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

PG-BGA

Staphylococcus spp. ISOLADOS DE MASTITE SUBCLÍNICA BOVINA EM DIFERENTES ESTADOS BRASILEIROS: RESISTÊNCIA ANTIMICROBIANA, DETECÇÃO DOS FATORES DE VIRULÊNCIA E IDENTIFICAÇÃO DO PERFIL CLONAL

PRISCILA LUIZA MELLO

Tese apresentada ao Instituto de Biociências, Câmpus de Botucatu, UNESP, para obtenção do título de Doutor no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração *Biologia de Parasitas e Micro-organismos.*

Maria de Lourdes Ribeiro de Souza da Cunha

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Palavras-chave: Biofilme; Enterotoxinas; Estafilococos; Mastite bovina; Perfil clonal.

“É pelo saber que o corpo se purifica, é procurando o saber que ele se eleva. Para o sabedor, todos os instintos tornam-se sagrados; no homem que se elevou, a alma torna-se alegre."

Friedrich Nietzsche

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SUMÁRIO

RESUMO

A mastite é considerada um grande problema na pecuária leiteira e o uso frequente de antimicrobianos contribui para o aumento da pressão seletiva de micro-organismos resistentes aos principais fármacos. A Organização Mundial da Saúde Animal tem alertado para o risco da resistência antimicrobiana resultante do uso indevido de antimicrobianos no tratamento de animais doentes. O presente trabalho tem como objetivos identificar e caracterizar o perfil clonal, bem como os fatores de virulência e de resistência aos antimicrobianos em *Staphylococcus* spp. isolados do leite de bovinos com mastite subclínica de várias regiões do Brasil. As amostras foram concedidas pela Embrapa Gado de Leite, provenientes de 6 estados brasileiros, incluindo Paraná, Santa Catarina, Rio Grande do Sul, Minas Gerais, São Paulo e Pernambuco. Foram incluídas 181 amostras de *Staphylococcus* spp. no estudo. A identificação fenotípica revelou um total de 82 (45,3%) *Staphylococcus aureus* e 99 (54,7%) *Staphylococcus* coagulase negativa (CoNS). Destes, a espécie mais isolada foi *S. chromogenes* com 27 (14,9%) amostras, seguido de *S. epidermidis* com 26 (14,3%). Realizou-se a confirmação genotípica utilizando a técnica Internal Transcribed Spacer - PCR (ITS-PCR) de todas as amostras, demonstrando um total de 98% de concordância com o teste fenotípico. Quanto à determinação da Concentração Inibitória Mínima (CIM), *S. aureus* revelou 99% de sensibilidade à oxacilina, enquanto os CoNS apresentaram 32% de resistência à esse antimicrobiano. Já para a vancomicina, todas as amostras foram sensíveis, apresentando apenas susceptibilidade reduzida pelo método de triagem, onde 13 amostras cresceram em BHI ágar com concentrações de 4µg/mL e 6 µg/mL de vancomicina. Com referência à produção de beta-lactamase foi possível observar que 96% das amostras de *Staphylococcus* spp. foram positivas. O gene *mec*A foi detectado em 8 amostras, todas do grupo CoNS e a tipagem do SCC*mec* revelou isolados do tipo I e tipo IV. Nas amostras em que não foram encontrados o gene *mecA*, foi realizada pesquisa do *mecA* homólogo (LGA_{251}) e um total de 12 amostras apresentaram um produto de PCR com tamanho semelhante ao esperado para o gene *mec*C. No entanto o sequenciamento das amostras revelou similaridade de 99% com um gene ancestral do *mec*A. Através da técnica de PCR foram pesquisados os genes do operon *ica* responsáveis pela produção de biofilme,

sendo detectados o gene *ica*A em 79 amostras (43,6%), o *ica*B em 24 (13,2%), o *ica*C em 57 (31,4%) e o *ica*D em 127 (70,1%). O gene *bap* foi detectado em 66 amostras (36,4%), enquanto o gene *bhp* foi encontrado apenas em 9 (4,9%) amostras de *S. epidermidis*. Já os ensaios de expressão por RT-qPCR comprovaram a expressão do gene *ica*A em 60 amostras (33%) do *ica*B em 6 (3,3%), do *ica*C em 26 (14,3%) e do *ica*D em 80 (44%). Quanto aos genes de enterotoxinas, o estudo revelou o gene *sea* como o mais frequente, sendo detectado em 18,2% das amostras, o gene *seb* em 7,7%, o *sec* em 14,9%, o *sed* 0,5%, o *see* em 8,2%, o *sei* em 6,6%, e o *ser* em 1,6%. Os genes *ses, set e tst* não foram detectados em nenhuma das amostras. A tipagem do perfil clonal das amostras pela técnica de *Pulsed Field Gel Electrophoresis* permitiu a identificação de oito clones de *S. aureus* que incluíram simultaneamente, ≥3 amostras, com similaridade ≥80%. Em relação às outras espécies estudadas, houve a formação de três grupamentos para a espécie *S. chromogenes*, enquanto para *S. epidermidis* quatro clusters foram detectados. Já ao analisar *S. saprophyticus*, foi possível observar apenas um grupamento. Quanto ao *Multilocus Sequence Type* (MLST) para o sequenciamento dos sete genes *housekeeping*, os ST prevalentes em *S. aureus* foram ST126 e ST1 enquanto em *S. epidermidis*, os ST foram todos distintos.

Palavras-chave: bovino de leite, estafilococos, mastite, biofilme, enterotoxinas, fatores de virulência.

ABSTRACT

Mastitis is considered a major problem in dairy farming and the frequent use of antimicrobials contributes to increased selection pressure of resistant micro-organisms to the main drugs. The World Organisation for Animal Health are aimed at the protection of animal and human health against the risk of antimicrobial resistance resulting from the treatment of sick animals. This study aims to identify and characterize the clonal profile and the virulence as well as the virulence factors and the antimicrobial resistance to *Staphylococcus* spp. isolated from bovine milk with subclinical mastitis from several regions of Brazil. The samples were provided by Embrapa Gado de Leite (Embrapa Dairy Cattle), from 6 Brazilian states, including Paraná, Santa Catarina, Rio Grande do Sul, Minas Gerais, São Paulo and Pernambuco. A total of 181 samples of *Staphylococcus* spp. were included in the study. Phenotypic identification revealed a total of 82 (45.3%) *Staphylococcus aureus* and 99 (54.7%) Coagulase-Negative *Staphylococci* (CoNS) and, of these, the most isolated species was *S. chromogenes* with 27 (14.9%) samples, followed by *S. epidermidis* with 26 (14.3%). We realized the genotypic confirmation by using the technique Internal Transcribed Spacer - PCR (ITS-PCR) of all samples, demonstrating a total of 98% of concordance with the phenotypic test. As for the determination of Minimum Inhibitory Concentration (MIC), *S. aureus* showed 99% of susceptibility to oxacillin, while CoNS showed 32% resistance to this drug. As for vancomycin, all samples were susceptible, by displaying only reduced susceptibility screening method, where 13 samples grown on BHI agar at concentrations of 4μg/mL and 6 μg/mL of vancomycin. With reference to the production of beta-lactamase was observed that 96% of *Staphylococcus* spp. strains were positive. The *mec*A gene was detected in 8 samples, all of CoNS group and typing of isolates revealed SCC*mec* type I and type IV. In the samples that were not found *mec*A gene, an homologous *mec*A (LGA_{251}) survey was conducted and a total of 12 samples showed a PCR product with size similar to that expected for *mec*C gene. However, the sequencing of the samples revealed a similarity of 99% with an ancestral gene *mec*A. Through PCR technique were investigated genes of the *ica* operon responsible for the production of biofilm, being detected *ica*A gene in 79 samples (43.6%), the *ica*B in 24

(13.2%), *ica*C in 57 (31 , 4%) and *ica*D in 127 (70.1%). The *bap* gene was detected in 66 samples (36.4%), while *bhp* gene was found only in 9 (4.9%) samples of *S. epidermidis.* Yet, the expression assays by RT-qPCR demonstrated the expression of *ica*A gene in 60 samples (33%), of *ica*B 6 (3.3%) of *ica*C in 26 (14.3%) and *ica*D 80 (44 %). As for enterotoxin genes, the study revealed the *sea* gene as the most frequent, being detected in 18.2% of samples, the *seb* gene in 7.7%, *sec* in 14.9%, *sed* 0.5% , *see* in 8.2%, *sei* at 6.6% and the *ser* in 1.6%. The *ses*, *set* and *tst* genes were not detected in any of the samples. The typing of clonal profile of the samples by the Pulsed Field Gel Electrophoresis technique, permitted the identification of eight clones of *S. aureus*, included both ≥3 samples, with ≥80% similarity. For the other species studied, there was the formation of three groups for the *S. chromogenes* species, while for *S. epidermidis*, four clusters were detected. Have to analyze *S. saprophyticus*, we observed only one grouping. As for the Multilocus Sequence Type (MLST) that is the sequencing of seven *housekeeping* genes, the ST prevalent in *S. aureus* were ST126 and ST1 while in *S. epidermidis*, the ST were all different.

Key words: dairy cattle, staphylococci, mastitis, biofilm, enterotoxins, virulence factors.

1. INTRODUÇÃO

Considerado um dos produtos de maior importância da agropecuária brasileira, o leite e seus derivados desempenham relevante papel econômico e social, gerando renda e emprego à população (CARVALHO et al., 2003).

O manejo inadequado dos animais de produção pode desencadear processos inflamatórios na glândula mamária, conhecidos como mastite, ocasionado por diversos micro-organismos que influenciam diretamente as características físico-químicas, composição e celularidade do leite, trazendo como consequência prejuízos financeiros ao criador devido à rejeição do produto final (GOTTARDI et al., 2008).

Com o surgimento de patógenos e introdução de agentes antimicrobianos na medicina veterinária, acreditou-se inicialmente que em pouco tempo as doenças infecciosas seriam prontamente curadas e não mais representariam uma ameaça às vidas humanas e animal. No entanto, constatou-se que o emprego terapêutico de um determinado fármaco era seguido, em curto espaço de tempo, pelo aparecimento de estirpes bacterianas resistentes e capazes de sobreviver ao tratamento (ACAR; ROSTEL, 2001).

Existe grande preocupação internacional a respeito do risco da transferência de bactérias resistentes entre animais e humanos e da transferência desses genes de resistência de bactérias de animais para patógenos humanos. Há preocupação também com a resistência antimicrobiana em relação à saúde animal. A Organização Mundial da Saúde Animal (OIE) reconhece que os países devem proteger a saúde animal e humana. Isso inclui também a proteção contra o risco da resistência antimicrobiana resultante de tratamento de animais doentes (DEHAUMONT, 2004).

Diversos micro-organismos estão envolvidos na gênese de processos infecciosos da glândula mamária de animais de produção. Estudos epidemiológicos em âmbito nacional e internacional têm demonstrado a presença do gênero *Staphylococcus* em aproximadamente 50% dos casos de mastite em bovinos, destacando-o como principal grupo de patógeno isolado (RADOSTITIS et al., 2007; BRABES et al., 1999).

Diversas pesquisas têm enfatizado a importância de *Staphylococcus* coagulase-negativa (CoNS) toxigênicos isolados de alimentos. Portanto, embora *Staphylococcus aureus* seja o principal agente envolvido em intoxicações alimentares, existe grande preocupação da comunidade científica com relação aos CoNS, reconhecidos como patógenos oportunistas em diversas afecções em humanos e animais, aliado aos riscos de linhagens toxigênicas na casuística de toxinfecção alimentar em humanos (CUNHA et al., 2006).

Micro-organismos que exibem resistência às penicilinas resistentes à penicilinase e classificados como *Staphylococcus* meticilina (oxacilina) – resistentes (MRSA) são frequentemente resistentes à maioria dos agentes microbianos (MANDELL et al., 1995). Atribui-se a resistência estafilocócica à meticilina, devido a aquisição do cassete cromossômico *mec*, considerado uma ilha de resistência e composto pelo gene estrutural *mec*A. A sua expressão resulta na produção de PBP 2ª (Proteínas Ligadoras de Penicilina), uma proteína de alto peso molecular e baixa afinidade a antibióticos betalactâmicos (MARTINEAU et al., 1998).

Estudos genéticos demonstram que a atual prevalência de MRSA resultou principalmente da disseminação de alguns clones, com a descrição de apenas cinco clones no mundo. Atualmente, para a investigação de surtos, podem-se utilizar as caracterizações fenotípica e genotípica para avaliação da disseminação dessas cepas.

De acordo com publicações, até agora, há 11 alótipos diferentes de SCC*mec*, os tipos I a XI, que foram revelados entre as cepas de MRSA. O SCC*mec* apresenta componentes genéticos do complexo gene *mec* e o gene responsável pela recombinação do cassete cromossômico (*ccr*). Variações dentro desses complexos gênicos servem como base primária para classificação dos vários tipos de SCC*mec* (OLIVEIRA et al., 2001).

Historicamente o MRSA fez o caminho inverso das demais bactérias, ou seja, iniciou como um problema em medicina e, posteriormente, emergiram como problema significativo na área veterinária (WEESE; DUIJKEREN, 2010). O MRSA já foi detectado em animais de produção, aves, equinos, cães, gatos (SASAKI et al., 2007) e alimentos (WEESE; DUIJKEREN, 2010).

Nos últimos anos, numerosas técnicas moleculares têm sido aplicadas na investigação epidemiológica da resistência antimicrobiana, com a finalidade de identificar e rastrear padrões de infecção. A epidemiologia molecular dos genes de resistência permite discriminar entre disseminação clonal de uma estirpe bacteriana em particular, e transferência horizontal de determinantes de resistência entre bactérias. A técnica de PCR (*Polimerase Chain Reaction*) é necessária para confirmação da resistência para meticilina, após detecção da resistência à oxacilina *in vitro* e vem sendo empregada para detectar e estudar resistência a outros antimirobianos (LARS; AARESTRUP, 2005; SCHMITT-VAN DE LEEMPUT; ZADOKS, 2007).

A tipagem molecular ou genotipagem é essencial para determinar a fonte de transmissão, avaliar o padrão de transmissão na emergência da resistência e estudar a evolução e organização molecular de bactérias resistentes. A genotipagem permite distinguir entre a disseminação clonal da resistência ou a disseminação horizontal e é usada para monitorar a distribuição global de

bactérias resistentes (AARTS et al., 2001). Tais informações podem contribuir para o desenvolvimento de estratégias mais eficientes com o intuito de reduzir os casos de infecção, uma vez que a partir dos perfis moleculares é possível inferir relações genéticas entre os diferentes clones, detectar o fluxo gênico e traçar rotas de dispersão da infecção no rebanho (KAPUR, et al., 1995).

Nas últimas décadas, uma grande variedade de técnicas moleculares baseadas no estudo da similaridade dos cromossomos foi desenvolvida, dentre elas se destacam o *Pulsed-Field Gel Eletrophoresis* (PFGE) e o *Multilocus sequence Typing* (MLST) (SCHWARTZ et al., 1983; MAIDEN et al., 1998).

A técnica de eletroforese em campo pulsado (PFGE, *Pulsed-Field Gel Eletrophoresis*) se tornou importante método, considerado padrão-ouro, para a tipagem molecular e caracterização de clones bacterianos, principalmente para os estafilococos (MIYAZAKI, 2006; SAIDA et al., 2006). Esta técnica tem como objetivo detectar variações genéticas entre isolados bacterianos filogeneticamente e epidemiologicamente relacionados (ZADOKS et al., 2002). Portanto, o PFGE pode ser considerado bom método para estabelecimento de relações clonais em estudos de epidemiologia molecular (TENOVER et al., 1995).

O MLST é uma técnica de caracterização molecular e investigação epidemiológica proposta por Maiden et al. (1998), a qual também tem sido amplamente utilizada para interferência filogenética de espécies bacterianas (MAIDEN et al., 1998; COOPER; FEIL, 2004; HARBOTTLE et al., 2006). O conjunto de alelos dos diferentes *loci* estudados no MLST determina o perfil ou *sequence type* (ST) de uma cepa bacteriana. Os ST encontrados dentro de uma população bacteriana permitem o estudo comparativo de suas cepas, bem como a inferência filogenética de seus membros (MAIDEN et al., 1998).

Além dos mecanismos de resistência, os Estafilococos podem apresentar diversos fatores de virulência. Um desses fatores de grande importância está relacionado à capacidade destes micro-organismos de produzirem biofilmes (AGUIAR et al., 2001). O biofilme protege a bactéria da ação dos componentes do sistema imunológico, pois dificultam a ação dos fagócitos (FOX et al., 2005), além de funcionar como uma barreira que dificulta a penetração de agentes antimicrobianos (STEWART, 1996).

A proliferação das células para aderir e formar biofilme é mediada pela produção do polissacarídeo intercelular de adesão (PIA) ou poly-N-succinil-β-1,6-glucosamina. A síntese é codificada pelos genes do operon *icaADB*C e os genes e produtos do locus *ica* foram demonstrados fundamentais para a formação de biofilmes e virulência dos micro-organismos (MELO, 2008).

Além da possibilidade das linhagens de *Staphylococcus* spp. apresentarem resistência aos antimicrobianos – principalmente do grupo dos beta-lactâmicos – estas bactérias também podem veicular toxinas aos humanos pela ingestão de alimentos (SALES et al., 2000; RADOSTITIS et al., 2007; WINN JR., et al., 2008).

Além da Toxina 1 da síndrome do choque tóxico (TSST-1), atualmente são conhecidos 23 tipos de enterotoxinas estafilocócicas (SE) e enterotoxinas-like (SE-like), juntamente com seus genes (se) correspondentes (OMOE et al., 2004; EUZÉBY, 2011). Os tipos A (*sea*), B (*seb*), C_{1-3} (*sec*), D (*sed*) e E (*see*), representam as toxinas estafilocócicas clássicas, que possuem atividade emética e geralmente encontram-se associadas a surtos de intoxicação alimentar (OMOE et al., 2004).

É possível verificar a presença das enterotoxinas, bem como sua expressão, com base na técnica da RT-PCR em tempo real (qRT-PCR), que possibilita a quantificação da expressão de genes induzidos em resposta a diferentes condições, apresentando boa reprodutibilidade. A quantificação da expressão dos genes é feita de forma relativa, isto é, pela comparação da expressão em um grupo exposto a um estímulo a um outro grupo não exposto, denominado grupo controle (ZARLENGA; HIGGINS, 2001). Essa é uma alternativa que vem sendo amplamente utilizada, por ter elevada sensibilidade e acurácia, sendo amplamente utilizada em diversas áreas biomédicas. Um crescente volume de artigos publicados demonstra a utilidade da PCR em tempo real relacionada à área microbiológica (ESPY et al., 2006).

Levando em consideração os diversos fatores de patogenicidade envolvidos no quadro da mastite, são de extrema importância o isolamento dos Estafilococos e o estabelecimento de perfil patogênico mais abrangente possível. Portanto, para uma melhor compreensão da dinâmica dos micro-organismos em rebanhos brasileiros, torna-se oportuno caracterizar as cepas de MRSA, visto que a tipagem molecular é essencial para determinar a fonte de transmissão, avaliar o padrão de transmissão na emergência da resistência aos antimicrobianos e estudar a evolução da organização molecular de bactérias resistentes.

2. OBJETIVO

2.1 Objetivo Geral

- Caracterizar o perfil clonal e os fatores de virulência e resistência aos antimicrobianos em *Staphylococcus* spp. isolados do leite de bovinos com mastite subclínica.

2.2 Objetivos específicos:

- Identificação fenotípica e genotípica de *Staphylococcus* spp. de rebanhos de várias regiões do Brasil.
- Determinar a resistência à oxacilina e vancomicina com base na Concentração Inibitória Mínima.
- Detectar a heterorresistência à vancomicina pelo método de triagem.
- Determinação da produção de beta-lactamase e resistência por hiperprodução dessa enzima.
- Detectar o gene *mec*A e *mec*A homólogo (*mec*C) de resistência à oxacilina em *Staphylococcus* spp. e tipagem do SCC*mec.*
- Detectar a presença dos genes *icaA, icaB, icaC, icaD, bap* e *bhp* envolvidos na formação de biofilme e verificar a expressão gênica das amostras com genes de operon *ica*ADBC.
- Detectar a presença dos genes *sea, seb, sec, sed, see, seg, seh, sei, sej, ser, ses, set e tst-1* responsáveis pela produção das enterotoxinas e da toxina da síndrome do choque tóxico (TSST) nas amostras de *Staphylococcus* spp.
- Realizar a tipagem molecular por PFGE para determinação do perfil clonal dos isolados de *Staphylococcus* spp.
- Realizar a tipagem dos clones prevalentes pela técnica de MLST.

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* ABNT(NBR6023)

4. APRESENTAÇÃO DA TESE

Os resultados e a discussão dos dados obtidos encontram-se apresentados na forma de artigos científicos.

4.1 Artigo Científico I (*Short communication*): Beta-lactam resistance and vancomycin heteroresistance in *Staphylococcus* spp. isolated from bovine subclinical mastitis.

Revista: Journal of Dairy Science

Fator de Impacto: 2.408. Submetido em: 21 de novembro de 2016. Aceito para publicação em 11 de abril de 2017.

4.2 Artigo Científico II: *Staphylococcus spp.* isolated from bovine subclinical mastitis in different regions of Brazil: Molecular typing and biofilm gene expression analysis by RT-qPCR
Revista: Veterinary Microbiology
Fator de Impacto: 2.564. Submetido em: 27 de dezembro de 2016.

4.3 Artigo Científico III: Detection of enterotoxigenic potential and determination of clonal profile in *Staphylococcus aureus* and coagulase-negative Staphylococci isolated from bovine subclinical mastitis in different Brazilian states.
Revista: Toxins
Fator de Impacto: 3.571. Publicado em 15 de abril de 2016.

Artigo Científico I

BETA-LACTAM RESISTANCE AND VANCOMYCIN HETERORESISTANCE IN *Staphylococcus* spp. ISOLATED FROM BOVINE SUBCLINICAL MASTITIS

Priscila Luiza Mello*, Luiza Pinheiro*†, Lisiane de Almeida Martins‡, Maria Aparecida Vasconcelos Paiva Brito §, Maria de Lourdes Ribeiro de Souza da Cunha*#

*Department of Microbiology and Immunology, Institute of Biosciences of Botucatu, Universidade Estadual Paulista - UNESP, Botucatu 18618-970, Brazil.

†Departament of Anatomic Pathology, Instituto Lauro de Souza Lima, Bauru, 17034-971, Brazil

‡Universidade Paranaense – UNIPAR, Umuarama 87502-210, Brazil.

§Embrapa Dairy Cattle, Juiz de Fora 36038-330, Brazil.

Correspondence: priscila_mello@msn.com Tel.: +55-14-3880-0428

ABSTRACT

The use of antimicrobial agents has led to the emergence of resistant bacterial strains over a relatively short period of time. Furthermore, *Staphylococcus* spp. can produce beta-lactamase, a fact that explains the survival of these strains in an infection focus despite the use of a beta-lactam antibiotic. The aim of this study was to evaluate the resistance of *Staphylococcus* spp. isolated from bovine subclinical mastitis to oxacillin and vancomycin by the Minimum Inhibitory Concentration (MIC) and to detect vancomycin heteroresistance by a screening method. Beta-lactamase production and resistance due to hyperproduction of this enzyme were also evaluated, in addition to investigation of the *mec*A and *mec*C genes and SCC*mec* typing. For this purpose, 181 *Staphylococcus* spp. isolated from mastitis subclinical bovine were analyzed. Using the phenotypic method, 33 (18.2%) of *Staphylococcus* spp. were resistant to oxacillin. In contrast, all isolates were susceptible to vancomycin and heteroresistance was detected by the screening method in 13 isolates. Beta-lactamase production was observed in 174 (96%) of the *Staphylococcus* spp. isolates. The *mec*A gene was detected in 8 isolates, all of them belonging to the species *S. epidermidis*. SCC*mec* typing revealed the presence of type I and type IV isolates.

Keywords: Beta-lactam; resistance; *mec*C; *mec*A; Staphylococci; cattle

SHORT COMMUNICATION

As one of Brazil's most important agricultural products, milk and milk products play a relevant economic and social role, generating income and employment for the population (Carvalho et al., 2003). The indiscriminate use of antibiotics, including vancomycin, one of the last therapeutic options for staphylococcal infections, has been selecting strains with reduced susceptibility to this drug (Schwalbe, 1987). According to Natoli (2009), the heteroresistant phenotype of these strains may be associated with treatment failure and/or may be a precursor of glycopeptide resistance.

The objective of the present study was to evaluate resistance of *Staphylococcus* spp. isolated from bovine subclinical mastitis to oxacillin and vancomycin by a phenotypic method (Minimum Inhibitory Concentration - MIC) and to detect vancomycin resistance by a screening method. Additionally, the *Staphylococcus* spp. isolates were submitted to the determination of beta-lactamase production and hyperproduction and investigation of the oxacillin resistance *mec*A and *mec*C genes. SCC*mec* typing was performed on isolates carrying the resistance gene.

A total of 181 *Staphylococcus* spp. strains isolated from bovine subclinical mastitis cases in six Brazilian states, maintained in the collection of Embrapa Dairy Cattle, were studied. Bacterial isolation and identification were performed according to the National Mastitis Council (NMC, 2004) using coagulase and sugar (trehalose, maltose, and mannitol) fermentation tests. Strains belonging to the CoNS group were submitted to biochemical tests as proposed by Cunha et al., (2004) for phenotypic identification of the species. Total DNA was extracted using the illustra® kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Genotypic identification of CoNS was performed using primers targeting conserved sequences adjacent to the 16S and 23S genes by the internal transcribed spacer-polymerase chain reaction (ITS-PCR) described by Couto (2001), using primers G1 and L1. PCR using the Staur-4 and Staur-6 primers developed

by Straub et al., (1999) was used for *S. aureus*. The amplification efficiency was monitored by 2% agarose gel electrophoresis stained with Saber Safe DNA Gel Strain® (São Paulo, SP, Brazil) viewed under a UV transilluminator.

The *in vitro* susceptibility of the isolates to oxacillin and vancomycin was tested by determining the MIC of these drugs using E-test® as follows: Mueller-Hinton culture medium, inoculum size matching the turbidity of a 0.5 McFarland standard, incubation at 35ºC, MIC determination as the value corresponding to the intersection of the ellipse of bacterial growth inhibition. The interpretation followed the breakpoints proposed by CLSI (2015), which suggests: Oxacillin breakpoints for *S. aureus*: susceptibility ≤ 2 μg/mL; resistance ≥ 4 μg/mL. CoNS: susceptibility ≤ 0.25 μg/mL; resistance ≥ 0.5 μg/mL. Vancomycin breakpoints: *Staphylococcus* spp.: susceptibility < 4 μg/mL; resistance ≥ 4 μg/mL.

PCR for the detection of the *mec*A was performed using primers mecA1 (AAAATCGATGGTAAAGGTTGG) and mecA2 (AGTTCTGCAGTACCGGATTTG) - 533 (bp) according to the parameters described by Murakami et al. (1991). International reference strains were included in all reactions: positive control (*S. aureus* ATCC 33591) and negative control (*S. aureus* ATCC 25923). The presence of the *mec*C gene was investigated in isolates in which the *mec*A gene was not detected. The primers and parameters described by Garcia-Alvarez et al. (2011) were used. SCC*mec* typing was performed using the method of Machado et al. (2007), which characterizes types I, II, III and IV isolates considering the corresponding molecular weights. As control for the SCC*mec* typing, COL strains for SCC*mec* type I were used; N315 for SCC*mec* type IA; PER34 for SCC*mec* type II; AN546 for SCC*mec* type III; HU25 for SCC*mec* type IIIA and MW2 for SCC*mec* type IV. The reactions were using the parameters consisting of: 92°C for 3 minutes followed by 30 cycles of 92° C for 1 minute, 56°C for 1 minute and 72°C for 1 minute and 30 seconds.

Production of beta-lactamase was detected using nitrocefin-impregnated disks placed on *Staphylococcus* spp. colonies previously incubated at 35ºC for 24 h on a Mueller-Hinton agar plate with the E-test oxacillin strip. The positive reaction was evidenced by the development of a red color and the negative color change. International reference strains were used as positive (*S. aureus* ATCC 33591) and negative (*S. xylosus* ATCC 29979) controls.

Isolates that tested negative for the *mec*A gene and exhibited oxacillin resistance by the phenotypic method were analyzed to determine whether they were hyperproducers of beta-lactamase using a disk containing amoxicillin (20 μg) and clavulanic acid (10 μg). The susceptibility breakpoint was the formation of an inhibition halo of 20 mm after incubation for 24 h at 35ºC (Ghoshal et al., 2004). The screening medium was prepared with Brain Heart Infusion agar (BHI) containing 4 μg/mL (Hiramatsu, 2001) and 6 μg/mL vancomycin (CLSI 2015). *Staphylococcus aureus* (ATCC 29213) susceptible to vancomycin and vancomycin-resistant *E. faecalis* (ATCC 51299) were used as negative and positive control, respectively.

Among the 181 isolates studied, 82 (45.3%) were identified as *S. aureus* and 99 (54.7%) were classified as CoNS: 27 (14.9%) *S. chromogenes*, 26 (14.4%) *S. epidermidis*, 17 (9.4%) *S. saprophyticus*, 6 (3.3%) *S. warneri*, 6 (3.3%) *S. simulans*, 6 (3.3%) *S. haemolyticus*, 2 (1.1%) *S. xylosus*, 4 (2.2%) *S. hominis*, and 5 (2.8%) *S. hyicus*.

Interpretation of the results according to the breakpoints defined by the CLSI (2015) revealed a rate of 33 (18.2%) of *Staphylococcus* spp. resistant to oxacillin (Table 1). The MIC_{50} was 0.25 μg/ml and the MIC_{90} was 1.5 μg/mL (Table 2). In contrast, for vancomycin, *S. aureus* exhibited MIC_{50} and MIC_{90} values of 0.5 and 1.5 μg/mL, respectively. In CoNS, the MIC_{50} and MIC_{90} values were 1.0 and 1.5 μg/mL, respectively. All *Staphylococcus* spp. analyzed were susceptible to vancomycin.

The *mec*A gene was detected in 8 (4.4%) CoNS isolates; two of them did not exhibit phenotypic resistance (Table 3). All isolates carrying the *mec*A gene belong to the species *S.*

epidermidis and originated from Paraná, Santa Catarina or Minas Gerais. The gene was not detected in the remaining isolates, although 27 (14.9%) strains were resistant by the phenotypic method. Thus, the presence of the *mec*A homolog, called *mec*C, was investigated. A PCR product with a size similar to that expected for this gene was detected in 12 of these 27 isolates. To confirm the presence of *mec*C, the 12 isolates were sent for sequencing. Sequence analysis showed that the products were not *mec*C but a gene with 99% identity to *mec*A, which was considered an ancestor of the gene (GenBank accession number: AB547234.1). Five (62.5%) of the 8 isolates analyzed were classified as SCC*mec* type I. The other 3 (37.5%) isolates were classified as type IV.

Regarding beta-lactamase production, 174 (96.13%) of the 181 isolates tested were positive. Among the negative isolates, only 2 (2.5%) belonged to the species *S. aureus*, while the other 5 (4.85%) were identified as CoNS. Hyperproduction of beta-lactamase was evaluated in isolates that were resistant to oxacillin by the phenotypic method, but that tested negative for detection of the *mec*A and *mec*C genes. Beta-lactamase hyperproduction was detected in 26 (96.3%) of the 27 isolates tested. *S. saprophyticus* was the species with the largest number of hyperproducers, with 9 (33.3%) isolates (Table 1).

Thirteen (7.18%) of the 181 isolates grew on BHI agar plates containing 4 or 6 µg/mL vancomycin. Seven isolates that grew at the lower concentration also exhibited growth when the vancomycin concentration was increased to 6 µg/mL (Table 1). The following species exhibited heteroresistance: *S. epidermidis* (n = 5), *S. haemolyticus* (n = 4), *S. saprophyticus* (n = 2), *S. warneri* (n = 1), and *S. aureus* (n = 1).

Thirty-three (18.2%) isolates were resistant to oxacillin by the phenotypic method, but only 8 (4.4%) of these isolates carried the *mec*A gene. The remaining isolates may have developed resistance through other mechanisms, such as hyperproduction of beta-lactamase (Geha et al., 1994). This result evidences the need to use phenotypic and genotypic methods for

the detection of resistance. Investigation of SCC*mec* types in the *mec*A-positive isolates revealed the presence of cassette type I in 5 isolates, while the 3 isolates carried type IV. Both cassette types (I and IV) carry the *mec*A gene as the single resistance determinant, conferring resistance only to this class of antimicrobials (Boyle-Vavra and Daum, 2007).

SCC*mec* typing is an important tool for the understanding of the epidemiology and clonal relationship of methicillin-resistant *Staphylococcus aureus* (MRSA), which can be classified into hospital-associated MRSA (HA-MRSA), community-acquired MRSA (CA-MRSA), and livestock-associated (LA-MRSA). Substantial differences exist between these types in terms of the distribution of toxins, risk factors and antimicrobial susceptibility (Vandenesch, 2003). SCC*mec* types IV, V and VII are frequently associated with CA-MRSA, which carry a smaller cassette that confers resistance only to beta-lactam antimicrobials. In contrast, HA-MRSA frequently harbor type I, II or III and are usually resistant to multiple drugs (Vandenesch, 2003) and LA-MRSA frequently harbor SCC*mec* type XI and are found in a variety of *Staphylococcus* spp. isolated from different animal species (Stefani et al., 2012).

The presence of the *mec*A gene in two isolates that were susceptible to oxacillin by the phenotypic method, both carrying SCC*mec* type IV, suggests the lack of phenotypic expression of oxacillin resistance or the presence of heterogenous resistance. The same was reported in a study conducted in China (Pu et al., 2014) in which 49 isolates carried *mec*A and 37 of them were susceptible to oxacillin by phenotypic methods. Taken together, these results suggest the need for genotypic methods to accurately identify oxacillin resistance.

A total of 33(18.2%) *Staphylococcus* spp. were resistant to oxacillin. Variation in the susceptibility profile of mastitis isolates has been observed between different regions and even between herds of the same region (Owens and Watts, 1988). The present study instead showed that, despite the widespread use of antimicrobial agents for mastitis treatment, most isolates were

susceptible to the antimicrobials tested. Additionally, the MIC of oxacillin and vancomycin are lower for *S. aureus* than for CoNS.

Regarding the screening method for detecting vancomycin-heteroresistant *Staphylococcus* spp., our results showed that 7.1% of the isolates were positive in media containing 4 and 6 µg/ml of the antibiotic. Of these positive isolates, 92.3% belonged to the group of CoNS. Hiramatsu (2001) suggested that, if growth is observed on BHI agar containing 4 µg/ml vancomycin, the strain can already be considered heteroresistant to vancomycin. According to Natoli (2009), the heteroresistance phenotype of these isolates may be associated with treatment failure and/or may be a precursor of glycopeptide resistance. A study carried out in Turkey analyzing 100 strains of staphylococci from cases of bovine mastitis, 10 strains were found to have reduced susceptibility to vancomycin with a MIC of 4-8 µg / ml. PCR was performed for the detection of the *van*A gene and the results showed that the strains did not carry this gene. Since the *van*A gene is not present, the mechanisms for heteroresistant strains can be explained with changes in the cell wall biosynthesis of the strains (Pehlivanoglu; Yardimci, 2012). The clinical significance of heteroresistance is not fully understood and a limiting factor in the understanding of this impact is the small number of studies involving bovine mastitis isolates.

Also regarding vancomycin heteroresistance, the association of these results with those of phenotypic resistance to oxacillin and presence of the *mec*A gene showed that only isolates carrying SCC*mec* type I exhibited heteroresistance. Although the mechanism of vancomycin resistance has not been studied in these heteroresistant subpopulations, with the confirmation of the presence or absence of the *van* genes, this resistance is probably due to thickening of the bacterial cell wall, preventing entry of the antibiotic into the cell (Billot-Klein, 1996).

In the present study, 174 (96.13%) of the isolates produced beta-lactamase. In addition, 26 (96.3%) of the 27 oxacillin-resistant isolates that did not carry the *mec*A or *mec*C gene were

confirmed to be hyperproducers of this enzyme. These findings indicate the presence of these beta-lactamase producers may favor the survival of other antibiotic-susceptible microorganisms in an infectious process (Tavares, 2001).

The present results demonstrate the need to evaluate the microbiological and molecular aspects of bovine mastitis isolates since the presence of the *mec*A gene in *S. epidermidis* confirms that cow milk can harbor resistant strains, even though these strains may have come from humans.

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Table 1. Phenotypic resistance to oxacillin and vancomycin, vancomycin heteroresistance, and hyperproduction of beta-lactamase in *Staphylococcus* spp. isolated from bovine subclinical mastitis

Species	Resistance to oxacillin (N)	Resistance to vancomycin (N)	Vancomycin heteroresistance (N)	Beta-lactamase hyperproduction (N)
S. aureus	1	0	1	2
S. chromogenes	5	0	0	4
S. epidermidis	9	0	5	3
S. haemolyticus	1	0	4	1
S. saprophyticus	10	0	2	9
S. simulans	3	0	0	3
S. xylosus	2	0	0	2
S. warneri	2	0	1	2
Total	**33**	**0**	**13**	**26**

Table 2. Interpretative breakpoints of MIC of Staphylococci strains isolated from bovine mastitis subclinical.

	Antimicrobial					
	Oxacillin			Vancomycin		
Species	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	Resistant (N)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	Resistant (N)
S. aureus	0.094	0.25	1	0.50	1.0	0
CoNS	0.25	1.5	32	1.0	1.5	0

Oxacillin breakpoints: *S. aureus*: susceptibility ≤ 2 µg/mL; resistance ≥ 4 µg/mL. CoNS: susceptibility ≤ 0.25 µg/mL; resistance ≥ 0.5 µg/mL. Vancomycin breakpoints: *Staphylococcus* spp.: susceptibility < 4 µg/mL; resistance ≥ 4 µg/mL (CLSI, 2015).

Table 3. Comparison of the presence of the *mec*A gene with the phenotypic method.

PCR	Phenotypic method: Oxacillin (1 µg/mL)			
	Susceptible		Resistant	
	N	(%)	N	(%)
*mec*A +	2	(1.1)	6	(3.3)
*mec*A -	146	(80.6)	27	(14.9)
Total	148	(81.7)	33	(18.2)

Artigo Científico II

Staphylococcus spp. isolated from bovine subclinical mastitis in different regions of Brazil: Molecular typing and biofilm gene expression analysis by RT-qPCR

Priscila Luiza Mello[1]; Danilo Flávio Moraes Riboli[1]; Lisiane de Almeida Martins[2]; Maria Aparecida Vasconcelos Paiva Brito[3]; Cassiano Victória[4]; Maria de Lourdes Ribeiro de Souza da Cunha[1*]

[1]Departamento de Microbiologia e Imunologia da Universidade Estadual Paulista – UNESP, Botucatu, SP 18618-970, Brasil; priscila_mello@msn.com

[2]Universidade Paranaense – UNIPAR, Umuarama 87502-210, Brasil; lisiane.almeida.martins@gmail.com

[3]Empresa Brasileira de Pesquisa Agropecuária – EMBRAPA, Juiz de Fora – MG, Brasil; maria.brito@embrapa.br

[4] Departamento de Higiene Veterinária e Saúde Pública da FMVZ - Botucatu - SP.

* Correspondence: cunhamlr@ibb.unesp.br; Tel.: +55-14-3880-0428

ABSTRACT

Bovine mastitis is mainly caused by bacteria of the genus *Staphylococcus* spp., which possess different virulence factors, including the capacity for biofilm formation. The biofilm protects the bacteria against the action of immune system components and serves as a barrier that impairs the penetration of antimicrobial agents. The objective of this study was to characterize 181 *Staphylococcus* spp. strains isolated from bovine subclinical mastitis by molecular methods and to verify by RT-qPCR the expression of genes of the *ica* operon, which are responsible for biofilm formation. The strains were provided by Embrapa Gado de Leite and had been isolated in six Brazilian states: Paraná, Santa Catarina, Rio Grande do Sul, Minas Gerais, São Paulo, and

Pernambuco. The *ica*A gene was detected in 79 (43.6%) isolates, *ica*B in 24 (13.2%), *ica*C in 57 (31.4%), and *ica*D in 127 (70.1%). The *bap* gene was identified in 66 (36.4%) isolates, while the *bhp* gene was found in 9 (4.9%). RT-qPCR confirmed expression of the *ica*A gene in 60 (75.9%) isolates, of *ica*B in 6 (25%), of *ica*C in 26 (45.6%), and of *ica*D em 80 (63%). Clonal typing of the isolates by PFGE permitted the identification of eight *S. aureus* clusters that simultaneously included ≥ 3 strains, with similarity ≥ 80%. Regarding the other species studied, three clusters were observed for *S. chromogenes* and four clusters for *S. epidermidis*. Only one cluster each was identified for *S. saprophyticus* and *S. simulans*, while the other species did not form any cluster. With respect to MLST, ST126 and ST1 were the prevalent sequence types in *S. aureus*, while in *S. epidermidis* all sequence types were different.

Keywords: CoNS, *Staphylococcus aureus*, cattle, biofilm, gene expression, pulsed-field gel electrophoresis, multilocus sequence typing

1. Introduction

Inadequate handling of livestock can trigger inflammatory processes in the mammary gland, known as mastitis, which is caused by different microorganisms that directly influence the physicochemical characteristics, composition and cellularity of milk (Gottardi et al., 2008). Epidemiological studies have demonstrated the presence of the genus *Staphylococcus* in approximately 50% of cases of bovine mastitis (Radostitis et al., 2007). These microorganisms possess different virulence factors, including the capacity for biofilm formation (Aguiar et al., 2001). The biofilm protects the bacteria against the action of immune system components by blocking phagocyte activity (Fox et al., 2005), and serves as a barrier that impairs the penetration of antimicrobial agents (Stewart, 1996).

The proliferation of cells to adhere and form a biofilm is mediated by the production of polysaccharide intercellular adhesin (PIA). This adhesin is encoded by the gene product of the *ica* locus of the *ica*ADBC operon, which is essential for biofilm formation and virulence of the

microorganisms (O'Toole et al., 2000). Another important gene that also regulates biofilm formation is biofilm-associated protein (*bap*), which encode the Bap surface protein. Unlike PIA which only seems to be involved in intercellular adhesion (Cucarella et al., 2004), this protein promotes primary binding to abiotic surfaces and intercellular adhesion. In addition to *bap*, the *bhp* gene is also related to biofilm formation irrespective of the presence of the *ica* operon. The presence and expression of biofilm genes can be analyzed by RT-qPCR, which permits to quantify the genes expressed.

A variety of molecular techniques based on chromosome similarity have been developed over the last decades, including pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) (Schwartz et al., 1983; Maiden et al., 1998). PFGE has become an important method and is considered the gold standard for the molecular typing and characterization of bacterial clusters, detecting genetic variations between phylogenetically and epidemiologically related isolates (Saida et al., 2006). MLST has been proposed by Maiden et al. (1998) for molecular characterization and epidemiological investigation and has been widely used for phylogenetic interference of bacterial species. The set of alleles at the different loci studied by MLST determines the allelic profile or sequence type (ST) of a bacterial strain. The STs found within a population permit the comparison of its strains, as well as phylogenetic inference of its members (Maiden et al., 1998).

Molecular typing is essential to determine the source of transmission and to study the evolution and molecular organization of these bacteria. Genotyping permits to distinguish between clonal or horizontal dissemination of resistance and is used to monitor the global distribution of resistant bacteria (Aarts et al., 2001). In this respect, the objective of the present study was to molecularly characterize *S. aureus* and coagulase-negative staphylococci (CoNS) isolated from bovine subclinical mastitis cases in herds from different Brazilian regions and states, and to analyze biofilm formation by qRT-PCR to increase our understanding of these

isolates. The findings will contribute to establishing measures to prevent and control the dissemination of pathogenic *Staphylococcus* spp. clusters in Brazil.

2. Materials and Methods

2.1 Isolation and origin of the strains

A total of 181 strains were studied; of these, 82 (45.3%) were identified as *S. aureu*s and 99 (54.7%) as CoNS, including 27 (14.9%) *S. chromogenes*, 26 (14.4%) *S. epidermidis*, 17 (9.4%) *S. saprophyticus*, 6 (3.3%) *S. warneri*, 6 (3.3%) *S. simulans*, 6 (3.3%) *S. haemolyticus*, 2 (1.1%) *S. xylosus*, 4 (2.2%) *S. hominis*, and 5 (2.8%) *S. hyicus*. These strains were isolated from bovine subclinical mastitis cases in six Brazilian states [Rio Grande do Sul (RS), Santa Catarina (SC), Paraná (PR), São Paulo (SP), Minas Gerais (MG), and Pernambuco (PE)] and maintained in the bacterial collection of Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA Gado de Leite). The internal transcribed spacer-PCR (ITS-PCR) technique described by Couto et al. (2001) was used to confirm the identification of the CoNS species.

2.2 Detection of the genes involved in biofilm formation

Genomic DNA was extracted using the Illustra® Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). PCR for amplification of the genes of the *ica*ADBC operon was performed according to Arciola et al. (2005). The parameters described by Cucarellas et al. (2004) were used for amplification of the *bap* gene and the method described by Qin et al. (2007) for amplification of the *bhp* gene. This screening was performed to identify which strains carried genes of the operon in their genomic DNA. Next, RT-qPCR using complementary DNA (cDNA) as template was used to determine whether these genes were expressed.

2.3 Expression analysis of the genes of the *ica*ADBC operon by RT-qPCR

The isolates carrying any gene of the *ica*ADBC operon were submitted to RT-qPCR for the analysis of expression and relative quantification of biofilm gene compared to the reference strain.

2.3.1 RNA extraction and cDNA synthesis

Total RNA was extracted using the Illustra RNAspin® Kit. Possible DNA residues were eliminated by treatment with RNase-free DNAse® (Promega). The concentration and purity of RNA in the samples were determined in a NanoDrop 2000® spectrophotometer (Thermo Fisher Scientific) and A260/A280 and A260/230 ratios, respectively, of approximately 2 were considered adequate for inclusion in the study. Complementary DNA was synthesized with the Superscript VILO® Kit (Life Technologies) using 1 μg RNA according to manufacturer instructions.

2.3.2 Standardization of the reaction and primers

RT-qPCR was performed in a StepOne Plus® thermocycler (Applied Biosystems). The reaction mixture contained 10 μL SYBR Green PCR Master Mix, 1.5 μL cDNA, 0.5 pMol/μL forward and reverse primers, and nuclease-free water in a total volume of 20 μL. The reactions were run in duplicate for each target and the level of gene expression was calculated based on the threshold cycle (CT), in which the gene encoding the 16S rRNA was used as normalizer (endogenous control) and the genes of the *ica* operon (*ica*ADBC) as calibrators. The reaction for standardization of the primers showed amplification of a single product for all genes tested in the absence of contamination or background noise. Melting curves were constructed for each gene studied and each curve had only one peak where the variation in temperature was not greater than 0.5ºC per sample in each group of genes analyzed. The primers described by Vandecasteele et al. (2003) were used for the *ica*A gene, those described by Klug et al. (2003) for *ica*B, and the primers of the study Tan et al. (2012) for *ica*C and *ica*D.

2.3.3 Relative standard curve

The data of the cDNA curves for each target were obtained in reactions performed in triplicate and serially diluted 1:10, resulting in 150, 15, 1.5, 0.15 and 0,015 ng per reaction. The following parameters were considered: variation in CT less than 0.5 between triplicates, R^2 >

0.9, efficiency of 90 to 105%, and a slope of 3.32. The threshold of each primer pair was established from the standard curves.

2.3.4 Analysis of gene expression in the isolates

The cDNA of all isolates carrying genes of the *ica* operon detected by conventional PCR in genomic DNA was submitted to RT-qPCR for detection of the 16S rRNA gene used as endogenous control. Once the positivity for the 16S rRNA gene was confirmed, expression of the genes was also analyzed. Relative quantification of biofilm gene expression by each isolate was compared to the expression of reference strains (ATCC 35985: biofilm producer and ATCC 1222: non-biofilm producer). The ΔΔCT value was calculated for relative quantification (Yuan et al., 2006).

2.4 Pulsed-field gel electrophoresis

PFGE of the *Staphylococcus* spp. isolates was performed according to the modified protocol of McDougal et al. (2003). For similarity analysis, the Dice correlation coefficient was calculated and a dendrogram was generated by the UPGMA method (unweighted pair group method using arithmetic averages) using the BioNumerics® software (version 7.0; Applied Maths, Belgium, 2015). A similarity coefficient of 80% was chosen for definition of the clusters.

2.5 Geographic distribution of *Staphylococcus* spp. clones

For classification of the PFGE clusters, the strains were analyzed regarding their location and distribution across the different states included in the study (PR, SC, SP, RS, MG, and PE). The geographic coordinates of each farm were used, with the milking room serving as a reference. These data were extracted from the questionnaires applied in previous epidemiological studies conducted on these farms. The image was generated with the Arcgis program. The farms were georeferenced and imported to the software, using the Geographic Coordinates System GCS Sirgas 2000 and Datum planimetric Sirgas 2000.

2.6 Multilocus sequence typing

MLST was performed as described by Enright et al. (2000). After amplification and sequencing of the seven housekeeping genes, the sequences were analyzed with the BioNumerics® software (version 7.0; Applied Maths, Belgium, 2015) and compared with the online database (http://www.mlst.net) to obtain the identification number of each allele. The combination of these alleles indicates the ST to which the isolates belong.

3. Results

3.1 Detection of genes involved in biofilm formation

The presence of biofilm formation-related genes was investigated in genomic DNA of all 181 isolates of the study. The *ica*A gene was detected in 79 (43.6%) isolates, *ica*B in 24 (13.2%), *ica*C in 57 (31.4%), and *ica*D in 127 (70.1%). The *bap* gene was detected in 66 (36.4%) isolates, while the *bhp* gene was found in 9 (4.9%).

3.2 Expression analysis of genes of the *ica*ADBC operon by RT-qPCR

Based on the results of genomic DNA analysis, the expression of these genes was analyzed by RT-qPCR using cDNA of the isolates as template. The *ica*A gene was expressed in 60 (75.9%) isolates, *ica*B was expressed in six (25%), *ica*C was expressed in 26 (45.6%), and *ica*D was expressed in 80 (63%). Figure 1 shows the comparison of detection in genomic DNA and cDNA expression by RT-qPCR. The distribution of the expressed genes according to *Staphylococcus* spp. species is shown in Table 1.

Among the 82 *S. aureus* isolates analyzed, the most expressed genes were *ica*A in 58 (70.7%) isolates and *ica*D in 60 (73.1%). Fifty-two isolates (63.4%) simultaneously expressed the two genes. The least expressed gene was *ica*B, which was only expressed in four isolates, while *ica*C was found in 18 isolates. The frequency of genes of the *ica*ADBC operon was lower in CoNS. Six *S. chromogenes* isolates expressed some of the biofilm genes and expression was also observed in seven *S. epidermidis* isolates.

3.3 Pulsed-field gel electrophoresis

Molecular typing by PFGE permitted the identification of eight clusters of *S. aureus* isolates that simultaneously included ≥ 3 strains, with similarity ≥ 80% (Figure 2). The largest cluster (CSA1) comprises 31 isolates from SP or MG. This cluster includes isolates from seven different farms, indicating dissemination of this cluster in the region studied. This heterogeneity in the origin of strains was also observed for the other *S. aureus* clusters.

With respect to the other species studied, the formation of three clusters was observed for *S. chromogenes* (Figure 3), with cluster 1 (CSC1) comprising the largest number of isolates (11) that originated from seven different farms. Four clusters could be identified for *S. epidermidis* (Figure 4), with isolates of the same cluster originating from different states, such as cluster 1 (CSE1) that includes isolates from SC and PR and cluster 2 (CSE2) that includes isolates from MG, SC and SP. On the other hand, only one cluster each was observed for *S. saprophyticus* and *S. simulans* (Figures 5 and 6). Table 2 shows the distribution of species according to electrophoretic pattern, their origin and biofilm gene expression. The remaining CoNS species did not form clusters according to the criteria adopted.

Spatial analysis permitted to verify the distribution of the clusters across the region studied (Figure 7).

3.4 Multilocus sequence typing

A group of 10 *S. aureus* and 5 *S. epidermidis* isolates were selected for MLST according to the criterion that at least one strain of each cluster described by PFGE would be included.

Six different STs were identified in *S. aureus*. The most prevalent STs were ST126 and ST1, which belonged to CC126 and CC1, respectively. Regarding the geographic distribution of the STs, ST126 (CC126) was detected in isolates from MG and SP, ST1 (CC1) only in isolates from PR, ST746 (CC97) in isolates from RS and SC, and ST8 and ST188 only in isolates from SC.

For *S. epidermidis*, the STs were all different even when the isolates were clustered by PFGE. In the case of one isolate, no exact combination was found for its set of alleles, probably because it is a new ST. The sequence of the combination of alleles was therefore submitted to the curator of the mlst.net site for the addition of the new ST.

Tables 3 and 4 show the origin of the isolates, PFGE cluster to which they belong, and combination of alleles for *S. aureus* and *S. epidermidis*, respectively.

4. Discussion

The genes of the *ica* operon were investigated in genomic DNA of all isolates studied. A high percentage of these genes was detected in *S. aureus*, in which the *ica*D gene was the most common, followed by *ica*A. Based on these results, gene expression analysis was performed, which confirmed that the *ica*D gene was the most expressed in the isolates of the present study. Fifty-two (63.4%) of the isolates simultaneously expressed the *ica*A and *ica*D genes. Vasudevan et al. (2003), investigating 35 *S. aureus* isolates from bovine mastitis cases, detected biofilm formation in 91.4% of the isolates and 100% were positive for the *ica*A and *ica*D genes. A high frequency of *ica*D-positive isolates has been reported in other studies investigating *S. aureus* from mastitis. In the study of Krewer et al. (2015), 92.8% of the isolates exhibiting adherence in microplates carried the *ica*D gene.

The *bhp* gene was only detected in *S. epidermidis* (4.9%), while the *bap* gene was found in 36.4% of all isolates studied, most of them *S. aureus*. Tormo et al. (2005) observed that *Staphylococcus* strains carrying the *bap* gene were strong biofilm producers even when they did not carry the *ica*ADBC genes. In cases of mastitis, this virulence factor facilitates the persistence of *Staphylococcus* spp. in the udder due to the capacity of these bacteria to adhere to the mammary epithelium and to the formation of multiple layers of cells surrounded by the biofilm matrix (Baselga et al., 1993).

Analysis of the clonal profile revealed the formation of eight clusters for *S. aureus*, with the largest cluster (CSA1) comprising isolates from SP and MG. This cluster includes isolates from seven different farms, indicating dissemination of this cluster in the region studied. Among CoNS, the *S. epidermidis* strains formed four clusters and the *S. chromogenes* strains formed three clusters. Epidemiological studies on *S. aureus* in cattle have shown a large number of molecular profiles to be involved in the etiology of mastitis worldwide, but some profiles tend to predominate in certain geographic regions (Zadoks, et al., 2000; Akineden et al., 2001).

In the study by Tondo et al. (2000) conducted in Nova Petrópolis, Rio Grande do Sul, PFGE analysis revealed 42 different patterns among 48 *S. aureus* strains isolated from food handlers, raw milk and milk products, demonstrating the diversity of this microorganism in the processing plant. These findings show the lack of an endemic strain among the personnel, although they have worked together for years in the same area. In contrast, we found a large number of isolates that formed clusters with a similarity of 80% or higher, even when the isolates were from different farms and different states.

According to Buzolla et al. (2001), strains with identical genotypes can possess characteristics that confer advantages for their survival in the environment, to colonize the udder and to cause disease, such as biofilm expression which was observed in the present study. It can also be indicative of clonal dissemination due to the possible lack of adequate hygiene conditions during milking. Mechanical milking lines are an important source of *Staphylococcus* transmission between dairy herds since these machines can be contaminated with microorganisms originating from the skin of the animal, milk or the milker's hands (Almeida et al., 2009).

Molecular typing by PFGE serves as a screening for MLST. The prevalent STs in *S. aureus* were ST126 (CC126; 30%) and ST1 (CC1; 30%). MLST has been used to characterize

and investigate the distribution of *S. aureus* clones in human infections (Vivoni et al., 2006) and in bovine mastitis (Kwon et al., 2005). However, MLST data for bovine isolates are still sparse.

ST1 (CC1) is widely found in humans, in different animal species and in cases of bovine mastitis (Rabello et al., 2007; Pilla et al., 2012). An important association between this clonal complex and infection with *S. aureus* in humans is reported worldwide, including in Brazil. This finding indicates the possible transmission of strains between humans and cattle, although it does not seem to be a frequent event (Zadoks et al., 2000).

CC126, together with CC97, have been reported in the literature to be associated with bovine mastitis in different herds and countries. These complexes are rarely isolated from human strains (Zadoks et al., 2011). Isolates belonging to CC126 were among those most frequently recovered in this study, a finding showing that different clonal complexes can predominate in different geographic areas. Although few studies have analyzed *S. aureus* isolates in Brazil, all clonal complexes found in the present study were also identified in *S. aureus* strains isolated from mastitis cases in other countries (Smith et al., 2005).

For *S. epidermidis*, each cluster exhibited a different ST, with the observation of ST59, ST81, ST48, and ST575, while no exact combination was found for the set of alleles of one isolate, probably because it is a new ST. The isolate typed as ST59 and that typed as ST81 are closely related. *S. epidermidis* ST59 was previously isolated from bovine mastitis in Germany from a nasal swab in the Republic of Ireland, and from an animal handler in India (MLST database), demonstrating that this strain can spread to cows and humans. ST81 was isolated from the environment in Poland and from a human wound in Denmark (MLST database).

Taken together, these data reveal the identification of strains with the same evolutionary origin as other isolates around the world, which are known to cause infections in humans and animals, suggesting their ability to spread between these species. The results of the present study highlight the need to evaluate the microbiological and molecular aspects of bovine mastitis

isolates so that effective infection control measures and efficient laboratory tests can be implemented.

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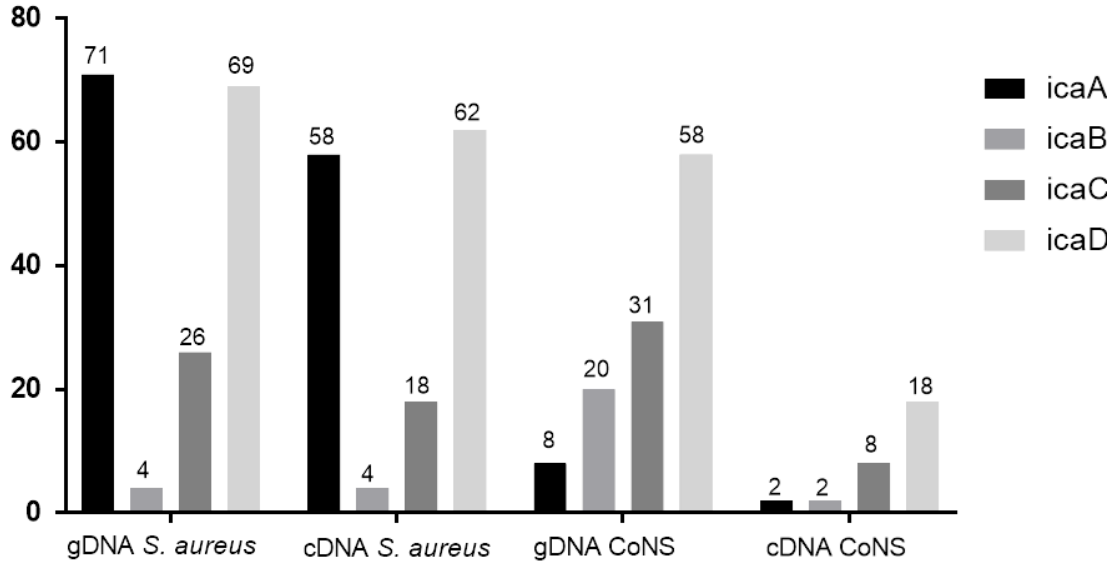

Figura 1. Comparação da detecção dos genes do operon *ica*ADBC por PCR convencional (gDNA) e ensaios de expressão por RT-qPCR (cDNA).

Tabela 1. Distribuição da detecção (gDNA) e expressão (cDNA) dos genes envolvidos na formação do Biofilme em relação às espécies de *Staphylococcus.*

Espécies (N)	**gDNA *ica*A**	**cDNA *ica*A**	**gDNA *ica*B**	**cDNA *ica*B**	**gDNA *ica*C**	**cDNA *ica*C**	**gDNA *ica*D**	**cDNA *ica*D**
S. aureus (82)	71	58	4	4	26	18	69	62
S. chromogenes (27)	0	0	4	1	6	1	12	4
S. epidermidis (26)	5	1	8	0	12	3	17	3
S. saprophyticus (17)	2	0	4	0	2	0	8	0
S. haemolyticus (6)	1	1	0	0	4	2	4	3
S. simulans (6)	0	0	1	1	3	0	5	0
S. warneri (6)	0	0	3	0	1	0	4	5
S. hyicus (5)	0	0	0	0	2	0	4	1
S. hominis (4)	0	0	0	0	0	0	2	0
S. xylosus (2)	0	0	0	0	1	0	2	2
Total (181)	**79**	**60**	**24**	**6**	**57**	**24**	**127**	**80**

Tabela 2. Características dos clones de *Staphylococcus spp.* isolados de mastite subclínica bovina em diferentes estados brasileiros, de acordo com o padrão eletroforético e quanto à expressão dos genes do operon *ica*ADBC.

Espécie	Cluster	Nº de amostras	Expressão dos genes *ica*ADBC	Origem
S. aureus	CSA1	31	*ica*A (23); *ica*B (0); *ica*C (4); *ica*D (23)	SP; MG
	CSA2	03	*ica*A (1); *ica*B (0); *ica*C (0); *ica*D (1)	SP; RS
	CSA3	07	*ica*A (5); *ica*B (2); *ica*C (5); *ica*D (7)	PR
	CSA4	05	*ica*A (3); *ica*B (0); *ica*C (2); *ica*D (4)	PR; SC; SP
	CSA5	06	*ica*A (5); *ica*B (0); *ica*C (1); *ica*D (5)	SC
	CSA6	05	*ica*A (4); *ica*B (0); *ica*C (2); *ica*D (5)	SC
	CSA7	03	*ica*A (2); *ica*B (0); *ica*C (2); *ica*D (2)	RS; SC; SP
	CSA8	04	*ica*A (3); *ica*B (1); *ica*C (0); *ica*D (3)	SC
S. chromogenes	CSC1	11	*ica*A (0); *ica*B (1); *ica*C (0); *ica*D (2)	SC; SP
	CSC2	04	*ica*A (0); *ica*B (0); *ica*C (0); *ica*D (0)	SC; SP
	CSC3	06	*ica*A (0); *ica*B (0); *ica*C (0); *ica*D (2)	PR; SC; SP
S. epidermidis	CSE1	04	*ica*A (0); *ica*B (0); *ica*C (0); *ica*D (1)	PR; SC
	CSE2	03	*ica*A (0); *ica*B (0); *ica*C (1); *ica*D (1)	MG; SC; SP
	CSE3	03	*ica*A (0); *ica*B (0); *ica*C (0); *ica*D (0)	SC
	CSE4	03	*ica*A (1); *ica*B (0); *ica*C (1); *ica*D (1)	PR
S. saprophyticus	CSS1	03	*ica*A (0); *ica*B (0); *ica*C (0); *ica*D (0)	SP
S. simulans	CSI1	03	*ica*A (0); *ica*B (1); *ica*C (0); *ica*D (0)	RS; SC

*CSA: Cluster *Staphylococcus aureus*; CSC: Cluster *Staphylococcus chromogenes*; CSE: Cluster *Staphylococcus epidermidis*; CSS: Cluster *Staphylococcus saprophyticus;* CSI: Cluster *Staphylococcus simulans*.

Tabela 3. Identificação do *sequence type* (ST) dos isolados de *Staphylococcus aureus.*

Origem	Cluster PFGE	Amostra	Alelos							
			arcC	***aroE***	***glpF***	***gmk***	***pta***	***tpi***	***yqiL***	**ST**
MG	CSA1	2	3	68	1	4	1	5	40	126
SP	CSA1	91	3	68	1	4	1	5	40	126
SP	CSA2	88	3	68	1	4	1	5	40	126
PR	CSA3	18	1	1	1	1	1	1	1	1
PR	CSA3	178	1	1	1	1	1	1	1	1
SC	CSA4	40	1	1	1	1	1	1	1	1
SC	CSA5	54	3	1	1	8	1	1	1	188
SC	CSA6	43	3	1	1	1	1	5	92	746
RS	CSA6	170	3	1	1	1	1	5	92	746
PR	CSA7	24	1	1	1	1	1	1	1	1
SC	CSA8	71	3	3	1	1	4	4	3	8

Tabela 4. Identificação do *sequence type* (ST) dos isolados de *S. epidermidis.*

Origem	Cluster PFGE	Amostra	Alelos							
			arcC	***aroE***	***gtr***	***mut***	***pyr***	***tpi***	***yqiL***	**ST**
PR	CSE1	16	1	1	1	6	2	1	7	575
SC	CSE1	75	2	17	1	1	2	1	1	81
MG	CSE2	1	2	1	1	1	2	1	1	59
SC	CSE3	81	7	1	2	6	2	1	1	Novo
PR	CSE4	11	7	1	2	2	4	1	4	48

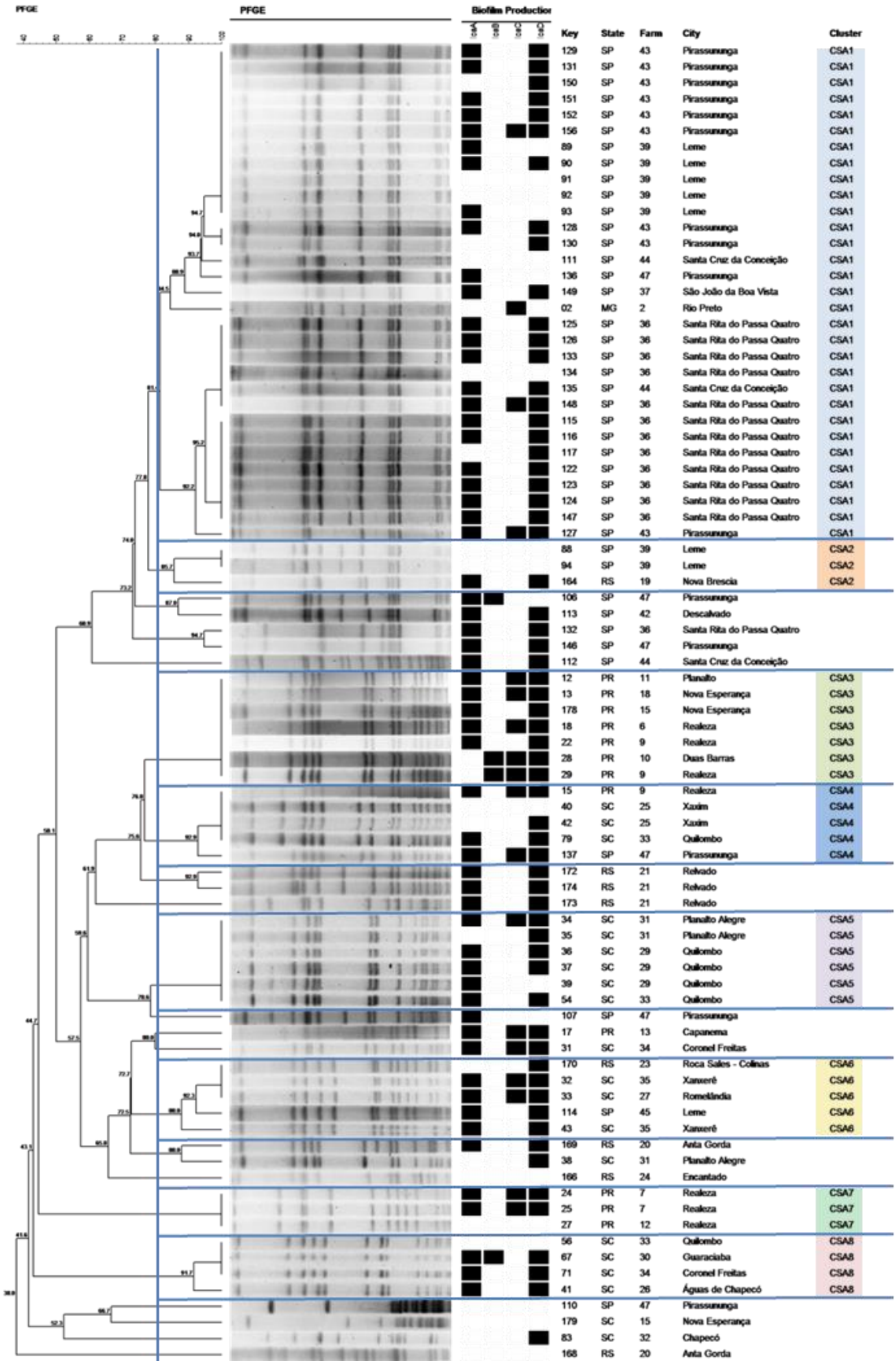

Figura 2. Dendrograma criado a partir do padrão eletroforético por PFGE de amostras de *S. aureus* (coeficiente de similaridade de DICE; método de clusterização: UPGMA).

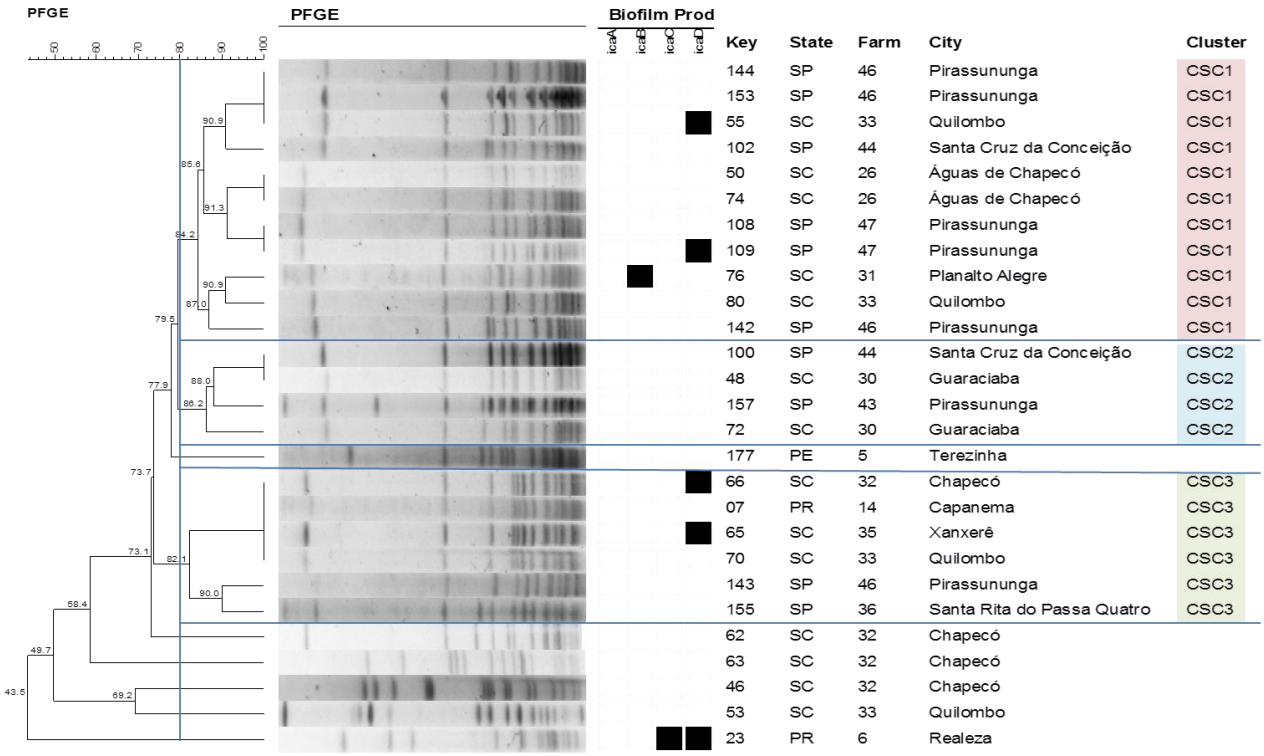

Figura 3. Dendrograma criado a partir do padrão eletroforético por PFGE de amostras de *S. chromogenes* (coeficiente de similaridade de DICE; método de clusterização: UPGMA).

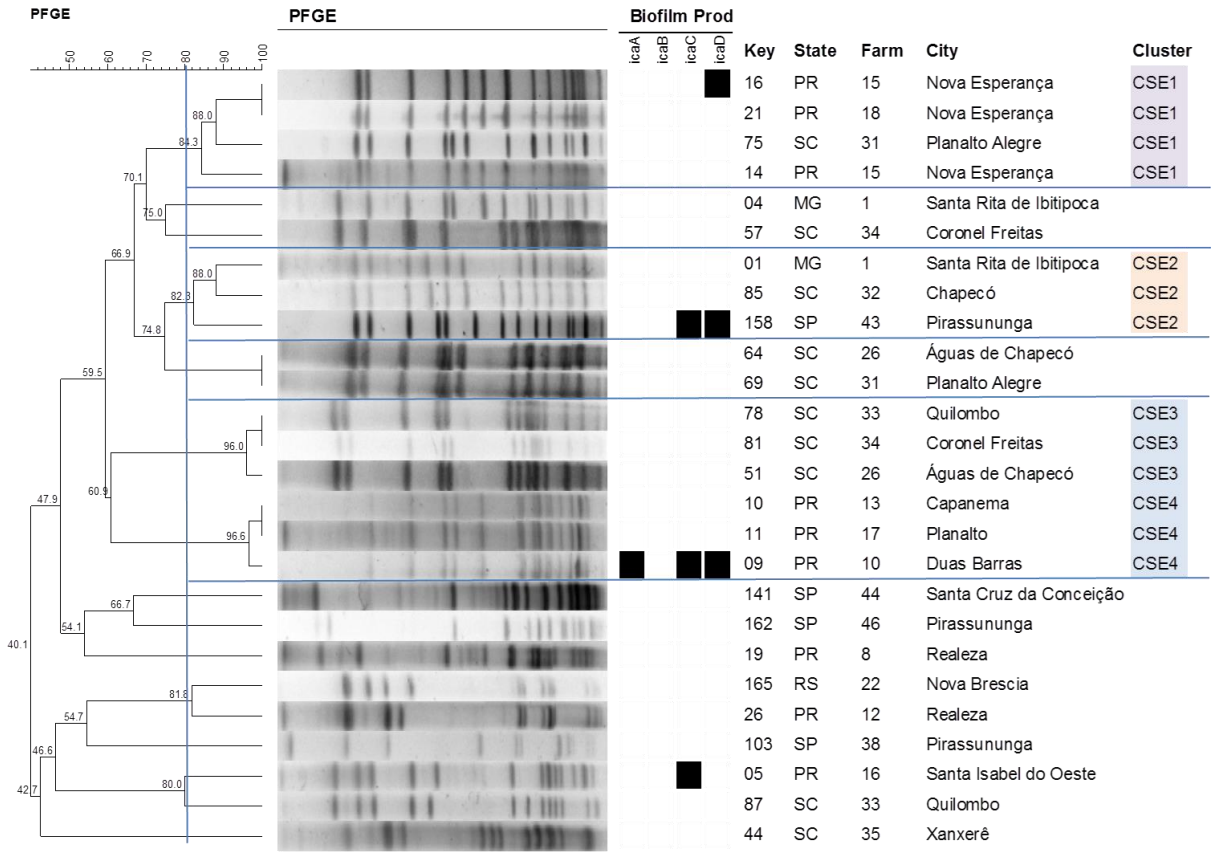

Figura 4. Dendrograma criado a partir do padrão eletroforético por PFGE de amostras de *S. epidermidis* (coeficiente de similaridade de DICE; método de clusterização: UPGMA).

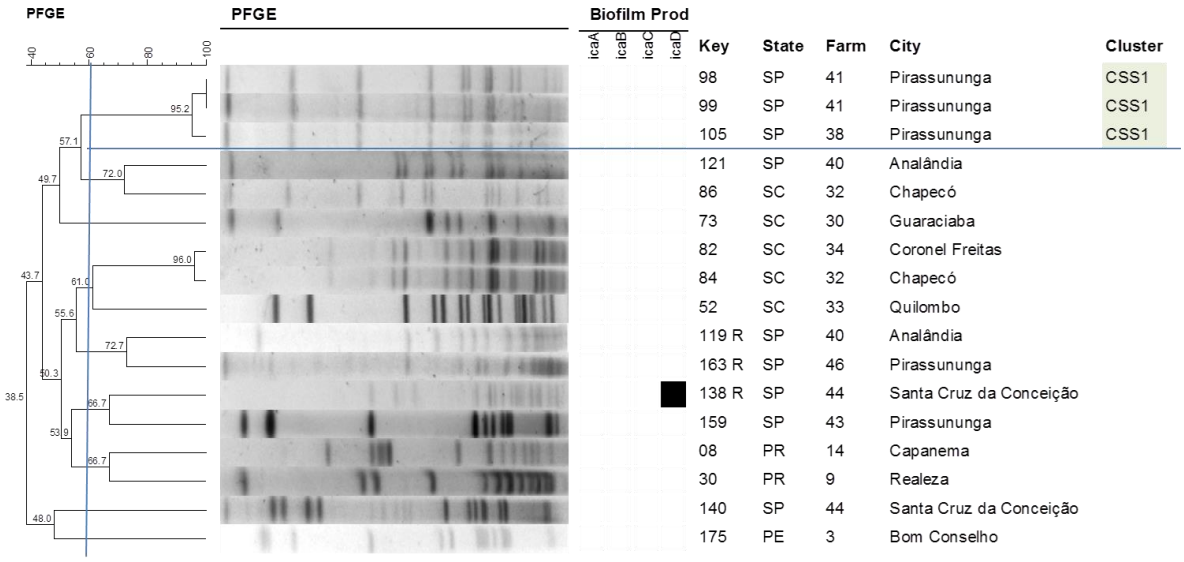

Figura 5. Dendrograma criado a partir do padrão eletroforético por PFGE de amostras de *S. saprophyticus* (coeficiente de similaridade de DICE; método de clusterização: UPGMA).

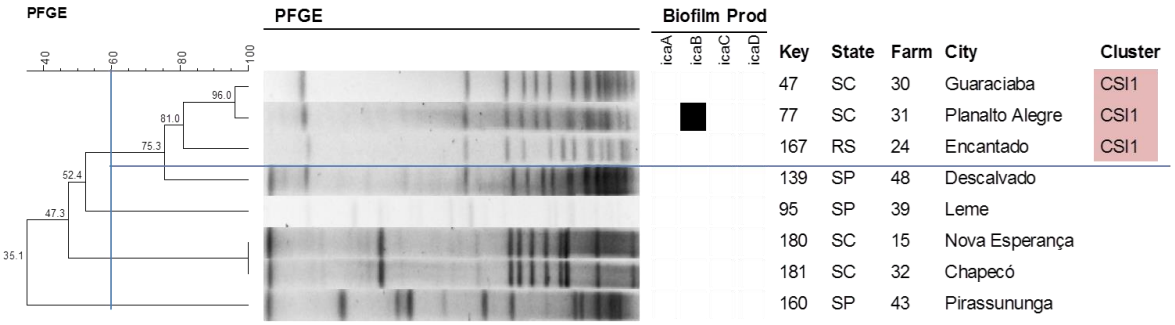

Figura 6. Dendrograma criado a partir do padrão eletroforético por PFGE de amostras de *S. simulans* (coeficiente de similaridade de DICE; método de clusterização: UPGMA).

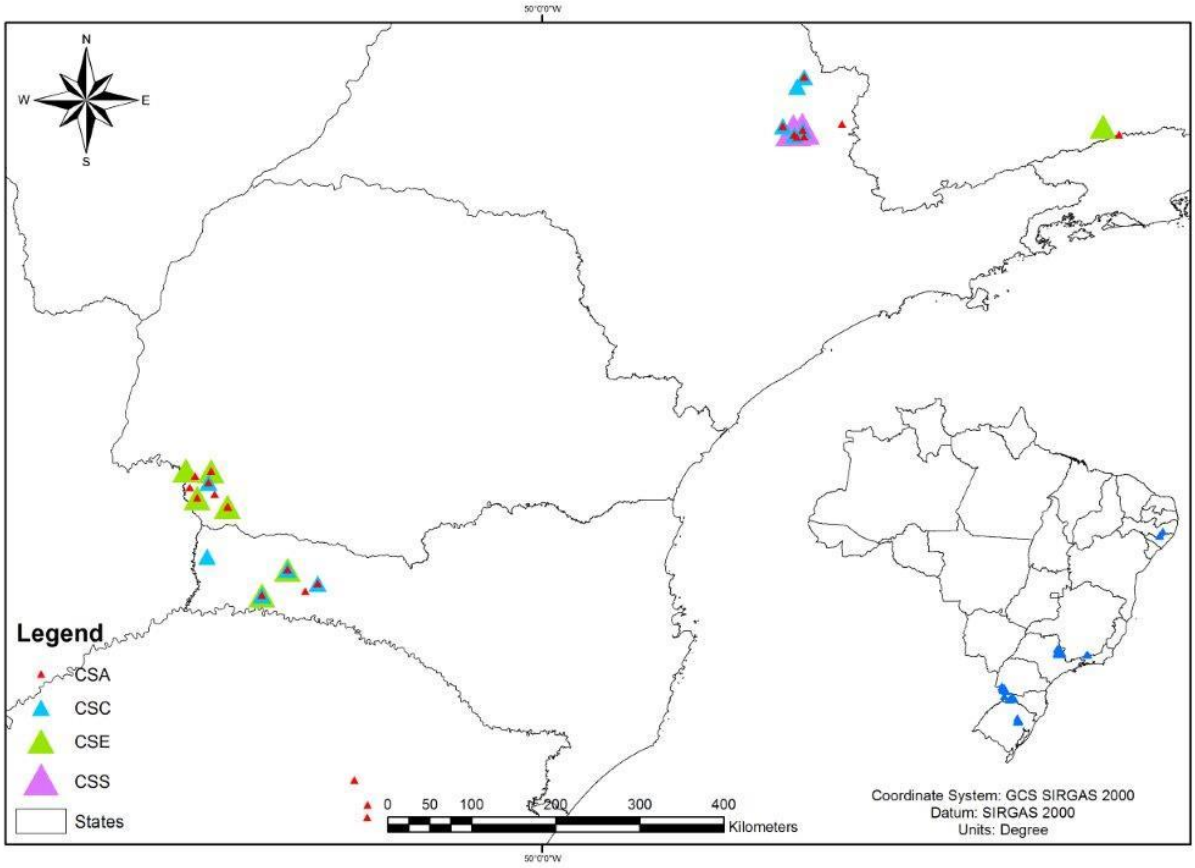

Figura 7. Análise espacial da distribuição dos clusters das diferentes espécies de *Staphylococcus* analisadas pelo PFGE. *CSA: Cluster *S. aureus*; CSC: Cluster *S. chromogenes*; CSE: Cluster *S. epidermidis*; CSS: Cluster *S. saprophyticus*

toxins

MDPI

Artigo Científico III

Detection of Enterotoxigenic Potential and Determination of Clonal Profile in *Staphylococcus aureus* and Coagulase-Negative Staphylococci Isolated from Bovine Subclinical Mastitis in Different Brazilian States

Priscila Luiza Mello [1], Danilo Flávio Moraes Riboli [1], Luiza Pinheiro [1,2], Lisiane de Almeida Martins [3], Maria Aparecida Vasconcelos Paiva Brito [4] and Maria de Lourdes Ribeiro de Souza da Cunha [1,*]

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[1] Department of Microbiology and Immunology, Institute of Biosciences of Botucatu, UNESP-Univ Estadual Paulista, Botucatu 18618-970, Brazil; priscila_mello@ibb.unesp.br (P.L.M.); danilo.riboli@ibb.unesp.br (D.F.M.R.); luizapinheiro@ibb.unesp.br (L.P.)
[2] Department of Anatomic Pathology, Instituto Lauro de Souza Lima, Bauru 17034-971, Brazil.
[3] Universidade Paranaense-UNIPAR, Umuarama 87502-210, Brazil; lisiane.almeida.martins@gmail.com
[4] Embrapa Dairy Cattle, Juiz de Fora 36038-330, Brazil; maria.brito@embrapa.br
* Correspondence: cunhamlr@ibb.unesp.br; Tel.: +55-14-3880-0428

Abstract: Epidemiological studies have identified *Staphylococcus aureus* as the most common agent involved in food poisoning. However, current research highlights the importance of toxigenic coagulase-negative staphylococci (CoNS) isolated from food. The aim of this study was to characterize *Staphylococcus* spp. isolated from cows with bovine subclinical mastitis regarding the presence of genes responsible for the production of staphylococcal enterotoxins and of the *tst-1* gene encoding toxic shock syndrome toxin 1, and to determine the clonal profile of the isolates carrying any of the genes studied. A total of 181 strains isolated in different Brazilian states, including the South, Southeast, and Northeast regions, were analyzed. The *sea* gene was the most frequent, which was detected in 18.2% of the isolates, followed by *seb* in 7.7%, *sec* in 14.9%, *sed* in 0.5%, *see* in 8.2%, *seg* in 1.6%, *seh* in 25.4%, *sei* in 6.6%, and *ser* in 1.6%. The *sej*, *ses*, *set*, and *tst-1* genes were not detected in any of the isolates. The typing of the isolates by pulsed-field gel electrophoresis revealed important *S. aureus* and *S. epidermidis* clusters in different areas and the presence of enterotoxin genes in lineages isolated from animals that belong to herds located geographically close to each other.

Keywords: staphylococcal enterotoxin; bovine mammary gland; Brazilian States; mastitis; pulsed-field gel electrophoresis; clonal profile

1. Introduction

Several microorganisms are involved in mammary gland infections in production animals. National and international epidemiological studies have demonstrated the presence of the genus *Staphylococcus* in approximately 50% of bovine mastitis cases, highlighting the role of this group as the main causative agent of this infection [1]. Although *Staphylococcus aureus* is the most common agent involved in subclinical mastitis, coagulase-negative staphylococci (CoNS) have gained importance as causative agents of intramammary infections. In a study conducted in Brazil, Rall *et al.* [2] found a prevalence of these microorganisms of 27.4%, while 28.6% of the isolates were *S. aureus* in a study conducted in Turkey [3].

Studies [4,5] have emphasized the importance of toxigenic CoNS isolated from food. Therefore, although *S. aureus* is the most common agent involved in food poisoning, there is current concern in the scientific community regarding CoNS, which have been recognized as opportunistic pathogens in human and animal infections, allied to risks of toxigenic lineages in cases of food poisoning in humans [5].

The staphylococcal enterotoxins (SE) type A (*sea*), B (*seb*), C (*sec*), D (*sed*), and E (*see*) are the classical staphylococcal toxins. These toxins have emetic activity and are usually associated with outbreaks of food poisoning [6]. In addition, recently described enterotoxins (*ses* and *set*), enterotoxin-like (SE-like) toxins that do exert emetic activity, and toxic shock syndrome toxin 1 (*tst-1*) are known [6,7]. The staphylococcal enterotoxin R (*ser*) was first described as an enterotoxin-like toxin by Omoe *et al*. [6], but experimental studies later proved its emetic activity [8].

The objective of this study was to characterize the enterotoxigenic potential of *Staphylococcus* spp. isolated from cattle with subclinical mastitis in six Brazilian states: Paraná (PR), Santa Catarina (SC), Rio Grande do Sul (RS), São Paulo (SP), Minas Gerais (MG), and Pernambuco (PE). Additionally, we determined the clonal profile and geographic distribution of the *S. aureus* and *S. epidermidis* strains that carried any of these genes.

2. Results

2.1. Identification of the Strains

Among the 181 strains studied, 82 (45.3%) were identified as *S. aureus* and 99 (54.7%) as CoNS, including 27 (14.9%) *S. chromogenes*, 26 (14.4%) *S. epidermidis*, 17 (9.4%) *S. saprophyticus*, 6 (3.3%) *S. warneri*, 6 (3.3%) *S. simulans*, 6 (3.3%) *S. haemolyticus*, 5 (2.8%) *S. hyicus*, 4 (2.2%) *S. hominis*, and 2 (1.1%) *S. xylosus.*

2.2. Detection of Enterotoxin and tst-1 Genes

Seventy-six (41.9%) of the 181 strains were positive for at least one of the genes studied. The *sea* gene was detected in 33 (18.2%) of the isolates, *seb* in 14 (7.7%), *sec* in 27 (14.9%), *sed* in 1 (0.5%), *see* in 15 (8.2%), *seg* in 3 (1.6%), *seh* in 46 (25.4%), *sei* in 12 (6.6%), and *ser* in 3 (1.6%). The *sej*, *ses*, *set*, and *tst*-1 genes were not detected in any of the strains studied (Table 1). Figure 1 shows the comparison of toxin gene detection in the *Staphylococcus aureus* and CoNS isolates.

Table 1. Number of strains evaluated (*n*) and detection of enterotoxin genes in *Staphylococcus* species isolated from cows with bovine subclinical mastitis in different Brazilian states.

Species	(*n*)	*sea*	*seb*	*sec*	*sed*	*see*	*seg*	*seh*	*sei*	*sej*	*ser*	*ses*	*set*	*tst-1*
S. aureus	(82)	15	9	6	0	4	1	21	9	0	3	0	0	0
S. chromogenes	(27)	5	0	5	0	1	0	10	1	0	0	0	0	0
S. epidermidis	(26)	3	2	8	1	4	1	6	0	0	0	0	0	0
S. saprophyticus	(17)	5	1	1	0	2	0	4	0	0	0	0	0	0
S. haemolyticus	(6)	1	1	2	0	1	0	0	0	0	0	0	0	0
S. simulans	(6)	1	0	1	0	1	0	1	0	0	0	0	0	0
S. warneri	(6)	0	0	2	0	1	0	2	1	0	0	0	0	0
S. hyicus	(5)	2	1	1	0	1	1	1	1	0	0	0	0	0
S. hominis	(4)	0	0	0	0	0	0	1	0	0	0	0	0	0
S. xylosus	(2)	1	0	1	0	0	0	0	0	0	0	0	0	0
Total	181	33	14	27	1	15	3	46	12	0	3	0	0	0

sea: staphylococcal enterotoxin A gene; *seb*: staphylococcal enterotoxin B gene; *sec*: staphylococcal enterotoxin C gene; *sed*: staphylococcal enterotoxin D gene; *see*: staphylococcal enterotoxin E gene; *seg*: staphylococcal enterotoxin G gene; *seh*: staphylococcal enterotoxin H gene; *sei*: staphylococcal enterotoxin I gene; *sej*: staphylococcal enterotoxin toxin J gene; *ser*: staphylococcal enterotoxin R gene; *ses*: staphylococcal enterotoxin S gene; *ser*: staphylococcal enterotoxin R gene; *set*: staphylococcal enterotoxin T gene; *tst*: toxic shock syndrome toxin 1 gene.

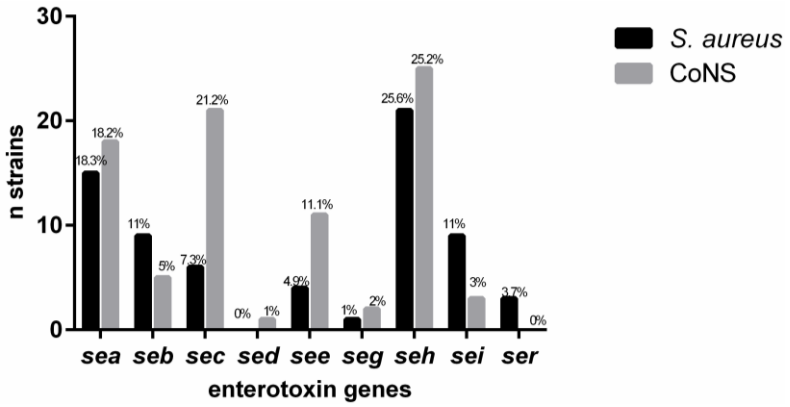

Figure 1. Detection of staphylococcal enterotoxins in *Staphylococcus aureus* and Coagulase-negative Staphylococci (CoNS) isolated from cows with bovine subclinical mastitis.

2.3. Distribution of Staphylococcal Enterotoxin Genes According to the Region Studied

Figure 2 shows the geographic distribution of enterotoxin gene-positive *Staphylococcus* spp. isolates. The *sea* and *seb* genes were found to coexist with the other genes studied and were detected in *Staphylococcus* spp. isolates from all Brazilian states, except for the state of Minas Gerais where only isolates carrying the *sec* gene were detected.

The *sei* and *ser* genes were only detected in *Staphylococcus* spp. from the southern states (PR, SC, and RS).

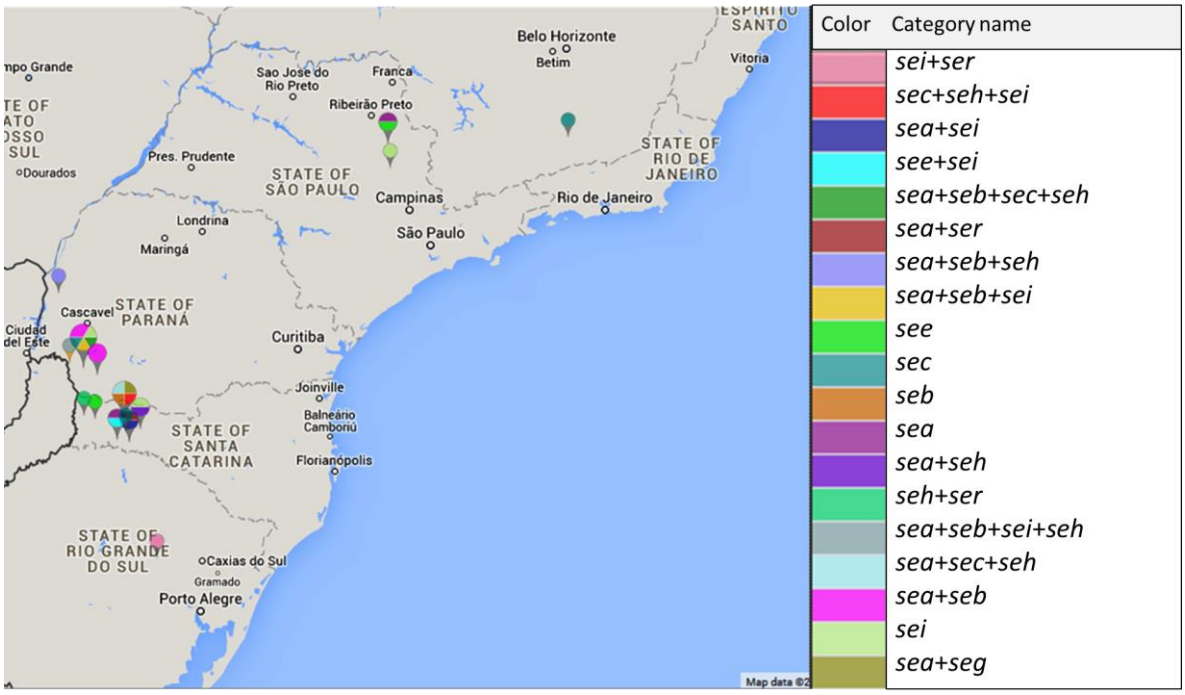

Figure 2. Geographic distribution of enterotoxin gene-positive *Staphylococcus* spp. strains isolated from cows with bovine subclinical mastitis in different Brazilian states.

2.4. Determination of Clonal Profile

Molecular typing by Pulsed-field gel electrophoresis (PFGE) was performed only on the *S. aureus* and *S. epidermidis* isolates that in which any enterotoxin genes had been detected. Therefore, among the 181 strains included in the study, 27 *S. aureus* isolates and 15 *S. epidermidis* isolates were analyzed, corresponding to the species with the highest prevalence of strains carrying enterotoxin genes.

Analysis permitted the identification of three *S. aureus* clusters that simultaneously included ≥3 isolates with similarity ≥80% (Figure 3, Table 2). Similarity was 100% in all groups. Cluster A, consisting of seven strains, contained a larger number of isolates with enterotoxigenic potential. This cluster comprised six isolates from Paraná and one isolate from São Paulo. The other clusters (B and C) also contained isolates from the South region, but from the state of Santa Catarina (Table 2). Cluster B comprised isolates originating from different states (SC and RS).

When the profile of *S. epidermidis* was analyzed, two clusters (A and B) were found. Each cluster contained three isolates from the South region (PR and SC, respectively) (Figure 4).

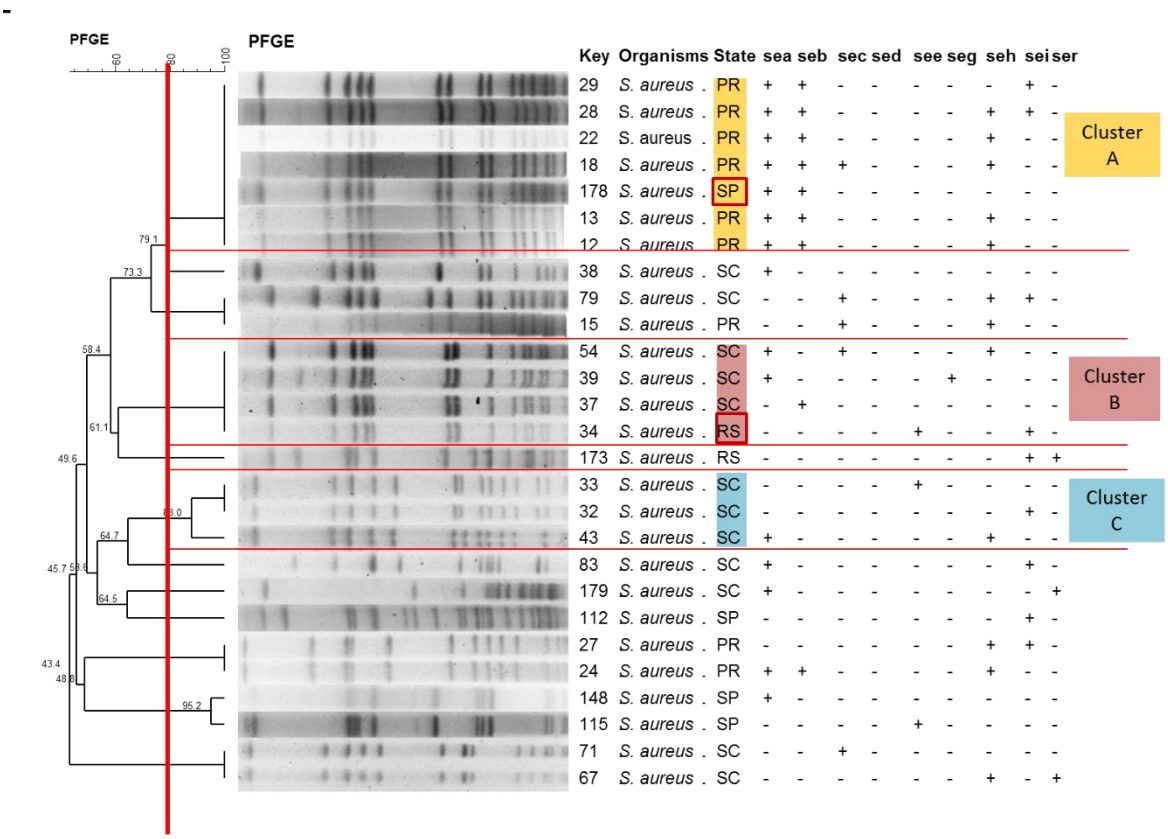

Figure 3. Determination of the clonal profile of *Staphylococcus aureus* strains carrying staphylococcal enterotoxin genes. The highlighted strain is interesting because of its similarity to isolates with origin in different states of Brazil (cluster A). The red lines highligth the division of the clusters and the red boxes highlight samples from different states within the cluster. Dendrogram generated by Dice/Unweighted pair group method using arithmetic averages (UPGMA) analysis (BioNumerics, Applied Maths). * PR: Paraná; SP: São Paulo; SC: Santa Catarina; RS: Rio Grande do Sul.

Table 2. Detection of enterotoxin genes in *Staphylococcus aureus* and *Staphylococcus epidermidis* clusters according to the region studied (PR: Paraná; SP: São Paulo; SC: Santa Catarina; RS: Rio Grande do Sul).

Species	Cluster	No. of Isolates	Enterotoxin Genes	Origin of Isolates
S. aureus	A	7	*sea* (7); *seb* (7); *sec* (1); *seh* (5); *sei* (2);	PR; SP
	B	4	*sea* (2); *seb* (1); *sec* (1); *see* (1); *seg* (1); *seh* (1); *sei*: (1)	SC; RS
	C	3	*sea* (1); *see* (1); *seh* (1); *sei (1);*	SC
S. epidermidis	A	3	*sea* (2); *seb* (1); *sec* (2); *see* (1); *seh* (2)	SC
	B	3	*seb* (1); *sec* (1); *see* (2);	PR

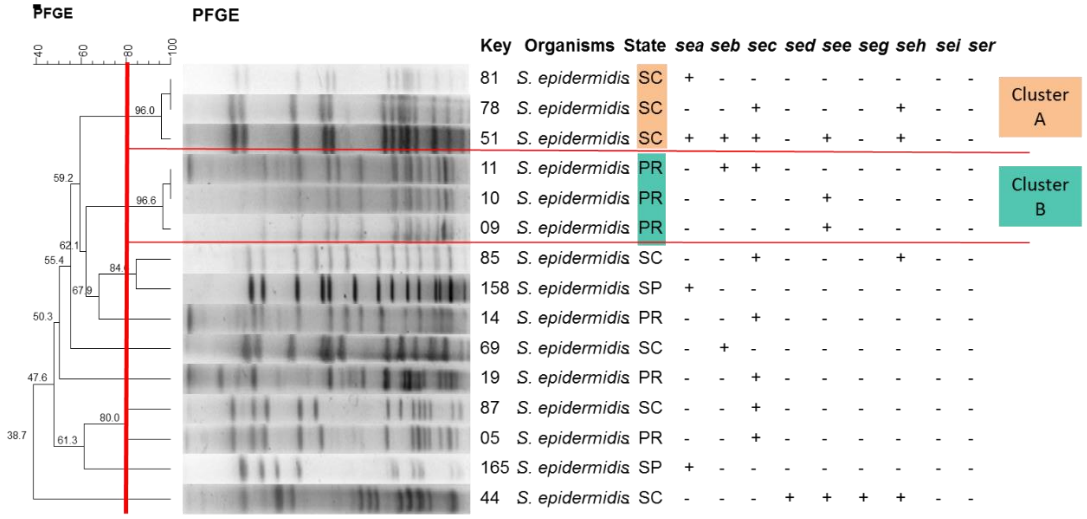

Figure 4. Determination of the clonal profile of *Staphylococcus epidermidis* strains carrying staphylococcal enterotoxin genes. Dendrogram generated by Dice/UPGMA analysis (BioNumerics, Applied Maths). The red lines highligth the division of the clusters.

3. Discussion

The *sea* gene, which exhibited the highest prevalence in this study (18.2%) and was detected in *S. aureus* and CoNS strains, is carried by a prophage [9] and can be easily disseminated among *Staphylococcus* spp. strains. Its product, enterotoxin A, is frequently associated with food poisoning since it is toxic at low concentrations [10,11]. Enterotoxin A is produced at the beginning of the exponential phase and its expression is not regulated by the accessory gene regulator (*agr*), different from enterotoxins B, C, and D, which depend on the *agr* system for maximum expression [11,12]. The *sec* gene is located on pathogenicity islands and can be divided into three subtypes (*sec*1, *sec*2, and *sec*3) based on antigenic differences and on the animal host with which it is associated. Some studies suggest that the heterogeneity of enterotoxin C is related to selection for modified *sec* sequences that facilitate the survival of *S. aureus* in their respective hosts [11,13]. In the present study, *sec* was the second most common classical enterotoxin after *sea*.

The *sed* gene was detected in only 0.5% of the strains studied, in an *S. epidermidis* isolate. This gene was not detected by Calsolari *et al*. [3] in toxigenic CoNS isolates, with only one *S. aureus* strain being positive. The *sed* gene is located on plasmid pIB485 [14] and enterotoxin D is the second most common toxin associated with food poisoning [11]. A small amount of this enterotoxin is able to cause illness, mainly in children and the elderly [15,16]. Nonetheless, the near absence of *sed* in the strains studied here suggests that it is scarcely related with *Staphylococcus* spp. isolates from mastitis cases in these regions of Brazil and consequently with possible events of food poisoning.

The data of a study on isolates from dairy products responsible for food poisoning in the state of Minas Gerais [16] are consistent with studies demonstrating that *sea* and *seb* are the most prevalent among the toxins identified [5]. The same study [16] showed the production of staphylococcal enterotoxins by CoNS using immunological assays.

The *see* gene was also detected in a small percentage (8.2%) and was not found in the study of Srinivasan *et al*. [17] who investigated *S. aureus* strains isolated from milk of cows with mastitis in the region of Tennessee, USA. None of the 78 isolates was positive for that gene. The *see* gene is carried by a prophage and studies have shown that the *see*, *sed*, and *sea* genes are closely related, with 81% sequence homology between *see* and *sea* [11,18].

Although the *sei* gene was only detected in a small percentage (6.6%), it was present in CoNS strains, which is an important fact rarely described in the literature. In contrast, in the study of Srinivasan *et al*. [17], 47 (60.3%) of the 78 *S. aureus* isolates studied carried the gene.

The *ser* gene was detected in three *S. aureus* isolates, all from the South regions. This gene was found to coexist with some other enterotoxins since two strains from SC carried the combination *ser*+*sea* or *ser*+*sec*, while

in the third isolate from RS, *ser* was associated with the enterotoxin I gene (*sei*). These data highlight the importance of knowledge on movable genetic elements that can transfer these genes among *Staphylococcus* spp. The same has been described by Lawrynowicz-Paciorek *et al*. [19] who also evaluated the frequency of coexisting genes.

Regarding the clonal profile, several epidemiological studies on *S. aureus* in cattle have demonstrated the involvement of a large number of molecular profiles in the etiology of mastitis in the world. However, certain profiles tend to predominate in different geographic regions [19,20]. In the present study, a large number of *S. aureus* strains grouped with ≥80% similarity were observed, even when the region studied included two different states, such as clusters A and B. In both cases, the source of one isolate differed from that of the others. This similarity among isolates obtained from animals that belong to herds from different states can be explained by the trade of animals between farms.

According to Buzolla *et al*. [21], strains with identical genotypes may possess characteristics that convey advantages for their survival in the environment, to colonize the udder, and/or to cause diseases. This fact was proven in the present study of enterotoxin genes, which demonstrated the presence of clusters with the same profile in different locations. Mechanical milking machines are important sources of transmission of staphylococci in dairy herds since they can be contaminated with microorganisms derived from the animal's skin and milk, or even from the hands of the operators [22].

Taken together, the results of clonal profile analysis showed the existence of important genetic similarity among the isolates, particularly among *S. aureus* isolated from intramammary infections of herds from different Brazilian states. Furthermore, PFGE was found to be an excellent technique to detect these clones, permitting the establishment of emergency control measures to prevent subsequent dissemination of virulent strains.

4. Experimental Section

4.1. Origin and Identification of the Strains

The 181 *Staphylococcus* spp. strains isolated from cows with bovine subclinical mastitis were provided by Embrapa Dairy Cattle. The strains were isolated in six Brazilian states: Paraná (PR), Santa Catarina (SC), and Rio Grande do Sul (RS); São Paulo (SP) and Minas Gerais (MG); and Pernambuco (PE), corresponding to the South, Southeast, and Northeast regions of Brazil, respectively.

The phenotypic and genotypic identification of these strains was performed for confirmation of the genus and correct identification of the species. The genus *Staphylococcus* was identified as described by Koneman *et al*. [23] using coagulase and sugar (trehalose, maltose, and mannitol) fermentation tests. Strains belonging to the CoNS group were submitted to biochemical tests as proposed by Cunha *et al*. [24] for phenotypic identification of the species. Total DNA was extracted using the illustra® kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Genotypic identification of CoNS was performed using primers targeting conserved sequences adjacent to the 16S and 23S genes by the internal transcribed spacer-polymerase chain reaction (ITS-PCR) described by Barry *et al*. [25] and Couto *et al*. [26], using primers G1 and L1. PCR using the Staur-4 and Staur-6 primers developed by Straub *et al*. [27] was used for *S. aureus*. The *S. aureus* ATCC 33591 reference strain was used as the positive control. The amplification efficiency was monitored by 2% agarose gel electrophoresis stained with Saber Safe DNA Gel Strain® (São Paulo, SP, Brazil) viewed under a UV transilluminator.

The reference strains used for ITS were: *S. chromogenes* (ATCC 43764), *S. epidermidis* (ATCC 12228), *S. saprophyticus* (ATCC 15305), *S. xylosus* (ATCC 29979), *S. hyicus* (ATCC 11249), *S. hominis* (ATCC 27844), *S. warneri* (ATCC 10209), *S. simulans* (ATCC 27851), and *S. haemolyticus* (ATCC 29970).

4.2. Detection of Enterotoxin and tst-1 Genes

PCR for the detection of the enterotoxin and *tst-1* genes was performed according to the parameters described by Johnson *et al*. [28] and Cunha *et al*. [5].

The following toxigenic *S. aureus* reference strains were used as positive control: ATCC 13565 (*sea*; *ser*), ATCC 14458 (*seb*), ATCC 19095 (*sec*), ATCC 23235 (*sed*), ATCC 27664 (*see*), *S. aureus* Food Research

Institute-FRI 361 (*sei*), and ATCC 51650 (*tst*). *Staphylococcus xylosus* ATCC 29971 was used as the negative control. The primer sequences are shown in Table 3.

Table 3. Sequence of the primers used and amplicon size.

Name	Product	5′ to 3′ Nucleotide Sequence	Reference	Amplicon Size (bp)
sea1 *sea2*	Enterotoxin A	TTGGAAACGGTTAAAACGAA GAACCTTCCCATCAAAAACA	[28]	120
seb1 *seb2*	Enterotoxin B	TCGCATCAAACTGACAAACG GCAGGTACTCTATAAGTGCC	[28]	478
sec1 *sec2*	Enterotoxin C	GACATAAAAGCTAGGAATTT AAATCGGATTAACATTATCC	[28]	257
sed1 *sed2*	Enterotoxin D	CTAGTTTGGTAATATCTCCT TAATGCTATATCTTATAGGG	[28]	317
see1 *see2*	Enterotoxin E	CAAAGAAATGCTTTAAGCAATCTTAGGCCAC CTTACCGCCAAAGCTG	[29]	170
seg1 *seg2*	Enterotoxin G	AATTATGTGAATGCTCAACCCGATC AAACTTATATGGAACAAAAGGTACTAGTTC	[29]	642
seh1 *seh2*	Enterotoxin H	CAATCACATCATATGCGAAAGCAG CATCTACCCAAACATTAGCACC	[30]	375
sei1 *sei2*	Enterotoxin I	CTCAAGGTGATATTGGTGTAGG AAAAAACTTACAGGCAGTCCATCTC	[29]	576
selj1 *selj2*	Enterotoxin J	CATCAGAACTGTTGTTCCGCTAG CTGAATTTTACCATCAAAGGTAC	[31]	146
ser1 *ser2*	Enterotoxin R	AGATGTGTTTGGAATACCCTAT CTATCAGCTGTGGAGTGCAT	[32]	123
ses1 *ses2*	Enterotoxin S	TTCAGAAATAGCCAATCATTTCAA CCTTTTTGTTGAGAGCCGTC	[8]	195
set1 *set2*	Enterotoxin T	GGTGATTATGTAGATGCTTGGG TCGGGTGTTACTTCTGTTTGC	[8]	170
tst1 *tst2*	Toxic shock syndrome toxin	ATGGCAGCATCAGCTTGATA TTTCCAATAACCACCCGTTT	[28]	350

4.3. Analysis by Pulsed-Field Gel Electrophoresis

The *Staphylococcus* spp. isolates that were positive for the enterotoxins by PCR were submitted to clonal profile analysis by pulsed-field gel electrophoresis (PFGE) according to the modified protocol of McDougal *et al*. [33].

The BioNumerics software (version 7.0; Applied Maths, Belgium, 2015) was used for similarity analysis. Dice correlation coefficients were calculated and a dendrogram was generated using the UPGMA method (unweighted pair group method using arithmetic averages). Band position tolerance and optimization were set at 1.25% and 1%, respectively. A similarity coefficient of 80% was chosen for the definition of clusters.

4.4. Spatial Distribution of Staphylococcal Enterotoxin Genes in Different Regions of Brazil

The addresses of all isolates were geocoded and added to the BioNumerics program (version 7.0; Applied Maths, Belgium, 2015). Only strains carrying some staphylococcal enterotoxin genes or toxic shock syndrome were adopted as inclusion criteria on the map.

5. Conclusions

The enterotoxin A gene exhibited the highest prevalence in the present study, suggesting its easy dissemination among *Staphylococcus* spp. strains, since it was widespread in the georeferencing study. The recently described enterotoxin R gene is already found in isolates from some of the regions in Brazil

and coexists with previously described enterotoxins. This fact poses a risk for the dissemination of this new type of enterotoxin among cattle herds. A limitation of the present study was the fact that we did not perform gene expression analysis since the simple presence of enterotoxin genes in *Staphylococcus* spp. isolates does not imply the occurrence of food poisoning. However, the present results contribute to the understanding of the enterotoxigenic profile of isolates from cows with bovine subclinical mastitis in different Brazilian herds, especially when considering that they are one of the most common microorganisms involved in intramammary infections in cattle and that milk is an excellent growth medium for *Staphylococcus* spp. The study also identified important *S. aureus* and *S. epidermidis* clusters in different regions and the presence of enterotoxin genes in lineages isolated from animals that belong to herds located geographically close to each other.

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Danilo Flávio Moraes Riboli: Participated in the laboratory experiments, performed the pulsed-field analysis, and contributed to the analysis of the georeferenced data; Luiza Pinheiro: Contributed to the molecular experiments and to the analysis of the georeferenced data; Lisiane de Almeida Martins: Participated in the design of the study and in sending part of the strains; Maria Aparecida Vasconcelos Paiva Brito: Participated in the design of the study and coordinated the main project that allowed the isolation of strains from different Brazilian states; Maria de Lourdes Ribeiro de Souza da Cunha: Coordinated the study, participated in the design of the study, and revised the manuscript. All authors have read and approved the final version of the manuscript.

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5. CONCLUSÕES

- Quanto à identificação das amostras, verificou-se que os CoNS foram mais prevalentes do que os *S. aureus*, sendo que *S. chromogenes* e *S. epidermidis* foram os micro-organismos isolados em maior frequência.

- A determinação da resistência à oxacilina pelo método fenotípico demonstrou que os CoNS apresentaram maiores taxas de resistência. Ao analisar a resistência à vancomicina, todas as amostras foram sensíveis.

- Todas as amostras foram sensíveis à vancomicina pelo método de triagem, no entanto, 13 amostras apresentaram heterorresistência a essa droga, sendo *S. epidermidis* a espécie com maior número de amostras com essa característica.

- A hiperprodução de beta-lactamase foi detectada em 96% dos isolados testados e *S. saprophyticus* foi a espécie com o maior número de amostras com essa característica.

- O gene *mec*A foi detectado apenas em 8 amostras do estudo, todas *S. epidermidis*. Dois tipos de SCC*mec* foram detectados (tipo I e tipo IV), sendo que o SCC*mec* tipo I foi encontrado com maior frequência.

- A presença e expressão dos genes codificadores do biofilme estiveram mais presentes em *S. aureus*, sendo que os genes mais expressos foram *ica*A e o *ica*D, onde 52 (63,4%) amostras expressaram esses dois genes simultaneamente.

- Quanto à detecção dos genes das enterotoxinas estafilocócicas, o gene *seh* foi o mais encontrado, seguido do gene *sea*. Não encontramos amostras com genes para *ses*, *set* e *tst*.

- A tipagem molecular por PFGE revelou um grande cluster de *S. aureus* que inclui amostras provenientes de diferentes propriedades e localidades. Nas outras espécies também foi possível verificar a formação de clusters com essas características.

- Quanto à tipagem dos clones prevalentes pela técnica de MLST, o ST126 e ST1 foram os mais encontrados em *S. aureus*. Em *S. epidermidis* todos os ST foram distintos, com a possível descoberta de um novo sequence type.