Instituto de Biociências de Botucatu.

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

PG-BGA

Caracterizaçao molecular e diversidade clonal de *Staphylococcus aureus*, isolados de leite de vacas com mastite subclinica no estado de São Paulo.

ERIKA CAROLINA ROMÃO BONSAGLIA

Tese apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do titulo de Doutor no Programa de Biologia Geral e Aplicada, Área de concentração Biologia dos Micro-organismos.

Orientadora: Profa. Dra. Vera Lúcia Mores Rall

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Aos meus pais,

Lúcia e Benedito

(in memorian)

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Epígrafe

“Você não pode mudar o vento, mas pode ajustar

as velas do barco para chegar aonde quer”

Confúcio

SUMÁRIO

RESUMO

Staphylococcus aureus é um agente comum de mastite bovina, responsável por grandes perdas econômicas na pecuária mundial. Esse micro-organismo possui fatores de virulência bastante conhecidos como a produção de hemolisinas, leucotoxinas e superantígenos como a toxina do síndrome do choque tóxico e enterotoxinas. O objetivo do estudo foi caracterizar molecularmente os isolados de *S. aureus* provenientes de leite de vacas com mastite subclínica, de várias regiões do estado de São Paulo, através de Multilocus Sequence Typing (MLST), *spa* typing, Pulse Field Gel Electrophoresis (PFGE) e agr. Também fizeram parte dos objetivos, testes fenótipicos e genotípicos de resistência a antimicrobianos, além da pesquisa de genes de alguns fatores de virulência como o *pvl* (toxina de Panton Valentine), *tst* (toxina da Síndrome do Choque Tóxico, , *sea-see*, *seg*, *seh* e *sei*, a fim de verificar quais os fatores de virulência mais envolvidos nesse quadro. Todos os isolados de *S. aureus* foram sensíveis a meticilina (MSSA), pois os genes *mecA* e *mecC* não foram encontrados. Entre os 12 antibióticos testados, observou-se resistência intermediaria à eritromicina e nenhuma das cepas foram resistentes à oxacilina, vancomicina e gentamicina. Os isolados resistentes à tetraciclina apresentaram o gene *tetK* . Na caracterização molecular, observou-se 23 *spa* types diferentes com prevalência dos tipos t605 e t127. A maioria das cepas (48,1%) eram pertencentes ao grupo *agr* II, seguido de 20,1% do grupo III e 8,1%, do I, sendo que o grupo *agr* IV não foi encontrado.Entre os fatores de virulência estudados, o gene *pvl* não foi observado. Em relação aos superantígenos, o gene *tst* foi observado em 105 das 285 cepas (37,1%) e a distribuição de genes que codificavam a produção de enterotoxinas foi muito variável. Entre os 285 isolados, 126 (44,2%) foram positivos para, pelo menos, um dos genes pesquisados e o mais frequente foi *seg*, ocorrendo isoladamente ou em combinações diferentes em 275 (96,5%), seguido por *seh* (25,/90%), sec (227/79,6%), *sei* (44; 15,4%), *sea* (31; 10,8%) e *sed* (10; 3,5%). Os genes *seb* e *see* não foram observados. Foram observados 23 *spa* type diferentes (t605, t127, t458, t521, t342, t318, t693, t177, t11659, t021, t2164, t1192, t7335, t114, t6811, t6980, t002, t3324, t559, t138, t321, t456, t2066). O mais frequente foi t605, ocorrendo em 168 cepas (58,9%), seguido t127, em 27 (9,5%) e a presença do t321 em vacas causando mastite não havia sido relatado anteriormente. Os *sequence types* (ST) mais frequentes foram o ST126, correspondendo a 59% dos isolados, seguidos por ST1 (9%), pertencentes ao complexo clonal (CC) 126 e 1, respectivamente. Os resultados mostraram diferenças entre os perfis entre as diversas técnicas moleculares, uma vez que o PFGE apresentou diferentes perfis entre cepas de mesmo *spa* type sugerindo que o *spa* typing não é um bom marcador molecular.

Palavras-chave: *S. aureus*, t321, *agr*, *Spa* typing, MLST.

ABSTRACT

Staphylococcus aureus is a common agent of bovine mastitis, causing economic losses in Brazilian livestock. This microorganism has well known virulence factors such as the production of hemolysins, leukotoxins and superantigens such as toxic shock syndrome toxin and enterotoxins. The objective of the study was to characterize molecularly isolates of *S. aureus* from milk of cows with subclinical mastitis, of the several regions of São Paulo State, through *Multilocus sequence typing* (MLST), *spatyping*, *Pulse Field Electrophoresis Gel* (PFGE) and *agr*. Phenotypic and genotypic tests of antimicrobial resistance, as well as the search for genes of some virulence factors such as pvl (Panton Valentine toxin), tst (Toxic Shock Syndrome toxin). In the present study, all isolates of *S. aureus* were sensitive to methicillin (MSSA), as the *mec*A and *mec*C genes were not found. About 12 antibiotics tested, intermediate resistance to erythromicyn was observed and none resistance to oxacillin, vancomycin and gentamicin. The tetracycline resistant isolates showed the *tet*K gene. Molecular characterization showed 23 different spa types with prevalence of t605 and t127. The most of the strains (48.1%) presented as belonging to the *agr* II group, followed by 20.1% of the *agr* III group and 8.1% of the *agr*I group, and the *agr* IV group was not found. About the virulence factors studied, the pvl gene was not observed. In relation to super antigens, the tst gene was observed in 105 of the 285 strains (37.1%) and the distribution of genes coding for enterotoxin production was very variable. Among the 285 isolates, 126 (44.2%) were positive for at least one of the genes. The most frequent was *seg*, occurring alone or in different combinations in 275 (96.5%) isolates, followed by *seh* (250/90%), *sec* (227/ 79,6%), *sei* (44/15,4%), *sea* (31/10,8) and *sed* (10/3.5%). The *seb* and *see* genes were not observed as well as *pvl* gene.There were observed 23 different spa types (t605, t127, t458, t521, t342, t318, t693, t177, t11659, t021, t2164, t1192, t7335, t114, t6811, t6980, t002, t3324, t559, t138, t321, t456, T2066). The most frequent was t605, occurring 168 strains (58.9%), followed by t127, in 27 (9.5%) and the presence of t321 in cows causing mastitis had not been previously reported. The most frequent sequence types (ST) were ST126, corresponding to 59% of the isolates, followed by ST1 (9%), belonging to the clonal complex (CC) 126 and 1, respectively. The results showed notable differences between profiles in PFGE compared to other techniques, strains of the same spa type with different profiles of PFGE suggesting that *spatyping* is not a good molecular marker.

Key words: *S.aureus*, t321, agr, s*pa* typing, MLST.

1. INTRODUÇÃO

A mastite bovina é uma doença com grande prevalência nos rebanhos leiteiros em todo o mundo e representa uma das mais significantes perdas econômicas na indústria leiteira, associadas à redução da qualidade do produto, redução da produção, descarte do leite, reposição de animais, além dos gastos com medicamentos e serviços veterinários (AIEMSAARD et al., 2011; JASIELSKI et al., 2014). Essa inflamação das glândulas mamárias de vacas que pode ser causada por traumas físicos ou infecção por micro- organismos gera alterações fisiológicas e metabólicas que leva a uma redução da área funcional do teto e, muitas vezes, à perda do teto ou descarte do animal (NAWROTEK, 2011). Para estabelecer a infecção, a bactéria precisa adentrar o úbere da vaca, vencendo as barreiras anatômicas, químicas e o sistema imunológico. Se as defesas imunes não forem capazes de eliminar o patógeno, a concentração de bactérias aumenta, passando a danificar o epitélio mamário e a produtividade leiteira é afetada negativamente (ZHAO & LACASSE, 2008).

A mastite pode ser classificada quanto à forma de manifestação em clínica ou subclínica. O primeiro tipo é caracterizado por alterações macroscópicas do leite e na glândula mamária, podendo evoluir para mastite crônica. O teste da caneca de fundo escuro é utilizado para detectar essa doença, sendo recomendado a cada início de ordenha (RIBEIRO e FURLONG 2007; COSER et al., 2012). A mastite subclínica não causa alterações macroscópicas no leite nem na glândula mamária, mas determina a elevação das contagens de células somáticas (CCS) e persistência das bactérias na glândula mamária, podendo ser diagnosticada pelo Califónia Mastitis Tests (CMT) ou pela contagem eletrônica de CCS (HALASA et al., 2007).

Quanto à classificação baseada no agente causador, a mastite pode ser classificada em mastite ambiental e contagiosa. A mastite contagiosa é caracterizada pela maior ocorrência de casos subclínicos. São geralmente de longa duração, com alta CCS e causados por micro-organismos como *Staphylococcus* spp e *Streptococcus* spp (OVERESCH et al., 2013) dos quais *S. aureus* é responsável por causar infecções persistentes (MARTINS et al., 2010). Já a mastite ambiental é causada por agentes cujo reservatório é o próprio ambiente, como os coliformes, *E. coli* e *Klebsiella* spp, podendo acometer vacas lactantes, secas ou novilhas, ao contrário da forma contagiosa, que é mais comum nas vacas em lactação. (SANTOS e FONSECA, 2007; SCHUKKEN et al., 2012).

1.1. Mastite e *S. aureus*

S. aureus está entre os três mais importantes patógenos causadores de mastite, com *E.coli* e *Streptococus agalactie* (HALASA et al., 2009). Pertence *à* família Staphylococcaceae, são cocos Gram-positivos, anaeróbios facultativos, imóveis e se agrupam em formato semelhante a cachos de uva. (TRABULSI et al., 2015). É composto por 52 espécies e 29 subespécies (DSMZ, 2017), mas apenas *S. aureus*, *S. schleiferi* subesps. *coagulans*, *S. intermedius, S. hyicus* e *S. delphini* sintetizam a enzima coagulase, sendo denominados estafilococos coagulase positiva (ECP), enquanto os demais são considerados estafilococos coagulase negativa (ECN) (BANNERMAN, 2003).

S. aureus é considerada a espécie mais virulenta desse gênero, conseguindo sobreviver em ambientes variados, são mesófilos que se desenvolvem entre 7ºC e 47,8ºC, crescem em meios hipertônicos (em concentrações de até 15% NaCl) e numa ampla variação de pH (4,2 a 9,3). Apresenta natureza ubíqua, tendo como reservatório

primário a pele e membranas mucosas, especialmente a região naso-faríngea de mamíferos e aves (LELOIR et al., 2003).

Estima-se que de 25 a 40% da população humana carreie *S. aureus*, representando importante indicador de higiene pessoal e da qualidade dos alimentos (INGAVALE et al., 2005; LUONG et al., 2006). É a principal espécie associada aos casos de intoxicação alimentar, responsável por 98% dos surtos (SANTANA et al., 2010).

Embora *S. aureus* possa sobreviver por longo tempo no ambiente, é necessária a colonização de tecidos para garantir sua sobrevivência e multiplicação (PETON & Le LOIR, 2014). Esses patógenos são transmitidos entre as vacas durante a ordenha, que pode ser mecânica ou manual, entre os animais do rebanho, pelas mãos dos ordenhadores ou equipamentos de ordenha (SAKWINSKA et al., 2011). Em rebanhos leiteiros, *S. aureus* é responsável por um terço dos casos de mastite clínica e subclínica (BOTREL et al., 2010). No Brasil, a ocorrência de *S. aureus* em isolados de leite de vacas com mastite vem sendo descrito desde a década de 50 por Lacerda Jr. et al. (1953). Subsequentemente, vários estudos em diferentes estados foram registrados como Arcuri et al. (2006), no norte do estado do Rio de Janeiro, onde foi a espécie predominante em leite de tanque de refrigeração. Em 2012, Mendonça e colaboradores, avaliando *Staphylococcus* spp. isolados de mastite bovina no estado do Rio de Janeiro, encontraram 36,2% de *S. aureus*. No estado de São Paulo, Rall et al. (2014) estudaram mastite por *Staphylococcus* spp. e encontraram a presença de *S. aureus* em 17.5% dos isolados.

A mastite causada por *S. aureus* é um problema para a saúde pública, pois, frequentemente, mais da metade das cepas isoladas de leite de glândulas infectadas apresentam, pelo menos, um gene que codifica para a produção de enterotoxinas, com

significantes variações, dependendo da região e país. A concentração bacteriana no leite de glândulas infectadas é geralmente moderada (menos de 10.000 UFC/mL), mas a contaminação do leite a granel pode levar à intoxicação estafilocócica, através dos produtos lácteos fabricados com leite cru. (Le LOIR et al., 2003; Le MARECHAL et al., 2011).

1.2. Toxinas estafilocócicas

Algumas cepas produzem exoproteínas responsáveis por manifestações clínicas específicas, como as enterotoxinas estafilocócicas (SEs), Toxina-1 da Síndrome do Choque Tóxico (TSST-1), Toxina Esfoliativa e a Panton Valentine Leucocidina (PVL) (DINGES et al., 2000; BENIĆ et al., 2012; RAHIMI & ALIAN, 2013).

Os superantigenos estafilocócicos (SAgs), incluindo SEs e TSST-1, foram originalmente identificados em *S. aureus*. As SEs foram nomeadas de acordo com as suas atividades eméticas, após a administração oral em um modelo primata. Por isso, várias SEs foram designadas como SE-like (SEl), uma vez que não apresentaram qualquer atividade emética, nos modelos animais já testados (LINA et al., 2004). Existem cinco SEs clássicas, SEA até SEE, antigenicamente distintas (BERGDOLL, 1989). Ao longo dos anos, novos tipos de SEs foram descritos e seus genes, sequenciados (SEG, SEH, SEI, SElJ, SelK, SElL, SElM, SElN, SElO, SELP, SElQ, SElR, SES, SET, SElU, SElV e SElX) (ONO et al., 2008; SEO & BOHACH, 2007; THOMAS et al., 2006, WILSON et al., 2011). Em 2017, Ono e colaboradores descreveram atividade emética em SeK, SeL, SeM, SeN e SeO, em modelos primatas. Os genes que codificam para a produção de SEs podem estar localizados nos cromossomos, plasmídeos, transposons ou integrados ao genoma de fagos, conferindo

uma vantagem seletiva às bactérias que os possuem (Le LOIR, BARON, & GAUTIER, 2003).

A PVL é uma toxina leucocitolítica associada com lesões necróticas e envolvida em severas infecções cutâneas e pneumonias (GILLET et al., 2002; KANEKO & KAMIO, 2004). Pode ser encontrada em cepas de *S. aureus* meticilina sensíveis (MSSA) ou resistentes (MRSA), de diferentes fontes como alimentos, infecções em humanos e animais (SUDAGYDAN e AYDIN 2010; CIRKOVIC et al., 2012; ROSSI et al., 2016).

A síndrome do choque tóxico (TST) é causada pela TSST-1 e pode estar presente simultaneamente às enterotoxinas, principalmente, as dos tipos B e C (Schmitz et al., 1998). Juntas (TSST-1 e enterotoxinas estafilocócicas) são capazes de atuar como superantígenos nas células do sistema imune bovino, contribuindo potencialmente com os mecanismos patogênicos da mastite bovina (YOKOMIZO et al., 1995; FERENS et al., 1998).

1.3. Resistência a Antimicrobianos

A resistência aos β-lactâmicos tem sido reportada na literatura como um dos principais problemas relacionados à mastite, principalmente pelas taxas elevadas de resistência à penicilina (SHI et al., 2010; KALMUS et al., 2011; JAGIELSKI et al., 2014). A multirresistência também vem ocorrendo e, recentemente, Wang et al. (2014) observaram que 69% dos isolados provenientes de mastite bovina foram resistentes a mais de 10 antimicrobianos utilizados no tratamento da doença. *Staphylococcus* spp é capaz de adaptar-se à pressão de seleção e sofrer mutações nos seus genes ou pode adquirir genes de outras espécies. Os mecanismos de resistência são variados, incluindo alteração da permeabilidade de membrana ou da parede celular, expulsão ativa do

antibiótico para o exterior da célula e inibição ou inativação do antibiótico (Paiva et al., 2010). A via mais usual de tratamento da mastite é a intramamária, pois se espera que o antimicrobiano atinja uma concentração no úbere maior ou semelhante à concentração inibitória mínima (CIM) para o patógeno. Porém, a CIM é definida com base em testes de suscetibilidade realizados *in vitro* e não simula as condições encontradas *in vivo* pelo patógeno, o que pode contribuir para diminuir o efeito do tratamento (APPARAO et al., 2009). Além disso, se nenhuma terapia com antibiótico é realizada no período seco, a maioria destas infecções ainda estará presente após o parto. A emergência da resistência aos antimicrobianos e a dificuldade na identificação de novas classes de agentes antimicrobianos tem impulsionado a investigação de novas estratégias terapêuticas, como o uso de moléculas capazes de silenciar genes de virulência e terapia gênica em infecções. A interferência na regulação dos fatores de virulência, como no locus *agr*, também parece ser uma abordagem promissora (COSTERTON et al., 2009).

1.4. Caracterização molecular dos grupos *agr, spa* typing, MLST e PFGE

Estudos com *S. aureus*, isolados do gado leiteiro com mastite, vem utilizando vários métodos de tipagem molecular como o *Mult Locus Sequence Typing* (MLST) s*pa* typing e *Pulsed Field Gel Electrophoresis* (PFGE), que possibilitam caracterizar esse patógeno através de comparações entre sequencias descritas em diferentes partes do mundo, sendo possível traçar rotas de dispersão e demonstrar a disseminação global de vários clones. Vários estudos revelaram que apenas alguns clones específicos são responsáveis pela maioria dos casos de mastite em uma única fazenda e que alguns destes clones podem ter uma ampla distribuição geográfica (ZADOCKS et al., 2000; KATSUDA et al., 2005; SMITH et al., 2005).

A expressão da virulência em *S. aureus* é estreitamente regulada e a intensidade da infecção, associada a algumas cepas, depende da sua capacidade de mobilizar e expressar seus fatores de virulência. (Le MARECHAL et al., 2011a). Esse patógeno pode causar doenças em humanos e animais, colonizando diferentes sítios do hospedeiro. Para que isso ocorra, o micro-organismo utiliza um sistema de detecção de *quorum sensing* que, por meio de comunicação célula-a-célula, controla a regulação de vários desses fatores de virulência. Este sistema, conhecido como *locus agr* secreta um peptídeo auto indutor (AIP) de maneira dependente da densidade celular e possibilita que uma população bacteriana possa responder em conjunto, quando a densidade celular crítica é atingida (CAFISO et al., 2007). O *locus agr* é constituído por quatro genes (*agrA, agrB, agrC, agrD*), sendo ativado durante a transição da fase de multiplicação exponencial para a fase estacionária (VUONG et al., 2004). São conhecidos quatro grupos distintos desse *locus*, I, II, III e IV, todos determinados pelo polimorfismo da sequência de aminoácido de *Agr*D e *Agr*C (SHOPSIN et al., 2003). Estudos relacionaram isolados nesses grupos com diversos fatores de virulência e infeções. O grupo *agr* I está associado com casos de mastite; a resistência a antimicrobianos, com os grupos II e III; a formação de biofilme, aos grupos *agr* I e III e o grupo *agr* IV parece estar relacionado com isolados produtores de esfoliatina, envolvidas na síndrome da pele escaldada (JARRAUD et al., 2000; SAKOULAS et al., 2003; MOISE-BRODER et al., 2004; VAUTOR et al., 2007).

Os tipos de spa podem ser determinados com a amplificação e o sequenciamento da região X do gene da proteína A (*spa*), que contém repetições polimórficas diretas. Atualmente encontram- se disponíveis 17.006 *spa* types diferentes, em um servidor de livre acesso (spaserver.ridom.de). Eventualmente, observou-se estreita relação entre o tipo de *spa* e doenças em hospedeiros específicos, como o t034, mais encontrado em

infecções ligadas a suínos (HASMAN et al., 2010) e o t605, mais frequentemente associados a bovinos (Silva et al., 2013). O *software* Ridom StaphType (versão 1.4; Ridom GmbH, Wu¨rzburg, Alemanha) fornece uma ferramenta de software que permite a análise direta de sequências e designação de tipos de *spa* por sincronização através de um servidor central (STROMMENGER et al., 2006)

A técnica de MLST detecta variações que definem linhas clonais relativamente estáveis, através da presença de sete genes conservados (*arc*C, *aro*E, *glp*F, *gm*K, *pta*, *tpi* e *yqi*L) (ENRIGHT et al., 2000). Pela variabilidade e diversidade nas sequencias desses nucleotídeos, o MLST apresenta mais de 2.400 ‘sequencias tipo’ (ST) para *S. aureus* (<www.mlst.net>). A grande maioria desses STs é agrupada em um número limitado de complexos clonais (CC) ou linhagens, os quais parecem estar distribuídos mundialmente. Os CC predominantes em *S. aureus* são CC1, 5, 8, 15, 22, 30, 45, 59, 80, 97 e 121 (NUBEL et al., 2011).

A técnica de PFGE desenvolvida em 1984 por Scharwartz and Canter. Consiste na digestão do genoma total bacteriano com enzimas de restrição, seguido de eletroforese em campo pulsado, que apresenta alto poder discriminatório, pois a alternância entre os sentidos do campo elétrico permite separar fragmentos que não seriam diferenciados em gel de agarose convencional (MASLOW & MULLIGAN, 1996). O padrão de bandas gerado (pulsotipo) pode ser analisado através de comparações dos perfis visualmente (TENOVER et al., 1995) ou através de softwares específicos, como o Bionumerics (CHURCH et al., 2011). É uma técnica com alta reprodutibilidade e considerada “padrão ouro” para uso em estudos epidemiológicos (PETERS, 2009).

2. OBJETIVOS

O objetivo desse trabalho foi caracterizar molecularmente isolados de *S. aureus*, provenientes de leite de vacas com mastite subclínica, de várias regiões do estado de São Paulo, através das técnicas de MLST, *agr* type, *spa* type e PFGE, além de identificar os genes para produção da toxina Panton Valentine Leucocidina (*lukPV*), toxina da síndrome do choque tóxico (*tst*), algumas enterotoxinas (*sea-see*, *seg*, *seh* e *sei*) e realizar o perfil fenotípico e genotípico de resistência dos isolados.

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Capítulo 1

Este Capítulo gerou o artigo: Epidemiology molecular and virulence genes of methicillin-susceptible *Staphylococcus aureus* (MSSA) isolated from milk of subclinical mastitis cows, a ser submetido ao Journal of Dairy Science.

Molecular epidemiology of methicillin-susceptible *Staphylococcus aureus* (MSSA) isolated from milk of subclinical mastitis cows.

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ABSTRACT

Bovine mastitis has been a concern for dairy herd for decades and the adaptation capacity of one of the main species responsible for the disease, *Staphylococcus aureus* (*S. aureus),* plays a pivotal role in this issue. The aim of this study was to establish a molecular and phenotypic profile of 285 *S. aureus* strains isolated from the milk of subclinical mastitis cows in 18 different farms in São Paulo State using spa typing, Multilocus Sequencing Type (MLST), Pulsed Field Gel Electrophoresis (PFGE), *agr* cluster (I, II, III and IV) typing, PCR for certain genes such as enterotoxins (*sea, seb, sec, sed, see, seg, seh, sei*), toxic shock syndrome toxin (*tsst*-1), and Panton-Valentine Leukocidin (*pvl*) and *in vitro* resistance assays for 12 antibiotics. The results showed a wide variety of strains with a high toxigenic potential, and the concomitant presence of *sec*, *seg* and *seh* were prevalent. In addition, we observed a predominance of the spa types t605 (ST 126, CC126) and t127 (ST1, CC1) and the unusual presence of t321 causing bovine mastitis, which has been previously only reported in swine. The most frequent ST were ST126 (59%) and ST1 (9%). Regarding PFGE, we observed 4 major groups and 6 profile patterns. The isolates were resistant to tetracycline, erythromycin, tobramycin, and ciprofloxacin. The *tsst*-1 gene was detected in 36.8% of isolates and *pvl* was not observed. A hundred and thirty-seven (48%) isolates possessed the *agr* type III followed by type II (20%) and I (8%), with type IV not being detected. Our results showed that there were notable differences in *S. aureus* profiles patterns between typing techniques, with PFGE presenting different profiles in strains with the same spa, showing that spa typing is not a good molecular marker.

Key words: *S. aureus*, t321, PFGE, *agr*, MLST

INTRODUCTION

Staphylococcus aureus is one the most important pathogens causing mastitis (Bardiau, 2014), and is a public health concern due to the presence of at least one gene encoding enterotoxin production in most isolates. Also, these staphylococcal enterotoxins (SEs) may play an important role in the pathogenesis of mastitis (Park et al., 2006) such as toxic shock syndrome toxin-1 (TSST-1) and Panton-Valentine Leukocidin (PVL) (Dinges et al., 2000; Benic et al., 2012; Rahimi & Alian, 2013).

The resistance to β-lactam antibiotics has been also reported as one of the main problems related to the persistence of mastitis, especially due to the high rates of penicillin resistance (Kalmus et al., 2011; Jagielski et al., 2014). Multidrug resistance is also reported to be increasing. Wang et al. (2014) found that 69% of the *S. aureus* isolates collected from milk of bovine mastitis animals were resistant to more than 10 drugs used in the treatment of mastitis. The most common form of treatment is the intra-mammary administration of the antibiotics, because it leads to a greater or similar antimicrobial concentration in the udder compared to systemic antibiotics, allowing an adequate minimum inhibitory concentration (MIC) at site of the infection (Apparao et al., 2009).

S. aureus isolates collected from dairy cattle with mastitis have been studied by various molecular methods such as Multi-Locus Sequence Typing (MLST), Pulsed-Field Gel Electrophoresis (PFGE), and *agr* cluster and spa typing which allow the characterization of this pathogen through comparisons between sequences described in different parts of the world, as well as tracing dispersal routes and demonstrating the global spread of several clones. Many studies have shown that only a few specialized clones are responsible for most cases of mastitis on a single farm and some of these

clones may have a wide geographical distribution (Zadocks et al., 2000; Katsuda et al., 2005; Smith et al., 2005).

Due its variability and diversity in the nucleotide sequences and even in the genetic loci, MLST has over 2,400 "sequences type" (ST). Most of those STs are grouped into a limited number of clonal complexes (CCs) which appear to be distributed worldwide. The predominant CCs in *S. aureus* are CC1, CC5, CC8, CC15, CC22, CC30, CC45, CC59, CC80, CC97 and CC121 (Nubel et al., 2011).

Therefore, molecular studies of *S. aureus* isolated from dairy farms can be an alternative to enable the selection of the main clones that cause mastitis, developing more effective treatments and preventive measures to contain the dissemination of the pathogen.

The aim of the present work was to assess the molecular-epidemiological relationships between MSSA isolated from milk mastitis cows through spa typing, MLST, PFGE, and *agr* typing and to verify the presence of virulence factors and their resistance to drugs.

MATERIALS AND METHODS

Samples: Two-hundred and eighty-five *S. aureus* isolates collected from milk from cows diagnosed with subclinical mastitis were studied in 18 different dairy farms in São Paulo State, Brazil. The diagnosis of subclinical mastitis was based on the California Mastitis Test (CMT), according to Schalm and Noorlander (1957), and confirmed by somatic cell counting (SCC) using flow cytometry (Somacount 300, Bentley Instruments, Chaska, MN). The positive milk samples were plated onto blood agar plates (Oxoid Brasil Ltda, São Paulo, Brazil) and incubated at 37°C for up to 72 h. *S. aureus* isolates were identified based on colony morphology, Gram staining, catalase,

coagulase, and DNase activities (Koneman et al., 2008). Molecular confirmation was performed using a multiplex PCR, looking for a species-specific staphylococcal nuclease (*nuc*) gene, as well as the staphylococcal methicillin-resistance genetic determinant (*mecA*) (CRL-AR, 2009).

PCR Testing for Genes Encoding Staphylococcal Virulence Factors: The Minispin Kit (GE Healthcare, Little Chalfont, UK) was used for DNA extraction according to the manufacturer's instructions. We performed PCR for the detection of Staphylococcal super-antigens *(Sags)* genes (Johnson et al., 1991; Omoe et al., 2002 and Jarraud et al., 2002), and *lukF*/*S-PV* (Lina et al., 1999). *S. aureus* USA 100 (*lukF*/*S-PV*), ATCC 13565 (*sea*), ATCC 14458 (*seb*), ATCC 19095 (*sec*), FRI 361 (*sed*, *seg*, *sei*), ATCC 27664 (*see*), FRI 137 (*seh*), and ATCC 51650 (*tst*) were used as positive controls and ultrapure water was used as a negative control.

Molecular Typing of S. aureus Isolates: The strains were tested for the *spa* gene (http://spaserver.ridom.de), *agr* allotype (Shopsin et al., 2003), and MLST (http://www.mlst.net) on one strain per *spa* type, assessing the most frequent ones. PFGE of genomic DNA, digested with the macro-restriction *Sma*I enzyme (PulseNet, 2016), was performed in 10 random strains isolated from four different farms. The band profiles were compared according to Bionumérics (BioNumerics software, 5.0) and Tenover et al. (1995).

Antimicrobial Susceptibility Testing and Detection of Resistance Genes: Antimicrobial susceptibility was performed using the disk-diffusion agar method, according to recommendations of the Clinical and Laboratory Standards Institute (CLSI,

2015); the drugs tested were oxacillin, cefoxitin, tetracycline, erythromycin, clindamycin, gentamycin, tobramycin, streptomycin, trimethoprim sulfamethoxazole, ciprofloxacin, cephalothin and vancomycin. The detection of resistance genes was investigated in resistant isolates by PCR, using specific primers such as *ant(4') (*tobramycin), *aac(6')-Ie-aph(2")-Ia and aph(3')-IIIa (*gentamycin*),* tet (tetracycline), cfr and *fexA* (clindamycin), str, *aadE* and *aadA* (streptomycin), *erm cluster* and *msrA* (erythromycin *), grl (A)* and *gyr (A)* (ciprofloxacin) (Van de Klundert et al., 1993; Schmitz et al., 1998; Clark et al., 1999; Aarestrup et al., 2000; Kehrenberg and Schwarz, 2005; Kehrenberg and Schwarz, 2006; Schnellmann et al., 2006)

RESULTS

Typeability and Diversity of Spa Types: All 285 strains were confirmed as *S. aureus* using a PCR assay, but none were identified to be MRSA. We found 23 different spa types (t605, t127, t458, t521, t342, t318, t693, t177, t11659, t021, t2164, t1192, t7335, t114, t6811, t6980, t002, t3324, t559, t138, t321, t456, t2066); the most frequent were t605, occurring in 168 isolates (58.9%), and t127, in 27 isolates (9.5%).

Clonal Lineages*:* The most frequent ST was ST126, occurring in 59% of the isolates, followed by ST1 (9%), belonging to CC 126 and 1, respectively. The Ridom Staph Type software also identified five strains belonging to CC 30, with the spa types t138 (1) and t318 (4), and 1 strain identified as t177 belonging to CC1.

Pulsed Field Gel Electrophoresis (PFGE): We performed PFGE in ten strains, with 8 being t605/ST126 and 2 being t693/ST1. The isolates were subdivided into 6 PFGE patterns, clustered into 4 PFGE types dominant. A dendrogram of percentage similarity

was calculated and defining clonality in PFGE using a similarity value of 80% as a cut-off, which is considered the gold standard (McDougal et al., 2003).

The strains analyzed in PFGE were from four different farms (Figure 1). According to Tenover et al. (1995), only three strains of farm I were closely related patterns showing variations in two to three bands. It is interesting to note that strain 18 is closely related to strain 08 (90.2%), but these harbor different spa types and MLSTs, yet the same *agr* cluster. Equally, strains 128 and 89 are closely related according to PFGE and harbor the same differences, but were isolated from different farms. We also observed 6 unrelated PFGE profiles considering the same spa type in different farms, such as t605.

Cluster agr: The type II *agr* was the most frequent, occurring in 137 (48%) out of 285 strains, followed by type III (57/20%) and type I (23/8%). The type IV *agr* was not found and 68 (24%) were not typable.

Virulence Factors: With regard to virulence factor, the *tsst-1* gene was found in 37.1% (105) of isolates, and the SE gene distribution was very variable, since all isolates (285) were positive for at least one of those genes. The most frequent was *seg*, occurring alone or in different combinations in 275 (96.5%) isolates, followed by *seh* (250/90%), *sec* (227/79.6%), *sei* (44/15.4%), *sea* (31/10.8%) and *sed* (10/3.5%). The *seb* and *see* genes were not observed, and neither was the *pvl* gene. A large number of strains were sensitive to the tested drugs and 37 out of 285 (13%) were resistant to at least one antibiotic; also, 6% (16/285) presented an intermediate resistance (Table 1).

DISCUSSION

The interest in MSSA has increased in recent years, since it may also be involved in important infections and could help to explain the occurrence and development of successful and different strains of MRSA. There are few data regarding MSSA genetic strains in food products of animal origin and derivatives such as milk (Silva et al., 2013). This study can provide data to help with understanding of the facts, as knowledge of the distribution of *S. aureus* will make it easier to formulate strategies to reduce the spread of infection.

We found a high diversity of spa type (23) in the 285 isolates of MSSA with subclinical mastitis. Aires-de-Sousa et al. (2007) and Silva et al. (2013) also reported the prevalence of t605 and t127 in Brazil. Said et al. (2010) observed MSSA t605 spa type in Canada and t127 has already been found in Switzerland (Huber et al., 2010) and Korea (Hwang et al., 2010) in isolates from bovine mastitis.

As far as we know, this is the first report of the detection of spa type t321 isolates in milk samples from cows with mastitis. Previously, it has only been isolated from humans, swine, and farm environments, such as in dust and milking parlors (Székely et al., 2012; Huang et al., 2014, Locatelli et al., 2017), showing that the host specificity of clones may be questioned. This suggests the need for further investigations, as host-specific clones may reduce the chance that human-derived antibiotic resistant *S. aureus* isolates are transmitted to cattle, although bovine mastitis may occasionally be caused by human-derived isolates (Ikawaty et al., 2008). In Brazil, Baptistão et al. (2016) isolated *S. aureus* t002 and t127 from food handlers, with t127 being one of the most frequently isolated in this study. This shows that food chain flaws have been usual and food handlers may be a source of these clones for the community. These strains can spread and develop a variety of host adaptations, which are capable of

causing active symptomatic infections in both species, such as *S. aureus* CC398 (Graveland et al., 2011).

MLST characterization was performed and the clones found were ST126, corresponding to 59% of the isolates, followed by ST1 (9%) belonging to CC 126 and 1, respectively. The Ridom Staph Type software also identified five strains belonging to CC 30, but these were spa types t138 (1) and t318 (4). One strain was identified as t177 belonging to CC1; it is important to note that its CC is most commonly isolated from humans (Lozano et al., 2012; Argudin et al., 2012). The population of *S. aureus* seems to be highly clonal, as suggested by Musser et al. (1990). As noted in other studies (Hata et al., 2010; Nubel et al., 2011; Chua et al., 2013), those strains can be represented by several clonal complexes, suggesting that there is no link between the specific genotype of MLST and propensity to cause disease (Feil et al., 2003).

In PFGE results, we observed different profiles in isolates within the same spa type. According to Tenover et al. (1995), a clone is a group of related isolates belonging to the same PFGE pattern; therefore, strains of the same spa type cannot be called a clone. The current study clearly shows that molecular profile analyses only in *S. aureus* isolated from mastitis are not able to determine the virulence potential. Also, the presence of the same profiles carrying different molecular markers and virulence genes can explain the difficulty in identifying effective medicines against this pathogen (Kimet al., 2011; Pereyra et al., 2017). According to Feil et al. (2003), the gain and loss of virulence genes, carried out through moving elements, play an important role in determining the virulence of an isolate. The movement of these genes can occur so rapidly that either the presence or absence is weakly related to clonal stability. Therefore, as reported by Moen et al. (2014), we strongly suggest that spa typing can be

useful to complement genotyping results in studies to compare mastitis bovine isolates; however, there is no higher specificity when used alone.

One of the most well-known regulatory systems involved in the expression of virulence genes in *S. aureus* is the *agr* group. Studies have shown that *agr* type I is more prevalent in humans than in animals (van Leeuwen et al., 2000). Instead, *agr* II has been found more frequently in animals (Melchior et al. 2009; Silva et al. 2013; Marques et al. 2017). These results were similar to those of our study, with positivity of 48% for *agr* II (136/285), followed by 20% for type III (57/285) and 8% for type I (23/285). The presence of *agr* type IV was not detected, and 69 (24%) strains were negative for all known types. Melchior et al. (2009) suggested that *agr* type II isolates are better adapted to the dairy environment than *agr* type I isolates.

Regarding virulence factor, the *tst* gene was found in 37% (105/285) of isolates, which was similar (37.5%) to the result found by Nader Filho et al. (2007) also in Brazil. On the other hand, Sá et al. (2004) did not observe this gene in *S. aureus* isolated from mastitis cows in the same region as the current study, but their study was performed 13 years ago, which suggests that in the recent period there may have been a diversification of strains or the spread of a new clone into these regions, demonstrating the genetic evolution of *S. aureus* towards a more virulent strain.

S. aureus strains can encode more than one enterotoxin gene simultaneously and over 50% of the isolates assessed showed this property (Zschock et al., 2005; Srinivasan et al., 2006). In current study, all 285 strains were positive for at least one gene and the combination *sec+seg+seh* was the most frequent, occurring in 56.8% of isolates. Zschock et al. (2005) analyzed milk from cows with mastitis and also observed that some strains of *S. aureus* can encode several genes, with 26.9% carrying two genes. According to these authors, the simultaneous production of different types of

enterotoxins can increase the toxigenic effect, suggesting that this co-production may play an important role in mastitis. Enterotoxin A (SEA) is one of the most frequently observed (Al-Bahry et al., 2014), although the literature shows highly variable results in the prevalence of *S. aureus* enterotoxin genes, depending on the kind of food and the biovar investigated (Normanno et al., 2005). In the current study, we found 31 (10.8%) isolates encoding the *sea* gene. On the other hand, Hata et al. (2006) evaluated this gene in isolates from mastitic milk and its presence was negative.

The *pvl* gene was not observed. Likewise, Pajic et al. (2014) did not observe this gene. According to Ćirković et al. (2012), the presence of the *pvl* gene is often associated with methicillin resistance, and all of our isolates were MSSA, which could explain this finding.

The increase of multidrug-resistant *S. aureus* isolated from bovine mastitis is a serious problem, increasing morbidity and costs against the disease. In addition, the indiscriminate use of antibiotics can lead to their accumulation in food, which can ultimately affect human health (Pol & Ruegg, 2007). Since antimicrobial therapy is one of the main tools for the control of mastitis caused by *S. aureus*, antibiogram assays can indicate the best treatment for each case of mastitis (Moroni et al., 2006). According to our results, 230 (80%) isolates were sensitive to all drugs tested. For the resistant and intermediate strains, we tested the presence of genes responsible for resistance to tetracycline (*tet*K, *tet*L and *tet*M), erythromycin (*erm*A, *erm*B, *erm*C, *mrs*A), tobramycin (*ant*4) and ciprofloxacin (*glr*, *gyr*). The resistance of the strains to tetracycline was confirmed by the presence of the *tetK* gene and both genes responsible for resistance to ciprofloxacin were present. With regard to the strains with intermediate resistance to erythromycin, only 4 were positive for the *erm*A gene and no gene encoding resistance for tobramycin was found. The discrepancy observed between the

genotype and phenotype in the four strains with intermediate resistance but which did not harbor any erythromycin genes was also observed by Goudarzi et al. (2016), where nine *S. aureus* isolates were resistant to erythromycin, but did not carry any of the tested erythromycin-resistant genes. The authors presumed that other variants of the *erm* genes, efflux pump (*msrB*) and a high rate of horizontal gene transfer between them is involved in this finding (Paiva et al.2010; Abdollahi et al. 2013; Kareen et al. 2015). Grando et al. (2008) also found *S. aureus* isolated from the hands of foodhandlers, showing intermediate resistance to erythromycin in the absence of the genes responsible for that behavior.

Molecular analysis has been important to show the dispersion and adaptation capacity of clones around the world. However due to the discrepancy observed between the different typing techniques such as PFGE, MLST and *spa* typing in the current study, we can question the exclusive use of *spa* typing as strains with the same type may have no phylogenetic relation, as shown by PFGE. On the other hand, it is useful as a marker due to its inexpensiveness, and exportability, as we noted some t321 strains causing mastitis, which had previously only been isolated in pigs and humans.

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Table 1. Sensibility profile to antimicrobial agents of *S. aureus*, isolated from milk of cows with subclinical mastitis.

Antibiotic	Susceptible % (N)	Intermediate % (N)	Resistant % (N)
oxacillin	100% (285)	0	0
cefoxitin	98.5% (281)	0	1.5% (4)
tetracycline	96.5% (275)	0	3.5% (10)
tobramycin	99.2% (283)	0.4% (1)	0.4% (1)
erythromycin	97.1% (277)	2.9% (8)	0
streptomycin	90.5% (258)	0	9.5% (27)
gentamicin	100% (285)	0	0
clindamycin	96.9% (276)	0	3.1% (9)
Trimethoprim-sulfamethoxazole	99.6% (284)	0	0.4% (1)
cephalothin	99.6% (284)	0	0.4% (1)
ciprofloxacin	98.9% (282)	0	1% (3)
vancomycin	99.6% (284)	0.4% (1)	0

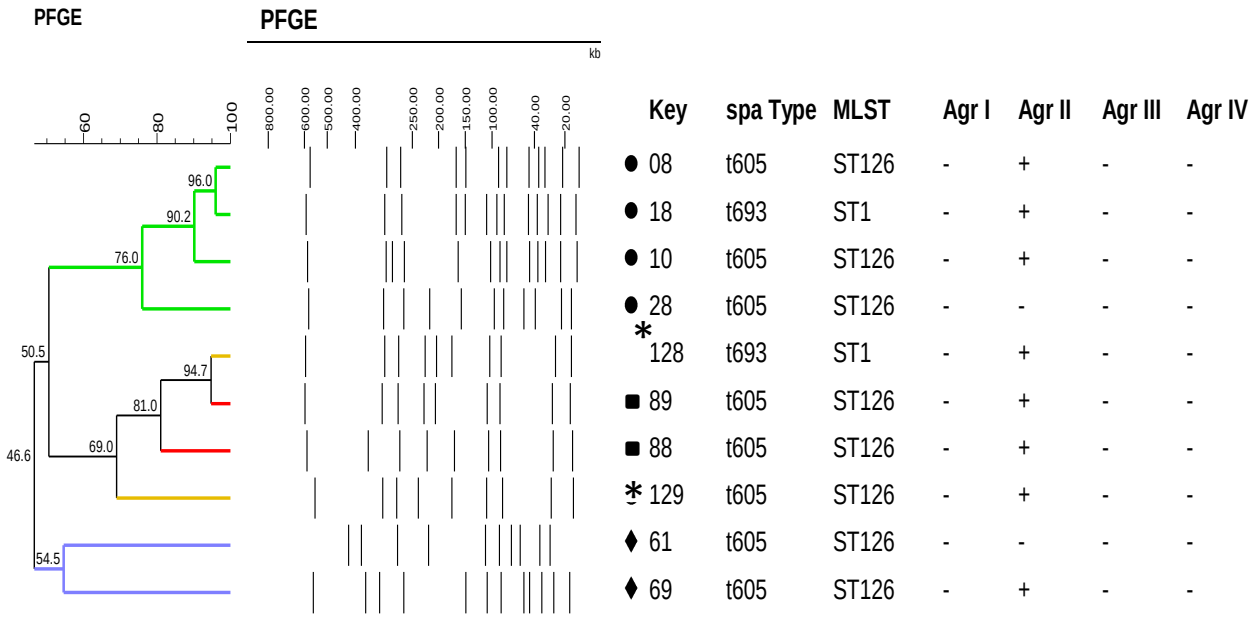

Figure 1. Dendrogram of pulsed-field gel electrophoresis (PFGE). PFGE types were determined by unweighted pair group methodology based on Dice coefficients (BioNumerics software, 5.0), with 1.25% band position tolerance and optimization of 0.5%. A similarity coefficient of 80% was selected to define PFGE types. ● Farm I, * Farm II, ■ Farm III, ♦ Farm IV.