

Phenotypical and Genotypical Assessment Techniques for Identification of Some Contagious Mastitis Pathogens

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Abstract—Mastitis is one of the most economic disease affecting dairy cows worldwide. Its classic diagnosis using bacterial culture and biochemical findings is a difficult and prolonged method. In this research, using of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) permitted identification of different microorganisms with high accuracy and rapidity (only 24 hours for microbial growth and analysis). During the application of MALDI-TOF MS, one hundred twenty strains of *Staphylococcus* and *Streptococcus* species isolated from milk of cows affected by clinical and subclinical mastitis were identified, and the results were compared with those obtained by traditional methods as API and VITEK 2 Systems. 37 of totality 39 strains (~95%) of *Staphylococcus aureus* (*S. aureus*) were exactly detected by MALDI TOF MS and then confirmed by a nuc-based PCR technique, whereas accurate identification was observed in 100% (50 isolates) of the coagulase negative staphylococci (CNS) and *Streptococcus agalactiae* (31 isolates). In brief, our results demonstrated that MALDI-TOF MS is a fast and truthful technique which has the capability to replace conventional identification of several bacterial strains usually isolated in clinical laboratories of microbiology.

Keywords—Identification, mastitis pathogens, mass spectral, phenotypical.

I. INTRODUCTION

MASTITIS represents one of the most significant problems in modern dairy production from the economic, diagnostic and public-health related point of view [1]. About 150 species of microorganisms, mostly bacteria is able to cause mastitis [2]. They are divided in two groups: contagious and environmental udder pathogens. The most common contagious pathogens causing mastitis in dairy cattle worldwide and 50-100 % of herds may be infected with these pathogens are *S. aureus* and *Streptococcus agalactiae* (*Strept. agalactiae*) [3], [4]. These pathogens have adapted to survive within the mammary gland and are spread from cow to cow at or around the time of milking [5]-[7]. Although the group of contagious pathogens is small regarding the number of species

included, in many countries this group is more often isolated from udder secretions [1]. The majority of intra-mammary infections due to *S. aureus* and *Strep. agalactiae* are subclinical and hence the response of this infection to treatment is comparatively poor and it causes premature culling and elimination of animals from herd [8], [9]. Furthermore, the presence of *S. aureus* in milk may present a degree of risk to the consumer because of the organism's capacity to produce enterotoxins and a toxic shock syndrome toxin-1 (TSST-1) which causes serious food poisoning [3], [9].

Concerning the importance of *S. aureus* and *Strept. agalactiae*, examining of flock for diagnosis of the initial cases of infection is necessary for prevention of infection spreading. Although the culture of milk is considered as a gold standard test for mastitis diagnosis, there are several disadvantages associated with bacterial culture, including no bacterial yielding from truly subclinically infected cows due to presence of high number of leukocytes or presence of preservatives or residual therapeutic antibiotics in submitted samples, intermittent shedding of organisms and cycling through low and high shedding patterns during lactation [10]-[12]. Moreover, microbiological culture of milk is time consuming and species identification by standard biochemical methods requires more than 48 hours to be completed. Due to the above-mentioned limitations of cultural methods, polymerase chain reaction (PCR) has been developed to identify various mastitis pathogens [3], [12], [13]. The progress of PCR based techniques supplies a talented option for the rapid detection of different microorganisms. Using of this technique, most of bacterial species can be identified in hours, instead of days needed for classical culture methods. PCR has high sensitivity and specificity and can improve the level of detection [3], [14], [15].

Both biochemical and genetic analyses are truthful, but their use may be prolonged and their cost comparatively high. Therefore, there is a still need for more rapid and cheap but accurate methods of identifying pathogens of various communicable diseases (Overall, novel skills for particular and rapid identification of microorganisms are a very important step toward a appropriate treatment of infectious diseases in veterinary and medical diagnostics and are of major concern today [6], [17]. Identification of different pathogens with mass spectrometry has been revealed as a technique for rapid identification [4], [18]. Consequently, the most commonly applied technique for mass spectral

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identification of microorganisms is matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) [19]. Detection of pathogens by MALDI-TOF-MS can be valid to a broad range of bacteria, yeasts, and moulds. In recent times, bacterial identification at the molecular level has been investigated using MALDI-TOF MS [20], [21]. Many microorganisms have been identified by MALDI-TOF MS via characteristic “chemical signatures” in a high-throughput mode using simple extraction and sample work-up protocols. After initial bacterial growth that requires about one day, and using crude bacterial extracts or lysate supernatants of whole cells, bacterial determination has been performed by MALDI-TOF MS in a few minutes [21]-[24]. This technique has therefore improved the field of bacterial screening by providing a much faster, more reliable, cheaper, and highly sensitive technique for bacterial identification. These features are particularly attractive for bacterial isolates screening in contaminated milk at subclinical levels [21]. In addition, MALDI-TOF-MS bacterial identification has also shown high interlaboratory reproducibility [25], and is rapidly being incorporated into human clinical microbiological routines. This technique requires little mass spectral (MS) skill, the use of simple instruments, and relatively fast operator training. When compared with DNA analysis based technologies, MALDI-TOF MS requires smaller amounts of biological material and involves simpler sample preparation protocols with no initial assessment, such as Gram staining [25]-[27]. In this study, we evaluated the performance of MALDI-TOF-MS for bacterial screening of subclinical mastitis in dairy cows based on searching the database and software analysis.

II. MATERIALS AND METHODS

A. Samples

Over a 12-weeks period quarter milk samples were collected from 4 dairy herds with high incidence of contagious mastitis pathogens (*S. aureus*, *Strept. agalactiae*) in El-Behira and Alexandria Governorates, Egypt and Al-Qassim region, Kingdom of Saudi Arabia, using standard measures explained by the National Mastitis Council [28]. From every herd, 100 cows in various phases of lactation and different age groups were selected for sampling. Prior to collection of sample; teat ends were clean with warm water and dried before 10 to 15 ml of milk was drawn and discarded. The teat ends were then scrubbed with cotton or paper towel containing 70% ethanol, and then the milk samples were transported on ice, frozen, and kept at -20°C until analysis.

B. Bacterial Isolation

120 isolates of bacteria isolated from samples of milk were collected from the four dairy herds. Isolation of all isolates was carried out according to the National Mastitis Council recommendations on examination of quarter-milk samples. The isolates were stored at -80°C for further investigation. To isolate bacteria from milk samples, 10µL of sampled milk was

streaked onto blood agar plates and incubated at 37°C for 24h to allow for bacterial growth.

C. Classical Identification

Standard identification of different pathogens was carried out by a variety of phenotypic tests, as the coagulase test (Becton, Dickinson and Company, Sparks, Maryland, USA), the catalase test (bioMérieux, Marcy l’Etoile, France), tests with the API and VITEK 2 Systems (bioMérieux). Quality control of commercial systems was carried out by periodically testing a range of ATCC strains. The criteria used for the approval of the identifications obtained with the API and VITEK 2 systems were that the isolate was identified with >90% probability.

D. Identification with API System

The API system strip (BioMérieux, Paris, France) consists of 32 cupules, 26 of which contain dehydrated biochemical media for colorimetric tests. The tests included acid production from urea (URE), L-arginine (ADH), L-ornithine (ODC), esculin (ESC), D-glucose (GLU), D-fructose (FRU), D-mannose (MNE), D-maltose (MAL), D-lactose (LAC), D-trehalose (TRE), D-mannitol (MAN), D-raffinose (RAF), D-ribose (RIB), D-cellobiose (CEL), potassium nitrate (NIT), sodium pyruvate (VP), 2-naphthyl-β-D-galactopyranoside (βGAL), L-arginine β-naphthamide (ArgA), 2-naphthyl phosphate (PAL), pyroglutamic acid-β-naphthylamide (PyrA), novobiocin (NOVO), sucrose (SAC), N-acetylglucosamine (NAG), D-turanose (TUR), L-arabinose (ARA) and 4-nitrophenyl-β-D-glucuronide (βGUR). The manufacturer’s recommended procedures (API System, BioMérieux) were followed. Briefly, the bacterial suspensions were prepared from overnight cultures on blood agar plates (5% horse blood). They were standardized with a turbidity equivalent to 0.5 McFarland standards in 6 ml of sterile distilled water. The ampule of inoculated API suspension medium was homogenized and 55µl of the suspension were dispensed in each cupule of the strip. The tests URE, ADH and ODC were covered with 2 drops of mineral oil. After an incubation period of 24h at 37°C, reagents were added for the nonspontaneous tests. Strain profiles were read and identified with Automatic Testing Bacteriology (ATB) Expression and were interpreted with API laboratory (LAB) software. This software gives the probability of the identification result in a range of 10 to 100%.

E. Identification with VITEK 2 System

Identification of all isolates were detected using the automated systems Vitek2® (bioMérieux– software 4.03), panel gram positive (GP) card. The manufacturer’s instructions were followed for the preparation of the inoculum and incubation of the isolates. The card was automatically filled by a vacuum device, sealed and inserted into the VITEK 2 reader-incubator module (incubation temperature, 35.5°C), and subjected to a kinetic fluorescence measurement every 15 min. The results were interpreted by the ID-GPC database, and final results were obtained automatically. All cards used were automatically discarded into a waste container.

F. MALDI-TOF MS Identification

Mass spectral identification of different isolates of microorganisms using MALDI-TOF MS was performed on a Microflex LT device (Bruker Daltonics GmbH, Bremen, Germany) with FlexControl (version 3.0) software (Bruker Daltonics) for the mechanical achievement of mass spectra in the linear positive mode in a range of 2 to 20 kDa, according to the instructions of the manufacturer. To prepare the samples for MALDI-TOF MS, cells from a single colony of fresh overnight culture (Columbia agar supplemented with 5% horse blood (bioMérieux), incubated 24 H at 37°C) were used for each isolate to prepare samples according to the microorganism profiling ethanol/acid formic extraction procedure, as recommended by the manufacturer. As can be seen from Fig. 1, the identification was first achieved by touching the surface of the investigated colony with a sterile pipette tip and directly applying the small amount of sample on polished or ground steel MSP 96 target plates (Bruker Daltonics).

The sited bacteria were enclosed with 1µL of a chemical matrix (saturated solution of α -cyano-4-hydroxy-cinnamic acid in 50% acetonitrile/2.5% trifluoroacetic acid) and air desiccated at room temperature to permit co-crystallization with the investigational sample. The spectra were then obtained by the mass spectrometer and were compared by using the BioTyper software. Each spectrum was the sum of the ions obtained from 200 laser shots performed in 5 different regions of the same well. The spectra have been analyzed in a range of 2000 to 20000 m/z. According to the measure designed by the manufacturer, a result was supposed right when the score value was >2.0 .

A typical analysis of staphylococcal and streptococcal isolates isolated from clinical and subclinical cases of bovine mastitis by MALDI-TOF MS, 10 to 20 prominent ion peaks were noted in the spectra from the region between 2,000 and 18,000 Da, with the highest-intensity peaks being consistently in the range of 3,000 to 10,000 Da. On this basis, the log (score) values obtained by MALDI-TOF MS correctly identified all but two staphylococcal isolates at the species level [$\log(\text{score}) \geq 2.0$], were not identified. As can be noticed from Fig. 2, ~95% (37/39) of *S. aureus* and 100% (31/31) of

Strep. agalactiae isolates were accurately identified by MALDI-TOF-MS. Among the five species of minor pathogens causing mastitis in cattle, *S. xyloso* ($n = 11$), *S. haemolyticus* ($n = 13$), *S. epidermidis* ($n = 7$), *S. chromogenes* ($n = 10$) and *S. saprophyticus* ($n = 9$) MALDI-TOF MS gave a right identification in 100% (50/50) (Table 1).

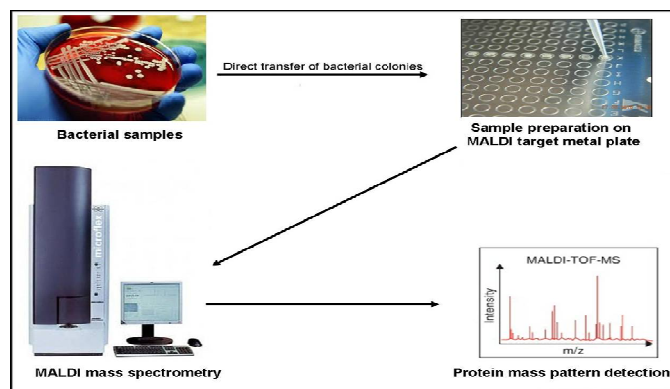


Fig. 1 Steps usually utilized in MALDI-TOF MS for identification of bacterial strains

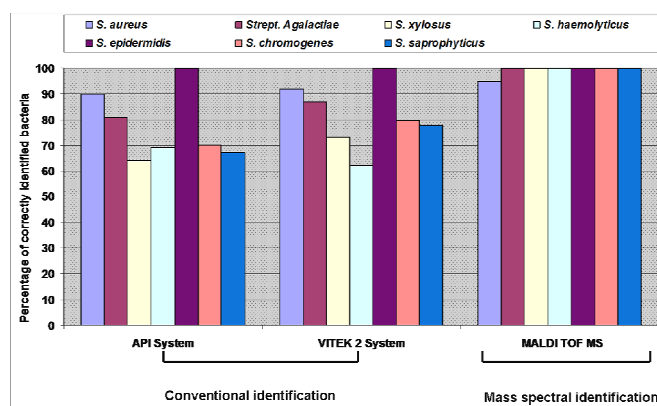


Fig. 2 Comparing the percentage of correctly identified *S. aureus*, *Strep. agalactiae* and CNS by using conventional and mass spectral methods

TABLE I
MASS SPECTRAL (MALDI-TOF MS) AND BIOCHEMICAL IDENTIFICATION OF 120 BACTERIAL STRAINS ISOLATED FROM DIFFERENT MILK SAMPLES FROM COWS WITH CLINICAL AND SUB-CLINICAL MASTITIS

No. of samples	Species	Biochemical identification								Mass spectral identification (MALDI-TOF-MS)	
		API ID System		VITEK 2 System		Coagulase test		Catalase test		+ve	-ve
		+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve		
39	<i>S. aureus</i>	35	4	36	3	36	3	39	0	37	2
31	<i>Strep. agalactiae</i>	25	6	27	4	0	31	0	31	0	0
11	<i>S. xyloso</i>	7	4	8	3	0	11	11	0	11	0
13	<i>S. haemolyticus</i>	9	4	8	5	0	13	13	0	13	0
7	<i>S. epidermidis</i>	7	0	7	0	0	7	7	0	7	0
10	<i>S. chromogenes</i>	7	3	8	2	0	10	10	0	10	0
9	<i>S. saprophyticus</i>	6	3	7	2	0	9	9	0	9	0

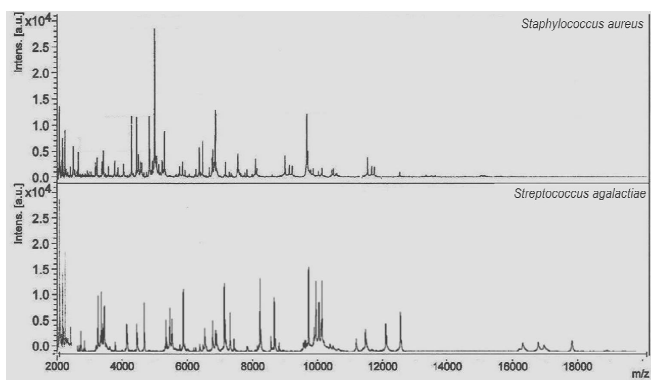


Fig. 3 MALDI-TOF-MS profiles of *S. aureus* and *Strept. agalactiae* cultured overnight at 37°C on Columbia blood agar plates

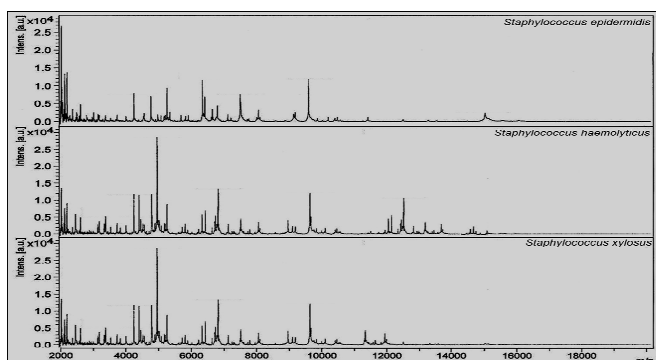


Fig. 4 MALDI-TOF-MS profiles of three isolates of Coagulase negative staphylococci (*S. epidermidis*, *S. haemolyticus* and *S. xylosus*) cultured overnight at 37°C on Columbia blood agar plates

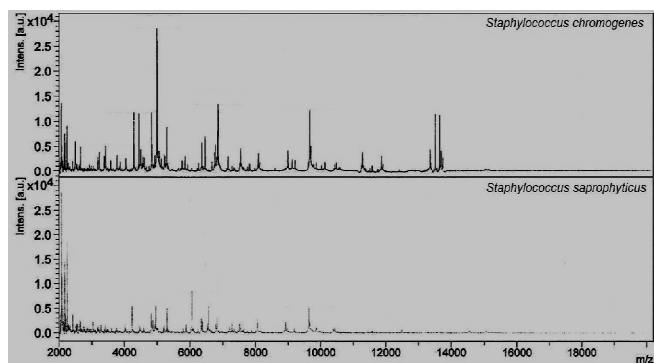


Fig. 5 MALDI-TOF-MS profiles of 2 isolates of CNS (*S. chromogenes* and *S. saprophyticus*) cultured overnight at 37°C on Columbia blood agar plates

One hundred twenty isolates of different types of contagious mastitis pathogens were analyzed by conventional (as API ID System and VITEK 2 System) and mass spectral (MALDI-TOF-MS) methods as described in materials and methods. For each spectrum, a value matching to the force was given to each peak. The peak with the highest intensity was arbitrarily set to 3; all the other peaks had a value matching to the relative intensity of this highest peak (Fig. 3, 4, and 5). It should be pointed out that minor peaks (relative intensity below 0.1) were inconstantly present. We reasoned that peaks that are species specific are likely to correspond to

bacterial components created in high quantity and that such components would therefore generate conserved peaks of high relative intensity. We consequently concentrated on peaks with a relative intensity above 0.1. In general, our data illustrate that, by selecting a suitable set of strains and keeping only the conserved peaks with an m/z above 0.1, a database can be engineered and used for species identification of *Micrococcaceae*.

Usually, the MALDI Biotyper pattern-matching algorithm considers the matches of the unknown sample spectrum against the reference database and the reverse matches of the major spectrum with the unidentified spectrum; it moreover evaluates the relative intensities of nameless and database spectra [29]. As well, MALDI-TOF MS is capable of recognize mixed bacterial cultures, as demonstrated by two of the samples of *S. aureus* that identified as by biochemical testing *S. epidermidis*. The MALDI-TOF MS and conventional identifications were carried out for all 39 *S. aureus* samples. Each sample was cultured twice and similar results were observed. Thirty five out of thirty nine bacterial isolates presented the same results through both identification methods. The MALDI Biotyper Database comprises data from strain collections (e.g., American Type Culture Collection, ATCC) and sequenced bacterial strains, and has been optimized for *Strept. agalactiae* identification [29].

In the current study, four samples of *S. aureus* were identified as *S. epidermidis* by the biochemical measures previously declared, while two samples of the four isolates documented by MALDI-TOF MS were identified as *S. aureus*. To confirm some identification results provided by MALDI-TOF MS as compared with the classical microbiology results, the 16S rRNA and *nuc* genes of certain samples were sequenced. Therefore, *nuc*-based PCR detection was used as a reference method for MALDI-TOF MS analysis in this study. The results of *nuc*-based PCR detection demonstrated that all the 39 isolates in this study were *S. aureus* (Fig. 6).

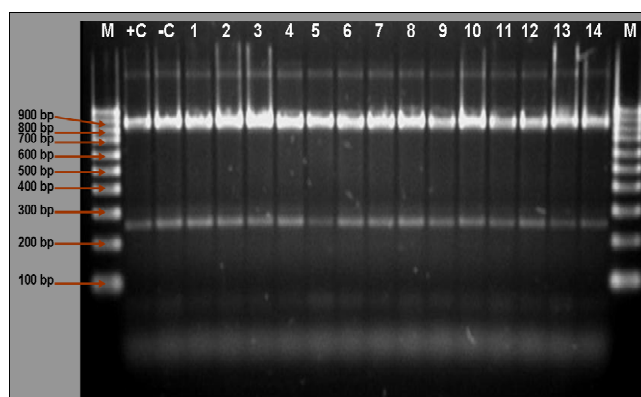


Fig. 6 Interpretation of PCR results after electrophoresis on an agarose gel (1.5%); Lane M, molecular size marker (MBI Fermentas ladder mix, Germany); Lane +C, *S. aureus* reference strain ATCC 29213 (positive control); Lane -C, negative control containing water; Lanes 1-14 positive 16S rRNA and *nuc* genes (Positive *S. aureus* strains. A strong signal of DNA band was detected in all strains from 1- 14

IV. DISCUSSION

Identification of microorganisms is usually accomplished using phenotypic based methods. Nevertheless, those methods still long-standing and infrequently of limited value as for example for CNS where commercial identification kits detected only 37% of 177 CNS strains with the Staph ID 32 API system [30]. In addition, ribotyping and PCR amplicon-sequencing based methods for identification of different types of bacteria still time consuming, expensive and technically demanding. In contrast, using of MALDI-TOF-MS technique, sample preparation and analysis are easy and can be achieved within minutes. No particular lysis stage is needed beyond the exposure to the matrix solution, and the device does not need a professional handler. Just a loopful of cells is required for MALDI-TOF-MS analysis, and the side view is produced with smallest consumables and cost [19]. For one sample of bacteria, mass spectral identification is achieved in a few minutes versus 5-7 day for phenotypical identification. Numerous samples can be achieved per day, and moreover the cost of the analysis is cheap compared to other techniques (in the range of a few cents).

The mass spectral data were evaluated with software supplied with the instrument. The bacterial isolates under study were recognized by comparing their spectral profiles with the database, which is composed of more than 300 strains of ~60 genera from national collection of type cultures (NCTC), including 18 species of genus *Staphylococcus*. The pattern identification algorithm uses all the mass intensity data in the mass spectrum to give the most excellent database match [31]. Therefore, 100% of CNS was accurately recognized by MALDI-TOF MS (50 isolates of CNS yielded strong signals in the mass spectrum, which led to correct identification). In addition 100% of 31 isolates of *Strep. Agalactiae* were accurately identified without any weak signal in the mass spectrum. While ~ 95% of 39 isolates of *S. aureus* were correctly identified by MALDI-TOF MS (only 2 isolates yielded weak signals in the mass spectrum, which led to failure of identification). Under the present experimental conditions, most spectral peaks noticed were in the mass range of m/z 2000-18000 Da, this range is somewhat resembling some studies of bacterial identification using MALDI-TOF-MS. for instance, previous MALDI-TOF-MS studies had reported the analysis of protein samples from solvent suspension or whole cell extraction resulting number of peaks in the 3000-18000 Da mass range. This range is relatively wide compared with the results obtained from [31]-[33]. They noticed that most spectral peaks were in the mass range of m/z 800-3500 Da. The difference in mass range of spectra peaks might be due to the difference in methods of sample preparation.

In this study, we revealed that MALDI-TOF-MS is a powerful instrument for the identification of different strains of contagious mastitis pathogens as *S. aureus*, *Strep. agalactiae* and CNS isolated from milk of cows affected by clinical and subclinical mastitis. The plan reported in this part is presently being extended to other major groups of bacteria isolated in clinical microbiology laboratories, thus allowing

for the suggestion of a scheme for bacterial identification in clinical veterinary laboratories depending on a two-step process. The first one is a fast classification of the isolated bacteria to be identified based on standard phenotypic examination such as growth conditions, gram staining, and morphology, therefore permitting classification of the pathogen within a group of bacteria frequently isolated in clinical microbiology laboratories, e.g., *Micrococcaceae*, catalase-positive, aerobic gram positive cocci. The second step depends on a MALDI-TOF-MS analysis, allowing the rapid identification of the species. We suppose that such an approach may permit the substitute in the near future of the conventional techniques of identification, which are time-consuming and sometimes not reliable.

The standard biochemical measures for bacteria identification are more vulnerable to inaccurate human evaluation or faults throughout sample work-up, as showed by the identification of *Strep. uberis* isolated from milk samples of cow with subclinical mastitis [21], [34]. Approaches at the molecular level have long been known as significant tools in microbiology [35], [36], and MALDI-TOF-MS utilizing "molecular fingerprints" seems to supply a trustworthy tool for identification of different types of microorganisms. This seems to open a novel era in microbiology screening in which traditional phenotypic techniques will change to molecular techniques able to providing quicker and more correct responses and similar genome-related methods of taxonomic and phylogenetic analysis [21].

The results presented in this study, supported by similar and extensive data for clinical human microbiology [16], [21], [26], [27], [29], [37]-[41], confirmed that MALDI-TOF MS provides rapid identification of bacteria (1 day against 5-8 days for conventional identification) and consequently the chance of an earlier treatment of subclinical and clinical mastitis with suitable antimicrobial agents. In addition, in the dairy industry, MALDI-TOF MS can provide a faster, cheaper, and more reliable identification of microorganisms for a more comprehensive microbiological quality control of milk. As MALDI-TOF MS is a new method that will certainly change the way of functioning of microbiology laboratories, additional future researches aimed at evaluating its cost-effectiveness and time to results in comparison to those for conventional methods will be required.

V. CONCLUSIONS

A successful control program for clinical and subclinical bovine mastitis can be established with an effective monitoring system for all dairy herds and accurate identification of microorganism that cause mastitis. Molecular methods may help for rapid and correct identification of different pathogens. Direct MALDI-TOF-MS analysis of bacterial cells requires little effort for sample preparation and can be accomplished within a few minutes. Therefore, MALDI-TOF-MS is shown to be a more fast and influential tool for the fast identification of different strains of *S. aureus*, *Strep. agalactiae* and CNS. We hope that MALDI-TOF MS technology can be regularly used in veterinary laboratories for

identification of different species of bacteria, particularly when failure of phenotypic methods forces clinical microbiologists.

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