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Characterization of biofilms and antimicrobial resistance of coagulase-negative *Staphylococcus* species involved with subclinical mastitis

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Research Article

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Abstract

Biofilm formation is a central feature to guarantee staphylococcal persistence in hosts and is associated with several diseases that are difficult to treat. In this research paper, biofilm formation and antimicrobial susceptibility were investigated in staphylococcal strains belonging to several species. These strains were isolated from the milk of cows with subclinical mastitis and most of them were coagulase-negative, with the prevalence of *Staphylococcus chromogenes*. High genetic diversity was observed among the strains by pulsed field gel electrophoresis. Antimicrobial resistance was assessed by disk diffusion and more than 50% of the strains were resistant to ampicillin and penicillin G, with multi-resistance profiles (13.6%) also being observed. Most strains (65.9%) formed biofilms when cultivated in BHI supplemented with 1% glucose. Most strains (72.7%) carried the intercellular adhesion gene (*icaA),* while less than half (36.3%) carried the biofilm-associated protein gene (*bap*). Concentrations of up to 10xMIC of erythromycin and tetracycline were not sufficient to suppress cell viability in preformed biofilms. Our results revealed that a genetically diverse group of biofilm-forming *Staphylococcus* species can be involved in subclinical mastitis. Since high antimicrobial concentrations cannot eradicate biofilm cells *in vitro*, their use in dairy animals may be ineffective in controlling infections, while supporting selection of resistant microorganisms. These data reinforce the need for alternative therapies aiming at disrupting biofilms for effective disease control.

Unlike the clinical form, subclinical mastitis does not cause significant changes in milk appearance, nor does it show the typical signs of inflammation in infected udders. However, in addition to increasing somatic cell counts, milk production decreases, making this form of mastitis responsible for most of the economic losses in the dairy industry, due to its high prevalence (Busanello *et al*., 2017). Studies have shown that the prevalence of subclinical mastitis in dairy cows is close to 48% on Brazilian farms (Acosta *et al*., 2016; Busanello *et al*., 2017).

Staphylococcus aureus is one of the main causes of this disease (Gomes *et al*., 2016) and the severity of mastitis caused by this bacterium is related to the production of several virulence factors involved in tissue adhesion, evasion of host defenses and injuring host cells (Cote-Gravel and Malouin, 2019). The frequency of *S. aureus* in Brazilian dairy farms is over 70% (Mesquita *et al*., 2019) and bacteria of this genus can represent 1 to 90% of the microorganisms causing both clinical and subclinical mastitis. However, coagulase negative staphylococci (CoNS) exceed the frequency of *S. aureus* causing the disease in all regions of Brazil (Acosta *et al*., 2016).

The persistence of CoNS is related to the high levels of antimicrobial resistance genes carried by them, and to the fact that many strains remain in the form of biofilms, making infections difficult to eradicate and, therefore, recurrent (Becker *et al*., 2014; Marsilio *et al*., 2018). The dense matrix-embedded communities of biofilms makes it difficult for antibiotics to reach their targets and provides an environment of increased availability of mobile genetic elements, through which antibiotic resistance can be exchanged between staphylococci (Gomes *et al*., 2016; Rossi *et al*., 2020).

The development of biofilms by *S. aureus* is influenced by the production of the multidomain and cell-surface anchored *biofilm-associated protein* (Bap) and is dependent on the synthesis of the polysaccharide intercellular adhesin (PIA), encoded by the *icaADBC* operon (Cucarella *et al*., 2001; O'Gara, 2007). Even though CoNS are the main cause of bovine subclinical mastitis in Brazil, the vast majority of studies still focus on the clinical form of the disease and on *S. aureus* isolates. In this study we aimed to characterize biofilm formation by different

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Staphylococcus species, mainly CoNS, isolated from bovine subclinical mastitis, analyzing the genetic diversity between the strains, the distribution of the *bap* and *icaA* genes and the effect of biofilm establishment on antimicrobial resistance.

Materials and methods

Microorganisms and culture conditions

We studied a diverse collection of *Staphylococcus* strains ($n = 44$) isolated from 22 different farms that comprise the main milk-producing areas of southeastern Brazil (online Supplementary Table S1), provided by EMBRAPA Dairy Cattle (Juiz de Fora, MG, Brazil). All strains were isolated from the milk of cows with subclinical mastitis, according to recommendations of the National Mastitis Council (Oliver *et al.*, 2004). These strains belong to eight species, identified by RFLP-PCR of the *groEL* gene (Santos *et al.*, 2008): the coagulase-positive *S. aureus* ($n = 5$) and *S. hyicus* ($n = 1$), and the coagulase-negative *S. capitis* ($n = 3$), *S. caprae* ($n = 1$), *S. chromogenes* ($n = 27$), *S. epidermidis* ($n = 4$), *S. haemolyticus* ($n = 2$), and *S. xylosus* ($n = 1$). Culture stocks maintained in Brain Heart Infusion (BHI, Difco, USA) and glycerol 30% at −20°C were activated on blood agar plates and inoculated in BHI and incubated at 37°C for 24 h before the experiments.

Pulsed-field gel electrophoresis

The clonal diversity of the strains was evaluated by means of their chromosomal restriction band patterns, produced after digestion with the restriction enzyme *Sma*I, separated by pulsed-field gel electrophoresis (PFGE), as suggested by Nunes *et al.* (2005). Restriction band patterns were analyzed visually, as recommended by van Belkum *et al.* (2007), and with the *Gel Compar II* software *version 4.01* (Applied Maths, Belgium) using the unweighted pair-group method with arithmetic mean (UPGMA) algorithm and the Jaccard coefficient. Strains that were the only ones in their species were not included in the analysis.

Biofilm formation assay

Biofilm formation by the strains was evaluated by the crystal violet method, as described by Ahn *et al.* (2005). Briefly, overnight cultures were diluted (1:50) in fresh BHI and grown at 37°C to an $OD_{600\ nm}$ of 0.4. Then, the cultures were diluted (1:100) in BHI supplemented with 1% glucose in polystyrene microplates to a final volume of 200 µl. After incubation at 37°C for 24 h, planktonic cells were removed, cells that were adhered to the wells were gently washed three times with distilled water and the plates were dried at 60°C for 1 h. The adherent bacteria were stained for 15 min with 0.1% crystal violet, and the microtiter plates were gently immersed in water to rinse the wells, followed by drying at 60°C for 30 min. Stained cells were resuspended in 200 µl of ethanol and the $OD_{570\ nm}$ was measured. Tests were performed in triplicate. Strains were classified as non, weak, moderate or strong biofilm producers, as suggested by Stepanovic *et al.* (2007).

Detection of bap and icaA genes by PCR

Total DNA was extracted from all strains by the guanidinium thiocyanate method (Pitcher *et al.*, 1989). The genes *bap* and *icaA* were amplified with the oligonucleotides bap2F/bap2R and icaAF/icaAR, respectively (Potter *et al.*, 2009). Amplification was performed with 100 ng of DNA, 0.2 mM of each primer, 0.5 mM of each dNTP, 1.5 mM $MgCl_2$, and 1 U of *Taq* DNA polymerase in amplification buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4, Biotools, USA). Amplicons (598 bp for *bap* and 287 bp for *icaA*) were observed after electrophoresis on 1.5% agarose gel and staining with ethidium bromide 0.5 µg/ml. *S. epidermidis* ATCC 35 984 was used as a control for *icaA* amplification (Arciola *et al.*, 2001), and *S. aureus* V329, as a control for *bap* amplification (Cucarella *et al.*, 2001).

Detection of bap and icaA genes by dot blotting

The detection of the *bap* and *icaA* genes was complemented by dot blotting. The probe consisted of PCR products obtained for each gene from their respective positive controls (described above), which were purified with the *QIAquick PCR purification kit* (QIAgen, Germany), and tagged with digoxigenin using the *Dig DNA Labeling and detection kit* (Roche, Switzerland), according to the manufacturer's instructions. Total DNA of all strains were applied separately as dots to a *Hybond N^+ nylon membrane* (GE Healthcare Life Sciences, USA) and fixated by UV radiation, following the procedures suggested by Sambrook and Russell (2001). For *icaA* probes, hybridization was performed at 56°C and washes were carried out under high-stringency conditions; for *bap* probes, hybridization was performed at 46°C and under low-stringency conditions. The same positive controls mentioned above were used in this procedure.

Antimicrobial resistance tests

The antimicrobial susceptibility of the strains was tested in triplicate by the disk diffusion method, against the following antimicrobial agents (Cecon, Brazil): ampicillin (10 µg), cefoxitin (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), clindamycin (2 µg), erythromycin (15 µg), gentamicin (10 µg), oxacillin (1 µg), penicillin G (10 UI), rifampicin (5 µg), trimethoprim-sulfamethoxazole (23.75/1.25 µg), tetracycline (30 µg) and mupirocin (200 µg). The minimum inhibitory concentrations (MIC) of erythromycin and tetracycline for selected strains were determined by the broth microdilution method. Tests were performed and interpreted according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2016).

Effects of minimum inhibitory concentrations on preformed biofilms

Strong biofilm-producing strains were selected to evaluate the effect of the antibiotics erythromycin and tetracycline on biofilm suppression, following the procedures suggested by Flemming *et al.* (2009). Biofilms were preformed as described above, and incubated for 24 h and 48 h. After incubation, the culture medium was removed from the wells and replaced by 200 µl of fresh Mueller Hinton broth (Difco, USA) containing concentrations of each antibiotic equivalent to their respective MIC, 5xMIC and 10xMIC. Microplates were incubated again for 24 h at 37°C. Then, the supernatant was removed, and wells were carefully washed twice with sterile PBS. To quantify viable cells, we added 200 µl of BHI supplemented with 1% glucose and 5% of the redox indicator *AlamarBlue* (Invitrogen, USA). After 1 h of incubation at 37°C, the $OD_{570\ nm}$ was measured.

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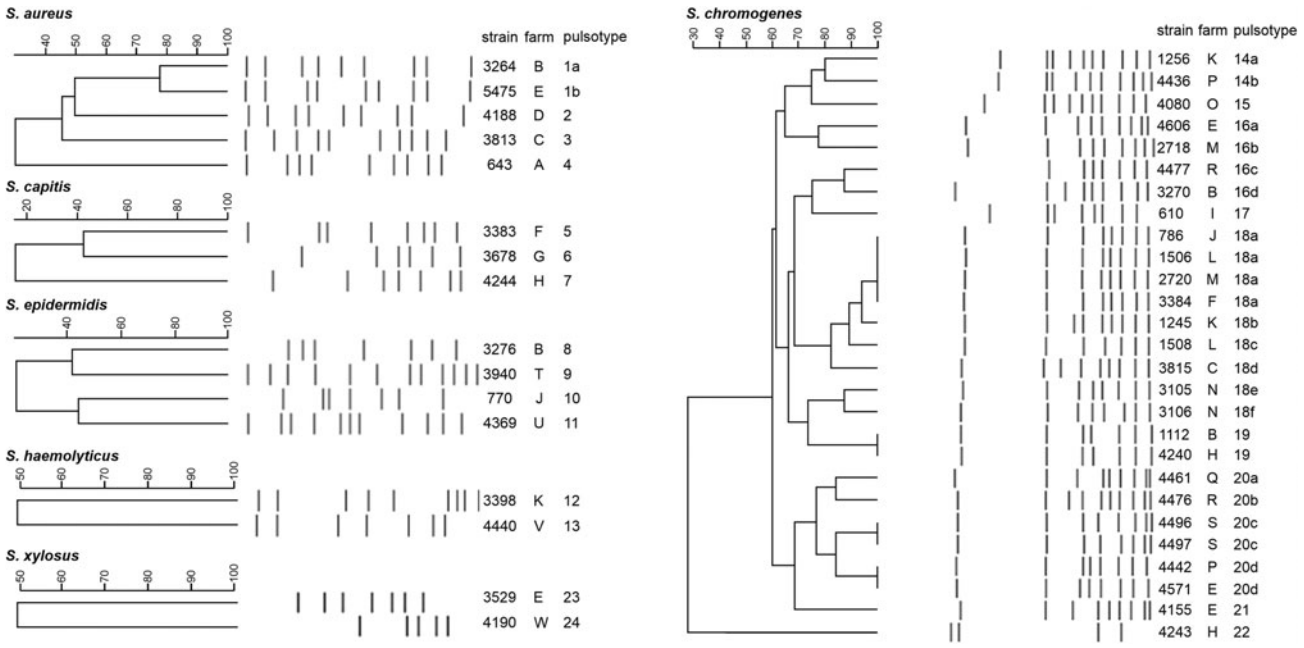

Fig. 1. Pulsed-field gel electrophoresis (PFGE) profiles of the genomic DNA of *Staphylococcus* strains causing subclinical mastitis, digested with the *Sma*I restriction enzyme.

The negative control (NC) of these experiments consisted only of culture medium, while the positive control (PC) consisted of biofilms that were not subjected to antimicrobial treatment. Tests were performed in triplicate and results were interpreted as follows: strong biofilm suppression if the antimicrobial caused a ≥75% reduction in $OD_{570\ nm}$ when compared to the PC; complete suppression if the antimicrobial caused a reduction ≤ the NC, and no suppression if none of the previous criteria were met.

Statistical analysis

The Spearman's correlation and the χ^2 tests were performed to investigate if there was a correlation between the presence of *bap* or *icaA* genes and biofilm formation. All analyses were done using Graphpad Prism 6 (available at https://www.graphpad.com/) and the degree of statistical significance was 95% ($P < 0.05$).

Results

Clonal diversity of strains

The strains studied here comprise a genetically diverse group, with 24 different genotypes and 18 subtypes obtained by PFGE (Fig. 1). *S. chromogenes*, the largest group, included nine pulsotypes and 16 subtypes. Considering them, only two strains (4496 and 4497, possibly siblings) isolated from the same farm (S) were classified with the same pulsotype (20C). Two strains from the N farm were classified with pulsotypes 18d and 18e, and showed a similarity closer to 80%. Pulsotype 18 was the most common among *S. chromogenes*. Considering the other species, only two isolates of *S. aureus*, classified as pulsotypes 1a and 1b, obtained from different farms, showed approximately 80% similarity (strains 3264 and 5475).

Phenotypic and genetic characteristics of biofilms

Most strains studied (65.9%) formed biofilms in the conditions tested, but at different levels (Fig. 2). They were classified as strong (29.5%), moderate (15.9%), weak (20.5%) or non-biofilm producers (34.1%). Regardless of their ability to form biofilms, most strains harbored the *ica*A gene (72.7%), while 36.3% contained the biofilm-associated *bap* gene. PCR results were confirmed by dot blotting, but the second was more sensitive (Fig. 2); while 15.9% of strains were PCR-positive for *icaA*, 63.6% were positive by dot blotting. The same was observed for the *bap* gene; 13.6% were PCR-positive, 34.1% were positive by dot blotting. There was no statistically significant ($P < 0.05$) correlation between biofilm formation and the presence of *bap* and *icaA* gene in these strains, as non-biofilm producers, such as *S. aureus* 643, *S. capitis* 4244 and *S. chromogenes* 610 were positive for both genes, while strong biofilm producers, like *S. haemolyticus* 3398 and 4440 were negative for both.

Antimicrobial resistance profile

More than half (56.8%) of the strains were resistant to at least one of the 13 antimicrobials tested (Fig. 2). Resistance to penicillin (54.5%) and ampicillin (52.3%) were the most common. Additionally, 13.6% of the strains were classified as multidrug resistant due to resistance to at least three classes of antibiotic. All strains were susceptible to rifampicin.

Based on their strongly attached biofilms and antimicrobial resistance profiles, we chose four strains to conduct the subsequent experiments: *S. capitis* 3383, *S. chromogenes* 4442 and *S. haemolyticus* 3398, which were susceptible to all antimicrobials tested, and *S. epidermidis* 770, resistant to ampicillin, penicillin G and tetracycline. For trials with preformed biofilms and drugs, erythromycin and tetracycline were chosen given the low resistance rate among the strains, 15.9% and 11.3%, respectively. Erythromycin MIC ranged from 0.25 to 0.5 µg/ml and tetracycline MIC ranged from 0.03125

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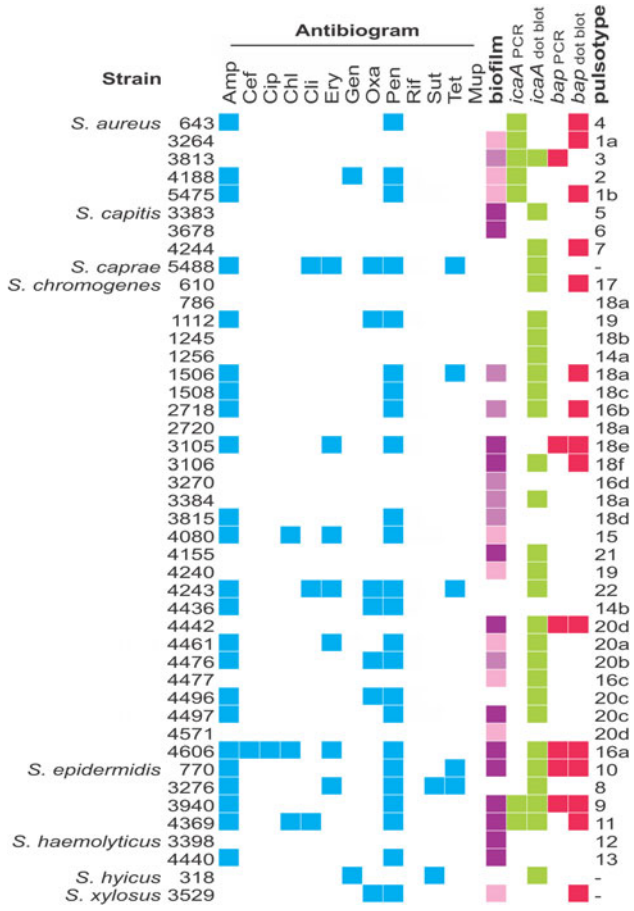

Fig. 2. Characterization of the *Staphylococcus* strains used in this work. Blue squares show resistance to the antimicrobials used; green and pink squares designate positive results for the presence of *icaA* and *bap* genes, respectively. Classification of biofilm formation is displayed in different shades of purple, which represent, from the lightest to the darkest tone, weak, moderate and strong biofilm formation. Pulsotypes were defined based on PFGE patterns, analyzing each species separately. Antimicrobials: ampicillin (Amp), cefoxitin (Cef), chloramphenicol (Chl), ciprofloxacin (Cip), clindamycin (Cli), erythromycin (Ery), gentamicin (Gen), oxacillin (Oxa), penicillin G (Pen), rifampicin (Rif), trimethoprim-sulfamethoxazole (Sut) and tetracycline (Tet).

to 0.125 µg/ml for susceptible strains; for the tetracycline-resistant *S. epidermidis* 770, MIC was 64 µg/mL (Table 1).

Effects of MIC, 5MIC and 10xMIC on preformed biofilms

We did not observe any complete or strong suppression (over 70%) of the viability of bacterial cells in their biofilms using inhibitory concentrations of erythromycin or tetracycline (Table 1). In biofilms, all strains tested were able to grow at the concentrations of their respective MICs and concentrations of 5x and 10x their MICs. The highest rates of biofilm suppression were observed in younger (24 h) biofilms of *S. epidermidis* 770 using different concentrations of tetracycline (35% for MIC, 52% for 5xMIC and 55% for 10xMIC). In general, suppression rates were higher as the concentrations of antibiotics increased, when considering biofilms of the same age (Table 1).

Discussion

Although subclinical mastitis is highly prevalent in dairy herds and is caused mainly by coagulase-negative staphylococci, most studies still focus on *S. aureus* and the clinical form of the disease, with many of them not even addressing the species of CoNS involved in the infectious processes (Acosta *et al.*, 2016; Busanello *et al.*, 2017). While this must be related to the fact that CoNS have historically been referred to as less pathogenic, since they lack the most of the virulence factors of *S. aureus,* the realization that CoNS can cause several diseases suggests the opposite may be true (Becker *et al.*, 2014).

In addition to being the main cause of subclinical mastitis, studies have shown that CoNS species can act as reservoirs of antimicrobial resistance genes, which can be transferred to more pathogenic species, such as *S. aureus*, thus increasing their potential for resistance to drug therapy. This, therefore, represents a major threat (Coimbra-e-Souza *et al.*, 2019; Rossi *et al.*, 2020). The exchange of genetic material is greatly facilitated by bacterial adhesion to biotic and abiotic surfaces, forming biofilms, that is, dense communities embedded in a self-produced matrix of exopolysaccharides (Madsen *et al.*, 2012). The high cell density of biofilms leads to increased concentration of exogenous DNA, enhanced cell competence to receive mobile genetic elements, and stabilization of cell-cell contact by the matrix, which may facilitate horizontal gene transfer and dispersion of antimicrobial resistance, while protecting bacteria from hosts' immune defenses and environmental stresses (Flemming *et al.*, 2016).

In this study, we analyzed the phenotypic and genotypic characteristics of biofilm production by a collection of staphylococcal isolates causing subclinical mastitis, most of which are CoNS. Their diversity was verified by PFGE, and is explained by the fact that small independent dairy farmers are responsible for most of the milk produced in Brazil, which increases diversity, while restricting strains to the animals of the same farm (Balcão *et al.*, 2016). Many of the strains studied in this work are resistant to multiple drugs, as they have acquired non-susceptibility to at least one agent in three or more categories of antimicrobials. This may be a consequence of the prophylactic use of antimicrobial drugs, a common practice in animal production in several countries, with the aim of promoting growth or preventing the development of diseases when animals are subjected to stressful conditions (Aarestrup, 2015). Resistance to oxacillin and cefoxitin is particularly alarming, since it indicates that strains are resistant to methicillin and cannot be combatted with last-resort antibiotics. In addition, resistance to most of the other antibiotics is frequently associated with the acquisition of genes located in mobile genetic elements, as recently reviewed by Schwarz *et al.* (2018), which can be exchanged in biofilms (Rossi *et al.*, 2020).

As noted here, most *Staphylococcus* involved with subclinical mastitis can form biofilms. While biofilm formation is known to be a multifactorial feature, it is strongly related to the production of two of the most studied factors: (i) the surface protein Bap (biofilm-associated protein) and (ii) the products of the *ica* operon, which coordinate the synthesis and export of the most important adhesive molecule of staphylococcal biofilms, the (exo)polysaccharide intercellular adhesion (PIA), also called poly-N-acetylglucosamine or PNAG (Otto 2013; Salina *et al.*, 2020). Even though *bap* and *icaA* are widespread among staphylococci, we found no significant correlation between genotype (the presence of these genes) and phenotype (biofilm production). This lack of correlation has been reported before for CoNS causing mastitis in dairy animals (Coimbra-e-Souza *et al.*, 2019), and indicates that other factors must be involved in biofilm formation by non-*aureus* staphylococcal species. These factors can include extracellular DNA (eDNA) released

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Table 1. Minimum inhibitory concentration and biofilm suppression by erythromycin (Ery) and tetracycline (Tet) in *Staphylococcus* strains

			Biofilm suppression (%)											
			Ery						Tet					
	MIC (µg/ml)		MIC		5xMIC		10xMIC		MIC		5xMIC		10xMIC	
Strain	Ery	Tet	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
S. capitis 3383	0.5	0.125	–	18	–	23	–	25	–	7	–	–	–	13
S. chromogenes 4442	0.25	0.03125	–	–	–	4	–	9	18	2	18	3	30	15
S. epidermidis 770	0.5	64	3	11	9	14	23	18	35	–	52	32	55	38
S. haemolyticus 3398	0.5	0.0625	3	2	3	4	7	18	9	–	22	–	29	–

from lysed bacteria and several other adhesive proteins, such as the accumulation-associated protein (Aap), protein A, fibrinogen-binding proteins FnbpA and FnbpB, among others (Otto, 2013). In addition, discrepancies between the detection of *bap* and *icaA* by PCR and dot blotting indicate that these genes may present polymorphisms among staphylococcal species, and the lack of sequences available for understudied CoNS makes it difficult to elaborate universal molecular markers for screening these genes. Most of the dozens of *icaA* sequences currently available on Genbank were obtained from *S. aureus*, *S. epidermidis* and *S. haemolyticus* and have only 75% of identity (data not shown), and there are only a few complete *bap* sequences available, mostly from *S. aureus*.

Although we still do not fully understand the nature of biofilms formed by non-*aureus Staphylococcus* species, here we showed that its production is an important protective barrier for CoNS, interfering with the activity of erythromycin and tetracycline, since a concentration equivalent to 10 × of each antibiotic MIC was not enough to suppress cell viability. Biofilms can act as a barrier to prevent antibiotics from reaching their molecular targets by various mechanisms, including drug chelation and precipitation, modification and degradation of the antibiotic, and tolerance to due to slower growth (Flemming *et al.*, 2016).

In conclusion, our study shows that a variety of *Staphylococcus* species are involved in subclinical mastitis, most of which can form biofilms and carry a high rate of resistance to ampicillin and penicillin G, but also to several other antibiotics commonly associated with mobile genetic elements in *Staphylococcus*. This work also reinforces the concern that high concentrations of antimicrobials may not be sufficient to eradicate biofilm cells, so their use in dairy animals may not only may be ineffective in controlling infections, but also may support selection of resistant microorganisms. Although biofilms do play an important role in the development of infections in mammary glands (Gomes *et al.*, 2016), *in vivo* studies with pathogens causing bovine mastitis are challenging and limited, therefore, we do not know to what extent the results obtained here *in vitro* translate *in vivo*. Nevertheless, new studies and searching for alternative therapies capable of disrupting biofilms are needed and should be encouraged.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029921000285

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