UNIVERSIDADE FEDERAL DE MINAS GERAIS
Instituto de Ciências Agrárias
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Leonardo Ferreira Oliveira

CARACTERIZAÇÃO GENOTÍPICA DE RESISTÊNCIA A ANTIMICROBIANOS E FORMAÇÃO DE BIOFILME EM CEPAS DE *Staphylococcus aureus* ISOLADAS DE LEITE DE VACAS COM MASTITE SUBCLÍNICA

MONTES CLAROS
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Orientadora: Anna Christina de Almeida
Coorientadores: Demerson Arruda Sanglard
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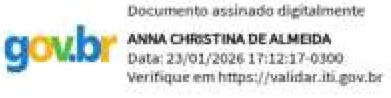
ATA DE DEFESA DE DISSERTAÇÃO

No dia 23 do mês de janeiro de 2026 às 09:30 horas, sob a Presidência da Professora Anna Christina de Almeida, D. Sc. (Orientadora – UFMG/ICA) e com a participação da Professora Eliane Macedo Sobrinho Santos, D. Sc. (Coorientadora - IFNMG), de Geziella Aurea Aparecida Damasceno Souza, D. Sc. (Pós-Doutoranda/Unimontes) e de Débora Martins Paixão, D. Sc. (Pós-Doutora/UFV), reuniu-se, por videoconferência, a Banca de defesa de dissertação de **Leonardo Ferreira Oliveira**, aluno do Curso de Mestrado em Produção Animal. O resultado da defesa de dissertação intitulada **"Caracterização genotípica de resistência a antimicrobianos e formação de biofilmes em cepas de *Staphylococcus aureus* isoladas de leite de vacas com mastite subclínica"**, sendo o aluno considerado **aprovado.** E, para constar, eu, Professora Anna Christina de Almeida, Presidente da Banca, lavrei a presente Ata que depois de lida e aprovada, será assinada por mim e pelos demais membros da Banca examinadora.

OBS.: O aluno somente receberá o título após cumprir as exigências do ARTIGO 53 do regulamento e da resolução 05/2016 do Curso de Mestrado em Produção Animal.

Montes Claros, 23 de janeiro de 2026.



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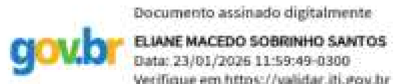

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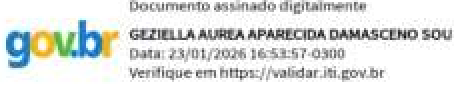

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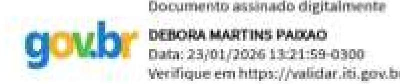

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RESUMO

A mastite bovina constitui um dos principais desafios sanitários e econômicos da pecuária leiteira mundial, caracterizando-se pela inflamação das glândulas mamárias decorrente principalmente de infecções bacterianas. Este estudo objetivou pesquisar e caracterizar marcadores moleculares de resistência antimicrobiana e produção de biofilme em cepas de *Staphylococcus aureus* isoladas de amostras de leite de vacas com mastite subclínica, além de determinar a diversidade genética e os perfis de resistência das espécies presentes. Foram analisados 30 isolados de *S. aureus* provenientes de rebanhos leiteiros, identificados por MALDI-TOF e submetidos à caracterização fenotípica e molecular. A análise molecular identificou elevada prevalência do gene *bla*Z (70%), explicando a resistência generalizada à penicilina, enquanto a ausência dos genes *mec*A, *mec*C e carbapenemases (*bla*KPC, *bla*OXA_{23}) indicou mecanismos alternativos de resistência não associados ao MRSA clássico. Quanto ao potencial de formação de biofilme, os genes *ica*A e *ica*D foram detectados em 80% dos isolados, o operon *ica* completo em 10% e o gene *bap* em 30%, evidenciando forte predisposição genética para esse fator de virulência. A análise da diversidade genética mediante oligonucleotídeos ISSR demonstrou expressiva variabilidade entre os isolados, com 48% dos primers gerando padrões polimórficos e ampla dispersão geográfica sem agrupamento municipal definido, sugerindo disseminação intermunicipal do patógeno. Os resultados evidenciam que as cepas analisadas representam uma dupla ameaça à saúde animal: elevada resistência aos β-lactâmicos mediada principalmente por *bla*Z e forte capacidade de formação de biofilmes, combinação que contribui para infecções persistentes e de difícil tratamento. A ausência de estruturação geográfica definida corrobora a hipótese de disseminação regional influenciada pela movimentação animal e deficiências nos protocolos de biossegurança. Este estudo valida os oligonucleotídeos ISSR como ferramenta viável para caracterização genética de *S. aureus*, oferecendo suporte à vigilância epidemiológica e ao desenvolvimento de estratégias terapêuticas mais eficazes para o manejo da mastite bovina subclínica.

PALAVRAS-CHAVE: PECUÁRIA LEITEIRA; SAÚDE ANIMAL; BLAZ; ISSR; EPIDEMIOLOGIA MOLECULAR; BETA-LACTÂMICOS.

ABSTRACT

Bovine mastitis represents one of the major health and economic challenges in dairy farming worldwide, characterized by inflammation of the mammary glands resulting primarily from bacterial infections. This study aimed to investigate and characterize molecular markers of antimicrobial resistance and biofilm production in *Staphylococcus aureus* strains isolated from milk samples of cows with subclinical mastitis, as well as to determine the genetic diversity and resistance profiles of the species present. Thirty *S. aureus* isolates from dairy herds were analyzed, identified by MALDI-TOF, and subjected to phenotypic and molecular characterization. Molecular analysis identified a high prevalence of the *bla*Z gene (70%), accounting for the widespread penicillin resistance, whereas the absence of *mec*A, *mec*C, and carbapenemase genes (*bla*KPC, *bla*OXA_{23}) indicated alternative resistance mechanisms not associated with classical MRSA. Regarding biofilm formation potential, the *ica*A and *ica*D genes were detected in 80% of isolates, the complete *ica* operon in 10%, and the *bap* gene in 30%, demonstrating a strong genetic predisposition for this virulence factor. Genetic diversity analysis using ISSR oligonucleotides revealed substantial variability among isolates, with 48% of primers generating polymorphic patterns and broad geographic dispersion without defined municipal clustering, suggesting inter-municipal dissemination of the pathogen. The results demonstrate that the analyzed strains represent a dual threat to animal health: high β-lactam resistance mediated primarily by *bla*Z and strong biofilm formation capability, a combination that contributes to persistent and difficult-to-treat infections. The absence of defined geographic structuring corroborates the hypothesis of regional dissemination influenced by animal movement and deficiencies in biosecurity protocols. This study validates ISSR oligonucleotides as a viable tool for genetic characterization of *S. aureus*, providing support for epidemiological surveillance and the development of more effective therapeutic strategies for managing subclinical bovine mastitis.

KEYWORDS: DAIRY CATTLE; ANIMAL HEALTH; BLAZ GENE; ISSR MARKERS; MOLECULAR EPIDEMIOLOGY; BETA-LACTAMS.

LISTA DE FIGURAS

Figura 1 - Figure 1. Phenotypic resistance and sensitivity of *S. aureus* isolates achieved against β-lactam antimicrobials 38

Figura 2 - Figure 2. Frequency of the blaZ gene and the number of isolated individuals per sampled municipality 39

Figura 3 - Figure 3. Detection of biofilm-forming genes among *S. aureus* isolates in this study by PCR 41

Figura 4 - Figure 4. Heatmap: Correlation matrix between molecular resistance markers and biofilm formation 41

Figura 5 - Figure 1. Distribution of results obtained from temperature gradient tests with 52 ISSR primers in *S. aureus* DNA 58

Figura 6 - Figure 2. Dendogram 62

LISTA DE TABELAS

Tabela 1 - Table 1. List of primers, sequence, expected amplicon size, and references for PCR analysis for detection of genes related to β-lactam resistance and biofilm formation..... 37
Tabela 2 - Table 2. Phenotypic and genotypic resistance profiles antimicrobials of bacterial isolates 38
Tabela 3 - Table 3. Gene profiles associated with biofilms of bacterial isolates 40
Tabela 4 - Table 1. Results of total number of bands amplified per temperature involving *Staphylococcus aureus* DNA amplified with ISSR oligonucleotides (UBC primer set #9, Vancouver, Canada) 59
Tabela 5 - Table 2. Results of total number of bands amplified per temperature involving *S. aureus* DNA amplified with ISSR oligonucleotides 60
Tabela 6 - Table 3. Dissimilarity matrix (%) (Jaccard index) among 30 *S. aureus* isolates, estimated from 58 fragments (loci), using 12 ISSR oligonucleotides developed by the University of British Columbia 61

LISTA DE ABREVITURAS

EPS	Extracellular Polymeric Substance
MRSA	Methicillin-Resistant *Staphylococcus aureus*
PIA	Polysaccharide Intercellular Adhesin
femA	Factor Essential for Methicillin
PBP2a	Penicillin-Binding Protein 2a
PCR	Reação em Cadeia da Polimerase
ISSR	Inter-Simple Sequence Repeats

SUMÁRIO

1. **INTRODUÇÃO** 11
2. OBJETIVOS 12
2.1. Objetivo Geral 12
2.2. Objetivos Específicos 12
3. **REFERENCIAL TEÓRICO** 13
3.1. Mastite bovina: Uma preocupação sanitária e econômica 13
3.2. Mecanismos de resistência bacteriana 14
3.3. Importância do gênero *Staphylococcus* 15
3.4. Genes de resistência em *Staphylococcus* 16
3.5. Biofilmes - Estrutura e Formação 18
3.6. Diversidade genética e as inter-relações de cepas 20
REFERÊNCIAS 21
4. ARTIGO CIENTÍFICO 1 - GENOTYPIC CHARACTERIZATION OF BIOFILM-FORMING AND ANTIMICROBIAL-RESISTANT S*taphylococcus aureus* IN BOVINE SUBCLINICAL MASTITIS 30
ABSTRACT 31
RESUMO 32
INTRODUCTION 33
MATERIALS AND METHODS 33
RESULTS 36
DISCUSSION 42
CONCLUSIONS 45
REFERENCE 46
5. ARTIGO CIENTÍFICO 2 - GENETIC DIVERSITY OF *Staphylococcus aureus* ISOLATES FROM SUBCLINICAL BOVINE MASTITIS USING INTER-SIMPLE SEQUENCE REPEAT OLIGONUCLEOTIDES 51
ABSTRACT 52
RESUMO 53
INTRODUCTION 54
MATERIAL AND METHODS 54
RESULTS 57
DISCUSSION 62
CONCLUSION 65
REFERENCES 66
6. CONSIDERAÇÕES FINAIS 71
ANEXO 1 – Tabela de identificação, caracterização feno-genotípica e localização geográfica das cepas 72
ANEXO 2 – Resultados da quantificação por espectrofotometria de DNA's extraídos de cepas de *Staphylococcus aureus* 73
ANEXO 3 – Fotodocumentação de teste de gradiente utilizando oligonucleotídeos ISSR 74
ANEXO 4 - Comprovante de submissão artigos 1 e 2. 79

1. INTRODUÇÃO

A mastite bovina subclinica representa um dos principais desafios sanitários e econômicos da pecuária leiteira mundial. Caracterizada pela inflamação das glândulas mamárias, essa enfermidade resulta principalmente de infecções bacterianas que comprometem simultaneamente a saúde animal e a produtividade do rebanho, acarretando perdas econômicas substanciais. Nesse contexto, a identificação precisa dos patógenos causadores e a caracterização detalhada dos perfis de resistência antimicrobiana constituem pilares fundamentais para o desenvolvimento de estratégias terapêuticas eficazes, que assegurem tanto o bem-estar animal quanto a sustentabilidade da produção leiteira (Aliyev; Karpuschenko, 2023; Souza *et al*., 2020).

A emergência e disseminação da resistência bacteriana aos antimicrobianos configuram uma questão crítica em saúde pública, com impactos significativos tanto na medicina humana quanto na veterinária. No cenário da produção animal, a presença de biofilmes formados por cepas bacterianas multirresistentes representa um desafio complexo. Estas estruturas microbianas contribuem para o estabelecimento de infecções persistentes de difícil controle, comprometendo não apenas a saúde dos animais e a produtividade das propriedades rurais, mas também a segurança alimentar da população (Silva *et al*., 2020; Morais *et al*., 2023).

Os biofilmes constituem comunidades bacterianas complexas, organizadas em estruturas tridimensionais e envoltas por uma matriz extracelular autoproduzida. Esta organização confere proteção substancial às células bacterianas, permitindo sua sobrevivência mesmo sob condições adversas, incluindo a exposição a agentes antimicrobianos. No contexto da pecuária leiteira, biofilmes podem se estabelecer em diversas superfícies, tais como equipamentos de ordenha, sistemas de abastecimento de água e recipientes de alimentação, funcionando como reservatórios persistentes de patógenos com potencial para infectar os animais. No interior das glândulas mamárias, a formação de biofilmes agrava significativamente o quadro de mastite, dificultando o tratamento e comprometendo tanto a quantidade quanto a qualidade da produção leiteira (Pedersen *et al*., 2021; Tartor *et al*., 2022; Gajewska; Chajęcka-Wierzchowska, 2020).

A formação de biofilmes apresenta estreita associação com a resistência antimicrobiana, fenômeno em grande parte atribuído à intensificação da troca genética horizontal no interior dessas estruturas e à ativação de genes que conferem resistência. A identificação e caracterização molecular desses determinantes genéticos em cepas formadoras de biofilme são

essenciais para a compreensão dos mecanismos de defesa bacteriana e para o desenvolvimento de estratégias de controle direcionadas e eficazes (Pajohesh *et al*., 2022; Ali, 2022).

O estudo dos fatores de virulência, particularmente a capacidade de formação de biofilme, evidencia a resiliência bacteriana frente aos tratamentos convencionais e às respostas imunológicas do hospedeiro, aumentando a complexidade do manejo das infecções. A investigação sistemática desses aspectos fundamenta o desenvolvimento de soluções precisas e sustentáveis para a preservação da saúde e produtividade animal (Li *et al*., 2020; Minich *et al*., 2022).

As abordagens analíticas fenotípicas e genotípicas constituem ferramentas complementares e fundamentais no estudo da resistência antimicrobiana e da formação de biofilme em bactérias. A análise fenotípica examina características observáveis, como padrões de crescimento bacteriano na presença de diferentes antimicrobianos, enquanto a análise genotípica permite a identificação de genes específicos associados à resistência e à formação de biofilme. Vale ressaltar que discrepâncias entre fenótipo e genótipo são comuns, especialmente devido à presença de genes silenciados ou não expressos sob determinadas condições, o que torna a abordagem molecular ainda mais relevante para uma caracterização precisa. Esta estratégia integrada proporciona compreensão abrangente sobre a resiliência bacteriana e os mecanismos de formação de biofilme. Apesar da relevância, os mecanismos moleculares que ligam a formação de biofilme à resistência em isolados de rebanhos da região norte de Minas Gerais permanecem pouco explorados, justificando investigações que possam subsidiar o desenvolvimento de terapias e medidas de controle mais eficazes para o enfrentamento deste problema na produção animal (Gomes et al., 2023; Freitas; Werner, 2022; İnce; Müştak, 2023).

2. OBJETIVOS

2.1. Objetivo Geral

Caracterizar molecularmente os determinantes genéticos de resistência aos antimicrobianos e de formação de biofilme em cepas de *Staphylococcus aureus* isoladas de leite de vacas com mastite subclínica.

2.2. Objetivos Específicos

- Identificar a presença de marcadores genéticos relacionados à formação de biofilme e resistência a antimicrobianos betalactâmicos;
- Correlacionar os perfis fenotípicos e genotípicos de resistência com a capacidade potencial de formação de biofilme;
- Avaliar a diversidade e a similaridade genética entre as cepas isoladas utilizando marcadores moleculares ISSR (*Inter Simple Sequence Repeat*).

3. REFERENCIAL TEÓRICO

3.1. Mastite bovina: Uma preocupação sanitária e econômica

A mastite em vacas leiteiras constitui uma enfermidade de impacto multifacetado, afetando tanto a produção leiteira quanto o bem-estar animal. Nos casos clínicos, a sintomatologia inclui manifestações evidentes como dor, edema mamário e alterações macroscópicas no leite, tais como presença de pus ou sangue. Já nos casos subclínicos, embora os sinais externos sejam sutis ou ausentes, a doença ainda compromete a produção e a qualidade do leite, elevando as contagens de células somáticas e reduzindo os teores de proteína e gordura. Esta condição compromete a eficiência produtiva, resultando em perdas econômicas substanciais decorrentes do descarte de leite e do aumento dos custos com tratamentos veterinários (Biscarini *et al*., 2020; Franco *et al*., 2022; Morales-Ubaldo *et al*., 2023; Caciano *et al*., 2023; Pereira *et al*., 2024).

A etiologia da mastite bovina é diversificada, englobando diversos microrganismos patogênicos. Entre as bactérias Gram-positivas, destacam-se os gêneros *Streptococcus* spp. e *Staphylococcus* spp., com especial ênfase para *Staphylococcus aureus*. Estes patógenos podem ser classificados em duas categorias principais: agentes contagiosos, que se disseminam predominantemente durante o processo de ordenha através do contato direto entre animais, e agentes ambientais, que são adquiridos mediante exposição a ambientes contaminados. A compreensão das particularidades epidemiológicas destes diferentes patógenos é fundamental para a implementação de protocolos terapêuticos adequados e estratégias preventivas eficazes no controle dos riscos de infecção no rebanho (Franco *et al*., 2022; Nascimento *et al*., 2023).

A emergência de microrganismos multirresistentes representa um desafio adicional significativo no tratamento da mastite. Estas cepas apresentam resistência aos antimicrobianos convencionalmente empregados, demandando a utilização de terapias alternativas ou

combinadas que elevam os custos de tratamento, prolongam os períodos de recuperação e aumentam os períodos de descarte de produtos de origem animal. A disseminação da resistência antimicrobiana dentro do rebanho configura um risco à saúde pública, reforçando a necessidade imperativa do uso racional e responsável de antimicrobianos no manejo sanitário animal (Oliveira *et al*., 2022; Zhao *et al*., 2024).

Numerosas bactérias causadoras de mastite apresentam capacidade de formar biofilmes. Estas estruturas conferem proteção adicional às células bacterianas, permitindo sua persistência na glândula mamária e estabelecendo infecções crônicas de difícil resolução terapêutica. O reconhecimento destes fatores de virulência e da importância dos biofilmes na patogênese da mastite é essencial para o desenvolvimento de estratégias aprimoradas de tratamento e controle, fundamentais para minimizar os impactos desta enfermidade sobre a saúde animal e a produção leiteira (Pedersen *et al*., 2021; Andrade *et al*., 2021; Vijayakumar; Jose, 2021).

3.2. Mecanismos de resistência bacteriana

Os agentes antimicrobianos desempenham papel fundamental no combate às infecções bacterianas, atuando sobre alvos moleculares específicos essenciais para a viabilidade bacteriana. Diferentes classes de antimicrobianos apresentam mecanismos de ação distintos: os beta-lactâmicos inibem a síntese da parede celular bacteriana, as quinolonas interferem na replicação do DNA, enquanto os aminoglicosídeos e as tetraciclinas atuam como inibidores da síntese proteica através de sua interação com os ribossomos bacterianos. Esta especificidade de ação permite que os antimicrobianos exerçam efeitos bactericidas ou bacteriostáticos sobre as células bacterianas, minimizando os impactos sobre as células do hospedeiro (Aminov, 2021; Devi *et al*., 2024; Belay *et al*., 2024).

A resistência bacteriana frequentemente emerge como resposta adaptativa às pressões seletivas exercidas por estes compostos antimicrobianos. Um dos mecanismos mais prevalentes é a produção de enzimas modificadoras, tais como as beta-lactamases, que promovem a inativação enzimática de determinados antibióticos. Estratégias adicionais de resistência incluem a alteração dos sítios-alvo dos antimicrobianos, a redução da permeabilidade da membrana celular e a expressão de sistemas de efluxo ativo que expelem os antimicrobianos antes que possam exercer seus efeitos deletérios (Kaderabkova *et al*., 2022; Agarwal *et al*., 2023; Zhuang *et al*., 2023).

As mutações genéticas espontâneas e os mecanismos de transferência horizontal de genes constituem vias adicionais através das quais as bactérias adquirem resistência antimicrobiana. A transferência horizontal de determinantes genéticos de resistência pode ocorrer através de três mecanismos principais: conjugação (transferência mediada por plasmídeos), transformação (captação de DNA livre do ambiente) e transdução (transferência mediada por bacteriófagos). Estes mecanismos possibilitam a rápida disseminação de genes de resistência entre populações bacterianas, representando desafios substanciais para o tratamento de infecções, particularmente em ambientes com elevada pressão seletiva por antimicrobianos (Uddin *et al*., 2021; Zeng *et al*., 2022; Halawa *et al*., 2024).

3.3. Importância do gênero *Staphylococcus*

O gênero *Staphylococcus* compreende cocos Gram-positivos caracterizados por seu arranjo morfológico em agrupamentos semelhantes a cachos de uvas. Estes microrganismos constituem parte da microbiota comensal natural da pele e das mucosas de mamíferos, podendo, entretanto, causar enfermidades quando ocorre comprometimento da imunidade do hospedeiro ou ruptura das barreiras tegumentares. No contexto da pecuária leiteira, *Staphylococcus* destaca-se como agente etiológico primário da mastite, uma inflamação da glândula mamária que compromete a qualidade e o volume da produção leiteira, impactando negativamente a economia do setor (He *et al*., 2020; Nascimento *et al*., 2023; Morales-Ubaldo *et al*., 2023).

O gênero *Staphylococcus* demonstra notável potencial para desenvolver resistência antimicrobiana, incluindo a resistência à meticilina, que resulta da produção de beta-lactamases e de modificações nas proteínas de ligação à penicilina, particularmente a PBP2a (Penicillin-Binding Protein 2a). Estas alterações estruturais reduzem a afinidade dos antibióticos beta-lactâmicos por seus alvos moleculares, tornando-os ineficazes. Adicionalmente, diversas cepas apresentam resistência a macrolídeos, lincosamidas e aminoglicosídeos, complexificando as estratégias de controle das infecções estafilocócicas (Foster, 2017; Vestergaard *et al*., 2019; Mlynarczyk-Bonikowska *et al*., 2022).

A capacidade de *S. aureus* formar biofilmes representa um desafio na produção animal. A matriz extracelular dos biofilmes atua como estrutura protetora, permitindo que as células bacterianas adiram firmemente às superfícies e resistam tanto aos antimicrobianos quanto aos mecanismos de defesa imunológica do hospedeiro. Esta proteção multifatorial torna as infecções mais persistentes e refratárias à erradicação terapêutica. Em condições como a mastite

crônica, os biofilmes desempenham papel crítico, favorecendo a recorrência de infecções e dificultando o controle, o que agrava as perdas econômicas e compromete a qualidade dos produtos lácteos (Peng *et al*., 2022; Tuon *et al*., 2023).

3.4. Genes de resistência em *Staphylococcus*

A caracterização molecular dos genes de resistência em *Staphylococcus* proporciona conhecimento fundamental sobre os mecanismos que conferem resistência aos antimicrobianos convencionais. Os genes *mec*A e *mec*C, associados ao fenótipo MRSA (*Methicillin-Resistant Staphylococcus aureus*), conferem resistência à meticilina através da codificação de proteínas de ligação à penicilina alteradas (PBP2a e PBP2c), que apresentam baixa afinidade pelos antibióticos beta-lactâmicos, comprometendo sua eficácia terapêutica. O gene *fem*A (*factor essential for methicillin resistance*) complementa este mecanismo ao estabilizar estas proteínas modificadas, potencializando a síntese da parede celular e aumentando a resiliência bacteriana. Adicionalmente, genes como *bla*Z contribuem para este fenótipo através da codificação de beta-lactamases que promovem a hidrólise enzimática da penicilina, restringindo ainda mais as opções terapêuticas (Nobrega *et al*., 2018; Mlynarczyk-Bonikowska *et al*., 2022; Heuser *et al*., 2022).

Outros determinantes genéticos relevantes incluem os genes *erm*A, *erm*B e *ermC*, que conferem resistência aos macrolídeos, lincosamidas e estreptograminas (fenótipo MLS) através da metilação do RNA ribossômico 23S, alterando os sítios de ligação ribossômica destes antimicrobianos. Estes genes podem apresentar expressão constitutiva ou induzível, sendo esta última ativada apenas após exposição inicial aos antibióticos. Esta variabilidade de expressão influencia diretamente as estratégias terapêuticas, uma vez que diferentes cepas bacterianas podem apresentar padrões distintos de resposta às diversas classes de antimicrobianos (Khodabandeh *et al*., 2019; Lund *et al*., 2022; Ma *et al*., 2022).

A resistência às tetraciclinas, governada pelos genes *tet*K e *tet*M, envolve mecanismos moleculares distintos. O gene *tet*K codifica uma proteína de efluxo ativo que expele o antibiótico da célula bacteriana, enquanto *tet*M codifica proteínas de proteção ribossômica que impedem a ligação do antimicrobiano ao seu alvo. A prevalência destes genes em ambientes de produção animal reflete o uso frequente de tetraciclinas como opções terapêuticas economicamente acessíveis para o controle de infecções bacterianas (Zeng *et al*., 2022; Wang *et al*., 2024; Zhang *et al*., 2024).

Os genes *fus*A e *fus*C conferem resistência ao ácido fusídico, antimicrobiano frequentemente utilizado no tratamento de infecções estafilocócicas em bovinos. A resistência resulta de modificações nos fatores de elongação da síntese proteica (EF-G), criando obstáculos adicionais significativos no manejo terapêutico das infecções. Similarmente, os genes de resistência aos glicopeptídeos *van*A e *van*B promovem alterações nos precursores peptidoglicanos, modificando os sítios de ligação da vancomicina. Esta resistência representa ameaça grave, dado o papel da vancomicina como antimicrobiano de última escolha no tratamento de infecções por Gram-positivos multirresistentes (Oliveira *et al*., 2022; Afshari *et al*., 2022; Memili *et al*., 2022).

A presença do gene *nor*A, que codifica uma bomba de efluxo de fluoroquinolonas, constitui outro fator de resistência significativo na pecuária, onde as fluoroquinolonas são amplamente empregadas. Este gene reduz a concentração intracelular do antimicrobiano, dificultando o controle das infecções e enfatizando a necessidade de administração antimicrobiana criteriosa (Palazzotti *et al*., 2019).

Diversos genes estão associados à resistência aos aminoglicosídeos, codificando enzimas modificadoras que promovem a inativação destes antibióticos através de acetilação, fosforilação ou adenilação, dificultando o tratamento de infecções bacterianas graves. O gene *aac(6')-Ie-aph(2")-Ia*, prevalente em MRSA, confere resistência a múltiplos aminoglicosídeos, incluindo gentamicina, tobramicina e amicacina. O gene *aph(3')-IIIa* codifica uma aminoglicosídeo fosfotransferase que confere resistência a canamicina, amicacina e neomicina. O gene *ant(4')-Ia* está associado à resistência à estreptomicina através de adenilação do antibiótico. Adicionalmente, os genes *aph(2")-Ib*, *aph(2")-Ic* e *aph(2")-Id* codificam fosfotransferases que conferem resistência a diversos aminoglicosídeos, especialmente em contextos com elevada pressão seletiva. Outro determinante importante é o gene *dfr*A, que confere resistência ao trimetoprim, antimicrobiano frequentemente utilizado em combinações sinérgicas com sulfonamidas. O *dfr*A atua modificando a enzima dihidrofolato redutase, alvo do trimetoprim, reduzindo sua eficácia terapêutica (Plattner *et al*., 2020; Özdemir, 2022; Shoaib *et al*., 2023; El Zowalaty *et al*., 2023).

Os genes *qacA* e *qacB* conferem resistência a compostos quaternários de amônio, agentes antimicrobianos amplamente utilizados em desinfetantes para manutenção da higiene nas instalações de produção animal. O gene *qac*A apresenta maior prevalência em *Staphylococcus*, enquanto *qac*B é predominantemente encontrado em *Enterococcus*. Ambos os

genes codificam proteínas de efluxo que exportam ativamente os agentes antimicrobianos para o exterior da célula bacteriana (Hong *et al*., 2019; McNeil *et al*., 2023).

3.5. Biofilmes - Estrutura e Formação

Bactérias produtoras de biofilme frequentemente abrigam múltiplos genes de resistência antimicrobiana, fenômeno que exerce efeitos profundos na produção animal, principalmente através do aumento da persistência e recorrência de infecções. Estas infecções tornam-se progressivamente mais desafiadoras de tratar, considerando a proteção combinada conferida pelas estruturas de biofilme e a resistência a múltiplos antibióticos. As consequências incluem aumento no consumo de antimicrobianos, elevação dos custos terapêuticos, diminuição da produtividade animal e aumento do risco de exposição ambiental e humana a patógenos resistentes. Coletivamente, estes resultados comprometem a eficiência produtiva e a segurança alimentar, apresentando riscos de contaminação ao longo de toda a cadeia de produção (Gajewska; Chajęcka-Wierzchowska, 2020; Schiffer *et al*., 2021; Pajohesh *et al*., 2022; Ali, 2024).

A formação do biofilme bacteriano desenvolve-se através de uma série de fases sequenciais e bem caracterizadas. Inicialmente, as células bacterianas estabelecem aderência reversível às superfícies, ligação que pode se tornar irreversível mediante a produção de proteínas adesivas específicas. Subsequentemente, forma-se uma microcolônia à medida que as células bacterianas se multiplicam e secretam componentes da matriz extracelular, incluindo proteínas, polissacarídeos e ácidos nucleicos, conferindo estabilidade estrutural ao biofilme. Durante a fase de maturação, o biofilme adquire maior complexidade estrutural e organizacional, desenvolvendo canais aquosos que facilitam a circulação de nutrientes e a remoção de metabólitos. Finalmente, na fase de dispersão, células bacterianas individuais se desprendem da estrutura madura para colonizar novas superfícies, facilitando assim a disseminação do biofilme para outras áreas (Schilcher; Horswill, 2020; Rather *et al*., 2021;).

Em bactérias causadoras de mastite bovina, a substância polimérica extracelular (EPS - *Extracellular Polymeric Substance*) constitui componente fundamental da estabilidade estrutural do biofilme. Funcionando como uma matriz adesiva composta por polissacarídeos, proteínas e ácidos nucleicos, o EPS envolve as células bacterianas, proporcionando proteção contra agentes antimicrobianos e contra a resposta imunológica do hospedeiro, tornando o tratamento da mastite mais complexo. Adicionalmente, o EPS permite que estas bactérias

adiram às superfícies de equipamentos de ordenha e recipientes de armazenamento de leite, perpetuando a contaminação e a transmissão de infecções entre animais e produtos lácteos, comprometendo a qualidade do leite e a segurança do consumidor (Schiffer *et al*., 2019; Pedersen *et al*., 2021; Jyoti *et al*., 2024).

As proteínas amiloides bacterianas desempenham papel central na arquitetura do biofilme, fornecendo um arcabouço resiliente e estável que protege contra desafios ambientais diversos, tais como dessecação, predação e exposição a antimicrobianos. Estas proteínas apresentam capacidade de automontagem em fibras altamente estáveis, reforçando a integridade estrutural do biofilme. Identificadas em diversas espécies bacterianas, as proteínas amiloides exercem funções essenciais na adesão celular e na maturação do biofilme. A matriz extracelular - uma rede complexa composta por proteínas, polissacarídeos e DNA extracelular - deve grande parte de sua coesão às fibras amiloides, que amplificam a estabilidade e a resiliência do biofilme (Ruhal; Katarina, 2021; Sønderby *et al*., 2022).

A produção de biofilme em *Staphylococcus* envolve a expressão coordenada de genes específicos que promovem a adesão celular e a formação da matriz extracelular. O operon *ica* (*intercellular adhesion*), responsável pela síntese da PIA (*Polysaccharide Intercellular Adhesin*, compreende quatro genes essenciais: *ica*A, *ica*B, *ica*C e *ica*D. Estes genes são vitais para este processo, codificando proteínas essenciais para a adesão intercelular, componente primário da matriz do biofilme. Este operon constitui alvo frequente de pesquisas direcionadas ao controle da formação de biofilme em ambientes clínicos e de produção animal (Mirzaei *et al*., 2021; Zheng *et al*., 2021; Jyoti *et al*., 2024).

A Proteína Associada ao Biofilme (Bap - *Biofilm Associated Protein*) é crucial para o desenvolvimento de biofilme em algumas cepas de *S. aureus* e outras espécies de *Staphylococcus*, promovendo agregação bacteriana e estrutura densa de biofilme. A presença de Bap está associada à capacidade da bactéria persistir em infecções crônicas, com a matriz amiloide conferindo proteção adicional (Schiffer *et al*., 2019; Schiffer *et al*., 2021; Shivaee *et al*., 2019). Coletivamente, estas proteínas amiloides, incluindo TasA, Curli, Fap, Esp e Bap, se organizam em fibras que conferem aos biofilmes resistência e durabilidade, propriedades essenciais para a adaptação bacteriana em condições adversas (Ruhal & Katarina, 2021; Sønderby *et al*., 2022; Khalil *et al*., 2022; Nicastro *et al*., 2022).

Em termos de composição molecular, o EPS do biofilme de *S. aureus* é constituído principalmente por adesina intercelular polissacarídica, que desempenha papel fundamental na colonização, no desenvolvimento do biofilme, na evasão imunológica e na resistência

antimicrobiana. O EPS em *S. aureus* também interage com proteínas como as proteínas associadas à acumulação (Aap - *Accumulation-Associated Protein*), que auxiliam na maturação do biofilme, além de outras proteínas de ligação que suportam a fixação à superfície, a infecção e a resiliência do biofilme (Mirzaei *et al*., 2021; Nasser *et al*., 2022; Hernández-Cuellar *et al*., 2023; Abdulqader & Abood, 2024).

3.6. Diversidade genética e as inter-relações de cepas

O rastreamento da linhagem genética de cepas de *S. aureus* isoladas de casos de mastite bovina constitui campo de estudo crítico com relevância substancial para o controle da resistência antimicrobiana na pecuária. Investigações epidemiológicas moleculares indicam que as cepas de *S. aureus* isoladas de vacas com mastite frequentemente albergam repertórios diversos de genes de resistência e virulência, cuja prevalência e variação são influenciadas pelas práticas de manejo local e por fatores ambientais específicos. Estes determinantes genéticos podem se disseminar através de elementos genéticos móveis, tais como plasmídeos e ilhas de patogenicidade (PAIs - *Pathogenicity Islands*), mecanismos que permitem adaptação e resposta rápida das populações bacterianas às pressões seletivas específicas presentes nos ambientes de produção leiteira. Consequentemente, agrupamentos genéticos distintos de *S. aureus* têm sido identificados em diferentes regiões leiteiras, apresentando adaptações únicas a rebanhos locais e evidências de fluxo gênico em áreas geograficamente interconectadas (Campos *et al*., 2022; Khasapane *et al*., 2024).

A disseminação de genes de resistência depende do grau de conectividade regional entre as propriedades rurais. Em áreas onde as fazendas estão geograficamente próximas, o compartilhamento de equipamentos, o transporte de animais e o contato direto entre rebanhos facilitam o intercâmbio genético entre populações de *S. aureus*. Em regiões geograficamente separadas, porém conectadas por rotas comerciais, determinados clones resistentes têm sido documentados com distribuição global, ilustrando como características de resistência podem propagar-se por longas distâncias através de redes comerciais. Inversamente, regiões geograficamente isoladas podem apresentar padrões genéticos mais distintos devido ao fluxo gênico restrito de fontes externas, embora introduções periódicas de genes de resistência ainda sejam possíveis através de interações limitadas, incluindo contato ocasional com humanos ou movimentação animal restrita (Sila *et al*., 2021; Campos *et al*., 2022; Silva *et al*., 2023).

Para investigar a diversidade genética e as inter-relações evolutivas entre cepas de *S. aureus*, pesquisadores frequentemente empregam marcadores moleculares ISSR (*Inter Simple Sequence Repeat*), que são eficazes na amplificação de sequências repetitivas específicas do DNA genômico. Esta técnica molecular permite a construção de dendrogramas detalhados que mapeiam visualmente as relações filogenéticas entre isolados bacterianos. Através destes dendrogramas, os investigadores podem identificar padrões de linhagem clonal e conexões evolutivas entre cepas provenientes de diferentes localidades geográficas, proporcionando informações críticas sobre a disseminação geográfica e o desenvolvimento evolutivo de linhagens de *S. aureus*. Os marcadores ISSR oferecem, portanto, uma ferramenta molecular de alta resolução, essencial para estudos epidemiológicos onde a distinção entre cepas relacionadas é fundamental para compreender a dinâmica de transmissão e estabelecer estratégias de controle eficazes (Polido *et al*., 2020; Naushad *et al*., 2020; Achek *et al*., 2021; Campos *et al*., 2022).

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4. ARTIGO CIENTÍFICO 1 - GENOTYPIC CHARACTERIZATION OF BIOFILM-FORMING AND ANTIMICROBIAL-RESISTANT S*taphylococcus aureus* IN BOVINE SUBCLINICAL MASTITIS

Artigo escrito de acordo com as normas da Revista **Journal of Antimicrobial Chemotherapy**

GENOTYPIC CHARACTERIZATION OF BIOFILM-FORMING AND ANTIMICROBIAL-RESISTANT *Staphylococcus aureus* IN BOVINE SUBCLINICAL MASTITIS

ABSTRACT

Background: *Staphylococcus aureus* is a main etiological agents of bovine mastitis. It leads to significant economic losses and challenging treatments. The increase of antimicrobial resistance and biofilm formation in isolated strains from dairy herds lead infection control difficulties, especially in subclinical cases.

Objective: This study aimed to characterize the molecular markers of antimicrobial resistance and biofilm formation in *S. aureus* isolates recovered from cases of subclinical bovine mastitis in regional dairy herds.

Methods: Thirty *S. aureus* isolateds were identified by MALDI-TOF mass spectrometry. Antimicrobial susceptibility testing was performed against a panel of β-lactam antibiotics. PCR-based assays were conducted to detect resistance genes (*bla*Z, *mec*A, *mec*C, *bla*KPC, *bla*OXA_{23}) and biofilm-associated genes (the complete *ica* operon, and *bap*), using ATCC reference strains as controls.

Results: Antimicrobial susceptibility testing revealed high resistance rates: 93.3% to penicillin and ampicillin, 90% to amoxicillin, 70% to oxacillin, and 63.3% to meropenem. Cefoxitin was the most effective, with 76.7% susceptibility. The blaZ gene was detected in 70% of isolates, correlating with widespread penicillin resistance. All isolates tested negative for *mec*A, *mec*C, and the carbapenemase genes, indicating non-MRSA and non-carbapenemase-mediated mechanisms for oxacillin and meropenem resistance. Biofilm gene analysis showed a high genetic potential: *ica*A and *ica*D were present in 80% of isolates, the complete *ica* operon in 10%, and the *bap* gene in 30%.

Conclusions: The findings demonstrate a dual threat in *S. aureus* from subclinical bovine mastitis: high β-lactam resistance, primarily mediated by *bla*Z, and associated with a strong genetic predisposition for biofilm formation. This combination likely contributes to persistent, treatment-refractory infections, significantly complicating disease management in the region.

Keywords: animal production; antibiotic; dairy cattle; Brazil.

CARACTERIZAÇÃO GENOTÍPICA DE *Staphylococcus aureus* FORMADOR DE BIOFILME E RESISTENTE A ANTIMICROBIANOS EM MASTITE BOVINA SUBCLÍNICA

RESUMO

Introdução: *Staphylococcus aureus* é um dos principais agentes etiológicos da mastite bovina, contribuindo para perdas econômicas significativas e desafios terapêuticos. O aumento da resistência antimicrobiana e da formação de biofilme em isolados provenientes de rebanhos leiteiros dificulta o controle da infecção, especialmente em casos subclínicos.

Objetivo: Este estudo teve como objetivo caracterizar os marcadores moleculares de resistência antimicrobiana e formação de biofilme em isolados de *S. aureus* recuperados de casos de mastite bovina subclínica em rebanhos leiteiros regionais.

Métodos: Trinta isolados de *S. aureus* foram identificados por espectrometria de massas MALDI-TOF. O perfil de suscetibilidade antimicrobiana foi determinado frente a um painel de antibióticos β-lactâmicos. Ensaios baseados em PCR foram realizados para detecção de genes de resistência (*bla*Z, *mec*A, *mec*C, *bla*KPC, *bla*OXA$_{23}$) e genes associados à formação de biofilme (operon *ica* completo e bap), utilizando cepas de referência ATCC como controles.

Resultados: Os testes de suscetibilidade antimicrobiana revelaram elevadas taxas de resistência: 93,3% para penicilina e ampicilina, 90% para amoxicilina, 70% para oxacilina e 63,3% para meropeném. A cefoxitina apresentou maior eficácia, com 76,7% de suscetibilidade. O gene *bla*Z foi detectado em 70% dos isolados, correlacionando-se com a resistência disseminada à penicilina. Todos os isolados testaram negativos para *mec*A, *mec*C e genes codificadores de carbapenemases, indicando mecanismos de resistência à oxacilina e ao meropeném não mediados por MRSA ou carbapenemases. A análise de genes relacionados ao biofilme demonstrou elevado potencial genético: *ica*A e *ica*D estavam presentes em 80% dos isolados, o operon *ica* completo em 10%, e o gene *bap* em 30%.

Conclusões: Os achados demonstram uma dupla ameaça em *S. aureus* provenientes de mastite bovina subclínica: elevada resistência a β-lactâmicos, mediada principalmente por blaZ, associada a uma forte predisposição genética para formação de biofilme. Esta combinação provavelmente contribui para infecções persistentes e refratárias ao tratamento, complicando significativamente o manejo da doença na região.

Palavras-chave: produção animal; antibiótico; gado leiteiro; Brasil.

INTRODUCTION

Bovine mastitis represents a prevalent challenge in dairy production, characterized by inflammation of the mammary glands caused primarily by bacterial infections [1]. This condition adversely affects both animal health and milk productivity, resulting in substantial economic losses [2]. Efforts to identify causative pathogens and characterize antimicrobial resistance profiles are essential for developing effective treatment strategies that promote animal welfare and sustainable production practices [3].

Multiple microorganisms can cause mastitis, with predominant pathogens including Gram-positive bacteria such as *Streptococcus* spp. and *Staphylococcus* spp., particularly *Staphylococcus aureus* [4]. These pathogens are categorized as either contagious agentes - transmitted primarily during milking - or environmental agents acquired through contact with contaminated surroundings [5].

The escalating concern regarding bacterial antimicrobial resistance constitutes a critical public health challenge, impacting both human and veterinary medicine. In livestock production, biofilm formation by multidrug-resistant bacterial strains represents a particularly complex challenge [6]. Biofilms contribute to persistent, difficult-to-control infections that affect animal health, farm productivity, and food safety [7].

Integrated phenotypic and genotypic analyses serve as valuable tools for investigating antimicrobial resistance and biofilm formation in bacteria [8]. Phenotypic analysis examines observable characteristics such as growth patterns in the presence of antimicrobials, while genotypic analysis identifies specific resistance genes and biofilm-associated determinants [9]. This combined approach provides comprehensive insights into bacterial resistance mechanisms and biofilm formation capabilities, supporting the development of more effective therapeutic and control strategies [10].

This study investigated molecular markers of β-lactam antimicrobial resistance and biofilm formation in 30 *S. aureus* isolates from subclinical bovine mastitis collected from dairy herds in the northern region of Minas Gerais, Brazil.

MATERIALS AND METHODS

Sample Collection and Bacterial Isolation

Bacterial strains were isolated from milk samples collected from the teats of dairy cows diagnosed with subclinical mastitis. Approximately 15 ml of milk was collected from each teat using aseptic techniques to prevent contamination. Samples were promptly transported to the

Animal Health Laboratory at the Institute of Agricultural Sciences, Federal University of Minas Gerais (ICA-UFMG). Animals originated from dairy herds on farms distributed across seven municipalities in the northern region of Minas Gerais, Brazil: Janaúba (15°48'05"S, 43°18'20"W), Montes Claros (16°43'41"S, 43°51'03"W), Icaraí de Minas (16°13'06"S, 44°54'13"W), São Pedro das Garças (16°10'15"S, 43°51'57"W), Juramento (16°51'08"S, 43°33'56"W), Nova Esperança (16°34'57"S, 43°56'14"W), and Matias Cardoso (14°51'21"S, 43°55'01"W).

Samples were inoculated onto blood agar medium (HiMedia, India) supplemented with 5% sheep blood and incubated at 35°C ± 2°C for 24-48 hours. Following primary culture, bacterial isolates were characterized based on colony morphology and definitively identified to species level by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry. Only isolates confirmed as *Staphylococcus aureus* were included in this study.

Antimicrobial susceptibility testing for β-lactam antibiotics was performed using the disk diffusion method (Kirby-Bauer). Biofilm formation capacity was assessed using the Congo Red Agar method. These procedures were conducted as part of ongoing research at the Animal Health Laboratory [11,12].

Confirmed bacterial isolates were transferred to 1.5 ml cryovials containing Brain Heart Infusion (BHI) broth (HiMedia, India) supplemented with 20% (v/v) glycerol (Êxodo Cientifica, Brasil) as a cryoprotectant. Samples were stored at -20°C to maintain strain viability and genetic stability.

DNA Extraction

S. aureus strains were selected based on two key phenotypic characteristics: resistance to β-lactam antimicrobials and biofilm production capacity. DNA extraction and subsequent molecular analyses were conducted at the Biotechnology Laboratory, ICA-UFMG.

DNA extraction was performed following the protocol described by Gu *et al.* [13] with minor modifications. Briefly, bacterial pellets were obtained after overnight activation in BHI broth at 35°C for 24 hours followed by centrifugation. Pellets were resuspended in 100 µL of Solution I (1× PBS, sucrose, and lysozyme [250 mg/ml]) to facilitate cell wall lysis, then incubated at 37°C for 35 minutes. Subsequently, 5 µl of Proteinase K (20 mg/mL) and 500 µl of Solution II (containing Tris/HCl pH 8.0, EDTA, 20% SDS, and ultrapure water) were added to induce cell membrane lysis, with incubation at 37°C for an additional 30 minutes. Following

lysis, 600 µl of chloroform/isoamyl alcohol (24:1, v/v) was added, and the mixture was vigorously vortexed for 30 seconds. The aqueous phase was separated by centrifugation at 10.000 rpm for 10 minutes at room temperature, and approximately 400 µl of the upper phase was carefully transferred to a fresh microcentrifuge tube.

For nucleic acid precipitation, 20 µl of 5 M NaCl (final concentration 0.1 M) and 800 µl of ice-cold absolute ethanol were added, mixed by inversion, and incubated at -20°C for 30 minutes. The DNA was pelleted by centrifugation under the same conditions, the supernatant was carefully removed, and the pellet was resuspended in 200 µl of nuclease-free water. DNA concentration and purity were determined spectrophotometrically (TECAN Infinite M Plex, Switzerland) by measuring absorbance at 260 nm and 280 nm. DNA samples exhibiting A_{260}/A_{280} ratios between 1.8 and 2.0 were considered acceptable and were subsequently diluted to a working concentration of 10 ng/µl for PCR amplification.

PCR Detection of Resistance and Biofilm-Associated Genes

PCR amplification was performed in a final reaction volume of 25 µl containing: 12.4 µl ultrapure water, 3.5 µl $MgCl_2$ (2.8 mM final concentration), 2.5 µL Tris/KCl buffer (pH 8.3; 10 mM Tris-HCl, 50 mM KCl), 1.0 µl dNTP mix (10 mM each), 1.3 µl of each primer (forward and reverse, 0.5 µM final concentration), 0.1 µl Taq DNA polymerase (1 U/µl), and 3.0 µl template DNA (10 ng/µl). Each isolate was screened with all primer pairs listed in Table 1 using a Nexus Gradient thermocycler (Eppendorf, Germany).

Amplified products were resolved by electrophoresis on 1.2% (w/v) agarose gels in 1× TAE buffer. Prior to loading, 3 µl of loading dye [0.125% (w/v) bromophenol blue, 10% (w/v) sucrose] and 5 µL of GelRed™ nucleic acid stain were added to each sample. Electrophoresis was conducted at a constant voltage of 120 V for 70 minutes. DNA bands were visualized and documented using a gel imaging system (L-PIX, Loccus Biotecnologia, Brazil).

Thermal cycling conditions and primer references are detailed in Table 1. Quality control measures included positive and negative controls in all reactions. For detection of *bla*Z, *mec*A, and *mec*C genes, *S. aureus* ATCC 43300 served as the positive control. Detection of *bla*KPC and *bla*OXA_{23} genes utilized DNA from well-characterized nosocomial isolates of *Klebsiella pneumoniae* and *Acinetobacter baumannii*, respectively. For *ica* operon and *bap* gene detection, *S. aureus* ATCC 25923 was employed as the positive control. Nuclease-free water served as the negative control in all PCR runs.

Statistical Analysis

To investigate linear relationships between the genes of interest, we employed an integrated analytical approach combining correlation analysis with visualization techniques. Initially, a correlation matrix was constructed for *bla*Z and all members of the *ica* operon (*ica*A, *ica*B, *ica*C, *ica*D), as well as the *bap* gene. This matrix was visualized as a heatmap to enable preliminary identification of co-occurrence patterns among genes.

To quantify and assess the statistical significance of observed correlations, particularly among genes within the *ica* operon, Pearson correlation coefficients were calculated between *ica*A and each of the other operon members (*ica*D, *ica*B, and *ica*C). Statistical significance was determined at $\alpha=0.05$. All statistical analyses and visualizations were performed using R software (version 4.x) with the corrplot and ggplot2 packages.

Table 1: List of primers, sequence, expected amplicon size, and references for PCR analysis for detection of genes related to β-lactam resistance and biofilm formation

Primer	Primer sequence (5' ... 3')	Amplicon size	References
*mec*A	F: AGTTCTGAGTACCGGATTTGC R: AAATCGATGGTAAAGGTTGGC	~ 533 bp	[14]
*mec*C	F: TCACCAGGTTCAAC[Y]CAAAA R: CCTGAATC[W]GCTAATAATATTTC	~ 356 bp	[15]
*bla*Z	F: TTAAAGTCTTACCGAAAGCAG R: TAAGAGATTTGCCTATGCTT	~ 377 bp	[16]
*bla*KPC	F: TGTCACTGTATCGCGTC R: CTCAGTGCTCTACAGAAAACC	~ 879 bp	[17]
blaOXA_{23}	F: ATGTGTCATAGTATTCGT R: TCACAACAACTAAAAGCACTG	~ 1057 bp	[18]
*ica*A	F: ACACTTGCTGGCGCAGTCAA R: TCTGGAACCAACATCCAACA	~ 188 bp	[19]
*ica*B	F: AGAATCGTGAAGTATAGAAAATT R: TCTAATCTTTTTCATGGAATCCGT	~ 900 bp	[20]
*ica*C	F: ATGGGACGGATTCCATGAAAAAGA R: TAATAAGCATTAATGTTCAATT	~ 1100 bp	[19]
*ica*D	F: ATGGTCAAGCCCAGACAGAG R: AGTATTTCAATGTTTAAAGCAA	~ 198 bp	[20]
bap	F: CCCTATATCGAAGGTGTAGAATTGCAC R: GCTGTTGAAGTTAATACTGTACCTGC	~ 971 bp	[21]

RESULTS

Phenotypic Antimicrobial Resistance Profiles

Antimicrobial susceptibility testing revealed alarmingly high resistance rates among the 30 *S. aureus* isolates examined (Figure 1, Table 2). Penicillin (PEN) and ampicillin (AMP) demonstrated the lowest efficacy, with 93.3% (28/30) of isolates exhibiting resistance to each antibiotic. Similarly, amoxicillin (AMO) showed limited effectiveness, with 90% (27/30) of strains classified as resistant. Extended-spectrum β-lactam antimicrobials also exhibited

concerning resistance profiles. Oxacillin (OXA), a key phenotypic marker for methicillin resistance, was ineffective against 70% (21/30) of isolates.

Table 2 - Phenotypic and genotypic resistance profiles antimicrobials of bacterial isolates

Isolated ID	Antimicrobials	*mec*A	*mecC*	*bla*Z	*bla*KPC	*bla*OXA_{23}
1	CFO, ASB, OXA, MER	-	-	+	-	-
2	CFO, ASB, OXA, MER	-	-	-	-	-
3	CFO, ASB, OXA, MER	-	-	+	-	-
4	CFO, ASB, OXA, MER	-	-	-	-	-
5	ASB, OXA, MER	-	-	+	-	-
6	ASB, OXA, MER	-	-	+	-	-
7	CFO, ASB, OXA, MER	-	-	+	-	-
8	ASB, OXA, MER	-	-	+	-	-
9	ASB, OXA, MER	-	-	+	-	-
10	ASB, OXA	-	-	+	-	-
11	ASB, OXA	-	-	+	-	-
12	ASB, OXA, MER	-	-	+	-	-
13	ASB, OXA, MER	-	-	-	-	-
14	ASB, OXA	-	-	+	-	-
15	ASB, OXA, MER	-	-	+	-	-
16	ASB, OXA, MER	-	-	+	-	-
17	ASB, OXA, MER	-	-	+	-	-
18	ASB, OXA, MER	-	-	+	-	-
19	ASB, MER	-	-	+	-	-
20	ASB, OXA, MER	-	-	+	-	-
21	ASB, OXA	-	-	+	-	-
22	ASB, MER	-	-	+	-	-
23	OXA, MER	-	-	-	-	-
24	AMO, PEN, CFO	-	-	-	-	-
25	AMO, PEN, AMP	-	-	-	-	-
26	AMO, PEN, AMP	-	-	-	-	-
27	AMP	-	-	-	-	-
28	AMO, PEN, AMP	-	-	+	-	-
29	PEN	-	-	+	-	-
30	AMO, PEN, CFO, AMP	-	-	-	-	-

Legend: CFO - cefoxitin ; ASB - ampicillin plus sulbactam ; OXA - oxacillin ; MER - meropenem ; AMO - amoxicillin; PEN - penicillin; AMP - ampicillin. “+”: Positive (present); “-”: Negative (absent).

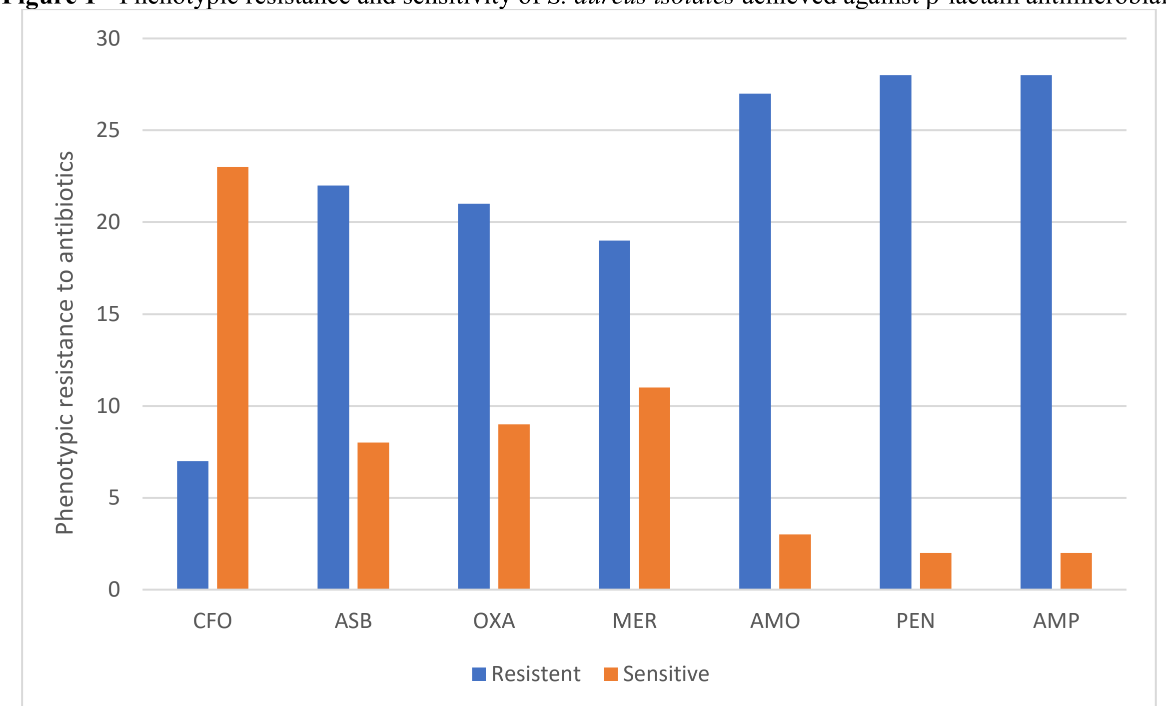
Comparable resistance rates were observed for ampicillin-sulbactam (ASB) at 73.3% (22/30). The carbapenem meropenem (MER) showed resistance in 63.3% (19/30) of strains, representing a particularly worrisome finding given the critical importance of carbapenems as last-resort antibiotics. The high resistance to meropenem (63.3%) is particularly striking, as carbapenems are not used in veterinary routine, suggesting potential co-selection or

environmental contamination. Notably, cefoxitin (CFO) emerged as the most effective agent in the antimicrobial panel, with 76.7% (23/30) of isolates remaining susceptible, corresponding to a resistance rate of only 23.3% (7/30).

Molecular Characterization of Resistance Determinants

Molecular screening revealed that the β-lactamase gene *bla*Z was present in 70% (21/30) of *S. aureus* isolates, indicating widespread dissemination of this penicillin resistance mechanism (Figure 2). This high prevalence provides a molecular basis for the observed phenotypic resistance to penicillin and related β-lactams.

Figure 1 - Phenotypic resistance and sensitivity of *S. aureus isolates* achieved against β-lactam antimicrobials



Legend: CFO - cefoxitin ; ASB - ampicillin plus sulbactam ; OXA - oxacillin ; MER - meropenem ; AMO - amoxicillin; PEN - penicillin; AMP - ampicillin.

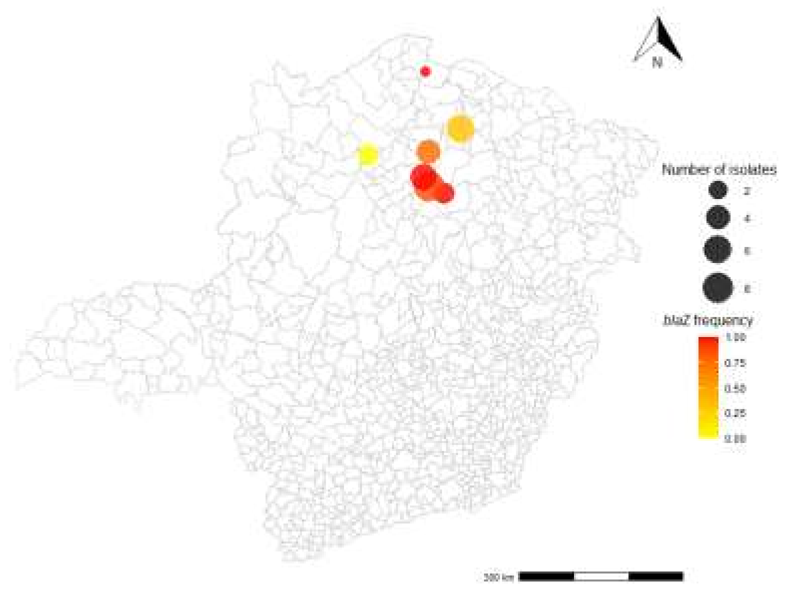
In stark contrast, all isolates tested negative for other critical resistance determinants. The *mec*A gene - the canonical marker for methicillin-resistant *S. aureus* (MRSA) - was not detected in any isolate, nor was its homologue *mec*C. The absence of *mec*A and *mec*C in oxacillin-resistant isolates suggests that the observed phenotype may be attributed to the hyperproduction of β-lactamases (encoded by *bla*Z) or alterations in native PBPs, characterizing these strains as Borderline Oxacillin-Resistant *S. aureus* (BORSA) rather than MRSA. Similarly, carbapenemase genes *bla*KPC and *bla*OXA_{23} were absent from all samples. These findings indicate that the observed phenotypic resistance to oxacillin and meropenem

likely stems from alternative mechanisms rather than classical MRSA determinants or carbapenemase production.

Geographic distribution analysis revealed heterogeneous prevalence patterns across municipalities. The majority of isolates originated from Montes Claros (n=8, 26.7%), followed by Janaúba (n=6, 20.0%) and Nova Esperança (n=4, 13.3%). Juramento, São Pedro das Garças, and Icaraí de Minas each contributed three isolates (10.0%), while Matias Cardoso provided one isolate (3.3%).

The *bla*Z gene showed 100% prevalence in isolates from Nova Esperança, Juramento, and São Pedro das Garças. Although Montes Claros harbored the highest absolute number of positive isolates (n=6), this represented only 75% prevalence within that municipality. Substantially lower frequencies were observed in Janaúba and Icaraí de Minas (33.3% each). The single isolate from Matias Cardoso tested positive for *bla*Z.

Figure 2 - Frequency of the *bla*Z gene and the number of isolated individuals per sampled municipality



Source: Creted by R software

Genetic Determinants of Biofilm Formation

Molecular screening for biofilm-associated genes revealed high genetic potential for biofilm formation among the isolates (Figure 3, Table 3). The *ica*A and *ica*D genes, encoding essential components of polysaccharide intercellular adhesin (PIA) biosynthesis, demonstrated the highest prevalence, with each detected in 80% (24/30) of isolates. In contrast, the remaining *ica* operon members showed substantially lower frequencies: *ica*C was present in 20% (6/30) and *ica*B in 13.3% (4/30) of strains.

The complete *ica* operon (*ica*ADBC) was detected in only 10% (3/30) of isolates, suggesting that while individual genes are common, intact operons are relatively rare in this population. The *bap* gene, encoding an alternative biofilm-associated protein pathway, was identified in 30% (9/30) of strains. Notably, a single isolate (3.3%) harbored both the complete *ica* operon and the *bap* gene, potentially conferring enhanced biofilm-forming capacity through redundant mechanisms.

Gene Co-occurrence and Correlation Analysis

Table 3 - Gene profiles associated with biofilms of bacterial isolates

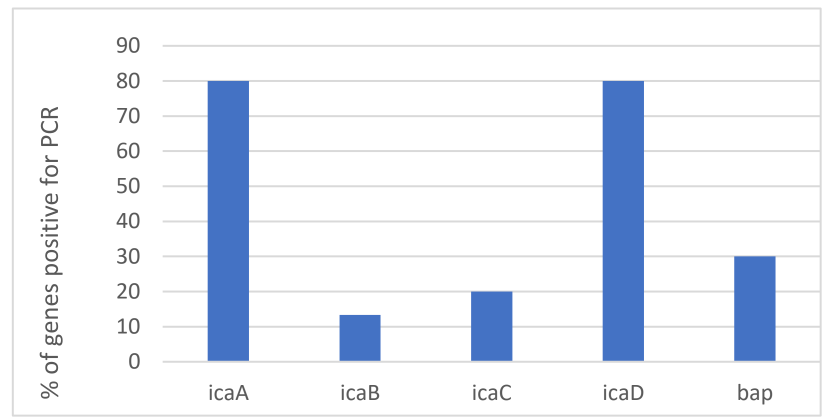
Isolated ID	*ica*A	*ica*B	*ica*C	*ica*D	*bap*
1	+	-	+	+	+
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-
5	+	+	-	+	-
6	-	-	-	-	+
7	-	-	-	-	-
8	+	-	-	+	-
9	+	+	+	+	+
10	-	-	-	-	+
11	+	-	-	+	-
12	+	-	-	+	-
13	+	+	+	+	-
14	+	-	-	+	+
15	+	-	+	+	+
16	+	-	-	+	-
17	+	+	+	+	-
18	+	-	-	+	-
19	+	-	+	+	+
20	+	-	-	+	-
21	+	-	-	+	+
22	+	-	-	+	+
23	+	-	-	+	-
24	+	-	-	+	-
25	+	-	-	+	-
26	+	-	-	+	-
27	+	-	-	+	-
28	+	-	-	+	-
29	+	-	-	+	-
30	+	-	-	+	-

Legend : "+": Positive (present); " -" : Negative (absent).

Correlation analysis revealed distinct patterns of gene co-occurrence (Figure 4). A perfect positive correlation was observed between *ica*A and *ica*D ($r = 1.00$, $p < 0.01$), indicating complete synchrony in the presence of these genes across all isolates. This strong association likely reflects their adjacent genomic location and coordinated regulation within the ica operon.

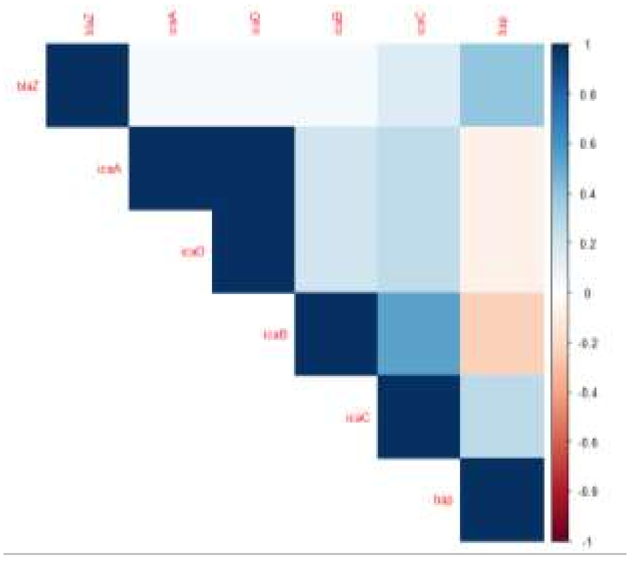
In contrast, correlations between *ica*A and other operon members were weak and statistically non-significant. The correlation coefficient for *ica*A and *ica*B was 0.20 ($p = 0.299$), while *ica*A and *ica*C showed $r = 0.25$ ($p = 0.183$). The correlation heatmap corroborated these findings, clearly demonstrating the robust *ica*A/*ica*D association while revealing minimal correlation among other gene pairs. These data suggest that while *ica*A and *ica*D are consistently co-inherited, the complete operon assembly is disrupted in most isolates, potentially through gene deletion or rearrangement events.

Figure 3 - Detection of biofilm-forming genes among *S. aureus isolates* in this study by PCR.



Source: Authors

Figure 4 - Heatmap: Correlation matrix between molecular resistance markers and biofilm formation.



Source: Creted by R software

No significant correlations were observed between biofilm-associated genes (*ica* operon members or *bap*) and the resistance marker *bla*Z, indicating that biofilm formation capacity and β-lactam resistance are likely independently acquired or maintained traits in this population.

DISCUSSION

Bovine mastitis caused by *S. aureus* represents a multifaceted challenge to the dairy industry, severely compromising both milk production and animal welfare. The pathogen's capacity to form biofilms and develop antimicrobial resistance substantially complicates infection control strategies. Clinical mastitis manifests through overt signs including mammary edema, pain, and visible milk alterations such as purulent discharge or hemolactia. Subclinical infections, though less apparent, insidiously elevate somatic cell counts (SCC) while reducing milk protein and fat content, thereby compromising product quality [22, 23]. The ability of *S. aureus* to establish chronic infections within mammary tissue generates substantial economic losses through milk discardment, escalated treatment costs, and premature culling of affected animals.

Staphylococcal resistance genes provide critical insights into antimicrobial evasion strategies. The *mec*A and *mec*C genes, hallmarks of the MRSA phenotype, encode modified penicillin-binding proteins (PBPs) with reduced β-lactam affinity. The *fem*A gene stabilizes these altered PBPs, enhancing cell wall integrity and resilience. Conversely, *bla*Z encodes β-lactamases that hydrolyze penicillin's β-lactam ring, effectively inactivating the antibiotic and severely limiting therapeutic options [24, 25].

Our analysis of *S. aureus* isolates from northern Minas Gerais revealed alarming resistance rates to penicillin and ampicillin (93.3%; 28/30). These findings align with previous reports from the Zona da Mata region, where penicillin resistance reached 78.9% (56/71), and other Brazilian studies documenting rates of 75.68% (28/37) [26,27]. This widespread resistance across Minas Gerais confirms endemic circulation of resistant strains and underscores the declining utility of traditional β-lactams as first-line therapeutic agents in bovine mastitis management.

Phenotypic oxacillin resistance, a surrogate marker for MRSA, was observed in 70% (21/30) of isolates. This prevalence approximates previous reports of universal resistance in northern Minas Gerais isolates but contrasts starkly with the markedly lower frequency (2.8%; 2/71) documented in the Zona da Mata region [26]. Such geographic heterogeneity likely

reflects regional variations in livestock management practices, antimicrobial stewardship protocols, and differential selective pressures exerted by diverse treatment regimens.

Cefoxitin demonstrated superior efficacy, with 76.7% (23/30) of isolates remaining susceptible. This finding diverges from earlier studies reporting complete resistance (100%; 16/16) within the same macroregion, potentially attributable to distinct resistance mechanisms, heterogeneous *mec*A expression patterns, or clonal lineage differences [11]. These discrepancies underscore the critical importance of integrating phenotypic and genotypic characterization for accurate MRSA identification.

The high prevalence of *bla*Z (70%; 21/30) aligns with established literature identifying this gene as the primary determinant of penicillin resistance in *S. aureus*. Our observed frequency is intermediate between previously reported ranges of 43.75-95.7% across diverse geographic contexts [1, 28, 29], likely reflecting regional variations in antimicrobial usage patterns and selective pressures.

Remarkably, despite substantial phenotypic oxacillin resistance, all isolates tested negative for *mec*A and *mec*C - the canonical genetic markers of MRSA. This genotype-phenotype discordance diverges from studies detecting *mec*A in 37.5% of northern Minas Gerais isolates and up to 50% in other contexts [11, 29], yet corroborates recent findings that similarly reported mec gene absence [30]. This paradox suggests alternative resistance mechanisms independent of the SCCmec cassette, including: (i) hyperproduction of β-lactamases, (ii) mutations in native PBPs reducing β-lactam affinity, (iii) upregulation of efflux pump systems, or (iv) cell wall thickening reducing antibiotic penetration. These possibilities warrant comprehensive genomic and transcriptomic investigations to elucidate the molecular underpinnings of *mec*-independent methicillin resistance.

The universal absence of carbapenemase genes (*bla*KPC, *bla*OXA$_{23}$) represents an encouraging finding, confirming that these critically important resistance determinants remain confined outside the bovine *S. aureus* population in this region [11]. However, vigilant surveillance remains imperative given the potential for horizontal gene transfer from healthcare-associated reservoirs.

Biofilm-associated gene profiling revealed high prevalence of *ica*A and *ica*D (80% each), encoding essential enzymes for polysaccharide intercellular adhesin (PIA) biosynthesis. This finding closely parallels previous reports documenting *ica*A (86.6%) and *ica*D (84.1%) prevalence, with 63.4% co-occurrence in bovine isolates [31], suggesting that PIA-dependent

biofilm formation constitutes a highly conserved and widespread mechanism in Brazilian bovine *S. aureus* populations.

Biofilm development proceeds through sequential, interdependent stages. Initial reversible adhesion mediated by surface proteins transitions to irreversible attachment through adhesin production. Subsequent microcolony maturation involves extensive extracellular matrix deposition comprising polysaccharides, proteins, and extracellular DNA (eDNA), conferring structural integrity. Mature biofilms develop complex three-dimensional architectures with water channels facilitating nutrient distribution and waste removal. Terminal dispersal releases planktonic cells that seed new infection foci, perpetuating the infectious cycle [32, 33].

The extracellular polymeric substance (EPS) matrix serves as a critical virulence determinant in mastitis-causing pathogens. This protective scaffold shields embedded bacteria from both antimicrobial agents and host immune effectors, substantially impeding therapeutic efficacy. Moreover, EPS-mediated adhesion to milking equipment and storage vessels establishes persistent environmental reservoirs, facilitating continuous cross-contamination cycles that compromise milk quality and food safety [34, 35].

Biofilm-associated protein (Bap) provides an alternative, PIA-independent biofilm formation pathway. Its amyloid-like structure promotes cell-cell aggregation and confers additional protection against environmental stresses and antimicrobial challenge [36]. The *bap* gene prevalence of 30% in our isolates approximates previously reported frequencies of 25% [37], though some studies detected no *bap*-positive isolates despite universal *ica*A presence [38].

Notably, *ica*B and *ica*C exhibited markedly reduced prevalence (13.3% and 20%, respectively), contrasting with reports of complete operon detection in 100% of veterinary hospital isolates [39] yet consistent with dairy herd studies showing *ica*B (4.9%) and *ica*C (31.7%) frequencies [31]. This heterogeneity suggests that while *ica*A and *ica*D are highly conserved, the complete ica operon frequently undergoes genetic erosion, potentially through deletion or rearrangement events. Critically, *ica*A/*ica*D presence does not guarantee functional PIA biosynthesis, as *ica*B and *ica*C encode essential glycosyltransferase and export functions [40, 41, 42].

The paradox concerning the MRSA phenotype and the absence of the *mec*A and *mec*C genes, suggests alternative resistance mechanisms independent of the SCCmec cassette. The observed profile strongly suggests the BORSA (Borderline Oxacillin-Resistant *S. aureus*)

phenotype, characterized by either the hyperproduction of β-lactamases (encoded by *bla*Z) or point mutations in native pbp genes that slightly reduce binding affinity to oxacillin, without the presence of the *mec*A gene [30, 43].

The rarity of the complete *ica* operon (10%) in our study suggests that biofilm architecture in these isolates might be protein-mediated rather than polysaccharide-dependent. This is supported by the 30% prevalence of the *bap* gene. Such a shift in biofilm composition is clinically relevant, as protein-based biofilms may respond differently to enzymatic dispersal agents or traditional sanitizers used in milking parlors [44].

The low frequence of the complete *ica* operon (10%; 3/30) reinforces that biofilm formation in bovine *S. aureus* frequently relies on alternative or compensatory mechanisms. The exceptional isolate (3.3%) harboring both complete *ica* operon and *bap* gene represents a particularly concerning finding, potentially conferring enhanced biofilm robustness through redundant pathways and heightened resistance to disruption strategies.

The confluence of *bla*Z-mediated penicillin resistance and robust biofilm-forming genetic capacity creates a "perfect storm" scenario for persistent, treatment-refractory infections. Biofilm encasement reduces antimicrobial penetration by up to 1000-fold, while the protected microenvironment facilitates horizontal gene transfer of resistance determinants. This synergistic combination explains the frequent therapeutic failures observed in subclinical mastitis management and underscores the urgent need for alternative intervention strategies including biofilm-disrupting agents, targeted immunotherapies, and antimicrobial stewardship programs.

CONCLUSIONS

This comprehensive molecular characterization of *S. aureus* isolates from subclinical bovine mastitis in northern Minas Gerais reveals a concerning dual-threat phenotype combining high-level β-lactam resistance with substantial biofilm-forming potential. The predominance of *bla*Z-mediated resistance (70%) explains widespread penicillin treatment failures, while the conspicuous absence of *mec*A/*mec*C despite phenotypic oxacillin resistance highlights the importance of elucidating alternative resistance mechanisms through whole-genome sequencing approaches.

The high prevalence of *ica*A and *ica*D (80%), coupled with *bap* gene detection (30%), indicates that biofilm formation capacity is deeply entrenched in this bacterial population. The perfect correlation between i*ca*A and *ica*D suggests these genes are maintained as a conserved

functional unit, while incomplete operon architecture in most isolates points to dynamic genetic fluidity within biofilm-associated loci.

The co-occurrence of antimicrobial resistance and biofilm-forming capacity in these isolates represents a formidable challenge for mastitis control programs. Effective management strategies must transcend conventional antimicrobial therapy to encompass: (i) enhanced biosecurity and hygiene protocols to limit pathogen transmission, (ii) implementation of antimicrobial stewardship programs to reduce selective pressure, (iii) development of biofilm-disrupting therapeutic adjuvants, and (iv) establishment of comprehensive molecular surveillance systems to track emerging resistance mechanisms.

These findings underscore the critical necessity for integrated "One Health" approaches recognizing the interconnected nature of animal, human, and environmental health in combating antimicrobial resistance and biofilm-associated infections in agricultural systems.

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5. ARTIGO CIENTÍFICO 2 - GENETIC DIVERSITY OF *Staphylococcus aureus* ISOLATES FROM SUBCLINICAL BOVINE MASTITIS BY INTER-SIMPLE SEQUENCE REPEAT OLIGONUCLEOTIDES

Artigo escrito de acordo com as normas da Revista BMC Microbiology

GENETIC DIVERSITY OF *Staphylococcus aureus* ISOLATES FROM SUBCLINICAL BOVINE MASTITIS BY INTER-SIMPLE SEQUENCE REPEAT OLIGONUCLEOTIDES

ABSTRACT

Staphylococcus aureus is one of the main etiological agents of bovine mastitis, affecting animal health and causing significant economic losses in dairy farming. The genetic variability of this species is associated with virulence factors and antimicrobial resistance, complicating the control and treatment of infections. This study aimed to assess the applicability of ISSR oligonucleotides as an alternative tool for genetic diversity analysis of *S. aureus*. Thirty isolates from milk samples of cows with subclinical mastitis were used, exhibiting multidrug resistance to antimicrobials and biofilm-forming capacity. The analysis consisted of applying 52 primers from the ISSR UBC Primer Set #9, with temperature gradient tests to optimize annealing. Amplification profiles were evaluated using GelAnalyzer 23.1.1 software and organized into binary matrices. Diversity studies and biometric analyses were conducted using GENES software, estimating genetic dissimilarity through the arithmetic complement of Jaccard's index (Sj). Cluster analysis employed the UPGMA hierarchical method, with results represented in dendrogram and scatter plot formats. Of the primers tested, 48% generated polymorphic patterns, 15% displayed monomorphic bands, 33% produced no amplification, and 4% were inconclusive. The 25 polymorphic primers revealed substantial genetic variability among isolates and wide geographic dispersion, with no clustering restricted by municipality. The absence of defined geographic structuring corroborates the hypothesis of intermunicipality dissemination of the pathogen, possibly influenced by animal movement and lack of uniform biosecurity protocols. The significant presence of polymorphisms demonstrates that ISSR oligonucleotides are viable for genetic characterization of *S. aureus*, providing support for epidemiological surveillance and management of strains with clinical and zootechnical relevance.

KEYWORDS: Annealing. Antimicrobials. ISSR. Multidrug-resistant.

DIVERSIDADE GENÉTICA DE *Staphylococcus aureus* ISOLADOS DE MASTITE BOVINA SUBCLÍNICA POR MEIO DE OLIGONUCLEOTÍDEOS INTER-SIMPLE SEQUENCE REPEAT

RESUMO

Staphylococcus aureus é um dos principais agentes etiológicos da mastite bovina, afetando a saúde animal e provocando perdas econômicas significativas na pecuária leiteira. A variabilidade genética dessa espécie está associada a fatores de virulência e resistência antimicrobiana, dificultando o controle e tratamento das infecções. Este estudo objetivou analisar a aplicabilidade de oligonucleotídeos ISSR como ferramenta alternativa para análise da diversidade genética de *S. aureus*. Foram utilizados 30 isolados provenientes de leite de vacas com mastite subclínica, apresentando multirresistência a antimicrobianos e capacidade de formação de biofilme. A análise consistiu na aplicação de 52 primers do conjunto ISSR UBC Primer Set #9, com testes de gradiente de temperatura para otimização do anelamento. Os perfis de amplificação foram avaliados pelo software GelAnalyzer 23.1.1 e organizados em matrizes binárias. O estudo de diversidade e análises biométricas foram conduzidos no software GENES, estimando-se a dissimilaridade genética pelo complemento aritmético do índice de Jaccard (Sj). A análise de agrupamento empregou o método hierárquico UPGMA, com resultados representados em dendrograma e gráfico de dispersão. Dos primers testados, 48% geraram padrões polimórficos, 15% apresentaram bandas monomórficas, 33% não produziram amplificação e 4% foram inconclusivos. Os 25 primers polimórficos evidenciaram expressiva variabilidade genética entre os isolados e ampla dispersão geográfica, sem agrupamento restrito por município. A ausência de estruturação geográfica definida corrobora a hipótese de disseminação intermunicipal do patógeno, possivelmente influenciada pela movimentação animal e carência de protocolos uniformes de biossegurança. A presença significativa de polimorfismos demonstra que oligonucleotídeos ISSR são viáveis para caracterização genética de *S. aureus*, oferecendo suporte à vigilância epidemiológica e ao manejo de cepas com relevância clínica e zootécnica.

PALAVRAS-CHAVE: Anelamento. Antimicrobianos. ISSR. Multirresistente.

INTRODUCTION

Staphylococcus aureus (*S. aureus*) has a significant impact on dairy farming, being one of the main causative agents of bovine mastitis. This infection compromises cow health and generates substantial economic losses (Maity *et al*., 2020; Tucker *et al*., 2023). Reduced milk production, increased treatment and management costs, and marketing difficulties are some of the consequences (El-Sayed and Kamel, 2021). Additionally, milk contamination may require product disposal, exacerbating losses (Hemati *et al*., 2023; Muzammil *et al*., 2023).

The species' diversity is closely related to resistance and virulence factors, which influence its ability to cause infections and evade treatment (Vaughn *et al*., 2020). Given this scenario, understanding the genetic diversity of *S. aureus* enables the development of more effective control strategies, benefiting animal health and the sustainability of dairy production (Campos *et al*., 2022; Majumder *et al*., 2023).

Among the methodologies available for diversity analysis, ISSR (*Inter Simple Sequence Repeat*) oligonucleotides represent a promising approach (Al-Khayri *et al.,* 2022). ISSRs are based on the presence of microsatellite sequences, short and repetitive DNA segments found in non-coding regions of the genome. These markers have been widely used in investigating the genetic variability of organisms, particularly eukaryotes (Saghir *et al*., 2022).

ISSRs offer several advantages, such as operational simplicity, low cost, and high capacity for polymorphism detection (Ismail *et al*., 2016; Chen *et al*., 2020). However, their application in prokaryotic studies remains limited, mainly due to the need for annealing temperature optimization, a critical parameter for ensuring result reproducibility and reliability (Carvalho *et al*., 2017).

Considering ISSR limitations in prokaryotes, this study proposes the validation of these oligonucleotides for *S. aureus* as an alternative approach to identify genetic markers for diversity studies. Given the scarcity of investigations applying this technique to prokaryotic organisms and the absence of prior reports of its application in *S. aureus*, this study aims to fill this methodological gap. Thus, we seek to demonstrate the viability of ISSR use in this context, contributing to the expansion of methodologies applicable to prokaryotic genotyping.

MATERIAL AND METHODS

Acquisition of Bacterial Strains

Bacterial strains were isolated from milk samples collected from teat quarters of cows from herds on farms in the northern region of Minas Gerais with subclinical mastitis. Sample

collection was performed using aseptic techniques to prevent contamination, and samples were sent to the Animal Health Laboratory at ICA-UFMG. Samples were inoculated on Blood Agar Base medium, supplemented with 5% sheep blood, and incubated at 35°C ± 2°C for 24 to 48 hours for bacterial growth.

After primary culture, bacterial isolates were morphologically characterized at the species level and subsequently identified at the species level by mass spectrometry using the MALDI-TOF technique.

Antimicrobial susceptibility testing against β-lactam antibiotics was performed using the disk-diffusion technique (Kirby-Bauer), as well as detection of biofilm-forming capacity using the Congo Red Agar technique. These procedures were conducted in the context of previous research developed at the Animal Health Laboratory (Tavares *et al*., 2024; Souza *et al*., 2019). Identified bacterial isolates were transferred to 1.5 ml microtubes containing a solution of BHI broth in 20% (v/v) glycerol to preserve bacterial viability. Samples were stored at -20°C to ensure strain stability and integrity.

Selection and DNA Extraction from *S. aureus* Strains

Thirty strains previously identified as *S. aureus* were selected based on two phenotypic characteristics of interest: multidrug resistance to antimicrobials and biofilm production capacity. Strains were transferred to a tube containing 3 ml of BHI broth and incubated at 35 ± 2°C for 24 hours. After growth and purity verification, DNA extraction was performed.

DNA extraction and analyses were conducted at the Biotechnology Laboratory of ICA-UFMG. The DNA extraction protocol described by Gu *et al*. (2005) was adopted with adaptations. Initially, one colony from each strain was inoculated into 5 ml of BHI broth and incubated at 35°C ± 2°C for 24 hours. Cultures were transferred to microcentrifuge tubes and centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded, and the process was repeated twice.

The bacterial pellet was resuspended in 100 µl of Solution I (5 ml of 1x PBS, 1 g of sucrose, and 50 µl lysozyme [250 mg/ml]) for cell wall lysis and incubated at 37°C for 35 minutes. Subsequently, 5 µl of a Proteinase K solution (20 mg/ml) and 500 µl of Solution II (120 µl of Tris/HCl [pH 8.0; 10 mM/50 mM], 2.4 µl of EDTA [0.5 M], 600 µl of SDS [20%], and 11,200 µl of ultrapure water) were added for cell membrane lysis, with a new incubation at 37°C for 30 minutes. After this step, 600 µl of a chloroform/isoamyl alcohol solution (24:1) was added, and the mixture was vigorously agitated using a vortex mixer (Warmnes).

Centrifugation was performed at 10,000 rpm for 10 minutes, and approximately 400 µl of the aqueous phase (upper layer) was transferred to another tube using a micropipette.

To precipitate nucleic acids, 20 µl of 5M NaCl (final concentration of 0.1 M) and 800 µl of ice-cold ethanol (two 400 µl volumes) were added. Centrifugation was performed again at 10,000 rpm for 10 minutes, the supernatant was discarded, and the DNA was resuspended in 200 µl of water for injection. DNA concentrations of samples were quantified using a spectrophotometer (TECAN equipment, Infinite M Plex model) by reading absorbance at 260 nm for subsequent dilution to a concentration of 10 ng/µl.

Annealing Temperature Gradient Tests

Gradient tests to determine annealing temperatures were conducted using thermocyclers equipped with this functionality (Eppendorf equipment, Gradient models). Fifty-two ISSR primers were randomly selected from the UBC primer set #9, developed by the University of British Columbia (Vancouver, Canada). Thermocyclers were programmed to operate in a temperature range from 45°C to 65°C (45°C, 45.5°C, 46.7°C, 48.6°C, 51.2°C, 53.8°C, 56.8°C, 58.8°C, 61.4°C, 63.3°C, 64.5°C, and 65°C), using DNA from two randomly selected individuals within the analyzed population as template. Based on these assays, specific annealing temperatures were defined for each oligonucleotide, seeking to optimize the generation of polymorphic electrophoretic patterns.

Each PCR reaction was assembled in a final volume of 25 µl, containing: 12.4 µl of ultrapure water, 3.5 µl of $MgCl_2$ (2.8 mM), 2.5 µl of Tris/KCl buffer (pH 8.3; 10 mM/50 mM), 1.0 µl of dNTPs, 2.5 µl of the ISSR primer (0.4 µM), 0.1 µl of Taq DNA polymerase (1 U/µl), and 3.0 µl of DNA (10 ng/µl). The thermal program consisted of an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles comprising: denaturation at 94°C for 1 minute, annealing at temperatures ranging from 45°C to 65°C for 1 minute, and extension at 72°C for 2 minutes. At the end, a final extension step was performed at 72°C for 7 minutes. Reactions were maintained at 4°C and subsequently stored in a freezer at -20°C.

PCR products were subjected to horizontal electrophoresis in 1.2% (w/v) agarose gel, prepared and immersed in SB buffer (10 mM NaOH, 10 mM boric acid, pH ~8.0), according to procedures adapted from Sambrook *et al*. (1989). At the time of sample loading onto the gel, each sample received the addition of 3 µl of type IV dye [containing 0.125% (w/v) bromophenol blue and 10% (w/v) sucrose], plus 5 µl of GelRed™. Electrophoretic runs were performed under

constant voltage of 120V for 75 minutes. Gel visualization and image capture were performed using a photodocumentation system (Loccus Biotecnologia, L-PIX model).

Analysis of Total Number of Amplified Bands per Temperature with ISSR Primers in *S. aureus*

For analysis of the total number of bands amplified per temperature in *S. aureus* DNA in the context of gradient testing, images obtained by photodocumentation system were used, selecting primers that showed greater polymorphism and better band definition. Quantification and comparison of amplification profiles were performed using GelAnalyzer 23.1.1 software.

Diversity Study and Biometric Analyses Based on Molecular Data

The diversity analysis employed 11 ISSR oligonucleotides (808, 862, 838, 840, 861, 835, 885, 807, 851, 826, and 810). Diversity studies and biometric analyses were conducted using GENES software. Data were organized into binary matrices, assigning a value of 1 for the presence and 0 for the absence of bands observed in photodocumentation. Genetic dissimilarity among isolates was estimated using the arithmetic complement of Jaccard's index (Sj), defined as: $Sj = a / (a + b + c)$. In parallel, cluster analysis was performed using the UPGMA hierarchical method (Unweighted Pair-Group Method with Arithmetic Mean) from the dissimilarity matrix generated by Jaccard's index. Results were represented in dendrogram and scatter plot formats, enabling visualization of the structure of formed clusters.

RESULTS

DNA concentration values obtained after extractions, expressed in ng/µl, showed variation among different samples (mean: 81.74; median: 54.4; and mode: 13.5 ng/µl), reflecting differences in extraction process efficiency. To ensure standardization of subsequent analyses, all samples were diluted to a final concentration of 10 ng/µl. This standardization aims to ensure result reproducibility in subsequent experimental steps.

Results from annealing temperature gradient tests performed with *S. aureus* DNA samples using ISSR oligonucleotides showed efficient amplification of different ISSR primers at distinct annealing temperatures, ranging from 45°C to 58°C. Reactions at 45°C resulted in amplification of primers UBC845, UBC895, and UBC841. At 46°C, primers UBC835, UBC881, UBC816, and UBC862 showed amplification. At 49°C, amplified primers were UBC885, UBC808, UBC830, and UBC861. At 50°C, only primer UBC880 showed

amplification. The highest number of amplified primers was observed at 51°C, with primers UBC825, UBC840, UBC810, UBC851, and UBC836. At 52°C, only primer UBC811 presented an amplified product. For higher temperatures, results were as follows: 54°C (UBC850 and UBC818), 55°C (UBC807), 56°C (UBC826 and UBC886), 57°C (UBC855), and 58°C (UBC809) (Table 2).

Figure 1 presents a summary of results obtained from temperature gradient tests performed with the 52 evaluated ISSR primers. It was observed that 33% of primers (n = 17) did not promote amplification of *S. aureus* DNA fragments. A total of 4% (n = 2) generated inconclusive results, with no defined bands or inconsistent patterns among replicates. Among primers that promoted amplification, 15% (n = 8) exhibited monomorphic patterns, while 48% (n = 25) revealed evident polymorphisms in amplification profiles.

Figure 1 - Distribution of results obtained from temperature gradient tests with 52 ISSR primers in *S. aureus* DNA

Source: Authors

Results from analysis of *S. aureus* DNA amplification profiles using 25 ISSR primers at different annealing temperatures demonstrated that the number of amplified bands ranged from 2 to 8, evidencing the direct influence of experimental conditions on PCR efficiency. The highest number of bands (8) was obtained with primer UBC 885 at 49°C. Other highly efficient primers (6 bands) included UBC 855 (57°C), UBC 880 (50°C), UBC 835 (46°C), UBC 840 (51°C), UBC 851 (51°C), UBC 862 (46°C), and UBC 886 (56°C). Primers UBC 845, UBC 895 (both at 45°C), and UBC 834 (46°C) showed lower efficiency, with only 2 amplified bands.

Table 1 - Results of total number of bands amplified per temperature involving *Staphylococcus aureus* DNA amplified with ISSR oligonucleotides (UBC primer set #9, Vancouver, Canada)

Reference reaction	ISSR oligonucleotides (5' → 3')	Annealing temperature Ta (ºC)
Microplate #1	UBC 807 (AGA GAG AGA GAG AGA GT)	55ºC
	UBC 855 (ACA CAC ACA CAC ACA CYT)	57ºC
	UBC 875 (CTA GCT AGC TAG CTA G)	No amplification
	UBC 894 (TGG TAG CTC TTG ATC ANN NNN)	No amplification
Microplate #2	UBC 806 (TAT ATA TAT ATA TAT AG)	No amplification
	UBC 850 (GTG TGT GTG TGT GTG TYC)	54ºC
	UBC 870 (TGC TGC TGC TGC TGC TGC)	No amplification
	UBC 885 (BHB GAG AGA GAG AGA GA)	49ºC
Microplate #3	UBC 809 (AGA GAG AGA GAG AGA GG)	58ºC
	UBC 845 (CTC TCT CTC TCT CTC TRG)	45ºC
	UBC 880 (GGA GAG GAG AGG AGA)	50ºC
	UBC 890 (VHV GTG TGT GTG TGT GT)	Monomorphic
Microplate #4	UBC 808 (AGA GAG AGA GAG AGA GC)	49ºC
	UBC 815 (CTC TCT CTC TCT CTC TG)	Monomorphic
	UBC 825 (ACA CAC ACA CAC ACA CT)	51ºC
	UBC 835 (AGA GAG AGA GAG AGA GYC)	46ºC
Microplate #5	UBC 805 (TAT ATA TAT ATA TAT AC)	No amplification
	UBC 820 (GTG TGT GTG TGT GTG TC)	Inconclusive
	UBC 830 (TGT GTG TGT GTG TGT GG)	49ºC
	UBC 840 (GAG AGA GAG AGA GAG AYT)	51ºC
Microplate #6	UBC 810 (GAG AGA GAG AGA GAG AT)	51ºC
	UBC 853 (TCT CTC TCT CTC TCT CRT)	No amplification
	UBC 865 (CCG CCG CCG CCG CCG CCG)	Inconclusive
	UBC 895 (AGA GTT GGT AGC TCT TGA TC)	45ºC
Microplate #7	UBC 811 (GAG AGA GAG AGA GAG AC)	52ºC
	UBC 851 (GTG TGT GTG TGT GTG TYG)	51ºC
	UBC 861 (ACC ACC ACC ACC ACC ACC)	49ºC
	UBC 871 (TAT TAT TAT TAT TAT TAT)	No amplification
Microplate #8	UBC 821 (GTG TGT GTG TGT GTG TT)	No amplification
	UBC 831 (ATA TAT ATA TAT ATA TYA)	No amplification
	UBC 841 (GAG AGA GAG AGA GAG AYC)	45ºC
	UBC 860 (TGT GTG TGT GTG TGT GRA)	No amplification
Microplate #9	UBC 801 (ATA TAT ATA TAT ATA TT)	Monomorphic
	UBC 802 (ATA TAT ATA TAT ATA TG)	Monomorphic
	UBC 803 (ATA TAT ATA TAT ATA TC)	Monomorphic
	UBC 804 (TAT ATA TAT ATA TAT AA)	Monomorphic
Microplate #10	UBC 824 (TCT CTC TCT CTC TCT CG)	No amplification
	UBC 834 (AGA GAG AGA GAG AGA GYT)	No amplification
	UBC 881 (GGG TGG GGT GGG GTG)	46ºC
	UBC 891 (HVH TGT GTG TGT GTG TG)	Monomorphic
Microplate #11	UBC 816 (CAC ACA CAC ACA CAC AT)	46ºC
	UBC 826 (ACA CAC ACA CAC ACA CC)	56ºC
	UBC 836 (AGA GAG AGA GAG AGA GYA)	51ºC
	UBC 846 (CAC ACA CAC ACA CAC ART)	No amplification
Microplate #12	UBC 814 (CTC TCT CTC TCT CTC TA)	No amplification
	UBC 844 (CAC ACA CAC ACA CAC AG)	No amplification
	UBC 862 (AGC AGC AGC AGC AGC AGC)	46ºC
	UBC 886 (VDV CTC TCT CTC TCT CT)	56ºC
Microplate #13	UBC 818 (CAC ACA CAC ACA CAC AG)	54ºC
	UBC 838 (TAT ATA TAT ATA TAT ARC)	Monomorphic
	UBC 877 (TGC ATG CAT GCA TGC A)	No amplification
	UBC 892 (CAT GGT GTT GGT CAT TGT TCC A)	No amplification

N = (A, G, C, T); R = (A, G); Y = (C, T); B = (C, G, T) (I. e. not A); D = (A, G, T) (I. e. not C); H = (A, C, T) (I. e. not G); V = (A, C, G) (I. e. not T).

Genetic dissimilarity analysis (Table 3) based on Jaccard's index revealed marked variability among the 30 *S. aureus* isolates. Seventeen pairs of isolates with dissimilarities exceeding 80% were identified, indicating significant genetic divergence. Isolate 1 demonstrated high dissimilarity with isolates 20 (84%), 21 (79%), 22 (82%), and 24 (88%), suggesting a distinctive genetic profile. The 21-22 pair recorded the highest dissimilarity value (89%), reflecting possible independent evolutionary origins or distinct adaptive trajectories.

Isolates 11, 20, 21, 22, and 24 emerged as the most divergent, presenting multiple relationships with dissimilarity above 80%. This pattern indicates a polyclonal bacterial population with coexistence of genetically distant lineages in the same region. The presence of highly divergent genomic profiles suggests multiple introductions or extensive genetic recombination, possibly mediated by mobile genetic elements or differential selective pressure.

Table 2 - Results of total number of bands amplified per temperature involving *S. aureus* DNA amplified with ISSR oligonucleotides

ISSR	Annealing Temperature	Banding Pattern
UBC 807	55ºC	5
UBC 855	57ºC	6
UBC 850	54ºC	3
UBC 885	49ºC	8
UBC 809	58ºC	4
UBC 845	45ºC	2
UBC 880	50ºC	6
UBC 808	49ºC	3
UBC 825	51ºC	4
UBC 835	46ºC	6
UBC 830	49ºC	4
UBC 840	51ºC	6
UBC 810	51ºC	5
UBC 895	45ºC	2
UBC 811	52ºC	5
UBC 851	51ºC	6
UBC 861	49ºC	5
UBC 841	45ºC	4
UBC 834	46ºC	2
UBC 816	46ºC	4
UBC 826	56ºC	4
UBC 836	51ºC	5
UBC 862	46ºC	6
UBC 886	56ºC	6
UBC 818	54ºC	3

Hierarchical cluster analysis using the UPGMA method (Unweighted Pair Group Method with Arithmetic Mean), applied to 30 *S. aureus* strains isolated from bovine herds with subclinical mastitis in Northern Minas Gerais, revealed the formation of three main groups, reflecting distinct genetic similarity profiles among isolates (Figure 2). Samples encompassed

seven representative municipalities of the region - Janaúba, Montes Claros, Icaraí de Minas, São Pedro das Garças, Nova Esperança, Juramento, and Matias Cardoso.

Table 3 - Dissimilarity matrix (%) (Jaccard index) among 30 *S. aureus* isolates, estimated from 58 fragments (loci), using 12 ISSR oligonucleotides developed by the University of British Columbia

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**
1	0																													
2	68	0																												
3	62	35	0																											
4	76	61	69	0																										
5	52	52	44	59	0																									
6	63	63	57	80	62	0																								
7	59	42	29	59	35	62	0																							
8	68	49	46	60	51	66	43	0																						
9	64	49	50	71	56	66	51	67	0																					
10	68	48	62	61	60	67	63	64	23	0																				
11	73	69	74	74	72	74	74	53	75	66	0																			
12	70	74	68	58	66	75	72	62	76	74	64	0																		
13	70	67	56	74	62	57	61	58	69	74	75	47	0																	
14	67	56	57	70	69	50	62	66	47	51	78	72	65	0																
15	50	59	56	74	48	68	53	54	66	67	64	61	56	68	0															
16	79	56	66	65	64	70	59	56	68	65	57	67	53	70	48	0														
17	71	68	75	68	63	76	66	63	73	71	56	69	69	79	58	50	0													
18	69	62	63	66	61	71	64	58	68	66	45	64	68	74	52	58	33	0												
19	67	67	64	70	66	68	65	59	66	67	80	65	61	71	65	77	76	71	0											
20	84	76	77	72	70	77	77	78	70	71	86	69	69	73	69	67	70	77	73	0										
21	79	62	70	53	65	71	64	71	68	62	67	74	83	74	83	73	61	59	71	88	0									
22	82	78	67	79	69	76	72	69	76	78	84	63	63	76	63	75	77	71	68	61	89	0								
23	72	68	69	69	74	59	76	71	61	57	66	70	70	47	74	69	78	73	73	76	73	74	0							
24	88	77	78	74	68	79	79	68	76	77	83	80	76	75	80	74	84	85	82	67	82	65	73	0						
25	72	65	53	76	64	55	55	56	60	61	70	74	58	59	63	69	75	69	74	72	76	65	69	64	0					
26	76	61	58	73	68	67	63	60	64	65	70	71	58	67	63	60	59	62	77	67	69	70	72	64	44	0				
27	71	64	52	76	48	66	53	50	67	68	73	70	57	73	61	68	71	69	76	71	79	64	82	63	42	48	0			
28	58	50	51	75	57	67	53	46	50	58	62	73	67	60	59	57	57	59	70	71	72	74	71	69	48	44	47	0		
29	62	62	56	69	61	61	53	50	68	66	70	71	64	71	56	62	68	67	77	81	73	75	79	74	38	48	36	38	0	
30	62	58	63	57	71	70	56	58	58	58	62	63	73	63	70	68	64	66	70	79	59	74	58	76	61	53	64	47	51	0

Genetic diversity analysis of *S. aureus* isolates allowed their division into three main groups based on genetic distance. Group I was mainly constituted by isolates 28, 30, 10, 21, 7, and 9, which presented reduced genetic distance among themselves, indicating high molecular similarity. Group II, which included isolates 26, 29, 22, 27, 12, 14, 23, and 16, demonstrated an intermediate genetic similarity pattern among its members. Finally, Group III, composed of isolates 3, 11, 4, 8, 20, 18, 25, 6, 19, 5, 13, 2, 17, 15, and 1, was characterized by the greatest

genetic heterogeneity, with late fusion of its subgroups occurring under high dissimilarity values.

Figure 2 - Dendrogram

Source: Generated using the GENES software.

DISCUSSION

S. aureus is a pathogen of great relevance in both human and veterinary medicine, being responsible for a wide range of infections, from cutaneous conditions to severe systemic diseases (Zhu *et al*., 2023). Its ability to develop antibiotic resistance, including the emergence of methicillin-resistant strains (MRSA), represents a major public health challenge (González-Machado *et al*., 2024). Studying the genetic diversity of this species is essential for understanding its virulence mechanisms and tracking epidemiological outbreaks (Oliveira *et al*., 2022).

The application of ISSR markers in studying *S. aureus* and other bacterial species is a promising and innovative approach, due to the scarcity of supporting literature. Although its genome is relatively small, at approximately 2.8 Mb, *S. aureus* exhibits high genetic polymorphism, which directly impacts its antimicrobial resistance, host adaptation, and pathogenic potential (Baba *et al*., 2008). Investigating this variability significantly contributes to developing new strategies for diagnosis, control, and treatment of infections associated with this species (Aung *et al*., 2022).

Results presented in Table 1 show that amplification efficiency with ISSR primers in *S. aureus* varies according to annealing temperature, which was tested between 45°C and 65°C.

Intermediate temperatures, especially between 49°C and 51°C, resulted in the highest number of amplified primers, indicating better specificity under these conditions. Table 2 confirms this trend, showing that the highest number of amplified bands occurred with primer UBC 885 at 49°C, while very low or very high temperatures generally produced fewer bands. These data suggest that annealing temperature directly influences ISSR PCR efficiency, making it important to choose optimized conditions for each primer to ensure good amplification results.

Appropriate annealing temperature selection proved to be a determining factor for DNA amplification success. During tests performed between 45°C and 65°C, it was observed that 51°C showed polymorphic amplification in five distinct primers. Generally, higher temperatures tend to favor binding specificity between primers and target DNA, which contributes to reducing nonspecific products. In contrast, lower temperatures limited reaction efficiency, probably due to lower specificity and increased formation of unwanted secondary structures. These findings are consistent with evidence already described in the literature, which indicates that poorly adjusted PCR parameters can compromise result sensitivity and reliability, even leading to false positives or negatives (Karahan *et al*., 2009).

Annealing temperature is a critical factor that influences primer annealing efficiency in PCR reactions, being fundamental for process success and stringency. This temperature determines the ability of primers to bind to target DNA sequences, where very low temperatures may result in nonspecific binding and excessively high temperatures may inhibit proper hybridization, compromising amplification efficiency. Higher annealing temperatures generally increase specificity, while lower temperatures can lead to nonspecific amplification. The identification of 51°C as an optimal annealing temperature for multiple primers aligns with the need for high stringency in prokaryotic PCR. This temperature balances the kinetic energy required for primer-target hybridization while minimizing non-specific annealing in the relatively small 2.8 Mb genome of *S. aureus* (Sipos *et al*., 2007; Narina *et al*., 2011).

ISSR markers are widely employed in plant genetics to investigate genetic diversity and establish phylogenetic relationships (Desai *et al*., 2015). However, their application in prokaryotes faces several limitations. One of the main difficulties stems from the fact that prokaryotic genomes are more compact and have fewer repetitions compared to eukaryotes (Setubal *et al.,* 2018; Kimbrel *et al*., 2022).

Since ISSR oligonucleotides act in regions located between microsatellites, the low frequency of these sequences in prokaryotes compromises their efficiency in amplifying polymorphic regions (Fondon; Garner, 2004; Fatemifard *et al.,* 2024). Additionally, designing

ISSR primers for these organisms is challenging, as it depends on the presence of previously identified microsatellites, which are uncommon in prokaryotes. Another factor to consider is the existence of alternative methodologies, such as MLST (Multi-Locus Sequence Typing), RAPD (Random Amplified Polymorphic DNA), and whole-genome sequencing, which provide higher resolution and more detailed information about bacterial genetic variability (Ravaioli *et al.,* 2022; Elkady *et al.,* 2022).

Despite these barriers, there are reasons to reconsider the use of ISSR markers in prokaryotic genetic diversity analysis. In certain species where microsatellites are more frequent, this approach may be viable (Baysal *et al*., 2011). It is worth noting that the ISSR technique presents advantages such as simplicity, low cost, and accessibility, making it an interesting alternative for laboratories with limited resources (Kumar *et al*., 2007; Kerem *et al*., 2023; Felix *et al.,* 2020). With advances in molecular biology and bioinformatics, it is possible to develop more suitable ISSR oligonucleotides for specific bacterial genomes, expanding their applicability (Gemmill and Grierson, 2021; Gupta *et al*., 2021).

Furthermore, the efficiency obtained with ISSR primers under different temperatures can be contextualized against other molecular approaches applied in studying *S. aureus* genetic diversity. Techniques such as MLST and RAPD have been widely used to map genetic variability and understand the population structure of this species, including among strains isolated from asymptomatic individuals. While both RAPD and ISSR are low-cost techniques, RAPD suffers from low reproducibility due to its short primers (~10 bp) and low annealing temperatures. In contrast, ISSR employs longer primers (16-22 bp) and higher annealing temperatures (>45°C), resulting in greater specificity. This robustness makes ISSR significantly more reproducible and suitable for routine molecular typing. (Sakwinska *et al*., 2009). In this scenario, the use of ISSR markers emerges as a complementary strategy, allowing detection of genetic variations potentially linked to distinct antimicrobial resistance profiles and virulence factors.

Although bacterial DNA is generally smaller and more compact than eukaryotic DNA, which may make ISSR oligonucleotide use more complex, it was observed that *S. aureus* presents a significant amount of polymorphic ISSRs. This finding indicates that despite limitations imposed by reduced bacterial genome size, ISSR markers can offer valuable information about *S. aureus* genetic diversity and population structure.

The effectiveness of ISSR-PCR in genotyping *S. aureus* can be attributed to its capacity to detect polymorphisms not only within classical simple sequence repeats (SSRs) but also

across the broader landscape of genomic structural plasticity. *S. aureus* harbors numerous insertion sequences (IS elements) and transposons, along with highly variable intergenic regions that exhibit substantial strain-to-strain diversity. Because ISSR primers anneal to inverted repeats associated with these mobile genetic elements and polymorphic regions, the technique effectively captures genomic fingerprints reflecting the chromosomal rearrangements and insertional polymorphisms that define the species' evolutionary dynamics and epidemiological patterns (Malachowa; DeLeo, 2010; Jiang *et al*., 2025).

The high molecular homogeneity of Group I, detected in geographically distant municipalities, suggests a shared clonal origin among isolates. This pattern indicates that pathogen dissemination overcomes geographic barriers, probably mediated by interstate animal movement, wild animal or human transit, or shared use of milking equipment without proper disinfection (Phiri *et al*., 2022).

Group II showed intermediate genetic similarity, suggesting circulation of regional lineages. Genetic proximity among isolates from distinct but same macroregion localities indicates possible adaptation to predominant edaphoclimatic and management conditions in Northern Minas, such as high temperatures and manual milking systems. Moderate genetic distances may reflect local selective pressures, including varied antimicrobial use, favoring the emergence of sublineages with discrete divergences from a common ancestor (Souza *et al*., 2019; Gonçalves *et al*., 2023).

In contrast, Group III exhibited significant genetic heterogeneity, indicating distinct evolutionary dynamics. The observed phylogenetic divergence suggests multiple introduction sources or independent infection events. This diversified profile corroborates the existence of environmental reservoirs - milking equipment, fomites, and teat skin - that harbor and perpetuate genetically distinct lineages, directly contributing to the persistence and recurrence of subclinical mastitis in herds (Cremonesi *et al*., 2015; Touaitia *et al*., 2025).

CONCLUSION

It is concluded that among the 52 ISSR primers tested, 25 presented polymorphic profiles, evidencing their potential for genetic diversity studies in *S. aureus*. The presence of polymorphisms reinforces the applicability of these markers in epidemiological and evolutionary investigations. Furthermore, results demonstrate that *S. aureus* strains associated with subclinical mastitis in the studied region exhibit substantial genetic variability and wide geographic dispersion, with no evidence of municipality-restricted clustering. The absence of

defined geographic structuring corroborates the hypothesis of intermunicipality dissemination of the pathogen, highlighting the influence of animal movement and the lack of uniform biosecurity protocols.

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6. CONSIDERAÇÕES FINAIS

A caracterização molecular de *S. aureus* provenientes de mastite bovina subclínica no norte de Minas Gerais revelou um cenário epidemiológico desafiador, marcado pela tríade: elevada resistência antimicrobiana mediada pelo gene *bla*Z (70%), potencial significativo de formação de biofilme (*ica*A e *ica*D em 80% dos isolados) e expressiva diversidade genética com ampla dispersão intermunicipal.

As taxas de resistência aos β-lactâmicos (>90% para penicilina, ampicilina e amoxicilina) comprometem a eficácia terapêutica convencional, enquanto a ausência dos genes *mec*A, *mec*C e carbapenemases sugere mecanismos alternativos de resistência que demandam investigação futura. A coocorrência entre resistência antimicrobiana e capacidade de formação de biofilme, aliada à ausência de estruturação geográfica definida, evidencia disseminação ativa do patógeno, possivelmente facilitada pela movimentação animal e pela carência de protocolos uniformes de biossegurança.

A validação dos marcadores ISSR como ferramenta molecular para caracterização de *S. aureus* representa avanço metodológico relevante, permitindo monitoramento epidemiológico de cepas circulantes e rastreamento de rotas de disseminação. Esses achados estabelecem bases científicas para implementação de estratégias regionais integradas de controle, incluindo vigilância epidemiológica molecular contínua, uso racional de antimicrobianos orientado por antibiogramas, aprimoramento de protocolos de biossegurança na cadeia produtiva leiteira e desenvolvimento de terapias alternativas direcionadas a biofilmes.

Perspectivas futuras incluem a investigação dos mecanismos moleculares alternativos de resistência à oxacilina e meropenem, estudos longitudinais de transmissão inter-rebanhos, avaliação da eficácia de agentes antibiofilme em protocolos terapêuticos, e desenvolvimento de programas regionais de certificação sanitária. A integração entre vigilância molecular, boas práticas de manejo e uso criterioso de antimicrobianos é essencial para conter a disseminação de cepas multiresistentes e preservar a sustentabilidade da pecuária leiteira regional, com impactos diretos na saúde pública, segurança alimentar e viabilidade econômica do setor.

ANEXO 1 – Tabela de identificação, caracterização feno-genotípica e localização geográfica das cepas

ID	Código	Resistencia a antimicrobianos	Município	*mec*A	*mec*C	*bla*Z	*bla*KPC	*bla*OXA$_{23}$	*ica*A	*ica*D	*ica*B	*ica*C	*bap*
1	269A	CFO, IPM, ASB, OXA, MER	Janaúba	-	-	+	-	-	+	+	-	+	+
2	60A	CFO, ASB, OXA, MER	Janaúba	-	-	-	-	-	-	-	-	-	-
3	LM99	CFO, ASB, OXA, MER	Montes Claros	-	-	+	-	-	-	-	-	-	-
4	110G	CFO, ASB, OXA, MER	Icaraí de Minas	-	-	-	-	-	-	-	-	-	-
5	A6	ASB, OXA, MER	Montes Claros	-	-	+	-	-	+	+	+	-	-
6	A9	ASB, OXA, MER	Montes Claros	-	-	+	-	-	-	-	-	-	+
7	LM302	CFO, ASB, OXA, MER	Montes Claros	-	-	+	-	-	-	-	-	-	-
8	M1	ASB, OXA, MER	São Pedro das Garças	-	-	+	-	-	+	+	-	-	-
9	R6	ASB, OXA, MER	Nova Esperança	-	-	+	-	-	+	+	+	+	-
10	B8	ASB, OXA	Juramento	-	-	+	-	-	-	-	-	-	+
11	R9	ASB, OXA	Nova Esperança	-	-	+	-	-	+	+	-	-	-
12	M4	ASB, OXA, MER	São Pedro das Garças	-	-	+	-	-	+	+	-	-	-
13	MF4	ASB, OXA, MER	São Pedro das Garças	-	-	-	-	-	+	+	+	+	-
14	LM173	ASB, OXA	Montes Claros	-	-	+	-	-	+	+	-	-	+
15	A1	ASB, OXA, MER	Montes Claros	-	-	+	-	-	+	+	-	+	+
16	R13	ASB, OXA, MER	Nova Esperança	-	-	+	-	-	+	+	-	-	-
17	R15	IPM, ASB, OXA, MER	Nova Esperança	-	-	+	-	-	+	+	+	+	-
18	R19	ASB, OXA, MER	Nova Esperança	-	-	+	-	-	+	+	-	-	-
19	B4	ASB, MER	Juramento	-	-	+	-	-	+	+	-	+	+
20	LM270	IPM, ASB, OXA, MER	Montes Claros	-	-	+	-	-	+	+	-	-	-
21	M2	ASB, OXA	São Pedro das Garças	-	-	+	-	-	+	+	-	-	+
22	B18	ASB, MER	Juramento	-	-	+	-	-	+	+	-	-	+
b23	LM261	IPM, OXA, MER	Montes Claros	-	-	-	-	-	+	+	-	-	-
24	NP3	AMO, PEN, CFO	Janaúba	-	-	-	-	-	+	+	-	-	-
25	C1	AMO, PEN, AMP	Icaraí de Minas	-	-	-	-	-	+	+	-	-	-
26	C2	AMO, PEN, AMP	Icaraí de Minas	-	-	-	-	-	+	+	-	-	-
27	NP5	AMP	Janaúba	-	-	-	-	-	+	+	-	-	-
28	GU1	AMO, PEN, AMP	Matias Cardoso	-	-	+	-	-	+	+	-	-	-
29	NP2	PEN	Janaúba	-	-	+	-	-	+	+	-	-	-
30	AG1	AMO, PEN, CFO, AMP	Janaúba	-	-	-	-	-	+	+	-	-	-

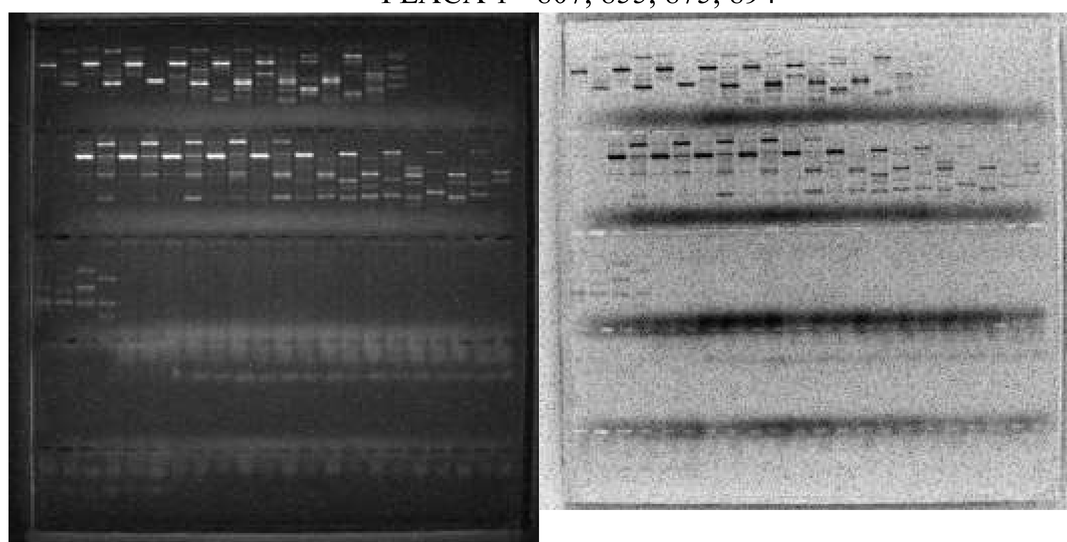
ANEXO 2 – Resultados da quantificação por espectrofotometria de DNA's extraídos de cepas de *Staphylococcus aureus*

ID	Código	A260nm	A280nm	Ratio	[DNA] (ng/ul)	DNA (ul)*	H2O (ul)*
1.	269A *S. aureus*	0,0524	0,0257	2,04	52,4	38	162
2.	60A *S. aureus*	0,0431	0,0256	1,68	43,1	46	154
3.	LM99 S. aureus	0,1362	0,0813	1,68	136,2	15	185
4.	110G *S. aureus*	0,0135	0,0124	1,09	13,5	148	52
5.	A6 *S. aureus*	0,0127	0,0133	0,95	12,7	157	43
6.	A9 *S. aureus*	0,0152	0,009	1,69	15,2	132	68
7.	LM302 *S. aureus*	0,0185	0,0219	0,84	18,5	108	92
8.	M1 *S. aureus*	0,1028	0,0481	2,14	102,8	19	181
9.	R6 *S. aureus*	0,0907	0,044	2,06	90,7	22	178
10.	B8 *S. aureus*	0,0283	0,012	2,36	28,3	71	129
11.	R9 *S. aureus*	0,2087	0,0981	2,13	208,7	10	190
12.	M4 *S. aureus*	0,0794	0,0388	2,05	79,4	25	175
13.	MF4 *S. aureus*	0,0926	0,0435	2,13	92,6	22	178
14.	LM173 *S. aureus*	0,0564	0,0265	2,13	56,4	35	165
15.	A1 *S. aureus*	0,0589	0,0297	1,98	58,9	34	166
16.	R13 *S. aureus*	0,0495	0,0267	1,85	49,5	40	160
17.	R15 *S. aureus*	0,0565	0,0295	1,92	56,5	35	165
18.	R19 *S. aureus*	0,1105	0,0526	2,1	110,5	18	182
19.	B4 *S. aureus*	0,2513	0,1153	2,18	251,3	8	192
20.	LM270 *S. aureus*	0,014	0,007	2	14	143	57
21.	M2 *S. aureus*	0,0611	0,0331	1,85	61,1	33	167
22.	B18 *S. aureus*	0,0245	0,0195	1,26	24,5	82	118
23.	LM261 *S. aureus*	0,0221	0,0171	1,29	22,1	90	110
24.	NP3 *S. aureus*	0,0304	0.0135	2,25	30,4	66	134
25.	C1 *S. aureus*	0,0408	0,0142	2,87	40,8	49	151
26.	C2 *S. aureus*	0,2702	0,1212	2,23	270,2	7	193
27.	NP5 *S. aureus*	0,3038	0,1367	2,22	303,8	7	193
28.	GU1 *S. aureus*	0,0315	0,012	2,63	31,5	63	137
29.	NP2 *S. aureus*	0,1632	0,075	2,18	163,2	12	188
30.	AG1 *S. aureus*	0,0135	0,0124	1,09	13,5	148	52

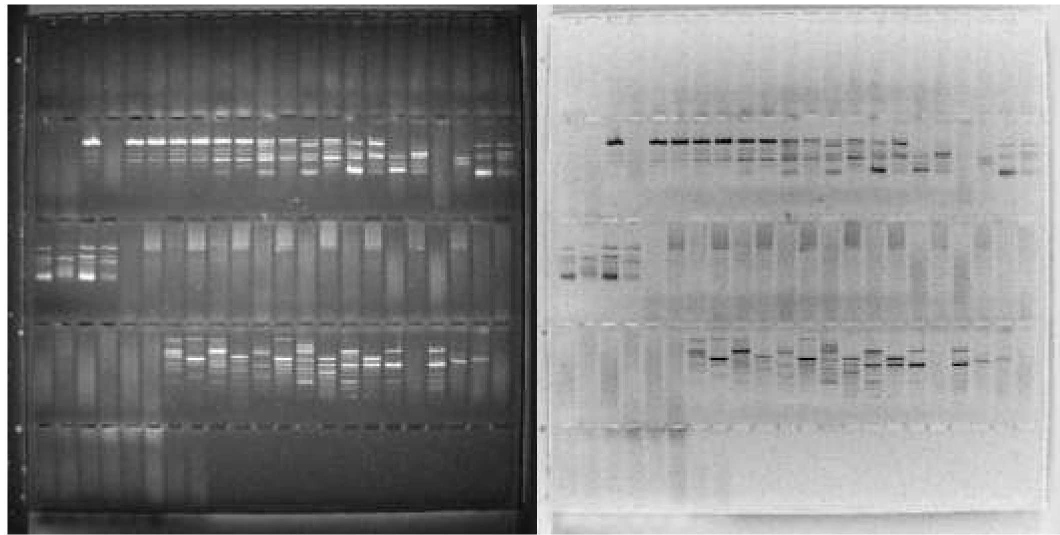
*Volumes em diluições compatíveis à preparação de 200 uL de DNA (10 ng/uL).

ANEXO 3 – Fotodocumentação de teste de gradiente utilizando oligonucleotídeos ISSR

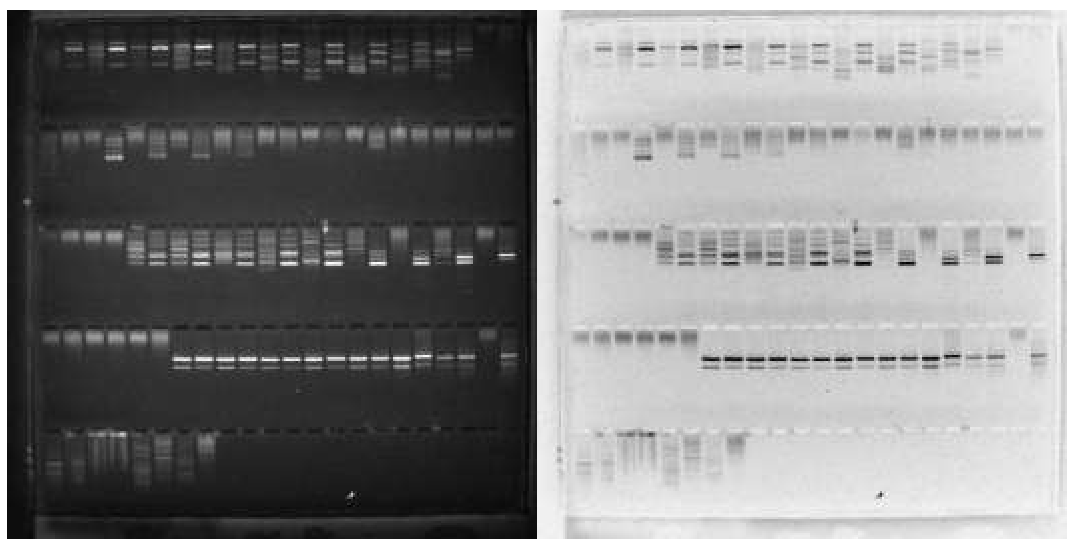
PLACA 1- 807, 855, 875, 894



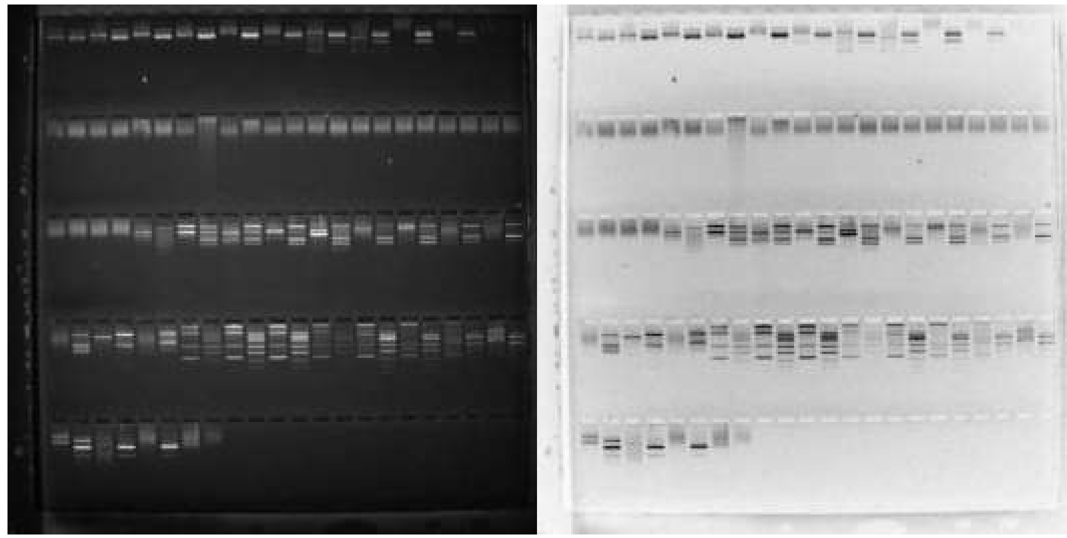
PLACA 02 - 806, 850, 870, 885



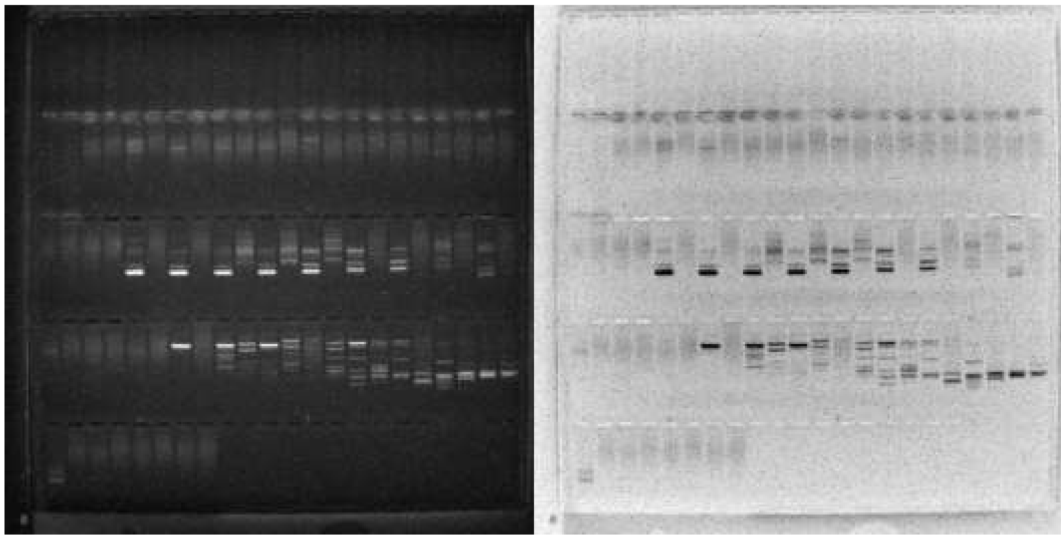
PLACA 03 - 809, 845, 880, 890



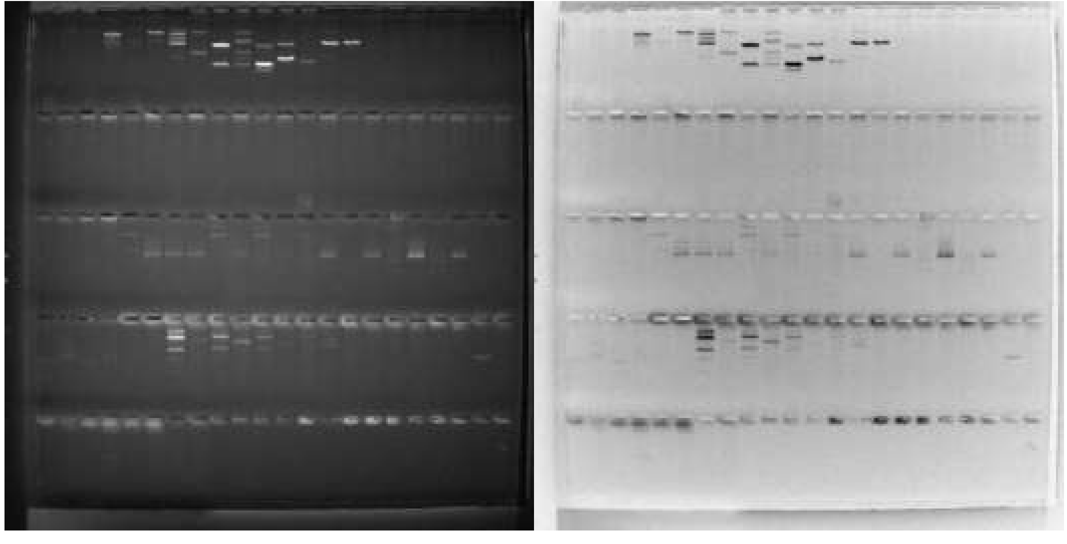
PLACA 04 - 808, 815, 825, 835



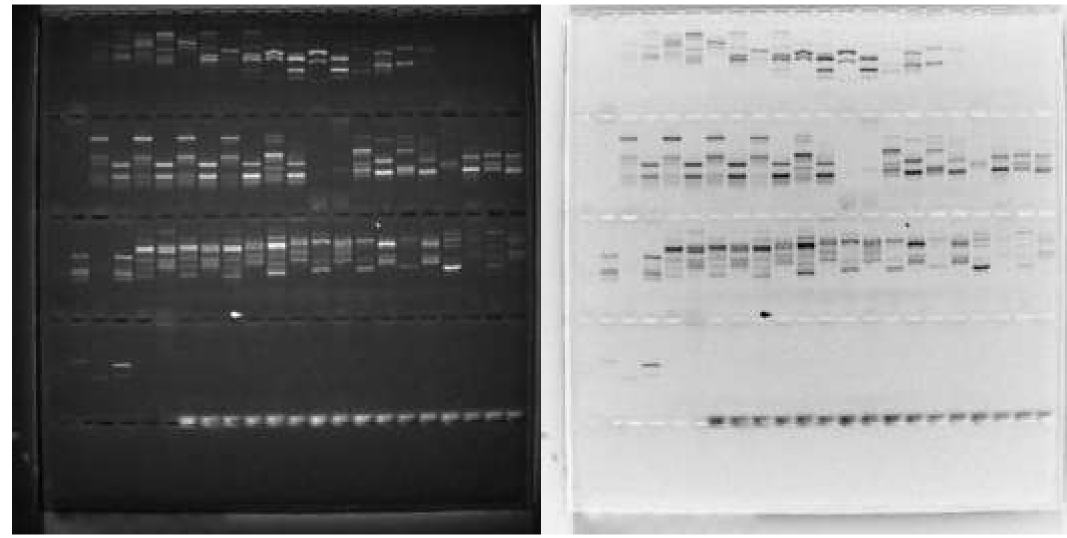
PLACA 05 - 805, 820, 830, 840



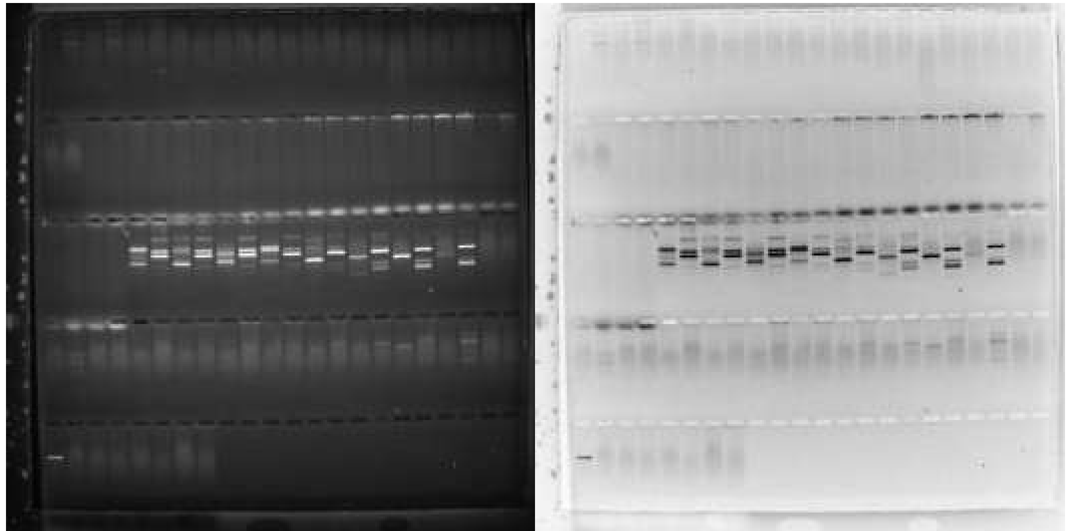
PLACA 06 - 810, 853, 865, 895



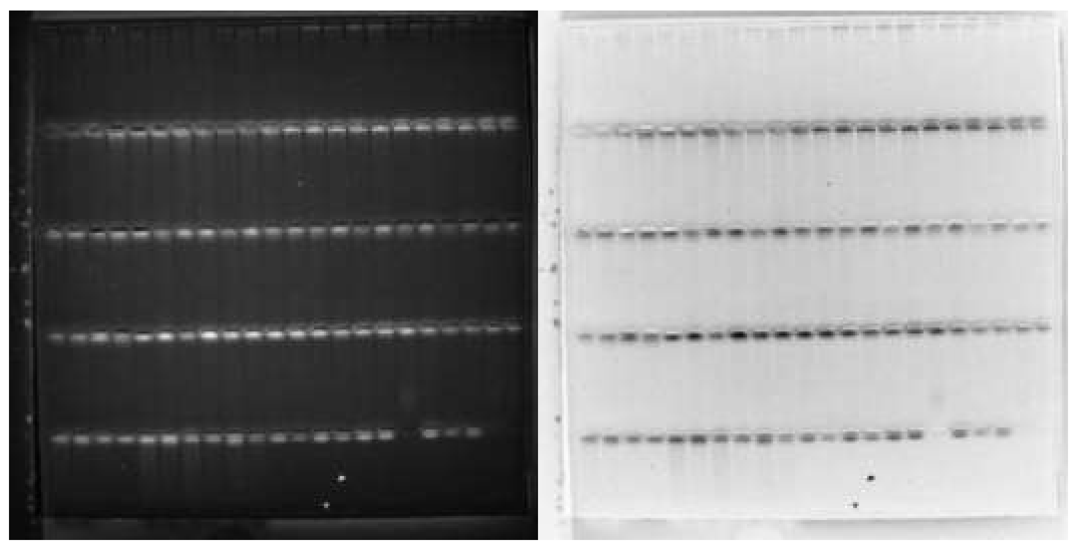
PLACA 07 - 811, 851, 861, 871



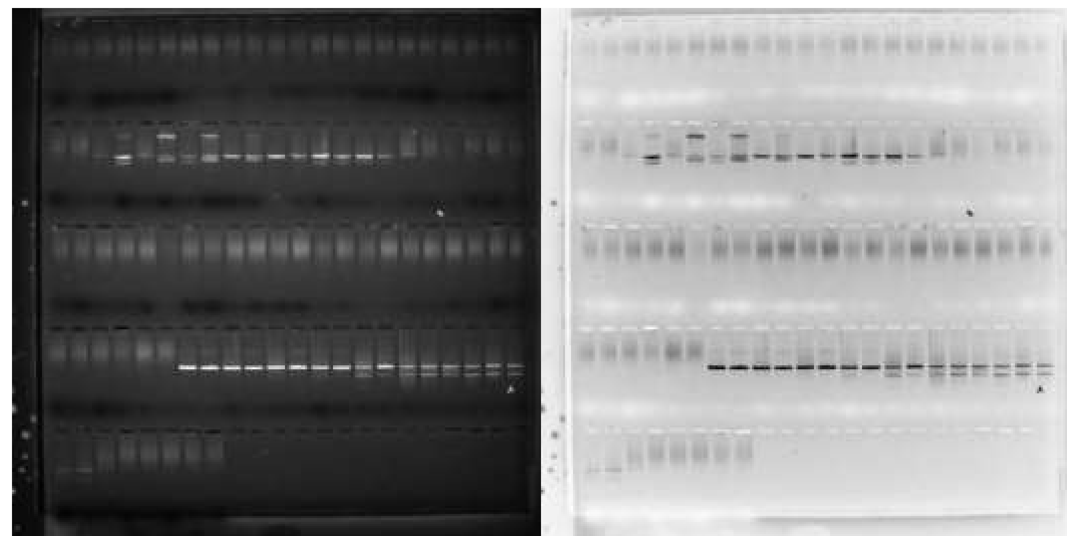
PLACA 08 - 821, 831, 841, 860



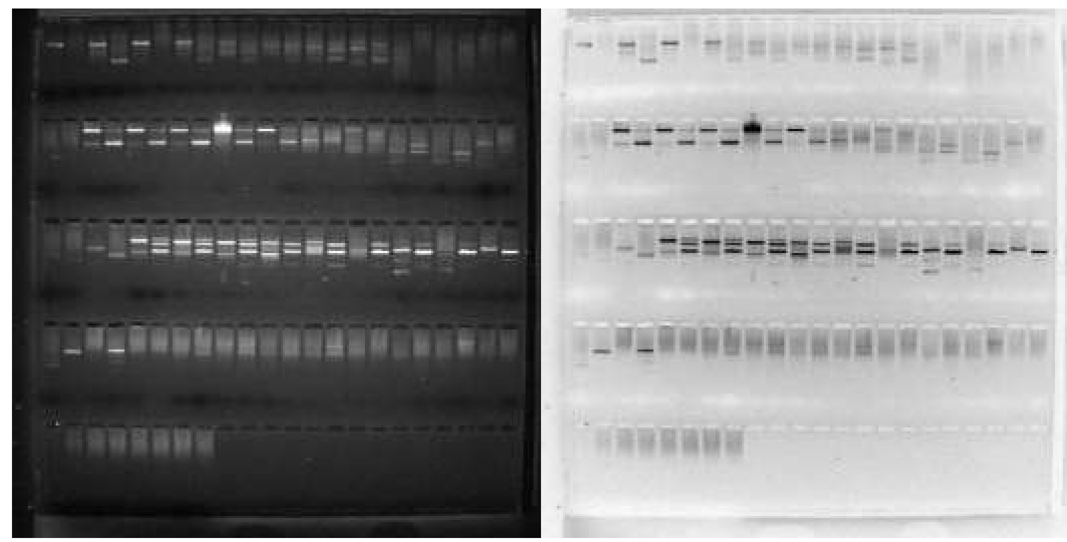
PLACA 09 - 801, 802, 803, 804



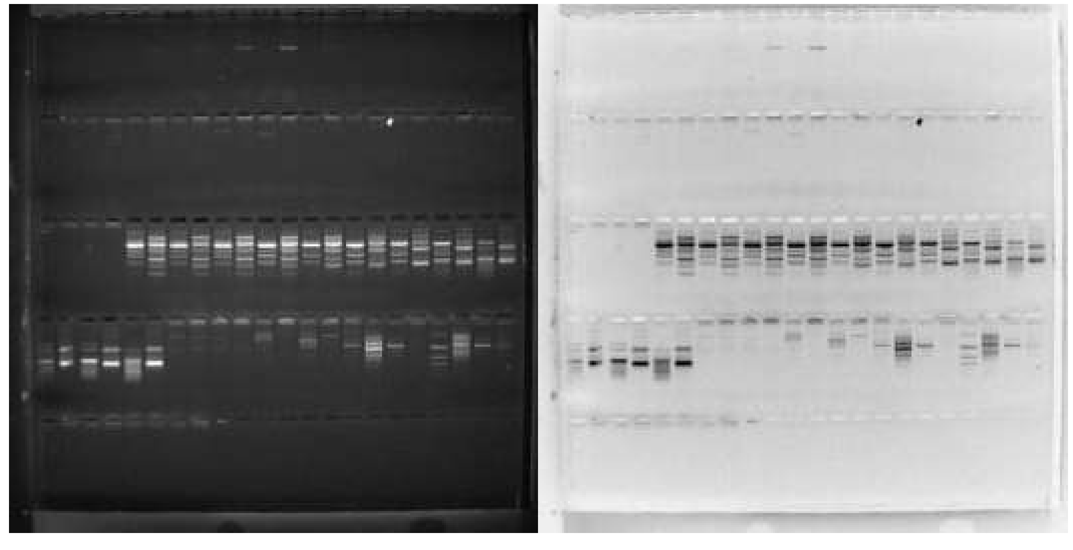
PLACA 10 - 824, 834, 881, 891



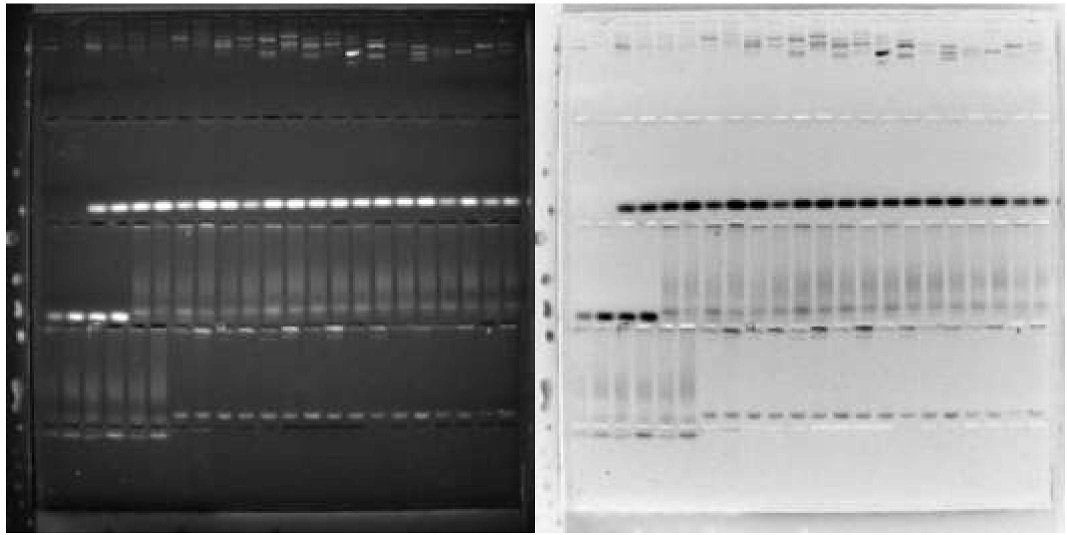
PLACA 11 - 816, 826, 836, 846



PLACA 12 - 814, 844, 862, 886



PLACA 13 - 818, 838, 877, 892



ANEXO 4 - Comprovante de submissão artigos 1 e 2