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Prevalence and molecular characterization of resistant *Staphylococcus aureus* strains in bulk milk tanks of dairy cattle in Northern Egypt

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Abstract

Bovine subclinical mastitis represents a major cause of severe economic losses in dairy farms. This research aimed to detect the antimicrobial resistance trends of *Staphylococcus aureus* and to determine the presence of *mecA, mphC, lnuA, tetK* and *tetL* antimicrobial resistance genes in raw bulk milk in the period between December 2023 and February 2024. One hundred raw bulk cow milk samples were gathered from different dairy farms in Egypt. The prevalence of subclinical bovine mastitis was 65.00% using California mastitis test. The prevalence of isolated *S. aureus* was 46.15% via bacterial culturing and all isolates (n = 30) were confirmed via hemolytic activity, catalase and coagulase test, and gram staining followed by polymerase chain reaction targeting *nuc1* gene. Antimicrobial sensitivity test was applied on all confirmed *S. aureus* isolates utilizing the disk diffusion method on Mueller-Hinton agar. The highest resistance was verified for tetracycline at 100% followed by erythromycin and clindamycin at 56.66 and 16.66%, respectively. The highest sensitivity at 100% was verified for amikacin, ampicillin, amoxicillin plus clavulanic acid, ampicillin plus sulbactam, ciprofloxacin, colistin, gentamicin, imipenem, tobramycin, doxycycline and vancomycin. Multidrug resistance was found in 20.00% of the total isolates. Methicillin resistant *S. aureus* represented by *mecA* gene was identified in 83.33% of isolates. Macrolides resistant *S. aureus* represented by *mphC* gene was identified in 16.66% of isolates. Lincosamide resistant *S. aureus* represented by *inuA* gene was identified in 66.66% of isolates. Tetracycline resistant *S. aureus* represented by *tetK* and *tetL* genes was detected in 23.33 and 53.33% of isolates, respectively. This study provided antibiotic-resistant *S. aureus* profiles to dairy farms to avoid treatment failure, adverse effects on animal health and economic impact for the owner of the animal.

Introduction

Mastitis, an inflammatory response in cow udder tissue, is a main cause of financial losses for dairy farms globally due to microbial infections or physical trauma.[1] *Staphylococcus* species, particularly *Staphylococcus aureus*, are widely recognized as the primary etiological agents of bovine mastitis.[2] It is a facultative anaerobic, gram-positive, catalase-positive, coagulase-positive and oxidase-negative. The spherical form of *S. aureus* has a diameter of 0.50 - 1.50 µm.[3]

Staphylococcus aureus caused mastitis is challenging to control due to its toxins, which disrupt cell membranes and harm milk-producing tissue.[4] *Staphylococcus aureus* is capable of acquiring resistance and releasing a range of pathogenic factors such as endotoxins and other dangerous proteins. It is extremely resistant to β-lactams antibiotics and causes both chronic and recurrent infections. In recent years, the rate of resistance has been increased significantly.[3]

Infections caused by *S. aureus*, especially those that are methicillin-resistant,[5] are contagious and difficult to treat leading to global bovine mastitis and increasing antibiotic use often resulting in multidrug resistance.[6] The World Health Organization[2] maintains that bacterial changes can lead to antimicrobial resistance (AMR) by decreasing the efficacy of medications and extending the duration of illnesses in the body which increases the chance of spreading to others so that AMR is characterized worldwide as a severe risk to public healthcare.[3]

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Multidrug resistance among *S. aureus* in Holstein mastitic cows is quite prevalent in Egypt resulting in substantial financial losses for the dairy industry.[5] high prevalence of *S. aureus* resistance to antibiotics, particularly β-lactams, due to uncontrolled use of antibiotics, poses a significant clinical and public health hazard in Egypt.[5] Resistance to drugs like tetracycline (TE), macrolides and methicillin that are expressed by the genes (*tetK* and *tetA*), (*ermB* and *ermC*) and (*mecA*, *mec1* and *mecC*), respectively, make them ineffective against *S. aureus* strains located in Egypt.[7]

Culturing of bulk tank milk is an inexpensive and effective technique for identifying the microbial cause of mastitis in a herd.[4] Bacterial identification is typically achieved through morphological, biochemical features and molecular biology techniques like polymerase chain reaction (PCR) and real time PCR.[8]

The present investigation utilized bacterial isolation and molecular identification to screen specific antibiotic resistance genes (*mec1, mphC, inuA, tetk* and *tetL*) from *Staphylococcus* isolates recovered from cow milk in northern Egypt.

Materials and Methods

Study period and location. This study was conducted in the period between December 2023 and February 2024 at the Veterinary Research Institute, National Research Center, Dokki, Cairo, Egypt. Ethical approval The Medical Research Ethics Committee approved this study (No. 13010121) at the National Research Center, Egypt.

Samples. One hundred bulk milk samples were aseptically collected from five dairy farms involved different breeds in Cairo, Giza and Kafr El-Sheikh. Kalyobia and Fayoum governorates, 20 samples from each farm and then analyzed for subclinical mastitis utilizing California mastitis test (CMT), in addition to bacteriological and molecular examination for isolation and identification of *S. aureus*. The history of the antibiotic usage in the all five farms was taken. The primary issue with these farms is their low milk yield which persists even after regular antibiotic treatment. Tanks collected from animals undergoing antibiotic treatments were excluded from sampling.

California mastitis test. The milk samples were checked for any detectable abnormalities and were exposed to screening using CMT (Weizur India Pvt Ltd., Anand, India). Two mL of 3.00% CMT component was added together with equal volume of milk sample from each tank and mixed thoroughly in a goblet made of white plastic paddle. Visual interpretations of reactions were made within a 20-sec timeframe. Negative reactions were assigned a value of 0, positive reactions were observed, with values ranging from +1 to +3, indicating the degree of gel formation. Milk samples that yielded positive results for CMT scores underwent a bacteriological testing.[5]

Microbiological examination. All positive samples for CMT were grown on Mannitol Salt Agar (Oxoid, Basingstoke, UK). We followed the National Mastitis Council recommendations for the isolation and identification of *S. aureus.* Raw milk samples were streaked onto mannitol salt agar plates and then incubated at 37.00 ˚C for 45 - 48 hr, during which time bacterial growth was monitored. To obtain pure cultures, bacterial colonies were sub-cultured on the same selective media plates and placed in an incubator for 48 hr at a temperature of 37.00 ˚C. Following the process of purification, the colonies were kept for later use in both nutrient agar and a 50.00% glycerol stock solution.[9] The purified colonies were cultured overnight at 37.00 ˚C in nutrient broth (Hi-Media®, Mumbai, India). The testing for hemolytic activity, catalase, coagulase and gram staining was performed to establish the presence of *S. aureus* according to Wehr and Frank[10] and followed by PCR for simultaneous detection of relevant antibiotic resistance genes in *S. aureus.*[11,12]

Antibiotic sensitivity test. An antibiotic sensitivity test was conducted on each confirmed *S. aureus* isolates using 14 standard antibiotic discs (Bioanalyse, Ankara, Türkiye) including (ciprofloxacin (CIP) 5.00 µg concentration, clindamycin (CD) 2.00 µg, colistin (CL) 10.00 µg, ampicillin (AM) + sulbactam (SAM) 10.00 + 10.00 µg, amikacin (AK) 30.0 µg, AM 10.00 µg, amoxicillin + clavulanic acid (AMC) 20.00 + 10.00 µg, TE 30.00 µg, tobramycin (TOB) 30.00 µg, vancomycin (VA) 30.00 µg, imipenem (IPM) 10.00 µg, doxycycline (DO) 30.00 µg, erythromycin (E) 15.00 µg and gentamicin (GN) 10.00 µg. Each isolate was subjected to an analysis utilizing the diffusion method on Mueller-Hinton agar following the parameters outlined by the Clinical and Laboratory Standards Institute.[13] The findings were categorized as resistant, intermediate and susceptible for each medication examined in accordance with the Oxoid Manual.[14]

DNA extraction. The purified colonies were extracted by the GF-1 Bacterial DNA Extraction Kit (Cat.no. GF-BA-100, Vivantis, Subang Jaya, Malaysia) in line with the company protocol. The nucleic acid was extracted using 50.00 µL of elution buffer. Extracted DNA was observed at 1.50% agarose gel electrophoresis.

Molecular identification using PCR. Polymerase chain reaction was done in GS-96 gradient thermocycler (Hercuvan, Kuala Lumpur, Malaysia) with a volume of 25.00 µL. The reaction mixture included. A volume 75.00 µL (10.00 µM) of each primer (Vivantis), 9.00 µL of deionized distilled water, 12.50 µL of Master Mix (Willofort Co. Ltd., London, UK) and 2.00 µL of extracted DNA. *Staphylococcus aureus* ATCC 25923 obtained from Zoonoses Department, National Research Centre was served as a positive control, and distilled water served as a negative control. The PCR products were documented by agarose gels electrophoresis at 1.50% and stained with

ViSafe Red Gel Stain (Vivantis). Subsequently, the samples were captured and examined utilizing the InGenius 3 gel documentation system (Syngene, Cambridge, UK). The primers utilized plus the exact PCR reaction are scheduled in Tables 1 and 2.

Statistical analysis. The obtained data were analyzed by Chi-square using the SPSS Software (version 15.0; SPSS Inc., Chicago, USA) and probability (*p*-values) of less than 0.01 was considered significant.

Results

Prevalence of *S. aureus* subclinical bovine mastitis (SCM) based on CMT. Using bulk milk samples from apparently normal cattle, the overall prevalence of SCM via CMT was 65.00%. Using 65 bulk tank milk samples from cattle with SCM, 30/65 (46.15%) *S. aureus* isolates were identified. Out of 20 bulk milk tank samples which were collected from each farm, the prevalence of *S.* aureus isolation was 25.00% (5/20) in Giza, 45.00% (9/20) in Cairo, 25.00% (5/20) in Fayoum, 35.00% (7/20) in Kafr El-Sheikh and 20.00% (4/20) in Kalyobia. The total prevalence was 30/100 (30.00%) in all farms (Table 3).

Antibiotic sensitivity test. The highest resistance was found to TE 30/30 (100%), followed by E, 17/30 (56.66%) and CD 5/30 (16.66%). On the other hand, 100% sensitivity was observed to AK, AM, AMC, SAM, CIP, CL, GN, IPM, TOB, DO and VA (Table 4).

Molecular identification using PCR. All 30 (100%) bacteriologically positive *S. aureus* isolates were confirmed using PCR targeting *nuc1* gene of *S. aureus*. Thereafter, we screened all the thirty isolates for AMR genes using PCR (Fig. 1). Methicillin resistance of *S. aureus* which was represented by *mecA* gene, was detected in 25/30 (83.33%) of isolates (Fig. 2). Lincosamide resistance of *S. aureus* represented by *inuA* gene was detected in 20/30 (66.66%) of isolates (Fig. 3). Macrolides resistance of *S. aureus* which was represented by *mphC* gene was detected in 5/30 (16.66%) of isolates (Fig. 4). Tetracycline resistance of *S. aureus* represented by *tetK, tetL genes* was detected in 7/30 (23.33%) and 16/30 (53.33%) of isolates (Figs. 5 and 6), respectively, (Table 4).

Table 1. Polymerase chain reaction primers used in the present study.

Target genes	Primer sequences (5´-3´)	Product size (bp)	References
InuA	F- GGTGGCTGGGGGGTAGATGTATTAACTGG R- GCTTCTTTTGAAATACATGGTATTTTTCGA	323 bp	11
Tetk	F- GTAGCGACAATAGGTAATAGT R- GTAGTGACAATAAACCTCCTA	169 bp	12
Nuc1	F- CTG GCA TAT GTA TGG CAA TTG TT R- TAT TGA CCT GAA TCA GCG TTG TCT	664 bp	15
Mec1	F- GTAGAAATGACTGAACGTCCGATAA R-CCAATTCCACATTGTTTCGGTCTAA	310 bp	16
MphC	F- GAGACTACCAAGAAGACCTGACG R- CATACGCCGATTCTCCTGAT	722 bp	17
TetL	F- TCGTTAGCGTGCTGTCATTC R- GTATCCCACCAATGTAGCCG	267 bp	18

Table 2. Polymerase chain reaction conditions for genes identification.

Genes	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Cycles
Nuc1	95.00 ˚C; 2 min	95.00 ˚C; 20 sec	60.00 ˚C; 30 sec	72.00 ˚C; 45 sec	72.00 ˚C; 10 min	35
MecA	95.00 ˚C; 2 min	95.00 ˚C; 30 sec	50.00 ˚C; 30 sec	72.00 ˚C; 45 sec	72.00 ˚C; 10 min	35
MphC	94.00 ˚C; 2 min	94.00 ˚C; 30 sec	60.00 ˚C; 30 sec	72.00 ˚C; 45 sec	72.00 ˚C; 7 min	35
InuA	94.00 ˚C; 2 min	94.00 ˚C; 30 sec	62.00 ˚C; 30 sec	72.00 ˚C; 45 sec	72.00 ˚C; 7 min	35
Tetk	95.00 ˚C; 2 min	95.00 ˚C; 30 sec	54.00 ˚C; 30 sec	72.00 ˚C; 45 sec	72.00 ˚C; 10 min	35
TetL	95.00 ˚C; 2 min	95.00 ˚C; 30 sec	60.00 ˚C; 30 sec	72.00 ˚C; 45 sec	72.00 ˚C; 10 min	35

Table 3. Epidemiologic data and genotypic traits of antibiotic resistant *Staphylococcus aureus* examined in different farms. Data are presented as number of samples *per* total positive (%).

Farm locations	Positive *S. aureus*	Prevalence of antibiotic resistance genes				
		Methicillin *Mec 1* gene	Macrolides *Mphc gene*	Lincosamide *Inua*	Tetracycline	
					Tetk	*TetL*
Giza	5/20 (25.00%)	5/5 (100%)	1/5 (20.00%)	5/5 (100%)	4/5 (80.00%)	5/5 (100%)
Cairo	9/20 (45.00%)	9/9 (100%)	3/9 (33.33)	2/9 (22.22%)	1/9 (11.11%)	4/9 (44.44%)
Fayoum	5/20 (25.00%)	3/5 (60.00%)	0/5 (0.00%)	5/5 (100%)	2/5 (40.00%)	5/5 (100%)
Kafr El-Sheikh	7/20 (35.00%)	7/7 (100%)	1/7 (14.28%)	5/7 (71.42%)	0/7 (0.00%)	2/7 (28.57%)
Kalyobia	4/20 (20.00%)	1/4 (25.00%)	0/4 (0.00%)	3/4 (75.00%)	0/4 (0.00%)	0/4 (0.00%)
Total	30/100 (30.00%)	25/30 (83.33%)	5/30 (16.66%)	20/30 (66.66%)	7/30 (23.33%)	16/30 (53.33%)
***p*-value**	0.0509	0.0001	0.0017	0.0003	0.0002	0.0001

Table 4. Phenotypic antimicrobial sensitivity pattern of 30 *Staphylococcus aureus* isolates from subclinical mastitic dairy cows.

Antibiotics	Concentration (µg)	No. isolates	No. resistant isolates (%)
Tetracycline	30.00	30	30/30 (100%),
Erythromycin	15.00	30	17/30 (56.66%),
Clindamycin	2.00	30	5/30 (16.66%).
Amikacin	30.00	30	0 (0.00%)
Ampicillin	10.00	30	0 (0.00%)
Amoxicillin plus clavulanic acid	20.00 + 10.00	30	0 (0.00%)
Ampicillin plus sulbactam	10.00 + 10.00	30	0 (0.00%)
Ciprofloxacin	5.00	30	0 (0.00%)
Colistin	10.00	30	0 (0.00%)
Gentamicin	10.00	30	0 (0.00%)
Imipenem	10.00	30	0 (0.00%)
Tobramycin	30.00	30	0 (0.00%)
Doxycycline	30.00	30	0 (0.00%)
Vancomycin	30.00	30	0 (0.00%)

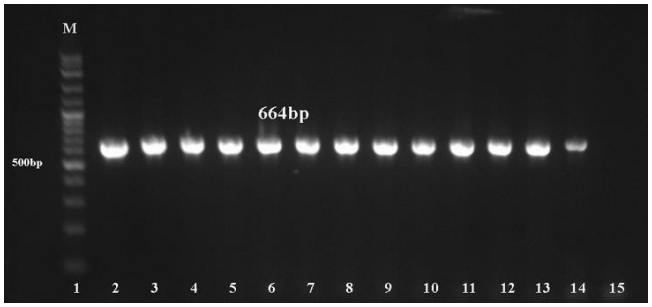

Fig. 1. Polymerase chain reaction product amplified from the *nuc1* gene (664 bp) of *Staphylococcus aureus*. Lane 1: 100 bp DNA marker, Lane 2: Control positive, Lanes 3-14: Representative positive samples, Lane 15: Control negative.

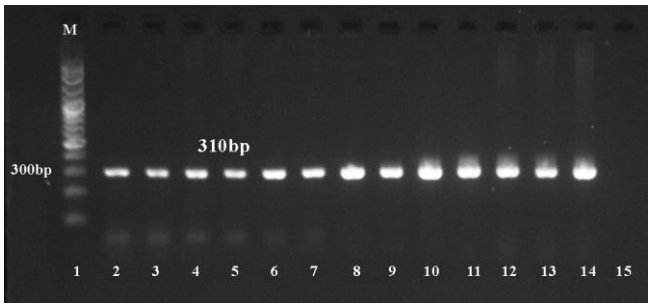

Fig. 2. Polymerase chain reaction product amplified from the *mecA1* gene (310 bp) of methicillin resistant *Staphylococcus aureus*. Lane 1: 100 bp DNA marker, Lanes 2 - 14: Representative positive samples, Lane 15: Control negative.

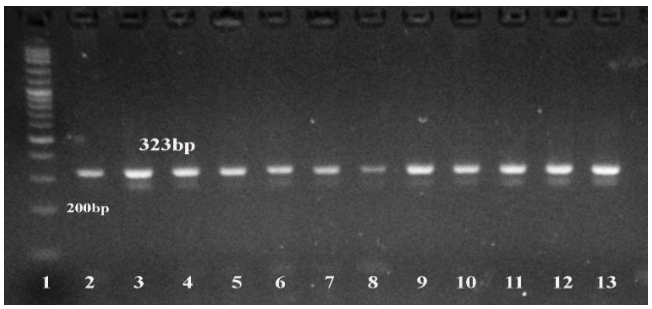

Fig. 3. Polymerase chain reaction product amplified from the *inuA* gene (323 bp) of lincosamide resistant *Staphylococcus aureus*. Lane 1: 100 bp DNA marker, Lanes 2 - 13: Representative positive samples.

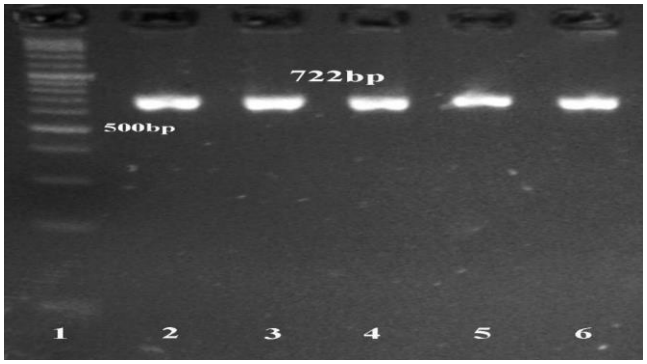

Fig. 4. Polymerase chain reaction product amplified from the *mphC* gene (722 bp) of macrolides resistant *Staphylococcus aureus*. Lane 1: 100 bp DNA marker, Lanes 2 - 6: Positive samples.

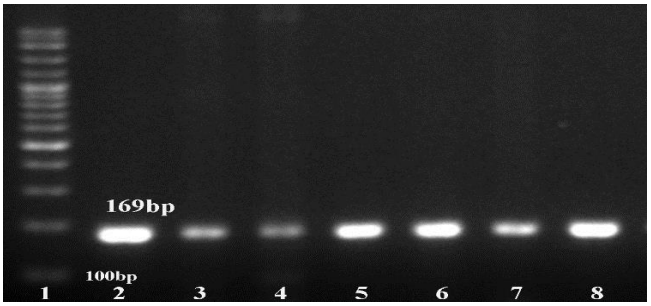

Fig. 5. Polymerase chain reaction product amplified from the *tetK* gene (169 bp) of tetracycline resistant *Staphylococcus aureus*. Lane 1: 100 bp DNA marker, Lanes 2 - 8: Positive samples.

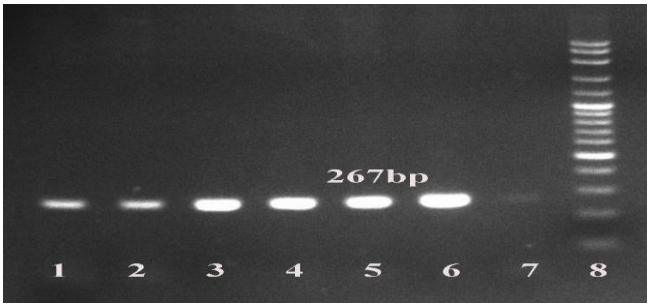

Fig. 6. Polymerase chain reaction product amplified from the *tetL* gene (267 bp) of tetracycline resistant *Staphylococcus aureus*. Lanes 1 - 6: Representative positive samples, Lane 7: control negative, Lane 8: 100 bp DNA marker.

Discussion

Bovine mastitis, a disease affecting dairy cows globally, is mainly caused by bacterial infections, particularly *S. aureus*, which can cause inflammation of the mammary gland and persist in the udder making it a significant economic concern.[1] *Staphylococcus aureus* has various virulence factors that help it survive and evade the immune response of the host. Additionally, the rise and spread of antibiotic-resistant *S. aureus* have raised significant concerns in recent years.[19]

Antibiotic-resistant *S. aureus* can cause mastitis in dairy cows which can lead to long-lasting infections, less milk output and worsened animal welfare.[20] To fight *S. aureus* antibiotic resistance, most of the work has been targeted to make sure that antibiotics are used safely in veterinary medicine that good biosecurity measures are put in place and that new treatment approaches are developed. These plans try to lower the selective pressure that makes drug-resistant genes appear and spread.[21] The objective of this work was to discover *S. aureus* genes associated with antibiotic resistance which could potentially be important for the management of mastitis.

It was indicated that the prevalence of subclinical mastitis in the milk samples collected from bulk tank milk using CMT was 65.00%. This was higher than the prevalence rate of SCM reported in a study conducted in Egypt (52.10%), which was published by Algammal *et al.*[5] Variations in geographic distribution, immunological status, unhygienic environments and contaminated milking utensils can all provide elevated rates of SCM in dairy farms which might be responsible for the high rate of *S. aureus* prevalence in the current report.

Among 100 bulk tank milk samples, the prevalence of *S. aureus* in mastitic milk samples was 30.00%, χ^2= 3.81, and the *p*-value was 0.0509 which was not statistically different among the different farms. This finding was lower than the research performed in Egypt by El-Razik *et al.,*[22] who revealed a *S. aureus* prevalence rate of 66.66%. In addition to molecular tests using the *nuc1* gene, cultural and biochemical tests were used to recognize *S. aureus.*

Rapid identification of *S. aureus* is possible through the use of molecular techniques like PCR, which are essential for treatment and the carrying out of infection control measures to prevent the spread of diseases and outbreaks.[23] All 30 (100%) bacteriologically positive *S. aureus* isolates were confirmed using *nuc1* gene for *S. aureus* using PCR.

Regarding antibiotic sensitivity test, the highest resistance was found to TE 30/30 (100%), followed by E, 17/30 (56.66%) and CD 5/30 (16.66%). On the other hand, 100% sensitivity was observed to AK, AM, AMC, SAM, CIP, CL, GN, IPM, TOB, DO and VA. Antibiogram analysis identified 6/30 (20.00%) of the total isolates as multidrug resistance which was similar to the result of Lubna *et al.*[3] The prolonged and random use of certain antimicrobials may potentially lead to the emergence of resistance.[24]

The highest resistance of TE in this report was in line with those of other studies in different countries. The findings of Jamali *et al.,*[25] in Iran indicated that 56.20% of *S. aureus* isolates recovered from raw cow milk were resistant to TE. Gao *et al.*,[26] conducted a comparable study in China and found that 98.10% of *S. aureus* isolates from raw milk were TE -resistant. Farmers in several nations treat dairy cattle with TE regularly to get rid of *S. aureus* infections. The main factor influencing TE resistance, particularly in cases where the *tetK* gene was involved, was staphylococci efflux mechanism.[27] The most often detected staphylococci resistance gene was the *tetK* gene encoded for TE.[28]

Regarding the antibiotic resistance genes, 25/30 (83.33%) of isolates were positive for the methicillin-resistant *S. aureus* encoding the *mecA* gene. These results were lower than those of Talaat *et al.,*[29] who revealed that all the *S. aureus* isolates (100%) carried the *mecA* gene in Holstein dairy cows in Egypt and higher than those of Hnini *et al.*,[30] who revealed that 3.80% of *S. aureus* isolates involved the *mecA* gene in bovine mastitis in the North-west of Portugal. The improper use of β-lactam antibiotics and inadequate hygienic environments during milking were the main cause of increased MRSA prevalence.

Regarding the macrolides *mphC* and lincosamide *lnuA,* resistance genes, 5/30 (16.66%) and 20/30 (66.66%) of isolates were positive, respectively.

When β-lactam resistance develops, macrolides and lincosamides are the recommended antibiotics.[31] The Staphylococci SCM isolates in Türkiye were shown to be resistant to macrolides and lincosamides -associated resistance genes either individually or in different combinations.[31]

Regarding the TE resistance genes *(tetK), tetL),* 7/30 (23.33%) and 16/30 (53.33%) of isolates were positive, respectively. These results were lower than those of Talaat *et al.,*[29] who revealed that all the *S. aureus* isolates (50.00%) carried the *tetK* gene in Holstein dairy cows in Egypt and higher than those of Hnini *et al.*,[30] who revealed that 15.40% of *S. aureus* isolates causing bovine mastitis carried the *tetK* gene in the Northwest of Portugal. The antibiotics used in this study were statistically different among the different examined farms ($p < 0.01$). The five farms varied in the hygiene applied in each farm and in proper use of the antibiotics. Poor hygiene practices and antimicrobial misusage take the form of saving an antibiotic for the next time a cow becomes ill, skipping dosages, failing to complete the entire prescribed course of treatment, administering antibiotics prescribed for diseases or other conditions and overusing antimicrobials without consulting a veterinarian. This could explain the high prevalence of drug resistance in some of these farms

and this is why it is imperative to discover new methods to diagnose it.[32]

This work concluded that *S. aureus* mastitis was more resistant to TE, E and CD, and more sensitive to AK, AM, AMC, SAM, CIP, CL, GN, IPM, TOB, DO and VA. According to this result, we advise dairy farms using these antibiotics in an adequate dose to avoid antibiotic resistance and for achieving high control measures. More investigations are recommended to understand the mechanism of transferring antibiotic resistant genes to avoid the impact of these genes on animal and human.

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Conflict of interest

The authors declare no conflict of interest.

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