Original Article

Investigation of bacterial pathogens in milk from mastitic dairy cattle by matrix-assisted laser desorption ionization-time of flight mass spectrometry

Gokben Ozbey[1*] Baris Otlu[2] Yusuf Yakupogullari[2] Betul Celik[2] Elif Seren Tanriverdi[2]

Neslihan Kelestemur[1] Tarik Safak[3] Ali Risvanli[4] Anil Persad[5] Emma Sproston[6]

Abstract

The scope of the present study was to assess the use of Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry (MS) as a quick technique for the identification of bacterial species in mastitis. In this study, milk samples from each udder quarter from a total of 250 dairy cattle were aseptically collected and tested. The samples were grouped into California Mastitis Test (CMT) positive, CMT negative and clinical mastitis. The samples were streaked on blood agar and the bacterial isolates were analysed using MALDI-TOF MS. Using MALDI-TOF MS, certain species such as *Staphylococcus chromogenes* (44/188, 23.4%), *Aerococcus viridans* (40/188, 21.3%) and *Staphylococcus haemolyticus* (19/188, 10.1%) were identified at a higher proportion in milk samples from cattle that were CMT positive. Moreover, the most common bacteria isolated from CMT negative milk samples were *A. viridans* (56/161, 34.8%), *S. haemolyticus* (24/161, 14.9%) and *S. chromogenes* (17/161, 10.6%). Only one isolate of *S. chromogenes* (1/4, 25%), *A. viridans* (1/4, 25%), *S. haemolyticus* (1/4, 25%) and *Enterococcus faecium* (1/4, 25%) was detected from milk samples with clinical mastitis using MALDI-TOF MS. There was a concurrence between the MALDI-TOF and biochemical bacterial identification method in 325 of 353 samples (92.06%). This study concludes that MALDI-TOF can be applied for quick determination of bacterial isolates once the bacterial colony has been isolated in milk samples.

Keywords: Cattle, California Mastitis Test, MALDI-TOF, milk samples

[1]*Firat University, Vocational School of Health Services, Elazig, Turkey*
[2]*Inonu University, Faculty of Medicine, Department of Medical Microbiology, Malatya, Turkey*
[3]*Kastamonu University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology, Kastamonu, Turkey*
[4]*Kyrgyz-Turkish Manas University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology, Bishkek, Kyrgyzstan*
[5]*The University of the West Indies, Faculty of Medical Sciences, School of Veterinary Medicine, St. Augustine, Eric Williams Medical Sciences Complex, Mount Hope, Trinidad and Tobago*
[6]*University of Aberdeen, School of Biological Sciences, Aberdeen, Scotland, UK*
***Correspondence**: gokben.ozbey@yahoo.com* (G. Ozbey)
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Introduction

Bovine mastitis remains a major problem that is frequently encountered in the dairy industry (Gomes and Henriques, 2016). In addition to being potentially fatal to dairy animals, the disease can result in severe economic loss to farmers because of decreased milk quality and production (Barkema *et al.,* 2006). Milk condemnation, the cost of treatment and premature culling of animals can also further exacerbate these losses (Barkema *et al.,* 2006; Petrovski *et al*., 2006). In the United States bovine mastitis is estimated to result in an over two billion dollars loss in revenue annually (Jones and Bailey, 2009). Bovine mastitis can be separated as clinical or subclinical depending on its presentation in the animal (Hossain *et al*., 2017). Clinical mastitis can present with a spectrum of different clinical signs ranging from swelling, redness, warmth and pain in the udder, to discoloration and gangrene of the affected quarter, and even death (Sharma *et al*., 2018). Subclinical mastitis infections, however, as the name suggests do not exhibit any clinical signs but can result in a reduction of milk yield and potentially progression towards clinical disease (Peters *et al*., 2015). Mastitis can occur by multiple aetiologies. However, bacterial pathogens including *Staphylococci*, *Streptococci* and *Gram*-negative species are the predominant causes of infection (Bogni *et al*., 2011).

Quick and reliable methods are necessary to identify the bacterial pathogens responsible for bovine mastitis. Traditional species characterization of bacterial pathogens using microbial culture and subsequent biochemical assays can take up to one week. The identification of bacterial pathogens can be performed via a variety of methods including culture based, genotypic or immunoassay methods, with each method having its own advantages and drawbacks (Duarte *et al*., 2015). Historically, bacterial culture followed by biochemical tests and other phenotypic assays have been the gold standard for the determination of bacterial species (Duarte *et al*., 2015). However, this method can be laborious, time consuming and requires a myriad of biochemical and other testing reagents (Carbonnelle *et al*., 2011). One alternative is the utilization of MALDI-TOF MS for a quick determination of bacterial species. The main advantage of using MALDI-TOF MS for bacterial identification is that it reduces the identification time from six days to less than an hour once the bacterial colony has been isolated. It also has higher discriminatory power and confidence levels than traditional biochemical methods (Rodrigues *et al*., 2017). MALDI-TOF MS uses the mass-to-charge ratio profile of bacterial microbial proteins and peptides where the resulting unique spectral profile is used to identify the bacteria. This profile is then compared to a known library and the organism identified (Bizzini *et al.,* 2010; Kliem and Sauer, 2012; Sandrin *et al*., 2013).

Currently, there are a lack of published studies reporting the utilization of MALDI-TOF MS for the determination of bacterial pathogens in Turkey. The scope of this study was to investigate the feasibility of using MALDI-TOF MS for the identification of bacterial species in milk samples from cattle that were CMT positive, samples from cattle with CMT negative and those showing signs of clinical mastitis from different farming conditions.

Materials and Methods

Samples: The current study was performed according to ethical standards and accepted by the Firat University Ethics Committee under protocol number: FU-2018/2. Milk samples were collected from 1000 quarters from a total of 250 dairy cattle (187 Simmental, 18 Brown Swiss, and 45 Holstein; between two and seven years old) during the period March 01-June 30, 2018. Samples were taken from the cattle at the Firat University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology Clinic in Elazig and from five private farms. The presence of mastitis was detected using a combination of the California Mastitis Test (CMT), clinical observation and microbiological assays according to protocols described by the National Mastitis Council (NMC) guidelines (NMC, 2005).

The California Mastitis Test was performed as described by Schallm *et al*. (1971). California Mastitis Test score of 0 and trace (±) was accepted as negative, while CMT scores of +, ++, and +++ were accepted as indicators of subclinical mastitis (Schallm *et al*., 1971). Study groups were planned as reported to Seker *et al*. (2009). The quarters which tested positive for both CMT and microbiological growth were considered mastitis positive. Following the screening for mastitis, the teat and teat orifice were cleaned using a cotton swab and 70% alcohol and a milk sample was aseptically collected from each quarter into individually labelled containers. These samples were kept in a cooler containing ice and immediately transported to Inonu University, Department of Microbiology laboratory for microbiological analysis.

Microbial culture and isolation: Ten microliters of each sample were cultured onto (a) blood agar, (b) Edwards Medium Modified Agar (Oxoid, UK) for *Streptococcus agalactiae* (*S. agalactiae)*, (c) Eosine Methylene Blue Agar (Oxoid, UK) for *Escherichia coli* (*E. coli)* and (d) MacConkey agar (Oxoid, UK) for Gram-negative bacteria and *Enterococcus* species (Shell *et al*., 2017). The inoculated plates were incubated aerobically at 37 °C for 24 h and for those plates with no growth, the incubation was extended for up to 72 h. After incubation, the plates were observed for growth and individual colonies with different morphologies were selected for further identification. Using a sterile loop, selected colonies were transferred from blood agar plates into bank tubes containing 700 µl of Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, UK), and incubated at 37 °C for 24 h. After incubation, 300 µl of buffered glycerol was added and the sample was stocked at -80 °C until further analysis.

One loopful of the frozen broth was cultured onto blood agar and incubated at 37 °C for 24 h after which, individual colonies were identified using a combination method including colony phenotypic properties such as the colony shape, size and color; haemolytic properties on blood agar; Gram-staining; catalase; oxidation-fermentation and bacitracin

susceptibility tests (Markey *et al*., 2013; Persson *et al*., 2011). Additionally, we performed catalase, oxidase tests and coagulase production for identification of *Micrococcus* and *Staphylococcus* spp. Identification of *Streptococcus spp.* was done based on carbohydrate fermentation, hippurate hydrolysis and hydrolysis of esculin, Christie-Atkins-Munch Peterson (CAMP) test, and their culture properties on Mannitol Salt Agar (MSA) (Oxoid, UK) plates.

Staphylococcus aureus (*S. aureus*) was further confirmed with Gram-staining, colony morphology, α- and β-hemolysis, catalase, coagulase test (positive), MSA test and the DNase test. *Bacillus* spp. was identified by Gram-staining and typical colony morphology (Markey *et al*. 2013; Persson *et al*. 2011). Gram-negative isolates were subjected for the indole, methyl red, voges-proskauer, citrate (IMViC) test to identify *E. coli* and oxidase, motility, MacConkey agar (Oxoid, UK), triple sugar iron agar, urea agar and ornithine decarboxylase, as described by Markey *et al*. (2013) and Persson *et al*. (2011).

A milk sample was accepted as positive if at least 10 colony-forming units (CFUs) of environmental pathogens such as Coagulase-negative Staphylococci (CoNS) and *Streptococcus uberis* (*S. uberis)* was observed. However, if at least one CFU of the pathogen responsible for contagious mastitis was observed, this sample was considered as mastitis positive (Persson *et al*., 2011). For other bacteria species, growth of at least three CFUs was required for positive categorization (Persson *et al*., 2011).

MALDI-TOF MS

Sample method: From the previously isolated bacterial samples, one colony was selected from the blood agar plate via sterile loop and transferred onto two separate spots of the MALDI-TOF MS disposable plate. The spots were then coated with 1 µL of matrix solution (α-Cyano-4-hydroxycinnamic acid, CHCA) and allowed to air dry for 1-2 mins at room temperature (Dubois *et al*., 2012; Westblade *et al*., 2013). Following the manufacturer`s instruction, *E. coli* ATCC 8739 was utilized for calibration and as an internal control and was utilized on the MALDI-TOF MS disposable plate for each group analyzed. The samples were examined on the VITEK MALDI-TOF MS (BioMerieux, France). Each spot was irradiated with 320-400 laser shots that were performed in five shot steps (automatic mode). Each spectrum was gathered using In Vitro Diagnostic (IVD) module V3.0 database. After the spectra acquisition, the results were transported from the Acquisition Station software to the VITEK MS Analysis Server (Myla version 4.4) where the identification results were listed (Dubois *et al*., 2012; Westblade *et al*., 2013).

Data analysis: The spectrum obtained from unknown samples was compared to the spectrum characteristics of known bacterial species on the VITEK MS v3.0 reference database. The software compares the peaks within representative data spectrums obtained from known species to those from each unknown species/sample. Each peak within the spectrum from the known data is weighted according to its specificity to the known species (Westblade *et al*., 2013).

The best match between the spectrum from the sample (unknown) and the spectrum of a single (known) organism or organism group was applied as a confidence value. The majority of confidence values were 99.9% and considered as a strong species identification. When confidence values were <99.9%, the spectrum was often adequately adjacent to that of a reference spectrum so that an obvious single organism with the highest confidence value (60-99.8%) could be identified. If a sole ID pattern was not identified, a list of potential organisms was provided with confidence values of <60%, often two different species/genus would be assigned the same confidence value (e.g. 50:50) and both identifications are listed in the results. Occasionally the strain tested was beyond the scope of the database and was considered as nonidentifiable (Westblade *et al*., 2013).

Results

Biochemical Methods and MALDI-TOF Results: Overall, 43.2% (108/250) of cows had at least quarter testing positive for mastitis via CMT with 52.4% (131/250) of the quarters sample testing negative for subclinical mastitis via CMT. Furthermore, 4.4% (11/250) of the cattle surveyed had at least one quarter positive for clinical mastitis. In this study, bacterial growth was observed in 353 of 1000 (35.3%) milk samples cultured. Of these 353, samples 188 milk samples came from CMT positive quarters.

Using MALDI-TOF, we identified 29 different bacterial species from these 188 samples (Table 1 and Figure 1a). In addition, although animals tested CMT negative, we still recovered bacterial isolates from 161 milk samples. From these, 30 different bacterial species were determined by MALDI-TOF (Table 2 and Figure 1b). From milk samples (n=11) taken from quarters with clinical mastitis, 4 bacterial species were recovered (Table 3 and Figure 1c).

From the CMT positive samples, the most isolated organism was *Staphylococcus chromogenes* (*S. chromogenes*) at 23.4% (44/188) followed by *Aerococcus viridans* (*A. viridans*) at 21.3% (40/188) and *S. haemolyticus* at 10.1% (19/188) as identified by MALDI-TOF.

The other main organisms were *S. aureus* at 9.6% (18/188), *Bacillus altitudinis/pumilis (B. altitudinis/pumilis)* at 3.2% (6/188) and *Bacillus licheniformis* at 2.7% (5/188) (Table 1). The singletons that made up 10.6% (20/188) of bacteria identified in CMT positive samples, consisted of 20 different species ID's that occurred on a single occasion; one of these was a double *Staphyloccoccus* spp. that occurred once, one was a triplicate *Bacillus* spp. and 3 isolates were assigned as 2 different genera. The remaining 15 were assigned as a single species on a single occasion. Figure 1a shows the percentages of genus's isolated where the majority belonged to *Staphylococcus* (57.4%), followed by *Aerococcus* (21.3%), singletons (10.6%) and *Bacillus* (5.9%).

From the CMT negative cultures the most commonly isolated organism was *A. viridans* which makes up 34.8% (56/161) of the bacterial species identified. The next most frequent organisms recovered were *S. haemolyticus* at 14.9% (24/161) and *S.*

chromogenes at 10.6% (17/161). *S. epidermidis* (10/161, 6.2%) and *S. aureus* (6/161, 3.7%) were isolated at a lower frequency (Table 2 and Figure 1b). Singletons contributed to 12.4% of all isolates from CMT negative samples and consisted of 20 unique species ID's. Of these, 3 isolates were assigned as 2 different *Staphylococcus* spp. combinations and one isolate was assigned to 2 different genera. The remaining 16 were assigned as a single species on a single occasion. In CMT negative samples, *Staphylococcus* was still the main genus isolated but at a lower percentage (44.7%) than in CMT positive samples (57.4%). Again, *Aerococcus* was the second most commonly isolated genus but this time at a higher percent (34.8%) than in CMT positive samples (21.3%) (Figure 1b).

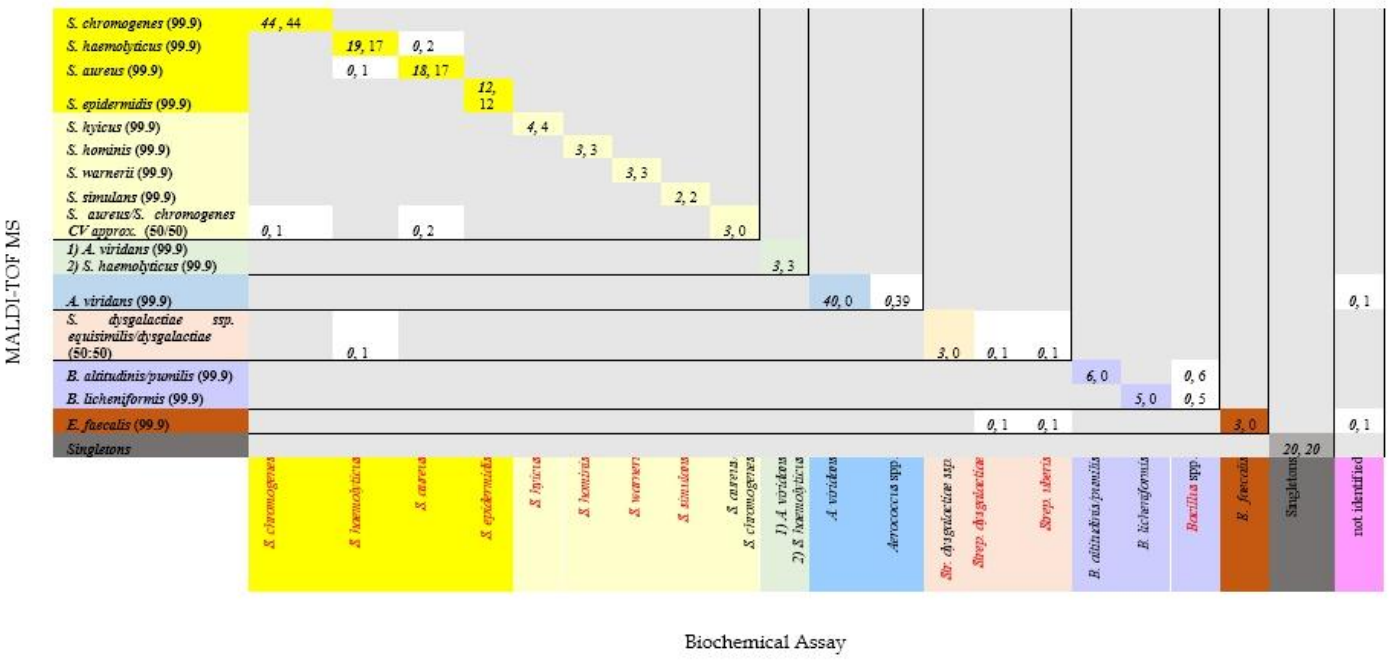
From animals with clinical mastitis, the organisms isolated were *S. chromogenes*, *A. viridans*, *S. haemolyticus* and *Enterococus faecium (E. faecium)*. Each of the 4 species was isolated on a single occasion (25%, 1/4) and identified using MALDI-TOF (Table 3 and Figure 1c). The numbers are too low to suggest any comparison or trends.

The MALDI-TOF MS technique represented further identification of *Brevibacterium luteolum (B. luteolum), Enterococcus faecalis (E. faecalis), E. faecium, Enterococcus hirae (E. hirae), Lactococcus garviae (L. garviae), Parvimonas micra (P. micra), Paenibacillus pabuli (P. pabuli), Pseudomonas fleurescens (P. fleurescens), Staphylococcus arlettae (S. arlettae), S. cohnii ssp. urealyticus, Streptococcus pluranimalium (Str. pluranimalium)* and *Shawenella putrefaciens (S. putrefaciens)* which had remained unidentified or misidentified using traditional biochemical tests from CMT positive and CMT negative samples.

From all the 353 isolates tested, 28 isolates (7.9%) were in disagreement between MALDI-TOF MS and biochemical methods. Of these, 12 (3.4%) isolates were misidentified at the genus level and the remaining 16 isolates (4.5%) failed to match at the species level but were in concordance at the genus level (Table 4). There was a consistency between the MALDI-TOF and biochemical bacterial identification method in 325 of 353 samples (92.06%). This high level of agreement supports the application of MALDI-TOF as an alternative to traditional bacterial culture and biochemical techniques for identifying bacteria.

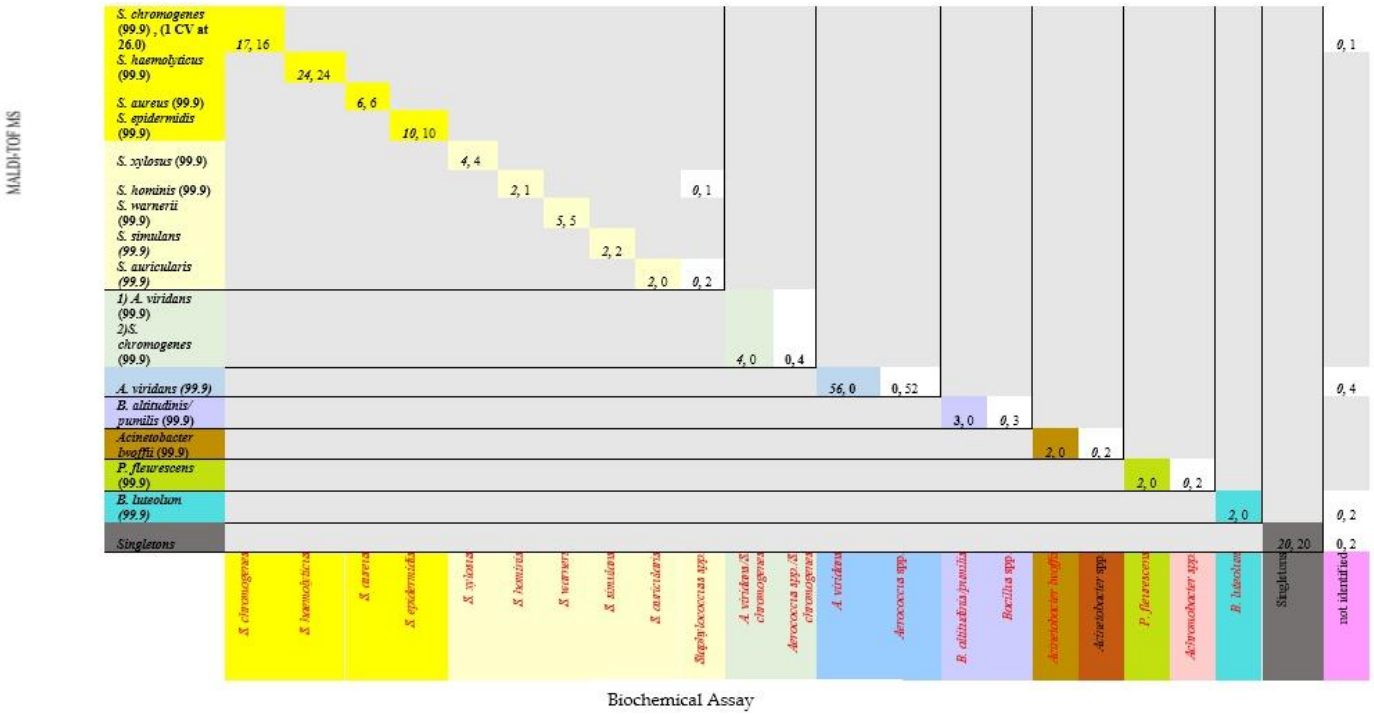
Figure 2 indicates the peptide mass fingerprint spectra of major bacteria (*A. viridans*, *S. chromogenes, S. haemolyticus* and *S. aureus*) compared with the reference spectra.

Table 1 Species assignment of bacterial isolates obtained from cattle milk samples that were CMT positive, using MADI-TOF MS techniques and biochemical assays.



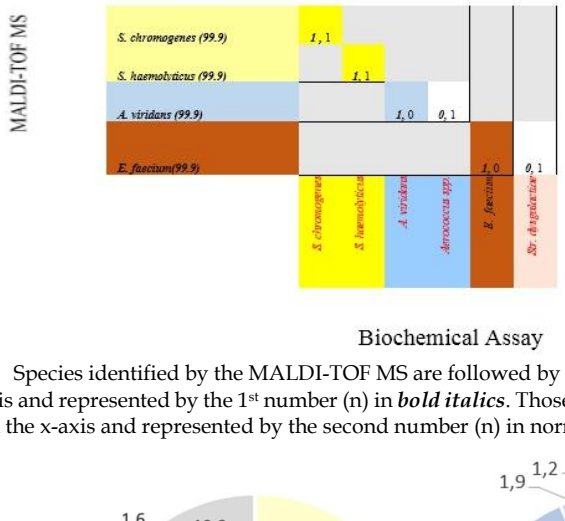
Species identified by the MALDI-TOF MS are followed by confidence values (CV). Those identified by MALDI-TOF MS are on the y-axis and represented by the 1st number (n) in ***bold italics***. Those identified using Biochemical assays correspond to the species/genera on the x-axis and represented by the second number (n) in normal text. Singletons are species that were only identified on a single occasion and grouped together. The total number of positive samples analysed was 188.

Table 2 Species assignment of bacterial isolates obtained from cattle milk samples that were CMT negative using MADI-TOF MS techniques and biochemical assays.



Species identified by the MALDI-TOF MS are followed by confidence values (CV). Those identified by MALDI-TOF MS are on the y-axis and represented by the 1st number (n) in ***bold italics***. Those identified using Biochemical assays correspond to the species/genera on the x-axis and represented by the second number (n) in normal text. Singletons are species that were only identified on a single occasion and grouped together. The total number of positive samples analysed was 161.

Table 3 Species assignment of bacterial isolates obtained from cattle milk samples that had clinical mastitis, using MADI-TOF MS techniques and biochemical assays.



Species identified by the MALDI-TOF MS are followed by confidence values (CV). Those identified by MALDI-TOF are on the y-axis and represented by the 1st number (n) in ***bold italics***. Those identified using Biochemical assays correspond to the species/genera on the x-axis and represented by the second number (n) in normal text. The total number of positive samples analysed was four.

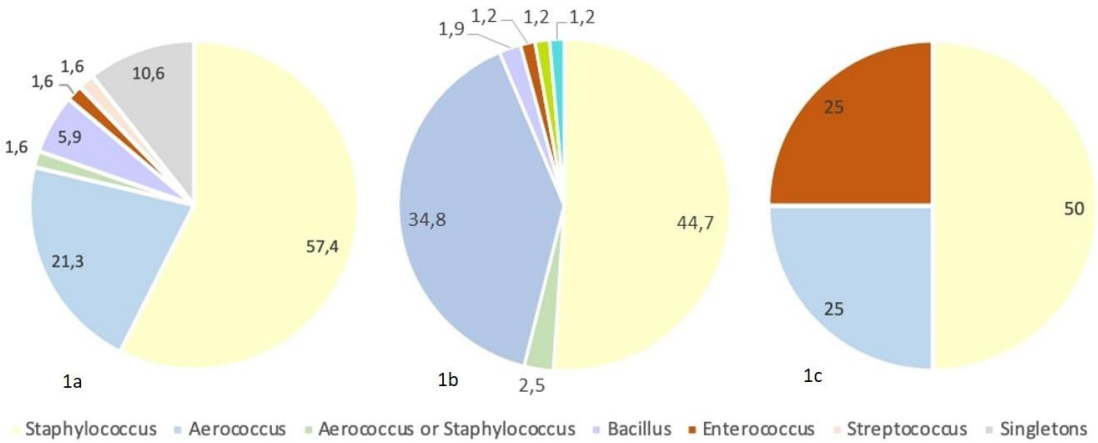


Figure 1 The percentage of bacterial genus cultured from milk samples obtained from cattle that were CMT positive, CMT negative and clinical mastitis using MALDI-TOF MS. 1a: CMT positive, 1b: CMT negative, 1c: clinical mastitis.

Table 4 Identification results of bacterial strains obtained from all milk samples with CMT positive, CMT negative, and clinical mastitis by MALDI-TOF MS *versus* the biochemical methods.

MALDI-TOF MS	n (%)	BIOCHEMICAL METHODS	n (%)
Achromobacter denitrificans	2 (0.6)	*Achromobacter* spp.	2 (0.6)
Acinetobacter iwoffii	2 (0.6)	*Acinetobacter* spp.	2 (0.6)
Aerococcus viridans	92 (26.1)	*Aerococcus* spp.	92 (26.1)
Aerococcus viridans	5 (1.4)	not identified	0
Bacillus altitudinis/pumilis	9 (2.5)	*Bacillus* spp.	9 (2.5)
Bacillus circulans	2 (0.6)	*Bacillus* spp.	2 (0.6)
Bacillus licheniformis	5 (1.4)	*Bacillus* spp.	5 (1.4)
Bacillus simplex	2 (0.6)	*Bacillus* spp.	2 (0.6)
Bacillus subtilis	2 (0.6)	*Bacillus* spp.	2 (0.6)
Brevibacterium luteolum	2 (0.6)	not identified	0
Corynebacterium amycolatum	1 (0.3)	*Corynebacterium* spp.	1 (0.3)
Corynebacterium confusum	1 (0.3)	*Corynebacterium* spp.	1 (0.3)
Corynebacterium freneyi	2 (0.6)	*Corynebacterium* spp.	2 (0.6)
Corynebacterium tuberculostearicum	1 (0.3)	*Corynebacterium* spp.	1 (0.3)
Corynebacterium xerosis	2 (0.6)	*Corynebacterium* spp.	2 (0.6)
Enterococcus faecalis	1 (0.3)	*Streptococcus dysgalactiae*	1 (0.3)
Enterococcus faecalis	1 (0.3)	*Streptococcus uberis*	1 (0.3)
Enterococcus faecalis	1 (0.3)	not identified	0
Enterococcus faecium	1 (0.3)	*Streptococcus dysgalactiae*	1 (0.3)
Enterococcus hirae	1 (0.3)	*Streptococcus dysgalactiae*	1 (0.3)
Escherichia coli	2 (0.6)	*Escherichia coli*	2 (0.6)
Klebsiella pneumoniae	2 (0.6)	*Klebsiella pneumoniae*	2 (0.6)
Lactococcus garviae	1 (0.3)	*Streptococcus uberis*	1 (0.3)
Micrococcus terreus	1 (0.3)	*Micrococcus* spp.	1 (0.3)
Micrococus lutea	2 (0.6)	*Micrococus* spp.	2 (0.6)
Paenibacillus pabuli	1 (0.3)	not identified	0
Parvimonas micra	2 (0.6)	not identified	0
Pseudomonas fleurescens	2 (0.6)	*Achromobacter* spp.	2 (0.6)
Serratia liquefaciens	1 (0.3)	*Serratia* spp.	1 (0.3)
Shawenella putrefaciens	2 (0.6)	*Pseudomonas* spp.	2 (0.6)
Staphylococcus arlettae	1 (0.3)	*Staphylococcus simulans*	1 (0.3)
Staphylococcus aureus	24 (6.8)	*Staphylococcus aureus*	24 (6.8)
Staphylococcus auricularis	2 (0.6)	*Staphylococcus* spp.	2 (0.6)
Staphylococcus chromogenes	61 (17.3)	*Staphylococcus chromogenes*	61 (17.3)
Staphylococcus chromogenes	1 (0.3)	not identified	0
Staphylococcus cohnii ssp. *urealyticus*	1 (0.3)	*Staphylococcus* spp.	1 (0.3)
Staphylococcus epidermidis	22 (6.2)	*Staphylococcus epidermidis*	22 (6.2)
Staphylococcus haemolyticus	42 (11.9)	*Staphylococcus haemolyticus*	42 (11.9)
Staphylococcus haemolyticus	2 (0.6)	*Staphylococcus aureus*	2 (0.6)
Staphylococcus hominis	4 (1.1)	*Staphylococcus hominis*	4 (1.1)
Staphylococcus hominis	1 (0.3)	*Staphylococcus* spp.	1 (0.3)
Staphylococcus hyicus	4 (1.1)	*Staphylococcus hyicus*	4 (1.1)
Staphylococcus saprophyticus	2 (0.6)	*Staphylococcus saprophyticus*	2 (0.6)
Staphylococcus simulans	4 (1.1)	*Staphylococcus simulans*	4 (1.1)
Staphylococcus warneri	8 (2.3)	*Staphylococcus warneri*	8 (2.3)
Staphylococcus xylosus	4 (1.1)	*Staphylococcus xylosus*	4 (1.1)
Streptococcus acidominimus	1 (0.3)	*Streptococcus* spp.	1 (0.3)
Streptococcus dysgalactiae ssp. *equisimilis/dysgalactiae*	1 (0.3)	*Streptococcus uberis*	1 (0.3)
Streptococcus dysgalactiae ssp. *equisimilis/dysgalactiae*	1 (0.3)	*Streptococcus dysgalactiae*	1 (0.3)
Streptococcus dysgalactiae ssp. *equisimilis/dysgalactiae*	1 (0.3)	*Staphylococcus haemolyticus*	1 (0.3)
Streptococcus pluranimalium	1 (0.3)	*Streptococcus agalactiae*	1 (0.3)
Streptococcus uberis	1 (0.3)	*Streptococcus uberis*	1 (0.3)
Trueperella pyogenes	1 (0.3)	*Arcanobacterium* spp.	1 (0.3)
Staphylococcus aureus/ Staphylococcus chromogenes	2 (0.6)	*Staphylococcus aureus*	2 (0.6)
Staphylococcus aureus/ Staphylococcus chromogenes	1 (0.3)	*Staphylococcus chromogenes*	1 (0.3)
Staphylococcus chromogenes + Staphylococcus epidermidis	1 (0.3)	*Staphylococcus chromogenes Staphylococcus epidermidis*	1 (0.3)
Aerococcus viridans + *Staphylococcus chromogenes*	4 (1.1)	*Aerococcus* spp. *Staphylococcus chromogenes*	4 (1.1)
Aerococcus viridans + *Staphylococcus haemolyticus*	3 (0.8)	*Aerococcus viridans* *Staphylococcus haemolyticus*	3 (0.8)
Staphylococcus haemolyticus+ *Staphylococcus epidermidis*	1 (0.3)	*Staphylococcus haemolyticus*+ *Staphylococcus epidermidis*	1 (0.3)
Total	353 (35.3)		331 (33.1)

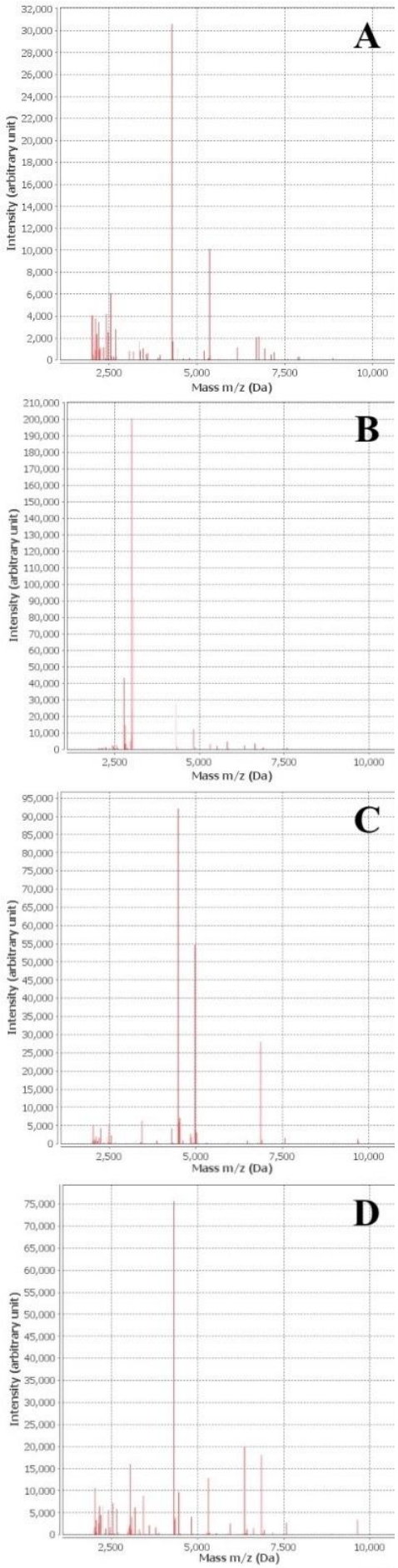


Figure 2 Peptide mass fingerprint spectra of *A. viridans*, *S. chromogenes, S. haemolyticus* and *S. aureus* compared with the reference spectra. A: *A. viridans*, B: *S. chromogenes,* C: *S. haemolyticus* and D: *S. aureus*

Discussion

Published research throughout different regions of the world has found different isolation rates for mastitis causing microorganisms. However, CoNS and *S. aureus* are the most widely found species in mastitis regardless of geographical location and despite the variation between studies and there is evidence that the prevalence of CoNS is increasing (Botrel *et al*., 2010; Nam *et al*., 2010; Pitkala *et al*., 2004).

In this study, using MALDI-TOF MS, we determined bacterial species in milk samples with similar accuracy as traditional techniques but in a faster time. The MALDI-TOF MS technique depends on initial microbiological culture which can still be time-consuming and arduous (Barreiro *et al*., 2017; Wieser *et al*., 2012) but using MALDI-TOF MS, we were able to avoid costly and time-consuming biochemical assays yet successfully and accurately identified the microbial organisms (Barreiro *et al*., 2010). Research carried out on 95 milk samples from mastitic cows from three dairy farms in Egypt has reported the prevalence of *S. aureus* at 26.3% (25/95), *E. coli* at 21.05% (20/95), *S. agalactiae* at 11.6% (11/95), and *Pseudomonas aeruginosa* (*P. aeruginosa)* at 5.3% (5/95) using MALDI-TOF MS (Shell *et al*., 2017). Sori *et al.* (2011), and Zeryehun and Abera (2017) reported that the least isolated ones were *B. cereus*, *C. bovis, Micrococcus* spp. and *S. uberis* from subclinical mastitic cows, these were also among the lower number of isolates in this study (Table 4).

In this study, *S. chromogenes* (23.4%) and *A. viridans* (21.3%) were the most frequently isolated bacterial species from CMT positive milk samples. This is consistent with previously published data which also indicated that *S. chromogenes* was the most common isolate obtained from bovine milk samples with mastitis (Barreiro *et al*., 2017; Dufour *et al*., 2012; Piessens *et al*., 2012; Rajala-Schultz *et al*., 2004; Taponen *et al*., 2006; Tomazi *et al*., 2014). *Staphylococcus chromogenes* belongs to the CoNS species and is more particularly adapted to the mammary glands of dairy cattle than other CoNS species. These are rarely isolated from extramammary sites and 54.5% of intramammary infection by *S. chromogenes* is maintained during lactation (Rajala-Schultz *et al*., 2004; Taponen *et al*., 2006; Thorberg *et al*., 2009; Tomazi *et al*., 2014). The cause of the high prevalence of *S. chromogenes* and potential persistence of infection of the mammary glands may indicate its adaptability and possibly decreased sensitivity to teat disinfectants and/or increased antibiotic resistance (Tomazi *et al*., 2014).

From 478 cows with clinical mastitis in Japan, 136 bacterial isolates were recovered; this included *E. coli* 32.8% (n=44), *A. viridians* at 28.3% (n=38), *Streptococcus* spp. other than *S. agalactiae* at 24.6% (n=33), CoNS at 11.2% (n=15), *P. aeruginosa* at 1.5% (n=2) and four *Gram*-negative bacteria, were unidentified. The same authors also reported that *A. viridans* was obtained between November and March which may suggest some degree of seasonality (Saishu*et al*., 2015).

The impact of *A. viridans* in the pathogenesis of bovine mastitis remains uncertain (Devriese *et al*., 1999), especially since *A. viridans* has commonly been isolated from cattle with clinical and subclinical mastitis (Spakova *et al*., 2012; Zadoks *et al*., 2004). Research conducted on 1,091 cows (4,364 quarters) from California, Minnesota, Iowa, and Wisconsin reported that *Aerococcus* spp. (15.5%) was the second most common of bacterial species isolated following by CoNS at 44.6%. This study used the API20E system (bioMérieux Vitek Inc.) (Arruda *et al*., 2013). Since *A. viridans* strains can tolerate moderately high ambient temperatures, it can survive in cattle manure for extended periods and this manure in turn can propagate the spread of this organism (Saishu *et al*., 2015).

Contrary to our results, a study performed in Germany using mastitic animals from 199 herds found the main bacterial pathogens isolated to be *E. faecalis* (53.8%, 64/119) and *E. faecium* (31.1%, 37/119). These 119 isolates were obtained from untreated bovine mastitis samples by conventional culture method and MALDI-TOF MS (Werner *et al*., 2012). Although *Bacillus* spp. is known to be capable of causing clinical mastitis (Nieminen *et al*., 2007), the role of *Bacillus* spp. in subclinical intramammary infections is not well documented. It has been found to be present in the normal bovine udder microflora (Al-Qumber and Tagg, 2006). However, it has been reported that 4.0% (Lago *et al*., 2014) and 10.0% (Arruda *et al*., 2013) of clinical mastitis have been caused by *Bacillus* spp. Consistent with the present results, CoNS species such as *S. chromogenes*, *S. epidermidis* and *S. haemolyticus* were the most predominant bacterial species in a study conducted on Canadian cows for clinical mastitis (Condas *et al*., 2017). In contrast with our study, *E. coli* was frequently isolated, followed by environmental Streptococci, *Klebsiella* spp. and CoNS from clinical mastitis in Wisconsin, USA (Oliveira *et al*., 2013).

Positive bacterial cultures from both CMT positive quarters (43.5%, 188/432) and CMT negative quarters (30.7%, 161/524) in this study demonstrate a high bacterial diversity. The CMT detects the body's inflammatory response to an infection and not the presence or absence of a bacterial colonies. Animals may have tested CMT negative despite recovery of bacterial isolates since the bacterial isolates had not colonized intramammary tissue sufficiently to induce an inflammatory response. These bacterial isolates may however induce mastitis in the future and as such identification of their presence is necessary to ensure healthy intramammary tissue and thus reduce the occurrence of mastitis and the consequent negative effects on the animal health, milk quality and farm profits.

Consistent with global data, variation in the mastitis causing organisms is also observed in the dairy industry in Turkey. In the Marmara region, the prevalence of *S. aureus* was at 28.1%, *S. epidermidis* at 23.1%, *S. agalactiae* at 18.9%, *E. coli* at 8%, *S. dysgalactiae* at 3.9% and *S. uberis* at 3.7% (Turutoglu *et al*., 1995). The reasons for a lower prevalence of *S. aureus* in our study compared to previously mentioned studies may be explained by variations in factors related to study population, herd size and hygiene and advances in hygienic milking practices, etc. Similar to our study, Ikiz *et al*., (2013) also reported a low isolation of *S. aureus* (4.44%) from milk samples obtained from

Turkish dairy cows with subclinical mastitis (Ikiz *et al*., 2013).

Consistent with the present study (21.3%, 40/188), *A. viridans* (20.19%, 21/104) was the 2nd most prevalent bacterial species from 104 catalase negative Gram-positive cocci isolated from 651 CMT positive subclinical mastitic dairy cows using VITEC 2 in a study carried out in the Konya region of Turkey (Hadimli *et al*., 2013).

Most previously published studies investigating the bacterial etiology of mastitis in Turkey have focused on biochemical and PCR methods. There is some current research utilizing MALDI-TOF MS in Turkey that reports *Staphylococcus* species as a major cause of cattle mastitis (Kırkan *et al*., 2018). *Staphylococcus* was present in 34.0% of mastitis positive milk samples and the *Staphylococcus* species identified using VITEK 2, VITEK (MALDI-TOF) MS, PCR, sequencing and biochemical methods showed that 29.4% were *S. simulans* (n=10) followed by *S. epidermidis* at 23.5% (n=8), *S. aureus* at 20.6 % (n=7), *S. saprophyticus* at 11.8% (n=4), *S. chromogenes* at 5.9% (n=2), *S. hyicus* at 5.9% (n=2), and *S. arlettae* at 2.95% (n=1) (Kırkan *et al*., 2018). The study also reported that 15.0% (n=15) of the samples were detected as *A. viridans* using the MALDI-TOF MS tool (Kırkan *et al*., 2018). *Streptococcus agalactiae*, *S. aureus* and *S. uberis* were correctly identified at 27.2%, 21.8%, 14.2% and 5.2%, respectively by direct-MALDI-TOF MS technique (Barreiro *et al*., 2018).

In our study, the MALDI-TOF MS technique was able to correctly detect all the microorganisms isolated but when using biochemical assays, 12 isolates were misidentified and 16 isolates remained unidentified. The MALDI-TOF MS technique identified these isolates as *B. luteolum, E. faecalis, E. faecium, E. hirae, L. garviae, P. micra, P. pabuli, P. fleurescens, S. arlettae, S. cohnii* ssp *urealyticus, S. pluranimalium* and *S. putrefaciens.* Other studies have detected a high concordance between these two techniques. Similar to our study (92.06%), 95.2% (n=1,116) of isolates tested using MALDI-TOF MS were in concordance with those previously identified in routine clinical testing (Eigner *et al*., 2009). Bizzini *et al.* (2010) also showed accurate identification of 95.1% (n=1,278) at species level and Rodrigues *et al*. (2017), described a 92.9% (n=170/183) consistency of MALDI-TOF MS with biochemical identification results. A slightly lower number (89.0%) of the identifications carried out using MALDI-TOF MS matched those from biochemical tests where 6.3% of the remaining isolates were misidentified at genus level and 4.7% were correct at only the genus level (Braga *et al*., 2018). In this study, most of the mismatches were related to *Enterococcus* and *Streptococcus* species, perhaps owing to the narrow phenotypic identity between these two genera (Braga *et al*., 2018). The disagreement results between MALDI-TOF MS and biochemical methods may also be due to the fact that biochemical assays can be prone to identification mistakes (Braga *et al*., 2018). For example, phenotypic similarities between *Streptococcus, Lactococcus and Enterococcus spp.* can result in misidentification or false interpretation of these species, a small percentage of biochemical assays since esculin and bile esculin tests performed may be variable (Braga *et al*., 2018; Murray *et al*., 2003).

This study provides valuable knowledge on the identification and proportion of bacterial species in milk samples from cattle with clinical and subclinical mastitis in Turkey. MALDI-TOF MS was able to detect the bacterial organisms with comparable accuracy to traditional culture and biochemical assays but in a shorter time and at a lower cost. The relatively quick and reliable determination of bacterial species by the MALDI-TOF MS method will not only aid in a better understanding of pathogens involved in mastitis cases but also provide better clinical treatment of animals and resolution of cases.

Conflicts of Interest: We declare that there is no conflict of interest

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