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Communication

[*Comunicação*]

Detection of *icaA*, *icaD*, and *bap* genes and biofilm production in *Staphylococcus aureus* and non-aureus staphylococci isolated from subclinical and clinical bovine mastitis

[*Detecção dos genes icaA, icaD e bap e produção de biofilme por Staphylococcus aureus e estafilococos não-aureus isolados de mastite clínica e subclínica bovina*]

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Staphylococcus spp. are the most prevalent bacteria isolated from bovine mastitis. The pathogenesis of *Staphylococcus* spp. can be attributed to virulence factors responsible for facilitating adhesion within the mammary gland, causing chronic infections (Fox *et al.,* 2005). Non-aureus staphylococci (NAS) species are reported in the literature as minor pathogens involved in mastitis but are very frequently isolated in intrammamary infections (Taponen and Pyörälä, 2009). However, they have been isolated in cases of mastitis, even in farms that have adopted effective programs to control *S. aureus* and *Streptococcus agalactiae*.

The presence of biofilms has been considered the basis for persistence of chronic infections by these strains (Cucarella *et al.,* 2004). Studies have demonstrated that NAS species are able to form biofilms like *S. aureus* does, but they are less invasive (Taponen and Pyörälä, 2009). Biofilm formation involves two steps: the adhesion of bacteria on the surface of the mammary gland mediated by a capsular antigen called capsular polysaccharide/adhesin (PS/A), followed by accumulation, maturation, and separation phases associated with the production of polysaccharide intercellular adhesion (PIA), essential to the spread of the staphylococci (Simojoki *et al.,* 2012).

Cell proliferation and biofilm formation are known virulence factors mediated by the presence of the *ica* locus, comprisings four genes, *icaA*, *icaD*, *icaB*, and *icaC*, organized in an operon (*icaADBC*) with the regulatory gene *ica*R. This operon is responsible for expression of PIA, present in the cell wall. The surface protein BAP (biofilm-associated protein) is responsible for promoting the primary fixation in inert surfaces. The expression of *bap* leads to biofilm formation even without the presence of the *icaADBC* operon. In dairy herds, there is an evidence of the importance of *bap* in mastitis caused by *S. aureus* (Cucarella *et al.,* 2004).

The aim of the present study was to verify the biofilm production by *S. aureus* and NAS isolates derived from subclinical and clinical bovine mastitis cases and evaluate the presence of *icaA*, *icaD,* and *bap* in these isolates by PCR. Biofilm production was evaluated on congo red agar and microtiter plate. We also determined whether the presence of biofilm production genes influenced milk somatic cell count (SCC). This research was approved by the Ethics Committee on Animal Use of São Paulo State University (Unesp) CEUA 167/2014.

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The samples were isolated from milk obtained from two dairy farms in the state of São Paulo, Brazil. A total of nine monthly visits were performed in order to obtain 100 strains of *S. aureus* from subclinical mastitis cases and 100 strains of NAS, being 92 from subclinical mastitis cases and eight from clinical mastitis cases. A total of 749 samples of bovine milk were analyzed. The California mastitis test (CMT) was performed to detect subclinical mastitis. The SCC of the milk samples was performed by flow cytometry on Somacount 300® equipment.

Ten microliters of the milk samples were plated on 5% blood agar and MacConkey agar and incubated at 37°C under aerobic conditions for 24–48h. Only pure *Staphylococcus* colonies were used. All isolates were identified as genus *Staphylococcus* based on colony morphology, Gram staining, and catalase reaction. After confirmation of the genus *Staphylococcus*, the enzyme coagulase was characterized in all isolates in tubes using tube methods. Other coagulase-positive *Staphylococcus* species were differentiated from *S. aureus* by mannitol fermentation and susceptibility to 5µg of novobiocin and 300IU of polymyxin B. Non-aureus staphylococci was differentiated from *Kokuria* based on an oxidation test, fermentation of glucose, by resistance to bacitracin (0.04U) and by sensitivity to furazolidone (100mg) (Baker, 1984).

Biochemical tests were performed in order to identify *Staphylococcus* spp., including sugar fermentation (xylose, arabinose, sucrose, trehalose, maltose, mannitol, lactose, xylitol, ribose, fructose, and mannose), production of hemolysin, nitrate reduction, presence of urease and ornithine decarboxylase, and resistance to novobiocin. *DNA Extraction.* Bacterial DNA was obtained after cultivation of the bacteria in 5mL BHI (brain heart infusion). The extraction was performed with an Illustra Blood GenomicPrep Mini Spin Kit (GE Healthcare, Chalfont St. Giles, UK) according to the manufacturer's specification.

For amplification of *icaA* and *icaD* genes, primers and the polymerase chain reaction (PCR) protocol was performed according to Vasudevan *et al.* (2003). For the *bap* primers and gene amplification, the method described by Cucarella *et al.* (2004) was used. *Staphylococcus aureus* ATCC 25923 was used as positive control for amplification of the *icaA* and *icaD* genes. An isolate obtained in this study was used as a positive control for the *bap* gene, which was positive by PCR with amplification of a 971 bp product and sequenced (GenBank accession number AY220730). For a negative control, *S. epidermidis* ATCC 12228 was used for all reactions.

Qualitative detection of biofilm formation by the isolated strains was evaluated on CRA plates according Freeman *et al.* (1989). Biofilm production on MtP was analyzed according to Vasudevan *et al.* (2003) with minor modifications. All *Staphylococcus* spp. previously grown in BHI were standardized using the MacFarland scale (150×10^6 cells/mL). For each sample for both *S. aureus* and NAS, 200µL of the diluted bacteria were inoculated into four wells, including positive and negative controls, and non-inoculated BHI, in order to extract the values of the non-inoculated BHI after reading in optical density. The optical density was determined using an ELISA reader (Labsystems, Multiskan EX) with a 540nm filter. Strains that produced mean absorbance values greater than 0.1 were considered positive for biofilm production.

Frequency distributions were used to describe the percentage of strains of *S. aureus* that contain the genes responsible for biofilm production. The chi-square or Fisher's exact test were used to compare the proportions of strains producing biofilms with the intensity of the subclinical cases. Analysis of variance was used to compare the means of SCC values in logarithmic scale of basis 10 ($\log_{(10)}$ cells/mL) between strains with or without the identified genes. Statistical analyses were performed with SAS 9.2 with statistical significance determined at the level of 0.05.

A CMT score of 3 was more often found in *S. aureus* (62%) as well as in NAS (55%) (P=0.02). The average of the results obtained for SCC were higher for *S. aureus* isolates (5.88±0.59) than for NAS isolates (5.71±0.36) with P=0.02.

All samples were classified in the same way using two phenotypic tests. The 100 isolates of *staphylococci* isolated from mastitic milk were

tested for biofilm genes and subsequent biofilm formation. Among of 100 NAS isolates, the most frequently identified species was *Staphylococcus xylosus* observed for 51% isolates, and *Staphylococcus simulans* in 24% of isolates, followed by: *Staphylococcus warneri* (9%), *Staphylococcus haemolyticus* (5%), *Staphylococcus hominis* subsp. *hominis* (4%), *Staphylococcus lugdunensis* (3%), *Staphylococcus capitis* (1%), S*taphylococcus cohnii* subsp. *urealyticum* (1%), *Staphylococcus epidermidis* (1%), *Staphylococcus caprae* (1%). The distribution of the frequencies of *icaA*, *icaD*, and *bap* genes in *S. aureus* isolates were 82, 83, and 58%, respectively. Among the 100 isolates of *S. aureus*, 56% contained all three genes.

A total of 25 (25%) *S. aureus* isolates produced biofilms on CRA, and 17 (68%) of these had the *ica*AD genes. Both bright black colonies and black colonies were a positive result on CRA. Two isolates of *S. aureus* produced biofilm on CRA without the presence of any of the investigated genes. The positive rate on MtP was 15%. Of the 82 *S. aureus* isolates possessing the *ica*A gene in the genotypic test, only 13 (15.8%) were positive for biofilm formation using MtP. For the 83 strains with the *ica*D gene, the positive rate was 13 (15.6%). There was no association between the presence of *ica*AD genes and biofilm formation in CRA or MtP (P=1.0). There was no relationship between the presence of the *bap* gene and biofilm formation in the MtP assay once only 12 strains with *bap* gene (20.7%) showed biofilm formation (P=0.08).

A total of 8% of the NAS isolates produced biofilms on CRA and 41% on MtP; however, none of them possessed the investigated genes. According to Fisher's test, the ability of the isolates of NAS to form biofilm was not different when comparing the results from CRA and MtP ($P > 0.05$) The agreement between the two phenotypic tests was poor (K=- 0.11). From the 75 *S. aureus* isolates negative on CRA, 62 (82.7%) were also negative for the MtP test. Among the 15 positive isolates in the adhesion test, only two, which represented 13.3% of the isolates were also positive in CRA.

The primers used for detection of *ica*A and *ica*D were described previously by Vasudevan *et al.* (2003) and were also used by Simojoki *et al.* (2012). These primers work well for recognition of *S. aureus* strains but failed to recognize *S. epidermidis* (ATCC 35984), a strain known to be positive for both genes. In the present study, only one isolate of *S. epidermidis* was obtained, and it is inappropriate to affirm that the primer used by Simojoki *et al*. (2012) is ineffective for the other species obtained too.

Thus, it emphasizes the need for use of standard strains for all species isolated in this present study in order to confirm the affirmation obtained by Simojoki *et al*. (2012). The same authors did not find the *bap* gene in 84 samples of *Staphylococcus* spp. isolated from bovine mastitis.

Two strains of *S. aureus* were able to produce biofilms without the presence of the investigated genes. Chaieb *et al.* (2005) previously found a strain that was negative for both genes (*ica*A and *ica*D) but produced some black colonies on CRA. It became apparent to Chaieb *et al.* (2005) that biofilm formation may be associated with other factors, not only the presence of the *ica*A and *ica*D genes. It also suggests that other regulatory genes or other genes involved in biofilm formation may be present in these strains, stimulating the production of PIA or other surface proteins can contribute to bacterial adhesion.

The *bap* gene, carried by a transposon present in the mobile pathogenicity island SaPIbov2 present only in *S. aureus* isolates of bovine origin, contributes to the pathogenic roles in persistent infections (Cucarella *et al.,* 2004). The presence of the *bap* gene confers chronicity to cases of mastitis, but *S. aureus* can form biofilms even without *bap*. These species may possess virulence factors, such as the expression of genes like *agr* that regulate staphylococcal membrane proteins that are able to promote the adhesion of these microorganisms to inert surfaces (Vautor *et al.,* 2007).

In the present study, the higher positive rate obtained for the *S. aureus* strains was on CRA compared to the MtP test, with 25% and 15%, respectively. This result agrees with the study by Los *et al.* (2010), which compared the results of positive strains on CRA and MtP, obtaining 58.9% positive on CRA and 35.6% on MtP. In Brazil, Costa Krewer *et al.* (2015) obtained positivity of 3.7% of isolates of *S. aureus*, other

coagulase-positive staphylococci and NAS on CRA and 96.3% of *Staphylococcus* spp. positives on MtP. The agreement between the phenotypic tests evaluated in this study was poor, with only two positive samples of *S. aureus* in both tests.

It is noteworthy that in the present study, biofilm production was evaluated by two phenotypic tests. Other methods of identifying strains of biofilm-positive staphylococci can be used, such as fluorescent in situ hybridization protocol (Simojoki *et al*., 2012).

The present study demonstrated that biofilm production in NAS isolates may be associated with factors other than the *icaA*, *icaD,* and *bap* genes, stimulating biofilm production *in vitro*. We did not observe a relationship between the presence of surveyed genes and SCC in *S. aureus* samples. Further studies can be performed in order to elucidate other factors involved in biofilm formation in NAS species.

Keywords: Staphylococcus aureus, non-aureus staphylococci, red agar congo, bovine mastitis

RESUMO

Algumas espécies de Staphylococcus *causam infecções crônicas intramamárias e podem levar à formação de biofilme. No presente estudo, levantou-se a hipótese de que as espécies de* Staphylococcus *isolados da mastite bovina são capazes de formar biofilme* in vitro *associado à presença dos genes* icaA, icaD *ou* bap. *Um total de 200 isolados de* Staphylococcus*, sendo 100* Staphylococcus aureus *de casos de mastite subclínica e 100 estafilococos não aureus (ENA) de casos de mastite subclínica e clínica, obtidos em duas fazendas leiteiras, no estado de São Paulo, foram avaliados quanto à capacidade de produzir biofilmes* in vitro*. A presença de* icaA*,* icaD *e* bap *foi confirmada por PCR, e a produção de biofilme em ágar vermelho congo (Congo Red Agar – CRA) e em teste de microplaca (Microtiter Plate – MtP) foi avaliada nos isolados de* S. aureus *e ENA. Os resultados mostraram a presença dos genes* icaA*,* icaD *e* bap *em* S. aureus*, mas não em ENA. A produção de biofilme pode estar associada à presença de outros fatores ou genes que estimulam a produção de biofilme* in vitro*. O ensaio de MtP serve como um modelo quantitativo para o estudo da aderência de espécies de estafilococos associados à mastite bovina.*

Palavras-chave: Staphylococcus aureus*, estafilococos não aureus, ágar vermelho congo, mastite bovina*

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