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VETERINARY MICROBIOLOGY - RESEARCH PAPER

Comparison of standard and on-plate extraction protocols for identification of mastitis-causing bacteria by MALDI-TOF MS

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Abstract

The objective was to compare standard versus on-plate sample preparation protocols for identification of mastitis bacteria by MALDI-TOF MS. A total of 186 bacterial isolates from cows with subclinical mastitis were identified by MALDI-TOF MS after preparation using two extraction protocols. On-plate protocol was performed by applying the bacterial colony directly from the culture plate onto the plate spot. For the standard protocol, lysis of bacterial colonies using reagents was performed in a cryotube, and the resulting extract was applied onto the plate spot for analysis. The on-plate protocol showed a similar bacteria identification rate (91.4%, $n = 170/186$) in comparison to the standard (94.6%, $n = 176/186$). Identification was higher for both protocols when scores used for species-level identification (≥ 2.0) was reduced to genus-level (≥ 1.7); genus-level identification score rate increased from 94.6 to 100% when using the standard protocol, and from 91.4 to 94.6% when using the on-plate protocol. However, when compared standard (as gold standard) versus on-plate protocol, genus-level identification score rate ranged from 87.1 to 89.8%. Therefore, when the on-plate protocol fails to identify any specie, the standard extraction may be more suitable as a reference protocol for use. Strategy for increasing identification with the on-plate protocol may include upgrading the reference database library. Choice of protocol for preparation may be influenced by the bacterial type to be identified. Standard and on-plate extraction protocols of bacterial ribosomal proteins associated with MALDI-TOF MS might be alternatives to conventional microbiology methods for identification of subclinical mastitis pathogens.

Keywords Mastitis · Subclinical case · MALDI-TOF MS · Standard and on-plate extraction

Introduction

The identification of mastitis-causing pathogens by means of conventional microbiology (CM) can take from 2 to 7 days for the complete diagnosis at the species level, since it involves plating milk samples on solid medium culture plates, incubation, and assessment of any microbial growth, and then subsequent application of biochemical tests [3]. In addition to the time spent, CM may retain identification limitations due to the high genotypic similarity between some mastitis-causing bacteria. As an example, a previous study showed that isolates of *Streptococcus* like-bacteria were mistakenly identified using CM when compared to gene sequencing methodologies [30].

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been utilized as a rapid and reliable methodology, complementary to CM, for the identification of mastitis-causing bacteria [1, 2, 4]. The MALDI-TOF MS method consists of the separation of ions according to the mass/charge ratio, enabling the high-sensitivity detection of a large variety of biomolecules [20]. The protein spectrum profile is generated using the mass spectrometry methodology and compared with reference databases for identification [3]. MALDI-TOF MS has been described as a tool for solving the discordant and poor discriminatory

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results found by CM which occur when related bacterial species have very similar spectral profiles [7]. A few studies have evaluated the standard MALDI-TOF MS protocol as a diagnostic tool in veterinary medicine [2–4].

In the on-plate protocol, the bacterial colony is applied directly onto the plate spot [3], whereas, when using the standard protocol, bacterial colonies are immersed in 70% ethanol in a separate tube and pelleting and drying the microorganism followed by extraction and spotting onto the plate for analysis by MALDI-TOF MS [28]. The on-plate protocol enables more rapid diagnosis, due to reduced sample preparation time when compared to the standard protocol [27]. Furthermore, the on-plate protocol uses smaller volumes of formic acid and fewer laboratory consumables than standard protocol, and it is a more environmentally friendly process for isolate preparation [28]. Overall, in a diagnostic laboratory setting, protein extraction by the standard protocol, which is considered a multi-step process, has been reported as an impractical and thus the use on-plate protocol may be preferred for sample preparation [19].

The standard protocol extraction for MALDI-TOF MS isolate preparation has already been reported for identification of ≥ 90% mastitis-causing bacteria [2, 14, 29], whereas, to the best of our knowledge, no studies compared different methods of extracting bacterial ribosomal proteins for identification of subclinical mastitis-causing bacteria, such as standard (tube extraction) and on-plate (i.e. direct transfer of colonies approach) by MALDI-TOF MS. For that reason, our hypothesis is that the on-plate protocol extraction may be used for identification of subclinical intramammary infection (IMI) cases in a similar way and with reliability as the manufacturer-recommended standard protocol. Therefore, the current study aimed to compare two MALDI-TOF MS sample preparation protocols (standard vs. on-plate) for identification of mastitis-causing bacteria.

Material and methods

A total of 186 cryopreserved bacterial isolates from quarter milk samples of subclinically infected dairy cows were evaluated in the present study. Considering the CM results, isolates of the genera *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., and other *Streptococcus*-like bacteria (e.g.; *Aerococcus* spp. and *Lactococcus* spp.) were selected. The isolates were cultured on blood agar with 5% defibrinated bovine blood and incubated aerobically at 37 °C for 24 h. Next, the isolates were submitted to Gram staining, catalase and KOH testing for morphology determination, and isolate differentiation [21]. The cryopreserved isolates pertaining to the bacterial genera identified by CM, and classified as *Streptococcus* like-bacteria (*Aerococcus* spp. and *Lactococcus* spp.), were previously confirmed by the partial sequencing of the 16S rRNA gene and carried out in a companion study [13].

Identification of the mastitis-causing pathogens by MALDI-TOF MS: standard and on-plate extraction protocols A loopful of bacteria from an isolated pure bacterial colony was added to 300 μL of autoclaved Milli-Q water (Millipore Corporation, Bedford, MA, USA) and 900 μL of HPLC grade ethanol, followed by homogenization using a K45- 2820 vortex mixer (Kasvi, Curitiba, Brazil) for 1 min. In order for the complete removal of the supernatant, centrifugation was performed (all centrifugations were carried out at 13,000×*g* for 2 min in an Eppendorf® 5417R centrifuge (Hamburg, Germany). The bacterial pellets were dried at room temperature for 5 to 10 min.

A 70% formic acid solution (10.0–50.0 μL) was added to the bacterial pellets, proportionally to the size of the sediment, for complete dissolution. Subsequently, 100% acetonitrile was added to each sample in equal volumes as the 70% formic acid solution, with a 10-min interval between reagents, thus producing bacterial extracts at a 1:1 ratio of 70% formic acid and 100% acetonitrile. A final centrifugation step was performed to separate bacterial cell debris from the supernatant containing the extracted ribosomal proteins [3].

A volume of 1.0 μL of each bacterial extract was applied to the spots on the steel plate (MSP 384 polished-steel target; Bruker Daltonik, Bremen, Germany) and left to dry at room temperature for 15 min. The dried spots were overlaid with 1.0 μL of matrix solution, consisting of α-cyano-4-hydroxycinnamic acid (HCCA) diluted in 50% acetonitrile and 2.5% trifluoroacetic acid. After matrix addition, the spots were dried at room temperature (~ 7 min) for analysis by matrix-assisted laser desorption ionization-time of flight mass spectrometry (Bruker Daltonics, Inc. Billerica, MA, USA).

Following the 24-h incubation of the bacterial culture, one colony was applied to the steel plate spot with the aid of a wooden stick. A volume of 1.0 μL of formic acid (70%) was applied to the spot and allowed to dry at room temperature. After drying, 1.0 μL of HCCA matrix solution was applied, and again left to dry at room temperature for 5 to 10 min.

Final stage of plate preparation A standard protein solution (Bacterial Test Standard, BTS; Bruker) was used for MALDI-TOF MS calibration. A positive control (*Escherichia coli*) and a negative control (formic acid and matrix) were analyzed on each plate. The analysis employing the MALDI-TOF mass spectrometry methodology was performed in FlexControl 3.4 software (Bruker Daltonik, Bremen, Germany), operated in linear mode, and equipped with a 337-nm nitrogen laser. The mass spectra were collected in a 2000 to 20,000 m/z mass range in automatic mode. Three thousand laser shots were

collected to generate each spectrum. Plate reading was performed according to the specifications for protein extraction identification (Bruker Daltonik, Bremen, Germany), and the spectral data processing was done using the MALDI Biotyper 4.1.70 (Bruker Daltonik, Bremen, Germany) computer software for microorganism identification (MBT version 7311 MPS library). Basically, scores ≥ 1.7 were considered reliable for genus identification, and scores ≥ 2.0 were considered reliable for genus and species identification. Isolates that presented identification disagreement between both methods were named misidentified. All the isolates classified as misidentified were tested three times by on-plate extraction protocol MALDI-TOF MS and were previously confirmed by the partial sequencing of the 16S rRNA gene as described previously in a companion study [13].

Comparisons of MALDI-TOF MS genus- or species-level identification, employing standard versus on-plate protocols, overall and stratified by groups of isolates (*Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Aerococcus* spp., and *Lactococcus* spp.) were carried out using McNemar's test of paired proportions, as suggested by Alatoom et al. [1]. *P* values < 0.05 were considered statistically significant. All analyses were performed using SAS, version 9.3 (SAS Institute, Cary, NC).

Results

Standard protocol associated with MALDI-TOF MS made possible identification at genus level of all bacterial isolates after CM ($n = 186$; score ≥ 1.7), and a total of 176 out of 186 (94.6%) subclinical mastitis-causing isolates were correctly identified at the species level (score ≥ 2.0). The on-plate protocol for the MALDI-TOF MS allowed the acquiring score ≥ 1.7 for 176 isolates out of 186 (94.6%), and score ≥ 2.0 for 170 (91.4%). However, considering the standard protocol as control (gold standard), the on-plate protocol correctly identified a total of 167 isolates out of 186 (89.8%) at the genus level (score ≥ 1.7), 162 of which (87.1%) were identified at the species level (score ≥ 2.0). Considering all of the bacterial isolates evaluated, the standard MALDI-TOF MS protocol enabled a similar identification at genus and species level when compared to the on-plate MALDI-TOF MS protocol (*P* value > 0.05; Table 1). However, we observed that when using the on-plate protocol, the identification of isolates varied according to the type of bacteria. For example, *Staphylococcus aureus* isolates had higher identification rate at species level (100%; $n = 68$) using on-plate MALDI-TOF MS protocol than *Streptococcus uberis* (90%; $n = 45/50$) (Table 1).

Table 1 Frequency of identification of mastitis-causing bacteria using on-plate extraction protocols associated with MALDI-TOF MS considering the standard protocol as control (correct identification at genus (score ≥ 1.7) and specie level (score ≥ 2)

Mastitis-causing pathogens	Total	MALDI-TOF MS extraction protocols			
		Standard		On-plate	
		Genus (≥ 1.7)	Species (≥ 2)	Genus (≥ 1.7)	Species (≥ 2)
Major pathogens					
Staphylococcus aureus	68	68	68	68	68
Streptococcus uberis	50	50	50	45	45
Streptococcus agalactiae	29	29	28	26	23
Streptococcus dysgalactiae	1	1	1	1	1
Subtotal	148	148	147	140	137
Infrequent pathogens					
Aerococcus viridans	14	14	9	6	5
Enterococcus faecalis	5	5	5	5	5
Staphylococcus chromogenes	4	4	1	3	3
Enterococcus faecium	3	3	3	3	3
Lactococcus garvieae	3	3	3	3	3
Enterococcus gallinarum	3	3	2	2	2
Lactococcus lactis	2	2	2	2	2
Staphylococcus epidermidis	1	1	1	1	1
Staphylococcus haemolyticus	1	1	1	1	0
Enterococcus hirae	1	1	1	1	1
Streptococcus lutetienties	1	1	1	0	0
Subtotal	38	38	29	27	25
Total	186	186	176	167	162

The standard protocol associated with MALDI-TOF MS for identification at the species and genus level resulted in a similar percentage of characterization when compared to the on-plate protocol, varying according to the bacterial group. Considering the standard protocol such as control, a total of 68 isolates of *Staphylococcus aureus* (100%) were identified at the species level using the on-plate protocol associated with MALDI-TOF MS. Regarding the cryopreserved isolates, such as *Streptococcus uberis* ($n = 50$), 45 (90%) were identified at the species level using the on-plate protocol associated with MALDI-TOF MS. Twenty-three isolates of *Streptococcus agalactiae* out of 29 (79.3% with score > 2.0 but 89.7% with score > 1.9) were identified at the species level by the on-plate protocol associated with MALDI-TOF MS, whereas the standard protocol enabled the identification at the species level of 28 out of 29 isolates (96.6%).

Additional infrequent isolates were submitted to both extraction protocols ($n = 38$) (Table 1). The standard protocol associated with MALDI-TOF MS allowed the identification of all 38 isolates (100%) at the genus level, which are considered infrequently associated with cases of subclinical IMI (e.g., *Aerococcus viridans* and *Lactococcus lactis*). In contrast, despite most infrequent pathogens had species confirmation with score > 2.0, three species of pathogens subclinical mastitis-causing (66.7% *Enterococcus gallinarum*, 64.3% *Aerococcus viridans*, and 25% *Staphylococcus chromogenes*) had lower percentage identification at species level by the standard protocol (Table 1). In turn, when using the on-plate MALDI-TOF MS protocol, infrequent pathogens identification at the genus level was reported in 27 out of the 38 (71.1%) and at the species level in 25/38 (65.8%) of these isolates. We observed that the percentage identification at the species level by the on-plate protocol was also lower for some bacteria considered infrequent (75% *Staphylococcus chromogenes*, 66.7% *Enterococcus gallinarum*, and 35.7% *Aerococcus viridans* and no confirmation of *Staphylococcus haemolyticus* and *Streptococcus lutetiensis*) similarly which was found by the standard protocol.

In the present study, mastitis-causing bacteria (*Staphylococcus haemolyticus*, *Lactococcus garvieae*, *Staphylococcus chromogenes*, *Staphylococcus epidermidis*, *Aerococcus viridans*, *Enterococcus faecalis*, *Lactococcus lactis*, *Enterococcus faecium*, *Enterococcus hirae*, *Enterococcus gallinarum*, and *Streptococcus lutetiensis*), unidentified at the species level by CM ($n = 38/186$), were correctly identified using the MALDI-TOF MS methodology associated with the standard protocol, since they exhibited unique fingerprint spectra (Fig. 1). Furthermore, major pathogens causing mastitis identified by CM

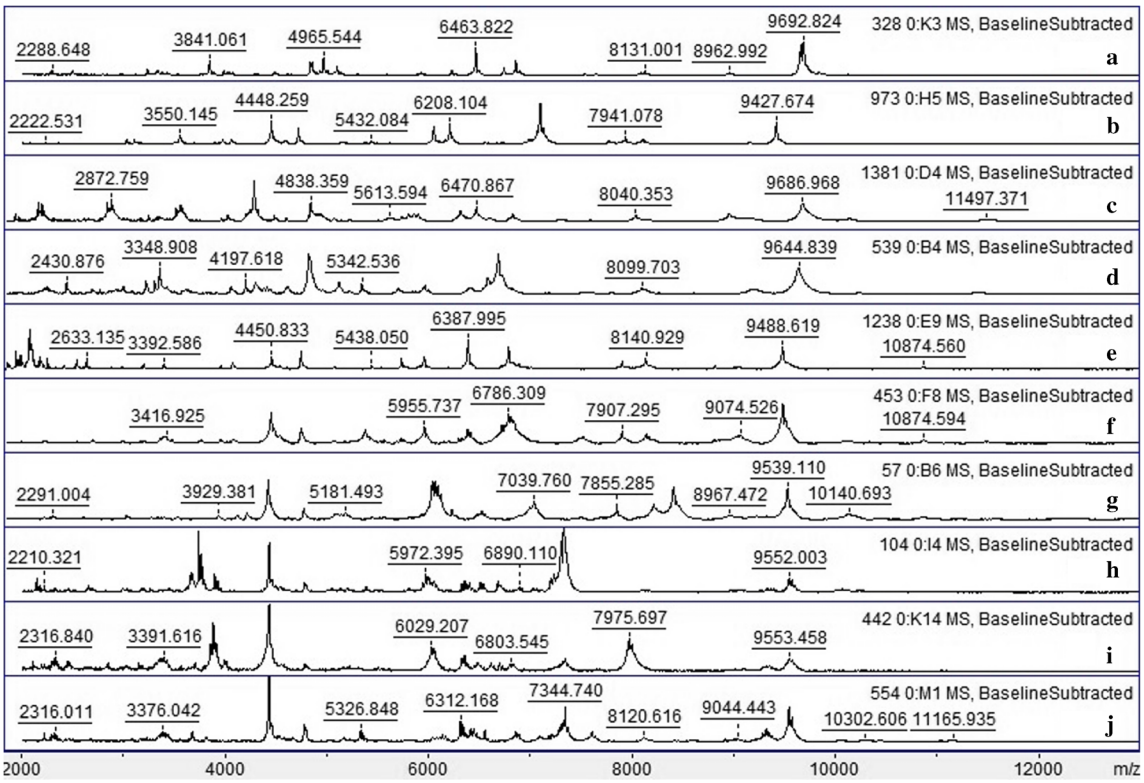

Fig. 1 MALDI-TOF mass spectra obtained using standard extraction protocol for identification of mastitis-causing bacteria, routinely unidentified at the species level by conventional microbiology. **a** *Staphylococcus haemolyticus*. **b** *Lactococcus garvieae*. **c** *Staphylococcus chromogenes*. **d** *Staphylococcus epidermidis*. **e** *Aerococcus viridans*. **f** *Enterococcus faecalis*. **g** *Lactococcus lactis*. **h** *Enterococcus faecium*. **i** *Enterococcus hirae*. **j** *Enterococcus gallinarum*

(e.g., *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus agalactiae*, and *Streptococcus dysgalactiae*; *n* = 148/186) were characterized using standard protocol.

Ten out of the 186 isolates were classified as "not reliable identification" (score < 1.7) by the on-plate protocol associated with MALDI-TOF MS. Additionally, a total of nine isolates were considered misidentified once they presented identification disagreement between standard and on-plate MALDI-TOF MS protocols extraction. However, we confirmed by the partial sequencing of the 16S rRNA gene all misidentified isolates and, three out of the nine were correctly identified using the on-plate MALDI-TOF MS protocol extraction (Table 2).

Discussion

In the present study, independent of both tested extraction protocols, our results indicated that the MALDI-TOF MS cut-off identification varied with the type of pathogen causing subclinical mastitis. Many factors can explain this variation such as bacterial cell wall characteristics that possibly interferes in the ribosomal protein extraction process [26]. Our results suggest that the standard protocol and the on-plate protocol are alternatives to traditional methods (CM) for the identification of subclinical mastitis-causing pathogens.

Previous studies described both advantages and disadvantages of using standard protocol and on-plate protocols for sample preparation [8, 9, 18, 19, 26, 28]. The standard protocol generates a pure protein extract, and the Biotyper library was created based on results of this first adopted protocol [1, 9]. Therefore, it was the reason why most of the studies have found highest identification scores as observed in our study when using the standard protocol. However, Alatoom et al. [1] emphasized that the standard protocol requires for the identification a greater concentration of bacteria as well as reagents since the extraction has been performed in a tube. According to Matsuda et al. [18], Theel et al. [28], and McElvania TeKippe et al. [19], the standard protocol has been more time-consuming due to the greater number of steps when compared to the on-plate protocol. For example, it was reported [18] that the standard extraction protocol consisted of approximately 13 steps, including completion of 2 rounds of centrifugation for 2 min each, requiring approximately 180 min for 48 samples. Likewise, the total hands-on time for processing 40 isolates was approximately 1.5 to 2 h for the standard protocol which had greater spent time than other extraction protocols (~45 min) [8]. Contrary to the standard protocol, we observed that the on-plate protocol requires smaller volumes of reagents (e.g., formic acid) and fewer laboratory consumables which is an environmentally friendly process for isolate preparation as mentioned by Theel et al. [28]. As a matter of fact, this may be explained since the on-plate protocol demands a smaller number of lab steps (e.g., not required the centrifugation) [8, 18]. On the other hand, the use of a less pure extracted protein preparation such as the on-plate protocol may not produce valid score because metabolites, pigments, and/or agar material on the surface of the bacteria cell may interfere with the crystallization process [9]. Additionally, Bizzini et al. [6] reported a disadvantage regarding the on-plate protocol, such as the small distance between the plate spots, which could lead to the inversion of the isolates and cross-contamination.

Our results corroborate with two studies that have already demonstrated the on-plate extraction protocol which offers species identification rates at least equivalent to the results of

Table 2 Identification disagreement between standard and on-plate MALDI-TOF MS protocols extraction by the partial sequencing of the 16S rRNA gene (all the isolates with identification disagreement were tested three times by on-plate extraction protocol MALDI-TOF MS and were previously confirmed by the partial sequencing of the 16S rRNA gene)

Conventional microbiology identification	MALDI-TOF MS protocols extraction				Sequencing of the 16S rRNA gene	
	Standard	Score	On-plate	Score	Biomolecular confirmation	Score (%)
Streptococcus uberis	*Aerococcus viridans*	2.01	*Enterococcus faecium*	2.11	*Aerococcus viridans*	100
Streptococcus uberis	*Aerococcus viridans*	1.95	*Enterococcus faecalis*	2.25	*Aerococcus viridans*	99
Streptococcus uberis	*Aerococcus viridans*	2.03	*Enterococcus faecalis*	2.04	*Aerococcus viridans*	99
Other Streptococcus	*Aerococcus viridans*	2.03	*Streptococcus uberis*	1.91	*Aerococcus viridans*	99
Streptococcus uberis	*Aerococcus viridans*	2.08	*Streptococcus uberis*	2.02	*Aerococcus viridans*	99
Other Streptococcus[1]	*Aerococcus viridans*	2.09	*Streptococcus uberis*	2.17	*Streptococcus uberis*	99
Enterococcus spp.	*Enterococcus gallinarum*	1.97	*Streptococcus uberis*	2.42	*Streptococcus uberis*	99
Streptococcus uberis	*Streptococcus uberis*	2.48	*Aerococcus viridans*	2.08	*Streptococcus uberis*	99
Staphylococcus aureus	*Staphylococcus chromogenes*	1.83	*Staphylococcus aureus*	2.32	*Staphylococcus aureus*	99

[1] The top 10 microorganism's identifications recovered for an isolate with identification disagreement between standard (*Aerococcus viridans*, score = 2.09) and on-plate (*Streptococcus uberis*, score = 2.17) MALDI-TOF MS protocols are presented in Table 3

the standard extraction protocol, when it was used for clinically relevant isolates of non-veterinary cases [18, 24]. In the present study, considering the species and genus-level identification score, the identification rates were 91.4% and 94.6%, respectively; when using independently the on-plate protocol. However, considering the standard protocol such as control, the on-plate protocol had identification rates of 87.1% and 89.8%, for species and genus-level, respectively. Our results of species and genus-level identification rate were higher than those described by Alatoom et al. [1], since they identified 168 isolates (56%) to the genus level and 60 isolates (20%) to the species level from multiple clinical sources (e.g., blood, tissue, and urine) but their protocol was the direct colony extraction (without formic acid usage).

Staphylococcus aureus isolates were 100% identified at the species level using both extraction protocols. In general, these identification rates of *Staphylococcus aureus* were higher than 84.6% described by Barreiro et al. [2] (in which only the standard protocol of ribosomal proteins was employed). Similar to our results, Dubois et al. [10] reported that the standard protocol resulted in 99.3% identification of the *Staphylococcus* spp. isolates, while Spanu et al. [27] described 100% identification of *Staphylococcus aureus* isolates. Matsuda et al. [18] described that *Staphylococcus aureus* had a higher identification rate than isolates of coagulase-negative staphylococci using standard and on-plate protocols from relevant clinical isolates but strains as *Staphylococcus caprae* and *Staphylococcus saprophyticus* shown higher identification rate by the on-plate protocol than the standard. In Table 2, *Staphylococcus aureus* cannot be considered misidentified as *Staphylococcus chromogenes* by MALDI-TOF using standard protocol since the obtained score was 1.83 which enable the identification only at the genus level (e.g., *Staphylococcus* spp.) but not at the species level. The partial sequencing of the 16S rRNA gene and MALDI-TOF using on-plate protocol made correctly possible to identify that isolate as *Staphylococcus aureus* which had been characterized as *Staphylococcus chromogenes* using standard protocol (score 1.83).

Regarding the isolates identified by CM as *Streptococcus agalactiae*, the standard protocol enabled the identification of 96.5% of the isolates at the species level, whereas the on-plate protocol identified only 79.3% with score > 2.0 and 89.7% with score > 1.9. Similar to that found in the present study, Barreiro et al. [2] identified 100% of the *Streptococcus agalactiae* isolates when using the standard protocol (score > 2.0). In addition, Lartigue et al. [16] described a percentage of identification of 99% when they submitted the *Streptococcus agalactiae* isolates to species-specific confirmation using the standard protocol, and it was similar to that reported in the present study. Benagli et al. [5] reported an equivalent species-level identification percentage (88.9%) of the cryopreserved isolates of *Streptococcus agalactiae* than those mentioned in the present study and studies cited herein. To the best of our knowledge, no results were found for comparison purposes regarding the identification of *Streptococcus agalactiae* isolated from cases of subclinical mastitis using the on-plate protocol. Factors such as the capsular polysaccharide, the lipoteichoic acid, and the surface proteins of bacterial wall could have interfered on on-plate extraction protocol for *Streptococcus agalactiae* identification by MALDI-TOF MS. For this reason, further studies are recommended to overcome this issue.

We could have been achieved higher identification scores at species level using the on-plate protocol if the manufacturer-recommended cutoff scores were lowered to ≥ 1.9. Seng et al. [25] and Risch et al. [22] used a score of ≥ 1.9 instead of the manufacturer's recommended score of ≥ 2.0 for species-level identification. In our study, we did not decrease the score cutoff for the on-plate protocol with the aim to obtain greater identification rate. However, different from our study, Fedorko et al. [11] noted a further increase in species-level identification of clinical isolates to 86% when the species-level cutoff score was lowered to ≥ 1.8. An intriguing question would be what should be the cutoff score which could be adopted for identifying mastitis-causing pathogens when using the on-plate protocol. A previous study has already evaluated a change of the identification score of ≥ 1.5 instead of the manufacturer's recommended score of ≥ 1.7 for genus-level identification [28]. Although, Alatoom et al. [1] and Schmitt et al. [24] reported that misidentifications possibly occurred when the manufacturer-recommended cutoff scores for identification were lowered, mainly at a score of ≥ 1.5 for genus-level identification. Overall, results from previous studies indicate that the application of genus-level score of ≥ 1.6 (instead of ≥ 1.7) and species-level score of ≥ 1.7 (instead of ≥ 2.0) may possibly be more appropriate when using the on-plate extraction protocol [1, 11, 24, 28].

Bacteria that are not routinely isolated using microbiological techniques and tests recommended by the National Mastitis Council [15], or by commercially available biochemical kits may be under-reported [12]. Werner et al. [30] performed the sequencing of *rpoB* and 16S rDNA genes from *Streptococcus* spp. identified by CM, and reported that they were *Lactococcus* spp. (70%), *Enterococcus* spp. (13%), *Streptococcus uberis* (7%), and *Streptococcus dysgalactiae* (1.5%). The top 10 microorganism's identifications recovered for an isolate with identification disagreement between standard (*Aerococcus viridans*, score = 2.09) and on-plate (*Streptococcus uberis*, score = 2.17) MALDI-TOF MS protocols are presented in Table 3. In the present study, the standard protocol identified 100% of *Aerococcus viridans*, *Lactococcus lactis*, and *Lactococcus garvieae* with score > 1.9.

Multiple facts may be used for explaining the inability of identifying all isolates to the species level, even using the

Table 3 The top 10 microorganism's identifications recovered for an isolate with identification disagreement between standard (*Aerococcus viridans*, score = 2.09) and on-plate (*Streptococcus uberis*, score = 2.17) MALDI-TOF MS protocols

Standard protocol				On-plate protocol			
Analyte name: E17				Analyte name: E09			
Analyte ID: 1238				Analyte ID: 1238			
Rank (quality)	Matched pattern	Score value	NCBI identifier	Rank (quality)	Matched pattern	Score value	NCBI identifier
1 (++)	*Aerococcus viridans* CCM 1914T CCM	2.091	1377	1 (++)	*Streptococcus uberis* DSM 20569T DSM	2.172	1349
2 (+)	*Aerococcus viridans* CCM 1915 CCM	1.883	1377	2 (+)	*Streptococcus uberis* GD71 GDD	1.864	1349
3 (+)	*Aerococcus viridans* CCM 1911 CCM	1.873	1377	3 (+)	*Streptococcus uberis* GD70 GDD	1.832	1349
4 (+)	*Aerococcus viridans* CCM 2439 CCM	1.844	1377	4 (−)	*Streptococcus canis* DSM 20715T DSM	1.407	1329
5 (−)	*Aerococcus viridans* DSM 20311 DSM	1.626	1377	5 (−)	*Lactobacillus delbrueckii* spp. *delbrueckii* DSM 20074T DSM	1.272	83684
6 (−)	*Aerococcus viridans* DSM 20340T DSM	1.625	1377	6 (−)	*Streptococcus thoraltensis* DSM 12221T DSM	1.263	55085
7 (−)	*Aerococcus viridans* CCUG 48207 CCUG	1.605	1377	7 (−)	*Streptococcus equi* ssp. *zooepidemicus* DSM 20727 DSM	1.257	149015
8 (−)	*Aerococcus viridans* CCUG 52146 CCUG	1.589	1377	8 (−)	*Streptococcus hyovaginalis* DSM 12220 DSM	1.252	59733
9 (−)	*Shewanella frigidimarina* DSM 12253T HAM	1.462	56812	9 (−)	*Chryscobacterium scophthalmum* LMG 13028T HAM	1.236	33962
10 (−)	*Pseudomonas putida* DSM 291T HAM	1.336	303	10 (−)	*Weissella minor* DSM 20014T DSM	1.221	1620

standard protocol. The manufacturer's spectral database contains a variable number of entries per species, usually being higher for common isolates [1]. We agreed with Lartigue et al.'s [16] affirmation that a higher number of entries for the same species will likely better reflect diversity within the species due to variations in protein expression between strains and potentially under different conditions. Additional reference strains of mastitis-causing pathogens considered as "infrequent" should more accurately represent the genetic diversity of the strain [17]. Hence, proper representatives from several genetic backgrounds for each species should improve the species-level identification rates [18]. For that reason, a potential solution would be the creation of reference database library including high quality main spectrum entries to be used when an on-plate protocol is used; instead of decreasing the cutoff [7, 16, 24].

Given the superior quality of the spectra generated by the standard protocol and the speed of the on-plate protocol, an implication of the present study is based on the usage of both methodologies of ribosomal protein extraction. In this case, as suggested by Bizzini et al. [6], the isolates would first be submitted to the on-plate protocol, followed by the standard protocol, in cases where species-level identification has not been obtained. In other words, the standard extraction may be more suitable as a reference protocol for use when the on-plate protocol fails to identify species. According to Bizzini et al. [6], when associating the usage of both extraction protocols, only 21% of the *Staphylococcus aureus* isolates were submitted to the standard MALDI-TOF MS protocol, since 79% identification of the *Staphylococcus aureus* isolates were initially obtained directly by using the on-plate protocol with score > 2.0. Moreover, Saffert et al. [23] showed that Gram-negative bacteria could be identified by the Biotyper software, with only 11% of bacteria requiring preparatory extraction (e.g., the usage of standard protocol), while the remaining of 89% were identified by the use of an extraction protocol more related with the colony being directly applied on-plate.

In conclusion, standard and on-plate extraction protocols of bacterial ribosomal proteins associated with MALDI-TOF MS are alternatives to CM method. Decisions as to which protocol to use depend on the type of bacteria. When the on-plate protocol fails to identify species, the standard extraction may be more suitable as a reference protocol for use. The on-plate MALDI-TOF MS protocol showed similar identification percentage to the standard MALDI-TOF MS.

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