


Identification of ovine-associated staphylococci by MALDI-TOF mass spectrometry

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ABSTRACT

The objective of this study was to use matrix-assisted laser desorption ionisation–time of flight mass spectrometry (MALDI-TOF MS) for the identification of ovine-associated staphylococci. Presumptive *Staphylococcus* isolates were recovered from ovine udder surface (US), individual raw milk, bulk tank milk, and cheese samples and were characterised by conventional phenotypic methods. A total of 69 bacterial isolates were further confirmed by MALDI-TOF MS. Forty-two (60.9%) of 69 isolates were successfully identified on genus and species level. Two thirds ($n = 28$) of the 42 identified isolates were shown to be *Staphylococcus* spp. These 28 staphylococcal isolates formed two clusters, one consisting of 22

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Staphylococcus aureus strains and the other composed of 6 non-*aureus* staphylococci, including *S. simulans* ($n = 3$), *S. auricularis*, *S. equorum*, and *S. haemolyticus*. MALDI-TOF MS has proven to be a reliable tool for the identification of staphylococci from raw sheep's milk, especially bulk tank milk; however, currently it appears to be less useful for the identification of bacterial isolates originating from ovine US samples. This is the first study to evaluate the applicability of MALDI-TOF MS for identification of *Staphylococcus* spp. in ovine raw milk, cheese, and US samples in Hungary.

KEYWORDS

MALDI-TOF MS, *Staphylococcus*, coagulase, sheep milk, cheese

1. INTRODUCTION

Staphylococci are Gram-positive and facultative anaerobic bacteria belonging to the family *Staphylococcaceae*. Their cells are spherical in shape and form grape-like clusters. The genus *Staphylococcus* currently contains 53 species and 24 subspecies (DSMZ, 2020). The coagulase-positive *Staphylococcus aureus* is a major pathogen responsible for intramammary infections in dairy ruminants (Bergonier et al., 2003; Peles et al., 2007). This species is found naturally on the mucous membranes and skin of warm-blooded animals and humans (Irlinger, 2008). It is highly prevalent in both individual and bulk-tank ovine milk (Marogna et al., 2010; de Garnica et al., 2011).

Coagulase-negative staphylococci (CNS) have long been viewed as opportunistic skin-borne microorganisms capable of contaminating milk samples (Pyörälä and Taponen, 2009). Currently, they are considered the most prevalent bacteria causing subclinical intramammary infections in dairy ewes (Bergonier et al., 2003). CNS infections in milking animals are associated with increased somatic cell counts in milk, reduced milk quality, and decreased milk yields (Pyörälä and Taponen, 2009; Leitner et al., 2019). Pilipčincová et al. (2010) reported *Staphylococcus epidermidis* and *Staphylococcus caprae* to be the most common CNS species in sheep milk.

The conventional culture-based identification of bacterial isolates is a time-consuming and labour-intensive process generally requiring a few days to produce a definitive result. Various manual and automated biochemical test systems for the phenotypic identification of staphylococci have also been commercially available for several decades. However, many of these tools fail to produce accurate results for CNS identification (Becker et al., 2014), because most phenotypic identification methods have been developed for microbes isolated from humans rather than animal pathogens (Zadoks and Watts, 2009). In addition, keeping phenotypic reference databases up to date is a major challenge. As a result, the accuracy of CNS identification is lower for phenotypic methods than for genotypic approaches, the latter being based on amplification, hybridisation, and sequencing procedures (Zadoks and Watts, 2009; Becker et al., 2014).

Similar to the emergence of polymerase chain reaction, the backbone of modern molecular biology, matrix-assisted laser desorption ionisation–time of flight mass spectrometry (MALDI-TOF MS) has also become a paradigm-shifting, high-throughput, and highly reliable method in microbiological diagnostics (Clark et al., 2013). It is increasingly used as a routine technique for



the universal identification of bacteria, including staphylococci, at the species level (Becker et al., 2014). MALDI-TOF MS identifies bacteria by determining their unique protein profiles, which are then matched to a reference database of known bacterial spectra (Cameron et al., 2018). This inexpensive rapid method is technically easy to perform (Kliem and Sauer, 2012; Patel, 2015).

The objective of this study was to use MALDI-TOF MS for the identification of *Staphylococcus* spp. isolated from ovine raw milk, cheese, and udder surface samples. To our knowledge, this is the first research dealing with MALDI-TOF MS-based identification of staphylococcal isolates recovered from udder surfaces of ewes.

2. MATERIALS AND METHODS

2.1. Sampling

In this study, 62 udder surface (US), 62 corresponding individual raw milk (IRM), 4 bulk tank milk (BTM), and 9 cheese samples were tested for the presence of *Staphylococcus* spp. Milk and US samples were collected between March and June in 2019 from three sheep farms located in the eastern part of Hungary (Hajdú-Bihar County and Jász-Nagykun-Szolnok County). Cheese samples were purchased in September 2019 at the 79th National Agriculture and Food Exhibition and Fair (79th OMÉK) in Budapest, Hungary. The samples, kept at 0–4 °C, were delivered to the microbiological laboratory at the Institute of Food Science of the University of Debrecen within 4 h of collection and were examined immediately.

2.2. Isolation of staphylococci

The isolation of staphylococci was performed according to the ISO 6888-1:1999 standard procedure of the International Organization for Standardization (ISO, 1999), as described by Peles et al. (2007), using Baird-Parker Agar supplemented with Egg Yolk Tellurite Emulsion (Oxoid, Basingstoke, UK). The plates were incubated aerobically at 37 °C for 24–48 h. A total of 69 *Staphylococcus* suspect colonies were picked for characterisation based on colony morphology, including size (large, medium, small), shape (circular, irregular, spindle), colour (black, grey, white), appearance (shiny, dull), and texture (smooth, rough). Presumptive *S. aureus* colonies were confirmed with a latex agglutination test (Prolex Staph Xtra Latex Kit; Pro-Lab Diagnostics, Bromborough, UK). Single colonies from the agar plates were inoculated into 7-ml aliquots of Tryptone Soya Broth (Oxoid), which were incubated at 37 °C for 24 h under aerobic conditions. Then 1 mL of broth and 0.5 mL of glycerol were transferred into cryogenic tubes (Cryotube; Thermo Fisher Scientific, Shanghai, China), which were stored at –80 °C until further examination. Sixty-nine isolates were further confirmed by MALDI-TOF MS using the MALDI Biotyper software 3.0 (Bruker Daltonik, Bremen, Germany).

2.3. Identification of staphylococcal species by MALDI-TOF MS

Staphylococcal isolates were individually subcultured on Plate Count Agar (Biolab Diagnostics, Budapest, Hungary) incubated aerobically at 37 °C for 24 h. Bacterial colonies were suspended in 300 µL of distilled water. Following the addition of 900 µL of 96% ethanol (Scharlab, Debrecen, Hungary), solutions were homogenised in a Stomacher 400 Circulator (Seward, Worthing, UK). All samples were then transported to the experimental bacteriology laboratory at the



AgroBioTech Research Center of the Slovak University of Agriculture in Nitra and were stored at $-20\text{ }^{\circ}\text{C}$ until examination. Samples were prepared for MALDI-TOF MS as previously described (Kačániová et al., 2020). Spectra were generated and analysed as described by Kačániová et al. (2019).

2.4. Data analysis

MALDI Biotyper 3.0 software (Bruker Daltonik) was used to process the raw spectral results acquired by the Biotyper system. Possible score outputs ranged between 0.000 (no match) and 3.000 (perfect spectrum match). Data analysis was performed using the manufacturer-recommended cutoff scores of 2.300–3.000 for highly probable species-level identification, 2.000–2.299 for probable species-level and highly probable genus-level identification, and 1.700–1.999 for probable genus-level identification. Scores lower than 1.700 were interpreted as no identification.

3. RESULTS AND DISCUSSION

As shown in Table 1, 42 (60.9%) of 69 bacterial isolates were successfully identified to genus and species level, whereas 27 isolates (39.1%) could not be reliably identified by MALDI-TOF MS. Two thirds ($n = 28$) of the 42 identified isolates were found to be *Staphylococcus* spp. and 14 of them (33.3%) belonged to other genera.

As for the distribution of staphylococcal isolates according to source, 10.7% were recovered from US ($n = 3$), 50% from IRM ($n = 14$), 28.6% from BTM ($n = 8$), and 10.7% from cheese samples (Table 2). The majority, i.e., 66.7–87.5%, of staphylococci isolated from US and both types of milk samples belonged to *S. aureus* ($n = 19$). In addition, CNS species such as *Staphylococcus auricularis* ($n = 1$, US), *Staphylococcus simulans* ($n = 3$, IRM), *Staphylococcus equorum* ($n = 1$, IRM), and *Staphylococcus haemolyticus* ($n = 1$, BTM) were also identified in these samples. In contrast, *S. aureus* was the only staphylococcal species detected in cheese ($n = 3$).

The highest MALDI score obtained in this study was 2.385, indicating the highly probable identification of an *S. aureus* isolate recovered from BTM on Farm 2 (Table 2). Fifteen out of 28

Table 1. Numbers and distribution of ovine-associated bacterial isolates

Type of sample	Number of				
	Total isolates	Unidentified isolates	Identified isolates	Isolates belonging to genera other than <i>Staphylococcus</i>	Isolates belonging to genus <i>Staphylococcus</i>
Udder surface	15	10	5	2	3
Individual raw milk	29	11	18	4	14
Bulk tank milk	8	0	8	0	8
Cheese	17	6	11	8	3
Overall	69	27	42	14	28



Table 2. Identification of ovine-associated *Staphylococcus* spp. by MALDI-TOF MS

Origin	Source of isolate	ID	<i>Staphylococcus</i> (S.) species	Result of latex agglutination test	MALDI score
Farm 1	US	CNS12	<i>S. aureus</i>	Positive	2.136
Farm 1	IRM	CNS15	<i>S. aureus</i>	Positive	2.018
Farm 1	US	CNS13	<i>S. aureus</i>	Positive	2.005
Farm 1	IRM	CNS5	<i>S. equorum</i>	Negative	1.907
Farm 1	IRM	SAA1	<i>S. aureus</i>	Positive	1.810
Farm 1	IRM	CNS11	<i>S. simulans</i>	Negative	1.803
Farm 2	BTM	SAA3	<i>S. aureus</i>	Positive	2.385
Farm 2	IRM	SAA9	<i>S. aureus</i>	Positive	2.245
Farm 2	IRM	CNS21	<i>S. aureus</i>	Positive	2.244
Farm 2	BTM	SAA4	<i>S. aureus</i>	Positive	2.240
Farm 2	BTM	SAA13	<i>S. aureus</i>	Positive	2.214
Farm 2	BTM	SAA5	<i>S. aureus</i>	Positive	2.186
Farm 2	IRM	SAA10	<i>S. aureus</i>	Positive	2.132
Farm 2	BTM	CNS19	<i>S. aureus</i>	Positive	2.123
Farm 2	BTM	SAA6	<i>S. aureus</i>	Positive	2.074
Farm 2	IRM	SAA8	<i>S. aureus</i>	Positive	2.049
Farm 2	BTM	SAA14	<i>S. aureus</i>	Positive	1.846
Farm 2	IRM	CNS20	<i>S. simulans</i>	Negative	1.820
Farm 2	IRM	SAA7	<i>S. aureus</i>	Positive	1.814
Farm 2	BTM	CNS18	<i>S. haemolyticus</i>	Negative	1.799
Farm 2	IRM	CNS23	<i>S. aureus</i>	Positive	1.758
Farm 3	IRM	CNS29	<i>S. simulans</i>	Negative	2.093
Farm 3	IRM	SAA12	<i>S. aureus</i>	Positive	1.919
Farm 3	IRM	SAA11	<i>S. aureus</i>	Positive	1.893
Farm 3	US	CNS31	<i>S. auricularis</i>	Negative	1.713
OMÉK	Cheese	CNS40	<i>S. aureus</i>	Positive	2.050
OMÉK	Cheese	CNS42	<i>S. aureus</i>	Positive	2.011
OMÉK	Cheese	SAA18	<i>S. aureus</i>	Positive	1.857

US: udder surface; IRM: individual raw milk; BTM: bulk tank milk; OMÉK: 79th National Agriculture and Food Exhibition and Fair held in Budapest, Hungary, in September 2019.

isolates (53.6%) had scores in the range of 2.000–2.299, which was highly probable genus-level and probable species-level identification. Furthermore, there were a total of 12 isolates in the score range of 1.700–1.999. The lowest MALDI Biotyper classification score determined for an identified isolate in the present research was 1.713. This value belonged to a *S. auricularis* strain isolated from a US sample on Farm 3.

The 28 staphylococcal isolates identified in this work formed two clusters, one consisting of 22 *S. aureus* strains from CNS19 to SAA3 (bottom of Fig. 1) and the other containing 6 non-*aureus* staphylococci (NAS) from CNS5 to CNS 20 (top of Fig. 1). Apart from this, because of the relatively low number of isolates successfully identified, no other major pattern was observed in the relatedness of staphylococci from various locations and sources.

This is the first study that has evaluated the applicability of MALDI-TOF MS for identification of *Staphylococcus* spp. in ovine raw milk, cheese, and udder surface samples in Hungary.



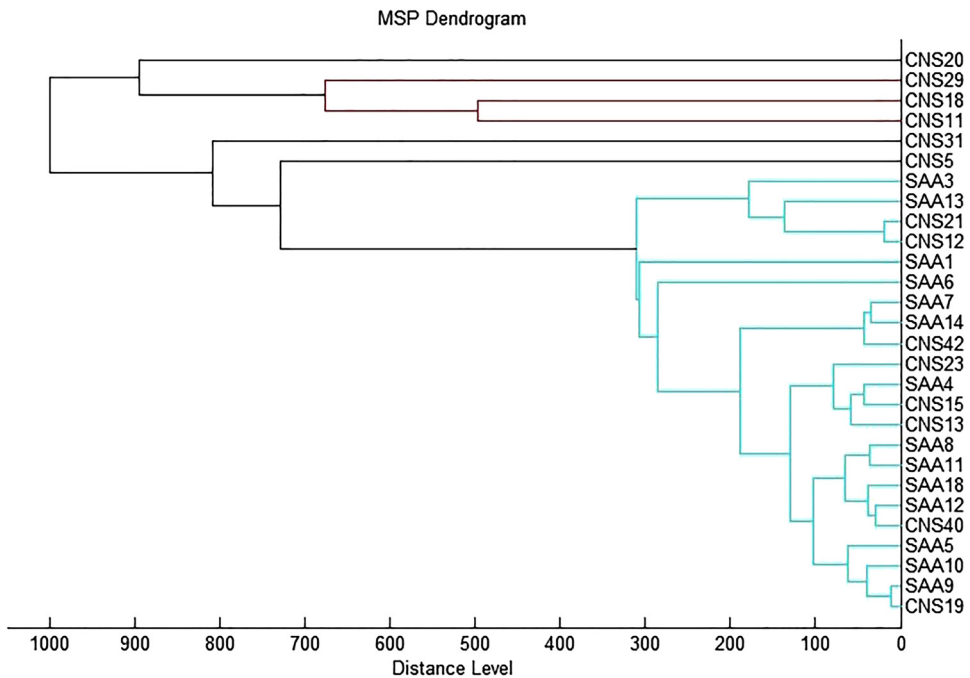


Fig. 1. MALDI-TOF MS-based main spectrum profile (MSP) dendrogram showing the relatedness of 28 ovine-associated *Staphylococcus* isolates examined in this study (identification numbers in the column to the right of the dendrogram are identical with those indicated in Table 2)

For this reason, and because similar work is scarce internationally as well, our results are partly discussed in relation to findings of bovine milk-based studies.

MALDI-TOF MS was able to identify approximately 61% (42/69) of our ovine-associated bacterial isolates. A higher typeability percentage (78%) was reported by [Mahmmod et al. \(2018\)](#) based on 511 NAS isolates, originating from bovine quarter milk and teat surface samples, using MALDI-TOF. Twenty-seven out of 69 isolates (39%) remained unidentified in our study. The most plausible explanation for this is that our reference database was limited in species coverage. The accuracy of MALDI-TOF MS analyses is entirely dependent upon the comprehensiveness of the reference database used to match protein profiles ([Schmidt et al., 2018](#)). Therefore, regular database updates are essential to provide reliable microbial identification ([Patel, 2015](#)). In addition, locally developed reference databases may be required for an efficient analysis of region-specific bacterial strains if geographic variations are expected in phenotypic and genotypic traits of certain genera, e.g., *Staphylococcus* or *Streptococcus* ([Benagli et al., 2011](#)). It should also be noted that experimental design, bacterial growth conditions, and sample preparation techniques may greatly influence the accuracy of identification of CNS species ([Tomazi et al., 2014](#)).

Nearly 80% of the 28 staphylococcal isolates recovered from ovine US, raw milk, and cheese samples were found to be *S. aureus*. Similarly, [Smith et al. \(2015\)](#) reported that IRM from mastitic suckler ewes was dominated by this species. In our study, 100% of the isolates originating from BTM were successfully identified to species level, and 87.5% of these isolates (7/8)



were determined to be *S. aureus*. Well over half, i.e. 62.1% and 64.7%, of the isolates from IRM and cheese samples, respectively, were also identified to species level by MALDI-TOF MS. Somewhat surprisingly, however, MALDI-TOF MS failed to identify two thirds of the isolates recovered from US samples. [Mahmmod et al. \(2018\)](#) likewise reported that unidentifiable NAS isolates originating from teat skin of cows outnumbered those from quarter milk samples by a factor of 11.5 to 1, following two rounds of MALDI-TOF analysis, indicating that although MALDI-TOF is a powerful tool for identification of NAS species from milk, it is less suitable for identification of isolates from nonmilk sources. A possible explanation may be that these unidentified staphylococci recovered from teat surfaces belonged to a commensal teat skin microbiota not included in commercial reference databases, which are typically derived from human isolates, and slight differences between animal and human isolates of identical bacterial species are thought to influence the results obtained when testing specific isolates from animals ([Randall et al., 2015](#); [Mahmmod et al., 2018](#)). Moreover, certain bacteria of environmental origin can develop an extra layer or protein-based capsule, providing protection against harsh environmental conditions, and this may affect the accuracy of MALDI-TOF MS analyses, which are based on the detection of abundant ribosomal and other housekeeping proteins ([Kliem and Sauer, 2012](#); [Mahmmod et al., 2018](#)).

4. CONCLUSIONS

A total of five *Staphylococcus* spp. (i.e. *S. aureus*, *S. simulans*, *S. auricularis*, *S. equorum*, and *S. haemolyticus*) were identified in ovine US, raw milk, and cheese samples by MALDI-TOF MS, with *S. aureus* and *S. simulans* being the most frequently isolated coagulase-positive and coagulase-negative species, respectively. MALDI-TOF MS has proven to be a reliable tool for the identification of staphylococci from raw sheep's milk, especially bulk tank milk; however, currently it appears to be less useful for the identification of bacterial isolates originating from US samples. The commercial reference database used in this study probably had no entries for several CNS species and was thus unable to correctly identify such isolates. Therefore, our MALDI-TOF MS database needs to be expanded with additional reference spectra to fill in gaps existing within the commercial library, even though new spectra are added every half a year from Bruker's database. In this regard, special emphasis should be placed on isolates recovered from ovine hosts and environmental sources.

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