Journal of Applied Microbiology, 2023, 134, 1–8
https://doi.org/10.1093/jambio/lxad249
Advance access publication date: 31 October 2023
Research Article

JOURNAL OF
APPLIED
MICROBIOLOGY
OXFORD
UNIVERSITY PRESS

MALDI–TOF bacterial subtyping for rapid detection of biomarkers in *Staphylococcus aureus* from subclinical bovine mastitis

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Abstract

Aims: This study aimed to evaluate matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) bacterial subtyping for the rapid detection of biomarkers in *Staphylococcus aureus* from subclinical bovine mastitis.

Methods and results: A total of 229 *S. aureus* isolates were obtained from milk samples collected from cows with subclinical mastitis using microbiological culture. *Staphylococcus aureus* isolates were also submitted to PCR analysis targeting the *mecA* and *mecC* genes, which are indicative of methicillin resistance. Confirmation of the species was achieved through MALDI–TOF MS analysis. To analyze antimicrobial resistance patterns, the MALDI BioTyper Compass Explorer and ClinProTools Bruker software were employed, and dendrograms were generated using Bionumerics software.

Conclusions: MALDI–TOF MS successfully identified *S. aureus* at the species level, but no methicillin resistance was observed. Moreover, spectral typing displayed limited similarity when compared to pulsed-field gel electrophoresis (PFGE).

Impact Statement

The inappropriate use of antibiotics for bovine mastitis treatment is a major concern because it can lead to the development of antimicrobial resistance and increase the risk of the presence of antibiotic residues in milk and dairy products. Thus, the timely detection of AMR is essential to promptly initiate suitable treatment while preventing unnecessary or excessive antibiotic usage.

Keywords: MALDI–TOF MS, resistance, biomarkers, mastitis, PCR, *Staphylococcus aureus*

Introduction

Antimicrobial resistance (AMR) poses a significant threat to global public health and the economy (OECD 2018). The World Health Organization (WHO) has estimated the potential for 10 million deaths by 2050 due to AMR, with an estimated cost of 100 trillion dollars (O'Neill 2016). Notably, foodborne pathogens have received increasing attention as they are responsible for significant proportions of emerging infectious diseases and express multidrug-resistant patterns along a globally connected food chain, which can compromise the health of consumers worldwide (Lammie and Hughes 2016, Feucherolles et al. 2019).

In dairy farms, excessive and inappropriate use of critically important antibiotics is common, particularly for clinical mastitis treatment (Kollef 2008, Gonçalves et al. 2018b). Mastitis, a disease primarily caused by bacterial infections, is a major concern in dairy herds worldwide. These infections can lead to both milk quality changes and reduced production of dairy cows (Ruegg 2017). The economic impact of subclinical mastitis is substantial, accounting for ∼70% of all mastitis costs and causing significant losses for both producers and the dairy industry (Gonçalves et al. 2018a,b; Gonçalves et al. 2020).

Staphylococcus aureus (*S. aureus*) is one of the major pathogens associated with intramammary infections in dairy cows (Gonçalves et al. 2020, Gonçalves et al. 2023). The transmission of *S. aureus* among cows is usually associated with poor hygiene practices during milking, including contamination from milking equipment, the hands of milkers, and teat skin (Langoni et al. 2011, Lee et al. 2012). The bacterium has a remarkable ability to invade the mammary gland, leading to the formation of fibrous tissue that reduces the effectiveness of antimicrobial treatment, resulting in persistent infections with low cure rates (Lee et al. 2014, Gonçalves et al. 2023). Subclinical infections caused by *S. aureus* are particularly concerning as they serve as a reservoir for the transmission of new infections and pose significant challenges for primiparous cows during the prepartum and immediate postpartum periods (Fox 2009, Beuron et al. 2014).

The public health risk associated with *S. aureus* infections is increased when considering the presence of methicillin-

Received 7 September 2023; **revised** 12 October 2023; **accepted** 30 October 2023

resistant strains (MRSA) carrying resistance genes (*mecA* or *mecC*) that confer resistance to most β-lactam antibiotics. This poses a substantial threat to both animal and human diseases caused by MRSA (Klimešová et al. 2017). β-lactam antibiotics share a common β-lactam ring structure, and the primary mechanism of resistance involves the expression of β-lactamases that inactivate the antibiotic by destroying the β-lactam ring (Kostrzewa et al. 2013). Additionally, *S. aureus* isolates may harbor virulence factors, including various enterotoxins, which can be excreted in milk and remain stable in consumer products (Fagundes and Oliveira 2004). Limited studies have focused on the production of delta-toxin, a virulence factor regulated by the *agr* accessory gene system. The *agr* system plays a crucial role in determining the overall virulence during the acute phase of infection, whereas the absence of *agr* is associated with chronic infections (Gagnaire et al. 2012).

To effectively control bovine mastitis in dairy herds and administer appropriate antibiotic treatment, it is essential to have rapid and reliable information about the identification and resistance status of the causative pathogen (Kuti et al. 2008, Bauer et al. 2014, Dik et al. 2016). In this regard, MALDI–TOF mass spectrometry has revolutionized pathogen identification and facilitated the detection of AMR through spectral biomarkers (Ballesté 2018). MALDI–TOF serves as an alternative to conventional methodologies, providing results within 24 hours after conventional bacterial culture (Barreiro et al. 2010, Abbott and Fang 2015, Jorgensen and Turnidge 2015). Thus, we hypothesized that rapid and accurate differentiation between MRSA and methicillin-susceptible *S. aureus* (MSSA) using mass spectrometry could offer therapeutic advantages in selecting antibiotics more effectively.

This study aimed to evaluate the MALDI–TOF bacterial subtyping for rapid detection of biomarkers in *S. aureus* from subclinical bovine mastitis. The specific objectives of this study were: (a) to detect spectral biomarkers associated with delta-toxin-producing *S. aureus* and MRSA using MALDI–TOF MS, based on previously reported biomarkers; and (b) to compare the spectral typing obtained through MALDI–TOF MS with the results of pulsed-field gel electrophoresis (PFGE) for *S. aureus* strains isolated from milk samples collected from cows with subclinical mastitis.

Materials and methods

The experimental procedures were approved by the Committee on Ethics in the Use of Animals (CEUA) of the Federal Rural University of Pernambuco (UFRPE), Recife, Brazil, and the Research Ethics Committee of the University of Pernambuco (UPE), Recife, Brazil. This study was conducted in collaboration with the UFRPE as part of the project titled “Genetic traceability and detection of AMR of *S. aureus*.” Ethical approval for the study was obtained from the CEUA on 18 April 2018 (license number: 037/2018; process number: 23082.006410/2018–34).

Selection of *S. aureus* strains

A total of 229 *S. aureus* isolates were obtained from cows with subclinical mastitis on dairy farms in Pernambuco, Brazil. *Staphylococcus aureus* isolates were collected on two separate occasions. The first collection comprised 38 *S. aureus* isolates from milk samples of primiparous cows from two farms. The second collection included 191 *S. aureus* isolates from milk samples of both primiparous and multiparous cows from five farms.

Table 1. Genetic information from the well-characterized isolates used as positive controls.

Positive control	ST[1]	Clonal complex
ATCC 33591	ST239	CC8
USA 100	ST5	–
USA 800	ST5	–
MW2	ST1	CC1
COL	–	CC8
HU25	–	CC8
PER 34	–	–
LGA251*	ST425	CC130

[1] Sequence type. *CC130 detected in 60% of isolates from isolated bovine strains (García-Álvarez et al. 2011).

For the study, eight well-characterized *S. aureus* strains were used as positive controls, providing genetic information regarding sequence types (STs) or clonal complexes (Table 1). The *S. aureus* strains that tested positive for *mecA* were obtained from hemoculture samples of human nosocomial infections (i.e. this is the only information provided by the hospital) and were included as internal controls in the construction of the mass spectrometer database. Finally, all mass spectra of 311 *S. aureus* saved in the Qualileite laboratory database relative to the veterinary diagnostics of 2020’s year were utilized for the final validation of the MRSA spectral traceability model.

Apparent phenotypic resistance to beta-lactam antibiotics

Staphylococcus aureus isolates from collection 1 were evaluated for phenotypic resistance to methicillin using the minimum inhibitory concentration technique described by Silva et al. (2020), whereas isolates from collection 2 were evaluated for phenotypic resistance to cefoxitin using the disk diffusion test described by Silva et al. (2023).

Identification of *S. aureus* using MALDI–TOF MS

Initially, the ribosomal protein extraction protocol described by Barcelos et al. (2019) was performed. Briefly, *S. aureus* isolates were inoculated onto blood agar plates (Becton Dickinson, MD, USA) and incubated for 24 hours.

On-board extraction protocol

After the 24-hour incubation of the bacterial culture, a bacterial colony was transferred to a spot on a steel plate using a disposable loop. Next, 1.0 μL of 70% formic acid was applied and allowed to dry at room temperature. Once dried, 1.0 μL of the matrix solution, comprising α-cyano-4-hydroxycinnamic acid diluted in 50% acetonitrile and 2.5% trifluoroacetic acid, was applied and dried again at room temperature. Plate reading was performed following the protein extraction identification instructions (Bruker Daltonik, Bremen, Germany), and the spectra were analyzed using Biotyper 3.0.

Final stage of plate preparation and confirmation of *S. aureus*

To calibrate the MALDI–TOF MS, a protein standard solution (Bacterial Test Standard, BTS; Bruker Daltonik, Bremen, Germany) was used. MALDI–TOF mass spectrometry analysis was performed using a Microflex Bruker instrument. Data were acquired using FlexControl 3.3 software (Bruker Daltonik, Bremen, Germany). Mass spectra were collected in the mass range of 2000 to 20 000 mz^{-1}, with a total of 240 laser shots accumulated for each spectrum. The obtained spectra were compared with data from the MALDI Biotyper (MBT) reference library (Bruker Daltonik, Bremen, Germany). Protein extracts were analyzed in automatic mode, generating a fingerprint (a set of protein peaks). This fingerprint was then compared with the reference library of the MBT 4.1.7 software, which contained 7311 entries. Results with scores between ≥ 1.7 and <2.0 were considered reliable for gender identification, while scores ≥ 2.0 were considered reliable for genus and species identification.

Detection of genes *mecA* and *mecC* by PCR

DNA extraction

Wild strains isolated from cases of subclinical mastitis ($n = 229$), previously identified as *S. aureus* by MALDI–TOF MS, were inoculated onto tryptic soy agar (TSA) to increase the bacterial quantity for genomic DNA extraction. Following incubation at 35–37°C for 48 hours, thermal DNA extraction was performed using the methodology described by Fan et al. (1995). The extracted DNA was quantified, and its purity was analyzed using a spectrophotometer with absorbance readings at 260 nm and a 260/280 ratio.

Detection of mecA and *mecC*

PCR-Multiplex was employed to detect the genes responsible for methicillin resistance, following the methodology described by Paterson et al. (2012).

Pulsed-field gel electrophoresis (PFGE) genotyping

PFGE was used as the gold standard genotyping technique to evaluate the randomly performed spectral typing using twelve *S. aureus* isolates (Patyi et al. 2011). In brief, the PFGE technique involved DNA digestion using the SmaI enzyme, which was performed on a 1% agarose gel with 0.5X tris-borate-EDTA (TBE) buffer, as described by McDougal et al. (2003). Voltage and ramp pulse times were applied following the methodology outlined by Lim et al. (2013). The resulting gels were exposed to ultraviolet light and documented. Dendrograms were generated using Bionumerics software (Applied Maths, Kortrijk, Belgium), allowing for comparison of the bands obtained from each strain following enzymatic digestion with the mass peaks obtained from the spectra acquired via MALDI–TOF MS.

Evaluation of spectral AMR using MALDI–TOF MS

The evaluation of AMR was carried out in two stages. Firstly, the captured spectra were imported into the Flex Analysis software to evaluate the peaks and load mass ratio. To identify potential spectral biomarkers associated with *S. aureus* causing mastitis, a systematic review was conducted, analyzing a total of 25 relevant studies. From these studies, five presented spectral biomarkers for the detection of delta toxin and MRSA (Table 2). Reference mass peaks for delta toxin (3005 and 3035 mz^{-1}) and methicillin resistance (4594 mz^{-1}) were identified (Gagnaire et al. 2012, Flores-Treviño et al. 2019). Secondly, the presence of spectral biomarkers associated with subclinical mastitis caused by *S. aureus* was assessed using machine learning algorithms ("QuickClassifier," QC; "Supervised Neural Network," SNN; and "Genetic Algorithm," GA) via Bruker's ClinProTools software. This involved the following:

(a) The evaluation of spectral biomarkers involves peak shifts that may be related to gene mutations associated with MRSA or delta toxin.
(b) The assessment of MRSA or delta toxin production requires unique biomarkers associated with the production of specific molecules (e.g. enzymes and porins).

The MRSA prediction model in ClinProTools was constructed using two groups: wild-type strains of *S. aureus* causing subclinical mastitis and positive controls (as previously described in Table 1). Finally, the MRSA spectral traceability model was based on the genetic algorithm (GA) via machine learning, using the parameters described by Zhang et al. (2015): no more than 50 generations, no more than 5 peaks in each model, 3 neighbors, a combination of 100 peaks, a mutation rate of 0.2, and a crossover rate of 0.5.

Results

Confirmation of strains and isolates using MALDI–TOF MS

A total of 229 isolates that were identified as *S. aureus* and associated with subclinical mastitis (collection 1: $n = 38$; collection 2: $n = 191$) obtained a MALDI–TOF MS score >2.0, enabling species-level identification as *S. aureus*. Additionally, eight standard strains (positive control, Table 1) and eight *mecA*-positive *S. aureus* strains (from human nosocomial infections) were also confirmed as *S. aureus* at the species level.

Apparent phenotypic resistance to Beta-lactam antibiotics

Among the *S. aureus* isolates from collection 1, 4 out of 38 (10.5%) were found to be oxacillin-resistant, while among the *S. aureus* isolates from collection 2, only 5 out of 191 (2.6%) exhibited cefoxitin resistance.

Detection of *mecA* and *mecC* genes

All 229 isolates were identified as *S. aureus* by MALDI–TOF MS with a score >2.0. However, methicillin resistance genes (*mecA* and *mecC*) were not detected, indicating that all *S. aureus* ($n = 229$) isolates were MSSA. *Staphylococcus aureus* was defined as MRSA when the presence of *mecA* or *mecC* was detected.

Spectral traceability of delta toxin and MRSA based on previous studies

Detection of delta toxin revealed that 15.3% ($n = 35/229$) of the *S. aureus* isolates exhibited a peak mass of 3005 mz^{-1}, while 3.9% ($n = 9/229$) displayed a peak mass of 3035 mz^{-1}. In the case of MRSA detection, 87.8% of the isolates ($n = 201/229$) showed a peak mass of 4594 mz^{-1}. Surprisingly, for MSSA detection, 97.8% ($n = 224/229$) of the isolates exhibited a peak mass of 2647 mz^{-1}. It is noteworthy that all MRSA strains used as positive controls had a peak mass of 2647 mz^{-1}.

Table 2. Percentage of *S. aureus* isolates observed in the present study that presented spectral biomarkers mentioned in 5 previous studies.

Biomarker	Peak mz^{-1}	Wild strains	%[1]	Standard strains	%[2]	References
Delta toxin	3005	35	15.3%	5	62.5%	Gagnaire et al. (2012); Paskova et al. (2020)
	3035	9	3.9%	0	0%	
MRSA	4594	201	87.8%	6	75%	Flores-Treviño et al. (2019)
MSSA	2647	224	97.8%	8	100%	Edwards-Jones et al. (2000); Feucherolles et al. (2019)

[1] Absolute frequency measured based on the total of 229 wild-type *S. aureus* strains.
[2] Absolute frequency measured based on the total of 8 *S. aureus* positive control strains.

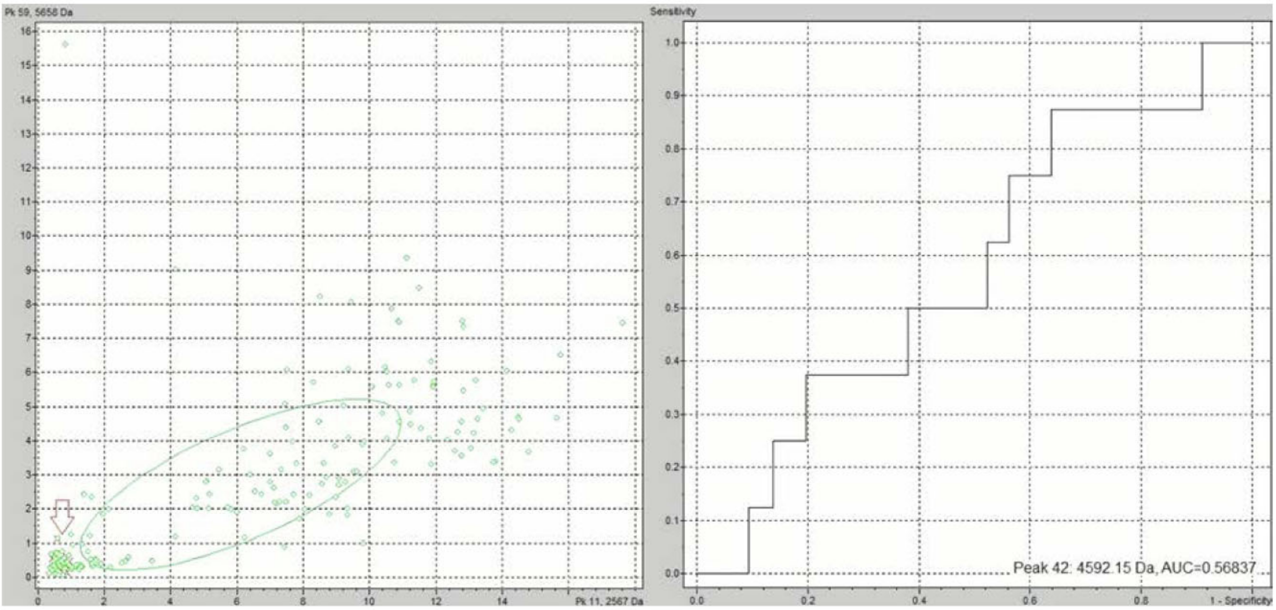

Figure 1. Comparison of standard strains and *S. aureus* isolates. (A) PCA analysis differentiating standard strains (red arrow) from *S. aureus* isolates grouped by the presence of the peak mass at 4594 ± 5 m/z (green). (B) ROC analysis of the 4594 ± 5 m/z peak for distinguishing between the standard strains and the *S. aureus* isolates characterized by the peak mass at 4594 ± 5 m/z.
[1] Principal Component Analysis.
[2] Operator Characteristics Curve.

Principal component analysis (PCA) was performed using the ClinProTools software to investigate whether the peak mass of 4594 ± 5 mz^{-1} could serve as a potential biomarker due to the high percentage of MRSA detected using MALDI. When comparing the wild-type strains grouped based on the presence or absence of the peak mass of 4594 ± 5 mz^{-1}, no statistical difference was observed between the two groups. Furthermore, the *S. aureus* isolates grouped based on the presence of a peak mass of 4594 ± 5 mz^{-1} showed no significant difference when compared to the group of standard strains (Fig. 1).

Spectral traceability of MRSA using genetic algorithm via machine learning

Given the absence of the *mecA* gene detection in all *S. aureus* isolates associated with subclinical mastitis, we focused on the standard strains and *S. aureus* isolates from hospital-acquired infections for the spectral traceability analysis. We did not observe any overlap in the PCA between the standard strains and the *S. aureus* isolates from hospital sources, despite both groups having *mecA* detection, suggesting some differences between the two groups. To further validate the GA through machine learning, wild *S. aureus* strains causing mastitis were included in the evaluation (Fig. 2). In general, the wild *S. aureus* isolates causing mastitis showed a dispersed distribution among both populations (control strains and hospital infection isolates), with some even overlapping with the standard strains.

Comparison of spectral typing and PFGE technique:

Twelve *S. aureus* isolates from cases of subclinical mastitis were randomly chosen based on their resistance to the antimicrobials oxacillin (17.2% resistance at the highest evaluated concentration of 16 µgmL^{-1}) and penicillin G (32% resistance at the highest evaluated concentration of 2 µgmL^{-1}). Interestingly, despite the high MIC values observed for both antimicrobials (oxacillin and penicillin G), only two isolates showed negative results for the detection of the *blaZ* gene (data not shown).

Using the PFGE technique, four distinct pulsotypes of *S. aureus* (A to D, Fig. 3) were identified. However, the dendrogram generated based on the mass spectrometer results showed low similarity compared to the dendrogram obtained using the standard PFGE methodology.

Overall, the comparison of the two dendrograms generated using the PFGE and MALDI–TOF MS techniques in the Bion-

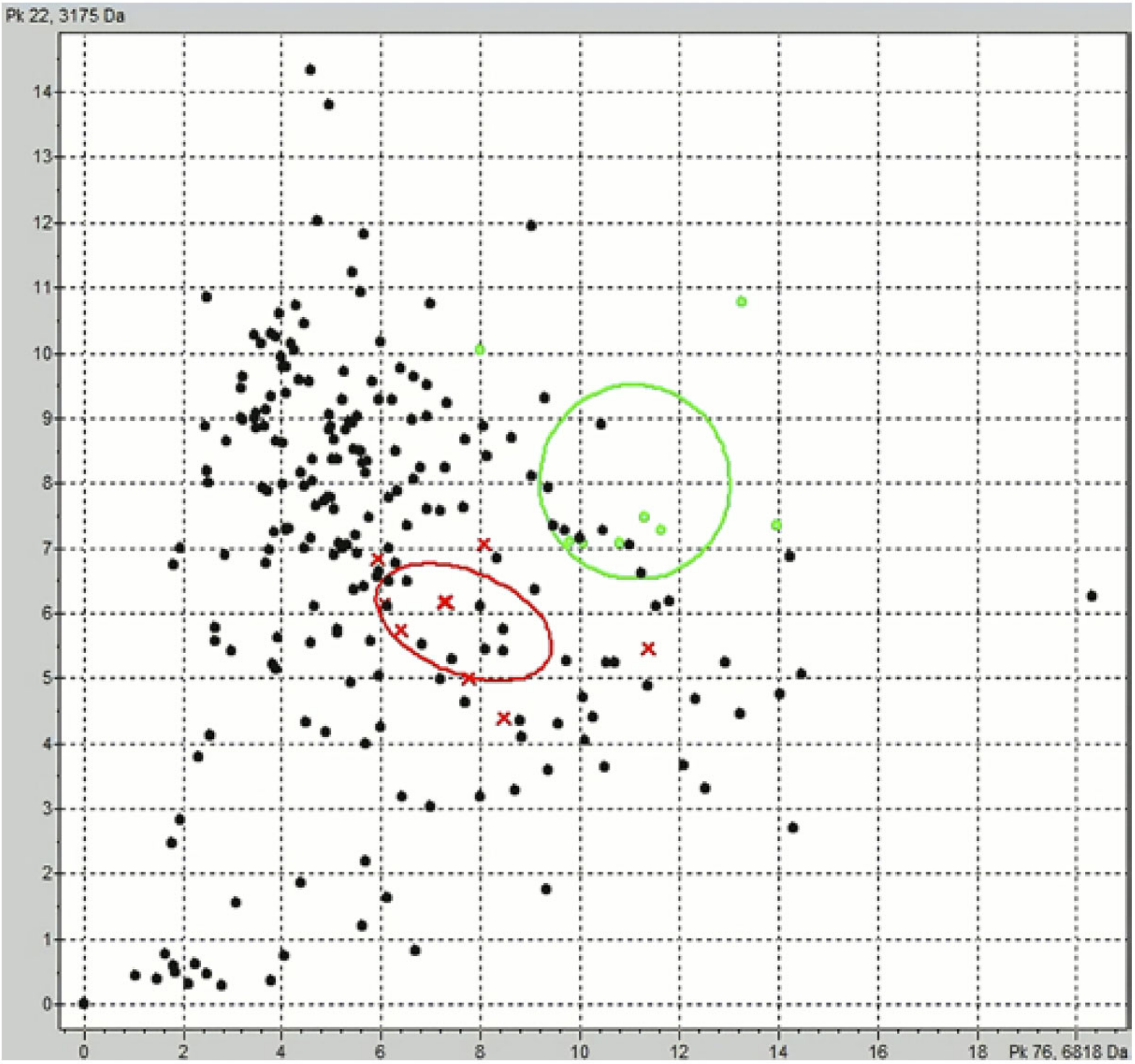

Figure 2. Comparison of PCA[1] between standard strains (red) and *S. aureus* strains derived from human nosocomial infections (green). Wild mastitis-causing *S. aureus* strains were used to validate the model (black).
[1] Principal Component Analysis.

umerics software revealed only an 11% similarity between the two methods. For instance, the C-pulsetype, which was the most frequent among the isolates (58.3%, $n = 7/12$) according to PFGE, was distributed across different spectral clusters in the proteomics-based dendrogram.

Discussion

In the context of AMR, there is increasing concern about foodborne pathogens due to the global emergence of multidrug-resistant patterns (Lammie and Hughes 2016). Therefore, this study aimed to develop a spectral traceability database for MRSA, as rapid detection of AMR allows for informed decision-making regarding antibiotic usage. To the best of our knowledge, there are limited studies on spectral traceability using spectral biomarkers for the rapid detection of resistant strains.

Confirmation of *S. aureus* species-level identification using MALDI–TOF MS was supported by the detection of the *nuc* gene through PCR (data not shown). These findings were consistent with a previous study that demonstrated the high accuracy of MALDI–TOF mass spectrometry for the rapid identification of *Staphylococcus* spp. (Barcelos et al. 2019).

Surprisingly, no MRSA (neither the *mecA* nor *mecC* genes) were detected in the *S. aureus* isolates associated with subclinical mastitis, despite 10.5% of *S. aureus* from collection 1 being found to be phenotypically oxacillin-resistant and 2.6% of *S. aureus* from collection 2 exhibiting phenotypic cefoxitin resistance. This finding aligns with previous studies that found zero livestock MRSA (neither *mecA* nor *mecC*) but 4.3% of methicillin-resistant *non-aureus* Staphylococci Mammaliicocci (MR-NASM; *mecA* gene detected) from bulk tank milk (BTM; $n = 300$ farms) located in Indiana, Ohio, and Michigan (Goncalves et al. 2023). Another study found lower than 1% of livestock MRSA from BTM ($n = 288$ farms) located in New York, Wisconsin, and Oregon (Cicconi-Hogan et al. 2014). However, our findings contrasted with those presented by Flores-Treviño et al. (2019), who reported 83.3% MRSA.

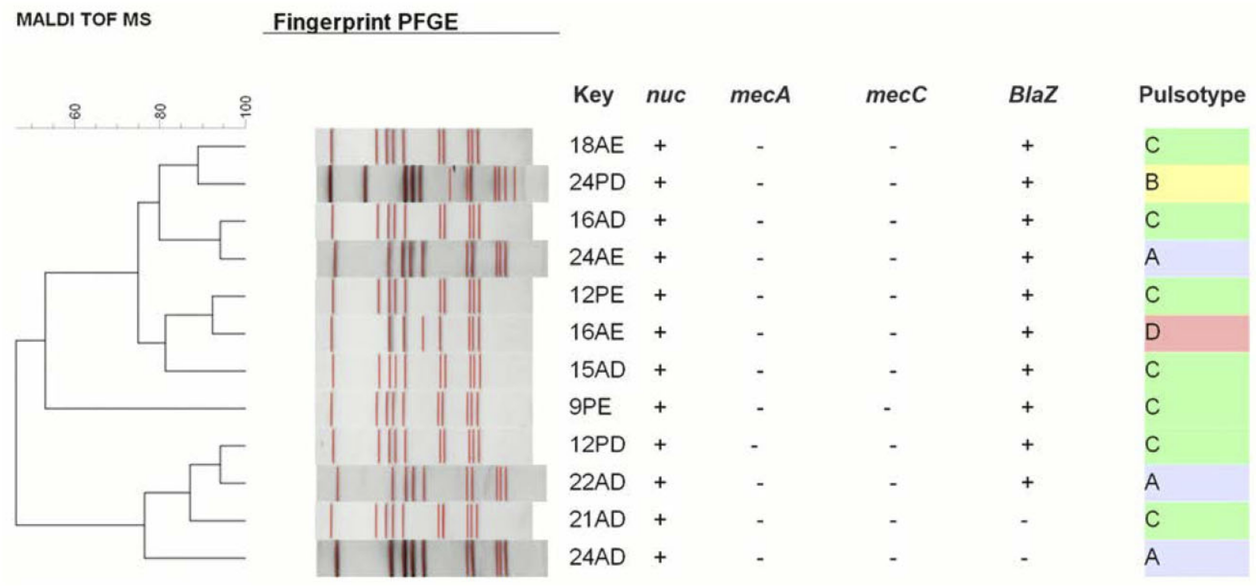

Figure 3. Dendrogram showing *S. aureus* pulsotypes (n = 12) obtained by MALDI–TOF MS. The bands shown represent *S. aureus* isolates after the PFGE technique based on the use of the restriction enzyme SmaI. Four distinct pulsotypes of *S. aureus* (A to D) were defined based on a cutoff of 80% similarity of DICE. Dendrograms (PFGE and MALDI–TOF) were prepared in the Bionumerics software using the UPGMA clustering method (0.5% optimization; 1.25% tolerance).

It is important to note that the high prevalence of MRSA in the Flores-Trevino et al. (2019) study was based on testing human hospital isolates with no connection to bovine subclinical mastitis cases. Further investigations are necessary to understand the low frequency of MRSA observed in our study, which focused on the detection of *mecA* and *mecC* genes in subclinical mastitis cases.

The absence of *mecA* gene expression prevented the genotypic characterization of AMR, specifically related to methicillin, in *S. aureus*. Previous studies have associated the peak at 4594 mz^{-1} with the 50S ribosomal protein L28, which is easily detected by MALDI–TOF MS and used for MRSA detection, as described by Flores-Treviño et al. (2019). However, the presence of different inhibitors of protein synthesis, such as macrolides, ketolides, lincosamides, and type B streptogramins, can inhibit the formation of the 50S particle in growing cells. Considering the use of this specific spectral biomarker alone, a total of 87.8% MRSA would have been identified. However, according to Flores-Treviño et al. (2019), it is necessary to detect the peak in the form of a doublet (two mass peaks together, 4594 and 4605 mz^{-1}) to achieve greater sensitivity in MRSA detection. In our study, the presence of doublets was investigated (data not shown), but all mastitis isolates were MSSA, showing negative results for *mecA* and *mecC* detection.

Detection of *S. aureus* delta-toxin was achieved through the identification of specific spectral biomarkers, namely peaks at 3005 or 3035 $\pm$ 5 mz^{-1}. In our study, 15.3% and 3.9% of *S. aureus* isolates displayed peaks corresponding to delta-toxin production, namely 3005 and 3035, respectively. Previous studies have linked delta toxin production to the status of the accessory regulatory gene (*agr*), the main regulator of virulence in *S. aureus*, and the expression of RNA III (Gagnaire et al. 2012). Deficiency in delta toxin has been associated with the chronicity of *S. aureus* infection and resistance to glycopeptides (GISA/heterogeneous GISA). Therefore, assessing the *agr* status could potentially predict infectious complications and guide the use of new therapies based on virulence factors. Furthermore, strains lacking both peaks (3005 and 3035 $\pm$ 5 mz^{-1}) may correspond to *agr*-defective strains that do not exhibit delta toxin production due to impaired RNAIII expression.

Overall, our findings suggest that the previously proposed spectral biomarkers 3005, 3035, and 4594 mz^{-1} cannot be used for rapid detection of delta-toxin-producing and MRSA strains, respectively. It is important to note that all of our standard MRSA strains (positive controls; Table 1) exhibited the spectral biomarker for MSSA (2647 mz^{-1}), which differs from the results reported by Feucherolles et al. (2019) regarding the use of this spectral biomarker for MSSA detection.

Our results demonstrate a low frequency of MRSA in cases of subclinical mastitis. Therefore, our database suggests that searching for spectral biomarkers focused on the detection of MSSA isolates would be more reliable than targeting resistant strains. Although we did not identify a spectral biomarker for MRSA detection using the GA, our results indicate that the peak mass of 3175 $\pm$ 5 mz^{-1} could be a potential biomarker for the detection of MSSA isolates.

These findings highlight a discrepancy between spectral biomarker detections and the *mecA* gene, underscoring the need for ongoing improvement in our database and detection models. Additionally, we recommend analyzing the SCC*mec* cassettes of the isolates, as this could potentially explain the observed differences among certain *S. aureus* isolates. We believe that these differences can be attributed to the fact that the PSM-*mec* peptide detected by MALDI is specifically associated with SCC*mec* cassette types II, III, and VIII, which were not assessed in our study (Schuster et al. 2018).

Regarding spectral typing compared to the PFGE technique, no similarities were observed between the two methodologies. PFGE is capable of detecting substantial intraspecies diversity to a greater extent than MALDI typing (Monecke et al. 2014). Lindgren et al. (2018) have also stated that the results of MALDI typing and PFGE cannot be easily compared between different laboratories. Therefore, MALDI typing is suggested for rapid screening, while methods like PFGE should be utilized when more comprehensive typing is required to obtain accurate epidemiological information. However, Ueda et al. (2015) reported successful identification of clones using mass spectrum analysis in clinically isolated outbreak strains, with typing results fully consistent with PFGE. While our findings are in line with those of Monecke et al. (2014) and Lindgren et al. (2018), they differ from those of Ueda et al. (2015). It is important to note that our results of PFGE focus on only 12 *S. aureus* isolates from mastitis, and the evaluation is limited to these isolates. Thus, we cannot conclusively infer that mass spectrometry would be inferior to molecular biology methodologies like PFGE for typing purposes.

The results of our study suggest that the MALDI–TOF MS methodology can be a rapid tool for bacterial identification in routine laboratory practice, enabling species-level identification of *S. aureus*. Our findings suggest a low prevalence of methicillin resistance among milk-associated *S. aureus* isolates, as determined by the detection of the mecA and mecC genes. Further studies are required to better understand the observed frequency. Therefore, our results emphasize that focusing on the detection of MSSA isolates would be more reliable. Additionally, our results indicate low repeatability in using the previously described spectral biomarkers 2647, 3005, 3035, and 4594 mz^{-1} for MSSA, delta-toxin, and MRSA detection, respectively. Finally, spectral typing showed limited similarity when compared to the standard PFGE technique.

Acknowledgments

We would like to thank the “Qualileite” Milk Quality Laboratory team at the School of Veterinary Medicine and Animal Science—USP, Brazil, as well as all the milk producers, for their assistance during the milk sampling period. Additionally, we are grateful to Prof. Dr Kevin Anderson from North Carolina State University for reviewing the English writing.

Conflict of interest: The authors have not stated any conflicts of interest.

Funding

The authors acknowledge FAPESP for the scholarship (Proc. 2013/23613-8 and Proc. BEPE 2015/04570-1) and project funding (Proc. 2014/17411-6).

Author contributions

Thainara Lopes (Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft), Carlos Eduardo Fidelis (Formal analysis, Methodology, Validation, Visualization, Writing – review & editing), Amanda Thaís Ferreira Silva (Formal analysis, Validation, Visualization, Writing – review & editing), Rinaldo Aparecido Mota (Investigation, Validation, Visualization, Writing – review & editing), Vera L. M. Rall (Investigation, Validation, Visualization, Writing - review & editing), Marcos Veiga dos Santos (Conceptualization, Investigation, Resources, Validation, Visualization, Writing – review & editing), and Juliano Leonel Gonçalves (Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing)

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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Received 7 September 2023; **revised** 12 October 2023; **accepted** 30 October 2023