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Virulence traits, *agr* typing, multidrug resistance patterns, and biofilm ability of MDR *Staphylococcus aureus* recovered from clinical and subclinical mastitis in dairy cows

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Abstract

Background Bovine mastitis caused by *Staphylococcus aureus* is considered a public health threat globally. Herein, we aimed to investigate the occurrence, *agr* typing, antimicrobial resistance patterns, biofilm production, and PCR-based detection of the virulence, biofilm, adhesion, and enterotoxins genes of *S. aureus* strains recovered from clinical and subclinical bovine mastitis.

Results The prevalence of *S. aureus* in the examined milk samples was 44.4%. Besides, 95% of the retrieved *S. aureus* strains were identified as MRSA. Herein, all the tested isolates were biofilm producers. PCR revealed that 85% of the retrieved *S. aureus* strains were positive for the *agr* I gene. Furthermore, the *clf*B, *clf*A, *fnb*B, *fnb*A, and *cna* genes were detected with a prevalence of 100%, 80%, 60%, 55%, and 30%, respectively. Also, all the tested *S. aureus* strains were positive for the *coa* gene (100%). Besides, 92.5% and 85% of the recovered strains harbored the *luk*F and *spa* genes, respectively. In addition, the prevalence of the *hla*, *hlb*, and *hlg* hemolysin genes was 70%, 50%, and 35%, respectively. Among the enterotoxin genes, the *seb* gene was detected in 30% of the tested strains. The prevalence of *eno* and *ica*A biofilm genes was 95% in the tested strains. Moreover, 15% of *S. aureus* strains were MDR to 8 antimicrobial agents and harbored the *mec*A, *erm*C, and *erm*B genes. As well, 12.5% of *S. aureus* strains were MDR to 8 antimicrobial agents and carried the *mec*A, *erm*C, *erm*B, *tet*K, and *tet*M genes. Also, 5% of *S. aureus* strains were XDR to 11 antimicrobial agents and carried the *mec*A, *erm*C, and *erm*B genes.

Conclusions The existence of MDR and XDR MRSA strains in bovine milk is a public health hazard. The *mec*A, *erm*C, *erm*B, *tet*K, and *tet*M resistance genes and the *coa, clf*B, *eno, ica*A, *lukF*, *spa*, *clf*A, and *hla* virulence genes are commonly associated with the MDR and XDR MRSA strains. Moreover, the *seb* gene was the predominant enterotoxin gene in the MRSA strains recovered from milk.

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Keywords MRSA, MDR, XDR, *agr* typing, Bovine mastitis, Virulence genes, Biofilm formation, Enterotoxin genes, MSCRAMM region

Background

Mastitis is one of the most vital threats affecting the dairy industry worldwide, causing productive losses by decreasing the quality and quantity of milk, and the recurrence of mastitis may shorten the productive life of affected animals [1]. Mastitis management requires collaboration between management processes (mainly milking) and the control of infectious agents, which are widely distributed in dairy farm environments [2]. Mastitis microorganisms are classified as "contagious" or "environmental" based on their reservoir, source of infection, and transmission mode. The infected mammary glands are the main source of infection and transmission of infectious pathogens during the milking process when clean areas are contaminated by infected milk droplets or when these droplets contaminate milking equipment, milking hands, and towels [3].

Among the variety of bacteria that are responsible for mastitis, *Staphylococcus aureus* (*S. aureus*) is among the major pathogens causing contagious mastitis in dairy farms [2]. There is limited data concerning the overall prevalence and status of *S. aureus* bovine mastitis in Egypt; however, previous investigations recorded a high prevalence of *S. aureus* mastitis (more than 40%) in different regions. [4, 5]. *S. aureus* is the most predominant and economically significant pathogen causing intramammary infection in bovine [6]. The pathogen could gain access to milk either by direct emission from infected mammary glands or by environmental contamination during the handling and processing of milk. Transmission of *S. aureus* frequently takes place during the milking procedures by the milking machine, milker's hands, and contaminated utensils. So, serious *S. aureus* infections are commonly associated with inadequate milking hygiene [7, 8].

S. aureus is one of the most important pathogenic bacteria because of the combination of toxin-mediated virulence, biofilm formation, antibiotic resistance, and invasion [9]. Communication between bacteria through quorum sensing is important for many cellular actions, such as biofilm formation, antibiotic resistance, sporulation, and the regulation of a set of virulence factors [10].

The agr (accessory gene regulator) system is a peptide quorum-sensing system found in all *Staphylococci* and is a dominant regulator of pathogenesis and biofilm development in *S. aureus* [11]. *S. aureus* strains have been divided into four agr specificity groups (I, II, III, and IV) [12]. *S. aureus* carries an arsenal of virulence factors that are responsible for pathogenesis via tissue adhesion, immune evasion, and host cell injury [13]. Microbial surface components recognizing adhesive matrix molecules (MSCRAMM) are a group of adhesin proteins that mediate the initial attachment of bacteria to host tissue, providing a critical step in establishing infection [14]. These proteins include fibronectin-binding proteins (FnbA and FnbB), fibrinogen-binding proteins (ClfA, ClfB, and Efb), capsule proteins (Capsule types 5 and 8), and collagen-binding proteins (Cna) that can bind to a variety of mammalian extracellular proteins and abiotic surfaces. These structures can bind to molecules such as collagen (mostly via Cna), fibronectin (via FnbA, B), and fibrinogen (with ClfA, B, and Fib) and thus evade the immune system and can then lead to infection. ClfA is the major staphylococcal fibrinogen (Fg) binding protein, and is responsible for the observed clumping of *S. aureus* in blood plasma, culminating in arthritis and endocarditis [15].

Other virulence factors encoded by different virulence genes play roles in infection by overcoming the immune system and causing host cell injury, such as *Staphylococcus* protein A on the cell wall (spa), Panton-Valentine leukocidin (PVL), hemolysins (HLs), *Staphylococcus* enterotoxins (SEs) and toxic shock syndrome (TSS) [16]. SpA plays a vital role in host immune evasion by altering innate and adaptive immune responses against *S. aureus* by inhibiting the classical complement pathway, blocking opsonophagocytosis, and inducing programmed B-cell death [13, 17] Moreover, PVL is one of the most potent, essential, and prevalent toxins and acts as a pore-forming leukotoxin that promotes the lysis and apoptosis of leukocytes [18]. Besides, *Staphylococcus* hemolysins are considered substantial virulence factors that confer bacterial invasion and escape from the host immune system [19].

Staphylococcal enterotoxins (STEs) are extracellular toxins (A, B, C, D, and E) that cause food poisoning with symptoms of vomiting, nausea, abdominal cramps, and diarrhea [20]. Together with the increase in the pathogenicity of *S. aureus*, the dramatic increase in antimicrobial resistance is a major warning that is considered one of the greatest challenges to global health and food security today [21]. According to the WHO, MRSA strains are coined 'superbugs', and more research and development of new antibiotics are urgently needed. Genetically encoded antimicrobial resistance can be considered a subtype of virulence,

as it increases host pathogenesis, allowing chronic or persistent disease [22]. Many factors are involved in the selective response of bacteria to antibiotic pressure, such as quorum sensing, adhesion, and biofilm development [23]. The regulation, expression, transfer, and exchange of virulence and antimicrobial resistance genes are mediated by biofilm formation [23]. This study aimed to investigate the occurrence, *agr* typing, antimicrobial resistance patterns, biofilm production, and PCR-based detection of the virulence, biofilm, adhesion, and enterotoxin genes of *S. aureus* strains recovered from clinical and subclinical bovine mastitis.

Materials and methods

Sampling and California mastitis test (CMT)

A total of 352 milk samples (320 apparently healthy quarter milk samples and 32 clinical mastitis milk samples) were collected from 112 dairy cows (80 apparently healthy cows and 32 clinically mastitis cows) from private farms in Damietta Governorate, Egypt (from January 2021–March 2021). Cows were clinically examined, and animals suffering clinical mastitis displayed reddish udder, increased udder temperature, udder pain, and bloody milk. The examined cows were 3–5 years old. Samples were collected during the mid-lactation. There was a history of using tetracycline, erythromycin, and amoxicillin-clavulanic acid.

The California mastitis test (CMT) was performed for the detection of subclinical mastitis in the apparently healthy quarter milk samples as described by Clements [24]. Milk samples were collected from each quarter after cleaning and disinfection of the udder with 70% ethanol. The milk samples were discarded, and the milk samples were collected from each quarter in sterile McCartney bottles and transported within 4–6 h in a cooler box maintained at 4–6 °C to the laboratory for bacteriological analysis.

Bacteriological examination

The milk samples were incubated for 24 h at 37°C and then centrifuged at 3000 rpm for 20 min, after which the cream and supernatant were discarded. A loopful of sediment was streaked on the surface of nutrient agar, blood agar, and mannitol salt agar (Oxoid, Hampshire, UK) and incubated aerobically at 37°C for 24–72 h. The suspected colonies were identified morphologically and biochemically, as described by Quinn [25]. The identification of the recovered isolates was confirmed via PCR detection of the 16Sr RNA gene, according to Monday [26]. Moreover, the identification of MRSA strains is performed based on cefoxitin resistance and the detection of the *mec*A gene [27].

Biofilm formation assay

The tube method was used as described by Christensen [28] for qualitative assessment of biofilm production. Briefly, BHI broth with 2% sucrose (10 ml) in plastic tubes was inoculated with a loopful of the isolates and incubated aerobically at 37°C for 24 h. The tubes were subsequently washed once with 9 ml of phosphate-buffered saline (pH 7.2). The biofilms were fixed with 10 ml of freshly prepared sodium acetate (2%) and stained with crystal violet (0.1%). The tubes were left at room temperature for 30 min, after which the stain was discarded. The washing step was repeated, and the tubes were left to dry in an inverted position at room temperature. Biofilm formation was detected by the presence of visible film on the wall and bottom of the tube. Isolates were scored as 0 for absence, + for weak, + + for moderate, and + + + for strong biofilm formation. The experiments were performed in triplicate and repeated three times.

Antimicrobial susceptibility testing of *S. aureus* isolates

The disc diffusion method was performed using 13 antimicrobial agents: cefoxitin (CFX; key of MRSA) (30 µg), ceftriaxone (CRO) (30 µg), ceftazidime (CAZ) (30 µg), amoxicillin-clavulanic acid (AMC) (20/10 µg), ampicillin-sulbactam (ASM) (10/10 µg), penicillin (P) (10 µg), levofloxacin (LEV) (5 µg), clindamycin (CD) (2 µg), chloramphenicol (C) (30 µg), rifampicin (RA) (5 µg), tetracycline (TE) (30 µg), gentamycin (GM) (10 µg), and erythromycin (E) (15 µg) (Oxoid). The antibacterial susceptibility test and interpretations of the inhibitory zones were performed according to the recommendations of the Clinical Laboratory Standard Institute (CLSI) [29]. The reference strain was supplied by the Animal Health Research Institute, Dokki, Egypt. The classification of isolates into multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains was performed according to Magiorakos [30]. The multiple antibiotic resistance (MAR) index was measured according to Blasco [31].

agr typing and PCR detection of virulence and antibiotic resistance genes

PCR was performed for to identify *S. aureus* via amplification of the 16S rRNA, *agr* typing of *S. aureus* (I, II, III, and IV), and detection of virulence and antimicrobial resistance genes. The nucleotide sequences of the primers used were listed in the supplementary Table S1 and the cycling conditions were illustrated in the supplementary Table S2. All primers were supplied by Metabion (Germany). The genomic bacterial DNA was extracted according to the QIAamp DNA Mini Kit instructions (QIAGEN, Germany), and the amount and quality of the DNA samples were measured using a NanoDrop ND-1000 spectrophotometer. All the reactions were

performed in triplicate and adjusted to a final volume of 25 µL (12.5 µL of Emerald Amp GT PCR Master Mix (Takara, Code No. RR310A kit), 1 µL of each reverse and forward primer, 5 µL of DNA template, and 5.5 µL of ultrapure PCR grade water). Reactions without template DNA (Negative controls) and positive control *S. aureus* strains (provided by Animal Health Research Institute, Egypt) were used. Amplified PCR products were detected by horizontal 1.5% (w/v) agarose gel electrophoresis (Applichem, Germany, GmbH), and the gel was photographed using the gel documentation system (Alpha Innotech, Biometra).

Statistical analyses

The Chi-square test was used in the analysis of the retrieved frequencies via SPSS software (version 16, SPSS Inc., Chicago, USA). Besides, Fisher's exact two-tailed test was used to determine the significance of the associations between resistance patterns, genotypic resistance, and biofilm formation. The differences were considered significant at values of $p < 0.05$. Moreover, correlation analysis was also carried out among the antibiotic resistance genes and the tested antibiotics (corrplot package, R-software; version 4.0.2; https://www.r-project.org/).

Results

Distribution of *S. aureus* among the examined milk samples

According to the CMT, the prevalence of subclinical mastitis in apparently normal quarter milk samples was 18.12% (58/320). *S. aureus* was isolated from subclinical mastitic and clinical mastitic milks samples with percentages of 46.55% (27/58) and 40.63% (13/32), respectively. The overall prevalence of *S. aureus* in the bacteriologically examined milk samples was 44.4% (40/90). A significant difference was recorded in the prevalence of *S. aureus* in subclinical mastitic and clinical mastitic milk samples ($p < 0.05$).

Antibiogram profiles of the retrieved *S. aureus* isolates

With respect to the susceptibility of the recovered isolates, the tested *S. aureus* isolates displayed a remarkable resistance to ceftazidime and ceftriaxone (100%, 40/40), followed by clindamycin (97.5%, 39/40), cefoxitin (95%, 38/40), penicillin (92.5%, 37/40), erythromycin (35/40, 87.5%), ampicillin-sulbactam (67.5%, 27/40), chloramphenicol (47.5%, 19/40), tetracycline (45%, 18/40), and amoxicillin-clavulanic acid (40%, 16/40).. Moreover, the retrieved *S. aureus* isolates revealed a notable sensitivity to rifampicin (90%, 36/40), gentamycin (85%, 34/40), and levofloxacin (77.5%, 31/40) (as illustrated in Table 1).

In this study, 95% (38/40) of the retrieved isolates were identified as MRSA (resistant to cefoxitin and harboring the *mec*A gene). Besides, there was a significant variance

Table 1 Antibiogram profiles of the recovered *S. aureus* strains

Antimicrobial class	Antimicrobial agents	*S. aureus* (*n* = 40)			
		Resistant		Sensitive	
		n	%	*n*	%
Penicillins	Penicillin G (P)	37	92.5	3	7.5
Cephalosporins	Cefoxitin (CFX)	38	95	2	5
	Ceftriaxone (CRO)	40	100	0	0
	Ceftazidime (CAZ)	40	100	0	0
β-Lactam-β-lactamase inhibitor combination	Amoxicillin-clavulanic acid (AMC)	16	40	24	60
	Ampicillin -sulbactam (ASM)	27	67.5	13	32.5
Fluoroquinolones	Levofloxacin (LEV)	9	22.5	31	77.5
Aminoglycosides	Gentamycin (GM)	6	15	34	85
Macrolides	Erythromycin (E)	35	87.5	5	12.5
Phenicols	Chloramphenicol(C)	19	47.5	21	52.5
Lincosamides	Clindamycin(CD)	39	97.5	1	2.5
Ansamycin	Rifampicin (RA)	4	10	36	90
Tetracycline	Tetracycline (TE)	18	45	22	55
Chi-square *P* value		90.616 $P < 0.0001$		154.8 $P < 0.0001$	

in the susceptibility of the retrieved *S. aureus* isolates to various tested antibiotics ($p < 0.05$).

Biofilm production by *S. aureus* isolates

Biofilm production was evaluated among the retrieved isolates, where 100% (40/40) of the isolates were positive for biofilm formation via the tube method. Among the biofilm producers ($n = 40$), three isolates were weak biofilm producers (7.5%), eleven isolates (27.5%) were moderate biofilm producers, and twenty-six isolates (65%) were strong biofilm producers (as described in Fig. 1).

agr genotyping and the distribution of virulence and antimicrobial resistance genes in *S. aureus* strains

PCR revealed that 85% of the retrieved *S. aureus* strains were positive for the *agr*I gene. In addition, the *agr*II and *agr*III genes were present among the tested MRSA strains with a prevalence of 10% and 5%, respectively, while no isolates harbored the *agr*IV gene, as illustrated in Table 2 and Fig. 2.

With respect to the adhesion genes, the *clf*B, *clf*A, *fnb*B, *fnb*A, and *cna* genes were detected with a prevalence of 100%, 80%, 60%, 55%, and 30%, respectively, as shown in Table 2 and Fig. 2.

Herein, all the tested *S. aureus* strains were positive for the *coa* gene (100%). Besides, 92.5% and 85% of the recovered strains harbored the *luk*F and *spa* genes, respectively. Moreover, the prevalence of the *hla*, *hlb*, and *hlg* hemolysin genes was 70%, 50%, and 35%, respectively. Among the enterotoxin genes, the *seb* gene was detected in 30% of the tested strains, whereas 25% of isolates harbor the *sea* gene. Regarding the biofilm genes, the prevalence of *eno* and *ica*A was 95% in the tested strains. Furthermore, all the retrieved *S. aureus* isolates harbor more than three virulence genes, 10% carrying twelve virulence genes, 7.5% carrying eleven virulence genes, 5% carrying ten virulence genes, and 27.5% carrying nine virulence genes. Only one isolate harbor all the virulence genes, and three isolates harbor thirteen virulence genes (Table 2 and Fig. 2).

Concerning the distribution of antimicrobial resistance genes, the tested *S. aureus* strains carried the *mec*A, *erm*B, *erm*C, *tet*K, and *tet*M genes with a prevalence of 95%, 97.5%, 92.5%, 62.5%, and 45%, respectively. Moreover, the *mec*A gene was detected in 38 isolates (95%), which were cefoxitin-resistant (MRSA strains) (as described in Table 2 and Fig. 2). Based on a dendrogram and hierarchical clustering, the isolates were classified into twelve clusters according to the dissemination of *agr* typing, antimicrobial resistance genes, and virulence genes (as illustrated in Fig. 3).

Table 2 Virulence, resistance genes, and *agr* typing of the retrieved *S. aureus* strains

Type	Gene	Positive isolates		Chi-square *P* value
		n	%	
Confirmatory	16 S rRNA	40	100	
agr typing	*agr*I	34	85	77.6 $p < 0.001$
	*agr*II	4	10	
	*agr*III	2	5	
	*agr*IV	0	0	
Coagulase gene	*coa*	40	100	**96.306** $p < 0.001$
Adherence genes	*clf*A	**32**	**80**	
	*clf*B	40	100	
	can	12	30	
	*fnb*B	24	60	
	*fnb*A	22	55	
Protein A gene	*spa*	34	85	
Panton–Valentine leucocidin	*luk*F	37	92.5	
Hemolysin genes	*hla*	28	70	
	hlb	20	50	
	hlg	14	35	
Biofilm genes	*eno*	38	95	
	*ica*A	38	95	
Enterotoxin genes	*sea*	10	25	
	seb	12	30	
Resistance genes	*mec*A	38	95	**11.248** 0.02391
	*erm*B	39	97.5	
	*erm*C	37	92.5	
	*tet*K	25	62.5	
	*tet*M	18	45	

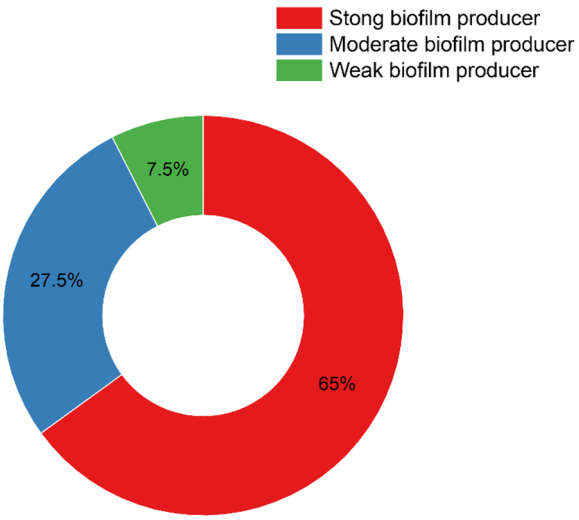

Fig. 1 Biofilm production of the recovered *S. aureus* isolates from milk

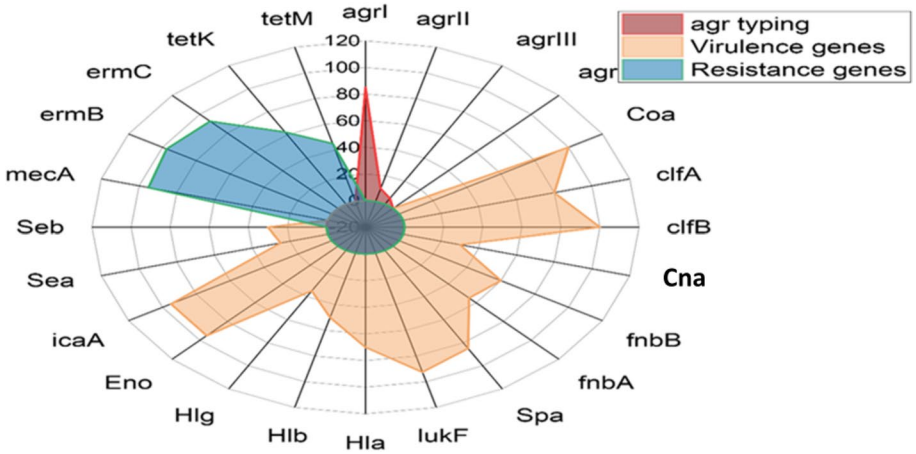

Fig. 2 *agr* typing and the distribution of virulence and antimicrobial resistance genes among the retrieved *S. aureus* isolates

Correlation between phenotypic and genotypic multidrug resistance

In this study, 15% (6/40) of *S. aureus* strains were MDR to 8 antimicrobial agents (five antimicrobial classes) and harbor the *mec*A, *erm*C, and *erm*B genes. Besides, 12.5% (5/40) of the *S. aureus* strains were MDR to 8 antimicrobial agents (6 classes) and carried the *mec*A, *erm*C, *erm*B, *tet*K, and *tet*M genes. Moreover, 5% (2/40) of the *S. aureus* strains were XDR to 11 antimicrobial agents (8 classes) and carried the *mec*A, *erm*C, and *erm*B genes. Additionally, the remaining isolates were MDR or XDR to 5–11 antimicrobial agents (as shown in Table 3 and Fig. 4). The MAR index values for the MDR and XDR *S. aureus* isolates ranged between 0.38 and 0.85 (>0.2), reflecting that the tested MDR *S. aureus* had recovered from a high-risk origin. Statistically, strong positive correlations were recorded between the *mec*A gene and CFX; *erm*C and E (r = 1); *erm*B and E (r = 0.99); *tet*M and TE (r = 1); and *tet*K and TE (r = 0.84) (as described in Fig. 5).

Association between the phenotypic antibiotic resistance pattern and biofilm formation

All strong biofilm-producing isolates (100%) were resistant to ceftriaxone and ceftazidime and were resistant to penicillin, clindamycin, and cefoxitin (96.15%). A high percentage of strong biofilm-producing isolates (88.46%) were resistant to erythromycin, followed by ampicillin-sulbactam (73.07%) (as shown in Table 4). There is a strong positive correlation between biofilm formation and antimicrobial resistance. Fisher's exact test revealed a significant correlation between strong biofilm formation and resistance to penicillin, cefoxitin, ceftriaxone, ceftazidime, erythromycin, and clindamycin ($p < 0.05$).

Associations between virulence genes, antibiotic resistance genes, and biofilm production

All strong biofilm-producing isolates (100%) harbor the *ica*A, *eno*, and *clf*B adhesion genes. Fisher's exact test revealed a significant relationship between strong biofilm producers and the occurrence of the *eno*, *ica*A, *sea*, *clf*A, *clf*B, *luk*F, and *hlb* genes ($P < 0.05$). However, there was no significant relationship between biofilm formation and the presence of other virulence genes ($P > 0.05$). All strong biofilm-producing isolates (100%) carried the *erm*B gene and almost harbored the *mec*A gene (96.15%). A high percentage of strong biofilm-producing strains (92.3%) carried the *erm*C gene (as illustrated in Table 5). Fisher's exact test revealed a significant correlation between the occurrence of antibiotic resistance genes (*mec*A, *erm*B, *erm*C, *tet*K, and *tet*M) and strong biofilm formation ($p < 0.05$).

Discussion

In this study, the prevalence of *S. aureus* was 46.55% and 40.63% in subclinical mastitic and clinical mastitic milk samples, respectively. In the present study, the total prevalence of *S. aureus* is nearly similar to a previous study in Egypt [5], which reported that the prevalence of *S. aureus* was 41.5%. Moreover, a lower prevalence (28.92%) was reported in cows suffering from mastitis in private farms in Giza Governorate, Egypt [32]. The high prevalence of *S. aureus* may be attributed to Milker's hands and contaminated milk utensils [7, 8]. A previous study in Brazil [9] reported that the prevalence of *Staphylococci* was 19.9% in the examined subclinical mastitic milk samples; of them, 68% were identified as *S. aureus*. Moreover, a previous investigation in China [19] reported that 65 *S. aureus* isolates (77.3%) were recovered from 84

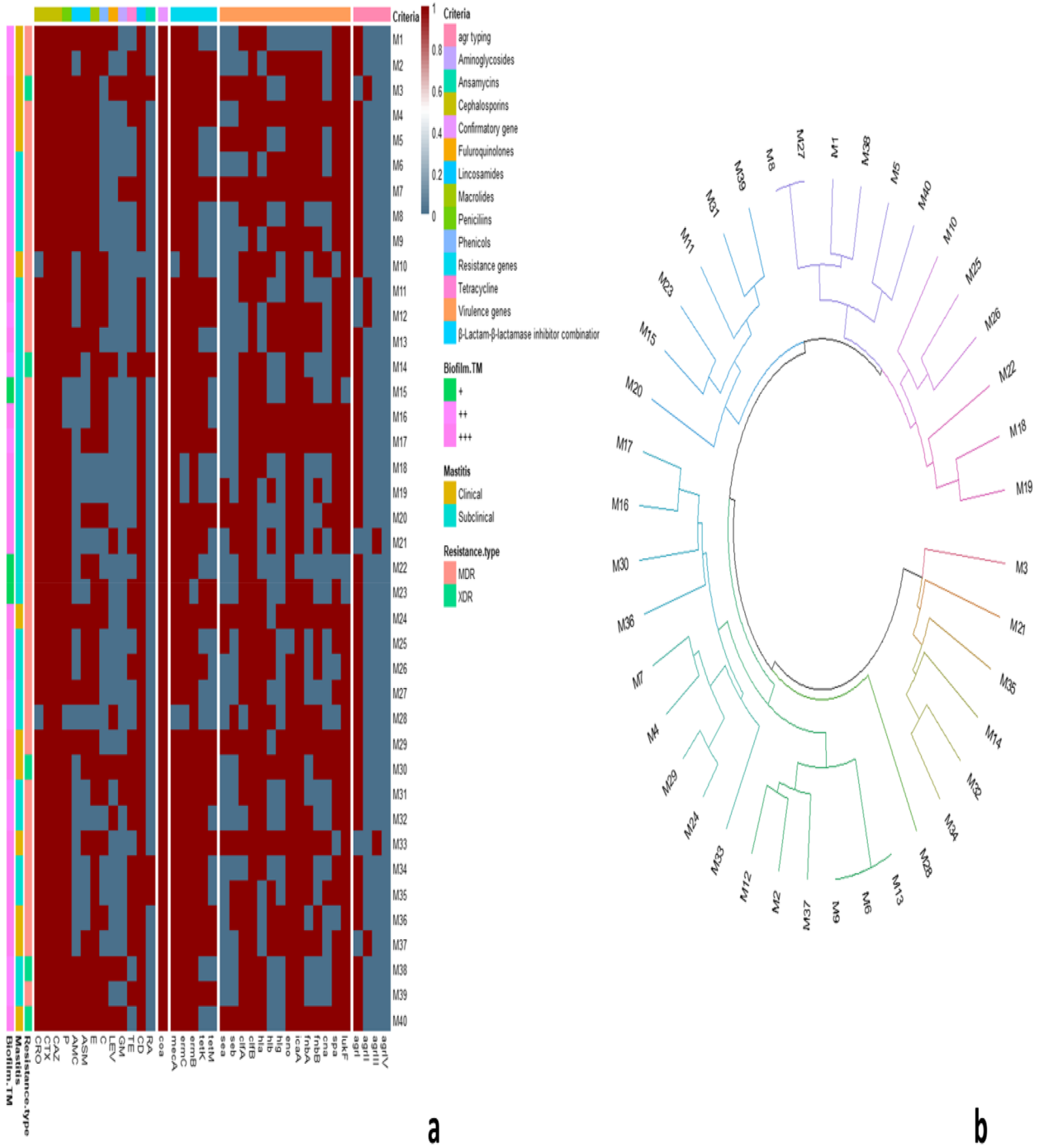

Fig. 3 **a** Heatmap showing the distribution of *agr* typing, multidrug-resistant phenotypes, antimicrobial resistance genes, and virulence genes in the recovered *S. aureus* isolates from milk. **b** Twelve clusters are illustrated in a dendrogram

bacteriologically examined quarter milk samples. In the present study, milk samples were obtained from clinically and subclinically mastitic cows. Approximately two-thirds of the *S. aureus* isolates originated from subclinical mastitis cases, whereas the remaining isolates were retrieved from cases showing clinical mastitis. In contrast, *S. aureus* isolates from animals suffering from clinical and subclinical mastitis were evenly distributed in other studies [33]. Host–pathogen interactions detect the form of mastitis, either clinical or subclinical, where

Table 3 Phenotypic and genotypic resistance patterns of the retrieved *S. aureus* isolates ($n=40$)

S. aureus		Type		Resistance patterns	Genotypic resistance	MARI
n	%					
6	15	MDR	MRSA	8 Antimicrobial agents/ 5 Classes: CFX, CRO, CAZ, P, AMC, ASM, E, CD	*mec*A, *erm*C, *erm*B	0.62
5	12.5	MDR	MRSA	8 Antimicrobial agents/ 6 Classes: CFX, CRO, CAZ, P, ASM, E, C, CD	*mec*A, *erm*C, *erm*B, *tet*K, *tet*M	0.62
2	5	XDR	MRSA	11Antimicrobial agents/ 8 Classes: CFX, CRO, CAZ, P, ASM, AMC, E, C, LEV, GM, CD	*mec*A, *erm*C, *erm*B	0.85
2	5	MDR	MRSA	10 Antimicrobial agents/ 7 Classes: CFX, CRO, CAZ, P, ASM, AMC, E, C, TE, CD	*mec*A, *erm*C, *erm*B, *tet*K, *tet*M	0.77
2	5	MDR	MRSA	8 Antimicrobial agents/ 6 Classes: CFX, CRO, CAZ, P, E, TE, CD, RA	*mec*A, *erm*C, *erm*B, *tet*K	0.62
2	5	MDR	MRSA	6 Antimicrobial agents/ 4 Classes: CFX, CRO, CAZ, E, C, CD	*mec*A, *erm*C, *erm*B, *tet*K	0.46
2	5	MDR	MRSA	5 Antimicrobial agents/ 3 Classes: CFX, CRO, CAZ, P, CD	*mec*A, *erm*B	0.38
2	5	MDR	MRSA	7 Antimicrobial agents/ 5 Classes: CFX, CRO, CAZ, P, TE, LEV, CD	*mec*A, *erm*C, *erm*B, *tet*K	0.54
2	5	MDR	MRSA	10 Antimicrobial agents/ 7 Classes: CFX, CRO, CAZ, P, AMC, ASM, E, TE, CD	*mec*A, *erm*C, *erm*B, *tet*K, *tet*M	0.77
2	5	MDR	MRSA	7 Antimicrobial agents/ 5 Classes: CFX, CRO, CAZ, P, ASM, E, CD	*mec*A, *erm*C, *erm*B	0.54
1	2.5	MDR	MRSA	8 Antimicrobial agents/ 6 Classes: CFX, CRO, CAZ, P, ASM, E, CD, TE	*mec*A, *erm*C, *erm*B, *tet*K, *tet*M	0.62
1	2.5	MDR	MRSA	8 Antimicrobial agents/ 6 Classes: CFX, CRO, CAZ, P, E, C, TE, CD	*mec*A, *erm*C, *erm*B, *tet*K	0.62
1	2.5	MDR	MRSA	9 Antimicrobial agents/ 6 Classes: CFX, CRO, CAZ, P, E, C, TE, CD	*mec*A, *erm*C, *tet*K, *tet*M	0.69
1	2.5	XDR	MRSA	11 Antimicrobial agents/ 9 Classes: CFX, CRO, CAZ, P, ASM, E, C, LEV, GM, TE, CD	*mec*A, *erm*C, *erm*B, *tet*K, *tet*M	0.85
1	2.5	MDR	MSSA	5 Antimicrobial agents/ 4 Classes: CRO, CAZ, ASM, LEV, CD	*erm*B	0.38
1	2.5	MDR	MRSA	8 Antimicrobial agents/ 6 Classes: CFX, CRO, CAZ, P, ASM, E, CD, TE	*mec*A, *erm*C, *erm*B, *tet*K, *tet*M	0.62
1	2.5	MDR	MRSA	9 Antimicrobial agents/ 7 Classes: CFX, CRO, CAZ, P, ASM, E, C, TE, CD	*mec*A, *erm*C, *erm*B, *tet*K, *tet*M	0.69
1	2.5	XDR	MRSA	11 Antimicrobial agents/ 8 Classes: CFX, CRO, CAZ, P, AMC, E, C, LEV, TE, CD, RA	*mec*A, *erm*C, *erm*B, *tet*K, *tet*M	0.85
1	2.5	MDR	MSSA	7 Antimicrobial agents/ 6 Classes: CRO, CAZ, P, ASM, E, GM, C	*erm*C, *erm*B	0.53
1	2.5	MDR	MRSA	10 Antimicrobial agents/ 7 Classes: CFX, CRO, CAZ, P, AMC, ASM, E, GM, TE, CD	*mec*A, *erm*C, *erm*B, *tet*K, *tet*M	0.77
1	2.5	XDR	MRSA	11 Antimicrobial agents/ 8 Classes: CFX, CRO, CAZ, P, AMC, ASM, E, RA, TE, GM, CD	*mec*A, *erm*C, *erm*B, *tet*K, *tet*M	0.85
1	2.5	MDR	MRSA	9 Antimicrobial agents/ 7 Classes: CFX, CRO, CAZ, P, E, LEV, C, TE, CD	*mec*A, *erm*C, *erm*B, *tet*K, *tet*M	0.69
1	2.5	MDR	MRSA	9 Antimicrobial agents/ 7 Classes: CFX, CRO, CAZ, P, AMC, E, C, LEV, CD	*mec*A, *erm*C, *erm*B	0.69

subclinical mastitis is the most common form in all dairy animals and is responsible for greater economic losses. *S. aureus* is responsible for nearly one-third of subclinical mastitis cases [34].

According to the WHO, numerous types of bacteria are rapidly developing resistance to antimicrobial agents, and these bacteria can be categorized into three priority groups, where methicillin-resistant *S. aureus* (MRSA) has been categorized as a priority 2 pathogen [35]. In developing countries, competent authorities do not sufficiently control antimicrobial agent use in animals and humans, so resistant strains of *S. aureus* are frequently isolated

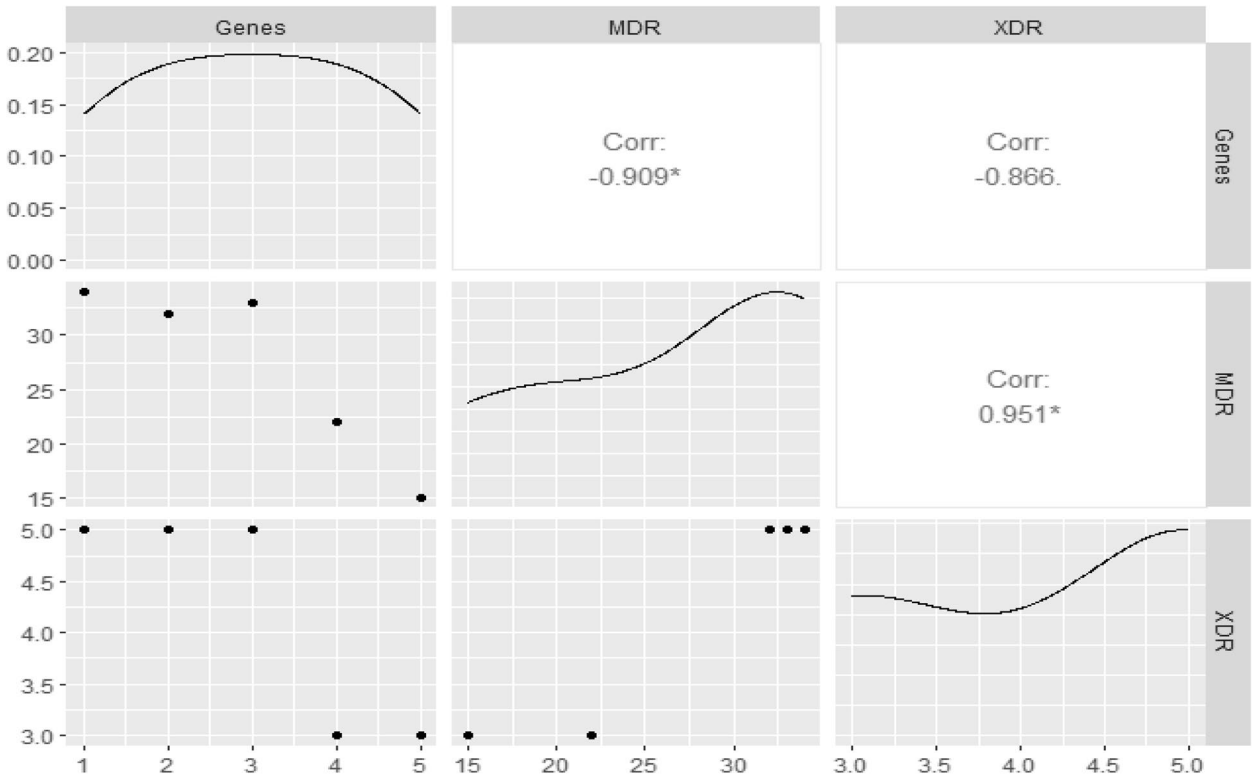

Fig. 4 The multidrug resistance patterns of the retrieved *S. aureus* strains from milk

from different cases [36]. Monitoring the virulence of livestock-associated MRSA is extremely important for public health. In this study, about 95% of the retrieved isolates were identified as MRSA. In Egypt, Tartor [5] identified all the recovered *S. aureus* isolates from mastitic milk as MRSA strains (100%) and Selim [4] reported that the prevalence of MRSA was 35.7%. Besides, El Faramaway [37] reported that the prevalence of MRSA was 67.4%. The differences in the prevalence may be attributable to the differences in sample size, seasons, and geographical area [4]. There have been extensive reports of antibiotic resistance recently, and all microorganisms exhibit varying degrees of resistance to the vast majority of antimicrobial agents. This poses a serious threat to the health of both humans and animals [16]. In this study, most *S. aureus* isolates (45%–100%) presented resistance to penicillin G, cefoxitin, clindamycin, ceftriaxone, ceftazidime, tetracycline, and erythromycin. Previous investigations revealed similar *S. aureus* resistance patterns [15, 38, 39]. *S. aureus* presented the highest penicillin G resistance rate of 92.5%. Algeria [40], Ethiopia [36], and China [41] presented penicillin resistance rates ranging from 76–91.3%. Penicillin resistance in *S. aureus* puts public health at risk, highlighting the need for strict regulation of animal infection treatment with these medications. Penicillin resistance may be more common than resistance to other antibiotics due to the susceptibility of *S. aureus* to β-lactam drugs [42]. This significant resistance in the current study may be due to Egypt's long-standing and indiscriminate use of these medications to treat dairy cow mastitis [43]. Additionally, 60% of the *S. aureus* strains were sensitive to amoxicillin-clavulanic acid, and 32.5% were sensitive to ampicillin-sulbactam. Although β-lactam antibiotics such as sulbactam and clavulanic acid exhibit limited bactericidal effects, they are potent β-lactamase inhibitors [44]. *S. aureus* strains were 45% tetracycline resistant in the current investigation, which aligns with the findings of previous studies [43, 45]. The prolonged use of broad-spectrum antibiotics, such as tetracycline, has led to the emergence of resistant bacteria. The ribosomal protection protein interferes with the resistance of *S. aureus* to tetracyclines. In India, tetracycline resistance was found in 56.2% of bovine raw milk [46]. A high percentage of cephalosporin-resistant strains were also reported by Chukwu [47]. Its low sensitivity to levofloxacin and gentamycin is similar to that of Feyissa [48].

In this investigation, the majority of the retrieved strains were MDR and 12.5% were XDR, indicating widespread antimicrobial resistance. Similarly, a previous study in Egypt reported that the majority of the retrieved *S. aureus* isolates were MDR and 14.8% were XDR [5],

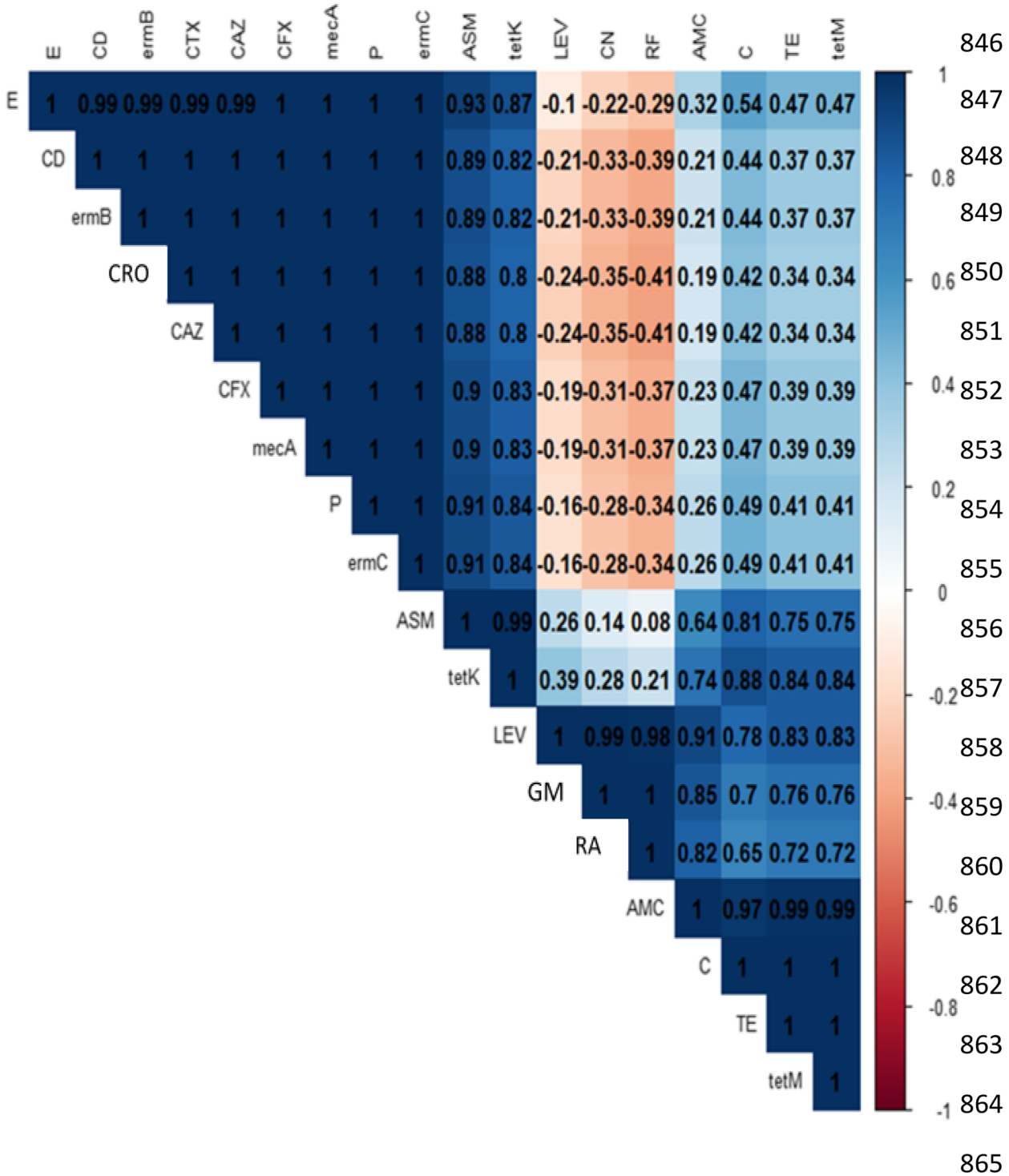

Fig. 5 Correlation between antibiotic resistance genes and the tested antibiotics

and a previous study in Malaysia reported a high prevalence of multidrug resistance, at 65.6% [49]. Moreover, a previous study reported a high prevalence of MDR *S. aureus* strains in Egypt (73%) [50]. The difference in the prevalence of MDR strains may be attributed to variations in geographical areas, seasons, and sample size [5]. The inappropriate use of antibiotics to treat animal diseases in the country may explain our study's increased

Table 4 Phenotypic antibiotic resistance patterns among *S. aureus* strains on the basis of biofilm production

Antibiotics	Biofilm producers		
	Strong biofilm producer	Moderate biofilm producer	Weak biofilm producer
Penicillin G	25 (96.15%)	10 (90.9%)	2 (66.66%)
Cefoxitin	25 (96.15%)	10 (90.9%)	3 (100%)
Ceftriaxone	26 (100%)	11 (100%)	3 (100%)
Ceftazidime	26 (100%)	11 (100%)	3 (100%)
Amoxicillin-clavulanic acid	11 (42.3%)	5 (45.45%)	0 (0%)
Ampicillin–sulbactam	19 (73.07%)	7 (63.63%)	1 (33.33%)
Levofloxacin	4 (15.38%)	5 (45.45%)	0 (0%)
Gentamycin	4 (15.38%)	2 (18.18%)	0 (0%)
Erythromycin	23 (88.46%)	9 (81.81%)	3 (100%)
Chloramphenicol	9 (34.61%)	8 (72.72%)	2 (66.66%)
Clindamycin	25 (96.15%)	11 (100%)	1 (33.33%)
Rifampicin	3 (11.53%)	1 (100%)	0 (0%)
Tetracycline	12 (46.15%)	5 (9.09%)	3 (100%)

MDR rate. In the present study, resistance to eight antibiotic classes was recorded, creating public health concerns. Handling and eating tainted milk and milk products could spread these resistant strains to people. Multidrug resistance complicates infection management, increases treatment failure, and prolongs hospital stays, resulting in poor patient outcomes and increased healthcare expenses for people and animals [51].

The Centers for Disease Control (CDC) stated that most bacterial species occur in communities protected via a secreted exopolysaccharide, slimy matrix (biofilm). All the isolates were able to form biofilms with different degrees, whereas the majority of the tested strains were strong biofilm producers. These results are nearly similar to a previous study [5], which reported that 55.6% of the tested *S. aureus* isolates were strong biofilm producers. Most *S. aureus* isolates that cause bovine mastitis are known to form biofilms [50, 52]. Among the five XDR *S. aureus* superbugs, 3 were strong biofilm producers, and the other two isolates were moderate biofilm producers. There is a strong correlation between the affinity of biofilm formation and phenotypic resistance to antibiotics. Fisher's exact test revealed a significant correlation between strong biofilm reactions and penicillin G, cefoxitin, ceftriaxone, ceftazidime, erythromycin, and clindamycin resistance ($p < 0.05$). The resistance of biofilm-protected bacteria to antimicrobial agents is 1000

Table 5 The occurrence of biofilm production in relation to the distribution of adherence, virulence, enterotoxin, and hemolysin of *S. aureus* strains

Type	Genes	Strong biofilm producer	Moderate biofilm producer	Weak biofilm producer
Biofilm Genes	*eno*	26 (100%)	10 (90.9%)	2 (66.66%)
	*ica*A	26 (100%)	10 (90.9%)	2 (66.66%)
Adherence genes	*clf*A	17 (65.38%)	4 (36.36%)	0 (0%)
	*clf*B	26 (100%)	11 (100%)	3 (100%)
	can	11 (42.3%)	1 (9.09%)	0 (0%)
	*fnb*B	16 (61.53%)	3 (27.27%)	0 (0%)
	*fnb*A	17 (65.38%)	4 (36.36%)	1 (33.33%)
Panton–Valentine leukocidin	*luk*F	26 (100%)	11 (100%)	0 (0%)
Virulence Genes	*spa*	22 (84.61%)	10 (90.9%)	2 (66.66%)
	coa	26 (100%)	11 (100%)	3 (100%)
Hemolysin genes	*hla*	13 (50%)	0 (0%)	0 (0%)
	hlb	11 (42.3%)	2 (18.18%)	0 (0%)
	hlg	8 (30.76%)	1 (9.09%)	0 (0%)
Enterotoxin genes	*sea*	3 (11.53%)	0 (0%)	0 (0%)
	seb	10 (38.46%)	2 (18.18%)	0 (0%)
Resistance genes	*mec*A	25 (96.15%)	10 (90.9%)	3 (100%)
	*erm*B	26 (100%)	11 (100%)	2 (66.66%)
	*erm*C	24 (92.3%)	10 (90.9%)	3 (100%)
	*tet*K	16 (61.53%)	7 (63.63%)	2 (66.66%)
	*tet*M	11 (42.3%)	6 (54.54%)	1 (33.33%)

times greater than that of planktonic bacteria, resulting in more persistent and difficult-to-treat infections [23].

In *Staphylococci*, the main system responsible for sensing bacterial cell density and responding with genetic adaptations is the accessory gene regulator (*agr*) [53]. The agr system can control and regulate some cell wall-associated proteins (coagulase, fibronectin-binding protein, and protein A) and several exoproteins, such as leucotoxins and hemolysins [54]. In the present study, the majority of the recovered *S. aureus* strains (85%) carried the highly invasive *agr* I gene. In this context, *S. aureus* strains belonging to *agr* group I can invade epithelial cells and continue to thrive in larger quantities in mammary gland tissue than other strains can [55]. These findings suggest that the greater the degree of invasiveness of the isolates is, the greater the likelihood of inducing subclinical mastitis. Moreover, this was shown in a recent study where *agr* type I was associated with *S. aureus* bovine subclinical mastitis cases; however, *agr* types II and III were associated with clinical mastitis [32, 56]

The ability of *S. aureus* to perform a wide range of infections is due to its arsenal of virulence factors, such as toxins, adhesins, and enzymes, many of which are regulated by the agr system. *S. aureus* depends on its pathogenesis in terms of tissue adhesion, immune evasion, and host cell injury [13]. At the beginning of bacterial pathogenesis, fimbriae, and pilli help bacteria attach loosely to the host cell surface, which is reversible. Once bacteria begin to produce an extracellular polymer matrix and adhesion molecules, attachment becomes irreversible [23]. MSCRAMM proteins are a group of adhesin proteins in which, among the MSCRAMM genes, *clf*B, *clf*A, *fnb*B, *fnb*A, and *cna* genes were detected with a prevalence of 100%, 80%, 60%, 55%, and 30%, respectively, especially in isolates producing strong or moderate biofilms. Ibrahim [32] reported that the prevalence of the *clf*A, *fnb*A, and *cna* genes in the recovered *S. aureus* strains was 89.5% for each. In Brazil, Rocha [57] detected three genes other than *cna* in all *S. aureus* strains from subclinical mastitis. In a previous study in Algeria, all the isolates harbor the MSCRAMM genes except the *cna* gene [58]. Clumping factors A and B inhibit host cell phagocytosis, and reports have shown that the expression of *fnb*B genes indicates increased invasiveness [59]. In addition to their role in antimicrobial resistance, biofilm extracellular adherence proteins and extracellular matrix protein-binding proteins help in the adhesion of *S. aureus* to the host cell surface [60]. The high prevalence of the *eno* and *ica* genes is similar to the results of Campoccia [61] and Tristan [62]. The *eno* and *ica* genes can collaborate to initiate and promote bacterial colonization and biofilm formation [63].

After adhering to the host cell surface, *S. aureus* escapes from the immune system of the host, which mainly involves *S. aureus* infection via three main mechanisms (phagocytes, antimicrobial peptides, enzymes, and the complement system) via the arsenal of virulence factors, such as hemolysins and leukocidins (related to phagocytic activity and leukocyte migration), clumping factor A (related to complement inactivation), protein A and clumping factors A and B (related to opsonization and immunoglobulins) [60].

In this study, the *spa* gene was detected in 85% of the isolates, indicating its pathogenicity (the greater the decrease in the *spa* gene is, the greater the increase in the number of complement C3b receptor-free sites on the cell surface of *S. aureus* for phagocytosis) [64]. Panton-Valentine leukocidin (PVL), which targets leukocytes to disrupt host cell function, is represented by *luk*F and is amplified in 92.5% of *S. aureus* isolates, allowing the escape *of S. aureus* from the immune system without eliciting either an inflammatory response or a systemic reaction [19]. Hemolysin is accused of cytokine release and controlling cell signaling pathways to initiate inflammatory responses and cell–cell interactions, causing mammary gland inflammation, cell edema, and necrosis in infected animals [65]. In this study, the prevalence of the *hla*, *hlb*, and *hlg* hemolysin genes was 70%, 50%, and 35%, respectively. In contrast, all isolates from animals with subclinical mastitis in Algeria carried alpha, beta, and delta hemolysins [58]. The differences in the prevalence of hemolysin genes could be attributable to the different locations where the studies were conducted [65, 66]. In this context, the presence of the *hla* and *hlb* genes leads to prolonged pathogen survival in the mammary gland, resulting in chronic infection [67]; hence, these genes are mostly associated with *S. aureus* from bovine mastitis. All XDR strains were positive for *spa*, *luk*F, and *clf*B, with variations in hemolysins and *clf*A, which indicate highly virulent strains. The coagulase protein encoded by the *coa* gene is one of several virulence factors produced by *S. aureus*, where it plays a crucial role in bacterial pathogenicity [68]. This protein is responsible for converting fibrinogen into fibrin, resulting in the formation of abscesses and allowing microorganisms to remain in the host's tissues. Identifying coagulase is a sign of virulence in *Staphylococci*. The data of the current study confirmed the presence of the *coa* gene in all the isolates (100%).

Herein, the *seb* gene was detected in 30% of the tested strains, whereas 25% of isolates harbor the *sea* gene**.** These heat-stable toxins are superantigens that are responsible for 90% of food poisoning in humans and are responsible for T-cell proliferation [69]. In this context, environmental factors such as temperature, pH, and moisture can impact enterotoxin production, and

management strategies in dairy facilities, such as inadequate hygiene practices and improper milking techniques or equipment, can influence toxin levels [70, 71].

Many researchers have identified MRSA in mastitis-affected dairy cow milk, indicating that it is a major public health issue. Staphylococcal cassette chromosomal *mec* acquisition causes *S. aureus* methicillin resistance. The cassette harboring the *mec*A gene encodes low-affinity penicillin-binding protein 2a, which confers pathogen resistance to β-lactam antibiotics [72]. Our analysis revealed *mec*A in 95% of the cattle mastitis *S. aureus* isolates aligned with Abd El-Hamid [50] and Ali et al. 2024, who reported 100% *mec*A in *S. aureus*, and Patel [73] reported *mec*A in 73.08% of *S. aureus* samples; moreover, Dan [74] and Marjory [75] reported lower *mec*A levels than those reported in this study. The difference in the prevalence of the *mec*A gene could be attributed to differences in geographical areas, seasons, and sample size [5]. The efflux mechanism of *Staphylococci* is crucial to tetracycline resistance, particularly from the *tet*K gene. The *tet*K gene was frequently detected in resistant *Staphylococci* (62.5%) in this study, similar to the findings of Aarestrup [76]. The genotype of MRSA isolates is usually *tet* (K) or *tet* (K, M) [77]. Our data revealed that the *erm*B gene was the most predominant resistance gene, which is similar to the findings of previous studies [78, 79]. Besides, the high prevalence of the *erm*C gene reported in this study is similar to the findings of El-Banna [78] and Spiliopoulou [80]. In addition to exhibiting β-lactam resistance, MRSA isolates are frequently resistant to other antimicrobial agents, such as tetracyclines, aminoglycosides, macrolides, fluoroquinolones, and chloramphenicol, which may be distributed rapidly in the environment through carriers or infected hosts and cause serious effects that are difficult to treat both humans and animals [81].

Study limitations

In the present study, we used California Mastitis Test (CMT) to detect subclinical mastitis. Although CMT is an inexpensive, easy-to-use, cow-side screening test that estimates the SCC of milk, it does not provide an exact SCC. Therefore, the lack of SCC information is still critical; indicating that several border samples might be non-inflammatory data (the high number of animals and objective issues in the geography might be helpful).

In conclusion, the evolution of MDR and XDR MRSA strains in bovine milk is considered a public health threat. The *mec*A, *erm*C, *erm*B, *tet*K, and *tet*M resistance genes are commonly associated with MDR and XDR MRSA strains. Moreover, the *seb* gene was the most predominant enterotoxin gene in the MRSA strains recovered from milk. Besides, the *coa, clf*B, *eno, ica*A, *luk*F, *spa*, *clf*A, and *hla* virulence genes are the most predominant virulence determinant genes associated with MRSA strains of milk origin. Strong positive correlations were recorded between resistance to certain antibiotics and the occurrence of their corresponding resistance genes. In addition, there was a significant relationship between strong biofilm producers and the occurrence of adhesion genes. Also, there is a strong positive correlation between biofilm formation and the occurrence of antimicrobial resistance. To monitor and control the emergence of MDR and XDR strains, mandatory antimicrobial susceptibility testing, proper antimicrobial use, and selecting appropriate antibiotics for efficient treatment are crucial. Identifying MRSA strains requires a synergistic combination of traditional and molecular assays.

Supplementary Information

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Supplementary Material 1.

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Authors' contributions
Conceptualization: A.M.A., N.H.E., and R.M.E.; Methodology: N.H.E., R.M.E., S.A.A., A.M.A., W.I.M., H.S.E., H.M.E., and A.A.; Software, investigation, resources, and data curation: A.M.A., R.M.E., N.H.E., W.I.M., A.M.Al, S.A., A.K., H.S.E., H.M.E., A.A., S.A.A., H.N., and A.A.M.; Writing and original draft preparation; A.M.A., N.H.E., R.M.E., W.I.M., S.A., A.K., A.M.Al, H.S.E., H.M.E., A.A., S.A.A., and A.A.M.; Writing-review and editing: A.M.A., N.H.E., and R.M.E. All authors have read and agreed to the published version of the manuscript.

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Data availability
All the data has been included in the manuscript.

Declarations

Ethics approval and consent to participate
This study was accomplished in compliance with the ARRIVE guidelines. All protocols were conducted according to relevant guidelines and regulations. The handling of animals, all experiments, and the collection of milk samples were approved by the Ethics Committee of the Faculty of Veterinary Medicine, Ismailia, Egypt (SCU-VET-REC- 2024037). Moreover, the collection of samples comply the institutional guidelines. The informed consent is not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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