Biofilm-producing ability of *Staphylococcus aureus* isolates from Brazilian dairy farms

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ABSTRACT

This study aimed to investigate the in silico biofilm production ability of *Staphylococcus aureus* strains isolated from milking parlor environments on dairy farms from São Paulo, Brazil. The *Staph. aureus* isolates were obtained from 849 samples collected on dairy farms, as follows: milk from individual cows with subclinical mastitis or history of the disease ($n = 220$); milk from bulk tank ($n = 120$); surfaces of milking machines and utensils ($n = 389$); and milk handlers ($n = 120$). Thirty-one *Staph. aureus* isolates were obtained and categorized as pulsotypes by pulsed-field gel electrophoresis and submitted to assays for biofilm formation on polystyrene, stainless steel, rubber, and silicone surfaces. Fourteen (45.2%) pulsotypes were considered producers of biofilm on the polystyrene microplate assay, whereas 13 (41.9%) and 12 (38.7%) pulsotypes were biofilm producers on stainless steel and rubber, respectively. None of the pulsotypes evaluated produced biofilms on silicone. Approximately 45% of *Staph. aureus* pulsotypes isolated from different sources on dairy farms showed the ability to produce biofilms in at least one assay, indicating possible persistence of this pathogen in the milking environment. The potential involvement of *Staph. aureus* in subclinical mastitis cases and its occurrence in milk for human consumption emphasize the need to improve hygiene practices to prevent biofilm formation on the farms studied.

Key words: *Staphylococcus aureus*, biofilm, milk, persistence

INTRODUCTION

Staphylococcus aureus is recognized worldwide as a frequent cause of subclinical IMI in dairy cows (Coelho

et al., 2011), and different sources of *Staph. aureus* in the dairy cow environment have been described (Zadoks et al., 2002). Intramammary infections in dairy cows are a major concern for the dairy industry, resulting in important loss of milk and suffering for the animals. They may also be potentially fatal, with major costs for dairy farmers. It is assumed that IMI are the result of cow-to-cow transmission, where infected animals in the herd, workers, and equipment and utensils used for milking are the main sources of the microorganism (De Vliegher et al., 2012).

Biofilms are structured communities of bacterial cells enclosed in a self-produced polymeric matrix and adhered to inert or living surfaces (Costerton et al., 1999). They are formed by adherence of the bacteria to the surface, followed by accumulation, maturation, and detachment, which are essential for dissemination and persistence of staphylococci (Fey and Olson, 2010). Biofilm formation is a potential virulence factor (Almeida and Oliver, 2001), especially for *Staph. aureus*; the ability to attach to surfaces has been demonstrated in the milk-processing environment, such as on rubber and stainless steel (Czechowski, 1990). These surfaces can be contaminated by spoilage or pathogenic bacteria that, under certain conditions, adhere to those surfaces, initiating cell growth and leading to biofilm formation (Fox et al., 2005). Thus, the present study aimed to evaluate the ability of *Staph. aureus* isolates obtained from milk and surfaces of the dairy environment to adhere to and produce biofilms on stainless steel, polystyrene, silicone, and rubber surfaces, as these materials are widely used in the milking environment as well in dairy plants.

MATERIALS AND METHODS

Sampling Design and Isolation of *Staphylococcus aureus*

Two hundred twenty samples of milk from individual cows (cows with subclinical mastitis or history of mas-

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titis), 120 samples of bulk tank milk, 389 swabs from milking machines and utensils (buckets and sieves), and 120 samples from workers' hands in 10 dairy farms located in the State of São Paulo, Brazil, were analyzed. The samples were collected monthly in the farms from August 2010 to January 2011. Samples were transported to the laboratory in coolers with ice ($5.0 \pm 1.2^\circ\text{C}$), and analyzed on the same day.

Staphylococcus aureus strains were detected and identified according to Lancette and Bennett (2001) by spread plates of Baird Parker agar with appropriate dilutions (0.1 mL), incubated at 35°C for 48 h. Black colonies with 2 halos were considered presumptive of *Staph. aureus*. These presumptive strains were confirmed by Gram staining, catalase reaction, clotting of rabbit plasma, presence of clumping factor (Staphyclin test; Laborclin, Pinhais, Brazil), and acetoin production. After identification, the isolates were maintained at -80°C until pulsotype determination by molecular typing using pulsed-field gel electrophoresis according to McDougal et al. (2003). Gels were analyzed using computer software (BioNumerics, version 6.1; Applied Maths Inc., Austin, TX) to define pulsotypes, as described by Lee et al. (2012).

Biofilm Formation Assays

The ability of the *Staph. aureus* pulsotypes to produce biofilms on flat-bottomed polystyrene microplates was determined according to the protocol described by Stepanović et al. (2000). Briefly, each pulsotype was resuspended in tubes containing tryptone soy broth (TSB; Oxoid Ltd., Basingstoke, UK), incubated at 37°C for 24 h and diluted until a concentration of 10^8 cells/mL (0.5 on MacFarland scale) was reached. Triplicate aliquots of 200 μL of each bacterial suspension were transferred to 3 wells of a sterile, 96-well, flat-bottomed plastic microplate and incubated at 37°C for 24 h. After incubation, the microplates were shaken, fixed, stained using crystal violet for 5 min, dried, and resuspended with 33% (vol/vol) glacial acetic acid. Biofilm formation in the microplates was measured in an ELISA reader set at 570 nm, and values were expressed in optical density values (OD). Triplicate negative controls with only sterile TSB were prepared exactly as described for the pulsotypes (Stepanović et al., 2000) and used as reference to determine the ability of *Staph. aureus* pulsotypes to produce biofilms. The mean OD value of negative controls (OD_{NC}) was 0.160 ± 0.021 . Pulsotypes were considered biofilm producers when their OD values were 3 times greater than standard deviation of the mean OD_{NC} . A positive control (*Staphylococcus epidermidis* ATCC 35.983) was also used. Additionally, pulsotypes showing ability to

produce biofilms were classified as weak ($\text{OD}_{\text{NC}} < \text{OD} \leq 2 \times \text{OD}_{\text{NC}}$), moderate ($2 \times \text{OD}_{\text{NC}} < \text{OD} \leq 4 \times \text{OD}_{\text{NC}}$) or strong ($\text{OD} > 4 \times \text{OD}_{\text{NC}}$) biofilm producers, according to Stepanović et al. (2000).

The ability to produce biofilms on stainless steel, rubber, and silicone surfaces was evaluated by epifluorescence microscopy using calcofluor white dye, as recommended by Shanks et al. (2005). Two square pieces (1.0×1.0 cm), one of stainless steel and one of rubber, and one silicone disc (diameter: 1.0 cm) were placed at the bottom of 3 wells of a 96-well flat-bottomed plastic microplate. Next, 2 mL of each TSB bacterial suspension were placed in each of the 3 wells and incubated at 35°C for 48 h without stirring. After incubation, the stainless steel and rubber coupons and silicone discs were removed from the wells with sterile forceps, rinsed with sterile water, placed on a glass slide, and allowed to dry at room temperature. One drop of calcofluor solution (0.05%, wt/vol) was added to each of the surfaces. The visualization of biofilms on stainless steel, rubber, and silicone discs was performed in an optical epifluorescence microscope (Olympus, Tokyo, Japan), with the wavelength set at 430 nm. *Staphylococcus epidermidis* (ATCC 35.983) and *Staph. epidermidis* (ATCC 12.228) strains were used as positive and negative controls, respectively, and served as the basis for image analysis using computer software (DP2-BSW, version 2.1; Olympus).

RESULTS AND DISCUSSION

Table 1 shows the ability of the 31 *Staph. aureus* pulsotypes to produce biofilms in the polystyrene microplate assays. Seventeen pulsotypes (54.8%) did not produce biofilms, whereas 14 (45.2%) pulsotypes were classified as weak ($n = 9$), moderate ($n = 2$), or strong ($n = 3$) biofilm producers, respectively. Biofilm production is recognized as an important virulence factor for bacteria of the genus *Staphylococcus* (Cucarella et al., 2002; Vasudevan et al., 2003; Fox et al., 2005). Hence, formation of biofilms by *Staph. aureus* is a major concern for the dairy industry and is frequently associated with a lack of monitoring of operational standards established for processing milk (Zadoks et al., 2002). The high frequency of *Staph. aureus* biofilm-producing isolates highlights the need for constant improvement of quality assurance systems in the dairy farms evaluated.

Although we conducted no genetic evaluation in the present study, previous reports have shown that the *IS257* gene is related to in vitro biofilm formation by *Staph. aureus* isolates from cows with mastitis, with 50% prevalence. Similarly, the *ica* gene cluster acts directly on the production of an extracellular adhesin polysaccharide, which is associated with pathogenic

Table 1. Biofilm formation by *Staphylococcus aureus* pulsotypes in the microplate assay¹

Category ²	No. (%) of pulsotypes	Optical density
Biofilm nonproducers	17 (54.8)	0.136 ± 0.017
Biofilm producers		
Weak	9 (29.0)	0.203 ± 0.040
Moderate	2 (6.5)	0.420 ± 0.039
Strong	3 (9.7)	0.695 ± 0.104

¹n = 31 pulsotypes, as defined by pulsed-field gel electrophoresis; values are expressed as mean ± SD of 3 replicates of each pulsotype.

²Classified by comparison of each pulsotype optical density (OD) with the mean OD value of negative controls (OD_{NC} = 0.160 ± 0.021), according to Stepanović et al. (2000), as follows: nonproducers (OD ≤ OD_{NC}), weak (OD_{NC} < OD ≤ 2×OD_{NC}), moderate (2×OD_{NC} < OD ≤ 4×OD_{NC}), or strong (OD > 4×OD_{NC}) biofilm producers.

Staph. aureus infections (Melchior et al., 2011). The *ica* gene was also present in *Staph. aureus* strains isolated from cows with mastitis in eastern Poland, showing a prevalence of 100% in all strains classified as biofilm producers (Szweda et al., 2012).

None of the *Staph. aureus* pulsotypes evaluated produced biofilm on silicone. *Staphylococcus aureus* pulsotypes isolated from different sources on dairy farms that showed ability to produce biofilms on microplates, stainless steel, and rubber are presented in Table 2. Thirteen (42.0%) pulsotypes were biofilm producers on stainless steel, and 12 (38.7%) produced biofilms on rubber. Only pulsotype P21, derived from an isolate from bulk tank milk and an isolate from a milking machine, produced biofilms in the polystyrene microplate, stainless steel, and rubber assays. Approximately 42% of the pulsotypes obtained from bulk tank milk, milk from individual cows, and environmental samples were biofilm producers in the stainless steel and rubber assays. However, many *Staph. aureus* pulsotypes from bulk tank milk or milk from individual cows were biofilm producers in at least one assay. This fact is of public health concern because it indicates a potential source for persistence of *Staph. aureus* contamination

on the dairy farms evaluated and emphasizes the difficulties in cleaning procedures of the milking equipment.

Possible explanations for the absence of biofilm formation on silicone, as observed in the present study, are difficult to assess at this time, because the initial adhesion of bacteria onto inert surfaces is a complex process resulting from physicochemical interactions between different components, including material surface properties, surface properties of bacteria, and environmental factors (An and Friedman, 1998; Oliveira et al., 2003). Silicone and stainless steel have similar hydrophobic characteristics (Ksontini et al., 2013); therefore, the *Staph. aureus* pulsotypes evaluated were expected to produce biofilm on both materials. Besides hydrophobicity and surface tension parameters, roughness of the material's surface is another factor that can influence bacterial adhesion to a given material (Katsikogianni et al., 2006). Rough surfaces, as found in rubber, have greater surface areas than smooth surfaces, such as in silicone, hence providing more favorable conditions for colonization (Baker and Greenham, 1988). However, the formation of biofilms on silicone surfaces has been reported for *Staph. epidermidis* (Sousa et al., 2009) and *Bacillus cereus* (Hamida et al., 2013). It remains to

Table 2. *Staphylococcus aureus* pulsotypes isolated from different sources in dairy farms and their ability to produce biofilms on microplates, stainless steel, or rubber¹

Source of isolates	Microplate (n = 14)	Stainless steel (n = 13)	Rubber (n = 13)
BULK MILK	P01 ^S , P15 ^W , P23 ^W , P25 ^W , P18 ^W , P30 ^W	P01, P12, P20, P23, P25	P13, P15, P30
Milk from individual cows	P16 ^S , P22 ^M , P03 ^W	P03, P16	P11, P22,
Milkers' hand and milking equipment ²	—	P07	
Milking equipment ²	P02 ^W , P24 ^S , P08 ^W	P08, P28, P24	P02, P08, P17, P28
Bulk milk, milk from individual cows, milkers' hands, and milking equipment	—	P09	—
Milking equipment and bulk milk	P21 ^M	P21	P21
Milk from individual cows and bulk milk	P29 ^W	—	P10
Milkers' hands	—	—	P31

¹Defined by pulsed-field gel electrophoresis; W = weak; M = moderate; S = strong.

²Surfaces of milking machine (internal surface of teat cups) and utensils (sieves and buckets).

be determined if variations in the surface properties of microorganisms could be involved in differences in the ability of *Staph. aureus* and other bacteria species to produce biofilm on silicone surfaces.

Comparison of data presented in our study with other previous reports is difficult because little information is available on biofilm formation by *Staph. aureus* isolates from dairy farms. Meira et al. (2012) observed that formation of biofilms by *Staph. aureus* isolates from the food industry in the same materials used in the present study was achieved after 3 d of cultivation. The authors also observed that peracetic acid (30 mg/L) and sodium hypochlorite (250 mg/L) were unable to remove the bacterial cells from the matrix of preformed biofilms, confirming that bacteria in biofilms are generally more resistant to environmental stress than their free-living counterparts (Costerton et al., 1999). In another study, carried out in 2 dairy factories producing UHT skim milk, the formation of biofilms by *Staph. aureus* occurred preferentially on polystyrene compared with stainless steel, suggesting that hydrophobicity was also a relevant factor in the formation of biofilms by *Staph. aureus* strains (Pagedar et al., 2010). However, no variation in biofilm formation by *Staph. aureus* pulsotypes on surfaces of polystyrene, stainless steel, or rubber was observed in the current study.

Even taking into consideration the limitations of this study in assessing adhesion, detachment, and biofilm formation of a monospecies inoculum under static conditions, our findings indicate that some of the *Staph. aureus* isolates assayed presented a clear ability to adhere and form biofilms on stainless steel, polystyrene, silicone, and rubber surfaces. This fact emphasizes the need for further studies for effective control of undesirable biofilms in the environment of dairy farms, including the type and nature of the contaminating residual materials (carbohydrates, fat, proteins, mineral salts) and how the natural mixed microbiota influences pathogen reduction during disinfection of surfaces (Simões et al., 2010). Integrated studies in regions that have dairy herds should include an initial investigation of the prevalence and identification of *Staph. aureus* strains with the ability to produce biofilms on materials commonly used in the dairy industry. In a second stage, different concentrations of sanitizers used in milk handling and processing lines should be evaluated, and predictive models should be generated to evaluate microbial growth parameters under the conditions in which these sanitizers are commonly used. Finally, the parameters obtained with application of these models should be evaluated to serve as an initial step to establish sanitizing procedures (Dias et al., 2012). These studies should be carried out on a regional basis using local dairy herds, because *Staph. aureus* strains found

in the milking environments show considerable variability in relation to various parameters of growth and metabolic activity.

Taking into account the variations in the ability of *Staph. aureus* pulsotypes to produce biofilms in different materials, further investigations are needed to determine the presence or absence of known genes involved in biofilm formation, such as *bap* and *ica* (Szweida et al., 2012). This might help to explain why some materials are more prone to promote biofilm formation than others commonly used in equipment and utensils in dairy farms.

CONCLUSIONS

Almost 45% of *Staph. aureus* pulsotypes isolated from different sources on dairy farms had the ability to produce biofilms on microplates, stainless steel, or rubber, indicating possible persistence of this pathogen in the milking environment. However, none of the pulsotypes evaluated was able to produce biofilms on silicone. Given the potential involvement of *Staph. aureus* in subclinical mastitis cases and its occurrence in milk for human consumption, hygiene practices must be improved to prevent biofilm formation on the farms studied.

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