

**UNIVERSIDADE FEDERAL DA PARAÍBA
CENTRO DE CIÊNCIAS AGRÁRIAS**

**SIMILARIDADE GENÉTICA E PADRÕES DE RESISTÊNCIA
ANTIMICROBIANA DE *Staphylococcus aureus* DE EQUINOS
SADIOS EM DIFERENTES REGIÕES DO BRASIL**

**Mauro de Mesquita Souza Saraiva
Médico Veterinário**

2015

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Mauro de Mesquita Souza Saraiva

Orientador: Celso José Bruno de Oliveira

Coorientador: Prof. Dr. Danilo Tancler Stipp

Coorientador: Dr. Denis Augusto Spricigo

Dissertação apresentada ao Programa de Pós-Graduação em Ciência Animal do Centro de Ciências Agrárias da Universidade Federal da Paraíba, como parte das exigências para obtenção do título de Mestre em Ciência Animal. Área de Concentração Saúde Animal do brejo paraibano

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APROVADA EM 25/02/2015

BANCA EXAMINADORA

Prof. Dr. Danilo Tancler Stipp
DCV/CCA/UFPB
Orientador

Profa. Dra. Suzana Aparecida Costa de Araújo
DCV/CCA/UFPB
Examinador

Dr. Felício Garino Junior
PPGMV/UFCG
Examinador

DADOS CURRICULARES DO AUTOR

MAURO DE MESQUITA SOUZA SARAIVA - Nascido em 19 de fevereiro de 1985, possui graduação em Medicina Veterinária pela Universidade Federal da Bahia (2009). Durante a graduação, foi estagiário bolsista da Secretaria de Administração da Bahia, vinculado ao Esquadrão de Polícia Montada do Estado da Bahia. Atuou como Médico Veterinário autônomo em clínica de equídeos e animais de companhia. Em 2013 ingressou no Programa de Pós-Graduação em Ciência Animal na área de Medicina Veterinária Preventiva sob a orientação do professor Dr. Celso José Bruno de Oliveira.

Epígrafe

“Família é quem você escolhe pra viver
Família é quem você escolhe pra você
Não precisa ter conta sanguínea
É preciso ter sempre um pouco mais de sintonia.”

Marcelo Yuka

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Sozinhos, nem sonhos se concretizam... Algumas pessoas foram muito importantes na construção e realização de mais um sonho em minha vida:

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**SIMILARIDADE GENÉTICA E PADRÕES DE RESISTÊNCIA
ANTIMICROBIANA DE *Staphylococcus aureus* DE EQUINOS SADIOS EM
DIFERENTES REGIÕES DO BRASIL**

RESUMO GERAL

Staphylococcus aureus é um dos mais importantes patógenos em medicina veterinária e o crescente surgimento de cepas resistentes a diversas classes de antimicrobianos eleva a necessidade de uma correta identificação e monitoramento desta espécie bacteriana. Este trabalho objetivou inicialmente avaliar a acurácia da PCR para os marcadores dos genes *nuc*, *femA* e *coa* na identificação de *S. aureus* obtidos de amostras de diferentes espécies animais. Foram analisados um total de 71 isolados de *Staphylococcus* spp. através do MALDI-TOF e foram identificadas 12 espécies de *Staphylococcus* diferentes. Dos *Staphylococcus aureus* (34) os genes *femA*, *nuc* e *coa* foram identificados em 30, 26 e 16 dos isolados, respectivamente. Embora a especificidade da PCR para todos os marcadores testados tenha sido de 100%, a sensibilidade encontrada foi de 88,2, 76,5 e 46,5 para os marcadores *femA*, *nuc* e *coa*, respectivamente. A sensibilidade foi de 100% quando utilizado os marcadores *femA* e *nuc* simultaneamente. Os resultados confirmam a PCR como uma técnica precisa para identificação de *S. aureus* e sugere o uso simultâneo dos *primers femA* e *nuc*. Posteriormente objetivou-se avaliar os perfis de resistência antimicrobiana e a relação genotípica entre *S. aureus* isolados a partir de fossas nasais de equinos sadios oriundos de dois Estados geograficamente distantes do Brasil (Paraíba e Rio Grande do Sul). De uma total de 123 *Staphylococcus* spp. analisados inicialmente, 21 isolados foram identificados como *Staphylococcus aureus* por testes bioquímicos e PCR utilizando-se marcadores espécie-específicos (*nuc*, *femA* e *coa*). Os resultados demonstraram quatro perfis de resistência diferentes. Foi observada multirresistência em 10 isolados de *S. aureus*. Embora nenhum isolado tenha sido encontrado carregando o gene *mecA*, seis isolados resistentes a meticilina foram encontrados, indicando a presença de *Staphylococcus aureus* meticilina-resistente (MRSA). A genotipagem por Rep-PCR demonstraram que os isolados não foram agrupados por sua origem geográfica, e indicam uma elevada diversidade de cepas de *Staphylococcus aureus* colonizando cavalos sadios no Brasil.

Palavras-Chave: Identificação de *Staphylococcus*, MALDI-TOF, MRSA, Multirresistencia, Rep-PCR

**GENETICS SIMILARITY AND ANTIMICROBIAL RESISTANCE PATTERNS OF
Staphylococcus aureus IN HEALTHY HORSES FROM DIFFERENT REGIONS OF
BRAZIL**

ABSTRACT

Staphylococcus aureus is a major pathogen in veterinary medicine and the increasing emergence of strains resistant to different classes of antimicrobial agents elevates the need for accurate identification and monitoring of this bacterial species. This study aimed to initially evaluate the accuracy of PCR for *nuc*, *femA* and *coa* genes for identification of *S. aureus* obtained from different animal species samples. A total of 71 *Staphylococcus* spp. isolates were analyzed through the MALDI-TOF and were identified 12 different species. Among *Staphylococcus aureus* (34) the *femA*, *nuc* and *coa* genes were identified in 30, 26 and 16 isolates respectively. Although the specificity of the PCR for all markers tested was 100%, the sensitivity was found 88.2%, 76.5% and 46.5% for *femA*, *nuc* and *coa* markers, respectively. The sensitivity was 100% when used the *femA* and *nuc* markers simultaneously. The results confirm the PCR technique as a accurate for *S. aureus* identification and suggests the simultaneous use of *femA* and *nuc primers*. Later aimed to evaluate the antimicrobial resistance patterns and genotypic relationship between *S. aureus* cultured from nasal cavities of healthy horses from two geographically distant states of Brazil (Paraíba and Rio Grande do Sul). A total of 123 *Staphylococcus* spp. analyzed initially, 21 isolates were identified as *Staphylococcus aureus* by biochemical tests and PCR using species-specific markers (*nuc*, *femA* and *coa*). The results showed four different resistance patterns. Multidrug resistance was observed in 10 *S. aureus* isolates. Although none *S. aureus* has been found harboring *mecA* gene, 6 methicillin-resistant isolates were found, indicating the presence of methicillin-resistant *Staphylococcus aureus* (MRSA). Genotyping by Rep-PCR showed that the isolates were not grouped by geographical origin, and indicate an enormous diversity of *Staphylococcus aureus* strains colonizing healthy horses in Brazil.

Keywords: MALDI-TOF, MRSA, Multiresistance, Rep-PCR, *Staphylococcus* identification.

INTRODUÇÃO

Bactérias do gênero *Staphylococcus* são comumente relacionados a doenças nosocomiais e infecções oportunistas em homens e animais. Colonizam pele e mucosas de mamíferos e aves, e podem causar infecções, normalmente resultante de baixas na imunidade do hospedeiro.

As bactérias deste gênero são divididas em dois grandes grupos de acordo com a produção da enzima coagulase: *Staphylococcus* coagulase-positivos (CoPS) e coagulase-negativo (CoNS). Destes, o grupo dos CoPS reúne as espécies mais patogênicas, sendo o *Staphylococcus aureus* (*S. aureus*) o mais patogênico do gênero e é capaz de adquirir resistência a diversos antimicrobianos utilizados em medicina humana e animal.

A identificação de *Staphylococcus* spp. é realizada por testes bioquímicos convencionais, sistemas comerciais e kits automatizados. Porém a ausência de marcadores fenotípicos específicos bem como as recentes mudanças na taxonomia de alguns *Staphylococcus* spp. dificultam a precisa identificação das espécies deste gênero. A reação em cadeia da polimerase (PCR), utilizando marcadores espécie-específicos e mais recentemente o uso de análise proteômica por espectrometria de massa (MALDI-TOF) vem sendo utilizados como alternativas de maior acurácia na identificação de diversos gêneros bacterianos.

Resistência a antimicrobianos é comumente encontrada em *Staphylococcus* spp., sobretudo a classe dos beta-lactâmicos, incluindo as oxacilinas e meticilinas. A resistência a esta classe deve-se por dois mecanismos principais: produção da enzima beta-lactamase, codificada pelo gene *blaZ*, e produção de proteína ligante de penicilina de baixa afinidade (*pbp 2a*), codificada pelo gene *mecA*.

Estudos comprovam que bactérias do gênero *Staphylococcus* spp., são frequentemente encontradas colonizando equinos saudáveis. Ao serem submetidos à situação de estresse, cavalos podem ser infectados por *Staphylococcus* spp. sobretudo as cepas resistentes, desenvolvendo desde lesões cutâneas localizadas a doenças sistêmicas graves, como endocardites e laminites.

Assim, o presente estudo objetivou, primeiramente, investigar a acurácia da PCR na identificação de *Staphylococcus aureus*, utilizando três marcadores específicos (*nuc*, *femA* e *coa*). Posteriormente investigar a resistência antimicrobiana e a similaridade genotípica de *Staphylococcus aureus* coletados de equinos saudáveis em diferentes regiões do Brasil.

FUNDAMENTAÇÃO TEÓRICA

O Gênero *Staphylococcus*

O gênero *Staphylococcus* pertence à família *Staphylococcaceae*, compreende bactérias Gram positivas em formatos de cocos que se agrupam usualmente em forma de cachos. São aeróbios facultativos, imóveis e não esporulados. Caracterizam-se por produzirem a enzima catalase, além de apresentarem diversos fatores de virulência como adesinas, cápsula, enzimas extracelulares, hemolisinas, proteína A, dentre outras (SPANU et al., 2012).

Bactérias do gênero *Staphylococcus* são habitantes naturais de pele e mucosas de homens e outros mamíferos, onde ocasionalmente são encontradas causando infecções oportunistas, exigindo cuidados no controle desses patógenos em hospitais, sejam humanos ou veterinários (GRIFFETH et al., 2008; WEESE, 2012).

As mais de 40 espécies desse gênero estão divididas quanto à produção da enzima coagulase em dois grandes grupos: estafilococos coagulase positivos (CoPS) e *estafilococos coagulase negativos* (CoNS). A coagulase é responsável por formar coágulos de fibrina ao redor das células bacterianas, dificultando a ação das células de defesa do hospedeiro (LUCE, 2010).

Dentre os CoPS incluem-se as espécies mais patogênicas do gênero, como o *Staphylococcus aureus*, *S. intermedius*, *S. pseudointermedius* e *S. hyicus*. Embora estas espécies possam apresentar especificidade em relação ao hospedeiro de onde são isoladas, é comum o isolamento de mais de uma espécie advinda de um mesmo hospedeiro, causando ou não infecções (PANTOSTI, 2012; SPOOR et al., 2013).

O *Staphylococcus aureus* (*S. aureus*) é um típico exemplar dos CoPS, considerado a espécie mais patogênica do gênero, causando desde pequenas lesões cutâneas a severas infecções sistêmicas (JARRAUD et al., 2002). É também a espécie mais associada a infecções bacterianas em humanos (SAKAI et al., 2004), mastites em bovinos (CIFTICI, et al., 2009), sinovites infecciosas em cavalos (WAUTERS et al., 2012) e ceratites em cães (TAJIMA et al., 2013). O *S. aureus* é capaz de desenvolver resistência a diversos antimicrobianos utilizados na clínica, dificultando controle e tratamento da infecção, podendo levar o paciente a óbito (PANTOSTI, 2012).

Identificação de *Staphylococcus*

A identificação das espécies de *Staphylococcus* é geralmente feita com base em suas características fenotípicas, iniciando pela coloração de Gram, realização das provas da catalase e oxidase para confirmação do gênero e teste da coagulase em tubo para distinção entre CoPS e CoNS. A identificação das espécies de *Staphylococcus* spp. é tradicionalmente realizada a partir de fermentação de açúcares e provas bioquímicas. Contudo, algumas espécies apresentam comportamento atípico, o que os torna um problema para correta identificação da espécie (LUCE, 2010).

Como alternativa, *kits* comerciais e sistemas automatizados têm sido utilizados por diversos laboratórios de microbiologia. No entanto, a interpretação dos resultados pode ser subjetiva, levando a erros, assim como o limitado número de testes bioquímicos que reduz consideravelmente a quantidade de espécies detectáveis (ZADOKS; WATTS, 2009).

O surgimento de novas espécies (DEVRIESE et al., 2005), reclassificação de outras já existentes (SASAKI et al., 2007), bem como a importância clínica de um diagnóstico rápido e preciso, fez com que os marcadores moleculares ganhassem cada vez mais espaço na rotina de diagnóstico, sendo a detecção dos mesmos considerada alternativa específica e sensível (BLAIOTTA et al., 2010).

Dentre os métodos de identificação de marcadores moleculares, destaca-se a reação em cadeia da polimerase (*Polymerase Chain Reaction*), conhecida como PCR. Muitos marcadores estão descritos na literatura para identificação de espécies do gênero *Staphylococcus*, sobretudo genes específicos de *S. aureus*, tais como o *nuc* (CIFTCI et al., 2009), *femA* (MEHROTRA; WANG; JOHNSON, 2000) e *coa* (HOOKEY; RICHARDSON; COOKSON, 1998).

A Espectrometria de Massa por Tempo de Voo de Ionização (MALDI-TOF) vem sendo descrita como uma excelente técnica para identificação de diferentes microorganismos a nível de espécie. Esta técnica apresenta como vantagens o baixo custo, obtenção de resultados em pouquíssimo tempo e baixo níveis de resíduos gerados (ALATOOM et al., 2011). O MALDI-TOF produz resultados de elevada acurácia na identificação de espécies em diversos genes bacterianos e por isso vem sendo introduzida em laboratórios de diagnósticos humanos e animais com excelente custo-benefício e abordagem rápida e precisa (BANNOEHR; GUARDABASSI, 2012, PIGNONE et al., 2006).

Resistência a Beta-Lactâmicos

Os *Staphylococcus* spp. são comumente relacionados à resistência a antimicrobianos, principalmente devido ao uso indiscriminado e sem critérios de prescrição destes fármacos (FIOL et al., 2010), tanto em seres humanos quanto em animais, comprometendo a eficiência do tratamento (BARBERIO et al., 2002).

A classe de antimicrobianos mais utilizada é a dos beta-lactâmicos. A este grupo pertencem todos os fármacos, naturais ou sintéticos, que possuem o anel beta-lactâmico em sua formulação. Dentre as diversas famílias, destaca-se a penicilina, cujas mais utilizadas são a penicilina G, ampicilina, amoxicilina e oxacilina (LOWY, 2003).

Nos anos 40, infecções causadas por *Staphylococcus*, em diferentes órgãos do corpo, eram completamente tratadas com o uso de penicilina. Porém em aproximadamente dois anos após a introdução dessa droga foram isoladas cepas resistentes (CASSETTARI; STRABELLI; MEDEIROS, 2005). Este fato levou ao desenvolvimento de outros beta-lactâmicos, como por exemplo, a meticilina, fármaco projetado para ser eficaz contra cepas resistentes. Contudo, em pouco tempo, também foram identificados isolados capazes de resistirem à droga, os quais receberam a denominação de MRSA, sigla oriunda do inglês, *methicillin resistant Staphylococcus aureus* (MRSA) (ROLINSON, 1961).

No ano de 1972 foi identificado o primeiro caso de MRSA obtido a partir de um animal. A bactéria foi isolada do leite de uma vaca com mastite (DEVRIESE; VAN DAMME; FAMEREE, 1972). Este achado pode ter ocorrido a partir de transferência horizontal da mão de ordenhadores ou mal uso de antimicrobianos no tratamento de mastites e doenças de pele (FIOL et al., 2010; MORGAN, 2008).

A resistência aos beta-lactâmicos ocorre por dois mecanismos distintos: produção da enzima beta-lactamase e produção da proteína ligante de penicilina adicional de baixa afinidade (PBP2a), codificadas pelos genes *blaZ* e *mecA*, respectivamente. A PBP2a, por possuir afinidade reduzida com os beta-lactâmicos, permite com que a bactéria prossiga com a síntese de peptidoglicano e formação da parede celular, enquanto que as outras PBPs estão bloqueadas pelo antimicrobiano (PEHLIVANO 龔 LU; YARDIMCI, 2012).

O gene *mecA* localiza-se em um elemento genético móvel conhecido como *Staphylococcal Chromosomal Cassette mec* (SCC*mec*). O SCC*mec* pode ser transferido por transposons ou plasmídeos e integrado a ilhas de patogenicidade do genoma de outras bactérias (KATAYAMA; ITO; HIRAMATSU, 2001). O cassete *mec* possui diferentes tipos e carrega

outros elementos genéticos que codificam resistência a outras classes de antimicrobianos, como pUB110, pT181 e Tn554 (ROBERTS, 2008).

A transferência horizontal do gene *mecA* e os demais elementos do SCC*mec* entre os *Staphylococcus* spp., resultou numa disseminação de clones multiresistentes ao redor do mundo, representando um perigo a saúde pública devido ao difícil controle de infecções causadas por estes agentes (COHN; MIDDLETON, 2010).

Infecções estafilocócicas em equinos

Infecções causadas por *Staphylococcus* spp. são comuns em diversas espécies de mamíferos. Em equinos, várias espécies de *Staphylococcus* podem ser comumente encontradas aderidas à pele. No entanto, não se sabe ao certo quais os fatores determinantes associados à colonização e ao desenvolvimento de infecções cutâneas em cavalos (AKRIDGE et al., 2013).

Cavalos com longo período de hospitalização são normalmente colonizados por MRSA. Pele e fossas nasais são os locais onde comumente são isoladas cepas resistentes de *S. aureus* em equinos e devem ser levadas em consideração durante a aplicação de medidas de prevenção e controle de infecções nosocomiais em clínicas de equino (VAN DEN EEDE et al., 2012).

Normalmente, cepas MRSA indistinguíveis geneticamente são isoladas em cavalos e funcionários de clínicas veterinárias, indicando que há transmissão direta entre humanos e animais hospitalizados. Medidas de controle que evitem a disseminação devem ser adotadas, tais como limitar o uso de penicilinas, adoção de medidas higiênicas, principalmente no manejo entre pacientes, e isolamento de cavalos infectados com MRSA em baias especiais (PANCHAUD et al., 2010; SIEBER et al., 2011).

Lesões dermatológicas em equinos são frequentemente causadas por *Staphylococcus* spp. unicamente ou associada a outros gêneros bacterianos. Em dermatites na quartela, os sinais clínicos iniciam com pápulas ou pústulas, em um ou mais membros, e podem desaparecer em lesões crônicas. Nestes casos o diagnóstico deve ser realizado por cultura a partir de uma biópsia, já que *swabs* superficiais podem levar a erros, devido à contaminação superficial (YU, 2013).

Doenças menos comuns em cavalos, como endocardites, também são causadas por *Staphylococcus* spp. A origem da doença está relacionada com uma infecção crônica

(SPONSELLER; WARE, 2001), onde fatores de virulência da bactéria facilitam a adesão em válvulas cardíacas, em casos de bacteremia (PEACOCK et al., 1999). Os *Staphylococcus* spp. ficam protegidos do sistema imune e ação de antimicrobianos, devido à capacidade de estimular o organismo a agregar plaquetas a seu redor (CLAWSON; WHITE, 1971).

OBJETIVOS

Objetivo Geral:

- Avaliar similaridade genotípica entre *Staphylococcus aureus* encontrados colonizando fossas nasais de equinos saudáveis em diferentes estados do Brasil.

Objetivos Específicos:

- Avaliar a ocorrência de MRSA, a partir de marcador molecular, colonizando equinos saudáveis;

- Avaliar a acurácia de diferentes marcadores moleculares para identificação de *Staphylococcus aureus*;

- Determinar o perfil genotípico dos *S. aureus* de acordo com a frequência dos marcadores moleculares *nuc*, *femA* e *coa*;

- Determinar o perfil de resistência dos *Staphylococcus aureus* a antimicrobianos utilizados em medicina veterinária.

CAPITULO I: Confirming *Staphylococcus aureus* from animal sources using PCR targeting *femA*, *nuc* and *coa* genes: a comparative study using MALDI-TOF as gold standard identification system

Abstract

Staphylococcus aureus is considered a major pathogen in veterinary medicine and the emergence of multidrug resistant strains potentially pathogenic to humans, such as methicillin resistant *S. aureus* (MRSA), warranty diagnostic methods to be reliable and fast. This study aimed to test the accuracy of PCR targeting the genes *nuc*, *femA* and *coa* in identifying *S. aureus* from veterinary sources. A total of 71 *Staphylococcus* spp. strains were assessed by matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) and 12 different staphylococci species were identified. Out of 34 *S. aureus* isolates, the genes *femA*, *nuc* and *coa* were respectively found in 30, 26 e 16. Although the PCR assays targeting any of those genes showed 100% specificity, their sensitivity values were 88.2, 76.5 and 47.1% for *femA*, *nuc* and *coa*, respectively. Absolute sensitivity was observed when *femA* and *nuc* markers were targeted simultaneously. These findings confirm PCR as an accurate method to identify *S. aureus* species from animal sources and strongly suggest the simultaneous use of primers targeting *femA* and *nuc* genes.

Keywords: Molecular identification, PCR, *Staphylococcus* identification,

INTRODUCTION

Staphylococcus is a ubiquitous microbial genus comprised by more than forty species colonizing humans, animals and environment. Based on their capacity to produce the coagulase enzyme, those organisms are generally classified in two major groups: coagulase positive (CoPS) and coagulase negative (CoNS) staphylococci.

Staphylococcus aureus (*S. aureus*) is a typical CoPS and has been frequently associated with nosocomial infections in humans. Although recent reports have highlighted the increasing importance of CoNS species as opportunistic pathogens, *S. aureus* remains a major disease-causing agent in veterinary medicine. It is the most important mastitis-causing agent in dairy animals (1) leading to extensive economic losses worldwide. Furthermore, the increasing role of animals as sources of methicillin resistant *S. aureus* (MRSA) potentially pathogenic to humans (2) reinforces the high importance of accurately identifying *S. aureus* species as a basis for releasing reliable data for antimicrobial resistance surveillance of this pathogen in livestock.

Staphylococcus aureus isolation normally doesn't represent a challenge for microbiologists and its identification is traditionally based on conventional phenotypic tests, normally exploring species-related metabolic pathways for carbohydrate fermentation. However, strain variations related to the capacity of metabolizing certain sugars have been identified as a cause of misidentification (3, 4). Moreover, the identification of *Staphylococcus* using fermentation-based panels are expensive, laborious and time-consuming.

Amongst innumerable microbial identification systems currently available, Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) has consolidated as a reference identification system in clinical microbiology since it provide accurate and very rapid results at a low cost per sample (5). However, the high cost of the equipment has been a limiting factor for its use in veterinary diagnostic laboratories.

Alternatively, the polymerase chain reaction (PCR) has been extensively used and recommended as a reliable approach in the identification of *Staphylococcus* species (6, 7). In our laboratory, we have been reported some inconsistent findings between PCR targeting *S. aureus* gene markers and phenotypic traits typically attributed to this organism, such as coagulase. For instance, the presence of specific *S. aureus* markers in coagulase negative staphylococci is intriguing and concerning since it could lead to false negative results if coagulase is performed as the first screening step for *S. aureus* isolation in many laboratories. While those findings could be easily attributed to limitations of biochemical or phenotypical tests in accurately, we investigated the accuracy of PCR using three different markers (*nuc*, *femA*, and *coa*) using MALDI-TOF as gold standard for identification of *S. aureus* originated from animal sources.

MATERIAL AND METHODS

Bacteria

A total of 71 *Staphylococcus* spp. isolates, including CoPS (n=40) and CoNS (n=31), were randomly selected from the microbiological bank of The Laboratory for Food Safety at the College of Agricultural Sciences of The Federal University of Paraiba (UFPB). The isolates originated from cows (n=5), goats (n=14), horses (n=13), pigs (n=38) and bird (n=1), and were cultured by means of conventional microbiological procedures using mannitol salt agar and blood agar. Presumptive isolates were analyzed under optic microscopy after Gram stained, tested for catalase, oxidase and coagulase in tubes, according to reference procedures (8). The sources of the bacteria are shown in Table S1.

Identification using Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF)

Detailed information about MALDI-TOF functioning, accuracy and applications in clinical microbiology have been reported elsewhere (9, 10). MALDI-TOF was used as gold standard in the present investigation. Isolates were analyzed using the equipment Vitek MS (BioMérieux[®], France) at the Department of Clinical and Toxicological Sciences, University of São Paulo (USP). Shortly, isolates were streaked onto tryptone soy agar (TSA) for 24 hours at 37°C and three to five colonies transferred to one of the 48 wells of the standard steel blade. The samples were covered by the matrix solution of α -cyano-4-hydroxycinnamic acid and dried at room temperature. Finally, prepared blades were placed into the equipment and the mass spectrum was analyzed and compared with a reference database (11).

Polymerase chain reaction (PCR)

DNA extraction. DNA was extracted by boiling-centrifugation method according to Adwan (12), with some modifications. Shortly, three to five colonies were resuspended in 100 μ L purified water, frozen for 10 minutes and immediately boiled at 100°C for 10 min. Afterwards, samples were centrifuged (12000 rpm at a 4°C for 3 min) and supernatant (approximately 85 μ L) was carefully pipetted and transferred to a new DNase free tube and stored at 4°C until use (within 1 week).

Master mix and cycling conditions. Mastermix was prepared in a 23 μ L volume using 1 U of Taq DNA polymerase (LGC Biotecnologia, USA), 2 mM MgCl₂, 200 μ M of each dNTPs (Thermo Fisher Scientific), 10 pmol of each oligonucleotide primer and 2 of μ L DNA template.

The oligonucleotide primer sequences targeting the staphylococcus-specific genes *nuc*,

femA and *coa*, and the respective cycling conditions used for *S. aureus* identification are shown in Table 1. The sequences were analyzed using *Primer Blast* (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and alignment was observed for *Staphylococcus aureus* species only. No homology of the sequences *primers* was detected in *Staphylococcus* species other than *S. aureus*.

PCR targeting *nuc* and *femA* genes were performed simultaneously in duplex-PCR assay (dPCR). A reference strain harboring the three genes (USA 400) kindly provided by the Infectious Diseases Molecular Epidemiology Laboratory (IDMEL) of The Ohio State University was used as control in all PCR assays.

Amplification products were electrophoresed in 2% agarose gel, stained with Gelred (Biotium, USA) visualized under u.v. and documented by Gel Logic 212 PRO (Carestream Molecular Imaging Software - Version 5.0, Carestream Health, Inc, USA).

Data analyses

The correlation amongst PCR targeting the genes *nuc*, *femA* and *coa* was determined by Cohen's Kappa index of concordance using interpretation criteria (Table S2) previously established by Landis and Koch (13).

Considering the results obtained by Maldi-tof, sensitivity and specificity of the PCR-assays targeting the genes *nuc*, *femA* and *coa* were calculated using a commercial software (Excel, Microsoft).

RESULTS AND DISCUSSION

Out of the 40 CoPS isolates, 34 were identified as *S. aureus* by MALDI-TOF and six CoPS other than *S. aureus* were identified as *S. intermedius* (2), *S. pseudointermedius* (2), and *S. hyicus* (2). Amongst the 31 CoNS isolates, the following species were identified: *S. sciuri* (12), *S. chromogenes* (4), *S. cohnii* subs. *cohnii* (4), *S. simulans* (4), *S. xylosus* (2), *S.*

saprofiticus (2), *S. capitis* (1), *S. lentus* (1), and *S. epidermidis* (1).

Amongst *S. aureus*, 30/34 (88.2%) harbored the gene *femA* and 26/34 (76.5%) were positive for the gene *nuc*. However, only 16/34 (47.0%) of the *S. aureus* isolates were positive for the *coa*.

All *Staphylococcus* species other than *S. aureus* identified by means of MALDI-TOF didn't harbor any of the three genes, indicating PCR assay targeting those markers to be highly specific (100%), regardless the gene markers used. The complete list of isolates, their respective sources and the presence of the molecular markers are shown in Table S1.

Based on the targeted genes, six different patterns were detected amongst the isolates, as shown in Table 2. The *nuc-femA-coa* was the most frequent pattern identified (41.2%) amongst the *S. aureus* isolates, while *femA-coa* and *nuc-coa* were detected in just one (2.9%) sample (Table 2).

The Cohen's kappa index of concordance was moderate (55.4%) between *femA-coa*, and substantial (60.4%, 61.7% and 70.1%) between *nuc-coa*, *nuc-femA-coa* and *nuc-femA*, respectively (Table S3).

The sensitivity of the PCR targeting *femA* and *nuc* gene were 88.2% and 76.5% respectively, while PCR-*coa* showed 47.1% sensitivity only. When more than one marker was used, PCR targeting *femA-nuc* showed 100% sensitivity, while PCR assays targeting *femA-coa* and *nuc-coa* showed 91.2% and 79.4% sensitivity, respectively (Table 3).

The high frequency of *femA* in *S. aureus* isolates is in accordance to previous studies showing that 72.5 to 100% of *S. aureus* isolates harbored the gene (14, 15). This gene is related to the synthesis of specific proteins of the *S. aureus* cell wall (16). A mutation in this gene might affect the glycine contents of the wall and ultimately causing instability (17).

The gene *nuc*, which encodes the enzyme thermonuclease, has been shown to be *S. aureus* specific (18) and has been reported to occur in all *S. aureus* isolates (19, 20). Although the

frequency of *nuc* amongst *S. aureus* in our study was high, some isolates were not positive for the gene, suggesting that misidentification by PCR could be related to putative deletions or mutations occurring in the gene, as previously indicated (21). However, point mutations are not supposed to interfere with amplification and the causes of false-negative results by PCR are still unknown.

Interestingly, however, was the low frequency of *coa* gene amongst *S. aureus* investigated in our study, considering that the frequency of coagulase production is regulated by this gene in *S. aureus* (19). The gene is associated with the capacity of the pathogen to coagulate fibrin and therefore it plays a key role in the pathogenesis of the agent by conferring protection against host immune defense mechanisms (22). Unlike our results, recent research suggest a high frequency of *coa* gene in *S. aureus* (23). Again, the low frequency of this gene might be associated with putative *coa* gene polymorphism. *S. aureus* strains from different regions or animal species are were shown to harbor more than one coagulase gene, with predominance of one or two types (24, 25).

While genome sequencing of higher number of *S. aureus* from animal sources are still not available to elucidate those questions, the results of the present investigation confirm the high specificity of PCR targeting *nuc*, *femA* and *coa* and strongly indicate the use of *nuc* and *femA* as a means to achieve optimal sensitivity in identifying *S. aureus* species. These findings are of special importance for clinical veterinary diagnostic laboratories, where PCR technique has been extensively used considering the continuous decrease in cost. Again, the precise identification of *Staphylococcus aureus* is of key importance for the appropriate monitoring of antimicrobial resistance in this species, especially in the context of the emergence of MRSA in livestock and companion animals potentially pathogenic to humans. In conclusion, PCR targeting the markers *femA* and *nuc* can accurately identify *S. aureus* of animal origin.

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Table 1. Primers and cycling conditions used in the identification of *Staphylococcus aureus*

Gene	Amplicon size (pb)	Oligonucleotide primer sequences (5'-3')	Cycling	Reference
<i>nuc</i>	(279pb)	F: GCG ATT GAT GGT GAT ACG GTT R: AGC CAA GCC TTG ACG AAC TAA AGC	2	CIFTCI <i>et al.</i> , 2009
<i>femA</i>	(132pb)	F: AAA AAA GCA CAT AAC AAG CG R: GAT AAA GAA GAA ACC AGC AG	2	MEHROTRA <i>et al.</i> , 2000
<i>coa</i>	(variable)	F: ATA GAG ATG CTG GTA CAG G R: GCT TCC GAT TGT TCG ATG C	1	HOOKEY <i>et al.</i> , 1998

1. 94°C 4 min (94°C 1 min, 60°C 1 min, 72°C 1 min) x 30 e 72°C 5 min; (Techne TC5000, Inghilterra)

2. 94°C 5 min (94°C 40s, 58° 40s, 72°C 1 min) x10 (94°C 1 min, 50° 1min, 72°C 2 min) x 25 e 72°C 10 min
(Techne TC5000, England).

Table 2. Genotypic patterns of *Staphylococcus aureus* based on the harboring of the genes *nuc*, *femA* and *coa* genes

Genotypic pattern	<i>nuc</i>	<i>femA</i>	<i>coa</i>	Number of isolates and relative frequency (%)
1	+	+	+	14 (41.2)
2	+	+	-	08 (23.5)
3	-	+	-	07 (20.6)
4	+	-	-	03 (08.8)
5	-	+	+	01 (02.9)
6	+	-	+	01 (02.9)
Total				34 (100)

+ = positive; - = negative

Table 3. Sensitivity and specificity values of PCR assays targeting *femA*, *nuc*, *coa* genes in identification of *Staphylococcus aureus* from animal sources. Accuracy was assessed based on a panel of 71 isolates including *Staphylococcus aureus* (n=34) and coagulase positive (CoPS) and negative (CoNS) species other than aureus (n=37)

Targeted gene(s) in the PCR	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative Predictive negative value (%)
<i>femA</i>	88.2	100	100	11.8
<i>nuc</i>	76.5	100	100	23.5
<i>coa</i>	47.1	100	100	52.9
<i>femA-nuc</i>	100	100	100	100
<i>femA-coa</i>	91.2	100	100	8.8
<i>nuc-coa</i>	79.4	100	100	20.6
<i>femA-nuc-coa</i>	100	100	100	100

CAPITULO II: Genotypic relatedness and antimicrobial resistance patterns of *S. aureus* cultured from nostrils of healthy horses from distant geographical regions in Brazil

Summary

The aim of this study was to determine the genotypic relatedness even as antimicrobial susceptibility patterns of *Staphylococcus aureus* cultured from nostril swabs of horses from two geographically distant States (Paraíba and Rio Grande do Sul), located more than 3.700 km from each other. Out of a total of 123 staphylococci organisms initially screened, 21 isolates were confirmed as *S. aureus* by biochemical tests and polymerase chain reaction (PCR) targeting *S. aureus* species-specific genes (*femA*, *nuc* and *coa*). These isolates showed four different antimicrobial resistance patterns. Multiresistance was observed in ten *S. aureus*. Although resistance to ceftiofur was observed in six isolates, indicating the presence of methicillin resistant *S. aureus* (MRSA), none harbored *mecA* gene. Genotyping analysis by Rep-PCR revealed that isolates were not clustered according to their geographical origin, and indicate a large genetic diversity of *S. aureus* colonizing horses in Brazil.

Keywords: Rep-PCR, Multiresistance, MRSA

Introduction

Staphylococcus spp. comprises a group of mesophilic bacteria commonly found in the environment and also colonizing skin and membranes of human and animals. Organisms of this genus can be assigned in two major groups according to their ability to synthesize coagulase enzyme: coagulase negative (CoNS) and coagulase positive staphylococci (CoPS).

Staphylococcus aureus (*S. aureus*) are typically CoPS and considered the main causative agent of opportunistic staphylococcal infections in both humans and animals, including ranging from located self-limiting cutaneous lesions to severe systemic infections, such as osteomyelitis, endocarditis e pneumonia [1,2]. A major concern about staphylococcal infections is related to the emergence of multidrug resistant strains, especially methicillin resistant *Staphylococcus aureus* (MRSA). Reports have shown that farm [3] and pet [4] animals can be asymptomatic carriers of MRSA. Besides, genetic approaches traced those bacteria as the causative agents of infections in humans directly in contact to livestock [5] and might represent a major risk to public health [6] *S. aureus* and MRSA have been detected in nostrils of healthy horses worldwide [7,8], but there is a lack of data about the occurrence of those bacteria in horses of South America.

The aim of this study was to investigate the antimicrobial susceptibility and the genotypic relatedness of *S. aureus* colonizing nostrils of horses from geographically distant areas in Brazil.

Material and methods

Bacteria and Staphylococcus aureus confirmation

Samples of nasal swabs of clinically healthy horses originate from different Brazilian states were collected: seven cities of Rio Grande do Sul State (identified as AI to AVII) and a only

city of Paraiba State (identified as B). The approximate distance between them is 3,700 km. A total of 123 *Staphylococcus* spp. were used in this study, 87 isolates of State A, and 36 isolates of State B. The correct identification of *S. aureus* species has been shown not to be a simple task [9] and identification are supposed to be a major bias in studies aiming to assess antimicrobial resistance in *S. aureus*. Therefore, besides morphological and phenotypical analysis of isolates, *S. aureus* species were confirmed by polymerase chain reaction (PCR) targeting three specific *S. aureus* genes (*nuc*, *femA* and *coa*). After DNA extraction (protocol provided as supplementary material), mastermix was prepared in a 23 μ L volume using 1 U of Taq DNA polymerase (LGC Biotechnologies, USA), 2mM MgCl₂, 200 μ M of each dNTPs (Thermo Fisher Scientific), and 2 of μ L DNA template.

The oligonucleotide primer sequences targeting the staphylococcus-specific genes *16S rRNA*, *nuc*, *femA*, *mecA* and *coa*, and the respective cycling conditions used for *S. aureus* identification are shown in Supplementary Table S1. PCR targeting *16S rRNA*, *nuc*, *mecA* and *femA* genes were performed simultaneously in multiplex-PCR assay (mPCR). Two strains (USA 400 and ATCC 25923) were used as positive control.

Amplification products were electrophorized in 2% agarose gel, stained with Gelred (Biotium, USA) visualized under UV and documented by Gel Logic 212 PRO (Carestream Molecular Imaging Software - Version 5.0, Carestream Health, Inc, EUA).

Antimicrobial susceptibility test

Antimicrobial susceptibility test was performed by Kirby-Bauer disk-diffusion method according to the Clinical and Laboratory Standards Institute [10]. The antimicrobial drugs used and their respective concentrations were as follow: ampicillin (AMP, 30 μ g), cefoxitin (CFO, 30 μ g), penicillin G (PEN, 10 UI), chloramphenicol (CLO, 30 μ g), gentamicin (GEN, 10 μ g), oxacillin (OXA, 1 μ g), clindamycin (CLI, 2 μ g), tetracycline (TET, 30 μ g), amoxicillin/clavulanate (AMC, 10 μ g), azithromycin (AZI, 15 μ g), vancomycin (VAN, 30

µg) e enrofloxacin (ENO, 10 µg) (CECON®, São Paulo, Brazil).

Isolates were considered as multiresistant when showing resistance against three or more drugs derived different antimicrobial classes.

Genotyping

Confirmed *S. aureus* isolates were extracted by the technique of phenol: chloroform: isoamyl alcohol, according to Sambrook et al. [11], and after genotyped by Repetitive extragenic palindromic-PCR (Rep-PCR) targeting the RW3A, according to Van Der Zee et al. [12]. Amplification products were electrophorized in 2% agarose gel, stained with Gelred (Biotium, USA) visualized under u.v. and documented by Gel Logic 212 PRO (Carestream Molecular Imaging Software - Version 5.0, Carestream Health, Inc, EUA).

Data analyses

The concordance amongst the presence of the genes *nuc*, *femA* and *coa* was determined by the Cohen's Kappa index of concordance according to the criteria previously established by Landis and Koch [13] (Supplementary Table S2).

The genetic relatedness amongst *S. aureus* was determined by analyzing the similarity amongst Rep-PCR fingerprints using the Dice coefficient at 2% tolerance for genetic distance measurement calculations. Cluster analysis was performed using the unweighted pair-group method with average linkages (UPGMA) by means of a commercial software (BioNumerics 7.1, Applied Maths, Belgium) and data was presented in a dendrogram. A 80% similarity coefficient was used as threshold for clustering.

The discriminatory power (*D* value) was calculated as described by Hunter [14]

Results and discussion

Out of the 123, 90 (73.17%) isolates were confirmed as CoPS isolates by tube-coagulase test, but only 21 were shown to be positive for at least of the three *S. aureus* species-specific

genes. The other isolates neither produced free coagulase or were positive for the *S. aureus* markers and therefore were identified as CoNS (Supplementary Table S3).

The low frequency of *S. aureus* amongst staphylococci might be related to the fact that only healthy animals were sampled. Similar frequencies of *S. aureus* carriage in horses have been reported in USA and Canada [15,16].

The genes *nuc* and *femA* were identified in 16 isolates, whereas only 9 isolates were positive for *coa*.

As shown in Supplementary Table S4, the concordance amongst the presence of these genes used to confirm *S. aureus* was low to fair. Besides, none of the genes were found in all *S. aureus* isolates and Table 1 presents the frequency patterns observed in the present study. The most common patterns were *nuc-femA* and *nuc-femA-coa*, shared by 33% and 28.5% *S. aureus*, respectively. The gene *coa* was the less frequent gene found in *S. aureus*. The low frequency of *coa* gene amongst isolates, even in coagulase-producing bacteria, could be related to putative polymorphism of this gene. In fact, variation in the *coa* gene have been observed amongst *S. aureus* [17,18]. Importantly, although PCR targeting *nuc* gene has been usually used for *S. aureus* confirmation purposes [19], our findings suggest that the use of more *S. aureus* markers might contribute to improve *S. aureus* detection in samples taken from horses.

The higher rates of antimicrobial resistance were observed for ampicillin (47.6%), penicillin G (47.6%), tetracycline (38.10%) and vancomycin (38.10%). The lowest resistance rates were observed for amoxicillin-clavulanate and enrofloxacin (4.76%). The others rates were clindamycin (33.3%), cefoxitin (28.57%), oxacillin (23.8%), chloramphenicol (14.28%), azithromycin (9.52%) and gentamicin (9.52). Pan-susceptibility was observed in 6 (28.57%) isolates.

Total of four different antimicrobial resistant patterns were observed and ten (47.6%)

multiresistant isolates were identified. Others results were six (28.6%) pan-susceptible, three (14.3%) isolates resistant to two antimicrobial, and two (9.5%) resistant to only one antimicrobial. Nosocomial infections in horses have been attributed to multiresistant *S. aureus* [20] and the high resistant rates against drugs commonly used are of great concern because of therapeutic implications associated to multiresistant strains in both horses and humans [21,22].

A total of nine *Staphylococcus* isolates (7.3%) out of 123 were positive for *mecA* gene, but this gene was not found among the *S. aureus* isolates. In spite of this fact, six *S. aureus* were resistant against cefoxitin, indicating MRSA. Recent studies have been demonstrated that MRSA does not necessarily require the presence and expression of *mecA* and other putative mechanisms for methicillin resistance, such as *mecC* has been shown to occur in MRSA [23]. This is particularly important for animal-derived *S. aureus*, since *mecA* gene was originally targeted as a marker for methicillin resistance in *S. aureus* from humans [24]. In this sense, is possible that pleomorphism in *mec*-operon might occur [25] and making it difficult to confirm MRSA by means of PCR-*mecA*. Importantly, the six cefoxitin-resistant isolates identified in the present study were also multiresistant isolates (Table 2), which has invariably being associated with multiresistance [26].

The Rep-PCR using the primer RW3A showed a D-value of 0.96, indicating to be highly discriminatory. The *S. aureus* isolates were clustered in five major groups (A, B, C, D and E), as shown in Figure 1.

Cluster A was comprised by two *S. aureus* isolates from State B and the control MRSA USA400 were closely related. Except for this cluster, which indicate the presence of MRSA amongst the *S. aureus* investigated in the present study, isolates were not clustered according to their geographical origin, indicating a large genotypic diversity of *S. aureus* colonizing nasal cavity of horses in Brazil.

Isolates from different geographical regions were identified in all clusters in the present study. Unlike our results, studies in Europe show that different regions might carry genetically related *S. aureus* [27].

In conclusion, our findings suggest the presence of methicillin-resistant *Staphylococcus aureus* (MRSA) and indicate a large genetic diversity of *S. aureus* colonizing the nasal cavity of horses in Brazil.

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Table 1. Frequency patterns of isolated formed from *nuc*, *femA* and *coa* genes

Patterns of <i>S. aureus</i> Markers (N°)	%	<i>nuc</i>	<i>femA</i>	<i>coa</i>
1 (7)	33.34	+	+	-
2 (6)	28.57	+	+	+
3 (3)	14.29	-	+	-
4 (2)	9.52	+	-	-
5 (2)	9.52	-	-	+
6 (1)	4.76	+	-	+
TOTAL (21)	100			

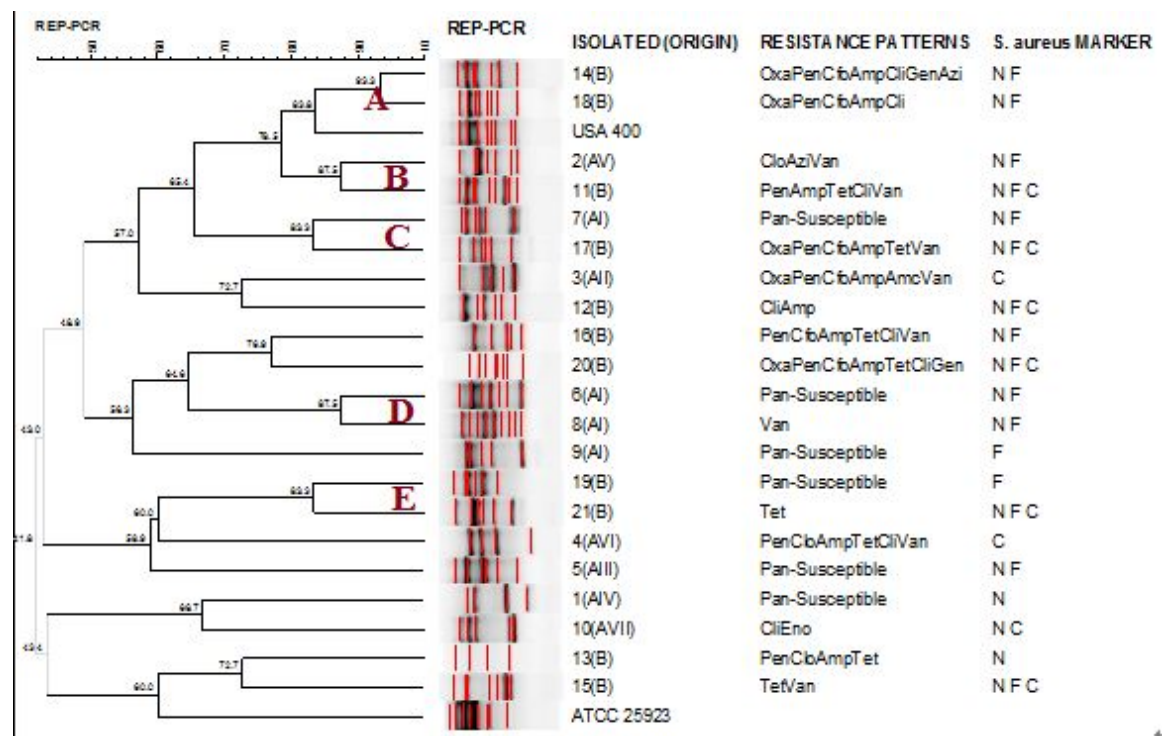
* + = Positive for specific primer; - = negative for specific primer

Table 2. Resistance and gene patterns of 21 *S. aureus* as well origins

Isolated	Origin	Resistance Patterns	Gene Patterns
1	A(IV)	Pan-Susceptible	<i>nuc</i>
2	A(V)	CloAziVan	<i>nuc-femA</i>
3	A(II)	OxaPenCloCfoAmpAmcVan	<i>coa</i>
4	A(VI)	PenCloAmpTetCliVan	<i>coa</i>
5	A(II)	Pan-Susceptible	<i>nuc-femA</i>
6	A(I)	Pan-Susceptible	<i>nuc-femA</i>
7	A(I)	Pan-Susceptible	<i>nuc-femA</i>
8	A(I)	Van	<i>femA</i>
9	A(I)	Pan-Susceptible	<i>femA</i>
10	A(VII)	CliEno	<i>nuc-coa</i>
11	B	PenAmpTetCliVan	<i>nuc-femA-coa</i>
12	B	CliAmp	<i>nuc-femA-coa</i>
13	B	PenCloAmpTet	<i>nuc</i>
14	B	OxaPenCfoAmpCliGenAzi	<i>nuc-femA</i>
15	B	TetVan	<i>nuc-femA-coa</i>
16	B	PenCfoAmpTetCliVan	<i>nuc-femA</i>
17	B	OxaPenCfoAmpTetVan	<i>nuc-femA-coa</i>
18	B	OxaPenCfoAmpCli	<i>nuc-femA</i>
19	B	Pan-Susceptible	<i>femA</i>
20	B	OxaPenCfoAmpTetCliGen	<i>nuc-femA-coa</i>
21	B	Tet	<i>nuc-femA-coa</i>

Amp = ampicillin; Cfo = cefoxitin; Pen = penicillin G; Clo = chloramphenicol; Gen = gentamicin; Oxa = oxacillin; Cli = clindamycin; Tet = tetracycline; Amc = amoxicillin/clavulanate; Azi = azithromycin; Van = vancomycin; Eno = enrofloxacin

Figure 1. Dendrogram of genotypic analysis made for RW3A from 21 *S. aureus* isolated



Amp = ampicillin; Cfo = cefoxitin; Pen = penicillin G; Clo = chloramphenicol; Gen = gentamicin; Oxa = oxacillin; Cll = clindamycin; Tet = tetracycline; Amc = amoxicillin/clavulanate; Azi = azithromycin; Van = vancomycin; Eno = enrofloxacin; N = Positive for *nuc* Marker; F = Positive for *femA* Marker; C = Positive for *coa* Marker

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APÊNDICES

APÊNDICE A

Material Suplementar do Capítulo I

TABLE S1. Staphylococcus isolates, coagulase test results, sources, genotypic patterns according to PCR targeting the genes *coa*, *nuc* and *femA* and species identification by Malfi-Tof

Isolate	Coagulase test	Source	<i>nuc</i>	<i>femA</i>	<i>coa</i>	Maldi-tof identification
1	NEG	Horse	0	0	0	<i>S. lentus</i>
2	POS	Horse	0	0	0	<i>S. pseudointermedius</i>
3	POS	Horse	0	0	0	<i>S. pseudointermedius</i>
4	NEG	Horse	0	0	0	<i>S. saprofiticus</i>
5	POS	Bird	1	1	0	<i>S. aureus</i>
6	POS	Cow	1	1	1	<i>S. aureus</i>
7	POS	Cow	1	1	1	<i>S. aureus</i>
8	POS	Goat	1	1	1	<i>S. aureus</i>
9	POS	Goat	1	1	1	<i>S. aureus</i>
10	POS	Goat	1	1	1	<i>S. aureus</i>
11	POS	Goat	1	1	1	<i>S. aureus</i>
12	POS	Goat	1	1	0	<i>S. aureus</i>
13	POS	Goat	0	1	0	<i>S. aureus</i>
14	POS	Goat	1	1	0	<i>S. aureus</i>
15	POS	Goat	1	1	0	<i>S. aureus</i>
16	POS	Goat	1	1	0	<i>S. aureus</i>
17	POS	Goat	0	0	0	<i>S. intermedius</i>
18	NEG	Goat	0	0	0	<i>S. chromogenes</i>
19	POS	Goat	0	1	0	<i>S. aureus</i>
20	POS	Cow	1	1	1	<i>S. aureus</i>
21	NEG	Cow	0	0	0	<i>S. capitis</i>
22	POS	Cow	1	1	1	<i>S. aureus</i>
23	NEG	Horse	0	0	0	<i>S. xylosus</i>
24	NEG	Horse	0	0	0	<i>S. sciuri</i>
25	NEG	Horse	0	0	0	<i>S. sciuri</i>
26	NEG	Horse	0	0	0	<i>S. sciuri</i>
27	NEG	Horse	0	0	0	<i>S. sciuri</i>
28	NEG	Horse	0	0	0	<i>S. sciuri</i>
29	NEG	Horse	0	0	0	<i>S. sciuri</i>
30	NEG	Horse	0	0	0	<i>S. sciuri</i>
31	POS	Goat	0	1	0	<i>S. aureus</i>
32	POS	Goat	0	1	0	<i>S. aureus</i>
33	POS	Horse	0	0	0	<i>S. intermedius</i>
34	NEG	Pig	0	0	0	<i>S. sciuri</i>

35	POS	Pig	0	0	0	<i>S. hyicus</i>
36	NEG	Pig	0	0	0	<i>S. chromogenes</i>
37	NEG	Pig	0	0	0	<i>S. simulans</i>
38	POS	Pig	0	0	0	<i>S. hyicus</i>
39	POS	Pig	1	1	1	<i>S. aureus</i>
40	NEG	Pig	0	0	0	<i>S. sciuri</i>
41	NEG	Pig	0	0	0	<i>S. xylosus</i>
42	NEG	Pig	0	0	0	<i>S. sciuri</i>
43	NEG	Pig	0	0	0	<i>S. chromogenes</i>
44	POS	Pig	1	0	1	<i>S. aureus</i>
45	POS	Pig	1	0	0	<i>S. aureus</i>
46	POS	Pig	1	1	1	<i>S. aureus</i>
47	POS	Pig	0	1	0	<i>S. aureus</i>
48	NEG	Pig	0	0	0	<i>S. saprofiticus</i>
49	NEG	Pig	0	0	0	<i>S. sciuri</i>
50	POS	Pig	0	1	1	<i>S. aureus</i>
51	POS	Pig	1	0	0	<i>S. aureus</i>
52	POS	Pig	1	1	1	<i>S. aureus</i>
53	POS	Pig	1	0	0	<i>S. aureus</i>
54	POS	Pig	1	1	1	<i>S. aureus</i>
55	POS	Pig	1	1	0	<i>S. aureus</i>
56	POS	Pig	1	1	1	<i>S. aureus</i>
57	POS	Pig	0	1	0	<i>S. aureus</i>
58	POS	Pig	1	1	0	<i>S. aureus</i>
59	POS	Pig	1	1	0	<i>S. aureus</i>
60	POS	Pig	0	1	0	<i>S. aureus</i>
61	POS	Pig	1	1	1	<i>S. aureus</i>
62	NEG	Pig	0	0	0	<i>S. cohnii cohnii</i>
63	NEG	Pig	0	0	0	<i>S. simulans</i>
64	NEG	Pig	0	0	0	<i>S. cohnii cohnii</i>
65	NEG	Pig	0	0	0	<i>S. sciuri</i>
66	NEG	Pig	0	0	0	<i>S. chromogenes</i>
67	NEG	Pig	0	0	0	<i>S. simulans</i>
68	NEG	Pig	0	0	0	<i>S. simulans</i>
69	NEG	Pig	0	0	0	<i>S. cohnii cohnii</i>
70	NEG	Pig	0	0	0	<i>S. cohnii cohnii</i>
71	NEG	Pig	0	0	0	<i>S. epidermidis</i>
Total			26	30	16	

POS = positive by the coagulase test; NEG = negative by the coagulase test; 1 = positive by PCR targeting the gene; 0 = negative by PCR targeting the gene

TABLE S2. Interpretation criteria for the Kappa-Cohen index of correlation, as originally published by LANDIS & KOCH (1977)

Kappa values	Concordance
<0	No Agreement
0-0.19	Poor Agreement
0.20-0.39	Fair Agreement
0.40-0.59	Moderate Agreement
0.60-0.79	Substantial Agreement
0.80-1.00	Almost Perfect Agreement

TABLE S3. Correlation values calculated by Kappa Cohen index (*K*) and their confidence intervals (CI) amongst PCR assays targeting the *nuc*, *femA* and *coa* genes used to identify *S. aureus*

Target gene in PCR	Correlation (<i>K</i>)	P- values	Confidence Interval (CI)
<i>nuc-femA</i>	0.70	< 0.001	0.469 – 0.934
<i>nuc-coa</i>	0.60	<0.001	0.384 – 0.823
<i>femA-coa</i>	0.55	<0.001	0.339 – 0.769
<i>nuc-femA-coa</i>	0.62	<0.001	0.483 – 0.751

APÊNDICE B

Material Suplementar do Capítulo II

DNA Extraction

The extraction of genomic DNA of *Staphylococcus* spp., Was conducted by boiling-centrifugation method. Three at five colony of each isolate was suspended in 100 µL of ultrapure water, than frozen for 10 minutes, and after boiled at 100 ° C followed by more 10 minutes. The samples were briefly centrifuged for 3 minutes at 12,000 rpm at 4 ° C to remove cellular debris. The supernatant was removed carefully to another tube (approximately 70 uL) and stored at 4 ° C until use.

Supplementary Table S1. *Primers* and cycling conditions used in the identification of *Staphylococcus aureus*

Gene	Amplicon Size (bp)	Primer Sequence (5'-3')	Cycling	Reference
<i>16S</i>	(756bp)	F: AAC TCT GTT ATT AGG GAA GAA CA R: CCA CCT TCC TCC GGT TTG TCA CC	2	ZHANG <i>et al.</i> , [27]
<i>rRNA</i>				
<i>nuc</i>	(279bp)	F: GCG ATT GAT GGT GAT ACG GTT R: AGC CAA GCC TTG ACG AAC TAA AGC	2	CIFTCI <i>et al.</i> , [28]
<i>femA</i>	(132bp)	F: AAA AAA GCA CAT AAC AAG CG R: GAT AAA GAA GAA ACC AGC AG	2	MEHROTRA <i>et al.</i> , [29]
<i>coa</i>	(variable)	F: ATA GAG ATG CTG GTA CAG G R: GCT TCC GAT TGT TCG ATG C	1	HOOKEY <i>et al.</i> , [30]
<i>mecA</i>	(168bp)	F: GTA GAA ATG ACT GAA CGT CCG ATAA R: CCA ATT CCA CAT TGT TTC GGT CTAAA	2	OLIVEIRA; LENCASTRE, [31]

1. 94°C 4 min (94°C 1 min, 60°C 1 min, 72°C 1 min) x 30 e 72°C 5 min; (Techne TC5000, England)

2. 94°C 5 min (94°C 40s, 58° 40s, 72°C 1 min) x10 (94°C 1 min, 50° 1min, 72°C 2 min) x 25 e 72°C 10 min (Techne TC5000, England)

Supplementary Table 2. Interpretation criteria for the Kappa-Cohen index of correlation according to Landis and Koch (1977)

Values Kappa	Concordance
<0	No Agreement
0-0.20	Poor Agreement
0.21-0.40	Fair Agreement
0.41-0.60	Moderate Agreement
0.61-0.80	Substantial Agreement
0.81-1.00	Almost Perfect Agreement

Supplementary Table 3. Genotypic patterns associated with the presence of the genes *16S* *rRNA*, *coa*, *nuc*, *femA* and *mecA* in *Staphylococcus aureus* from nasal swabs of horses

Isolated	Old	Origin	Coagulase	16S	<i>nuc</i>	<i>femA</i>	<i>coa</i>	<i>mecA</i>
1	20	PELOTAS	NEG	1	0	0	0	0
2	5	PELOTAS	NEG	1	0	0	0	1
3	3	PELOTAS	NEG	1	0	0	0	0
4	3	PELOTAS	POS	1	0	0	0	0
5	8	PELOTAS	NEG	1	0	0	0	0
6	18	PELOTAS	NEG	1	0	0	0	0
7	18	PELOTAS	POS	1	0	0	0	0
8	18	PELOTAS	NEG	1	0	0	0	0
9	19	PELOTAS	NEG	1	0	0	0	0
10	10	PELOTAS	POS	1	0	0	0	0
11	4	PELOTAS	NEG	1	0	0	0	1
12	5	RIO PARDO	POS	1	0	0	0	0
13	15	ALEGRETE	POS	1	0	0	0	0

14	15	ALEGRETE	POS	1	0	0	0	0
15	8	ALEGRETE	NEG	1	0	0	0	0
16	20	ALEGRETE	POS	1	0	0	0	0
17	2	ALEGRETE	POS	1	0	0	0	0
18	10	CRUZ ALTA	NEG	1	0	0	0	0
19	14	CRUZ ALTA	NEG	1	0	0	0	0
20	12	CRUZ ALTA	POS	1	0	0	0	0
21	4	CRUZ ALTA	POS	1	0	0	0	0
22	8	CRUZ ALTA	POS	1	0	0	0	0
23	4	CRUZ ALTA	POS	1	0	0	0	0
24	19	CRUZ ALTA	NEG	1	0	0	0	0
25	19	CRUZ ALTA	NEG	1	0	0	0	0
26	20	SAO LUIZ GONZAGA	POS	1	0	0	0	0
27	7	SAO LUIZ GONZAGA	POS	1	0	0	0	0
28	6	SAO LUIZ GONZAGA	POS	1	0	0	0	0
29	4	SAO LUIZ GONZAGA	POS	1	0	0	0	0
30	15	SAO LUIZ GONZAGA	POS	1	0	0	0	0
31	8	SAO LUIZ GONZAGA	POS	1	0	0	0	0
32	5	PORTO ALEGRE	NEG	1	0	0	0	0
33	6	PORTO ALEGRE	POS	1	1	0	0	0
34	20	PORTO ALEGRE	POS	1	0	0	0	0
35	20	PORTO ALEGRE	NEG	1	0	0	0	0
36	20	PORTO ALEGRE	POS	1	0	0	0	0
37	4	PORTO ALEGRE	NEG	1	0	0	0	0
38	4	PORTO ALEGRE	POS	1	0	0	0	0
39	4	PORTO ALEGRE	NEG	1	0	0	0	0
40	20	PORTO ALEGRE	NEG	1	0	0	0	0
41	2,5	PORTO ALEGRE	POS	1	0	0	0	0

42	18	RIO PARDO	POS	1	0	0	0	0
43	18	RIO PARDO	NEG	1	0	0	0	0
44	14	RIO PARDO	POS	1	0	0	0	0
45	10	RIO PARDO	NEG	1	0	0	0	0
46	25	RIO PARDO	POS	1	0	0	0	0
47	25	RIO PARDO	POS	1	1	1	0	0
48	10	RIO PARDO	POS	1	0	0	0	0
49	15	RIO PARDO	POS	1	0	0	0	0
50	9	CRUZ ALTA	POS	1	0	0	0	0
51	7	CRUZ ALTA	POS	1	0	0	0	0
52	7	CRUZ ALTA	POS	1	0	0	1	0
53	12	RIO PARDO	NEG	1	0	0	0	0
54	13	SAO LUIZ GONZAGA	POS	1	0	0	0	0
55	20	SAO LUIZ GONZAGA	POS	1	0	0	0	0
56	7	SAO LUIZ GONZAGA	POS	1	0	0	0	0
57	8	SAO LUIZ GONZAGA	NEG	1	0	0	1	0
58	12	SAO LUIZ GONZAGA	POS	1	0	0	0	0
59	6	SAO LUIZ GONZAGA	NEG	1	0	0	0	0
60	6	SAO LUIZ GONZAGA	NEG	1	0	0	0	0
61	10	PELOTAS	NEG	1	0	0	0	0
62	30	PELOTAS	POS	1	0	0	0	0
63	30	PELOTAS	POS	1	0	0	0	0
64	11	PELOTAS	NEG	1	0	0	0	0
65	6	PELOTAS	POS	1	0	0	0	0
66	12	PELOTAS	NEG	1	1	1	0	0
67	10	RIO PARDO	POS	1	0	0	0	0
68	10	ALEGRETE	POS	1	0	0	0	0
69	10	ALEGRETE	POS	1	0	0	0	0

70	10	ALEGRETE	POS	1	1	1	0	0
71	10	ALEGRETE	POS	1	0	0	0	0
72	10	ALEGRETE	NEG	1	0	0	0	0
73	10	ALEGRETE	POS	1	1	1	0	0
74	10	ALEGRETE	POS	1	0	1	0	0
75	10	ALEGRETE	NEG	1	0	1	0	0
76	6	ALEGRETE	POS	1	0	0	0	0
77	4	ALEGRETE	POS	1	0	0	0	0
78	4	ALEGRETE	POS	1	0	0	0	0
79	11	ALEGRETE	POS	1	0	0	0	0
80	12	URUGUAIANA	POS	1	0	0	0	0
81	31	URUGUAIANA	NEG	1	0	0	0	0
82	10	URUGUAIANA	POS	1	0	0	0	0
83	10	URUGUAIANA	NEG	1	0	0	0	0
84	16	URUGUAIANA	POS	1	0	0	0	0
85	2,5	URUGUAIANA	POS	1	0	0	0	0
86	6	URUGUAIANA	NEG	1	1	0	1	0
87	5	URUGUAIANA	NEG	1	0	0	0	0
88	3	PATOS	POS	1	0	0	0	0
89	3	PATOS	POS	1	0	0	0	0
90	3	PATOS	POS	0	1	1	1	0
91	4	PATOS	POS	1	0	0	0	0
92	5	PATOS	POS	1	0	0	0	0
93	5	PATOS	POS	1	0	0	0	0
94	4	PATOS	POS	1	1	1	1	0
95	3.5	PATOS	POS	1	0	0	0	0
96	4	PATOS	POS	1	0	0	0	0
97	5	PATOS	POS	1	1	0	0	0

98		PATOS	POS	1	0	0	0	1
99	4	PATOS	POS	1	0	0	0	1
100	4	PATOS	POS	0	1	1	0	0
101	3.5	PATOS	POS	1	0	0	0	0
102	4	PATOS	POS	1	1	1	1	0
103	4	PATOS	POS	0	1	1	0	0
104	5	PATOS	POS	1	0	0	0	0
105	4	PATOS	POS	1	1	1	1	0
106	5	PATOS	POS	1	0	0	0	1
107	5	PATOS	POS	0	1	1	0	0
108	4	PATOS	POS	1	0	0	0	1
109	6	PATOS	POS	1	0	1	0	0
110		PATOS	POS	1	0	0	0	1
111	4	PATOS	POS	0	1	1	1	0
112	4	PATOS	POS	1	0	0	0	0
113	5	PATOS	POS	1	0	0	0	0
114	5	PATOS	POS	0	1	1	1	0
115	5	PATOS	POS	1	0	0	0	0
116	6	PATOS	POS	1	0	0	0	1
117	4	PATOS	POS	1	0	0	0	0
118	5	PATOS	POS	1	0	0	0	0
119	4	PATOS	POS	1	0	0	0	0
120	5	PATOS	POS	1	0	0	0	0
121	5	PATOS	POS	1	0	0	0	0
122	6	PATOS	POS	1	0	0	0	0
123	5	PATOS	POS	1	0	0	0	1
TOTAL				117	16	16	9	9

Coagulase = tube-coagulase; 1= Positive for specific primer; - = negative for specific primer

Supplementary Table S4. Kappa *Cohen* concordance amongst of *nuc*, *femA* and *coa* genes, thus as these genes and tube-coagulase

Patterns	Values of		
	Kappa	P- Values	Confidence Interval
			*sup: 0.719
Coagulase- <i>femA</i>	0.295	0.172	inf: -0.128
			sup: 0.719
Coagulase- <i>nuc</i>	0.295	0.172	inf: -0.128
			sup: 0.64
<i>nuc-femA</i>	0.213	0.33	inf: -0.215
			sup: 0.361
<i>nuc-coa</i>	0.025	0.882	inf: -0.31
			sup: 0.199
<i>nuc-femA-coa</i>	-0.048	-	inf: -0.295
			sup: 0.255
coagulase- <i>coa</i>	-0.05	-	inf: -0.355
			sup: 0.184
<i>femA-coa</i>	-0.152	-	inf: -0.487

* Sup = head limit; inf = lower limit; coagulase = tube-coagulase