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Research Article
Identification of *Staphylococcus aureus* Causing Bovine Mastitis using MALDI-TOF Fingerprinting

[1]Waleed S. Shell, [1]M.L. Sayed, [2]Aatia A. El-Gedawy, [1]Ghada M. El Sadek, [3]A.A. Samy and [1]Abdelhakam M.M. Ali

[1]Central Laboratory for Evaluation of Veterinary Biologics (CLEVB),
[2]Animal Health Research Institute (AHRI),
[3]Department of Microbiology and Immunology, National Research Center (NRC), Dokki, Egypt

Abstract

Background: Reliable and rapid methods for identification of clinical bacterial isolates are mainly dependent on phenotypic and genotypic characteristics of the bacteria. As an alternative identification methods, mass spectral (proteomics) analysis for identification of clinical bacterial isolates including *Staphylococcus aureus* has been recognized. This study was aimed to evaluate and compare the performance, reliability and sensitivity of conventional bacteriology, phenotypic methods and MALDI-TOF MS in identification of clinical *Staphylococcus aureus* isolated from bovine mastitis cases. **Materials and Methods:** Ninety five milk samples were collected from three dairy farms (Giza governorate-Egypt) with high incidence of contagious bovine mastitis and examined for isolation and detection of the causative agents using classical identification, API-Staph kit and matrix-assisted laser desorption-ionization-time-of-flight mass spectrometry (MALDI-TOF MS). The MALDI-TOF MS were carried out using standard ethanol-formic acid extraction method. **Results:** Twenty five cases from these 95 cases were detected by conventional methods and API-Staph kit to be due to *Staphylococcus aureus*. All *Staphylococcus aureus* isolates with 4 *Staphylococcus aureus* reference strains (control positive) and one *E. coli* isolate revealed from bovine mastitic case (control negative) were examined using MALDI-TOF MS. The MADI-TOF MS identified all clinical bacterial samples as *Staphylococcus aureus* with the exception of the control negative sample which was *E. coli* with 100% agreement with bacteriological and phenotypical examination. Also, MALDI-TOF MS gave a valid score of 100% when used in identification of tested *Staphylococcus aureus*, control positive and control negative samples with 100% sensitivity in comparison to results obtained by ABI system and conventional methods. **Conclusion:** This study concluded that according to its fast, accurate and reliable nature, MADLI-TOF MS could be used as alternative diagnostic tool for routine differentiation and identification of *Staphylococcus aureus* isolates in the clinical bacteriological laboratory. The MALDI-TOF MS need more verification and validation using more samples to detect reliability, sensitivity and performance of this type of bacterial identification.

Key words: Bovine mastitis, *Staphylococcus aureus*, MALDI-TOF MS, Bruker daltonics

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Corresponding Author: Waleed S. Shell, Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Egypt

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

A reliable and rapid method of identification of pathogenic bacterial isolates is mainly dependent on phenotypical characters (morphology, culture and serology) and genotypical characters (DNA and RNA) of the bacteria. Traditional characterization and identification of these isolates is often a time waste procedures needing also long incubation periods, biochemical reactions and requires considerable expertise[1,2].

Mastitis is a worldwide disease in dairy cows throughout the world and is responsible for significant economical losses to the dairy farms due to loss in milk production, discarded abnormal milk, degrading milk price and quality due to high somatic cell or bacterial count, high treatment cost, increased birth costs, increased percentage of herd replacement, subsequent mastitis and hazardous of antibiotics residues in milk[3]. Mastitis usually classified according to the causative pathogens, into contagious mastitis caused by *Staphylococcus aureus* and *Streptococcus agalactiae* which are widespread from the infected quarters during milking and environmental mastitis caused by *Streptococcus dysgalactiae*, *Escherichia coli* and *Streptococcus uberis* which are present in the environment (droppings, flooring, bedding) and generally transmitted at any period of cow's life either during milking or during dry period[4].

Identification of *Staphylococcus aureus* isolates revealed from milk of mastitic cows depends on classical phenotypic identification as morphological and cultural methods, Gram stain, antibiotics sensitivity testing, pbp2a latex agglutination test kit, oxacillin agar screen test and cefoxitin disk diffusion test[5-7].

As an alternative identification method, mass spectral (proteomics) analysis for identification of clinical bacterial isolates including *Staphylococcus aureus* has been accepted. Matrix-assisted laser desorption-ionization-time-of-flight mass spectrometry (MALDI-TOF MS) can be used as a sensitive, reliable and rapid procedure for identification of deferent clinical bacterial isolates[8], such as Gram-positive bacteria[9], mycobacteria[10], *Brucella*, Enterobacteriaceae[11], yeast[12], mold[13] and non-fermenting bacteria[14].

The MALDI-TOF MS profiling for binomial identification of microorganisms has been demonstrated by Holland *et al.*[15] and Claydon *et al.*[16] in the 1990s but has evolved to the first-line identification method just in the past 5 years. The technique, combined with reference peptide databases and advanced software has revolutionized microbial characterization[17]. It is consistent with 16S rRNA gene sequencing and is expected to substitute for classic biochemical tests[18]. Its quickness and reliability makes it fit for counter-bioterrorism, epidemiological tracing of field strains and detection of food contamination[19]. The MALDI-TOF MS is approximately two-thirds less expensive than conventional bacteriological methods[20].

Aim of this study is to evaluate and compare the performance, reliability and sensitivity of classical bacteriological and phenotypic methods in comparison to MALDI-TOF MS in identification of clinical *Staphylococcus aureus* isolated from bovine mastitis cases.

MATERIALS AND METHODS

Physical examination of mastitis

Clinical examination of udder: Udder attachments, any physical abnormalities such as anatomical malformations, presence of lesions, swelling of the udder and tick infestation were recorded[21].

Physical examination of milk: The milk was examined for its color, odor, consistency and other abnormalities prior to milking[21].

Sampling (milk samples collection): A total of 95 milk samples were collected by Animal Health Research Institute (AHRI) from clinically and physically examined mastitic cows from three dairy farms (Giza governorate-Egypt) with high incidence of contagious bovine mastitis using standard protocols and measures mentioned by the national mastitis council and Suleiman *et al.*[22]. Briefly, these measures are based on disinfection of the teats with cotton moistened with 70% alcohol. After discarding the first few milk squirts, about 20 mL of milk sample were collected in sterile universal bottles and kept in an ice box for transportation to the laboratory for bacteriological examination according to Suleiman *et al.*[22].

Bacterial isolation: Bacteriological examination of isolates was performed following standard protocols of the national mastitis council, Quinn *et al.*[5] and Sears *et al.*[6]. Briefly, A loopful of each milk sample was cultured on sheep Blood Agar (BA). In parallel, MacConkey agar (MaC) plates were used to isolate *Enterococcus* species and also any Gram-negative bacteria. Inoculated plates were incubated aerobically at 37°C for 24-48 h.

Classical bacterial identification: A variety of phenotypic tests used for standard and presumptive identification of bacterial isolates. These tests included colony morphologic characteristics on culture plates, Gram stain, haemolytic

features on blood agar, oxidation-fermentation test and catalase test[5,6,23]. Identification of *Micrococcus* and *Staphylococcus* species were based on catalase and oxidase tests, coagulase production by using staphylect plus reagent (Oxoid) and their growth characteristics on Mannitol Salt Agar (MSA), where *Streptococcus* and Gram negative isolates were identified[5,6,23].

Oxacillin screening agar test was carried out according to Jain *et al*.[7] and the Mueller-Hinton agar plates, supplemented with 4% (w/v) Nacl containing oxacillin at concentration of 6 ug mL^{-1}, were spot inoculated with a cotton swab according to outlines documented by Clinical and Laboratory Standards Institute (CLSI)[24]. After 24 h incubation at 35°C, oxacillin resistance was detected by bacterial growth.

API-Staph kit identification (BioMerieux): API-Staph kit was used to confirm the results of classical identification methods of *Staphylococcus aureus*. The API system strip (BioMe´rieux, Paris, France) consists of 32 cupules, 26 of which contain dehydrated biochemical agents for colorimetric examinations. The tests based mainly on acid production from different reagents as urea, L-arginine, D-glucose, D-fructose and D-mannose.

Bacterial reference strains: Four referances *Staphylococcus aureus*, as shown in Table 1, obtained from Boehringer Ingelheim Vetmedica, Inc.

***Staphylococcus aureus* culture conditions:** *Staphylococcus aureus* isolates and reference strains (control positive) and *E. coli* isolate from bovine mastatic case (control negative) were cultivated onto brain heart infusion agar medium or 7% sheep blood agar. Plates were incubated at 37°C for 24 h till the appearance of streaks[5]. These fresh pure cultures were used for MALDI-TOF MS identification.

MALDI-TOF MS protocol for *Staphylococcus aureus* isolates: The two standard methods recommended by Bruker Daltonics for the bacterial identification using MALDI-TOF MS: The direct transfer procedure[25] and the standard ethanol-formic acid extraction method[26] which was carried out in this study as follow: Approximately 10 colonies of fresh culture on brain heart infusion agar were harvested using sterile needles into tube containing 300 µL of sterile distilled water. These tubes were vortexed then 900 µL of absolute ethanol was added. Vortexing to homnogenize the sample then the tubes were centrifuged at 13,000×g for 2 min. The supernatant was removed by pipetting. Samples were dried for 2 min at room temperature, each pellet was suspended in 150 µL of a solution containing 50% acetonitrile and 1% aqueous trifluoroacetic acid in a 2 mL sterile tube containing 20 mg of acid washed glass beads. The cells were lyses mechanically. The supernatants were transferred to new tubes after centrifugation at 13,000×g for 2 min. Two extractions were performed for each strain and of each extract 1 µL was deposited on MALDI-TOF steel target plate in six replicates (Bruker daltonics). The plates were dried at room temperature and then overlaid with 1 µL of matrix solution containing-cyano-hydroxycinnamic acid saturated with high-performance liquid chromatography (HPLC)-grade water, 2.5% trifluoroacetic acid and 50% acetonitrile. The sample was allowed to co-crystallize with this mixture. Results were analyzed with MALDI-TOF MS spectrometer using flex control software (Bruker Daltonics). According to the guidelines of the manufacturer, a score of ≥2 depicts identify the sample to the species level and an intermediate log score between <2 and ≥1.7 for identify the sample to the genus level. A low score of <1.7 was considered as unreliable for identification.

RESULTS AND DISCUSSION

All collected milk samples (95 samples) were examined for detection of bacterial causes of contagious bovine mastitis. From the milk samples, Gram-positive cocci (*Staphylococcus* and *Streptococcus* species) were the most causative pathogens isolated from bovine milk samples in addition to *Escherichia coli* species. Twenty five *S. aureus* were isolated from bovine mastitis cases of 35.7% from total isolated bacteria. These isolates were identified phenotypically using classical methods, oxacillin screening agar test and API-Staph kit (BioMerieux). Comité de l'antibiogramme de la société française de microbiologie[27] suggested using oxacillin instead of methicillin in identification of *Staphylococcus aureus*. Ten isolates out of 25 *S. aureus* isolates were methicillin-resistant. Four *Staphylococcus aureus* reference strains were used in this study as a control positive (Boehringer Ingelheim Vetmedica, Inc.) (Table 1), these references strains were used in production and evaluation of mastitis vaccine.

Escherichia coli isolate revealed from bovine mastatic case were used in this study as a control negative. Out of 95

Table 1: *Staphylococcus aureus* references' strain

Phage type	*Staphylococcus aureus* strain No.	Capsular serotype
I	584763	8
II	596535	5
III	596510	336
Miscellaneous type	6032	8

Table 2: Identification of *Staphylococcus aureus* reference strains (R) and field isolates (F) using MALDI-TOF

Analyte name	Analyte ID	Organism	Matched pattern	Score value	NCBI identifier
R1 (+++) (A)	584763	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33591 THL+ *Staphylococcus simiae* DSM 17639 DSM	2.327	1280
R2 (+++) (A)	596510	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33591 THL+ *Staphylococcus simiae* DSM 17638 DSM	2.5	1280
R3 (++) (A)	596535	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33862 THL	2.222	1280
R4 (++) (A)	6032	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33862 THL	2.064	1280
F1 (+++) (A)	1	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33591 THL+ *Staphylococcus epidermidis* ATCC 14990T THL	2.406	1280
F2 (++) (A)	2	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33591 THL	2.267	1280
F 3 (++) (A)	3	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33591 THL	2.171	1280
F4 (++) (A)	4	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33862 THL	2.234	1280
F5 (++) (A)	5	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33862 THL+ *Bacillus flexus* DSM 1320T DSM	2.24	1280
F6 (++) (A)	6	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33591 THL	2.42	1280
F7 (++) (A)	7	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33591 THL	2.491	1280
F8 (++) (A)	8	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33591 THL+ *Staphylococcus simiae* DSM 17639 DSM	2.011	1280
F9 (+++) (A)	9	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33591 THL+ *Staphylococcus simiae* DSM 17639 DSM	2.033	1280
F10 (+++) (A)	10	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33591 THL	2.434	1280
F11 (+++) (A)	11	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33591 THL	2.288	1280
F12 (++) (A)	12	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33591 THL+ *Staphylococcus simiae* DSM 17639 DSM	2.233	1280
F13 (++) (A)	13	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33591 THL+ *Staphylococcus epidermidis* ATCC 14990T THL	2.323	1280
F14 (++) (A)	14	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33591 THL	2.3	1280
F15 (+++) (A)	15	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33591 THL	2.333	1280
F16 (+++) (A)	16	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33862 THL+ *Bacillus flexus* DSM 1320T DSM	2.1	1280
F17 (++) (A)	17	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33591 THL	2.012	1280
F18 (++) (A)	18	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33591 THL+ *Staphylococcus simiae* DSM 17639 DSM	2.213	1280
F19 (+++) (A)	19	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33591 THL+ *Staphylococcus simiae* DSM 17639 DSM	2.112	1280
F20 (++) (A)	20	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33862 THL+ *Bacillus flexus* DSM 1320T DSM	2.423	1280
F21 (+++) (A)	21	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33591 THL+ *Staphylococcus epidermidis* ATCC 14990T THL	2.354	1280
F22 (+++) (A)	22	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33862 THL+ *Bacillus flexus* DSM 1320T DSM	2.221	1280
F23 (+++) (A)	23	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33591 THL	2.21	1280
F24 (+++) (A)	24	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33591 THL	2.33	1280
F25 (++) (A)	25	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 33591 THL	2.4	1280
F26 (+++) (A)	26	*Escherichia coli*	*Escherichia coli* DH5alpha BRL+ *Enterobacter cloacae* MB_8779_05 THL	2.362	562

mastitic cases, 20 cases were caused by *E. coli* where *Streptococcus agalactiae* and *Pseudomonas arigenosa* were recovered from 11 and 5 cases, respectively. *Escherichia coli* and *Streptococcus agalactiae* was the causative agent of mastitis in 9 mixed infection cases. Twenty five cases were due to non bacterial cause.

Using MADI-TOF MS, all phenotypical and bacteriological identified *Staphylococcus aureus* and control negative and positive samples were checked. The MADI-TOF MS identified all clinical bacterial samples as *Staphylococcus aureus* with the exception of the control negative sample which was *E. coli* with 100% agreement with bacteriological and phenotypical examination (Table 2).

The MALDI-TOF MS has successfully been used successfully for identification of many bacterial species. However, only a few researches have evaluated the efficacy of MALDI-TOF MS based identification to be used as routine in diagnostic laboratory[28]. In this study, MALDI-TOF MS could accurately identify all ATCC strains, references strains (positive control) and *E. coli* isolate recovered from bovine mastitis case as a negative control as well as tested samples. The results obtained from this study concluded that identification

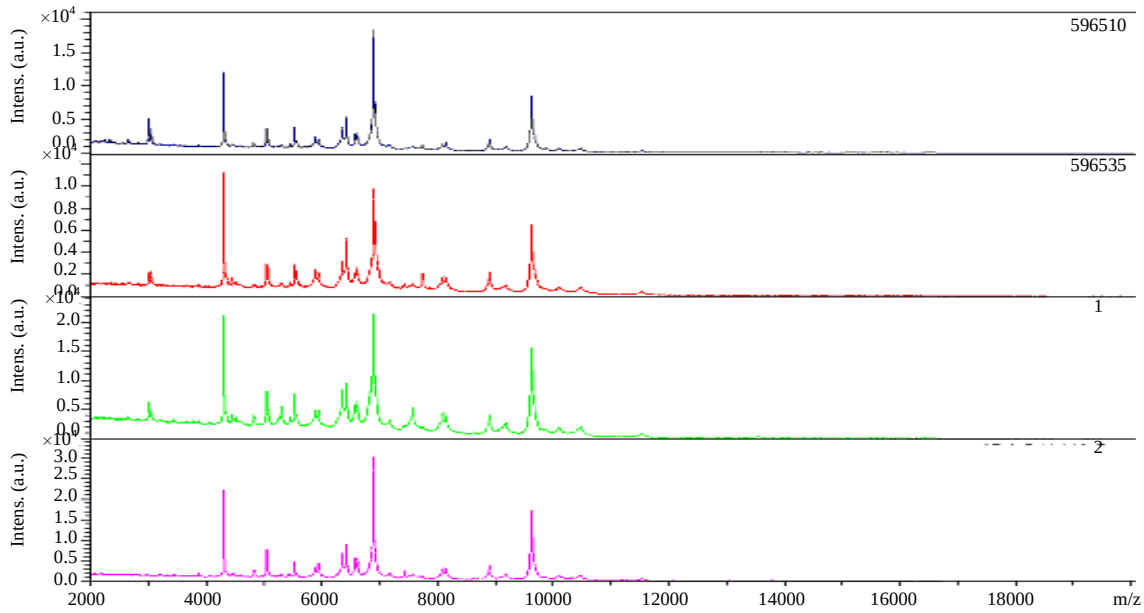

Fig. 1: Overview of the MALDI-TOF mass spectra of four *Staphylococcus aureus*: reference strains 596510 and 596535 and field isolate No. 1 and 2, respectively revealed from bovine mastitic cases

of different *Staphylococcus aureus* strains isolated from bovine mastitis cases was possible using MALDI-TOF MS. The samples being processed and run in duplicates for more results accuracy so the reproducibility of the apparatus was examined and subsequently found to be consistent for all samples. The MALDI-TOF MS mass spectral patterns were also reproducible for bacterial strains and isolates belonging to the same genus and species[29,30].

By examination of staphylococcal isolates and strains revealed from cases of bovine mastitis by MALDI-TOF MS, 10-20 prominent ion peaks were detected in the mass spectra. These prominent ion peaks were from the region between 3,000 and 11,000 Da, with the highest-intensity peaks being consistently in the range of 4,000-10,000 Da. On this basis, the score values obtained by MALDI-TOF MS correctly identified all *Staphylococcus aureus* isolates at the species level (score ≥2.0). Inspection of mass spectra (Fig. 1) reveals strain-specific peaks at 4240, 6900 and 9700 Da for all *Staphylococcus aureus*.

In this study, MALDI-TOF MS gave a valid score of 100% when used in identification of *Staphylococcus aureus* causing bovine mastitis with 100% sensitivity in comparison to results obtained by ABI system and conventional methods this agree with Da Motta *et al.*[31], Barreiro *et al.*[32] and Dubois *et al.*[33] which reviewed that MALDI-TOF is suitable assay for the differentiation of *Staphylococcus* isolates at the species level. Also, similar finding agreed with that recorded by Rohokale *et al.*[34] who mentioned that MALDI-TOF MS technique proved equally (100%) effective in identification of *Staphylococcus aureus* with highest accuracy as compared with bacterial culture and PCR assay which demonstrating the reliability and accuracy of MALDI-TOF MS in *Staphylococcus aureus* and other bacterial identification. Other study[35] revealed that out of 222 Staphylococcaceae isolates from 29 species, 165 (74.3%) were assigned correctly using MALDI-TOF MS. Only one isolate was incorrectly identified to *Staphylococcus aureus* while it actually belonged to the just recently described coagulase-positive species *S. argenteus*. The remaining isolates species (56 isolates) decision was not achieved. For all *S. aureus* isolates, a 100% match rate was obtained with the commercial and the extended database. Also, detection and typing of *S. aureus* strains recovered from bovine mastitis cases were carried out by proteomic analysis (MALDI-TOF MS) and the frequency of precise identification at the genus and species levels was 97.97%[36].

Valid identification scores as explained by the manufacturer is 2.0 or more were enough for a reliable identification to the species level (green color) (i.e., species score cutoff of 2.0) and scores of ≥1.7 but <2.0 were reliable and accepted for detection to the genus level (yellow color). Scores under 1.7 were considered unsatisfactory and unreliable identification (red color)[37,38] as shown in Fig. 2 and Table 2.

This technique is depend upon the presence of highly abundant proteins in a mass range between 2 and 20 kDa by calculating their mass (m) to charge (z), m/z values. Thus, for each clinical bacterial isolates a standard fingerprint is generated which is used for comparison with the stored

Meaning of score value

Rang	Description	Symbols	Color
2.300...3.000	Highly probable species identification	(+++)	Green
2.000...2.299	Secure genus identification, probable species identification	(++)	Green
1.700...1.999	Probable genus identification	(+)	Yellow
0.000...1.699	Not reliable identification	(-)	Red

Analyte Name: B23
Analyte description:
Analyte ID: *Staphylococcus aureus* isolate No. 5
Analyte creation Date/Time: 2016-03T12:43:41.395
Applied MSP Library(ies):
Applied taxonomy tree: Bruker taxonomy

Rank (Quality)	Matched pattern	Score value	NCBI Identifier
1 (++)	*Staphylococcus aureus* ATCC 33862 THL+*Bacithus flexus* DSM 1320T DSM	2.24	1280
2 (++)	*Staphylococcus aureus* ATCC 33591 THL+*Bacithus flexus* DSM 1320T DSM	2.224	1280
3 (+)	*Staphylococcus aureus* ATCC 33591 THL	1.911	1280
4 (+)	*Staphylococcus aureus* ATCC 33862 THL	1.879	1280
5 (+)	*Staphylococcus aureus* ssp., *aureus* DSM 3463 DSM	1.776	46170
6 (+)	*Staphylococcus aureus* ssp., *aureus* DSM 4910 DSM	1.72	46170
7 (-)	*Staphylococcus aureus* ATCC 29213 THL	1.69	1280
8 (-)	*Staphylococcus aureus* ssp., *aureus* DSM 346 DSM	1.609	46170
9 (-)	*Staphylococcus aureus* DSM 17639 DSM	1.557	308354
10 (-)	*Staphylococcus aureus* THL 25923 THL	1.55	1280

Fig. 2: Meaning of MALDI-TOF MS score value

reference spectra and thereby providing identification for the sample. The most important advantage of mass spectral approach over phenotypic and genotypic procedures is a simple straight forward sample preparation procedure and the short time required for analysis as it can carried out using one of these two methods: Identification from direct culture or after extraction of samples using ethanol: Formic acid method[29,30].

The identification using MALDI-TOF MS method could analyze samples in short time (may be within minutes) after culture positivity especially when direct cultural identification methods used rather than ethanol: formic acid extraction method. However, identification by conventional methods needs more facilities, chemicals, time and experiences. In contrast, the non requirement of high technical expertise, the simple extraction procedure and low running cost provide MALDI-TOF MS provide more advantages over other methods for identification. But the applications have to be carefully carried out, as the results accuracy decrease by using of too much of materials and chemicals. The samples have to be overlaid with the matrix solution with care to avoid incidence of the liquid smear between spots, which increase possibility of cross contamination[39].

CONCLUSION

A successful management and plan for diagnosis of contagious bovine mastitis in Egypt can be established with accurate, strong and effective monitoring system for all dairy flocks and farms in a parallel with an accurate and fast identification of microorganisms that cause bovine mastitis. Mass spectral (proteomics) identification tool of bacterial isolates by MALDI-TOF-MS is fast and took about 30 min per isolate from target plate to gain the final results and also requires little effort for sample preparation. In this study, MALDI-TOF-MS is demonstrated to be a most fast and sensitive tool for identification of *S. aureus* isolates causing contagious bovine mastitis when compared with the results of classical identification and ABI system. Also, from above results, It could be concluded that *S. aureus* is one of the major pathogen causing bovine mastitis in Egypt so, according to its fast, reliable and accurate nature, MADLI-TOF MS could be introduced as a regular diagnostic tool for routine identification and differentiation of *Staphylococcus aureus* isolates and bacterial causing bovine mastitis in the clinical bacteriological veterinary laboratory in Egypt in order to provide more precise identification on clinical specimens. Anyhow, this assay needs more verification and validation using more samples to detect reliability, specificity, sensitivity and performance of this type of bacterial identification.

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