

Caracterização molecular de *Staphylococcus* sp, isolados de leite de vacas com mastite em diferentes regiões do estado de São Paulo

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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 microrganismos (BPM).

*Vera Lúcia Mores Rall
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**BOTUCATU – SP
2013**



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FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉCNICA DE AQUISIÇÃO E TRATAMENTO DA INFORMAÇÃO
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CAMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: **ROSEMEIRE APARECIDA VICENTE**

Silva, Nathália Cristina Cirone.

Caracterização molecular de *Staphylococcus* sp., isolados de leite de vacas com mastite em diferentes regiões do estado de São Paulo / Nathália Cristina Cirone Silva. – Botucatu : [s.n.], 2013

Tese (doutorado) - Universidade Estadual Paulista, Instituto de Biociências de Botucatu

Orientador: Vera Lúcia Mores Rall

Coorientador: João Pessoa Araújo Júnior

Capes: 20100000

1. Infecções estafilocócicas. 2. Bovino de leite – Doenças. 3. Mastite.
4. Leite – Contaminação.

Palavras-chave: MLST; Spatyping; *Staphylococcus aureus*.

AGRADECIMENTOS

À Deus, que esteve ao meu lado para me acompanhar, a minha frente para me conduzir, atrás de mim para me proteger, acima de mim para me guiar e dentro de mim para me iluminar.

Aos meus pais e meu irmão que sempre me apoiaram e me ajudaram a vencer os desafios.

À minha orientadora Vera por todo apoio, ensinamentos, incentivos e paciência.

Ao meu co-orientador João Pessoa pela ajuda e paciência.

Aos meus colegas de laboratório que me ensinaram e me ajudaram muito durante o curso de doutorado.

À Felipe pela ajuda, amor e estímulo.

À Professora Carmen Torres e colegas de laboratório na Espanha que me receberam e me ensinaram técnicas que foram muito importantes ao meu trabalho.

Aos amigos pelo apoio e carinho nas horas difíceis.

E à todos que não foram citados mas de alguma maneira contribuíram para a concretização desse sonho.

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RESUMO

Staphylococcus sp é agente comum da mastite bovina, causando grandes perdas econômicas na pecuária brasileira. Esse micro-organismo possui fatores de virulência bastante conhecidos como a produção de hemolisinas, leucotoxinas e superantígenos como a toxina do síndrome do choque tóxico e enterotoxinas. Além disso, várias espécies de *Staphylococcus* podem adquirir genes de resistência aos antibióticos β lactâmicos. O objetivo do estudo foi caracterizar molecularmente isolados de *Staphylococcus* sp provenientes de leite de vacas com mastite clínica e subclínica de várias regiões do estado de São Paulo. Foram realizadas coletas de leite de vacas com mastite clínica e subclínica de diferentes fazendas no estado de São Paulo e realizados testes fenotípicos de resistência a antimicrobianos e de virulência (Toxina de Panton Valentine, síndrome do choque tóxico e toxinas esfoliativas), além de testes moleculares como Pulse Field Gel Electrophoresis (PFGE), Multilocus Sequence Typing (MLST) e Spa typing para comparação entre as cepas epidemiologicamente importantes e da tipagem do cromossomo cassette estafilocócico em cepas metilina resistentes. As cepas resistentes a metilina apresentaram amplo perfil de resistência e genes de resistência importantes e pouco relatados, sendo observados genes como *fexA*, *lsaE*, *lnuB*. Foram detectados dois novos spatyping (t10852 e t10856), bem como um novo alelo *yqiL* e um novo ST (2493). Os ECNs apresentaram resistências à maioria dos antibióticos estudados e foi observado a uma deleção no gene *ermC*, que codifica a resistência à eritromicina.

ABSTRACT

Staphylococcus sp. is the common agent of bovine mastitis, causing big economic losses in Brazilian cattle. This micro-organism have virulence factors have been well known as the production of hemolysin, and leucotoxinas toxin superantigens such as toxic shock syndrome and enterotoxins. In addition, several *Staphylococcus* species can acquire antibiotic resistance genes β lactamics. The objective of the study is to characterize molecularly *Staphylococcus* sp. from milk of cows with subclinical mastitis in various regions of the state of São Paulo. Samples were coleted from milk of cows with subclinical and clinical mastitis from different farms in the state of São Paulo. Tests were performed phenotypic antimicrobial resistance and virulence (toxin Panton Valentine, toxic shock syndrome and exfoliative toxins), molecular tests as Eletrophoresis Pulse Field Gel Electrophoresis (PFGE), Multiloccus Sequence Typing (MLST) and spa typing for comparison between epidemiologically important strains, as well as the typing of strains in staphylococcal cassette chromosome methicillin reistentes. Methicillin-resistant strains showed broad resistance profile and resistance genes important and underreported were detected as *fexA*, *lsaE*, *lnuB*. Two new spatyping (t10852 and t10856) were detected as well as a new allele *yqiL* and a new ST (2493). The ECN showed resistance to most antibiotics and was observed the deletion in *ermC* gene encoding resistance to erythromycin.

Introdução

1. INTRODUÇÃO

A mastite bovina é uma das doenças que mais causam gastos às propriedades leiteiras, representando 70% das suas perdas econômicas, pela queda na produção, devido ao descarte de leite contaminado, assistência veterinária, gastos com medicamentos e mão de obra e reposição do plantel (MARTINS et al., 2006).

No Brasil, vários estudos relataram o isolamento de *Staphylococcus* de amostras de leite de vaca com mastite (ZAFALON et al., 2007, SANTOS et al., 2008). Entre as espécies de estafilococos coagulase-positivas (ECP), as que têm maior ocorrência são *S. intermedius*, *S. hyicus* e *S. aureus*, sendo esta última, a de maior importância, pela elevada prevalência e virulência (ZAFALON et al., 2009) e quando estabelecida nas glândulas mamárias do animal lactante, é difícil de ser erradicada (BRITO et al., 2000).

Devido à grande quantidade de nutrientes, o leite é um excelente meio de cultura para o desenvolvimento de micro-organismos, podendo ser responsável pela transmissão de importantes zoonoses ao homem (AMARAL et al., 2004). Vários micro-organismos são causadores de mastite bovina, estando presentes no leite ainda no úbere bovino ou podem contaminar o leite durante ou após a ordenha, devido à contaminação por equipamentos e manipuladores (CLUTTERBUCK, 2007).

O gênero *Staphylococcus* possui 47 espécies e 24 subespécies (DSMZ, 2012) e está incluído na família Staphylococaceae. Esse gênero é colonizador frequente de muitas espécies animais, incluindo os seres humanos. Pode causar infecções como dermatites, septicemias e meningite em humanos e mastite em gado leiteiro (QUINN et al., 2000). Este micro-organismo pode produzir biofilme, que contribui para a sua capacidade de colonizar glândula mamária e pode comprometer a eficácia do tratamento de antibiótico intramamário (SALASIA et al., 2004; VIRDIS et al., 2010). O biofilme é

constituído pela adesina polissacarídica intercelular (PIA), que é mediada por locus *ica* (adesão intercelular) (ZIEBUHR et al. 1999).

O *S. aureus* é um micro-organismo coagulase e DNase positivas, o que o diferencia das outras espécies, coagulase negativas. Produz ácido a partir da fermentação da lactose, maltose e do manitol, reduz nitrato e azul de metileno e hidrolisa a ureia. A maioria das cepas é hemolítica. É um patógeno oportunista que normalmente faz parte da microbiota humana e de animais. O local mais comum de colonização são as fossas nasais, mas também podem colonizar a pele (especialmente se lesionada), orofaringe, vagina, axilas, períneo e trato intestinal. Acredita-se que há três tipos de indivíduos segundo a colonização estafilocócica: as pessoas não colonizadas, os colonizados intermitentes se os indivíduos onde a colonização se estabeleceu definitivamente. Cerca de um terço da população humana está temporariamente colonizado e outro terço é portador nasal (GORDON; LOWY, 2008).

Outras espécies de estafilococos, tanto coagulase positiva (ECP) como coagulase negativa (ECN), estão presentes na mucosa e da pele de seres humanos e também podem causar infecções. *S. epidermidis* é a espécie mais frequente de ECN e a mais importante devido ao seu papel como um patógeno oportunista. Outras espécies de ECN causadores de infecções humanas são, principalmente, *S. saprophyticus*, *S. haemolyticus* e *S. lugdunensis* (GORDON; LOWY, 2008).

1.1. Fatores de virulência dos Estafilococos

A capacidade patogênica dos estafilococos é caracterizada pela produção de várias enzimas (coagulase, hialuronidase, catalase, termolipase, etc) e a possibilidade de produção de certas toxinas. Além disso, entre os determinantes de virulência estão os componentes da parede celular (cápsula polissacarídica mucóide, adesinas, proteína A, ácido teicóico). A expressão destes fatores de virulência ajuda a bactéria a se adaptar a ambientes hostis, facilitando a sua subsequente sobrevivência. (GORDON; LOWY, 2008).

As toxinas são divididas em três grupos principais: leucocidinas, hemolisinas e superantígenos. O último grupo inclui as toxinas exfoliativas, TSST e enterotoxinas (LINA et al, 1999).

As leucotoxinas são toxinas formadoras de poros que tem como alvo as células do sistema imunitário tais como neutrófilos polimorfonucleares (PMN) (PANTON et al., 1932). Incluem a gama hemolisina e Leucocidina de Panton-Valentine (PVL), compostas de duas proteínas distintas secretadas independentemente (PRÉVOST et al., 2001). A produção de PVL tem sido preferencialmente ligada a quadros de furúnculos, abscessos cutâneos, infecções cutâneas necróticas (COUPPIÉ, 1995; PRÉVOST, 1995) e mastites bovinas (BARRIO et al., 2006). As hemolisinas alfa, beta e delta também são toxinas citolíticas, lesando hemácias e outras células do organismo (BOHACH; FOSTER, 2000).

Algumas cepas de *S. aureus* produzem toxinas esfoliativas (A e B) que tem sido associadas a doenças como a síndrome da pele escaldada, ocasionando esfoliação da pele. A atividade biológica e o grau de similaridade genética entre os dois tipos de toxinas esfoliativas são semelhantes, porém o gene da toxina esfoliativa A é cromossômico enquanto o da B é plasmidial (MARRAK et al., 1990).

A proteína estafilocócica A, codificada pelo gene *spaA*, é uma exoproteína ligada à membrana conhecida pela sua capacidade de se ligar à região Fc de imunoglobulinas, que ativa o sistema imune da maioria das espécies de mamíferos (ALONSO; DAGGET 2000). O gene dessa proteína se encontra num *locus* único, estável, altamente discriminatório que reflete macro e microvariação da população de *S.aureus* e que pode ser utilizado na epidemiologia desta bactéria (SHOPSIN et al., 1999)

A proteína coagulase, presente em *S.aureus* e em outros SCP, tem a capacidade de converter o fibrinogênio em fibrina (PALMA et al. 1999). Apresenta-se também como um fator de virulência na infecção intramamária. Esta proteína é codificada pelo gene *coa* e é usada como na identificação fenotípica das espécies (SHOPSIN et al. 2000, REINOSO et al. 2004)

A toxina da síndrome do choque tóxico (TSST-1) é produzida por um número significativo de cepas de *S. aureus* (30 a 60%) e são conhecidas por suas propriedades pirogênicas, tóxicas e de superantigenicidade. A TSST-1 é capaz de romper barreiras mucosas e agredir células endoteliais. (DINGES, 2000; BOYLE, 2007).

Quando o *S. aureus* é confrontado pelo sistema imune. Ele pode expressar várias proteínas imuno-moduladoras. Uma delas é o inibidor do complemento de estafilococos (Scin), que é um eficiente inibidor do complemento da lectina, da via clássica e da via alternativa (ROOIJAKKERS et al., 2005). Van Wamel et al. (2006) observaram que o gene que codifica a Scin (*scn*) é parte de um "cluster de imune evasão" (IEC) e além desse gene, foram identificados o *sak*, *chp*, *sea* e *sep* (VAN WAMEL et al., 2006).

1.2. Resistência a antibióticos

Os β -lactâmicos são os antibióticos de escolha para o tratamento de infecções por estafilococos, embora outros antibióticos também possam ser utilizados, mas o uso prolongado e repetido pode levar a resistência a estes antibióticos. *Staphylococcus* sp é capaz de adaptar-se à pressão de seleção e sofrer mutações nos seus genes ou pode adquirir genes de outras espécies. Os mecanismos de resistência são variados e entre eles estão a alteração da permeabilidade de membrana ou da parede celular, expulsão ativa do antibiótico para o exterior da célula, inibição ou inativação do antibiótico (MILLER et al., 1996). Na tabela 1, estão listados os antibióticos estudados nesse trabalho e seus respectivos genes de resistência.

Em 1942, dois anos após a introdução da penicilina para uso médico, foi isolada a primeira amostra hospitalar de *S.aureus* resistente a esse medicamento. Após esse episódio, cepas resistentes à penicilina também foram observadas na comunidade. Desde 1960, cerca de 80% das cepas de *S. aureus* são resistentes à penicilina. Dois anos após a introdução da meticilina, em 1961, cepas de *S.aureus* resistente a esse antibiótico foram detectadas, devido à presença do gene *mecA*. Nesses últimos anos, vários clones de *S. aureus* meticilina resistente (MRSA) foram associados a infecções hospitalares (LOWY, 1998; DEURENBERG, 2008).

Tabela 1: Antibióticos e seus respectivos genes de resistência em *Staphylococcus*

Antibióticos	Genes de resistência	Referências
Oxacilina e Cefoxitina	<i>mecA, mecLGA251</i>	Gomez-Sanz et al. (2010); Cuny et al. (2011)
Penicilina	<i>BlaZ</i>	Gomez-Sanz et al. (2010)
Tetraciclina	<i>tet(K), tet(L), tet(M)</i>	Gomez-Sanz et al. (2010)
Estreptomicina	<i>str, ant6, ant3(9)</i>	Gomez-Sanz et al. (2010)
Cloranfenicol	<i>fexA, pc221, pc224</i>	Kehrenberg and Schwarz (2005)
Ciprofloxacina	Mutação nos genes <i>gyrA</i> e <i>grlA</i>	Lozano et al. (2012b)
Clindamicina e eritromicina	<i>erm(A), erm(B), erm(C), erm(F), erm(T), msr(A), cfr, vga(C), lnu(A/A'), lnu(B), lsa(B), lsa(E)</i>	Gomez-Sanz et al. (2010) Lozano et al. (2012a)
Sulfatomexazol	<i>dfr(A), dfr(D), dfr(G), dfr(K)</i>	Gomez-Sanz et al. (2010)
Gentamicina	<i>aac2' aph6'</i>	Gomez-Sanz et al. (2010)
Tobramicina	<i>ant4</i>	Lozano et al. (2012b)

1.3. Caracterização molecular

A fim de compreender a epidemiologia dos MRSAs, de *S. aureus* metilina sensíveis (MSSAs) e ECN, conhecer os clones predominantes implicados em surtos e infecções em hospitais e na comunidade, conhecer as variantes associadas a certos ambientes e a determinados animais e transmissibilidade, desenvolveram-se diferentes técnicas de tipagem molecular.

A maior vantagem das técnicas de tipagem baseadas na análise de DNA reside na estabilidade dos marcadores genéticos utilizados e da sua aplicabilidade universal para diferentes gêneros e espécies de micro-organismos (DOMINGUEZ et al. 2005).

1.3.1. Tipagem pelo SCCmec

É uma técnica baseada na análise do polimorfismo do cassete SCCmec contendo o gene *mecA*. Não se sabe exatamente o mecanismo de aquisição do mesmo, mas os genes *ccrA* e *ccrB*, presentes em elemento SCCmec, codificam recombinases capazes de integrar o SCCmec no cromossomo. O SCCmec é uma ilha genômica que, além das recombinases, tem também elementos de inserção como IS431 e IS1217 (Figura 1). Assim, pode-se encontrar, no interior do SCCmec, genes de resistência a outros antibióticos como a eritromicina (por adquirir o transposon Tn554 transportando o gene *erm* (A)) ou amino glicosídeos (aquisição de plasmídeo pUB110 que possui o gene *aphA3* e/ou o plasmídeo p1258 portador do gene *aadD*), entre outros (HIRAMATSU et al., 1999).

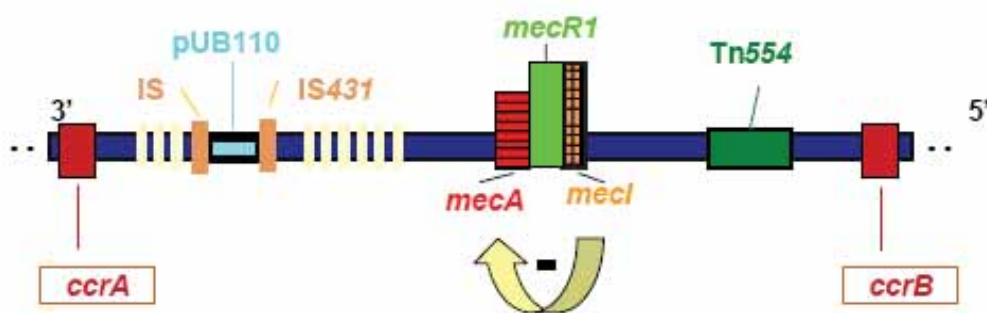


Figura 1: Representação de um cassete SCCmec tipo (Borraz. 2006).

Atualmente, 11 tipos de SCCmec já foram descritos (IWG-SCC, 2012), sendo os tipos I a V, os mais frequentes. Os tipos I, IV, V, VI e VII estão ligados à resistência, principalmente, aos antimicrobianos β lactâmicos, enquanto II e III podem resultar em resistência a outras drogas, devido a genes de resistência adicionais integrados (OLIVEIRA et al, 2006; TAKANO et al, 2008).

De acordo como se encontram os genes regulatórios e as seqüências de inserção IS1272 e IS431, as diferentes classes do complexo *mec* são diferenciadas (Tabela 2).

Tabela 2: Estrutura do complexo *mec*

Complexos gene <i>mec</i>	Estrutura do complexo <i>mec</i>	Tipos de SCC<i>mec</i>
class A	IS431- <i>mecA</i> - <i>mecR1</i> - <i>mecI</i>	II, III, VIII
class B	IS431- <i>mecA</i> - Δ <i>mecR1</i> -IS1272	I, IV, VI
class C1	IS431- <i>mecA</i> - Δ <i>mecR1</i> -IS431 (duas IS431s estão inseridas na mesma direção)	VII, X
class C2	IS431- <i>mecA</i> - Δ <i>mecR1</i> -IS431 (duas IS431s estão inseridas em direções opostas)	V, IX
class D	IS431- <i>mecA</i> - Δ <i>mecR1</i>	
class E	<i>blaZ</i> - <i>mecA</i> LGA251- <i>mecR1</i> LGA251- <i>mecI</i> LGA251	XI

Adaptado de <http://www.sccmec.org>

Assim, o gene *mecA* é parte de uma ilha genômica conhecida como SCCmec, que está integrada no cromossomo de *S. aureus*.

Foram descritos diferentes tipos de SCCmec (Tabela 3), dependendo das características do gene *ccr* e as sequências adjacentes e a sequência da região de *mec* e seus genes de regulação e determinantes genéticos adquiridos como uma consequência da integração de plasmídeos e transposons (ITO et al. 2001).

Tabela 3: Tipos de SCCmec

TIPO SCCmec	TIPO <i>ccr</i>	COMPLEXO <i>mec</i>
I	1	B
II	2	A
III	3	A
IV	2	B
V	C	C
VI	4	B
VII	C2	C2, C8
VIII	4	A
IX	1	C2
X	7	C1
XI	9	E

Adaptado de <http://www.sccmec.org>

Na Figura 2, os tipos de SCCmec de I a VI são apresentados.

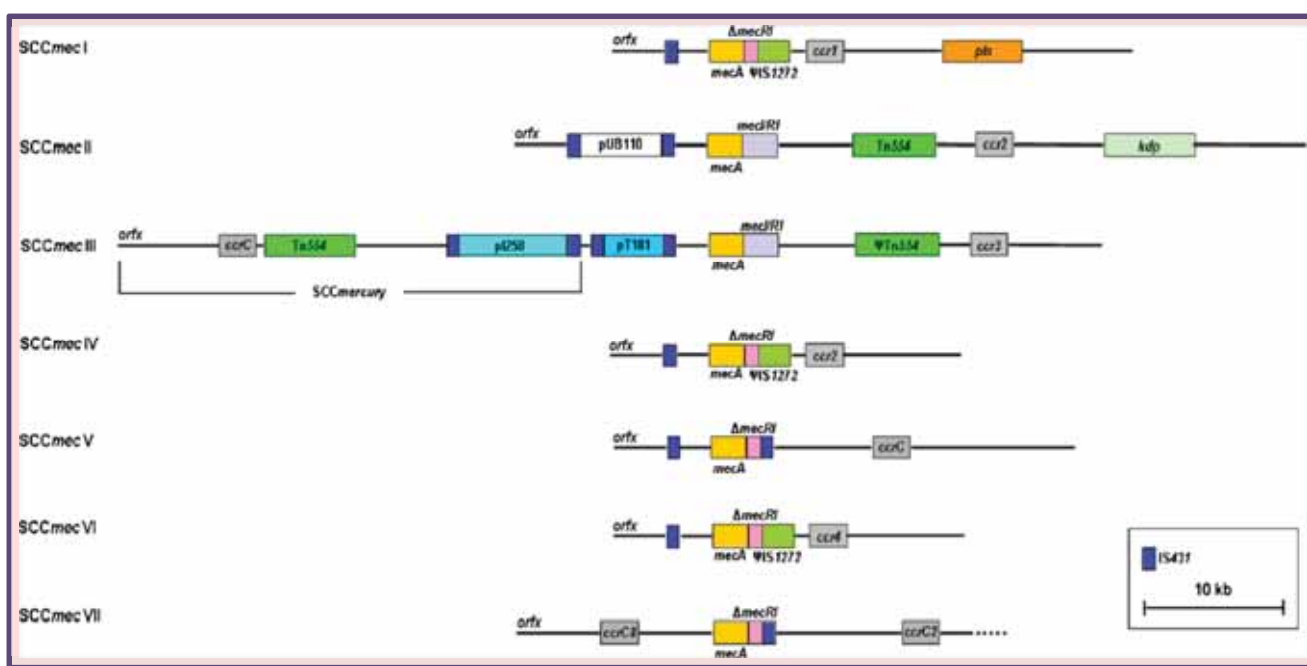


Figura 2: Representação dos tipos de SCCmec I, II, III, IV, V e VI (Deurenberg et al. 2008).

1.3.2. Tipagem pelo *locus agr*

O *locus agr* (accessory gene regulator) foi descrita pela primeira vez em *S. aureus*, mas é um sistema global de regulação de virulência no gênero *Staphylococcus*. Tem sido sugerido também que o *locus agr* pode influenciar a capacidade de *S. aureus* para colonizar o hospedeiro e competir com outras bactérias, incluindo outras espécies de *Staphylococcus* (WRIGHT et al., 2005).

As cepas de *S. aureus* podem ser classificadas entre quatro tipos de *agr*. Cepas que pertencem ao mesmo grupo podem ativar a resposta de *agr* em outras cepas do mesmo grupo, mas inibem as que pertencem a outro grupo, sendo uma forma de interferência entre bactérias (SHOPSIN et al., 2003).

1.3.3. Tipagem pelo gene *spa*

Esta técnica é baseada na amplificação e sequenciamento da região polimórfica do gene que codifica a proteína A, o *spa*, que pode sofrer acréscimos e/ou perda dos fragmentos repetidos e sofrer mutações espontâneas nessas regiões. Assim, a análise do número de repetições e variabilidade pode ser atribuído a diferentes tipos (*spa*-type) (HARMSEN et al. 2003).

Esta técnica é de grande interesse devido à sua rapidez e simplicidade. Além disso, verificou-se uma boa correlação entre a técnica de Multi Locus Sequence Typing (MLST) e *spat*ype (OLIVEIRA et al. 2002) em muitos casos.

1.3.4. Tipagem por Multilocus Sequence Typing (MLST)

O MLST é uma técnica desenvolvida em 1998 (MAIDEN et al., 1998) e caracteriza cepas bacterianas utilizando sequências de fragmentos internos de sete genes constitutivos (*housekeeping*), imprescindíveis ao metabolismo do micro-organismo, pois codificam enzimas metabólicas. Esta análise detecta variações que definem linhas clonais relativamente estáveis e permite que diversas cepas isoladas em diferentes países possam ser identificadas e relacionadas (DOMINGUEZ et al., 2005). No caso de *S. aureus*, esta técnica foi executada a partir de 2000 e os sete genes *housekeeping* (Figura 3) utilizados são: *arcC* (quinase de carbamato), *aroE* (desidrogenase chiquimato), *glpF* (glicerol cinase), *gmK* (guanilato-quinase), *pta* (fosfato aciltransferase), *tpi* (triosefosfato) e *yqiL* (acetil CoA acetiltransferase), (ENRIGHT et al., 2000). A sequência destes genes pode ser comparada com alelos conhecidos e acessíveis numa base de dados na página <http://www.mlst.net>. O perfil alélico permite se obter uma sequência tipo (ST). O programa *eBurst* relaciona os diferentes ST em

complexos clonais (CC). Pertencem a um mesmo complexo os STs que diferem em apenas um alelo do ST principal.

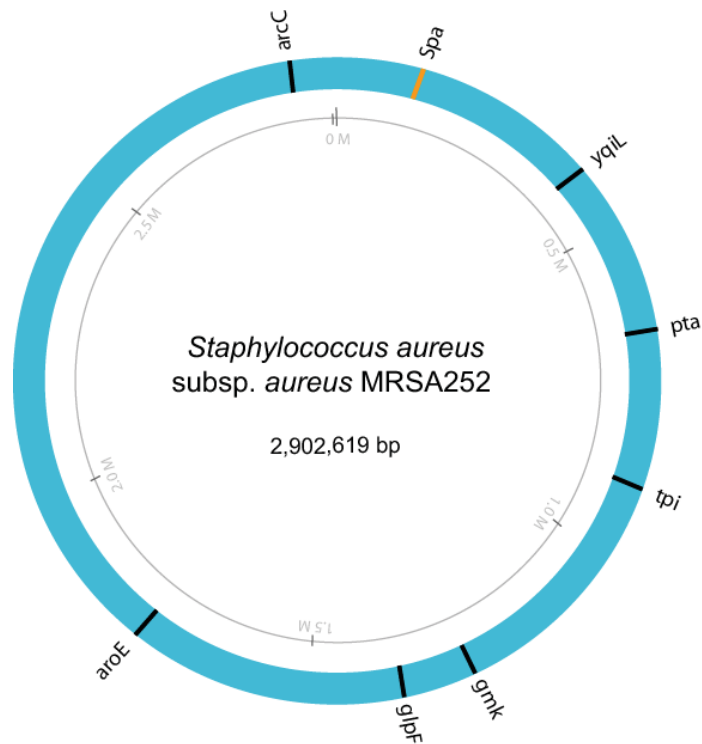


Figura 3: Ilustração dos genes *spa* e genes constitutivos do MLST

1.3.5. Eletroforese em Gel de campo pulsado

O campo pulsado em gel de eletroforese (*Pulsed-Field Gel Electrophoresis*-PFGE) consiste na digestão do genoma total bacteriano com enzimas de restrição (em *S. aureus*, geralmente usa-se a *SmaI*, por exemplo), seguida de eletroforese em uma cuba com campo pulsátil (que permite a discriminação de bandas de alto peso molecular). O padrão de bandas gerado (pulsotipo) pode ser analisado visualmente ou através de softwares específicos (TENOVER et al. 1995; CHURCH et al., 2011). Essa técnica é utilizada para identificar o parentesco dos isolados em contextos epidemiológicos mais restritos, como, por exemplo, em surtos dentro de um hospital. Para análise de isolados

com distribuição mais ampla e a longo prazo, é necessária a utilização de algum procedimento com maior poder discriminatório, que tenha a capacidade de identificar variações que se acumulam lentamente.

1.4. Importância epidemiológica

As origens dos principais clones de MRSA são ainda pouco entendidas. Kreiswirth et al.(1993) propuseram que os MRSA descendem de um único ancestral de *S. aureus*, que adquiriu o gene *mecA*. Entretanto, Fitzgerald et al. (2001) mostraram que há diferenças entre alguns MRSA, o que implica que o gene *mecA* foi transferido entre diversas linhagens ancestrais de *S. aureus*.

A primeira cepa de *S.aureus* associada a animais de criação resistente a meticilina (Livestock-associated Methicillin-Resistant *Staphylococcus aureus* LA – MRSA) foi isolada a partir de uma vaca leiteira, há aproximadamente 40 anos atrás (DEVRIESE et al., 1972). Posteriormente, houve vários relatos de MRSA em vacas (JUHASZ-KASZANYITZKY et al., 2007; VANDERHAEGHEN et al., 2010).

O aparecimento do clone ST398, isolado de porco, com capacidade de causar doença em seres humanos iniciou a especulação sobre o potencial de cepas de origem animal ser transmitidas aos seres humanos, resultando em morbidade e mortalidade (WULF; VOSS, 2008).

Sung e Lindsay (2007) demonstraram que o clone ST151, específico de bovino e comumente associado à mastite, é virulento e susceptível a aquisição de resistência a vancomicina. Foi sugerido que esse clone pode ser uma ameaça à saúde pública, se transmitido e adaptado às populações humanas (GUINANE et al., 2008).

A identificação de sequências tipo (ST) específicas em aves, bovinos e equinos idênticos ou semelhantes aos STs encontrados em humanos implica que a transferência de *S. aureus* entre espécies pode ocorrer (LOWDER et al., 2009; GARCIA-ALVAREZ et al., 2011; SAKWINSKA et al., 2011). O uso inadequado de antibióticos no setor pecuário, a longo prazo, provavelmente selecionou bactérias resistentes e os animais podem ser reservatórios dessas cepas (SUNG; LINDSAY, 2007).

Assim, é importante uma vigilância epidemiológica rigorosa para identificar eventuais patógenos que possam interferir na produtividade, bem-estar animal e na segurança alimentar, em todo o mundo.

2. OBJETIVOS

Na mastite bovina, é importante a caracterização de micro-organismos como o gênero *Staphylococcus*, identificando-se os fatores de virulência (importantes na patogenicidade) e os clones, num estudo epidemiológico da disseminação das cepas, pois no Brasil, há escassez dessas informações.

2.1. Objetivo Geral

Caracterizar molecularmente e fenotipicamente isolados de *Staphylococcus* sp, provenientes de leite de vacas com mastite de várias regiões do estado de São Paulo.

2.2. Objetivos específicos

- Isolar cepas de *Staphylococcus* sp. metilina resistente e *Staphylococcus aureus* metilina sensível em leite de vacas com mastites, clínica ou subclínica, de várias regiões do estado de São Paulo.
- Verificar a diversidade clonal das cepas através das técnicas de MLST, PFGE e spatyping.
- Identificar o tipo do SCCmec dos isolados resistentes a metilina.
- Identificar, nas cepas isoladas, os genes para produção de toxina Pantone-Valentine Leucocidina (lukPV), toxinas esfoliativas (eta e etb) e para a toxina da síndrome do choque tóxico (tst).
- Realizar o perfil fenotípico e genotípico de resistência dos isolados.

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*Capítulo 7. Detection, Molecular
Characterization, and Clonal Diversity of
Methicillin-susceptible Staphylococcus aureus in
milk of cows with mastitis in Brazil*

Artigo submetido á revista Journal Dairy Science

Detection, Molecular Characterization, and Clonal Diversity of Methicillin-susceptible *Staphylococcus aureus* in milk of cows with mastitis in Brazil

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Running title: Molecular Characterization of MSSA in cows in Brazil

Abstract

The objective of this study was to determine the frequency of methicillin-susceptible *Staphylococcus aureus* (MSSA) in milk of cows with clinical and subclinical mastitis in Brazil, to characterize the obtained isolates and the circulating lineages. A total of 1171 cows from 11 farms in the state of São Paulo, Brazil, were evaluated. In total, 4684 milk samples (one per mammary gland) were obtained and 1484 milk samples were positive for California-Mastitis-Test (CMT), being studied for MSSA recovery. MSSA isolates were characterized by *spa*, *agr* and multilocus-sequence-typing (MLST). Resistance and virulence traits were investigated by PCR. Fifty-six MSSA were recovered from 32 cows of 10 farms (2.7%). Seven *spa*-types were identified (% of isolates): t002 (1.8), t127 (44.6), t605 (37.5), t1784 (1.8), t2066 (1.8) and two new ones t10852 (1.8) and t10856 (10.7). Five distinct sequence-types (ST) were detected (% isolates): ST1 (46.4), ST126 (37.5), ST133 (10.7), ST5 (3.6), and a novel ST registered as ST2493 (1.8). Resistances were detected for streptomycin, chloramphenicol and tetracycline. One strain contained *fexA* gene (included within Tn558) and three strains *tet(K)* gene. In general, strains were susceptible to most antibiotics studied and lacked genes encoding important virulence factors. MSSA was detected in a low proportion of milk samples of cows with mastitis (2.7%) and recovered isolates presented high diversity of genetic lineages, CC1 and CC126 as the predominant ones, and CC133 was also detected. Further epidemiological studies with molecular characterization of isolates are required to gain knowledge on the circulating genetic lineages among the cow population with mastitis.

Keywords: *Staphylococcus aureus*, *spa* typing, mastitis

Introduction

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

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

S. aureus can acquire methicillin-resistance (MRSA) due to the acquisition of the *mecA* gene. MRSA represents an important therapeutic problem when implicated in human or animal infections, and numerous studies have focused on the characterization of these isolates. Nevertheless, the interest on methicillin-susceptible *S. aureus* (MSSA) is increasing in the last years, given that they can be also implicated in important infections and may help understand the appearance and evolution of the different and

successful MRSA lineages. Few data do exist on the circulating genetic lineages of MSSA in food-producing animals or in derived food products, as is the case of milk.

The objectives of this study were to analyze the prevalence of *S. aureus* in milk of cows with mastitis and to perform the molecular typing and genetic characterization of MSSA isolates recovered.

Materials and Methods

Origin of samples

A total of 1171 cows from 11 different farms in the state of São Paulo, Brazil (Figure 1), were evaluated. In total, 4684 milk samples (one per mammary gland) were obtained and 1484 of them were positive for mastitis. The methodology and interpretation criteria used for diagnosis of clinical and subclinical mastitis were based on examination of animals before each milking by the California Mastitis Test - CMT (SCHALM & NOORLANDER, 1957). Samples were collected in sterile tubes after sterilization of the zone (ostium) with iodized alcohol (2.5%), and they were transported to the laboratory under refrigeration (4-8 ° C) in cool boxes with ice packs.

Bacterial isolates

The samples were plated on blood agar (5%) (Oxoid) and incubated under aerobic conditions at 37° C, and readings were performed after 24, 48 and 72 hours of incubation. Identification of *S. aureus* was based on colony morphology, Gram staining, catalase, coagulase and DNase activities (KONEMAN et al., 2008). Molecular identification was performed by a multiplex PCR that also allows the discrimination of MSSA and MRSA by amplification of the species-specific *nuc* gene and the

staphylococcal methicillin resistance genetic determinant (*mecA*) (CRL-AR, 2009). One MSSA isolate per mammary gland was further investigated.

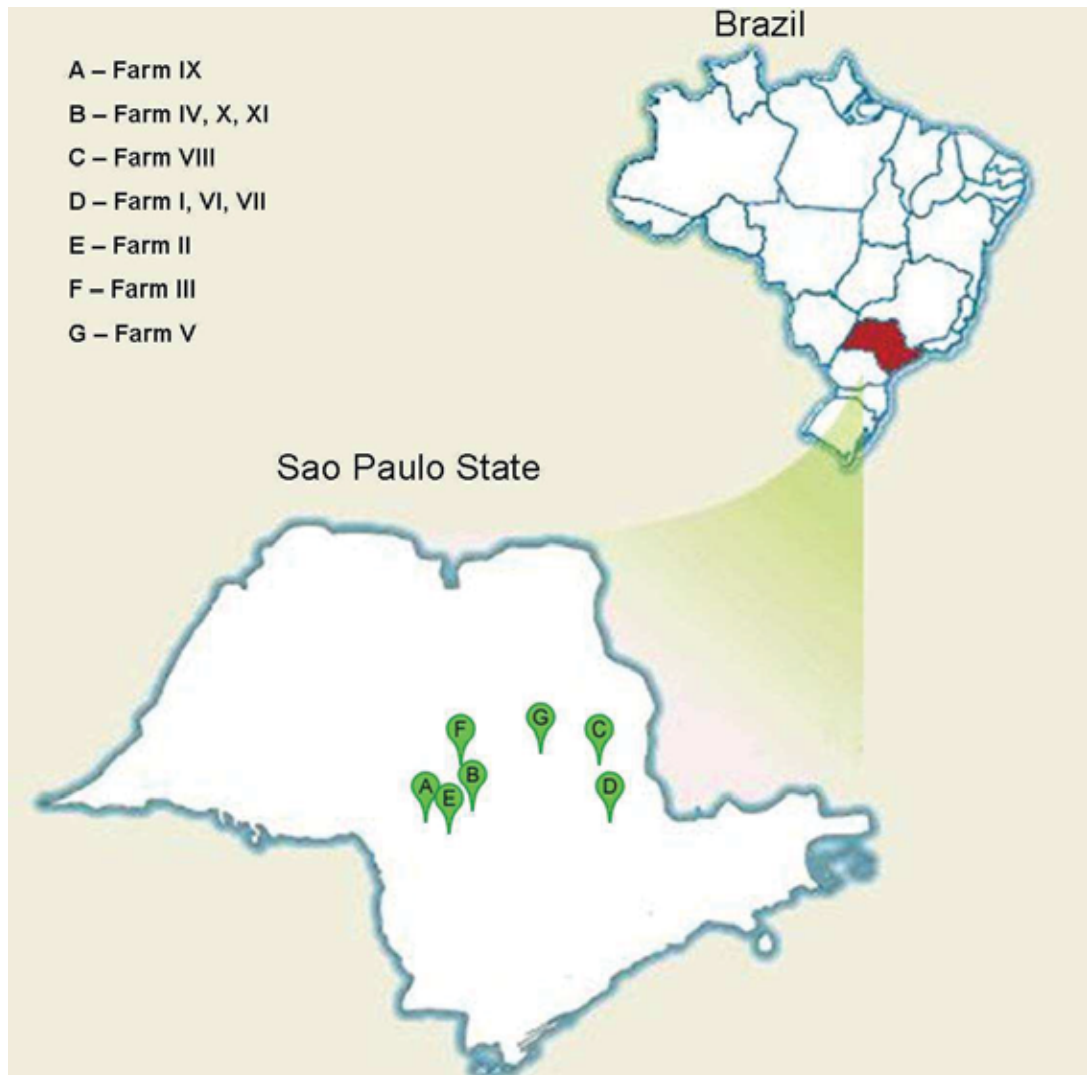


Figure 1. Sampling regions of the State of Sao Paulo, Brazil.

Molecular typing and clonal relatedness of S. aureus isolates

All *S. aureus* isolates were characterized by *spa*-typing (<http://spaserver.ridom.de>) and *agr*-allotype (SHOPSIN et al., 2003) by specific PCRs. Multi-locus-sequence-typing (MLST) was performed as previously described (www.mlst.net) on one representative strain per *spa*-type. Pulsed-Field-Gel-

Electrophoresis (PFGE) of genomic DNA previous digestion with the macrorestriction *Sma*I enzyme was performed in one strain per *spa*-type per cow. PFGE band profiles were compared according to Tenover criteria (TENOVER et al., 1995).

Antimicrobial susceptibility testing and detection of resistance genes

Antimicrobial susceptibility testing to oxacillin, cefoxitin, tetracycline, erythromycin, clindamycin, gentamicin, tobramycin, streptomycin, trimethoprim-sulphamethoxazole, ciprofloxacin, and chloramphenicol was performed by disk-diffusion agar method in accordance with the Clinical and Laboratory Standards Institute recommendations (CLSI, 2012). Detection of antimicrobial resistance genes was investigated in resistant isolates by specific PCRs (KEHRENBURG & SCHWARZ, 2006; GÓMEZ-SANZ et al., 2010). Positive and negative controls from the collection of the University of La Rioja were used in each PCR assay.

Virulence factors and immune evasion cluster (IEC) genes

The presence of the genetic determinants of Pantone-Valentine-Leukocidin (*lukF/S-PV*), Toxic-Shock-Syndrome-Toxin 1 (*tst*), and Exfoliative-Toxin A (*eta*) and B (*etb*) was analyzed by PCR (LINA, et al., 1999; JARRAUD, 2002). Presence of the human-associated immune evasion cluster (IEC) genes (*scn*, *chp*, *sak*, *hly*, *sea*, *sep*), which are enclosed within ϕ 3 bacteriophage, was analyzed as previously recommended (van WAMEL, 2006).

Results

Detection and isolation of S. aureus

S. aureus isolates were recovered from 97 of the 1484 milk samples tested (6.5%) and were obtained from 53 cows. Among these, fifty-six isolates were MSSA (obtained from 3.7 % of the tested samples) and were recovered from 32 cows (2.7% of tested animals), while the remaining 41 isolates were MRSA. In total, 57.7% of *S. aureus* obtained, recovered from distinct farms, corresponded to MSSA, and were further characterized in this study.

Among the 32 cows MSSA-positive, more than one mammary gland contained this microorganism in 17 of them (12 animals, two positive glands; 3 animals, three positive glands; 2 animals, four positive glands).

Molecular typing of MSSA isolates

Two new *spa*-types were identified among 7 of the 56 tested MSSA: a) One isolate presented the *spa*-type t10852, and was also ascribed to the novel sequence type ST2493, enclosed within clonal complex CC1; and b) Six isolates presented the *spa*-type t10856, and were ascribed to the sequence-type ST133 (CC133). The other *spa*/ST/CC types detected among the remaining 49 MSSA were as follows (number of isolates): t605/ST126/CC126 (25), t127/ST1/CC1 (21), t002/ST5/CC5 (1), t1784/ST1/CC1 (1), and t2066/ST5/CC5 (1). The following *agr*-allotypes were obtained (number of isolates): *agr*_I (6), *agr*_II (23) and *agr*_III (27), while *agr*_IV was not found (Table 1).

Table 1. Number and characteristics of MSSA isolates investigated and number of positive cows.

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

^aperformed one strain per *spa* type

The MSSA isolates recovered from different mammary glands presented similar genetic characteristics (*spa*/ST/CC) in 16 of the 17 animals that were infected by MSSA in more than one gland.

SmaI-PFGE performed with 29 representative MSSA isolates revealed that isolates that belonged to the same *spa*-type presented identical or closely related patterns (table 2), while those with distinct *spa*-type exhibited non-related PFGE profiles.

Antimicrobial resistance, toxin genes and IEC profile

Few resistances were detected among our MSSA isolates: streptomycin (50%), and chloramphenicol and tetracycline (3.5%). Three tetracycline resistant isolates harbored the *tet*(K) gene and one chloramphenicol-resistant isolate the *fexA* gene (table 2). The possible inclusion of the *fexA* gene within the transposon Tn558 was investigated (KEHRENBERG & SCHWARZ, 2006), revealing the presence of the

transposase genes (*tnpA*, *tnpB*, *tnpC*), and the *orf138*, characteristic of the conserved transposon. In addition, circular intermediates were detected, indicating functional activity. No streptomycin resistance genes were identified in the streptomycin-resistant staphylococci. All MSSA isolates were negative for the tested toxin genes as well as for the IEC genes.

Table 2. Antibiotic resistance characteristics of the investigated isolates divided by farm of procedence.

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

^a CHL, chloramphenicol; TET, tetracycline, STR, streptomycin; ^b the *fexA* gene was enclosed within the transposon Tn558.

Discussion

The occurrence of *S. aureus* among milk samples of cows with mastitis detected in this study is relatively low (6.5%). Coelho et al. (2009) observed 17.3% of MSSA in milk samples from bovine mastitis in Brazil. In 2010, other study detected 38% of milk samples positive for *S. aureus* (D'Amico and Donnelly, 2010), while Oikonomou et al. (2012) reported 12.5%. MSSA was detected in 62% of bulk tank milk from Minnesota dairy farms (Haran et al., 2012). The possibility that the low recovery rate observed in our study might be due, in part, to the methodology employed, cannot be discarded. Milk contamination with *S. aureus* is of concern because it puts at risk people working on the farm. Moreover, milk can reach the consumer in case of bad pasteurization or when milk is used as raw material for the production of Minas cheese or other dairy product. Lee (2003) suggested a possible transmission of *S. aureus* from cows to humans.

Only two cows presented the four teats infected with MSSA, while almost half presented only one mammary gland infected. These data evidences the importance of studying all mammary glands for an accurate evaluation of mastitis rates.

The most prevalent *spa*-type among our MSSA isolates from bovine source is t127 and t605, and it is in accordance with previous reports from Brazil (Aires-de-Sousa et al., 2007; Rabello et al., 2007). In Canada, t605 strains of MSSA were also observed (Said et al., 2010), while t127 isolates have been previously isolated from Switzerland and Korea (Huber et al., 2010, Hwang et al., 2010). Alternatively, other studies on milk samples from cows with mastitis showed *S. aureus* with different *spa*-types not detected among our MSSA isolates (Haran et al., 2012, Johler et al., 2011).

The database of the Ridom *spa* server network for *spa* typing (<http://spaserver.ridom.de>) shows that MLST ST1/*spa*-type t127 has a relative global frequency of 1.9% and t605 of 0.1% (data collected in January 2013). MSSA isolates with *spa*-types t127 and t605 were distributed in almost all regions studied of the state of Sao Paulo, while those with *spa*-type t10856 were found in two distinct regions (B and D). The 56 strains were distributed in four clonal complexes: CC1, CC5, CC126 and CC133. Among these, CC1 and CC126 were predominant. Rabello et al. (2007) observed the same clonal complexes in a study in Brazil from milk samples collected from cows with subclinical mastitis. The clonal complexes CC1 and CC5 are common among human isolates (Argudin et al., 2011; Lozano et al., 2012) and Juhász-Kasanyitzky et al. (2007) reported strains of CC1 in human and bovines. Franco et al. (2011) suggested that transmission of this lineage (CC1) from animals to human is possible, although there are differences in the host-specific genetic characteristics between the two clusters. The clonal complex CC126 is not common among isolates from human beings and several studies observed this CC in ruminants (Smith et al., 2005; Rabello et al., 2007; Aires-de-Sousa et al., 2007), suggesting that ruminants are reservoirs for this CC.

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

None of the investigated virulence factors yielded positive among our MSSA isolates, what indicates that MSSA isolates that lack important virulence determinants can still be responsible for mastitis. A recent study has shown strains from milk from animals with mastitis with the *etb* gene (Salasia et al., 2011).

Antibiotic susceptibility testing revealed that 27 isolates (48.2%) were susceptible to all antibiotics tested. Other studies reported susceptibility rates of 42% (Coelho et al., 2009) or 67% (Aires-de-Sousa et al., 2007) among bovine *S. aureus*, the later from farms located in Rio de Janeiro State. The detection in one isolate of the complete and conserved Tn558, which carries the chloramphenicol and florfenicol resistance gene *fexA*, is remarkable and, to the best of our knowledge, it represents the first description of this transposon in Brasil. Since its first description in a *S. lentus* from a cow with a respiratory tract infection, this transposon has been detected in *S. simulans*, *S. chromogenes* and *S. sciuri* from bovine and equine *S. aureus* isolates (Kehrenberg and Schwarz, 2006), *S. cohnii* isolates from swine farms in China (Wang et al., 2012) and in *Bacillus* from swine feces (Dai, 2010). Further molecular epidemiological studies are required to assess the burden of this mobile genetic element among the Brazilian cow population. Interestingly, streptomycin-resistant strains resistant did not contain the expected resistance genes suggesting the presence of unknown resistance genetic determinants to this antibiotic that should be characterized in the future.

Conclusion

S. aureus was isolated in approximately 6.5% of samples tested, with MSSA obtained in 2.7% of investigated animals (57.7% of all *S. aureus* recovered). The mayor

lineages detected among MSSA were CC126 and CC1, followed by the ruminant associated CC133. In general, MSSA strains were susceptible to most antibiotics studied; although the detection of the conserved Tn558 evidences potential acquisition capacities. Interestingly, the resistance genetic determinants to streptomycin among our MSSA revealed unknown. Although all isolates lacked genes encoding important virulence factors, all were recovered from cases of mastitis, evidencing their virulence capacities. Due to the scarce data available on molecular characteristics of *S. aureus* isolates from cattle in Brazil, continued epidemiological surveillance is essential to gain knowledge on the circulating lineages responsible for cow mastitis in this mayor milk producer country, due to its potential implications in both animal and human health.

Acknowledgments

Silva N. C. C. has a fellowship from Capes - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Process - 9877-11-8. Gómez-Sanz E. has a predoctoral fellowship of the Gobierno de La Rioja (Spain) and Benito D. has a predoctoral fellowship of the Ministerio de Economía y Competitividad of Spain. Part of this work was financially supported by Project SAF2012-35474 from the Ministerio de Economía y Competitividad of Spain.

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*Capítulo 77: Methicillin-resistant
Staphylococcus aureus of lineage ST398 as a
cause of mastitis in cows on a farm in São
Paulo State, Brazil*

*Artigo submetido como Short communication à revista Research
in Veterinary Science*

**Methicillin-resistant *Staphylococcus aureus* of lineage ST398 as a cause of mastitis
in cows on a farm in Sao Paulo State, Brazil**

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Running title: MRSA ST398 in cows on a farm in Brazil

Summary

The objective was to analyze the prevalence and molecular characteristics of methicillin-resistant *Staphylococcus aureus* (MRSA) in milk of mastitis cows. Milk samples of 100 cows from one farm were evaluated for mastitis (California-mastitis-test) and MRSA recovery and isolates were characterized using molecular techniques, antimicrobial resistance and virulence profile. Four MRSA were isolated, all typed as t011-ST398. Antimicrobial resistances were observed (antimicrobial-resistance gene) penicillin/oxacillin/cefoxitin-*mecA/blaZ* (n=4), tetracycline-*tet(K)/tet(M)* (n=4), streptomycin-*aadE* (n=4), ciprofloxacin, with substitutions in GyrA (S84L) and GrlA (S80F) (n=3), gentamicin-*aac2'-aph6* (n=2), tobramycin-*ant4* (n=2) and clindamycin-*lnuB* (n=1). MRSA of lineage ST398 is worldwide spread, normally multidrug resistant and responsible for cow mastitis.

Key words: MRSA, ST398, *mecA*, *S. aureus*

Sponsorships: Capes - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Process - 9877-11-8.

Introduction

Bovine mastitis is a disease that causes economic prejudice in the dairy industry. *Staphylococcus aureus* is considered one of the most important pathogens in bovine clinical and subclinical mastitis (Kumar et al., 2011, Radtke et al., 2012).

The expression of the *mecA* gene confers methicillin resistance in *S. aureus* (MRSA) and also resistance to almost all type of β -lactams, agents frequently used for mastitis treatment (Sawant et al., 2005).

MRSA of the clonal complex CC398 has been observed in different geographical regions and is considered an important livestock-associated lineage (Fluit, 2012). In addition, it has been detected in human and other animal populations, such as bovine, horses, poultry and dogs (Nemati et al., 2008; van denEede et al., 2009; Floras et al., 2010; Feßler et al., 2012).

The objectives of this study were to analyze the prevalence of MRSA in milk of cows with mastitis and perform the molecular typing and genetic characterization of the isolates obtained.

Material and Methods

Origin of samples: A total of 400 samples of milk of 100 cows (one milk sample per mammary gland) from one farm in the State of São Paulo, Brazil, were evaluated. The California Mastitis Test (CMT) was used for the diagnosis of clinical and subclinical mastitis (Schalm and Noorlander, 1957). Mastitis was considered when at least one of the 4 milk samples obtained from each cow was positive by CMT, and all milk samples positive for mastitis were further studied for *S. aureus* recovery.

Bacterial isolates: Samples were plated on blood agar-5%, incubated at 37°C and readings were taken after 24, 48 and 72 hours of incubation. Characteristic colonies were preliminary identified using Gram staining, catalase, coagulase and DNase tests (Koneman et al., 2008). Molecular identification and methicillin resistance was performed by a multiplex PCR of the species-specific *nuc* gene and the methicillin-resistance genetic determinant *mecA* (Gómez-Sanz et al., 2010).

Molecular typing of MRSA strains: All MRSA isolates were characterized by *agr*-allotype, *spa*-typing, determination of staphylococcal cassette chromosome *mec* (SCC*mec*) and multi-locus sequence typing (MLST) by specific PCRs (Shopsin et al., 2003, <http://spaserver.ridom.de>; IWG-SCC, 2009, www.mlst.net).

Virulence gene profiling and immune evasion cluster (IEC): Presence of the genes encoding the Pantone-Valentine-Leukocidin (*lukF/lukS*), Toxic-Shock-Syndrome-Toxin 1 (*tst*), and Exfoliative-Toxin A (*eta*), and B (*etb*) was analyzed by PCR (Jarraud, 2002). Detection of genes of the IEC was analyzed as previously recommended (van Wamel, 2006).

Antimicrobial susceptibility testing and detection of resistance genes: penicilin, oxacilin, cefoxitin, tetracycline, erythromycin, clindamycin, gentamicin, tobramycin, streptomycin, trimethoprim-sulphamethoxazole, ciprofloxacin, and chloramphenicol were tested by disk-diffusion agar method (CLSI, 2012).

Detection of the following antimicrobial resistance genes (*mecA*, *blaZ*, *aadE*, *ant3(9)*, *str*, *tetK*, *tetM*, *tetL*, *aac2'*, *aph6*, *ant4*, *lnuA*, *lnuC*, *vgaA*, *vgaC*, *lnuB* and *lsaE*) was investigated by specific PCRs and in some cases by sequencing and analysis of amino acid substitutions (*gyrA*, *glaA*) (Gómez-Sanz et al., 2010).

In addition, mapping PCR was implemented to detect specific antimicrobial resistance gene clusters (Lozano et al., 2012). Positive and negative controls from the collection of the University of Rioja were used.

Results

Detection, isolation of *S. aureus* and molecular typing: 115 milk samples were positive for CMT and they were recovered from 36 of the 100 tested cows (36%). Thirty-two *Staphylococcus* spp. were isolated (from 18 different cows), and 15 of them were identified as *S. aureus* (from 8 cows). Four of these *S. aureus* isolates were MRSA, and they were detected in 4 cows (only one mammary gland was positive), representing 4% of tested animals and 11.1% of those with mastitis. The four MRSA isolates were further characterized.

The 4 MRSA isolates were typed as *spa*-type t011, which is associated to lineage ST398, of clonal complex CC398, and presented *agr* type I and *SCCmec* V.

Toxin gene profile, immune evasion cluster and antimicrobial resistance pheno- and genotypes: All MRSA isolates were negative for the tested toxin genes as well as for the genes of the IEC. All isolates exhibited a multidrug-resistant profile (resistance to at least three classes of antimicrobials). In addition to β -lactams, isolates were resistant to tetracycline (100%), streptomycin (100%), ciprofloxacin (75%), gentamicin (50%), tobramycin (50%), and clindamycin (25%). The resistance genes are shown in Table 1. The genetic determinant for streptomycin (*aadE*) resistance was only observed in one strain (C6129) (Table 1). In addition, this isolate harbored the clindamycin resistance gene *lnu*(B) and the novel *lsa*(E), that together with *aadE*, were enclosed within the

same antimicrobial gene cluster as that recently described for the first time in staphylococci (Lozano et al., 2012, Wendlandt et al., 2013a).

Table 1: Characterization of the MRSA isolates in this study

Isolate number	<i>spa-agr-MLST</i>	SCC <i>mec</i>	Resistance phenotype ^a	Resistance gene detected	Amino acid substitutions within the QRDR ^b of:	
					GyrA	GrIA
C5960	t011-I-ST398	V	PEN, OXA, FOX, TET, STR, CIP	<i>mecA, blaZ, tetK, tetM</i>	S84L	S80F
C6129	t011-I-ST398	V	PEN, OXA, FOX, TET, CLI, TOB, GEN, STR, CIP	<i>mecA, blaZ, tetK, tetM, ant4, aac2'aph6, [lnuB, lsaE, aadE]^c</i>	S84L	S80F + R44H
C6130	t011-I-ST398	V	PEN, OXA, FOX, TET, TOB, GEN, STR, CIP	<i>mecA, blaZ, tetK, tetM, ant4, aac2'aph6</i>	S84L	S80F
C6128	t011-I-ST398	V	PEN, OXA, FOX, TET, STR, CIP	<i>mecA, blaZ, tetK, tetM</i>	S84L	S80F

^a PEN: penicillin, OXA: oxacilin, FOX: ceftiofur, TET: tetracycline, CLI: clindamycin, TOB: tobramycin, GEN: gentamicin, STR: streptomycin, CIP: ciprofloxacin.

^b Quinolone Resistance Determining Region.

^c Genes expected to be physically linked based on PCR mapping and bibliography. The recently characterized *spw* gene was also detected within the cluster, based on mapping PCR.

Discussion

Mastitis is economically the most relevant disease of dairy cattle. Presence of MRSA as causative agent of this diseases is worrisome because the cow does not present symptoms in subclinical mastitis and milk can be marketed.

Few studies have already detected MRSA that belong to lineage ST398 in milk from bovine mastitis (Feßler et al., 2010; Vanderhaeghen et al., 2010).

MRSA ST398 has obtained special attention as colonizers and causative agents of infections in pigs (Gómez-Sanz et al., 2010; Fluit et al., 2012). Further reports have

shown that ST398 isolates are not restricted to these animals, but can also be isolated from humans, bovines, poultry, horses and dogs (Nemati et al., 2008; Floras et al., 2010; Feßler et al., 2010; Lozano et al., 2011, van den Eede et al., 2009; Fluit et al., 2012). MRSA of this lineage is considered an important zoonotic clone, given that transmission between different animal hosts and humans has been suggested in numerous occasions (Lozano et al., 2011; Feßler et al., 2012; Fluit et al., 2012).

None of the strains observed in this study harbored any of the virulence genes studied, or any of the human-associated IEC genes, which is a common characteristic of MRSA isolates of this lineage (Fluit et al., 2012). In contrast, all isolates were multidrug-resistant and exhibited tetracycline resistance, also typical of MRSA ST398 (Feßler et al., 2010; Gómez-Sanz et al., 2010; Fluit et al., 2012). Tetracycline resistance in MRSA ST398 has been reported as a consequence of the extensive use of this antimicrobial in livestock and seems to be endemic in MRSA of this lineage (Lozano et al., 2012; Fluit et al., 2012).

One strain presented *lnu*(B) and the recently characterized *lsaE* genes, both of which confer resistance to lincosamides (Wendlandt et al., 2013a). The genetic environment of these two genes revealed to be identical to that recently described for the first time in staphylococci in few Spanish MRSA ST398 isolates (Lozano et al., 2012). Interestingly, this cluster also carries a recently characterized spectinomycin resistance gene, designated *spw* (Wendlandt et al., 2013b), and evidences the antimicrobial resistance acquisition capacities of geographically-distinct MRSA ST398 isolates.

To our knowledge, this is the first detection of MRSA ST398 from bovine in Brazil. The resistance gene properties of the investigated isolates are relevant, due to its

capacity to be present in various animal and human hosts. More epidemiological studies on MRSA on animals should be conducted for a better understanding on the transmission routes of MRSA of different lineages among animals and humans.

Conclusion

MRSA was isolated in 4% of the initial investigated animals and in 11.1% of those animals with mastitis (by CMT). All MRSA isolates belonged to the lineage ST398, were multidrug-resistant and lacked important virulence genes. In Brazil, epidemiological studies of *S. aureus* in cattle are scarce, but are essential to gain knowledge on the circulating lineages responsible for cow mastitis in this milk producer country. This is the first description on MRSA ST398 in milk of cows with mastitis in Brasil.

Acknowledgments

Silva N. C. C. has a fellowship from Capes-Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Process - 9877-11-8. Part of this work was financially supported by Project SAF2012-35474 from the Ministerio de Economía y Competitividad of Spain.

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*Capítulo 111. Characterization of methicillin-
resistant coagulase-negative staphylococci in milk
from cows with mastitis in Brazil*

Artigo submetido como Short communication à revista

Veterinary Microbiology

Characterization of methicillin-resistant coagulase-negative staphylococci in milk from cows with mastitis in Brazil

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Abstract

Staphylococci are one of the most prevalent microorganisms in bovine mastitis. *Staphylococcus* spp is widespread in the environment, can infect animals and humans as saprophytic or pathogens. The objective of this study was to determine the frequency of methicillin-resistant coagulase-negative staphylococci (CNS) in milk of cows with mastitis in Brazil and to characterize their antimicrobial resistance profile and their SCC*mec*. Milk samples of 4684 mammary glands of 1171 cows obtained from 11 different farms in the state of São Paulo, Brazil, were evaluated and 1484 (31.7%) milk samples were positive for mastitis, by California Mastitis test. Identification of methicillin-resistant CNS was based on biochemical and molecular methods. Susceptibility testing for 11 antimicrobials was performed by disk-diffusion agar. Antimicrobial resistance genes and SCC*mec* were investigated by specific PCRs. Twenty-seven *mecA* positive CNS were detected (1.8% of samples) from 24 animals: *S. chromogenes* (7 isolates), *S. epidermidis* (8), *S. hyicus* (5), *S. warneri* (6) and *S. simulans* (1). The SCC*mec* IVa was identified in 10 *mecA* positive CNS, while the remaining 17 isolates harbored non-typeable SCC*mec*. In addition to oxacilin and cefoxitin resistance, CNS showed resistance to tetracycline (n=8), streptomycin (n=6), tobramycin (n=6), and gentamicin (5), and harbored the genes: *tetK* (n=8), *aac2'**aph6'* (n=5), *ant4* (n=6), and *str* (n=3). One isolate presented intermediate erythromycin resistance and harbored an *ermC* gene with an uncommon 89 bp deletion. *mecA* positive CNS can be implicated in cow mastitis and they constitute a reservoir of resistance genes that can be transferred to other pathogenic bacteria.

Key words: *mecA*, *Staphylococcus*, *ermC*, resistance

Introduction

Staphylococcus have 47 species and 24 subspecies and the coagulase-negative *Staphylococcus* (CNS) are the majority in the group (DSMZ, 2012). The most important and pathogenic species in this genus is *S. aureus*, but the CNS, increasingly, have a special attention due to many reports about the involvement of this species in human and animal infections (Feßler et al., 2010, Guimarães et al., 2013; Cui et al., 2013).

CNS are present on the body of cow, and is very common in teat apices (Taponen et al., 2008), also is an opportunistic bacteria that can cause subclinical or clinical mastitis in cows (Taponen and Pyörälä. 2009). Several studies have reported the isolation of *Staphylococcus* sp. from samples of cow milk with mastitis (Santos et al., 2008; Feßler et al., 2010; Guimarães et al., 2013). CNS can be involved in mastitis infection, resulting on reduced production of milk and its quality, causing the most important economic losses in the dairy industry (Feßler et al., 2010). Besides, the multi resistance in CNS is common (Nam et al., 2010, Viridis et al, 2010) and worrying because, as a reservoir, they can also transfer antimicrobial resistance genes to *S.aureus* (Archer and Climo, 1994).

The CNS can produce biofilm that compromises the efficacy of intramammary antibiotic treatment (Viridis et al., 2010). The polysaccharide intercellular adhesion production (PIA) is mediated by locus *ica* (intercellular adhesion), encoding *icaA*, *icaB*, *icaC* and *icaD* genes (Ziebuhr et al. 1999).

The objective of this study was to determine the frequency of methicillin-resistant coagulase-negative staphylococci (CNS) in milk of cows with mastitis in Brazil and to characterize their antimicrobial resistance profile and their SCC*mec*.

Material and methods

Origin of samples

A total of 1171 cows from 11 different farms in the state of São Paulo, Brazil were evaluated. In total, 4684 samples (one per mammary gland) were obtained and 1484 milk samples were positive for mastitis. The methodology and interpretation criteria used for diagnosis of clinical and subclinical mastitis were based on examination of animals before each milking by the California Mastitis Test - CMT (Guimarães et al., 2013). Samples were collected in sterile tubes after disinfection of the zone (ostium) with iodized alcohol (2.5%), and they were transported to the laboratory under refrigeration (4-8°C) in cool boxes with ice packs.

Bacterial isolates.

In laboratory, the samples with two crosses or more in CMT were also submitted to Somatic Cell Count (SCC) in order to confirm the infection. This analysis was performed by flow cytometry, with Somacount 300 (Bentley Instruments).

The samples were plated on blood agar (5%), and incubated under aerobic conditions at 37°C and readings taken after 24, 48 and 72 hours of incubation. Identification CNS species was based on colony morphology, Gram staining, catalase, coagulase and biochemical tests (Koneman et al., 2008) and molecular methods (Poyart et al., 2001).

Antimicrobial susceptibility testing and detection of resistance genes and SCCmec

Antimicrobial susceptibility testing to oxacillin, ceftiofur, tetracycline, erythromycin, clindamycin, gentamicin, tobramycin, streptomycin, trimethoprim-sulphamethoxazole, ciprofloxacin, and chloramphenicol was performed by disk-diffusion agar method in accordance with the Clinical and Laboratory Standards Institute recommendations (CLSI, 2012). Detection of antimicrobial resistance genes and

SCCmec was investigated in resistant and intermediate isolates by specific PCRs (Gómez-Sanz et al., 2010, Wendlandt, 2012). Positive and negative controls from the collection of the University of La Rioja were used in each PCR assay.

Investigation of ica operon

The investigation of operon *ica* were performed by PCR in accord of Ziebuhr et al. (1999).

Results

CNS were detected in 142 out 1484 milk samples (9.6%) and 27 (1.8%) were positive for *mecA*, from 24 animals. It was isolated 8 strains of *S. epidermidis* 7 of *S. chromogenes*, 6 of *S. warneri*, 5 of *S. hyicus* and 1 *S. simulans*.

The SCCmec IVa was identified in 10 *mecA* positive CNS, while the remaining 17 isolates harbored non-typeable SCCmec, but 2 presented *ccr2* and 1 *ccr1*. In addition to oxacilin and cefoxitin resistance, CNS showed resistance to tetracycline (8), streptomycin (6), tobramycin (7), and gentamicin (5), and harbored the genes: *mecA* (27), *tetK* (8), *aac2'aph6'* (5), *ant4* (6), and *str* (3), *lnuB*, *lsaE* (Table 1). One strain showed an intermediate resistance phenotype to both erythromycin and clindamycin, harbored the deletion in *ermC* gene.

PCR of the *erm(C)* gene revealed an amplicon slightly smaller than the expected size (89bp). Sequencing of such amplicon revealed a deletion of 89bp within the *ermC* gene, what may explain the low level of resistance to ery-cli (Figure 1).

Table 1: Phenotype and genotype profile of resistance in CNS, isolated from subclinical and clinical mastitis in Brazil

Specie	Resistance phenotype (isolates)	Resistance Genotype (isolates)	SCCmec
<i>S. chromogenes</i>	OXA (7), FOX (7), CC ^I (2), TET (1), STR (1), TOB ^I (1)	<i>mecA</i> (7) , <i>InuB</i> (2), <i>tetK</i> (1), <i>ant4</i> (1), <i>IsaE</i> (2)	non-typeable (5), Iva (1), non-typeable with <i>ccr2</i> (1)
<i>S. hycus</i>	OXA (5), FOX (5), CC ^I (3), TET (1), STR (1)	<i>mecA</i> (5) , <i>str</i> (1)	non-typeable (5)
<i>S. epidermidis</i>	OXA (8), FOX (8), CC ^I (2), TET (5), STR (2), TOB (3), GEN (4), TOB ^I (1)	<i>mecA</i> (8) , <i>tetK</i> (5), <i>ant4</i> (4), <i>aac2'</i> <i>aph6'</i> (4), <i>str</i> (1), <i>ermC</i> (1)*	non-typeable (1), Iva (7)
<i>S. warneri</i>	OXA (6), FOX (6), CC ^I (1), TET (2), STR (2), TOB (1), GEN (1), TOB ^I (1)	<i>mecA</i> (6) , <i>tetK</i> (2), <i>ant4</i> (2), <i>aac2'</i> <i>aph6'</i> (1), <i>str</i> (1)	non-typeable (2), Iva (2), non-typeable with <i>ccr2</i> (1), non-typeable with <i>ccr1</i> (1)
<i>S. simulans</i>	OXA (1), FOX (1)	<i>mecA</i> (1)	non-typeable

OXA: oxacilin, FOX: cefoxitin, STR: streptomycin, TET: tetracycline, CIP: ciprofloxacin, PEN: penicillin, CC: clindamycin, TOB: tobramycin, GEN: gentamycin.

* deletion of 89 bp, ^I: Intermediate resistance

Figure 1: Comparison of sequence observed and genbank (*Staphylococcus aureus* strain 7504026-1 plasmid ErmC (*erm(C)*) gene, complete cds)

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deletionermC      1 ----- 0
strain7504026    1 TTATACTAATTTTATAAGGAGGAAAAAATATGGGCATTTTTAGTATTTTT 50
deletionermC      1 ----- 0
strain7504026    51 GTAATCAGCACAGTTCATTATCAACCAAACAAAAATAAGTGGTTATAAT 100
deletionermC      1 ----- 0
strain7504026    101 GAATCGTTAATAAGCAAATTCATTATAACCAAATTAAGAGGGTTATAA 150
deletionermC      1 ----- 0
strain7504026    151 TGAACGAGAAAAATATAAAACACAGTCAAACCTTTATTACTTCAAACAT 200
deletionermC      1 -----aactataaggaTTTAATTGATCATGATAAT 30
strain7504026    201 AATATAGATAAAAATAATGACAAATATAAG--ATTAAATGAACATGATAAT 248
deletionermC      31 ATCTTTG-AATCGGCTCAGGAAAAGGGCATTTTACCCTTGAATTAGTACA 79
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strain7504026	249	ATCTTTGAAATCGGCTCAGGAAAAGGGCATTTTACCCCTGAATTAGTACA	298
deletionermC	80	GAGGTGTAATTTTCGTAAGTCCATTGAAATAGACCATAAAATTATGCAAAA	129
strain7504026	299	GAGGTGTAATTTTCGTAAGTCCATTGAAATAGACCATAAAATTATGCAAAA	348
deletionermC	130	CTACAGAAAATAAACTTGTGATCACGATAATTTCCAAGTTTAAACAAG	179
strain7504026	349	CTACAGAAAATAAACTTGTGATCACGATAATTTCCAAGTTTAAACAAG	398
deletionermC	180	GATATATTGCAGTTTAAATTTCTAAAAACCAATCCTATAAAAATTTGG	229
strain7504026	399	GATATATTGCAGTTTAAATTTCTAAAAACCAATCCTATAAAAATTTGG	448
deletionermC	230	TAATATACCTTATAACATAAGTACGGATATAATACGCAAAATGTTTTTG	279
strain7504026	449	TAATATACCTTATAACATAAGTACGGATATAATACGCAAAATGTTTTTG	498
deletionermC	280	ATAGTATAGCTGATGAGATTTATTTAATCGTGAATACGGGTTTGCTAAA	329
strain7504026	499	ATAGTATAGCTGATGAGATTTATTTAATCGTGAATACGGGTTTGCTAAA	548
deletionermC	330	AGATTATTAATAACAAAACGCTCATTGGCATTATTTTTAATGGCAGAAGT	379
strain7504026	549	AGATTATTAATAACAAAACGCTCATTGGCATTATTTTTAATGGCAGAAGT	598
deletionermC	380	TGATATTTCTATATTAAGTATGGTTCCAAGAGAATATTTTCATCCTAAAC	429
strain7504026	599	TGATATTTCTATATTAAGTATGGTTCCAAGAGAATATTTTCATCCTAAAC	648
deletionermC	430	CTAAAGTGAATAGCTCACTTATCAGATTAATAGA-----	464
strain7504026	649	CTAAAGTGAATAGCTCACTTATCAGATTAATAGAAAAATCAAGAATA	698
deletionermC	465	-----	464
strain7504026	699	TCACACAAAGATAAACAGAAAGTATAATTATTCGTTATGAAATGGGTAA	748
deletionermC	465	-----AAAAATCAATTTAACAATTCCTTAA	490
strain7504026	749	CAAAGAATACAAGAAAATATTTACAAAAATCAATTTAACAATTCCTTAA	798
deletionermC	491	AACATGCAGGAATTGACGATTTAAACAATATTAGCA-----	526
strain7504026	799	AACATGCAGGAATTGACGATTTAAACAATATTAGCTTTGAACAATTCTTA	848
deletionermC	527	----- 526	
strain7504026	849	TCTCTTTTCAATAGCTATAAAATTATTTAATAAGTAA 884	

Discussion

Mastitis is a problem for dairy industry and the antibiotic resistance of the bacteria that cause this disease should be study for designates the better treatment. Among the *Staphylococcus* spp., *S. aureus* is the most important pathogen and the coagulase-negative staphylococci isn't very studied.

But, it is increasingly common coagulase-negative staphylococci in mastitis cow despite be considered an opportunistic pathogen. In Brazil, some studies related the presence of these species in milk of mastitis bovine (Nascimento et al., 2005, Guimarães et al., 2013).

The biofilm production can interfere in the antibiotic treatment. In this study the strains didn't present the locus *ica*, a important *cluster* of biofilm production (Viridis et al., 2010).

Our study observed low percent of methicillin-resistant coagulase-negative staphylococci, but the strains presented resistance to others antibiotics, with exception of *S. simulans*. In others studies about bovine milk were observed species of CNS as *S. chromogenes*, *S. epidermidis*, *S. hycus*, *S. simulans* with a multi-resistance profile (Sawant et al. 2009, Sampimon et al., 2011).

The SCCmec typing was not possible on the majority of strain, but others studies also presented nontypeable CNS (Barbie et al., 2010; Zong et al., 2011). This suggest that is necessary a nomenclatura of SCCmec for CNS or more specific primers. The SCCmec IVa is preferentially associated with *S. epidermidis* as were observed in this study (Feßler et al., 2010; Zong et al., 2011).

Two strains presented resistance of clindamycin, by the presence of *lnu(B)* and the recently characterized *lsaE* genes. These genes confer resistance to lincosamides (Wendlandt et al., 2012). Lozano et al. (2012) described recently the genetic environment of these two later genes for the first time in staphylococci (MRSA ST398) in Spain, and our strains of *S. chromogenes* revealed to be identical of this.

Erythromycin is a macrolide antibiotic witch blocks the protein synthesis (Spížek & Rezanka, 2004). One strain was intermediary to erytromicin on the disk test and presented a deletion in *ermC* gene that can interfere in the resistance of this

antibiotic. It is interesting to remark that one MR *S. epidermidis* presented a deletion in the central part of the *ermC* gene that still conferred intermediate resistance to ery-cl. This result evidences partially deleted *ermC* genes can be still functional.

In Brazil, there are few studies about resistance of *Staphylococcus* spp. in milk of bovine mastitis but is necessary more studies for characterization of these species and improve the control and treatments of mastitis.

Conclusion

Although coagulase-negative staphylococci is not the principal pathogen of mastitis, they need a special attention because they can be implicated in cow mastitis. In this study, the strains presented much resistance and important genes as *lnuB*, *lsaE* and the deletion in *ermC*. Furthermore, CNS constitutes a reservoir of resistance genes that can be transferred to other pathogenic bacteria.

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4. CONCLUSÕES

Em geral, as cepas de *S.aureus* resistentes a metilina apresentaram perfil de resistência maior a outros antibióticos que os sensíveis. Genes de resistência importantes e pouco relatados foram detectados como *fexA*, *lsaE*, *lnuB*. Foram detectados dois novos *spatyping* (t10852 e t10856), bem como um novo alelo *yqiL* e um novo ST (2493). Os ECNs apresentaram resistências à maioria dos antibióticos estudados e foi observado a deleção do gene *ermC* que codifica a resistência a eritromicina.

Apêndice

Figura 1: Multiplex para os genes 16s, mecA e nuc (*S.aureus*)

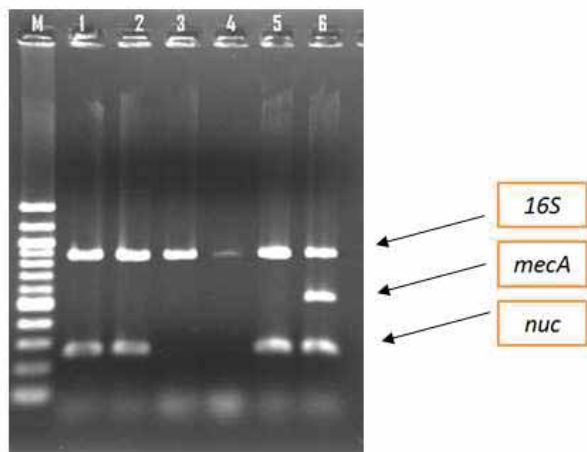


Figura 2: Perfil de resistência a Tobramicina



Figura 3: Perfil de resistência a oxacilina, ceftoxitina, gentamicina, penicilina, estreptomicina

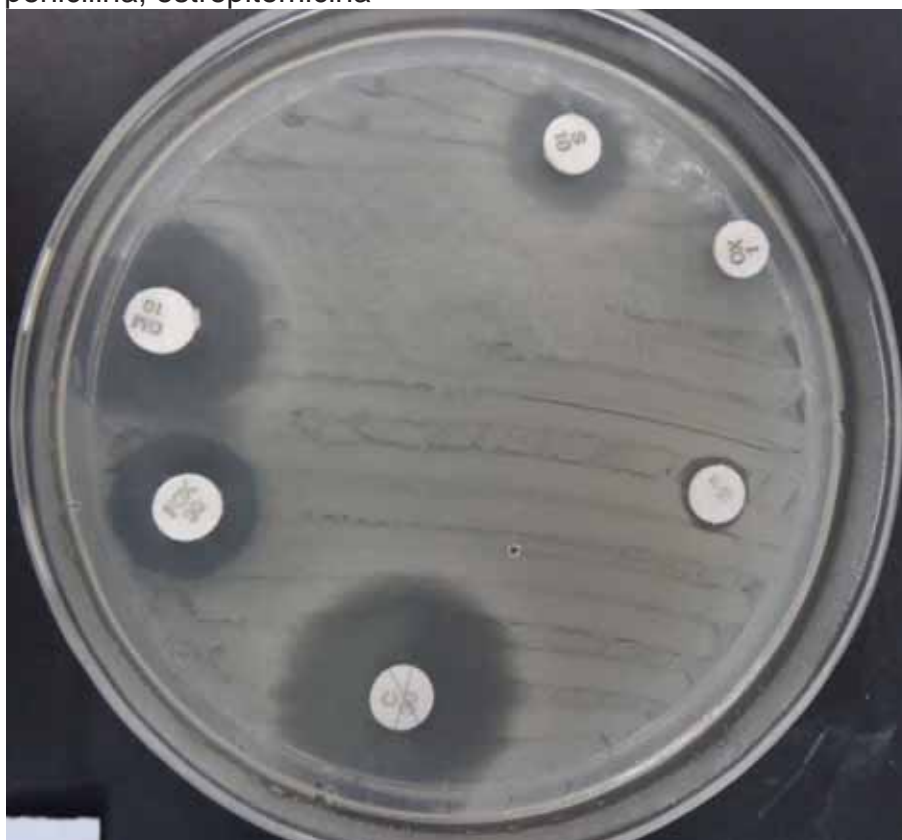


Figura 4: Perfil de resistência a tetraciclina e ciprofloxacina



Figura 5: Perfil de resistência a clindamicina, lincosamida

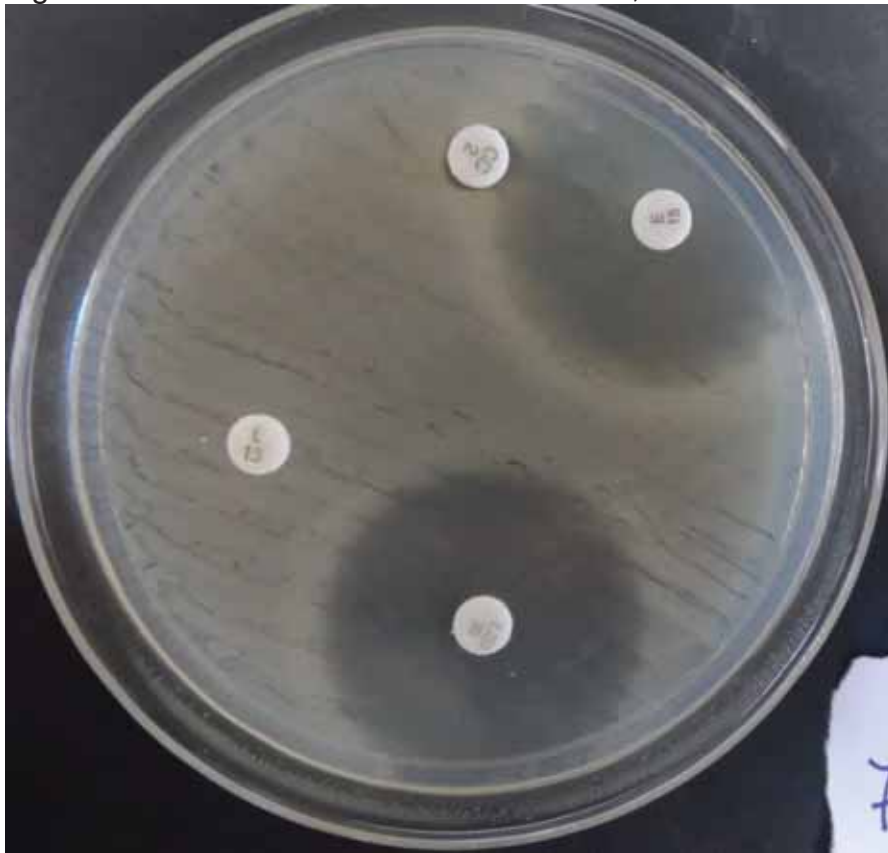


Figura 6: PFGE

