



## Molecular characterization and clonal diversity of methicillin-susceptible *Staphylococcus aureus* in milk of cows with mastitis in Brazil

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### ABSTRACT

Mastitis is an important disease for the dairy industry worldwide, causing economic losses and reducing milk quality and production. *Staphylococcus aureus* is a worldwide agent of this intramammary infection, which also causes foodborne diseases. The objective of this study was to determine the frequency of methicillin-susceptible *Staphylococcus aureus* (MSSA) isolates in milk of mastitis cows in Brazil and to analyze the genetic lineages and the content of antimicrobial resistance genes and virulence factors among these isolates. Fifty-six MSSA isolates were recovered from 1,484 milk samples (positive for the California mastitis test) of 518 cows from 11 different farms in Brazil (representing 51% of total *Staph. aureus* obtained), and they were further characterized. Methicillin-susceptible *Staphylococcus aureus* were isolated from 3.7% of California mastitis test-positive tested milk samples and from 6.2% of tested mastitic cows. Methicillin-susceptible *Staphylococcus aureus* isolates were characterized by *spa* typing, *agr* typing, and multilocus sequence typing, and resistance and virulence traits were investigated by PCR. Seven *spa* types were identified among MSSA (% of isolates): t127 (44.6), t605 (37.5), t002, t1784, t2066 (1.8), and 2 new ones: t10856 (10.7) and t10852 (1.8). Five distinct sequence types (ST) were detected (% of isolates): ST1 (46.4), ST126 (37.5), ST133 (10.7), ST5 (3.6), and a novel ST registered as ST2493 (1.8). Resistances were detected for streptomycin, chloramphenicol, and tetracycline. One strain contained the chloramphenicol resistance gene (*fexA*; included within transposon Tn558) and 3 strains contained the tetracycline resistance gene [*tet(K)*]. Methicillin-susceptible *Staphylococcus aureus* strains were susceptible to most of the antibiotics studied and lacked the virulence genes of Panton-Valentine leukocidin (*lukF/S-PV*), toxic shock syndrome toxin 1 (*tst*), exfoliative toxin A

(*eta*), and exfoliative toxin B (*etb*), as well as the genes of the immune evasion cluster. Methicillin-susceptible *Staphylococcus aureus* isolates were detected in a relatively low proportion of cows with mastitis (6.2%) and recovered isolates presented high diversity of genetic lineages, with CC1 and CC126 the predominant clonal complexes, and CC133 also being detected. Larger epidemiological studies with molecular characterization of isolates are required to deepen the knowledge on the circulating genetic lineages among the cow population with mastitis.

**Key words:** *Staphylococcus aureus*, methicillin susceptibility, *spa* typing, mastitis

### INTRODUCTION

Milk production in Brazil is one of the most important branches of the Brazilian agribusiness and the country is the sixth leading producer of milk (Klein et al., 2012). Dairy cow mastitis is the most important disease in the dairy industry worldwide, and it is associated with pain and reduced well-being of affected animals (Halasa et al., 2007). Mastitis causes economic losses due to reduced milk production, milk discard, premature slaughter, and antibiotic usage (McDougall et al., 2009).

In Brazil, several studies have reported the isolation of *Staphylococcus aureus* in milk samples from cows with mastitis (Zafalon et al. 2007; Santos et al. 2008; Klein et al., 2012); however, studies related to molecular characterization of *Staph. aureus* from these types of milk samples are scarce. This microorganism is one of the most important etiological agents of mastitis, which is of concern for humans and livestock (Capurro et al. 2010). *Staphylococcus aureus* can produce a wide range of extracellular toxins and virulence factors such as Panton-Valentine leukocidin (PVL), toxic shock syndrome toxin (TSST), and several exfoliatins and enterotoxins, which represent a risk for humans and animals, being associated with severe infections (Jarraud et al., 2002; Francis et al., 2005).

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*Staphylococcus aureus* can acquire methicillin resistance (MRSA) due to the acquisition of the *mecA* gene. Methicillin resistance represents an important therapeutic problem when implicated in human or animal infections, and numerous studies have focused on the characterization of these isolates. Nevertheless, the interest on methicillin-susceptible *Staph. aureus* (MSSA) has increased in recent years, given that they can also be implicated in important infections and may help to explain the appearance and evolution of the different and successful MRSA lineages. Few data exist in the circulating genetic lineages of MSSA in food-producing animals or in derived food products, as in the case of milk.

Coagulase-positive and coagulase-negative staphylococci were recovered in a previous study from milk of cows with mastitis from 10 farms in Brazil and enterotoxin genes were studied in these isolates (de Freitas Guimarães et al., 2013). The present work focused on the molecular characterization of MSSA isolates recovered in milk of mastitic cows in Brazil, analyzing the genetic lineages of the isolates as well as their content in antimicrobial resistance genes and in specific virulence genes, including, among others, the MSSA isolates obtained in the previous study (de Freitas Guimarães et al., 2013).

## MATERIALS AND METHODS

### Samples and Bacterial Isolates

A total of 4,684 milk samples from 1,171 cows (from the 4 teats) from 11 different farms in the state of São Paulo, Brazil, were evaluated for mastitis detection. The methodology and interpretation criteria used for diagnosis of clinical and subclinical mastitis were based on examination of animals before each milking by the California mastitis test (CMT; Schalm and Noorlander, 1957). From these samples, 1,484 of them, obtained from 518 cows, were CMT positive and were studied for staphylococci detection. The CMT-positive samples were plated on blood agar plates (Oxoid Brasil Ltda, São Paulo, Brazil) and incubated under aerobic conditions at 37°C, and readings were performed after 24, 48, and 72 h of incubation. Identification of *Staph. aureus* was based on colony morphology, Gram staining, and catalase, coagulase, and DNase activities (Koneman et al., 2008). Molecular identification was performed by a multiplex PCR that also allows the discrimination of MSSA and MRSA by amplification of the species-specific staphylococcal nuclease (*nuc*) gene and the staphylococcal methicillin-resistance genetic determinant (*mecA*; CRL-AR, 2009). One MSSA isolate per positive mammary gland was characterized. The MSSA

isolates from 10 of the studied farms (n = 50) were obtained in a previous study (de Freitas Guimarães et al., 2013), and those from 1 additional farm (n = 6) were obtained in the present study; all 56 MSSA isolates of the 11 farms were characterized in the present study.

### Molecular Typing and Clonal Relatedness of MSSA Isolates

All MSSA isolates were characterized by *spa* gene typing (<http://spaserver.ridom.de>) and *agr* allotype (Shopsin et al., 2003) by specific PCR. Multilocus sequence typing was performed as previously described (<http://www.mlst.net>) on 1 representative strain per *spa* type. Pulsed-field gel electrophoresis (PFGE) of genomic DNA, previously digested with the macrorestriction *Sma*I enzyme, was performed on 1 strain per *spa* type per cow. Pulsed-field gel electrophoresis band profiles were compared according to previously reported criteria (Tenover et al., 1995).

### Antimicrobial Susceptibility Testing and Detection of Resistance Genes

Antimicrobial susceptibility testing to oxacillin, cefoxitin, tetracycline, erythromycin, clindamycin, gentamicin, tobramycin, streptomycin, trimethoprim-sulfamethoxazole, ciprofloxacin, and chloramphenicol was performed by the disk-diffusion agar method in accordance with the Clinical and Laboratory Standards Institute recommendations (CLSI, 2012). Detection of antimicrobial resistance genes was investigated in resistant isolates by specific PCR (Table 1). Positive and negative controls from the collection of the University of La Rioja (Logroño, Spain) were used in each PCR assay.

### Virulence Factors and Immune Evasion Cluster Genes

The presence of the genetic determinants of Panton-Valentine leukocidin (*lukF/S-PV*), toxic shock syndrome toxin 1 (*tst*), and exfoliative toxin A (*eta*) and B (*etb*) was analyzed by PCR (Lina et al., 1999; Jarraud et al., 2002). The presence of the human-associated immune evasion cluster (IEC) genes (*scn*, *chp*, *sak*, *hly*, *sea*, and *sep*), which were enclosed within the  $\phi$ 3 bacteriophage, was determined as previously recommended (van Wamel et al., 2006).

## RESULTS

*Staphylococcus aureus* isolates were recovered from 97 of the 1,484 CMT-positive milk samples tested (6.5%)

**Table 1.** Primers used for PCR detection of antibiotic resistance genes and the transposon Tn558

Primer	Sequence (5'→3') <sup>1</sup>	Amplicon size (bp)	Reference
<i>mecA</i>	F: TGCTATCCACCCTCAAACAGG R: AACGTTGTAACCAACCCCAAGA	287	Kondo et al. (2007)
<i>tet(K)</i>	F: TTAGGTGAAGGGTTAGGTCC R: GCAAACCTCATTCCAGAAGCA	697	Aarestrup et al. (2000)
<i>tet(M)</i>	F: GTTAAATAGTGTCTTGGAG R: CTAAGATATGGCTCTAACAA	576	Aarestrup et al. (2000)
<i>tet(L)</i>	F: CATTGGTCTTATTGGATCG R: ATTACACTTCCGATTTCCG	456	Aarestrup et al. (2000)
<i>cfr</i>	F: TGAAGTATAAAGCAGGTTGGGAGTCA R: ACCATATAATTGACCACAAGCAGC	746	Kehrenberg and Schwarz (2006)
<i>aadE</i>	F: ACTGGCTTAATCAATTGGG R: GCCTTTCCGCCACCTCACCG	597	Clark et al. (1999)
<i>str</i>	F: TATTGCTCTCGAGGGTTC R: CTTTCTATATCCATTCATCTC	646	Schnellmann et al. (2006)
<i>aadA</i>	F: TGATTGCTGGTTACGGTGAC R: CGCTATGTTCTCTTGCTTTTG	284	Clark et al. (1999)
<i>fxaA</i>	F: GTACTTGTAGGTGCAATTACGGCTGA R: CGCATCTGAGTAGGACATAGCGTC	1,272	Kehrenberg and Schwarz (2005)
<i>tnpA</i>	F: GGTTCAGAAAGTAATTGTGGAGGA R: TGTGGCATGCGTATGACGTA	930	Kehrenberg and Schwarz (2006)
<i>tnpB</i>	F: GGA ACA GTG ATA AGC CGG ATG R: ACATGGTCCTGCAATTACTGGTA	1,419	Kehrenberg and Schwarz (2006)
<i>tnpC</i>	F: CAG CTA GCT AAA GAC AAG TCG GA R: GTTCATTCTCTTCTTCTAACGCCT T	289	Kehrenberg and Schwarz (2006)
Almost complete Tn558	F: ACC AGA TTA ACA TCA GGC CAA G R: TTACGGTCAGAAGAATGTACCACA	5,741	Kehrenberg and Schwarz (2006)

<sup>1</sup>F = forward; R = reverse.

and were obtained in 10.2% of cows tested with mastitis. Among these, 56 isolates were MSSA (57.7% of total *Staph. aureus*), obtained from 3.7% of the tested CMT-positive milk samples and from 6.2% of mastitic cows (32 animals), and they were further characterized in this study (1 isolate per positive sample). Among the 32 cows positive for MSSA detection, 17 of them presented this microorganism in milk samples of more than 1 teat (12 animals with 2 positive teats, 3 animals with 3 positive teats, and 2 animals with 4 positive teats).

### Molecular Typing of MSSA Isolates

Two new *spa* types were identified among 7 of the 56 tested MSSA isolates: (1) 1 isolate presented the *spa* type t10852 and was also ascribed to the novel sequence type (ST)2493, enclosed within clonal complex (CC)1, and (2) 6 isolates presented the *spa* type t10856 and were ascribed to ST133 (CC133). The other *spa*/ST/CC types detected among the remaining 49 MSSA isolates were as follows (number of isolates in parentheses): t605/ST126/CC126 (25), t127/ST1/CC1 (21), t002/ST5/CC5 (1), t1784/ST1/CC1 (1), and t2066/ST5/CC5 (1). The following *agr* allotypes were obtained (number of isolates in parentheses): *agr*-I (6), *agr*-II (23), and *agr*-III (27), whereas *agr*-IV was not found (Table 2).

The MSSA isolates recovered from different teats presented similar genetic characteristics (*spa*/ST/CC) in 16 of the 17 animals that carried MSSA in more than 1 teat. The *Sma*I PFGE performed with 29 representative MSSA isolates (1 per *spa* type and per cow) revealed that isolates that belonged to the same *spa* type presented identical or closely related patterns (less than 3 bands of difference; for example, patterns A1-A2-A3; Table 2), whereas those with a distinct *spa* type exhibited unrelated PFGE profiles (more than 6 DNA bands of difference; for example, patterns A, B, or C; Table 2).

### Antimicrobial Resistance, Toxin Genes, and IEC Profile of MSSA Isolates

Few resistances were detected among our MSSA isolates: streptomycin (50%), and chloramphenicol and tetracycline (3.5%). Three tetracycline-resistant isolates harbored the tetracycline-efflux protein (*tetK*) gene and 1 chloramphenicol-resistant isolate the florfenicol-chloramphenicol exporter gene (*fxaA*; Table 3). The possible inclusion of the *fxaA* gene within the transposon Tn558 was investigated (Kehrenberg and Schwarz, 2006), revealing the presence of the transposase gene (*tnpA*, *tnpB*, and *tnpC*) and *orf138*, characteristics of the conserved transposon. In addition, circular intermediates were detected, indicating functional activity.

**Table 2.** Number and characteristics of methicillin-susceptible *Staphylococcus aureus* (MSSA) isolates investigated and number of positive cows

No. of MSSA isolates	No. of animals	<i>agr</i> type <sup>1</sup>	<i>spa</i> type <sup>2</sup>	ST/CC <sup>3</sup>	PFGE pattern <sup>4</sup> (no. of isolates)
25	15	III	t127	ST1/CC1	A1 (12), A2 (2), A3 (1)
21	10	II	t605	ST126/CC126	B1 (5), B2 (3), B3 (2)
6	4	I	t10856 <sub>(new)</sub>	ST133/CC133	C1 (3), C2 (1)
1	1	III	t10852 <sub>(new)</sub>	ST2493 <sub>(new)</sub> /CC1	—
1	1	I	t1784	ST1/CC1	—
1	1	II	t002	ST5/CC5	—
1	1	II	t2066	ST5/CC5	—

<sup>1</sup>*agr* = accessory gene regulator.<sup>2</sup>*spa* = *Staphylococcus* protein A-encoding gene.<sup>3</sup>ST = sequence type; CC = clonal complex. Performed in 1 strain per *spa* type.<sup>4</sup>PFGE = pulsed-field gel electrophoresis.

No streptomycin resistance genes were identified in the streptomycin-resistant staphylococci. All MSSA isolates were negative for the tested toxin genes (*lukF/S-PV*, *tst*, *eta*, and *etb*) as well as for the IEC genes (*scn*, *chp*, *sak*, *hbl*, *sea*, and *sep*).

## DISCUSSION

The occurrence of MSSA isolates among milk samples of cows with mastitis detected in our study was relatively low (6.2% of cows with mastitis tested). Nevertheless, other staphylococci (both coagulase-positive and coagulase-negative) were present in these samples (up to 20% of CMT-positive samples contained staphylococci), as has been previously reported (de Freitas Guimarães et al., 2013), and could be implicated in clinical or subclinical mastitis, with *Staph. aureus* the most important species among coagulase-positive staphylococci. Milk contamination with *Staph. aureus* is of concern because it puts at risk people working on the farm. Moreover, contaminated milk can reach the consumer in case of inefficient pasteurization or when

raw milk is used illegally for the production of Minas cheese or other dairy product. Lee (2003) suggested a possible transmission of *Staph. aureus* from cows to humans. Other studies refer higher prevalences of *Staph. aureus* or MSSA in milk samples (Coelho et al., 2009; D'Amico and Donnelly, 2010; Haran et al., 2012; Oikonomou et al., 2012).

The most prevalent *spa* types among our MSSA isolates from bovine sources were t127 and t605, and it is in accordance with previous reports from Brazil (Aires-de-Sousa et al., 2007; Rabello et al., 2007). In Canada, MSSA strains of lineage t605 were also observed (Said et al., 2010), whereas t127 isolates have been previously isolated from Switzerland and Korea (Huber et al., 2010; Hwang et al., 2010). Alternatively, other studies on milk samples from cows with mastitis showed *Staph. aureus* with different *spa* types to those detected among our MSSA isolates (Johler et al., 2011; Haran et al., 2012).

The database of the Ridom *spa* server network for *spa* typing (<http://spaserver.ridom.de>) shows that *spa* type t127 has a relative global frequency of 1.9% and t605

**Table 3.** Antibiotic resistance characteristics and *spa* types of the investigated methicillin-susceptible *Staphylococcus aureus* (MSSA) isolates according to the farm of origin

Farm	<i>spa</i> type of isolates (no. of isolates)	Phenotype of resistance (no. of isolates) <sup>1</sup>	Resistance gene detected (no. of isolates)
I	—	—	—
II	t2066 (1)	STR (1)	—
III	t002 (1)	CHL (1), STR (1)	<i>fecA</i> <sup>2</sup> (1)
IV	t127 (5), t10856 (1)	STR (2)	—
V	t127 (1), t10852 (1)	STR (1)	—
VI	t605 (9), t1784 (1)	TET (1), STR (8)	<i>tet</i> (K) (1)
VII	t127 (2), t10856 (4)	STR (1)	—
VIII	t127 (3)	STR (2)	—
IX	t127 (3), t605 (9)	TET (1), STR (6)	<i>tet</i> (K) (1)
X	t605 (3)	TET (1)	<i>tet</i> (K) (1)
XI	t127 (11), t10856 (1)	STR (6)	—

<sup>1</sup>STR = streptomycin; CHL = chloramphenicol; TET = tetracycline.<sup>2</sup>The florfenicol-chloramphenicol exporter gene (*fecA*) was enclosed within the transposon Tn558.



of 0.1% (data collected in January 2013). Methicillin-susceptible *Staph. aureus* isolates with *spa* types t127 and t605 were distributed in almost all regions studied of the state of São Paulo, whereas those with *spa* type t10856 were found in 2 distinct regions (B and D). The 56 strains were distributed in 4 clonal complexes: CC1, CC5, CC126, and CC133. Among these, CC1 and CC126 were predominant. Rabello et al. (2007) observed the same clonal complexes in a study in Brazil from milk samples collected from cows with subclinical mastitis. The clonal complexes CC1 and CC5 are common among human isolates (Argudín et al., 2011; Lozano et al., 2012) and Juhász-Kaszanyitzky et al. (2007) reported strains of CC1 in humans and bovines. Franco et al. (2011) suggested that transmission of this lineage (CC1) from animals to humans is possible, although differences exist in the host-specific genetic characteristics between the 2 clusters. The clonal complex CC126 is not common among isolates from human beings and several studies observed this CC in ruminants (Smith et al., 2005; Aires-de-Sousa et al., 2007; Rabello et al., 2007), suggesting that ruminants are reservoirs for this CC.

The novel *spa* type t10856 belongs to CC133. Previous studies have reported the existence of CC133 in clinical *Staph. aureus* isolates from bovines (Rabello et al., 2007; Schlotter et al., 2012) and this lineage is very common in small ruminants (Guinane et al., 2010) and also in ungulates (Gharsa et al., 2012). It has been suggested that the CC133 clone is a result of a host jump from humans to these animals by an adaptive genome diversification from allelic variation, gene decay, and horizontal acquisition of mobile genetic elements containing virulence genes (Guinane et al. 2010).

The *agr* types I, II, and III were detected among our MSSA isolates, with *agr* types II and III the most frequently detected. Isolates of lineage ST1/CC1 were included in *agr* type III but 1 strain was included in *agr* type I.

The 4 virulence factors studied in this work (related to the Panton-Valentine leukocidin, toxic-shock-syndrome-toxin 1, and exfoliative toxin A and B) in MSSA isolates were negative. Nevertheless, these isolates could contain other virulence genes as is the case for enterotoxins (de Freitas Guimarães et al., 2013). A recent study showed *Staph. aureus* strains from milk from animals with mastitis with the *etb* gene encoding the exfoliative toxin B (Salasia et al., 2011).

Antibiotic susceptibility testing revealed that 27 of the 56 isolates (48.2%) showed susceptibility to all antibiotics tested. Other studies reported susceptibility rates of 42% (Coelho et al., 2009) or 67% (Aires-de-Sousa et al., 2007) among bovine *Staph. aureus*, the latter from farms located in Rio de Janeiro State. The

detection in 1 isolate of the complete and conserved Tn558, which carries the chloramphenicol and florfenicol resistance gene *fxrA*, is remarkable and, to the best of our knowledge, it represents the first description of this transposon in staphylococci in Brazil. Since its first description in a *Staphylococcus lentus* isolate from a cow with a respiratory tract infection (Kehrenberg and Schwarz, 2004), this transposon has been detected in *Staphylococcus lentus simulans*, *Staphylococcus lentus chromogenes*, and *Staphylococcus lentus sciuri* from bovines and in equine *Staph. aureus* isolates (Kehrenberg and Schwarz, 2006), *Staphylococcus cohnii* isolates from swine farms in China (Wang et al., 2012), and in *Bacillus* from swine feces (Dai et al., 2010). Further molecular epidemiological studies are required to assess the burden of this mobile genetic element among the Brazilian cow population. Interestingly, streptomycin-resistant strains did not contain the expected resistance genes, suggesting the presence of mutations in specific ribosomal proteins or unknown resistance mechanisms to this antibiotic that should be characterized in the future.

## CONCLUSIONS

Methicillin-susceptible *Staph. aureus* isolates were recovered in approximately 3.7% of milk samples tested, representing 57.7% of all *Staph. aureus* recovered. The major lineages detected among MSSA were CC126 and CC1, followed by the ruminant-associated CC133. In general, MSSA strains were susceptible to most antibiotics studied, although the detection of the conserved transposon Tn558 evidences potential acquisition capacities. In spite of the absence of streptomycin resistance genes studied, 50% of MSSA strains were resistant to this antibiotic in the diffusion disk test, suggesting that other genes could be implicated. Due to the scarce data available on molecular characteristics of *Staph. aureus* isolates from cattle in Brazil, continued epidemiological surveillance is essential to gain knowledge on the circulating lineages responsible for cow mastitis in this major milk-producing country, due to its potential implications in both animal and human health.

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