Methicillin-Resistant Staphylococcus *Aureus* Causing Bovine Mastitis in Egypt

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Abstract

Increasing the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in bovine milk, characterized by multidrug resistance (MDR) and the presence of resistance genes, poses significant challenges for mastitis treatment and raises public health concerns. This study investigated the antimicrobial resistance profiles of *S. aureus* isolates from clinical and subclinical mastitis cases in Egyptian dairy cattle. A total of 480 milk samples (120 cows, one sample per udder quarter) were analyzed. Mastitis was detected in 32.5% (156/480) of samples, with 27 *S. aureus* isolates recovered. All isolates harbored the *mec*A gene and exhibited resistance to 8–10 antibiotics, with multiple antibiotic resistance (MAR) indices ranging from 0.61 to 0.82. Resistance to erythromycin, oxacillin, and methicillin was universal (100%), while ceftaroline showed the lowest resistance rate at 11.1%. These findings highlight the high risk of MRSA contamination in milk, emphasizing the need for stringent hygiene measures and antimicrobial resistance monitoring in dairy production systems.

Keywords: MRSA, MDR, MAR, bovine mastitis, antimicrobial resistance, *mec*A gene.

Introduction

Bovine mastitis is a major economic burden in dairy production worldwide, leading to reduced milk yield, increased culling rates, and higher veterinary costs ***(Romero et al., 2018)***. Clinical mastitis is characterized by visible signs such as udder swelling, heat, pain, and abnormal milk secretion, whereas subclinical mastitis lacks overt symptoms but is detectable through elevated somatic cell counts (SCC) ***(Hussain et al., 2013)***. *Staphylococcus aureus* is a predominant pathogen in both forms of mastitis and a potential zoonotic agent **(*Rollin et al., 2015*)**.

The development of antimicrobial resistance, particularly in MRSA, complicates mastitis management. MRSA strains resist β-lactam antibiotics through *mec*A mediated production of penicillin-binding protein 2a (PBP2a) ***(Cameron et al., 2016)***, and frequently exhibit cross-resistance to other classes, including aminoglycosides, macrolides, and fluoroquinolones (***Vestergaard et al., 2019***). The spread of such strains is exacerbated by antimicrobials selective pressure and antibiotic misuse in livestock (***Guo et al., 2020***). This study aimed to (i) determine the prevalence of *S. aureus* in bovine mastitis cases in Egypt, (ii) assess the antimicrobial resistance patterns of MRSA isolates from clinical and subclinical mastitis.

Material and methods

Samples Collection:

From October 2018 to April 2020, 480 aseptically collected quarter milk samples were obtained from 120 lactating cows across commercial farms in Sharkia and Dakahlia Governorates, with additional samples obtained from clinical cases referred to the Animal Health Research Institute, Zagazig. Each cow provided four samples (one per udder quarter).The farms had extensive management strategies and hand milking practices; before sample collection, the udder of each animal was palpated for the detection of any abnormalities such as swelling, hotness, asymmetry, or physical changes, then the udder, teats, and hands of the examiner were washed using running water and soap and were dried with a clean towel, were then sterilized with 70% ethyl alcohol to prevent external contamination, the first strips of milk were excluded and thrown away as they may be contaminated from the teat orifice, then 15–20 mL of milk samples were collected from each quarter into sterile screw-capped McCartney tubes (Thermo Fisher Scientific, Waltham, MA, USA). The milk samples were immediately transported in an ice container to the laboratory at the Animal Health Research Institute, Zagazig, Sharkia, Egypt, with minimal delay for SCC and bacteriological examination.

Mastitis Diagnosis:

Fresh collected milk samples were evaluated within one hour of milking using California Mastitis Test (CMT) **(*Quinn et al., 2011*)** and somatic cell count (SCC) **(*Hisira et al., 2023*)**. In CMT, 2 mL of milk sample was mixed with CMT reagent (Chimertech, India) and gently agitated in a four-well plastic paddle, and the SCC was also measured automatically using MT05 somatic cell counter (MT05, Slovakia) after mixing the milk sample (10 mL) with 5 mL of the 20% S4 reagent. The SCC is scored on a scale of 0 to 3, with scores of 2 or 3 considered based on changes in milk viscosity. Samples with SCC

<200.000 cells/mL of milk were considered normal, while ≥250000 cells/mL were considered positive for subclinical mastitis.

Isolation and Identification of *S. aureus* isolates:

Milk samples were centrifuged at 3000 rpm for 5 min, the cream layer was discarded, and sediments were inoculated onto Baird-Parker agar (BPA) (Oxoid, UK), and the characteristic black colonies surrounded by a clear halo zone were subcultured on mannitol salt agar medium (Oxoid, UK) and 5% sheep blood agar. The colonies were identified by Gram Staining, and biochemical tests as (Catalase and Coagulase) **(*Quinn et al., 2011*)**, and for molecular confirmation; the *Sa0836* gene was amplified with specific primers listed in **(Table 1)**. For further examination, the identified isolates were stored at -20 °C in brain heart infusion broth (BHI)(Oxoid, UK) containing 30% glycerol.

Antimicrobial Susceptibility Testing:

Kirby-Bauer disc diffusion technique was used for testing *S. aureus* isolates susceptibility to 12 antibiotics representing 9 classes, according to CLSI guidelines **(*CLSI, 2022*)**, included: amikacin (AK, 30 µg), amoxicillin/clavulanic acid (AMC, 20 µg/10 µg), ceftaroline (CPT, 30 µg), chloramphenicol (C, 30 µg), erythromycin (E, 15 µg), gentamicin (CN, 10 µg), methicillin (ME, 5 µg), norfloxacin (NOR, 10 µg), oxacillin (OX, 30 µg), quinupristin/dalfopristin (QD, 15 µg), tetracycline (TE, 30 µg), and vancomycin (VA, 30 µg). These antimicrobial agents were selected to monitor MDR among isolates (***Magiorakos et al., 2012***). MAR index for each isolate was estimated to assess the level of resistance.

Confirmation the methicillin resistance gene (*mec*A):

Genomic DNA was extracted from 24-h cultures of MRSA isolates in BHI broth using the QIAampDNA Mini Kit (Qiagen, Germany), using the NanoDropTM 1000 spectrophotometer (ThermoFisher Scientific, USA) to measure the quantity and purity of DNA, and using oligonucleotide primers listed in (Table 1) for identification *mec*A gene by PCR (***Spanu et al., 2004***). PCR amplification was carried out in a T3 Thermal cycler (Biometra, Germany) with 5 µL extracted DNA, 12.5 µL 2X EmeraldAmpGT PCR master mix (Takara, Japan), 1 µL (20 pmol) of both forward and reverse primers (Metabion, Germany), and nuclease-free water to a final volume of 25 µL, and the PCR fragments were visualized using agarose gel electrophoresis and ethidium bromide staining.

Table (1): *Oligonucleotide primer sequences used in PCR amplification assays.*

Target gene	Primer	Primer sequence (5'-3')	Amplified segement (bp)	Reference
Sa0836	*S. aureus*-F	TCGAAATTAAATGTTGTCGTGTCTTC	573	**Goto *et al.*, 2007**
	S. aureus-R	TCATTTTTGACATGRAGAGAAACATC		
***mec* A**	*mec*A - 1	GTAGAAATGACTGAACGTCCGATAA	310	**McClure *et al.*, 2006**
	*mec*A - 2	CCAATTCCACATTGTTTCGGTCTAA		

Results

Prevalence of Bovine Mastitis

Mastitis was detected in 39 cases (32.5%) of the samples, with 25% classified as subclinical cases (Table 2).

Isolation of *S. aureus* from Bovine Mastitis

A total of 27 *S. aureus* isolates were recovered, including 8 isolates from clinical mastitis, and 19 isolates from subclinical mastitis (Table 3, Fig. 1).

Antibiotic Susceptibility of *S. Aureus* Isolates

All 27 isolates demonstrated high MAR indices (0.61 to 0.82) and resistance to 8–10 antibiotics. Resistance to erythromycin, oxacillin, and methicillin was universal (100%), while ceftaroline showed the lowest resistance rate at 11.1% (Table 4).

PCR Confirmation

PCR amplification targeting the *Sa0836* gene produced a 573 bp fragment, confirming the identification of *S. aureus* (Fig. 2).

Resistance genes in MRSA isolates

PCR analysis confirmed that every MRSA isolate carried the *mec*A gene (Fig. 3).

Table (2) Incidence of the Mastitis.

Examined Cattle (Total No.)	Total Mastitis (CMT)	SCC No. (%)	
		Subclinical Mastitis	Clinical Mastitis
120	39 (32.5)	30 (25.0)	9 (0,075)

No. = number

Table (3) Incidence of the isolates *S. aureus*.

Mastitis	Mastitis Cases (No.)	The isolates No. (%)
Clinical	9	8 (20.4)
Subclinical	30	19(48.7)
Total	39	27 (69.2)

No.= number

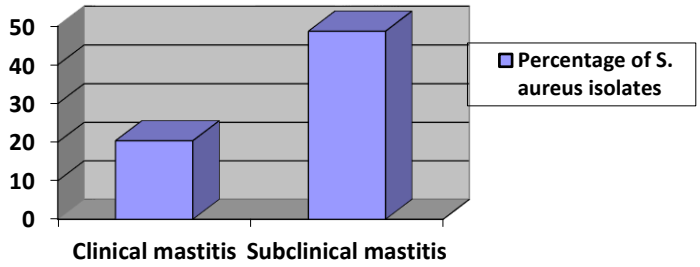

Fig. (1): Frequency of the isolates *S. aureus*.

Table (4): The Antibiotic Susceptibility of S. *Aureus* Isolates.

Class	Antimicrobial Agent	The isolates No. (%)		
		R	I	S
Aminoglycosides	AK	10 (37.1)	8 (29.6)	9 (33.3)
	CN	23 (85.2)	1 (3.7)	3 (11.1)
β-Lactam/ β-Lactamase Ihibitors	AMC	24 (88.9)	0.0	3 (11.1)
Cephalosporins	CPT	3 (11.1)	0.0	24 (88.9)
Fluoroquinolones	NOR	14 (51.9)	1 (3.7)	12 (44.4)
Glycopeptides	VA	23 (85.2)	0.0	4 (14.8)
Macrolides	E	27 (100)	0.0	0.0
Penicillinase - Stable Penicillins	ME	27 (100)	0.0	0.0
	OX	27 (100)	0.0	0.0
Phenicols	C	15 (55.6)	10 (37.0)	2 (7.4)
Streptogramins	QD	12 (44.5)	5 (18.5)	10 (37.0)
Tetracyclines	TE	25 (92.6)	2 (7.4)	0.0

No., number of isolates; I, intermediate; S, sensitive; R, resistant; AMC, moxicillin/clauvalanic acid; AK, amikacin; CN, gentamycin; C, chloroamphenicol; CPT, ceftarolline; E, erythromycin; ME, methicillin; OX, oxicillin; NOR, norfloxacin; QD, quinopristin/ dalfopristin; TE, tetracycline; VA, vancomycin.

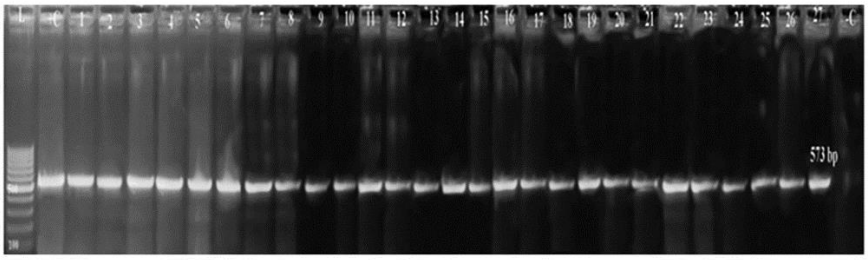

Fig. (2): Agarose gel electrophoresis of the amplified PCR products of *Sa0836* gene at 573 bp. Lane (L) : 100 - 600 bp DNA Ladder, Lanes 1- 27 : positive isolates, Lane +C: Positive control and Lane -C: Negative control.

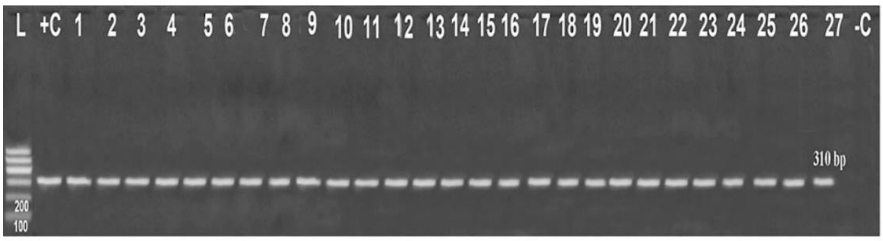

Fig. (3): Agarose gel electrophoresis of the amplified PCR products of *mec*A gene at 310 bp. Lane (L):): 100-600bp DNA Ladder, Lanes 1-27: Positive isolates, Lane +C: Positive control, and Lane -C: Negative control.

Discussion

Bovine mastitis is a significantly important disease, causing a pronounced reduction in milk production and quality, and causing economic losses in the livestock industry, not only in Egypt, but also worldwide **(*Stevens et al., 2016*)**. This study emphasizes the importance of including subclinical mastitis cases, as milk from these animals is often consumed raw or unpasteurized, posing a risk of pathogen transmission to humans and this is a critical public health consideration, especially in regions where raw milk consumption is common.The total prevalence of mastitis in the collected milk samples from each quarters was 32.5% that was comparable to findings from other countries like Ethiopia (39.9%; ***Kitila et al., 2021***), but lower than some figures reported in Egypt (52.1%; ***Algammal et al., 2020*)**, and Uganda (86.2%; ***Abrahmsén et al., 2014*)**. The recovery rate of *S. aureus* (69.2%) was consistent with findings from India (79.71%; ***Neelam et al., 2022***), and was higher than reports from China (46.2%; ***Wang et al., 2018***), but was lower than in Bangladesh (100%; ***Jahan et al., 2015***). Antibiotic resistance has increased among various bacterial pathogens, which is considered an emerging problem with a major public health concern due to the risk of resistance transmission to human as well as its influence on the effectiveness of the current antibiotic therapy(***Vestergaard et al., 2019***). *S. aureus* has gained multidrug resistance, making it easier for it to infiltrate the immune system of the host, MRSA has shown resistance to all types of β-lactam antibiotics currently available for mastitis therapy ***(Vanderhaeghen et al., 2010)***. Therefore, MRSA infection is more difficult to treat with various types of antibiotics and thus more dangerous. The frequency of MRSA among our study was (100%), and markedly greater than rates in China (47.6%; ***Wang et al., 2015***), and in earlier Egyptian studies (67.4%; ***Elfaramawy et al.,***

2019), that may be influenced by sample size, seasonal factors, and regional differences **(*Klibi et al., 2018***). Further, the identified MRSA strains were confirmed using PCR for detection of *mec*A gene, where all the tested strains (100%) harbored this gene. Most MRSA isolates possessed a MDR phenotype and carried many resistant determinants in chromosome and plasmids as the resistance to methicillin is attributed to the existence of *mec*A gene on the *S. aureus* chromosome, which is encoded for the synthesis of PBP2a, and its incidence confirms that these isolates are indeed MRSA, which poses significant treatment challenges and public health risks due to their multidrug-resistant nature **(*Ito et al., 2003*)**.Overall, the high frequency of MDR isolates in the study area has been related to excessive use and imprudent use of antibiotics, including in livestock husbandry, and substandard infection control and prevention practices ***(Garoy et al., 2019)***. Accordingly, governments and individuals should pay attention to prevent further spread of MRSA.

Conclusion

MRSA causes a major hazard to together animal and public health. Routine screening and molecular characterization of MRSA strains are critical for effective control. Implementing stringent hygiene practices and monitoring antibiotic use are imperative for the dairy industry.

Ethics Approval Knowledgeable agreement was found from farm owners, and all measures were permitted by the Institutional Animal Care and Use Committee at Suez Canal University (Ethical approval no.: SZUC 2018119).

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الملخص العربي

المكورات العنقودية الذهبية المقاومة للميثيسيلين المسببة لالتهاب الضرع البقري في مصر

تم إجراء هذه الدراسة على 480 عينة من الحليب، جُمعت من 120 بقرة حلوب، حيث تم أخذ عينات فردية من كل ربع من الضرع. تم جمع العينات من مزارع مختلفة في محافظتي الشرقية والدقهلية، وبلغ معدل انتشار التهاب الضرع في العينات 32.5% (480/156)، منها 25% حالات التهاب ضرع خفي. تم عزل 27 سلالة من المكورات العنقودية الذهبية المقاومة للميثيسيلين من 39 عينة مصابة بالتهاب الضرع، بنسبة 69.2%. أظهرت العزلات خصائص نمو مميزة على أطباق العزل البكتيري، وكانت إيجابية لاختبار التخثر، مما أكد تصنيفها على أنها مكورات عنقودية ذهبية. كما تم التحقق منها بواسطة اختبار تفاعل البلمرة المتسلسل (PCR)، حيث تم الكشف عن جين *Sa0836* بحجم 573 bp. أما فيما يخص اختبار الحساسية للمضادات الحيوية، فقد أظهرت جميع العزلات الـ 27 مقاومة لـ 8 إلى 10 مضادات ميكروبية، حيث تراوح مؤشر المقاومة المتعددة للمضادات (MAR) بين 0.61 و 0.82. بالإضافة إلى ذلك، كانت جميع العزلات تحتوي على جين *mec*A، مما يؤكد مقاومتها للمثيسيلين.