MALDI-TOF MS PARA TRIAGEM DE BIOMARCADORES DE RESISTÊNCIA ANTIMICROBIANA DE *Staphylococcus aureus* ISOLADOS DE CASOS DE MASTITE SUBCLÍNICA

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Objetivos

O presente estudo teve como objetivos: a) identificar a espécie *S. aureus*, utilizando MALDI- TOF MS e PCR; b) confirmar se as cepas de *S. aureus* são portadoras de genes de resistência por PCR; e c) detectar os biomarcadores de *S. aureus* produtor de delta toxina e de resistência à meticilina (MRSA) via MALDI-TOF MS, relatados em estudos prévios.

Métodos e Procedimentos

Um total de 229 *S. aureus* causadores de mastite subclínica, isolados de amostras de leite de quartos mamários e oriundos de 14 propriedades leiteiras de Pernambuco, foram utilizados no presente estudo. Adicionalmente, oito cepas padrões foram utilizadas como controle positivo. Para construção final do banco de dados, oito cepas de *S. aureus* positivas para *MecA*, oriundas de casos de infecção hospitalar humana, foram incluídas.

Inicialmente, os isolados de *S. aureus* foram reinoculados em placas de Ágar sangue, seguido de incubação por 24 h. As análises de MALDI-TOF MS foram realizadas de acordo com o protocolo de extração das proteínas ribossomais como descrito por Barcelos et al. (2010). Os espectros foram analisados no Biotyper 3.0. e os dados foram adquiridos por meio do FlexControl 3.3.

As cepas selvagens receberam a técnica de extração térmica do DNA de acordo com a metodologia de Fan et al. (1995). Para a confirmação da espécie *S. aureus* foi realizada à amplificação da região específica do gene *nuc* utilizando a técnica conforme Brakstad, Aasbakk e Maeland (1992). Para detecção dos genes responsáveis pela resistência à meticilina foi realizada PCR-Multiplex, seguindo a metodologia de Paterson et al. (2012).

A avaliação da resistência antimicrobiana (AMR) foi realizada por meio de duas etapas. Primeiramente, com base em uma revisão sistemática dos possíveis biomarcadores espectrais associados à *S. aureus* causador de mastite, selecionamos possíveis biomarcadores espectrais: 3005 e 3035 m/z para a delta-toxina; 4594 m/z para MRSA; e 2647 m/z para MSSA. Os espectros captados foram importados para o Flex Analysis, para avaliação dos picos e razão massa carga. Num segundo momento, foi avaliado a presença dos biomarcadores espectrais pelo ClinProTools, via algoritimo genético, utilizando os parâmetros descritos por Zhang et al. (2015).
CEUA UFRPE (nº 037/2018).

Resultados

Todos os isolados presentes no estudo apresentaram escore > 2.0 via MALDI-TOF MS e detecção do gene *nuc* positivo, o que possibilitou a confirmação em nível de espécie como *S. aureus*. Entretanto, não houve detecção dos genes de resistência (*mecA* e *mecC*), totalizando 100% do isolados sensíveis a meticilina (MSSA).

A detecção dos possíveis biomarcadores espectrais descritos na literatura (delta toxina, MRSA e MSSA) está apresentada na Tabela 1, mostrando a porcentagem dos isolados com a respectiva presença dos picos de massa relatados. Todas as cepas controle, MRSA positivas, apresentaram o pico de massa 2647 m/z, que tinha sido reportado em estudos prévios como biomarcador para MSSA.

Tabela 1. Percentual dos isolados de *S. aureus* observados no presente estudo que apresentaram biomarcadores espectrais mencionados em estudos prévios.

Biomarcador	Pico m/z	Cepas selvagens	%1	Referências
Delta toxina	3005	35	15,3%	Julie Gagnaire et al., 2012; Veronika Paskova et al., 2020
	3035	9	3,9%	Julie Gagnaire et al., 2012
MRSA	4594	201	87,8%	Samantha Flores-Treviño et al., 2019
MSSA	2647	224	97,8%	Maureen Feucherolles et al., 2019; Valerie Edwards-Jones et al., 2000

[1] Frequência absoluta mensurada com base no total das 229 cepas selvagens de *S. aureus*.

A análise de componente principal (PCA) via ClinProTools foi realizada para verificar se o pico 4594 ±5 m/z poderia ser adotado como biomarcador. As cepas selvagens agrupadas quanto à presença ou ausência de 4594 ±5 m/z foram comparadas e não apresentaram diferença estatística. Portanto, a análise de rastreabilidade espectral incluiu as cepas padrões em comparação aos isolados de *S. aureus* positivos para *MecA*, oriundos de infecção hospitalar. Os isolados de *S. aureus* causadores de mastite subclínica foram utilizados para validação do modelo (Figura 1). No geral, não observamos intercepção no PCA entre as cepas controle e as cepas *MecA* positivas. Além disso, todos os isolados selvagens de *S. aureus,* MRSA negativos, ficaram dispersamente distribuídos entre ambas as populações avaliadas no modelo.

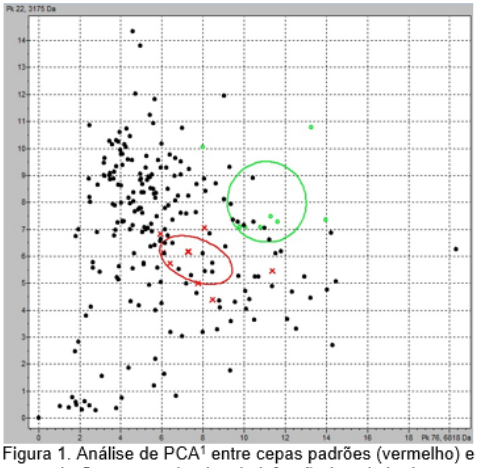

Figura 1. Análise de PCA[1] entre cepas padrões (vermelho) e cepas de *S. aureus* oriundas de infecção hospitalar humana (verde). As cepas selvagens de *S. aureus* causadoras de mastite foram utilizadas para validação do modelo (preto).
[1] Análise de Componente Principal.

Conclusões

A MALDI-TOF MS possibilita identificação de *S. aureus* em nível de espécie, similar aos resultados de detecção do gene *nuc*. Provavelmente, existem poucos MRSA oriundos de casos de mastite subclínica, com base nos resultados encontrados para detecção dos genes *MecA* e *MecC*. Nossos resultados sugerem baixa repetibilidade quanto ao uso dos biomarcadores espectrais 2647, 3005, 3035, e 4594 m/z, previamente descritos em estudos científicos, para detecção rápida de MSSA, produção de delta-toxina e MRSA. No geral, a procura de biomarcadores espectrais para MSSA seria a forma mais assertiva diante da baixa frequencia de MRSA causadores de mastite subclínica.

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MALDI-TOF MS FOR BIOMARKERS SCREENING OF ANTIMICROBIAL RESISTANCE OF *Staphylococcus aureus* ISOLATED FROM SUBCLINICAL MASTITIS CASES

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Objectives

The present study aimed to: a) confirm the *Staphylococcus* species, using MALDI-TOF MS and PCR; b) detect whether *S. aureus* isolates carry resistance genes by PCR; and c) describe the spectral biomarkers frequency observed for delta toxin-producing as well as for methicillin resistance (MRSA) from *S. aureus* isolates via MALDI-TOF MS.

Materials and Methods

A total of 229 *S. aureus* causing subclinical mastitis were isolated from mammary quarter milk samples from 14 dairy farms in Pernambuco. Additionally, eight standard strains were used as a positive control. The final database contained eight *MecA*-positive strains of *S. aureus* from cases of human nosocomial infection.

Initially, the *S. aureus* isolates were re-inoculated on blood agar plates, followed by 24 h incubation. MALDI-TOF MS analyzes were performed according to the ribosomal protein extraction protocol as described by Barcelos et al. (2010). Spectra were analyzed in Biotyper 3.0. and data were acquired using FlexControl 3.3.

The wild strains received the technique of thermal DNA extraction according to the methodology described by Fan et al. (1995). The amplification of the *nuc* gene to confirm the *S. aureus* at the species level were performed as described by Brakstad, Aasbakk and Maeland (1992). The genes responsible for resistance to methicillin were detected by PCR-Multiplex, following the described methodology of Paterson et al. (2012).

The assessment of antimicrobial resistance (AMR) was carried out in two steps. First, based on a systematic review of possible spectral biomarkers associated with *S. aureus* mastitis-causing, we selected possible spectral biomarkers: 3,005 and 3,035 m/z for delta-toxin; 4,594 m/z for MRSA; and 2,647 m/z for MSSA. The captured spectra were imported into Flex Analysis, for the evaluation of mass peak and mass-to-charge ratio. In a second moment, the presence of spectral biomarkers was evaluated by ClinProTools, via genetic algorithm, using the parameters described by Zhang et al. (2015). CEUA UFRPE (No. 037/2018).

Results

All 229 *S. aureus* isolates had score > 2.0 via MALDI-TOF MS and detection of the *nuc* gene, which enabled the confirmation of *S. aureus* at the species level. However, there was no detection of resistance genes (*mecA* and *mecC*), totaling 100% of methicillin-sensitive isolates (MSSA).

The spectral biomarkers frequency of delta-toxin producing, MRSA and MSSA were described in Table 1. Surprisingly, all MRSA positive control strains had a mass peak of 2,647 m/z, which had been reported in previous studies as a biomarker for MSSA.

Table 1. The spectral biomarkers frequency of delta-toxin producing, MRSA and MSSA.

Biomarker	Peak m/z	*S. aureus* isolates	%[1]	References
Delta toxin	3005	35	15.3%	Julie Gagnaire et al., 2012; Veronika Paskova et al., 2020
	3035	9	3.9%	Julie Gagnaire et al., 2012
MRSA	4594	201	87.8%	Samantha Flores-Treviño et al., 2019
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[1] Absolute frequency measured based on the total of 229 wild strains of *S. aureus*.

Principal component analysis (PCA) via ClinProTools was performed to verify whether the 4,594 ±5 m/z mass peak could be adopted as a MRSA biomarker. All *S. aureus* isolates were grouped based on the presence or absence of 4,594 ±5 m/z. The two groups were compared and were similar statistically. Therefore, the spectral traceability analysis included the standard strains in comparison to those *MecA*-positive *S. aureus* isolates from nosocomial infection. The *S. aureus* isolates causing subclinical mastitis were used to validate the model (Figure 1). Overall, we did not observe PCA interception between standard strains and *MecA*-positive *S. aureus* isolates. In addition, all 229 *S. aureus* isolates that had MRSA-negative results were dispersed among both populations evaluated in the model.

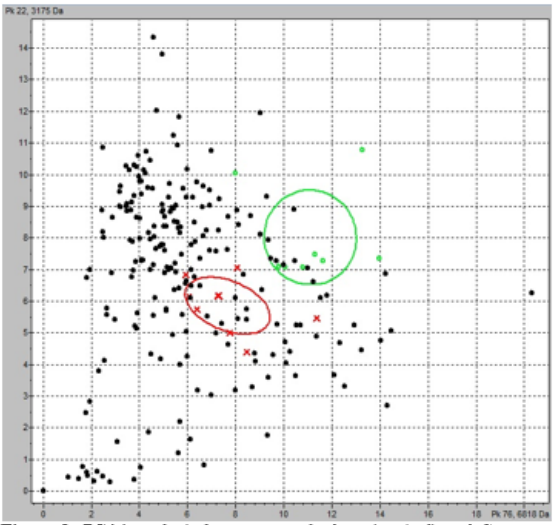

Figure 1. PCA1 analysis between standard strains (red) and *S. aureus* strains from human nosocomial infection (green). The wild strains of *S. aureus* causing mastitis were used for model validation (black).
1 Principal Component Analysis.

Conclusions

MALDI-TOF MS allows identification of *S. aureus* at the species level as similarly to the observed results of *nuc* gene detection. There are probably few MRSA from cases of subclinical mastitis, based on our results found for the detection of *MecA* and *MecC* genes. Our results suggests low repeatability regarding the use of the previous reported spectral biomarkers 2,647, 3,005, 3,035, and 4,594 m/z, for the rapid detection of MSSA, delta-toxin producing and MRSA. Overall, the search for spectral biomarkers for MSSA would be the most assertive way in view of the found low frequency of MRSA causing subclinical mastitis.

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