RAPHAEL CONTELLI KLEIN

CARACTERIZAÇÃO MOLECULAR DE ESTIRPES DE *Staphylococcus aureus* DE MASTITE BOVINA E ATIVIDADE ANTI-BIOFILME DE UMA LECTINA TIPO C

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Bioquímica Agrícola, para obtenção do título de *Doctor Scientiae*.

VIÇOSA
MINAS GERAIS-BRASIL
2014

Ficha catalográfica preparada pela Seção de Catalogação e Classificação da Biblioteca Central da UFV

T

K64c
2014

Klein, Raphael Contelli, 1983-

Caracterização molecular de estirpes de *Staphylococcus aureus* de mastite bovina e atividade anti-biofilme de uma lectina tipo c / Raphael Contelli Klein. – Viçosa, MG, 2014.

xii, 120f. : il. ; 29 cm.

Inclui apêndice.

Orientador: Andréa de Oliveira Barros Ribon.

Tese (doutorado) - Universidade Federal de Viçosa.

Inclui bibliografia.

1. *Staphylococcus aureus*. 2. Mastite bovina. 3. Caracterização molecular. 4. Lectina. I. Universidade Federal de Viçosa. Departamento de Bioquímica e Biologia Molecular. Programa de Pós-graduação em Bioquímica Agrícola. II. Título.

CDD 22. ed. 579.353

RAPHAEL CONTELLI KLEIN

CARACTERIZAÇÃO MOLECULAR DE ESTIRPES DE *Staphylococcus aureus* DE MASTITE BOVINA E ATIVIDADE ANTI-BIOFILME DE UMA LECTINA TIPO C

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Bioquímica Agrícola, para obtenção do título de *Doctor Scientiae*.

APROVADA: 18 de fevereiro de 2014

_____	_____
Juliana Rocha Lopes Soares Ramos	Maria Aparecida V. de Paiva Brito
_____	_____
Hilário Cuquetto Mantovani (Coorientador)	Leandro Licursi de Oliveira (Coorientador)

_____

Andrea de Oliveira Barros Ribon
(Orientadora)

A Deus,
Aos meus pais, Urânia e Gervásio
A meus avós Solange e Antônio
A minha esposa Mary Hellen
A minha irmã Thiara

Dedico

AGRADECIMENTOS

Agradeço muito a Deus pelo dom da vida e por estar sempre presente, iluminando meu caminho, guiando meus passos e me dando saúde, fé e força de vontade para seguir em frente.

Aos meus pais pelo amor, carinho e dedicação incondicionais e por estarem sempre torcendo por mim e fazendo de tudo para que meus sonhos pudessem se realizar.

Ao meu amado avô Contelli e minha amada avó Solange, que me deram amor, carinho, atenção e apoio durante toda minha vida.

A minha linda e inteligente esposa Mary Hellen e sua fiel escudeira Nina, minha filha de 4 patas, pelo amor, paciência, atenção, carinho e pela eterna disposição em me ajudar e apoiar em todos os momentos. E também pelas maravilhas que Mary fez na cozinha.

A minha irmã preferida Thiara e seu marido Antônio, pela amizade, companheirismo e apoio.

A toda minha família que cresceu e ficou mais perfeita após meu casamento. Agradeço especialmente meus tios Daniela e Maurício, as primas mais lindas e fofas do mundo, Ana Paula e Catarina. Agradeço também a minha querida e amada sogra Sandra, meu fino cunhado Júnior e minha cunhada gatíssima Bianca. Agradeço a fiel e maravilhosa companhia de Elaine, Patrícia, Tio Júnior, Tia Penha, Juninho. Agradeço a Rodrigo e Patrícia pela atenção na visitas a Vitória, a "vó" Maria e Luzia e "vô" Arlindo, Graça, Rosa, Léo, Carla, Maria.

A Universidade Federal de Viçosa por ter oferecido toda a estrutura necessária e pelo seu belo *campus* que serve de inspiração para trabalhar, mesmo nos finais de semana e feriados.

A Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pelas bolsas concedidas.

Ao Programa de Pós-Graduação em Bioquímica Agrícola pela oportunidade de crescimento, realização profissional e aprendizado durante o desenvolvimento deste curso.

Aos funcionários do Departamento de Bioquímica e Biologia Molecular pelos trabalhos prestados.

A minha orientadora professora Andréa de Oliveira Barros Ribon, por ter tido a coragem de me aceitar depois do mestrado, para continuar comigo por mais esses 4 anos, me conduzindo com competência, dedicação, paciência e pelo imenso apoio em todos os momentos. Um agradecimento especial por ter nos incentivado a fazer o doutorado sanduíche no Canadá, o que acabou se tornando a melhor experiência da minha vida. Agredeço ao Rômulo pelo companherismo nos momentos de descontração e por entender os dias de excesso de trabalho de sua mulher dedicados aos seus orientados.

Ao meu queridíssimo e amado François Malouin simplesmente por ser um orientador perfeito, por saber motivar seu grupo de trabalho, por cativar cada um de seus orientados e guiar os experimentos com muita cautela e sabedoria. Pelos momentos de críticas e pelas conversas que tanto me acalmaram e motivaram a tomar as decisões corretas na ciência e na vida. Agradeço também a sua esposa Suzane por ser sempre tão agradável e pelos belos jantares oferecidos em sua casa reagados bons vinhos e ótimas conversas.

A Dr[a]. Maria Aparecida V. Paiva Brito pela disposição e apoio nos experimentos de microbiologia, por ceder os isolados utilizados neste trabalho e pela participação na banca examinadora proporcionando discussões que enriqueceram muito esta tese.

Ao professor Hilário Cuquetto Mantovani pela co-orientação deste trabalho e pela participação na banca examinadora com sugestões que servirão para o crescimento desta pesquisa.

Ao querido professor Leandro Licursi de Oliveira, pela disposição em ajudar, discutir e muitas vezes colocar a "mão na massa", me ajudando e ensinando muito sobre ciência. Agradeço também a Silvia pelos válidos ensinamentos sobre cromatografia e pela paciência em esperar o marido quando este estava com o autor dessa tese.

A professora Juliana Rocha Lopes Soares Ramos pela receptividade, simpatia e por ter aceito participar na banca examinadora.

Ao Núcleo de Biomoléculas da UFV pelo suporte nos experimentos de microscopia, em especial ao professor Humberto Ramos e a Edvaldo Barros pelo direcionamento nos ensaios de espectrometria de massa. Agradeço ao Núcleo de Microscopia e Microanálise, em especial a Karla pela super ajuda e simpatia nos ensaios de microscopia.

Ao nosso casal modelo Luciano e Juliana, por serem pessoas amáveis, por nos transmitirem paz e sabedoria e estarem sempre disponíveis para nos ajudar em todas as situações, e aos seus filhos, Matheus e Rafinha, por nos trazerem momentos de felicidade.

A prof[a]. Elizabeth Pacheco Batista Fontes, por ser um exemplo de pesquisadora a ser seguido e por estar sempre disposta a ajudar.

A chefe do departamento prof[a]. Márcia Rogéria de Almeida Lamêgo, e todos os demais professores pela disponibilização de equipamentos, apoio e momentos de sabedoria.

Aos amigos e colegas do Laboratório de Biotecnologia Molecular Aline, Amanda, Ananda, Carlos, Dani Arruda, Dani Mendes, Gabriella, Gilza, Luiza, Mônica, Murilo, Patrícia, Silvana e Vanessa pela boa convivência do dia a dia e demais colegas de outros laboratórios, Matheus e Thiago, por estarem sempre dispostos a ajudar.

Ao nosso amigo Eduardo por sua competência, agilidade, paciência, atendendo a todos os alunos com muito bom humor e por ser nosso guia nesses quatro anos. Um funcionário exemplar, e um ótimo amigo e cozinheiro.

Aos meus amigos Arthur, André, Brunella, Paulo, Débora, Diego, Diógenes, Gabi, Guilherme e Luís Eugênio, Michelle, pela amizade verdadeira, apoio e adição de felicidade aos momentos de descontração. A todos meus amigos que, de perto ou de longe, torceram para que este trabalho fosse concluído.

A minhas queridas vizinhas, Ariádine, Débora, Camila, Mariane, que não moram mais nos apartamentos ao lado, mas continuam em meu coração.

Aos amigos do poker Fernando, Felipe, Gillian, Hiroshi, Issac, Igor, Miltão, Naroga, Pedro, Rafael pelas noites de descontração em ótimas companhias.

Agradeço demais da conta aos grandes amigos do Canadá Jack e Ágata, Ceiça e François, Douglas e Sophie, Alessandro e Fernanda, Kauã, Nina, Isabella, Rafael. Quero agradecer especialmente ao Rodrigo e a Milene por terem sidos amigos irmãos.

Aos amigos do laboratório do Canadá, Julie, Eric, Rolland, Koui, Abdel, Michael, Catherine, Veronic, Maxime, Gabriel.

Um agradecimento especial ao meu amigo e companheiro de laboratório, esportes, festas e trabalho, Alexandre Fugere, um parceiro pra vida toda.

A nossa corretíssima e adorável Celine pela amizade e ajuda em todos os momentos, desde a nossa chegada até nossa partida.

A todos os colegas do curso de bacharelado em bioquímica que contribuíram nos momentos de estudos e de diversão.

E a todos aqueles que contribuíram direta ou indiretamente para a realização deste trabalho.

BIOGRAFIA

Raphael Contelli Klein filho de Gervásio Klein e Urânia Contelli Klein, nasceu em 14 de novembro de 1983, em Eunápolis, Bahia.

Em fevereiro de 1999 foi aceito no Centro Federal de Educação Tecnológica (CEFET) de Eunápolis, onde concluiu o ensino médio em dezembro de 2001.

Em março de 2003 ingressou na Universidade Federal de Viçosa, concluindo o curso de Bacharelado em Bioquímica em janeiro de 2008.

Em março de 2008 iniciou o mestrado no Programa de Pós-Graduação em Bioquímica Agrícola, na Universidade Federal de Viçosa, finalizando a dissertação em fevereiro de 2010. No mês subsequente começou a longa jornada no doutorado na mesma instituição e Programa de Pós-Graduação, submetendo-se a defesa de tese no dia 18 de fevereiro de 2014.

SUMÁRIO

RESUMO..... xi
ABSTRACT..... xii
1. INTRODUÇÃO GERAL 1
1.1 Mastite bovina..... 1
1.2. *Staphylococcus aureus*..... 2
1.3. Biofilme..... 4
2. REFERÊNCIAS..... 6

CAPÍTULO 1: Caracterização fenotípica e molecular de *Staphylococcus aureus* presentes em rebanhos leiteiros do Brasil……..…… 12
RESUMO..... 13
1.INTRODUÇÃO..... 14
2. MATERIAL E MÉTODOS..... 17
2.1. Micro-organismos utilizados e condições de cultivo……..…..... 17
2.2. Extração de DNA genômico ……..……..... 17
2.3. PCR *multiplex* ……..…… 17
2.4. Determinação do grupo *agr*……...…….. 18
2.5. Polimorfismo do gene *spa*……..…….. 19
2.6. Produção de biofilme……..…….. 20
2.7. Determinação da Concentração Inibitória Mínima (CIM)..... 20
3. RESULTADOS..... 22
4. DISCUSSÃO..... 29
5. CONCLUSÕES..... 32
6. REFERÊNCIAS..... 33

CAPÍTULO 2: Genotypic and phenotypic characterization of *Staphylococcus aureus* causing persistent and non-persistent subclinical bovine intramammary-quarter infections during lactation and at dry-off..... 42
ABSTRACT..... 45
1.INTRODUCTION..... 47
2.MATERIALS AND METHODS..... 50
2.1. Bacterial Isolates Collection..... 50

2.2. Preparation of Genomic DNA..... 51
2.3. Validation of Persistence by VNTR Typing..... 51
2.4. Detection of Genotypic Markers by PCR..... 52
2.5. Biofilm Production..... 52
2.6. Quantitative Expression of *hld* (qRT-PCR)..... 53
2.7. Spa Typing..... 54
2.8. Statistical Analysis..... 55
3. RESULTS..... 56
3.1. Validation of Persistence for Lactational and Dry-off Isolates by VNTR Typing……... 56
3.2. Detection of Genotypic Markers…..……. 57
3.3 Biofilm Production…….……...……. 58
3.4. *hld* Expression……..…….. 58
3.5. Spa Typing……..…….. 58
4. DISCUSSION..... 61
5. ACKNOWLEDGEMENTS…….....……...…… 69
6. REFERENCES……..…….....…….…… 70
Tables……..…….……. 79
Figures legends.……..…..…… 85
Figures……..…… 87

CAPÍTULO 3: A C-type lectin from *Bothrops jararacussu* venom disrupts staphylococcal biofilms…….……..…….. 92
ABSTRACT…….…….……..……. 94
1. INTRODUCTION…….…….….. 95
2. MATERIALS AND METHODS……...…..… 97
2.1. Bacterial isolates and venom source ……..….... 97
2.2. Biofilm production assay ……..……...…….. 97
2.3. Size exclusion and affinity FPLC chromatography ……..…. 98
2.4. Protein in-gel digestion …….…….…..….. 98
2.5. Mass spectrometry ……..……..…. 99
2.6. Criteria for protein identification ……..…….…..... 99
2.7. Scanning electron microscopy of biofilms...……..……..…. 100

3. RESULTS…… 101
4. DISCUSSION…… 110
5. CONCLUSION…… 113
6. ACKNOWLEDGMENTS…… 114
7. REFERENCES…… 115
8. APPENDIX - SUPPORTING INFORMATION …… 119

RESUMO

KLEIN, Raphael Contelli, D.Sc., Universidade Federal de Viçosa, fevereiro de 2014. **Caracterização molecular de estirpes de *Staphylococcus aureus* de mastite bovina e atividade anti-biofilme de uma lectina tipo C.** Orientadora: Andréa de Oliveira Barros Ribon. Coorientadores: Leandro Licursi de Oliveira e Hilário Cuquetto Mantovani.

Staphylococcus aureus é um dos principais patógenos responsáveis à mastite bovina subclínica que, frequentemente, evolui para a forma crônica. A caracterização de cepas circulantes em rebanhos leiteiros é importante para o desenvolvimento de métodos de diagnóstico precoces e identificação de marcadores de prognóstico. Esse trabalho foi dividido em três capítulos. No capítulo I, isolados bovinos coletados em diversas fazendas dos Estados de Minas Gerais e Rio de Janeiro foram caracterizados com base em características moleculares e fisiológicas. Observou-se a predominância do *spa* t605 e do grupo *agr* tipo II sobre os demais. A maioria dos isolados apresentou moderada produção de biofilme e uma alta sensibilidade aos antimicrobianos testados. Esses resultados aumentam o conhecimento sobre as cepas disseminadas em rebanhos brasileiros e contribuem para definição de estratégias de combate à mastite no país. No capítulo II, realizou-se uma busca por marcadores de prognóstico em 285 isolados de *S. aureus* originários do Canadá. Os isolados foram coletados de animais com manifestação de mastite clínica ou subclínica, ao longo do ciclo lactacional da vaca, sendo a persistência validada por método molecular. Os isolados que persistiam do período seco até a próxima lactação produziram mais biofilme que os não persistentes. Os isolados clínicos foram menores produtores de biofilme quando comparados aos isolados subclínicos. A produção de biofilme foi inversamente porporcional à expressão do gene *hld*. Dezoito tipos de *spa* foram encontrados, sendo o t529 o mais predominante. Para os isolados em lactação, o gene *seg* foi associado com a redução da chance da bactéria ser persistente. No capítulo III, uma busca por substâncias com atividade antipatogênica em veneno de *Bothrops jararacussu* foi realizada. Frações obtidas por cromatografia de exclusão molecular mostraram atividade anti-biofilme quando testadas sobre *S. aureus* e *S. epidermidis*. Espectrometria de massas identificou uma lectina que foi purificada por cromatografia de afinidade, cuja atividade anti-biofilme foi comprovada sobre diferentes espécies de bactérias causadoras de mastite bovina. Esse trabalho revelou uma nova atividade biológica para lectinas do tipo C, que pode ser testada como estratégia complementar no combate de infecções causadas por *S. aureus*.

ABSTRACT

KLEIN, Raphael Contelli, D.Sc., Universidade Federal de Viçosa, February of 2014. **Molecular characterization of *Staphylococcus aureus* from bovine mastitis and antibiofilm activity of a C-type lectin** Advisor: Andréa de Oliveira Barros Ribon. Co- Advisors: Leandro Licursi de Oliveira and Hilário Cuquetto Mantovani.

Staphylococcus aureus is a major pathogen responsible for subclinical bovine mastitis that often progresses to a chronic form. Molecular characterization of strains circulating in dairy herds is important to develop methods for early diagnosis and to identify prognostic markers which may help in the definition of appropriate mastitis control. This work was divided into three chapters. In Chapter I, bovine isolates collected from several farms in the states of Minas Gerais and Rio de Janeiro were characterized based on molecular and physiological characteristics. The *spa* type 605 and the *agr* type II predominated over other groups. Most isolates showed a moderate biofilm production and a high sensitivity to the tested antibiotics. These results increase our knowledge of the strains disseminated in Brazilian herds and improve herd management and disease control in the country. In Chapter II, we performed a search for prognostic markers in 285 isolates of *S. aureus* originated from Canada. The isolates were collected from animals showing clinical or subclinical mastitis during the lactational cycle of the cow, and persistence was validated by a molecular approach. The isolates that persisted during dry period through freshening produced more biofilm than non-persistent ones. Clinical isolates produced less biofilm than subclinical isolates. Biofilm production inversely correlated to *hld* expression level. Eighteen *spa* types were found, but t529 was more prevalent. For subclinical lactational isolates, the gene *seg* was associated with a reduction in the odds of the bacteria being persistent. In Chapter III, a search for substances found with anti-pathogenic activity was performed in the venom of *Bothrops jararacussu*. Fractions obtained by size exclusion chromatography showed anti-biofilm activity when tested on *S. aureus* and *S. epidermidis*. Mass spectrometry identified a lectin that was purified by affinity chromatography, whose anti-biofilm activity was demonstrated on different species of bovine mastitis bacteria. This work revealed a new biological activity for C-type lectins that can be tested as a complementary strategy to combat infections caused by *S. aureus*.

1. INTRODUÇÃO GERAL

1.1. *Mastite Bovina*

O Brasil é um dos principais produtores de leite, ocupando a quarta posição do *ranking* mundial, além de ser o principal produtor da América do Sul (FAO, 2013). Esse reconhecimento poderia ser maior se a produtividade brasileira não fosse tão baixa (1,3 t/cabeça/ano) quando comparada a de outros países como Estados Unidos (9,7 t/cabeça/ano), Canadá (8,0 t/cabeça/ano) e Alemanha (7,2 t/cabeça/ano) (EMBRAPA Gado de Leite, 2013). Porém, o Brasil ainda apresenta boas condições de alavancar sua capacidade produtiva graças ao tamanho de seu rebanho leiteiro e a riquezas naturais, como solo e água.

A mastite bovina é reconhecidamente uma das doenças que mais causa prejuízos a rebanhos leiteiros no mundo (Raza, 2013). Ela é caracterizada por uma infecção da glândula mamária causada principalmente por micro-organismos (Pyörälä, 2002; Prestes *et al.,* 2003). A mastite é a doença de maior impacto econômico na pecuária leiteira com prejuízos estimados em US$ 35 bilhões/ano (Ruegg, 2005), representados por gastos diretos com o tratamento (serviços e medicamentos) e prejuízos indiretos decorrentes da morte dos animais, decréscimo da produção e descarte do leite (Rainard, 2005; Petrovski, 2006). A doença tem ainda impacto negativo na indústria de laticínios devido à redução significativa da qualidade do leite e derivados. O leite produzido apresenta menor teor de lactose, gordura, proteínas e minerais, além de um número elevado de proteínas séricas e células somáticas o que compromete seu valor nutricional e sua aceitabilidade pelo consumidor (Bruckmaier *et al.,* 2004; Oliveira e Timm, 2006).

Quanto à forma de apresentação, a mastite é classificada em clínica ou subclínica. O edema do quarto mamário, a sensibilidade ao toque, além da presença de coágulos e, algumas vezes, de sangue no leite, são sintomas mais comuns da mastite clínica. Nos casos mais severos o que se observa é uma reação generalizada, na qual o animal apresenta febre, perda de apetite, desidratação e septicemia, que pode evoluir para morte (Freitas *et al.,* 2005). Na mastite subclínica, a doença se manifesta sem sintomas visíveis, sendo o diagnóstico feito por testes indiretos que se baseiam na elevação da contagem de células somáticas (CCS), tais como células leucocitárias, presentes no leite. Estimativas calculam que para cada caso clínico

existem de 20 a 40 casos subclínicos (Wattiaux, 1996), que são geralmente detectados em 50-70% do rebanho (Gruet *et al.,* 2001).

O controle da mastite bovina é feito com base em programas de manejo onde são adotadas medidas preventivas, princípios de higiene e o tratamento de animais doentes (National Mastitis Council, 2001). Estudos confirmam que a adoção dessas medidas reduz significativamente a prevalência e a incidência da mastite no rebanho leiteiro. Porém, as práticas de controle preventivas, baseadas em higiene e manejo, são muitas vezes desconhecidas ou mal aplicadas pelos produtores (Carvalho, 2004; Dias, 2007).

1.2. *Staphylococcus aureus*

Staphylococcus aureus é um dos principais agentes causadores da mastite bovina (Godden *et al*., 2002; Zadoks e Fitzpatrick, 2009). A bactéria é encontrada no úbere de vacas infectadas, sendo disseminada para quartos mamários não infectados e para animais sadios principalmente no momento da ordenha. *S. aureus* pode causar infecções clínicas ou subclínicas, a forma de manifestação mais comum. A infecção subclínica pode se tornar crônica, devido a capacidade da bactéria em sobreviver intracelularmente, o que dificulta a ação de antimicrobianos e do sistema imune (Sears e McCarthy, 2003). A falha na detecção de animais infectados é um fator que contribui para a disseminação de *S. aureus* no rebanho, impondo uma ameaça a outros animais sadios e uma preocupação aos produtores. Isso tem motivado a caracterização molecular de um número crescente de isolados de *S. aureus*, visando identificar marcadores que possam associar um grupo de bactérias a um determinado tipo de infecção.

S. aureus possui um repertório de fatores de virulência nem sempre presente em todas as estirpes. Isso contribui para a grande diversidade genética de bactérias encontradas nos rebanhos, mas ao mesmo tempo dificulta uma correlação entre o tipo de manifestação e fatores específicos (Haveri *et al*,, 2008; Vautor *et al*., 2009; Klein *et al*., 2013). Não se pode deixar de mencionar os fatores associados ao animal, como sistema imune, idade e números de gestações, muitas vezes deixados de lado, mas que podem influenciar a evolução da infecção (Barkema *et al*., 2006).

Tem sido sugerido que alguns clones são responsáveis pela maioria das infecções intramamárias observadas em rebanhos do mundo (Kapur *et al*., 1995; Fitzgerald *et al*., 1997; Rabello *et al*., 2005). O CC97, por exemplo, é mundialmente

distribuído sendo composto quase que exclusivamente por isolados bovinos, o que revela uma preferência desses isolados por um hospedeiro específico (Smith *et al*., 2005) e, possivelmente, características próprias que podem ser usadas no controle da mastite bovina.

Por outro lado, vários autores têm demonstrado a existência de mais de um clone por rebanho ou fazendas, embora haja sempre a predominância de um sobre os demais (Myllys *et al*., 1997; Joo, *et al*., 2001; Haveri *et al*., 2008). As estirpes dominantes seriam mais adaptadas ao hospedeiro e, por isso, causariam infecções persistentes (Schukken *et al*., 2011). Diferentemente das associadas às infecções não persistentes, essas estirpes estariam associadas um quadro clínico menos severo e com maior capacidade de infecção, o que aumentaria a possibilidade de sua disseminação no rebanho.

Na tentativa de diferenciar isolados de casos clínicos e subclínicos Fitzgerald *et al*., (1997) utilizando a técnia de RAPD, conseguiram discriminar dois grupos com maiores chances de estarem associados à mastite clínica em animais da Irlanda, entretanto, não foi possível encontrar um grupo que possuísse isolados exclusivamente causadores de mastite clínica. O grupo RAPD tipo 7 foi mais sensível à penicilina, característica que o separou dos demais grupos encontrados.

Haveri *et al*., (2008) mostraram que genes que codificam algumas toxinas e resistência à ampicilina estão geralmente ligados a infecções intramamárias persistentes registradas em rebanhos da Finlândia. Algumas dessas toxinas (*txt* e *sec*) já haviam sido reportadas como importantes para a severididade das manifestações (Matsunaga *et al*., 1993).

Na última década, abordagens proteômicas têm sido empregadas como estratégia para verificar a expressão de fatores de virulência de *S. aureus* e relacionar a presença de determinadas proteínas à severidade das infecções intramamárias bovinas (Le Maréchal *et al*., 2011; Wolf *et al*., 2011). Porém, até o momento não foi possível definir um panorama completo entre as características genéticas dos isolados bovinos de *S. aureus* e os diferentes tipos de manifestações apresentados pelos animais. Esse fato não deixa dúvida que os mecanismos moleculares envolvidos na patogênese de *S. aureus* são mais complexos do que se pensava.

1.3. Biofilme

S. aureus é um patógeno reconhecido por sua notória capacidade de se tornar resistente aos antimicrobianos e a relação entre a capacidade de formação de biofilme e essa resistência a antimicrobianos tem sido demonstrada (Melchior *et al*., 2009; Chamber e DeLeo, 2009). A formação de biofilmes vem sendo considerada um dos mais importantes fatores de virulência de *S. aureus* auxiliando no processo de infecção causada por este micro-organismo (Cucarella *et al*., 2004). Biofilmes são formados por uma associação de bactérias imersa numa matriz polimérica composta por exopolissacarídeos, proteínas e DNA extracelular. Essa estrutura auxilia na adesão a diferentes superfícies e protege a bactéria da desidratação, do sistema imune do hospedeiro e também dos antimicrobianos (Hall-Stoodley *et al*., 2004; Melchior *et al*., 2006; Parra-Ruiz *et al*., 2012). O biofilme tem sido visto como um dos principais motivos para a falha dos tratamentos de infecções causadas por *S. aureus* (Melchior *et al*., 2009; Singh *et al*., 2010).

O uso de antimicrobianos em associação com drogas direcionadas ao biofilme tem sido apontado como uma nova estratégia para o combate das infecções bacterianas (Chen e Wen, 2011). O alvo preferido na abordagem anti-biofilme é a matriz polimérica extracelular, devido à sua importância para a estrutura do biofilme (Fux *et al*., 2003; Marcato-Romain *et al*., 2012). Estirpes de *S. aureus* mutadas nos genes que codificam duas nucleases apresentaram produção limitada de biofilme *in vitro* e *in vivo*, o que foi correlacionado com um aumento na sensibilidade à daptomicina (Benekken *et al*., 2012). DNase I causou uma diminuição de 51,6 % no biofilme formado por *S. aureus* e aumentou a susceptibilidade a antimicrobianos (Tetz *et al*., 2009). Lisostafina combinada a antimicrobianos também foi muito eficiente no tratamento *in vivo* de *S. aureus* meticilina-resistentes (Walencka *et al*., 2005).

A busca por fatores anti-biofilme vêm sendo feita a partir das mais variadas fontes biológicas (Kaplan *et al*., 2004; Huigens *et al*., 2008; Quave *et al*., 2012). Extratos de *Rhodiola crenulata*, *Epimedium brevicornum* e *Polygonum cuspidatum* reduziam a formação de biofilme de *Propionibacterium acnes* em 64.8 %, 98.5 %, e 99.2 %, respectivamente (Coenye *et al*., 2012). Dentre esses, o composto mais eficaz foi o resveratrol, isolado de *P. cuspidatum*, foi capaz de reduzir 80 % do biofilme formado. Um exopolissacarídeo purificado de *Vibrio* inibiu a produção de biofilme em bactérias Gram-negativas e positivas e afetou biofilme pré-formado de

Pseudomonas aeruginosa (Jiang *et al*., 2011). Um produto natural extraído de *Rubus ulmifolius* reduziu a formação de biofilme em *S. aureus*, aumentando significativamente a sensibilidade da bactéria a vários antimicrobianos, comprovando que a estratégia anti-biofilme têm um potencial terapêutico (Quave *et al.*, 2012).

2. REFERÊNCIAS BIBLIOGRÁFICAS

Barkema, H.W., Schukken, Y.H., Zadoks, R.N., 2006. Invited Review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. Am. Dairy Sci. Assoc. 89, 1877–1895.

Beenken, K.E., Dunman, P.M., McAleese, F., Macapagal, D., Murphy, E., Projan, S.J., Blevins, J.S., Smeltzer, M.S. 2004. Global gene expression in *Staphylococcus aureus* biofilms. J. Bacteriol. 186, 4665-4684.

Bruckmaier, R.M., Ontsouka, C.E., Blum, J.W., 2004. Fractionized milk composition in dairy cows with subclinical mastitis. Vet. Med. 49, 283-290.

Carvalho, G.F., Molina, L.R., Cunha, R.P.L., Cruz, J.C.M. 2004. Efeito da implementação de um programa de mastite. 3f. Disponível em: http://www.rehagro/com.br/siterehagro/artigostécnicos/cd noticia=713.

Chambers, H.F.; Deleo, F.R. 2009. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat. Rev. Microbiol., 7, 629-41.

Chen, L., Wen, Y.M. 2011. The role of bacterial biofilm in persistent infections and control strategies. Int. J. Oral. Sci. 3, 66-73.

Coenye, T., Brackman, G., Rigole, P. 2012. Eradication of *Propionibacterium acnes* biofilms by plant extracts and putative identification of icariin, resveratrol and salidroside as active compounds. Phytomed., 19, 409–412.

Cucarella, C., Tormo, A. M., Ubeda, C., Trotonda, P. M., Monzon, M., Peris, C., Amorena, B., Lasa, I., Penade, R. J. 2004. Role of Biofilm-Associated Protein Bap in the Pathogenesis of Bovine *Staphylococcus aureus*. Infection and Immunity, 72, 2177-2185.

Dias, R.V.C., 2007. Principal methods for diagnostics and control of dairy mastitis. Acta Vet. Bras., 1, 23-27.

Embrapa. 2013. Panorama do leite. Ano 6, n°67 (Abril/2013).

FAO (Food and Agriculture Organization). 2013. FAO Statistical Yearbook 2013. http://www.fao.org/docrep/018/i3107e/i3107e00.pdf

Fitzgerald, J.R., Meaney, W.J., Hartigan, P.J., Smyth, C.J., Kapur, V. 1997. Fine-structure molecular epidemiological analysis of *Staphylococcus aureus* recovered from cows. Epidemiol. Infect., 119, 261-269.

Freitas, M.F.L., Pinheiro Junior, J.W., Stamford, T.L.M., Rabelo, S.S.A., Silva, D.R., Silveira Filho, V.M., Santos, F.G.B., Sena, M.J., Mota, R.A., 2005. Perfil de sensibilidade antimicrobiana *in vitro* de *Staphylococcus* coagulase positivos isolados de leite de vacas com mastite no agreste do estado de Pernambuco. Arq. Inst. Biol., 72, 171-177.

Fux, C.A.; Stoodley, P.; Hall-Stoodley, L.; Costerton, J.W. 2003. Bacterial biofilms: a diagnostic and therapeutic challenge. Expert. Rev. Anti. Infect. Ther. 1, 667-683.

Godden, S.M., Jansen, J.T., Leslie, K.E., Smart, N.L., Kelton D.F., 2002. The effect of sampling time and sample handling on the detection of *Staphylococcus aureus* in milk from quarters with subclinical mastitis. Can. Vet. J. 43, 38-42.

Gruet, P., Maincent, P., Berthelot, X., Kaltsatos, V., 2001. Bovine mastitis and intramammary drug delivery: review and perspectives. Adv. Drug Deliv. Rev. 50, 245-259.

Hall-Stoodley, L.; Costerton, J.W.; Stoodley, P. 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol. 2, 95-108.

Haveri, M.; Hovinen, M.; RoslöF, A.; Pyörälä, S. 2008. Molecular types and genetic profiles of *Staphylococcus aureus* strains isolated from bovine intramammary infections and extramammary sites. J. Clin. Microb., 46, 3728-3735

Huigens, R.W., Ma, L., Gambino, C., Moeller, P.D., Basso, A., Cavanagh, J., Wozniak, D.J., Melander, C. 2008. Control of bacterial biofilms with marine alkaloid derivatives. Mol. Biosyst. 4, 614-621.

Jiang, P., Li, J., Han, F., Duan, G., Lu, X., Gu, Y., Yu, W. Antibiofilm activity of an exopolysaccharide from marine bacterium *Vibrio sp*. QY101. PLoS One. 2011 7;6(4):e18514.

Joo, Y.S., Fox, L.K., Davis, W.C., Bohacn, G.A., Park, Y.H. 2001. *Staphylococcus aureus* associated with mammary glands of cows: genotyping to distinguish different strains among herds. Vet. Microbiol. 80, 131-138

Kaplan, J.B., Ragunath, C., Velliyagounder, K., Fine, D.H., Ramasubbu, N. 2004. Enzymatic detachment of *Staphylococcus epidermidis* biofilms. Antimicrob Agents Chemother. 48, 2633-2636.

Kapur, V., Sischo, W.M., Greer, R.S., Whittam, T.S., Musser, J.M. 1995. Molecular population genetic analysis of *Staphylococcus aureus* recovered from cows. J. Clin. Microbiol. 33, 376-380.

Klein, R. C., M. H. Fabres-Klein, M. A. V. P. Brito, L. G. Fietto, A. O. B. Ribon. 2012. *Staphylococcus aureus* of bovine origin: Genetic diversity, prevalence and the expression of adhesin-encoding genes. Vet. Microbiol. 160,183-188.

Le Maréchal, C., Seyffert, N., Jardin, J., Hernandez, D., Jan, G., Rault, L., Azevedo, V., François, P., Schrenzel, J., van de Guchte, M., Even, S., Berkova, N., Thiéry, R., Fitzgerald, J.R., Vautor, E., Le Loir, Y. 2011. Molecular basis of virulence in *Staphylococcus aureus* mastitis. PLoS One, 6, 27354.

Marcato-Romain, C.E., Pechaud, Y., Paul, E.; Girbal-Neuhauser, E., Dossat-Letisse, V. 2012. Removal of microbial multi-species biofilms from the paper industry by enzymatic treatments. Biofouling, 28, 305-314.

Matsunaga T., Kamata S., Kakiichi N., and Uchida K., 1993. Characteristics of *Staphylococcus aureus* isolated from peracute, acute and chronic bovine mastitis. J. Vet. Med. Sci. 55, 297-300.

Matsuzaki, K., Sugishita, K., Harada, M., Fujii, N., Miyajima, K., 1997. Interactions of an antimicrobial peptide, magainin 2, with outer and inner membranes of Gram-negative bacteria. Biochim. Biophys. Acta. 327, 119-130.

Melchior, M.B., Vaarkamp, H., Fink-Gremmels, J. 2006. Biofilms: A role in recurrent mastitis infections? The Veterinary Journal, 171, 398-407.

Melchior, M. B.; van Osch, M. H. J.; Graat, R. M.; van Duijkeren, E.; Mevius, D. J.; Nielen, M.; Gaastra, W.; Fink-Gremmels, J. 2009. Biofilm formation and genotyping of *Staphylococcus aureus* bovine mastitis isolates: evidence for lack of penicillin-resistance in Agr-type II strains. Vet Microbiol. 137, 83-89.

Myllys, V., Ridell, J., Björkroth, J., Biese, I., Pyörälä, S. 1997. Persistence in bovine mastitis of *Staphylococcus aureus* clones as assessed by random amplified polymorphic DNA analysis, ribotyping and biotyping. Vet. Microbiol. 51, 245-251.

National Mastitis Council. 2001. Current concepts of bovine mastitis. 4th ed. Madison. 64 p.

Oliveira, D.S., Timm, C.D., 2006. Composição do leite com instabilidade da caseína. Cienc. Tecnol. Aliment. 26, 259-263.

Parra-Ruiz, J., Vidaillac, C., Rybak, M.J. 2012. Macrolides and staphylococcal biofilms. Rev. Esp. Quimioter. 25, 10-16.

Petrovski, K. R., Trajcev, M., Buneski, G., 2006. A review of the factors affecting the costs of bovine mastitis. J. S. Afr. Vet. Assoc. 77, 52-60.

Prestes, D.S., Filati, A., Cecim, M.S., 2003. Suscetibilidade à mastite: Fatores que a influenciam – uma revisão. Zootec.Vet. Agro. 9, 48-59.

Pyörälä, S., 2002. New strategies to prevent mastitis. Reprod. Domest. Anim. 37, 211-216.

Quave, C.L., Estévez-Carmona, M., Compadre, C.M., Hobby, G., Hendrickson, H., Beenken, K.E., Smeltzer M.S. 2012. Ellagic acid derivatives from Rubus ulmifolius inhibit *Staphylococcus aureus* biofilm formation and improve response to antibiotics. PLoS One. 7(1):e28737.

Rabello, R.F., Souza, C.R., Duarte, R.S., Lopes, R.M., Teixeira, L.M., Castro, A.C., 2005. Characterization of *Staphylococcus aureus* isolates recovered from bovine mastitis in Rio de Janeiro, Brazil. J. Dairy Sci. 88, 3211-3219.

Rainard, P., 2005. Tackling mastitis in dairy cows. Nature Biotech. 23, 430-432.

Raza1, A., Muhammad, G., Sharif, S., Atta, A. 2013. Biofilm Producing *Staphylococcus aureus* and Bovine Mastitis: A Review. Molecular Microbiology Research (Online), 3, 1-8.

Ruegg, P., 2005. Premiums, production and pails of discarded milk how much money does mastitis cost you? Resources Milk Money 3, 50-56.

Sears, P.M., McCarthy, K.K. 2003. Management and treatment of staphylococcal mastitis. Vet. Clin. North Am. Food Anim. Pract., 19, 171–185.

Singh, R.; Ray, P.; Das, A.; Sharma, M. 2010. Penetration of antibiotics through *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. J Antimicrob Chemother., 65, 1955-1958.

Schukken, Y.H., Günther, J., Fitzpatrick, J., Fontaine, M.C., Goetze, L., Holst, O., Leigh, J., Petzl, W., Schuberth, H.J., Sipka, A., Smith, D.G.E., Quesnelld, R., Wattsd, J., Yancey, R., Zerbe, H., Gurjara, A., Zadoks, R.N., Seyfert, H.M. 2011. Host-response patterns of intramammary infections in dairy cows. Vet. Immunol. Immunopathol. 144, 270-289.

Tetz, G.V., Artemenko, N.K., Tetz, V.V. 2009. Effect of DNase and antibiotics on biofilm characteristics. Antimicrob. Agents and Chemother., 53, 1204-1209.

Vautor, E., Magnone, V., Rios, G., Le Brigand, K., Bergonier, D., Lina, G., Meugnier, H., Barbry, P., Thiéry, R., Pépin, M., 2009. Genetic differences among *Staphylococcus aureus* isolates from dairy ruminant species: a single-dye DNA microarray approach. Vet. Microbiol. 133, 105-114.

Walencka, E., Sadowska, B., Rózalska, S., Hryniewicz, W., Rózalska, B. 2005. Lysostaphin as a potential therapeutic agent for staphylococcal biofilm eradication. Pol.J. Microbiol. 54, 191-120.

Watiaux, M. 1996. Lactancia e ordeno http://babcock.cals.wisc.edu/des /lacS/mastitis.html.

Wolf, C., Kusch, H., Monecke, S., Albrecht, D., Holtfreter, S., von Eiff, C., Petzl, W., Rainard, P., Bröker, B.M., Engelmann , S. 2011. Genomic and proteomic characterization of *Staphylococcus aureus* mastitis isolates of bovine origin. Proteomics 11, 2491-2502.

Zadoks, R.; Fitzpatrick, J. 2009. Changing trends in mastitis. Ir Vet J. 62, 59-70.

CAPÍTULO 1

CARACTERIZAÇÃO FENOTÍPICA E MOLECULAR DE *Staphylococcus aureus* PRESENTES EM REBANHOS LEITEIROS DO BRASIL

RESUMO

A mastite bovina é principal doença dos rebanhos leiteiros mundiais e acarreta um grande prejuízo à indústria de laticínios. *Staphylococcus aureus* é o principal patógeno associado à mastite subclinica crônica. Essa bactéria possui grande heterogeneidade genética e forma biofilme, características que dificultam o controle das infecçoes no rebanho. O objetivo desse trabalho foi caracterizar isolados bovinos de *S. aureus* quanto ao tipo de *spa* (proteína A estafilocócica) e determinar a prevalência de 16 genes considerados importantes no processo de infecção. Essas características foram relacionadas ao grupo *agr*; ao perfil de sensibilidade a antimicrobianos e à produção de biofilme. Pela tipagem do gene *spa* foi possivel identificar 12 tipos, sendo t605 o mais prevalente com 75,3 % dos isolados, t127 e t267 com 5,8 % e os nove tipos restantes (t002, t10856, t1130, t11521, t1402, t2279, t337, t4173 e t518) com 1,4 % cada. Nenhum dos genes avaliados foi encontrado em 100 % dos isolados, mas, entretanto, todos os genes apresentaram um alta prevalencia, sendo que os menos prevalentes foram os genes *eap*, *hla* e *sacol1912* com 87 %, 93 % e 95 %, respectivamente. Todos os demais genes foram idenficados em mais de 95 % dos isolados. O grupo *agr* II foi encontrado em 80 % dos isolados, seguido do grupo I (11 %) e do grupo III (8,6 %). Não foram identificados isolados do grupo *agr* IV. A maioria dos isolados apresentou uma moderada produção de biofilme, tendo os isolados classificados como *agr* II como uma maior média quando comparada com os demais grupos *agr*. A determinação da concentração inibitória mínima (CIM) permitiu a identificação de dois isolados resistentes para tetraciclina, entretanto, a maioria dos isolados apresentou alta susceptibilidade para eritromicina (94 %), gentamicina (98 %), oxacilina (100 %) e tetraciclina (88 %). Os resultados de caracterização adquiridos nesse estudo forneceram dados importantes que permitiram um melhor entendimento de características de *S. aureus* causador de mastite bovina em Minas Gerais.

1. INTRODUÇÃO

O Brasil possui uma posição de destaque no cenário leiteiro internacional, ocupando o quarto lugar entre os maiores produtores mundais (Embrapa Gado de Leite, 2013). Porém, a produtividade é considerada extremamente baixa (1,3 t/cabeça/ano) quando comparada a outros países produtores de leite, como Estados Unidos (9,7 t/cabeça/ano), Alemanha (7,2 t/cabeça/ano) e Canadá (8,0 t/cabeça/ano) (Embrapa Gado de Leite, 2012). Um dos motivos para essa baixa produtividade é a mastite bovina, uma inflamação da glândula mamária causada principalmente por bactérias, dentre as quais destaca-se *Staphylococcus aureus* (Tiwari, *et al*., 2013; Hogeveen, *et al*., 2011).

S. aureus é um patógeno contagioso encontrado no úbere de vacas infectadas que pode ser disseminado para quartos mamários não infectados e para animais sadios no momento da ordenha. As manifestações causadas por essa bactéria podem ser clínica ou subclínica, tipo que mais preocupa pelo o potencial que tem de evoluir para uma infecção crônica (Barkema *et al*., 2006).

Alguns estudos sugerem que as infecções intramamárias são causadas por um pequeno número de clones especializados, como o CC 97 (complexo clonal), que tem distribuição mundial com STs exclusivamente relacionadas a estirpes bovinas (Kapur *et al*., 1995; Smith *et al*., 2005). Os ST são definidos pela técnica MLST (*Multilocus Sequencing Type*) que consiste no sequenciamento de fragmentos internos de sete genes conservados envolvidos no metabolismo de *S. aureus*. Isso torna a técnica dispendiosa, embora facilite a comparação entre resultados obtidos em diferentes laboratórios. A tipagem pela técnica de *spa typing* se baseia no polimorfismo da região codificante 3' da proteína A específica de *S. aureus* (*spa*). Essa região polimórfica é composta por conjuntos de repetições de nucleotídeos. A composição de bases dessas repetições encontradas em uma estirpe específica é designada como um código de repetição única. A combinação dessas repetições para uma dada estirpe determina o tipo de spa. Cada tipo de *spa*, geralmente, contém conjuntos de 2 a 16 repetições únicas e o comprimento individual de cada uma dessas repetições possui geralmente 24pb, mas exceções de 21 a 30 também são econtradas. Embora o *spa typing* seja uma técnica de tipagem que utiliza um único locus, ela oferece uma resolução de subtipagem equiparável com técnicas mais caras e/ou mais trabalhosas, tais como MLST e PGFE. Sendo assim, essa técnia destaca-se

por ser um método rápido, robusto e preciso e que tem sido utilizada com sucesso para discriminar isolados presentes em rebanhos leiteiros (Lange *et al*., 1999; Aires de Souza *et al*., 2006)

A presença de diferentes fatores de virulência auxilia no estabelecimento das infecções estafilocócicas (Sutra e Poutrel, 1994) e também contribui para a diversidade genética de *S. aureus* (Klein *et al*., 2012), já que nem todos eles são encontrados necessariamente em todos isolados do rebanho. A esses fatores são atribuídos os mais diversos papéis como reconhecimento e ligação a proteínas da matriz extracelular do hospedeiro, resistência à fagocitose, lise de células eucariotas e capacidade de metabolizar substratos presentes no leite (Chhatwal, 2002; Patti *et al.,* 1994). A investigação da distribuição de genes relacionados ao processo de infecção fornece informações importantes para o desenvolvimento de estratégias de identificação e controle das infecções, e por isso, muitos estudos têm sido realizados numa tentativa de correlacionar a presença de fatores específicos a um determinado tipo de manisfestação causada por *S. aureus* (Haveri *et al*., 2008, Veh, *et al*., 2014, submetido).

A expressão de diversos fatores de virulência é finamente ajustada por reguladores transcricionais globais que são ativadados ou reprimidos de maneira dependente da fase de crescimento e da densidade populacional. Em *S. aureus* essa regulação é feita principalmente por meio do sistema *agr* (gene regulador acessório) que tem sob o seu controle mais de 150 genes, incluindo adesinas e muitas toxinas (Dunman *et al*., 2001). Além de reger o processo de infecção, o *regulon agr* pode ser dividido em grupos específicos, denominados *agr* I, II, III e IV (Harraghy, *et al.,* 2007). Essa divisão é baseada em diferenças genéticas observadas no gene *agr*. Alguns autores sugerem que o grupo *agr* está relacionado a determinados tipos de infecções (Jarraud *et al*., 2002), outros correlacionam determinadas características genéticas à grupos *agr* específicos (Jarraud *et al*., 2000; Sakoulas *et al.*, 2003). Nesse contexto, foi demonstrado que o grupo *agr* I tem uma melhor capacidade de internalização nas células epitelias de glândulas mamárias, enquanto que isolados do grupo *agr* II são mais propensos à produção de biofilme e à resistência a penicilinas (Melchior *et al*., 2009).

A formação de biofilmes vem sendo considerada um dos mais importantes fatores de virulência de *S. aureus* auxiliando sobremaneira no processo de infecção (Cucarella *et al*., 2004). Biofilmes são formados por uma associação de bactérias

imersa numa matriz polimérica composta por exopolissacarídeos, proteínas e DNA extracelular. Essa estrutura auxilia na adesão a diferentes superfícies e protege a bactéria contra desidratação e o sistema imune do hospedeiro (Melchior *et al*., 2006; Parra-Ruiz *et al*., 2012). O biofilme também tem sido visto como um dos principais motivos para a falha dos tratamentos de infecções causadas por *S. aureus* (Melchior *et al*., 2009; Singh *et al*., 2010).

Nesse trabalho, setenta isolados bovinos foram tipados de acordo com o gene *spa*. A presença de de fatores de virulência dentro dos diferentes tipos de *spa* obtidos foi avaliada, bem como a classificação em grupos *agr*, a produção de biofilme e o perfil de sensibilidade a antimicrobianos. Verificou-se a predominância do *spa* t605 e do grupo *agr* II sobre os demais, uma alta sensibilidade da maioria dos isolados aos antimicrobianos e uma prevalência elevada dos genes de virulência estudados.

2. MATERIAL E MÉTODOS

2.1. Micro-organismos utilizados e condições de cultivo

Staphylococcus aureus NRS155, NRS133, RF122 e ATCC29213 foram utilizados como controles nos ensaios. Os demais 70 *S. aureus* foram isolados de casos de mastite subclínica em rebanhos de quinze cidades dos Estados de Minas Gerais e Rio de Janeiro. As bactérias foram cultivadas em meio infusão de cérebro e coração (BHI), a 37 °C, sob agitação e os estoques foram mantidos - 80 ºC, no mesmo meio, adicionado de 40% glicerol. BHI contendo 0,25% de glicose (BHIg) foi usado para avaliar a produção de biofilme. O meio Mueller-Hinton foi usado na determinação da concentração inibitória mínima (CIM).

2.2. Extração de DNA genômico

O DNA genômico dos isolados foi extraído a partir de cultivo celular crescido por 16 h sob agitação de 225 rpm, a 35 °C. As células foram coletadas por cetrifugação e ressuspendidas em TE (10 mM Tris-HCl, 1mM EDTA, pH 8,0) contendo 45 mg/mL de lisozima (SIGMA-L6876, Oakville, ON, Canadá) e 200 µg/mL de lisostafina (SIGMA-L7386). Após 1 h de incubação a 37 °C, o DNA foi extraído utilizando GenElute™ Bacterial Genomic DNA Kit (Sigma-NA2110) seguindo as recomendações do fabricante. A qualidade e a concentração do DNA extraído foi avaliada por espectrofotometria em leitora de microplacas Epoch (BioTek, Winooski, VT, USA). As amostras foram armazenadas a -20 ºC para uso posterior.

2.3. PCR *multiplex*

A prevalência dos genes foi determinada por meio de PCR *multiplex* para cada um dos grupos descritos na Tabela 1. A reação de amplificação continha 200 ng de DNA genômico, 2,0 µL de tampão de amplificação 10X, 3,0 mM de $MgCl_2$, 0,2 mM de cada desoxirribonucleotídeo (dNTP), 1,25 U de *Taq* DNA Polimerase Thermopol (New England Biolabs-M0267, Whitby, ON, Canadá) e uma mistura contendo 0,1 µM de cada um dos pares de oligonucleotídeos (*primers*) de cada grupo (Tabela 1), totalizando um volume final de 20 µL. As condições de amplificação consistiram de uma etapa de pré-desnaturação a 94 °C por 5 min, seguida por 30 ciclos de 30 seg a 94 °C, 40 seg a 57 °C (ou 58 °C para o grupo 4) e 90 seg a 68 °C, com uma extensão final a 68 °C por 10 min. Os amplicons foram

analisados por eletroforese em gel de agarose 1,5% contendo 1 µL de Gel Red (Biotium- 41003, Hayward, CA, USA). As imagens foram registradas em sistema de fotodocumentação Gel Doc™ XR+ (BIO-RAD, Hercules, CA, USA).

Tabela 1. Lista de *primers* utilizados neste trabalho.

Genes	Posições no genoma[¥] (Inicial - Final)	Grupos *multiplex*	Tamanhos dos amplicons	Sequências dos *primers* (5'-3')	Tm (°C)
aux	1241229 - 1241972	G1	634	F - CTTTTAGTTCTGTGTGATGGTATGG R - CAATAGCCGCGAGTATGAAAGT	57
sacol2365	2426716 - 2426087	G1	569	F - TTTAGCTGCATGTGGTCAAGA R - CAGTAACCATTGCTTGTCCAGT	57
sacol2295	2356052 - 2356552	G1	445	F - AATCGGCACAGCATTAGTAGGT R - TTACCTGCTTCACTTGCAGAAA	57
eap	993585 - 994019	G1	330	F - CGCTACTGTGGCAGGTAATGA R - TCAAACAAGTTTGCTGTGTAGC	57
sab0166	206321 - 206629	G1	214	F - CAATTCAGACCGAAAAGAAAGC R - GATGATATTGGAACCCCACCT	57
hla	1180074 - 1181033	G2	831	F - TCCAGTGCAATTGGTAGTCATC R - CAACAACACTATTGCTAGGTTCCA	57
lp309	713907 - 714743	G2	690	F - AAAGTACGATTGTCGCACCACT R - TAGGGATAGTTTGTGGTACAGTTGG	57
sacol1912	1967095 - 1966496	G2	484	F - GATACAGATCAAGCGCCAGTT R - AAGCAAAGCGTGACGTAAAGT	57
fib	1176734 - 1177231	G2	383	F - CAGATGCGAGCGAAGGATAC R - TCGCTCTTGTAAGACCATTTTC	57
sacol2325	2387560 - 2388444	G3	741	F - GCCAGCCATCTTTAACTGCT R - GTTCGGAGTGTTTGCGTTTT	57
isaa	2648751 - 2649452	G3	619	F - GTTGGTCAACAGTGTTTGTTGG R - TCATTAGCAGTGGCATTAGGTG	57
nuc	1363130 - 1363663	G3	430	F - AAATCGCTTGCTATGATTGTGG R - TCTCTCTAGCAAGTCCCTTTTCC	57
sacol2385	2444267 - 2444695	G3	168	F - TCAAGCTACTAGAAGCGCAAAA R - CTTGGTTTGATTTTAGGCAAGG	57
sacol2171	2251354 - 2253330	G4	1395	F - CTGGAGAACACGCCTTTGAT R - CGCCTAAATGGTTTTGAACG	58
isda	1145889 - 1147826	G4	1006	F - CGTAGCTGGAGTAGCTTCCTTC R - ACCAAACTCTCGTCCAATTGAT	58
lip	2767125 - 2769167	G4	1587	F - CGTTGCTTCACCAGTGTATGTT R - GCAGCTGAGAAGCAAGTGAATA	58
pan-agr		agr	-	F - ATGCACATGGTGCACATGC	57
agr I[£]		agr	439	R - GTCACAAGTACTATAAGCTGCGAT	57
agr II[£]		agr	572	R - GTATTACTAATTGAAAAGTGC-CATAGC	57
agr III[£]		agr	321	R - CTGTTGAAAAAGTCAACTAAAAGCT	57
agr IV[£]		agr	657	R - CGATAATGCCGTAATACCCG	57
spa-1113			-	F-TAA AGA CGA TCC TTC GGT GAG C	63
spa-1514			110 - 422	R-CAG CAG TAG TGC CGT TTG CTT	63

¥ Todos os *primers* acima descritos foram desenhados baseado no genoma de *S. aureus* COL (taxid: 93062), à excessão dos *primers* sab0166, que foi desenhado baseado no genoma de *S. aureus* RF 122 (taxid: 273036).

£ Todos os *primers* utilizados foram desenhados nesse trabalho, à exceção dos primers *spa* (Aires de Sousa et al., 2006) e *agr* (Shopsin et al., 2003). Os amplicons apresentados para o gene *agr* são resultado de cada um dos *primers agr* R com o *primer pan-agr* F.

2.4. Determinação do grupo *agr*

Os grupos *agr* foram identificados por meio da amplificação por PCR do domínio hipervariável do *locus agr* utilizando oligonucleotídeos específicos para cada grupo (Shopsin *et al*., 2003). Foi utilizado um oligonucleotídeo *forward* único (*pan-agr*), específico para uma região conservada e comum aos quatro grupos. Oligonucleotídeos *reverse*, específicos para cada um dos grupos *agr* também foram

utilizados (*agr* I, *agr* II, *agr* III e *agr* IV) (Tabela 1). Os cinco *primers* foram adicionados em uma única reação de PCR e a especificidade do grupo *agr* foi identificada de acordo com os tamanhos esperados dos amplicons. A reação de PCR foi preparada com 200 ng de DNA genômico, 2,0 µL de tampão de amplificação 10X, 2,0 mM de $MgCl_2$, 0,2 mM de cada dNTP, 1 U de *Taq* DNA Polimerase Thermopol (New England Biolabs-M0267, Whitby, ON, Canadá) e um mistura contendo 0,1 µM de cada um dos 5 pares de *primers,* totalizando um volume final de 20 µL. As condições de amplificação consistiram de uma etapa de pré-desnaturação a 94 °C por 5 min, seguidos por 30 ciclos de 30 seg a 94 °C, 60 seg a 57 °C e 60 seg a 68 °C, com uma extensão final a 68 °C por 5 min. Os amplicons foram analisados por eletroforese em gel de agarose 1% contendo 1 µL de Gel Red. As imagens foram registradas em sistema de fotodocumentação Gel Doc™ XR+.

2.5. Polimorfismo do gene *spa*

A região polimórfica X da proteína A (gene *spa*) foi amplificada utilizando *primers spa*-1113-f e *spa*-1514r (Tabela 1), como descrito por Aires de Sousa *et al*., (2006). A reação de amplificação continha 100 ng de DNA, 0,2 mM de cada dNTP (dATP, dCTP, dGTP, e dTTP), 10 pmol de cada *primer*, 5 µL of tampão de reação II 10X, 1.5 mM de $MgCl_2$, e 1,25 U de AmpliTaq DNA polimerase (Applied Biosystems- N808-0130, Foster City, CA, USA), em um total de 20 µL de reação. As condições de amplificação consistiram de uma etapa inicial de desnaturação a 95 °C por 5 min, seguido de 30 ciclos de 30 s a 95 °C, 30 s a 63°C e 45 s a 72 °C, e mais uma etapa final de extensão a 72 °C por 7 min. Cinco microlitros da reação foram utilizados para avaliação dos amplicons em gel de agarose 1,2% contendo 1 µL de Gel Red. As imagens foram registradas em sistema de fotodocumentação Gel Doc™ XR+. Outros 2,5 µL da reação foram diluídos no mesmo volume de água ultrapura e enviados pra sequenciamento na *Plate-forme d'Analyses Génomiques de l'Université Laval* (Université Laval, QC, Canadá). As amostras foram sequenciadas com os mesmos *primers* descritos anteriormente. Os resultados oriundos do sequencimento em formato ab1 foram submetidos à análise pelo programa BioNumerics v5.1 (Applied Maths) e por meio de aplicativo de *spa typing*, todas as sequências foram comparadas com aquelas presentes no banco de dados do servidor Rindom SpaServer (http://www.*spa*server.ridom.de/). Para cada isolado, um tipo de *spa* específico foi identificado.

2.6. Produção de biofilme

A produção de biofilme foi avaliada em microplacas de poliestireno de 96 poços com fundo chato. As bactérias foram estriadas em meio sólido contendo BHIg e posteriormente inoculadas em meio BHIg e incubadas a 35 °C *overnight*. A cultura foi utilizada para a preparação de uma suspensão bacteriana padrão 0,5 McFarland no mesmo tipo de meio. Um volume de 100 µL dessa suspensão foi adicionado em cada poço da microplaca, seguido da adição de 100 µL do meio de cultura (Mitchell *et al*., 2013). As microplacas foram incubadas a 35 °C por 22 h. Após esse período, os poços foram lavados por três vezes em tampão salina fosfato (PBS), secos em capela de exaustão e corados com cristal violeta por 30 min. Posteriormente, as placas foram lavadas três vezes em água destilada e etanol 95% (200 µL) foi adicionado aos poços para dissolução do biofilme e após 1 h a densidade óptica (DO_{560nm}) foi medida em leitor de microplacas (VersaMax Molecular Devices). Os isolados que apresentaram valores de DO_{560nm} menor que 0,25 foram considerados fracos produtores de biofilme, enquanto que os isolados com DO_{560nm} entre 0,25 e 0,50 foram classificados com moderados e aqueles com DO_{560nm} maior que 0,50 como fortes produtores (Khan *et al*., 2011). Os ensaios foram realizados em triplicata biológica cada um com três repetições técnicas.

2.7. Determinação da Concentração Inibitória Mínima (CIM)

A determinação da CIM de antimicrobianos foi feita pelo método de microdiluição, de acordo com as recomendações do *Clinical Laboratory Standard Institute* (CLSI 2003). Os antimicrobianos utilizados para os testes foram eritromicina, gentamicina, oxacilina e tetraciclina. As bactérias retiradas do estoque a -80 °C foram estriadas em placas de Petri contendo meio BHI ágar e incubadas a 35 °C, *overnight*. Preparou-se um inóculo padrão, ressuspendendo as colônias diretamente em um tubo de ensaio contendo PBS até densidade equivalente a na escala 0,5 McFarland. Esse inóculo padrão foi diluído 150 vezes em meio Mueller-Hinton ajustado com 20 mg/L de cálcio e 10 mg/L de magnésio (MHCA). Cinquenta microlitros desse preparado foram adicionados a cada um dos poços de uma microplaca de 96 poços. Uma diluição seriada variando de 128 µg/mL até 0,12 µg/mL foi feita para cada um dos antimicrobianos e outros 50 µL de cada uma das concentrações acrescentadas aos mesmos poços. Todos os testes foram feitos utilizando-se os valores de CIM da estirpe referência *S. aureus* ATCC 29213 como

controle. Os valores de CIM utilizados como limites de suscetibilidade para os antimicrobianos foi o seguinte: eritromicina ≤ 0,5 μg/mL, gentamicina ≤ 4,0 μg/mL, oxacilina ≤ 2,0 μg/mL e tetraciclina ≤ 4,0 μg/mL (Woods e Washington, 1995).

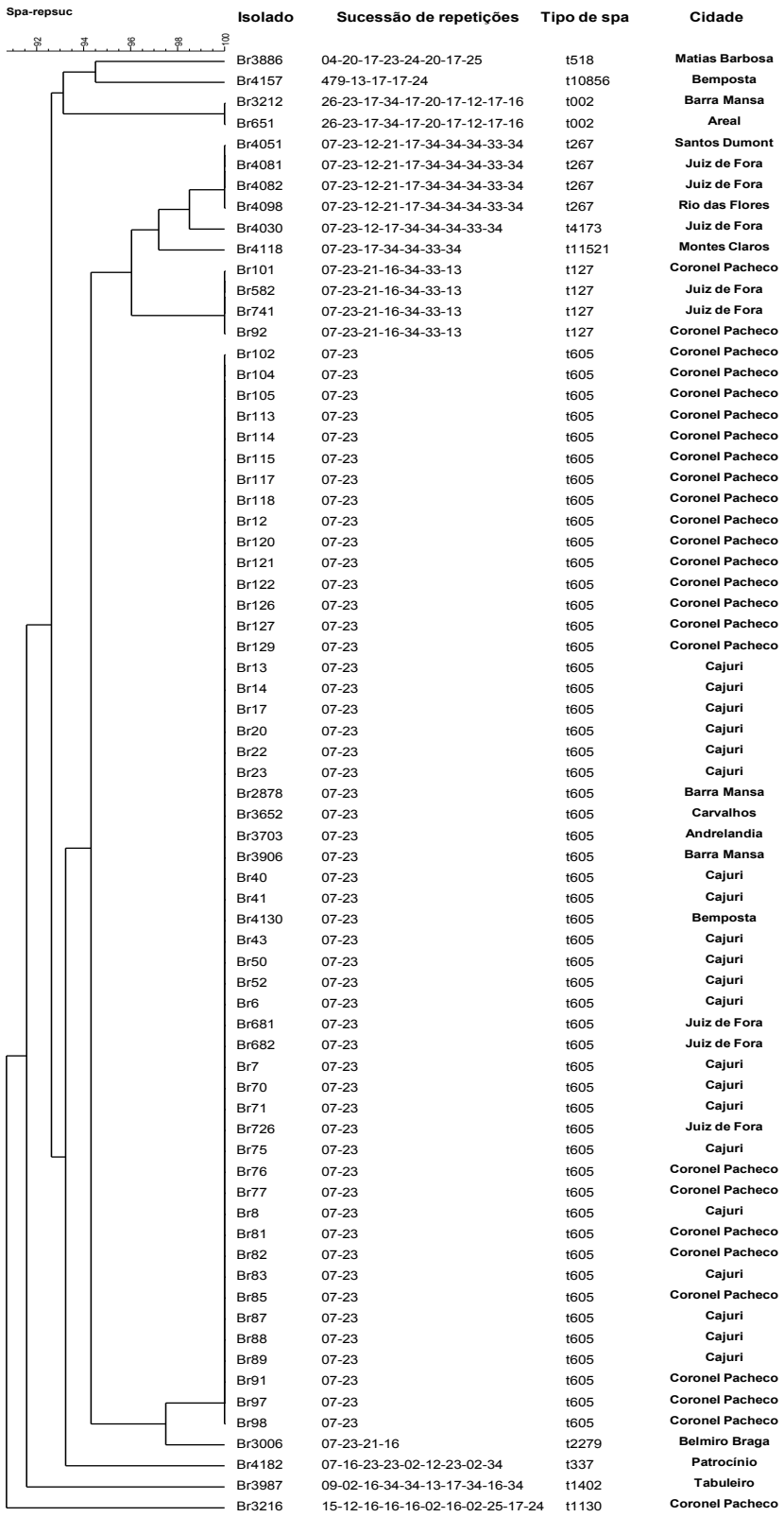
3. RESULTADOS

Setenta isolados de *S. aureus* provenientes de casos de mastite bovina de 11 municípios do estado de Minas Gerais e 4 municípios do estado do Rio de Janeiro foram selecionados para a tipagem molecular pelo polimorfismo do gene *spa*, o que revelou que eles pertenciam a 12 tipos diferentes. Um dendrograma foi gerado para mostrar as similaridades entre os isolados estudados, bem como, os tipos de repetições que geraram cada um dos tipos de *spa* e as cidades de onde eles foram isolados (Figura 1).

Cinquenta e dois isolados (75,3%) foram classificados como o tipo t605, enquanto apenas quatro (5,8%) pertenceram ao grupo t267, quatro ao grupo t127 e dois, ao grupo t002 (2,8%). Os demais oito grupos restantes contiam apenas uma bactéria. Os isolados do grupo t605 foram coletados de sete cidades, sendo cinco de Minas Gerais e duas do Rio de Janeiro. O grupo t267 apresentou isolados de duas cidades mineiras e uma do Rio de janeiro, enquanto que as bactérias do grupo t127 foram coletadas nas cidades de Juiz de Fora e Coronel Pacheco. Já o grupo t002 tinha dois isolados, sendo um da cidade de Barra Mansa e o outro de Areal.

Os isolados também foram classificados de acordo com o grupo *agr*. A classificação foi feita por meio da amplificação de regiões hipervariáveis do locus *agr* de *S. aureus* e determinada de acordo com o tamanho da banda, a saber, 439 pb (*agr* I), 572 pb (*agr* II), 321 pb (*agr* III) e 657 pb (*agr* IV). A grande maioria dos isolados (80%) foi classificada como *agr* II, seguido por *agr* I (11,4%) e III (8,6%). Não foi encontrado nenhum isolado pertencente ao grupo *agr* IV.

Comparando-se os tipos de *spa* com os grupos *agr*, constatou-se que todos os isolados dentro do *spa* t605 pertenciam ao grupo *agr* II. O mesmo tipo de relação ocorreu entre t127 e *agr* III e t267 e *agr* II.



Isolado	Sucessão de repetições	Tipo de spa	Cidade
Br3886	04-20-17-23-24-20-17-25	t518	Matias Barbosa
Br4157	479-13-17-17-24	t10856	Bemposta
Br3212	26-23-17-34-17-20-17-12-17-16	t002	Barra Mansa
Br651	26-23-17-34-17-20-17-12-17-16	t002	Areal
Br4051	07-23-12-21-17-34-34-34-33-34	t267	Santos Dumont
Br4081	07-23-12-21-17-34-34-34-33-34	t267	Juiz de Fora
Br4082	07-23-12-21-17-34-34-34-33-34	t267	Juiz de Fora
Br4098	07-23-12-21-17-34-34-34-33-34	t267	Rio das Flores
Br4030	07-23-12-17-34-34-34-33-34	t4173	Juiz de Fora
Br4118	07-23-17-34-34-33-34	t11521	Montes Claros
Br101	07-23-21-16-34-33-13	t127	Coronel Pacheco
Br582	07-23-21-16-34-33-13	t127	Juiz de Fora
Br741	07-23-21-16-34-33-13	t127	Juiz de Fora
Br92	07-23-21-16-34-33-13	t127	Coronel Pacheco
Br102	07-23	t605	Coronel Pacheco
Br104	07-23	t605	Coronel Pacheco
Br105	07-23	t605	Coronel Pacheco
Br113	07-23	t605	Coronel Pacheco
Br114	07-23	t605	Coronel Pacheco
Br115	07-23	t605	Coronel Pacheco
Br117	07-23	t605	Coronel Pacheco
Br118	07-23	t605	Coronel Pacheco
Br12	07-23	t605	Coronel Pacheco
Br120	07-23	t605	Coronel Pacheco
Br121	07-23	t605	Coronel Pacheco
Br122	07-23	t605	Coronel Pacheco
Br126	07-23	t605	Coronel Pacheco
Br127	07-23	t605	Coronel Pacheco
Br129	07-23	t605	Coronel Pacheco
Br13	07-23	t605	Cajuri
Br14	07-23	t605	Cajuri
Br17	07-23	t605	Cajuri
Br20	07-23	t605	Cajuri
Br22	07-23	t605	Cajuri
Br23	07-23	t605	Cajuri
Br2878	07-23	t605	Barra Mansa
Br3652	07-23	t605	Carvalhos
Br3703	07-23	t605	Andrelandia
Br3906	07-23	t605	Barra Mansa
Br40	07-23	t605	Cajuri
Br41	07-23	t605	Cajuri
Br4130	07-23	t605	Bemposta
Br43	07-23	t605	Cajuri
Br50	07-23	t605	Cajuri
Br52	07-23	t605	Cajuri
Br6	07-23	t605	Cajuri
Br681	07-23	t605	Juiz de Fora
Br682	07-23	t605	Juiz de Fora
Br7	07-23	t605	Cajuri
Br70	07-23	t605	Cajuri
Br71	07-23	t605	Cajuri
Br726	07-23	t605	Juiz de Fora
Br75	07-23	t605	Cajuri
Br76	07-23	t605	Coronel Pacheco
Br77	07-23	t605	Coronel Pacheco
Br8	07-23	t605	Cajuri
Br81	07-23	t605	Coronel Pacheco
Br82	07-23	t605	Coronel Pacheco
Br83	07-23	t605	Cajuri
Br85	07-23	t605	Coronel Pacheco
Br87	07-23	t605	Cajuri
Br88	07-23	t605	Cajuri
Br89	07-23	t605	Cajuri
Br91	07-23	t605	Coronel Pacheco
Br97	07-23	t605	Coronel Pacheco
Br98	07-23	t605	Coronel Pacheco
Br3006	07-23-21-16	t2279	Belmiro Braga
Br4182	07-16-23-23-02-12-23-02-34	t337	Patrocínio
Br3987	09-02-16-34-34-13-17-34-16-34	t1402	Tabuleiro
Br3216	15-12-16-16-16-02-16-02-25-17-24	t1130	Coronel Pacheco

Figura 1. Similaridade entre os tipos de *spa* identificados nesse estudo. Os 70 isolados bovinos de *Staphylococcus aureus* foram *agr*upados de acordo com a classificação do tipo de *spa*. A sucessão de repetições que determina de um tipo específico também está apresentada.

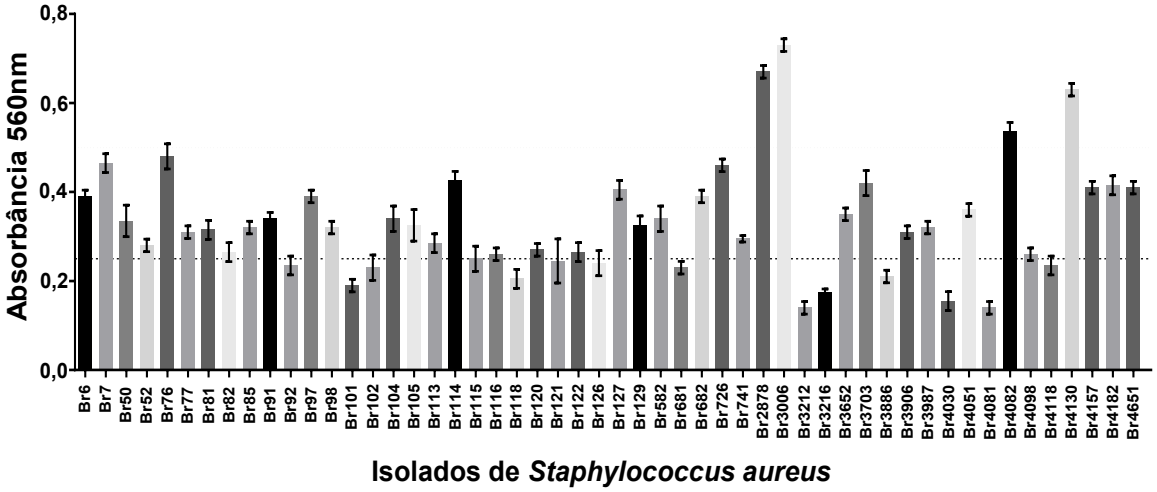


Figura 2. Produção de biofilme por diferentes isolados bovinos de *Staphylococcus aureus*. Cinquenta e dois isolados de *S. aureus* foram incubados em microplacas de 96 poços em meio BHI com 0,25% de glicose a 37 °C por 22 h. O cristal violeta foi utilizado para a coloração do biofilme e a quantificação foi obtida através da leitura de DO_{560nm}. Os resutados são provenientes da média de três experimentos independentes.

Feitas essas análises, foram selecionados isolados abrangendo todos os tipos de *spa* e *agr* encontrados previamente, para a determinação da produção de biofilme e sensibilidade para quatro antimicrobianos. A quantificação da produção de biofilme foi estratificada em três níveis: baixa (DO_{560nm} < 0,25); média (0,2 5< DO_{560nm} < 0,5); alta (DO_{560nm} > 0,5) (Figura 2). Desta forma, 12 isolados foram classificados como baixos produtores de biofilme e quatro, como altos produtores. Os demais foram considerados médios produtores de biofilme. Dentre os com baixa produção de biofilme pode-se destacar Br3212, Br3216, Br4030, Br4081 e dentre os fortes, Br2878 e Br3006.

Comparando-se a produção de biofilme com o tipo *agr*, foi possível observar que a produção média de biofilme dos isolados pertencentes ao grupo *agr* II foi a maior que os demais grupos e que a média do grupo *agr* I foi a menor, embora as diferenças entre os três grupos sejam pequenas (Figura 3).

Após a quantificação do biofilme, o perfil de sensibilidade à eritromicina, gentamicina, oxacilina e tetraciclina foi determinado para os mesmos 52 isolados. Todos os isolados foram sensíveis a oxacilina, enquanto 98%, 94% e 88% mostraram

suscetibilidade à gentamicina, eritromicina e tetraciclina, respectivamente (Tabela 2). Dentre os quatro antimicrobianos testados, a gentamicina e eritromicina tiveram CIM de até 64 µg/mL e maior que 128 µg/mL, respectivamente. A variação da CIM para a oxacilina foi a menor, ficando entre 0,12 µg/mL e 0,5 µg/mL. A CIM de 0,25 µg/mL para eritromicina ocorreu em 84,6% e para gentamicina 78,8%. Para a tetraciclina, 47,1% dos isolados apresentaram uma sensibilidade intermediária. No caso deste antimicrobiano a CIM foi 16 µg/mL e 32 µg/mL para 2 % e 9,8% das bactérias, respectivamente. Os isolados Br4051 e Br3006 mostrararm um perfil de sensibilidade similar entre si e diferente dos demais. Eles foram os únicos a apresentar valores de CIM elevados para dois antimicrobianos. Ambos tiveram os valores de CIM de 32 µg/mL e maior que 128 µg/mL, para tetraciclina e eritromicina, respectivamente.

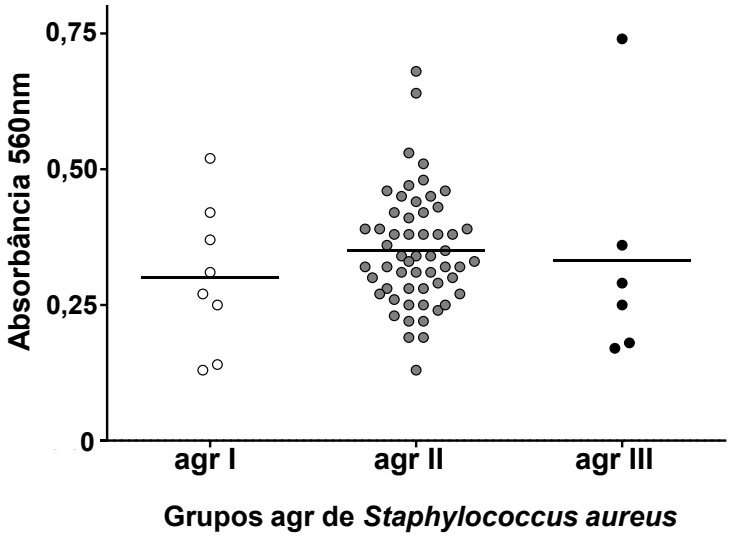


Figura 3. Produção de biofilme em *Staphylocoocus aureus* em relação aos grupos *agr*. Os valores obtidos da produção de biofilme foram plotados de acordo com o grupo *agr* de 52 isolados. Cada círculo representa um isolado e a média de cada grupo está apresentada pelo traço entre os isolados.

Tabela 2. Concentração Inibitória Mínima (CIM) e perfil de suscetibilidade de 52 *Staphylococcus aureus* isolados de mastite bovina.

Antimicrobiano	CIM_{50} (µg/mL)¥	CIM_{90} (µg/mL)£	Variação (µg/mL)	Suscetibilidade (%)
Eritromicina	0,25	0,25	0,12 - 128	94
Gentamicina	0,25	0,25	0,12 - 64	98
Oxaciclina	0,12	0,25	0,12 - 0,5	100
Tetraciclina	0,12	0,25	0,12 - 32	88

¥ CIM_{50} : Concentração inibitótia mínima para 50% dos isolados testadas

£ CIM_{90} : Concentração inibitótia mínima para 90% dos isolados testadas

A determinação da CIM para os antimicrobianos testados também foi analisada levando-se em conta os três grupos *agr* encontrados. De forma geral, a maior parte dos isolados apresentou valores de CIM igual ou menores que 0,5 µg/mL para os três grupos *agr*. Os grupos *agr* I e III apresentaram, respectivamente, três e dois isolados com CIM de 32 µg/mL para tetraciclina e um isolado com CIM maior que 128 µg/mL para eritromicina um dos grupos *agr* I e *agr* III (Figura 4). O grupo *agr* II apresentou um isolado com um valor de CIM de 64 µg/mL para gentamicina, dois isolados com CIM de 16 µg/mL para eritromicina e outro com mesmo valor para tetraciclina.

Comparando-se os valores de CIM dentro do *spa* do tipo t605, notou-se que todos os isolados foram considerados sensíveis para todos os antibiótiocos, à exceção de Br726 que apresentou uma CIM de 32 µg/mL tetraciclina.

A comparação entre produção de biofilme e o tipo de *spa* também foi realizada (Figura 5). Os grupos de *spa* que tinham quatro ou mais isolados foram avaliados quanto à produção média de biofilme.

Os 52 *S. aureus* isolados de casos de mastite bovina de 11 cidades do estado de Minas Gerais e 4 cidades do estado do Rio de Janeiro foram utilizados para a determinação da prevalencia de 16 genes por meio de PCR multiplex. Os 16 pares de primers utilizados foram distribuídos em 4 grupos multiplex como mostrado na tabela 3. Nenhum dos genes avaliados foi encontrado em 100% dos isolados, mas, entretanto, todos os genes apresentaram um alta prevalencia, sendo que os menos prevalentes foram os genes *eap*, *hla* e *sacol1912* com 87%, 93% e 95%, respectivamente. Todos os demais genes foram idenficados em mais de 95% dos isolados (Tabela 3).

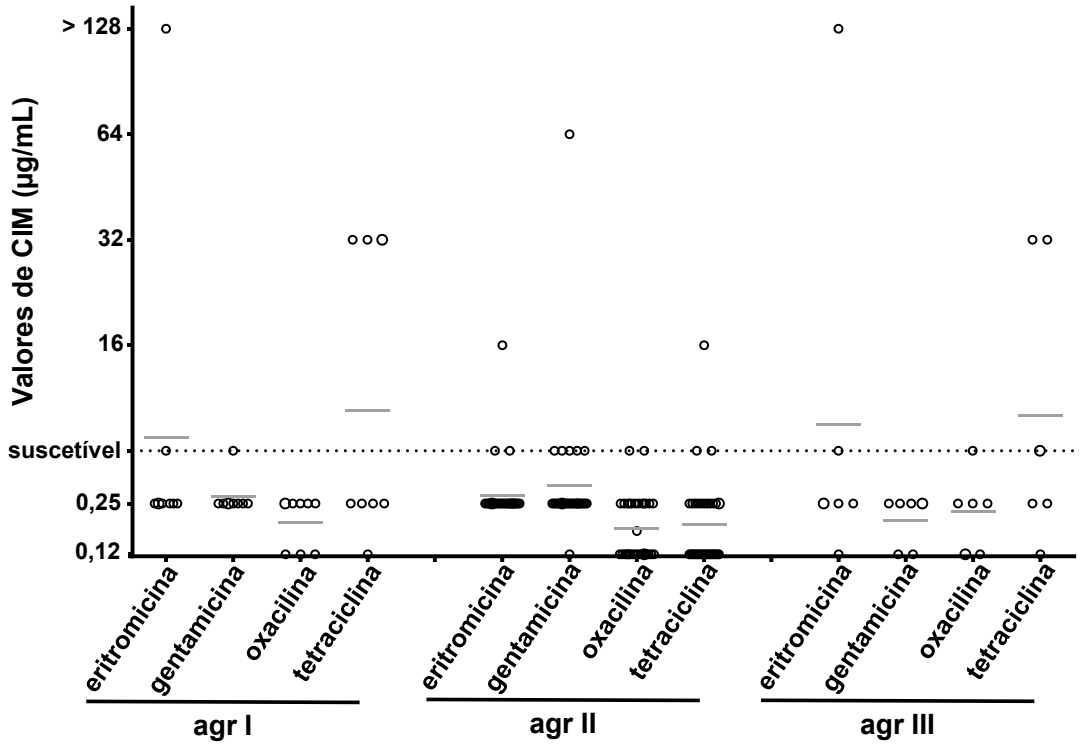


Figura 4. Concentração inibitória mínima para quatro antimicrobianos em relação aos grupos *agr*. Os valores de CIM para os quatro antimicrobianos testados estão apresentados levando-se em consideração o grupo *agr* dos isolados. Cada círculo representa um isolado. Todos isolados abaixo da linha pontilhada são considerados suscetíveis. A determinação da CIM foi realizada pelo método da microdiluição em caldo de acordo com as recomendações da CLSI, 2003.

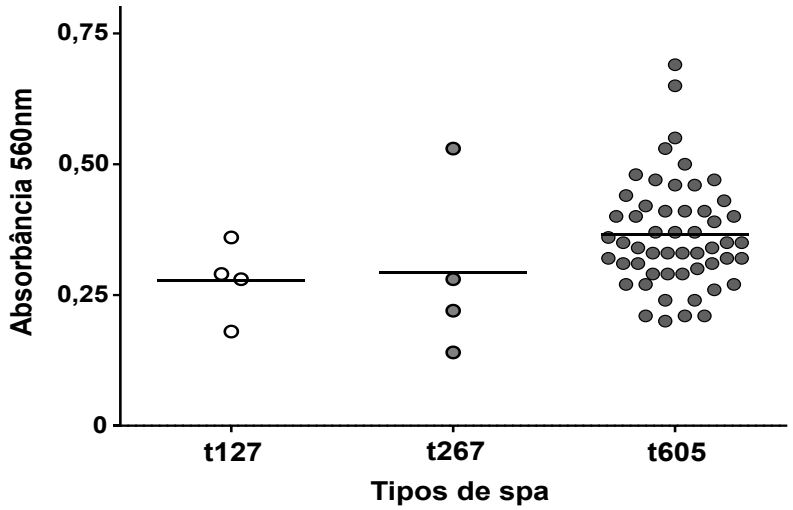


Figura 5. Produção de biofilme por *Staphylococcus aureus* pertencentes aos *spa* t127, t267 e t605. Cada círculo representa um isolado e a média de cada grupo está apresentada pelo traço entre os isolados.

Tabela 3. Prevalência dos genes de *S. aureus* estudados.

Gene	Função celular	Prevalência (%) (n=94)
aux	Proteína relacionada com resistência à meticilina	98
eap	Proteína de aderência extracelular	87
fib	Proteína de ligação à fibrinogênio	96
hla	Alfa-hemolisina	93
isaa	Antígeno imunodominante A	96
isda	Proteína de parede regulada por ferro	98
lip	Lipase	98
lp309	Transportador ABC regulado por ferro	97
nuc	Termonuclease	96
sab0166	Proteína hipotética	98
sacol1912	Proteína hipotética	95
sacol2171	Proteína da síntese de aerobactina	98
sacol2295	Proteína da biossíntese de estafiloxantina	98
sacol2325	Proteína da família lysR	96
sacol2365	Proteína hipotética	98
sacol2385	Proteína da família hsp20	96

4. DISCUSSÃO

A disseminação geográfica de *S. aureus* em rebanhos leiteiros pode ser estudada por diversos métodos moleculares e fenotípicos, embora esses últimos tenham menor poder discriminatório que os primeiros. O polimorfismo do gene *spa,* que se baseia na presença de um número variável de repetições encontradas em um região conservada do gene, é uma forma robusta de revelar a diversidade genética de estirpes de *S. aureus*, e pode se reverter em uma informação importante para delinear medidas de controle da mastite bovina.

Considerando a quantidade de rebanhos leiteiros no Brasil, poucos estudos foram conduzidos usando a tipagem do *spa* como ferramenta de análise, o que dificulta na definição de um cenário de distribuição de *S. aureus* nos rebanhos nacionais. Nesse trabalho foram identificados 12 tipos de *spa*, sendo t605 o mais predominante (75,3%), seguido pelos grupos t127 e t267. Esses resultados estão de acordo com aqueles encontrados por Aires de Sousa (2007) e Silva *et al*. (2013), que estudaram rebanhos do estado do Rio de Janeiro e São Paulo. Porém, neste estudo, t605 está presente principalmente em fazendas de Minas Gerais, com dois registros apenas para uma fazenda de Barra Mansa (RJ). Isso permite aumentar a área de distribuição do t605 para os três estados da região sudeste, reflexo provavelmente à proximidade geográfica entre eles. O mesmo raciocínio pode ser considerado para t127 e t267. t267 (Aires de Sousa *et al*., 2007) e t10856 e t002 (Silva *et al*., 2013) também foram encontrados nesse trabalho, esses últimos exclusivamente em fazendas do Rio de Janeiro. t267 e t127 foram encontrados em rebanhos leiteiros da Índia, Korea e Suiça (Hubber *et al*., 2010; Hwang *et al*., 2010; Mitra *et al*, 2010), mostrando a disseminação desses grupos de isolados em outros países. Um estudo com 195 isolados bovinos do Canadá identificou a predominância do t529 (47,7%) sobre os outros tipos como t267 (19,5%) (Veh, *et al*., submetido).

A genotipagem molecular de isolados de *S. aureus* vem sendo realizada como forma de associar determinadas características do patógeno ou doença a grupos específicos (Smith *et al*., 2005; Buzzola *et al*., 2007; Mechior *et al*., 2009; Silva *et al*., 2013). Nesse trabalho, todos os isolados classificados como t605 pertenceram ao grupo *agr* II, o que pode sugerir uma correlação entre eles. Resultado semelhante foi observado entre t127 e *agr* III, e t267 e *agr* I, embora a análise de mais isolados seja necessária para validar esse achado. Outros autores descreveram estirpes t267

representadas apenas por *agr* I (Mitra *et al*., 2013) e t127 por pelo grupo *agr* III (Silva *et al*., 2013). Juntos, esses resultados podem realmente indicar uma ligação entre tipos de *spa* com grupos *agr*, assim como correlações que existem entre *spa* e MLST (Rabelo *et al*., 2007).

A maioria dos isolados (80%) de *S. aureus* analisados foi classificada como grupo *agr* II e apenas 11,4% e 8,6% aos grupos I e III, respectivamente, o que também foi relatado por Veh *et al*., (submetido). Nenhum isolado pertenceu ao grupo IV, que parece estar pouco representado em isolados de *S. aureus* associados à mastite bovina (Gilot e Leewen *et al*., 2004; Buzzola *et al*., 2007). Já havia sido sugerida uma associação entre o grupo *agr* e o tipo de infecção. Assim, os vários trabalhos de caracterização molecular de *S. aureus* de origem bovina vem mostrar uma relação entre *agr* tipo II e mastite bovina.

Segundo Buzzola *et al*., (2007), isolados de mastite bovina do grupo *agr* I possuem uma maior capacidade de internalização em células MAC-T quando comparados a isolados dos demais grupos, sendo portanto mais associados a infecções persistentes. Melchior *et al*., (2009) descreveram que isolados pertencentes ao grupo II preferem nichos extracelulares, e por isso, produzem mais biofilme. Os autores também mostraram a correlação entre o grupo *agr* II e resistência à penicilina. Isso não foi demonstrado neste trabalho, uma vez que os isolados bovinos do grupo *agr* II produziram mais biofilme em relação à média dos grupos I e III, embora de forma não significativa (Figura 3).

A maioria dos isolados estudados nesse trabalho foi susceptível aos antimicrobianos oxacilina (100%), eritromicina (94%), gentamicina (98%) e tetraciclina (88%), perfil similar aos relatados por Ferreiro *et al*. (1985) e Brito *et al*. (2001). Uma menor porcentagem de isolados bovinos sensíveis à gentamicina e tetraciclina já foi relatada nos rebanhos brasileiros (Nader Filho *et al*., 1991). Apenas 66,1% dos isolados estudados por Langoni *et al*. (1992) foram susceptíveis à gentamicina e e apenas 23,7% à tetraciclina. Essa variação pode ser devido à presença de diferentes isolados nos rebanhos ou aos diferentes métodos empregados nos ensaios (Brito *et al*., 2001). No Brasil não existe uma legislação restritiva quanto ao uso de antimicrobianos de uso veterinário. Diversos antimicrobianos com uma variedade de princípios ativos são vendidos sem restrição. Aliado a essa disponibiliade os produtos são, muitas vezes, empregados sem o acompanhamento adequado de um veterinário ou zootecnista. Nesse panorama é possível vislumbrar

que a possibilidade de emprego de diferentes antimicrobianos em diferentes regiões pode ocasionar um perfil sensibilidade bastante heterogêneo (De Oliveira *et al*., 2000, Brito *et al*., 2001; Ochoa-Zarzosa *et al*., 2008). Países com regulamentação adequada para o uso de antimicrobianos têm demonstrado um baixo nível de resistência quando comparados com outros países (Aarestrup *et al*., 1998; Grave *et al*., 1999; de Oliveira *et al*., 2000). Porém, é interessante notar que a alta sensibilidade dos isolados bovinos coletados nas fazendas de Minas Gerais deveria refletir em uma maior eficácia dos tratamentos com antimicrobianos aplicados no combate às infecções estafilocócicas, o que nem sempre acontece.

Todos os genes de virulência avaliados por PCR multiplex foram encontrados com uma alta prevalência nos 52 isolados bovinos, sendo os genes *eap*, *hla* e *sacol1912*, os menos encontrados, com 87%, 93% e 95%, respectivamente. Todos os demais estavam presentes em mais de 95% do isolados. Logo, não foi possível a associar a presença ou ausência de um gene ao um tipo de *spa* ou a um grupo *agr* específico.

5. CONCLUSÕES

Os dados apresentados confirmam a presença de vários tipo de *spa* em rebanhos leiteiros do Brasil, com especial destaque para t605, tipo mais predominante e disseminado em fazendas dos estados de Minas Gerais e Rio de Janeiro. Outros 11 tipos foram encontrados o que revela uma circulação de *S. aureus* geneticamente distintos nos rebanhos analisados. Os isolados bovinos caracterizados são predominantemente do grupo *agr* tipo II, e com menor frequência do grupo I e III. Sugere-se uma ligação entre t605 e *agr* II.

Em geral, os isolados são sensíveis aos antimicrobianos testados, com poucos resistentes à tetraciclina, informação que pode ajudar na definição de terapia com antimicrobianos. Porém, a maioria dos isolados foi considerada moderado a forte produtores de biofilme, característica que pode influenciar no sucesso terapêutico. Muitos fatores de virulência foram identificados em todos os isolados e podem representar potenciais marcadores para o diagnóstico de *S. aureus* de origem bovina.

6. REFERÊNCIAS BIBLIOGRÁFICAS

Aarestrup, F.M.; Bager, F.; Jensen, N.E.; Madsen, M.; Meyling, A.; Wegener, H.C. 1998. Resistance to antimicrobial agents used for animal therapy in pathogenic, zoonotic and indicator bacteria isolated from different food animals in Denmark: A baseline study for the Danish Integrated Antimicrobial Resistance Monitoring Programme (DANMAP). APMIS, 106, 745-70.

Aires-de-Sousa, M.; Boye, K.; de Lencastre, H.; Deplano, A.; Enright, M.C.; Etienne, J.; Friedrich, A.; Harmsen, D.; Holmes, A.; Huijsdens, X.W.; Kearns, A.M.; Mellmann, A.; Meugnier, H.; Rasheed, J.K.; Spalburg, E.; Strommenger, B.; Struelens, M.J.; Tenover, F.C.; Thomas, J.; Vogel, U.; Westh, H.; Xu, J.; Witte, W. 2006. High interlaboratory reproducibility of DNA sequence-based typing of bacteria in a multicenter study. J Clin Microbiol., 44, 619-21.

Aires-de-Sousa, M.; Parente, C.E.; Vieira-da-Motta, O.; Bonna, I.C.; Silva, D.A.; de Lencastre, H. 2007. Characterization of *Staphylococcus aureus* isolates from buffalo, bovine, ovine, and caprine milk samples collected in Rio de Janeiro State, Brazil. Appl Environ Microbiol., 73, 3845-9.

Allard, M.; Ster, C.; Jacob, C.L.; Scholl, D.; Diarra, M.S.; Lacasse, P.; Malouin, F. 2013. The expression of a putative exotoxin and an ABC transporter during bovine intramammary infection contributes to the virulence of *Staphylococcus aureus*. Vet Microbiol., 162, 761-70.

Barkema, H.W.; Schukken, Y.H.; Zadoks, R.N. 2006. Invited Review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. Am. Dairy Sci. Assoc., 89, 1877-95.

Blumberg, H.M.; Rimland, D.; Kiehlbauch, J.A.; Terry, P.M.; Wachsmuth, I.K. 1992. Epidemiologic typing of *Staphylococcus aureus* by DNA restriction fragment length polymorphisms of rRNA genes: elucidation of the clonal nature of a group of bacteriophage-nontypeable, ciprofloxacin-resistant, methicillin-susceptible *S. aureus* isolates. J. Clin. Microbiol., 30, 362–9.

Bovinocultura de Leite. 2010 - Parte 1, Volume 1. Banco do Brasil – BB *e o* Instituto Interamericano de Cooperação para Agricultura.

Brito, M.A.V.P.; Brito, J.R.F.; Produção científica brasileira sobre mastite bovina. In: Brito, J.R.F., Bressan, M. (ed.). 1996. Controle integrado da mastite bovina. Juiz de Fora: Embrapa-CNPGL, 68-96.

Brito, M.A.V.P.; Brito, J.R.F.; Silva, M.A.S.; Carmo, R.A. 2001. Concentração mínima inibitória de dez antimicrobianos para amostras de *Staphylococcus aureus* isoladas de infecção intramamária bovina. Arq. Bras. Med. Vet. Zootec., 53, 531-7.

Buzzola, F. R.; Barbagelata, M. S.; Caccuri, R. L.; Sordelli, D.O. 2007. Attenuation and persistence of and ability to induce protective immunity to a *Staphylococcus aureus* aroA mutant in mice. Infect Immun., 74, 3498-506.

Chambers, H.F.; Deleo, F.R. 2009. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat Rev Microbiol., 7, 629-41.

Cheung, A.L.; Eberhardt, K.J.; Chung, E.; Yeaman, M.R.; Sullam, P.M. 1994. Diminished virulence of a sar-/agr- mutant of *Staphylococcus aureus* in the rabbit model of endocarditis. J. Clin. Invest., 94, 1815–22.

Chhatwal, G.S. 2002. Anchorless adhesins and invasins of Gram-positive bacteria: a new class of virulence factors. Trends Microbiol., 10, 205-8.

Costerton J. W.; Stewart P. S.; *et al*. 1999. Bacterial biofilms: a common cause of persistent infections, Science 284, 1318-22.

Cucarella, C.; Tormo, A. M.; Ubeda, C.; Trotonda, P. M.; Monzon, M.; Peris, C.; Amorena, B.; Lasa, I.; Penade, R. J. 2004. Role of Biofilm-Associated Protein Bap in the Pathogenesis of Bovine *Staphylococcus aureus*. Infection and Immunity, 72, 2177-85.

De Oliveira, A. P.; Watts, J.L.; Salmon, S. A. Aarestrup, F.M. 2000. Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Europe and United States. J. Dairy Sci., 83, 855-62.

Dunman, P.M.; Murphy, E.; Haney, S.; Placios, D.; Tucker-Kellogg, G.; Wu, S. 2001. Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the agr and/or sarA loci. J. Bacteriol., 183, 7341-53.

Embrapa. 2012. Produtividade animal em países selecionados-2010. Tabela 02.19. http://www.cnpgl.embrapa.br/nova/informacoes/estatisticas/producao/tabela0219.php. Acessado em 11/02/2014.

Embrapa. 2013. Panorama do leite. Ano 6, n°67 (Abril/2013).

Ferreiro, L.; Ferreiro, C.L.R.; Bangel JR, J.J. *et al*. 1985. Mastite bovina na grande Porto Alegre, RS - Brasil. II Susceptibilidade antimicrobiana dos mais freqüentes agentes etiológicos isolados durante o período 1982-1985. Arq. Fac. Vet. UFRGS., 13, 89-94.

Francois, P.; Huyghe, A.; Charbonnier, Y.; Bento, M.; Herzig, S.; Ivan Topolski, I.; Fleury, B.; Lew, D.; Vaudaux, P.; Harbarth, S.; Leeuwen, W.; Belkum, A.; Blanc, D.; Pittet, D. e Schrenzel, J. 2005. Use of an automated multiple-locus, variable-number tandem repeat-based method for rapid and high-throughput genotyping of *Staphylococcus aureus* isolates. J. Clin. Microbiol., 43, 3346-55.

Gillet, Y.; Issartel, B.; Vanhems, P.; Fournet, J.C.; Lina, G.; Bes, M.; Vandenesch, F.; Piémont, Y.; Brousse, N.; Floret, D.; Etienne, J. 2002. Association between Staphylococcus aureus strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. Lancet., 359, 753-9.

Gilot, P.; van Leeuwen, W. 2004. Comparative analysis of agr locus diversification and overall genetic variability among bovine and human *Staphylococcus aureus* isolates. J. Clin. Microbiol., 42, 1265-9.

Godden, S.M.; Jansen, J.T.; Leslie, K.E.; Smart, N.L.; Kelton D.F. 2002. The effect of sampling time and sample handling on the detection of *Staphylococcus aureus* in milk from quarters with subclinical mastitis. Can. Vet. J., 43, 38-42.

Grave, K.; Greko, C.; Nilsson, L.; Odensvik, K.; Mork, T.; Ronning, M. 1999. The usage of veterinary antibacterial drugs for mastitis in cattle in Norway and Sweden during 1990–1997. Prev. Vet. Med., 42, 25–55.

Harraghy, N.; Kerdudou, S; Herrmann, M. 2007. Quorum-sensing systems in staphylococci as therapeutic targets. Anal Bioanal. Chem., 387, 437-44.

Haslinger-Loffler, B.; Kahl, B.C.; Grundmeier, M.; Strangfeld, K.; Wagner, B.; Fischer, U.; Cheung, A.L.; Peters, G.; Schulze-Osthoff, K.; Sinha, B. 2005. Multiple virulence factors are required for *Staphylococcus aureus*-induced apoptosis in endothelial cells. Cell. Microbiol., 7, 1087-97.

Haveri, M.; Hovinen, M.; Roslof, A.; Pyorala S. 2008. Molecular Types and Genetic Profiles of *Staphylococcus aureus* Strains Isolated from Bovine Intramammary Infections and Extramammary Sites, Journal of Clinical Microbiology Vol. 46, No. 11, p. 3728-3735.

Hogeveen, H.; Huijps, K.; Lam, T.J.G.M. 2011. Economic aspects of mastitis: New developments. Veterinary Journal, 59, 16-23.

Huber, H.; Koller, S.; Giezendanner, N.; Stephan, R.; Zweifel, C. 2010. Prevalence and characteristics of meticillin-resistant *Staphylococcus aureus* in humans in contact with farm animals, in livestock, and in food of animal origin, Switzerland, 2009. Euro Surveill, 15.

Hwang, S. Y.; Park, Y.K.; Koo, H.C.; Park, Y.H.. 2010. spa typing and enterotoxin gene profile of *Staphylococcus aureus* isolated from bovine raw milk in Korea. J. Vet. Sci., 11, 125-31.

Jarraud, S.; Mougel, C.; Thioulouse, J.; Lina, G.; Meugnier, H.; Forey, F.; Nesme, X.; Etienne, J.; Vandenesch, F. 2002. Relationships between *Staphylococcus aureus* genetic background, virulence factors, agr groups (alleles), and human disease. Infect. Immun. 70, 631-41.

Kapur, V.; Sischo, W.M.; Greer, R.S.; Whittam, T.S.; Musser, J.M. 1995. Molecular population genetic analysis of *Staphylococcus aureus* recovered from cows. J. Clin. Microbiol., 33, 376-80.

Klein R.C; Fabres-Klein, M.H.; Brito, M.A.; Fietto, L.G.;, Ribon, A.O.B. 2012. *Staphylococcus aureus* of bovine origin: genetic diversity, prevalence and the expression of adhesin-encoding genes.Vet Microbiol. 9, 183-8.

Lange, C.; Cardoso, M.; Senczek, D.; Schwarz, S. 1999. Molecular subtyping of *Staphylococcus aureus* isolates from cases of bovine mastitis in Brazil. Vet. Microbiol., 67, 127-41

Langoni, H.; Pinto, M.P.; Domingues, P.F.; *et al*. 1991. Etiologia e susceptibilidade da mastite bovina subclínica. Arq. Bras. Med. Vet. Zootec., 43, 507-15.

Melchior, M. B.; Vaarkamp, H.; Fink-Gremmels, J. 2006. Biofilms: a role in recurrent mastitis infections? The Veterinary Journal, 171, 398-407.

Melchior, M. B.; van Osch, M. H. J.; Graat, R. M.; van Duijkeren, E.; Mevius, D. J.; Nielen, M.; Gaastra, W.; Fink-Gremmels, J. 2009. Biofilm formation and genotyping of *Staphylococcus aureus* bovine mastitis isolates: evidence for lack of penicillin-resistance in Agr-type II strains. Vet Microbiol,, 137, 83-9.

Melles, D.C.; van Leeuwen, W.B.; Snijders, S.V.; Horst-Kreft, D.; Peeters, J.K.; Verbrugh, H.A.; van, B.A. 2007. Comparison of multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and amplified fragment length polymorphism (AFLP) for genetic typing of *Staphylococcus aureus*. J. Microbiol. Methods, 69, 371–5.

Mitra, D.S.; Velu, D.; Bhuvana, M.; Krithiga, N.; Banerjee, A.; Shome, R.; Rahman, H.; Ghosh, K.S.; Shome, R.B. 2013. *Staphylococcus aureus* spa type t267, clonal ancestor of bovine subclinical mastitis in India. Journal of Applied Microbiology, 114, 1604-15.

Monecke, S.; Kuhnert, P.; Hotzel, H.; Slickers, P.; Ehricht, R. 2007. Microarray based study on virulence-associated genes and resistance determinants of *Staphylococcus aureus* isolates from cattle. Vet. Microbiol., 125, 128–40.

Murchan, S.; Kaufmann, M.E.; Deplano, A.; de Ryck, R.; Struelens, M.; Zinn, C.E.; Fussing, V.; Salmenlinna, S.; Vuopio-Varkila, J.; El Solh, N.; Cuny, C.; Witte, W.; Tassios, P.T.; Legakis, N.J.; van Leuwen, W.; van Belkum, A.; Vindel, A.;

Laconcha, I.; Garaizar, J.; Haeggman, S.; Olsson-Liljequist, B.; Ransjo, U.; Coombes, G.; Cookson, B. 2003. Harmonization of pulsed-field gel electrophoresis protocols for epidemiological typing of strains of methicillin-resistant *Staphylococcus aureus*: a single approach developed by consensus in 10 European laboratories and its application for tracing the spread of related strains. J. Clin. Microbiol., 41, 1574-85.

Nader Filho, A.; Amaral, L.A.; Rossi Jr, O.D.; *et al*. 1992. Sensibilidade dos *Staphylococcus* coagulase positiva e dos *Staphylococcus* coagulase negativa isolados de casos de mastite bovina à ação de antibióticos e quimioterápicos. Ars Vet., 8, 142-7.

NCCLS 2003. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Sixth Edition. NCCLS document M7-A6 [ISBN 1-56238-486-4]. NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA.

Ochoa-Zarzosa, A.; Loeza-Angeles, H.; Sagrero-Cisneros, E.; Villagómez-Gómez, E.; Lara-Zárate, L.; López-Meza, J.E. 2008. Antibacterial activity of thionin Thi2.1 from Arabidopsis thaliana expressed by bovine endothelial cells against *Staphylococcus aureus* isolates from bovine mastitis. Vet. Microbiol., 127, 425-30.

Parra-Ruiz, J.; Vidaillac, C.; Rybak, M.J. 2012. Macrolides and staphylococcal biofilms. Rev Esp Quimioter. 25, 10-6.

Patti, J.M.; Bremell, T.; Krajewska-Pietrasik, D.; Abdelnour, A.; Tarkowski, A.; Ryden, C.; Höök, M. 1994. The *Staphylococcus aureus* collagen adhesin is a virulence determinant in experimental septic arthritis. Infect. Immun., 62, 152-61.

Pitkala, A.; Haveri, M.; Pyorala, S. 2004. Bovine mastitis in Finland 2001-prevalence, distribution of bacteria, and antimicrobial resistance. J. Dairy Sci., 87, 2433-41.

Rabello, R.F.; Moreira, B.M.; Lopes, R.M.; Teixeira, L.M.; Riley, L.W.; Castro, A.C. 2007. Multilocus sequence typing of *Staphylococcus aureus* isolates

recovered from cows with mastitis in Brazilian dairy herds. J Med Microbiol., 56, 1505-11.

Rabello, R.F.; Souza, C.R.; Duarte, R.S.; Lopes, R.M.; Teixeira, L.M.; Castro, A.C. 2005. Characterization of *Staphylococcus aureus* isolates recovered from bovine mastitis in Rio de Janeiro, Brazil. J Dairy Sci., 88, 3211-9.

Reinoso, E.B.; El-Sayed, A.; Lammler, C.; Bogni, C.; Zschock, M. 2008. Genotyping of *Staphylococcus aureus* isolated from humans, bovine subclinical mastitis and food samples in Argentina. Microbiol. Res., 163, 314-22.

Ruegg, P. 2005. Premiums, production and pails of discarded milk how much money does mastitis cost you? Resources Milk Money, 3, 50-6.

Sabat, A.; Krzyszton-Russjan, J.; Strzalka, W.; Filipek, R.; Kosowska, K.; Hryniewicz, W.; Travis, J.; Potempa, J. 2003. New method for typing *Staphylococcus aureus* strains: multiple-locus variable-number tandem repeat analysis of polymorphisms and genetic relationships of clinical isolates. J. Clin. Microbiol., 41, 1801-4.

Said, K. B.; Ismail, J.; Campbell, J.; Mulvey, R.M.; Bourgault A-M.; Messier, S.; Zhao, X. 2010. Regional Profiling for Determination of Genotype Diversity of Mastitis-Specific *Staphylococcus aureus* Lineage in Canada by Use of Clumping Factor A, Pulsed-Field Gel Electrophoresis, and spa Typing. J. Clin. Microbiol., 48, 375-86.

Sakoulas, G.; Eliopoulos, G.M.; Moellering, JR.R.C.; Novick, R.P.; Venkataraman, L.; Wennersten, C.; Degirolami, P.C.; Schwaber, M.J.; Gold, H.S. 2003. *Staphylococcus aureus* accessory gene regulator (agr) Group II: is there a relationship to the development of intermediate-level glycopeptide resistance? T. J. Infect. Dis., 187, 929-38.

Shopsin, B.; Mathema, B.; Alcabes, P.; Said-Salim, B.; Lina, G.; Matsuka, A.; Martinez, J.; Kreiswirth, B.N. 2003. Prevalence of agr specificity groups among *Staphylococcus aureus* strains colonizing children and their guardians. J. Clin. Microbiol., 41, 456-9.

Silva, N.C.; Guimarães, F.F.; Manzi, M.P.; Budri, P.E.; Gómez-Sanz, E.; Benito, D.; Langoni, H.; Rall, V.L.; Torres, C. 2013. Molecular characterization and clonal diversity of methicillin-susceptible *Staphylococcus aureus* in milk of cows with mastitis in Brazil. J Dairy Sci., 96, 6856-62.

Singh, R.; Ray, P.; Das, A.; Sharma, M. 2010. Penetration of antibiotics through *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. J Antimicrob Chemother., 65, 1955-8.

Smith, E.M.; Green, L.E.; Medley, G.F.; Bird, H.E.; Fox, L.K.; Schukken, Y.H.; Kruze, J.V.; Bradley, A.J.; Zadoks, R.N.; Dowson, C.G. 2005. Multilocus sequence typing of intercontinental bovine *Staphylococcus aureus* isolates. J. Clin. Microbiol., 43, 4737-43.

Sutra, L.; Poutrel, B. 1994. Virulence factors involved in the pathogenesis of bovine intramammary infections due to *Staphylococcus aureus*. J. Med. Microbiol, 40, 79-89.

Tiwari, J.G.; Babra, C.; Tiwari, H.K.; Williams, V.; Wet, S.D. 2013. Trends In Therapeutic and Prevention Strategies for Management of Bovine Mastitis: An Overview. J Vaccines Vaccin 4: 176.

Vancraeynest, D.; Hermans, K.; Haesebrouck, F. 2004. Genotypic and phenotypic screening of high and low virulence *Staphylococcus aureus* isolates from rabbits for biofilm formation and MSCRAMMs. Vet. Microbiol., 103, 241-7.

Veh, K.A.; Klein, R.C., Keefe, G.; Lacasse, P.; Scholl, D.; Roy, J.P.; Haine, D.; Dufour, S.; Talbot, B.G.; Ster, C.; Ribon, A.O.B.; Malouin, F. (submetido) Genotypic and phenotypic characterization of *Staphylococcus aureus* causing persistent and non-persistent subclinical bovine intramammary-quarter infections during lactation and at dry-off.

von Eiff, C.; Friedrich, A.W.; Peters, G.; Becker, K. 2004. Prevalence of genes encoding for members of the staphylococcal leukotoxin family among clinical isolates of *Staphylococcus aureus*. Diagn. Microbiol. Inf. Dis., 49, 157-62.

Woods, G.I.; Washington, J.A. Antibacterial susceptibility tests: dilution and disk diffusion methods. In: Murray, P.R.; Baron, E.J.; Pfaller, M.A. *et al*. (ed.) Manual of clinical microbiology. Washington: American Society For Microbiology Press, 1995. P.1327-1341.

CAPÍTULO 2

GENOTYPIC AND PHENOTYPIC CHARACTERIZATION OF *Staphylococcus aureus* CAUSING PERSISTENT AND NON-PERSISTENT SUBCLINICAL BOVINE INTRAMAMMARY-QUARTER INFECTIONS DURING LACTATION AND AT DRY-OFF

INTERPRETIVE SUMMARY

Characterization of intramammary-quarter persistent *Staphylococcus aureus*. *by Veh and Klein et al.* We compared the phenotypes and virulence gene patterns of persistent and non-persistent subclinical *S. aureus* isolates from intramammary-quarter infections (qIMI) during lactation or at dry-off. We found that biofilm production, *hld* expression, the *spa* type and the presence of the enterotoxin genes *sen*, *seg* and *tst* are markers that can help determining the likelihood of an isolate to produce a subclinical or persistent qIMI. The present study significantly expanded our current knowledge on the genotypic and phenotypic traits of *S. aureus* isolates associated with qIMI persistence.

Genotypic and Phenotypic Characterization of *Staphylococcus aureus* Causing Persistent and Non-Persistent Subclinical Bovine Intramammary-Quarter Infections During Lactation and at Dry-Off

K. A. Veh*[†1], R. C. Klein[‡1], C. Ster*[†], G. Keefe[§†], P. Lacasse[#†], D. Scholl[‖†2], J.-P. Roy[‖†], D. Haine[‖†], S. Dufour[‖†], B. G. Talbot*[†], A. O. B. Ribon[‡3], and F. Malouin*[†3]

*Centre d'étude et de valorisation de la diversité microbienne (CEVDM), Département de biologie, Faculté des sciences, Université de Sherbrooke, Sherbrooke, QC, Canada J1K 2R1.

[†]Canadian Bovine Mastitis and Milk Quality Research Network, C.P. 5000, St-Hyacinthe, QC, Canada, J2S 7C6.

[‡]Departmento de Bioquimica e Biologia Molecular, University Federal de Viçosa, Viçosa 36570-000, MG, Brazil.

[§]Centre for Veterinary Epidemiological Research, University of Prince Edward Island, Charlottetown, Prince Edward Island, Canada C1A 4P3.

[#]Dairy and Swine Research and Development Centre, Agriculture and Agri-Food Canada, Sherbrooke, QC, Canada J1M 0C8.

[‖]Département de Pathologie et Microbiologie and Département de Sciences Cliniques, Faculté de Médecine Vétérinaire, Université de Montréal, C.P. 5000, St-Hyacinthe, QC, Canada J2S 7C6.

[1]Have equally contributed to the work,

[2]Current address: South Dakota Agricultural Experiment Station, SD, USA,

[3]Co-Corresponding authors:

FM Phone: 819-821-8000 ext. 61202, FM e-mail: francois.malouin@usherbrooke.ca
AOBR Phone: 55 31 38992837, AOBR e-mail: abribon@ufv.br

ABSTRACT

Staphylococcus aureus (*S. aureus*) is a significant pathogen that frequently causes clinical and subclinical mastitis in dairy cows. *S. aureus* involved in persistent and unapparent (subclinical) intramammary-quarter infections (qIMI) represent a reservoir and a risk for dissemination of the pathogen within the herd. To compare the phenotypes and virulence gene patterns of a total of 285 persistent and non-persistent subclinical qIMI, quarter milk samples were systematically collected from asymptomatic cows across Canada. A collection of 81 clinical qIMI isolates were also studied for comparison. VNTR typing (variable number of tandem repeats) was used to validate the persistence of the same *S. aureus* strain in three consecutive quarter milk samples collected at intervals of 3 wk during lactation or before and after dry-off. These isolates were compared genotypically (*seg*, *sen*, *sec* and *tst*) by using PCR and *spa* typing. Biofilm production and *hld* expression levels were quantified using a 96-well plate colorimetric method and qPCR, respectively. The genes *seg* and *sen* were associated with a reduction in the likelihood of the bacteria to be in a clinical mastitis case or being persistent in subclinical lactational isolates and dry-cow isolates, respectively. On the other hand, gene *tst* was associated with increase odds for isolates to be in a clinical case. We found that the odds of being persistent according to biofilm production were not significant for the lactation period or the dry-off period. However, both dry-off and lactational isolates produced significantly more biofilm than isolates collected from cows with clinical mastitis during lactation and isolates producing more biofilm were more likely to be subclinical by 83%. Also, we showed that isolates expressing more *hld* were more likely to be non-persistent during either lactation or the dry period. Three *spa* types were predominant (t529, t267, and a novel type: t13401). The *spa* type t529 was the

most frequent and strains carried several superantigen genes, notably both *sen* and *seg* in 75.5% of the isolates. The *spa* type t529 was found across all the groups of isolates. The novel *spa* type (t13401) produced significantly more biofilm than t529 and t267 and only one isolate (6%) was of this *spa* type in clinical isolates. The present study significantly expanded our current knowledge on the genotypic and phenotypic traits of *S. aureus* isolates associated with qIMI persistence.

Keywords: dairy cow, mastitis**,** *Staphylococcus aureus*, persistence

1. **INTRODUCTION**

Staphylococcus aureus (*S. aureus*) is a worldwide pathogen causing many serious diseases in humans and animals. It is an important etiological agent of clinical and subclinical bovine mastitis and causes major economic losses to the dairy industry as it reduces both milk quality and production (Awale *et al*., 2012; Bar *et al*., 2008). Of particular interest are the *S. aureus* isolates responsible for subclinical and persistent intramammary-quarter infections (qIMI) that usually respond poorly to antimicrobial treatments and that can remain undetected because they often only cause minor or unapparent clinical symptoms (Sol *et al*., 2000). The presence of such strains represents a reservoir that maintains the occurrence of *S. aureus* infections in herds (Anderson and Lyman, 2006; Sutra and Poutrel, 1994). An epidemiological study on dairy farms in Canada between 2007-2008 by the Canadian Bovine Mastitis Research Network (CBMRN) showed that *S. aureus* was the predominant microorganism isolated from milk and mastitis cases all together (Reyher *et al*., 2011).

Staphylococcus aureus potentially produces several virulence factors that can contribute to persistence (Dinges *et al*., 2000; Oliveira *et al*., 2011a; Sutra and Poutrel, 1994). These factors can be divided into different groups, including surface-associated factors, degradative enzymes, staphylococcal enterotoxins (such as SEC, SEG, SEN and others) and toxic shock syndrome toxin-1 (TSST-1) (Haveri *et al*., 2008; Oliveira *et al*., 2011a). The enterotoxins are known as superantigen toxins because of their ability to polyclonally activate T cells in a nonspecific manner (Dinges *et al*., 2000). This ultimately causes the host immune system to be inefficient

and may explain in part the existence of chronic and recurrent infections (Fitzgerald *et al*., 2000; Akineden *et al*., 2001; Anderson and Lyman, 2006; Xie *et al*., 2011).

Biofilm formation by *S. aureus* is considered an important virulence factor, which may influence the persistence of the pathogen during bacterial-induced bovine mastitis (Cucarella *et al*., 2004). It is estimated that 80% of chronic infections are associated with biofilm-producing bacteria (Davies, 2003). Biofilms reduce opsonization by antibodies and phagocytosis (Otto, 2013). In addition, the bacteria within biofilms are less susceptible to antibiotics and very difficult to remove (Costerton *et al*., 1999; Melchior *et al*., 2006, 2007).

Most bovine staphylococcal strains can produce hemolysins as shown by their hemolytic activity on sheep blood agar (Aarestrup *et al*., 1999). These pore-forming exotoxins induce pro-inflammatory changes in mammalian cells, inactivate the immune system by their direct cytotoxic effect and degrade tissues, providing bacteria with nutrients and facilitating spreading to new sites (Dinges *et al*., 2000). The delta-hemolysin or δ-toxin is produced by 97% *S. aureus* strains and mainly causes cytolysis of red and white blood cells as well as other mammalian cells (Dinges *et al*., 2000; Larsen *et al*., 2002). It is translated from the transcript RNAIII, a key player in the quorum sensing system (*agr*) of staphylococci (Novick and Geisinger, 2008). The RNAIII/*hld* transcript plays an important regulatory role and inversely controls the production of bacterial cell-surface associated proteins and secreted virulence factors. Expression of RNAIII/*hld* is itself controlled and permitted by *agr* (Dinges *et al*., 2000; Novick and Geisinger, 2008). Agr thus regulates the level of transcription and translation of several groups of virulence genes (Novick and Geisinger, 2008). Once *agr* is activated by bacterial density through secretion of an autoinducing peptide, transcription of the regulatory

RNAIII/*hld* allows reduction of surface proteins such as adhesins required for colonization. At the same time, RNAIII/*hld* allows the production of secreted proteins such as nucleases and proteases thus helping the release of bacteria from biofilms (Novick, 2003; Otto, 2013)

Staphylococcus aureus characteristics and the importance of some virulence factors in the ability of isolates to cause persistent qIMI have not been completely investigated. A few studies have reported predominating *S. aureus* clonal types that can modulate the outcome of mastitis (Buzzola *et al*., 2001; Fitzgerald *et al*., 2000; Matsunaga *et al*., 1993; Smith *et al*., 1998; Zadoks *et al*., 2002; Haveri *et al*., 2005). It has already been shown that *S. aureus* involved in bovine mastitis present specific virulence genotypes and/or phenotypes that include the capacity to produce specific toxins and/or biofilm (Cucarella *et al*., 2004; Haveri *et al*., 2008) but additional information is needed for a complete understanding of the disease and more specifically for the persistence of qIMI.

The aim of the present study was to determine whether genotypic markers such as *seg*, *sen*, *sec*, *tst* and the *spa* type as well as the phenotypic expression of biofilm and the level of RNAIII/*hld* expression could be of prognostic value to predict the likelihood that *S. aureus* subclinical qIMI, either during lactation or at dry-off, would become persistent. This study systematically compares those virulence markers in well-characterized and validated persistent isolates. These strains were also compared to those causing typical clinical mastitis.

2. MATERIALS AND METHODS

2.1. Bacterial Isolates Collection

Staphylococcus aureus strain Newbould (ATCC 29740) was used as the reference strain for comparison to a total of 366 *S. aureus* isolates from subclinical and clinical cases of mastitis. Isolates were obtained from the CBMRN Mastitis Pathogen Culture Collection (MPCC) (Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, QC, Canada). All isolates were collected from 29 herds in 2007 and 2008, and were obtained from four different areas of Canada (Atlantic provinces, Quebec, Ontario and Western Canada). Systematic sampling of quarter milk samples allowed lactational isolates to be collected from subclinical intramammary-quarter infections (qIMI). Isolates that could be cultured from the 3 successive 3-weekly quarter milk samplings (S1, S2, S3) during the lactation period and that shared the same VNTR type (variable number of tandem repeats for 5 loci, as evaluated by multiplex PCR, see subsequent description) were defined as persistent isolates (P-SUB, n = 139). Those defined as non-persistent lactational isolates (NP-SUB, n = 38) were present at the first sampling S1 but not at both S2 and S3. In addition, lactational isolates causing moderate to severe clinical mastitis were also collected (CLIN, n = 81) for comparison. More details on sampling procedures, herd and cow characteristics and the CBMRN isolate collection is provided in Reyher *et al*. (2011).

Dry cow-fresh cow isolates (DC-FC) were retrieved from milk of apparently normal milking quarters (i.e. subclinical qIMI) before dry-off and after calving. Isolates that could be retrieved at drying-off and freshening, that shared same VNTR type and that, therefore, apparently survived antibiotic therapy were defined as

persistent (DC-FC, n = 44). Non persistent dry cow isolates were collected at dry-off but were not re-isolated at calving (NP-DC, n = 64).

2.2. Preparation of Genomic DNA

Staphylococcus aureus isolates were grown in brain heart infusion (BHI) broth (BD, ON, Canada) overnight at 35˚C. The recommendations from the manufacturers of the Gene Elute kit (Sigma Aldrich Canada, Oakville, ON) were followed for chromosomal DNA extraction. The exception was the use of lysis buffer composed of 1X TE buffer, lysozyme (45 µg/ml) and lysostaphin (200 µg/ml) to achieve efficient lysis of *S. aureus* cells. DNA extracted was stored at -20˚C until used.

2.3. Validation of Persistence by VNTR Typing

The variable numbers of tandem repeat (VNTR) loci typing was described previously by Sabat *et al*. (2003). VNTR was used to validate the persistence of isolates. Persistence was validated if DC and FC isolates or lactational S1, S2 and S3 isolates had an identical VNTR profile (Fig. 1). Briefly, VNTR is based on analysis of five tandem repeat loci. Repetitive DNA from the V8 serine protease (*ssp*), protein A (*spa*), Ser-Asp-rich fibrinogen-binding protein (*sdrCDE*), clumping factor B (*clfB*) and clumping factor A (*clfA*) genes were amplified in a single multiplex PCR. Amplified products were separated by electrophoresis on 2% agarose gel, stained with ethidium bromide and then visualized by UV transilluminator and photographed. The sequences of primers for VNTR are listed in Table 1. For each validated series of persistent isolates, the first isolate of the series (DC or S1 isolates) was used for detailed characterization and comparison to the non-persistent isolates.

2.4. Detection of Genotypic Markers by PCR

All of the selected subclinical and clinical, persistent and non-persistent, *S. aureus* isolates (n = 367) were used to perform PCR analysis to detect the superantigen genes *seg*, *sen*, *sec*, *tst* as well as the positive control gene *femA* (Table 1). Oligonucleotide primers were synthesized by Sigma Aldrich Canada. One multiplex PCR and two simplex PCR were performed for the detection of toxin genes. The PCR reactions were performed in a final volume of 25 µl reaction mixture consisted of 2.5 µl of 10X PCR buffer (NEB, Ipswich, MA), 0.2 µM or 0.4 µM of each primer, 0.4 mM dNTP each, 3 mM $MgCl_2$ (NEB), 0.1 U *Taq* DNA polymerase (NEB) and 300 ng template DNA. All PCR reactions were performed using a GeneAmp PCR system 2700 (Life technologies, Burlington, ON). The cycling conditions were the following: an initial denaturation at 95°C for 4 min; 35 cycles of 95°C for 30 s, a specific temperature for 30 s (Table 1), 68°C for 30 s; and a final extension at 68°C for 10 min. One negative control and one positive control strain were included in each series of PCR amplifications. Amplified products were resolved by electrophoresis on 1.5% agarose gel, stained with ethidium bromide (0.5 µg/ml) and visualized by an UV transilluminator.

2.5. Biofilm Production

Isolates were grown on BHI agar supplemented with 0.25% glucose and incubated at 35˚C for 18-24 h. The culture was used to prepare a bacterial suspension in BHI 0.25% glucose broth (0.5 McFarland standard). A 50-µl volume of the bacterial suspension was added to each well of a flat-bottom polystyrene microtiter plate containing a half volume of the same medium as previously described (Mitchell *et al*., 2010). The plates were incubated at 35˚C for 24 h. After incubation, the wells

were washed three times with phosphate buffered saline (PBS), air dried and stained with a 0.1% crystal violet solution for 30 min. The plates were washed and the adherent biofilm was then dissolved in ethanol. The absorbance was measured at 560 nm by spectrophotometry (Bio-Tek Instruments) and normalized according to the biofilm production measured for the reference *S. aureus* strain Newbould (ATCC 29740). Each experiment was repeated three times (each having four technical replicates).

2.6. Quantitative Expression of *hld* (qPCR)

Bacterial cultures grown overnight were used to inoculate BHI broth to an optical density (OD) of 0.1 at 600 nm. The bacteria were then grown at 35°C until they reached 0.4 OD and treated with RNAprotect (QIAGEN, Mississauga, ON). RNA was extracted from the pellets after bacterial lysis with lysostaphin (200 µg/ml) for 1 h at 35˚C using the RNeasy Mini kit and the RNase-free DNase set (QIAGEN). One microgram of total RNA was reverse transcribed with 0.5 mM dNTP, 50 ng of random hexamers and 200 U of Invitrogen Superscript II reverse transcriptase according to the manufacturer's recommendations (Life Technologies). RNA was hydrolyzed and the cDNAs were purified with the QIAquick PCR purification kit (QIAGEN). One microlitre of the cDNA preparation was amplified on the Stratagene MX3000P Real-Time PCR instrument with the Jump Start *Taq* DNA polymerase (Sigma Aldrich Canada), SYBR Green and 10 µM of the primers (Table 1). Reaction mixtures were denatured for 10 min at 95°C, followed by 35 cycles of 30 s at 95°C, 1 min at 60°C and 1 min 30 s at 72°C. Dissociation and standard curves were obtained to insure the specificity and the efficiency of reactions. Relative expression was calculated by using the cycle threshold (Ct) of *gyrB* as the calibrator gene for each

isolate. RNAIII (*hld* gene) expression (ΔCt) was calculated by using the difference between the (Ct) of RNAIII and (Ct) of *gyrB* for each isolate. We used *gyrB* as a calibrator because *gyrB* expression did not vary significantly in the exponential growth phase. The fold expression of the *hld* gene from each experiment was then normalized with the general value of Newbould (ΔCt) expression (fold expression = $2^{-\Delta\Delta Ct}$).

2.7. Spa typing

The polymorphic X region of the protein A gene (*spa*) was amplified using the primers spa-1113f and spa-1514r (Table 1), as described by Aires de Sousa *et al*. (2006). The PCR was performed in a total volume of 20 µl containing 100 ng of DNA, 0.2 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), 10 pmol of each primer, 5 µl of 10-fold concentrated PCR Buffer II (Life Technologies), 1.5 mM $MgCl_2$, and 1.25 U of Ampli*Taq* DNA polymerase (Life Technologies). Thermal cycling reactions consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30s, annealing at 63°C for 30 s, and extension at 72°C for 45 s, with a single 7 min final extension. Five microliters of the PCR reaction were used to check the amplicons on a 0.8% agarose gel. Another 2.5-ul volume of the PCR reaction was diluted with the same volume of nanopure water and sent for sequencing at the *Plate-forme d'Analyses Biomoléculaires de l'Université Laval* (Université Laval, QC, Canada). Samples were sequenced using the same primers described before. The ab1 files from sequencing were submitted to BioNumerics software v5.1 (Applied Maths) and using the *spa* typing plug-in, all *spa* sequences were compared against the *spa* database server (http://www.spaserver.ridom.de/). One specific *spa* type was assigned to each isolate.

2.8. Statistical Analysis

Logistic regression models were developed to predict persistence of isolates according to each genotypic markers *sen*, *sec*, *seg*, and *tst*, biofilm production and *hld* expression during lactation, over the dry-period, and between clinical and chronic lactation samples respectively. The models included province of sampling as a fixed effect covariate and herd as a random effect. Pairwise differences between provinces were realized with Tukey's multiple comparisons test. Multiple comparisons were also realized between the different isolates for mean biofilm production and *hld* expression from a linear mixed model including herd as a random effect and province as a fixed effect. The dependent variable was log-transformed in these models due to their skewed distribution. Genetic diversity within each isolate groups (CLIN, P-SUB, NP-SUB, DC-FC, NP-DC) was determined with Simpson's index (Simpson, 1949). Bootstrap significance between each diversity indices was computed using 2000 boostrap resamples. All statistics were realized with R version 3.0.2 (R Core Team, 2012).

3. RESULTS

3.1.Validation of Persistence for Lactational and Dry-off Isolates by VNTR Typing

In this study, isolates were considered as "persistent" if the isolates from all successive samplings showed the same VNTR profile on agarose gel (Fig. 1). Among a collection of 153 putative cases of persistent lactational isolates (3 isolates per case), 139 cases were validated as true persistent by VNTR typing (91% of the original set of isolates showing presence of *S. aureus* in S1, S2 and S3 samples). The non-validated strains were excluded from the study and the validated 139 isolates from S1 were the subclinical persistent strains retained for the study. These 139 validated persistent isolates were compared to 38 non-persistent isolates (*S. aureus* only present in S1).

From an initial collection of 53 DC-FC isolates (*i.e*. isolates present before and after dry-off), 44 (83%) *S. aureus* were validated as true persistent cases by VNTR typing. The non-validated strains were excluded from the study and the true 44 DC-FC isolates were compared to 64 non-persistent isolates (NP-DC) only found at dry-off.

There was no difference in the distribution of the different groups of strains (NP-DC, DC-FC, NP-SUB, P-SUB and CLIN) across the four geographic regions ($P = 0.222$). Statistical analysis revealed that persistent P-SUB strains were isolated more frequently in cows which had more days in milk during the lactation period ($P < 0.001$). In addition, cows with persistent isolates presented higher somatic cell counts (SCC) in milk ($P < 0.001$).

3.2. Detection of Genotypic Markers

The distribution of the four superantigen-encoding genes investigated among the different groups of isolates is reported in Table 2. The *sen* and *seg* genes were the most frequently detected. The majority of NP-SUB isolates showed both *sen* and *seg* genes (65.8%). In contrast, P-SUB isolates that did not possess any of the genes *sen* and *seg* or that possessed only one of either gene were predominant (59%) in that group of isolates (Table 2). As for P-SUB lactational isolates, there were more persistent DC-FC isolates negative for or carrying only one of the *sen* or *seg* gene (63.6%) in comparison to NP-DC isolates (53.1%). Overall, statistical analysis for subclinical isolates recovered during lactation revealed that the presence of gene *seg* is associated with a reduction in the possibility that the bacteria persist by 66% (OR = 0.34, 95%CI: 0.14-0.84, $P = 0.019$). This suggests that the presence of *seg* may be associated with non-persistent isolates found in subclinical infections during lactation. The same reduction in odds of being persistent was present during the dry-off period for the gene *sen* (OR = 0.39, 95%CI: 0.16-0.97, $P = 0.044$).

Interestingly, for isolates causing clinical mastitis during lactation, *seg* (42.0%) was the most frequent gene detected but only 18.5% of clinical isolates had both *sen* and *seg* toxin genes, whereas 81.5% carried none or only one of *sen* or *seg* genes (Table 2). Genes *seg* and *sen* were also associated with a reduction in the odds for bacteria to be in a clinical mastitis case (OR = 0.54, 95%CI: 0.30-0.96, $P = 0.036$; and OR = 0.19, 95%CI: 0.10-0.37, $P < 0.001$ respectively). On the other hand, gene *tst* was associated with increase odds for isolates to be in a clinical case (OR = 3.24, 95%CI: 1.48-7.13, $P = 0.003$).

3.3. Biofilm Production

The ability to form biofilm was investigated for each isolate group (Fig. 2). Data show that persistent DC-FC isolates produced significantly more biofilm than strains that do not persist after calving (NP-DC) or isolates that caused moderate to severe clinical mastitis during lactation (CLIN). Isolates which caused persistent or non-persistent subclinical mastitis (P-SUB or NP-SUB) also produced significantly more biofilm than clinical strains (CLIN). The odds of being persistent according to biofilm production were however not significant for the lactation period or the dry-off period (OR = 1.33, 95%CI: 0.73-2.42, P = 0.349; and OR = 1.44, 95%CI: 0.95-2.19, P = 0.086 respectively). However, isolates producing more biofilm were more likely to be subclinical by 83% (OR = 0.17, 95% CI: 0.08-0.36, P <0.001).

3.4. *hld* Expression

The expression level of *hld* was also compared among the different groups of isolates (Fig. 3). No statistical differences were found between any pairwise comparisons. Interestingly however, isolates expressing more *hld* were more likely to be non-persistent during either lactation or the dry period (OR: 0.93, 95%CI = 0.87-0.99, P = 0.027; and OR = 0.85, 95%CI: 0.75-0.96, P = 0.007 respectively).

3.5. Spa Typing

A total of 18 different *spa* types were identified from a random selection of 195 isolates that included 45 clinical isolates and 150 subclinical isolates (78 and 72 from the lactation and dry-off period, respectively). All had 2 to 13 repeats and the numeric code of the *spa* types were assigned based on the repeat succession. Sixteen isolates however revealed a novel *spa* type, which is now registered as t13401 after

submission to the Ridom Spa database server. The distribution of these 18 *spa* types among the isolates is reported in Table 3. The *spa* type t529 (n = 93) was the most common and was present in all groups of isolates although it was less frequent in DC-FC isolates. The second and third predominant *spa* types were t267 (n = 38) and the novel type t13401 (n = 16), respectively. Figures 4 and 5 show some properties (biofilm production and *hld* expression, respectively) of the most frequent *spa* types ($n \geq 5$). Interestingly, t529 isolates produced significantly less biofilm than other *spa* types such as t605, t1166 or the t13401 isolates (Fig. 4, $P < 0.025$). Also, the novel t13401 *spa* type isolates (only 1 found in the CLIN group, Table 3) produced more biofilm than types t267, t359 and t529 ($P < 0.001$) (Fig. 4). Inversely, the t529 isolates expressed higher *hld* levels than types t267, t605, t3380 or t13401 (Fig. 5, $P < 0.001$). Type t529 thus produced less biofilm and had elevated *hld* expression, albeit the range of *hld* levels was quite large for this *spa* type.

Table 4 shows the distribution of the enterotoxin genes studied among isolates of the three major *spa* types. Noteworthy, all superantigen genes were most frequent in t529 and 75.3% of t529 isolates were positive for both *sen* and *seg* genes. Table 5 reports the distribution of genes among t529 and across the different groups of strains. Strikingly, most of the t529 isolates which were positive for both *sen* and *seg* genes were distributed in all the subclinical isolates (P-SUB, NP-SUB, DC-FC and NP-DC) in contrast to those seen in CLIN isolates (only 22.2% of *sen*+/*seg*+). The CLIN group also differed from the other groups by their higher frequency of the *tst* gene (Table 5).

Simpson diversity index represents 1 – probability that any two species drawn from the isolate population are the same, and ranges from zero (100% probability) to one (0% probability). Genetic diversity increased from 0.59 (clinical isolates) to 0.68

(P-SUB), 0.73 (P-SUB and NP-DC), and 0.79 (DC-FC). The only statistical difference between Simpson's indices was between DC-FC and CLIN isolates (P = 0.009).

4. DISCUSSION

Bovine mastitis is the most frequent and costly disease of dairy cows (Bar *et al*., 2008; Reyher *et al*., 2011). Systematic screening of quarter milk samples in the CBMRN National Cohort of Dairy Farms (NCDF) over a two-year period revealed that *S. aureus* was present in 2.4% of apparently normal mammary quarters (qIMI) during lactation, and in 3% at dry-off (Reyher *et al*., 2011). These subclinical qIMI decrease milk quality and are a reservoir for transmission within the herd. As expected we can confirm that cows with subclinical and persistent qIMI presented higher somatic cells counts in milk ($P < 0.001$). The principal aim of this study was therefore to compare persistent and non-persistent *S. aureus* isolates from such subclinical qIMI in the hope of identifying bacterial characteristics associated with persistence. Since *S. aureus* was also found to be the predominant pathogen in clinical mastitis cases (Reyher *et al*., 2011), we also compare the properties of the subclinical isolates to those of the clinical isolates and examined any relationship or difference.

Visual expression of pathogen infection (i.e., subclinical vs. clinical disease) does not indicate qIMI persistence, as both types of infections may be either persistent or non-persistent. Persistence is a very important element of *S. aureus* epidemiology and our study was focused on identifying predictors of persistence by comparison with cases that were not persistent and that were eliminated. One difficulty in such an investigation is the precision in the definition of cases and whether or not the studied isolates can belong to one study group or another. *S. aureus* strains may be capable of causing subclinical or clinical manifestations at one point or another. To avoid as much as possible inclusion of transitory strains, our

selection criteria for lactational qIMI persistence demanded the presence of the same strain three consecutive times in the same quarter milk samples over a period of six weeks. We feel that this avoided inclusion of subclinical strains that could have been on a path to clinical qIMI while at the same time confirming qIMI persistence. Also included as a comparison in the study were *S. aureus* strains from clinical qIMI. The inclusion of the clinical strains in the study is of interest because such isolates might reveal characteristics linked to their ability to accentuate an inflammatory response from the host (clinical mastitis).

We feel that optimal data comparisons can be carried out between NP-SUB and P-SUB isolates (non-persistent and persistent lactational qIMI isolates, respectively) and between NP-DC and DC-FC isolates (non-persistent and persistent qIMI isolates recovered during the dry period, respectively). Comparing lactational isolates to dry-cow isolates is also tempting because one can anticipate that persistent isolates collected during lactation can be the same as those that persist through the dry period and vice versa despite the changing physiology of the mammary gland. It should be noted that all dry period isolates were submitted to antibiotherapy, which implies that dry-cow isolates that persisted through freshening were under an additional selective pressure. Note however that from antibiotic susceptibility testing of many isolates, equivalent susceptibility levels were found between DC-FC and NP-DC strains and it is unlikely that *in vitro* antibiotic resistance *per se* is involved in such persistence (unpublished data). A poor correlation between *in vitro* antibiotic susceptibility and treatment outcome was indeed previously reported (Apparao *et al*., 2009). On the other hand, it has been previously reported that lower cure rates may be more related to the bovine host adaptation of *S. aureus* strains rather than to antibiotic resistance (van den Borne *et al*., 2010).

This study thus used "validated" persistent and non-persistent isolates collected from cows with subclinical qIMI during the lactation and dry period. These isolates were therefore investigated for the presence of superantigen genes (*sen*, *sec*, *seg* and *tst*), biofilm production, *hld* expression level and *spa* type, in order to obtain valuable information on genotypic and phenotypic patterns that could be used to predict *S. aureus* persistence. Some specific genetic and phenotypic observations on isolates obtained from bovine mastitis have previously been reported by studies from different geographic regions such as the USA, Europe, Argentina, Brazil and India (Buzzola *et al*., 2001; Klein *et al*., 2012; Larsen *et al*., 2002; Mitra *et al*., 2013) and only few studies have investigated *S. aureus* isolated from persistent bovine mastitis (Anderson and Lyman, 2006; Günaydın *et al*., 2011; Haveri *et al*., 2008, 2005; Matsunaga *et al*., 1993; Oliveira *et al*., 2011a; Ote *et al*., 2011). These studies used a variety of methods of analysis, different groups and types of isolates, various definitions of subclinical cases, and various definitions or validation of persistence (*e.g*., clonal persistence within a herd *vs*. qIMI persistence). This variation in methods makes it very difficult to carry out comparisons across studies and between different types of infections.

In the present study, the presence of the superantigen toxin gene combination *seg*+/*sen*+ was more frequent in non-persistent NP-SUB and NP-DC isolates compared to that found in their respective persistent counterparts (P-SUB and DC-FC, respectively). Similarly to subclinical persistent strains, clinical isolates also showed a low prevalence of the *sen*+/*seg*+ gene combination (18.5%). Such coexistence of *seg* and *sen* in the same isolates has been reported (Piccinini *et al*., 2012). They attributed this phenomenon to their being in the same enterotoxin gene cluster (*seg*, *sei*, *sem*, *sen*, *seo*, and *seu*), located on the genomic island vSaß-1

(Lindsay and Holden, 2006; Malachowa and DeLeo, 2010). In addition, *sec* and *tst* genes or their combined presence suggested that some of the strains contained the pathogenicity island SaPIbov (Fitzgerald *et al*., 2001). Thus, the superantigen genes tested in our study (*seg*, *sen*, *sec* and *tst*) were most likely located on two pathogenicity islands (SaPIbov and νSaβ-1), which could appear either alone or together in the same isolate.

The presence of the *seg* gene has frequently been reported in *S. aureus* strains from bovine mastitis (Zschöck *et al*., 2005). In the present study, the presence of gene *seg* was associated with a reduction in the possibility that a bacteria will be persistent for subclinical isolates collected during lactation (NP-SUB *vs*. P-SUB). Similarly, the presence of gene *sen* was associated with a reduction in the odds of persistence in isolates collected during the dry-off period (NP-DC *vs*. DC-FC). Consequently, we observed that the majority of persistent isolates either mostly carried only one of the *sen* or *seg* genes or neither of them, indicating the absence of the genomic island vSaß-1. Genes *seg* and *sen* were also associated with a reduction in the odds of being a clinical mastitis case. On the other hand, gene *tst* was associated with increase odds of being a clinical case. Overall, it seems that genes *seg* and *sen* are associated with a subclinical manifestation of mastitis but not with persistence. Among other studies on persistent bovine mastitis, considerable variability has been reported in the prevalence of superantigen genotypes between farms and countries (Günaydın *et al*., 2011; Haveri *et al*., 2008; Piccinini *et al*., 2012; Wolf *et al*., 2011).

The ability of *S. aureus* to form biofilm helps the bacterium to survive hostile environments within the host, and is considered to be of major importance for chronic or persistent infections (Costerton *et al*., 1999). Biofilm has been proposed to

facilitate the adherence and colonization of staphylococci on the mammary gland epithelium, to help evasion from the immunological defenses, and to increase resistance to antimicrobial agents (Cucarella *et al*., 2004; Dubravka *et al*., 2010; Melchior *et al*., 2006). Biofilms can also release planktonic bacterial cells from their outer layers allowing recurrence of infections (Melchior *et al*., 2006). The present study showed that persistent DC-FC isolates from the dry period produced significantly more biofilm than isolates that do not persist in fresh cows (NP-DC). It is possible that antibiotic treatment removes *S. aureus* strains with low biofilm production during dry-off. Also, both dry-off and lactational isolates produced significantly more biofilm than isolates collected from cows with clinical mastitis during lactation (CLIN). The biofilm-forming ability of staphylococci isolated from bovine subclinical mastitis was also evaluated by Oliveira *et al*. (2006) and Dhanawade *et al*. (2010) and their findings are in accordance with ours. We found however that the odds of being persistent according to biofilm production were not significant for the lactation period or the dry-off period. On the other hand, isolates producing more biofilm were more likely to be subclinical by 83%. A positive correlation between biofilm production and the ability of strains to internalize in epithelial cells has also been previously observed and suggests their greater ability to establish chronic intramammary infections (Hensen *et al*., 2000; Oliveira *et al*., 2011b). Strains that show a strong biofilm production and adherence capacity are also the most likely to avoid removal by milk flow (Hensen *et al*., 2000).

In *S. aureus*, the *agr* system encodes a two-component quorum-sensing system that modulates production of a regulatory RNA molecule, RNAIII, which is the effector molecule of the *agr* system (Novick and Geisinger, 2008). The expression of many toxins and hydrolytic enzymes is under the control of this

RNAIII. Production of δ-toxin (Hld), which is encoded within the RNAIII locus, has been negatively correlated with biofilm production (Beenken *et al*., 2004; Novick and Geisinger, 2008) and it was used in this study as a surrogate to determine *agr* expression. A low expression of RNAIII would indicate a reduced production of exotoxins and a concomitant increase in the production of surface proteins (e.g., MSCRAMMs) which help colonization (Novick, 2003; Novick and Geisinger, 2008). Gilot *et al*. (2002) demonstrated that inhibition of RNAIII synthesis leads to a diminution of the virulence phenotype in *S. aureus*. In addition and as expected, Wesson *et al*. (1998) showed that a strain mutated in *agr* was more efficiently internalized by cultured bovine mammary epithelial cells than the wild-type strain. The *agr*-minus strain also showed increased adherence and biofilm formation and these properties were considered important for the development of chronic infections (Wesson *et al*., 1998). Accordingly, we showed here that isolates expressing more *hld* were more likely to be non-persistent during either lactation or the dry period.

The *spa* gene mediates host-pathogen interactions and the sequence repeats of its polymorphic region can be used for typing *S. aureus* isolates (Said *et al*., 2010). The *spa* gene has been indeed one of the most successful single genetic markers used for the typing of *S. aureus* strains (Aires-de-Sousa *et al*., 2006). In the present study, we used *spa* typing to investigate the possible associations between high biofilm production, low *hld* expression and/or persistence. The analysis of 195 *S. aureus* isolates by *spa* typing revealed 18 different *spa* types, of which t529 and t267 were found in the majority of isolates. Although statistical differences in term of genetic diversity could not be demonstrated, it is noteworthy to mentioned that genetic diversity appeared to increase with level of persistence (i.e. from clinical to non-persistent lactational, then non-persistent at drying-off, persistent during the

lactation, and, finally, to persistent over the dry-off period). These differences should, however, be further explored and confirmed in a future study. Nevertheless, this observation suggests that persistency is achieved through the development of various mechanisms leading to a more diverse isolates population rather than through a common shared specialization mechanism among the isolates.

Type t529 isolates were frequently positive for both *sen* and *seg* (75.5%), and compared to t267, t529 isolates activated significantly more *agr* (*i.e*., increased *hld* expression). It is noteworthy that only 22.2% of the CLIN isolates of the *spa* type t529 showed the *sen*+/*seg*+ gene combination. The t529 CLIN group also differed from the other groups by the higher frequency of the *tst* gene. A similar study has reported a high prevalence of t267 in subclinical mastitis isolates from India (Mitra *et al*., 2013). Our results are in general agreement with Said *et al*. (2010) who have observed a predominance of *spa* type t529 and t267 in Canada's clinical bovine mastitis isolates. It should be noted that we also revealed the presence of a significant number of isolates with a novel *spa* type in Canada (t13401). This novel *spa* type was poorly represented in clinical isolates and these isolates produced significantly more biofilm and showed lower *agr* activation than that seen with the t529 isolates. This novel *spa* type might have been overlooked in other studies since it is not frequent in symptomatic mastitis cases. More work is needed to characterize this group of isolates.

S. aureus qIMI strains that persist during lactation and those strains that persist from dry-off to freshening are especially problematic because they may not be readily detected in daily work routines and they provide a reservoir of infective bacteria within herds. The present study significantly expanded our current knowledge of the genotypic and phenotypic traits of *S. aureus* isolates associated

with persistence. Identification of efficient markers that distinguished between those *S. aureus* more likely to persist and those less likely to persist will help udder health management decision-making will require additional analyses and studies.

5. ACKNOWLEDGEMENTS

This research was supported by a contribution from the Dairy Research Cluster initiative [Dairy Farmers of Canada (Ottawa, ON, Canada), Agriculture and Agri-Food Canada (Ottawa, ON, Canada), and the Canadian Dairy Commission (Ottawa, ON, Canada)] through the Canadian Bovine Mastitis and Milk Quality Research Network (St-Hyacinthe, QC, Canada). The Canadian Bovine Mastitis and Milk Quality Research Network is supported by the Natural Sciences and Engineering Research Council (NSERC) of Canada, Alberta Milk, Dairy Farmers of New Brunswick, Nova Scotia, Ontario and Prince Edward Island, Novalait Inc., Dairy Farmers of Canada, Canadian Dairy Network, AAFC, PHAC, Technology PEI Inc., Université de Montréal, and University of Prince Edward Island. This work was also supported by Discovery grant 89758-2010 from NSERC to FM. RCK was supported by a Ph.D. scholarship from CAPES-Brazil.

6. REFERENCES

Aarestrup, F.M., H.D. Larsen, N.H. Eriksen, C.S. Elsberg, and N.E. Jensen. 1999. Frequency of alpha- and beta-haemolysin in *Staphylococcus aureus* of bovine and human origin. A comparison between pheno- and genotype and variation in phenotypic expression. *APMIS*. 107:425–430.

Aires-de-Sousa, M., K. Boye, H. de Lencastre, A. Deplano, M.C. Enright, J. Etienne, A. Friedrich, D. Harmsen, A. Holmes, X.W. Huijsdens, A.M. Kearns, A. Mellmann, H. Meugnier, J.K. Rasheed, E. Spalburg, B. Strommenger, M.J. Struelens, F.C. Tenover, J. Thomas, U. Vogel, H. Westh, J. Xu, and W. Witte. 2006. High interlaboratory reproducibility of DNA sequence-based typing of bacteria in a multicenter study. *J. Clin. Microbiol.* 44:619–621.

Akineden O, C. Annemüller, A.A. Hassan, C. Lämmler, W. Wolter, and M. Zschöck. 2001. Toxin genes and other characteristics of *Staphylococcus aureus* isolates from milk of cows with mastitis. *Clin. Diagn. Lab. Immunol.* 8:959–964.

Anderson, K.L., and R.L. Lyman. 2006. Long-term persistence of specific genetic types of mastitis-causing *Staphylococcus aureus* on three dairies. *J. Dairy Sci.* 89:4551–4556.

Apparao, M.D., P.L. Ruegg, A. Lago, S. Godden, R. Bey, and K. Leslie. 2009. Relationship between in vitro susceptibility test results and treatment outcomes for gram-positive mastitis pathogens following treatment with cephapirin sodium. *J. Dairy Sci*. 92:2589-97.

Awale, M.M, G.B. Dudhatra, K. Avinash, B.N. Chauhan, and D.R. Kamani. 2012. Bovine mastitis: a threat to economy. *Scientific reports* 1:295. doi:10.4172/.295

Bar, D., L.W. Tauer, G. Bennett, R.N. González, J.A. Hertl, Y.H. Schukken, H.F. Schulte, F.L. Welcome, and Y.T. Gröhn. 2008. The cost of generic clinical mastitis in dairy cows as estimated by using dynamic programming. *J. Dairy Sci.* 91:2205–2214.

Beenken, K.E., P.M. Dunman, F. McAleese, D. Macapagal, E. Murphy, S.J. Projan, J.S. Blevins, and M.S. Smeltzer. 2004. Global gene expression in *Staphylococcus aureus* biofilms. *J. Bacteriol.* 186:4665–4684.

Van den Borne, B.H.P., M. Nielen, G. van Schaik, M.B. Melchior, T.J.G.M. Lam, and R.N. Zadoks. 2010. Host adaptation of bovine *Staphylococcus aureus* seems associated with bacteriological cure after lactational antimicrobial treatment. *J. Dairy Sci.* 93:2550–2558.

Buzzola, F.R., L. Quelle, M.I. Gomez, M. Catalano, L. Steele-Moore, D. Berg, E. Gentilini, G. Denamiel, and D.O. Sordelli. 2001. Genotypic analysis of *Staphylococcus aureus* from milk of dairy cows with mastitis in Argentina. *Epidemiol. Infect.* 126:445–452.

Costerton, J.W., P.S. Stewart, and E.P. Greenberg. 1999. Bacterial biofilms: a common cause of persistent infections. *Science*. 284:1318–1322.

Cucarella, C., M.A. Tormo, C. Ubeda, M.P. Trotonda, M. Monzón, C. Peris, B. Amorena, I. Lasa, and J.R. Penadés. 2004. Role of biofilm-associated protein

bap in the pathogenesis of bovine *Staphylococcus aureus*. *Infect. Immun.* 72:2177–2185.

Davies, D. 2003. Understanding biofilm resistance to antibacterial agents. *Nat. Rev. Drug Discov.* 2:114–22.

Dhanawade, N.B., D.R. Kalorey, R. Srinivasan, S.B. Barbuddhe, and N. V Kurkure. 2010. Detection of intercellular adhesion genes and biofilm production in *Staphylococcus aureus* isolated from bovine subclinical mastitis. *Vet. Res. Commun.* 34:81–89.

Dinges, M.M., P.M. Orwin, and P.M. Schlievert. 2000. Exotoxins of *Staphylococcus aureus*. *Clin. Microbiol. Rev.* 13:16–34.

Dubravka, M., S. Lazic, B. Vidic, J. Petrovic, D. Bugarski, and Z. Seguljev. 2010. Slime production and biofilm forming ability by *Staphylococcus aureus* bovine mastitis isolates. *Acta Vet. Brno.* 60:217–226.

Fitzgerald, J.R., P.J. Hartigan, W.J. Meaney, and C.J. Smyth. 2000. Molecular population and virulence factor analysis of *Staphylococcus aureus* from bovine intramammary infection. *J. Appl. Microbiol.* 88:1028–1037.

Fitzgerald, J.R., S.R. Monday, T.J. Foster, G.A. Bohach, P.J. Hartigan, W.J. Meaney, and C.J. Smyth. 2001. Characterization of a putative pathogenicity island from bovine *Staphylococcus aureus* encoding multiple superantigens. *J. Bacteriol.* 183:63–70.

Gilot, P., G. Lina, T. Cochard, and B. Poutrel. 2002. Analysis of the genetic variability of genes encoding the RNA III-activating components Agr and

TRAP in a population of *Staphylococcus aureus* strains isolated from cows with mastitis. *J. Clin. Microbiol.* 40:4060–4067.

Günaydın, B., Ö. Aslantaş, and C. Demir. 2011. Detection of superantigenic toxin genes in *Staphylococcus aureus* strains from subclinical bovine mastitis. *Trop. Anim. Health Prod.* 43:1633–1637.

Haveri, M., M. Hovinen, A. Roslöf, and S. Pyörälä. 2008. Molecular types and genetic profiles of *Staphylococcus aureus* strains isolated from bovine intramammary infections and extramammary sites. *J. Clin. Microbiol.* 46:3728–3735.

Haveri, M., S. Taponen, J. Vuopio-Varkila, S. Salmenlinna, and S. Pyörälä. 2005. Bacterial genotype affects the manifestation and persistence of bovine *Staphylococcus aureus* intramammary infection. *J. Clin. Microbiol.* 43:959–961.

Hensen, S.M., M.J. Pavicić, J.A. Lohuis, and B. Poutrel. 2000. Use of bovine primary mammary epithelial cells for the comparison of adherence and invasion ability of *Staphylococcus aureus* strains. *J. Dairy Sci.* 83:418–429.

Jarraud, S., C. Mougel, J. Thioulouse, G. Lina, H. Meugnier, F. Forey, X. Nesme, J. Etienne, and F. Vandenesch. 2002. Relationships between *Staphylococcus aureus* genetic background, virulence factors, agr groups (alleles), and human disease. *Infect. Immun.* 70:631–641.

Klein, R.C., M.H. Fabres-Klein, M.A.V.P. Brito, L.G. Fietto, and A. de O.B. Ribon. 2012. *Staphylococcus aureus* of bovine origin: genetic diversity, prevalence and the expression of adhesin-encoding genes. *Vet. Microbiol.* 160:183–188.

Larsen, H.D., F.M. Aarestrup, and N.E. Jensen. 2002. Geographical variation in the presence of genes encoding superantigenic exotoxins and beta-hemolysin among *Staphylococcus aureus* isolated from bovine mastitis in Europe and USA. *Vet. Microbiol.* 85:61–67.

Lindsay, J.A., and M.T.G. Holden. 2006. Understanding the rise of the superbug: investigation of the evolution and genomic variation of *Staphylococcus aureus*. *Funct. Integr. Genomics*. 6:186–201.

Malachowa, N., and F.R. DeLeo. 2010. Mobile genetic elements of *Staphylococcus aureus*. *Cell. Mol. Life Sci.* 67:3057–3071.

Matsunaga, T., S. Kamata, N. Kakiichi, and K. Uchida. 1993. Characteristics of *Staphylococcus aureus* isolated from peracute, acute and chronic bovine mastitis. *J. Vet. Med. Sci.* 55:297–300.

Melchior, M.B., J. Fink-Gremmels, and W. Gaastra. 2007. Extended antimicrobial susceptibility assay for *Staphylococcus aureus* isolates from bovine mastitis growing in biofilms. *Vet. Microbiol.* 125:141–149.

Melchior, M.B., H. Vaarkamp, and J. Fink-Gremmels. 2006. Biofilms: a role in recurrent mastitis infections? *Vet. J.* 171:398–407.

Mitchell, G., C.-A. Lamontagne, E. Brouillette, G. Grondin, B.G. Talbot, M. Grandbois, and F. Malouin. 2008. *Staphylococcus aureus* SigB activity

promotes a strong fibronectin-bacterium interaction which may sustain host tissue colonization by small-colony variants isolated from cystic fibrosis patients. *Mol. Microbiol.* 70:1540–1555.

Mitchell, G., D.L. Séguin, A.-E. Asselin, E. Déziel, A.M. Cantin, E.H. Frost, S. Michaud, and F. Malouin. 2010. *Staphylococcus aureus* sigma B-dependent emergence of small-colony variants and biofilm production following exposure to *Pseudomonas aeruginosa* 4-hydroxy-2-heptylquinoline-N-oxide. *BMC Microbiol.* 10:33-47.

Mitra, S.D., D. Velu, M. Bhuvana, N. Krithiga, A. Banerjee, R. Shome, H. Rahman, S.K. Ghosh, and B.R. Shome. 2013. *Staphylococcus aureus spa* type t267, clonal ancestor of bovine subclinical mastitis in India. *J. Appl. Microbiol.* 114:1604–1615.

Moisan, H., E. Brouillette, C.L. Jacob, P. Langlois-Bégin, S. Michaud, and F. Malouin. 2006. Transcription of virulence factors in *Staphylococcus aureus* small-colony variants isolated from cystic fibrosis patients is influenced by SigB. *J. Bacteriol.* 188:64–76.

Novick, R.P. 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol. Microbiol.* 48:1429–1449.

Novick, R.P., and E. Geisinger. 2008. Quorum sensing in staphylococci. *Annu. Rev. Genet.* 42:541–564.

Oliveira, L., A.C. Rodrigues, C. Hulland, and P.L. Ruegg. 2011a. Enterotoxin production, enterotoxin gene distribution, and genetic diversity of

Staphylococcus aureus recovered from milk of cows with subclinical mastitis. *Am. J. Vet. Res.* 72:1361–1368.

Oliveira, M., R. Bexiga, S.F. Nunes, C. Carneiro, L.M. Cavaco, F. Bernardo, and C.L. Vilela. 2006. Biofilm-forming ability profiling of *Staphylococcus aureus* and *Staphylococcus epidermidis* mastitis isolates. *Vet. Microbiol.* 118:133–140.

Oliveira, M., R. Bexiga, S.F. Nunes, and C.L. Vilela. 2011b. Invasive potential of biofilm-forming Staphylococci bovine subclinical mastitis isolates. *J. Vet. Sci.* 12:95–97.

Ote, I., B. Taminiau, J.-N. Duprez, I. Dizier, and J.G. Mainil. 2011. Genotypic characterization by polymerase chain reaction of *Staphylococcus aureus* isolates associated with bovine mastitis. *Vet. Microbiol.* 153:285–292.

Otto, M. 2013. Staphylococcal infections: mechanisms of biofilm maturation and detachment as critical determinants of pathogenicity. *Annu. Rev. Med.* 64:175–188.

Piccinini, R., R. Tassi, V. Daprà, R. Pilla, J. Fenner, B. Carter, and M.F. Anjum. 2012. Study of *Staphylococcus aureus* collected at slaughter from dairy cows with chronic mastitis. *J. Dairy Res.* 79:249–255.

R Core Team. 2012. R: a language and environment for statistical computing. R Foundation for statistical Computing, Vienna, Austria. ISBN 3-900051-07-0.

Reyher, K.K., S. Dufour, H.W. Barkema, L. Des Côteaux, T.J. Devries, I.R. Dohoo, G.P. Keefe, J.-P. Roy, and D.T. Scholl. 2011. The National Cohort of Dairy

Farms--a data collection platform for mastitis research in Canada. *J. Dairy Sci.* 94:1616–1626.

Sabat, A., J. Krzyszton-russjan, W. Strzalka, R. Filipek, K. Kosowska, W. Hryniewicz, J. Travis, and J. Potempa. 2003. New method for typing *Staphylococcus aureus* Strains: multiple-locus Variable-Number Tandem Repeat analysis of polymorphism and genetic relationships of clinical isolates. *J. Clin. Microbiol.* 41:1801–1804.

Said, K.B., J. Ismail, J. Campbell, M.R. Mulvey, A.-M. Bourgault, S. Messier, and X. Zhao. 2010. Regional profiling for determination of genotype diversity of mastitis-specific *Staphylococcus aureus* lineage in Canada by use of clumping factor A, pulsed-field gel electrophoresis, and spa typing. *J. Clin. Microbiol.* 48:375–386.

Simpson, E.H. 1949. Measurement of diversity. *Nature* 163:688.

Smith, T.H., L.K. Fox, and J.R. Middleton. 1998. Outbreak of mastitis caused by one strain of *Staphylococcus aureus* in a closed dairy herd. *J. Am. Vet. Med. Assoc.* 212:553–556.

Sol, J., O.C. Sampimon, H.W. Barkema, and Y.H. Schukken. 2000. Factors associated with cure after therapy of clinical mastitis caused by *Staphylococcus aureus*. *J. Dairy Sci.* 83:278–284.

Sutra, L., and B. Poutrel. 1994. Virulence factors involved in the pathogenesis of bovine intramammary infections due to *Staphylococcus aureus*. *J. Med. Microbiol.* 40:79–89.

Wesson, C.A., L.E. Liou, K.M. Todd, G.A. Bohach, W.R. Trumble, and K.W. Bayles. 1998. *Staphylococcus aureus* Agr and Sar global regulators influence internalization and induction of apoptosis. *Infect. Immun.* 66:5238–5243.

Wolf, C., H. Kusch, S. Monecke, D. Albrecht, S. Holtfreter, C. von Eiff, W. Petzl, P. Rainard, B.M. Bröker, and S. Engelmann. 2011. Genomic and proteomic characterization of *Staphylococcus aureus* mastitis isolates of bovine origin. *Proteomics*. 11:2491–2502.

Xie, Y., Y. He, A. Gehring, Y. Hu, Q. Li, S.-I. Tu, and X. Shi. 2011. Genotypes and toxin gene profiles of *Staphylococcus aureus* clinical isolates from China. *PLoS One*. 6:e28276.

Zadoks, R.N., W.B. van Leeuwen, D. Kreft, L.K. Fox, H.W. Barkema, Y.H. Schukken, and A. van Belkum. 2002. Comparison of *Staphylococcus aureus* isolates from bovine and human skin, milking equipment, and bovine milk by phage typing, pulsed-field gel electrophoresis, and binary typing. *J. Clin. Microbiol.* 40:3894–3902.

Zschöck, M., B. Kloppert, W. Wolter, H.P. Hamann, and C. Lämmler. 2005. Pattern of enterotoxin genes *seg*, *seh*, *sei* and *sej* positive *Staphylococcus aureus* isolated from bovine mastitis. *Vet. Microbiol.* 108:243–249.

Table 1: Target genes, primer sequences, expected sizes of amplicons, and PCR conditions.

Genes	Primer sequence	Product size (bp)	PCR (hybridization temperature)	Reference
seg	Fwd: AATTATGTGAATGCTCAACCCGATC Rev: AAACTTATATTGGAACAAAAGGTACTAGTTC	642	50˚C	Jarraud *et al*., 2002
sec	Fwd: GTAAAGTTACAGGTGGCAAAACTTG Rev: CATATCATACCAAAAAGTATTGCCGT	297	50˚C	Jarraud *et al*., 2002
tst	Fwd: TTCACTATTTGTAAAAGTGTCAGACCCACT Rev: TACTAATGAATTTTTTTATCGTAAGCCCTT	180	50˚C	Jarraud *et al*., 2002
sen	Fwd: ATTGTTCTACATAGCTGCAA Rev: TTGAAAAAACTCTGCTCCCA	681	50˚C	Jarraud *et al*., 2002
femA	Fwd: ACAGCTAAAGAGTTTGGTGCCTIIIIIGATAGCATGC Rev:TTCATCAAAGTTGATATACGCTAAAGGTIIIIICACACGGTC	729	50˚C	This study
hld	Fwd: TAATTAAGGAAGGAGTGATTTCAATG Rev: TTTTTAGTGAATTTGTTCACTGTGTC	100	60˚C	Mitchell *et al*., 2008
gyrB	Fwd: GGTGCTGGGCAAATACAAGT Rev: TCCCACACTAAATGGTGCAA	107	60˚C	Moisan *et al*., 2006
sspA	Fwd: ATCMATTTYGCMAAYGATGACCA Rev: TTGTCTGAATTATTGTTATCGCC	132	55˚C	Sabat *et al*., 2003
spa	Fwd: AGCACCAAAAGAGGAAGACAA	290	55˚C	Sabat *et al*., 2003

	Rev: GTTTAACGACATGTACTCCGT			
clfA	Fwd: GATTCTGACCCAGGTTCAGA	945	55˚C	Sabat *et al*., 2003
	Rev: CTGTATCTGGTAATGGTTCTTT			
clfB	Fwd: ATGGTGATTCAGCAGTAAATCC	880	55˚C	Sabat *et al*., 2003
	Rev: CATTATTTGGTGGTGTAACTCTT			
sdrCDE	Fwd: GTAACAATTACGGATCATGATG	648/580/622	55˚C	Sabat *et al*., 2003
	Rev:TACCTGTTTCTGGTAATGCTTT			
spa typing	spa-113f: TAAAGACGATCCTTCGGTGAGC	110 to 422	63˚C	Aires-de-Sousa *et al*., 2006
	spa-1514r: CAGCAGTAGTGCCGTTTGCTT			

Table 2: Distribution (%) of target genes among all *Staphylococcus aureus* isolates.

	Lactation period (n = 258)			Dry-off period (n = 108)	
Gene	CLIN (n=81)	P-SUB (n=139)	NP-SUB (n=38)	DC-FC (n=44)	NP-DC (n=64)
seg+	42.0	49.6	68.4	34.1	46.9
sen+	26.0	51.8	68.4	45.6	60.9
sec+	12.4	5.0	10.5	9.1	6.3
tst+	24.7	8.6	15.8	15.9	9.4
sen+ *seg*+	18.5	41	65.8	34.1	46.9
sen- and/or *seg*-	81.5	59	34.1	63.6	53.1

CLIN, isolates from IMI with moderate to severe clinical signs during lactation; P-SUB, persistent isolates from subclinical IMI during lactation; NP-SUB, non-persistent isolates from subclinical IMI during lactation; DC-FC, dry cow-fresh cow isolates persisting during the dry-off period through freshening; NP-DC, dry cow isolates not found in fresh cows.

Table 3: Distribution (%) of *Staphylococcus aureus* isolates among the different *spa* types.

			Lactation period (n = 123)			Dry-off period (n = 72)	
Spa type	n	Spa repeats	CLIN (n = 45)	P-SUB (n = 40)	NP-SUB (n = 38)	DC-FC (n = 35)	NP-DC (n = 37)
078	2	04-21-12-41-20-17-12-12-17	-	-	2.6	-	2.7
127	1	07-23-21-16-34-33-13	-	-	-	-	2.7
224	1	07-23-12-21-17-34-33-34	-	-	2.6	-	-
267	38	07-23-12-21-17-34-34-34-33-34	20	20	10.5	28.6	18.9
359	11	07-23-12-21-17-34-34-33-34	8.9	5	2.6	11.4	-
605	8	07-23	2.2	2.5	-	2.8	13.5
529	93	04-34	60	45	55.3	28.6	45.9
1166	5	03-16-21-17-23-13-17-17-17-23-24	-	2.5	5.3	2.8	2.7
1182	1	07-23-12-21-17-34-34-34-34-34-34-33-34	-	2.5	-	-	-
1190	1	07-23-21-17-34-34-34-34-33-34	-	2.5	-	-	-
1965	2	07-17-34-34-34-33-34	-	2.5	2.6	-	-
2211	1	04-34-34	-	2.5	-	-	-
2445	2	07-16	-	-	-	2.8	2.7
3380	9	07-23-12-21-17-34-34	4.4	5	2.6	8.6	2.7
9129	1	07-16-21-17-34-34-34-33-34	-	-	2.6	-	-
10610	1	99-20-69-31-70-13-17-16-16-16	-	-	-	-	2.7
12186	2	07-23-21-17-34-34	2.2	-	2.6	-	-
13401	16	07-23-12-21-12-21-17-34-34-34-34-33-34	2.2	10	10.5	14.3	5.4

CLIN, isolates from IMI with moderate to severe clinical signs during lactation; P-SUB, persistent isolates from subclinical IMI during lactation; NP-SUB, non-persistent isolates from subclinical IMI during lactation; DC-FC, dry cow-fresh cow isolates persisting during the dry-off period through freshening; NP-DC, dry cow isolates not found in fresh cows.

Table 4: Distribution (%) of target genes among isolates of the three major *spa* types.

Gene	t529 (n = 93)	t267 (n = 38)	t13401 (n = 16)
seg+	84.9	11.4	18.6
sen+	80.6	13.2	37.5
sec+	15.0	2.6	0
tst+	22.6	7.9	18.8
sen+ *seg*+	75.3	2.6	12.5

Table 5: Distribution (%) of target genes among isolates of the major *spa* type t529.

	Lactation period (n = 66)			Dry-off period (n = 27)	
Gene	CLIN (n = 27)	P-SUB (n = 19)	NP-SUB (n = 20)	DC-FC (n = 10)	NP-DC (n = 17)
seg+	55.6	100	100	100	88.2
sen+	37.0	100	100	100	94.1
sec+	22.2	5.2	20	20	5.9
tst+	40.7	5.2	25	20	11.8
sen+ *seg*+	22.2	100	100	100	88.2

CLIN, isolates from IMI with moderate to severe clinical signs during lactation; P-SUB, persistent isolates from subclinical IMI during lactation; NP-SUB, non-persistent isolates from subclinical IMI during lactation; DC-FC, dry cow-fresh cow isolates persisting during the dry-off period through freshening; NP-DC, dry cow isolates not found in fresh cows.

Veh and Klein *et al*. FIGURE LEGENDS

Figure 1: Examples of persistence validation by VNTR typing. The three first cases were validated as persistent because isolates showed the same VNTR profile in the lactational samples S1, S2 and S3 taken at 0, 3 and 6 weeks, respectively. The last case was not validated as persistent because the S1 isolate profile differed from that of S2 and S3.

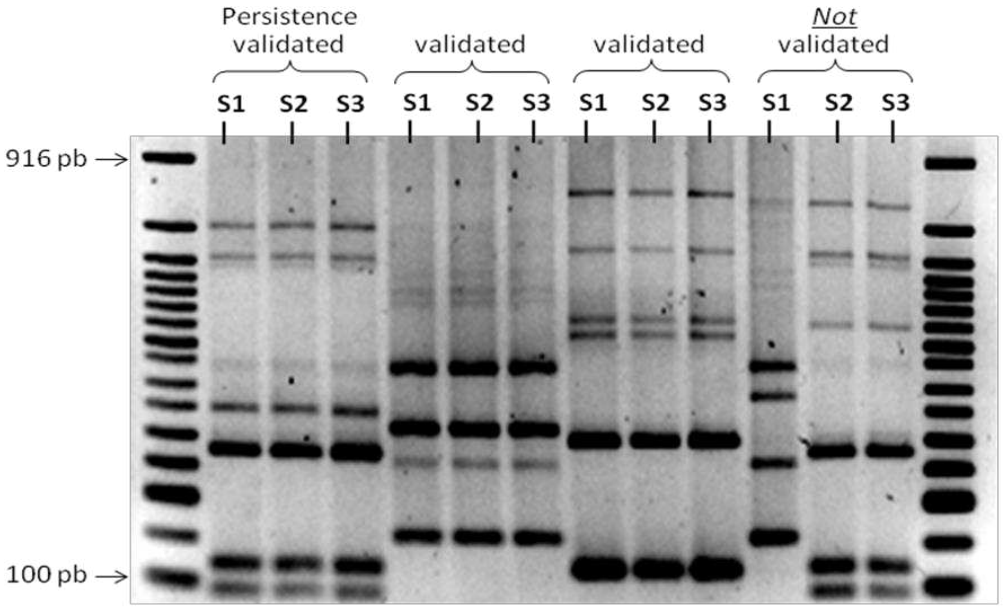
Figure 2: Boxplots of biofilm production of the different groups of *Staphylococcus aureus* isolates. Each symbol represents one strain. The horizontal bar represents the median. DC-FC, dry cow-fresh cow isolates persisting during the dry-off period through freshening; NP-DC, dry cow isolates not found in fresh cows; Clinical, lactational isolates from IMI with moderate to severe clinical signs; NP-SUB, non-persistent isolates from subclinical IMI during lactation; P-SUB, persistent isolates from subclinical IMI during lactation.

Figure 3: Boxplots of *hld* expression level of the different groups of *Staphylococcus aureus* isolates. Each symbol represents one strain. The horizontal bar represents the median. DC-FC, dry cow-fresh cow isolates persisting during the dry-off period through freshening; NP-DC, dry cow isolates not found in fresh cows; Clinical, lactational isolates from IMI with moderate to severe clinical signs; NP-SUB, non-persistent isolates from subclinical IMI during lactation; P-SUB, persistent isolates from subclinical IMI during lactation.

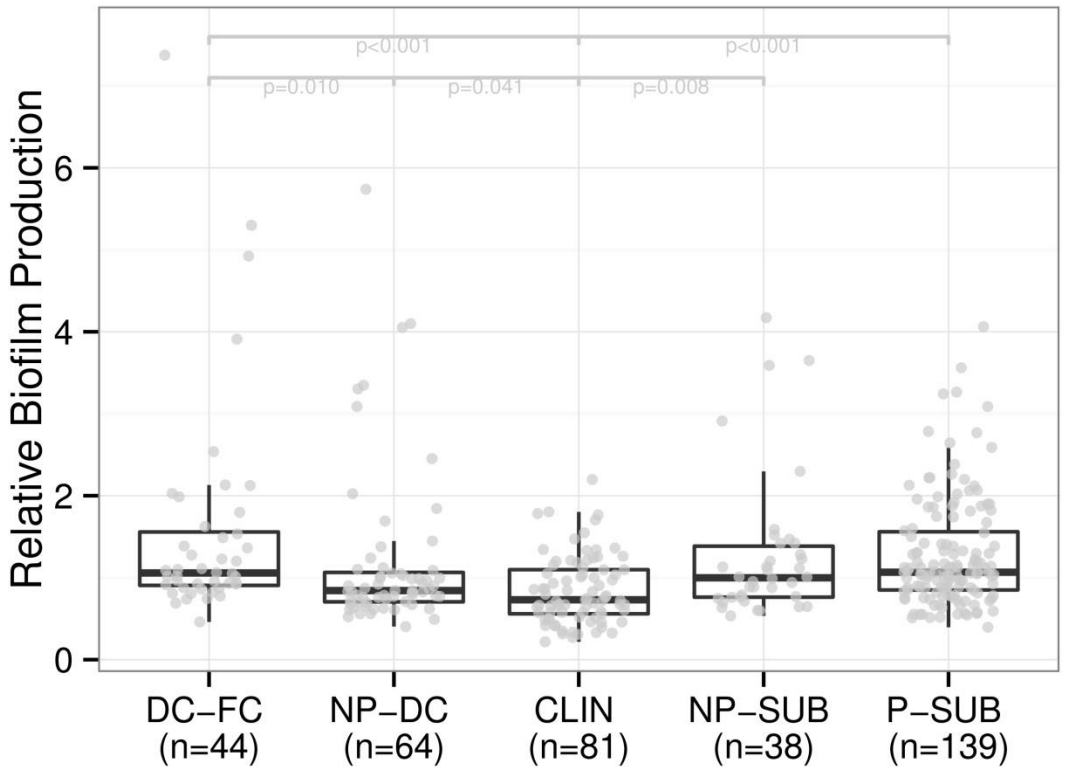
Figure 4: Boxplots of biofilm production of the predominant *spa* types of *Staphylococcus aureus*. Each symbol represents one strain. The horizontal bar represents the median. Statistical analysis showed that the isolates of *spa* t1166, t605 and the novel t13401 group produce more biofilm than *spa* t529 isolates. Also, the t13401 strains produce more biofilm than the t267 and t359 isolates.

Figure 5: Boxplots of *hld* expression level of the predominant *spa* types of *Staphylococcus aureus*. Each symbol represents one strain. The horizontal bar represents the median. Statistical analysis showed that *spa* type t529 strains have higher *agr* activation compared to types t267, t3380, t605 and the novel t13401 isolates.

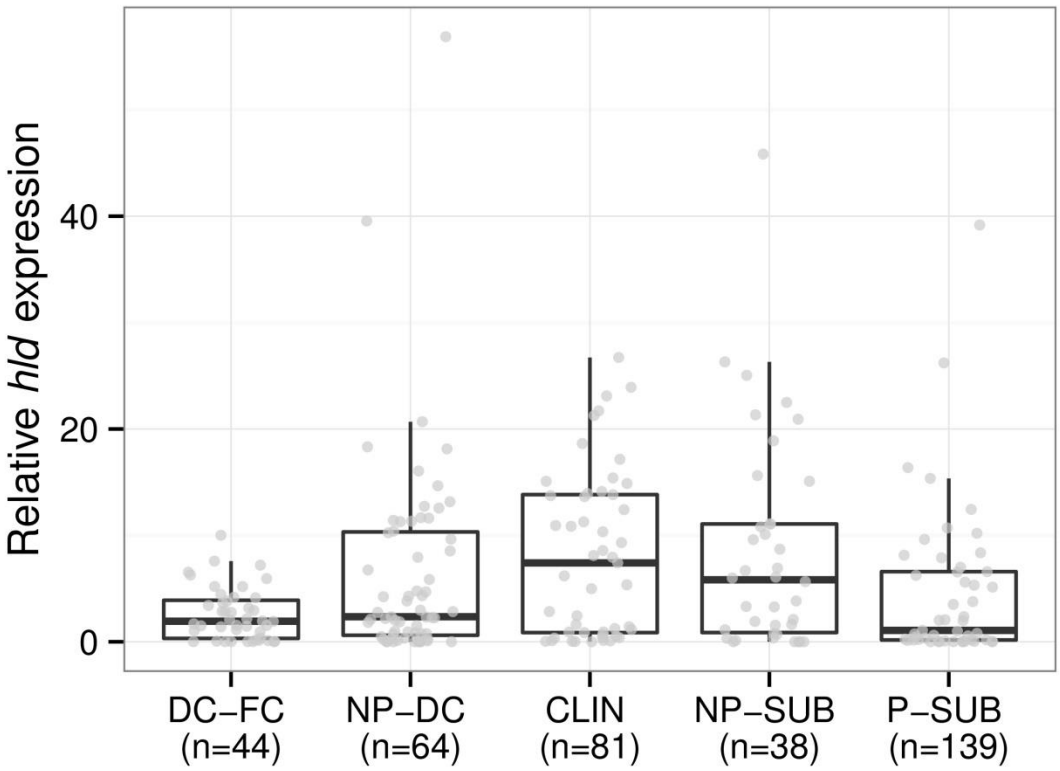
Veh and Klein *et al*. Figure 1



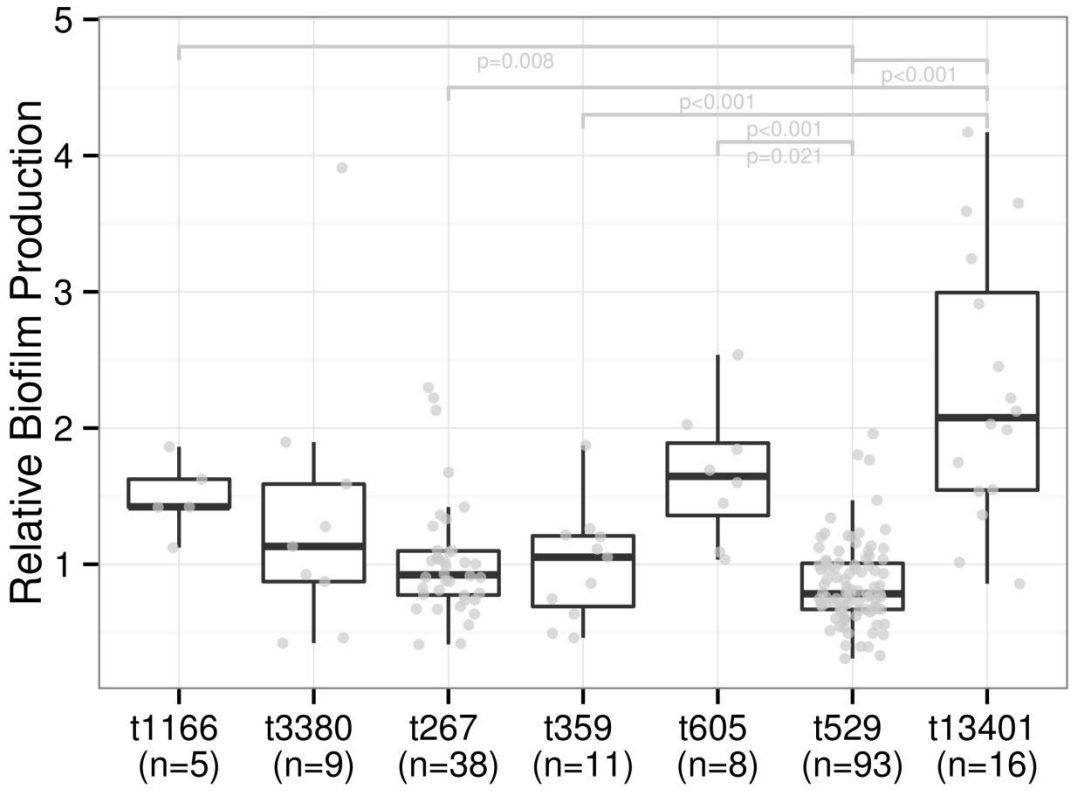
Veh and Klein *et al*. Figure 2



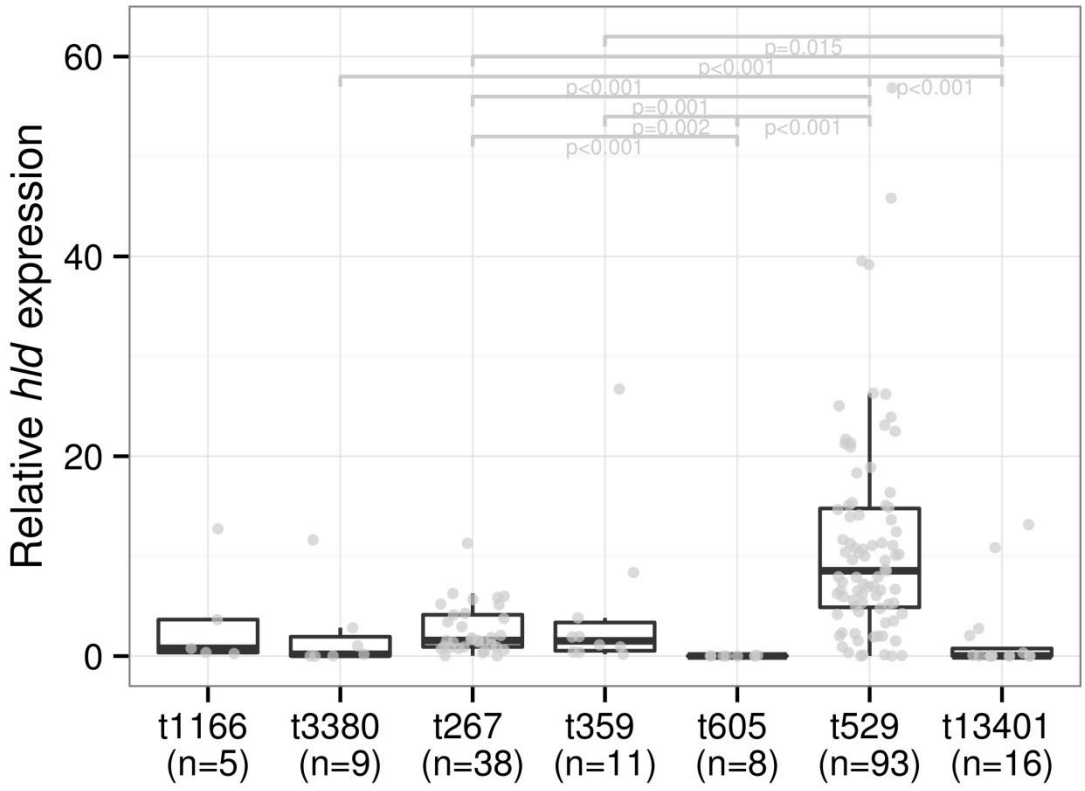
Veh and Klein *et al*. Figure 3



Veh and Klein *et al*. Figure 4



Veh and Klein *et al*. Figure 5



CAPÍTULO 3

A C-TYPE LECTIN FROM *Bothrops jararacussu* VENOM DISRUPTS STAPHYLOCOCCAL BIOFILMS

A C-type lectin from *Bothrops jararacussu* venom disrupts staphylococcal biofilms

Raphael Contelli Klein[1], Mary Hellen Fabres-Klein[1], Leandro Licursi de Oliveira[2], Renato Neves Feio[3], François Malouin[4*], Andréa de Oliveira Barros Ribon[1*].

[1] Laboratório de Biotencologia Molecular, Departmento de Bioquímica e Biologia Molecular, Universidade Federal de Viçosa, 36570.000, Viçosa, Minas Gerais, Brazil.

[2] Laboratório de Glicobiologia, Departamento de Biologia Geral, Universidade Federal de Viçosa, 36570.000, Viçosa, Minas Gerais, Brazil.

[3] Museu de Zoologia João Moojen – Departamento de Biologia Animal, Universidade Federal de Viçosa, 36570.000, Viçosa, Minas Gerais, Brazil.

[4] Centre d'étude et de valorisation de la diversité microbienne (CEVDM), Département de biologie, Faculté des sciences, Université de Sherbrooke, Sherbrooke, QC, Canada J1K2R1.

[*]Co-corresponding authors: e-mails: abribon@ufv.br / francois.malouin@usherbrooke.ca

ABSTRACT

Bovine mastitis is a major threat to animal health and the dairy industry. *Staphylococcus aureus* is a contagious pathogen that is usually associated with persistent intramammary infections, and biofilm formation is a relevant aspect of the outcome of these infections. Several biological activities have been described for snake venoms, which led us to screen secretions of *Bothrops jararacussu* for antibiofilm activity against *S. aureus* NRS155. Crude venom was fractionated by size-exclusion chromatography, and the fractions were tested against *S. aureus*. Biofilm growth, but not bacterial growth, was affected by several fractions. Two fractions (15 and 16) showed the best activities and were also assayed against *S. epidermidis* NRS101. Fraction 15 was identified by MALDI-TOF mass spectrometry as a galactose-binding C-type lectin with a molecular weight of 14.4 kDa. The lectin was purified from the crude venom by D-galactose affinity chromatography, and only one peak was observed. This pure lectin was able to inhibit 75% and 80% of *S. aureus* and *S. epidermidis* biofilms, respectively, without affecting bacterial cell viability. The lectin also exhibited a dose-dependent inhibitory effect on both bacterial biofilms. The antibiofilm activity was confirmed using scanning electron microscopy. A pre-formed *S. epidermidis* biofilm was significantly disrupted by the C-type lectin in a time-dependent manner. Additionally, the lectin demonstrated the ability to inhibit biofilm formation by several mastitis pathogens, including different field strains of *S. aureus, S. hyicus, S. chromogenes, Streptococcus agalactiae*, and *Escherichia coli*. These findings reveal a new activity for C-type lectins. Studies are underway to evaluate the biological activity of these lectins in a mouse mastitis model.

1. INTRODUCTION

A bacterial biofilm is defined as a complex and structured community of organisms enclosed in a self-produced polymeric matrix that contains exopolysaccharides, proteins, teichoic acids, enzymes, and extracellular DNA [1, 2]. Biofilms provide a firm attachment to different surfaces [3] and also provide physical protection against diverse environmental conditions, such as UV exposure, dehydration, salinity, the host immunological system, and antimicrobial agents [4, 5].

Previous studies have demonstrated that bacteria inside biofilms can be up to 1000 times more resistant to antibiotics than free-living bacteria [6, 7]. Taken together, these features make infections caused by biofilm-producing pathogens difficult to treat; thus, biofilms are an area of concern in human and animal infections.

New strategies to disrupt biofilm formation have shown efficacy *in vitro*, and they usually target the different components that form the extracellular matrix [8, 9, 10]. Mutations in genes encoding extracellular nucleases reduced biofilm formation and were correlated with an increase in daptomycin susceptibility in a murine model, revealing the therapeutic relevance of this strategy [11]. Currently, there is a race to identify substances with antibiofilm activity; which has previously been observed in plant extracts, algae polysaccharides, DNaseI, and proteases [12].

Bovine mastitis is an important disease that affects dairy cattle and can be caused by several microorganisms, mainly bacteria [13]. *Staphylococcus aureus* is one of the most important pathogens that cause clinical and subclinical mastitis [14]. The contagious nature of the bacteria help them to spread from one animal to the other during milking, which makes preventative management an effective method to control the disease [15].

S. aureus is quite resistant to antibiotic therapy [16] and its ability to form a well-structured biofilm may contribute to this resistance [17]. Several authors have reported the *in vitro* production of biofilms by bovine mastitis isolates [18, 1, 19], and their various biofilm-producing potentials suggest that biofilms are vital virulence factors, at least for some strains.

Snake venoms are a rich source of substances that affect different biological processes, such as neurotransmission, coagulation, and inflammation. Captopril, the first oral angiotensin-converting enzyme (ACE) inhibitor, was isolated from

Bothrops jararaca and is currently used commercially for the treatment of hypertension [20]. The venom from *Bothrops jararacussu* is rich in metalloproteases, serine proteases, phospholipase A_2, L-amino acid oxidases, and other components that could be promising new drugs for the treatment of several diseases. In the current study, we report a new biological activity for a snake venom compound. A purified component was able to interfere with biofilm formation in *Staphylococcus* sp. and was also efficient against pre-formed biofilms. The antibiofilm substance was identified as a C-type lectin. This is the first report describing the antibiofilm activity of a C-type lectin.

2. MATERIAL AND METHODS

2.1. Bacterial isolates and venom source

The reference strains *Staphylococcus epidermidis* NRS101 (ATCC 35983) and *S. aureus* NRS155 (RN 9120) were obtained from NARSA (Network on Antimicrobial Resistance in *Staphylococcus aureus*) and were used as controls in biofilm assays. The other staphylococcal isolates used in this study were kindly provided by Embrapa Dairy Cattle, Juiz de Fora, Minas Gerais. Bacteria were grown in Brain Heart Infusion broth (BHI, HiMedia, Mumbai, India) supplemented with 0.25% glucose (BHIg) at 37°C with agitation. All of the isolates used in this study were stored at -80°C in BHI containing 40% glycerol.

Bothrops jararacussu venom was manually extracted from snakes collected at Mata da Biologia (20°45'S, 42°52'W), Universidade Federal de Viçosa. The venom was stored frozen at -20°C until use.

2.2. Biofilm production assay

Biofilm formation was assessed in sterile flat-bottomed 96-well polystyrene microtiter plates. The bacterial isolates were inoculated into BHIg at 37°C and grown for 16 h on a rotary shaker at 180 rpm. A cell suspension adjusted to 0.5 McFarland scale was prepared for each bacterial strain and added to the wells containing snake venom fractions or purified C-type lectin. The wells were then filled with BHIg to a final volume of 200 µL. The plates were incubated at 37°C without agitation. Growth was monitored by the optical density (O.D.) at 600nm with a microplate reader (VersaMax Molecular Devices, Sunnyvale, USA). After 22 h, the medium was discarded, the wells were gently washed three times with 200 µL of sterile PBS (pH 7.4), and staining was performed with 200 µL of 0.1% crystal violet for 30 min. Each well was re-washed three times with 200 µL of sterile distilled water prior to the addition of 200 µL of 95% ethanol and measurement of the OD_{560nm}. Biofilms incubated with PBS only were used as controls. Each isolate was tested in triplicate, and the assay was repeated three times.

For the pre-formed biofilm assay, the biofilm was allowed to grow for 22 h as described above. The compounds were added into the wells and incubated at 37°C for 2 and 3 h. The biofilm disruption was then evaluated.

2.3. Size exclusion and affinity FPLC chromatography

Crude venom was centrifuged at 10,000 xg for 10 min to remove insoluble material. The resulting supernatant was maintained at -20°C for further assays. For size-exclusion chromatography, one aliquot of this sample was suspended in a saline buffer (150 mM NaCl) to a final concentration of 5 mg/mL and applied onto a Superdex Peptide HR 10/30 (GE Healthcare, Buckinghamshire, England) in an FPLC System (GE Healthcare). The material was eluted using the same buffer at a constant flow rate of 0.5 mL/min and monitored by absorbance measurements at 280 nm. Fractions (400 µL) were collected, pooled, and kept at -20°C until the determination of biological activity.

For the C-type lectin purification, the centrifuged venom was suspended in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) supplemented with 0.5 M NaCl (pH 7.4) to a final concentration of 25 mg/mL and applied onto an agarose-D-galactose column (GE Healthcare) connected to an FPLC system (GE Healthcare). The column was washed with the same buffer at a flow rate of 0.5 ml/min until the absorbance at 280 nm had returned to baseline. The C-type lectin bound to the column was eluted with PBS containing 300 mM D-galactose. The elution profile was monitored by reading the absorbance at 280 nm. The purified lectin was dialyzed against PBS and maintained at -20°C.

All samples were analyzed by SDS-PAGE using 12 % polyacrylamide gels (12 x 10 cm, 0.75 mm thickness) under reducing conditions [36].

2.4. Protein in-gel digestion

Bands of interest were extracted from gels, placed in 96-well plates, and washed with water. Tryptic digestion was performed using a MassPrep liquid handling robot (Waters, Milford, USA) according to the manufacturer's specifications, and the protocol of Shevchenko *et al*. [37] was followed with the modifications suggested by Havlis *et al*. [38]. Briefly, proteins were reduced with 10 mM DTT and alkylated with 55 mM iodoacetamide. Trypsin digestion was performed using 126 nM modified porcine trypsin (sequencing grade, Promega, Madison, WI, USA) at 58°C for 1 h. The digestion products were extracted using 1% formic acid and 2% acetonitrile followed by 1% formic acid and 50% acetonitrile. The recovered extracts were pooled, vacuum centrifuge-dried and subsequently

resuspended in 10 µl of 0.1% formic acid. Aliquots (2 µl) of the extracts were then analyzed by mass spectrometry.

2.5. Mass spectrometry

Mass spectrometry analysis was performed at the Proteomics Platform of the Quebec Genomics Center (Centre de recherche du CHU de Québec, CHUL, Québec, QC, Canada) on a TripleTOF 5600 mass spectrometer fitted with a nanospray III ion source (ABSciex, Concord, ON) and coupled to an Agilent 1200 HPLC. Samples (2 uL) were injected via the Agilent 1200 autosampler onto a 0.075 mm (internal diameter) self-packed PicoFrit column (New Objective, Woburn, MA, USA) packed with an isopropanol slurry of Jupiter C18 (5 µm; Phenomenex), which served as the stationary phase, using a pressure vessel set at 700 p.s.i. The column length was 15 cm. The samples were run using a 65 min gradient from 5-35% solvent B (solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in acetonitrile) at a flow rate of 300 nL/min. Data were acquired using an ion spray voltage of 2.4 kV, curtain gas of 30 PSI, nebulizer gas of 8 PSI, and an interface heater temperature of 125ºC. An information-dependent acquisition (IDA) method was set up with the MS survey range set between 400 amu and 1250 amu (250 msec), followed by dependent MS/MS scans with the mass range set between 100 and 1800 amu (50 msec) of the 20 most intense ions in high sensitivity mode with a 2+ to 5+ charge state. Dynamic exclusion was set for a period of 3 sec and a tolerance of 100 ppm. MGF peak list files were created using Protein Pilot software (version 4.5; ABSciex) utilizing the Paragon and Progroup algorithms [39]. MGF sample files were then analyzed using Mascot (version 2.4.0; Matrix Science, London, UK). Mascot was set up to search the Uniref100-*Homo sapiens* database assuming trypsin digestion. Mascot was searched with a fragment ion mass tolerance of 0.10 Da and a parent ion tolerance of 0.10 Da. The oxidation of methionine was specified as a variable modification, and carbamidomethylation (C) was specified as a fixed modification. Two missed cleavage sites were allowed.

2.6. Criteria for protein identification

Scaffold (version 4.0.1; Proteome Software Inc., Portland, OR, USA) was used to validate the MS/MS-based peptide and protein identifications. The FDR rate of proteins/peptides was set to 1% or less based on decoy database searching. Protein

probabilities were assigned by the Protein Prophet algorithm [40]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

2.7. Scanning electron microscopy of biofilms

S. aureus NRS155 and *S. epidermidis* NRS101 biofilms were prepared in polystyrene supports (0.3 x 0.3 x 0.1 cm). The supports were immersed into 96-well microtiter plates containing 100 µL of a 0.5 McFarland bacterial suspension and 100 µL of BHIg. Biofilms were treated using 100 µg/mL of lectin per well. The control consisted of biofilms treated with PBS. The microplates were incubated at 37°C for 22 h without agitation. The medium was discarded, and the wells were gently washed three times with 200 µL of sterile PBS (pH 7.4) and fixed with a 2.5% (w/v) solution of glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 2 h. After fixing the cells, the samples were dehydrated using an ethanol series (30%, 50%, 70%, 80%, 95%; 15 min each), followed by three washes in 100% ethanol. The samples were then dried in a critical point dryer (CPD, Bal-tec 030) using liquid CO_2 and subsequently coated with gold (approximately 15 nm thickness) using a sputter coater (Balzers, FDU 010). The polystyrene supports were examined on a scanning electron microscope (Leo, 1430 VP) at an accelerating voltage of 20 kV [41].

3. RESULTS

Crude venom of *B. jararacussu* was centrifuged and diluted to a concentration of 5 mg/mL. Subsequently, 100 µL aliquots were injected into an FPLC coupled to a Superdex peptide column to separate substances with biological activity against bacteria. Four main peaks were observed, and the first three peaks contained the majority of the proteins (Figure 1). A total of 48 fractions were recovered and screened for biological activity. The effect of 34 fractions that contained detectable amounts of protein on bacterial cell viability was evaluated by measuring OD_{600nm}; however, none of the fractions showed significant activity against *S. aureus* NRS155 (Figure 2A). Interestingly, some fractions showed strong antibiofilm activity (Figure 2B). Fractions 15 and 16 were selected for the subsequent assays.

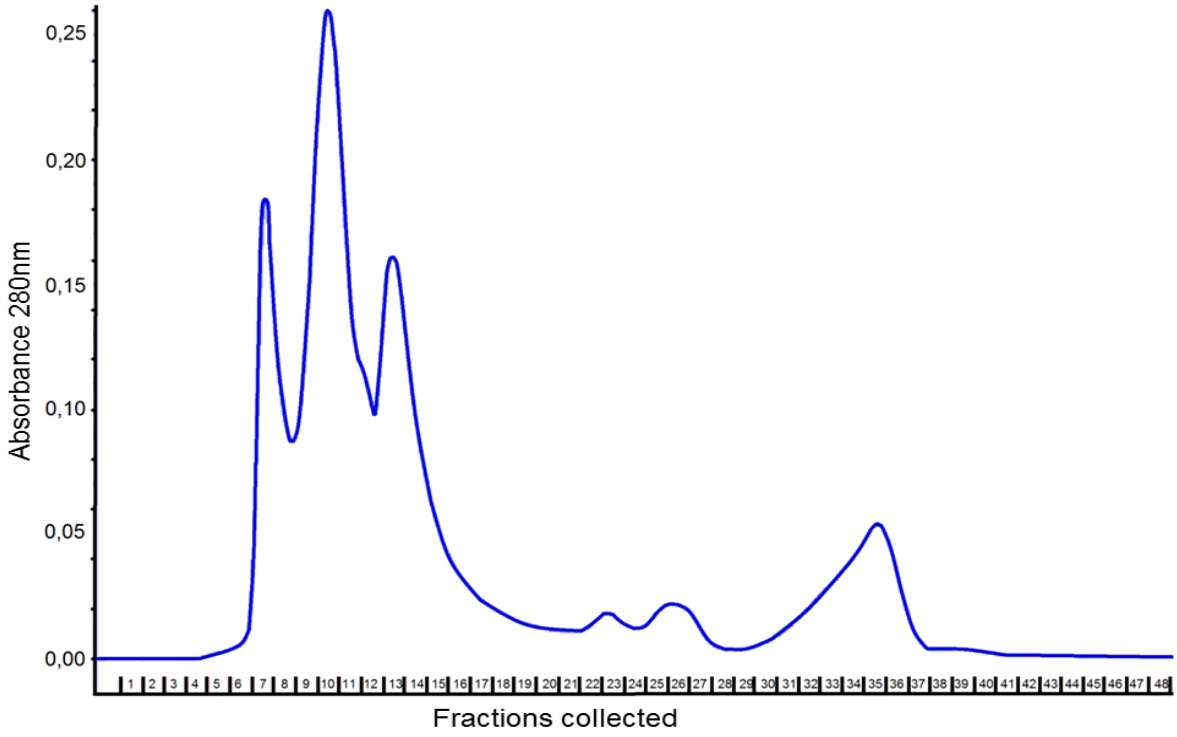


Figure 1. Chromatography of *Bothrops jararacussu* venom. Size-exclusion chromatography of *B. jararacussu* venom on a Superdex Peptide HR 10/30 column equilibrated with 150 mM NaCl. The fractions (0.4 mL/tube) were collected at flow a rate of 0.5 mL/min.

A microplate biofilm assay and a growth curve analysis were performed to evaluate whether fractions 15 and 16 were affecting bacterial growth in a way that

could promote a reduction in biofilm production (Figure 3). No significant effect on *S. aureus* or *S. epidermidis* growth was observed, although the OD_{600nm} value obtained in the stationary phase was slightly lower than that observed in the control group. A comparison of the specific growth rates between cells treated with saline and cells treated with fractions 15 and 16 were performed, and no effect was observed (Table S1). These results indicate that the fractions may have been inhibiting biofilm formation by other mechanisms that were not greatly affecting bacterial growth.

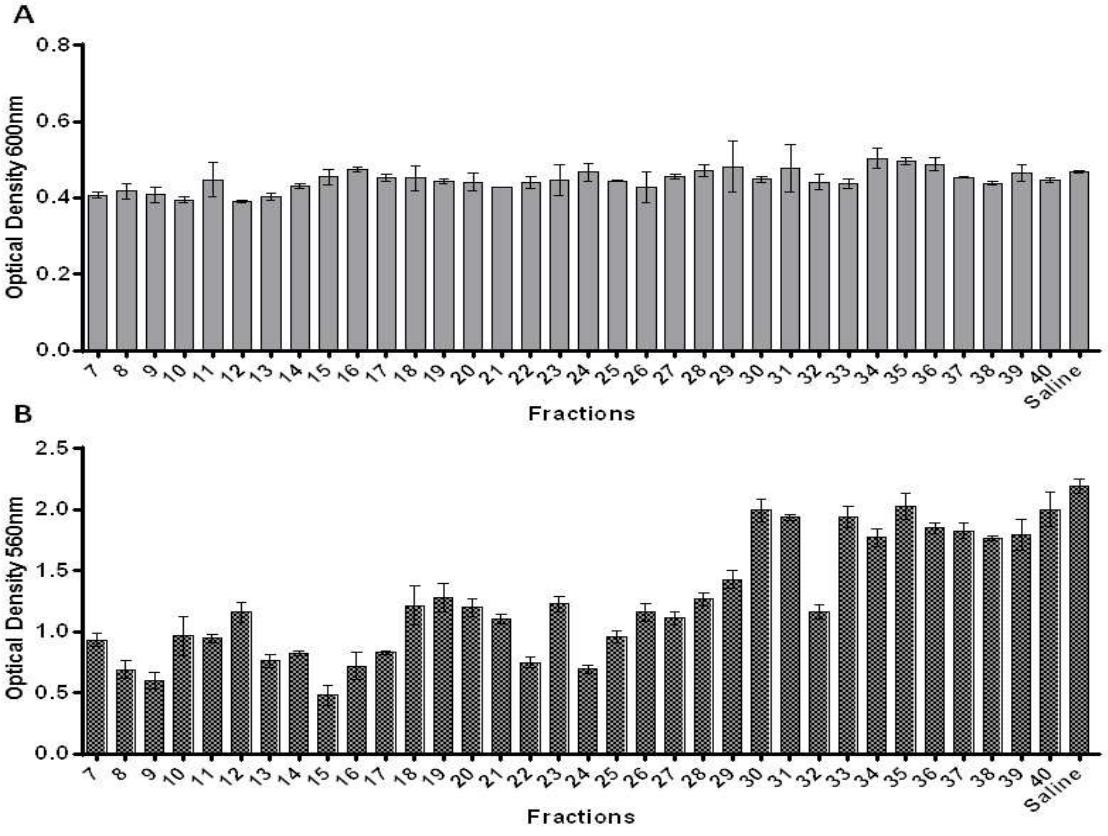


Figure 2. Effect of fractions purified from *Bothrops jararacussu* venom on *Staphylococcus aureus* growth and biofilm biomass. *Staphylococcus aureus* NRS155 was grown in BHI with 0.25 % glucose at 37 °C for 22 h in contact with the fractions. Bacterial growth was determined by measuring OD_{600nm} (A), and the biofilm biomass was determined by measuring OD_{560nm} (B). The results are the average of three independent experiments ± SD.

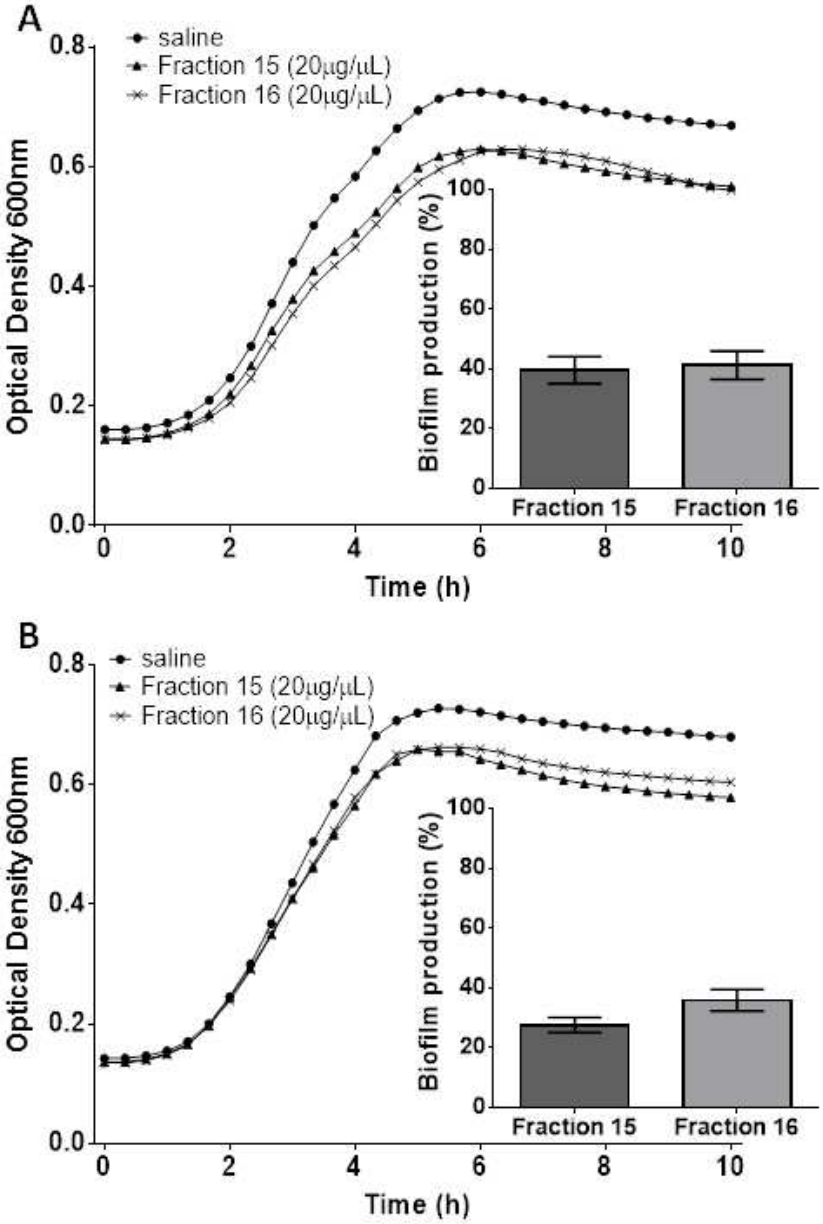


Figure 3. Effect of fractions 15 and 16 on bacterial growth and biofilm production. (A) *Staphylococcus aureus* NRS155 and (B) *S. epidermidis* NRS101 were grown in BHI medium with 0.25 % glucose (control) or in the same medium containing fractions 15 and 16 at 37 ºC. The bacterial growth (OD_{600nm}) and biofilm biomass (OD_{560nm}) were measured using a multidetection microplate reader. The percentage of biofilm disruption was calculated relative to that observed with the saline solution. Representative results of three independent experiments are shown.

Fractions 15 and 16 were analyzed by SDS-PAGE, and a band of approximately 15 kDa was observed (data not shown). This band was extracted from the gel, trypsinized, and analyzed on a TripleTOF 5600 mass spectrometer. A stronger band

of the same size (15 kDa) obtained from fraction 15 of whole venom was also sequenced. The ions obtained from the peptides identified by mass spectrometry are shown in Figure 4. Considering a minimum of 5 peptides with a threshold of 95%, 27 exclusive unique peptides were found along with 42 exclusive unique spectra. Using these criteria, the protein Q7T228 was identified as a C-type lectin with 94% coverage (Figure 4B). The same results were obtained for the two sequenced bands.

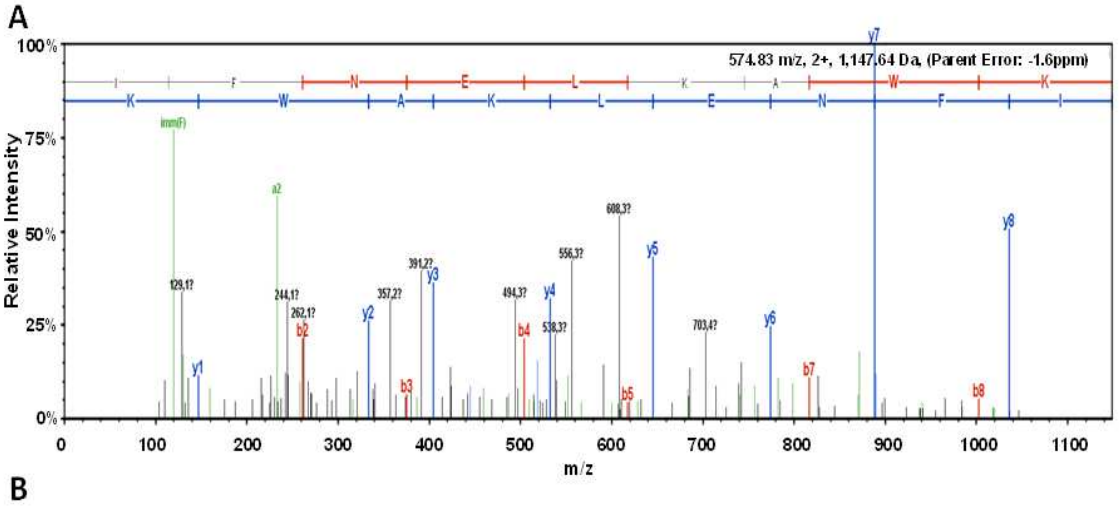


Figure 4. A C-type lectin was identified by MALDI-TOF MS. Mass spectrometry was performed on a TripleTOF 5600 mass spectrometer fitted with a nanospray III ion source and coupled to an Agilent 1200 HPLC. (A) Ions generated from representative digested peptide (K)IFNELKAWK(D), m/z 574.83. (B) Coverage percentage and protein sequence of C-type lectin.

C-type lectins have galactose-binding properties. For this reason, we performed a single chromatographic step using an agarose-D-galactose affinity column coupled to an FPLC AKTA Purifier UPC10 to isolate the lectin identified by mass spectrometry. The crude venom was centrifuged, diluted in PBS, and applied to the column using a 10 mL loop. Only one peak was obtained when 300 mM galactose was used as the elution buffer (Figure 5A). A single strong band of ≈15 kDa was found when the eluate was analyzed by SDS-PAGE (Figure 5B). The waste contained all other proteins initially present in the crude venom.

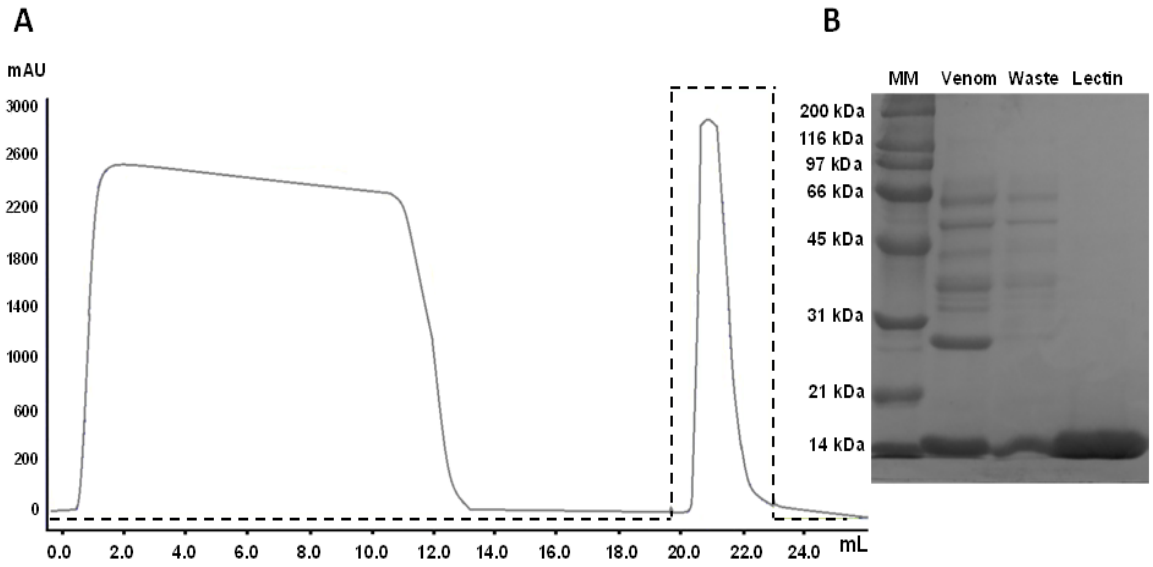


Figure 5. Affinity chromatography of the C-type lectin. (A) The lectin from *Bothrops jararacussu* venom was purified using a D-galactose column (3 cm x 1 cm, I.D.). The adsorbed proteins (lectin) were eluted with PBS buffer (pH 7.4) containing 300 mM galactose (dashed line). The protein concentration was monitored by reading the OD_{280nm}. (B) SDS-PAGE analysis of the purified lectin from *B. jararacussu* venom.

The antibiofilm activity of the purified lectin was again assayed to evaluate the effect of increased lectin concentrations on *S. aureus* (Figure 6A) and *S. epidermidis* (Figure 6B). A significant dose-dependent effect on the formation of *S. aureus* and *S. epidermidis* biofilms was observed. The purified lectin did not have an effect on bacterial growth at any concentration tested.

To evaluate the effect of the lectin on established biofilms, *S. aureus* and *S. epidermidis* were allowed to grow in BHIg for 22 h (Figure 7). The cells were then gently washed and incubated with 100 µg/mL lectin for 2 or 3 h. More than 50% of the pre-formed biofilm was disrupted when compared to the control. Also, biofilm disruption was accentuated when the incubation time was extended from 2 h to 3 h.

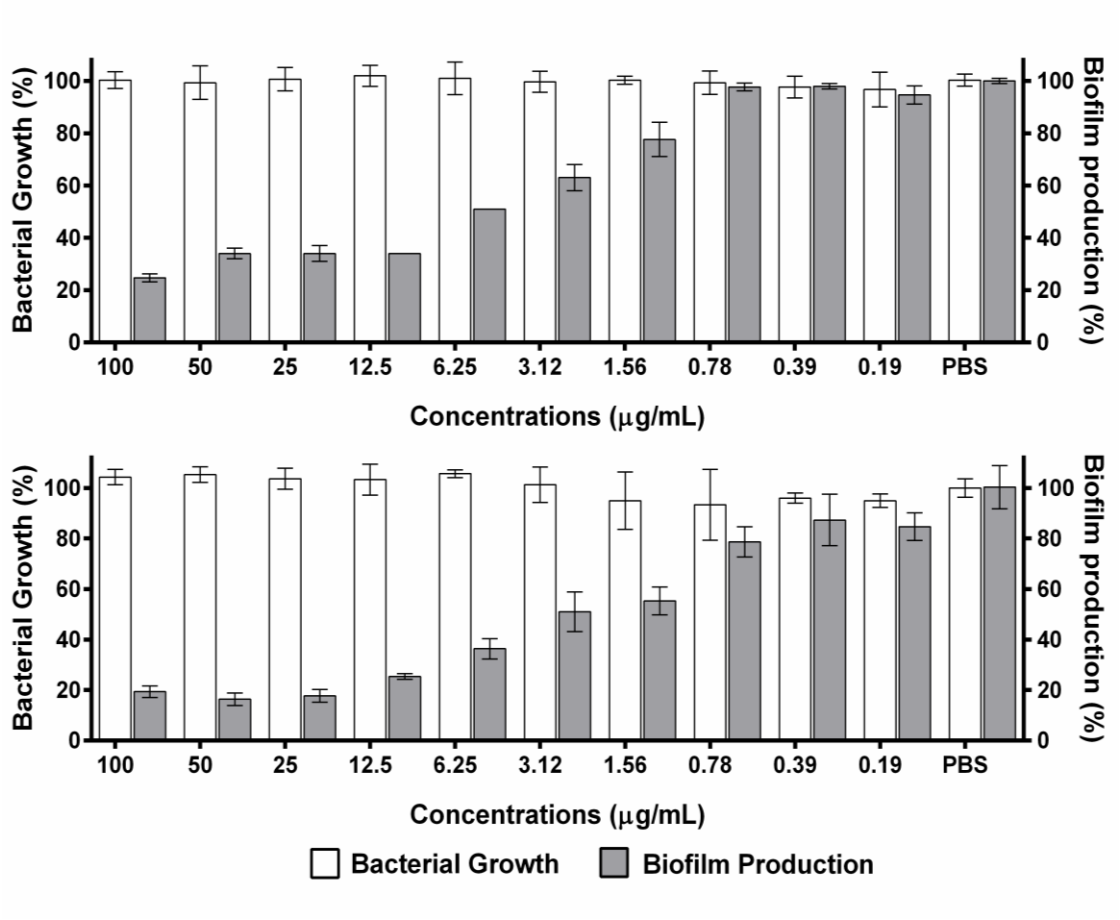


Figure 6. The C-type lectin prevented biofilm formation in a dose-dependent manner. The C-type lectin was incubated at 37 °C for 22 h in serial dilutions from 100 µg/mL to 0.19 µg/mL. The bacterial growth (OD_{600nm}) and biofilm production (OD_{560nm}) of *S. aureus* NRS155 (A) and *S. epidermidis* NRS101 (B) were monitored. The percentage of bacterial growth and biofilm production was calculated relative to that observed with PBS buffer and expressed in percentage. The results are the average of three independent experiments ± SD.

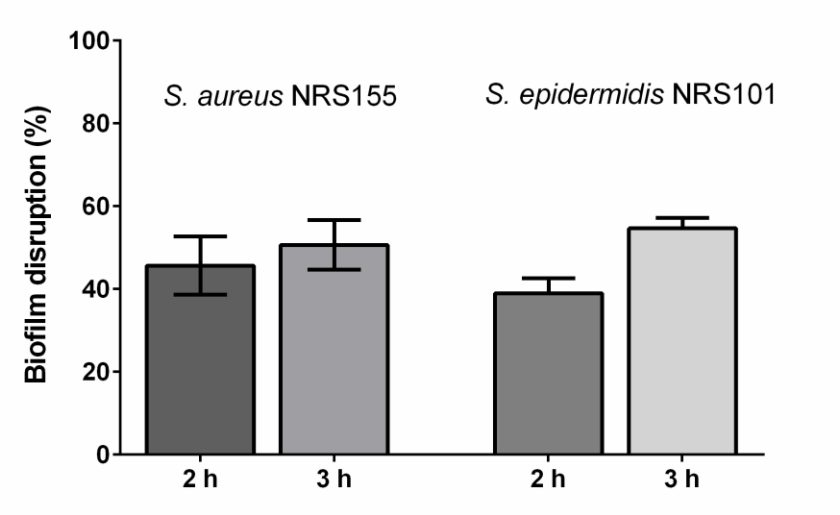


Figure 7. The C-type lectin disrupts pre-formed staphylococcal biofilms. *S. aureus* and *S. epidermidis* were cultivated in microplates for 22 h at 37 °C. The bacteria were washed, and the biofilm cells attached to the microtiter plate wells were incubated with the C-type lectin for an additional 2 or 3 h. After staining with crystal violet, the OD_{560nm} was measured. The percentage of biofilm disruption was calculated relative to that observed with PBS, which served as a control. The values are the means ± SD from three independent experiments.

Scanning electron microscopy was performed as an alternative means of evaluating the antibiofilm effect of the lectin (Figure 8). Bacterial cells were treated with 100 µg/mL lectin (Figure 8C and 8D) or PBS as a control (Figure 8A and 8B). Cell clusters on polystyrene supports were only seen with the control treatment and were accompanied with an extracellular polymeric substance (EPS) matrix that typically surrounds staphylococcal biofilms (Figure 8A, arrows). Biofilm production by both bacteria was drastically reduced using the lectin.

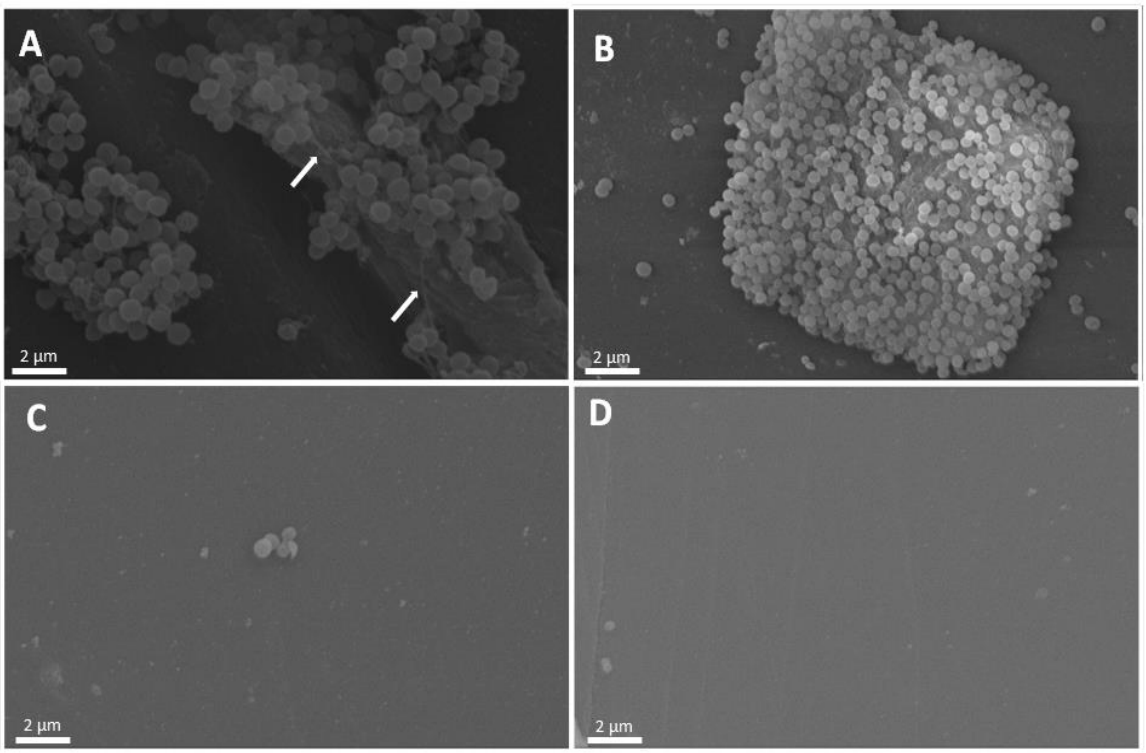


Figure 8. Scanning electron microscopy reveals disruption of staphylococcal biofilms by lectin. *Staphylococcus aureus* NRS 155 (A – C) and *S. epidermidis* NRS 101 (B – D) were grown on a polystyrene surface for 22 h at 37 °C in the presence (C – D) or absence (A – B) of lectin. Arrows indicate staphylococcal extracellular polymeric substance.

We lastly evaluated whether the lectin was also inhibiting biofilm formation in different bacterial species. The lectin showed promising activity against all bacteria studied, including *S. hyicus, S. chromogenes, Escherichia coli*, and other bovine mastitis clinical isolates (Figure 9). Although it showed antibiofilm activity, the lectin did not affect bacterial growth. The growth curves (Figure S1) for all tested bacteria were similar, and the comparison of specific growth rates did not reveal any significant differences (Table S1).

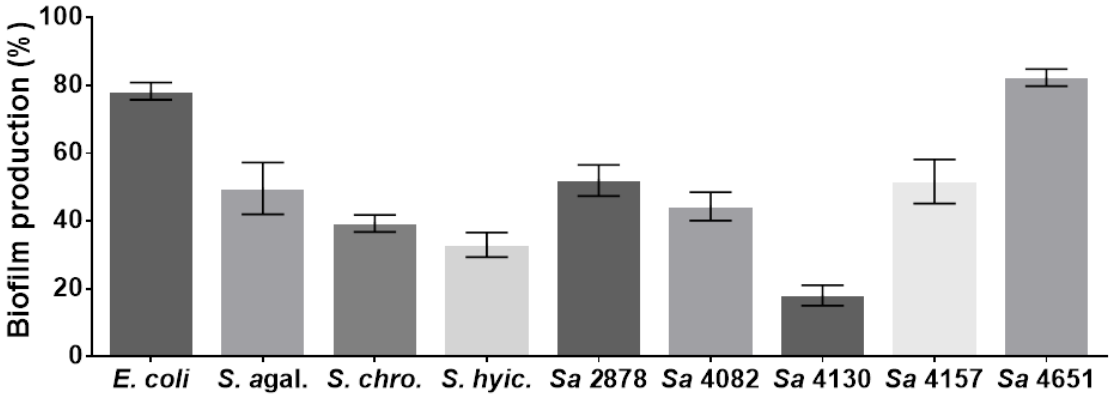


Figure 9. The C-type lectin prevents biofilm production in different bacterial species. The bacteria *E. coli* (*Escherichia coli*), *S. agal*. (*Streptococcus agalactiae*), *S. chro*. (*Staphylococcus chromogenes*), *S. hyic.* (*Staphylococcus hyicus*), *Sa* 2878 (*Staphylocccus aureus* 2878), *Sa* 4082 (*S. aureus* 4082), *Sa* 4130 (*S. aureus* 4130), *Sa* 4157 (*S. aureus* 4157) and *Sa* 4651 (*S. aureus* 4651) were grown in BHIg medium containing 50 µg/mL lectin for 22 h at 37 °C. Biofilm production was monitored by reading the OD_{560nm} after staining with crystal violet. The percentage of biofilm disruption is shown relative to that observed with PBS, which was used as a control. The values are the means (±SD) of three independent experiments.

4. DISCUSSION

Mastitis is considered the most important disease of dairy cow herds [1]. Although it can be caused by several bacterial species, *S. aureus* is recognized as the major cause of bovine mastitis [21, 22]. Infections caused by *S. aureus* are difficult to treat, have a low cure rate, and frequently evolve to a chronic state [23]. Different mechanisms have been suggested for pathogen persistence in the udder and their high resistance to antimicrobials and the ability of bovine strains to produce biofilms [17].

A promising strategy for combating staphylococcal infections involves the targeting of bacterial virulence rather than killing the bacteria [24]. Use of such a strategy would make the pathogen less virulent, thus making infections easier to treat [25]. Biofilms are important virulence factors for *S. aureus* and other bacteria; thus, they can be considered to be good drug targets. Drugs aimed at extracellular polymeric substances (EPS) are of special interest due to their importance in the arrangement of biofilms formed by diverse microorganisms [26, 27].

Snake venom consists of a pool of different constituents that makes them a rich source of compounds with diverse biological functions [28, 23]. The main contribution of this work was the demonstration of a new biological activity for a protein purified from the venom of *Bothrops jararacussu*, a common species found in the Atlantic Forest of southeastern Brazil. This is also the first time that a lectin has been shown to disrupt bacterial biofilms.

Using size-exclusion chromatography we identified four major peaks with a considerable quantity of proteins. It is well documented that snake venoms are a reservoir of proteins and peptides [29]; however, their antibiofilm activity has not been reported. At the concentration tested, some fractions were able to degrade biofilm without affecting bacterial growth. The fractions that corresponded to the major peaks had more activity than the other fractions. These results may be due to the different protein concentrations that were used in the preliminary screening. At this step, an equal volume of each fraction was used instead of an equal concentration. Thus, fractions that contained greater amounts of active proteins may have shown more pronounced effects. In size-exclusion chromatography, the larger proteins are collected in the earlier tubes, whereas the smaller proteins are collected in the later tubes. However, using this fractionation approach, there is not a complete

separation of proteins present in the crude venom. Larger amounts of a specific protein may be present in one tube, while its quantity may be lower in the adjacent tubes. This phenomenon can explain the high antibiofilm activity of fraction number 15 and the reduced activity of the adjacent fractions.

The antibiofilm activity against *S. aureus* biofilms encouraged us to evaluate the activity against different microorganisms. Fractions 15 and 16 did not affect bacterial cell viability, and despite the slight difference in the time to reach the stationary phase, the growth rate was similar. The C-type lectin purified by affinity chromatography also very effectively inhibited the biofilms of other pathogens (Figure 9), although it did not have any effect on the growth of other species. This result suggests that the slight effect on growth caused by fractions 15 and 16 could be due to proteins other than lectin that were present in these fractions.

Biofilm is reduced proportionally to the increase of lectin concentrations. In the presence of 100 µg/mL lectin, the biofilm formation of *S. aureus* NRS155 and *S. epidermidis* NRS101 was inhibited by 75% and 80%, respectively. Scanning electron microscopy confirmed the antibiofilm effect of lectin, revealing that *S. aureus* and *S. epidermidis* biofilms were diminished by treatment with 100 µg/mL lectin. The typical biofilm structure, with exopolysaccharides surrounding the cells, was observed only with PBS treatment.

Using a TripleTOF 5600 mass spectrometer coupled to an Agilent 1200 HPLC, we identified a C-type lectin from *Bothrops jararacussu* with 94% coverage. Lectins are proteins with well-characterized properties and many biological activities [29]. The SDS-PAGE excised band that was identified by mass spectrometry was ≈15 kDa, which is the expected molecular weight of *B. jararacussu* monomers (15,021 Da). C-type lectins from snake venom are usually described as disulfide-linked dimers containing two homologous polypeptides of approximately 15 kDa that are capable of binding to carbohydrates in a non-covalent manner [30]. Usually, these lectins have a carbohydrate recognition domain (CRD) that binds a sugar moiety and are calcium dependent [31]. This sugar-binding property was used to purify the protein using a D-galactose affinity column and may be part of the mechanism by which C-type lectins disrupt biofilms. Bacterial biofilms contain many different carbohydrates, mainly glucose and galactose, and the compositions vary among species [32]. Thus, considering the variation in the percentage of galactose moieties on biofilms formed by different bacteria, it is reasonable to

hypothesize that the antibiofilm activity of lectin can be variable depending on how many galactose residues are present.

The large amount of lectin purified by affinity chromatography suggests that the lectin has a high specificity for galactose; however, this result does not exclude a lower binding affinity for other sugars. The differential affinity of the lectin for different sugars and the variable composition of sugars among bacteria may explain the variable effect on bacterial biofilm disruption.

We suggest that C-type lectins may interfere with biofilms via two possible mechanisms. The first mechanism could involve the binding of the CDR domain to carbohydrates present in the biofilm. This binding could disturb biofilm production during bacterial growth. The second mechanism could involve disturbance via a domain that has not been described. Reports describing variable lectin functions have been published [33, 34]. Dos Reis Almeida *et al*. (2010) described the N-acetyl-β-D-glucosaminidase activity of paracoccin, a lectin from *Paracoccidioides brasiliensis*. Similarly, the lectin purified in this work could be a N-acetylglucosamine-degrading protein that acts on the biofilm extracellular matrix. This compound is considered ubiquitous [35], which can explain its effect on biofilms formed by different species.

It should be highlighted that aside from biofilm disruption, the purified lectin did not affect bacterial growth, which is an important feature of compounds that target bacterial virulence. Compounds not affecting growth would not apply any selective pressure for resistance. Compounds with these features have been considered as alternative strategies for the control of persistent infections, such as those caused by biofilm-forming bacteria [24]. Evaluation of the therapeutic activity of C-type lectin in animal models is underway.

5. CONCLUSIONS

This study was the first to reveal that compounds in snake venom have effective antibiofilm activity. A C-type lectin was purified by affinity chromatography, and different assays were used to demonstrate a biological activity that has never been described previously for this family of proteins. The C-type lectin had a significant inhibitory effect on several Gram-positive and Gram-negative biofilms but did not have effects on the growth of these species. Because biofilms are important virulence factors that enable bacteria to survive antibiotic therapy and the host immune response, C-type lectin appears to be a promising tool that can be used against biofilm-forming bacteria. Our study provides a new perspective for research on lectins, and studies are under way to evaluate their antibacterial activity *in vivo*.

6. ACKNOWLEDGMENTS

The authors are grateful to Embrapa Dairy Cattle for providing all other staphylococcal and streptococcal isolates used in the study. We also acknowledge Universidade Federal de Viçosa microscopy core facility for the use of the scanning electron microscopy. *Staphylococcus aureus* NRS155 and *S. epidermidis* NRS101 were obtained through the Network of Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) program supported under NIAID/NIH Contract # HHSN272200700055C.

The access to genetic resources was provided by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq - nº 010548/2013-0) and the collection of venom *in situ* was provided by MMA-SISBIO (39126-1). This research was supported by Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG) CBB-APQ-01054-10. http://www.fapemig.br/ and Natural Sciences and Engineering Research Council of Canada. Grant 89758-2010. http://www.nserc-crsng.gc.ca/. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

7. REFERENCES

1. Melchior MB, Vaarkamp H, Fink-Gremmels J (2006) Biofilms: A role in recurrent mastitis infections? Vet J 171: 398-407.
2. Parra-Ruiz J, Vidaillac C, Rybak MJ (2012) Macrolides and staphylococcal biofilms. Rev Esp Quimioter 25: 10-16.
3. Sutherland IW (2001) Biofilm exopolysaccharides: a strong and sticky framework. Microbiol 147: 3-9.
4. Hall-Stoodley L, Costerton JW, Stoodley P (2004) Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol 2: 95-108.
5. Høiby N, Ciofu O, Johansen HK, Song Z, Moser C, Jensen PO, Molin S, Givskov M, Tolker-Nielsen T, Bjarnsholt T (2011) The clinical impact of bacterial biofilms. Int J Oral Sci 3: 55-65.
6. Hamilton MA (2002) Testing antimicrobials against biofilm bacteria. J AOAC Int 85: 479-485
7. Cerca N, Martins S, Cerca F, Jefferson KK, Pier GB, Oliveira R, Azeredo J (2005) Comparative assessment of antibiotic susceptibility of coagulase-negative staphylococci in biofilm versus planktonic culture as assessed by bacterial enumeration or rapid XTT colorimetry. J Antimicrob Chemother 56: 331-336.
8. McDougald D, Rice SA, Barraud N, Steinberg PD, Kjelleberg S (2011) Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. Nat Rev Microbiol 10: 39-50.
9. Bjarnsholt T, Ciofu O, Molin S, Givskov M, Høiby N (2013) Applying insights from biofilm biology to drug development – can a new approach be developed? Nat Rev Drug Discov 12: 791-808.
10. Kostakioti M, Hadjifrangiskou M, Hultgren SJ (2013) Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. Cold Spring Harb Perspect Med 3: a010306.
11. Beenken KE, Spencer H, Griffin LM, Smeltzer MS (2012) Impact of extracellular nuclease production on the biofilm phenotype of *Staphylococcus aureus* under *in vitro* and *in vivo* conditions. Infect Immun 80: 1634-1638.
12. Taraszkiewicz A, Fila G, Grinholc M, Nakonieczna J (2013) Innovative strategies to overcome biofilm resistance. Biomed Res Int 2013: 150653.

13. Hillerton J, Berry E (2005) Treating mastitis in the cow – a tradition or an archaism. J Appl Microbiol 98: 1250-1255.
14. Zadoks R, Fitzpatrick J (2009) Changing trends in mastitis. Ir Vet J 62: 59-70.
15. Barkema HW, Green MJ, Bradley AJ, Zadoks RN (2009) Invited review: The role of contagious disease in udder health. J Dairy Sci 92: 4717-4729.
16. Chambers HF, Deleo FR (2009) Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat Rev Microbiol 7: 629-641.
17. Raza A, Muhammad G, Sharif S, Atta A (2013) Biofilm Producing *Staphylococcus aureus* and Bovine Mastitis: A Review. Molecular Microbiology Research 3: 1-8.
18. Vasudevan P, Nair MKM, Annamalai T, Venkitanarayanan KS (2003) Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. J Vet Microbiol 92: 179-185.
19. Dhanawade NB, Kalorey DR, Srinivasan R, Barbuddhe SB, Kurkure NV (2010) Detection of intercellular adhesion genes and biofilm production in *Staphylococcus aureus* isolated from bovine subclinical mastitis. Vet Res Commun 34: 81-89.
20. McCleary RJ, Kini RM (2013) Non-enzymatic proteins from snake venoms: a gold mine of pharmacological tools and drug leads. Toxicon 62: 56-74.
21. Reyher KK, Dufour S, Barkema WH, Des Côteaux L, DeVries JT, Dohoo RI, Keefe PG, Roy JP, Scholl TD (2011) The National Cohort of Dairy Farms—A data collection platform for mastitis research in Canada. J Dairy Sci 94: 1616-1626.
22. Athar M (2007) Preparation and evaluation of inactivated polyvalent vaccines for control of mastitis in dairy buffaloes, Ph.D. Thesis, University of Agriculture Faisalabad. Avaiable: http://prr.hec.gov.pk/Thesis/1813.pdf. Accessed 3 February 2014.
23. Sutra L, Poutrel B (1994) Virulence factors involved in the pathogenesis of bovine intramammary infections due to *Staphylococcus aureus*. J Med Microbiol 40: 79-89.
24. Papa R, Artini M, Cellini A, Tilotta M, Galano E, Pucci P, Amoresano A, Selan L (2013) A new anti-infective strategy to reduce the spreading of

antibiotic resistance by the action on adhesion-mediated virulence factors in *Staphylococcus aureus*. Microb Pathog 63: 44-53.

25. Tateda K, Ishii Y, Kimura S, Horikawa M, Miyairi S, Yamaguchi K (2007) Suppression of *Pseudomonas aeruginosa* quorum-sensing systems by macrolides: a promising strategy or an oriental mystery? J Infect Chemother 13: 357-367.
26. Fux CA, Stoodley P, Hall-Stoodley L, Costerton JW (2003) Bacterial biofilms: a diagnostic and therapeutic challenge. Expert Rev Anti Infect Ther 1: 667-683.
27. Marcato-Romain CE, Pechaud Y, Paul E, Girbal-Neuhauser E, Dossat-Letisse V (2012) Removal of microbial multi-species biofilms from the paper industry by enzymatic treatments. Biofoulin 28: 305-314.
28. Fox JW, Serrano SM (2008) Exploring snake venom proteomes: multifaceted analyses for complex toxin mixtures. Proteomics 8: 909-920.
29. Koh DC, Armugam A, Jeyaseelan K (2006) Snake venom components and their applications in biomedicine. Cell Mol Life Sci 63: 3030-3041.
30. Elífio-Esposito SL, Hess PL, Moreno AN, Lopes-Ferreira M, Ricart CAO, Souza MV, Hasselman-Zielinski F, Becker JA, Pereira LF (2007) A c-type lectin from *Bothrops jararacussu* venom can adhere to extracellular matrix proteins and induce the rolling of leukocytes. J Venom Anim Toxins incl Trop Dis 13: 782-799.
31. Sakakura M, Puthinan S, Moriyama C, Kimura T, Moriya J, Irimura T, Shimada I (2008) Carbohydrate binding mechanism of the macrophage galactose-type C-type lectin 1 revealed by saturation transfer experiments. J Biol Chem 283: 33665-33673.
32. Bales PM, Renke EM, May SL, Shen Y, Nelson DC (2013) Purification and Characterization of Biofilm-Associated EPS Exopolysaccharides from ESKAPE Organisms and Other Pathogens. PLoS One 21: e67950.
33. Lu J, Teh C, Kishore U, Reid KBM (2002) Collectins and ficolins: sugar pattern recognition molecules of the mammalian innate immune system. Biochim Biophys Acta 1572: 387-400.
34. Dos Reis Almeida FB, de Oliveira LL, Valle de Sousa M, Roque Barreira MC, Hanna ES (2010) Paracoccin from *Paracoccidioides brasiliensis*;

purification through affinity with chitin and identification of N-acetyl-beta-D-glucosaminidase activity. Yeast 27: 67-76

35. Jabbouri S, Sadovskaya I (2010) Characteristics of the biofilm matrix and its role as a possible target for the detection and eradication of *Staphylococcus epidermidis* associated with medical implant infections. FEMS Immunol Med Microbiol. 9: 280-291.
36. Laemli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.
37. Havlis J, Thomas H, Sebela M, Shevchenko A (2003) Fast-response proteomics by accelerated in-gel digestion of proteins. Anal Chem 75: 1300-1306.
38. Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal Chem 68: 850-858.
39. Shilov IV, Seymour SL, Patel AA, Loboda A, Tang WH, Keating SP, Hunter CL, Nuwaysir LM, Schaeffer DA (2007) The Paragon Algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. Mol Cell Proteomics 6: 1638-1655.
40. Nesvizhskii AI, Keller A, Kolker E, Aebersold R (2003) A statistical model for identifying proteins by tandem mass spectrometry. Anal Chem 75: 4646-4658.
41. Wang R, Neoh KG, Shi Z, Kang E-T, Tambyah PA, Chiong E (2012) Inhibition of *Escherichia coli* and *Proteus mirabilis* adhesion and biofilm formation on medical grade silicone surface. Biotechnol Bioeng 109: 336-345.

8. APPENDIX - SUPPORTING INFORMATION

Table S1. Comparison of the specific growth rate μ (h^{-1}) of multiple bacterial strains grown under different conditions.

Organism	Conditions				
	Saline	**Fraction 15**	**Fraction 16**	**PBS**	**Lectin**
Staphylococcus epidermidis NRS101	0.1898	0.1603	0.16989	0.1251	0.1204
Staphylococcus aureus NRS155	0.1484	0.1248	0.1217	0.1578	0.1544
Staphylococcus aureus 4082				0.1125	0.1186
Staphylococcus aureus 4157				0.1860	0.1977
Staphylococcus aureus 4130				0.0612	0.0593
Staphylococcus aureus 4651				0.1524	0.1523
Staphylococcus chromogenes				0.0878	0.0883
Staphylococcus hyicus				0.1229	0.1209
Staphylococcus agalactiae				0.0448	0.0454
Escherrichia coli				0.1728	0.1722

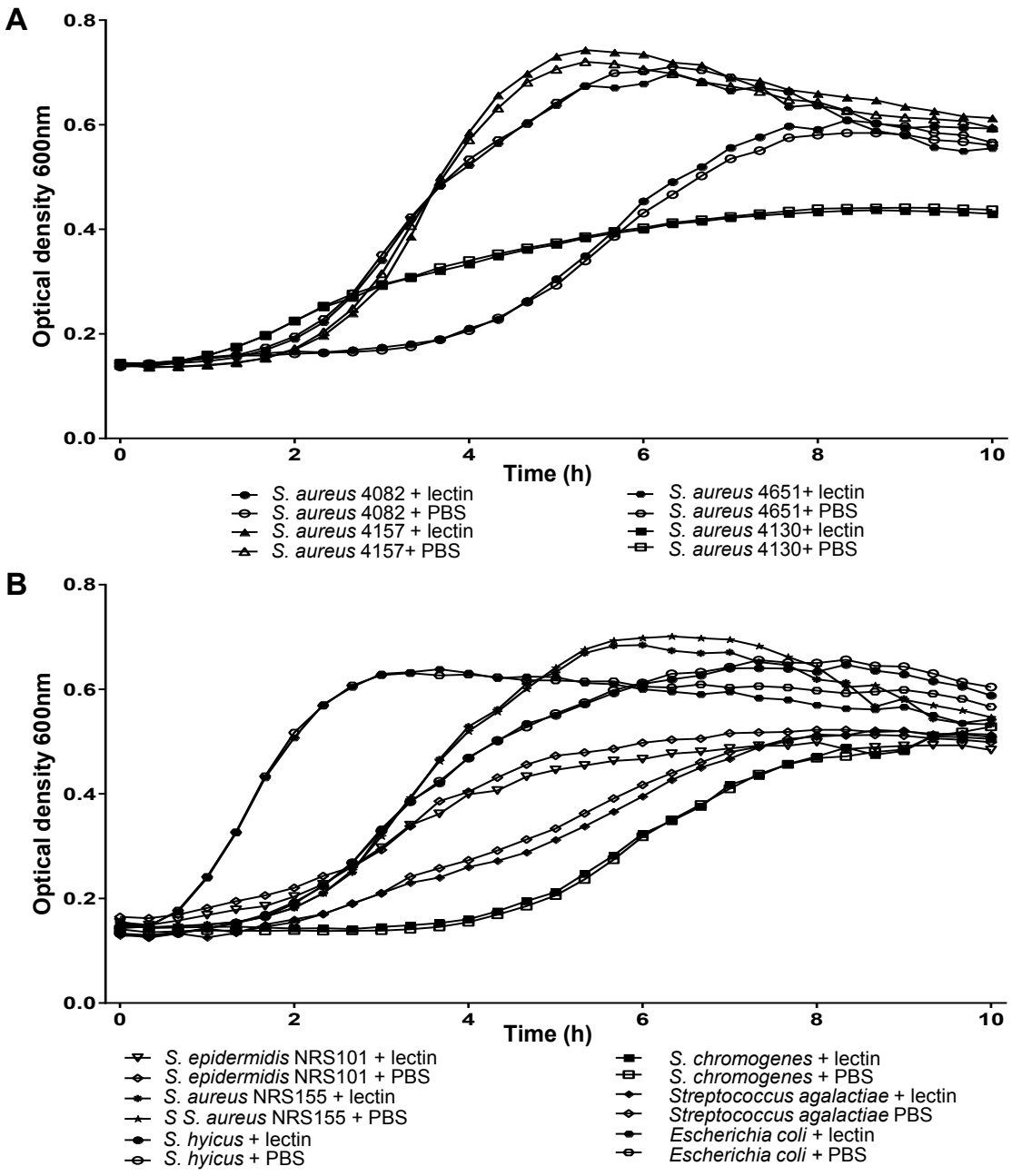


Figure S1. Effect of lectin on bacterial growth. (A) *S. aureus* NRS155, *S. epidermidis* NRS101, *S. hyicus, S. chromogenes, Streptococcus agalactiae, Escherichia coli*, and (B) *S. aureus* 4082, *S. aureus* 4157, *S. aureus* 4651, and *S. aureus* 4130 were grown in BHI medium with 0.25 % glucose (control) or in the same medium containing 50 µg/mL lectin at 37 ºC. The bacterial growth (OD_{600nm}) was determined using a multidetection microplate reader. The representative results of three independent experiments are shown.