprimido más de 300 veces comparado con las cepas meticilino sensibles, lo que conlleva a la formación de una biopelícula a base de proteínas (Pozzi et al., 2012).

Estudios sobre caracterización de cepas de *S. aureus* formadoras de biopelículas

La presencia de biopelícula y la capacidad patogénica de las cepas que expresan esta característica se ha demostrado *In vitro* e *In vivo* mediante diferentes estudios. Desde principios de los años 90 hay evidencia de esta conformación. Baselga et al. (1993) evidenció en tejido mamario a través de técnicas histoquímicas la presencia de una matriz de exopolisacáridos que rodeaba a la cepa de *S. aureus* responsable de la infección. Otros estudios también han demostrado en forma indirecta la presencia de

biopelícula mediante la detección de anticuerpos contra algunos de sus componentes, información que ha servido como base para el desarrollo de vacunas contra esta estructura (Cucarella et al., 2004; Pérez et al., 2009; Prenafeta et al., 2010). Uno de los estudios más representativos sobre el tema fue el realizado por Cucarella et al. (2004), pues en este se incluyó cepas de ambos fenotipos y se relacionaron con el RCS, la tolerancia a antibióticos, la capacidad de formación de biopelícula, persistencia en glándula mamaria y la producción de anticuerpos contra Bap. Los resultados mostraron que las cepas formadoras de biopelícula fueron más persistentes, presentaron menos RCS y mayor tolerancia a los antibióticos *In vitro* que las cepas sin esta característica. Además, se evidenció que las cepas *bap* positivas fueron mejores formadoras de biopelículas que las cepas *ica* positivas; lo que llevó a concluir que la sola presencia de *bap* es suficiente para formar biopelícula; mientras que, las cepas que solo tenían el operón *ica* probablemente necesitan de otros genes o estímulos para su formación (Cucarella et al., 2004). Fox et al. (2005) determinó que las cepas de *S. aureus* aisladas de leche formaron biopelícula con mayor frecuencia que las cepas aisladas de los sitios extramamarios. En el trabajo realizado por Oliveira et al. (2007) se reportó que el 80,8% de las cepas de *S. aureus* evaluadas generaron biopelícula a las 72 horas y solo un 34,6% lo había hecho a las 24 horas. Un estudio realizado en Polonia y publicado en el año 2012, identificó que el 57,6% de los aislados formaron biopelícula (Szweda et al., 2012). Veh et al. (2015b) publicó que las cepas de *S. aureus* que fueron más persistentes durante el periodo seco fueron las que mejor formaron biopelícula.

En cuanto a la identificación de proteomas relacionados con la formación de biopelículas por *S. aureus* aislado de mastitis bovina, no se tiene suficiente información sobre el tema. Algunos de los estudios donde se han identificado proteomas relacionados con la infección, se han realizado directamente en leche de animales infectados con el patógeno y el objetivo no ha sido la formación de biopelícula. Es el caso del estudio realizado por Kim et al. (2011) donde observaron diferencias en las proteínas detectadas en leche de animales infectados con tres diferentes cepas SCV de

S. aureus (Verma & Ambatipudi, 2016). Huang et al. (2014) también reportó diferencias en la expresión de al menos 55 proteínas después de analizar los proteomas de la leche de animales sanos e infectados con *S. aureus*. Uno de los estudios revisados que tuvo como objetivo identificar proteínas producidas durante la formación de biopelícula, fue el realizado por den Reijer et al. (2016); sin embargo, en este trabajo solo se incluyeron cepas aisladas de humanos y las células empleadas en el modelo fueron extraídas de la epidermis de pacientes; es decir, no tuvo relación con mastitis bovina. Los resultados mostraron diferencias entre las proteínas producidas durante la formación de biopelícula en microplaca y en cultivo celular. Las proteínas identificadas que mostraron diferencias entre los modelos evaluados se relacionaron con la adhesión, la modulación inmune y toxinas.

RESULTADOS POR OBJETIVO

Resultados del objetivo 1

Objetivo 1: Caracterizar genotípica y fenotípicamente cepas de *S. aureus* y evaluar diferencias en susceptibilidad a meticilina y capacidad formadora de biopelículas.

Para cumplir con este objetivo se ejecutaron las siete actividades que se describen a continuación:

1. Se confirmó mediante la amplificación del gen *nuc*, especie específico, que las cepas a emplear en el estudio fueran *S. aureus*.
2. Se determinó mediante PCR convencional en las 277 cepas de *S. aureus* la presencia de los locus *ica* y *bap*, asociados con formación de biopelícula.
3. Se evaluó en cada una de las cepas la capacidad de formación de biopelícula sobre microplacas de poliestireno.
4. Se determinó mediante PCR convencional en las 277 cepas de *S. aureus* la presencia de los locus *mecA* y *mecC*, asociados con meticilino resistencia.
5. Se tipificaron las 277 cepas mediante la técnica *spa typing*.
6. Se secuenció el genoma de una de las cepas fuerte formadora de biopelícula y se comparó con dos cepas de referencia de *S. aureus* (RF122 y Newbould 305).
7. Se evaluó la capacidad de invasión de tres cepas con diferentes genotipos y fenotipos en términos de formación de biopelículas.

Resultado de la actividad 1 del objetivo 1

El estudio inició con 300 cepas almacenadas como *S. aureus* en el banco de cepas del laboratorio de microbiología de la Unidad diagnóstica de la facultad de Ciencias Agrarias de la Universidad de Antioquia. Estas cepas fueron identificadas inicialmente empleando técnicas microbiológicas convencionales (Coloración de Gram, detección de Catalasa y prueba de la coagulasa).

La extracción de ADN de cada cepa fue realizada empleando el estuche comercial DNeasy Blood & Tissue kit (Qiagen, Germany), siguiendo las recomendaciones del fabricante para bacterias Gram positivas.

En total, 277 cepas de las 300 incluidas fueron confirmadas como *S. aureus* y continuaron en el estudio, debido a que se obtuvo amplificado del gen *nuc*. De manera que, las cepas restantes (23/300) fueron descartadas, sugiriendo inconvenientes en la identificación inicial con las técnicas empleadas.

Resultados de las actividades 2 y 3 del objetivo 1

Los resultados de las dos actividades se presentan en el artículo 1 titulado "**Genotypic and phenotypic characterization of biofilm production by *Staphylococcus aureus* strains isolated from bovine intramammary infections in Colombian dairy farms"**.

Este artículo se encuentra publicado en la revista "**Heliyon**" 5 (2019) e02535 (https://doi.org/10.1016/j.heliyon.2019.e02535).

Sin embargo, es importante anotar que en este artículo se trabajó con 229 cepas, debido a que 48 de las 277 cepas incluidas presentaron problemas que dificultaron su evaluación fenotípica en cuanto a capacidad de formación de biopelícula. Las dificultades evidenciadas fueron: crecimiento inadecuado, probablemente por pérdida de viabilidad (proceso de congelación y descongelación) y contaminación del cultivo madre con otras bacterias durante la manipulación en los ensayos previos.

Resultados de las actividades 4 y 5 del objetivo 1

Los resultados de estas actividades se relacionan en el artículo 2 titulado "**Molecular epidemiology of bovine mastitis caused by *Staphylococcus aureus* in Colombia reveals high genetic diversity and presence of clones associated with human infections".**

Resultados de las actividades 6 y 7 del objetivo 1

Los resultados de estas actividades se relacionan en el artículo 3 titulado "**Phenotypic characterization and whole genome analysis of a strong biofilm-forming *Staphylococcus aureus* strain associated with subclinical bovine mastitis in Colombia".**

Este artículo se encuentra publicado en la revista "**Frontiers in Veterinary Science**" 7:530 (2020) (https://doi:10.3389/fvets.2020.00530).

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UNIVERSIDAD DE ANTIOQUIA
Facultad de Ciencias Agrarias

Artículo 1

Genotypic and phenotypic characterization of biofilm production by *Staphylococcus aureus* strains isolated from bovine intramammary infections in Colombian dairy farms

El artículo completo se adjunta con el documento final (anexo) o puede ser descargado en el siguiente link: https://doi.org/10.1016/j.heliyon.2019.e02535

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Genotypic and phenotypic characterization of biofilm production by *Staphylococcus aureus* strains isolated from bovine intramammary infections in Colombian dairy farms

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ABSTRACT

The ability of *Staphylococcus aureus* to form biofilms is an important virulence factor because this has been associated with persistent bovine intramammary infections. Different mechanisms of biofilm formation have been described in *S. aureus*; however, the process has been found to be mainly driven by the *ica* and *bap* genes. The presence of the *ica* and *bap* genes, as well as the biofilm formation *in vitro* were evaluated in 229 *S. aureus* strains isolated from bovine milk collected from different regions of Department of Antioquia, Colombia. Three different genotypes grouped into three separate clusters were identified from *in vitro* assays. Genotype 1 (*ica* positive and *bap* negative) was the most prevalent (78.17%), followed by genotype 2 (*ica* and *bap* positive) (12.66%) and genotype 0 (*ica* and *bap* negative) (9.17%). Biofilm formation was observed in 81.26% of the strains from which 100% of genotype 2 isolates showed biofilm formation. The biofilms formed by genotype 2 isolates were also found to have the highest optical density (>2.4). These results showed that most of the *S. aureus* strains were capable of biofilm formation, suggesting the virulence potential particularly in *bap*-positive strains.

Artículo 2

Molecular epidemiology of bovine mastitis caused by *Staphylococcus aureus* in Colombia reveals high genetic diversity and presence of clones associated with human infections

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Abstract

Staphylococcus aureus is one of the most common pathogens that cause intramammary infections in cattle. These infections are generally characterized by their poor response to antibiotics and their persistence in the mammary gland. Genotyping of *S. aureus* strains isolated from mastitis has become a fundamental tool to understand the complex epidemiology of this pathogen. Despite of the importance of this pathogen in Colombia, no information is available on the genotypes that circulate within the herds of Antioquia (Colombia). A total of 277 strains of *S. aureus* isolated from intramammary infections in cattle from Antioquia were described and molecular characterization was performed.

Factors such as cattle breed, type and frequency of mastitis, and antibiotic susceptibility profile were considered for the epidemiological description. Spa typing was also performed. Of the 38 different spa types found in this investigation (more than 25% of the genotypes identified per municipality were different), 29 were grouped into 16 clonal complexes. The most frequent spa types were t267 and t521, both with 41 isolates. Furthermore, it was determined that 51 (18%) of the genotypes described had been unreported so far (new) and 26 (68%) had also been previously reported in humans. Knowledge on the spa types of *S. aureus* strains isolated from cattle with mastitis within the region increased knowledge on their characteristics and possible behaviors at clinical, epidemiological, and therapeutic levels. These factors are fundamental for their control and prevention.

Keywords: Bovine intramammary infection; Clonal complexes; Genotyping; Mastitis; *Staphylococcus aureus*; Spa type

Introduction

It is considered that bovine intramammary infection (IMI) is one of the diseases that causes the highest economic losses for milk producers throughout the world because it leads to decreases in milk production, increases in drug costs, and the discarding of productive cows (Keefe, 2012).

Staphylococcus aureus is one of the main pathogens that cause IMI in cattle (Olde Riekerink et al., 2006). Control of this microorganism in the mammary gland is limited as it is difficult to identify in most cases, it responds poorly to the antibiotics used for treatment and tends to give rise to persistent infections (Olde Riekerink et al., 2006; Zadoks et al., 2011). In a study conducted in Antioquia, two veterinary diagnostic

laboratories reported that *S. aureus* was the second most common microorganism isolated from milk samples between 2013 and 2015 (Vidal et al., 2016).

Different studies and reported genomes of *S. aureus* have demonstrated much diversity between strains. Evidence has demonstrated that approximately 20%–30% of its genome is diverse because of specific lineage genes and mobile genetic elements (Matuszewska et al., 2020). This variability has been associated with virulence, response to antibiotic treatment, transmission between hosts, geographic distribution, and type of mastitis, which makes management and control in herds difficult (Haveri et al., 2008).

The genotyping of *S. aureus* strains isolated from IMI has become a fundamental tool for understanding the complex epidemiology of this pathogen (Boss et al., 2016; Smith et al., 2016; Vanderhaeghen et al., 2014; Zadoks et al., 2011). Pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and spa typing are some of the most widely used methods to typify *S. aureus* strains (Boss et al., 2016). Spa typing is based on the sequencing of the spacer variable region (region X) of the *spa* gene. It provides comparable resolution but is less expensive and laborious than the other two methods mentioned above. It is also possible to infer clonal complexes (CCs) with this methodology, which groups genotypes related to a common ancestor (Feltrin et al., 2016; Haveri et al., 2008; Olde Riekerink et al., 2006; Sakwinska et al., 2011). Some of the most important spa types reported in bovine IMIs are t521, t267, and t543, which have been associated with clinical and subclinical mastitis. CC97 is the most prevalent CC in bovine mastitis worldwide and CC398 is associated with the presence of methicillin-resistant *S. aureus* clones (MRSA) (Angel-Andrés et al., 2014; Hata et al., 2010). The genotypes of the circulating strains and their association with sensitivity profiles to antibiotics *in vitro* were unknown in Antioquia, which is one of the main dairy regions of Colombia, and where *S. aureus* is one of the main causes of IMI in cattle. This information is essential for understanding the possible behaviors of these strains at

clinical and epidemiological levels, as well as their response to antibiotic treatments. Thus, the aim of this study was to identify the genotypes of the *S. aureus* strains isolated from IMI in Antioquia, Colombia, by means of spa typing.

Materials and Methods

S. aureus isolates

A total of 277 strains of *S. aureus* stored in the strain bank of the Biogenesis Research Group of the Department of Agricultural Sciences of the University of Antioquia (Medellín, Colombia) were characterized. The strains were isolated from bovine milk of cows suffering from IMI, between July and December 2015, obtained from dairy farms located in different regions of the department of Antioquia (Table 1). Isolates were initially classified as *S. aureus* using conventional microbiological techniques, based on the guidelines of the National Mastitis Council of the United States (National Mastitis Council, 2004). Isolates were stored at −80°C in Trypticase soy agar (TSA) (Oxoid, United Kingdom) supplemented with 10% glycerol until use.

Table 1. Number of *S. aureus* strains isolated in each region

Region	Municipality	No. of isolates (%)
North	San Pedro de los Milagros, Santa Rosa de Osos, Belmira, Bello, Entrerríos, Don Matías, Carolina del Príncipe, San José de la Montaña	228 (82%)
East	La Unión, Rionegro, Abejorral, La Ceja, Santuario	27 (10%)
Southeast	Urrao	20 (7.2%)

	West	San Jerónimo	2 (0.7%)
	Total	15	277 (100%)

No: Number

Clinical and epidemiological characteristics

Clinical and epidemiological information was obtained from the history and sample entry records for each animal. The information collected included place of origin, type of infection (clinical or subclinical), frequency of infection (first time or recurrent), and cattle breed.

DNA extraction

The stored *S. aureus* strains were thawed and cultured in TSA medium (Oxoid, United Kingdom) and incubated at 37°C for 24 h. DNA was extracted using the DNeasy Blood & Tissue Kit (Quiagen, Germany) according to the manufacturer's recommendation for Gram-positive bacteria. A NanoDrop kit (ThermoFisher Scientific, USA) was used to measure the purity and concentration of the extracted DNA. The DNA was then stored at −20°C until use.

Molecular confirmation of S. aureus and mecA gene detection

The strains used in the study were confirmed as *S. aureus* through polymerase chain reaction (PCR). The PCR proposed by Graber et al. (2009) allowed the amplification of a fragment of the *nuc* gene, recognized as being species-specific Sommerhäuser et al. (2003). The *mecA* gene was detected according to the methodology described by Zehra et al. (2017). The PCR reactions were conducted in a PTC 200 thermocycler (Perkin-Elmer Inc., USA).

Determination of susceptibility to antibiotics

The antibiotic susceptibility profile of the isolated *S. aureus* strains was determined with the Kirby–Bauer methodology, following the recommendations of the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2020). The disc-diffusion antibiotic used (Oxoid, United Kingdom) were Penicillin G, Cefoperazone, Cloxacillin, Ampicillin/Sulbactam, Amoxicillin clavulanic acid, Lincomycin and Trimethoprim Sulfamethoxazole. *S. aureus* ATCC 25922 strain was used as a control. The resistance to each of the antibiotics was recorded as a binary attribute for each isolate. Moreover, two variables were derived from the resistance profiles. The first one was the number of antibiotics to which the isolate was resistant and the second was the resistance to at least one of the tested antibiotics.

Genotyping using spa typing

The polymorphic region of the gene coding for protein A (*spa* gene), known as region X, was amplified by PCR for each strain. The primers used in the reaction were those described by Shopsin et al. (1999), while the thermal profile followed was that reported by Jiménez et al. (2012), with denaturation at 94°C for 30 s, alignment at 60°C for 1 min, and 72°C extension for 1 min, for 30 cycles. Expected band size was approximately 1.000 bp, which was later sequenced by Macrogen (USA). Spa types were assigned in two stages; eGenomics software was used in the first stage (Shopsin et al., 1999) and the Ridom SpaServer website (http://www.spaserver.ridom.de/), developed by Ridom GmbH, was used in the second. CCs were inferred through analysis of the repetition pattern of the spa types obtained (Strommenger et al., 2008) or by consulting the Ridom SpaServer website.

Statistical analysis

Descriptive analyses were performed for all the variables of interest. Further bivariate comparisons using Chi square and Fisher's exact tests were assessed to determine the relationship between antibiotic resistance, municipalities, and genotypes (CC). A

statistical threshold of $P < 0.05$ was considered. All the analyses were carried out on Rstudio statistical software program (version 3.6.0) (https://cran.R-project.org).

Results

Geographical distribution and epidemiological and clinical characteristics

The municipality of San Pedro de los Milagros contributed a total of 60 isolates, the majority in this study, followed by the municipality of Santa Rosa de Osos with 49 isolates. The municipality with the least isolates was San Jerónimo with two (Table 2).

The highest proportion of *S. aureus*-positive samples, 243 (88%), were obtained from Holstein cattle, followed by the Jersey breed with 30 (11%). The subclinical stage prevailed (96%) in terms of the presentation of the infection, as did the report of recurrent infections (69%) (Table 2).

Table 2. Distribution and characteristics of the strains under study.

Municipality	Number of isolations	Breeds (n)	Frequency of mastitis (n)	Type of mastitis (n)
San Pedro de los Milagros	60	Holstein (59), Jersey (1)	P (14) R (46)	Subclinical (59) Clinical (1)
Santa Rosa de Osos	49	Holstein (40), Jersey (9)	P (16) R (33)	Subclinical (42) Clinical (7)
Belmira	34	Holstein (30), Jersey (4)	R (34)	Subclinical (34)

Bello	32	Holstein (28), Jersey (4)	P (9) R (23)	Subclinical (32)
Entrerríos	23	Holstein (16), Jersey (7)	P (4) R (19)	Subclinical (23)
Urrao	20	Holstein (15), Bon (3), Jersey (2)	P (18) R (2)	Subclinical (20)
Don Matías	17	Holstein (14), Jersey (3)	P (11) R (6)	Subclinical (16) Clinical (1)
La Unión	9	Holstein (9)	P (5) R (4)	Subclinical (9)
Carolina del Príncipe	8	Holstein (7), Girolando (1)	P (4) R (4)	Subclinical (8)
Rionegro	6	Holstein (6)	R (6)	Subclinical (6)
Abejorral	5	Holstein (5)	P (1) R (4)	Subclinical (5)
San José de la Montaña	5	Holstein (5)	P (2) R (4)	Subclinical (5)
La Ceja	4	Holstein (4)	R (4)	Subclinical (4)
Santuario	3	Holstein (3)	P (2) R (1)	Subclinical (1) Clinical

				(2)
San Jerónimo	2	Holstein (2)	R (2)	Subclinical (2)

(n): number of strains; P: first time; R: recurring

S. aureus strains Typing

A total of 38 different spa types were identified. Santa Rosa de Osos was the municipality with the highest number of different genotypes, 18 in total (38% of genotypes found in the municipality). The municipalities that followed were San Pedro de los Milagros with 15 (25% of those found in the municipality) and Belmira with 13 (38% of those found in the municipality). A wide diversity of spa types was generally present in all municipalities as more than 25% of the genotypes identified by municipality were different (Table 3).

Table 3. Spa type and clonal complexes by municipality.

Municipality	**Clonal complex identified**	**Spa type identified***	**Different spa type (%)**
San Pedro de los Milagros	CC1, CC479, CC97, CC398, CC8	New, t605, t543, t527, t2207, t267, t3626, t521, t571, t2112, t8845, t7753, t398, t345, t064	15 (25%)
Santa Rosa de Osos	CC45, CC97, CC8, CC479, CC1, CC98, CC5, CC522, CC084	New, t521, t543, t267, t2207, t445, t024, t2143, t008, t1106, t1236, t149, t17143, t2112, t2803, t4103, t527, t692	18 (38%)

Belmira	CC479, CC8, CC30, CC1, CC97, CC59, CC98, CC398	New, t543, t267, t2112, t008, t064, t1135, t2207, t4911, t521, t1236, t5051, t571	13 (38%)
Bello	CC97, CC15, CC479, CC 1, CC5, CC98	t267, t521, t605, t1885, t543, t1107, t1236, t2112, t2207, New	10 (31%)
Entrerríos	CC97, CC30, CC 1, CC164, CC97, CC479	t267, t521, t1135, t2207, New, t1429, t2112, t543, t605	10 (44%)
Urrao	CC 1, CC398, CC97, CC037, CC22, CC479	t189, t527, t605, t571, t267, t1190, t515, t521, t543, New	10 (50%)
Don Matías	CC97, CC1, CC4118	t521, t2207, t527, t1106, t426, New	6 (35%)
La Unión	CC97, CC8	New, t521, t2413, t064	4 (44%)
Carolina del Príncipe	CC97, CC479, CC97	t267, t543, t521, New	4 (50%)
Rionegro	CC1, CC97, CC479	New, t2207, t267, t543	4 (67%)
Abejorral	CC97, CC5	New, t267, t458	3 (80%)
San José de la Montaña	CC97, CC1, CC22	t2112, t2207, t521, t515	4 (80%)
La Ceja	CC479, CC4118, CC15	t543, t426, t1885	3 (75%)
Santuario	CC1	New, t2207, t6280	1 (33%)

San Jerónimo	CC97	t521	2 (99%)

*The identified spa types are listed from highest to lowest frequency

The most frequent spa types were t267 and t521, both with 41 isolates, which were represented in almost all municipalities (12/15 municipalities). We were unable to assign a spa type to 51 (18%) of the 277 isolates because no comparable patterns were found, and they were classified as new (Table 4). Only 6 of the 277 strains evaluated were isolated from clinical mastitis and the t521 (2), t008 (1), t2207 (1), t3626 (1), and t426 (1) genotypes were identified. 26 (68%) of the 38 identified spa types had also been previously reported in humans (Supplementary table 1).

Table 4. Most frequent clonal complexes and spa type.

Clonal complex	Spa type (n)	Total
CC97	t267 (41), t521 (41), t2112 (10), t1236 (7)	99
CC1	t605 (28), t2207 (21), t527 (8), t1190 (2), t345 (1)	60
CC479	t543 (21)	21
CC359	t189 (4)	4
CC8	t064 (3), t008 (2), t024 (2)	7
CC5	t1107 (1), t149 (1), t458 (1)	3
CC30	t1135 (3)	3
CC4118	t426 (3)	3
CC398	t571 (3)	3
CC45	t445 (2), t2143 (1)	3

CC15	t1885 (2)	2
CC22	t515 (2)	2
CC164	t1429 (1)	1
CC522	t17143 (1)	1
CC084	t4103 (1), t692 (1)	2
CC59	t4911 (1)	1
N/A	t1106 (2), t3626 (2), t2413 (1), t2803 (1), t398 (1), t5051 (1), t6280 (1), t7753 (1), t8845 (1)	11
N/A	New (51)	51

N/A (not assigned): it was not possible to assign them a CC.

A total of 16 CCs were inferred from the spa types obtained. The most frequent CC was CC97. It was represented by spa types t521, t267, t1236, and t2112. CC1 constituted of more than three different genotypes (t605, t527, t1190, t2207, and t345) (Table 5).

Both the 51 new genotypes and nine other identified genotypes were not possible to be assigned to a specific clonal complex (Table 4).

Antibiotic susceptibility and presence of the mecA gene

The *S. aureus* strains evaluated showed resistance to the antibiotic penicillin and lincomycin (37%), followed by ampicillin/sulbactam (10%), cefoperazone (7%), trimethoprim/sulfa (4%), cloxacillin (3%), and amoxicillin/clavulanic acid (2%). Out of the 277 strains evaluated, only 2 were positive for the *mecA* gene. Figure 1 shows the number of strains per CC and their resistance against to antibiotic tested.

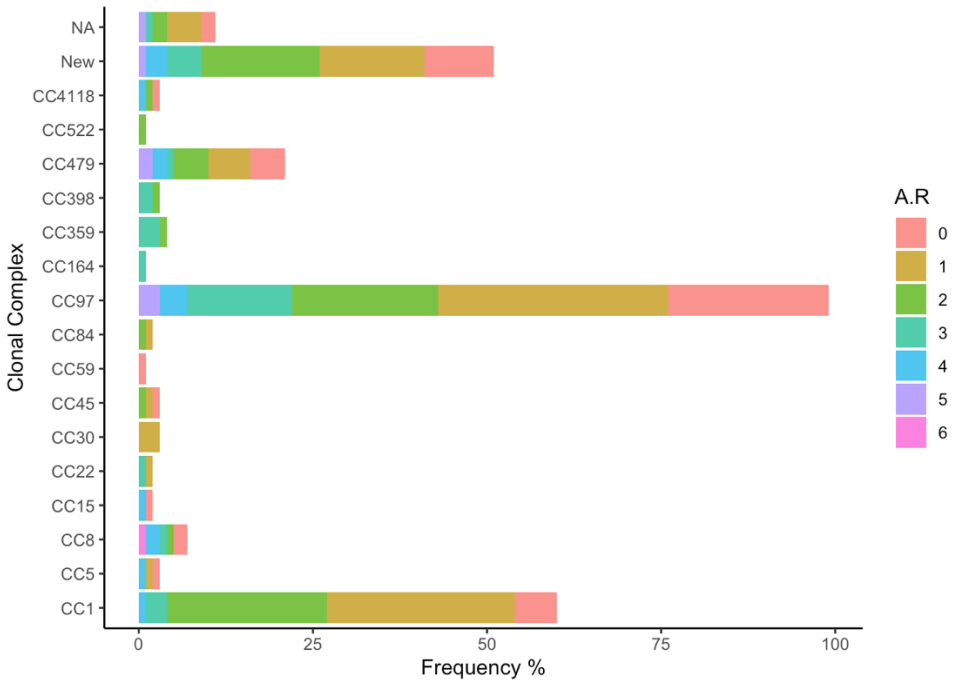

Figure 1. Number of strains (frequency) per clonal complex (CC) and number of antibiotics to which they were resistant. A.R: Number of antibiotics to which the strains evaluated showed resistance. NA: refers to an unassigned CC.

The bivariate analysis performed between resistance to at least one antibiotic and municipality was significant (*P*=0.01799) (Figure 2), and between antibiotic resistance to at least one antibiotic and genotype (CC) did not show significant association (*P*=0.387).

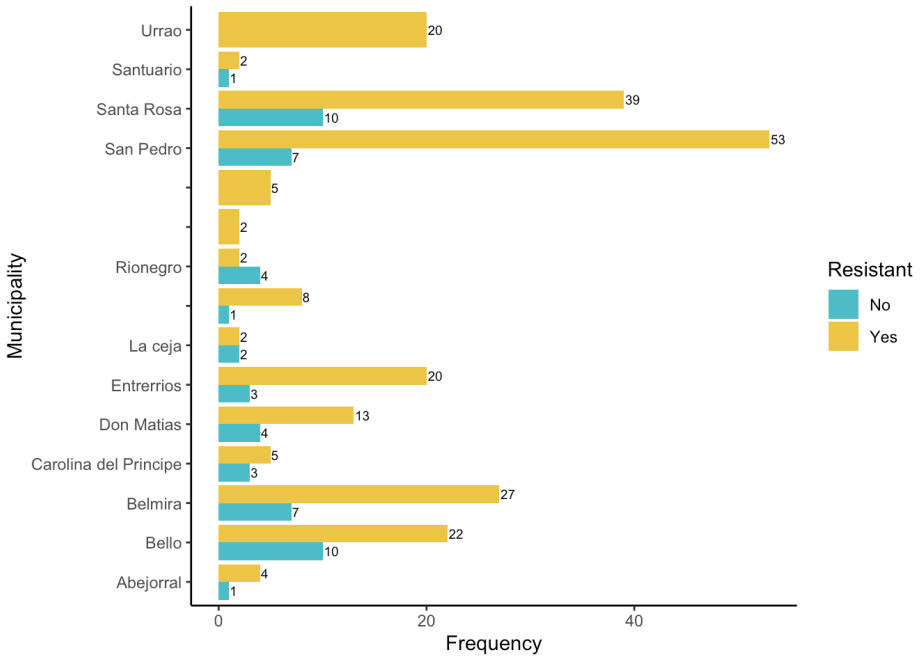

Figure 2. Distribution of the resistance to at least one antibiotic by municipality.

Discussion

A total of 38 different spa types were found in this study, of which 29 were grouped into 16 CCs. More than 25% of the genotypes identified by municipality were different, indicating the high diversity of circulating strains in this part of the country. These results were consistent with what was reported by Tokajian et al. (2010), who determined a diversity of 37%, but differ from those reported by Omar et al. (2014) and Strommenger et al. (2008), where diversities of 30% and 18% were found, respectively. The variability found in our study could be explained by the management practices that can change between areas, geography, and the season of the year, as well as the relationship between hosts (Boss et al., 2016; Kosecka-Strojek et al., 2016).

One of the main findings of this investigation was the detection of spa types t064, t024, and t008 in cattle, which had previously only been reported in humans in Colombia (Jiménez et al., 2012). According to Fenner et al. (2008), t008 has been reported as a

community-associated clone, whereas t024 was associated with the hospital environment by Bartels et al. (2007). These last two genotypes have been widely reported as MRSA (Boss et al., 2016); however, in this study they were methicillin sensitive because the *mecA* gene was not found in them. t024 was first reported as the cause of human infections associated with the community in the city of Medellín (Antioquia, Colombia) (Jiménez et al., 2012). Our study is the first to report the presence of these clones in cattle in Colombia. The above-mentioned spa types are classified as CC8, which has a clade known as CC8bov. Some studies have associated t024 specifically with the bovine clade, whereas others report their origin in humans, demonstrating that they have adapted to both species (Boss et al., 2016).

Genotypes t2207, t1135, t2143, t445, t571, t1885, t4911, t3626, and t149, were also found in this study, and they have been mainly associated with hospital infections in humans (Israel et al., 2018). Nevertheless, t2207 has also been reported as the cause of sporadic infections in both cattle and rabbits (Haveri et al., 2008). t571, which has only been reported in humans, has been strongly associated with the handling of production animals (cattle). This genotype is classified as CC398, a production livestock-associated group (Boss et al., 2016) and whose main reservoir is pigs, although clones adapted to other hosts such as cattle and humans have also been identified (Wang et al., 2018).

The two most common spa types in our study were t521 and t267, both genotypes commonly reported as causing subclinical mastitis in cattle (Schmidt et al., 2017). t267 was only found to cause subclinical mastitis in this study, which is consistent with reports from Brazil, Canada, and Japan (Boss et al., 2016; Hata et al., 2010; Israel et al., 2018; Olde Riekerink et al., 2006), whereas t521 was found to cause both clinical and subclinical mastitis. t543, one of the other most frequent genotypes in this study, has been associated with both clinical and subclinical mastitis. However, t543 was only found in subclinical infections in this study, agreeing with what was reported in other

investigations worldwide (Israel et al., 2018; Smith et al., 2016; Wang et al., 2016). T521 and t267 are part of CC97, which is recognized as the most reported complex in genotyping studies of strains obtained from cattle (Wang et al., 2018). These data agree with the findings of this study as it was the most frequent CC. An investigation conducted by Budd et al. (2016), where they compared the adhesion capacity of three CCs, with CC97 among them, found that this formed stronger bonds by means of fibronectin than CC8 and CC1, suggesting that this behavior could make a difference in terms of persistence capacity in the bovine mammary gland (Peña & Uffo, 2013).

The CC30 is relatively old and has spread worldwide. Clones belonging to this CC caused a pandemic after World War II (Cheung et al., 2014) and, over time. CC30 strains have become the main clones associated with hospital settings (Sharma-Kuinkel et al., 2015). Spa type t1135, a member of this group, was found in this study. CC45 has been recognized for being successful in colonization processes in humans, mainly in children, and CC22 has been reported in humans as strains that can be infectious and colonizing (Rodríguez-Tamayo et al., 2016). CC1 has been reported in strains isolated from humans and cattle. Evidence suggests that this CC can be transmitted from animals to humans, an indication that it adapts to the two species (Silva et al., 2013).

This investigation determined that 68% of the identified genotypes have also been previously implicated in human infections. This situation requires attention from public health authorities, because in Colombia between 43.6% and 77% of milk harvest continues to be manual (Ramírez et al., 2014; Reyes et al., 2017), increasing the risk for milkers to acquire this pathogen and spread it among individuals in their environment. Furthermore, approximately 41% of raw (unpasteurized) milk is commercialized informally (Business Bridge, 2015), which may also facilitate its spread among individuals who are not exposed to livestock. Therefore, this situation turns bovine into potential sources of the pathogen for humans (Shepheard et al., 2013).

Another relevant finding of this study was that the presence of 51 previously unreported spa types (18%). These, and another nine genotypes, could not be assigned to any CC.

S. aureus strains showed the highest resistance to antibiotics penicillin and lincomycin in the susceptibility profile obtained. These results are consistent with that reported by other studies previously carried out in the same region, where these antibiotics showed the same behavior against the evaluated strains (Ramírez et al., 2018; Ramírez et al., 2011). The association found between the resistance and the municipality is due to high frequency of strains resistant at least one antibiotic isolated in several municipalities.

Conclusions

Our research is the first in Colombia to perform molecular identification of the circulating *S. aureus* strains in one of the main dairy regions of the country. These results showed the wide diversity of circulating strains in cattle. Furthermore, 68% of the genotypes identified from IMI have been also described as causing infections in humans, making dairy cattle an important reservoir of the pathogen for people in proximity, such as milkers, and for individuals who are not from their environment. These findings highlight the need for active surveillance of circulating *S. aureus* strains in order to identify strains with zoonotic potential and the occurrence of new clones with high public health risk.

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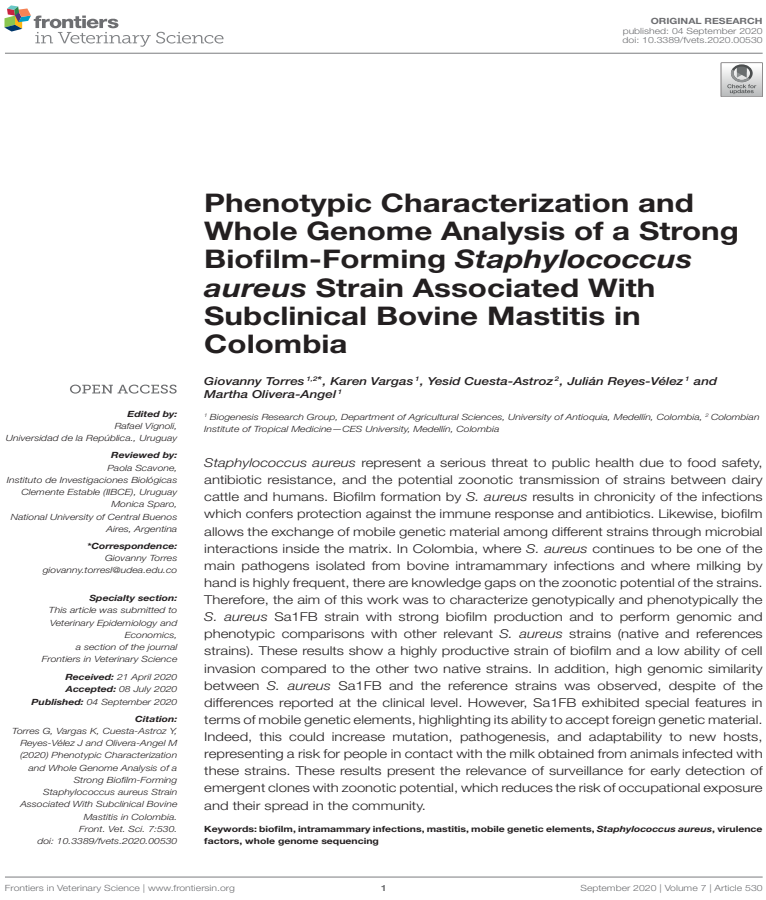
Artículo 3

Phenotypic characterization and whole genome analysis of a strong biofilm-forming *Staphylococcus aureus* strain associated with subclinical bovine mastitis in Colombia

El artículo completo se adjunta con el documento final (anexo) o puede ser descargado en el siguiente link: https://doi:10.3389/fvets.2020.00530



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Phenotypic Characterization and Whole Genome Analysis of a Strong Biofilm-Forming *Staphylococcus aureus* Strain Associated With Subclinical Bovine Mastitis in Colombia

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Staphylococcus aureus represent a serious threat to public health due to food safety, antibiotic resistance, and the potential zoonotic transmission of strains between dairy cattle and humans. Biofilm formation by *S. aureus* results in chronicity of the infections which confers protection against the immune response and antibiotics. Likewise, biofilm allows the exchange of mobile genetic material among different strains through microbial interactions inside the matrix. In Colombia, where *S. aureus* continues to be one of the main pathogens isolated from bovine intramammary infections and where milking by hand is highly frequent, there are knowledge gaps on the zoonotic potential of the strains. Therefore, the aim of this work was to characterize genotypically and phenotypically the *S. aureus* Sa1FB strain with strong biofilm production and to perform genomic and phenotypic comparisons with other relevant *S. aureus* strains (native and references strains). These results show a highly productive strain of biofilm and a low ability of cell invasion compared to the other two native strains. In addition, high genomic similarity between *S. aureus* Sa1FB and the reference strains was observed, despite of the differences reported at the clinical level. However, Sa1FB exhibited special features in terms of mobile genetic elements, highlighting its ability to accept foreign genetic material. Indeed, this could increase mutation, pathogenesis, and adaptability to new hosts, representing a risk for people in contact with the milk obtained from animals infected with these strains. These results present the relevance of surveillance for early detection of emergent clones with zoonotic potential, which reduces the risk of occupational exposure and their spread in the community.

Keywords: biofilm, intramammary infections, mastitis, mobile genetic elements, *Staphylococcus aureus*, virulence factors, whole genome sequencing

Resultados del objetivo 2

Objetivo 2: Caracterizar y comparar los proteomas generados *In vitro* durante la formación de biopelícula por cepas de *S. aureus* aisladas de mastitis.

Para cumplir con este objetivo se ejecutaron las cinco actividades que se describen a continuación:

1. Se seleccionaron tres cepas genotípica y fenotípicamente diferentes de acuerdo con los resultados obtenidos en el objetivo 1.
2. Se co-cultivaron las tres cepas con células epiteliales mamarias bovinas durante 12 y 24 horas.
3. Se identificaron las proteínas bacterianas extraídas después de cumplido los tiempos establecidos mediante nano-LC-MS/MS.
4. Se compararon las proteínas obtenidas de las cepas formadoras de biopelículas con los controles.
5. Se seleccionaron tres proteínas expresadas en las cepas productoras de biopelículas relacionadas con la formación de esta matriz.

Los resultados de todas las actividades relacionadas se encuentran en el artículo 4 titulado "**Proteomic analysis of the interaction between biofilm-forming *Staphylococcus aureus* strains and bovine mammary epithelial cells**".

Artículo 4

Proteomic analysis of the interaction between biofilm-forming *Staphylococcus aureus* strains and bovine mammary epithelial cells

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Abstract

Several proteomic approaches have been used to study the complex interactions between the bovine host and *Staphylococcus aureus*. However, most of studies on biofilms have used bacterial cultures on abiotic surfaces, which probably do not reflect the behavior of bacteria *in vivo*. Biofilm produced by *S. aureus* on bovine biotic surfaces has been less studied. The aim of this study was to identify *S. aureus* proteins associated with the biofilm formation during interaction with bovine mammary epithelial cells. Three *S. aureus* strains with different genotypic and phenotypic characteristics in terms of biofilm formation were included. The 1BF strain carried the *ica* and *bap* genes and exhibited the highest ability to form biofilm. Whereas, 2BF was only *ica* positive and showed a weaker phenotype. The 3NF strain was used as a control, which it did not have any of these genes and was classified as non-biofilm former. The strains were co-

cultured with bovine mammary epithelial cells for 12 h and 24 h. Bacterial proteins were extracted and analyzed with nano-LC-MS/MS. Six proteins were identified associated with the biofilm formation that were only expressed during the co-culture with mammary epithelial cells and were not identified in the controls. IsdA and Eap were expressed by both biofilm-forming strains, enolase, ClfA and SdrD were also produced by 2BF strain, while TRAP by 1BF. IsdA, ClfA and SdrD were the identified proteins involved with biofilm production that exhibited the highest predicted antigenicity (> 90%). These results confirm that IsdA, ClfA and SdrD are potential candidates for vaccines and could be used as immunogens in immunoassays, since are involved in the early stage of the infection, in the biofilm formation and have the ability of elicit host immune response.

Keywords: Biofilm; Intramammary infections; Mastitis; Proteins; Proteomic; *Staphylococcus aureus*; Virulence factors

Introduction

Staphylococcus aureus is one of the main causing agent of intramammary infections (IMI) worldwide (Keefe, 2012). The infections caused by this pathogen are characterized due to their persistence and the poor antibiotic response, which represents an important economic problem for the dairy industry (Veh et al., 2015).

Different phenotypic characteristics from *S. aureus* have been associated with persistent IMI, being the ability to form biofilm one of the most important due to confers protection against antibiotics and the host's immune response (Gomes et al., 2016). This pathogen can form biofilm through several described mechanisms such as polysaccharide intercellular adhesion (PIA)-dependent or PIA-independent, and in five stages: attachment, multiplications, exodus, maturation, and dispersal (McCarthy et al., 2015; Moormeier & Bayles, 2017). However, proteins involved in this process have not yet been fully defined.

Proteomics approach and the utilization of bioinformatic tools have been used to study the complex interactions between host and *S. aureus* (Huang et al., 2014). Studies have revealed several proteomic profiles in both milk and blood serum of cows with IMI, as well as in the bacterial cells (Huang et al., 2014; Kim et al., 2011; Smith et al., 2016; Zhang et al., 2015). Most proteomic studies on biofilms have used bacterial cultures on abiotic surfaces, which probably have not reflected the actual behavior of bacteria under *in vivo* conditions (Lei et al., 2017). Evidence has shown that studies that have been carried out on abiotic surfaces to evaluate the biofilm forming process can show discrepancies with respect to those that have used cellular or animal models (biotics) (Brady et al., 2006; den Reijer et al., 2016). Brady et al. (2006) found that only 26 of the more than 100 proteins identified from biofilm matrix under *in vitro* conditions were recognized in the biofilms formed *in vivo* (Lei et al., 2017).

The biofilm forming process by *S. aureus* on bovine biotic surfaces (e.g. mammary epithelial cells) has been less studied. Therefore, increasing knowledge about this process is essential to understand its performance under *in vivo* conditions. The aim of this study was to identify *S. aureus* proteins associated with the biofilm formation during interaction with bovine mammary epithelial cells.

Materials and methods

Staphylococcus aureus strains

Three *S. aureus* strains were selected from 229 isolates recovered from bovine IMI in the Department of Antioquia (Colombia), which were previously characterized genotypically and phenotypically in terms of biofilm formation (Torres et al., 2019). A strain of each of the three groups of identified genotypes was randomly chosen. Table 1 describes the characteristics of the selected strains. The 3NF strain was used as a non-biofilm formation control.

Table 1. Genotypic and phenotypic characteristics of the selected strains.

Strain	Genotypic characteristic*	Phenotypic characteristic†
1BF	*ica*(+)–*bap*(+)	Strong biofilm former
2BF	*ica*(+)–*bap*(-)	Weak biofilm former
3NF	*ica*(-)–*bap*(-)	Not biofilm former

* Genes amplified associated with biofilm formation. (+) amplified marker; (-) marker not amplified. †Capacity of biofilm formation on abiotic surfaces (microplates).

Bovine mammary epithelial cells

A clonal bovine mammary epithelial cells (MEC) (Yoder et al., 2019) were initially cultured into a T25 polystyrene culture flask (TrueLine, USA) using DMEM medium (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, USA), 5 μg/mL insulin (Sigma-Aldrich, USA), and 1 μg/mL hydrocortisone (Sigma-Aldrich, USA). The flask with MEC was incubated for five days at 37ºC with 5% CO^2, until to reach confluency.

After reviewing the purity of culture, the MEC monolayer was washed with sterile PBS (VWR, USA) and treated with 0.25% trypsin (AMRESCO, USA) until cells detached. Then, the MEC were resuspended in fresh DMEM medium and seeded (~$2X10^5$ cells/well) in 24-well polystyrene tissue culture-treated microtiter plates (TrueLine, USA). The plates were incubated for 24 h at 37ºC with 5% CO^2.

Cell infection

The confluent monolayer of MEC was co-cultured with each *S. aureus* strain at a multiplicity of infection (MOI) of 10:1 (Valle et al., 2012). Briefly, the bacterial strains were transferred from stock culture into Trypticase soy agar (TSA) (Oxoid, United Kingdom) and incubated at 37ºC overnight under aerobic conditions. The colonies were suspended in sterile distilled water until a turbidity comparable to 0.5 MacFarland scale (~10^8 CFU/mL) was reached. This suspension was diluted 1:10 in DMEM medium to reach a bacterial concentration of approximately 10^7 CFU/mL. Then, 100 µL from the

diluted suspension was aliquoted in each well that contained the MEC monolayer. Likewise, the same strain was only cultured with DMEM in absence of MEC (control on abiotic surface – CAS). Two plates were prepared, one for each time of assay (12 and 24 h). The plates were incubated for 12 h and 24 h at 37ºC with 5% CO^2. Experiments were performed in duplicates and were repeated two times.

Proteins extraction

Once the incubation times were completed, the culture medium was removed and discarded. The proteins were extracted using RNA/Protein Purification Plus Kit (Norgen, Canada) according to protocol for Gram-positive bacteria with some modifications. Briefly, the MEC monolayer was detached following the manufacturer recommendations for cultured animal cells. This material was transferred to a tube and bacteria were lysate according to protocol. Finally, the genomic DNA was removed, and total proteins were isolated.

Protein identification by nano–LC–MS/MS

Samples were analyzed by Creative Proteomics (USA) using an Ultimate 3000 nano UHPLC system coupled with Q Exactitive HF mass spectrometer (Thermo Fisher Scientific, USA) with Nanospray Ion Source.

Samples (5 μL) were loaded onto a nanocolumn: rapping column (PepMap C18, 100Å, 100 μm x 2 cm, 5 μm) and an analytical column (PepMap C18, 100Å, 75 μm x 50 cm, 2 μm) at a flow rate of 250 nL/min. Peptides were separated using a linear gradient from 2 to 8% buffer B (80% ACN, 0.1% formic acid) in 3 min, from 8% to 20% buffer B in 50 min, from 20% to 40% buffer B in 26 min, then from 40% to 90% buffer B in 4 min.

The peptides were subjected to nanospray ionization (2.2 kV) followed by tandem mass spectrometry (MS/MS). Full scan spectra were measured between *m/z* 300 – 1.800 at a resolution of 70.000 at 400 *m/z*. Peptides were selected for MS/MS using collision-induced dissociation (CID) with a normalized collision energy of 40%. Data dependent

MS/MS allowed to obtain up to top 15 most intense peptide ions from the preview scan in the Orbitrap.

The raw file was searched using Maxquant (1.5.6.5) against bovine and *S. aureus* protein database from Uniprot. Peptides with a probability score < 0.01 were considered for protein identification.

Data analysis

The identified bacterial proteins from each biofilm former strain were contrasted with the proteins identified from not biofilm former strain (3NF) and with the results obtained from the same strain cultured without MEC (CAS) in order to determine the proteins that were only expressed during the interaction with MEC. Those proteins found in the three lists were removed and only the identified proteins from biofilm producer strains co-cultured with MEC were selected. These analyses were performed individually for each time evaluated. Finally, selected proteins by each biofilm-forming strain were compared between them to find common or unique proteins.

Once the common or unique proteins were recognized between the biofilm-forming strains, these were submitted to bioinformatic analysis using the websites SCRATCH Protein Predictor (http://scratch.proteomics.ics.uci.edu/) and UniProt (https://www.uniprot.org/) in order to assess their sequence, antigenicity, function, and subcellular location. Additionally, it was verified by literature review if these proteins were involved in the biofilm formation process.

Statistical analysis

Due to the lack of normality of the outcome variable (number of proteins produced), a non-parametric analysis was performed using the Kruskal-Wallis test. Comparing the number of proteins and the given groups formed by genotype and time. A statistical

significance threshold of $P < 0.05$ was used to assess the overall differences between groups.

Results

Proteins identified in the biofilm-forming strains during contact with MEC

Proteins that were only identified from the strains co-cultured with MEC but were not identified from strains cultured without MEC (CAS) are shown in Table S1 (Supplementary table 1).

The results showed that the 2BF isolate was expressed more proteins (33) during the first 12 h of contact with MEC, followed of the 1BF strain (20). The percentages of virulence factors expressed by 1BF and 2BF strains were similar at 12 h and 24 h. Comparable results were observed at 24 h, although in this time, the 1BF strain presented more proteins (48) than 2BF (Table 2). The Kruskal-Wallis analysis did not find significant differences among the number of proteins by genotype, times, and virulence factors ($P > 0.05$).

Table 2. Proteins number identified from biofilm-forming strains during contact with MEC.

Strain	Genotype	Time (h)	No. proteins identified in contact with MEC	No. virulence factors (%)
1BF	*ica*(+)–*bap*(+)	12	20	3 (15.0)
2BF	*ica*(+)–*bap*(-)	12	33	5 (15.2)
1BF	*ica*(+)–*bap*(+)	24	48	8 (16.7)
2BF	*ica*(+)–*bap*(-)	24	36	8 (22.2)

(+) amplified marker; (-) marker not amplified; No: number

Of the 53 proteins identified at 12 h between biofilm-forming strains (1BF and 2BF), only 6 (11.3%) were common between these. Whereas, 14 (26.4%) and 27 (50.9%) of the 53 proteins were exclusive of 1BF and 2BF strains, respectively. On the other hand, 14 (16.7%) of the 84 proteins identified at 24 h were common between both strains, 34 (40.1%) were unique of the 1BF strain and 22 (26.2%) of the 2BF strain (Fig. 1).

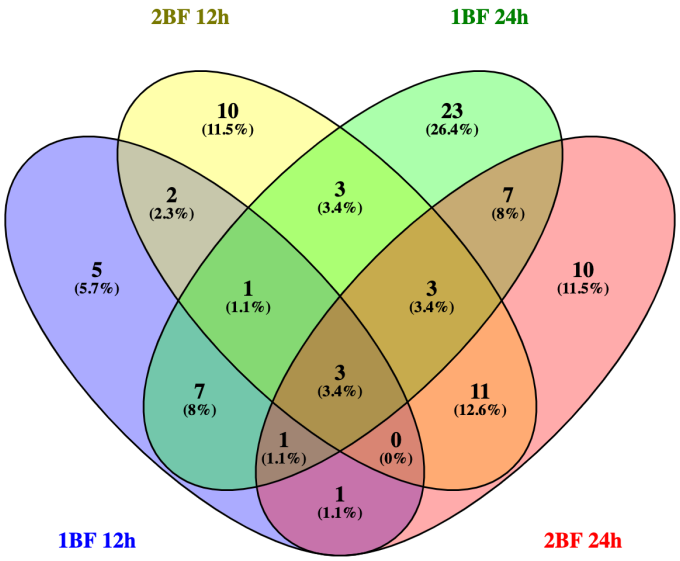

Figure. 1. Venn diagram. Each oval represents a strain by time evaluated. The numbers in intersections indicate common proteins between strains by time evaluated. The numbers outside of the intersections are unique proteins identified in each strain by time evaluated.

Virulence factors expressed only by biofilm-forming strains

Once selected the proteins expressed from two strains producer of biofilm during interaction with MEC, these were filtered again. In this phase, only the proteins recognized as virulence factors and expressed from biofilm-forming strains, but not expressed by the control (3NF strain), were selected (Table 3).

Table 3. Proteins only identified from biofilm-forming strains by time evaluated.

Id protein	Protein	Strain		Time	
		1BF	2BF	12h	24h
Q99UX4	Iron-regulated surface determinant protein A	X	X	X	X
Q99SN8	Uncharacterized leukocidin-like protein 1	X	X	X	X
Q99QS1	Protein map/Extracellular adherence protein (Eap)	X	X		X
Q5HEI1	Phospholipase C	X		X	X
Q99W46	Serine-aspartate repeat-containing protein E	X		X	X
Q8NVW1	Signal transduction protein TRAP	X			X
Q6GHV7	Iron-regulated surface determinant protein B	X			X
Q8NVL8	Uncharacterized leukocidin-like protein 2		X	X	X
Q6GE14	Gamma-hemolysin component A		X	X	X
Q6GIL4	Enolase		X	X	X
Q931F4	Immunoglobulin-binding protein sbi		X		X
Q2G015	Clumping factor A		X		X
O86488	Serine-aspartate repeat-containing protein D		X		X

Id: Uniprot identification; X: presence

Two of the three proteins identified in both strains were present from 12 h of contact with MEC. Similar results were observed with three of the six proteins detected in 2BF strain

and two of the four identified in 1BF strain, which were recognized from 12 h of contact. The rest of proteins were only detected at 24 h (Table 3).

Proteins identified associated with the biofilm formation

According to literature reviewed and the information obtained from the Uniprot website, six of the 13 (46.2%) proteins selected have been associated with the biofilm formation (Table 4). Two of the three proteins expressed in both strains, Iron-regulated surface determinant protein A (IsdA) and Protein map (Map), also referred as Extracellular adherence protein (Eap), were involved in the biofilm formation process, since these have the ability to bind to different molecules and to form biofilms under iron–limiting conditions (in the following part of the article, the Map is only referred to as Eap).

Enolase, Clumping factor A (ClfA), and Serine-aspartate repeat-containing protein D (SdrD) were identified as proteins related to biofilms. These were only detected in the 2BF strain and play an important role during the process of adhesion and aggregation of the bacterial cells.

Regarding to the proteins detected only in the 1BF strain associated with biofilm, the signal transduction protein TRAP acts as an activator of the Agr system, which regulates the expression of some genes necessary for biofilm formation.

Table 4. Main characteristics of the identified proteins involved in the biofilm formation process.

Protein (Strain)	**Gen**	**Length (Da)**	**Subcellular location**	**Key role in biofilm formation process**	**Predicted antigenicity**
IsdA (1BF-	*isdA*	350 (38,756)	Cell wall	Fibronectin and fibrinogen binding	0.94

2BF)				Biofilm formation under iron-limiting conditions	
Map/Eap (1BF-2BF)	*map/eap*	476 (53,377)	Secreted Extracellular surface	Fibronectin, fibrinogen, and eDNA binding Biofilm formation under iron-limiting and normal conditions	0.66
TRAP (1BF)	*traP*	167 (19,563)	Membrane	Regulator of the expression of some genes involved in biofilm formation	0.86
Enolase (2BF)	*eno*	434 (47,117)	Cytoplasm Secreted Cell surface	Laminin and eDNA binding	0.37
ClfA (2BF)	*clfA*	927 (96,448)	Cell wall	Fibrinogen, AnnexinA2, and fibrin binding	0.94
SdrD (2BF)	*sdrD*	1,315 (142,776)	Cell wall	Extracellular matrix adhesion	0.98

1893

Discussion

Bacterial biofilms have become in an important subject of study in both the medical and industrial areas, because biofilm gives the bacteria high ability to evade the host immune system and resist the action of antibiotics and disinfectants. *S. aureus* IMI have been characterized by their chronicity, which biofilms are one of main virulence factor associated with this course of infection.

The initial phase in the biofilm formation process is the attachment on an abiotic or biotic surface (Moormeier & Bayles, 2017). There is not biofilm without adequate and strong adhesion to surfaces, since non-adhered bacteria could be removed from the host (Gong et al., 2010). Several studies that blocked or eliminated (mutants) some proteins involved in the initial adherence of *S. aureus* to the cell matrix, showed a reduction or avoidance of the biofilm formation, which demonstrated the importance of these molecules in the development of biofilm matrix (Cheung et al., 2009; Clarke et al., 2004; Gong et al., 2010). *S. aureus* attaches the surface using different proteins that belong to a well-characterized group of proteins known as microbial surface components recognizing adhesive matrix molecules (MSCRAMM), which are part of cell wall-anchored (CWA) proteins (Foster et al., 2014). Several of these proteins share a common motif (LPXTG). However, they have different binding target on the host cell matrix (Moormeier & Bayles, 2017). Some of the main proteins described that have been involved in the initial adherence to biotic surfaces and the subsequent biofilm formation are Fibronectin-binding proteins (FnBPA and FnBPB), Clumping factors (ClfA and ClfB), Serine-aspartate repeat family proteins (SdrC, SdrD, and SdrE), Iron-regulated surface determinants (IsdA, IsdB, IsdC, and IsdH), and biofilm associated protein (Bap) (Moormeier & Bayles, 2017).

IsdA and Eap were the two proteins identified in both biofilm-forming strains that have been associated with the development of biofilm (Lin et al., 2012). Both IsdA and Eap play an important role in adhesion process and in the biofilm formation in iron limited

environment. Iron is an essential component for bacterial growth and the biofilm formation, but its availability in the host is generally restricted (Hammer & Skaar, 2011). Therefore, bacteria can use different mechanisms to iron acquisition from environment, being the cell invasion (greater than 90% of the iron is located intracellularly) and the siderophores production some of these (Hammer & Skaar, 2011; Lin et al., 2012). Other of the way reported that *S. aureus* use to capture iron is the Isd system, which is formed by a group of proteins encoded by *isd* operon (eight genes). Among these proteins is IsdA, a surface receptor that participates in the acquisition of iron, especially when its availability is limited (Maresso & Schneewind, 2006).

The culture media used in this work presented iron-deplete conditions, something similar to what is found in the cow`s milk. Several studies have shown that *S. aureus* expresses IsdA in the presence of the iron chelating agent or when iron availability is limited. For example, Clarke, Wiltshire, & Foster (2004) found evidence that related the IsdA expression under iron-limited conditions. Likewise, Lin et al. (2012) also observed the increase on the expression of this protein in the presence of the iron chelating agent 1,2,3,4,6-penta-O-galloyl-b-D-glucopyranose (PGG). Although, the results of our study are in agreement with those observed by these authors, presence of IsdA when iron availability was limited, these were different because the protein was not identified in the strains cultured without MEC (abiotic surface with only culture medium). This was only found in the biofilm producing strains cultured with MEC (biotic surface). The results could indicate that the main function of this protein under the assay conditions used was the adhesion to the surface of the MEC.

IsdA protein has also been involved with the adhesion process of *S. aureus* to the host cells (Moormeier & Bayles, 2017). Fibronectin and fibrinogen are some of the proteins to which IsdA can bind during the initial stage of the colonization (Clarke et al., 2004). Lei, Gupta, & Lee (2017) reported the presence of IsdA in the surfactome of the *S. aureus* strain evaluated during the acute phase of infection of bone implants used in a rat

model. According to the results obtained in our study, this protein was expressed by biofilm forming strains from 12 h after of MEC inoculation, confirming its participation in the early stage of the infection. On the other hand, studies that achieved to inactivate *isdA* gene or block the protein (monoclonal antibodies) observed a decrease in the ability of *S. aureus* to colonize the host tissue (Bennett et al., 2019; Cheung et al., 2009; Clarke et al., 2004; Hammer & Skaar, 2011).

Regarding to Eap, other of the proteins identified in the biofilm-forming strains, has also been reported with a dual function (Yonemoto et al., 2019). Like IsdA, Eap is involved in the process of adhesion and biofilm formation, but differs from this, because Eap belong to the group of the secretable expanded repertoire adhesive molecules (SERAM) and not to the group of MSCRAMM (Geraci et al., 2017). This kind of proteins are secreted and after re-bind to the bacterial cell surface (Geraci et al., 2017; Thompson et al., 2010). Released Eap can bind to a number of host cell matrix components such as fibronectin, as well as to plasma proteins (fibrinogen) and extracellular DNA (eDNA) (Eisenbeis et al., 2018; Thompson et al., 2010).

Reports have suggested that Eap plays an important role in later infection process due lack of expression in the stationary growth phase (Geraci et al., 2017; Hussain et al., 2001). We found Eap at 24 h of contact with MEC, suggesting its participation from initial stage of infection. Our results are similar to those studies that have demonstrated the importance of this protein in the biofilm development, since this structure could not be formed without an adequate adhesion process from the early phase. Johnson, Cockayne, & Morrissey (2008) and Thompson et al. (2010) achieved a reduction of biofilm formation when inactivated the *eap* gene. Likewise, Yonemoto et al. (2019) recently reported the role of Eap in the biofilm formation by methicillin-resistant isolates, founding a large amount of this protein in the bacterial extracellular matrix. Unlike IsdA, Eap can also be found in the biofilm development under iron-replete conditions (Thompson et al., 2010).

Signal transduction protein TRAP was the protein detected in the 1BF strain, which has participation in the development of biofilm by *S. aureus*. TRAP is a major regulator of pathogenesis in *S. aureus*, because its phosphorylation leads to the activation Agr system, which mediates up-regulation the expression of virulence factor and genes associated to biofilm formation (Gilot et al., 2002; UniProt Consortium, 2019). Several studies have concluded that mutations or inhibitions of *traP* locus can limit induction of *agr*, and consequently reducing the capacity to form biofilm (Tsang et al., 2007). Moreover, Tsang et al. (2007) reported that the mutations performed on this gene from *S. aureus* strains did not affect expression of *agr* or the ability to produce biofilm. The fact of have found this molecule only in the 1BF strain, and not in the 2BF (weak biofilm former) and 3NF (non-biofilm former) strains, could suggest its importance in the ability to form a strong biofilm.

Enolase was one of proteins identified in the 2BF strain that has been associated with biofilm formation. This protein is encoded by the *eno* gene, which functions as a glycolytic enzyme within the cytoplasm, but can also be released from the cell and attached at the surface of the bacterial wall (Foulston et al., 2014). Enolase has the capacity of bind to laminin, mediating the attach to the extracellular matrix during the early phase of infection (Carneiro et al., 2004; Kot et al., 2018). Several works have demonstrated the participation of enolase in the development of the matrix biofilm, since this protein is expressed from the first hours of the colonization. In our study, enolase was found in the biofilm forming strain from 12 h, but was not identified in the controls (3NF and CAS), agreeing with the results presented by Kot et al. (2018), who found a significant increase in the expression level of *eno* gene from biofilm-forming strains compared to planktonic cells. The gene was expressed from 3 h under biofilm conditions used, achieving the highest levels at 6 and 8 h. The authors proposed that the significantly higher transcript level of *eno* gene in the first hour of growth of biofilm producer strains suggest that this molecule is important for the first stage of biofilm formation, because bacterial cells interact with host extracellular ligand. Yoshii et al.

(2017) also demonstrated the important role of enolase in the biofilm formation process, since they achieved to inhibit the staphylococcal biofilm formation using Norgestimate (acetylated progestin). This product repressed the expression of enolase protein in the extracellular matrix compared to the control.

The second protein associated with biofilm formation found in the 2BF strain was ClfA. This protein is an important adhesin, usually covalently anchored to the bacterial cell wall, belonging to the MSCRAMM group and encoded by the *clfa* gen (Gong et al., 2010). Although, ClfA protein generally has been associated with the binding to plasmatic fibrinogen, a study showed that this molecule also has a direct mechanism of binding to the MEC in the absence of fibrinogen (Ashraf et al., 2017). The authors confirmed that ClfA interacted with AnnexinA2, a phospholipid-binding protein found on the surface of MEC. It has described that most of the isolates express ClfA and that this participates in the initial stage of the infection (Lacey et al., 2017; Zecconi & Scali, 2013). However, in this study, ClfA protein was only present at 24 h and was no expressed by all strains evaluated, since it was not identified in the 1BF and 3NF strains under the assay conditions used.

Regarding to the role that ClfA plays in the biofilm formation process, Zapotoczna et al. (2015) informed about a biofilm phenotype mediated by coagulase and ClfA, since the latter can bind to fibrin formed by coagulase or fibrinogen available in plasma, leading to an immediate clumping of bacterial cells (Zapotoczna et al., 2016).

The SdrD protein is another of the molecules that is part of the MSCRAMM group, which is encoded by the *sdrD* gene located within the *sdr* locus (Josefsson et al., 1998). This protein plays an important role in the bacterial adhesion to host tissues and the subsequent biofilm formation (Ma et al., 2012). Both Ma et al. (2012) and Vaishampayan et al. (2018) observed in their works a down-regulation of *sdrD* gene and a reduction in the biofilm formation when the strains analyzed were in contact with the evaluated products. Askarian et al. (2017) carried out another study where demonstrated its

potential pathogenic. They reported that SdrD contributed to *S. aureus* survival and the ability to escape the innate immune system in human blood. Some studies have shown that all strains are no carriers of the *sdrD* gene, being found in about 60% of the isolates tested (Liu et al., 2015; Sohail & Latif, 2018). We only identified this protein in the 2BF, which is agree with those results. However, since this gene was not tested in the 3NF isolate, it is not possible assure that this were not carriers of *sdrD*.

The predicted antigenicity of the identified proteins associated with the development of biofilm ranged between 37% to 98%. SdrD presented the highest antigenicity (98%), followed by IsdA and ClfA (94%), TRAP (86%), Eap (66%), and enolase (37%). Stranger-Jones et al. (2006) showed the antigenic potential of SdrD and IsdA, since they generated significant protective immunity in a murine model against lethal challenge with human clinical isolates of *S. aureus*. These animals were immunized with a vaccine composed of four antigens (IsdA, IsdB, SdrD, and SdrE). In addition, Bennett et al. (2019) evidenced a reduction of bacterial burden in a murine septic model after of the injection of monoclonal antibodies against IsdA.

Several studies have proposed the ClfA protein as a good vaccine candidate, because it is an important virulence factor, most of bacterial strains are gene carriers, and show a high predicted antigenicity, such as was reported in this study (94%). According to published by Camussone et al. (2014) the vaccine composed of lysed cell of a CP5 *S. aureus* supplemented with different recombinant antigens, among these ClfA, elicited antibodies that promoted neutrophil phagocytosis and inhibited internalization into MEC. Similar results were shown by Nour El-Din et al. (2006), who immunized cows with a DNA vaccine against ClfA and observed a decrease in adherence of *S. aureus* to MEC. In addition, a recent work also demonstrated that this protein is a potent human T cell activator, which could be crucial for the generation of vaccines anti-*S. aureus* (Lacey et al., 2017).

Despite TRAP presented a predicted antigenicity of 86%, we did not find works related to this characteristic, probably because this is an intracellular protein. Regarding Eap, which achieved an antigenicity of 66%, Joost et al. (2011) reported significant differences between antibodies titers against Eap from infected and healthy individuals, finding the highest titers in infected patients than controls. On the other hand, enolase was that showed the lowest predicted antigenicity (37%). This result could be explained because enolase present an identity around 50% with bovine enolase, which are enzymes essentials for the degradation of carbohydrates.

Conclusions

This study identified proteins involved in biofilm production that were only expressed during interaction with MEC and were not found in the not biofilm formation strain, demonstrating their importance in the infectious process and, probably, in the subsequent *in vivo* biofilm formation. Despite of evaluating three genotypically and phenotypically different strains, not significant differences were found amongst different attributes (genotype, times, number of proteins and virulence factors).

Furthermore, the results of our study confirmed that SdrD, IsdA and ClfA proteins are good candidates for vaccines and could be used as immunogens in immunoassays, since these are involved in the early stage of the infection, in the biofilm formation and have the ability of elicit an immune response.

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Resultados del objetivo 3

Objetivo 3: Determinar anticuerpos en muestras de leche y sangre frente a las proteínas recombinantes de interés.

Para cumplir con este objetivo se ejecutaron las tres actividades que se describen a continuación:

1. Se sintetizaron de forma recombinante las tres proteínas seleccionadas según los resultados del objetivo 2.
2. Se tomaron muestras de leche y sangre de animales positivos y negativos a *S. aureus*.
3. Se evaluaron todas las muestras frente a las proteínas recombinantes mediante una prueba de ELISA.

Los resultados de todas las actividades relacionadas se encuentran en el artículo 5 titulado "**Utility of an indirect ELISA test based in recombinants proteins IsdA, ClfA and SdrD of *Staphylococcus aureus* to detect bovine intramammary infections**".

Artículo 5

Utility of an indirect ELISA test based in recombinants proteins IsdA, ClfA and SdrD of *Staphylococcus aureus* to detect bovine intramammary infections

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Abstract

Fast and accurate diagnosis is one of the key strategies in the successful control of intramammary infections caused by *Staphylococcus aureus*. Among the diagnostic tools that have been proposed for the detection of *S. aureus* infection are the immunoassays, because these offer advantage in terms of costs, fast, and ease compared to other diagnostic tools. However, the main challenge of this type of test is to identify immunogens that allow to accurately discriminate between infected and uninfected cows with *S. aureus*, since this bacterium can colonize naturally different sites of animal body. Therefore, the aim was to detect antibodies in bovine samples against three *S. aureus* proteins (IsdA, ClfA, SdrD) involved in biofilm formation. Ninety-eight cows in lactation and not vaccinated were included. Forty-eight of these cows were infected with *S. aureus*, while the rest (48 cows) were uninfected. Blood and milk samples were taken from each animal to collect serum. IgG titers against the three proteins and a mixture of them were measured in each sample using an ELISA test. The result showed significant

differences in the IgG response against proteins evaluated, highlighting the antigenic potential of IsdA and demonstrating that some antigens can be better indicators of infection than others. According to ROC curves analysis, the protein mixture was the one that showed the greatest capacity (sensitivity of 79% and specificity of 77%) to differentiate between infected and uninfected cows when the blood samples were used. In addition, this mixture also showed the highest specificity (94%) using milk samples, suggesting its possible use as a diagnostic complement to somatic cell counts. Our finding will contribute to the knowledge about the identification of potential antigens and their possible usefulness as indicator of infections in diagnostic test or as vaccine targets.

Keywords: Biofilm; ELISA; IgG; Immunoassays; Mastitis; Proteins; *Staphylococcus aureus*.

Introduction

Bovine mastitis caused by *Staphylococcus aureus* is a common disease, and is characterized by the onset of chronic and subclinical intramammary infections (IMI) (Keefe, 2012). These infections can impact animal welfare and also lead to economic losses for farmers (Halasa et al., 2007; Hernández-Castellano et al., 2017).

The ability of *S. aureus* to form biofilms is one of the key mechanisms of pathogenesis, conferring protection against antibiotics and the host's immune response (Gomes et al., 2016). Therefore, this structure will allow bacteria to persist in the mammary gland and a potential pathogen reservoir (Cucarella et al., 2004). Several studies have shown that the biofilm formation process is a diverse and complex process, which involves different proteins belonging to a group known as microbial surface components recognizing

adhesive matrix molecules (MSCRAMM) (McCarthy et al., 2015; Zapotoczna et al., 2016). Some of the proteins in this group that have been involved in the initial adherence and the subsequent biofilm formation are Fibronectin-binding proteins (FnBPA and FnBPB), Clumping factors (ClfA and ClfB), Serine-aspartate repeat family proteins (SdrC, SdrD, and SdrE), Iron-regulated surface determinants (IsdA, IsdB, IsdC, and IsdH), and biofilm associated protein (Bap) (Moormeier & Bayles, 2017). In addition, several studies have also shown the immunogenic potential (vaccines) of different MSCRAMM and their usefulness as targets for screening tests (Bennett et al., 2019; Stranger-Jones et al., 2006).

Early detection of *S. aureus* is crucial to optimize treatments, segregate infected animals, and therefore, interrupt the pathogen transmission`s cycle (Hernández-Castellano et al., 2017). Mastitis diagnosis is regularly performed using somatic cell counts (SCC) and bacteriological culture (Rainard et al., 2018). However, a recent study showed that a number significative (30.8%) of cows could remain undetected due to the low SCC values that can have the infected animals with this pathogen (Petzer et al., 2017). On the other hand, culture methods could be complex, time consuming, and with variable sensitivity (Rainard et al., 2018). Hence, the interest in finding efficient diagnostic tools that allow to improve the pathogen detection process or even complement the conventional methods.

The immunogenic potential of some of the proteins expressed during biofilm production could be used for diagnostic purposes, since evidence has shown that infected patients can generate higher antibody titers against these proteins than healthy individuals (den Reijer et al., 2017; Joost et al., 2011). Therefore, the objective of this study was to evaluate three *S. aureus* proteins (IsdA, ClfA, SdrD) involved in biofilm formation as indicators of intramammary infections.

Materials and methods

Animals

Ninety-eight cows in lactation and not vaccinated against *S. aureus* from dairy herds located in eight municipalities of the Department of Antioquia (Colombia) were included in this study. Forty-eight of these cows were infected with *S. aureus*, while the rest (48 cows) were non-infected with this bacterium (non-*S. aureus* control). Regarding uninfected, 16 were infected with other pathogens and 32 did not present IMI (Table 1). A cow was considered infected if a sample had ≥ 10 CFU/10 µl. On the other hand, animals without IMI were those that had three negative results of bacteriological culture from independent samples taken weekly. Bacteriological culture was performed based on standard protocols recommended by National Mastitis Council (National Mastitis Council, 2004).

Table 1. Cows included in the study infected with a pathogen different to *S. aureus.*

No. of cows included infected with a pathogen different to *S. aureus*	**Pathogen isolated**
6	Non-aureus *Staphylococcus* spp.
3	*Streptococcus agalactiae*
4	*Streptococcus uberis*
3	*Escherichia coli*

No: number

Sampling and serum collection

Blood (coccygeal vein) and milk samples (morning milking) were aseptically collected from cows using standard procedures. Both blood and milk were centrifuged for 10 min at 3,000 *g* to collect serum. All samples were stored at -20 until use.

The procedures used in this study were approved by the Ethics Committee for Animal Experimentation at the University of Antioquia (Medellín, Colombia).

Staphylococcus aureus molecular typing

The isolates recovered from sampled cows, which initially were identified as *S. aureus* through conventional bacteriological methods, were confirmed by amplification of *nuc* gene using the Polymerase chain reaction (PCR) protocol described by Fournier et al. (2008).

Once confirmed the isolates as *S. aureus*, two different PCRs were carried out to detect the *ica* and *bap* genes, following the conditions published by Cucarella et al. (2004) and Torres et al. (2019). These genes have been described as being involved in the biofilm formation process (Cucarella et al., 2004). *S. aureus* strain V329 was used as positive control, since this strain harbors both markers.

Recombinant proteins of S. aureus

Proteins included were selected based on the results obtained in the objective number two from doctoral thesis. The criteria taken into account to select the proteins were: expression by *S. aureus* strains evaluated; involvement with biofilm formation process; display on the cell wall; and exhibit a predicted antigenicity higher than 90% calculated in the websites SCRATCH Protein Predictor (http://scratch.proteomics.ics.uci.edu/). Using these criteria, IsdA, ClfA, and SdrD proteins were chosen.

The three recombinant proteins were generated by CUSABIO (www.cusabio.com) under the protocols of expression and purification established for them. The expression system used to IsdA and SdrD were *Escherichia coli*; whereas in ClfA was a yeast. Both ClfA (AA 229–559) and SdrD (AA 36–330) were generated partially in comparison with mature proteins. Instead, IsdA was produced complete (full length). Purity obtained for these proteins were greater than 85% and the predicted antigenicity were higher than 89%.

Immunoassay

Bovine IgG levels against the three recombinant proteins (RP) were measured in the serum samples through the indirect Enzyme-linked immunoSorbent assay (ELISA). Initially, the ELISA was standardized using different concentrations of each RP and sample dilutions to determine the best assay conditions. The RP concentrations tested per well were 0.1 and 0.5 µg; whereas serum dilutions were 1:100, 1:200, and 1:500. The RP concentrations and sample dilution that showed the best results were 0.1 µg for IsdA and 0.5 µg for the other two proteins, ClfA and SdrD. Regarding sample dilutions, this was 1:100.

Once the assay conditions were defined, each RP was diluted using coating buffer (4.42 g of Na_2CO_3 and 5.04 g of $NaHCO_3$ in a liter of distilled water, pH 9.6) until to reach the concentration established. Then, 100 µl from each diluted protein was dispensed into 96-well plates (Greiner Bio-One, USA) and incubated at 4 ºC overnight. At the same time, a protein mixture was prepared based on the defined concentrations. We prepared two plate for each protein and mixture tested, one per each sample type (blood and milk serum). The next day, the wells were washed four times with Tris-buffered saline (50 mM Tris-Cl, 150 mM NaCl in a liter of distilled water, pH 7.6) with Tween 20 at 0.05% (TBS-T). After washing, the plates were blocked using 200 µl of blocking buffer (TBS with 5% non-fat milk) and incubated at 37 ºC for 1 h. Plates were washed again as mentioned previously. Next, 50 µl of each serum samples, diluted 1:100 in blocking buffer, were dispensed to each well and incubated at 37 ºC for 1 h. Completion of incubation, plates were washed four times with TBS-T to remove of excess antibodies. After 50 µl the anti-bovine IgG (Sigma-Aldrich, USA) was added at wells, which was previously diluted 1:5000 in blocking buffer. Plates were incubated again at 37 ºC for 1 h. Finally, a fourth wash was performed and subsequently 100 µl from a substrate solution (1-Step™ Turbo TMB-ELISA; ThermoFisher Scientific, USA) was dispensed according to protocol suggested by manufacturer. The reaction was stopped by the addition of 100 µl of 1 M sulfuric acid, and absorbance was read at 450 nm in a plate

reader for ELISA (Bio-Rad, USA). Antibody levels were expressed as optical density (OD). Experiments were performed in duplicate and repeated two times.

Statistical analysis

Descriptive analysis was performed for all the variables of interest. Normality assessment of the outcome variable was performed. Due to the lack of normality of the outcome variable (optical densities) obtained from infected and uninfected animals, a non-parametric analysis was performed using the Kruskal-Wallis test and non parametric multiple comparisons (Wilcoxon test). A statistical significance threshold of $P < 0.05$ was used to assess the overall differences between groups.

The diagnostic accuracy (sensitivity and specificity) was evaluated using receiver operating characteristic (ROC) curves. These curves were plotted, and area under curve (AUC) was used to determine the capacity of test to discriminate between animals infected and uninfected with *S. aureus*. ROC curves were performed using R package "pROC" (version 1.15.3).

Results

Genotypes of S. aureus strains isolated from infected cows

The strains evaluated were confirmed as *S. aureus*, since the *nuc* gene was amplified in all of these.

The PCR analysis for the two markers associated with the biofilm formation evaluated, showed the *ica* locus amplification in 42 (87.5%) strains, and 10 (20.8%) of these also carried the *bap* gene. On the other hands, in six (12.5%) isolates none of the markers were amplified. According to these results, we classified the strains into three genotypes as follows: genotype 1 those strains that only harbored *ica* locus, genotype 2 harboring

both loci, and genotype 0 for strains where the none of the markers were amplified (Table 2).

Table 2. Genotypes identified in *S. aureus* isolates.

Genotype	Markers	No. Of strains (%)
1	*ica*(+)–*bap*(-)	32 (66.7)
2	*ica*(+)–*bap*(+)	10 (20.8)
0	*ica*(-)–*bap*(-)	6 (12.5)
	Total	**48 (100)**

(+) amplified marker; (-) marker not amplified

Characterization of protein-specific IgG levels in blood and milk serum samples

The IgG concentration showed a wide variation of values (OD) according to the protein evaluated (IsdA, ClfA, and SdrD), cows status (infected and uninfected), and sample type (blood and milk serum). The values of OD ≤ 0.06 (Blank) were interpreted as the absence of specific antibodies. The levels of total IgG are summarized in Table 3.

Table 3. IgG levels against the proteins evaluated by sample type and cows status.

Protein	Sample	Cows status	Min. OD value	Mean OD value	Median OD value	Max. OD value
IsdA	Blood serum	Infected	**0.3110**	**0.5826**	**0.6125**	**0.7150**
		Uninfected	0.1690	0.4885	0.5170	0.7010
	Milk serum	Infected	0.0511	0.0887	0.0730	0.2370
		Uninfected	0.0520	0.0633	0.0590	0.1000
ClfA	Blood	Infected	0.0155	0.2705	0.2445	0.6100

	serum	Uninfected	0.0019	0.1869	0.1310	0.6920
	Milk	Infected	0.0520	0.0678	0.0628	0.1575
	serum	Uninfected	0.0565	0.0716	0.0710	0.1240
SdrD	Blood	Infected	0.0570	0.2051	0.1760	0.4895
	serum	Uninfected	0.0035	0.1898	0.1625	0.4495
	Milk	Infected	0.0445	0.0557	0.0548	0.0780
	serum	Uninfected	0.0535	0.0677	0.0678	0.1025
Mix	Blood	Infected	0.1270	0.4590	0.4855	0.6240
	serum	Uninfected	0.0960	0.2745	0.2665	0.5390
	Milk	Infected	0.0510	0.0768	0.0665	0.2100
	serum	Uninfected	0.0510	0.0568	0.0560	0.0700

Min: minimum; Max: maximum; Mix: assay performed with a mixture of the three proteins; OD: optical density. The bold number highlight the best results obtained.

According to these results, the levels of IgG in blood serum against IsdA were higher compared to other proteins and mixture of them, especially those found in the infected animals. Whereas, the milk serum concentrations were similar among assays performed.

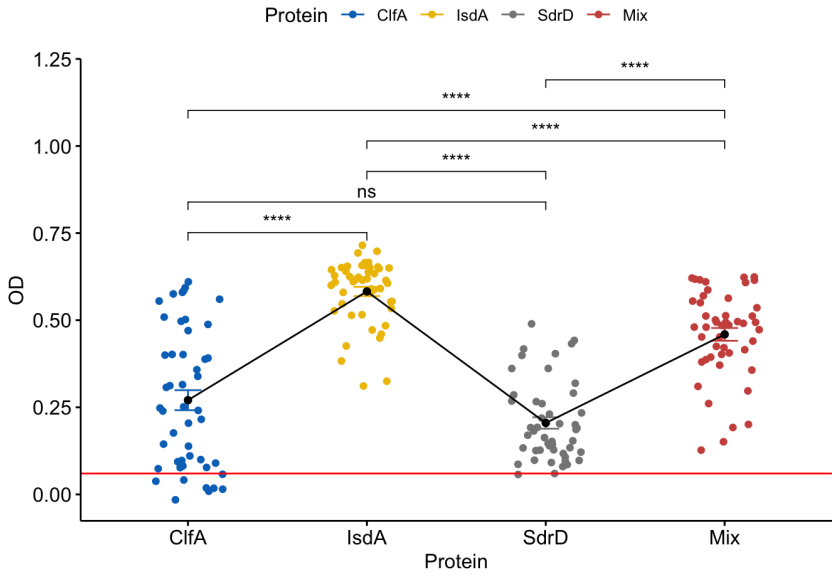
Differences in the IgG response according to protein evaluated, sample type and cows status

IgG levels in blood serum samples

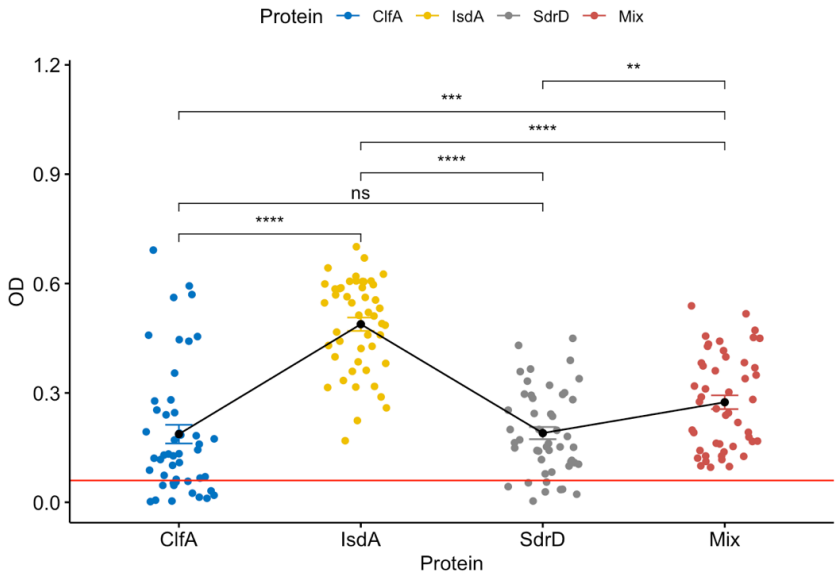
Among the infected animals a higher response against IsdA protein compared to the other proteins and mixture of them was observed. The Wilcoxon test analysis showed significant differences among the proteins tested, except between ClfA and SdrD proteins (Figure 1A). Amongst the uninfected animals, the results showed differences in the response for all proteins (Figure 1B).

When were compared the results between infected and uninfected animals for each assay, these presented differences for IsdA, ClfA, and the mixture, but not for SdrD. The highest differences between the two groups of animals were observed in the mixture and IsdA (*P* <0.0001) (Figure 2).

A



B



2520

2521 **Figure 1**. **A.** Total IgG response against the proteins identified in blood serum samples
2522 from infected animals (n = 48). **B**. Total IgG response against the proteins identified in
2523 blood serum samples from uninfected animals (n = 48). The blue points represent the
2524 optical densities (OD) obtained in each sample for ClfA protein, yellow points for IsdA,
2525 grey points for SdrD, and red points for the mixture of proteins. Asterisks indicate
2526 statistically significant differences among proteins (ns: *P* >0.05; * *P* <0.05; ** *P* <0.01; ***
2527 *P* <0.001; **** *P* <0.0001). Horizontal red line corresponds to the blank measurement
2528 (OD = 0.06).

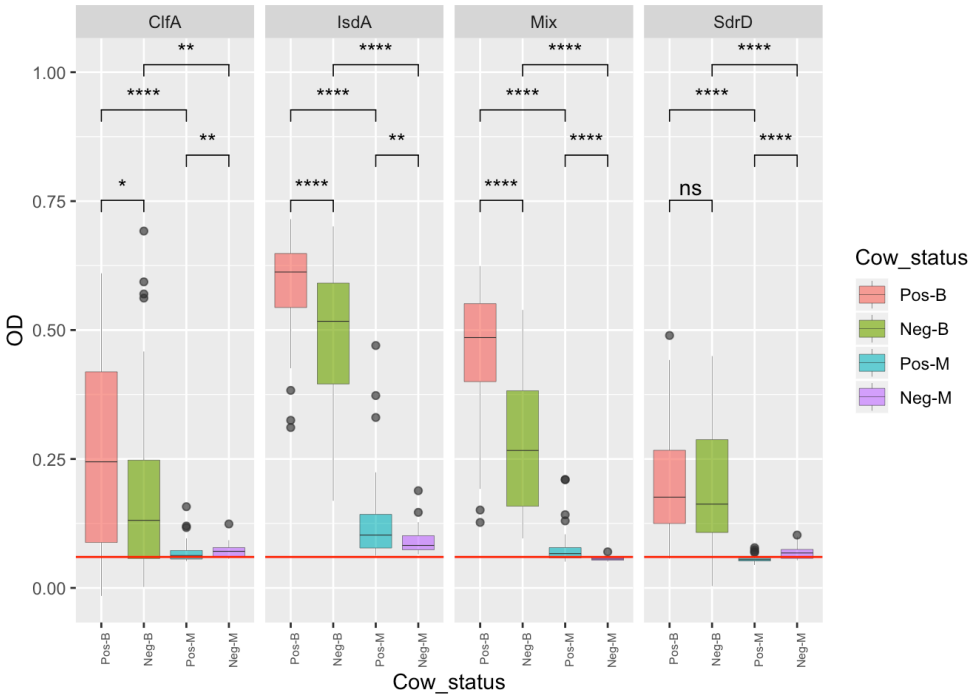


Figure 2. Total IgG response against the proteins evaluated (n = 48 infected and 48 uninfected). Pos-B correspond to the optical densities (OD) obtained in the blood samples collected from infected animals; Neg-B correspond to the OD obtained in the blood samples collected from uninfected animals; Pos-M correspond to the OD obtained in the milk samples collected from infected animals; Neg-M correspond to the OD obtained in the milk samples collected from uninfected animals. Asterisks indicate statistically significant differences among proteins (ns: P >0.05; * P <0.05; ** P <0.01; *** P <0.001; **** P <0.0001). Horizontal red line corresponds to the blank measurement (OD = 0.06).

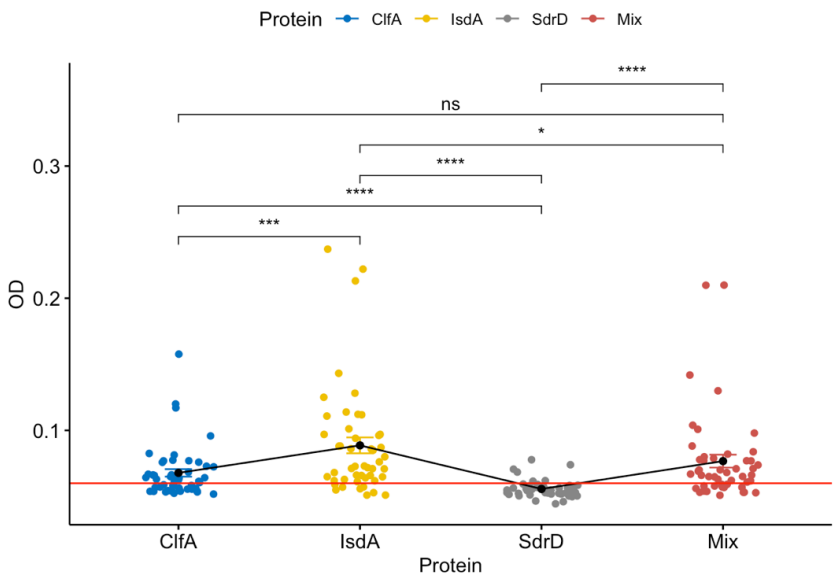
IgG levels in milk serum samples

The concentrations of IgG in milk were lowest with respect to those found in blood, but were significantly different among most of these in both infected and uninfected animals (Figure 3). The Kruskal-Wallis analysis did not show differences between the IgG titers

generated by infected cows for ClfA and the mixture of proteins (Figure 3A). Whereas in the uninfected, no differences were observed between IsdA and SdrD (Figure 3B).

The comparison of the response among infected and uninfected cows identified differences between these groups for all proteins and the mixture evaluated. As was observed in blood samples, the protein mixture was the one that showed the highest differences between infected and uninfected animals (*P* <0.0001). Although statistically significant differences of *P* <0.0001 also were observed in SdrD. The lowest IgG titers against SdrD in milk were observed in the cows infected (Figure 2).

A



B

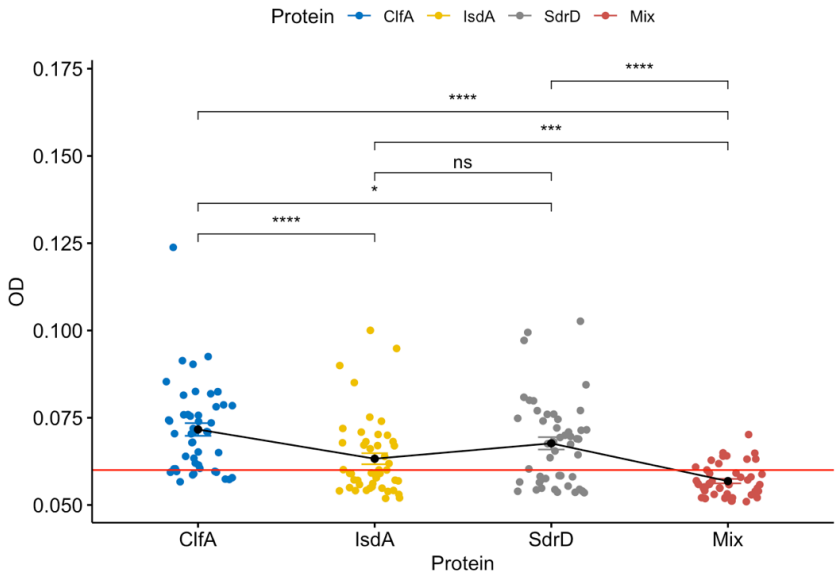
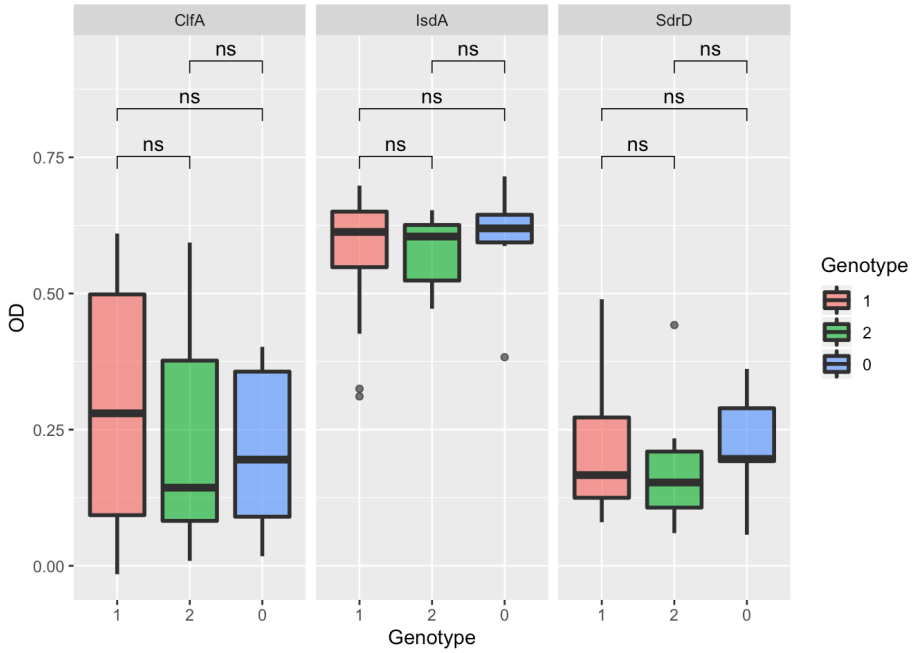


Figure 3. **A.** Total IgG response against the proteins identified in milk serum samples from infected animals (n = 48). **B**. Total IgG response against the proteins identified in milk serum samples from uninfected animals (n = 48). The blue points represent the optical densities (OD) obtained in each sample for ClfA protein, yellow points for IsdA, grey points for SdrD, and red points for the mix of proteins. Asterisks indicate statistically significant differences among proteins (ns: P >0.05; * P <0.05; ** P <0.01; *** P <0.001; **** P <0.0001). Horizontal red line corresponds to the blank measurement (OD = 0.06).

Total IgG titers versus genotype

According to the analysis carried out to determine the association between the IgG concentrations obtained and the identified genotypes, no statistically significant differences were found among the comparison performed for both the blood and milk serum samples (Figure 4).

A


ClfA
IsdA
SdrD
ns
OD
0.75
0.50
0.25
0.00
1
2
0
Genotype
Genotype
1
2
0

B

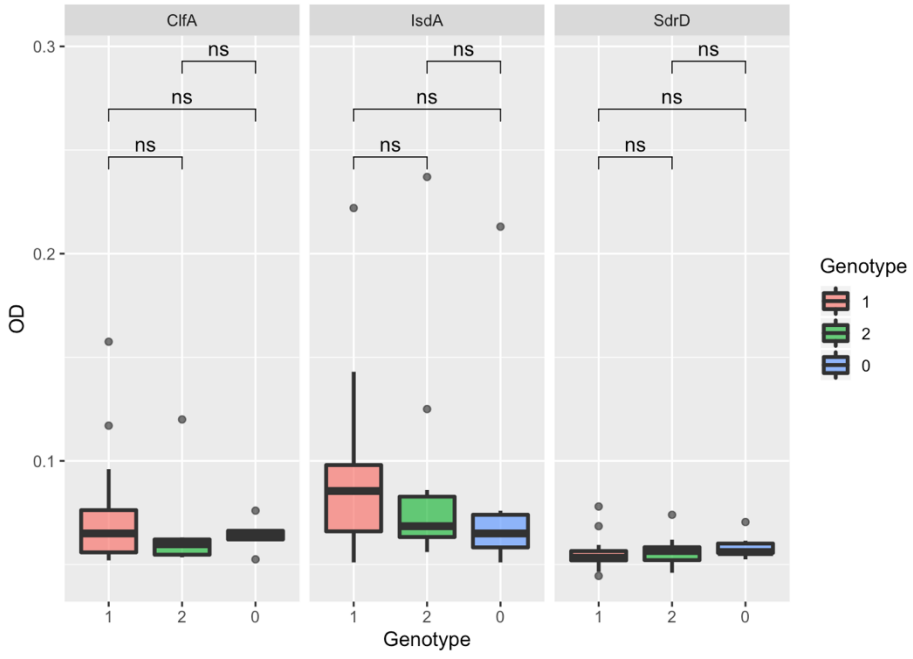

ClfA
IsdA
SdrD
ns
OD
0.3
0.2
0.1
1
2
0
Genotype
Genotype
1
2
0

Figure 4. **A.** Total IgG titers in blood serum versus genotype isolated. **B.** Total IgG titers in milk serum versus genotype isolated. The data are presented as optical densities (OD). The red box represents the genotype 1 (n = 32), green box to genotype 2 (n = 10), and blue box to genotype 0 (n = 6). The statistical analysis is interpreted as follow (ns: P >0.05; * P <0.05; ** P <0.01; *** P <0.001; **** P <0.0001).

Sensitivity and specificity analysis

The diagnostic use potential of each protein and their mixture were determined by sensitivity and specificity analysis using ROC curves estimation. The calculated thresholds, sensitivity and specificity for each protein are shown in the Table 4.

Table 4. Results obtained in the sensitivity and specificity analysis for each protein and sample type.

Protein	Sample	AUC	CI (95%)	Threshold (OD)	Sensitivity	Specificity
IsdA	Blood serum	0.74	0.63 – 0.83	0.61	54%	90%
	Milk serum	0.75	0.65 – 0.85	0.061	83%	60%
ClfA	Blood serum	0.61	0.50 – 0.73	0.2	58%	71%
	Milk serum	0.67	0.55 – 0.77	0.068	71%	60%
SdrD	Blood serum	0.54	0.42 – 0.65	0.056	100%	17%
	Milk serum	0.79	0.72 – 0.86	0.063	92%	60%
Mix	Blood	**0.82**	**0.72 – 0.88**	**0.385**	**79%**	**77%**

	serum					
	Milk serum	**0.84**	**0.76 – 0.91**	**0.065**	**60%**	**94%**

AUC; area under the curve; CI: confidence interval; Mix: assay performed with a mixture of the three proteins; OD: optical densities. The bold number highlight the best results obtained.

Overall, the protein mixture evaluated with the blood samples presented the best results, since the AUC calculated based on a threshold of 0.385 allowed to achieve a sensitivity and specificity with which was possible to obtain the higher true positive rate with the lower false positive rate (Figure 5).

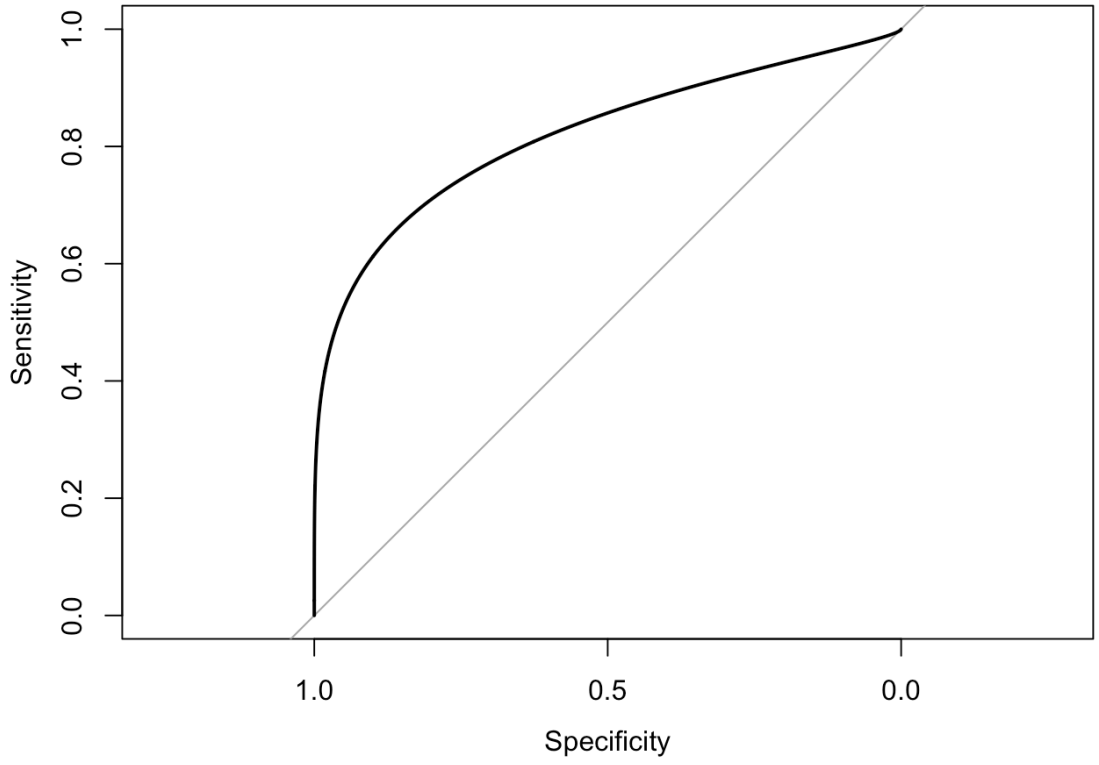


Figure 5. ROC curve derived from analysis performed between the protein mixture and the blood serum samples. The AUC, as well as the best sensitivity and specificity calculated were 0.82, 79%, and 77%, respectively.

Discussion

Early detection of IMI caused by *S. aureus* is essential to interrupt the chain of transmission, to optimize antibiotic treatment and to adjust management measures within herds. On a regular basis, IMI diagnosis uses SCC detection in milk. However, according to a study performed in 95,228 quarters milk samples and 386,031 composite milk samples, 20.5% of quarters and 30.8% of cows infected with *S. aureus* could remain undetected when using the thresholds of 200.000 cell/ml in quarters samples and 150.000 cell/ml in composite samples, since these infections can occur with low SCC (Petzer et al., 2017). Regarding to bacteriological culture, other ways that can be used to detect *S. aureus* from cows with IMI, this has some limitations for its routine use, because it time consuming, has a highest cost than SCC, and the sensitivity can be variable due to an intermittent or low number of bacteria in the samples (Britten, 2012; Fabres-Klein et al., 2014; Rainard et al., 2018). Misdiagnosis of IMI caused by *S. aureus* will allowed its fast spreading and persistence in dairy herds (Costa et al., 2018; Cucarella et al., 2004). Hence, the importance and need to find alternative diagnostic tools to improve the diagnosis of this pathogen or complement those that exist.

The detection of specific antibodies against antigens of several pathogens have also been implemented as effective diagnostic tools (Fabres-Klein et al., 2014). However, this type of diagnosis in *S. aureus* infections still represents a challenge to solve, especially those immunoassays that can be developed for identification of IMI caused by this pathogen (Rainard et al., 2018). Several proteins with immunogenic potential that are expressed during early stage of biofilm-forming process have been evaluated as vaccine targets and serological markers (Bennett et al., 2019; Stranger-Jones et al., 2006). In this study, we tested three proteins involved in the biofilm production by *S. aureus*. IsdA

protein play an important role in the adhesion process and in the biofilm formation under iron limited conditions (Clarke et al., 2004; Moormeier & Bayles, 2017). Studies that blocked this protein or inactivate the gene achieved decreases the capacity of *S. aureus* to colonize the host tissue, and therefore, the ability to form biofilm (Bennett et al., 2019; Hammer & Skaar, 2011). ClfA has generally been associated to the binding of plasmatic fibrinogen; nevertheless, it was also reported that this protein can attach directly to MEC in the absence of fibrinogen (Ashraf et al., 2017). Zapotoczna et al. (2015) described a biofilm phenotype mediated by coagulase and ClfA, since that their interaction with formed fibrin or fibrinogen lead to a clumping of bacterial cells. As with IsdA and ClfA, some of the roles described of the SdrD protein are the adhesion to extracellular matrix of host cells and subsequent biofilm formation (Ma et al., 2012). Several authors observed that the down-regulation of *sdrD* gene caused a reduction in the biofilm formation (Ma et al., 2012; Vaishampayan et al., 2018).

We observed that the specific IgG levels varied according to the protein tested, sample type and cows status (infected or uninfected). These results agree with Nishitani et al. (2015) study, who also found a response broad and variable between the patients groups (Infected and control) and antigens repertoire evaluated. Main IgG function is the opsonization of microorganisms and therefore promote their phagocytosis by macrophages and neutrophils (Schukken et al., 2011). Studies have reported the increase of the IgG_1 and IgG_2 subclass in the gland after challenging it with different immunogens (Schukken et al., 2011). However, the IgG_1 is the predominant subclass in milk of health bovines, whereas in animals with IMI is IgG_2, becoming it in the main opsonin for the phagocytosis process mediated by neutrophils in the infected mammary gland (Boerhout et al., 2016; Pastoret, 1998; Schukken et al., 2011). In this study, we did not discriminate by subclass, on the contrary, we measured the total IgG against each protein in the samples evaluated, since the objective was to obtain the highest antibodies concentration per antigen tested.

Comparisons of IgG levels in blood from infected cows for each protein showed significant differences in the response among almost all, except between ClfA and SdrD. Highest IgG titers evidenced were against IsdA, even above of the protein mixture. This result is in accordance with what was found in a study conducted in patients with osteomyelitis. Authors reported that IsdA was one of the 14 proteins evaluated that presented the highest IgG levels, demonstrating its immunogenic potential (Nishitani et al., 2015). Other of the studies that highlighted the potential of this protein, was the performed by den Reijer et al. (2017), who also tested the IgG response against 50 proteins in humans with osteomyelitis. They detected that IsdA was one of the 10 antigens that had highest titers. On the other hand, Stranger-Jones et al. (2006) generated significant protective immunity in a murine model challenged with pathogens *S. aureus* strains and previously immunized with a vaccine composed of four antigens, among these IsdA, confirming also the antigenic capacity of this protein.

Regarding to IgG detected in blood from uninfected animals, we observed the same differences among proteins. However, when we compared the results between the two groups of animals, differences were identified for IsdA, Mix and ClfA, but not for SdrD. Similar results were observed in some immunoassays performed with human samples, since these also showed differences in IgG levels against several antigens between patients and controls (den Reijer et al., 2017; Nishitani et al., 2015). Likewise, the result obtained with SdrD (no differences between groups) has also been reported, which suggest that there are better markers (immunodominant) than others (Nishitani et al., 2015). The IgG titers detection in uninfected animals could be explained by natural colonization with *S. aureus*. This pathogen could be naturally found in the teat skin and external orifices from healthy cows, where it triggers an immune response (Boerhout et al., 2016). Indeed, this has been one of the main limitations in the development of diagnostic techniques based on the detection of specific antibodies.

IgG levels reported in milk samples were significantly lowest than in blood in both infected and uninfected, which are in accordance with what have been previously reported. Increased blood antibodies do not necessarily lead to an increase of these in milk (Boerhout et al., 2016). In addition, evidence has shown that the increase of IgG in milk is probably pathogen-dependent, since its transfer has been related to disrupt of the blood-mammary barrier caused by the inflammatory process. Because *S. aureus* commonly causes subclinical IMI, this barrier could be not affected, and therefore the exudation of IgG from plasma to milk would be limited (Hernández-Castellano et al., 2017; Rainard et al., 2018; Renna et al., 2019).

Different IgG responses against *S. aureus* have been reported in milk. For example, Doymaz et al. (1988) did not find statistically significant differences in the IgG concentration in milk from infected and uninfected quarters. Instead, Renna et al. (2019) identified differences in milk obtained from cows during early involution of the mammary gland. Although, they observed IgG titers higher in uninfected than in infected cows. This data differs from most of our results, because we found that the highest mean IgG titers in infected animals in three (IsdA, Mix, ClfA) of the four assays performed. Some causes that could explain these discrepancies are the stage of lactation (dry period vs milking), the infection progress (acute or chronic; new or re-infection) or the specific IgG measured (Rainard et al., 2018; Renna et al., 2019). In this study, we did not take into account the stage of the infection of the animals, our inclusion criteria were cows infected with *S. aureus* and not vaccinated against this pathogen, since the objective was to determine if the immunoassay had the ability to discriminate between infected and uninfected animals independently of the infection stage. This could be other limitation in our work due to a study reported that the increasing of antibodies in milk could take two weeks (Fox & Adams, 2000). Therefore, the cows with early infections could not be detected.

Regarding to the diagnostic usefulness of the immunoassay based on ROC curves analysis, the best AUC obtained was 0.82 corresponding to the evaluation performed between the protein mixture and the blood samples. The calculated sensitivity (79%) and specificity (77%) calculated allowed us to achieve the higher true positive rate with the lowest false positive rate. An immunoassay (Multiplex Luminex assay) carried out with 14 proteins *S. aureus*, which were tested with samples from human with osteomyelitis, achieved an AUC of almost 0.90, as well as a sensitivity and specificity of 80% and 92.5%, respectively. Authors combined the IgG titers obtained with 14 proteins (Nishitani et al., 2015). The results obtained with blood samples are not totally comparable with those reported by Fabres-Klein et al. (2014) in their review on immunodiagnosis of staphylococcal IMI, because the data included by them correspond to milk evaluation. The sensitivity of the immunoassays tested was between 75% - 89% and the specificity between 70% - 90%. In our work, the sensitivity and specificity with an AUC above 0.80 obtained in milk were of 60% and 94% for Mix, respectively.

Taking into account the *S. aureus* infections are difficult to treat and in most of cases animals are culled because of the lack of response to antibiotics, it is necessary to identify the highest number of positive animals within the herd in order to timely interrupt the transmission chain. In this case, it is recommended to use a highly sensitive screening test, such as is the SCC, because animals with the disease rarely are lost (Maxim et al., 2014). According to Petzer et al. (2017), the sensitivity and specificity of the SCC to detect this pathogen when a threshold of >150 000 cell/ml was used in composite milk was 84.2% and 52.8%, respectively. However, a highly sensitive test but not so specific can also include a high number of false positives, which should be discharged using a highly specific test such as bacteriological culture. Nevertheless, the use of bacteriological culture in the routine is limited due to the reasons mentioned above. Therefore, our highly specific test (94%) could be used in milk as a complement to SCC, since it would allow to confirm, quickly and inexpensively, the true negatives (uninfected) and those infections caused by other pathogens. A highly specific test

would help optimize treatments (adequate use of antibiotics), since it is widely known that animals infected with *S. aureus* generally do not respond to antibiotic therapy.

Recently a research reported that the host cell immune response varied according to *S. aureus* lineage. They found that strains belonging to clonal complex 97 (CC97), the major bovine *S. aureus* complex, were highly immunogenic compared to other lineages evaluated (Murphy et al., 2019; Spoor et al., 2013). In contrast to these results, we did not observe an influence of the identified genotypes on the IgG titers in both in blood and milk.

Conclusions

We found significant differences in the IgG response against proteins involved in the biofilm formation by *S. aureus*, highlighting the antigenic potential of IsdA compared to the other proteins and demonstrating that some antigens can be better indicators of infection than others.

Although, the highest IgG titers in both blood and milk were obtained against IsdA, the protein mixture showed the greatest capacity to differentiate between infected and uninfected cows when blood samples were used. Furthermore, this mixture also showed the highest specificity using milk samples, suggesting its possible use as a diagnostic complement to SCC.

These results will contribute to the knowledge about the identification of potential antigens, as well as their behavior *in vivo* and possible utilization as indicators of infection in diagnostic test or as vaccine targets, both essential tools for the successful control of any pathogen.

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Conclusiones generales

- La mayoría (81,3%) de las cepas de *S. aureus* aisladas de bovinos con infecciones intramamarias en la región presentaron capacidad para formar biopelículas.
- Las cepas de *S. aureus* portadoras el gen *bap* (genotipo 2) fueron las que presentaron mayor capacidad para formar biopelículas comparadas con los genotipos 0 y 1, sugiriendo su virulencia y potencial para generar infeciones persistentes difíciles de eliminar.
- La tipificación de las cepas de *S. aureus* mediante la técnica "*spa typing*" mostró una amplia diversidad (38 spa tipos) de genotipos circulantes en la región.
- El 68% de los genotipos identificados también han sido previamente reportados como causantes de infecciones en humanos, demostrando la influencia de los ordeñadores en la transmisión y convirtiendo a los bovinos en resorvorios importantes del patógeno. Además, la cercanía de estos genotipos con cepas propias de los bovinos incrementa el riesgo de recombinación genética entre estas, por consiguiente, la posible aparición de clones con potencial zoonótico.
- El 18,4% de los aislados de *S. aureus* presentaron genotipos nuevos, previamente no reportados, sugiriendo la posibilidad de que sean cepas propias de la región y mostrando el potencial de variación de este patógeno.
- El 37% de las cepas de *S. aureus* fueron resistentes a los antibióticos Penicilina y Lincomicina, tal como había sido reportado en trabajos previos, en los cuales se demostró que estos son los antibióticos a los que la mayoría de cepas presentan resistencia en la región.
- No se encontró asociación entre los genotipos de *S. aureus* identificados y la resistencia a los antibióticos.
- El genoma de la cepa de *S. aureus* Sa1FB, la cual fue reconocida como fuerte formadora de biopelícula y causante de mastitis subclínica, mostró alta identidad con los genomas de las cepas de referencia RF122 y Newbould 305, ambas cepas causantes de mastitis clínicas en bovinos.

- La presencia de material genético móvil (profagos, transposasas, isla de patogenicidad) en el genoma de la cepa Sa1FB secuenciada, resalta su capacidad para aceptar material genético externo, el cual podría modificar su genoma, por lo tanto, incrementar su patogenicidad y capacidad de adaptación a nuevos huéspedes.
- En este estudio, a medida que incrementó la capacidad de formación de biopelícula disminuyó la capacidad de invasión celular.
- Se comprobó mediante microscopía electrónica que las cepas portadoras del gen *bap* muestran un fenotipo de biopelícula independiente de PIA (a base de proteínas), a pesar de la presencia del locus *ica*, sugiriendo que en presencia de *bap* este es el que conduce la formación de biopelícula. Mientras que, las cepas portadoras solo del operón *ica*, generaron una biopelícula donde, probablemente, predominó PIA, debido a que se observó una matriz en la cual estaban embebidas las células bacterianas.
- La identificación de proteínas implicadas en el proceso de formación de biopelícula que solo fueron expresadas por las cepas formadoras durante la interacción con las CEM, pero ausentes en los controles, demuestran su importancia en las primeras horas del proceso infeccioso y, probablemente, en la posterior formación de biopelícula *in vivo*.
- Los resultados confirmaron que las proteínas IsdA, ClfA y SdrD de *S. aureus* son potenciales candidatos para vacunas contra infecciones intramamarias y podrían ser usadas en inmunoensayos como marcadores de infección, debido a que están implicadas en la fase temprana de la infección, en la formación de biopelículas y tienen la capacidad (antigenicidad) de desencadenar la respuesta inmune.
- Los títulos de IgG encontrados contra las proteínas evaluadas en el inmunoensayo demostraron que algunos antígenos son mejores marcadores de infección que otros, resaltando el potencial antigénico de IsdA, dado que fue la proteína que presentó los mayores títulos de anticuerpos con respecto a las otras.

- A pesar de que fue IsdA la proteína que tuvo los títulos más altos de IgG específica, fue la mezcla de las tres proteínas la que exhibió, según el análisis de curvas ROC, la más alta sensibilidad (79%) y especificidad (77%) en conjunto cuando se emplean muestras de sangre, o la mayor especificidad (94%) al usar muestras de leche.
- Los resultados obtenidos en el inmunoensayo ampliará el conocimiento sobre potenciales antígenos, su comportamiento *in vivo* y posible utilidad como marcadores de infección en pruebas diagnósticas.

Anexos

1. Artículo 1

Se anexa el artículo publicado en la revista Heliyon (Q1) (https://doi.org/10.1016/j.heliyon.2019.e02535)

2. Artículo 3

Se anexa el artículo publicado en la revista Frontiers in Veterinary Science (Q1) (https://doi:10.3389/fvets.2020.00530)

3. Archivo con material suplementario de los artículos

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