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ORIGINAL ARTICLE

Genotypic and phenotypic detection of capsular polysaccharide and biofilm formation in *Staphylococcus aureus* isolated from bovine milk collected from Brazilian dairy farms

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Abstract *Staphylococcus aureus* is a pathogen that frequently causes mastitis in bovine herds worldwide. This pathogen produces several virulence factors, including cell-associated adhesins, toxic and cytolytic exoproteins, and capsular polysaccharides. The aim of the present study was to test for the presence of genes involved in capsular polysaccharide production and biofilm formation in *S. aureus* isolated from bovine mastitis samples collected from 119 dairy herds located in three different Brazilian regions, as well as to assay the production of capsular polysaccharides and biofilm, in vitro. The detection of the *cap*, *icaAD*, and *bap* genes was performed using PCR. The detection and quantification of capsular polysaccharide production was performed using ELISA assays. The ability of the isolates to form a biofilm was examined using the polystyrene surface of microtiter plates. All 159 *S. aureus* isolates investigated harboured the *cap* gene: 80 % carried the *cap5* gene and 20 % carried the *cap8* gene. Sixty-nine percent of the isolates expressed capsular polysaccharide (CP) in vitro, 58 % expressed CP5 and 11 % expressed CP8. All of the isolates harboured the *icaA* and *icaD* genes, and 95.6 % of the isolates carried the *bap* gene. Of the 159 isolates analysed, 97.5 % were biofilm producers. A significant association between the capsular genotype and phenotype and the amount of biofilm formation was detected: *cap5*/CP5 isolates tended to form more biofilm and to produce a thinner CP layer than *cap8*/CP8 isolates. The results indicate a high potential for pathogenicity among *S. aureus* isolated from bovine milk collected from three different regions in Brazil.

Keywords *cap* · *icaAD* · *bap* · Capsule · Adhesin

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Introduction

Staphylococcus aureus is a pathogen that is responsible for a diverse spectrum of human and animal diseases. It is regarded as a very common pathogen that causes bovine mastitis, and many virulence factors contribute to the pathogenesis of staphylococcal infections (O'Riordan and Lee 2004; Fournier et al. 2008). The knowledge of these virulence factors is of fundamental importance for the control of disease, and some of them have been targeted as components for vaccine development (Cocchiaro et al. 2006; Scali et al. 2015).

Capsular polysaccharide (CP) or the capsule is a cell wall bacterial component that protects bacteria from phagocytic uptake and enhances microbial virulence. Although eleven capsular polysaccharide serotypes have been identified in *S. aureus* isolates, only the polysaccharides from serotypes 1, 2, 5, and 8 have been purified and chemically characterized. Among them, types 5 and 8 are prevalent in *S. aureus* isolated from human and bovine infections (O'Riordan and Lee 2004). Serotype 336 has been reported by Guidry et al. (1998), but it is a cell wall

surface antigen, specifically, a polyribitol phosphate N-acetylglucosamine, which resembles teichoic acid from the cell wall (Verdier et al. 2007).

The distribution of capsular serotypes among *S. aureus* isolates from bovine mastitis from different countries shows great variability (Poutrel et al. 1988; Guidry et al. 1998; Tollersrud et al. 2000; Han et al. 2000; Hata et al. 2006; Camussone et al. 2012). According to Guidry et al. (1998), the extracellular polysaccharide capsule is particularly relevant to bovine mastitis, because 94 to 100 % of *S. aureus* strains isolated from bovine mastitis are encapsulated.

A biofilm is a structured community of bacterial cells that are enclosed in a self-produced, polymeric matrix that adheres to an inert or living surface and constitutes a protected mode of growth that allows survival in a hostile environment (Costerton et al. 1999). Biofilms are inherently tolerant to host defences and antibiotic therapies and are the root of many persistent and chronic bacterial infections (Costerton et al. 1999), including bovine mastitis (Cucarella et al. 2004; Fox et al. 2005). There is a consensus that the primary determinant of the accumulation phase of staphylococcal biofilm formation is polysaccharide intercellular adhesin (PIA). Production of PIA is mediated by the *icaADBC* operon, and strains harbouring this cluster are potential biofilm producers (Heilmann et al. 1996; Cramton et al. 1999).

Some proteins involved in biofilm formation have also been identified in staphylococci, including biofilm associated protein (Bap), which is encoded by the *bap* gene (Götz 2002). Cucarella et al. (2001) demonstrated that Bap is not only involved in the primary attachment step but is also, together with PIA, involved in cell-to-cell aggregation and thus biofilm maturation. Lasa and Penades (2006) observed that staphylococcal isolates harbouring the *bap* gene were strong biofilm producers, even in the absence of the *icaADBC* operon.

Because there is scarce information about these virulence factors in *S. aureus* isolates from Brazilian dairy herds, taking into consideration the continental size of the country and the growing importance of the dairy sector, the aim of the present study was to evaluate the presence of genes involved in CP production and biofilm formation in *S. aureus* isolated from bovine milk from three different Brazilian regions and to access the production of both CPs and biofilms in vitro. This information is important to estimate the usefulness of incorporating both components, capsules and biofilm, in a future vaccine formulation appropriated for our country.

Material and methods

Bacterial isolates

A total of 159 *S. aureus* isolates was included in this study. The isolates were randomly selected from a collection of bovine milk bacteria maintained at the Embrapa Dairy Cattle Research Center (Juiz de Fora, Brazil). They were isolated from 119 dairy herds located in south, southeast, and north Brazil, over a nineteen-year period (1994–2013) (Fig. 1). The map shown in Fig. 1 was generated using the ArcGIS 10.2.2 program. The isolates were identified by Gram staining and catalase, coagulase, and acetoin production according to the National Mastitis Council (2004). Phenotypic identification of *S. aureus* was confirmed by PCR amplification of the *femA* gene, which is specific for *S. aureus*, according to Mehrotra et al. (2000). Samples were stored at −80 °C in skim milk (Difco, Sparks, MD, USA) containing 10 % glycerol (Cromoline, Diadema, SP, Brazil). Bacterial cultures obtained from frozen stocks were grown on plates containing BHI agar (Brain Heart Infusion Agar, Himedia, Mumbai, India) for 24 h at 37 °C.

PCR assays

The detection of two genes involved in polysaccharide production (i.e., *cap5* and *cap8*) and three genes involved in biofilm formation (i.e., *icaA*, *icaD*, and *bap*) was performed using PCR. Whole cell DNA was extracted using a standard phenol-chloroform procedure as described by Hesselbarth and Schwarz (1995). The primers used in the PCR reactions were described by Verdier et al. (2007) for amplification of *cap5* and *cap8*, by Vasudevan et al. (2003) for amplification of *icaA* and *icaD*, and by Potter et al. (2009) for amplification of the *bap* gene. The primers used, the size of the amplified products and the PCR cycling conditions are described in Supplementary Table 1.

For amplifications, PCR mixtures containing PCR Master Mix [10 x (100 mM Tris-HCl pH 8.8 and 500 mM KCl), 1.7 mM $MgCl_2$, 0.2 mM dNTPs, and 1.5 U of Taq DNA polymerase] (Ludwig Biotech, Alvorada, RS, Brazil), 10 pmol/μL of each primer and 100 ng of target DNA were used. For *cap* amplifications, the prototype *S. aureus* strains CP5 (Reynolds) and CP8 (Becker) were used as positive controls. The PCR reactions were performed on a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). The PCR products were analysed by electrophoresis on 1.5 % agarose gels stained with ethidium bromide.

Sequence analysis of the amplicons

Some amplicons obtained in the PCR assays were sequenced by commercial sequencing facilities (i.e., Myleus Biotecnologia, Belo Horizonte, MG, Brazil and Setor de Sequenciamento de DNA da Universidade de São Paulo, SP, Brazil) to check their identity with the *cap5*, *cap8*, *icaA*, *icaD*, and *bap* genes. The primers used for the sequencing reactions were the same as those for the PCR amplifications. Sequence

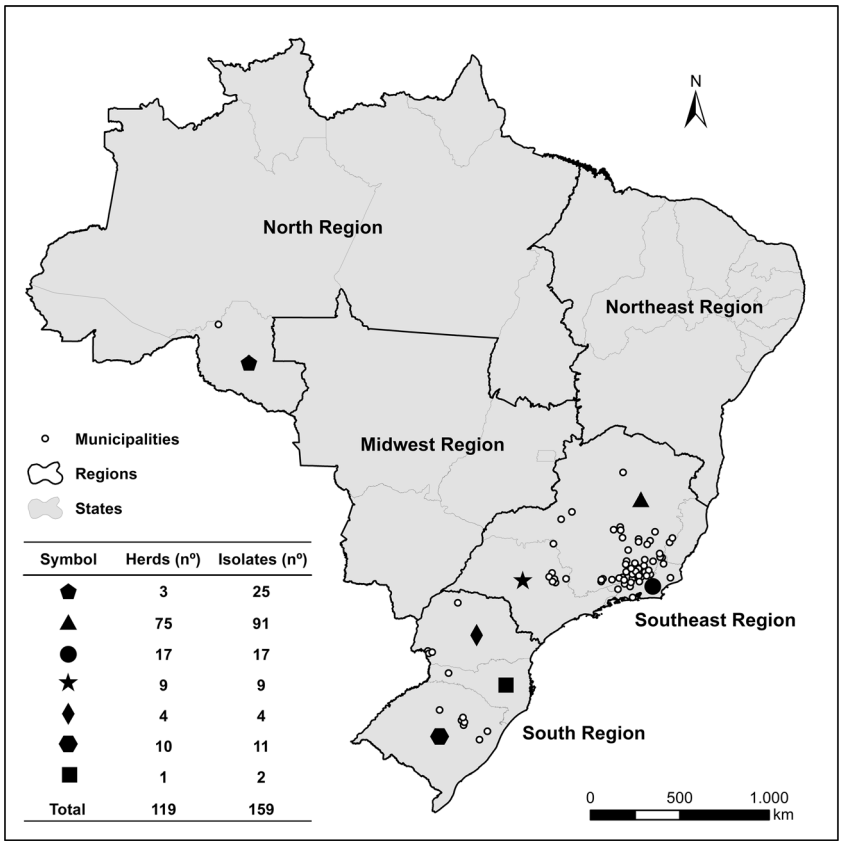

Fig. 1 Map illustrating the origin of the 159 *Staphylococcus aureus* investigated in the present study, isolated from dairy herds located in south, southeast, and north Brazil, over a nineteen-year period (1994–2013)

comparisons were made using the DNA Baser Sequence Assembler v3.5.4 (Heracle BioSoft SRL, www.DnaBaser.com). Contigs were blasted against the *Staphylococcus* spp. nucleotide database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST).

Detection of polysaccharide production

Sera from mice immunized with CP5 and CP8 bound to human serum albumin were used. Sera were kindly provided by Nazarena Pujato (Pujato et al. 2015). The CP from the prototype *S. aureus* CP5 (Reynolds) and CP8 (Becker) and from all isolates carrying *cap5* or *cap8* genes were extracted as described by Fattom et al. (1990). The polysaccharide concentration was determined using the phenol-sulphuric acid method (Dubois et al. 1956), and CPs were visualized using SDS-Page and silver staining. The absence of proteins was verified using the bicinchoninic acid assay (Smith et al. 1985) and SDS-Page followed by Coomassie Blue staining. ELISA assays were performed as follows: 5 μg of purified CPs from isolates genotyped as carrying *cap5* and *cap8* were used as antigens to sensitize 96-well plates. Plates were blocked with PBS-low-fat milk (5 %) and incubated with CP5 or CP8 antisera (1/200), respectively. Finally, a goat anti-rabbit IgG conjugated to alkaline peroxidase was used as the secondary antibody, and the reaction was developed with TMB (Thermo Fisher Scientific, Waltham, MA,USA). All incubations were carried out at 37 °C, for 60 min. Optical Densities (OD) were measured at 450 nm using an ELISA plate reader (Infinite® F50, Tecan, Grödig, Austria).

Detection of biofilm formation

The ability of the *S. aureus* isolates to form a biofilm was examined according to Stepanović et al. (2007) using the polystyrene surface of flat-bottomed 96-well microtiter plates. Bacterial cultures were incubated in 5 mL of Trypticase Soy Broth (TSB, Difco, Sparks, MD, USA) at 37 °C for 24 h. After incubation, the cultures were diluted with sterile TSB to obtain a turbidity similar to the 0.5 McFarland scale (i.e., ~10^8 CFU/mL) and diluted again 1:100 in TSB supplemented with 1 % glucose (Merck, Rio de Janeiro, RJ, Brazil) to obtain an inoculum of approximately 10^6 CFU/mL. Diluted bacteria was vortexed and then inoculated in a microtiter plate (200 μL per well). Each isolate was tested in triplicate, and the assay was repeated three times. Negative controls were represented by wells containing 200 μL of TSB. Inoculated plates were covered with a lid and incubated for 24 h at 37 °C. After incubation, the content of the wells was discarded and each well was washed three times with 300 μl of sterile phosphate-buffered saline (PBS, pH 7.2). After washing, the remaining attached bacteria were heat-fixed by incubating at 60 °C for

60 min. The adherent biofilm layer formed in each well was stained with 150 μL of 2 % crystal violet for 15 min at room temperature. The stain was then aspirated and the plate was rinsed under running tap water until the washings were free of stain. After the plate was air-dried at room temperature, the dye bound to the cells was eluted using 150 μL of 95 % ethanol (v/v) per well. Lastly, the covered plate was left at room temperature for 30 min without shaking. The absorbance at 570 nm was measured using a microplate reader (Microplate Spectrophotometer Eon™, BioTek® Instruments, Winooski, VT, USA). To analyse the results of biofilm formation on polystyrene microtiter plates, the bacteria adhesion capacity was classified as one of the four following categories: a non-biofilm producer, weak biofilm producer, moderate biofilm producer, or strong biofilm producer according to Stepanović et al. (2007).

Statistical analyses

Fisher's exact test was used to test for an association between genotype (i.e., *cap5* or *cap8*) and biofilm formation (i.e., strong, moderate, weak, or non-biofilm categories) and between the biofilm formation and polysaccharide production. When a significant association was detected, the measures of association for ordinal variables, Goodman and Kruskal's gamma coefficient (γ_{GK}) and Stuart's tau-c statistic (τ_c), together with their 95 % confidence interval (CI), were used to report the strength and direction of the association.

For analyses and comparisons involving the optical density measures, nonparametric methods were employed given the non-normality of the data. Comparisons of optical densities values for both CP production and biofilm formation between genotypes were made using the Wilcoxon-Mann-Whitney test and comparisons among CP phenotypes by the Kruskal-Wallis nonparametric ANOVA followed by Dunn's pairwise multiple comparison test. The existence of a correlation between the optical densities for CP and biofilm was assessed by means of the Spearman's rank correlation and the Pearson's linear correlation coefficients; the latter was calculated for the logarithm of the densities. All analyses were conducted using R software version 3.1.3 (R Core Team 2015), with the exception of the estimation of the measures of association, which were conducted using the FREQ procedure of SAS version 9.2 (SAS Institute Inc. 2009). For all tests, statistical significance was considered when p was less than or equal to 0.05.

Results

Polysaccharide production

Amplification of *cap* genes resulted in amplicons with the expected sizes. DNA sequencing of the fragments obtained from two isolates carrying *cap5* or *cap8* confirmed that the fragments were representative of these genes.

All 159 isolates amplified one of the two *cap* genes: 128 isolates (80 %) amplified *cap5* and 31 isolates (20 %) amplified *cap8* gene (Table 1). Thus, the majority of the isolates analysed were of the *cap5* genotype. The *cap5* genotype predominated in the isolates from the north (96 %) and southeast (82 %) regions, while only 47 % of the isolates from the south were *cap5*, and the remaining isolates were *cap8*.

Isolates genotyped as *cap5* and *cap8* were tested with rabbit sera anti-CP5 and anti-CP8, respectively. Of the 159 *S. aureus* analysed, 92 (58 %) expressed CP5 and 17 (11 %) expressed CP8. Fifty isolates (31 %) expressed neither CP5 nor CP8 (Table 1). Of the 128 isolates genotyped as *cap5*, 92 (72 %) expressed CP5 and 36 (28 %) did not express CP5 and were considered to be phenotypically non-typeable. Of the 31 isolates genotyped as *cap8*, 17 (55 %) expressed CP8 and 14 (45 %) did not and were considered to be phenotypically non-typeable.

Globally, all 159 isolates from this study harboured one of the searched genes, *cap5* or *cap8*; however, only 109 isolates (69 %) expressed CP in vitro. A higher percentage of isolates carrying the *cap8* gene did not express CP8 in vitro (45 %) compared with isolates carrying the *cap5* gene that did not

Table 1 The distribution of the capsular polysaccharide genotypes and phenotypes for 159 *Staphylococcus aureus* isolates obtained from bovine milk collected from three geographical regions in Brazil

	Geographical regions							
	North		Southeast		South		Total	
	Genotype	Phenotype	Genotype	Phenotype	Genotype	Phenotype	Genotype	Phenotype
cap5 (%)	24 (96 %)	19 (76 %)	96 (82 %)	67 (57 %)	8 (47 %)	6 (35 %)	128 (80 %)	92 (58 %)
cap8 (%)	1 (4 %)	1 (4 %)	21 (18 %)	16 (14 %)	9 (53 %)	0	31 (20 %)	17 (11 %)
NT[1] (%)	0	5 (20 %)	0	34 (29 %)	0	11 (65 %)	0	50 (31 %)
Total	25		117		17		159	

[1] *NT* non-typeable

express CP5 (28 %). This difference, however, was not statistically significant.

Isolates harbouring *cap8* produced more CP (optical density – OD median of 0.21) than *cap5* isolates (OD median of 0.11) (pWMW <0.0001), as shown in Fig. 2a. A similar result was observed for the phenotypic detection of CP, since CP8 producing isolates presented an OD median of 0.23 and CP5 isolates produced an OD median of 0.13 (pDunn =0.0031) (Fig. 2b). Phenotypically non-typeable isolates presented an OD median of 0.09 for CP production.

Isolates harbourig *cap5* presented an OD median for biofilm formation of 1.77, whereas *cap8* isolates presented an OD median for biofilm formation of 0.84 ($p_{WMW} < 0.0001$) (Fig. 2c). Similarly, CP5 isolates presented an OD median for biofilm formation of 1.67 and CP8 isolates presented an OD median for biofilm formation of 0.84 (pDunn =0.0003) (Fig. 2d). Phenotypically non-typeable isolates presented an OD median of 1.51 for biofilm production.

Biofilm formation

All 159 *S. aureus* isolates showed amplification products corresponding to the *icaA* and *icaD* genes. One-hundred-fifty-two isolates (95.6 %) amplified a 598-bp product, corresponding to the *bap* gene. DNA sequencing of the fragments obtained by PCR from the three isolates carrying *icaA*, *icaD*, or *bap* confirmed that the fragments were a part of the amplified genes.

Of the 159 isolates analysed, 155 (97.5 %) were biofilm producers: 73 isolates (45.9 %) were considered to be strong biofilm producers, 47 isolates (29.6 %) were classified as moderate biofilm producers and 35 isolates (22 %) were classified as weak biofilm producers. Four isolates (2.5 %) did not produce a biofilm. There was a predominance of the strong biofilm producer phenotype among isolates from the north (68 %) and southeast (46.1 %), while in the south, moderate (47 %) and weak (41 %) biofilm producers predominated (Table 2).

Of the 159 isolates tested, 152 had the three genes related to biofilm production, *icaA*, *icaD*, and *bap*. These isolates were classified as strong (72 isolates), moderate (44 isolates), weak (32 isolates), or non-biofilm-forming (4 isolates). The seven isolates that did not harbour the *bap* gene were biofilm producers. They were classified as strong (1 isolate), moderate (3 isolates), and weak (3 isolates) biofilm formers.

Polysaccharide production versus biofilm formation

Fifty-six percent of the *cap5* isolates (and 53 % of the CP5 isolates) were strong biofilm producers, whereas 93.6 % of the *cap8* isolates (and 88 % of the CP8 isolates) were moderate or weak producers (Table 3). There was evidence of a statistically significant association between the genotype (i.e., *cap5* or *cap8*) and amount of biofilm produced (i.e., strong, moderate, weak, or non-biofilm categories) ($p_{Fisher} < 0.0001$; $\gamma_{GK} = -0.73$, 95 % CI = −0.87 to −0.59; $\tau_c = -0.36$, 95 % CI = −0.48 to −0.24) and between the phenotype (i.e., CP5 or CP8) and amount of biofilm produced ($p_{Fisher} = 0.0004$; $\gamma_{GK} = -0.69$, 95 % CI = −0.90 to −0.48; $\tau_c = -0.28$, 95 % CI = −0.43 to −0.14). We also found a statistically significant negative correlation of moderate intensity ($r_{Pearson} = -0.42$ for the logarithm of the variables; $p < 0.0001$; $r_{Spearman} = -0.42$; $p < 0.0001$) between the optical densities for CP production and biofilm formation.

Discussion

Polysaccharide production

Capsule production by *S. aureus* enhances microbial virulence by rendering the bacteria resistant to phagocytosis. The prevalence of encapsulated *S. aureus* among bovine mastitis samples is variable and influenced by the geographic source of the isolate (Cocchiaro et al. 2006).

In the present study, all *S. aureus* isolates carried *cap* genes and were classified based on the presence or absence of either the *cap5* or *cap8* genes. The presence of *cap* genes in all of the isolates analysed was also reported in the studies conducted by Tollersrud et al. (2000); Salasia et al. (2004); Bardiau et al. (2014), and Khichar and Kataria (2014), with *S. aureus* isolated from bovine mastitis, as well as by Verdier et al. (2007), with *S. aureus* isolated from human infections. Nevertheless, Reinoso et al. (2008) and Camussone et al. (2012) in Argentina, Babra et al. (2013) in Australia and Marques et al. (2013) in Brazil reported lower percentages of *S. aureus* isolates collected from bovine mastitis samples carrying the *cap* gene.

Eighty percent of the isolates from this study were classified as *cap5* and the remainder as *cap8*. A higher proportion of *cap5* isolates (compared to *cap8* isolates) has also been reported by Salasia et al. (2004) in Indonesia, Reinoso et al. (2008) and Camussone et al. (2012) in Argentina, and Khichar and Kataria (2014) in India. Other studies conducted in different countries reported that *cap5* and *cap8* were evenly distributed among *S. aureus* isolates from bovine mastitis (Bar-Gal et al. 2015) or that there was a higher proportion of *cap8* than *cap5* isolates (Salasia et al. 2004; Babra et al. 2013; Marques et al. 2013; Bardiau et al. 2014).

In our study, 58 % of the isolates expressed CP5 and 11 % expressed CP8. A higher proportion of isolates expressing CP5 has also been reported in studies conducted by Poutrel et al. (1988) in France and by Camussone et al. (2012) in Argentina. However, several studies that examined the CP serotype among *S. aureus* from bovine mastitis samples reported a higher proportion of serotype 8 in relation to serotype

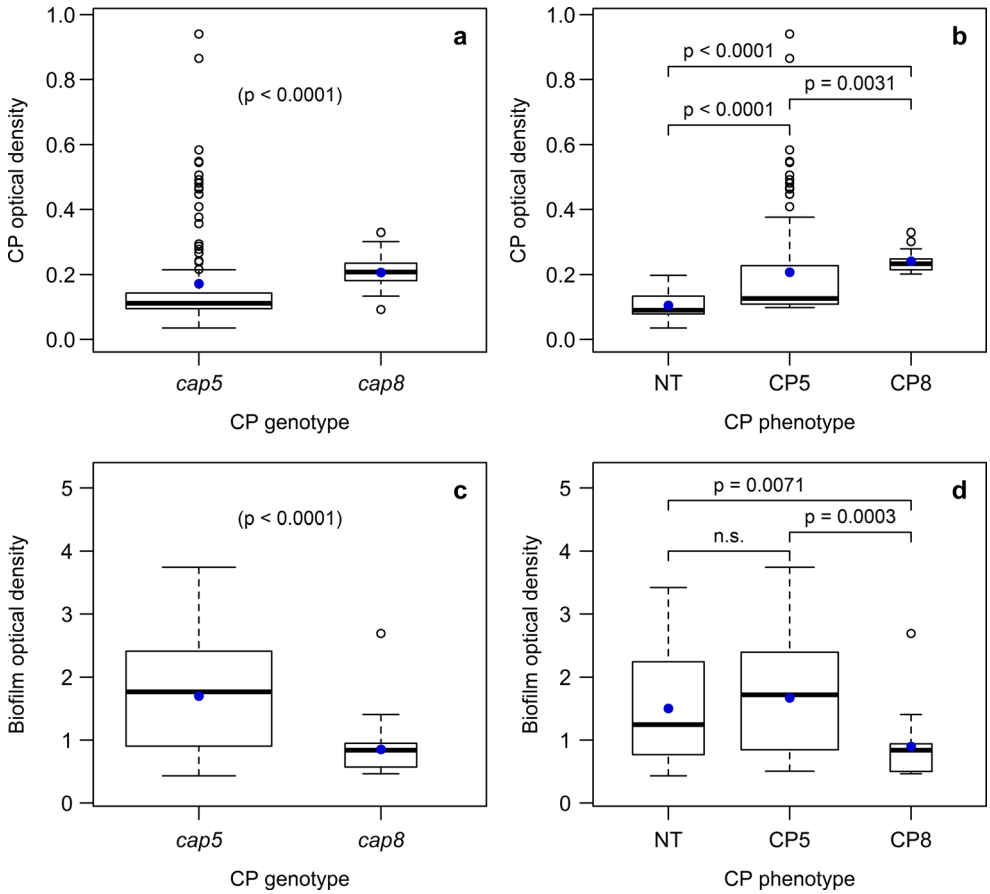

Fig. 2 Box plot representation of capsular polysaccharide (CP) and biofilm optical densities distributions by CP genotype and phenotype, with boxes' widths proportional to sample sizes. Top line graphs **a** and **b**: CP optical densities by CP genotype and phenotype, respectively; bottom line graphs **c** and **d**: biofilm optical densities by CP genotype and phenotype, respectively. Each box spans from the first to the third quartile (i.e., the interquartile range or IQR); the bold segment inside the box and the filled circle indicate the median's and the mean's locations, respectively; whiskers above and below the box extend either to the minimum/maximum data value or to the most extreme value falling within the extent equivalent to 1.5 × IQR starting from the box; points exceeding these limits are considered suspected outliers and are marked with unfilled circles. NT: non-typeable; n.s.: statistically not significant

5 or similar proportions between the two phenotypes in different countries in Europe, the USA, Korea, Japan, and Belgium (Guidry et al. 1998; Tollersrud et al. 2000; Han et al. 2000; Hata et al. 2006; Bardiau et al. 2014).

According to O'Riordan and Lee (2004), the expression of *S. aureus* CP5 and CP8 in vitro is highly sensitive to various environmental signals and is probably influenced by the in vivo environment as well. Bacterial growth conditions, such as the culture medium, have been shown to influence CP production. Capsule production in vitro is inhibited by yeast extract, alkaline growth conditions and anaerobiosis, but is enhanced by the growth of the bacterium in milk or in medium supplemented with up to 5 % NaCl. The growth conditions could be the reason for the different results observed among the several studies and for the large number of phenotypically non-typeable isolates observed in this and other studies.

Table 2 The distribution of biofilm production phenotypes for 159 *Staphylococcus aureus* isolates from bovine milk collected from three geographical regions in Brazil

Biofilm production	North	Southeast	South	Total (%)
Strong producer	17 (68 %)	54 (46.1 %)	2 (12 %)	73 (45.9 %)
Moderate producer	7 (28 %)	32 (27.4 %)	8 (47 %)	47 (29.6 %)
Weak producer	1 (4 %)	27 (23.1 %)	7 (41 %)	35 (22 %)
Non producer	0 (0 %)	4 (3.4 %)	0 (0 %)	4 (2.5 %)
Total	25	117	17	159 (100 %)

Mutations or deletions within the *cap* locus may be the reason for the failure to react to capsular antisera, as suggested previously. Cocchiaro et al. (2006) demonstrated that strains that were positive for *cap* genes but that were phenotypically non-typeable had mutations in essential *cap* genes, in regulatory loci or in the promoter upstream the *cap 5*(*8*) *A* gene, resulting in a CP-negative phenotype. These authors also reported bovine *S. aureus* strains that did not hybridize to any of the capsule genes, which indicates that the *cap 5*(*8*) gene cluster is absent. Tuchscherr et al. (2007) detected a variant of IS257 that displaced the capsule genes within phenotypically non-typeable *S. aureus* isolated from bovine mastitis in Argentina. The authors hypothesized that the deletion of the *cap5*(*8*) locus occurred in the past and that non-typeable isolates may have enhanced the ability to persist in subclinical intramammary infections in cows.

Biofilm formation

The ability of *S. aureus* to form biofilm helps the bacteria to survive in hostile environments within the host and is

Table 3 A comparison between capsular polysaccharide genotype and phenotype and biofilm production for 159 *Staphylococcus aureus* isolated from bovine milk in Brazil

Biofilm production	Polysaccharide genotype and phenotype				
	cap5	CP5	*cap8*	CP8	NT[1]
Strong producer	72 (56 %)	49 (53 %)	1 (3.2 %)	1 (6 %)	23 (46 %)
Moderate producer	33 (26 %)	23 (25 %)	14 (45.2 %)	6 (35 %)	18 (36 %)
Weak producer	20 (16 %)	17 (18.5 %)	15 (48.4 %)	9 (53 %)	9 (18 %)
Non producer	3 (2 %)	3 (3.5 %)	1 (3.2 %)	1 (6 %)	0
Total	128	92	31	17	50

[1] *NT* non-typeable

considered to be responsible for chronic and persistent infections, including bovine mastitis (Costerton et al. 1999; Cucarella et al. 2004).

Regarding the presence of *icaA* and *icaD* genes, 100 % of the isolates from this study contained both genes, indicating that all harboured the *icaADBC* locus. Studies conducted with *S. aureus* isolated from bovine mastitis in different countries reported the concomitant presence of both genes in most of the *S. aureus* isolates analysed (Vasudevan et al. 2003; Cucarella et al. 2004; Szweda et al. 2012; Melo et al. 2013; Prenafeta et al. 2014; Bar-Gal et al. 2015; Castelani et al. 2015; Fabres-Klein et al. 2015). Moreover, other studies (Ciftci et al. 2009; Dhanawade et al. 2010; Coelho et al. 2011; Darwish and Asfour 2013; Marques et al. 2013) reported that lower percentages of *S. aureus* of bovine origin harboured these genes.

Regarding the presence of the *bap* gene, it was present in 95.6 % of the isolates from this study, a very high percentage compared with previous studies. Sung et al. (2008); Vautor et al. (2008), and Szweda et al. (2012) did not find this gene among *S. aureus* isolated from bovine mastitis specimens, whereas Darwish and Asfour (2013) found only one isolate harbouring the *bap* gene. Cucarella et al. (2004) in Spain and Zuniga et al. (2015) in Brazil reported that 25.6 % and 15.8 % of *S. aureus* isolates from bovine mastitis samples harboured this gene, respectively.

Vautor et al. (2008) reported that the prevalence of the *bap* gene among *S. aureus* isolates, including bovine mastitis isolates, was very low. Conversely, most of the isolates analysed in the present study harboured this gene. The nature of the discrepancies between studies may be due to geographical differences. The *bap*-positive isolates from this study were obtained from farms located at different geographical regions and over a long time period, so a clonal origin of the isolates was discarded.

Tremblay et al. (2013) found biofilm-associated genes in coagulase-negative staphylococci (CNS) isolated from milk samples from Canadian dairy farms, including the *bap* gene. The distribution of the *bap* gene among CNS species varied considerably; in the species *Staphylococcus xylosus*, *bap* was detected in 92 % of the isolates, a result similar to ours, although the isolates belonged to different staphylococcal species.

Concerning biofilm formation on polystyrene microtiter plates, only four isolates were not able to produce a biofilm in vitro. The percentage of biofilm producer isolates found in this study was similar to those reported by Babra et al. (2013); Darwish and Asfour (2013); Melo et al. (2013), and Krewer et al. (2015) in studies conducted with *S. aureus* isolated from bovine mastitis samples from Australia, Egypt and Brazil. In these studies and in our study, the percentage of biofilm producing isolates was high, ranging from 97 % to 100 % positivity. The percentage of biofilm producing isolates in our study was, however, higher than those reported by Vasudevan et al. (2003); Fox et al. (2005); Dhanawade et al. (2010); Coelho et al. (2011); Szweda et al. (2012); Marques et al. (2013); Bardiau et al. (2014), and Fabres-Klein et al. (2015) with *S. aureus* from bovine mastitis samples. In studies conducted in Brazil, Coelho et al. (2011); Marques et al. (2013), and Fabres-Klein et al. (2015) found that 80 %, 81 % and 87 % of isolates produced biofilms, respectively.

In this study almost half of the isolates were strong biofilm producers; the remainder of the isolates were moderate and weak biofilm producers. The number of strong biofilm producers was higher than that reported by Krewer et al. (2015) in *S. aureus* isolated from bovine mastitis samples from northeast Brazil.

The microtiter plate method is frequently used to detect biofilm production, but different protocols have been used in these investigations. The different culture conditions and biofilm quantification in vitro and also the interpretation criteria can contribute to different results between studies (Götz 2002; Stepanović et al. 2007; Prenafeta et al. 2014). Prenafeta et al. (2014) obtained different results for biofilm production by methicillin-resistant bovine *S. aureus* using two different interpretation criteria. Xue et al. (2014) reported that milk and lactose increased biofilm formation in two bovine mastitis *S. aureus* isolates. Fabres-Klein et al. (2015) demonstrated that milk and slime production are factors that have a positive effect on biofilm production by bovine *S. aureus*. In addition, they suggested that bovine isolates of *S. aureus* adapt to the milieu found in the udder, with milk influencing biofilm

production and consequently promoting bacterial survival. According to Götz (2002), the failure of *ica*-positive staphylococcal isolates to form a biofilm in vitro could be due to point mutations in the locus or to the insertion of sequence elements which can turn on or off *ica* expression.

In the present study, we found a significant association between the capsular genotype and phenotype and the amount of biofilm formation, i.e., *cap5* (or CP5) isolates tended to form more biofilm and to produce a thinner CP layer than *cap8* (or CP8) isolates. We also observed a negative correlation between the optical densities for CP production and biofilm formation, which may suggest that isolates that produce a thicker CP layer produce a thinner biofilm layer, and vice-versa.

Bardiau et al. (2014) investigated and correlated properties that may be associated with persistent mastitis, including the expression of CP5 and CP8 and biofilm production. They also observed a correlation between the capsular profile and biofilm production. As in the present study, these authors also reported that more *cap8* isolates were weak biofilm producers. On the other hand, Babra et al. (2013) investigated the association between biofilm formation and capsular phenotypes from *S. aureus* isolated from bovine mastitis samples from Australia and did not find an association between both features. However, isolates in the latest study were not categorized in relation to the amount of biofilm formed as they were in the present study and in the study from Bardiau et al. (2014).

We observed that isolates from the Brazilian southern region differed in the capsular genotype and the presence of the *bap* gene from isolates from the southeastern and northern regions. Nevertheless, due to the small number of isolates from the southern region that were analysed, this observation should be confirmed using a larger number of isolates from this region.

The great number of biofilm producer isolates observed in this study could explain the failure to treat mammary infections caused by *S. aureus* and the persistence of the infection by these organisms that is frequently reported in the literature and also by field veterinarians and producers. Staphylococcal cells embedded in a biofilm or in microcolonies are much more resistant to antibiotics than planktonic cells, and once a biofilm is formed, treatment with currently available antibiotics is difficult (Götz 2002).

According to Scali et al. (2015) to date available vaccines do not provide consistent protection against *S. aureus* intramammary infection. Despite the unsatisfactory results, the increasing concerns on antibiotics usage in food production and the need of sustainable dairy farming underscore the interest in developing new tools, including vaccines, to control widespread diseases such as *S. aureus* mastitis. Capsular polysaccharides and biofilm are two virulence factors used as vaccine targets against *S. aureus* bovine intrammamary infection. Once all *S. aureus* isolates analysed in this study harboured genes involved in capsule production and in biofilm formation and a large proportion of these isolates produced capsule and/or biofilm in vitro, a vaccine against bovine staphylococcal mastitis containing these components would be suitable for use in Brazil.

Conclusion

All of the *S. aureus* isolates analysed in this study harboured genes involved in capsule production and in biofilm formation. Sixty-nine percent of the isolates produced capsular polysaccharide in vitro and 97.5 % formed a biofilm on polystyrene microtiter plates. The great majority of isolates harboured the *bap* gene. A significant association between the capsular genotype and phenotype and the amount of biofilm formation was detected, i.e., *cap5* (or CP5) isolates tended to form more biofilm and to produce a thinner CP layer than *cap8* (or CP8) isolates. The results indicate a high pathogenicity potential among *S. aureus* isolated from bovine milk in three different Brazilian regions and suggest that both capsule production and biofilm formation play an important role in the virulence of *S. aureus* isolated from bovine mammary glands in Brazil.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent This article does not contain any studies with human participants or animals performed by any of the authors.

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