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Identification of bovine mastitis pathogens using MALDI-TOF mass spectrometry in Brazil

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Research Article

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Abstract

In this Research Communication we evaluate the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to identify 380 bacteria isolated from cases of bovine mastitis in Brazil. MALDI-TOF MS identifications were compared to previous identifications by biochemical tests and 16S rRNA sequencing. MALDI-TOF MS achieved a typeability of 95.5%. The accuracy of MALDI-TOF MS for the identification of *Staphylococcus* isolates was 93.2%. The agreement between MALDI-TOF MS and biochemical identification of *Streptococcus agalactiae* was 96%, however, the agreement between these techniques for identifying other catalase-negative, Gram-positive cocci was lower. Agreement in identifying Gram-negative bacteria at the genus level was 90.5%. Our findings corroborate that MALDI-TOF MS is an accurate, rapid and simple technique for identifying bovine mastitis pathogens. The availability of this methodology in some research institutions would represent a significant step toward increasing the diagnosis and epidemiological studies of bovine mastitis and other animal infectious diseases in Brazil.

Mastitis is a significant disease in dairy cattle that causes substantial economic loss. Several bacteria can cause bovine mastitis (NMC, 2004), and differentiation between species is sometimes challenging (Cameron *et al*., 2017). In many studies of bovine mastitis, there is no species-level identification of *Staphylococcus* species (other than *S. aureus), Streptococcus* species and other catalase-negative, Gram-positive cocci. Accurate identification at the species level is essential for epidemiological studies. The lack of accuracy of traditional methods and the cost of molecular methods (Cameron *et al*., 2017) are among the probable causes for the lack of speciation of these groups of microorganisms. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a rapid and sensitive method for identifying microorganisms (Singhal *et al*., 2015), however, it is not yet available in many diagnostic laboratories. Because of the scarcity of information concerning the bacterial species that cause bovine mastitis in Brazil, the present study aimed to evaluate the use of MALDI-TOF MS for species-specific identification of bacteria isolated from cases of bovine mastitis and to compare the results to those obtained by conventional biochemical tests and partial 16S rRNA gene sequencing.

Materials and methods

We used 380 isolates, including 203 *Staphylococcus* spp., 156 catalase-negative, Gram-positive cocci and 21 Gram-negative bacteria. They were isolated from milk samples obtained from mammary quarters of cows with or without signs of mastitis and are mantained in a biological collection. The isolates were identified at least at the genus level and were isolated and identified according to the procedures recommended by the National Mastitis Council (NMC, 2004).

All isolates were evaluated by MALDI-TOF MS using a direct transfer protocol. The spectra of each sample were generated using a mass spectrometer (MALDI-TOF LT Microflex, Bruker®, Billerica, MA, USA) equipped with a 337-nm nitrogen laser in a linear path and controlled by the FlexControl 3.3 (Bruker®) program. The spectra were collected in a mass range between 2000 and 20 000 m/s, and they were analyzed using the MALDI Biotyper 3.1 (Bruker®) program with the standard configuration for bacteria identification. Values for matching greater than or equal to 2.0 (reliable genus identification and probable species identification) were accepted. The isolates that were not identified with the direct transfer protocol with a score equal to or greater than 2.0 in two attempts were subjected to an extended protocol, as described in the online Supplementary Materials and Methods. The morphological and biochemical tests thate were performed are also described there.

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To control identification by MALDI-TOF MS and biochemical tests, 35 standard strains were used (online Supplementary Table S1). *Staphylococcus* spp. were analyzed using MALDI-TOF MS, and the results were compared to previously performed 16S rRNA gene sequencing results (Lange *et al*., 2015). Catalase-negative, Gram-positive cocci and Gram-negative bacteria were identified using MALDI-TOF MS, and the results were compared to biochemical identification results. The performance of MALDI-TOF MS was evaluated in terms of its typeability, i.e., the technique's ability to assign a species name to a sample. For staphylococci, the technique was also evaluated in terms of accuracy, i.e., obtaining the correct species name for a sample, considering the 16S rRNA gene sequencing identification to be the gold standard. Details of the protocols are included in the online Supplementary Materials and Methods.

Results

In total, 12 genera and 37 species of bovine mastitis pathogens were identified using MALDI-TOF MS, including 13 species of the *Staphylococcus* genus, 15 species of catalase-negative, Gram-positive cocci and nine species of Gram-negative bacteria. Of the 380 isolates analyzed, 363 isolates (95.5%) were identified with scores equal to or above 2.0, indicating probable species identification; 14 isolates (3.7%) were identified with scores between 1.7 and 1.9, indicating probable genus identification; and MALDI-TOF MS did not identify three isolates (0.8%). Thus, MALDI-TOF MS showed a typeability of 95.5%.

All reference strains were correctly identified using MALDI-TOF MS with scores equal to or above 2.0; however, there were some differences in nomenclature depending on the taxonomic changes that occurred (online Supplementary Table S1).

Of the 203 staphylococci subjected to MALDI-TOF MS, 201 (99%) were identified by this technique as follows: 192 (94.6%) had scores equal to or above 2.0, nine (4.4%) had scores between 1.7 and 1.9, and MALDI-TOF MS did not identify two *Staphylococcus* isolates. For staphylococci, MALDI-TOF MS showed a typeability of 94.6%. The identification results of the staphylococci by MALDI-TOF MS and by 16S rRNA gene sequencing are shown in Table 1 and online Supplementary Table S2. All isolates identified as *S. aureus*, *S. simulans*, *S. pasteuri* or *S. succinus* by 16S rRNA sequencing were also identified by MALDI-TOF MS. *S. sciuri*, *S. haemolyticus,* and *S. auricularis* also were identified using the two techniques, however, some isolates had scores below 2.0. Divergent identifications occurred with *S. chromogenes*, *S. epidermidis*, *S. hyicus*, *S. agnetis*, *S. caprae,* and *S. intermedius*. Four isolates identified as *S. agnetis* by 16S rRNA sequencing were identified as *S. hyicus* by MALDI-TOF MS. The only *S. devriesei* isolate was not identified by MALDI-TOF MS. Isolates with more than one possible identification by 16S rRNA sequencing were identified as *S. xylosus* or *S. haemolyticus* by MALDI-TOF MS. Of the three isolates not identified by 16S rRNA sequencing, one was also not identified by MALDI-TOF MS, and two were identified as *S. aureus* but with scores below 2.0. These three isolates were not unambiguously identified at the species level either by 16S rRNA sequencing or by MALDI-TOF MS. Considering the 193 *Staphylococcus* isolates with unequivocal identification by 16S rRNA sequencing and excluding the nine isolates identified with scores between 1.7 and 1.9, the agreement between 16S rRNA sequencing and MALDI-TOF MS for *Staphylococcus* was 93.2% (180/193). Thus, the accuracy of MALDI-TOF MS in the identification of *Staphylococcus* isolates was 93.2%.

Of 156 catalase-negative, Gram-positive cocci, 150 (96.2%) had scores equal to or above 2.0. Five isolates (3.2%) had scores between 1.7 and 1.9, and one isolate was not identified using this technique. For catalase-negative, Gram-positive cocci, MALDI-TOF MS showed a typeability of 96.2%. The 156 catalase-negative, Gram-positive cocci identification results are shown in Table 2 and online Supplementary Table S2. The genera *Streptococcus*, *Enterococcus*, *Lactococcus,* and *Aerococcus,* were identified by MALDI-TOF MS: eight species of *Streptococcus*, four species of *Enterococcus*, two species of *Lactococcus,* and one species of *Aerococcus*. In total, four genera and 15 species of catalase-negative, Gram-positive cocci were identified by MALDI-TOF MS. Table 2 compares the results obtained by MALDI-TOF MS and biochemical identification and shows that MALDI-TOF MS provided more accurate identification of catalase-negative, Gram-positive cocci than biochemical identification. Using MALDI-TOF MS, 50, 43, and 17 isolates were identified as *S. agalactiae*, *S. uberis,* and *S. dysgalactiae,* respectively. The agreement between the results of MALDI-TOF MS and the biochemical characterization was 96% for the identification of *S. agalactiae,* 88.4% for the identification of *S. uberis,* and 82.4% for the identification of *S. dysgalactiae*, which was satisfactory for the species *S. agalactiae,* but lower for *S. uberis* and *S. dysgalactiae*. The three isolates identified as *S. equinus*/*S. lutetiensis* by MALDI-TOF MS had identification scores below 2.0, however, the isolate identified as *S. gallolyticus* had a score above 2.0.

The identification of Gram-negative bacteria by MALDI-TOF MS was achieved on the first attempt with the direct transfer protocol with a score equal to or above 2.0. MALDI-TOF MS identified the 21 Gram-negative bacteria in seven genera and eight species (Table 2 and online Supplementary Table S2). The typeability of MALDI-TOF MS for Gram-negative bacteria was 100%. Using MALDI-TOF MS, the bacteria were identified at the species level, except for the *Salmonella* genus. Using biochemical tests, the bacteria were identified at the genus level, except for *Escherichia coli*. At the genus level, the agreement between MALDI-TOF MS and biochemical identification results was 90.5%.

Discussion

The divergent identifications and low discrimination (scores between 1.7 and 1.9) of *Staphylococcus* species by MALDI-TOF MS observed in the present study were also reported in previous studies (Cameron *et al*., 2016, 2017; Nonnemann *et al*., 2019). According to Nonnemann *et al*. (2019), low discrimination indicates that the database is inadequate for species identification, suggesting the need for continuous data expansion in the databases. It may be the case that the origin of an isolate (human, animal, or environmental sample) or the source of the sample may cause differences in the spectra of the same species. According to Singhal *et al*. (2015) and Cameron *et al*. (2017), databases should contain reference mass fingerprints of all species of interest and mass fingerprints of multiple strains of each species due to the substantial genetic heterogeneity within species.

MALDI-TOF MS did not identify the species *S. agnetis* and *S. devriesei* because they are not included in the identification software database (Cameron *et al*., 2017; Schmidt *et al*., 2018; Wilson *et al*., 2019). Four isolates identified as *S. agnetis* by 16S rRNA sequencing were misidentified as *S. hyicus* by MALDI-TOF MS, probably because *S. agnetis* is closely related to *S. hyicus*.

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Table 1. Identification of 203 *Staphylococcus* spp. by MALDI-TOF MS compared to 16S rRNA sequencing

		Identification by MALDI-TOF MS			
Identification by 16S rRNA sequencing[a]	N° isolates	Unidentified[b]	Genus level[c]	Species level[d]	Agreement between both techniques [n° isolates (%)]
S. chromogenes	82	0	0	79	79/82 (96.4)
S. aureus	35	0	0	35	35/35 (100)
S. epidermidis	28	0	0	26	26/28 (92.9)
S. sciuri	11	0	2	9	11/11 (100)
S. hyicus	10	0	1	8	9/10 (90)
S. haemolyticus	9	0	2	7	9/9 (100)
S. simulans	8	0	0	8	8/8 (100)
S. agnetis	4	0	0	0	0/4 (0)
S. auricularis	1	0	1	0	1/1 (100)
S. caprae	1	0	0	0	0/1 (0)
S. devriesei	1	1	0	0	0/1 (0)
S. intermedius	1	0	0	0	0/0
S. pasteuri	1	0	0	1	1/1 (100)
S. succinus	1	0	0	1	1/1 (100)
S. xylosus/S. saprophyticus	4	0	0	4	–
S. haemolyticus/S. devriesei	1	0	0	1	–
S. haemolyticus/S. warneri/S. auricularis	1	0	1	0	–
S. haemolyticus/S. devriesei/S. auricularis/S. warneri	1	0	0	1	–
Unidentified	3	1	2	0	–
TOTAL	203	2	9	180	180/193 (93.2)

[a]Results obtained by Lange *et al.* (2015).
[b]Score ≤1.7.
[c]Score 1.7 to ≤2.0.
[d]Score ≥2.0; – = comparison not performed.

The percentage of agreement of 93.2% between MALDI-TOF MS and 16S rRNA sequencing in the present study was slightly less than reported by Cameron *et al.* (2017) (99.5%) for the identification of *Staphylococcus* species isolated from bovine and by Wilson *et al.* (2019) (98%) for the identification of bovine mastitis microorganisms. The agreement between the results of MALDI-TOF MS and the biochemical characterization of the most frequently observed catalase-negative, Gram-positive cocci involved in bovine intramammary infections was 96% for the identification of *S. agalactiae* and 88.4 and 82.4% in the identification of *S. uberis* and *S. dysgalactiae,* respectively. The 96% concordance presented by *S. agalactiae* was close to the value reported by Cherkaoui *et al.* (2011) (88%), who studied *S. agalactiae* isolates of human origin, and by Raemy *et al.* (2013) (100%), who studied bovine mastitis isolates. The 88.4% concordance presented by *S. uberis* was lower than the value reported by Raemy *et al.* (2013) (100%), and the 82.4% concordance presented by *S. dysgalactiae* at species level was higher than the level reported by Cherkaoui *et al.* (2011) (39%).

According to Raemy *et al.* (2013), the identification of *S. dysgalactiae* at the subspecies level is not satisfactory by MALDI-TOF MS, as we verified in the present study: reference strain *Streptococcus dysgalactiae* subsp. *equisimilis* ATCC 12388^T was identified only at the species level. Alnakip *et al.* (2020), however, achieved differentiation between *S. dysgalactiae* subspecies isolated from bovine mastitis using MALDI-TOF MS fingerprinting with a detailed spectral analysis of characteristic genus and species-specific biomarkers. The three isolates identified as *S. equinus*/*S. lutetiensis* by MALDI-TOF MS had identification scores below 2.0. Cameron *et al.* (2016) also reported low discrimination of *S. equinus*/*S. lutetiensis* and *S. gallolyticus* using MALDI-TOF MS. On the other hand, Alnakip *et al.* (2020) reported successful differentiation of all streptococcal isolates at the species and subspecies level, including *S. equinus* and *S. gallolyticus*, using MALDI-TOF MS fingerprinting and Data Explorer® analysis software (v. 4.0).

The MALDI-TOF MS technique allowed for differentiation between the genera *Enterococcus*, *Lactococcus* and *Aerococcus* and the identification of species of these genera, which was not possible with the biochemical tests. According to Cameron *et al.* (2016), isolates of *Lactococcus* and *Enterococcus* genera were identified as other streptococci until recently. However, with the application of MALDI-TOF MS, accurate and easy speciation of these and other members of the environmental streptococci is now possible.

In the present study, the agreement between the results of MALDI-TOF MS and biochemical identification of

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Table 2. Identification of 156 catalase-negative, Gram-positive cocci and 21 Gram-negative bacteria by MALDI-TOF MS compared to biochemical characterization

Identification by MALDI-TOF MS					
Identification	N° isolates	Unidentified[a]	Genus level[b]	Species level[c]	Agreement between MALDI-TOF MS and biochemical characterization [n° isolates (%)]
Catalase-negative Gram-positive cocci					
Streptococcus					
S. agalactiae	50	0	0	50	48/50 (96)
S. uberis	43	0	1	42	38/43 (88.4)
S. dysgalactiae	17	0	0	17	14/17 (82.4)
S. pluranimalium	5	0	0	5	–
S. equinus/lutetiensis	3	0	3	0	–
S. gallolyticus	1	0	0	1	–
S. parauberis	1	0	0	1	–
S. oralis	1	0	0	1	–
Enterococcus					
E. faecalis	13	0	0	13	–
E. faecium	5	0	0	5	–
E. avium	1	0	0	1	–
E. hirae	1	0	0	1	–
Lactococcus					
L. garvieae	6	0	1	5	–
L. lactis	6	0	0	6	–
Aerococcus					
A. viridans	2	0	0	2	–
Unidentified	1	1	0	0	–
SUBTOTAL	156	1	5	150	–
Gram-negative bacteria					
Escherichia coli	9	0	0	9	9/9 (100)
Salmonella sp.	3	0	3	0	3/3 (100)
Pseudomonas aeruginosa	3	0	0	3	3/3 (100)
Klebsiella oxytoca	1	0	0	1	1/1 (100)
Klebsiella pneumoniae	1	0	0	1	1/1 (100)
Klebsiella variicola	1	0	0	1	1/1 (100)
Citrobacter freundii	1	0	0	1	1/1 (100)
Enterobacter cloacae	1	0	0	1	0/1 (0)
Leclercia adecarboxylata	1	0	0	1	0/1 (0)
SUBTOTAL	21	0	3	18	19/21 (90.5)

[a]Score ≤1.7.
[b]Score 1.7 to ≤2.0.
[c]Score ≥2.0; – = comparison not performed.

Gram-negative bacteria was 90.5% at the genus level, which was similar to the value reported by Rodrigues *et al.* (2016) (92.9%), who compared the two techniques for the identification of enterobacteria isolated from dairy cattle environments. The genera and species identified in the present study were also identified from milk samples of cows with mastitis using MALDI-TOF MS and other techniques in previous studies (Raemy *et al.*, 2013; Rodrigues *et al.*, 2016; Cameron *et al.*, 2016, 2017; Nonnemann *et al.*, 2019). The rate of identification of bovine mastitis pathogens by MALDI-TOF MS was 95.5% at the species level (referred by us as typeability) and 3.7% at the genus level, similar to those reported by Cameron *et al.* (2017) (92% at the species level) and by Nonnemann *et al.* (2019) (93.5% at the species level and 6.5% at the genus level). In the present study, a repeat of the direct

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transfer protocol or an extended protocol was necessary to identify some staphylococci and catalase-negative, Gram-positive cocci. Cameron *et al.* (2017) obtained their results using a direct transfer protocol. By contrast, Nonnemann *et al.* (2019) used an extended protocol in addition to the direct transfer protocol to identify bovine mastitis bacteria using MALDI-TOF MS.

In conclusion, our findings add to the body of evidence suggesting that MALDI-TOF MS is an accurate, fast, and simple technique for identifying bovine mastitis pathogens. The availability of this methodology in research institutions throughout Brazil would represent a significant step toward increasing the diagnosis and epidemiological studies of bovine mastitis and other infectious diseases of animals. Pathogen-specific diagnosis is important for the management of bovine mastitis because modes of transmission and infection, as well as treatments, differ between different mastitis-causing microorganisms (Cameron *et al.*, 2017; Condas *et al.*, 2017). To ensure rapid initiation of correct treatment and prudent use of antimicrobials, timely and accurate identification of the causative agent of bovine mastitis is crucial and MALDI-TOF MS could provide such rapid diagnosis for mastitis control. Thus, this is of special interest for the dairy industry, particularly for the dairy diagnostic sector.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029921000595

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