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VETERINARY MICROBIOLOGY - RESEARCH PAPER

Virulence factors and antimicrobial resistance in *Staphylococcus aureus* isolated from bovine mastitis in Brazil

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Abstract

This study aimed to evaluate virulence factors and genetic markers of antimicrobial resistance in 400 *Staphylococcus aureus* strains isolated from bovine mastitis in four Brazilian states, as well as to assess the association between these characteristics and field information. Virulence factors and drug resistance genes were identified by PCR screening. Biofilm-forming and hemolytic phenotype were detected using Congo red Tryptic Soy Broth and defibrinated sheep blood agar, respectively. Of all isolates, 83.5% were biofilm-forming and 98.5% strains exhibited biofilm gene *icaAD*, and a significant association between phenotype and genotype for biofilm was observed ($P = 0.0005$). Hemolysin genes were observed in 82.85% ($hla^{+}hlb^{+}$), 16.5% (hla^{+}) and 0.75% (hlb^{+}) isolates, whereas the hemolytic phenotype exhibited was complete and incomplete hemolysis in 64.25%, complete in 28.25%, incomplete in 4.75%, and negative in 2.75% of the strains. Virulence factors genes *luk*, *seb, sec*, *sed*, and *tst* were observed in 3.5%, 0.5%, 1%, 0.25%, and 0.74% isolates, respectively. The gene *blaZ* was detected in 82.03% of penicillin-resistant isolates, whereas *tetK* and *aac(6')-Ie–aph(2')-Ia* were observed in 33.87% and 45.15% of the tetracycline and aminoglycosides-resistant isolates, respectively. Fluoroquinolone resistance gene *mepA* was detected for the first time in *S. aureus* from bovine mastitis. Resistance genes *tetM* (3.22%), *tetL* (1.61%), *ermA* (14.29%), *ermB* (14.29%), *ermC* (33.3%), *ermT* (9.52%), *ermY* (4.76%), *msrA* (9.52%), and *mphC* (9.52%) were also detected among resistant isolates. No association between virulence factors or antimicrobial-resistant genes and year of isolation, geographic origin, or antimicrobial resistance profile was observed. Our results showed that *S. aureus* strains isolated from bovine mastitis in the four Brazilian states sampled are mainly biofilm-forming and hemolytic, whereas virulence genes associated with enterotoxins, *luk* and *tst*, were less frequently observed. Moreover, a wide variety of resistance genes that confer resistance to almost all classes of antimicrobial agents approved for use in animals and humans were found. Overall, the data point to a great pathogenic potential of *S. aureus* associated with bovine mastitis and to the non-negligible risks to public health of staphylococcal infections from animal origin.

Keywords Staphylococci · Intramammary infection · Hemolysins · Biofilm · *mepA*

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Introduction

Bovine mastitis is one of the most common diseases that affects the world dairy production, being responsible for decreasing quantity and quality of milk produced [1]. *Staphylococcus aureus* is one of the main pathogens isolated from bovine mastitis in different parts of the world where dairy farming is expressive [1]. Indeed, studies conducted in countries from Europe, Africa, and America have reported a high prevalence of *S. aureus* in dairy cows reaching 41% in France (1995–2012), 47.2% in herds from Italy (2012–2013) [2], 74% in Ethiopia (2014–2015) [3], and Canada (2003–2005) [4]. In Brazil, several studies have showed the importance of *S. aureus* in the epidemiology of mastitis in cattle [5–8]. Although it can be involved in clinical mastitis (especially after calving), the infection is usually subclinical, causing no visible changes in milk or udder [1].

The *S. aureus*' ability to cause infections are related to the expression of various virulence factors, which are structures, products, or mechanisms frequently acquired by mobile genetic elements [9–11]. It is well documented that *S. aureus* strains from bovine mastitis can produce a wide variety of extracellular toxins, such as enterotoxins, encoded mainly by *sea*, *seb*, *sec*, *sed*, and *see* genes, toxic shock syndrome toxin 1 (*tst*), Panton–Valentine leukocidin (PVL) (*luk*), alpha, and beta hemolysins (*hla* and *hlb*), among others [10, 12, 13]. The ability to produce toxins by *S. aureus* strains from animal origin is not only of animal health but also of public health concern, since some of these toxins, such as enterotoxins, hemolysins and toxic shock syndrome toxin 1, in addition to favoring the infection, are also thermostable and remain active even after the thermal treatments used in milk [14]. Furthermore, the production of extracellular polymeric substances (EPS), mainly exopolysaccharide, appears to play a crucial role in the infection, adhesion, and colonization of the mammary glandular epithelium [15]. Thus, formation of biofilm also ensures the successful colonization and maintenance of *S. aureus* in the host tissues.

In addition to being an important factor for the colonization, biofilm formation by *S. aureus* strains isolated from bovine mastitis, which expresses the *icaA* and *icaD* genes, may be associated with antimicrobial resistance [15]. The mechanisms responsible for drug resistance include physical and chemical diffusion barriers formed by the exopolysaccharide matrix, which makes the penetration of antimicrobials difficult, besides creating microenvironments that antagonize the drug [5]. Antimicrobial resistance is a major problem in animal and public health, and an alarming increase in drug resistance among *S. aureus* strains has been reported worldwide [16–18]. Antimicrobial resistance genes (ARG) commonly reported in *Staphylococcus* spp. isolated from cattle are mainly *mecA* and *blaZ* (β-lactam resistance) [18, 19]; *tetK*, *tetL*, and *tetM*, (tetracycline resistance) [20]; *ermA*, *ermB*, *ermC*, *ermT*, *ermY*, *msrA*, and *mphC* [macrolide, lincosamide, streptogramin B (MLSB), and macrolide phosphotransferase resistance] [17, 21]; *aac(6')-Ie–aph(2')-Ia* [aminoglycoside modifying enzyme (AME)] [17]; and *mepA*, *grlA/grlB*, and *gyrA/gyrB* (fluoroquinolone resistance) [22]. Staphylococci carrying such resistance genes play a key role in the spread of resistance as they can easily exchange their resistance genes by horizontal transfer with staphylococci or other Gram-positive bacteria from animal or human origin, which increases the risk of antimicrobial resistance transmission from animals to humans [16, 22].

A further understanding of the potential for damage of *S. aureus* isolates from milk is of great importance considering its zoonotic potential, since this agent is considered as one of the main pathogens causing food poisoning [23] and the principal agent responsible for the contagious mastitis worldwide [1]. From animal and also public health's points of view, it is essential to determine which virulence factors and, chiefly, resistance genes are carried by *S. aureus* isolates from a certain region. Therefore, the aims of this study were to evaluate (*i*) virulence factors and (*ii*) genetic markers of drug resistance of *S. aureus* strains isolated from bovine mastitis in four Brazilian states (Minas Gerais, São Paulo, Rio de Janeiro, and Goiás), as well as (*iii*) the temporal and spatial association of these characteristics.

Material and methods

Bacterial strains and culture conditions

A total of 400 *S. aureus* strains isolated from cows with mastitis were used in the present study. These are representative *S. aureus* strains from the Collection of Microorganisms of Agribusiness Interest from Empresa Brasileira de Pesquisa Agropecuária (Embrapa) Gado de Leite, isolated between 1994 and 2016 from different Brazilian states. Embrapa is a Brazilian reference center for bovine mastitis research, and its collection comprises *S. aureus* strains from great temporal and geographic distributions, covering the main milk-producing regions in Brazil. The isolates were randomly sampled from the 1168 *S. aureus* strains of the Embrapa's collection to obtain about 33% of all *S. aureus* in the collection and a minimum of 30% of strains from each state (supplementary Table S1). In addition, a minimum of 20% of isolates was sampled per year, with the exception of 5 years (2005, 2006, 2007, 2008, and 2013) of the 22 years sampled (1994 to 2016), for which some strains did not grow on thawing. The distribution of the isolates sampled per year and state is shown in the Fig. 1a. Of the 400 strains, 31 were isolated from bulk tank milk samples, whereas the remaining (369) were obtained from individual cow's milk samples (combined sample from all quarters or from individual mammary quarters). The

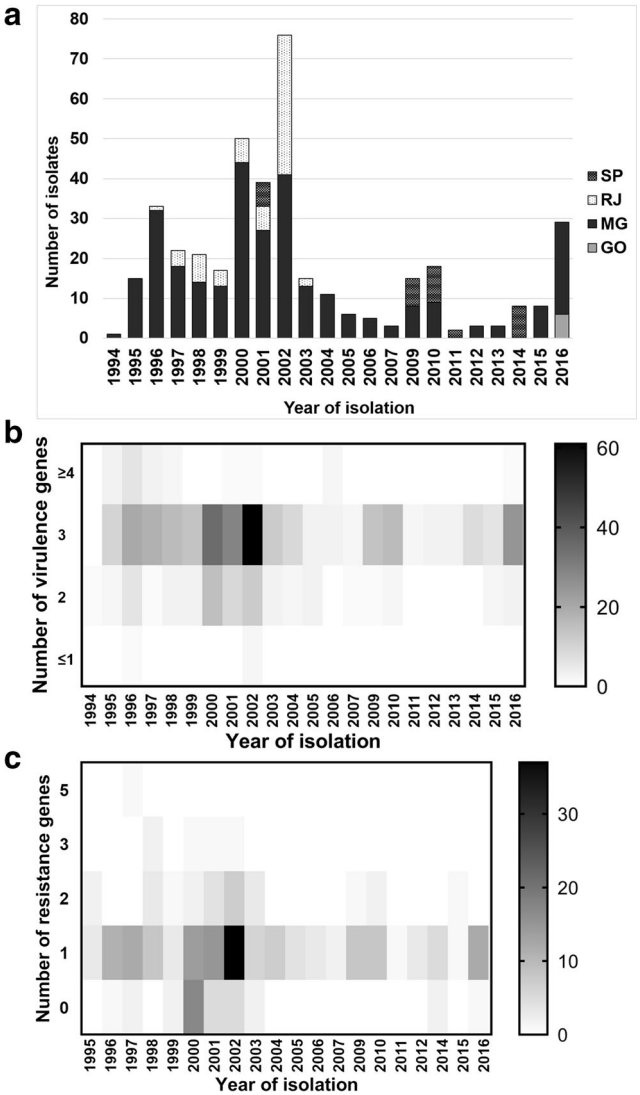

Fig. 1 Distribution, virulence, and antimicrobial-resistant profile of *Staphylococcus aureus* isolated from bovine mastitis in Brazil, 1994–2016. **a** Distribution of *S. aureus* according to the year of isolation and Brazilian state (*SP* São Paulo, *RJ* Rio de Janeiro, *MG* Minas Gerais, and *GO* Goiás). **b** Distribution of the number of virulence genes among *S. aureus* isolated from bovine mastitis in São Paulo, Rio de Janeiro, Minas Gerais, and Goiás states, Brazil, 1994–2016, according to the year of isolation. **c** Distribution of the number of antimicrobial resistance genes among *S. aureus* isolated from bovine mastitis in São Paulo, Rio de Janeiro, Minas Gerais, and Goiás states, Brazil, 1994–2016, according to the year of isolation

bacteriological analysis was carried out by culturing 10 μL of milk on 5% defibrinated sheep blood agar at 37 °C for 24 to 48 h. Colonies were identified by Gram test, morphology, size, pigmentation, and biochemical tests [24].

The antimicrobial susceptibility profile of all studied strains was carried out previously by Aizawa et al. [25] using agar disk diffusion method and the following antimicrobial disks (Sensifar®, Brazil): cefoxitin (30 μg), oxacillin (1 μg), ampicillin (10 μg), enrofloxacin (5 μg), ciprofloxacin (5 μg), cephalothin (30 μg), ceftiofur (30 μg), amoxicillin + clavulanic acid (20/10 μg), erythromycin (15 μg), neomycin (30 μg), gentamicin (10 μg), tetracycline (30 μg), sulfamethoxazole + trimethoprim (1.25/23.75 μg), and penicillin-novobiocin (10UI/30 μg). Only isolates that exhibited a resistance phenotype to any of the antimicrobials previously tested were evaluated for the presence of drug resistance genes, according to the profile observed. The search for genes associated with antimicrobial resistance was restricted to strains that showed resistant phenotype to allow more robust inferences about the pathogenic potential of *S. aureus* strains assessed. The search for genes associated with virulence was carried out in all isolates.

The isolates were cultured on Brain Heart Infusion (BHI) agar (Merck, Germany) plates, at 37 °C, for 24 h, in aerobic conditions. All strains were stored in BHI broth (Merck, Germany) supplemented with 20% glycerol (Sigma-Aldrich, USA) at − 80 °C.

DNA extraction

After growth on BHI plates, all strains were suspended in phosphate-buffered saline (PBS) (0.01 M, pH 7.4, all reagents from Merck, Germany) and centrifuged, and the pellet was submitted to genomic DNA extraction with guanidium thiocyanate (Merck, Germany), according to Pitcher et al. [26]. The quantity and quality of DNA extracted were assessed by spectrophotometry using the NanoVue™ spectrophotometer (GE Healthcare, USA). DNA samples were kept at − 20 °C until the analysis.

Identification of *S. aureus*

In addition to phenotypic identification [24], all strains were also tested for the presence of conserved thermonuclease gene (*nuc*) by PCR (Table 1) to be confirmed as *S. aureus* [27]. Visualization of the amplified PCR products was performed in 1.0% agarose gel in tris-borate-EDTA buffer (TBE) (89 mM Tris Base, 89 mM boric acid, and 2 mM EDTA; pH 8.0; all from Sigma-Aldrich, USA) and stained with ethidium bromide (0.5 mg/mL) (Ludwig Biotecnologia Ltda, Brazil). Following electrophoresis, the gels were visualized under ultraviolet light and photographed (L-PIX EX, Loccus Biotechnology, Brazil). The molecular weight marker 100 bp DNA ladder (Kasvi, Brazil) was used in all electrophoresis.

Phenotypic detection of biofilm and hemolysin production

For phenotypic identification of biofilm-forming strains, four colonies of each strain were inoculated in trypticase broth (TSB) (Merck, Germany) supplemented with Congo Red (0.8 g/L) and sucrose (36 g/L) (Sigma-Aldrich, USA), and

Table 1 Genes investigated in *Staphylococcus aureus* isolated from bovine mastitis in this study by PCR

Target	Gene	Sequence (5′ to 3′)	Amplicon size (bp)[a]	Positive control (ATCC)[b]	Reference	Modifications
Thermostable nuclease (*S. aureus* species-specific)	*nuc*	F AGTTCAGCAAATGCATCACA R TAGCCAAGCCTTGACGAACT	400	25,923	[27]	Annealing at 56 °C for 30 s and extension at 72 °C for 30 s
Staphylococcal enterotoxin A	*sea*	F GGTTATCAATGTGCGGGTGG R CGGCACTTTTTTCTCTTCGG	102	13,565	[28]	2.5 mM $MgCl_2$
Staphylococcal enterotoxin B	*seb*	F GTATGGTGGTGTAACTGAGC R CCAAATAGTGACGAGTTAGG	164	14,458	[28]	
Staphylococcal enterotoxin C	*sec*	F AGATGAAGTAGTTGATGTGTATGG R CACACTTTTAGAATCAACCG	451	19,095	[28]	
Staphylococcal enterotoxin D	*sed*	F CCAATAATAGGAGAAAATAAAAG R ATTGGTATTTTTTTTCGTTC	278	23,235	[28]	
Staphylococcal enterotoxin E	*see*	F AGGTTTTTTCACAGGTCATCC R CTTTTTTTTCTTCGGTCAATC	209	27,644	[28]	
Resistance to methicillin	*femA*	F AAAAAAGCACATAACAAGCG R GATAAAGAAGAAACCAGCAG	132	25,923	[28]	Annealing at 57 °C for 1 min and extension at 72 °C for 1 min
Toxic shock syndrome toxin 1	*tst*	F ACCCCTGTTCCCTTATCATC R TTTTCAGTATTTGTAACGCC	326	33,586	[28]	
Panton–Valentine leukocidin (PVL)	*luk*	F ATCATTAGGTAAAATGTCTG GACATGATCCA R GCATCAASTGTATTGGATAG CAAAAGC	433	25,923	[29]	Annealing at 62 °C for 1 min and extension at 72 °C for 1 min
Alpha-hemolysin	*hla*	F CTGATTACTATCCAAGAAAT TCGATTG R CTTTCCAGCCTACTTTTTTATCAGT	209	8096	[10]	Unchanged
Beta-hemolysin	*hlb*	F GTGCACTTACTGACAATAGTGC R GTTGATGAGTAGCTACCTTCAGT	309	13,565	[10]	
Biofilm	*icaAD*	F CCTAACTAACGAAAGGTAGG R TTAGCGTTGGGTATTCCCTC	1266	51,651	[11]	

[a] Base pairs (bp)

[b] American Type Culture Collection (ATCC)

incubated for 48 h at 37 °C, as described by Lee et al. [30]. *Staphylococcus aureus* ATCC 51651 and *S. chromogenes*, isolated from bovine mastitis belonging to the Collection of Microorganisms from Laboratório de Bacteriologia, Departamento de Medicina Veterinária, Universidade Federal de Lavras, were used as positive and negative controls in all assays, respectively (supplementary Fig. S1A).

Strains were tested for hemolysis production on 5% (*v*/*v*) defibrinated sheep blood agar (Merck, Germany) cultured at 37 °C for 24 h [24]. The hemolytic phenomenon was then observed, and zones of complete hemolysis were considered due to alpha-hemolysin, whereas darkening zones surrounding the colonies (incomplete hemolysis) were regarded as beta-hemolysin zone (supplementary Fig. S1B). *Staphylococcus aureus* ATCC 25923 (complete hemolytic phenotype) and *S. hyicus* (non-hemolytic), isolated from bovine mastitis belonging to the Collection of Microorganisms from Laboratório de Bacteriologia, Departamento de Medicina Veterinária, Universidade Federal de Lavras, were used as control for comparative analyses.

Detection of virulence genes

Detection of the biofilm genes *icaAD* [11]; enterotoxins *sea*, *seb*, *sec*, *sed*, and *see* [28]; hemolysins *hla* and *hlb* [10]; toxic shock syndrome toxin (TSST-1) *tst* and *femA* [28]; and Panton–Valentine leukocidin (PVL) *luk* [29] was performed by PCR screening using primers and conditions stated in Table 1. Positive controls are also described in Table 1. All reagents of the PCR mix without template DNA were routinely used in each assay, as negative control. Agarose gel electrophoresis for PCR products was performed as described in Section Identification of *S.aureus*.

Detection of antimicrobial resistance genes

Strains described as resistant, according to the phenotype previously observed [25] were screened for the presence of resistant genes. Positive controls, primers, and PCR conditions used in all PCR assays for detection of resistance genes are summarized in Table 2. Positive controls are also described in Table 2. All reagents of the PCR mix without template DNA

were routinely used in each assay, as negative control. Agarose gel electrophoresis for PCR products was performed as described in Section Identification of *S.aureus*.

Multidrug resistance was defined as resistance to three or more antimicrobial class. The antimicrobial groups were defined according to Clinical and Laboratory Standards Institute (CLSI) M100 manual (28th ed.) [38].

Screening for mutations in *gyrA* and *grlA* genes

For ciprofloxacin- and enrofloxacin-resistant strains, PCR amplification of quinolone resistance–determining regions (QRDRs) *gyrA* and *grlA* was carried out as described above. PCR-amplified DNA was separated by agarose gel electrophoresis (described in Section Identification of *S.aureus*) to verify the efficiency of amplification, purified using a PCR purification kit (Invitek, USA), and sequenced using Big Dye™ 3.1 (Applied Biosystems, USA) on an ABI-3500 automatic sequencer (Applied Biosystems, USA). The sequences obtained were submitted to quality evaluation by the Phred software (reliability index > 20) [39], grouped in a consensus with the CAP3 (Sequence Assembly Program) software [40]. Amino acid changes were identified by comparison with published wild-type sequences of GrlA encoded by the *grlA* gene [41] and GyrA encoded by the *gyrA* gene [42]. The comparisons were performed with aid of the software BioEdit 7.2 [43] using the sequences of *S. aureus* strains MRSA252 (ATCC BAA-1720™) and RN4220 (derived from NCTC8325-4) for identification of mutations potentially associated with antimicrobial resistance.

Statistical analyses

Prevalence was obtained in cross tabulations and expressed as percentage. Associations between the variables were carried out by univariate analysis using chi-square or Fisher's exact tests. In all cases, a P value ≤ 0.05 was defined as significant. All statistical analyses were performed using GraphPad Prism 8.1 (GraphPad Software, USA).

Results

Identification of *S. aureus* isolates and prevalence of biofilm production ability and biofilm-associated genes

All 400 isolates were confirmed as *S. aureus* by PCR amplification of gene *nuc*. Prevalence of biofilm formation was 83.5% (334/400) among the tested *S. aureus* isolates. PCR analysis for detection of the *icaAD* biofilm gene revealed that 98.5% (394/400) isolates harbored *icaAD* gene (Table 3). Interestingly, 83.25% (333/400) of the isolates that phenotypically were biofilm producers also exhibited *icaAD* gene; however, 15.25% (61/394) did not produce biofilm but harbored these genes. A significant association between the phenotype and genotype for biofilm production was observed ($P = 0.0005$). In contrast, no association between the biofilm phenotype or genotype and the other variables tested, such as year of isolation, geographic origin, and antimicrobial resistance profile, was observed.

Prevalence of virulence genes and hemolytic phenotype

Table 3 summarizes the frequency of virulence genes investigated among the *S. aureus* strains isolated from bovine mastitis. After the *icaAD* gene, the most common in the studied isolates were those coding for alpha and beta hemolysins. Double hemolytic phenotype (complete and incomplete) was exhibited by 64.25% (257/400) of the strains, while 28.25% (113/400) showed only complete hemolysis, 4.75% (19/400) only incomplete hemolysis, and 2.75% (11/400) were non-hemolytic. A significant association between the phenotype and genotype for hemolysin production was observed ($P < 0.05$).

In order to analyze the frequency of virulence genes according to the year of isolation, years were grouped based on the cumulative distribution in percentiles according to the number of isolates (25%, 50%, and 75%) (1994–1998, 1999–2001, 2002–2004, and 2005–2016), and the strains were also classified according to the number of virulence genes found into classes as follows ≤1, 2, 3, and ≥4 genes (Fig. 1). Most of the isolates showed at least three of the virulence genes tested [77.0% (308/400)], being *icaAD* and *hla* the most prevalent among the isolates. Distribution of *S. aureus* strains according to the number of virulence genes exhibited and the year of isolation is shown in the Fig. 1b). No pattern was observed for the presence of virulence genes over the years among the isolates tested. In addition, no association between virulence factor genes or hemolytic phenotype and year of isolation, geographic origin or antimicrobial resistance profile was observed.

Prevalence of antimicrobial resistance genes

Prevalence of ARG among resistant *S. aureus* isolated from bovine mastitis in the four sampled Brazilian states (Minas Gerais, São Paulo, Rio de Janeiro, and Goiás) is shown in Table 4. The most frequently found genes among resistant *S. aureus* isolates (only strains resistant to a given class were tested for the corresponding resistance gene) were *mepA* (fluoroquinolone resistance), *blaZ* (β-lactam resistance), *aac(6')-Ie–aph(2')-Ia* (aminoglycoside resistance), *tetK* (tetracycline resistance), and *ermC* (macrolide resistance) (Table 4). Although some mutations were observed in the comparison

Table 2 Antimicrobial resistance genes investigated in resistant *Staphylococcus aureus* isolated from bovine mastitis in this study by PCR

Target	Gene	Primer sequence (5′ to 3′)	Amplicon size (bp)[a]	Positive control[b]	Reference
β-Lactam resistance	*blaZ*	F CAGTTCACATGCCAAAGAG R TACACTCTTGGCGGTTTC	772	60	[31]
Macrolide resistance—rRNA erm methylase	*erm(A)*	F TCTAAAAAGCATGTAAAAGAA R CTTCGATAGTTTATTAATATTAG	645	75, 76, 78	[32]
Macrolide resistance—rRNA erm methylase	*erm(B)*	F GAAAAGTACTCAACCAAATA R AGTAACGGTACTTAAATTGTTTA	639	76, 60	[32]
Macrolide resistance—rRNA erm methylase	*erm(C)*	F TCAAAACATAATATAGATAAA R GCTAATATTGTTTAAATCGTCAAT	642	184, 398	[32]
Macrolide/lincosamide/streptogramin B (MLSB) resistance	*erm(T)*	F CCGCCATTGAAATAGATCCT R TTCTGTAGCTGTGCTTTCAAAAA	200	161	[33]
Macrolide resistance—rRNA erm methylase	*erm(Y)*	F AGGCCCCTTTTAAAGACGAAGGCA R GGCGCGATTGTTCATTTTAAGGCCC	320	60	[33]
Macrolide resistance—efflux pump	*msr(A)*	F GGCACAATAAGAGTGTTTAAAGG R AAGTTATATCATGAATAGATTGTCCT GTT	940	60, 352	[9]
Macrolide resistance—macrolide phosphotransferase	*mph(C)*	F ATGACTCGACATAATGAAAT R CTACTCTTTCATACCTAACTC	900	60, 352	[31]
Tetracycline resistance (efflux pump)	*tet(L)*	F CATTTGGTCTTATTGGATCG R ATTACACTTCCGATTTCGG	456	240	[34]
Tetracycline resistance (efflux pump)	*tet(K)*	F TTAGGTGAAGGGTTAGGTCC R GCAAACTCATTCCAGAAGCA	697	184, 82	[34]
Tetracycline resistance (ribosomal protection)	*tet(M)*	F GTTAAATAGTGTTCTTGGAG R CTAAGATATGGCTCTAACAA	657	75, 78	[34]
Aminoglycoside resistance—aminoglycoside-modifying enzyme (AME)	*aac(6′ -Ie–aph(2′)-Ia*	F CAGAGCCTTGGGAAGATGAAG R CCTCGTGTAATTCATGTTCTGGC	348	137, 386	[35]
Fluoroquinolone resistance (efflux pump)	*mepA*	F ATGTTGCTGCTGCTCTGTTC R TCAACTGTCAAACGATCACG	718	ATCC[c] 33,591	[36]
Fluoroquinolone resistance (topoisomerase IV mutation)	*grlA*	F TGCCAGATGTTCGTGATGGT R TGGAATGAAAGAAACTGTCTC	339	ATCC 33591	[37]
Fluoroquinolone resistance (DNA gyrase mutation)	*gyrA*	F TCGTGCATTGCCAGATGTTCG R TCGAGCAGGTAAGACTGACGG	394	ATCC 33591	[37]

[a] Base pairs (bp)

[b] Strains used as positive controls were from the collection of the Laboratório de Bacteriologia, Departamento de Medicina Veterinária, Universidade Federal de Lavras [60]

[c] American Type Culture Collection (ATCC)

of *gyrA* and *grlA* sequences of quinolone-resistant strains and the strain RN4220 (GenBank: AY661734.1), none of the changes has resulted in amino acid exchange and could not be associated with resistance to quinolones (supplementary Fig. S1). Thirty-two tested isolates exhibited two ($n = 26$), three ($n = 5$), and five ($n = 1$) ARG simultaneously (supplementary Table S2 and S3). No association between ARG and year of isolation, geographic origin, or antimicrobial resistance profile was observed.

Association between virulence and antimicrobial resistance

Combinations for the presence of virulence genes and ARG were observed, albeit not common [2.5% (10/400)] (supplementary Table S3 and Fig. 2). Furthermore, no pattern was observed for the presence of ARG over the years among the isolates tested (Fig. 1c).

Discussion

In the present study, we investigated some of the major virulence factors and genetic markers of antimicrobial resistance in *S. aureus* strains isolated from bovine mastitis. Our results showed that Brazilian staphylococci from animal origin have a great potential to cause severe infections, since they exhibited mainly a hemolytic and biofilm forming profile, in addition to a wide variety of resistance genes. Strains were tested for the biofilm production, and the presence of *icaAD* gene

was also investigated, as this gene is commonly detected in biofilm-forming *S. aureus* strains isolated from bovine mastitis. A high frequency (83.25%) of biofilm-forming strains that also harbored the *icaAD* gene was observed, as detected by others in studies with *S. aureus* isolates from bovine mastitis [44–46]. As expected, a significant association was observed between the phenotype and genotype for biofilm-forming ability. These results suggest that *icaAD* is a crucial gene for biofilm formation in *S. aureus*. However, the presence of *icaAD* gene in non-biofilm-forming strains (15.25%) could be explained considering that biofilm formation is regulated and influenced by the quorum sensing system [47], which can result in non-expression of the operon. On the other hand, expression of the biofilm phenotype by strains that did not carry *icaAD* genes, observed in low frequency, may be due to the existence of other ica-independent biofilm formation mechanisms like cell wall–anchored proteins: staphylococcal surface protein SasX and SasG, clumping factors A and B, serine-aspartate repeat protein SdrC, staphylococcal protein A, and fibronectin-binding proteins A and B [15, 48]. The ability to produce biofilm is important for bacterial pathogenesis since it allows the bacteria to form microbial communities, better able to survive the aggressions of the host and the environment, especially those from the immune system, antimicrobials, disinfectants, or even the lack of nutrients [48]. The high frequency in which the biofilm-forming phenotype/genotype was found among the *S. aureus* mastitis isolates tested indicates that this is a particularly important characteristic for the bovine mammary gland colonization, probably having a role in the spread of the pathogen and long-term/persistent infections.

Table 3 Prevalence of virulence factor genes in *Staphylococcus aureus* strains isolated from bovine mastitis in São Paulo, Rio de Janeiro, Minas Gerais, and Goiás states, Brazil, 1994–2016, assessed by PCR

Gene	No. of isolates	Prevalence (%)
Enterotoxins		
sea$^+$	0/400	0
seb$^+$	2/400	0.5
sec$^+$	4/400	1.0
sed$^+$	1/400	0.25
see$^+$	0/400	0
Toxic shock syndrome toxin – 1 (TSST)		
tst$^+$	3/400	0.74
Hemolysin		
hla$^+$*hlb*$^-$	66/400	16.5
hla$^-$*hlb*$^+$	3/400	0.75
hla$^+$*hlb*$^+$	329/400	82.85
hla$^-$*hlb*$^-$	2/400	0.5
Panton–Valentine leukocidin (PVL)		
luk$^+$	14/400	3.5
Biofilm		
icaAD$^+$	394/400	98.5

As observed for biofilm formation, the majority of *S. aureus* isolates also exhibited phenotype and genotype for hemolysin production, which were also significantly associated, clearly showing the pathogenic potential of these isolates. Indeed, our findings for the presence of hemolysin genes (*hla* and *hlb*) and hemolytic phenotype revealed that a large proportion (82.85% and 64.25%) can produce alpha and beta hemolysins, which has also been reported by others [49, 50]. Therefore, the community risk associated with infections acquired through contact or consumption of animal products considering the production of alpha and beta hemolysins by *S. aureus* from mastitis are even higher compared to the other toxins investigated in the present study, since both, but especially beta hemolysin, have stability against inactivation at high temperatures (thermostable below 90 °C for 30 min) [49, 51]. Moreover, the interaction between alpha and beta hemolysins increase the adherence to bovine mammary epithelial cells and the proliferation of *S. aureus* [52], which have implications also for the pathogenesis of bovine mastitis. In fact, as well as to the biofilm-forming ability, the results of the present study on the presence of genes encoding hemolysins also suggest that these toxins may be associated with the successful infection and great adaptation of *S. aureus* to the bovine mammary gland. Moreover, it has been suggested that hemolysins have a role in the formation of the *S. aureus* biofilm, suggesting a synergistic action of these virulence mechanisms [51].

In addition to the ability to form biofilm and produce hemolysis, *S. aureus* can also produce a wide variety of enterotoxins, frequently involved in foodborne disease outbreaks [23]. The majority (95%) of *S. aureus* food poisoning cases are caused by enterotoxins *sea*, *seb*, *sec*, *sed*, and *see* [53]; however, in this study enterotoxins genes were observed in low frequency among the *S. aureus* strains (Table 33). Similarly, studies conducted in Turkey and Brazil also found low frequency or absence of the *sec* gene in *S. aureus* isolated from cattle [8, 12]. With regard to the *seb* and *sed* genes, the low frequency was also corroborated by other findings, since the frequency of these genes seems to be low in *Staphylococcus* spp. or were not observed [54, 55]. Likewise, in the present study, neither *sea* nor *see* genes were detected, similar to the results found by Yang et al. [49] that did not observe any *sea*-positive *S. aureus* ($n = 39$) strains among isolates from bovine clinical mastitis in China. These findings point to a low risk of food poisoning associated with enterotoxins encoded by *sea*, *seb*, *sec*, *sed*, and *see* genes among *S. aureus* from cattle in the four Brazilian states sampled. However, it is worth to note that these staphylococcal enterotoxins keep their biological and immunological activities even following pasteurization, food processing, and exposure to gastrointestinal proteases [23], which indicates non-

Table 4 Prevalence of antimicrobial-resistant genes among resistant *Staphylococcus aureus* isolates from bovine mastitis in São Paulo, Rio de Janeiro, Minas Gerais, and Goiás states, Brazil, 1994–2016, assessed by PCR

Antimicrobial class	Gene	Number of resistant strains[a]	Number of strains showing ARG[a] (%)
Penicillin	*blaZ*	217	178 (82.03)
Tetracyclines	*tetK*	62	21 (33.87)
	tetL	62	1 (1.61)
	tetM	62	2 (3.22)
Macrolides	*ermA*	21	3 (14.29)
	ermB	21	3 (14.29)
	ermC	21	7 (33.30)
	ermT	21	2 (9.52)
	ermY	21	1 (4.76)
	msrA	21	2 (9.52)
	mphC	21	2 (9.52)
Aminoglycosides	*aac(6')-Ie-aph(2')-Ia*	13	6 (45.15)
Quinolones	*mepA*	7	7 (100)

[a] Previously determined by [25]

[b] Antimicrobial resistance genes

negligible public health risk, although with the low frequency observed. In addition, some enterotoxins have also been associated with an important role in the pathogenesis of bovine mastitis, since they can induce bovine mammary epithelial cell (bMEC) apoptosis [54].

Likewise, the *tst* gene responsible for the production of TSST-1 was also detected in the present study in low frequency (0.74%) in *S. aureus* isolates from bovine mastitis, similar to what was observed in China (2.6%) [49]. Nonetheless, it is interesting to note that the two *tst* positive strains identified also exhibited the *sec* gene. A comparable relationship between the presence of these two genes has been reported in the literature [56]. Both toxins can exhibit various biological activities and act as superantigens for cells of the bovine immune system, contributing to pathological mechanisms of bovine mastitis [51]. Moreover, considering that Staphylococcal enterotoxins (SEs) and TSST-1 can keep their biological and immunological activities after pasteurization [14], detection of strains able to produce both toxins in cow's milk samples also represents an important threat to public health. Although found in low frequency, the expression of these toxins could lead to episodes of food poisoning even in milk products subjected to heat treatment.

The *luk* gene was also exhibited by only a few isolates, which was not surprising, since in other studies the PVL gene was rarely detected in *S. aureus* strains isolated from bovine mastitis [13, 57]. However, as stated before for other virulence factors observed, despite of the low prevalence, PVL-positive strains from bovine milk could be a significant risk to the community, due to the pore-forming characteristic of PVL toxin, its association with necrosis of skin and soft tissues and with community-acquired methicillin-resistant *S. aureus* (MRSA) [13].

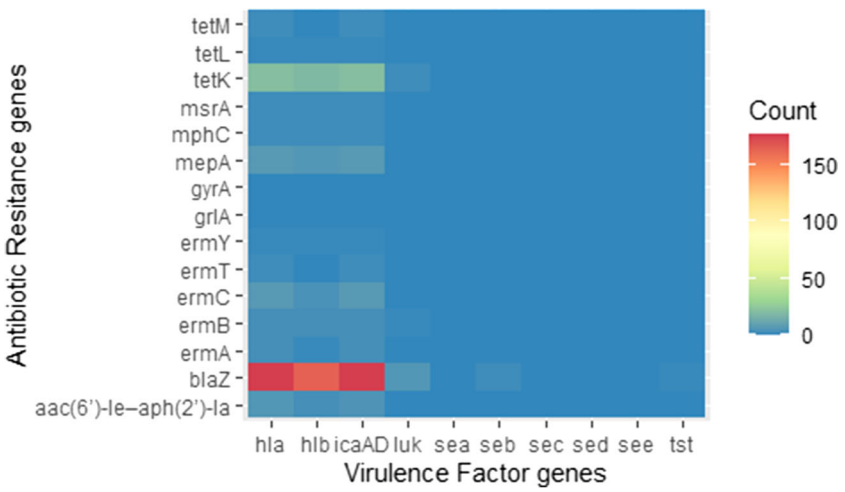

Fig. 2 Profile of virulence genes (*icaAD, hla, hlb, luk, sea, seb, sec, sed, see*, and *tst*) and antimicrobial resistance genes [*blaZ, tetK, tetl, tetM, ermA, ermB, ermC, ermT, ermY, mrsA, mphC, mepA*, and *aac(6')-Ie–aph(2')-Ia*] among *S. aureus* isolated from bovine mastitis in São Paulo, Rio de Janeiro, Minas Gerais, and Goiás states, Brazil, 1994–2016, assessed by PCR

The present study also analyzed the distribution of ARG among resistant *S. aureus* isolated from bovine mastitis in the Brazilian states sampled. The *blaZ* gene, screened among β-lactam–resistant strains (ampicillin, amoxicillin + clavulanic acid, penicillin-novobiocin, ceftiofur), was the main genetic marker of resistance for this antimicrobial class. The assessment of these ARG helps to determine strategies for treatment, control and prevention of dissemination of resistance between animals and humans, especially MRSA (*mecA*-positive strains), since the resistance to β-lactams are principally conferred by *mecA* and *blaZ* genes, and their presence implies resistance to almost all β-lactams agents [19]. Recently, the *mecC* gene has also emerged as an important mechanism of resistance to this antimicrobial group among *S. aureus* from mastitis [18]. Nonetheless, in contrast to *blaZ*, all strains were previously demonstrated negative for *mecA* and *mecC* genes [25]. Additionally, in a proportion of the isolates that exhibited resistance to β-lactams [39/217 (18%)], *blaZ* was also detected. Thereby, further studies are needed to identify the genetic determinant of resistance for these isolates that were phenotypically β-lactam resistant.

For tetracycline-resistant strains, by far the most frequent gene found was *tetK* ($n = 21$), followed by *tetM* ($n = 2$) and *tetL* ($n = 1$), which was also frequently observed in a study conducted by Martini et al. [20] in *S. aureus* isolated from bovine mastitis in Minas Gerais state, Brazil. The detection of these genes in *S. aureus* from animal origin is of high concern to public health, since these genes are transferred by mobile genetic elements, which may facilitate the spread of several resistance genes and consequently can lead to treatment failure in both veterinary and human medicine [16].

The investigation of the genetic determinants of resistance of other antimicrobial classes widely used for the treatment of staphylococcal infections, as macrolides, lincosamide, and streptogramin B (MLS_B), revealed that most of the isolates resistant to macrolides (erythromycin) carried the gene *ermC*, as well as observed by Lina et al. [9] in *S. aureus* isolates. On the other hand, low frequency of *ermA* and *ermB* genes was observed in the present study, as it has also been reported elsewhere [17], showing that these genes are not commonly detected in *S. aureus* from bovine mastitis. Similarly, the other genes related to macrolides resistance (*ermT*, *msrA*, and *mphC*) were found in lower frequency, suggesting that these mechanisms are probably less important in the resistance to this antimicrobial class in *S. aureus* from animal origin. Moreover, two isolates were detected harboring *msrA* and *mphC*, of which one also carried the *ermY, blaZ*, and *ermB* genes (supplementary Tables S2 and S3). Interestingly, *mphC* often occurs linked to *msrA*, and the presence of *mphC* gene alone confers only low-level resistance to macrolides [21].

Although aminoglycosides are widely used for mastitis treatment in cattle [1], few studies have been focused in the identification of mechanism of resistance against this antimicrobial class in *S. aureus*. In the present study, the aminoglycoside resistance gene *aac(6')-Ie-aph(2')-Ia* was detected in high proportion (45.15%) of aminoglycoside-resistant *S. aureus*, suggesting that this gene is an important mechanism of resistance to this antimicrobial group and could be associated with mastitis treatment failure in cattle. Indeed, a similar study has also reported a high prevalence of *aac(6')-Ie-aph(2')-Ia* in *S. aureus* isolated from mastitis [17].

In general, two important mechanisms are responsible for resistance to fluoroquinolones among *S. aureus* strains. The first one is attributed to mutations occurring in the QRDR of *grlA*/*grlB* (topoisomerase IV) and *gyrA*/*gyrB* (DNA gyrase), whereas the second is mediated by drug efflux [41, 42]. Several efflux pumps have been described in *S. aureus,* including those encoded by *norA, norB, norC, mdeA, mepA, sepA*, and *sdrM* genes [58]. In this study, it was identified for the first time that the fluoroquinolone resistance gene *mepA* in all isolates of *S. aureus* from bovine mastitis were phenotypically resistant to ciprofloxacin or enrofloxacin. The importance of *mepA* detection among bovine mastitis *S. aureus* goes beyond resistance to fluoroquinolones, as this gene also confers resistance to a wide range of compounds, including various dyes and biocides [59], such as iodine, quaternary ammonium and chlorhexidine, widely used in post-milking teat dipping. On the other hand, mutations in QRDR associated with resistance were not observed in quinolone-resistant strains, reinforcing that *mepA*-encoded efflux pump was responsible for the observed phenotype.

Albeit virulence factors and the ability to resist to antimicrobial drugs in *S. aureus* have been demonstrated to be closely associated, since both contribute to successfully host colonization and dissemination into a population and thereby are usually synergistically regulated [51], no significative association was observed between the virulence and antimicrobial resistance genes assessed. This result can be partly explained by the low frequency observed for some genes encoding virulence factors and ARG or even due to the non-probability sampling used. However, the findings of the present study call attention to the adoption of a One Health approach to the growing challenges in human and animal health, such as staphylococcal infections, mainly related to drug resistance, one of the key priorities of this initiative. Furthermore, although no livestock-associated MRSA (LA-MRSA) has been identified among the mastitis isolates investigated, which have been mainly implicated in severe infections and deaths in the humans [16], the resistance genes observed, all located in mobile elements, point to the risk of antimicrobial resistance transfer of these genes for other *Staphylococcus* spp. or even for other genera of medical importance. Additionally, despite the direct comparison of the results of the present study with others also conducted in Brazil is not possible, due to the difference in the distribution of the isolates (geographical and temporal) and different methodologies used to assess

virulence factors and resistance genes; other studies performed in *S. aureus* from different Brazilian states also point to the importance of biofilm production and presence of enterotoxins and antimicrobial resistance genes (*blaZ*) for the pathogenesis of mastitis, besides the potential food poisoning risk associated with dairy products [5–7].

Conclusions

Our results showed that *S. aureus* strains isolated from bovine mastitis in the four Brazilian states sampled carried mainly biofilm-forming and hemolytic genes, whereas virulence genes associated with enterotoxins, PVL and TSST-1 were less frequently observed. Moreover, a wide variety of resistance genes that confer resistance to almost all classes of antimicrobial agents approved for use in animals and in human population were found. Overall, the data point to a great pathogenic potential of *S. aureus* associated with bovine mastitis and to the non-negligible risks to public health of staphylococcal infections from animal origin.

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Availability of data and material The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Code availability Not applicable

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Compliance with ethical standards

Conflicts of interest/competing interests The authors declare that they have no conflict of interest.

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Consent to participate Not applicable

Consent for publication All authors gave the consent for publication.

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