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Research Article
Genetic Characterization, Antimicrobial Resistance Patterns and Virulence Determinants of *Staphylococcus aureus* Isolated form Bovine Mastitis

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Abstract

Background and Objective: *Staphylococcus aureus* is commonly associated with mastitis in dairy herds with potential public health implications. This study was conducted to investigate the existence of *S. aureus* in mastitic milk and to determine the antimicrobial resistance profiles of the isolated strains as well as the resistance and virulence associated genes. **Materials and Methods:** Two hundred quarter milk samples were collected from 3 dairy farms at Dakahliya (n = 2) and Damietta (n = 1) Governorates, Egypt from September to December 2016. Conventional culturing and Polymerase Chain Reaction (PCR) assays targeting *nuc* (thermonuclease) and *coa* (coagulase) genes were performed. Isolates were tested for its susceptibility against 14 antimicrobial agents using disk diffusion method. All the isolates were screened for the presence of β-lactamases (*blaZ, mec*A) and virulence associated (*pvl* and *tst*) genes by PCR. **Results:** The *S. aureus* was detected in 42% (84/200) of the total examined milk samples. Regarding the antibiogram results, *S. aureus* revealed a high resistance against ampicillin (95.2%) and penicillin (83.3%) and a lower resistance was observed against gentamicin (23.8%), amikacin (16.7%) and ciprofloxacin (14.3%). Multidrug resistances were detected in 83.3% of the isolated *S. aureus*. Of the 70 penicillin-resistant *S. aureus* isolates, *blaZ* gene was identified in 67 (95.7%) isolates. Fifty percent of *S. aureus* isolates harbored the specific amplicon of *mec*A gene. Markedly, all *mec*A positive strains displayed multidrug resistance and were also positive for *blaZ* gene. The virulence determinants *pvl* and *tst* were detected in 7.1 and 11.9% of the isolated *S. aureus*, respectively. **Conclusion:** Presence of multidrug resistant and toxin producing *S. aureus* in dairy farms pose a major risk to public health. Therefore, this study highlighted the importance of developing an efficient control program to inhibit the transmission of *S. aureus*, particularly multidrug resistant strains to humans.

Key words: *Staphylococcus aureus*, mastitis, antimicrobial resistance, virulence, zoonoses

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

INTRODUCTION

Staphylococcus aureus, a Gram-positive bacterium, constitutes part of the commensal microbiota in the skin, nose and respiratory tracts of both humans and animals. Other strains of *S. aureus* are highly pathogenic to human causing variety of diseases that includes skin infections, osteomyelitis, septic arthritis, septicemia and food poisoning[1]. Food intoxication in human is mainly caused by *S. aureus* thermostable enterotoxins that can resist the pasteurization temperature[2].

Mastitis is one of the most common infectious diseases affecting dairy herds, which lead to sever financial losses and attributed to decrease in milk yield and milk quality. The *S. aureus* is considered the most significant pathogen associated with mastitis in dairy herds[3,4]. Zoonotic potential of clinical and sub-clinical mastitis arises from the possibility of shedding the pathogens and their toxins into milk[5].

The emergence of antimicrobial resistance among *S. aureus* has been suggested to cause delay in antibiotic treatment of bovine mastitis. This resistance against wide range of antimicrobial classes may be attributed to the indiscriminate use of these agents in the treatment of bovine mastitis[6]. In Egypt, the over-prescription and misuse of antimicrobials used for the treatment of bacterial infections in humans and animals, have been associated with the exacerbation of antimicrobial resistance among these pathogenic bacteria[7,8].

The β-lactams have been frequently used for treatment of bovine mastitis but their efficiency is decreased due to development of β-lactamase encoded *bla*Z that hydrolyze penicillins[9]. Methicillin/oxacillin resistance is another β-lactam resistance mechanism, results from the production of low-affinity penicillin-binding protein (PBP2a) encoded by *mec*A gene[10]. Methicillin-resistant *Staphylococcus aureus* (MRSA) attracted a great concern by mid-1990s because they displayed multiple resistances to a wide range of antimicrobial classes other than β-lactams and disseminated continually through new communities[11].

There are wide arrays of virulence determinants associated with the pathogenicity of *S. aureus*[12]. These determinants consist of enzymes and cytotoxins that include hemolysins (α, β, γ and δ), nucleases, lipases, proteases, collagenases and hyaluronidases. Additionally, some *S. aureus* strains produce exoproteins, such as Panton-Valentine leukocidin (PVL), toxic shock syndrome toxin-1 (TSST-1), exfoliative toxins (ETA and ETB) and staphylococcal enterotoxins (SEA-E, G-I)[13].

This study aimed to investigate the occurrence of *S. aureus* in mastitic milk from three dairy herds at Dakahliya and Damietta Governorates, Egypt. Molecular characterization of the isolated strains was performed using PCR targeting thermonuclease (*nuc*) and coagulase (*coa*) genes, β-lactamases (*mec*A and *blaZ*) and virulence associated genes (TSST-1 gene, *tst* and Panton-Valentine leukocidin gene, *pvl*). The antimicrobial resistance profiles were also determined.

MATERIALS AND METHODS

Samples collection and bacteriological culturing: Cattle from three dairy farms located at Dakahliya (n = 2) (Latitude 31.04° N and longitude 31.37° E) and Damietta (n = 1) (Latitude 31.36° N and longitude 31.67° E) Governorates, Egypt were included in this study. A total of 200 individual quarter milk samples were taken from 120 cows during the period from September to December 2016. All the cattle investigated had a median age of 5-7 years. Physical examination of the udder was done before collection of each sample to estimate the existence of any signs of inflammation. All the milk samples were collected aseptically by hand milking in sterile tubes after discarding the first few streams of milk. The collected milk samples were transported aseptically in ice coolers to the Laboratory of Bacteriology, Mycology and Immunology Department, Faculty of Veterinary Medicine, Mansoura University for bacteriological evaluation. Informed consents were taken from the owners of the farms prior sampling.

Milk samples were subjected to *S. aureus* isolation procedures as previously described by Wang *et al.*[14]. Presumptive *S. aureus* colonies were picked up and purified by sub-culturing on the surface of tryptone soya agar (TSA; Oxoid, UK) plates. Then, the presumptive colonies were subjected to gram staining and standard biochemical tests[15].

Antimicrobial susceptibility testing: Antimicrobial susceptibility profiling was done by disc diffusion method for the isolated *S. aureus* against 14 antimicrobial agents (Oxoid, UK) and according to Clinical and Laboratory Standards Institute (CLSI) guidelines[16]. The *S. aureus* isolates were tested against penicillin (P; 10 IU), oxacillin (OX; 15 μg), amoxicillin (AX; 25 μg), tetracycline (TE; 30 μg), streptomycin (S; 10 μg), amikacin (AK; 30 μg), sulfamethoxazole-trimethoprim (SXT; 23.75/1.25 μg), rifampin (RA; 5 μg), erythromycin (E; 15 μg), ampicillin (AM; 15 μg), chloramphenicol (C; 30 μg), vancomycin (VA; 30 μg), gentamicin (CN; 10 μg) and ciprofloxacin

(CIP; 5 µg). Interpretation of the results was done following CLSI guidelines. A single strain was considered multidrug resistant if it exhibited resistance to 3 or more different antimicrobial classes.

Genotypic characterization of *S. aureus*: The DNA extraction from *S. aureus* isolates was performed using PureLink Genomic DNA extraction Kit (Invitrogen, Carlsbad, CA) according to the manufacturer guidelines. The PCR was performed on the isolated *S. aureus* to determine *nuc*, *mec*A and *blaZ* genes as previously reported by Oliveira *et al.*[17]. The *coa* gene was also investigated as previously outlined[18]. The virulence determinants *pvl* and *tst* genes encoding PVL and TSST-1 were determined according to Lina *et al.*[19] and Sallam *et al.*[20], respectively. The primers sequences and their PCR products are summarized in Table 1[17-20]. The PCR for each specific gene was performed using 96 well Bio-Rad (Munich, Germany) thermal cycler. Each PCR reaction mixture was done in a final volume of 25 µL containing 12.5 µL of 2X PCR master mix (Promega, Madison, USA), 1 µL of 20 pmol of each primer (Metabion, Germany), 5 µL DNA template and the volume of the reaction mixture was completed to 25 µL using DNase/RNase-free water. Thermocyclic condition for each PCR reaction was done as summarized in Table 2. The amplified products for each gene were separated by subjecting 3 µL aliquots to agarose (1.2%) gel for 30 min at 100 V followed by a 20 min staining in ethidium bromide solution. Gels were then visualized under UV light and photographed.

RESULTS AND DISCUSSION

In this study, of the 200 mastitic milk samples subjected to bacterial culturing, eighty four (42%) *S. aureus* isolates were identified based on the morphological and biochemical characters. A wide variety in the prevalence rate of *S. aureus* from bovine mastitis was detected in many previous studies[5,21-27]. Lower occurrence of *S. aureus* was reported in a study conducted by Anderson *et al.*[21], who determined a 13.6% prevalence rate of *S. aureus* from the lactating cows in three different areas in USA. In another study from Germany, Schlotter *et al.*[22] identified *S. aureus* in 15.5% of the total examined milk samples. However, higher recovery rates of *S. aureus* were determined from several countries; Zimbabwe (49.3%)[23], South Ethiopia (51.2%)[24] and Brazil (53%)[5]. In Egypt, variable isolation rates of *S. aureus* from mastitic milk were previously recorded by Elhaig and Selim[25] (38.3%), Elsayed *et al.*[26] (11.2%) and El-Ashker *et al.*[27] (5.6%). Since pasteurization process could eliminate *S. aureus* in milk, yet, the possibility of human infection with *S. aureus* might be attributed to the consumption of raw milk and dairy products particularly homemade cheese[2,28]. Moreover, the

Table 1: Sequences and amplified products size of primers used in polymerase chain reaction assay

Genes	Oligonucleotide sequences (5'-3')	Product size (bp)	References
nuc	GCGATTGATGGTGATACGGTT (F)	270	Oliveira *et al.*[17]
	AGCCAAGCCTTGACGAACTAAAGC (R)		
mecA	TCCAGATTACAACTTCACCAGG (F)	162	Oliveira *et al.*[17]
	CCACTTCATATCTTGTAACG (R)		
blaZ	TACAACTGTAATATCGGAGGG (F)	861	Oliveira *et al.*[17]
	CATTACACTCTTGGCGGTTTC (R)		
coa	CGAGACCAAGATTCAACAAG (F)	500-1000	Himabindu *et al.*[18]
	AAAGAAAACCACTCACATCA (R)		
pvl	ATCATTAGGTAAAATGTCTGGACATGATCCA (F)	433	Lina *et al.*[19]
	GCATCAACTGTATTGGATAGCAAAAGC (R)		
tst	CGTAAGCCCTTTGTTGCTTG (F)	543	Sallam *et al.*[20]
	CCACCCGTTTTATCGCTTGAAC (R)		

Table 2: Cyclic polymerase chain reaction conditions of the different primer sets

			Cycling condition			
Genes	Initial denaturation	No. of cycles	Denaturation	Annealing temperature	Extension	Final extension
nuc	94°C/5 min	35	94°C/30 sec	60°C/1 min	72°C /1 min	72°C/10 min
*mec*A	94°C/5 min	35	94°C/30 sec	56°C/1 min	72°C/1 min	72°C/10 min
blaZ	94°C/5 min	35	94°C/30 sec	57°C/1 min	72°C/1 min	72°C/10 min
pvl	94°C/5 min	35	94°C/30 sec	60°C/1 min	72°C/1 min	72°C/10 min
coa	94°C/5 min	35	94°C/30 sec	54°C/1 min	72°C/1 min	72°C/10 min
tst	94°C/5 min	35	94°C/30 sec	59°C/1 min	72°C/1 min	72°C/10 min

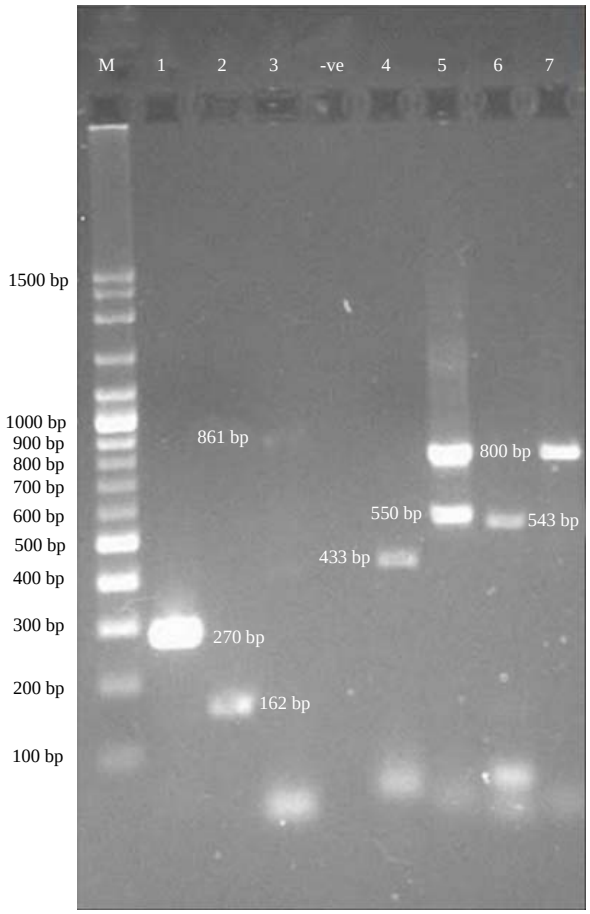

Fig. 1: Representative agarose gel electrophoresis of the expected amplified PCR products for *nuc* (Lane 1, 270 bp), *mec*A (Lane 2, 162 bp), *bla*Z (Lane 3, 861 bp), *pvl* (Lane 4, 433 bp), *coa* (Lane 5 and 7, variable products 500-1000 bp) and *tst* (Lane 6, 543 bp) genes, Lane -ve: Negative control and Lane M: 100 bp DNA ladder

incorporation of milk from mastitic cows into the bulk milk especially in developing countries implies a serious hazard to humans.

The *nuc* gene, which is considered a genetic marker used for the rapid and direct identification of *S. aureus*, was detected using PCR in all the biochemically identified isolates from mastitic milk[29,30]. Coagulase gene encoded by *coa* was also used for the confirmation of the isolated *S. aureus*. In this study, all the recovered *S. aureus* possessed the specific amplified products of *coa* gene (Fig. 1). Coagulase is a significant virulence determinant of *S. aureus* causes coagulation of plasma and its expression is assumed to resist phagocytosis, making the *S. aureus* more virulent[31].

Antimicrobials are considered the best choice for the treatment of mastitis; however, the improper usage of antimicrobial drugs poses great hazards to both human and animal health due to the emergence of antimicrobial resistance[32]. In this study, about 83% of the isolated *S. aureus* showed multidrug resistances to 3 or more antimicrobial agents. The isolated *S. aureus* displayed higher resistance to ampicillin (95.2%) followed by penicillin (83.3%). At the same time, the isolates exhibited lower antimicrobial resistance with gentamicin (23.8%), amikacin (16.7%) and ciprofloxacin (14.3%) (Table 3). Nearly similar results were previously determined by Al-Ashmawy *et al.*[28], Akindolire *et al.*[32] and Prashanth *et al.*[33], who found that the majority of *S. aureus* isolates from milk and dairy products showed higher antimicrobial resistance against penicillin.

The penicillin resistance in *S. aureus* is a well-recognized phenomenon worldwide due to the production of β-lactamases. Of the 70 penicillin-resistant *S. aureus* isolates, genotypic characterization revealed that 67 (95.7%) isolates had *blaZ* gene (Table 4). Similarly, Da Costa Krewer *et al.*[34] reported the higher occurrence of *bla*Z gene among β-lactam resistant *S. aureus* isolated from bovine mastitis in the

Table 3: Antimicrobial resistance of *S. aureus* isolates from mastitic milk

				Resistant		Sensitive	
Antibiotics	Disc codes	Concentrations	Antimicrobial classes	No.	%	No.	%
Amoxicillin	AX	25 µg	β-lactam	54	64.3	30	35.7
Sulfamethoxazole trimethoprim	SXT	23.75/1.25 µg	Potentiated sulfonamides	66	78.6	18	21.4
Gentamicin	CN	10 µg	Aminoglycosides	20	23.8	64	76.2
Ciprofloxacin	CIP	5 µg	Quinolones	12	14.3	72	85.7
Chloramphenicol	C	30 µg	Phenicols	58	69.0	26	31.0
Penicillin	P	10 IU	β-lactam	70	83.3	14	16.7
Rifampin	RA	5 µg	Rifampicin	32	38.1	52	61.9
Amikacin	AK	30 µg	Aminoglycosides	14	16.7	70	83.3
Vancomycin	VA	30 µg	Glycopeptide	64	76.2	20	23.8
Streptomycin	S	10 µg	Aminoglycosides	50	59.5	34	40.5
Tetracycline	TE	30 µg	Tetracycline	44	52.4	40	47.6
Erythromycin	E	15 µg	Macrolides	40	47.6	44	52.4
Ampicillin	AM	15 µg	β-lactam	80	95.2	4	4.8
Oxacillin	OX	15 µg	β-lactam	42	50.0	42	50.0

Table 4: Antimicrobial resistance phenotypes and genotypes of *S. aureus* isolates from mastitic milk

	Strain				
Profiles	No.	%	Resistance phenotypes	Antimicrobial resistance genes	Virulence genes
P1	12	14.3	P,RA,VA,S,TE,E,AM,AX,CIP,CN,SXT,C,OX	*blaZ, mec*	ND
P2	10	11.9	P,RA,VA,S,TE,E,AM,AX,SXT,C,OX	*blaZ, mec*	ND
P3	10	11.9	P,AK,VA,S,TE,E,AM,AX,SXT,C,OX	*blaZ, mec*	ND
P4	8	9.5	P,RA,VA,S,TE,E,AM,AX,CN,SXT,OX	*blaZ,mec*	ND
P5	2	2.4	P,RA,AK,VA,TE,AM,AX,SXT,C,OX	*blaZ, mec*	*tst, pvl*
P6	2	2.4	P,AK,VA,S,AM,AX,SXT,C	*blaZ*	*tst, pvl*
P7	4	4.8	P,VA,S,AM,AX,SXT,C	*blaZ*	ND
P8	2	2.4	P,VA,S,AM,AX,SXT,C	*blaZ*	*pvl*
P9	2	2.4	P,VA,S,AM,AX,SXT,C	*blaZ*	*tst*
P10	2	2.4	P,VA,AM,SXT,C	*blaZ*	*tst*
P11	10	11.9	P,VA,AM,SXT	*blaZ*	ND
P12	2	2.4	P,AM,SXT	*blaZ*	*tst*
P13	3	3.6	P,AM,C	ND	ND
P14	1	1.2	P,AM,C	*blaZ*	ND
P15	10	11.9	AM	ND	ND
P16	4	4.8	ND	ND	ND

ND: Not determined

Northeast of Brazil. This could explain that other mechanisms have a role in the resistance of staphylococci to β-lactams than *blaZ* gene[35].

All the isolated *S. aureus* were screened for the presence of *mec*A gene using PCR where 50% of the isolates harbored the specific amplicon of *mec*A gene at 162 bp (Fig. 1). The *mec*A gene was detected in all the phenotypically identified isolates that resist oxacillin. The existence of *mec*A-positive MRSA in bovine milk has been reported worldwide in many previous studies[36-38]. However, *mec*A-negative MRSA has been also recovered from bovine milk; the resistance revealed by *mec*A-negative MRSA isolates might be attributed to the presence of *mec*A homologues as *mec*C or other β-lactam resistance mechanisms[15-39,40].

The results of disk diffusion test for antibiotic resistance revealed that all MRSA isolates were resistant to 9 or more antimicrobial agents. Likewise, several studies reported that MRSA strains isolated from milk and some dairy products exhibited a multidrug resistance[28,41,42]. The ability of MRSA strains to resist a wide range of antimicrobial agents might be due to production of β-lactamases and PBP2a. Interestingly, all *mec*A-carrying strains were also positive for the *blaZ* gene. The MRSA is commonly known to be originated from humans and transmitted to animals under poor hygienic measures; however the existence of MRSA in milk and animal environment pose a major threat to the consumers and occupational contacts[37,43].

All the isolates were further assessed for the presence of two additional virulence genes, including *tst* and *pvl*. Toxic shock syndrome toxin-1, encoded by *tst* gene is a superantigen secreted by *S. aureus* in susceptible hosts and is responsible for toxic shock syndrome in humans[44]. In this

study, *tst* was detected in 11.9% (10/84) of the tested *S. aureus* isolates. Similarly, in a pan-European survey that involved 12 European countries and examined 456 *S. aureus* isolates from cow milk, 12.9% of the isolates harbored *tst* gene[45]. However, several previous studies revealed the absence of *tst* in milk and other dairy products[28,46].

The specific amplicon of *pvl* gene was detected only in 7.1% of the isolated *S. aureus*. This was opposite to that determined previously in many studies that reported the absence of *pvl* gene in *S. aureus* isolates from animal origin[46-49]. The *pvl* gene, which is responsible for the severe necrotic inflammation in soft tissues and skin, is commonly associated with the community-acquired MRSA (CA-MRSA) strains from humans[50]. Nonetheless, the presence of *pvl* gene in this study from mastitic cows might be attributed to the close contact of animals with its rearing community[33].

CONCLUSION

In conclusion, the existence of *S. aureus* carrying *pvl* gene indicates personnel sources of contamination to dairy farms. Special concern should be considered to prevent this source of contamination through prevention of unauthorized persons from entering dairy farms as well as the adoption of restrict hygienic measures. In addition, high resistance against β-lactams which are widely used in veterinary practice and the presence of MRSA constitutes another burden to the public health. Hence, monitoring the emergence of multidrug resistant strains of *S. aureus* in dairy farms is essential to control the spreading of this pathogen and the related zoonotic hazard.

SIGNIFICANCE STATEMENTS

This study determined the prevalence of *S. aureus* and some of its virulence determinants as well as antimicrobial resistance in mastitic cows from different dairy herds. Monitoring of *S. aureus*, which indicates the improper hygienic measures, in dairy herds is a prerequisite for the initiation of effective infection control measures. The *pvl* is one of *S. aureus* virulence associated genes that many researchers were not able to explore from animal origin. Thus a new principle on the existence of *pvl* from animal sources may be arrived at.

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