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# 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 Tn*558*) 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*, *hlb*, *sea*, and *sep*), which were enclosed within the φ3 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%)