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Biofilm production and beta-lactamic resistance in Brazilian *Staphylococcus aureus* isolates from bovine mastitis

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ABSTRACT

Staphylococcus spp. play an important role in the etiology of bovine mastitis. *Staphylococcus aureus* is considered the most relevant species due to the production of virulence factors such as slime, which is required for biofilm formation. This study aimed to evaluate biofilm production and its possible relation to beta-lactamic resistance in 20 *S. aureus* isolates from bovine mastitic milk. The isolates were characterized by pheno-genotypic and MALDI TOF-MS assays and tested for genes such as *ica*A, *ica*D, *bap*, *agr RNAIII*, *agr* I, *agr* II, *agr* III, and *agr* IV, which are related to slime production and its regulation. Biofilm production in microplates was evaluated considering the intervals determined along the bacterial growth curve. In addition, to determine the most suitable time interval for biofilm analysis, scanning electron microscopy was performed. Furthermore, genes such as *mec*A and *bla*Z that are related to beta-lactamic resistance and oxacillin susceptibility were tested. All the studied isolates were biofilm producers and mostly presented *ica*A and *ica*D. The Agr type II genes were significantly prevalent. According to the SEM, gradual changes in the bacterial arrangement were observed during biofilm formation along the growth curve phases, and the peak was reached at the stationary phase. In this study, the penicillin resistance was related to the production of beta-lactamase, and the high minimal bactericidal concentration for cefoxitin was possibly associated with biofilm protection. Therefore, further studies are warranted to better understand biofilm formation, possibly contributing to our knowledge about bacterial resistance *in vivo*.

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Introduction

Staphylococcus spp. play an important role in the etiology of intramammary infections of dairy cattle. *Staphylococcus aureus* stands out among the prevalent etiologic agents in this type of infection due to its ability to produce a wide array of virulence factors that contribute to the bacterial invasion.[1] Of them, the production of slime, an extracellular mucopolysaccharide, appears to play a crucial role in the adhesion and colonization of the microorganism on the mammary glandular epithelium; this not only favors biofilm formation and their extracellular persistence but also ensures success in their installation and maintenance in the host tissues.[2] Slime is composed of a high-molecular-weight polysaccharide intercellular adhesin. Its production is mediated by the intercellular adhesion operon (ica) formed by the genes *ica*A, *ica*B, *ica*C, and *ica*D and a regulator gene, *icaR*, which encodes the ICAA, ICAB, ICAC, and ICAD proteins.[3]

Furthermore, *ica*-independent mechanisms possibly play an essential role in bacterial biofilm formation. For example, the function of *bap*, which encodes for the surface protein Bap, is to assist in intercellular adhesion and biofilm formation. This gene has been primarily studied in isolates from bovine mastitis.[4]

The repression of *agr* quorum-sensing system is necessary for biofilm formation. Its reactivation in established biofilms through auto inducing peptides (AIPs) addition or glucose depletion triggers biofilm detachment.[5] The *agr* system includes AgrD, the signaling octapeptide produced in high cell density; AgrB, a transmembrane protein responsible for secretion, export, and processing of active AgrD; and AgrC, a membrane receptor that triggers AgrA phosphorylation mechanism when bound to AgrD. The phosphorylated AgrA positively regulates the production of the effector molecule RNA III.[6] *S. aureus* can be classified into four polymorphic Agr types (AgrI, AgrII, AgrIII, and AgrIV) based on the specificity of AIP to the signal receptor AgrC.[7]

Moreover, biofilm production in *S. aureus* from mastitis can be associated with antimicrobial resistance.[8] The mechanisms responsible for this resistance include the physical and chemical diffusion barrier formed by the exopolysaccharide matrix, which hinders antimicrobial penetration, the existence of microenvironments that antagonize the antibiotic action, the activation of stress responses that cause changes in bacterial physiology, and the stable and slower growth of these microorganisms due to nutrient limitation and the absence of antimicrobial targets.[2]

Antimicrobials such as beta-lactams are preferred for the treatment of staphylococcal infections. However, production of beta-lactamase enzymes, coded by *blaZ* that hydrolyzes the beta-lactamic ring, and production of low-affinity penicillin binding protein (PBP2a), coded by *mec*A, may lead to antimicrobial resistance.[9]

This study aimed to detect the phenotypic expression of biofilm and the presence of structural and regulatory genes involved in the production of this virulence factor. In addition, the stages of biofilm synthesis along the growth curve were evaluated by scanning electron microscopy (SEM), and pheno-genotypic resistance to beta-lactamic and its possible relation to biofilm production were evaluated.

Materials and methods

Sampling and pheno-genotypic and proteomic identification

Three dairy cattle farms located in an important milk production region of Rio de Janeiro, Brazil, were selected owing to the high prevalence of subclinical mastitis on the farms, identified through the California mastitis test and somatic cell count. In total, 120 milk samples were collected in October and November 2012. Fifty nine *Staphylococcus* spp. were isolated, of which 41 were *S. aureus* strains.

After phenotypic identification, all 41 strains were submitted to polymerase chain reaction (PCR) for 16S rRNA to confirm the *Staphylococcus* spp.[10] PCR for *coa*,[11] *nuc*,[12] and 23S rDNA[13] genes were performed to characterize *S. aureus*. The ATCC 29213 *S. aureus* was used as quality control. Furthermore, all isolates were evaluated by the matrix-assisted laser desorption ionization-time of flight mass spectrometry, as described by Motta et al.,[14] considering the accepted values for matches ≥2.

The *S. aureus* isolates were subjected to disk diffusion tests using amoxicillin (10 μg), ampicillin (10 μg), azithromycin (15 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), cefepime (30 μg), enrofloxacin (5 mcg), erythromycin (15 μg), streptomycin (10 μg), moxifloxacin (5 μg), neomycin (30 mcg), novobiocin (5 mcg), cotrimoxazole (25 μg), and tetracycline (30 μg) disks. After overnight incubation at 35 °C, followed by inhibition zone measurement, the results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) standards.[15–17] These isolates were subjected to DNA extraction and amplification of *hlA* and *hlB*,[18] *fbnA* and *fbnB*,[19] and *cap5* and *cap8*,[19] according to the protocol described by Marques et al.[20] and Tito et al.[21] To study the biofilm production, 20 *S. aureus* strains were selected considering their antibiotic resistance profiles and the presence of virulence genes.

Qualitative and quantitative biofilm assay

Biofilm production was measured using qualitative and quantitative assays, described by Marques et al.[20] All the 20 *S. aureus* isolates were transferred to sheep blood agar for 24 h at 35 °C. The grown colonies were inoculated into tryptic soy broth (TSB) containing 0.24% glucose to stimulate slime production for 24 h at 35 °C. The bacterial cultures were adjusted to a 0.5 McFarland scale and diluted 1:10 in TSB with the addition of 0.24% glucose. Aliquots of this suspension (200 μL) were inoculated into sterile polystyrene 96-wellmicroplates for 24 h at 35 °C without agitation. After discarding this material, the wells were washed twice with 200 μL sterile saline, oven dried at 65 °C for 1 h, and stained with 200 μL safranin 1% for 15 min. Subsequently, the wells were washed three times with distilled water and dried at room temperature. The absorbance was determined at 490 nm in an ELISA reader (BIO RAD MODEL 680). Uninoculated wells containing TSB broth with 0.24% glucose were used as controls. The tests were

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