J. Dairy Sci. 100:6414–6421
https://doi.org/10.3168/jds.2017-12700

Host-pathogen interactions in bovine mammary epithelial cells and HeLa cells by *Staphylococcus aureus* isolated from subclinical bovine mastitis

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ABSTRACT

Staphylococcus aureus is a common pathogen that causes subclinical bovine mastitis due to several virulence factors. In this study, we analyzed *S. aureus* isolates collected from the milk of cows with subclinical mastitis that had 8 possible combinations of *bap*, *icaA*, and *icaD* genes, to determine their capacity to produce biofilm on biotic (bovine primary mammary epithelial cells and HeLa cells) and abiotic (polystyrene microplates) surfaces, and their ability to adhere to and invade these cells. We also characterized isolates for microbial surface components recognizing adhesive matrix molecules (MSCRAMM) and *agr* genes, and for their susceptibility to cefquinome sulfate in the presence of biofilm. All isolates adhered to and invaded both cell types, but invasion indexes were higher in bovine primary mammary epithelial cells. Using tryptic soy broth + 1% glucose on abiotic surfaces, 5 out of 8 isolates were biofilm producers, but only the $bap^{+}icaA^{+}icaD^{+}$ isolate was positive in Dulbecco's Modified Eagle's medium. The production of biofilm on biotic surfaces occurred only with this isolate and only on HeLa cells, because the invasion index for bovine primary mammary epithelial cells was too high, making it impossible to use these cells in this assay. Of the 5 biofilm producers in tryptic soy broth + 1% glucose, 4 presented with the *bap*/*fnbA*/*clfA*/*clfB*/*eno*/*fib*/*ebpS* combination, and all were protected from cefquinome sulfate. We found no predominance of any *agr* group. The high invasive potential of *S. aureus* made it impossible to observe biofilm in bovine primary mammary epithelial cells, and we concluded that cells with lower invasion rates, such as HeLa cells, were more appropriate for this assay.

Key words: microbial surface components recognizing adhesive matrix molecules (MSCRAMM), *agr* allotype, mastitis, biofilm, resistance

INTRODUCTION

Staphylococcus aureus is one of the most common pathogens to cause subclinical bovine mastitis due to its multiple virulence factors (Bardiau et al., 2014). Bacterial interaction with host cells is the first step in the establishment of mastitis. Once the bacteria are attached, they can produce biofilm, which consists of an exopolysaccharidic matrix that aids in adherence and colonization to the epithelium of the mammary gland, protecting the bacteria from the host immune system and antimicrobial action (Pereira et al., 2009).

Staphylococcus aureus adheres to the surface via a capsular polysaccharide/adhesin. Then, bacterial multiplication occurs in multiple layers, associated with the production of 2 closely related polysaccharides—poly-*N*-acetylglucosamine and polysaccharide intercellular adhesin (Kristian et al., 2004)—encoded by the *ica* ADBC operon, in which *icaA* and *icaD* are responsible for encoding the most important proteins involved in biofilm formation by this pathogen (Vasudevan et al., 2003; O'Neill et al., 2007). Some isolates of *S. aureus* have the *bap* gene, encoding the protein associated with biofilm (Bap) that mediates initial adhesion to the surface and binding between bacterial cells, allowing these isolates to produce biofilm even in the absence of the *ica* operon (Cucarella et al., 2004).

Microbial surface components recognizing adhesive matrix molecules (**MSCRAMM**) is an additional *S. aureus* virulence factor associated with the adhesion and invasion of host cells (Klein et al., 2012). It expresses different proteins that can bind specifically to a variety of extracellular components in mammals, such as fibrinogen-binding proteins (Fib), clumping factors A and B (ClfA and ClfB), fibronectin binding proteins (FnBPA and FnBPB), and collagen binding protein (Cna; Khoramrooz et al., 2016).

Received February 8, 2017.
Accepted April 8, 2017.
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Adherence to the cell surface can allow *S. aureus* to invade epithelial cells, settling into deeper parts of the mammary gland (Hensen et al., 2000). Invasive potential is also an important virulence factor related to mastitis, allowing the microorganism to survive and multiply within the epithelial cells; be protected from host defense mechanisms and antimicrobial agent action; and persist in the host for a long period without causing apparent inflammation (Alva-Murillo et al., 2012; Bardiau et al., 2014).

Different molecular typing methods are used to establish the phylogenetic relationships among *S. aureus* isolates, including the *agr* system, which controls most of the virulence factors used to classify isolates into 4 allelic groups (I to IV; Zhang et al., 2016). As well, specific *agr* groups could be associated with the invasion phenotype (Bardiau et al., 2014).

The aims of this study were to test *S. aureus* isolates collected from the milk of cows with subclinical mastitis with 8 possible profiles, considering the *bap*, *icaA*, and *icaD* genes. We tested these isolates for the presence of MSCRAMM genes and ranked them molecularly by *agr* group. We also investigated their ability to produce biofilm on biotic and abiotic surfaces, their potential for adhesion and invasion in bovine primary mammary epithelial cells (**BMEC**) and HeLa cells, and their susceptibility to antimicrobials in biofilms.

MATERIALS AND METHODS

Staphylococcus aureus Isolates

The *S. aureus* isolates were recovered in a previous study (Silva et al., 2013) from 1,484 milk samples obtained from 518 cows on 11 farms in Brazil that had a positive on the California Mastitis Test, indicative of subclinical mastitis (Schalm and Noorlander, 1957). The isolates were identified as described by Murray et al. (2007), and we performed PCR amplification to confirm the presence of the species-specific staphylococcal nuclease gene (*nuc*; CRL-AR, 2009).

Detection of Genes Associated with Adhesion and Biofilm Formation agr Grouping

We verified the presence of *bap*, *icaA*, and *icaD* genes, according to the methods of Cucarella et al. (2001) and Vasudevan et al. (2003), respectively, using the Minispin kit (GE Healthcare, Little Chafont, Buckinghamshire, United Kingdom), according to the manufacturer's instructions (https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1314750913712/litdoc28916282_20161014140559.pdf). We used *S. aureus* ATCC 35983 as a positive control for the *ica* cluster, as well as a sequenced *bap*-positive isolate. *Salmonella* sp. was the negative control for all PCR tests. The tests were performed using a Gene Amp PCR System9700 (Applied Biosystems, Carlsbad, CA).

Eight isolates representing all possible genetic profiles taking account *bap*, *icaA*, and *icaD* were tested for the presence of MSCRAMM genes (*fnbA*, *fnbB*, *fib*, *clfA*, *clfB*, *cna*, *eno*, and *ebpS)*, according to the method of Tristan et al. (2003) and were characterized by *agr* allotype according to the method of Shopsin et al. (2003). The positive controls for *agr* I, II, III, and IV were *S. aureus* COL, N315, MW 2, and RN4850, respectively.

Cell Cultures

We used 2 different cells for cultures: BMEC and the established HeLa cell line. The HeLa cells were maintained in flasks with Dulbecco's Modified Eagle's Medium (**DMEM**) Max glucose (Sigma-Aldrich, St. Louis, MO) plus 10% fetal bovine serum (**FBS**; Sigma-Aldrich), at 37°C for 5 to 7 d in a 5% CO_2 atmosphere (Hensen et al., 2000).

Cultivation of BMEC. We isolated BMEC using tissue fragments from the mammary gland of a healthy cow, which were deposited in Petri dishes with a growth medium consisting of DMEM supplemented with antimicrobial agents and 10% FBS. The concentrations of antimicrobial agents were 10× the usual concentration (Sigma-Aldrich): gentamicin (50 μg/mL), penicillin (250 IU/mL), streptomycin (1 mg/mL), polymyxin B (50 IU/mL), and amphotericin B (2.5 mg/mL). After 48 h, the medium was replaced with DMEM plus 10% FBS and the same antibiotics, but in their usual concentrations (1×). After 72 h, the primary mammary epithelial cell monolayers formed islands with aggregated fibroblasts, and the epithelial cells were recovered by selective trypsinization. The BMEC were maintained in the same manner as HeLa cells.

To characterize the BMEC, we performed real-time PCR for the cytokeratin 18 gene (*krt18*) expressed by epithelial cells, and for the vimentin gene (*vim bos*) expressed by mesenchymal cells (Anaya-López et al., 2006).

Total RNA Extraction. Total RNA was obtained using the commercial Total RNA Purification Kit (Norgen Biotek Corp., Thorold. Ontario) according to the manufacturer's instructions (https://norgenbiotek.com/sites/default/files/resources/Total-RNA-Purification-Kit-Insert-PI17200-32-M14.pdf). Madin-Darby bovine kidney (MDBK) cells were used as a positive control and baby hamster kidney (BHK-21) cells as a negative control. The RNA samples were quantified using a NanoDrop 2000 Spectrophotometer (Thermo

Fisher Scientific, Waltham, MA), and appropriate values of the ratio of absorbance at 260 nm to 280 nm over 1.7 were achieved.

cDNA Synthesis. To obtain cDNA, 1 μL of random primer (Sigma-Aldrich) and 4 μL of RNA sample were incubated at 70°C for 5 min. Afterward, 15 μL of solution containing 1 μL of RNase Out (Invitrogen, Grand Island, NY), 4 μL of Impron II 5× reaction buffer (Promega, Madison, WI), 2.4 μL of $MgCl_2$ (25 m*M*), 1 μL of dNTP mix (10 m*M*), 0.5 μL of Impron II RT, and 6.1 μL of nuclease-free water (20 m*M* $MgCl_2$) was added. The mixture was then incubated at 25°C for 5 min, 42°C for 60 min, and 70°C for 15 min and then cooled in Gene Amp PCR System 9700 (Applied Biosystems).

Qualitative Real-Time PCR. Real-time reactions were standardized to a final volume of 20 μL containing 0.3 μ*M* of each primer (Sigma-Aldrich), 10 μL of GoTaq qPCR Master Mix (Promega), 6.8 μL of RNase-free water, and 2 μL of sample. The conditions used were 1 cycle at 95°C for 2 min; 40 cycles at 95°C for 15 s and 60°C for 1 min; and 1 cycle of 60 to 95°C for the dissociation curve. The endogenous control was glyceraldehyde-3-phosphatase dehydrogenase (*gapdh*), a housekeeping gene (Cagnini, 2014). Primers for *vim bos* (fCAGGATGTTGACAATGCGTCTC, and rCCGCATCATGCAGTTTCTTCAA, annealing temperature at 60°C, 100 bp) and *krt18* (fATAATGCCCGTCTTGCTGCT, and rGCCCGTGTATGTCACTCTCC, annealing temperature at 60°C, 90 bp) were designed using the Primer Blast program (http://www.ncbi.nlm.nih.gov/nuccore).

S. aureus Adhesion and Invasion Assays

Adhesion Assay. Each isolate was grown in tryptic soy broth (**TSB**; Oxoid, Basingstoke, UK) at 37°C for 18 to 24 h to reach the stationary growth phase and diluted in DMEM to obtain a suspension of approximately 1.5×10^7 cfu/mL. Cells (HeLa and BMEC) were grown in 24-well plates. Before the adherence assay, each well was washed 3 times with PBS^+ buffer (0.01 *M* PBS, supplemented with 0.1 g/L $CaCl_2$ and 0.2 g/L $MgCl_2$; Sigma-Aldrich), and 1 mL of the bacterial suspension was added to each well. After incubation at 37°C for 1 h in 5% CO_2, the monolayers were washed 3 times with PBS^+ buffer to remove nonadherent bacteria. Next, the cells were resuspended in 500 μL of 0.1% trypsin + 0.04% EDTA (Fermentas, St. Leon Rot, Germany) in each well. The plates were incubated at 37°C for 10 to 15 min in 5% CO_2. After incubation, the suspensions were homogenized by pipetting, and cell detachment was checked using an inverted phase-contrast microscope. After resuspension, trypsinization was stopped by adding 500 μL of DMEM without antimicrobial agents. Serial 10-fold dilutions of the cells were performed and inoculated onto trypticase soy agar plates (**TSA**; Oxoid) and incubated at 35°C for 24 h. The growth of colonies on TSA was considered a positive result, demonstrating the recovery of adhered bacteria (Hensen et al., 2000).

Invasion Assay. The isolates were diluted as described for the adherence assay, cells were washed 3 times with PBS^+ buffer, and 1 mL of the bacterial suspension was added to each well. The plate was incubated at 37°C for 3 h in 5% CO_2. Then, the cell monolayers were washed 3 times with PBS^+ buffer to remove extracellular bacteria, 1 mL of Eagle's solution containing 5% FBS and 10 μg/mL gentamicin was added to each well, and the plate was reincubated at 37°C during 2 h in 5% CO_2. Afterward, 100 μL were plated onto TSA agar to verify the absence of growth and confirm the action of gentamicin. Then, the plates were washed 4 times with PBS^+, and each well received 1 mL of Triton X-100 (Sigma-Aldrich) at a final concentration of 0.1% (vol/vol) in distilled water at 37°C for 10 min to lyse the cells. Next, serial 10-fold dilutions were prepared from the lysed cells, inoculated onto TSA plates, and incubated at 35°C for 24 h. We determined the percentage of invasion by recovery and counting of the colonies and calculating the relative percentage, compared with the values determined in the adhesion assay (adhered bacteria/internalized bacteria × 100) (Hensen et al., 2000).

Biofilm Formation on Biotic and Abiotic Surfaces

We tested for biofilm production according to the method of Vasudevan et al. (2003) with modifications, using TSB, TSB + 1% glucose, and DMEM. Briefly, after overnight incubation at 35°C for 24 h, the cultures grown in TSB were diluted (0.5 MacFarland scale) with Densicheck (bioMeriéux, Marcy-l'Étoile, France), and aliquots of 200 μL were distributed in quadruplicate in 96-well polystyrene microplates (abiotic surface) and incubated at 35°C for 48 h. After being washed in PBS (pH 7.4), the wells were stained with 1% crystal violet for 15 min, washed 3 times with the same buffer, dried, and read in an Epoch Microplate Reader (Biotek, Winooski, VT) at 570 nm. Uninoculated TSB was used as a blank to correct the absorbance value. The assay was repeated 3 times.

To determine biofilm production in biotic surfaces, BMEC and HeLa cells were grown on glass coverslips in 24-well plates as described, until monolayer formation. Next, the wells were washed with PBS^+ to remove nonadhered cells. The bacterial inoculum was prepared following the methodology previously described in

DMEM, and 1 mL was inoculated into the wells. After 48 h of incubation, the coverslips were removed and fixed in glutaraldehyde for confirmation of biofilm production by scanning electron microscopy (Coenye and Nelis, 2010).

Susceptibility to Cefquinome Sulfate in the Presence of Biofilm

The isolates were incubated at 37°C for 24 h in Mueller-Hinton broth (Oxoid) and the susceptibility test to cefquinome sulfate (Sigma-Aldrich) was performed according to CLSI (2015), with modifications, to determine the minimum bactericidal concentration (**MBC**) in 3 h. The susceptibility to cefquinone was checked using a resazurin microtiter assay method at concentrations ranging from 1,024 to 0.125 μg/mL, and using bacterial standardized suspensions (10^5 cfu/mL). The microplate was gently agitated and incubated at 35°C for 3 h. After this, inocula with no visual growth were plated on TSA and incubated at 35°C for 24 h. The MBC value was determined by the highest dilution that completely inhibited bacterial growth on TSA plates.

After MBC determination, the biofilm assay was performed as described above, using only the producer isolates previously tested. Then, the wells were washed 4 times with sterile PBS (pH 7.4), 200 μL of cefquinome sulfate with a MBC value of 512 μg/mL was added, and the microplate was incubated at 35°C for 3 h. After this, all contents from the wells were plated onto TSA to confirm antimicrobial efficiency (noted by the absence of growth after 35°C for 24 h). The wells were filled with sterile PBS, and the biofilm was broken using an ultrasonic processor (UP100H; Hielscher, Teltow, Germany; 5 cycles of 5 s each, 30% amplitude) and again, the contents were plated onto TSA to observe the presence or absence of growth after incubation at 35°C 24 h. We considered the absence of bacterial growth before sonication and its presence after sonication as confirmation that the biofilm protected the cells below it.

Statistical Analysis

We used the Prism (GraphPad, La Jolla, CA) software program to perform statistical analysis. The data on biofilm production in 3 different culture media were nonparametric according to the Shapiro-Wilk test; therefore, the Kruskal-Wallis test was applied followed by Dunn's multiple comparison test ($\alpha = 5\%$). Regarding the comparison of the invasion index, and considering the 2 cell types, the groups were found to be nonparametric according to the Shapiro-Wilk test. Because the data were paired and nonparametric, we applied the Wilcoxon signed rank test ($\alpha = 5\%$).

RESULTS

Staphylococcus aureus Genetic Profiles

After testing 107 isolates of *S. aureus*, we obtained 8 possible different profiles for the presence of *bap*, *icaA*, and *icaD*: (1) $bap^+icaA^+icaD^+$; (2) $bap^+icaA^+icaD^-$; (3) $bap^+icaA^-icaD^-$; (4) $bap^+icaA^-icaD^+$; (5) $bap^-icaA^+icaD^+$; (6) $bap^-icaA^-icaD^+$; (7) $bap^-icaA^+icaD^-$; and (8) $bap^-icaA^-icaD^-$. We tested these isolates for the presence of MSCRAMM genes, and the *eno* gene encoding the laminin-binding protein was the only one present in all isolates. On the other hand, *cna* occurred only in 1 isolate (12.5%). The diversity and distribution of these genes among the isolates is listed in Table 1. The same table shows that no specific *agr* type predominated. One isolate was classified as no type, and isolate 4 presented simultaneously as gene types I and III.

Cultured Cells in Adhesion and Invasion Assays

The BMEC, obtained from an explant, grew with characteristic morphology over 5 d. We observed fibroblasts, which were eliminated using selective trypsinization. Madin-Darby bovine kidney cells were used as a positive control for expression in qualitative PCR, and the *gapdh* gene showed the highest expression, with a cycle threshold of 14.9. Cytokeratin 18 and vimentin presented cycle threshold values of 19.9 and 23.4, respectively, confirming their epithelial origin (Madin and Darby, 1958). These cycle threshold values presented similar patterns to those of BMEC.

The values for adhesion and invasion assays using BMEC and HeLa cells are also presented in Table 1. All isolates adhered to and invaded BMEC with an invasion index of 6.9 to 85.7%. Invasion in HeLa cells was not as efficient, though all adhered satisfactorily. We found a statistically significant difference ($P = 0.0156$) when comparing the invasion index between BMEC and HeLa cells.

Isolate 1, the largest producer of biofilm, presented the lowest invasion rate (0.02%). In contrast, isolate 4 showed the highest invasion rate (85.7%) but produced biofilm poorly on the culture media we used.

Biofilm Production in Both Biotic and Abiotic Surfaces

We tested all isolates for biofilm production in polystyrene microplates comparing 3 different media, as outlined in Table 2. Using TSB + 1% glucose, 5

Table 1. Adhesion and invasion assay of *Staphylococcus aureus* isolates obtained from the milk of cows with subclinical mastitis in bovine mammary epithelial cells and HeLa cells, considering the genetic profiles related to biofilm production, MSCRAMM proteins, and *agr* allotypes

	Biofilm			MSCRAMM[1]							*agr* allotypes			Bovine mammary epithelial cells			HeLa		
Isolate	*bap*	*icaA*	*icaD*	*fnbA*	*fnbB*	*clfA*	*clfB*	*cna*	*eno*	*fib*	*ebpS*	*agrI*	*agrII*	*agrIII*	Adhesion[2]	Invasion[2]	Invasion index, %	Adhesion[2]	Invasion[2]	Invasion index, %
1	+	+	+	+	−	+	+	−	+	+	+	−	+	−	13.0	0.9	6.9	9.5	0.002	0.02
2	+	+	−	+	+	+	+	+	+	+	+	−	−	+	11.0	0.8	7.3	5.5	0.002	0.04
3	+	−	−	+	−	+	+	−	+	+	+	−	+	−	1.9	1.6	84.2	4.5	0.001	0.02
4	+	−	+	+	−	+	+	−	+	+	+	−	−	+	3.5	3.0	85.7	1.0	0.008	0.8
5	−	+	+	−	−	−	−	−	+	−	−	+	−	+	3.1	1.2	38.7	0.07	0.01	14.3
6	−	−	+	−	−	−	−	−	+	−	−	−	−	−	0.1	0.04	40	0.06	0.006	10
7	−	+	−	+	−	+	−	−	+	+	+	+	−	−	0.05	0.007	14	0.05	0.001	2
8	−	−	−	−	+	+	−	−	+	+	−	+	−	−	0.03	0.005	16.7	0.02	0.003	15

[1]MSCRAMM = microbial surface components recognizing adhesive matrix molecules.
[2]Equivalent to 10^6 cfu.

out of 8 profiles (62.5%) produced biofilm. Isolate 1 (*bap*$^+$*icaA*$^+$*icaD*$^+$) was the strongest producer in TSB and TSB + 1% glucose, and it was the only isolate that also produced biofilm in DMEM. Statistical analysis by Kruskal-Wallis test (Dunn's test) showed that *S. aureus* produced biofilm more efficiently in TSB + glucose than in TSB and DMEM ($P < 0.05$).

Only isolate 1 was tested on biotic surfaces (HeLa cells and BMEC), because it was the only isolate that produced biofilm in DMEM (Figure 1). However, we were unable to observe biofilm production in BMEC because the bacteria invaded the cell in 6 h (time invasion assay), hindering visualization of biofilm production, which occurs in 48 h.

Antimicrobial Resistance Due to Biofilm

Regarding the isolates' susceptibility to cefquinome sulfate in the presence of biofilm, the MBC was 512 μg/mL in 3 h of incubation, and all producer isolates provided the same result, with growth after sonication showing biofilm protection.

DISCUSSION

We obtained BMEC cells using an explant from a mammary bovine gland fragment. We found fibroblast contamination, which we eliminated by selective trypsinization. This can occur in monolayers due to cells migrating from the stroma (Zhao et al., 2010). However, the qualitative PCR results showed higher expression of cytokeratin 18 than vimentin. As well, epithelial cells in vitro can express vimentin (Franke et al., 1979).

We carried out tests with HeLa cells to verify whether this cell type could replace primary cultures of bovine mammary epithelium in adhesion and invasion assays of *S. aureus* isolated from mastitis, because they are immortal lines and easily maintained. We found that these cells were ideal for observing biofilm production by *S. aureus* isolated from animals, because the invasion index can be extremely low and destruction of the cell monolayer does not occur, enabling the visualization of biofilm.

All isolates adhered to both cell types. Hensen et al. (2000) observed that 100% of 20 strains isolated from cows with mastitis also adhered to BMEC. We found no studies in the literature on adhesion assays conducted on HeLa cells using S. *aureus* from bovine sources.

Among the MSCRAMM genes, *eno* was the most frequent, occurring in 100% of isolates, followed by *clfA/fib* (75%), and *ebpS/fnbA* (62.5%); the *cna* gene was present in only 1 isolate (12.5%). Khoramian et al. (2015) observed lower percentages for *ebpS* and *fnbA* (55 and 45%, respectively) but obtained very similar

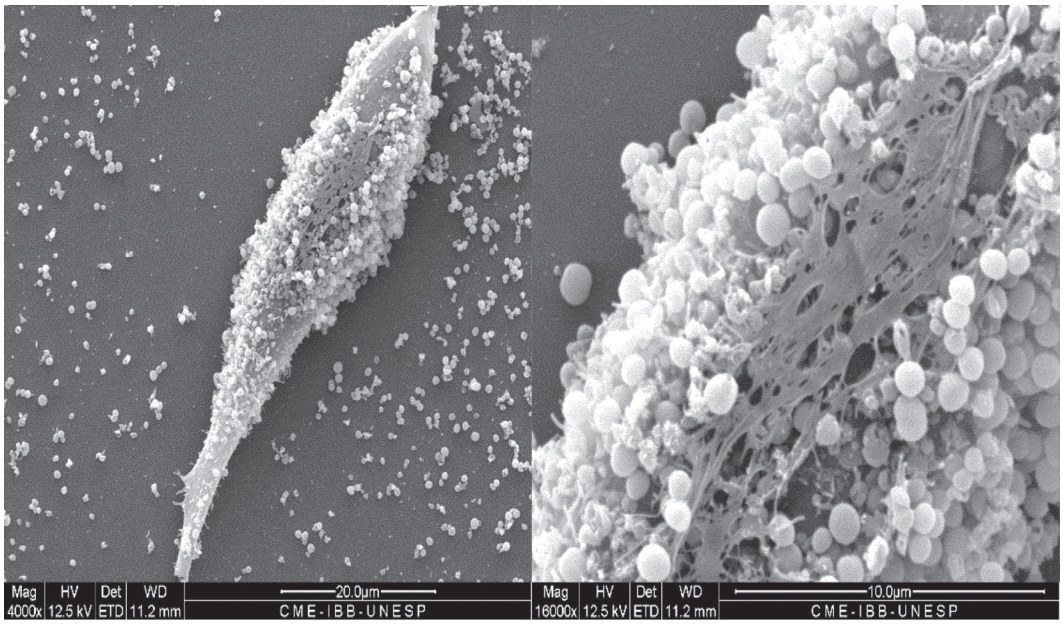

Figure 1. Scanning electron micrographs showing biofilm production in HeLa cells by *Staphylococcus aureus* isolated from the milk of cows with subclinical mastitis at (A) 4,000× and (B) 16,000× magnification.

results for *cna* (14%). Salaberry et al. (2015) found *cna* in none of 8 *S. aureus* isolates and concluded that its presence in the mammary gland was not important for this pathogenesis. The same authors also observed a high frequency of the *fnbA* gene (87.5).

The *clfA* and *clfB* genes occurred in 75% and 50% of isolates, respectively. Zmantar et al. (2008) affirmed that *clfA* is one of the main proteins that binds *S. aureus* to fibrinogen and fibrin. According to Kochut and Dersch (2013), *fnbA* and *fnbB* strongly favor *S. aureus* adhesion and invasion into mammary gland cells. In the present study, 2 isolates with a high invasion indexes in BMEC (isolates 3 and 4) possessed the *fnbA* gene.

Isolates 1 and 2 (bap^+) were the most adherent and produced biofilm the most strongly. This seemed to occur because adhesion and biofilm production are closely related. Moreover, the invasion rates of these isolates in BMEC were the lowest (6.9 and 7.3%, respectively). Valle et al. (2012) used Bap as a model to investigate interactions between biofilm matrix components and the mammary gland cells of rats. They observed that Bap allowed adherence to epithelial cells, and its expression appears to have reduced the invasion rate, with the bacteria remaining on the surface of the host cell, protected by biofilm. However, isolates 3 and 4 had 100% invasion rates, even in the presence of the *bap* gene, suggesting that Bap's ability to decrease invasion may depend on its interaction with other genetic factors, such as the MSCRAMM genes.

Isolate 4 (*agr* I and III allotypes) was a biofilm producer, and it showed an invasion index of 85.7% in BMEC. Bardiau et al. (2014) observed an association

Table 2. In vitro biofilm production (positive, +; negative, −) by *Staphylococcus aureus* strains isolated from milk of cows with subclinical mastitis, using 3 different media[1]

	TSB + 1% glucose			TSB			DMEM		
Strain	Production	OD	SD	Production	OD	SD	Production	OD	SD
1	+	0.5637	0.04635	+	0.2953	0.02941	+	0.1262	0.09907
2	+	0.2771	0.03889	−	0.0719	0.04525	−	0.0727	0.0424
3	+	0.1842	0.0554	−	0.0656	0.03958	−	0.0329	0.0192
4	+	0.1507	0.07311	−	0.0412	0.02738	−	0.0162	0.0102
5	+	0.1332	0.005485	−	0.0834	0.03154	−	0.0347	0.01626
6	−	0.04225	0.01956	−	0.0252	0.00455	−	0.0067	0.01112
7	−	0.089	0.01015	−	0.0343	0.01548	−	0.01398	0.02104
8	−	0.0430	0.00812	−	0.03775	0.008057	−	0.03725	0.006652

[1]TSB = tryptic soy broth; DMEM = Dulbecco's Modified Eagle's Medium; OD = optical density.

between the *agr* I allotype, high invasion rates in MAC-T cells, and biofilm formation by *S. aureus* strains isolated from bovine mastitis. Isolate 8, also *agr* I allotype, did not have any of the biofilm genes. However, those authors also reported that members of the *agr* II group had a low invasion index and weak or no biofilm production, an association not observed in isolate 1, which belonged to this group but presented a low invasion rate and was the strongest biofilm producer.

Biofilm production occurred in 5 out of 8 (62.5%) isolates, although 7 isolates presented at least 1 gene for producing biofilm, and TSB + 1% glucose was the best growth medium. In the literature, we found no data about biofilm production in DMEM, but its use would be necessary for biofilm production on a cell monolayer. Surprisingly, although DMEM is rich in nutrients and 4.5% glucose, biofilm formation occurred only in 1 isolate grown in this medium (isolate 1: $bap^{+}icaA^{+}icaD^{+}$) and, for this reason, only this isolate was tested in HeLa cells.

Isolate 1 had the 3 genes involved in biofilm production, and it was the only producer in unsupplemented TSB and in DMEM. Among the biofilm producers isolated in TSB + 1% glucose, isolate 1 was also the strongest producer. According to Brouillette et al. (2005), the presence of the *icaAD* gene cluster associated with the *bap* gene can lead to strong biofilm production and greater resistance to antimicrobial agents.

All isolates that produced biofilm in supplemented TSB, with the exception of isolate 5, possessed the *bap* gene as well as a major number of the MSCRAMM genes, also having in common the *eno/fnbA/fib/clfA/clfB and ebpS* genes. Zuniga et al. (2015) observed that *S. aureus* and other staphylococci isolated from subclinical bovine mastitis with the genetic profile *eno/fnbA/fib/bap* were related to a higher SCC because of its major persistence in the organism.

Isolate 3 did not present the cluster *ica* but still produced biofilm. On the other hand, $bap^{+}eno^{+}fnbA^{+}fib^{+}$ was related to high-intensity inflammation in mastitis (Zuniga et al., 2015). According to Cucarella et al. (2004), deletion of the *ica* cluster in a positive *bap* isolate did not change its ability to produce biofilm, leading them to conclude that the Bap protein can compensate for the lack of polysaccharide intercellular adhesin and polysaccharide/adhesin.

Strain 5 produced biofilm ($icaA^{+}icaD^{+}$), despite the presence of only *eno* among the MSCRAMM genes. The *icaA* gene alone presents low enzymatic activity, but its coexpression with *icaD* leads to a significant increase in its expression (Arciola et al., 2001). Szweda et al. (2012) also reported biofilm production by isolates with the genotype $ica^{+}bap^{-}$. Isolates 6 ($bap^{-}icaA^{-}icaD^{+}$) and 7 ($bap^{-}icaA^{+}icaD^{-}$) were not biofilm producers and showed only 1 gene at locus *ica* and the lowest presence of MSCRAMM genes. Isolate 6 was not classified as any *agr* allotype, an unusual event.

We observed the production of biofilm in cultured cells only with isolate 1 in HeLa cells, because it was the only isolate to produce biofilm in DMEM, and it invaded BMEC cells in 7 h, less time than was needed for biofilm production (48 h). For this isolate, adhesion was similar in both cells, but the invasion index was higher in BMEC ($P < 0.05$).

Regarding the susceptibility to cefquinome sulfate, in the presence of biofilm, no isolates grew after incubation with the antimicrobial agent, demonstrating its efficacy. However, after the biofilm was broken by sonication, all isolates were recovered in TSA agar, confirming the protective effect of biofilm.

The presence of genes related to the production of biofilm and to MSCRAMM protein synthesis can help their colonization and persistence. Biofilm is an important virulence factor, because it can provide resistance to antimicrobials used in the treatment of mastitis. *Staphylococcus aureus* has high invasive potential in BMEC cells, making biofilm observation unfeasible in this cell type; the use of different cells, such as HeLa cells, is more appropriate because of their lower invasion rates.

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