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RESEARCH ARTICLE

Phenotypic, Genotypic and Antibiogram among *Staphylococcus aureus* Isolated from Bovine Subclinical Mastitis

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This study was planned to determine the phenotypic, genotypic, and antibiogram characterization of *S. aureus* recovered from bovine subclinical mastitis in Menoufiya Governorate, Egypt. A total of 140 (28%) milk samples were positive by the California Mastitis Test (CMT) collected from 500 lactating cows. One hundred and five samples were positive on Baird parker agar and Mannitol salt agar which were confirmed by coagulase test and amplification of the *nuc* gene into 43 (30.7%) *S. aureus*. Sensitivity test against 9 antibiotics for *S. aureus* isolates revealed that the highest rate of antibiotics resistance was for penicillin (90.69%), oxacillin (81.39%), chloramphenicol (58.14%) and tetracycline (53.48%), and 33 (76.74%) of the *S. aureus* identified as MRSA strains and exhibited multidrug-resistant (MDR), while the highest sensitivity for gentamicin (76.76%), both amoxicillin/clavulanic acid and vancomycin (69.77% for each) and ciprofloxacin (62.79%). Furthermore, fifteen isolates were selected for detecting the presence of antibiotic-resistance and virulence genes among *S. aureus* strains. The *mec*A was the most prevalent gene (100%) among *S. aureus* strains followed by *bla*Z (80%), *tet*K (66.7%) and *erm*B (40%) genes with no detection of the *van*A gene. Moreover, *coa* and *spa* virulence genes were detected in all tested isolated. In conclusion, our results indicate the importance of the regular surveillance of phenotypic and genotypic profiles for *S. aureus* isolates to ensure effective control measures for bovine mastitis and minimizes the evolution of MDR strains.

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INTRODUCTION

Mastitis is defined as an inflammation of the mammary gland resulted in a reduction of milk yield and quality causing significant economic losses in the dairy industry worldwide (El-Faramaway *et al.,* 2019). *Staphylococci* are reported as the most common bacterial cause of subclinical mastitis (Pumipuntu *et al.,* 2019). It was categorized into coagulase-positive (CPS) and coagulase-negative (CoNS) *Staphylococci* based on the coagulase activity (Wald *et al.,* 2019). *Staphylococcus aureus* is one of the most famous CPS causing contagious intramammary infection (Monistero *et al.,* 2018).

The extensive use of antimicrobial therapy as a common strategy for treatment and control of mastitis, has led to emerging of more resistant pathogens and poor response to treatment (Cheng *et al.,* 2019). The misuse of antimicrobials to combat mastitis had led to emerging of resistant strains of *Staphylococcus* spp., undermining the efficacy of treatment (Cheng and Han, 2020). Moreover, Methicillin-resistant *Staphylococci* (MRSA) that encoded by the *mec*A gene which enhanced the resistance against β-lactam antimicrobials (Srednik *et al.,* 2017). Many surveillances studies have identified the resistance of many bovine MRSA strains to various antimicrobial agents that pose a major public health hazard and impeded effective treatment of bovine mastitis (Wang *et al.,* 2015;

Liu *et al.*, 2017 and El-Faramaway *et al.*, 2019). A recent study in Egypt (Youssif *et al.*, 2020) revealed that *S. aureus* strains conferred resistance to tetracycline, B-lactams, macrolides, methicillin, vancomycin, and norfloxacin that encoded by (*tet*K- *tet*A), (*bla*Z, bla_{TEM}), (*erm*B, *erm*C), (*mec*A, *mec*1, *mec*C), (*van*A) and (*nor*A) genes respectively. According to this information, this study was conducted to determine the phenotypic, genotypic, and antibiogram characterization of *S. aureus* recovered from bovine subclinical mastitis in Menoufiya Governorate, Egypt.

MATERIALS AND METHODS

Samples collection and Study area: Five-hundred dairy cows from individual cases in Menoufiya Governorate, Egypt were examined during the period from October 2018 to December 2019; these cows have phenotypically normal milk character, but some were positive by California Mastitis Test (CMT) which indicated as subclinical mastitis. The California Mastitis Test (CMT) was performed according to the **(**IMMUCELL CORPORATION, Portland). Positive California Mastitis Test (CMT) milk samples were collected aseptically and transferred in cold conditions as soon as to the laboratory where it subjected to bacterial isolation and identification.

Bacteriological isolation and identification: For isolation of *Staphylococcus* spp., milk samples were centrifuged, and the sediment was then cultured into Baird-Parker agar, Mannitol salt agar, and blood agar 10% (Oxoid Ltd.) then incubated for 1-2 days at 37°C. Confirmation for the *S. aureus* was done through Gram staining, catalase, coagulase (APHA, 1992), detection of hemolytic activity, DNase agar testing (Murray *et al.*, 2003) and for the biofilm activity onto Congo red medium (Arciola *et al.*, 2015).

Antibiogram profile of *S. aureus* isolates: The obtained isolates of *S. aureus* were tested *in vitro* using a Kirby–Bauer disk diffusion method against different antibiotic groups to detect the susceptibility and resistance pattern of these isolates to 9 antimicrobial discs (Oxoid). The used antibiotics were: Penicillin: P (100 IU), Chloramphenicol: C (30 µg), Oxacillin: OX (1 µg), Tetracycline: TE (30 µg), Erythromycin: E (15 µg), Ciprofloxacin: CIP (5 µg), Amoxicillin/clavulanic acid: AMC (30 µg), Gentamicin: CN (10 µg), Vancomycin: VA (30 µg). The obtained results were interpreted as resistant, intermediate, or susceptible according to the inhibitory zone diameter established by the Clinical Laboratory Standards Institute (CLSI, 2017).

Molecular detection of antibiotic resistance and virulence genes in *S.aureus isolates*: Freshly grown typical *S.aureus* colonies were harvested and the DNA extraction was performed according to the manufactures' guidelines using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

Primers were utilized in a 25-µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The primers (Metabion, Steinkirchen, Germany) used for detecting gene encodes the thermostable nuclease of *S. aureus* (*nuc*) (Louie *et al.*, 2002), methicillin resistance genes (*mec*A) (McClure *et al.*, 2006), β-lactams resistance genes (*blaZ*) (Duran *et al.*, 2012), tetracycline resistance genes (*tetK*) (Duran *et al.*, 2012), vancomycin resistance genes (*van*A) (Patel *et al.*, 1997), macrolides resistance genes (*erm*B) (Schlegelova *et al.*, 2003), *coa* (Iyer and Kumosani, 2011) and *spa* (Wada *et al.*, 2010) virulence genes are listed in Table 1.

The reaction was performed in an Applied biosystem 2720 thermal cycler (Applied Biosystems, Foster, CA). Fifteen microliter aliquots of all PCR products and Gelpilot 100 bp (Qiagen, Germany, GmbH) were used to determine the fragment sizes followed by electrophoresed through 1.5% agarose gels (Applichem, Germany, GmbH). The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data were analyzed through computer software.

RESULTS

Prevalence of *S. aureus* recovered from subclinical mastitis cases: Out of the examination of 500 dairy cows, 140 (28%) were diagnosed as subclinical mastitis depending on California Mastitis Test (CMT). From California Mastitis Test (CMT) positive samples, 105 were positive on a Baird parker medium and Mannitol salt agar which differentiated by coagulase test into 43 (24.28%) coagulase positive *staphylocci* (CPS).

Table 1: The PCR primers for virulence and antibiotic resistance genes of *S.aureus*

Target gene	Primer sequences	Amplified amplicon (bp)	Primary Denaturation	Secondary denaturation	Annealing	Extension	Final extension
nuc	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTA AAGC	270	94˚C 5 min	94°C 30 sec	55˚C 30 sec	72˚C 1 min.	72˚C 10 min
Coa	ATAGAGATGCTG GTACAGG GCTTCCGATTGTTCG ATGC	630	94˚C 5 min	94˚C 30 sec.	55˚C 40 sec	72˚C 45 sec.	72˚C 10 min
Spa	TCAACAAAGAACAACAAAATGC GCTTTCGGTGCTTGAGATTC	226	94˚C 5 min	94˚C 30 sec.	55˚C 40 sec	72˚C 45 sec.	72˚C 10 min
*mec*A	GTAGAAATGACTGAACGTCCGATAA CCAATTCCACATTGTTTCGGTCTAA	310	94˚C 5 min.	94˚C 45 sec.	50˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.
*bla*Z	ACTTCAACACCTGCTGCTTTC TGACCACTTTTATCAGCAACC	173	94˚C 5 min.	94˚C 30 sec.	54˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.
*tet*K	GTAGCGACAATAGGTAATAGT GTAGTGACAATAAACCTCCTA	360	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.
*van*A	CATGACGTATCGGTAAAATC ACCGGGCAGRGTATTGAC	885	94˚C 5 min.	94˚C 30 sec.	56˚C 40 sec.	72˚C 50 sec.	72˚C 10 min.
*erm*B	CATTTAACGACGAAACTGGC GGAACATCTGTGGTATGGCG	425	94˚C 5 min.	94˚C 30 sec.	51˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.

All genes were amplified for 35 cycles.

According to the biochemical and enzymatic activity of bacteriologically isolated coagulase positive staphylocci (CPS), for hemolysis on blood agar media, 31 (72.1%) isolates from CPS exhibited β-hemolysis, while 12 (27.9%) showed α- hemolysis. Ten isolates (23.25%) from CPS were positive DNase activity, and 15 isolates (34.88%) from CPS have a biofilm activity on the Congo red medium. All CPS isolates were confirmed as *S. aureus* through successful amplification and targeting the *nuc* gene (Fig. 1A), virulence genes, *coa* gene (Fig. 1B) and the *spa* gene (Fig. 1C).

Antimicrobial susceptibility test of *S. aureus* isolated from subclinical mastitis: Sensitivity test against 9 antibiotics for 43 *S. aureus* isolates revealed that the highest rate of antibiotics resistance was for penicillin (90.69%), oxacillin (81.39%), chloramphenicol (58.14%) and tetracycline (53.48%), Meanwhile, high susceptibility were noticed with for gentamicin (76.75%), amoxicillin/clavulanic acid and vancomycin (69.77%), and ciprofloxacin (62.79%) (Table 2).

Based on the results of phenotypic resistance of oxacillin and further molecular detection of *mec*A gene by PCR, 33(76.74%) out of 43 *S. aureus* isolates were identified as MRSA strains

Multidrug resistance (MDR) profiles of *S. aureus* from subclinical mastitis: The 33 identified MRSA strains showed MDR to 3-6 antibiotics groups, only two isolates (6.1%) were MDR to 6 antibiotics groups. Fourteen isolates (42.4 %) were MDR to 5 antibiotics groups, in addition to 10 isolates (30.3%) were MDR to 4 antibiotics groups and 7 isolates (21.2%) were MDR to 3 antibiotics groups (Table 3).

Molecular detection of *S. aureus* antibiotic resistance genes: The prevalence of the antibiotic-resistance genes among selected fifteen *S. aureus* isolates showed that the *mec*A gene was the most detected gene in all *S. aureus* isolates (100%) (Fig. 2A). Moreover, the *bla*Z gene was successfully amplified in 12 (80%) isolates in *S. aureus* (Fig. 2B). Concerning the *tet*K gene, it was detected in 10 isolates with a percentage of 66.7% in *S. aureus* (Fig. 2C). While the *erm*B gene was identified in 6 isolates with a prevalence of 40% in *S. aureus* (Fig. 2D) isolates. The *van*A gene was not detected in any isolates of *S. aureus*.

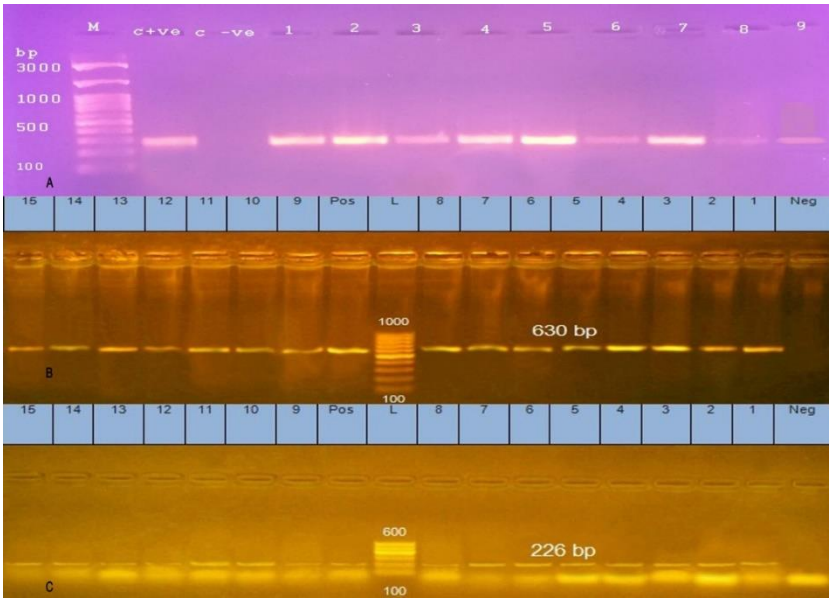

Fig. 1: Agarose gel electrophoresis for PCR products for the detection of *nuc*, (Fig-1A) at 270bp from Lane; 1-9; *coa* gene (Fig-1B) at 630 bp from Lane; 1-15 and *spa* gene (Fig-1C) at 226 bp Lane 1-15. Lane L: 100 bp DNA marker; lane Neg: Negative control; and lane Pos: Positive control.

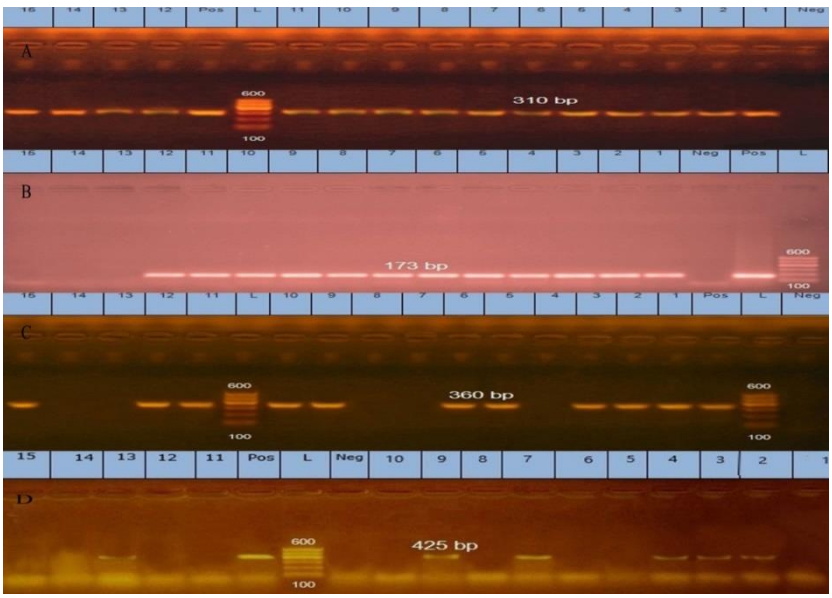

Fig. 2: Agarose gel electrophoresis for PCR products for the detection of antibiotic resistance target genes in 15 *S. aureus* strains; (A) All *S. aureus* strains (1-15) were positive for the *mec*A gene (310 bp). (B) Lanes 1-12 were positive for the *bla*Z (173 bp), while lanes 13-15 were negative. (C) Lanes 1-3, 5, 6, 9-12 and 15 were positive for the *tetK* (360 bp), while lanes 4, 7, 8, 13 and 14 were negative. (D) Lanes 2-4, 7, 9 and 13 were positive for the *erm*B gene (425 bp), while lanes 1, 5, 6, 8, 10, 11, 12, 14 and 15 were negative. Lane L: 100 bp DNA marker; lane Neg: Negative control and lane Pos: Positive control.

Table 2: Antimicrobial susceptibility test of *S. aureus* isolated from subclinical mastitis

Antimicrobial classes	Antimicrobial agents	S Mm	I mm	R Mm	No of *S. aureus* isolates (%)					
					R	%	I	%	S	%
Beta –lactams	Oxacillin (OX)	≥22	-	<21	35	81.39	3	6.98	5	11.63
	Penicillin (P)	≥29	-	<28	39	90.69	0	0	4	9.31
	Amoxicillin/Clavulanic acid (AMC)	≥20	-	<19	13	30.23	0	0	30	69.77
Tetracycline	Tetracycline (TE)	≥19	15-18	<14	23	53.48	5	11.63	15	34.89
Macrolides	Erythromycin (E)	≥23	14-22	<13	21	48.83	4	9.31	18	41.86
Fluoroquinolones	Ciprofloxacin (CIP)	≥21	16-22	<15	16	37.21	0	0	27	62.79
Chloramphenicol	Chloramphenicol (C)	≥18	13-17	<12	25	58.14	6	13.96	12	27.9
Aminoglycosides	Gentamycin (CN)	≥15	13-14	<12	10	23.25	0	0	33	67.75
Glycopeptides	Vancomycin (VA)	>2	4-8	<16	1	2.33	12	27.9	30	69.77

Table 3: Multidrug resistance (MDR) profiles of *S. aureus* from subclinical mastitis

S.aureus resistant isolates		Resistance profile	No. of resistance antimicrobial classes
No.	%*		
2	4.65	OX-P-AMC-E-CIP-TE-C-CN	6
4	9.30	OX-P-AMC-E-CIP-TE-C	5
2	4.65	OX-P-AMC-E-TE-C-CN	5
1	2.32	OX-P-E-CIP-VA-C-CN	5
1	2.32	OX-P-AMC-CIP-TE-C-CN	5
1	2.32	OX-P-E-CIP-TE-C	5
1	2.32	P-AMC-E-TE-C-CN	5
1	2.32	P-AMC-E-CIP-TE-C	5
1	2.32	P-E-CIP-TE-C	5
1	2.32	P-E-TE-C-CN	5
1	2.32	P-CIP-TE-C-CN	5
2	4.65	OX-P-AMC-E-CIP-C	4
1	2.32	OX-P-AMC-CIP-TE-C	4
1	2.32	OX-P-AMC-E-TE-C	4
1	2.32	OX-P-CIP-C-CN	4
1	2.32	P-E-TE-C-CN	4
1	2.32	P-E-TE-C	4
1	2.32	P-E-C-CN	4
1	2.32	P-CIP-TE-C	4
1	2.32	P-E-CIP-C	4
3	6.98	OX-P-TE-C	3
1	2.32	OX-P-C-CN	3
1	2.32	OX-P-E-TE	3
2	4.65	E-TE-C	3

OX=Oxacillin; P=Penicillin; AMC=Amoxicillin/Clavulanic acid; E= Erythromycin; CIP=Ciprofloxacin; TE=Tetracycline; C= Chloramphenicol; CN=Gentamycin; VA=Vancomycin. *Percentage was estimated according to the total number of isolates of *S. aureus* (43).

DISCUSSION

Mastitis is the major infectious disease in dairy herds, and it is responsible for major economic losses in the milk industry. The significant reduction in milk production attributed to subclinical mastitis constitutes great financial losses (Romero *et al.,* 2018). Besides financial implications, the importance of mastitis in public health should not be disregarded.

In this study, the overall prevalence of subclinical mastitis was 28%. Our result, nearly like those of other studies reported in Egypt by (Abdel-Tawab *et al.*, 2016) that reported a prevalence rate of subclinical mastitis in 26.7%. Our results are also consistent with the results of Kayesh *et al.* (2014) who reported subclinical mastitis in Bangladesh with an incidence rate 28.5%.

Meanwhile, higher prevalence rate has been reported by Mpatswenumugabo *et al.* (2017) in Rwanda, who demonstrated that the prevalence of subclinical mastitis was 50.4%. On the other hand, our results are higher than those that have been reported with Birhanu *et al.* (2017) who reported subclinical mastitis in lower incidence (16.1%). These differences in subclinical mastitis prevalence rates might attribute to differentiate of breeds of animals, management, and applicable health practices (Rathod *et al.*, 2017).

In our present study, forty–three samples showed microbial growth on a Baird parker medium, Mannitol salt agar and identified as *S. aureus* depending on the coagulase test and amplification of the *nuc* gene and virulence genes (*coa* and *spa*) genes. This was supported by David *et al.* (2010) illustrated that amplification of the *nuc* gene of *S. aureus* is considered as a gold standard method. Additionally, Elfaramawy *et al.* (2019) showed that all *S. aureus* isolates from bovine mastitis carried both *coa* and *spa* virulence genes which play a worthy role in bovine mastitis pathogenicity.

Moreover, *S. aureus* is one of the most commonly found pathogens in clinical mastitis. Our results agree with previous studies carried out by Mousa *et al.* (2015) recorded that *S. aureus* was prevalent with 32% recovered from bovine subclinical mastitis in Egypt. Moreover, in a study in Rwanda, Ndahetuye *et al.* (2019) reported *S. aureus* prevalence rate with 22%.

In the current study, most of the *S. aureus* strains showed a high antimicrobial resistance to penicillin, oxacillin, chloramphenicol, and tetracycline. Meanwhile, high susceptibility was noticed for amoxicillin/clavulanic acid, vancomycin, gentamicin, and ciprofloxacin. Similar findings in Egypt, Abdel-Tawab *et al.* (2016) reported that *S. aureus* exhibit phenotypic resistance to oxytetracycline and penicillin-G and were sensitive to amoxicillin/clavulanic acid. Moreover, Saidi *et al.* (2019) reported a high resistance to penicillin G and tetracycline. The results indicated a correlation between antibiotic use and antimicrobial resistance. Similar findings have been published by Liu *et al.* (2017), who indicates that herd-level use of certain antimicrobials for mastitis treatment was positively linked to antimicrobial resistance in isolates from a mastitis sample and most of the *S. aureus* strains demonstrate high antimicrobial resistance to classes beta lactams, sulphonamides and flour- quinolones.

In the present study, out of 43 *S. aureus* isolates, 33 (76.74 %) were classified as MRSA strains based on phenotypic resistance to Oxacillin in antimicrobial susceptibility test and molecular detection of *mec*A gene which in agreement with Quinn *et al.* (2011). The identified MRSA strains showed MDR to several antibiotics groups that clarify the role of MRSA strains in difficulties in treatment of *S. aureus* mastitis. This in accordance with (Wang *et al.,* 2015) who showed 34 (15.5%) multiple-drug resistance of MRSA strains out of 219 *S. aureus* from dairy cattle in China which constitute public health concern. In addition to, Elfaramawy *et al.* (2019) showed that (67.39%) of *S. aureus* bovine mastitis isolates in Egypt were classified as MRSA strains and showed resistance to several classes of antimicrobials.

The antibiotics have been prescribed in the past decades as an effective protocol in the treatment of bovine

mastitis. In our study, 33/43 (76.74%) of *S. aureus* isolates exhibited MDR to 3-6 antibiotics groups. Our result was similar to Wang *et al.* (2015) whose concluded that *S. aureus* particularly MRSA strains express resistance to several antimicrobial drugs and considered a newly emerged etiology in bovine mastitis with a public health problem. Moreover, Algammal *et al.* (2020) reported that more than 50% of *S. aureus* isolates from mastitis showed resistance to penicillin and tetracycline that minimized the benefits treatment.

Regarding to the molecular screening of five antibiotic-resistance genes (*mec*A, *bla*Z, *erm*B, *tet*K, and *van*A) among *S. aureus* revealed that *mec*A gene was the most predominant gene in 15 isolates (100%), followed by *bla*Z, *tet*K and *erm*B genes with prevalence rates 12 (80%), 10 (66.7%) and 6 (40%), respectively with no detection of *van*A gene. Our results related to the study reported by in Egypt (Abdeen *et al.,* 2015) illustrated the detection of *mec*A and *bla*Z genes among all *S. aureus* isolated from bovine mastitis. Another study in China (Qu *et al.,* 2019) demonstrated the detection of *bla*Z (95%), *tet*M (33%), *tet*K (31%), *erm*T (26%), *mec*A (16%) and *van*A (4%), respectively among *S. aureus* isolates in dairy cattle farms.

In conclusion, our study summarized that *S. aureus* was the most prevalent and common bacterial etiology of subclinical intramammary infection in dairy cattle. Moreover, the higher predominance of phenotypic and genotypic resistance of *S. aureus* indicates the potential economic loss and public health hazard. Thus, a recommendation for the dairy farm owners to review the hygiene regimen and regular investigation regarding MRSA screening to reduce the incidence of multidrug resistance and the use of antibiotics with great caution.

Authors contributions: WSM, UHA, EA, and AAT and involved in the conception of the research idea and methodology design, performed data analysis and interpretation, laboratory work, and prepared the manuscript, and these authors contributed equally to the manuscript, RF contributed in the samples collection and part of laboratory work. All authors read and approved the final manuscript.

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