Universidade Federal Rural de Pernambuco

Programa de Pós-graduação em Biociência Animal

Guilherme Santana de Moura

Dinâmica da infecção e caracterização molecular das mastites causadas por *Staphylococcus spp.* em ovelhas da raça Santa Inês

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Orientador: Prof. Dr. Rinaldo Aparecido Mota

Coorientadora: Profª. Drª. Luciana B. B. C. da Costa

Tese apresentada ao Programa de Pós-Graduação em Biociência Animal da Universidade Federal Rural de Pernambuco, como parte das exigências para a obtenção do título de Doutor em Biociência Animal.

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DEDICATÓRIA

A minha esposa Michele e meu filho Davi.

Aos meus pais e minha irmã

Que são, sempre foram e sempre serão a minha base.

Dedico.

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Assim, não desistamos de fazer o que é bom,

pois colheremos no tempo devido,

se não desanimarmos.

Gálatas 6:9

Caracterização biomolecular das mastites causadas por *Staphylococcus* spp. em ovelhas da raça Santa Inês

RESUMO

O presente trabalho traçou o perfil epidemiológico das infecções intramamárias (IIM) causadas por estafilococos em ovelhas Santa Inês através de ferramentas de biologia molecular contemplando os seguintes aspectos: 1) investigar a incidência de infecção intramamária (IIM) em ovelhas de corte no período pós-parto e a persistência da infecção durante a lactação, e seu efeito no crescimento de cordeiros. Neste estudo, 38 ovelhas e 44 cordeiros foram acompanhados do parto até o desmame aos 90 dias. Os cordeiros foram pesados ao nascimento e a cada 15 dias, e o exame microbiológico e identificação de *Mycoplasma agalactiae* pela reação da cadeia da polimerase (PCR) das amostras de leite das ovelhas foram realizados nos respectivos momentos. Observou-se que 14 (36,8%; 5 por *Mycoplasma agalactiae*; 5 por *Staphylococcus sciuri*, 2 por *Staphylococcus aureus* e 2 por *Staphylococcus simulans*) e 20 (31,6%) ovelhas apresentam IIM persistente e transiente, respectivamente. Portanto, 63,26% das IIM, todas por estafilococos não *aureus*, não persistiram durante a lactação, sugerindo perfil oportunistas de algumas espécies. A taxa de sobrevivência de cordeiros de ovelhas com IIM persistente (33,3%) foi bem menor que dos animais sadios (100%) ou com IIM transiente (91,7%). Além disto, houve diferença no ganho de peso para cordeiros de ovelhas persistentemente infectadas os 90 dias em relação aos demais grupos. Apesar do impacto negativo da mastite nas ovelhas Santa Inês sobre o desempenho e a morte de cordeiros, o agente etiológico e seu potencial para causar IIM persistente devem ser considerados. 2) relatar um caso esporádico de mastite gangrenosa causada por

Staphylococcus haemolyticus multirresistente em ovelha Santa Inês. O exame microbiológico, o perfil de resistência a antimicrobianos e presença de fatores de virulência foram investigados. Os isolados de *Staphylococcus haemolyticus* apresentaram multirresistência a antimicrobianos, e foram positivos para dois genes enterotoxigênicos estafilocócicos e proteína B de ligação à fibronectina (fnbA). 3) investigar se os estafilococos isolados de mastite clínica e subclínica, do ápice das ovelhas e tonsilas de cordeiros são geneticamente distintos, e possuem genes relacionados à produção de enterotoxinas e resistência a meticilina e penicilina. Foram utilizados 78 isolados de estafilococos proveniente de ovelhas Santa Inês (Estudo 1). Os isolados de estafilococos foram identificados por MALDI-TOF MS, e a PCR foi realizada para identificação dos genes de virulência e resistência a antimicrobianos. Além disso, os isolados foram genotipados através de amplificação aleatória de DNA polimórfico - RAPD. Observou-se que *S. aureus* isolados de amostras de leite diferem daqueles isolados de nichos extramamários, diferentemente das espécies de estafilococos não-*aureus*. *S. aureus* hospedou a maioria dos genes de virulência e resistência. Detectou-se um isolado de *S. aureus* e um *Staphylococcus sciuri* isolados do ápice do teto resistente a meticilina. Portanto, o presente estudo forneceu informações mais detalhadas da epidemiologia dos estafilococos associados à ovelha. 4) relatar a presença de *Staphylococcus sciuri* portadores do gene de resistência a meticilina *mecC*, sendo, portanto, o primeiro relato no Brasil. A resistência a cefoxitina e oxacilina foram determinadas pela concentração inibitória mínima, e os genes de resistência foram confirmados por PCR, e sequenciamento dos produtos da PCR.

Palavras-chave: Epidemiologia Molecular; MALDI-TOF; Performance de cordeiros.

Biomolecular characterization of mastitis caused by Staphylococcus spp. in Santa Inês sheep

ABSTRACT

The present work traced the epidemiological profile of intramammary infections (IMI) caused by staphylococci in Santa Inês sheep using molecular biology tools covering the following aspects: 1) to investigate the incidence of intramammary infection (IMI) in meat ewes in the post-partum and the persistence of infection during lactation, and its effect on lamb growth. In this study, 38 ewes and 44 lambs were followed from birth until weaning at 90 days. The lambs were weighed at birth and every 15 days, and the microbiological examination and identification of *Mycoplasma agalactiae* by polymerase chain reaction (PCR) of the sheep's milk samples were performed at respective times. It was observed that 14 (36.8%; 5 by *Mycoplasma agalactiae*; 5 by *Staphylococcus sciuri*, 2 by *Staphylococcus aureus* and 2 by *Staphylococcus simulans*) and 20 (31.6%) sheep present persistent and transient IIM, respectively. Therefore, 63.26% of IMIs, all due to *non-aureus staphylococci*, did not persist during lactation, suggesting opportunistic profiles of some species. The survival rate of lambs from sheep with persistent IMI (33.3%) was much lower than healthy animals (100%) or with transient IMI (91.7%). In addition, there was a difference in weight gain for lambs from sheep persistently infected at 90 days in relation to the other groups. Despite the negative impact of mastitis on Santa Inês sheep on lamb performance and death, the etiologic agent and its potential to cause persistent IMI should be considered. 2) to report a sporadic case of gangrenous mastitis caused by multi-resistant *Staphylococcus haemolyticus* in Santa Inês sheep. The microbiological examination, the antimicrobial resistance profile and the presence of virulence factors were investigated. *Staphylococcus haemolyticus* isolates showed multidrug resistance to antimicrobials and were positive for two staphylococcal enterotoxygenic genes and fibronectin-binding protein (*fnbA*). 3) investigate whether the staphylococci isolated from clinical and subclinical mastitis, from apex of sheep and lambs' tonsils are genetically distinct, and have genes related to the production of enterotoxins and resistance to methicillin and penicillin. 78 *staphylococcus* isolates from Santa Inês sheep were used (Study 1). *Staphylococcus* isolates were identified by

MALDI-TOF MS, and PCR was performed to identify the virulence and antimicrobial resistance genes. In addition, the isolates were genotyped by random amplification of polymorphic DNA (RAPD). It was observed that *S. aureus* isolated from milk samples differ from those isolated from extra-mammary niches, differently from non-aureus staphylococcus species. S. aureus hosted most virulence and resistance genes. An isolate of *S. aureus* and other of *Staphylococcus sciuri* shown methicillin-resistance. Therefore, the present study provided more detailed information on the epidemiology of staphylococci associated with sheep. 4) to report the presence of *Staphylococcus sciuri* carrying the methicillin resistance gene *mecC*, being, therefore, the first report in Brazil. Resistance to cefoxitin and oxacillin was determined by the minimum inhibitory concentration, and the resistance genes were confirmed by PCR, and sequencing of PCR products.

Keywords: Molecular epidemiology; MALDI-TOF; Lamb´s performance.

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ANEXO I

1. Introdução

A mastite tem um grande impacto na economia e no bem-estar das fêmeas criadas para produção de leite ou carne. Em ovelhas, ela é causada principalmente por bactérias Gram-positivas, incluindo estafilococos, estreptococos e enterococos. Bactérias Gram-negativas como as enterobactérias, também podem causar mastite nestes animais, embora com ocorrência significativamente mais baixa do que em bovinos (BERGONIER et al., 2003a). Outros agentes etiológicos relevantes são os micoplasmas, mas, uma vez que essa bactéria também causa outro sinais clínicos graves como claudicação, ceratoconjuntivite e problemas respiratórios, alguns autores não as consideram como agente causador primário de mastite (Pisanu et al. 2015). Embora vários microrganismos possam causar mastite em ovelhas, *Staphylococcus* spp. são os agentes mais comumente isolados (CONTRERAS et al., 2007; Vasileiou et al., 2019), tanto nas formas clínica, subclínica ou crônica. Rotineiramente, estes patógenos causadores de mastite são isolados e classificados de forma generalista (ex. estafilococos coagulase-negativo), ou seja, não discriminam as espécies causadoras de mastite, o que limita o entendimento e aprofundamento do estudo dos fatores de risco e alguns aspectos de causa e efeito da mastite. Os estudos baseados na biologia molecular e introdução da técnica de espectrometria de massa com fonte de ionização e dessorção a laser assistida por matriz e analisador de tempo-de-voo (MALDI-TOF) trouxeram novos avanços na identificação das espécies mais prevalentes nas mais diversas espécies (TOMAZI et al., 2014) o que melhorou a abordagem no controle e tratamento das infecções intramamárias. O presente trabalho visa caracterizar através de ferramentas de biologia e epidemiologia molecular, as mastites em ovelhas Santa Inês traçando o perfil epidemiológico, os fatores

de virulência e os impactos das infecções intramamárias nos rebanhos de corte. O nosso trabalho teve como objetivo levantar os aspectos epidemiológicos mais importantes das mastites em ovelhas da raça Santa Inês utilizando as ferramentas de biologia molecular, associando as perdas causadas pela doença e desdobrando as implicações na saúde humana.

2. Revisão de Literatura

2.1 Principais agentes etiológicos da mastite em ovelhas

A mastite é um problema comum em ovelhas, porém sua prevalência é variável entre os rebanhos principalmente quanto ao tipo de produção (leite ou corte) (ARSENAULT et al., 2008a). Estudos realizados em abatedouros da Grã-Bretanha mostraram prevalência muito alta variando de 13 a 50% (CONINGTON et al., 2008), indicando que a mastite clínica é uma importante causa de abate de ovelhas no Reino Unido. Nos Estados Unidos, a prevalência de mastite clínica varia de 1 a 3%, mas a mastite subclínica pode variar entre 5% e 30%,em alguns rebanhos leiteiros esse número pode alcançar 35% (BERGONIER; DE CREMOUX; et al., 2003). No Brasil, as prevalências em rebanhos de ovelhas da raça Santa Inês variam entre 28,5% (DOMINGUES et al., 2008) e 31,45% (COUTINHO et al., 2006) dependendo da região.

Staphylococcus aureus é uma bactéria frequentemente isolada de casos clínicos de mastite em ovinos, correspondendo a até 35% dos casos (BERGONIER et al., 2003a), além de estar diretamente relacionada aos casos de mastite gangrenosa (SEYFFERT et al., 2012). *Manhemmia haemolytica* é, também, uma causa muito importante de mastite clínica em ovinos (MAVROGIANNI; CRIPPS; FTHENAKIS, 2007; ARSENAULT et al.,

2008a; PEREIRA et al., 2014). Acredita-se que a fonte da infecção do úbere com *M. haemolytica* é o nariz e a garganta de cordeiros em aleitamento (MAVROGIANNI; CRIPPS; FTHENAKIS, 2007). Já os coliformes mais comumente isolados em casos de mastite clínica incluem *Escherichia coli*, *Pseudomonas aeruginosa* e *Klebsiella pneumonia*, sendo *Salmonella spp*. mais raramente isolada. *Staphylococcus* coagulase negativa (SCN) ou *Staphylococcus* não-*aureus* (SNA) são os principais agentes causadores de mastite em pequenos ruminantes (BERGONIER et al., 2003a). Essas bactérias constituem um grupo diverso e por isso, a apresentação clínica e a gravidade da infecção são bastante variáveis. Em ovelhas, elas são os isolados mais comuns da mastite subclínica em todas as fases da lactação, incluindo o período seco e o desmame (ONNI et al., 2010). Muitas espécies de SNA isoladas de ovelhas possuem baixa patogenicidade, no entanto, algumas espécies demonstraram causar uma mastite clínica tão grave quanto o *S. aureus* (EBRAHIMI et al., 2014).

Situações em que o quadro clínico de mastite em rebanhos ovinos incluir artrite e conjuntivite com ou sem pneumonia, a agalaxia contagiosa deve ser considerada um diagnóstico diferencial (GIADINIS et al., 2012). O agente causador desta síndrome é o *Mycoplasma agalactiae* sendo responsável por surtos de mastite em ovelhas no Brasil, nas regiões mediterrânea e alpina da Europa (DE AZEVEDO et al., 2006; GÓMEZ-MARTÍN et al., 2013).

Outro patógeno importante é o vírus Maedi-visna (MV-v), também conhecido como Ovine Progressive Pneumonia virus (OPP-v) que está associado a pneumonia progressiva crônica e perda de peso, bem como alterações na glândula mamária ovina (VAN DER MOLEN; VECHT; HOUWERS, 1985).

2.2 A biologia molecular no diagnóstico da mastite em ovinos de corte

O diagnóstico da mastite em ovelhas de corte, de um modo geral, é realizado da mesma forma que para a mastite em animais leiteiros, baseando-se no exame clínico geral do animal, para avaliar a glândula mamária e a secreção láctea (FRAGKOU; BOSCOS; FTHENAKIS, 2014). Os casos clínicos são de fácil diagnóstico, pois o animal apresenta sinais de alteração da glândula mamaria, pode apresenta sinais sistêmicos e o leite tem grumos, pus ou sangue, entretanto o diagnóstico da forma subclínica só é possível por meio de métodos complementares como contagem de células somáticas e a cultura microbiológica do leite (lactocultura) (BLAGITZ et al., 2014). Porém, fica evidente que em rebanhos numerosos, como são grande parte dos rebanhos de ovinos de corte, o diagnóstico fica comprometido já que na maioria das vezes esses animais são criados de forma extensiva com pouca rotina de manejo. Este detalhe muitas vezes faz com que o problema seja percebido tardiamente com a morte da ovelha, morte ou retardo no crescimento dos cordeiros (ARSENAULT et al., 2008a).

A cultura microbiológica do leite é o método de diagnóstico mais usado para determinar o agente etiológico envolvido nos casos de mastite (CLEMENTS; TAYLOR; FITZPATRICK, 2003). Na última década aumentou o número de trabalhos que aliaram o cultivo bacteriano às técnicas de biologia molecular, o que foi particularmente importante para o entendimento das mastites de pequenos ruminantes que anteriormente eram descritas como sendo, principalmente, causadas pelo grupo dos *Staphylococcus coagulase negativos* (SCN) hoje chamados de *Staphylococcus não-aureus* (SNA) (MAHMMOD et al., 2018). Com o uso da técnica de Reação em Cadeia da Polimerase (PCR) e o MALDI-TOF é possível determinar as espécies mais comumente envolvidas

nos casos clínicos e subclínicos das mastites auxiliando na compreensão dos fatores de risco e no controle da doença nos rebanhos (PEREYRE et al., 2013; SCHULTHESS et al., 2014; TOMAZI et al., 2014).Contudo estudos complementares que possam associar corretamente causa e efeito de cada microrganismo no processo infecioso da glândula mamária de ovelhas da raça Santa Inês, além das perdas associadas e o impacto na produção, ainda são necessários. Outro ponto que ainda é necessário avançar é relacionado ao diagnóstico precoce da doença e a padronização do manejo das matrizes antes mesmo da parição de forma preventiva como já ocorre em rebanhos leiteiros (DE VLIEGHER et al., 2012).

Uma grande variedade de métodos de tipagem molecular tem sido usada nas últimas duas décadas para investigar a epidemiologia da mastite bovina no nível de subespécies (PYÖRÄLÄ; TAPONEN, 2009; DE VLIEGHER et al., 2011; CAMERON et al., 2017), no entanto, nas mastites em ovelhas de corte este recurso continua limitado a poucos estudos(ARSENAULT et al., 2008a; ONNI et al., 2010). Métodos de tipagem comparativa baseados em padrões de bandas eletroforéticas, métodos de tipagem de bibliotecas baseados na sequência de genes selecionados, matrizes de genes de virulência e projetos de sequenciamento completo de genoma bacteriano são exemplos de técnicas utilizadas nos estudos de epidemiologia molecular da mastite (NEMEGHAIRE et al., 2014a; SILVA; ALCÂNTARA; MOTA, 2018).

A distribuição de linhagens de patógenos da mastite foi investigada isoladamente ou associando animais, rebanhos, países e espécies hospedeiras, levando em consideração a glândula mamária, outros nichos animais ou humanos e fontes ambientais(DE VLIEGHER et al., 2014; JÁCOME et al., 2014; MOURA et al., 2018).

Estudos epidemiológicos moleculares contribuíram consideravelmente para nosso entendimento de fontes, rotas de transmissão e prognóstico para muitos patógenos da mastite além de auxiliar na compreensão de mecanismos de adaptação ao hospedeiro e, consequentemente, no poder patogênico destes microrganismos(JAMROZY et al., 2012; BALLHAUSEN et al., 2017).

Métodos comparativos, como eletroforese em gel de campo pulsado (PFGE) e tipagem aleatória de DNA polimórfico amplificado (RAPD), têm sido utilizados para estudos dentro do rebanho em escalas espaço-temporais curtas, devido ao seu poder discriminatório relativamente alto, enquanto a tipificação por sequência de foco múltiplo (MLST) é mais adequado para estudos em larga escala espaço-temporal, como a comparação de populações entre rebanhos ou países, porque é um sistema de tipagem universal baseado em genes domésticos de evolução lenta (EL-JAKEE et al., 2013; MAHMMOD et al., 2018; MOURA et al., 2018).

2.3 Genes de resistência antimicrobiana e virulência

Um ponto bastante relevante nas pesquisas que utilizaram biologia molecular foi a detecção de genes de resistência antimicrobiana e de virulência em microrganismos potencialmente zoonóticos como *S. aureus*, que produzem toxinas superantigênicas, enzimas, citotoxinas, exotoxinas e toxinas esfoliativas responsáveis pelo agravamento do quadro clínico dos animais (ALMEIDA et al., 2013).

S. aureus produz uma grande variedade de fatores de virulência, associados à parede bacteriana ou secretados, que possuem como função principal, transformar os componentes do hospedeiro em nutrientes para o crescimento bacteriano. Contribuem

com a patogenicidade e são responsáveis pelos sintomas e severidade das infecções (MERZ; STEPHAN; JOHLER, 2016).

A expressão de fatores de virulência e mecanismos reguladores é controlada por genes de virulência específicos. Os fatores que contribuem para a patogenicidade dos estafilococos podem ser classificados como componentes bacterianos da superfície celular (fatores de aderência) e fatores secretados (LE MARÉCHAL et al., 2011).

Os fatores de aderência incluem várias proteínas que atuam principalmente durante a fase inicial da infecção. Sua principal função é facilitar a ligação da bactéria à superfície da célula hospedeira, levando simultaneamente à evasão da resposta imune em cascata do hospedeiro. São exemplos a proteína estafilocócica A (*SpA*), a proteína A de ligação à fibronectina e a proteína B de ligação à fibronectina (*FnbpA* e *FnbpB*), a proteína de ligação ao colágeno e as proteínas do fator de agregação A e B (MURAI et al., 2016).

Os genes *icaA, icaB, icaC, icaD* são responsáveis pela produção de adesão intercelular de polissacarídeo e produção de biofilme (FERREIRA et al., 2014). O gene *bap* também codifica uma proteína de superfície importante, denominada “proteína associada ao biofilme”; além de sua contribuição para a ligação bacteriana inicial, também foi considerado que esta proteína é capaz de induzir um processo de produção de adesão intercelular de polissacarídeo/biofilme independente de poli-N-acetil-glucosamina, especialmente em superfícies abióticas (MARTINS et al., 2015).

Os fatores de virulência secretados estão presentes principalmente durante a fase tardia da infecção e geralmente têm papel mais distinto na patogenicidade microbiana

(JOHLER et al., 2016). Com base em sua atividade principal, os determinantes de virulência secretada são ainda classificados em quatro categorias: superantigênicos, toxinas citolíticas, exoenzimas e proteínas diversas. A toxina 1 da síndrome do choque tecidual (TSST-1) e as enterotoxinas são os superantígenos mais proeminentes, geralmente causando condições clínicas de maior gravidade (LE MARÉCHAL et al., 2011).

As toxinas citolíticas (α-hemolisina, β-hemolisina, γ-hemolisina, toxinas da família leucocidina) são capazes de formar poros na parede celular do hospedeiro, causando vazamento osmótico do conteúdo celular e, portanto, citólise fornecendo os nutrientes necessários para um maior crescimento bacteriano (EBRAHIMI et al., 2014).

Os estafilococos são frequentemente resistentes aos antibióticos ß-lactâmicos devido à produção de ß-lactamase e/ou pela produção de uma proteína ligadora de penicilina (PBP2a) com baixa afinidade por estes antibióticos. ß-lactamase e PBP2a são codificadas por genes localizados nos operons *mec* e *bla*, respectivamente. A betalactamase é codificada pelo gene estrutural *blaZ*, este por sua vez é regulado pela proteína DNA ligante BlaI e pelo transdutor de sinal BlaR1 (HAO et al., 2012).

O termo MRSA é usado para designar as linhagens de *S. aureus* que não respondem ao tratamento com antibióticos β-lactâmicos. Caracterizam-se como cepas que possuem o gene *mecA* ou demonstram uma concentração inibitória mínima (CIM) à oxacilina mais alta que 4 mg/L. Entretanto, alguns isolados clínicos são *mecA*-positivos e suscetíveis à oxacilina. O gene *mecA* é responsável pela codificação da proteína de ligação à penicilina (PBP2a) que funciona como um alvo alternativo resistente à inibição pelo antibiótico, permitindo a formação da camada de peptídeoglicano da parede celular,

impedindo a morte bacteriana (Velasco et al., 2014). Em ovelhas da raça Santa Inês, ZAFALON e colaboradores (2012) investigaram a resistência à oxacilina em estafilococos coagulase-negativos (SCN) isolados do leite de ovelhas com mastite subclínica no Estado de São Paulo e encontraram quatro cepas com a presença do gene *mecA*, sem identificar as espécies que abrigavam esse gene. O surgimento da resistência antimicrobiana é mais comum entre as espécies de SCN, que também podem causar injúrias ao tecido mamário ocasionando queda da produção de leite (TAPONEN; PYÖRÄLÄ, 2009).

Em 2011, foi feita a descrição de isolados de MRSA do Reino Unido, Irlanda e Dinamarca, que abrigam um homólogo *mecA* divergente denominado *mecC* (anteriormente mecALGA251; GARCÍA-ÁLVAREZ et al., 2011). A proteína de ligação à penicilina codificada pelo *mecC* difere da *mecA*, por ter uma maior afinidade relativa pela oxacilina em comparação à cefoxitina. Estes isolados de MRSA *mecC*-positivos *(mecC*-MRSA) representam potencial problema de saúde pública, pois o grau de divergência de nucleotídeos entre *mecC* e *mecA* significa que são negativos ao testes de diagnóstico atuais, como ensaios de PCR e testes de aglutinação de látex que detectam *mecA* e PBP2a, respectivamente (STEGGER et al., 2012).

A descoberta inicial do *mecC*-MRSA no Reino Unido revelou que isolados de bovinos e humanos em proximidade geográfica estavam altamente relacionados, sugerindo transmissão entre os dois (GARCÍA-ÁLVAREZ et al., 2011). Outros trabalhos identificaram que o *mecC* está presente em complexos clonais (CCs) encontrados em humanos e em uma variedade diversificada de espécies animais em toda a Europa (PATERSON; HARRISON; HOLMES, 2014).

Contrastando com a mastite bovina, pouca informação está disponível no campo da epidemiologia molecular de *S. aureus* isolados de casos de mastite ovina, principalmente nas ovelhas destinadas a corte. Embora haja pouca dúvida de que o principal e mais importante reservatório de *S. aureus* é a própria glândula mamária infectada da ovelha, estudos mais amplos são necessários para aumentar o conhecimento sobre outros possíveis reservatórios, as vias de transmissão, a dinâmica da infecção, os fatores de risco e as associações entre os isolados clonais (Vautor et al., 2005).

Uma vez que *S. aureus* é considerado um agente patogênico oportunista, é possível que certos clones sejam mais propensos a aderir e colonizar o úbere das ovelhas devido a presença de determinados fatores de virulência, aumentando o seu potencial para a adesão e colonização em comparação a outros clones (Zastempowska; Lassa, 2012). Muitos fatores de virulência foram identificados no genoma de *S. aureus*, mas, diferenças na patogenicidade entre os isolados de campo permanecem em grande parte desconhecidos (Harrison et al., 2013).

Na investigação epidemiológica veterinária, o uso de tipagem molecular do *S. aureus* pode ser uma poderosa ferramenta para fornecer informações sobre o grau de clonalidade para a confirmação de um clone específico responsável pela mastite (Vautor et al., 2005).

2.4 Impactos econômicos e medidas de controle

Em muitos rebanhos de ovinos de corte, a mastite resulta em grandes perdas econômicas devido ao baixo desempenho de cordeiros ao desmame, alta mortalidade

da prole ou até perdas de ovelhas nos casos mais graves da doença (VERÍSSIMO et al., 2010), no entanto, os efeitos dessas perdas devem ser avaliadas longitudinalmente ao longo da lactação da ovelha e, assim, entender melhor como cada microrganismo afeta a performance do cordeiro ao desmame.

Estudos realizados em abatedouros mostraram que a mastite, identificada na linha de inspeção como alterações no úbere, é um achado comum em ovelhas descartadas, levando à especulação de que essa é uma razão importante para o abate (CONINGTON et al., 2008). Em um estudo escocês, em um levantamento de três anos sobre as perdas de ovelhas, a mastite foi responsável por 8,4% de todas as mortes de ovelhas (GRANT; SMITH; GREEN, 2016). A mastite em ovelhas tem um efeito bem documentado na produção de leite, com redução na produção de leite além de diminuição da concentração de lactose e, principalmente, da gordura presente no leite devido â infecção intramamária e pela contagem elevada de células somáticas, afetando negativamente a produção de queijo (GONZALO et al., 2002).

A mastite em ovinos de corte está diretamente ligada aos casos do complexo inanição/hipotermia que pode ser definido como uma condição em que o cordeiro não pode ser alimentado adequadamente pela ausência total de produção de leite ou pela produção de leite em qualidade inferior (ROOK, 2013). Essa condição é uma das principais causas de mortalidade de cordeiros na primeira semana de vida (GRANT; SMITH; GREEN, 2016).

A mastite de ovinos ainda não foi estudada tão intensamente quanto em vacas e alguns aspectos de sua etiologia, patogênese e epidemiologia não foram suficientemente

esclarecidos, e medidas precisas ainda não foram propostas para seu controle (GELASAKIS et al., 2015a), principalmente nos sistemas de criação extensiva.

Uma prática comum entre os criadores de ovinos de corte é verificar o úbere de cada ovelha no final da lactação ou seis semanas antes do início da estação de monta (GRANT; SMITH; GREEN, 2016). Ovelhas com alterações no úbere ou infecção intramamária são frequentemente, mas nem sempre, abatidas. O impacto dessa prática é desconhecido. As hipóteses possíveis incluem redução da transmissão progressiva de cepas bacterianas que causam mastite, redução do número de cordeiros de crescimento lento em um rebanho, redução do descarte prematuro de matrizes e diminuição da mortalidade de cordeiros (HUNTLEY et al., 2012).

O controle de infecções intramamárias em ovelhas precisa considerar as várias etiologias, bem como a diversidade dos fatores de risco. Na concepção e implementação de um programa de controle, existem fatores que devem ser abordados em todas as situações e fatores de relevância para fazendas específicas.

2.5 Conclusões e perspectivas futuras

Nos últimos anos, uma maior quantidade de artigos tem aumentado a literatura disponível sobre mastite em ovinos. No entanto, ainda são necessários mais estudos para a elucidação e o entendimento de todos os mecanismos e impacto da doença nos rebanhos. O presente artigo revisa a literatura publicada sobre a doença. Vários trabalhos destacaram a importância dos fatores de virulência dos agentes causadores da mastite, especialmente *S. aureus* e estafilococos *não aureus* que são as principais bactérias envolvidas na doença. Mecanismos patogênicos, com especial referência ao

papel das defesas locais no teto, também foram elucidados nos últimos anos e podem ser explorados na formulação de estratégias que induzam respostas imunes locais na glândula das ovelhas, como um meio de proteger os animais. Além dos testes bacteriológicos bem estabelecidos, a biotecnologia colabora para o diagnóstico rápido e preciso do problema, o que pode aumentar os esforços na descoberta de tratamentos efetivos. Embora os métodos de controle da doença tenham sido melhorados, no futuro, as abordagens que consigam alcançar o problema na sua origem e que sejam economicamente viáveis serão necessárias.

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CAPÍTULO 1

Persistence of intramammary infections in meat-producing ewes and their impact on lambs' growth

(Small Ruminant Research)

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Abstract

Santa Inês is an important Brazilian sheep breed due to its rusticity and adaptability to tropics climate. However, in many Santa Inês flocks, mastitis results in large economic losses mainly to sheep culling, treatments, low lambs' performance at weaning, or due to high offspring mortality. Here, we evaluated the incidence of intramammary infections (IMIs) during the immediate postpartum period and their persistence throughout lactation and estimate their effect on lambs' growth losses in a herd of meat-producing Santa Inês sheep. Thirty-eight ewes and forty-five Santa Inês lambs were followed from birth to weaning for 90 days. During the experiment, lambs were weighed at birth and every 15 days for a total of seven moments. At each moment, samples of milk from their respective dams were collected aseptically for conventional microbiological analysis and for identification of *Mycoplasma agalactiae* through Polymerase Chain Reaction (PCR). The ewes were divided into three groups according to the maintenance of infection status

observed through microbiological and PCR tests in: uninfected, transiently and persistently infected. The mean weight, survival curve and growth curve of lambs from persistently infected ewes were lower compared to the other two groups of ewes (transiently infected and uninfected). Lambs from persistently infected sheep were up to 50% lighter than lambs born from uninfected dams or dams with a transient IMI. The losses caused by persistent IMI accounted for 62% when we consider the total weight that was not produced by lambs. Thus, our study pointed out when we evaluated the impact of mastitis on lamb's performance, the etiological agent and their potential to cause persistent IMI should be considered.

Keywords: Mastitis, *Mycoplasma*, *Staphylococcus*, lambs´ mortality.

1. Introduction

Meat sheep production has been increasing in many countries around the world. Santa Inês is an important Brazilian breed mainly in the northeast of the country and is used to obtain early weaning lambs due to its rusticity and adaptability to tropics climate. Santa Inês sheep present estrus all year long with exceptional maternal ability, prolificacy, high weaning rates, great feed conversion and fast growing of the lambs, considered valuable characteristic of this breed. They are also less susceptible to ectoparasites and endoparasites compared to other breeds and have a lower incidence of hoof problems (ISSAKOWICZ et al., 2016).

The occurrence of sheep mastitis has been a cause for concern and has become particularly important in meat producing flocks due its relationship to a reduction in lamb

weight gain and an increase in mortality (Moroni et al., 2007; Veríssimo et al., 2010; Grant et al., 2016), however the effects of these losses need to be evaluated longitudinally throughout ewe lactation and so, we can better understand how each microorganism affect the result in lambs weaning. Mastitis can be caused by several etiologic agents. In sheep, notably *Staphylococcus* spp. are the most prevalent microorganisms (Souza et al., 2012; Vasileiou et al., 2019), but *Mycoplasma agalactiae*, *Mannheimia haemolytica* and even lentiviruses are also important etiologies (Souza et al. 2012; Santos et al., 2018). In this regard, the diagnosis of *M. agalactiae* mastitis on previous studies that evaluated the effect of mastitis on lamb's performance has been neglected, as the conventional milk culture alone usually did not allow us to detect this pathogen (Gioia et al., 2016). Furthermore, there is scarce information on the incidence of intramammary infections (IMIs) in the immediate postpartum period and their persistency throughout lactation (Takano et al., 2018), especially in meat-producing ewes.

Mastitis in meat sheep is directly linked to cases of the starvation/hypothermia complex that can be defined as a condition where the lamb cannot be fed properly by the total absence of milk production or by the production of low quantities of low quality milk (ROOK, 2013). This condition is one of the main causes of lamb mortality in the first weeks of life. Sheep mastitis has not yet been studied as intensively as in cows and some aspects of its etiology, pathogenesis and epidemiology have not sufficiently been clarified, and precise measures have not yet been proposed for its control (GELASAKIS et al., 2015a). Thus, the present work aimed to evaluate the incidence of IMIs during the immediate postpartum period and their persistence throughout lactation and estimate their effect on lambs' growth losses in a herd of meat-producing Santa Inês sheep.

2. Material and Methods

2.1 Animals and samplings

This experiment was carried out under the ethical guidelines of animal experimentation of the National Council of Animal Experimentation Control - CONCEA (Brazil). Thirty-eight ewes and forty-six Santa Inês lambs were followed from birth to weaning during a period of 90 days in a commercial sheep herd. Throughout the experiment, lambs were weighed at birth (M0) and every 15 days for a total of seven moments (M0 - M6), always in the morning. Milk samples from both halves were collected aseptically for conventional microbiological analysis and Polymerase Chain Reaction (PCR) analysis to detect *Mycoplasma agalactiae* (see further) at each of these moments. All animals were under the same flock management during the whole period; they were housed in stalls with food delivered three times a day (chopped Elephant grass as roughage, balanced concentrate, and minerals and vitamins supplements), fresh water *ad libitum*, and were grouped with a maximum of 15 animals per pen (ewes and lambs). A single purebred Santa Inês ram was the sire of all lambs. All ewes were tested negative for Small Ruminant Lentiviruses through agar gel immunodiffusion (Biovetech®, Recife, Brazil) before the experiment following the protocol described by Nascimento-Penido et al. (2017).

2.2 Isolation and identification of mastitis pathogens

For microbiological analysis, 10 µL from each milk samples (n = 460) were cultured on 5% sheep blood agar and incubated at 37°C for 24h-48h. An ewe was considered as having an intramammary infection (IMI) if $\geq$ 100 colony forming units/mL were detected in the bacteriological culture in one or both mammary halves. For species identification,

the isolates were further submitted to mass spectrometry through Matrix Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry (MALDI-TOF MS), as previously described by Cameron et al. (2017).

An aliquot of 1 mL of each milk sample (n = 460) was also submitted to PCR for detection of *Mycoplasma agalactiae* according to protocol described by Tatay-Dualde et al. (2015). An ewe was considered infected if one of mammary halves was positive at PCR.

2.3 Definition of transiently and persistently infected ewe

An ewe was considered as uninfected if had negative results in milk culture and PCR in all moments. An ewe was regarded as having a transient IMI if had a positive result in microbiological culture or PCR in only one or two moments, consecutive or not. A persistent IMI was assumed that an ewe had a positive result in microbiological culture or PCR in at least three consecutive moments by the same pathogen.

2.4 Losses estimative

In order to estimate the losses due persistent or transient IMIs, we first assume that all lambs born alive should be weaned at 90 days. In addition, the final weight at weaning should be the mean weight of lambs from uninfected ewes.

2.5 Statistical Analysis

Comparisons among lambs´ weight were performed using Analysis of variance (ANOVA) followed by Tukey test to look for significant differences from uninfected (healthy), transiently and persistently infected ewes. Data were expressed as arithmetic means. Observed survival was calculated with the Kaplan-Meier method for the 90 days period (birth - weaning). The proportion of death lambs over time was estimated comparing first among uninfected, transiently and persistently infected ewes and then, the proportion of death lambs over time among different group of microorganisms. Statistical difference was set at a *P* value ≤ 0.05. A linear regression model was conducted to determine the relationship between lamb weight and ewe infection status (uninfected, transiently and persistently infected) at each moment from birth to weaning and the differences among growth curves. Statistical analyses were performed with GraphPad Prism version 7.04 for Windows (GraphPad Software, La Jolla, California, USA).

3. Results and discussion

In the present study, *M. agalactiae* was detected through PCR in 5 (13.16%) ewes and they stayed infected during the entire experiment and so, all of them were considered persistently infected. These data step up the role of this pathogen in small ruminants' mastitis, as previously reported (Gómez-Martín et al., 2013; Santos et al., 2018), which treatment is not an option and may determine premature ewe culling. *M. agalactiae* is a microorganism associated to severe cases of agalactia, arthritis and conjunctivitis in sheep (GÓMEZ-MARTÍN et al., 2013), although none of these signs were observed in this flock.

Furthermore, biochemical tests that are widely used to identify bacteria isolated from mastitis did not allow us to accurately discriminate NAS species, and consequently important information were lose, as *non-aureus staphylococci* (NAS) represent the most prevalent pathogen associated with mastitis in small ruminants (Souza et al., 2012; Dore et al., 2016; Moura et al., 2018). Differently from other studies, MALDI-TOF MS was used here to identify microorganisms at species level, giving the possibility to follow the persistence of agents during the postpartum period and so, to better understand the relation of this factor in relation to lamb's losses/performance. In this regard, staphylococci were the only bacteria specie isolated in the present study (Table 1), and most of them were *Staphylococcus sciuri*. *S. sciuri* is an emerging microorganism and may present several virulence factors being identified as one of the important reservoirs for antimicrobial resistance and virulence genes (NEMEGHAIRE et al., 2014b).

Table 1. Frequency of detection of *staphylococci* per sampling moment from milk samples aseptically collected from Santa Inês ewes

	M0	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**
S. sciuri	13 (34.21%)	7 (18.42%)	5 (13.16%)	5 (13.16%)	5 (13.16%)	5 (13.16%)	5 (13.16%)
S. simulans	2 (5.26%)	2 (5.26%)	2 (5.26%)	2 (5.26%)	2 (5.26%)	2 (5.26%)	2 (5.26%)
S. aureus	2 (5.26%)	2 (5.26%)	2 (5.26%)	2 (5.26%)	2 (5.26%)	2 (5.26%)	2 (5.26%)
S. cohnii	-	2 (5.26%)	-	-	-	-	-
S. haemolyticus	1 (2.63%)	1 (2.63%)	-	-	-	-	-

S. xylosus	-	1 (2.63%)	-	-	-	-	-
S. saprophyticus	1 (2.63%)	-	-	-	-	-	-
S. warneri	-	1 (2.63%)	-	-	-	-	-
S. chromogenes	1 (2.63%)	-	-	-	-	-	-
S. devriesei	1 (2.63%)	-	-	-	-	-	-
Total	21 (55.26%)	16 (42.11%)	9 (23.68%)	9 (23.68%)	9 (23.68%)	9 (23.68%)	9 (23.68%)

Among these 38 sheep, 3 (8%) were primiparous and 35 (92%) were multiparous. From primiparous ewes, 2 (66%) and from multiparous ewes, 19 (54%) had an IMI at parturition. In this regard, while the study size is limited, we could identify two meat-producing primiparous ewes infected at parturition, which strengthen the idea that mastitis control should begin at younger stages, even before lactation, as it has already been stated by De Vliegher et al. (2012) in dairy cattle and by Takano et al. (2018) in dairy ewes. Thus, the occurrence of IMIs before the first lactation in meat-producing ewes could negatively impact the mammary gland development and future milk production.

All ewes delivered clinical healthy lambs, 45 in total. Among these, 30 were born from single births and seven from twin births. Interestingly, all twins were born from persistently infected sheep (two *M. agalactiae*, two *S. sciuri*, two *S. simulans* and one *S. aureus*). Fourteen ewes (36.8%) were considered persistently infected and twelve (31.6%) had transient infections and twelve (31.6%) were uninfected. Among the persistently infected ewes, 5 (36%) of ewes were positive for *M. agalactiae,* 5 (36%) had

IMIs by *S. sciuri*, 2 (14%) had IMIs by *S. aureus* and 2 (14%) had IMIs by *S. simulans*. There were 21 (45.6%) lambs born from persistently infected ewes, 13 (28.3%) from transiently infected ewes and 12 (26.1%) from uninfected dams. Moreover, most of the IMIs by NAS were identified at parturition, but most of them (63.26%) did not persist throughout lactation, which suggest the opportunist behavior of some NAS species, as previous reported in dairy cattle (De Visscher et al., 2014) and dairy goats (Jácome et al., 2014).

The survival rates between lambs from ewes persistently infected compared to lambs from ewes either recovered from the infection or negative showed a statistical difference ($P < 0.001$). Survival rates were 33.3% (7/21) for lambs of persistently infected ewes, 91.7% (11/12) of those who were transiently infected and 100% (12/12) of lambs from uninfected ewes reached at 90 days of age. Almost half (48%) of the lambs of persistently infected dams died within 45 days and 20% died within the first 15 days of life. Among microorganisms involved in persistent infections, *M. agalactiae* presented the highest mortality rate (57%) in the first 15 days of lamb life, a period at which lambs are highly dependent on milk, as well as a lower survival rate at the end of period (Figure 1). While the study size is limited, among staphylococci species, *S. sciuri* and *S. simulans* had similar survival rates (43%) and interestingly, lambs whose dams were persistently infected with *S. aureus* had similar survival rates to lambs born from uninfected dams (100%).

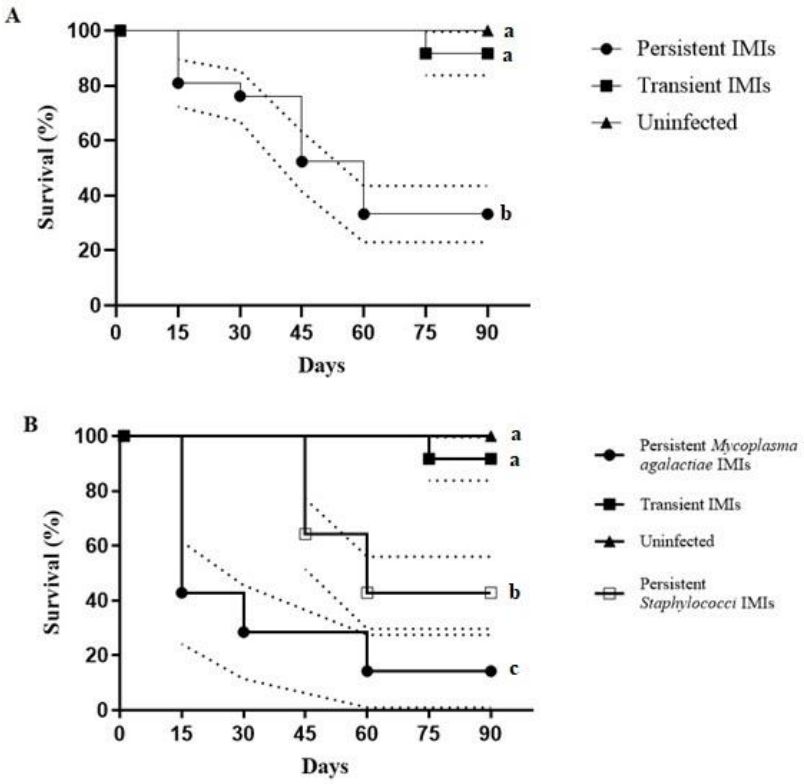

Figure 1. Survival rates of Santa Inês lambs (mean ± standard error) per group throughout days in life.

Survival rate within the total of 45 lambs. **A.** Kaplan Meier plot of lambs from uninfected, transiently and persistently infected dams. *P* value < 0,001; **B.** Kaplan Meier plot of lambs from uninfected, transiently and persistently infected dams (per microorganism group). Different letters indicated *P* ≤ 0.001. IMIs: intramammary infections.

In relation to the growth curve, there was no significant difference between lambs' performance of uninfected and transiently infected groups, however lambs from persistently infected ewes had inferior performance compared to the other two groups (uninfected and transiently infected). Overall there was a difference in weight gain for

lambs of persistently infected sheep of about 8 kg (around 50%) at 90 days in relation to the other two groups (Figure 2).

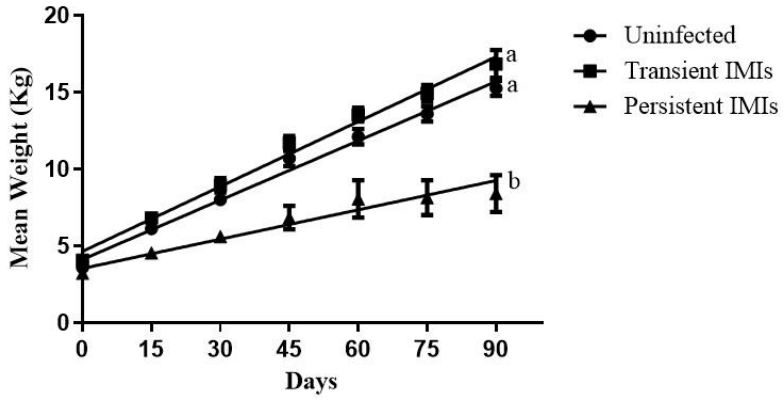

Figure 2. Santa Inês lambs' performance (mean weight ± standard error) from uninfected, transiently and persistently infected dams throughout days in life.

Different letters indicated $P \leq 0.001$. Equations of regression - Uninfected: Y = 0.1287*X + 4.117; Transient IMIs: Y = 0.1407*X + 4.646; Persistent IMIs: Y = 0.06358*X + 3.534. IMIs: intramammary infections.

4. Conclusions

Our study strengthens the idea of the negative impact of mastitis in meat-producing ewes on lambs' performance and deaths, nonetheless the etiological agent and their potential to cause persistent IMI should be regarded. However, more longitudinal studies still needed to increase our understanding of mastitis epidemiology in meat-producing ewes to help us establish more effective control strategies in these flocks.

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CAPÍTULO 2

Diversity and virulence genes from staphylococci isolated from milk samples, ewes' teat apex, and tonsils of suckling lambs in meat-producing sheep

(Veterinary Microbiology)

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ABSTRACT

Mastitis is an important disease in sheep, even in those used for meat production. The disease economically impacts the production system by increasing the lamb mortality and decreasing the lamb growth rates. Thus, our study investigated whether *staphylococci* isolated from clinical and subclinical mastitis, ewes' teat apex, and tonsils of suckling lambs in meat-producing sheep were genetically diverse and possess genes for enterotoxins, methicillin and penicillin resistances. A total of seventy-eight isolates of *Staphylococci* strains from Infectious Diseases Laboratory of Federal Rural University of Pernambuco database were used, from a study on mastitis in Santa Inês sheep. The isolates were selected based on their niche (clinical mastitis, subclinical mastitis, teat apex and lambs' tonsil). The isolates were submitted to MALDI-TOF for speciation, and PCR for virulence and resistance genes (*sea, seb, sec, sed, see, tsst, blaZ* and *mecA*). Additionally, the isolates were genotyped through RAPD. We observed 15 RAPD-types among 21 fingerprinted S. aureus isolates. The clusters differ among subclinical and clinical mastitis from lamb' tonsils and ewes' teat apex. The *S. aureus* hosted most of

virulence and resistance genes. Differently from *S. aureus*, all *Non-aureus Staphylococci* (NAS) isolated from extramammary niche did not differ from those isolated from milk samples. We found one MRSA and one MRNAS (i.e. *S. sciuri*) that were detected just in teat apex samples posing critical. One Health concerns considering the close contact of humans to the teat skin during routine clinical examination of the mammary gland. Our study provided more detail information of the epidemiology of ewe-associated staphylococci.

Keywords: mastitis, *Staphylococcus aureus*, non-*aureus* staphylococci, molecular characterization, small ruminant.

INTRODUCTION

Mastitis is an important disease in sheep, even in those used for meat production. The disease economically impacts the production system by increasing the lamb mortality and decreasing the lamb growth rates (Blagitz et al., 2014). Among several mastitis pathogens, there is a clear consensus that staphylococci are the primary etiological agents of ewes' intramammary infections (IMIs). While, *S. aureus* can cause both clinical and subclinical mastitis, this bacterium is the major causal agent of clinical mastitis in ewes. On the other hand, the heterogenous group of non-*aureus* staphylococci (NAS) are responsible for most of the cases of subclinical mastitis (Vasileiou et al., 2019).

It is well-known that staphylococci can be isolated from various sites of the body in ewes (Albenzio et al., 2003; Fragkou et al., 2007; Mavrogianni et al., 2007; Gougoulis et al., 2008; Fragkou et al., 2011), which suggest a potential dissemination of these bacteria into the mammary gland. However, no study has investigated the staphylococci cluster distribution in meat-producing ewes, which could allow us to legitimate a body site as a potential source of staphylococci to the mammary gland, helping us in monitor staphylococci spreading and outline critical control strategies (Piessens et al., 2011; Piessens et al., 2012; Wuytack et al., 2019). Furthermore, apart from one study using *S. aureus* isolates from clinical and subclinical mastitis in meat sheep, no study has examined whether staphylococci isolated from clinical and subclincal mastitis are genotype independent (Hoekstra et al., 2019).

Additionally, few studies have explored the presence of enterotoxins (Virmecati et al., 2006; Unal and Cinar, 2012; Unal et al., 2012; De Almeira, 2013; Azara et al., 2017; Zafalon et al., 2018) and methicillin and penicillin resistances (França et al., 2012; Unal and Cinar, 2012; Unal et al., 2012; Martins et al., 2017; Zafalon et al., 2018) in

staphylococci isolated from ewes' milk samples, and none of them have investigated if staphylococci isolated from extramammary niche can also harbor these genes, which are relevant concerns to public health (Moura et al., 2018a; Moura et al., 2018b).

Thus, our study investigated whether staphylococci isolated from clinical and subclinical mastitis, ewes' teat apex, and tonsils of suckling lambs in meat-producing sheep were genetically diverse and possess genes for enterotoxins, methicillin and penicillin resistances.

MATERIAL AND METHODS

Bacteria isolates identification

A total of seventy-eight isolates of *Staphylococci* strains from a previous study on mastitis in Santa Inês sheep, belonging to Infectious Diseases Laboratory of Federal Rural University of Pernambuco were used. The isolates were selected based on their niche (clinical mastitis, subclinical mastitis, teat apex and lambs' tonsil). The isolates were submitted to mass spectrometry through Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF). Bacterial samples were prepared as previously described by Schulthess et al., 2016. The colonies were transferred directly to a 96-spots polished steel plate (Bruker Daltonics) and with 1 µl of a saturated cyano-4-hydroxycinnamic acid (HCCA) matrix solution (Bruker Daltonics). The mass spectra were obtained and analyzed using a microflex mass spectrometer LT (Bruker Daltonics) in combination with RUO (Research-use-Only) versions of the MALDI Biotyper software package (version 3.0) and the reference database V. 3.1.2.0 (3,995 entries). The mass spectra of the samples were compared with the reference mass spectra in the database, calculating a value (score) between 0 and 3, reflecting the similarity between the sample and the

reference spectrum, showing the top 10 bank records corresponding data (SCHULTHESS et al., 2014).

Genomic DNA Extraction and Polymerase Chain Reaction (PCR)

Genomic DNA was extracted from isolates using the Wizard Kit SV Genomic DNA Purification System (Promega®-Madison, Wisconsin, USA) according to manufacturer's instructions. Polymerase chain reaction (PCR) was performed to access *blaZ* and *mecA* gene responsible for beta lactamic resistance (beta-lactamases production and protein binding protein modification, respectively) and for virulence genes (Staphylococcal enterotoxins and toxic shock syndrome toxin). Reactions were assembled separately for each gene in a final volume of 25µL per well, containing 1 uL DNA template added to a 24 uL master mix prepared by using a commercial kit (Illustra PuReTaq Ready-To-Go PCR beads - GE Healthcare, UK). PCR parameters for amplification of virulence factors genes fragments were as follows: 94 °C for 5 min. (initial denaturation); 32 cycles of 94 °C for 1 min., annealing temperature °C (specific for each fragments, see Table 1) for 1 min. and 72 °C for 1 min.; and 72 °C for 5 min. (final extension) and for resistance genes the thermal profile was 4 min. at 94 °C, followed by 32 cycles of denaturation at 94 °C for 30 sec., annealing at 50.5 °C for 30 sec. (*blaZ* gene) or 55 °C (*mecA* gene) and extended at 72 °C for 30 sec., with final extension at 72 °C for 5 min. Then 10µL of each reaction was electrophoresed for 40 minutes at 100V in 1.5% agarose gel stained with ethidium bromide, visualized and photo documented under ultraviolet light.

Table 1. Oligonucleotides used in this study

Target gene	Name	Oligonucleotide sequence (5´- 3´)	Expected size	At (ºC)[1]

sea[3]	sea_F	CCGAAGGTTCTGTAGAAGTATG	269pb	55
	sea_R	GCTTGTATGTATGGTGGTGTA		
seb[3]	seb_F	CCCGTTTCATAAGGCGAGTT	314pb	55
	seb_R	ACGTAGATGTGTTTGGAGCTAAT		
sec[3]	sec_F	AGATGAAGTAGTTGATGTGTATGG	451pb	57
	sec_R	CACACTTTTAGAATCAACCG		
sed[3]	sed_F	GTCACTCCACACGAAGGTAATAA	255pb	57
	sed_R	GAGACTTTAGACCCATCAGAAGAA		
see[3]	see_F	GCTGGAGGCACACCAAATA	301pb	55
	see_R	CATAACTTACCGTGGACCCTTC		
tsst[3]	tsst_F	ACCCCTGTTCCCTTATCATC	326pb	55
	tsst_R	TTTTCAGTATTTGTAACGCC		
blaZ[2]	blaZ_F	AAGAGATTTGCCTATGCTTC	102pb	55
	blaZ_R	GCTTGACCACTTTTATCAGC		
mecA[2]	mecA1	AAAATCGATGGTAAAGGTTGG	533bp	52
	mecA2	AGTTCTGCAGTACCGGATTTGC		

Staphylococal enterotoxins (*sea, seb, sec. sed* and *see*); Toxic shock syndrome toxin (*tsst*); Beta lactamase gene (*blaZ*), Methicillin resistance gene (*mecA*). [1]Annealing temperature °C. [2] PCR primers sequence were designed by Sabat et al. (2003). [3] PCR primers sequence were designed by Mehrotra et al. (2000).

Random Amplification of Polymorphic DNA (RAPD)

RAPD-PCR was carried out as described previously by Williams et al. (1990). The 23 nucleotide-long primer D11344 (5'-AGTGAATTCGCGGTGAGATGCCA-3') was used in these PCR reactions. The cycling programee was 4 cycles of [94 ºC, 5 min.; 36 ºC, 5 min. and 72 ºC], 30 cycles of [94 ºC, 1 min.; 36 ºC, 1 min.; and 72 ºC, 2 min.], and then 72 ºC, 10 min. After PCR, 20uL aliquots of products were eletrophoresed in 2% agaroses gels containing 0.5ug/ml ethidium-bromide in the gel and 1X Tris acetate running buffer and photographed under UV light. A 100-bp Plus DNA ladder (Thermos Scientific, USA) was used as a molecular size standard. Comparison of PCR fingerprinting profiles were performed using GelCompar II software, version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium). Similarity analysis of results was calculated using the Dice

coefficient/unweighted pair-group method with arithmetic mean - UPGMA (band matching tolerance 0.5% and optimization 0.5%) (SCHÄFER et al., 2019).

RESULTS

Staphylococcus aureus

Descriptive results and genotyping

Here, we observed 15 RAPD-types among 21 fingerprinted *S. aureus* isolates (Fig. 1). Our results showed that *S. aureus* isolated from clinical and subclinical mastitis (cluster 1) differ from those isolated from lamb' tonsils and ewes' teat apex (Fig. 1). From the other side of the coin, ewes' teat apex and lamb' tonsils strains are not genotype independent (Fig. 1). Therefore, while the cluster 2 is mainly represented by ewes' teat apex isolates (66.66%), the cluster 3 has 80% of isolates from lamb' tonsils.

Virulence genes

The *sea*, *seb*, *sec*, *sed*, *see*, *blaZ* and *mecA* genes were detected in 80.95%, 57.14%, 52.38%, 9.52%, 4.76%, 14.29% and 4.76% of all *S. aureus* strains, respectively (Fig. 1). No sample was positive for toxic shock syndrome toxin. All *S. aureus* isolates from milk samples habor sea gene, and 71.43%, 57.14% and 28.57% carried the *seb*, *sec* and *blaZ* genes (Fig. 1). Regarding *S. aureus* isolated from teat apex, 57.14%, 28.57%, 28.57%, 14.29%, 14.29% and 14.29% harbor *sea*, *seb*, *sec*, *sed*, *see* and *blaZ* genes. The *sea*, *seb*, *sec*, *sed* and *see* genes were detected in 85.71%, 85.71%, 57.14%, 14.29% and 14.29% of *S. aureus* strains originated from lamb' tonsils. The single methicillin-resistant *S. aureus* (MRSA) strain was found in the teat apex. Just three *S. aureus* strains (12.29%)

did not harbor any of the investigated genes. Overall, there was no association between any cluster and a specific virulence gene.

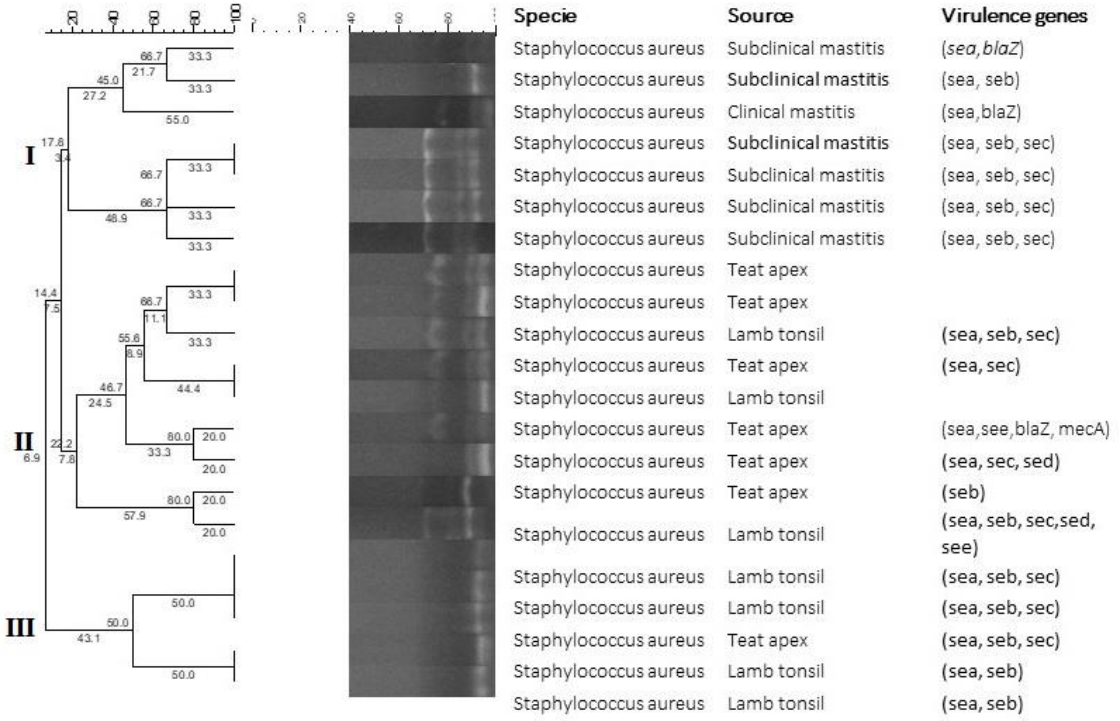

Figure 1. Dendrogram of RAPD profiles of sheep-associated *Staphylococcus aureus* strains.

Staphylococcus sciuri

Descriptive results and genotyping

We found 23 RAPD-types among 33 fingerprinted *S. sciuri* isolates (Fig. 2). From a different behavior from *S. aureus*, none of the clusters were exclusively represented by *S. sciuri* isolated from IMIs or extramammary niches (i.e. lamb' tonsils and ewes' teat apex) (Fig. 2). Nonetheless, the cluster 1 stood for *S. sciuri* strains isolated from subclinical mastitis (69.23%) and teat apex (30.77%) and covered a great proportion

(69.23%) of *S. sciuri* strains isolated from subclinical mastitis. The cluster 2 was represented by *S. sciuri* strains from clinical mastitis (20%), subclinical mastitis (20%), teat apex (20%) and lamb' tonsils (40% - all of them). The cluster 3 and 5 have only *S. sciuri* strains isolated from teat apex. The cluster 4 has *S. sciuri* strains isolated from milk samples (60%) and teat apex (40%).

Virulence genes

In the current study, 15 (45.45%) of all *S. sciuri* strains did not carry any investigated virulence genes (Fig. 2). The *sea*, *seb*, *sec*, *see* and *blaZ* genes were detected in 39.39%, 36.36%, 21.21%, 9.09% and 21.21% of *S. sciuri* strains. No sample was positive for *sed* and toxic shock syndrome toxin. The *S. sciuri* originated from milk samples carry *sea* (50%), *seb* (50%), *see* (25%) and *blaZ* (43.75%) genes, but none of them harbor *sec*, *sed* and *mecA* genes. The single methicillin-resistant NAS (MRNAS) strain was found in the teat apex, in contrast to no positive sample for *blaZ* gene. The enterotoxins genes for *sea* (23.08%), *seb* (23.08%) and *sec* (30.77%) were the only ones that were detected in *S. sciuri* isolated from teat apex. The *S. sciuri* isolates from the lamb' tonsils were positive for only *sea* (25%) and *sec* (75%) genes. There was no association between any cluster and a specific virulence gene. Overall, *S. sciuri* showed lower rates of the presence of the investigated virulence genes than *S. aureus*, but their presence was higher than the other NAS species.

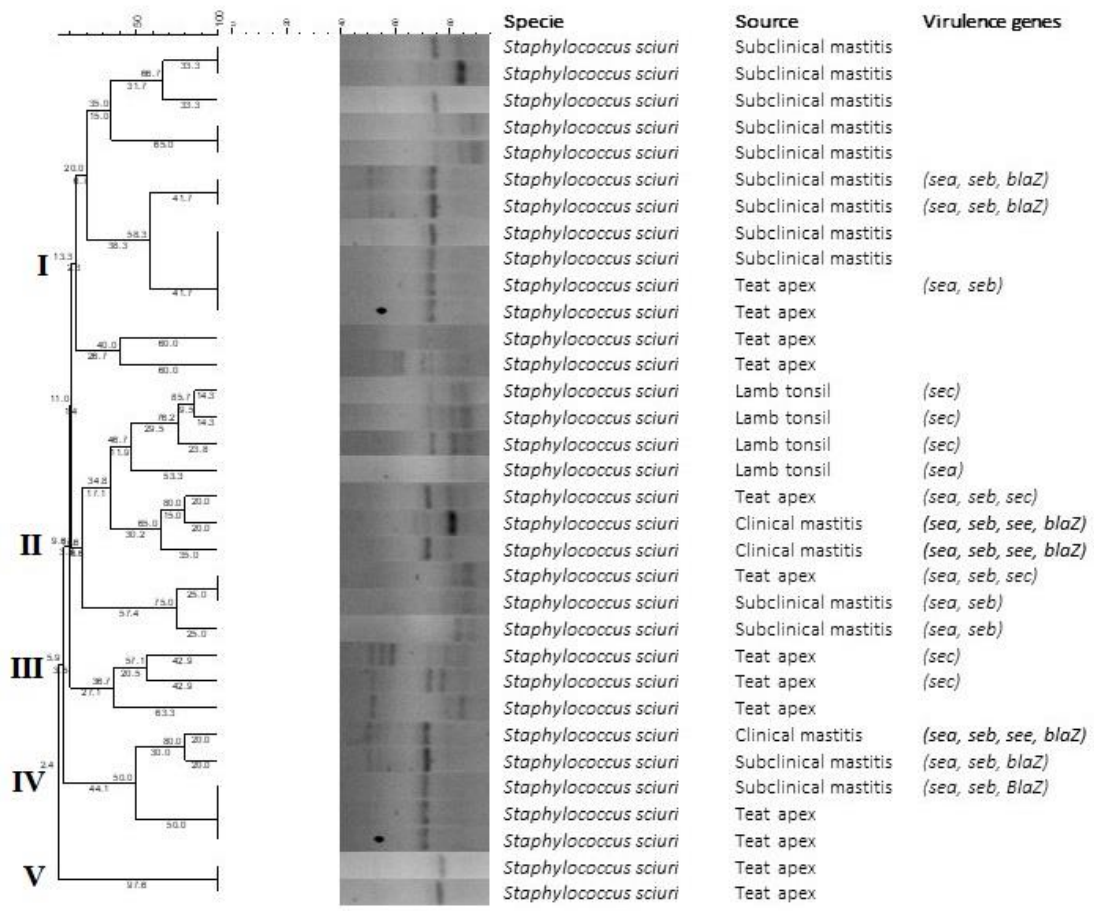

Figure 2. Dendrogram of RAPD profiles of sheep-associated *Staphylococcus sciuri* strains.

Staphylococcus simulans

Descriptive results and genotyping

We observed 10 RAPD-types among 16 fingerprinted *S. simulans* isolates (Fig. 3). Similarly to *S. sciuri*, none of the clusters were exclusively represented by *S. similans* isolated from IMIs or extramammary niches (Fig. 3). The cluster 1 stood for *S. sciuri* strains isolated from teat apex (60%), lamb' tonsil (30%) and subclinical mastitis (10%). The cluster 2 represent just one isolate from teat apex, while clusters 3 stood for one

strain isolated from all ecological niches, i.e. subclinical mastitis (milk sample), teat apex and lamb' tonsil. The cluster 3 was composed by two *S. simulans* strains isolated from lamb' tonsils. One *S. simulans* strain isolated from teat apex had a very lower level of similarity in comparison to other strains and were not assigned into clusters.

Virulence genes

The majority of *S. simulans* were negative (62.5%) for all searched virulence genes. Beyond that, just the *sea* (25%), *seb* (37.5%) and *sec* (37.5%) genes were detected (Fig. 3).

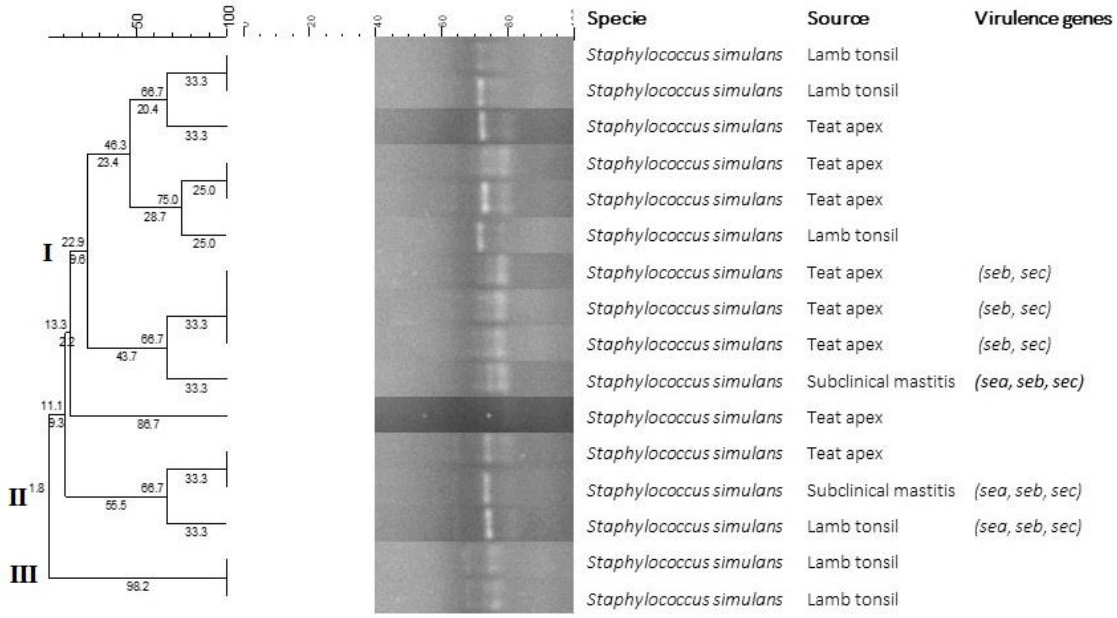

Figure 3. Dendrogram of RAPD profiles of sheep-associated *Staphylococcus simulans* strains.

Staphylococcus xylosus

Descriptive results and genotyping

In the current study, *S. xylosus* was not isolated from lamb' tonsils. As all other NAS species, a specific cluster of *S. simulans* were not exclusively associated with IMIs or extramammary niches (Fig. 4). One *S. xylosus* strain isolated from subclinical mastitis had a very lower level of similarity in comparison to other strains and were not assigned into clusters.

Virulence genes

In the present study, 45.45% of *S. xylosus* strains did not carry any investigated virulence genes. The *sea*, *seb*, *sec* and *blaZ* genes were detected in 45.45%, 36.36%, 36.35% and 9.09% of all *S. xylosus* strains (Fig. 4).

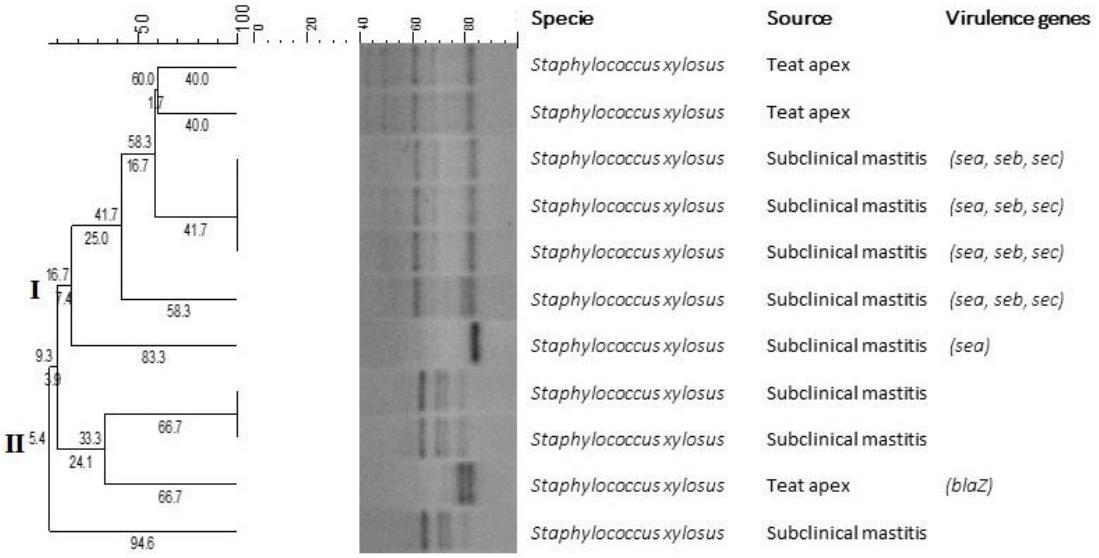

Figure 4. Dendrogram of RAPD profiles of sheep-associated *Staphylococcus xylosus* strains.

DISCUSSION

Our study did not strengthen the idea that the *S. aureus* strains isolated from extramammary niche (i.e. teat apex and lamb' tonsils) is an important source for *S. aureus* strains that cause IMIs in meat-producing ewes. Thus, even though *S. aureus* can colonize extramammary niches of ewes, our study showed that the udder is the most important reservoir of these bacteria, as all *S. aureus* isolated from milk samples were genotypically distinct from those isolated from lamb' tonsils and ewes' teat apex. In this regard, we postulated that *S. aureus* isolated from milk samples (mammary gland environment) are more likely to survive in this niche due to their long co-evolution and selective evolutionary pressure resulting in their adaptation to this environment with low oxygen availability and iron-restricted conditions (Le Maréchal et al., 2009), beyond their better ability to use lactose as a primary carbohydrate (Sharer et al., 2003) and evade the host immune defense mechanisms (Foster et al., 2014).

In agreement with our results, Leuenberger et al. (2019) showed that the epidemiology of *S. aureus* in dairy cows depended markedly on the genotype that are strictly related to its adaptation to a particular host and even to a unique body site. Conversely, Albenzio et al. (2003) considered that lamb's mouths were a major source of ewe udder and milk contamination, even though no genotype analysis were performed in this study. Furthermore, while the size is limit, *S. aureus* and *S. sciuri* strains isolated from clinical and subclinical mastitis did not appear to be genotypically distinct, as previous described for *S. aureus* isolated from both goats and sheep (Hoekstra et al., 2019), suggesting that clinical statuses of the staphylococcal IMIs in meat-producing ewes are mainly determined by host factors.

From other perspective, *S. aureus* isolated from teat apex did not differ from those isolated from lamb' tonsils, which suggest a potential transmission of this bacterium from the ewes' teat apex into the tonsils of lambs during suckling. On the other way around, Fragkou et al. (2011) demonstrated the transmission of *Mannheimia haemolytica* from the tonsils of lambs to the teat of ewes.

Differently from *S. aureus*, all NAS isolated from extramammary niche did not differ from those isolated from milk samples. Thus, we speculate that the same NAS present in the teat apex can colonize the teat canal and even the teat duct, which could afford protection under certain circumstances, nonetheless with reducing efficacy of teat and mammary gland defenses, the same NAS may ascend to the mammary gland parenchyma and, ultimately cause mastitis, as previously proposed (Fragkou et al., 2007; Vasileiou et al., 2019). Corroborating with the aforementioned, De Visscher et al. (2016) showed that prepartum teat apex colonization with *Staphylococcus chromogens* increased the likehood of IMI by this bacterium in dairy cows in corresponding quarter at parturition. Thus, milk can act as a source of NAS for teats during milking and the teat microbiota can serve as a source of NAS that cause IMI (De Visscher et al., 2014). Furthermore, the presence of the same RAPD-type NAS in lamb' tonsils, teat and milk reinforce the idea of a potential transmission of this bacterium from the ewes' teat apex into the tonsils of lambs during suckling, as previously reported (Vasileiou et al., 2019).

Here, we found one MRSA and one MRNAS (i.e. *S. sciuri*) that were detected just in teat apex samples posing critical One Health concerns considering the close contact of humans to the teat skin during routine clinical examination of the mammary gland in the herd studied, which are in accordance with our previous results in dairy goats (Moura

et al., 2018a). Apart from a study that identified MRSA isolated from udder skin (Carfora et al., 2016), to the best of our knowledge, ours is the first report on the detection of MRSA and MRNAS isolated from teat apex in sheep. Although, we did not find any MRNAS here in milk samples, the potential transmission of NAS from teat to the mammary could arise the concerns of milk contamination with MRNAS, beyond its possible risk of contamination of lambs and human with both MRSA and MRNAS during lamb suckling and contact persons during management processes. In this regard, *mecA*-positive NAS may act as potential donors for the rise in new MRSA clones (Garza-Gonzales et al., 2010).

Among the heterogenous group of NAS, we should highlight the important role of *S. sciuri* specie, which has low host specificity and may act as an important reservoir for virulence genes, including methicillin-resistance, as it can easily spread between host species by direct cross-infection (Nemeghaire et al., 2014; Khazandi et al., 2018). Considering our results, it can be observed that among NAS species, *S. sciuri* posed a higher rates of virulence genes, similarly to *S. aureus*, which has great implications for One Health issues. Thus, considering the potential transmission of virulence genes between staphylococcal from different ecological niches and even distinct hosts (especially *S. sciuri*), the staphylococcal virulence genes detected here should not be ignored. For instance, genes encoding enterotoxins have also distinct supports, most of which are mobile genetic elements (Le Loir et al., 2003).

Moreover, although the presence of staphylococcal enterotoxins has been previously studied in ewes (Virmecati et al., 2006; Unal and Cinar, 2012; Unal et al., 2012; De Almeira, 2013; Azara et al., 2017; Zafalon et al., 2018), the impact of sheep-associated

staphylococci (i.e. isolated from extramammary niches) has long been neglected. Furthermore, most of the studies available in the literature failure to provide information at specie level, as variations within and between the heterogenous group of NAS species exist. Here, we investigated the presence of the most common enterotoxins (Bergdoll and Lee Wong, 2005), and critical mechanisms associated with antimicrobial resistance that can occur by one of the two mechanisms: the first is related to the production of β-lactamase associated with *blaZ* gene (Lowy, 2003; Aragão et al., 2019), and the second involves the production of penicillin binding protein 2a (PBP2a), a protein that binds to β-lactams and is associated with the *mecA* gene (Guignard et al., 2005; Aragão et al., 2019). Thus, our study provided more detail information of the epidemiology of ewe-associated staphylococci with critical One Health concerns.

CONCLUSIONS

In our study, all *S. aureus* isolated from milk samples were genotypically distinct from those isolated from lamb' tonsils and ewes' teat apex, result of their long co-evolution and selective evolutionary pressure, thus, we can affirm that transmition between milk and lamb is less likely to occur. Among NAS group, we should highlight the important role of *S. sciuri* specie, which has low host specificity and may act as an important reservoir for virulence genes, including methicillin-resistance, similarly to *S. aureus*. About transmition of NAS between ewes and lambs, they show a different pattern with close related clusters that could indicate a cross infection through milk ingestion.

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CAPÍTULO 3

First report of livestock-associated *Staphylococcus aureus* and *Staphylococcus sciuri* harboring the mecC gene in a Brazilian sheep herd

(Emerging Infectious Diseases)

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Livestock-associated (LA) methicillin-resistant *Staphylococcus aureus* have globally emerging during the past decades (Lakhundi and Zhang, 2018). The resistance to methicillin involves the production of penicillin binding protein 2a (PBP2a), a protein that binds to β-lactams and is associated with the *mecA* gene. However, a homologous gene (only 69% and 63% identity at the DNA and amino acid levels, respectively), *mecC* gene (previously so-called $mecA_{LGA251}$) was described in United Kingdom (2007) during an epidemiological survey of bovine mastitis. Further, this gene was identified in humans, including an isolated obtained in 1975, indicating that the prevalence of MRSA containing *mecC* has been underestimated for a long time (Garcia-Alvarez et al., 2011; Lakhundi and Zhang, 2018). Beyond that, this gene was later identified in other staphylococcal species, including *Staphylococcus sciuri* (Khazandi et al., 2018; Lakhundi and Zhang, 2018). Thus, there are growing concerns about livestock animals acting as an important reservoir and source of the emergence of novel methicillin-resistance staphylococci clones threaten the human and animal health.

Although, several studies have identified *mecC*-containing staphylococci strains in Europe (Lakhundi and Zhang, 2018), reports outside Europe are scarce (Srednik et al.,

2017). To the best of our knowledge, this study described the first detection of methicillin-resistant *Staphylococcus aureus* and *Staphylococcus sciuri* harboring the *mecC* gene in a Brazilian sheep herd.

The current study was carried out in a herd from a teach and research institute (Center of Agrarian Sciences, Federal University of Paraíba, Bananeiras, Brazil). Samplings were collected from 40 Santa Ines lactating ewes at December 2016 until May 2017. Teat apex samples were acquired by rubbing a sterile moisted swab onto teats apex, placing them into sterile tubes containing 5 mL Muller-Hinton broth with 6.5% NaCl, and then streaked onto mannitol salt agar and incubated at 37 °C for 24 h. The bacteria colonies were further identified as previously described (Moura et al., 2018). The milk samplings were aseptically collected from each mammary half for bacteriological analysis as previously standardized by National Mastitis Council (Oliver et al., 2004). The bacteriological culture was performed by culturing 0.01 mL of each milk sample on 5 % sheep blood agar plates. The plates were incubated for 72 h at 37 ºC, followed by observation of colony morphology, Gram staining and biochemical testing (Oliver et al., 2004). All bacteria isolates were further submitted to mass spectrometry through Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF) for speciation. The isolates were initially tested using the Kirby Bauer disk diffusion technique with disks for oxacillin (1 µg) and cefoxitin (10 UI/30 µg) for the prediction of methicillin-resistant *S. aureus* (MRSA). Antimicrobial susceptibilities were also performed using a broad antimicrobial susceptibility profile based on the automated Vitek 2® compact system (BioMériux, Inc., Durham, NC, USA) by minimum inhibitory concentration (MIC) using the veterinary susceptibility AST-GP69 card (BioMériux, Inc., Durham, NC, USA). We

followed the recommendations and interpretative criteria defined by Clinical and Laboratory Standards Institute (CLSI, 2018) to define susceptibility/resistance to antimicrobials.

The *mecC* gene was detected by polymerase chain reaction (PCR), as previously described by Paterson et al. (2014). Briefly, reactions were assembled in a final volume of 15 µL per well, containing 100 ng of DNA template, 10 pmol of each primer (F-CATTAAAATCAGAGCGAGGC; R-TGGCTGAACCCATTTTTGAT), Taq buffer (10 mM Tris, 50 mM KCl, 2.5mM $MgCl_2$), 200 mM dNTPs and 1 U Taq DNA polymerase (Cenbiot, Taq DNA polymerase, Ludwig Biotec, Porto Alegre, Brazil). Thermocicler conditions were 4 min. at 94 °C, followed by 32 cycles of denaturation at 94 °C for 30 sec., annealing at 55 °C and extended at 72 °C for 30 sec., with final extension at 72 °C for 5 min. Then 10 µL of each reaction was electrophoresed for 40 minutes at 100 V in 1.5% agarose gel stained with BlueGreen, visualized and photodocumented under ultraviolet light.

Here, the *mecC* gene was detected in one *S. aureus* strain isolated from the teat apex and two *S. sciuri* strains - one isolated from teat apex and the other one isolated from a milk sample obtained from a subclinical case of mastitis. The staphylococci strains originated from two lactating Santa Ines. Curiously, the MRSA and methicillin-resistant *S. sciuri* isolated from a subclinical case of mastitis came from the same animal, which indicates a transference of resistance between these staphylococcal strains, as previously proposed (Lakhundi and Zhang, 2018). Thus, our study strengthens the idea that *mecC* gene may have originated from non-*aureus* staphylococci, as previously suggested for *mecA*. Nonetheless, this issue is still under debate, and further investigations including whole genome sequencing of *mecC*-positive staphylococci are

needed, which may offer hints into the origin and evolution of this resistant determinant (Khazandi et al., 2018; Lakhundi and Zhang, 2018). Also, both *S. sciuri* isolates shown resistance pattern to oxacillin in MIC test and were multidrug resistant (table 1).

Furthermore, the close contact between these animals during the milking routine and routine clinical examination of the mammary gland in herd studied pose a great risk to human health, as these animals were also used for teaching proposes. In addition, the consumption of raw milk is a common practice in the Northeast region of Brazil due to the cultural habits and lack of information on public health issues, despite that the marketing of raw milk is illegal in Brazil (Oliveira et al., 2011). Altogether, the detection of circulating staphylococci containing *mecC* gene in the Northeast region of Brazil brings a critical issue for the livestock animals as reservoir and source of clinically relevant superbug with a potential risk of transmission to humans. Thus, human and veterinary medicine professionals should implement collaborative efforts and health cooperation programs to determine and monitor the spread of these methicillin-resistance staphylococci in the human–animal interface.

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Table 1. Antibiotic resistance pattern of one *Staphylococcus aureus* isolated from ewe teat apex niche and two *Staphylococcus sciuri* isolated from ewe subclinical mastitis sample and teat apex.

Drugs	***Staphylococcus aureus* (Teat apex)**	***Staphylococcus Sciuri* (subclinical mastitis)**	***Staphylococcus Sciuri* (Teat apex)**
Fusidic Acid	S	R	R
Amoxicillin / Clavulanic Acid	S	S	S
Benzylpenicillin	S	R	R
Clindamycin	S	S	S
Chloramphenicol	S	S	S
Enrofloxacin	S	S	S
Gentamycin	S	S	S
Marbofloxacin	S	S	S
Nitrofurantoin	S	S	S
Oxacillin	S	R	R

Rifampicin	S	R	R
Tetracycline	S	R	R
Trimetropim / sulfamethoxazole	S	S	S
Vancomycin	S	S	S

R = Resistant; S = Sensitive.

Considerações finais

Demonstrou-se no presente estudo o impacto negativo da mastite nas ovelhas de corte Santa Inês sobre o desempenho e a morte de cordeiros, no entanto, o agente etiológico e seu potencial para causar infecção intramamária (IIM) persistente deve ser considerado. Estafilococos não-*aureus* (ENA) não devem ser considerados como um grupo homogêneo de bactérias, sendo que algumas espécies de ENA causam infecção IIM persistente, enquanto outras apresentam perfil mais oportunista, enfatizando a necessidade da determinação precisa da espécie bacteriana envolvida na etiologia da mastite. O nicho ecológico determina o agrupamento genético de *S.* aureus, porém, o mesmo não se aplica aos ENA. Algumas espécies de estafilococcos não-*aureus*, apesar de serem considerados patógenos secundários em bovinos, devem ser considerados como patógenos principais em ovinos podendo causar grandes perdas econômicas a ovinocultura de corte, e carrear genes de resistência a antimicrobianos e fatores de virulência críticos à saúde animal e humana.

ANEXOS

Anexo I – Artigo publicado na revista Brazilian Journal of Veterinary Research and Animal Science

CASE REPORT

Catarrhal mastitis by *Staphylococcus simulans* in a nulliparous goat

Mastite catarral causada por* Staphylococcus simulans *em uma cabra nulipara

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Catharral mastitis by *Staphylococcus simulans* in a nulliparous goat

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Resumo

Relatamos o caso de uma cabra nulípara da raça Parda Alpina, de 1 ano, pertencente ao Setor de Caprinocultura da Universidade Federal da Paraíba – Bananeiras - Brasil. Ambas as glândulas foram naturalmente infectadas por *Staphylococcus simulans* α-hemolítico. As glândulas mamárias apresentaram mastite aguda catarral com envolvimento sistêmico, respondendo positivamente ao tratamento sistêmico com gentamicina associada a amoxicilina. O presente relato sugere a importância de considerar o potencial patogênico de *Staphylococcus não-aureus* (SNA) com causador de mastite clínica também em animais nulíparos. O isolado mostrou resistência a tetraciclina e continha genes de produção de toxinas estafilocócicas (*sec*, *seg* e *TSST-1*). Além disso, tem sido relatado que *Staphylococcus simulans* é um patógeno emergente em seres humanos causando infecções cutâneas e osteoarticulares, principalmente naqueles que têm contato íntimo com animais de fazenda. Até onde sabemos, este é o primeiro relato de uma mastite clínica em uma cabra nulípara causada por *Staphylococcus simulans*.

Palavras-chave: infecção intramamária, estafilococos coagulase-negativos, small ruminants.

Abstract

We report a case of a nulliparous Alpine Goat, 1 year old, belonging to a dairy goat farm in semi-arid region of Brazil. Both glands were naturally infected by α-hemolytic *Staphylococcus simulans* and evolved similarly clinically signs. The mammary glands presented an acute catarrhal mastitis with systemic clinical signs that responded positively to treatment with gentamicin associated to amoxicillin. The present report suggests the importance of the pathogenic potential of *non-aureus Staphylococci* strains (NAS) as a cause of clinical mastitis also in nulliparous animals. The isolate showed resistance to tetracycline and contained staphylococcal toxin production genes (*sec, sec and TSST-1*). Moreover, it has been reported that *Staphylococcus simulans* is an emerging pathogen in humans causing cutaneous and osteoarticular infections mainly in those who have close contact to farm animals. To the best of our knowledge, it is the first report of a clinical mastitis in a nulliparous goat caused by *Staphylococcus simulans*.

Keywords: intramammary infection, coagulase-negative staphylococci, pequenos ruminantes.

CASE REPORT

Mastitis is the most important and costly disease in dairy goat production. Animals can present physical, chemical, pathological and bacteriological changes in milk and glandular tissue. Even though *Staphylococcus aureus* is the most common agent involved in clinical mastitis cases (BERGONIER et al., 2003), *non-aureus Staphylococci* (NAS) strains play a great role in goat mastitis. For instance, unlike in cows, NAS especially novobiocin-sensitive NAS, such as *Staphylococcus (S.) simulans*, lead to a great increase in somatic cell count in small ruminants, and then can be regarded as major pathogens. The importance of staphylococci in dairy goat herds is not only limited to animal production, but is also a relevant issue that should be regarded due to implications to public health and well-being (BERGONIER et al., 2003).

The present study reports a case of nulliparous Alpine Goat, one year of age, raised in intensive grazing management, housed in elevated goat shed with food (chopped Elephant grass + concentrate + minerals) and fresh water ad libitum. The animal showed episcleral injection, light fever (40 ºC) and signs of udder inflammation (local pain and swelling). Inflammation of both halves of mammary gland evolved similarly. They presented increased temperature, edema and clumps on milk, what was characterized as catarrhal mastitis (DELLA LIBERA et al., 2007). The goat was totally dried off and treated according to the following protocol: Gentamicin 4mg/kg + Amoxicillin 15 mg/kg (IM-12/12 h for three days) and Flunixin meglumin 2,2mg/kg (IV- single dose). After treatment, the animal clinically recovered from infection. Microbiological culture of both mammary gland secretions was performed before treatment. The samples were streaked directly onto Blood agar plates (BD, Heidelberg, Germany) and incubated at 37 °C for 24 h. Pure homogeneous colonies, circular, pinhead, convex, light grey were cultured showing α-hemolytic pattern. Those colonies were submitted to VITEK® 2 Compact (bioMérieux, Marcy-l'Étoile, France) for speciation and antimicrobial susceptibility test by the determination of the minimal inhibitory concentration (LIGOZZI et al., 2002), and the bacteria was considered as resistant or susceptible to antimicrobials according to CLSI (2013). *S. simulans* was identified and the results of antimicrobial susceptibility test was shown in Table 1. We also performed genotyping for important virulence genes (Table 2)

(MEHROTRA; WANG; JOHNSON, 2000). Briefly, the DNA was extracted from pure culture isolates, using a commercial kit (Wizard® Genomic DNA Purification Kit, Madison, Wisconsin, EUA) according to manufacturer's instructions and stored at -20 °C. The primers used for amplification of the virulence factors genes fragments are shown in Table 2. The PCR products were visualized by electrophoresis in 2 % agarose gel stained with Blue Green Loading Dye I (LGC Biotecnologia, São Paulo, Brazil) and photographed under UV illuminator (Molecular Imaging L.PIX Loccus biotecnologia, São Paulo, Brazil).

To the best of our knowledge, this is the first report of *S. simulans* strain causing clinical mastitis in goat. This bacteria isolate was positive for two staphylococcal enterotoxigenic genes (*sec* and *seg*) and also for toxic shock syndrome toxin-1 (*TSST-1*), but didn't show the virulence genes for clumping factor A (*clfA*), clumping factor B (*clfB*), fibronectin-binding protein A (*fnbA*) and fibronectin-binding protein B (*fnbB*). There is scarce information of potential risk of NAS isolated from goats for human health. One of the major concerns about staphylococcal infections is the potential to produce staphylococcal enterotoxins (SEs), such as *TSST-1*. *TSST-1* gene is one of the most prevalent in severe cases of bovine staphylococcal mastitis, and can cause disease in humans, although investigations of staphylococcal enterotoxins (SEs) in goats are rare (FREITAS et al., 2008); PEIXOTO; MOTA; COSTA, 2010).

Despite NAS being one of the most isolated pathogens in cases of subclinical mastitis in goats and cows with high persistence rates compared to other species (RUEGG, 2009), *S. simulans* association with a clinical case in goats had not been previously reported. Regarding that clinical mastitis needed to be carefully handle by veterinarians and dairy farms, as the potential of transmission of *S. simulans* to humans should not discarded. Thus, from the public health point of view, recent cases of septic osteoarthritis (MALLET et al., 2011) and cutaneous infections (TOUS ROMERO, 2016) in humans have been reported mainly in people who had intimate contact with production animals, suggesting that the zoonotic potential of this pathogen cannot be neglected.

Finally, although mastitis is a great problem for goat dairy production, there is a scarce information about mastitis in nulliparous goats (JÁCOME et al., 2014). Furthermore, beyond the animal and public health implications that should not be neglected, our results

strengthen the idea that the control measures for this disease in goats cannot wait until the beginning of lactation, since infection can occur before milk production and milking, as it has been discussed and highlighted in the last decade for dairy heifers (DE VLIEGHER et al., 2012).

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Table 1. Antibiotic resistance pattern of *Staphylococcus simulans* isolated from a catharral mastitis case in a nulliparous goat

Antibiotics	MIC	Interpretation	Antibiotics	MIC	Interpretation
Fusidic Acid	≥ 32	S	**Kanamycin**	≤ 4	S
Ampicillin/sulbactam	16	S	**Marbofloxacin**	≤ 0,5	S
Benzylpenicillin	≥ 0,5	S	**Nitrofurantoin**	256	S
Clindamycin	≥ 8	S	**Oxacillin**	≥ 4	S
Chloramphenicol	8	S	**Rifampicin**	≥ 32	S
Enrofloxacin	≤ 0,5	S	**Tetracycline**	≥ 16	R
Erythromycin	≥ 8	S	**Trimethoprim/Sulfamethoxazole**	≤ 10	S
Gentamicin	≤ 0,5	S	**Vancomycin**	≥ 32	S
Imipenem	2	S			

S = sensitive; R = resistant. MIC: minimum inhibitory concentration.

Table 2. Oligonucleotides for the different virulence genes used in the study.

Target gene	Name	Oligonucleotide sequence (5´- 3´)	Expected size	At (ºC)[1]
fnbA	fnbA_R	ACTTCACCTGTCGCCATTAC	539pb	61
	fnbA_F	GCAGTACAAGCACCACAAAC		
fnbB	fnbB_F	AGGCGACGGCAAAGATAAA	317pb	57
	fnbB_R	TAGTAACCTGACCACCACCT		
clfA[2]	clfA_F	GATTCTGACCCAGGTTCAGA	945pb	60
	clfA_R	CTGTATCTGGTAATGGTTCTTT		
clfB[2]	clfB_F	ATGGTGATTCAGCAGTAAATCC	880pb	55
	clfB_R	CATTATTTGGTGGTGTAACTCTT		
sea	sea_F	CCGAAGGTTCTGTAGAAGTATG	269pb	55
	sea_R	GCTTGTATGTATGGTGGTGTA		
seb	seb_F	CCCGTTTCATAAGGCGAGTT	314pb	55
	seb_R	ACGTAGATGTGTTTGGAGCTAAT		
sec[3]	sec_F	AGATGAAGTAGTTGATGTGTATGG	451pb	57
	sec_R	CACACTTTTAGAATCAACCG		
sed	sed_F	GTCACTCCACACGAAGGTAATAA	255pb	57
	sed_R	GAGACTTTAGACCCATCAGAAGAA		
see[3]	see_F	GCTGGAGGCACACCAAATA	301pb	55

	see_R	CATAACTTACCGTGGACCCTTC		
seg	seg_F	GCCAGTGTCTTGCTTTGTAATC	491pb	57
	seg_R	GAATGCTCAACCCGATCCTAA		
seh	seh_F	CACATCATATGCGAAAGCAGAAG	365pb	56
	seh_R	CCCAAACATTAGCACCAATCAC		
sei	sei_F	AGGCAGTCCATCTCCTGTATAA	568pb	60
	sei_R	TGCTCAAGGTGATATTGGTGTAG		
tsst-1[3]	tsst_F	ACCCCTGTTCCCTTATCATC	326pb	55
	tsst_R	TTTTCAGTATTTGTAACGCC		

[1]Annealing temperature °C. [2]PCR primers sequence were designed by SABAT et al., 2003. [3]PCR primers sequence were designed by MEHROTRA; WANG; JOHNSON, 2000. cflA: clumping factor A (cflA); cflB: clumping factor B; fnbA: fibronectin-binding protein A; fnbB: fibronectin-binding protein B; TSST-1: toxic shock syndrome toxin-1; sea: staphylococcal enterotoxin A; seb: staphylococcal enterotoxin B; sec: staphylococcal enterotoxin C; sed: staphylococcal enterotoxin D; see: staphylococcal enterotoxin E; seg: staphylococcal enterotoxin G; seh: staphylococcal enterotoxin H; and sei: staphylococcal enterotoxin I.

Anexo II – Resumo expandido publicado no suplemento da revista Pesquisa Veterinária Brasileira - Uso do MALDI-TOF como ferramenta de diagnóstico das mastites causadas por Staphylococcus aureus em ovinos de corte

224. Moura G.S., Marques M.F.S., Souza F.N., De Vliegher S., Da Costa L.B.C. & Mota R.A. **Uso da técnica de MALDI-TOF no diagnóstico das mastites causadas por *Staphylococcus aureus* em ovinos de corte.** *Pesquisa Veterinária Brasileira 38(Supl.):386-387.* Laboratório de Bacteriologia e Doenças Infecciosas, Universidade Federal Rural de Pernambuco, R. Manuel de Medeiros s/n, Dois Irmãos, Recife, PE 52171-900, Brasil. E-mail: guilhermesmoura@hotmail.com
Recursos provenientes da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)

Moura, G.S.; Marques, M.F.S.; Souza, F.N.; De Vliegher, S.; Da Costa, L.B.C.; Mota, R.A. Uso do MALDI-TOF como ferramenta de diagnóstico das mastites causadas por *Staphylococcus aureus* em ovinos de corte. *Pesquisa Veterinária Brasileira 38(Supl.):00-00*. Laboratório de Bacteriologia e Doenças Infecciosas, Universidade Federal Rural de Pernambuco, R. Manuel de Medeiros, S/N – Dois Irmãos, Recife, PE 52171-900, Brasil. E-mail: guilhermesmoura@hotmail.com
Projeto realizado com auxílio de recursos provenientes da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES.

Introdução: A mastite é uma enfermidade com grande impacto econômico e no bem-estar de ovinos de corte nos diversos sistemas de produção. Nestes rebanhos, a mastite é considerada a maior causadora das mortes em cordeiros até o desmame devido à dificuldade das ovelhas em fornecer as suas crias leite de boa qualidade nutricional e volume suficiente (GRANT; SMITH; GREEN, 2016), levando ao chamado complexo inanição-hiportermia. Ela é causada principalmente pela infecção por bactérias gram-positivas, sendo os *Staphylococcus não-aureus* as espécies mais prevalentes (ACOSTA et al., 2016), entretanto, o *Staphylococcus aureus* é o responsável pelos casos mais severos da doença (MØRK et al., 2007). Nos últimos anos vários trabalhos têm voltado as atenções para os rebanhos de corte devido a alta prevalência desta enfermidade, que neste caso determina a perda do principal produto deste tipo de exploração, o cordeiro (VERÍSSIMO et al., 2010). Deste modo, estudos que visem o diagnóstico rápido e eficiente de um dos mais perigosos agentes causadores de mastite em ovinos de corte é de grande importância sobretudo para região nordeste do Brasil que possui forte aptidão para produção destes animais, propondo o uso de ferramentas diagnósticas precisas e com capacidade de processar um número substancial de amostras em um curto período, possibilitando agilidade na tomada de decisão. Desta forma o presente trabalho se propõe a comparar o método tradicional de identificação de *Staphylococcus aureus* por testes fenotípicos e a técnica da espectrometria de massa por ionização e dessorção a laser assistida por matriz - tempo de voo (MALDI-TOF) em isolados oriundos de mastites em ovelhas Santa Inês.

Material e Métodos: Um total de 30 isolados de *Staphylococcus aureus* oriundos de casos de mastite clínica n=6) e mastite subclínica (n=24) foram utilizados nesse estudo. Estes isolados foram obtidos através de metodologia descrita por MARTINS et al., 2015 com adaptações. Resumidamente, foram inoculadas secreções lácteas de ovelhas Santa Inês em meio ágar sangue enriquecido com 5% de sangue de carneiro desfibrinado e encubadas em aerobiose a 37 ºC por 24h. As bactérias provenientes destas culturas foram submetidas a coloração de gram e depois inoculadas em meio Manitol Salgado. Colônias que apresentaram capacidade de fermentar o manitol, indicada pela alteração no indicador vermelho de fenol e modificando a cor do meio para amarelo, foram selecionadas e submetidas ao teste de coagulase e catalase. Foram consideradas como *Staphylococcus aureus* as colônias bacterianas positivas a fermentação do sal manitol e aos testes de coagulase e catalase. Estas mesmas bactérias foram submetidas à identificação pela técnica de espectrometria de massa por ionização e dessorção a laser assistida por matriz - tempo de voo (MALDI-TOF). As amostras bacterianas foram preparadas como descrito anteriormente por SCHULTHESS et al., 2014. As colônias foram transferidas diretamente para uma placa de aço polido com 96 alvos (Bruker Daltonics) e cobertas com 1 µL de uma solução saturada de matriz de ácido-ciano-4-hidroxicinâmico (HCCA) (Bruker Daltonics).

Os espectros de massa foram obtidos e analisados utilizando um espectrómetro de massa microflexo LT (Bruker Daltonics) em combinação com versões RUO (Research-use-Only) do pacote de software MALDI Biotyper (versão 3.0) e a base de dados de referência V.3.1.2.0 (3.995 entradas). Foram comparados os espectros de massa das amostras com os espectros de massa de referência no banco de dados, calculando um valor (escore) entre 0 e 3, refletindo a semelhança entre a amostra e o espectro de referência, exibindo os 10 principais registros de bancos de dados correspondentes. Foram utilizados um teste T não paramétrico para analisar as diferenças entre as médias dos scores de detecção dos isolados provenientes de mastites clínicas e subclínicas.

Resultados: Um total de 30 isolados de *Staphylococcus aureus* foram selecionados através do isolamento bacteriano e testes fenotípicos. Todos os isolados identificados pelos testes bioquímicos foram também identificados pelo MALDI-TOF como *Staphylococcus aureus* com scores maiores que 2,0. Os valores variaram de 2,03 a 2,46 com um valor de score médio de 2,26. Não houve diferença significativa entre as médias dos scores de identificação dos isolados provenientes de mastites clínicas e subclínicas pelo MALDI-TOF (P=0,18).

Discussão: Os métodos convencionais de identificação de bactérias são largamente utilizados no mundo todo nas rotinas laboratoriais. Elas têm como vantagem a fácil execução, o baixo custo e a alta reprodutibilidade, entretanto, a demora e quantidade de etapas para a obtenção dos resultados são os principais gargalos(RUEGG, 2009). Por isso, a busca por novas técnicas para identificação bacteriana com agilidade e acurácia vem a cada dia ganhando mais espaço como as técnicas moleculares. Dentre elas o MALDI-TOF se destaca pelo seu resultado preciso e interpretação inequívoca atribuída por scores (SCHULTHESS et al., 2014). No nosso estudo, todos os isolados de *S. aureus* foram corretamente identificados pela técnica em comparação ao método fenotípico com scores acima de 2,0. Além disso, não houve diferença significativa entre os scores de identificação para bactérias isoladas de amostras clínicas e subclínicas o que mostra que o MALDI-TOF pode ser usado em ambas ocasiões. Estudos realizados em vacas também demonstraram a eficácia da técnica na identificação de *Staphylococcus* não-aureus em amostras de infecções intramamárias em bovinos (TOMAZI et al., 2014).

Conclusão: A técnica da espectrometria de massa por ionização e dessorção a laser assistida por matriz - tempo de voo (MALDI-TOF) foi eficaz na identificação dos isolados de *Staphylococcus aureus* provenientes de infecções intramamárias de ovelhas Santa Inês. A ferramenta pode ser empregada na rotina de diagnóstico microbiológico das mastites em rebanhos ovinos, garantindo agilidade e acurácia nos resultados.

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TERMOS DE INDEXAÇÃO: Ovinocultura de corte, Complexo Inanição-Hipotermia, Mastite Subclínica, Diagnóstico, Perdas econômicas.

Anexo III – Trabalho apresentado no evento One Health Symposium – 2018 – The Ohio State University.

Gangrenous mastitis in sheep caused by multidrug resistant non-aureus staphylococci

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Abstract

Gangrenous mastitis is considered the most severe form of mastitis in ewes, frequently leading to sepsis and death in few hours, mainly caused by *Staphylococcus aureus*. In the last years, non-aureus staphylococci (NAS) has being matter of great concern due the ability to acquire virulence genes and, especially, antimicrobial resistance, being able to act as infectious agent capable to disseminates these genes for most diverse animals and to humans. Although NAS are the main species isolated from infected udder halves in ewes, no report of NAS causing gangrenous mastitis in ruminants was found. The aim of this work is report the first case of a gangrenous mastitis in a meat-producing ewe caused by multidrug resistant non-aureus staphylococci. An ewe in a sheep breeding facility shown signs of infection during the breeding season. On clinical examination, the animal had hypothermia, tachycardia, tachypnea, apathy, low reflex and injected episcleral vessels. The mammary gland had skin detachment and laceration, with bloody secretion. The diagnosis was gangrenous mastitis and sepsis. Bloody secretion samples were cultured under aerobic or microaerophilic incubation at 37°C on sheep blood agar for 24h and then submitted to MALDI-TOF for speciation. In both growth conditions, the isolates were identified in MALDI-TOF as *Staphylococcus haemolyticus*. They were submitted to antimicrobial susceptibility test through VITEK® automated system and performed genotyping virulence and resistance genes. *Staphylococcus haemolyticus* isolated showed antimicrobial multiresistance, being resistant to 9 of the 15 evaluated principles and positive for two Staphylococcal Enterotoxigenic genes (sec and see) and fibronectin binding protein B. Understanding mastitis etiology is extremely important because these animals are in direct contact with humans and may become an important infectious agent, causing occupational disease. Thus, to best of our knowledge, this is the first report of a gangrenous mastitis in sheep caused by multidrug resistant non-aureus staphylococci.

Anexo IV – Prêmio – 13th Annual International Scholar Research Exposition – The Ohio State University

THE OHIO STATE UNIVERSITY

Certificate of Recognition

GUILHERME SANTANA DE MOURA

13th Annual International Scholar Research Exposition

November 6, 2018

Through your research you contribute to the advancement of learning and scholarship across cultures, build respect among different peoples, and develop constructive leadership to serve society in the global community.

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Anexo V – Artigo técnico – OSU Sheep Team – Mastitis: An issue not to be taken lightly

Mastitis in Small Ruminants:

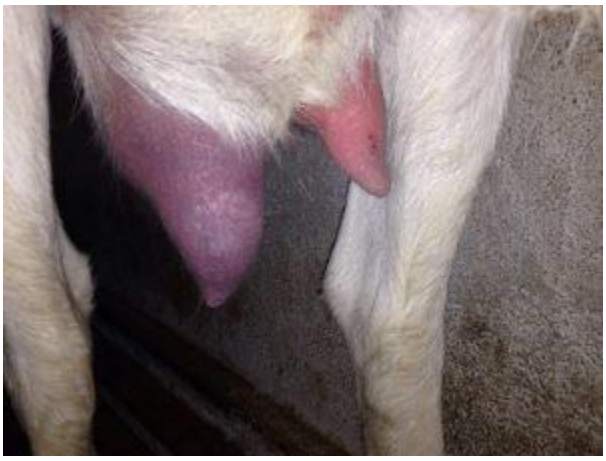
What is mastitis?

Mastitis in goats and sheep, similar to cows, is defined as inflammation of the mammary gland and can occurs due several factors, which may be **infectious** or **not** and may present in *clinical* or *subclinical* form. In clinical mastitis, it is possible to observe the signs of inflammation, such as:

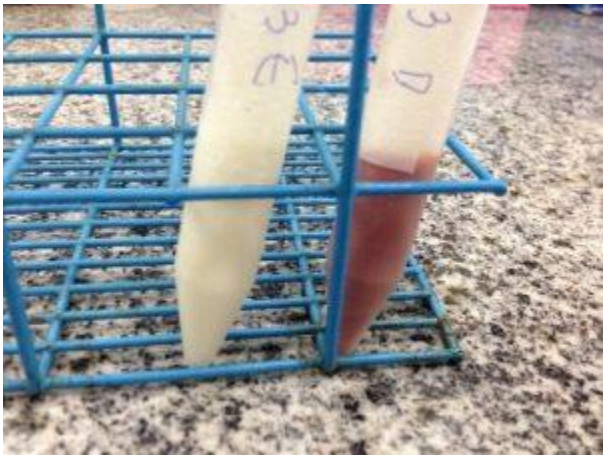
- pain,
- redness,
- swelling of the gland,

- and changes in milk characteristics, which may show lumps, pinkish/reddish coloration or even absence of secretion.
- Some severe cases could lead to udder necrosis (“blue bag”) and even death.

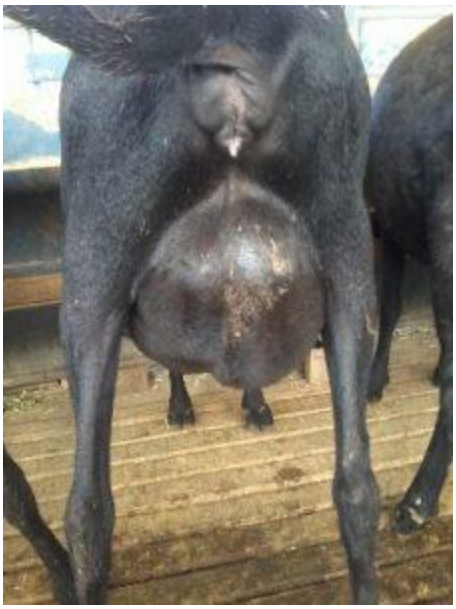
In subclinical mastitis, the female does not present inflammatory signs, however, due to presence of some microorganisms in the mammary gland milk quality can be decreased.



(Gangrenous mastitis in a goat.)



(Difference between normal milk and milk from gangrenous mastitis.)



(Sheep mastitis.)

Etiology

The inflammatory process of the mammary gland can have several origins. For example, traumas and lesions or it can be due to infectious agents, such as fungi, viruses, or in majority of cases bacterial agents. They can cause either environmental or contagious mastitis.

- Environmental mastitis is directly related to the hygiene of the places where these ewes and goats remain.
- Contagious mastitis are associated with transmission between animals and even between human-animal interactions.

Whereas most bacteria can cause either clinical or subclinical mastitis, *Staphylococcus aureus*, *Pasteurella hemolytica* and various yeasts and molds are often recovered from milk samples of ewes affected with clinical symptoms. "Blue bag" (clinical mastitis with a hard, cold swollen udder) is typically caused by *Pasteurella hemolytica* or *Staphylococcus aureus.* Coagulase-negative staphylococci have been frequently reported to be the most commonly isolated pathogens recovered from cases of subclinical mastitis of dairy ewes.

***Ewes with subclinical mastitis produce less quantities of milk and milk with lower quality.*

(Blood agar plate with Staphylococcus aureus colonies (contagious mastitis).

Management / Control

The correct management of the ewes and goats in any production system, dairy or meat, is the key point for mastitis control. Preventing mastitis in dairy herds will ensure milk quality, animal health and welfare.

Among the most important measures in management of dairy goats and ewes, we can point out the sanitary control of the animals, especially regarding clinical forms of mastitis, separating the positive animals and discarding the contaminated milk.

In addition, the adoption of a microbiological-based milking line, pre and post dipping usage, and regular maintenance and hygiene of milking machines are measures that also contribute to better milk quality and animal health.

In meat herds and flocks, mastitis control is mainly based on culling animals that present recurrent episodes of clinical mastitis, which directly affect kid and lamb growth. The other general managements are related to hygiene measures that should be part of the property routine, ensuring a clean environment on stables, maternity paddocks, milking parlor, material and equipment used in milking.

(Milk samples for culture in blood agar plates.)

(Dairy goat milking parlor.)

Highlights

- Mastitis is considered one of the mostly **costing diseases** in the world, because it directly affects milk quality and its products.
- In meat herds and flocks, it is notorious for the losses of kids and lambs due to mortality as a result of low nutrition caused by mastitis.
- Prevention of infection is the key to control mastitis.
- Good hygienic housing and consistent milking practices are crucial to minimize the impact of this disease.

https://u.osu.edu/sheep/2018/01/23/mastitis-in-small-ruminants/