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Bovine Mastitis Caused By *Streptococcus uberis*: Virulence Factors and Biofilm

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Abstract

Bovine mastitis is a multifactorial disease, commonly caused by microorganisms. The pathology affects dairy farms worldwide and causes significant economic losses. Different pathogens can cause the disease and they are classified as contagious, environmental and minor pathogens. *Streptococcus uberis* is a ubiquitous bacterium and is considered the main environmental agent. It is a very versatile microorganism able to use host factors to survive and colonize bovine mammary gland. Different virulence factors have been reported in *S. uberis* strains, such as proteoglycans and various proteins, which are secreting in milk facilitating the establishment of intramammary infections. Strategies for the control of environmental agents have less impact compared to those applied for contagious agents. Furthermore, intramammary infections are associated with biofilm formation which leads to antibiotic resistance making the treatment of recurrent infections hard. Thus, different alternative control methods have been proposed, as the use of bacteriocins and immunomodulatory compounds. The present review summarizes different studies about the characterization of *S. uberis* virulence factors and the importance of the studies to promote and design effective and novel therapeutic approaches.

Keywords: *Streptococcus uberis*; Mastitis; Virulence factors; Biofilm

Introduction

Bovine mastitis is the most common pathology that affects dairy farms around the world, causing significant economic losses due to reduced milk production and cow health, antibiotic therapy, slaughter and the death of livestock. Mastitis incidence differs locally and represents the highest cost in the dairy industry. The disease has been a reason of attention and to improve its control has been of high concern for several decades. It is the most common disease of antibiotic treatment in dairy farms. In the world, clinical mastitis losses are estimated from US$ 7561 to US$ 119 per cow, with variances among different farms [1,2]. In Argentina, it was estimated that the decrease in milk production and quality was about more than 220 million pesos per year in the 1980s [3]. Then efforts were focused on improving the hygiene quality and milk health through a high mastitis control [4].

Mastitis pathogens are categorized as contagious and environmental. Different reports about frequency and type of microorganism isolated reveal that *Streptococcus uberis* is globally recognized as one of the most important environmental pathogens implicated in intramammary infections [5]. On the other hand, *Staphylococcus aureus* is the most prevalent contagious pathogen present in dairy herds [6]. However, pathogen incidence can vary geographically.

The intensive administration of antibiotics in the treatment and control of mastitis is associated to an increase in the resistance of microorganisms to antibiotics, with their implications for human health due to the risk of passage of resistant strains to the food chain and then to man. In addition, application of antibiotic therapy during lactation, involves the removal of the animal from the productive circuit in order to avoid the presence of antibiotics in milk which leads to significant economic losses [7]. The economic losses produced by the disease have guided studies towards the search of strategies for the prevention and/or treatment of bovine mastitis in order to optimize milk production, ensure safety products and promote regional economic growth. One of the biggest challenges of the dairy industry is to reduce the use of antibiotics in food-producing animals, focusing the research studies on the search for alternative control methods. Bacteriocins offer an alternative as potential antibacterial agents for the treatment of mastitis [8-10]. In addition, the application of immunomodulatory compounds to stimulate the specific immune response of the mammary gland is one of the alternative therapies currently studied [11].

S. uberis, is the main environmental agent responsible for bovine mastitis, and it is characterized by virulence factors such as proteoglycans and several proteins, and when secreted in milk cause intramammary infections [12-14]. Furthermore, a high genetic diversity was found among the strains by using different techniques makes the searching of effective control strategies difficult. Pulse field gel electrophoresis (PFGE) is the current standard method used in order to characterize genetic patterns. Albuquerque et al. [15] showed the usefulness of dot blot and MLSA assays to evaluate *S. uberis* population structure.

Intramammary infections are difficult to eliminate due to their multifactorial nature, and their control requires a program based on the prevention of new infections and the elimination of existing ones [16]. The implementation of a 5-point control plan has allowed a reduction incidence of mastitis cases due to contagious pathogens, such as *S. aureus* and *Streptococcus agalactiae* [17]. However, these measures have shown less impact on the incidence of mastitis caused by environmental pathogens. Despite the economic impact caused by the high prevalence of environmental streptococci in most dairy herds with conventional management practices, there is still no in-depth knowledge of the

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virulence determinants associated with the pathogenesis of bovine mastitis caused by these microorganisms. Consequently, the strategies for the control of this type of mastitis are deficient and inadequate.

The present review summarizes different studies investigating *S. uberis* virulence factors and the importance of the studies to promote and design effective and novel therapeutic approaches.

Bovine mastitis

Bovine mastitis is an inflammatory disease of the mammary gland, most frequently caused by pathogen agents, to a lesser extent by trauma or chemical origin injuries [18]. The inflammatory process is generated by the presence of inflammatory mediators that lead to invasion of leukocytes into the mammary gland [19]. Mastitis is considered as a multifactorial disease because the risk of infection is related to the cow's immunity system, the inoculum and virulence of the microorganism, the environmental conditions and the milking management established in the herd [20].

Different pathogen agents can cause the disease and they are classified as contagious, environmental and minor pathogens. Contagious pathogens are associated with infected udders, nipple lesions and colonization of the nipple canal. The reservoir is the cow and the pathogens are transmitted by cow-cow contact, or from teat to teat during and after milking. The most representative microorganisms of this group are *S. aureus* and *S. agalactiae*. Environmental pathogens are widely distributed in different sites where the animals eat, sleep and transit, especially in humid places or with high content of organic matter. This group is formed by coliforms, *S. uberis* and *Pseudomonas aeruginosa*. Infections caused by these pathogens are more difficult to control because they occur in cow transition periods from three weeks pre-calving to four weeks postpartum. Among the minor pathogens *Corynebacterium bovis* and coagulase negative staphylococci (CNS) are included. CNS group has become important in the last few years, as commensal and opportunistic agent causing important infections [21,22].

Streptococcus uberis

By the year 1928, descriptions of streptococci with different properties compared to the common pathogens that cause mastitis were reported. Over the following years and until 1932, reports from several researchers in Europe contributed to the importance of differentiate these microorganisms in different species. The name *S. uberis* was proposed by Diernhofer in 1932 in order to identify streptococci associated with bovine mastitis [23]. The agent was characterized according to the following biochemical characteristics: presence of smooth and round colonies on agar, turbidity in broth medium, presence of diplococci, ability to ferment glucose, lactose, sucrose, mannitol and salicin, lack of raffinose and glycerol fermentation, positive hipurate and esculin hydrolysis, and presence of greenish colonies on blood agar plates. Then, Seeley in 1951 [24] contributed to the characterization of this agent demonstrating the nutritional requirements as nicotinic acid, pyridoxine, thiamine, riboflavin, folic acid, pantothenic acid and biotin; tryptophan, phenylalanine, arginine, valine, leucine and glutamic acid. Some years later, in 1997 Kitt and Leigh 1997 found *S. uberis* strains auxotrophic for 13 amino acids [25].

S. uberis is a gram-positive coccus, facultative anaerobe with high nutritional requirements. It is not a spore former and is negative for biochemical tests of oxidase and catalase. Currently it belongs to the Streptococcaceae family, which includes pathogenic, commensal and opportunistic species. Analysis of the *S. uberis* genome demonstrated the ability of *S. uberis* to live in different ecological niches due to its nutritional flexibility indicating that it can adapt to different types of environments as an opportunistic pathogen [26].

Phylogenetic analysis proposed by Bentley et al. placed *S.uberis* within the pyogenic group with *Streptococcus pyogenes*, *Streptococcus dysgalactiae* subsp. equisimilis, *Streptococcus agalactiae* and *Streptococcus dysgalactiae*, *Streptococcus equi*, *Streptococcus canis* and *Streptococcus iniae* [27]. Lancefield's classification system is used to classify streptococcal species, although *S. uberis* is not classifiable by this method because no group of antigens is kept among the strains [28]. Phenotypic identification of *S. uberis* is determined on conventional protocols such as examination of cultural and morphological characteristics, standard biochemical tests, and enzyme activity [29,30]. In order to confirm identification, molecular assays were designed [31-34]. Correct identification is necessary for an efficient therapeutic choice and for also supervising the mastitis control schemes in the herds. A scheme designed by Odierno et al. could biochemically identify typical and atypical *S. uberis* isolates [30]. Furthermore, this scheme showed a clear correlation with 16S rDNA RFLP for most streptococcal and streptococcal-like species [35].

S. uberis is a ubiquitous agent isolated from different parts of the body cow, bedding and soil and elements of the dairy herd environment as well. It has been associated with subclinical and clinical mastitis in lactating and non-lactating cows and can also live on the mammary gland leading to chronic intramammary infections [36-38]. Mastitis subclinical chronic infections are considered extremely costly and difficult to treat [39]. *S. uberis* has a great ability to colonize epithelial cells of the mammary gland, evading the host defense mechanisms leading to antibiotic resistance through the virulence factors.

Virulence factors

Phenotypic studies have allowed the identification and characterization of potential virulence determinants (capsule, plasminogen activating factor, uberis factor, M-like and R-like proteins, neutrophilic toxin, hyaluronidase, extracellular matrix binding proteins) in *S. uberis* strains [36]. However, it is known that virulence factor expression in bacteria could be controlled by signals from the environment. Therefore, the *S. uberis* genome has been sequenced complete, being a valuable resource to facilitate the study between *S. uberis* and the bovine host [26,40].

Different virulence factors of *S. uberis* have been described, such as proteoglycans and various proteins, which are secreting in milk facilitating the establishment of intramammary infections [41-43]. Briefly, virulence factors includes: plasminogen activator proteins such as PauA and PauB and SK resistance to phagocytosis presented by a hyaluronic acid capsule CAMP factor a surface dehydrogenase protein GapC sortases Opp proteins implicated in dynamic transport of solutes lactoferrin binding proteins, and adherence and invasion of epithelial cells mediated by SUAM [42-51].

Streptococci agents are able to activate plasminogen thanks to the action of secreted enzymes [52]. Bacterial plasminogen activators comprise streptokinase produced by different Streptococcus pathogen species and differ greatly in structure [53]. Streptokinase Esk was purified from *S. equisimilis* and its amino acid sequence is quite different from the classical streptokinases studied [44,54]. Likewise, a plasminogen activator, named PadA was identified in bovine isolates of *Streptococcus dysgalactiae* [55]. This activator could activate bovine, ovine, equine and rabbit but not human plasminogens. Furthermore, Wiles et al. (2010) described a plasminogen named skizzle (SkzL),

produced by *Streptococcus agalactiae*, which has a sequence identity lower than streptokinase and staphylokinase [56]. Reports have shown that *S. uberis* is able to activate bovine plasminogen [41]. Streptokinase was a plasminogen activator described in *Streptococcus* spp. [44]. Once plasminogen is activated to plasmin, it confers the access to deep tissues by its action on extracellular matrix proteins. Similarly, PauA activator (molecular weight of approximately 30 KDa) was the first plasminogen activator described in *S. uberis* strains capable of activating bovine, ovine and equine plasminogen, however it is not able to activate porcine or human plasminogen [42,57]. It is suggested that the activation of plasminogen by PauA could facilitate early colonization of the mammary gland because it promotes the removal of nutrients [41]. *In vitro*, it has been shown that PauA mediates the acquisition of plasmin in culture medium with the addition of plasminogen. During infection of the mammary gland, *S. uberis* is found predominantly in the luminal region of secretory alveoli and ductular tissue, indicating that bacterial growth occurs in residual and recently synthesized milk. This environment is probably deficient in free peptides and amino acids, so the activation of plasminogen by PauA could facilitate the growth of *S. uberis* because it indirectly affects the hydrolysis of casein to peptides with essential amino acids [44,57]. Binding of plasmin to the bacterial surface would also allow the availability of peptides near the cell surface [58]. In addition, plasmin allows the proteolytic to breakdown fibrin and connective tissue proteins, thus facilitating bacterial penetration of tissue barriers and their dissemination in the tissues around the infection. A second plasminogen activator called PauB, with a molecular weight of 45 kDa, was identified by Johnsen et al. in a *S. uberis* strain isolated from a case of clinical mastitis in Denmark [44]. Ward and Leigh determined the absence of the plasminogen activator PauA in this strain and found that *pauB* gene was present in the locus normally occupied by *pauA* [43]. The authors demonstrated its activity on bovine, ovine, equine, goat, porcine, rabbit and human plasminogen. Therefore, PauB represents another plasminogen activator with wide specificity but found at low frequency. Studies carried out by our research group found that *pauA* gene was found in 48 (61.5%) strains and no strain yielded *pauB* gene [59]. Most of the reported findings demonstrate that the PauA protein could also be used as an antigen for the possible development of a vaccine subunit [60].

In the different streptococci, including *S. pyogenes*, *Streptococcus milleri* and *Streptococcus suis* exopolysaccharide capsules in phagocytosis resistance and in bacterial virulence are crucial [61-65]. In addition, Okamoto et al. reported that *S. pyogenes* exopolysaccharide capsule increases the adherence to alveolar epithelial cells and stimulates the adhesion to keratinocytes via an M-protein-independent pathway [66,67]. The hyaluronic acid capsule is encoded by *hasABC* genes and is one of the main virulence factors due to the role that it plays in phagocytosis [68]. *has* genes homologues of *S. pyogenes* are implicated in the capsule formation of *S. uberis*. Nevertheless, *hasABC* operon structure is not conserved in *S. uberis* strains [45]. Even though this capsule does not prevent against macrophages and not all the *S. uberis* strains are able to produce the capsule [45,59]. Hyaluronic acid blocks the Fc receptors on the surface of phagocytic cells avoiding the binding of opsonic antibodies onto the membrane of phagocytes and, therefore, the union and envelopment of opsonized bacteria.

An additional potential virulence factor reported is CAMP factor encoding gene *cfu*. CAMP factor is a protein initially discovered in *S. agalactiae* which produces the synergistic lysis of ovine erythrocytes in the presence of a ß-toxin *S. aureus* [69,70]. The deduced amino acid sequence of CAMP factor of *S. uberis* was found to be homologous to CFB amino acid sequence of *S. agalactiae* [46]. Different studies reported that CAMP factor and CAMP factor-like genes are quite widespread among the streptococci group, leastwise in serogroups A, B, C, G, M, P, R and U [71]. Furthermore, a linkage among CAMP gene *cfa* of *S. pyogenes*, *cfb* gene of *S. agalactiae*, *cfu* gene of *S. uberis* and *cfg* gene of *S. canis* was described by Hassan et al. [69,70]. Gase et al. [72] and Hassan et al. [73] reported a lethal effect of this factor when was administered parenterally in mice and rabbit and the active substance showed a similar effect like the CAMP factor of *S. agalactiae*. Different studies have shown that not all *S. uberis* tested strains have a positive CAMP reaction although it could be a virulence factor [13,74,75].

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a ubiquitous enzyme found at the surface of several prokaryotic and eukaryotic organisms, it is involved in glycolysis converting glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate and it has been implicated as virulence factor [76,77].

The enzyme was firstly identified located on the surface of *S. pyogenes* and then it was found in streptococci groups B, C, E, G, H and L [78,79]. Winram and Lottenberg [80] described that the enzyme is expressed on the streptococcal cell surface and it is involved in different functions such as the attaching of *S. pyogenes* to plasmin. Pancholi and Fischetti showed the ability of GAPDH to bind several host proteins [79]. Madureira et al. demonstrated that *S. agalactiae* GAPDH is a virulence-associated immunomodulatory protein [81]. Oliveira et al. reported a new and different function of the secreted GAPDH as an inducer of apoptosis of murine macrophages [81]. As the enzyme has also different functions to the original, it has been called "moonlighting proteins" [82]. A recent study showed that GAPDH is an appropriate vaccine candidate against bacterial and parasitic infections [62]. Furthermore, homology among GapC products of *S. uberis*, *S. agalactiae* and *S. dysgalactiae* strains could lead to the design of a vaccine containing a specific chimeric protein covering protein regions of each species [83].

Sortase are enzymes involved in covalent attachment of enzymes, pilins and adhesion-mediating large surface glycoproteins to the bacterial cell wall contributing to the development and maintenance of the infection, for which they are considered as virulence determinants [48]. Egan et al. identified sortaseA (SrtA) substrates in *S. uberis* strains and Leigh et al. studying a SrtA deficient strain of *S. uberis* reported that a number of sortase anchored proteins have a significant function in the pathogenesis of *S. uberis* infection [84-86]. In accordance with phylogenetic analyses, two different studies suggested the categorization of sortases in four subfamilies (A-D) or five subfamilies (SrtA, SrtB and families 3 to 5) and GBS strains have sortases from two of these subfamilies [87,88].

One of the strategies of *S. uberis* to survive and colonize bovine mammary gland is through the binding to lactoferrin, which is normally found in milk and mammary gland secretions of non-lactating cows [89]. The binding to lactoferrin is mediated by an adhesion molecule of *S. uberis*, called SUAM, encoded by *sua* gene. SUAM has a molecular weight of approximately 112 KDa and has been identified and characterized [90]. It has been proposed that this molecule plays an important role in the pathogenesis of mastitis and is considered a potential virulence factor of this microorganism. Almeida et al. [91] and Patel et al. [92] suggested that SUAM, through its binding to lactoferrin (Lf) and subsequent binding to a receptor present on the surface of the mammary epithelial cell, would facilitate bacterial adhesion by triggering the internalization of this pathogen into the cellular cytoplasm [90,91]. The internalization provides a protective environment against phagocytosis by neutrophils and antimicrobials

present in milk. *In vitro* studies showed that SUAM plays a central role through adhesion and internalization in bovine mammary epithelial cells of *S. uberis* and consequently could be involved in biofilm formation. The adhesion molecule SUAM, together with the oligopeptide transport system (OppA) and a lipoprotein receptor (MutA antigen), would be involved in the infection as well as in the ability to adhere to the cells [91]. Consequently these factors could be involved in the formation of biofilm and have also been described as virulence factors.

A study carried out by Lasagno et al., characterized phenotypically *S. uberis* strains by the presence of virulence factors as plasminogen activator factor (PAF), hyaluronidase (HYA), capsule (CAP) and CAMP factor [13]. Sixty five percent, 56.3%, 59.4% and 25% of the strains expressed plasminogen activator factor, hyaluronidase or capsule and CAMP factor, respectively [13]. Taking into account the combination of virulence factors thirteen different virulence profiles were identified indicating a notable heterogeneity in their phenotypic characteristics. Similarly, other results determining the distribution of virulence-associated genes in 78 *S. uberis* strains by PCR showed that *hasC* gene was present in 89.7% of the strains, being the most common gene in the examined isolates. *sua* gene was found in 83.3%, *gapC* in 79.4%, *cfu* in 76.9%, *hasA* in 74.3%, *hasB* in 66.6%, *skc* in 65.3%, *oppF* in 64.1%, *pauA* in 61.5% and *lbp* in 11.5%. *hasABC* genotype was found in 61.5% of the strains [59]. Perrig et al. reported that *sua* and *pauA* are prevalent and highly conserved genes, being important candidates to be included in a mastitis vaccine against *S. uberis* [93]. Results of our group show that not all the virulence genes could be amplified in all the analyzed strains, nevertheless all of them were present in combination, indicating that other virulence factors may be implicated [12,59]. Furthermore, results revealed the absence of classical virulence factors such as those present in other species of Streptococcus [59].

Bovine mastitis and biofilm

Several species of streptococci, such as *Streptococcus mutans* and *Streptococcus pneumoniae*, have been recognized to have the ability to form biofilm [93]. *Streptococcus* spp. biofilm growth is particularly studied in *Streptococcus mutans* and *Streptococcus gordonii* [94-96]. A biofilm matrix is composed of microbial cells, polysaccharides, water and other extracellular products; which provide a sheltered and protected site for bacterial growth [97]. In this way the bacteria are more resistant to antibiotics, disinfectants and host defenses. Therefore, the difficulties to treat recurrent infections could be related to the capacity of the pathogens to produce biofilm [98]. Due to its size, biofilm is not susceptible to being phagocytosed by polymorphonuclear neutrophils or macrophages. In addition, it allows the bacteria to adapt to unfavorable conditions present in the surrounding environment as cold, heat, drying and particularly situations of rapid and constant flow, such as arteries and other living tissue structures or inert surfaces such as catheters or tubes used in mechanical milkers present in the livestock industry [99]. The literature reported that 65% of infections would be associated with biofilm formation in other mastitis agents, such as *S. aureus* [100]. *S. aureus* is one of agent most studied as a biofilm producer. The ability of this microorganism to persist in the mammary gland forming biofilm would be one of the possible sources of persistent or chronic infections [101]. Biofilm growth is more resistant to antibiotics than planktonic growth, and commonly a high concentration of antimicrobial agents is necessary to remove biofilm formation [102].

The concentration and type of nutrient available in the environment influence the development and chemical composition of the biofilm; in oligotrophic environments, microorganisms respond to nutritional stress through alterations in the morphology and cell surface [103]. Different studies about biofilm production by mastitis strains have been reported [104,105]. Previously, we studied the biofilm formation ability of *S. uberis* strains the effects of several factors as additives and bovine milk compounds on biofilm production and the genetic variation among *S. uberis* isolates to establish relationship between virulence profiles and PFGE patterns [106-108]. The results showed that *S. uberis* isolates had a notable ability to produce biofilm at different degrees [14]. Our results agree with those of Moore who reported that the majority of the strains were able to produce biofilm [109]. Likewise, Varhimo et al. reported that the biolfim was produced at different degrees [105]. In addition, to establish the optimal conditions for biofilm formation of *S. uberis* strains, biofilm assays were carried out under various representative conditions of the mammary gland and the most favorable conditions for biofilm formation were at pH 7 and at 37°C [106]. A decrease in biofilm formation was observed by the addition of bovine milk compounds as casein hydrolysate (3 mg/ml) and carbohydrates as glucose (5%) and lactose (0.5% and 5%). Furthermore, extrachromosomal ADN was observed in cell-free supernatants [106]. The *in vitro* biofilm formation of *S. uberis* strains has been investigated using different culture media and conditions, obtaining divergent results [105,106,109,110]. Similarly, Rossini et al. showed discrepancies between different studies in *S. agalactiae* (Group B Streptococcus) [111].

Biofilm is an example of group behavior where different genes are involved. It has been associated with the presence of *quorum sensing* and bacterial competition genes [111]. *Quorum sensing* is a process involved in cell-cell communication essential for biofilm formation, but also for bioluminescence, expression of virulence factors, sporulation, mating, production of antibiotics and DNA exchange [112]. *luxS* gene is involved in *quorum sensing*, and this system controls the behavior when the population of bacteria reaches certain cell density. This reaction becomes effective by the simultaneous action of a significant number of cells [113]. On the other hand, the genes *comEA* and *comEC* are involved in the bacterial competence, allowing the transformation of genomic DNA through the uptake of DNA strands. *comEA* gene acts as a DNA receptor and passes through a protein channel that regulates uptake. This channel is encoded by the *comEC* gene [114]. According to a study performed by Moore most of the strains evaluated yielded genes associated with biofilm formation as *luxS*, *comEA, comEC* and *comX* [109]. Our results showed that the rate of *luxS, com EA* in *S. uberis* isolates was 42.8% and 21.4%, respectively [14]. Similarly, more than half of the strains were positive for *comEC* and *comEX* genes [115,116]. Results suggest that these genes would be necessary for biofilm formation. A better understanding of the *quorum sensing* process may contribute to develop effective methods to avoid microbial biofilm formation.

Conclusion

Intramammary infections by *S. uberis* cause important economic losses in the dairy industry. Different studies have characterized phenotypically and genotypically *S. uberis* strains and aimed at researching to improve the knowledge of virulence factors and biofilm production in *S. uberis* strains. The studies could serve as basis for the future development of effective and appropriate treatment protocols that could alleviate the impact of mastitis caused by this environmental pathogen.

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