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RESEARCH ARTICLE

Comparison of MALDI-TOF mass spectrometry with phenotypic methods for identification and characterization of *Staphylococcus aureus* causing mastitis

Alharbi, A.[1,2], Al-Dubaib, M.[1], Elhassan M.A. Saeed[1,5], Elbehiry, A.[3,4*]

[1]Department of Veterinary Medicine, College of Agriculture and Veterinary Medicine, Qassim University, Saudi Arabia
[2]Ministry of Environment, Water and Agriculture, Saudi Arabia
[3]Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, University of Sadat City, Egypt
[4]Department of Public Health, College of Public Health and Health Informatics, Al Bukayriyah, Qassim University
[5]Department of Microbiology, Faculty of Veterinary Medicine, Khartoum University
[*]Corresponding author: aymanella2007@yahoo.com

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ABSTRACT

The emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) infection is one of the greatest threats to both animal and human health. Our investigation was aimed to identify and differentiate between MRSA and methicillin-sensitive *Staphylococcus aureus* (MSSA) recovered from mastitic milk using MALDI-TOF mass spectrometry compared with phenotypic methods and studying their susceptibility to various antibiotics. Four hundred milk samples from mastitic animals (cows, sheep, goats, and dromedary camels) were investigated. Phenotypic identification of *S. aureus* was made through MASTASAPH Latex test, STAPH ID 32, and Vitek 2 system. The proteomic characterization of *S. aureus* was done by MBT. The Kirby Bauer method was accomplished to detect the resistance of *S. aureus* strains to antibiotics. The results of the MASTASAPH Latex test, revealed that 54 (46%) were recognized as *S. aureus*. All *S. aureus* isolates were identified by MBT with a score of more or equal 2.00. Several peaks were identified in the mass of 4590 Da, 4863 Da, and 4938 Da for MSSA and in the mass of 2636 Da and 3009 Da for MRSA. The MSP dendrogram demonstrated that the *S. aureus* isolates were classified into one group with a distance level of less or equal 400. The percentage of *S. aureus* resistance against carbenicillin, erythromycin and kanamycin was 94.4%, 38.88%, and 33.33%, respectively. In conclusion, *S. aureus* bacteria are among the key triggers for mastitis in Saudi Arabia. MBT is reported to be not only the rapid tool to identify *S. aureus* but also able to discriminate MRSA from MSSA.

Keywords: *S. aureus* mastitis; MRSA; single peak; PCA; antimicrobial resistance

INTRODUCTION

Mastitis is one of the common obstacles in various dairy farms in many parts of the world from the investigative, pathogenic, cost-effective, and public health-related viewpoints (Varela-Ortiz *et al.,* 2018). Mastitis has severe economic effects and leads to a decrease in the quality and quantity of milk due to the change in milk composition; the milk volume becomes instable. Furthermore, the fertility of the animal is damaged, which causes an unsystematic estrus cycle and a problem in calving. On the other hand, other costs are incurred due to the need for intervention, herd treatment, and infection control (Rollin *et al.,* 2015; Pumipuntu *et al.,* 2019).

Many types of pathogenic microorganisms can cause mastitis, amounting to at least 137 microorganisms. They include *Escherichia coli* and *Streptococcus agalactiae*. However, *Staphylococci* remain the key factor that causes mastitis in a lot of dairy herds (Sender *et al.,* 2017; Varela-Ortiz *et al.,* 2018). Many forms of mastitis are caused by various bacteria such as *Staphylococcus* species. Both *Staphylococcus aureus* (*S. aureus*) and *coagulase-negative staphylococci* (CNS) and are two of the main microorganisms associated with clinical and subclinical mastitis (Elbehiry *et al.,* 2016; Pumipuntu *et al.,* 2019). *Staphylococcus* includes 49 species and 26 subspecies, divided into two clusters according to their capability to yield coagulase (Taponen *et al.,* 2012; Han *et al.,* 2015). *S. aureus* is a major cause of mammary infections in dairy animal (Zecconi & Scali, 2013; Wang *et al.,* 2018). About 10-40% of mastitis infections are caused by *S. aureus* in China and other countries (Basanisi *et al.,* 2017; Wang *et al.,* 2018). Moreover, this kind of bacteria is highly infectious to humans (Pollitt *et al.,* 2018). Remarkable alterations have befallen in the epidemiology of *S. aureus* over the last few years. For instance, methicillin-resistant *Staphylococcus aureus*, known as MRSA, which was essentially restricted to health facilities, has become an important pathogen in society. This enhances the significance of this pathogen to public health as well as animal health

(Guimarães *et al.,* 2017). Although *S. aureus* is a key reason for animals' mastitis, previous studies have shown a lower spread of cow MRSA, indicating that MRSA does not usually accompany mastitis (Hendriksen *et al.,* 2008; Guimarães *et al.,* 2017).

S. aureus mastitis can be analyzed by phenotypic and genotypic methods to offer the most consistent consequences. Phenotypic and genotypic techniques require significant effort and cost. Further, these methods have not yet been used to distinguish between MRSA and MSSA. Differentiation between MRSA and MSSA has been previously studied using different methods (Koivula *et al.,* 2007; Wolters *et al.,* 2010; Elbehiry *et al.,* 2016), especially with the spectral differences (Du *et al.,* 2002; Böhme *et al.,* 2012; Elbehiry *et al.,* 2016). However, slight strong proof has been existed on the application of peptide mass fingerprinting technique (PMFT) as a rapid and accurate method for identifying various *S. aureus* isolates at the species level. According to previous studies (Koivula *et al.,* 2007; Wang *et al.,* 2013; Cantekin *et al.,* 2015; Elbehiry *et al.,* 2016), one of the most prominent boundaries connected to the accurate detection of *S. aureus* is the lack of a precise, prompt, and appropriate tool that has the ability to distinguish the bacterial classes involving this pathogen.

However, almost all molecular methods approximately are effortful and expensive. Therefore, there is still a need for a fast, cost-effective, and accurate method to detect the various microorganisms of infectious diseases. Generally, PMFT, as a creative technique, provides an accurate and quick description of the various microbes and is considered a good step toward the proper management of infectious illnesses in veterinary and medical identification (Sauer *et al.,* 2008; Elbehiry *et al.,* 2016). As a new technology for identifying microorganisms, PMFT depends on the protein structure of the cells of the microbes. Although this technique appeared years ago, only recently did studies show a positive detection of the species in the research laboratory (Emonet *et al.,* 2010; Cherkaoui *et al*., 2011; Sun *et al.,* 2020). In the last two years, this technology has been used as a common technology for species identification because it is carried out in a short time, nearly one working day (Burckhardt & Zimmermann, 2011). This technology is characterized by being fast and cost-effective provided that there is a quality-managed database of the reference spectra that comprises all the microorganisms. Great endeavors have led to the standard sample arrangement protocols; thus, improved analysis and database tools have emerged (Cherkaoui *et al.,* 2011; Yan *et al.,* 2020). In the current study, the recent methods and the new-generation technologies are compared to the classical methods of bacteria identification.

The failure to treat *S. aureus*-associated mastitis goes back to the long period of treatment, and the infection resulting from *S. aureus* yielding beta-lactamase. In addition, *S. aureus* causes an abscess rounded by capsules of thick fibers in the mammary gland. This plays a role in the accumulation of antibiotics on the target location, and bacterial destruction is reduced (Basdew & Laing, 2011; Saðlam *et al.,* 2017). The development of MRSA strains has led to the application of intravenous antibiotics that have the potential for common side effects such as diarrhea, nausea, and headache and less common adverse effects (e.g. hypertension, lactic acidosis, and elevated liver enzymes) (Miller *et al.,* 2020). The ability of *S. aureus* to form a biofilm also increases its pathogenicity as well as resistance to many antibiotics (Luther *et al.,* 2018). The spread of this kind of bacteria as a species resistant to many antibiotics and causing wide infections is escalating, with minimal treatment options (Goudarzi *et al.,* 2020).

Based on the above facts, this study seeks to shed light on the using of MBT compared with the phenotypic methods for identification and discrimination of both MRSA and MSSA recovered from various dairy farms with a history of mastitis. A further objective was to examine the vulnerability of *S. aureus* strains to antibiotics commonly used to treat S. *aureus* mastitis.

MATERIALS AND METHODS

Sample Collection

In the current study, 400 mastitic milk samples from different animal species (100 from cows, 100 from sheep, 100 from goats, and 100 from dromedary camels) of selected farms with a high frequency of *S. aureus* in the Al-Qassim region of KSA. Strict hygienic measures were adopted during the collection of milk samples to avoid contamination via the microorganisms existing in the udder and teats and to avoid the accumulation of microorganisms on the hands of the sample collectors and in the environment.

Before the sample was taken, the ends of the teats were disinfected and washed using ethanol (70%). The initial milking was disposed of to considerably lower the infection of the teat canal (Quinn *et al.,* 1999). Also, a sterilized universal bottle and well-fitted cups were used. The universal bottle was marked with a long-lasting marker before the process of sampling. To minimize the possibility of contaminating the teat ends while taking the sample, the close teats were firstly sampled and then the far ones (Quinn *et al.,* 1999). Approximately 10 ml of sterile milk was gathered from each quarter utilizing a screw-lidded sterile analysis coded tube. All samples were transported as soon as possible to the Microbiology Laboratory. If direct inoculation wasn't suitable, samples were reserved at 4°C until cultivated for isolation within 24 h. Each sample was streaked on blood agar, Mannitol salt agar, Baird-Parker agar (BPA), and MacConkey agar media at 37°C for 24 h.

Isolation of *S. aureus*

Firstly, all milk samples were streaked on blood agar and then incubated at 37°C for 48 h. Based on the roles and regulations of National Mastitis Council (2011), isolation of all samples were carried out. The initial detection of *S. aureus* strains was carried out by the morphological features of the colonies, degree of hemolysis, as well as Gram staining technique. Colonies that appeared as grayish-white, creamy, and/or golden-yellow coloration were recognized as *S. aureus*. In addition, colonies that showed positive results to catalase and coagulase tests were initially identified as *S. aureus*. Afterward, a specific medium was used for examination of culture features.

Phenotypic identification of *S. aureus*

Catalase test

This test was predominantly utilized for differentiation between *Staphylococci* and *Streptococci*. In brief, 1-2 ml of H_2O_2 solution was dispensed into a sterile tube. A sterilized wooden stick was utilized to take numerous colonies of the 18 to 24 h test bacterium and then immersed in the H_2O_2 solution. Positive results were indicated by appearance of bubbles immediately after the addition of H_2O_2.

MASTASAPH Latex test

This test is a commonly putative technique for distinguishing *S. aureus* from other Micrococcaceae. *S. aureus* is recognized to release coagulase, which be able to clot plasma in the gel. This experiment is valuable in distinguishing *S. aureus* from other CNS. An agglutination test (MASTSTAPH) was carried out according to the method described by Macfaddin (2000). Briefly, the MASTASAPH Latex reagent was used to equilibrate room temperature and shaken well. Then, 1 drop of MASTASAPH Latex reagent was added to a circle on the MASTASAPH test card. From overnight culture plate, 2 to 4 colonies were emulsified and spread thoroughly over the circle of the latex cards and the results were then interpreted within 1 minute after gentle shaking the cards.

STAPH ID 32 (API system)

API Staph is considered a valuable test for rapid identification of *Staphylococcal* spp. The API Staph ID 32 test strip is composed of 32 capsules, of which (26) capsules encompass dehydrated biochemical agents for colorimetric analyses. The test was implemented and interpreted as stated by the company's instructions (bioMérieux, Lyon, France). In brief, the stored *S. aureus* strains were re-cultured on blood agar. The bacterial suspensions were prepared after one successive day of incubation for all isolates, to a density of 0.5 McFarland in 6 ml of purified water and dispersed into the wells of the strips. Afterword, the prepared strips were incubated at 37°C and inspected by naked eye after 24 h. The isolates of *S. aureus* were offered and recognized through Automatic Testing Bacteriology (ATB) Expression and the interpretation of these files was carried out through API laboratory (LAB) software. LAB allows for the likelihood of result detection in a range of 10% to 100% (Sampimon *et al.,* 2009).

Vitek 2 system

In the present research, the Vitek 2 system (ID-Gram Positive Cocci cards, ID-GPC), bioMérieux) was also used for the verification of *Staphylococcal* species following the instructions of the manufacturing corporation. The ID-GPC card is composed of plastic that encompasses sixty-four wells and is divided into 18 flatulent and 46 well for fluorimetric testing and inhibitory tests that encompass pH change tests and derivatives to find -oxidases and aminopeptidases. Normally, -oxidases detection substrates are paired with 4-methylumbelliferone (4MU); substrates for the finding of -oxidases are paired with (4MU). The twenty-one test substrates are: tyrosine-7AMC, pyroglutamic acid-7AMC, proline-7AMC, phenylalanine-7AMC, lysine-7AMC, threonine-7AMC, α-glutamic acid-7AMC, histidine-7AMC, aurease (butiloxicarbonyl-Val-Pro-Arg-AMC), arginine-7AMC, alanine-7AMC, 4MU-phosphate, 4MU-n-acetyl-β-d-glucosaminide, 4MU-β-d-mannoside, 4MU-β-d-glucuronide, 4MU-β-d-glucoside, 4MU-β-d-galactoside, 4MU-α-d-N-acetylneuraminic acid, 4MU-α-d-glucoside, 4MU-α-d-galactoside, and 4MU-α-l-arabinofuranoside. Moreover, the card encompasses sixteen fermentation tests, which are Salicin, N-acetyl-glucosamine, l-arabinose, lactose, glycerol, d-xylose, d-trehalose, d-sorbitol, d-raffinose, d-melibiose, d-mannitol, d-maltose, d-glucose, d-galactose, arbutine, and amygdaline), two decarboxylase tests (ornithine and arginine), and other six other tests (6.5% NaCl, polymyxin B sulfate, novobiocin, optochin, pyruvate, and urease). In brief, 2-3 fresh pure colonies were transferred by a sterile swab into a sterilized plastic test tube containing 3.0 mL of sterile saline. The tube was then vortexed and the turbidity was adjusted (GP: 0.50-0.63) using DensiChek™. The results were interpreted according to the identiûcation levels of the Vitek 2 system (Table 1).

Cefoxitin disc diffusion technique for identification of MRSA

According to CLSI (2009) standards, cefoxitin disc diffusion method was achieved for differentiation between MRSA and MSSA. Briefly, the bacterial suspension was adjusted **(**0.5 McFarland) and then scattered on the Mueller-Hinton agar plates. Cefoxitin discs (30 µg) were inserted in the plates and then all plates were incubated for 18-24 h at 35°C. the results were interpreted by measuring the inhibition zone (susceptible, ≥22 mm; resistant, ≤21 mm). This test was performed twice to obtain accurate results.

Proteomic identification of *S. aureus* isolates

Ethanol/Formic Acid Extraction protocol

To precisely detect all *S. aureus* strains, the PMFT (MALDI Biotyper, Bruker Daltonics, Germany) was used. All samples were analyzed using FlexControl software with Compass Flex Series version 1.3 software. It was determined that the isolates with score of ≥ 2.000 are set as species-level identification, whereas the isolates with the score of 1.70 to 1.99 are set as genus-level identification. Full-extraction controls (Ethanol/Formic Acid Extraction protocol) were accomplished as stated by Barreiro *et al*. (2010). In brief, all samples were prepared by culturing on blood agar, then incubated for 18-24 hours at 37°C.

For each sample, 1-2 fresh colony were transmitted to a clean Eppendorf tube and thoroughly mixed in 300 µl of distilled water. Nine hundred µl of pure ethanol was then added. The contents were prudently mixed, and then the centrifugation was carried out for all tubes for 2 minutes at 13,000 rpm. The pellet of each tube was left to dry in air after removal of the supernatant. The pellet was then blended with 50 µl of formic acid (70%) before the addition of an equal quantity of acetonitrile. Centrifugation of the mixture was then carried out for a couple of minutes at 13,000 rpm and then 1 µl of the supernatant was inoculated onto a target plate and permitted to dry for 5 minutes at 25°C. Subsequently, each isolate was covered with one µl of matrix solution (α-cyano-4 hydroxy-cinnamic acid, HCCA). Finally, the target plate was inserted in the MBT device for programmed running and data analysis. *Escherichia coli* was used as Bacterial test standard (BTS) throughout the experiment.

Data analysis, Discrimination, and Clustering

Within the range from 0 to 3, the score value of the unidentified spectrum was pinpointed through the concord between the anonymous spectrum and the well-known spectra kept in the reference library. The species and genus level are foreseeable in the range from 2.00 to 2.29 and 1.700 to 1.999, respectively. Moreover, a score of 0.00 to 1.69 indicates that the evidence of identification is unreliable.

Table 1. Identification levels of the Vitek 2 system

ID Massage Confidence Level	Choices	% of identification probability
Excellent	1	96 to 99
Very Good	1	93 to 95
Good	1	89 to 92
Acceptable	1	85 to 88
Low Discrimination	2 to 3	Sum of choices = 100; after resolution to one choice, percent probability reflects the number associated with the selected choice
Unidentified Organism	> 3 or 0	N/A

The different spectra made by the MBT software were detected in an m/z range from 2,000 Da to 20,000 Da. PCA and SPI were generated by the software to differentiate between MRSA and MSSA isolates. As per the MBT library, which contains more than 7000 bacterial and fungal sub-species, a dendrogram was also brought about from the (MSP) data set. The MSP dendrogram was brought about as per the assessment of the key spectra of the identified species. Primarily, the main spectra of the MBT taxonomy were matched with the resultant spectra in a cross-wise matrix.

Susceptibility of *S. aureus* strains to various antimicrobial agents

The Kirby-Bauer method was accomplished to detect the degree of sensitivity and resistance of *S. aureus* strains against various antimicrobial agents. This examination was carried out and the results were registered in keeping with the approvals and measures of the National Committee of CLSI (2016). As shown in Table 2, the following disks (Company, Mast Diagnostika, Germany) were applied in our current study: clindamycin (2 µg), erythromycin (10 µg), cefoxitin (30 µg), kanamycin (30 µg), gentamicin (120 µg), neomycin (30 µg), carbenicillin (100 µg), ciprofloxacin (5 µg), and chloramphenicol (10 µg).

In brief, all isolates were incubated at 37°C for 24 h to obtain fresh colonies. Then, 3-4 of these colonies were inoculated via a disposable inoculator loop onto a sterile tube containing 5 ml of sodium chloride (0.9%) and mixed carefully. The turbidity of the growing culture was equilibrated with NaCl (0.9%) using Sensititre (TREK Diagnostic Systems, Ashford, Kent, England) to obtain turbidity optically comparable to that of the 0.5 McFarland standards (ca 1×10^8 CFU/mL). Within 15 minutes after adjustment of the turbidity of the suspension, a clean cotton swab was dipped into the suspension and then firmly pressed against the inner side of the test tube, and then the swab was rotated to discard the excess liquid. After squeezing, the swab was speckled onto the whole surface of a Mueller-Hinton agar plate (Cat. No.: MDM 355) using a sterile swab 3 times. The Mueller-Hinton plate was rotated about 60 degrees after each use to confirm good spreading of the inoculum on the plate's surface.

Finally, the antimicrobial disks were directly pressed down onto the plates using sterilized forceps. All disks were incubated at 37°C for a couple of days. The zone's diameter was measured using a cylinder after incubation, and the results were interpreted in millimeters. Generally, no more than 6 disks were placed on each plate to prevent the inhibition zone from overlapping and causing a potential mistake in the measurement.

Table 2. Zone diameter interpretative references (NCCLS, 2013) for different antimicrobial drugs against *S. aureus* strains

Antimicrobial drug	Conc. (µg)	Zone diameter of *S. aureus* (mm)	
		R	S
Clindamycin	2	≤ 14	≥ 21
Erythromycin	10	≤ 13	≥ 23
Cefoxitin	30	≤ 21	≥ 22
Kanamycin	30	≤ 13	≥ 18
Gentamicin	120	≤ 19	≥ 27
Neomycin	30	≤ 16	≥ 26
Carbenicillin	100	≤ 23	≥ 29
Ciprofloxacin	5	≤ 15	≥ 21
Chloramphenicol	10	≤ 12	≥ 18

RESULTS

Preliminary identification of *S. aureus isolates*

S. aureus isolates were initially detected by colony morphology, degree of hemolysis, and the Gram staining technique. Colonies with a creamy or yellow color that gave positive results in the catalase test, and that also showed both complete and incomplete degrees of hemolysis, were recognized as *Staphylococcus*. Out of 400 milk samples involved in the current investigation, a total of 120 (30%) isolates of *Staphylococcus* were isolated. As can be seen in Table 3, 12 (10%) of these isolates were recovered from camels, 48 (40%) from cows, 28 (23.3%) from sheep, and 32 (26.6%) from goats. The positive results of the MASTASAPH Latex test were interpreted in 54 (45%) of 120 bacterial isolates, recognized as *S. aureus*, whereas the other 66 strains were recognized as CNS (16 *S. chromogens* strains, 11 *S. haemolyticus* strains, 24 *S. epidermidis* strains, and 15 *S. saprophyticus* strains). For further analysis, the preliminary identified isolates were deposited in Cryobank vials at -20°C.

Biochemical analysis of *S. aureus* strains

The Vitek 2 Compact system and API system (Staph ID 32 API system) were used as phenotypic techniques for the detection of *S. aureus*. The positive results for the Vitek 2 Compact system were interpreted in 51 (94.44%) of 54 *S. aureus* isolates, whereas the other 3 strains were recognized as CNS. Based on the results of the Staph ID 32 API system, 48 (88.89%) strains were properly recognized as *S. aureus*, whereas the other 6 strains were recognized as CNS.

Based on our findings, 54 isolates of *S. aureus* were assessed by various methods for phenotypic identification. All *S. aureus* isolates were correctly recognized by the MASTASAPH Latex test, 94.47% of *S. aureus* strains were properly recognized by the Vitek 2 Compact system, and 88.89% of *S. aureus* isolates were properly recognized by the Staph ID 32 system. From these results, it appears that the MASTASAPH Latex test is considered a good tool for phenotypic identification of various strains of *S. aureus*.

Standard detection of MRSA strains

A modified Kirby-Bauer method (agar disk diffusion) was carried out to test the isolates of *S. aureus* for their resistance

Table 3. Overall number of samples, number of positive *Staphylococcus,* and percentage (%) of positive samples

Animal species	No. of samples	No. of positive *Staphylococci*	% of positive *Staphylococci*	No. of positive *S. aureus*	% of positive *S. aureus*
Camels	100	12	12%	7	58.33%
Cows	100	48	48%	22	45.83%
Sheep	100	28	28%	11	39.28%
Goats	100	32	32%	14	43.75%
Total	**400**	**120**	**30%**	**54**	**45%**

to methicillin based on the guidelines recommended by NCCLS. A cefoxitin disk (30µg) was used as well as a Muller-Hinton agar comprising 4% of NaCl were used in the current investigation. The inhibition zone was measured after incubation at 37°C for 24 h. To calculate the occurrence of the MRSA stains recovered from all clinical samples, the total number of MRSA strains was divided by the total number of isolated *S. aureus* strains. Out of 54 *S. aureus* isolates, 17 (37.7%) strains presented a ≤21 mm inhibition zone for cefoxitin. Depending on the procedures of the Clinical Laboratory Standard Institute (CLSI), they were classified as Methicillin-Resistant *Staphylococcus Aureus* (MRSA).

Peptide Mass fingerprinting identification

In the current investigation, the isolated bacteria were examined by MBT and the resultant spectra were paralleled with the stored spectra in the MBT database. A typical exploration of several *S. aureus* strains recovered from mastitic samples was illustrated by the Compass software of Microflex LT. Based on our results, approximately 20 protuberant ion peaks were observed in the original bands from the zone ranging from 3,000 to 10,000 Daltons (Da) (Figure 1), and strong signals were demonstrated between 6500 and 7000 Da (Figure 2), which coordinated with 3 reference isolates of MRSA stored in the Compass software library (*S. aureus* ATCC 33591 THL, *S. aureus* DSM 3463, and *S. aureus* DSM 20232) and 5 reference strains of MSSA (*S. aureus* ATCC 29213, *S. aureus* ATCC 33862, *S. aureus* DSM 20231, *S. aureus* DSM 346, and *S. aureus* DSM 799).

In the current analysis, 40 MSSA and 14 MRSA isolates were properly recognized, with a log score fluctuating from 2300 to 3000 for 6 strains of MSSA and 2 strains of MRSA; 34 MSSA strains and 12 MRSA strains were appropriately documented with a log value fluctuating from 2.00 to 2.29. However, zero MSSA and MRSA strains were detected in score values from 1.7 to 1.99. Both MSSA and MRSA strains were detected by corresponding their spectra with the MBT database, which contains more than 300 strains of 16 genera from the ATCC and German Collection of Microorganisms and Cell Cultures GmbH (DSMZ).

According to the single peak analysis of various mass areas, frequent dissimilarities were recognized to distinguish between MSSA and MRSA strains. We observed many single peak signals within the range of 2636 to 4938 Da, which revealed numerous variations of intensities between the identified strains of MSSA and MRSA. Thus, the single peak represents a significant tool to distinguish between MRSA and MSSA. The specific results were enlarged into the area of approximately 4590 Da, 4863 Da, and 4938 Da, consistently. In such cases, the single peaks of attention exhibited several differentiations in intensity among MRSA and MSSA. A strong intensity of peaks was detected in MSSA (green color) in the mass of 4590 Da, 4863 Da, and 4938 Da (Figure 3) and was absent in MRSA (red color). In contrast, greater peaks of intensity were identified in MRSA (red color) in the mass of 2636 Da and 3009 Da (Figure 4) and were absent in MSSA (green color).

In addition, the PCA represents a supplementary calculated tool extracted from the Compass software of the MBT for analyzing data sets to illustrate the degree of resemblance and variety of various spectra of the protein profile. Likewise, PCA reduces the variances of a complex dataset, as stated by the different algebraic assessments. Several spectral proteins for MSSA and MRSA isolates were established in three-dimensional (3d) PCA in Figure 5. Every spectrum was represented via dot and the various colors demonstrate the reflected group contribution in which every dot was represented by one spectrum of the protein side view.

The cluster outlook of the 3d PCA illustrated that the majority of peaks for all strains of *S. aureus* were strictly correlated and harmonized (Figure 6). Regarding PCA calculation sets, every single peak may perhaps develop loading values derived from the PC calculation. In our investigation, every signal was identified with loading 1, loading 2, and loading 3 values resulting from the calculation of PC1, PC2, and PC3. In brief, the influences of the three PCs (PC1, PC2, and PC3) in generating the side view in a proportion plot of the explained variance were approximately 27%, 18%, and 10% for *S. aureus* (Figure 7).

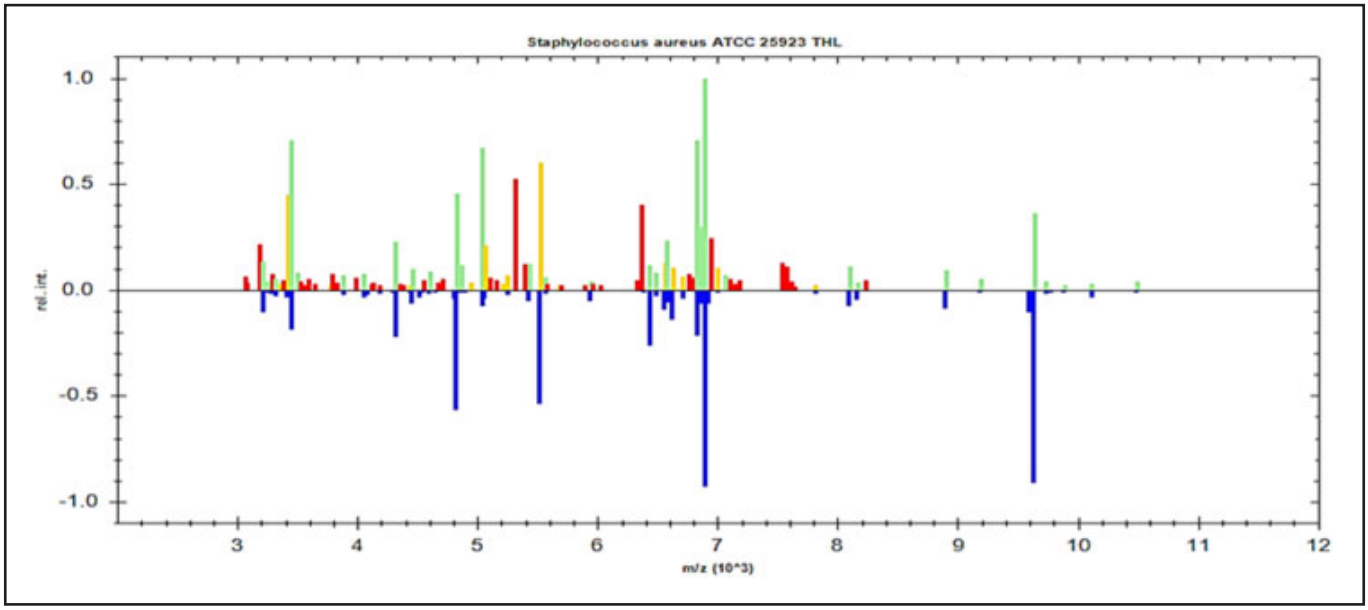

Figure 1. Evaluation of spectral protein profiles of *S. aureus* recovered from mastitic samples with *S. aureus* ATCC 25923 as a reference strain stored in the Compass Satellite software. The blue color in the lower part means the stored spectra in the library of the Compass software that was used for matching the pattern; the green color in the upper part of the spectra means excellently matched peaks, whereas the red and yellow colors mean mismatched and intermediate peaks, respectively.

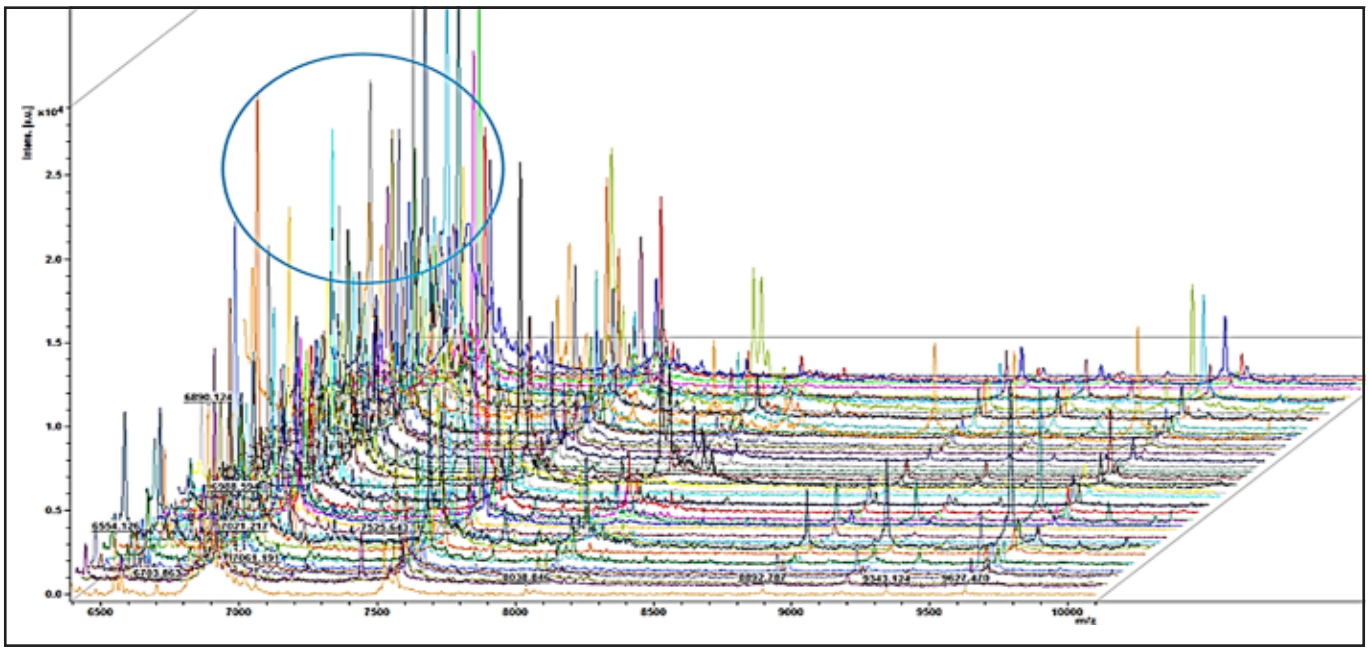

Figure 2. Higher peak intensities of 54 *S. aureus* strains recovered from milk samples were concentrated between 6,500 and 7,000 Da.

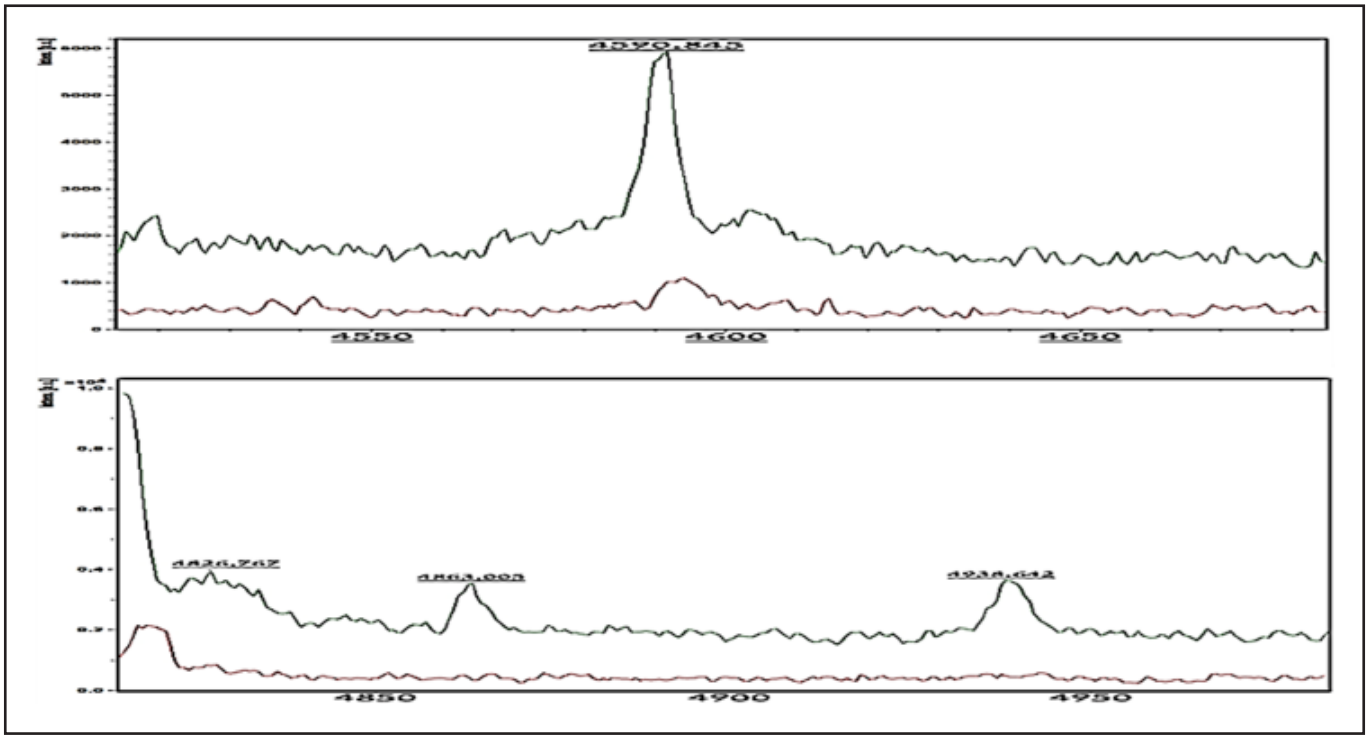

Figure 3. Single peak intensities (4590 Da, 4826 Da, 4863 Da, 4938 Da) were detected in MSSA (green color), while they were absent in MRSA (red color).

A real gel image was created by the Compass software programmed in the MBT for illustration of the protein profile for 54 identified isolates of MRSA. As can be seen in Figure 8, the spectra were distributed in the range from 3,000 Da to 10,000 Da with strong peaks demonstrated between 3,000 and 10,000 Da. To clarify whether the Compass software program can differentiate the clonally associated strains, bands from 54 strains of both MRSA and MSSA were investigated as described above. Figure 9 shows the MSP dendrogram for 54 isolates of *S. aureus.* Based on our results, the created dendrogram revealed that the evaluated *S. aureus* strains were strictly associated with 8 reference strains of *S. aureus*. As seen from Table 4, 11 strains were matched with *S. aureus* spp. aureus DSM 20231T DSM, 12 strains were matched with *S. aureus* spp. aureus DSM 346 DSM, 13 strains were matched with *S. aureus* ATCC 29213 THL, 3 strains were matched with *S. aureus* spp. *aureus* DSM 799 DSM, 1 strain was matched with *S. aureus* ATCC 33591 THL, 10 strains were matched with *S. aureus* spp. aureus DSM 3463 DSM, 1 strain was matched with *S. aureus* ATCC 33862 THL, and 3 strains

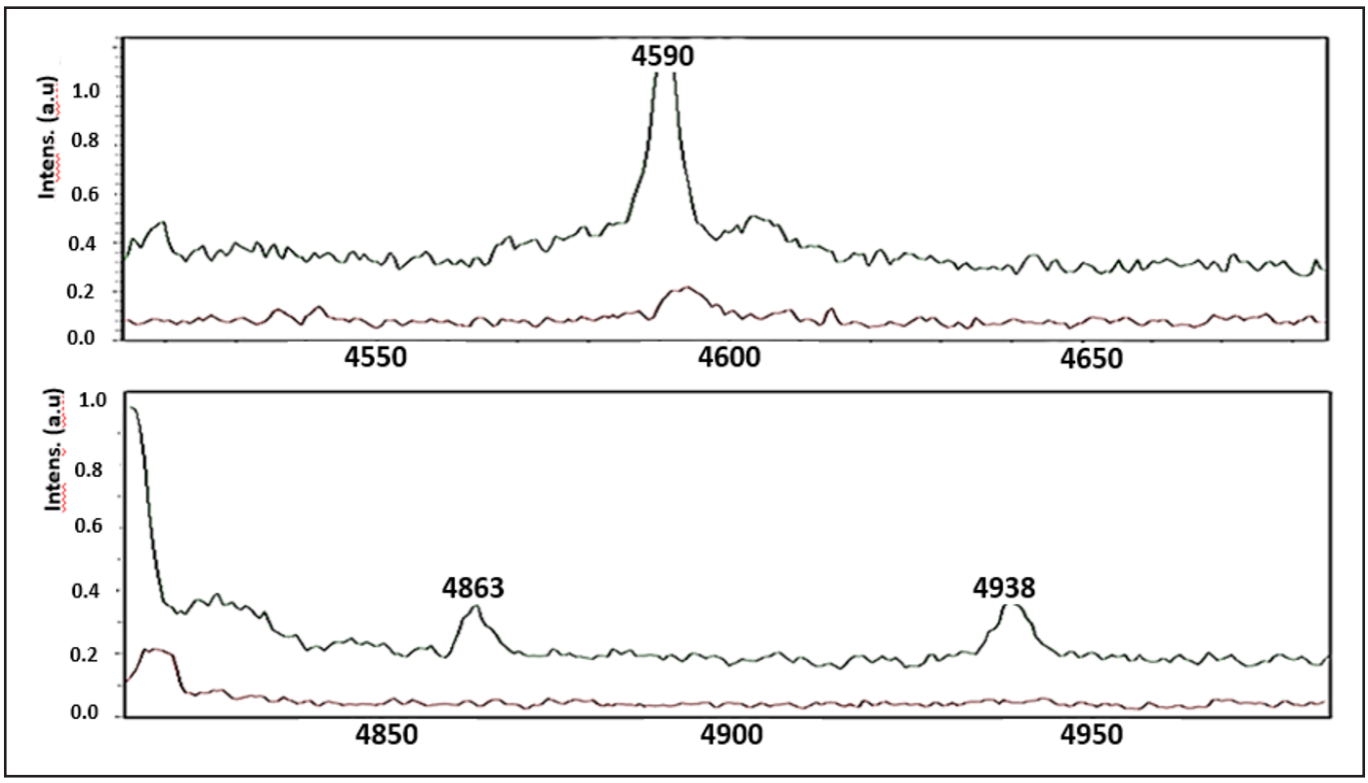

Figure 4. Single peak intensities (2636 Da, 3009 Da) were discovered in MRSA (red color), while they were absent in MSSA (green color).

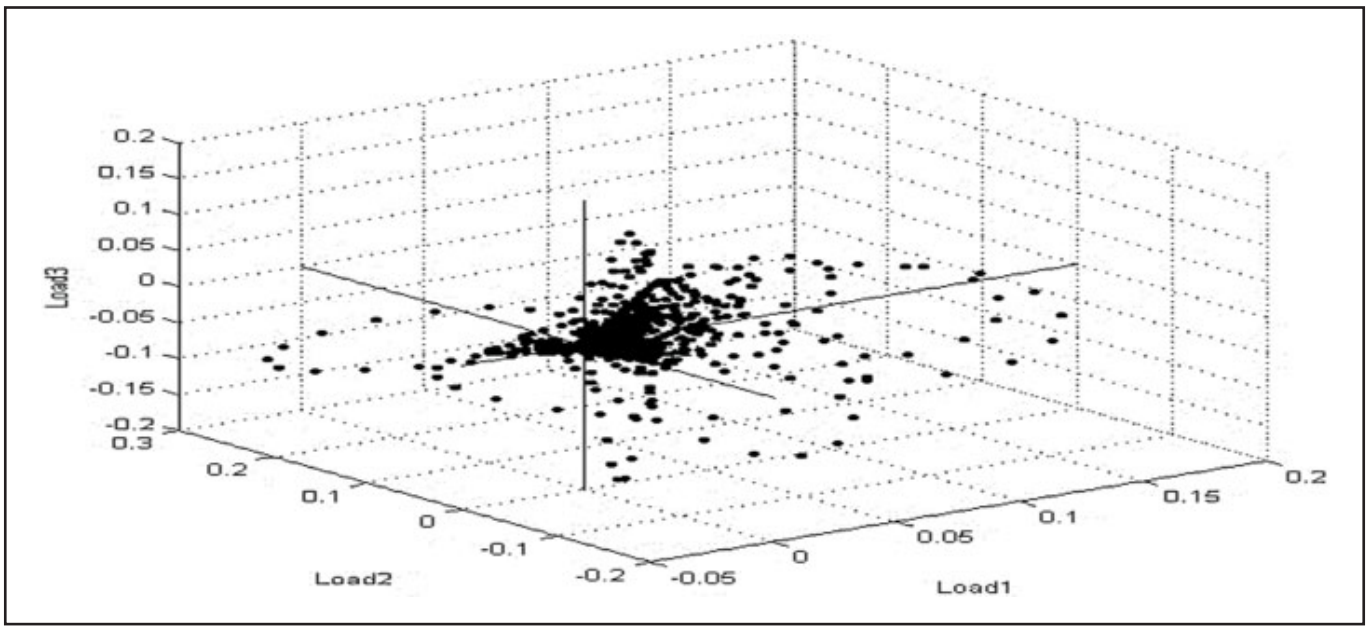

Figure 5. PCA dimensional image illustrating several spectra for *S. aureus* isolate recovered from mastitic samples. Each spot refers to the strength of single signals. The signa were altered up to the loading value consistent with loading 1, loading 2, and loading 3.

were matched with *S. aureus* spp. *aureus* DSM 20232 DSM. The results of the MSP dendrogram presented a strong relation among the isolates of *S. aureus* with certain *S. aureus* reference strains, particularly ATCC *S. aureus* 33591, ATCC *S. aureus* 29213, ATCC *S. aureus* 33862, and DSM *S. aureus* 799.

According to the interpreted results in Table 5, comparing the application PMFT with other conventional methods, it is clear that the PMFT was able to identify *S. aureus* and other CNS by 100%. In contrast, the other conventional methods were able to identify *S. aureus* and CNS at varying rates, ranging from 94% to 73%. Also, the use of MASTASAPH Latex was able to distinguish between *S. aureus* and other CNS by 100%.

Susceptibility of *S. aureus* strains to various antimicrobial agents

Our results were disclosed in keeping with the procedures of the CLSI. An antimicrobial susceptibility test for 54 *S. aureus*

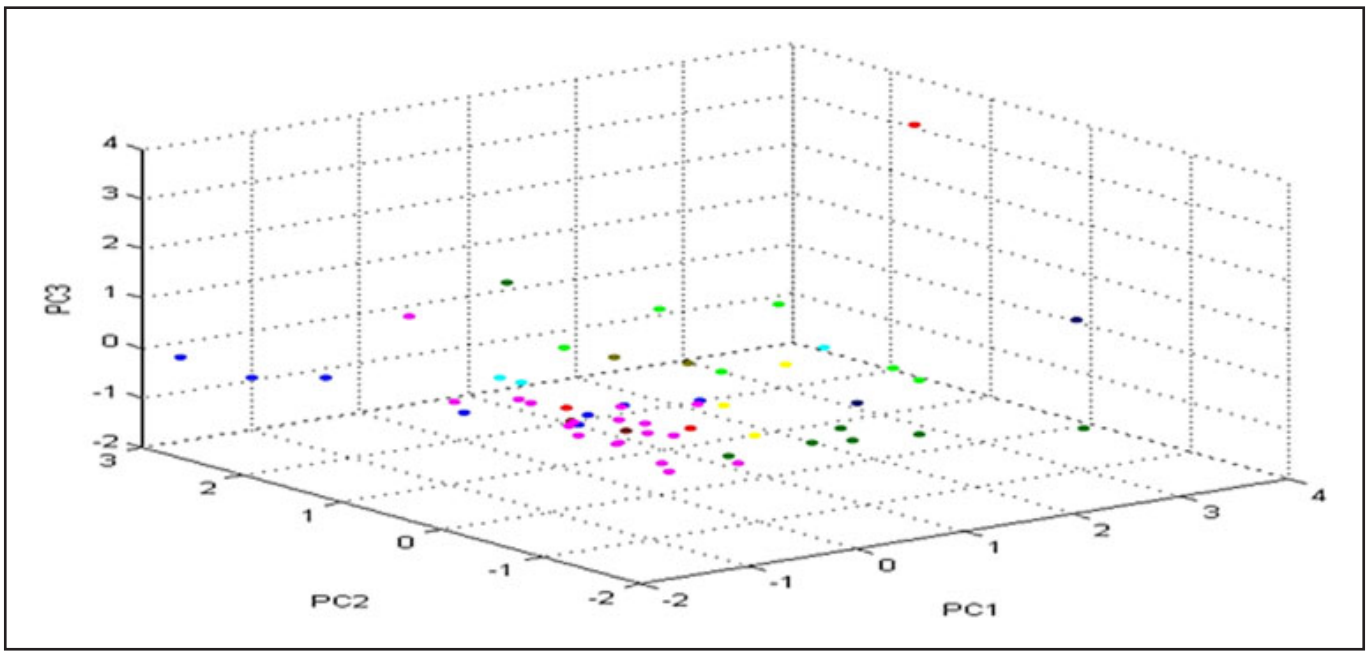

Figure 6. The cluster view of the 3d PCA for nearly all strains of *S. aureus* (except tw strains) was classified as one group (strictly correlated and harmonized).

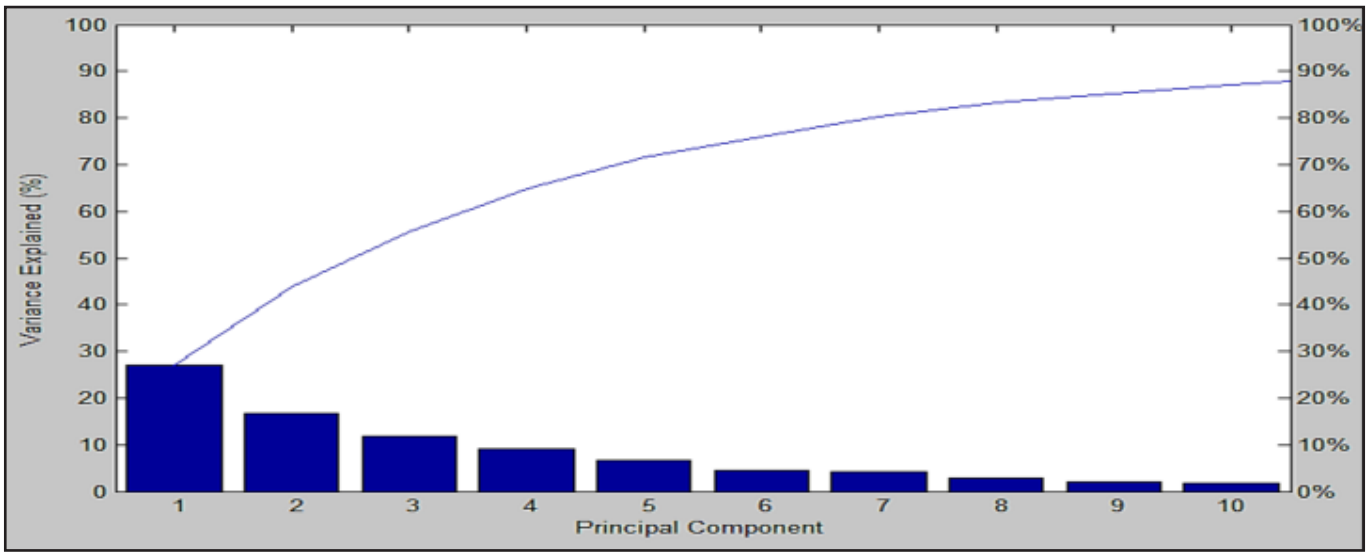

Figure 7. The effect of PCs on the grouping description in the conspiracy of percentage illuminated differences of PC for all strains of *S. aureus* recovered from mastitic samples.

Table 4. The rate of identified *S. aureus* strains recovered from mastitic milk samples that matched with 8 reference strains of *S. aureus* stored in the MBT library

No. of identified strains	Matched reference strains in Bruker library
12	*S. aureus* spp. aureus DSM 346 DSM
3	*S. aureus* spp. aureus DSM 799 DSM
10	*S. aureus* spp. aureus DSM 3463 DSM
1	*S. aureus* ATCC 33862 THL
3	*S. aureus* spp. aureus DSM 20232 DSM
11	*S. aureus* spp. aureus DSM 20231T DSM
1	*S. aureus* ATCC 33591 THL
13	*S. aureus* ATCC 29213 THL

strains exhibited that 94% (51/54), 39% (21/54), 33% (18/54), 31% (17/54) 31% (17/54), and 24% (12/54) were resistant to carbenicillin, erythromycin, Kanamycin, cefoxitin, ciprofloxacin and neomycin, respectively. In addition, 72% (39/54), 81% (44/54), and 94% (51/54) of the tested strains were positively affected by chloramphenicol, gentamicin and clindamycin, respectively. Details on the percentage of resistant and susceptibility of the *S. aureus* strains against various antibiotics are revealed in Table 6.

DISCUSSION

S. aureus remains one of the most important complex microbes causing mastitis in various animal farms, thereby creating constant headaches for breeders worldwide due to the

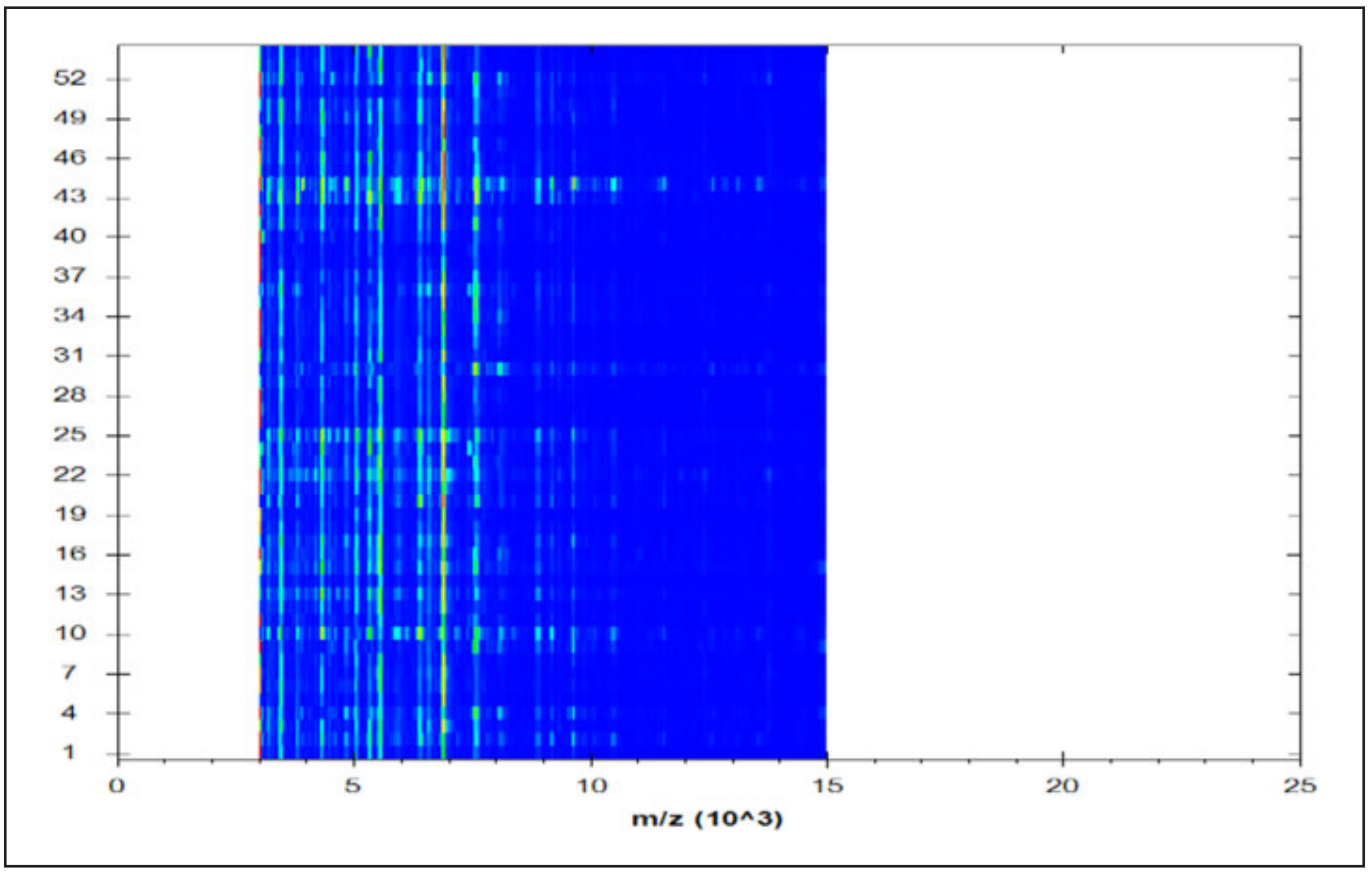

Figure 8. Spectra gel view of protein for 54 MRSA and MSSA strains. The yelllow dots are the gathering of various protein spectra with different insides.

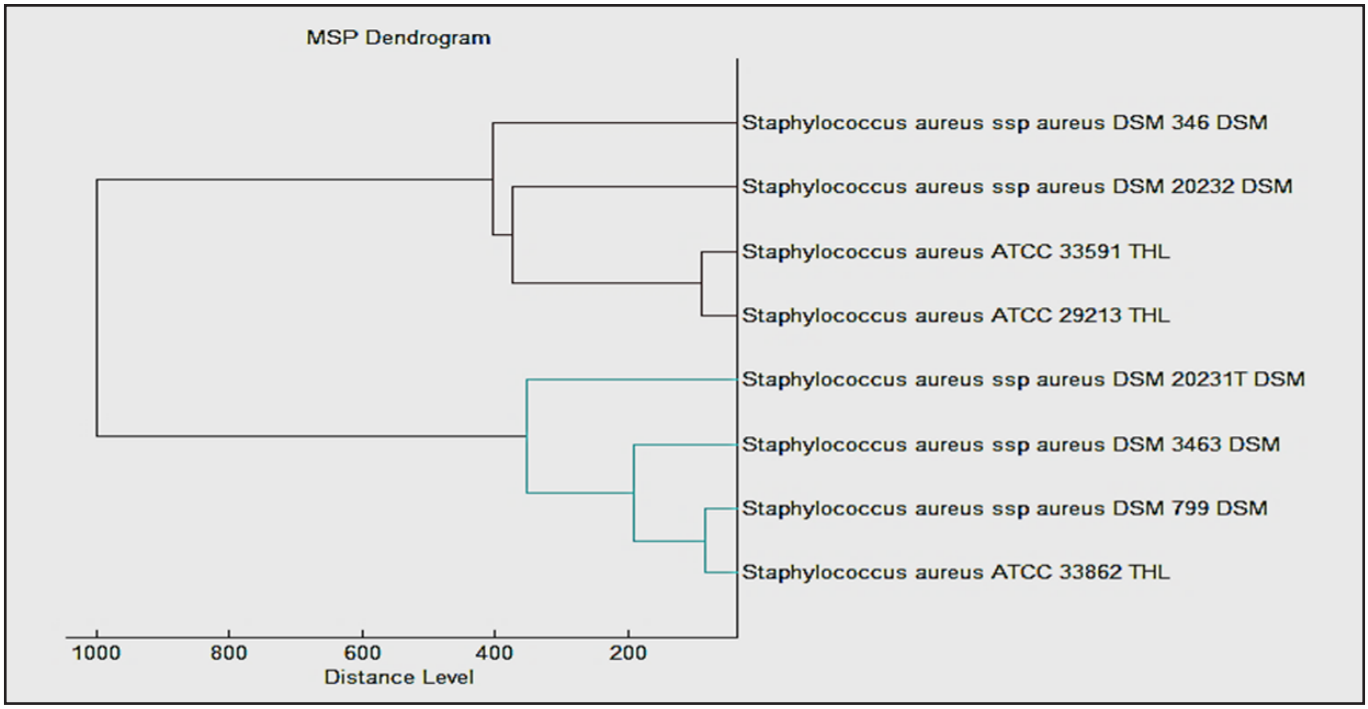

Figure 9. MSP dendrogram for 54 isolates of *S. aureus* matched with 8 reference strains of *S. aureus* stored in the Bruker library.

Table 5. Comparison between PMFT and phenotypic method for identification of *S. aureus* and other types of Staphylococcus species from different milk samples

Species	No. of isolates	Phenotypic identification			PMFT
		API ID system	Vitek 2 system	MASTASAPH Latex	
S. aureus	54	89%	94%	100%	100%
S. chromogens	16	75%	87.5%	0%	100%
S. haemolyticus	11	73%	91%	0%	100%
S. epidermidis	24	79%	87.5	0%	100%
S. saprophyticus	15	73%	93%	0%	100%

Table 6. Degree of resistance and susceptibility of identified *S. aureus* strains against various antibiotics

Drug class	*S. aureus*			
	Resistant		Sensitive	
	No.	%	No.	%
Chloramphenicol	5	9.25%	39	72.22%
Gentamicin	6	11.11%	44	81.48%
Carbenicillin	51	94.4%	1	1.85%
Erythromycin	21	38.88%	32	59.25%
Clindamycin	2	3.7%	51	94.44%
Kanamycin	18	33.33%	36	66.66%
Ciprofloxacin	17	31.4%	31	57.40%
Neomycin	13	24.07%	5	9.25%
Cefoxitin	17	31%	37	69%

resulting economic problems it causes, up to the eradication of infected animals from the herd. Because the milk produced from infected animals is contaminated with *S. aureus*, it may have a significant harmful effect on human health (Li *et al.,* 2017). It is already known that this bacterium is resistant to a very large number of antibiotics used on dairy farms, which represents a clear threat in the process of controlling this microbe (Barlow, 2009).

Because determining the type of microbe causing mastitis, as well as the methods used in early diagnosis, are important to the process of controlling disease in different animal farms, it is necessary to find a quick and effective way to identify the microbes that cause mastitis, so as to create a successful treatment process (El Behiry *et al.,* 2012; Deb *et al.,* 2013). For many years, genetic and other traditional methods have been considered among the most successful methods in the process of diagnosing the microorganisms causing mastitis, but these methods have many drawbacks, such as the long time spent on diagnosis as well as the high cost and the need for specialists trained in these techniques; additionally, false results may be detected (Bizzini *et al.,* 2010; Schröttner *et al.,* 2014).

From this perspective, the initial goal of the current investigation was to compare phenotypic methods (MASTAST Latex, STAPH ID 32, Vitek-2) with PMFT as techniques to identify the level of *S. aureus* species in various dairy animals suffering from intra-mammary infection. The current study showed that the number of *S. aureus* from 400 samples from various dairy animals infected with mastitis was 13.5% (54 isolates), through isolating the strains in the laboratory using fundamental protocols (e.g., color of colonies, colony morphology, Gram stain, catalase test, and MASTASAPH Latex test). Parallel findings have been obtained in previous studies in Ethiopia (Getahun *et al.,* 2007; Marama *et al.,* 2016). A similar study, conducted in China, showed that the spread of *S. aureus* in the raw milk of cows was 22% (Liu *et al.,* 2017). In general, our present data show that *S. aureus* often exists in the raw milk of mastitis-infected animals in the Central Region of the Kingdom of KSA.

The quick and precise identification of *S. aureus* strains in cultures taken from samples of milk is the mainstay of governing *S. aureus* mastitis. The coagulase test, as a standard tool, has high sensitivity and has been used to identify the existence of *S. aureus* in milk samples. However, only half of the isolates were tested positive after incubation for 4 h; incubation for a night is necessary to get accurate results (Dai *et al.,* 2019). Thus, the commercial tools utilized to determine the existence of *S. aureus* are widely used in clinical microbiology laboratories, as the analysis of tubular coagulation, which is commonly used as an ideal index for *S. aureus* exploration, requires up to 24 h to obtain concluding test results (Zschock *et al.,* 2004; Wang *et al.,* 2018).

Another study was carried out by Gülmez-Sağlam *et al*. (2017) on 235 milk cows infected with mastitis; a large number of both *S. aureus* and CNS were isolated. The percentage of *Staphylococcus* species was 49.7% (117/235). Out of 117 *Staphylococcus* isolates, 63.24% (74/117) and 36.75% (43/117) were identified as *S. aureus* CNS, respectively. In addition, a study conducted by Sevinti and Şahin (2009) on 79 samples of milk from cows with a history of clinical mastitis in Kars Province detected 23 *S. aureus* and 19 CNS isolates. In the same study, the tests were performed to isolate *S. aureus* from 100 cows suffering from clinical and subclinical mastitis. It was found that 48 (48%) *Staphylococcus* species were isolated as 22 (46%) CPS and 26 (54%) CNS. In this study, *S. aureus* is the bacterium that was isolated at the highest rate among the bacterial agents causing mastitis. This seems to be in harmony with the isolation results of other studies. Such variations in the levels of *S. aureus* prevalence are due to the varied populations investigated by the executed methodologies in addition to other elements (Li *et al.,* 2017; Wang *et al.,* 2018).

Because dromedary camels are considered to be among the most important sources of milk and meat for citizens in KSA, the current study also studies the prevalence of *S. aureus* among camels afflicted with mastitis. As stated by Jilo et al. (2017), the spread and causes of mastitis in camels have been reported as varied according to the geographical area and herd management. In the current investigation, the spread of *Staphylococcal* mastitis in camels reported in this study is shown as low, i.e., 12% (12/100 milk samples), particularly as compared to the previous studies of Al-Juboori *et al*. (2013) and Al-Dughaym *et al.* (2015), which demonstrated spreading rates from 36% to 41%. No clear-cut clarification of this relatively low spread of bacteria in camels is present in the current research. However, other factors, such as hygienic milking practices and a proper milking area, might contribute to this low rate.

Moreover, our study found that *S. aureus* is the prominent organism isolated from sheep and goats suffering from mastitis, at 39.28% (11/28 isolates) in sheep and 43.75% (14/32 isolates) in goats. Similar studies supported this finding, i.e., Alemu and Abraha (2017); Abdalhamed *et al.* (2018); and Haggag *et al.* (2019), who revealed that *S. aureus* is the primary microorganism found in dairy goats because it is detected in ranges from 6% to 40% in all extracted microorganisms.

While *Staphylococci* consist of forty-five species and twenty-one subspecies that are known to be the key causes of mastitis in dairy animals, there is still debate over the clinical relevance of *S. aureus* isolated from animals infected with mastitis. Some regard *S. aureus* as a main carrier of mastitis, while others view it as a minor carrier of the disease (Harjanti *et al.,* 2018). Thus, the present study examined seemingly healthy animals and others that presented symptoms of mastitis; 54 *S. aureus* isolates and 66 CNS isolates were correctly recognized by using tube coagulase test and later by the Vitek™ 2 compact system. The potential capability of the Vitek 2 system (ID-GPC card) to quickly and effectively identify Gram-positive cocci was initially evaluated by Basses *et al.* (1997), who revealed, via isolates of *Staphylococci*, *Streptococci*, and *Enterococci*, 98.0% total agreement (86.8% agreement to the species level without supplemental testing needed and 11.2% agreement to the species level after additional testing), while 1.7% of isolates were not correctly identified and 0.3% of isolates were not identified (Ligozzi *et al.,* 2002; Huh *et al.,* 2018).

In the present study, similar accuracy rates were shown by the Vitek 2 system in the uncovering of *Staphylococci*. The organisms that showed less discrimination identification comprised a limited count of strains and did not provide important information regarding the improvement by Vitek 2 system. As per the results of the current study, correct identification of 51/54 isolates of *S. aureus* (94%) was achieved. Typical results were accessed by past researchers, such as Spanu *et al*. (2003), who evaluated the ability of the Vitek 2 system in rapidly identifying 130 *S. aureus* and 275 CNS strains extracted from blood cultures and demonstrated that 90.5% of CNS and 99.2% of *S. aureus* strains were suitably marked out.

Other studies examined eleven *S. aureus* isolates via the Vitek 2 system and reported that the species were 100% identified (Da Silva Paim *et al.,* 2014; Gitau *et al.,* 2018). The Vitek 2 system contributed to the right identification of Gram-positive microorganisms; however, this method did not manage to detect some uncommon bacteria due to certain restrictions. Consequently, an extra method must be used to precisely detect the bacteria at the species level (Elbehiry *et al.,* 2016). The drawback of all the published research is the notion that the ID32 STAPH system is widely used as a comparative approach to evaluate the other systems of phenotypic identification, such as the Vitek 2 system (Layer *et al.,* 2006; Ambaraghassi *et al.,* 2019). Because the ID32 STAPH system is normally used as a reference approach, results attained with ID32 STAPH are added. In the study of El Behiry *et al*. (2014), 89% of the tested *S. aureus* strains were correctly identified via the ID32 STAPH system, which matched our study, in which the ID32 STAPH system was able to correctly identify (84%) (in all tests) the *Staphylococc*al species.

PMFT is one of powerful, fast, truthful, and correct technique of identifying microorganisms, especially in subclinical and clinical cases of mastitis (Carbonnelle *et al.,* 2007; El Behiry *et al.,* 2014; Elbehiry *et al.,* 2016). The application of PMFT in the diagnosis of mastitis has been demonstrated by Nonnemann *et al.* (2018); it was shown to be a cost-effective and quick method when applied to 500 isolates, and twenty-four genera and sixty-one specifies were identified. In this research, the *S. aureus* strain was identified by proteomic analysis and the accuracy of identification at the levels of genus and species was 54/54 (100%). Similar results were shown by Loonen *et al*. (2012), who found CNS isolates recovered from clinical cultures through 5 methods: *tuf* gene sequencing, 16S rRNA gene sequencing, Staph ID 32 API system, Vitek 2 system, and PMFT. Also, PMFT was found to be the method that delivered the highest rate of identification (99.3%).

In this research, the data were analyzed by MBT, which revealed that the range of the majority of spectral peaks of the examined *S. aureus* strains was between 3,000-10,000 Da. This finding is consistent with the findings of several similar studies that addressed microbial detection through MBT (Lartigue *et al.,* 2009; Barreiro *et al.,* 2010; Silva *et al.,* 2015). However, this range is comparable to those of previous studies, which revealed that the range of most of the peaks is from 800-3500 Da (Edwards-Jones *et al.,* 2000; Walker *et al.,* 2002). As concluded by Shell *et al*. (2017), on strains and isolates extracted from mastitis cases examined by MBT, the peaks ranged from 3,000 to 11,000 Da and the highest levels of intensity ranged between 4,000 and 10,000 Da. Such variations in the spectral peaks, range, and mass can be attributed to differences in the arrangement of sampling approaches. Although their variations in spectra were based on the source, there is a continuing order for use of the PMFT method to diagnose new subareas through which to identify microbes such as fish pathogens, environmental microbial isolates, and mastitis pathogens (Nonnemann *et al.,* 2018).

Also, this study aimed to identify the capability of PMFT to correctly differentiate between MRSA and MSSA. Many researchers investigated MBT typing profiles. Some researchers emphasized the connection between MBT profiles created by MSSA and MRSA. In a study by Du *et al*. (2002), 74% of 76 *S. aureus* strains and MRSA were exactly identified from MSSA by utilizing cluster analysis together with a dendrogram. Jackson *et al*. (2005) revealed that MRSA and MSSA in *S. aureus* showed varied intensities at m/z 3048, m/z 3086, and m/z 3124. Furthermore, Drake *et al.* (2011) suggested differentiation based on peaks noticed at m/z 2302 and m/z 3871. According to Tang *et al*. (2019), MSSA and MRSA can be differentiated by MBT with an accuracy of over 90%. In this study, the chosen peaks at the masses of 2,636 Da and 3,009 Da granted robust emphasis to distinguish between MSSA and MRSA. The time required to obtain results from MBT was clearly less than that required by molecular approaches and methods.

The remarkable differences between classical identification techniques and PMFT are represented in the anticipated time and cost required to identify the sample. The cost of using PMFT to identify bacteria amounts to about €1.43/sample, but sample identification with other classical methods costs €4.6-8.23/sample (Feucherolles *et al.,* 2019).

Moreover, Cherkaoui *et al*. (2010) demonstrated that the cost of the reagents required for phenotypic identification employing recent automated tools is approximately $10 per isolate, but does not exceed 0.50 per sample with PMFT. The cost and maintenance expenses of PMFT are among the potential pitfalls of this technology (Neville *et al.,* 2011; Chen *et al.,* 2013; Pavlovic *et al.,* 2013; Macori *et al.,* 2019).

In this study, the PMFT technique is compared to the colorimetric method. The results showed that the use of PMFT to identify the different strains of *Staphylococcus* is a superior technique because it entails a simple procedure, a large sampling volume, premium accuracy, and higher

sensitivity. Here, the *S. aureus* strains extracted from animals infected with mastitis were identified more quickly, economically, and precisely by MBT with compass software than by the Vitek 2 compact system and staph ID system. Also, MBT showed a better capability for discrimination between MRSA and MSSA in terms of peak intensities. Thus, the ability to solve the problems of mastitis, whether clinical or subclinical, was available when precise and early diagnostic tools were used.

The release of β-lactamase by *S. aureus* is the reason for the inability to treat subclinical mastitis using penicillin. This leads to hydrolysis of β-lactam rings. The majority of *S. aureus* strains release penicillinases, which then make *S. aureus* resistant to the β-lactam set of antibiotics (Algammal *et al.,* 2020). The prominent feature notice for *S. aureus* is the resistance to β-lactam or aminoglycosides (Virdis *et al.,* 2010). This is demonstrated in the current study, which elicited a resistance rate of 94% of the extracts toward carbenicillin of β-lactam group. This finding corresponds with the results of Zhang *et al*. (2012), who concluded a 90% resistance grade to penicillin. In contrast, no resistance to aminoglycosides was noticed in this research based on 81% susceptibility for gentamicin. About 60%-90% of the microorganisms recovered from clinical and subclinical mastitis showed resistance to β-lactam antibiotics (Barboza-Corona *et al.,* 2009; León-Galván *et al.,* 2015). Typical results were found by Zhang and Buckling (2012), who reported that 94.8% of the isolates resisted at least one antimicrobial agent, especially penicillin and ampicillin. Also, the resistance rate of erythromycin (85.6%) reported by Zhang *et al*. (2012) contradicts the results of the current study, which revealed a resistance rate of only 39%.

The resistance of *S. aureus* to various antimicrobials goes back to the attainment of moving genetic elements including plasmids and jumping genetic elements (insertion sequences and transposons). Such elements integrate the determinants of antimicrobial resistance and move among species or stains through horizontal gene transfer. The first resistance to penicillin caused by the release of β-lactamase, and that caused hydrolysis of the β-lactam ring was found in the 1940s (Varela-Ortiz *et al.,* 2018). According to Adzitey (2015) and Mansor *et al*. (2019), the resistance to antibiotics differs from one region to another and depends on many factors such as the time, the tested samples, the method of sampling, and the degree to which the antibiotics are used in different areas.

In conclusion, Successful management and planning to diagnose infectious mastitis in KSA requires an effective, robust, and accurate monitoring system for all dairy farms and flocks, together with rapid and exact identification of the microorganisms that cause this infection. The quick identification of microorganisms is achieved by PMFT, which takes about thirty minutes per isolate from the intended plate to obtain the ultimate results. Also, less effort is required to prepare the sample. In this research, PMFT is reported to be the most rapid and sensitive instrument by which to identify *S. aureus* isolates that cause infectious mastitis when it is benchmarked against other classical methods such as Vitek 2 and the ABI system. It was concluded that *S. aureus* bacteria are among the main microorganisms causing mastitis in KSA. PMFT is characterized by accuracy, quickness, and reliability. It could be used as a systematic tool to detect and distinguish *S. aureus* and mastitis-causing bacteria isolates in veterinary laboratories in KSA in order to accurately identify the clinical specimens. However, this assay requires more validation and verification utilizing more samples to determine the performance, reliability, specificity, and sensitivity of this kind of bacterial discrimination. *S. aureus* revealed a higher degree of resistance (94.4%) to carbenicillin of the β-lactam group. In contrast, 94.44% and 81.48% of *S. aureus* strains were sensitive to clindamycin and gentamycin, respectively.

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Conflict of interest

The authors declare that they have no competing interests.

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