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ANTIBIOTIC RESIDUES AND THEIR CORRESPONDING RESISTANCE GENES OF *STAPHYLOCOCCUS AUREUS* IN RAW MILK

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ABSTRACT

The extensive use of antibiotics created two of the biggest hazards to global health in the twenty-first century, namely, antibiotic residues and antimicrobial-resistant (AMR) bacteria. This research seeks to scrutinize the correlation between antibiotic residues and antibiotic-resistance genes (ARGs) of *Staphylococcus aureus* (*S. aureus*). 120 raw milk samples were systematically procured from diverse sources, involving dairy farms (40), farmers' houses (20), dairy shops (40), and street vendors (20). These samples underwent thorough screening for subclinical mastitis (SCM), *S. aureus* counts, and antibiotic residues. Coagulase-positive *S. aureus* (CPSA) was isolated and quantified in 15% (18/120) of the samples. Among these isolates, 94.4% displayed resistance to penicillin, 5.6% to trimethoprim, 22.2% to gentamycin, ofloxacin, and erythromycin, while 27.8% exhibited resistance to vancomycin. PCR amplification of *23S rRNA* confirmed the identity of all tested strains as *S. aureus* (100%), which were then found to harbor ARGs associated with β-lactams (*blaZ* gene), aminoglycosides (*aac*(6')*aph* (2") gene), and vancomycin (*Van A* gene). Milk samples that underwent reverse-phase high-performance liquid chromatography (HPLC) revealed residues of oxytetracycline, amoxicillin, and gentamicin at rates of 8.3%, 1.7%, and 6.7%, respectively, with maximum levels exceeding the European Union Maximum Residue Limits (EU-MRL) (2010). The ARGs related to multidrug-resistant (MDR) *S. aureus* were identified in 40% (8/20) of milk samples contaminated with antibiotic residues.

Keywords: antibiotic resistance genes (ARGs), MDR-*S. aureus*, raw milk, HPLC, PCR.

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INTRODUCTION

S. aureus, a common Gram-positive bacterium naturally residing on the epidermis and mucosal lining of humans and animals (Bierowiec *et al.*, 2016), is frequently implicated in food poisoning cases concomitant the intake of spoiled milk and other animal products (Padilla *et al.*, 2017). Contemporary global records of food poisoning have sparked worries about public health due to food contamination by this bacterium (Bissong *et al.*, 2020; WHO, 2020).

Regarding the milk industry, *S. aureus* can find its way to milk and milk products by being excreted in the milk of an infected milking animal. Pathogenic *S. aureus* is one of the most prevalent pathogens responsible for mastitis disease, causing udder inflammation. Mastitis can be graded as clinical mastitis or sub-clinical mastitis (SCM) (Rana *et al.*, 2022). Asymbotomatic SCM is significant, being a silent source of infection transmission among the herd or to humans through infected milk consumption. In Africa, the bovine SCM prevalence was 49.9% from 2015-2020, with 70-80% of total losses (Cobirka *et al.*, 2020; Schnitt and Tenhagen, 2020).

To control bacterial infections including mastitis in dairy farms, antibiotics were regularly used. However, the misuse of antibiotics, coupled with ignorance of the advisable withdrawal periods, and the illegitimate use of antibiotics, have exposed milk and dairy products to contamination by antimicrobial drug residues (Roope *et al.*, 2019; Theuretzbacher *et al.*, 2019), in addition to the emergence of AMR bacterial strains (Ghimpețeanu *et al.*, 2022). According to Feleke *et al.* (2022), antimicrobial drug residues may trigger adverse health effects, including allergic reactions, carcinogenic and mutagenic effects, bone marrow failure, effects on the genital organs, as well as AMR. Consequently, international institutions have settled MRLs for veterinary medicines and their presence in animal-derived food products (FAO, 2021; USDA-MRL, 2021). Actually, the origin of AMR bacterial strains started shortly after the penicillin discovery (Lee *et al.*, 2018). Furthermore, the bacteria have evolved diverse tactics to resist the antimicrobials, which eventually leads to the emergence of MDR bacteria (Zhang and Cheng, 2022). According to Kavya *et al.* (2023), most drug-resistance genes arise from inheritable gene mutations. Since the development of AMR is seen as a virulence determinant that strengthens host pathogenesis and allows persistent or chronic infections (Emaneini *et al.*, 2016; de Jong *et al.*, 2018), identifying ARGs becomes beneficial to recognize and assess the pathogenic potential of *S. aureus.* (Hodille *et al.*, 2017). In particular, multidrug-resistant (MDR) *S. aureus* strains that harbor diverse ARGs, especially animal-associated *S. aureus*, can be transferred to human beings via the food chain and become a critical global health issue, causing serious and difficult-to-treat infections (Haag *et al.*, 2019; Guo *et al.*, 2020; Lemma *et al.*, 2021). In 2019, 1.27 million people passed away directly from antibiotic resistance, mostly from antibiotic-resistant *Staph aureus* (GBD, 2022).

The bacteria gained resistance against commonly and extensively prescribed antibiotic groups, e.g., the β-lactam group, which includes penicillins, methicillin, oxacillin, nafcillin, cephalosporins, monobactams, carbapenems, and cephalosporins (Bush and Bradford, 2016). In the late 1950s, penicillin-resistant *S. aureus* turned into a pandemic, mainly due to the *blaZ* gene that encodes for the β-lactamase that hydrolysis β-lactams (Pinho; 2008; Ghabbour *et al.*, 2022). As well, the aminoglycosides resistance gene (*aac(6')/aph(2')* gene) is widely identified among enterococci and staphylococci (Akya *et al.*, 2020). Although the glycopeptide antibiotic vancomycin was a cornerstone for

MRSA infection treatment, vancomycin-resistant *S. aureus* (VRSA) was recorded for the first time in 2002 in a diabetic patient in the USA. Vancomycin resistance genes are *vanA, vanB,* and *vanC* genes (Bamigboye *et al*., 2018; Ghabbour *et al.,* 2022).

MATERIALS AND METHODS

1. Samples collection and preparation

120 milk samples were obtained randomly from different sources, including dairy farms, farmers` houses, dairy shops, and street vendors in Sohag Governorate. 50 ml of milk, including mixed quarters' samples from animals that underwent SCM testing, were collected in clean sterile falcon tubes labeled with the source, site, and sampling date. Each milk sample was obtained in pairs; the first sample was used for the isolation and enumeration of *S. aureus*, and the second was used to check for antibiotic residues. All samples were transported to the laboratory of the Microbiology Department at the Animal Health Research Institute, Sohag branch in ice boxes to be prepared and examined as soon as possible.

Samples from dairy farms and farmers' houses were collected from every quarter for detection of SCM by the California Mastitis Test (CMT, Bovivet®, Kruuse™, Denmark) according to MAC Campus FACC (2018), while samples collected from dairy shops and street vendors were tested for heat treatment by Storch's test according to Lampert (1975).

2. Enumeration & isolation of *S. aureus*

S. aureus enumeration was performed using Baird-Parker agar medium (Oxoid, CM0257), whereas the bacteria were isolated using sodium chloride broth 10% and mannitol salt agar medium according to (AOAC, 2000).

3. Phenotypic characterization of Staphylococci

For the biochemical identification of *S. aureus,* catalase, coagulase, oxidase, mannitol fermentation, and hemolysis tests were used as described by **Carter and Cole Jr, (2012).**

4. Antibiotic resistance profile of *S. aureus*

Using the Kirby-Bauer disk diffusion test, an overnight culture of each bacterial isolate was spread on the surface of Mueller-Hinton agar plates (Oxoid, CM0337). After 10 mins, different antibiotic disks were placed on the inoculated agar plate and then incubated at 37°C. After 24 hours, the diameters of the inhibition zone were measured in millimeters (mm) **(CLSI, 2020).**

5. Molecular identification of *S. aureus* and detection of ARGs

5.1. DNA extraction

The DNA was extracted using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) following the manufacturer's instructions.

5.2. Polymerase chain reaction (PCR) amplification using oligonucleotide primers

Table A: Primers sequence used in this study

Gene	Primer sequence (5'-3')	Length of amplified product	Reference
S. aureus 23S rRNA	AC GGAGTTACAAAGGACGAC AGCTCAGCCTTAACGAGTAC	1250 bp	**Bhati *et al.*, 2016**
blaZ	TACAACTGTAATATCGGAGGG CATTACACTCTTGGCGGTTTC	833 bp	**Bagcigil *et al.*, 2012**
aac(6')aph (2'')	GAAGTACGCAGAAGAGA ACATGGCAAGCTCTAGGA	491 bp	**Duran *et al.*, 2012**
VanA	CATGACGTATCGGTAAAATC ACCGGGCAGRGTATTGAC	885 bp	**Patel *et al.*, 1997**

Table B: PCR conditions used for target genes

Target gene	PCR conditions				
	Primary denaturation	Number of PCR cycles			Final extension
		Denaturation	Annealing	Extension	
S. aureus 23S rRNA	94˚C 5 min.	94˚C/ 30 sec.	55˚C 40 sec.	72˚C 1.2 min.	72˚C 10 min.
			35 cycles		
blaZ	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 50 sec.	72˚C 10 min.
			35 cycles		
aac(6')aph (2'')	95˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.
			40 cycles		
vanA	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 1.2 min.	72˚C 12 min.
			35 cycles		

For the amplification of *S. aureus*, the oligonucleotide primers with sequences listed in **Table (A)** were obtained from Midland Certified Reagent Company-oligos (USA) and utilized following the cycling conditions shown in **Table (B).**

5.3. Analysis of the PCR products

15 µl from each PCR product were examined by electrophoresis in 1.5% agarose gel stained with ethidium bromide under UV light.

6. Detection of antibiotic residues in raw milk samples using Reversed-phase high-performance liquid chromatography (RP-HPLC)

The analysis was carried out in the HPLC unit (AHRI, Dokki). Antibiotics were extracted from milk samples with dichloromethane. It was separated by HPLC using a C18 column and detected with a photo-diode array detector at 280 nm wavelength, according to the method described by Fennell *et al.* (1995) with modifications.

The quantitative analysis of amoxicillin, oxytetracycline, and gentamycin antibiotic residues in milk samples was carried out by RP-HPLC (Rahman *et al.,* 2021). Compounds were separated on a prepacked 250 mm, 4.6 mm internal diameter 5 µm particle size, LiChrospher C18. HPLC conditions were set for three microbiologically positive antibiotics.

6.1. Calculations

a standard curve of antibiotics standard solutions (concentrations versus peak area) was first prepared, and from the measured peak areas of test samples, antibiotic concentrations were calculated from a regression equation as follows: $y = ax + b$

Where: y= peak area, a= slope of curve, x= AB concentration, b= intercept of y.

7. Statistical Analysis

The descriptive statistics were performed by the Statistical Program for Social Science (SPSS), version v.26 computer software. SPSS Inc. Chicago, USA.

RESULTS

Table 1: Incidence and count of *S. aureus* in the examined milk samples.

Sample source	Positive samples		CPSA count (CFU/ml)			Samples above E.S.	
	No.	%	Min.	Max.	Average	No.	%
Dairy farms	6/40	15	$2x10^2$	$1.33x10^4$	$5.08x10^3$	6	15
Farmers house	4/20	20	$2x10^2$	$1.7x10^4$	$4.8x10^3$	4	20
Dairy shops	3/40	7.5	$1x10^2$	$3.7x10^3$	$1.87x10^3$	3	7.5
Street vendors	5/20	25	$8x10^2$	$7.9x10^3$	$3.54x10^3$	5	25
Total	**18/120**	**15**	**$1x10^2$**	**$1.7x10^4$**	**$4.06x10^3$**	**18**	**15**

No.: number of examined samples; E.S.: Egyptian Standard (ES: 7123/2010) "CPS should be $<10^2$cfu/ml milk".

Table 2: Frequency distribution of CPSA in the examined milk samples.

CPSA count	Sample source									
	Dairy farms		Farmers' house		Dairy shops		Street vendors		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%
$<10^2$	0/6	0	0/4	0	**0**	**0**	0	0	0	0
10^{-2}	3/6	50	3/4	75	1/3	33.3	1/5	20	8/18	44.4
10^{-3}	1/6	16.7	0/4	0	2/3	66.7	4/5	80	7/18	38.9
10^{-4}	2/6	33.3	1/4	25	0/3	0	0/5	0	3/18	16.7
Total	**6/6**	**100**	**4/4**	**100**	**3/3**	**100**	**5/5**	**100**	**18/18**	**100**

Table 3: Correlation between CMT-positive samples and CPSA in the examined milk samples.

Sample source	Positive CMT		Positive CPSA	
	No.	%	No.	%
Dairy farms	20/40	50	6/40	15
Farmers' house	4/20	20	4/20	20
Total	**24/60**	**40**	**10/60**	**16.7**

CMT: California Mastitis Test

Table 4: Antibiotic sensitivity of CPSA isolated from the examined milk samples.

Antibiotic class	Antibiotics	Sensitive		Resistant	
		No.	%	No.	%
β-lactams	Penicillin (10 U)	1	5.6	17	94.4
	Floxacillin (5µg)	18	100	0	0
Folate antagonist	Trimethoprim (5 µg)	17	94.4	1	5.6
Aminoglycosides	Gentamycin (10 µg)	14	77.8	4	22.2
Fluoroquinolones	Ofloxacin (5 µg)	14	77.8	4	22.2
	Ciprofloxacin (5 µg)	18	100	0	0
Lipopeptides	Polymyxin-B (300U)	18	100	0	0
Chloramphenicol	Chloramphenicol (30 µg)	18	100	0	0
Glycopeptides	Vancomycin (30 µg)	13	72.2	5	27.8
Macrolides	Erythromycin (15 µg)	14	77.8	4	22.2

Table 5: Molecular identification of CPSA isolated from the examined samples.

Sample sources	No. of identified *S. aureus*	No. of tested CPSA
Dairy farms	3	3
Farmers house	2	2
Dairy shops	2	2
Street vendors	3	3
Total	**10**	**10**

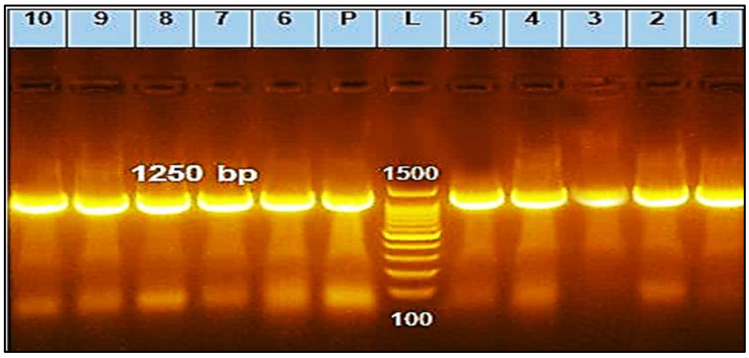

Fig. (1). Agarose gel electrophoresis of the PCR product of the *23S rRNA* gene of *S. aureus* (1250 bp). Lane L: 100 bp DNA ladder. Lane P: Control positive, lane N: Control negative, Lanes 1-10: positive samples for the *S.aureus 23S rRNA* (1250 bp).

Table 6: Detection of ARGs in *S. aureus* isolates.

ARGs	No. of tested *S. aureus*	No. of identified *S. aureus*
***bla Z* gene**	10	8
aac (6´) aph(2´´)gene	4	4
***Van A* gene**	5	1

bla Z: β-lactamase; aac(6´)aph(2´´): aminoglycoside-resistant gene; *Van A*: vancomycin-resistant gene.

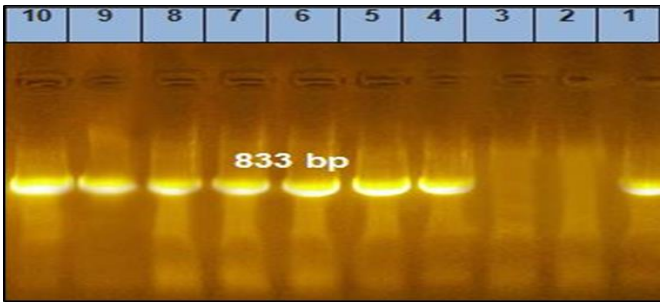

Fig. (2). Agarose gel electrophoresis of the PCR product of the blaZ gene of S. aureus (833bp). Lane L: 100 bp DNA ladder. Lane P: Control positive, lane N: Control negative, Lanes 1-10: Positive samples for the *S.aureus blaZ gene (*833 bp).

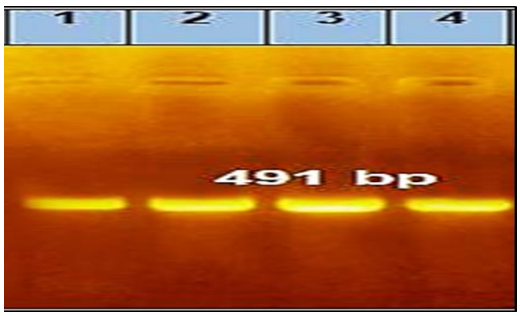

Fig. (3). Agarose gel electrophoresis of the PCR product of *aac (6´) aph(2´´)* gene of *S. aureus* (491 bp). Lane L: 100 bp DNA ladder. Lane P: Control positive, lane N: Control negative. Lanes 1-4: Positive samples for the *S.aureus aac (6´) aph (2´´)* gene (491 bp).

Fig. (4). Agarose gel electrophoresis of the PCR product of *Van A* gene of *S. aureus* (885 bp). Lane L: 100 bp DNA ladder. Lane P: Control positive, Lane N: Control negative, Lane 2: Positive sample for the *S.aureus Van A* gene (885 bp).

Table 7: Prevalence of different antibiotic residues in the examined milk samples.

Sample	Positive samples antibiotic-residues		Oxytetracycline		Amoxicillin		Gentamicin	
	No.	%	No.	%	No.	%	No.	%
Dairy farms	7/40	17.5	2/40	5	2/40	5	3/40	7.5
Farmers house	8/20	40	4/20	20	0/20	0	4/20	20
Dairy shops	1/40	2.5	0/40	0	0/40	0	1/40	2.5
Street vendors	4/20	20	4/20	20	0/20	0	0/20	0
Total	**20/120**	**16.7**	**10/120**	**8.3**	**2/120**	**1.7**	**8/120**	**6.7**

Table 8: Concentration of antibiotic residues in the examined samples using HPLC.

Antibiotics	Positive samples		Minimum conc. (µg/l)	Maximum conc. (µg/l)	MRL value (µg/l)
	No.	%			
Oxytetracycline	10/120	8.3	37.2	201.9	100
Amoxicillin	2/120	1.7	5.9	10	4
Gentamicin	8/120	6.7	1.02	108.03	100

MRL according to the European (EU) and Codex Alimentarius Commission standards (CAC) (European Commission, 2010); MRL: Maximum Residual limits; µg/l=ppb.

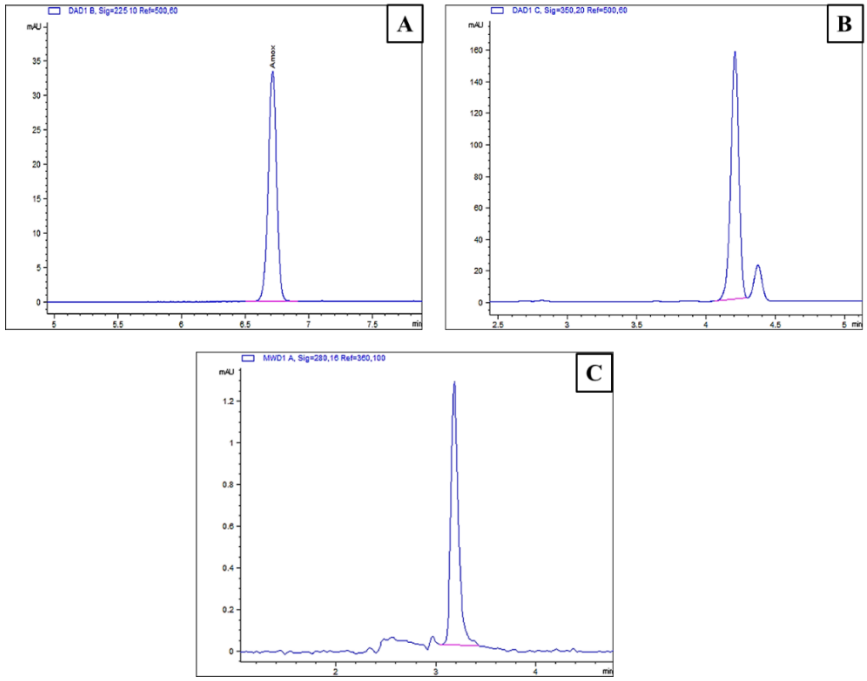

Fig. (5). Chromatogram of milk sample with antibiotic residues: A) amoxicillin at 5.9 ppb, B) oxytetracycline at 37.2 ppb, C) gentamicin at 1.2 ppb.

Table 9: Correlation between antibiotic residues in milk and the presence of ARGs in *S. aureus* isolates.

Sample sources	Positive samples antibiotic-residues		Positive samples containing *S. aureus* with ARGs	
	No.	%	No.	%
Dairy farms	7/40	17.5	3/40	7.5
Farmers house	8/20	40	3/40	15
Dairy shops	1/40	2.5	1/40	2.5
Street vendors	4/20	20	1/40	5
Total	**20/120**	**16.7**	**8/120**	**6.7**

Table 10: Coexistence of antibiotic residues, AR-*S. aureus* and ARGs in milk samples.

Antibiotic	Samples with residues		AR-*S. aureus*	*S. aureus* with ARGs
	No.	%		
Oxytetracycline	10/20	8.3	ND	ND
Amoxicillin	2/20	1.7	ND	ND
Gentamicin	8/20	6.7	4/20	4/20
Vancomycin	ND	0	5/20	1/20
Penicillin	ND	0	17/20	8/20

* ND: Not detected, AR: Antibiotic-resistant, ARGs: Antibiotic resistance genes

DISCUSSION

Coagulase (*Coa*) and von Willebrand factor binding protein (*vWbp*) known as staphylococcal coagulases, can coagulate human and rabbit plasma. They bind to prothrombin forming staphylothrombin which converts fibrinogen to fibrin. The *S.*

aureus is coagulase positive while most staphylococci are coagulase-negative. *S. aureus* can be protected inside an abscess by the action of *Coa* and *vWbp* where *Coa* can produce a pseudo capsule consisting of fibrin surrounding microcolonies of staphylococci, while *vWbp* can produce outer fibrin mesh. So, the action of *Coa* and *vWbp* together is important in the protection of *S. aureus* from neutrophil attack (Becker *et al.*, 2014).

The *S. aureus* counts (CFU/ml) in the examined milk samples using the direct surface plate method on Baired-Parker agar revealed that CPSA count was about 15% (6/40), 20 % (4/20), 7.5% (3/40), and 25% (5/20) in milk samples from dairy farms, farmers' houses, dairy shops, and street vendors, respectively, with a total incidence of 15% (18/120) (Table1). These results agree with those obtained by Abd El Tawab *et al.* (2016), and Meshref *et al.* (2019), who isolated *S. aureus* from 18.2%, and 16% of raw milk samples, respectively. Higher findings were indicated by Souza *et al.* (2017), Garas (2019), and Fawy (2022).

Whereas, the CPSA counts ranged from $2x10^2$ to $1.33x10^4$ with an average count of $5.08x10^3$ in milk samples from dairy farms, $2x10^2$ to $1.7x10^4$ with an average count of $4.8x10^3$ in farmers' houses, $1x10^2$ to $3.7x10^3$ with an average count of $1.87x10^3$ in dairy shops, as well as $8x10^2$ to $7.9x10^3$ with an average count of $3.54x10^3$ in street vendors with a total average count of $4.06x10^3$. All tested samples exceeded the Egyptian standard (ES, 2010) for CPSA (Table 1). Similar CPSA counts were reported in Italy by Iannetti *et al*. (2019) with values ranging from $2.9x10^3$ to $3.5x10^3$ CFU/ml with an average value of 3.5 Log CFU/ml. The microbial contamination of raw milk may originate from animals, due to endogenous or udder infection, from feces and skin, and/or from the environment during milking or storage (Verraes *et al.*, 2014).

As indicated in (Table 2), the highest frequency distribution of CPSA counts was 10^2 for dairy farms, and farmers' houses samples (50, and 75%), and 10^3 for dairy shops, and street vendors (66.7, and 80%) respectively.

- **Incidence of CPSA in positive SCM samples**

S. aureus mastitis has economic impacts, as well as food security and antibiotic consumption challenges. Udders infected with *S. aureus* mastitis act as a source of infection to other animals (Gomes *et al.*, 2016; Rainard *et al.,* 2018). *Staphylococcu*s accounted for 43.7% of bovine SCM cases (Khasapane *et al.,* 2023). In the current study, SCM was detected using a field test CMT at dairy farms and farmers' household dairy animals. SCM-positive samples were 50% (20/40) in dairy farms and 20% (4/20) in farmers houses, with a total of 40% (24/60) (Table 3). These values were in agreement with those demonstrated by Tripura *et al*. (2014) Mureithi and Njuguna (2016), Sağlam *et al*. (2017), and Saad *et al*. (2023), who detected SCM in dairy farms at incidences of 51.8, 49.7, 54.2, and 53.3%, respectively. Also, Krishnamoorthy *et al*. (2021) reported a 45% global prevalence of SCM, 46% in North America, 42% in Asia, in Europe 37%, in Oceania 36% and 34% in Latin America. Similarly, SCM was reported by Khasapane *et al.* (2023) at a rate of 41.02% in Africa.

However, higher incidences were mentioned by Pumipuntu *et al*. (2019), who found that 59% of dairy farms in Thailand were positive for SCM by CMT. Also, Shaker *et al.* (2019), Saad *et al.* (2023), and Bakr *et al.* (2019) stated even higher results at rates of 76.6, 90, and 92%, respectively. On the other hand, lower findings were reported in Assiut, Egypt by Sayed and Abdel-Hafeez (2009) who found that SCM at a rate of 31.82%. Also in Algeria, Zaatout *et al*. (2020) found SCM at a rate of 37.66%. Whereas, Aqib *et al.* (2018) explained that *S. aureus* prevalence is variable, ranging from less than 10% to as high as 65%.

In the present study, CPSA was detected in 15% (6/40) and 20% (4/20) of dairy farms and farmers' houses, respectively, with 16.7% (10/60) overall prevalence (Table 3). A higher rate was investigated by Sağlam *et al*. (2017), who found that 31.49% of milk samples were contaminated with CPSA. However, lower results were obtained in Thailand by Pumipuntu *et al*. (2019) and in Algeria by Zaatout *et al*. (2020) who found that 10.7% and 5.31% of CPSA were isolated from SCM milk, respectively.

- **Antibiotic-resistant profile of the isolated strains of *S. aureus***

MDR-*S. aureus* are strains that showed resistance to 3-4 antibiotics (Gajdács, 2019). The obtained results showed that 94.4% of the isolates were penicillin-resistant, 5.6% were trimethoprim-resistant, 22.2% were resistant to gentamycin, ofloxacin, and erythromycin, and 27.8% were vancomycin-resistant. On the other hand, the isolates were 100% sensitive to floxacillin, polymyxin B, ciprofloxacin, and chloramphenicol (Table 4).

These results are in line with those obtained in Sohag Governorate by Fawy (2022), who revealed that all *S. aureus* isolated strains were resistant to the β-lactam antibiotics. The resistance of *S. aureus* to penicillin can be explained according to Lamari *et al.* (2021), who demonstrated that β-lactams including penicillins, cephalosporins, monobactams, and carbapenems are still the most widely used antibiotics in lactating cows for the treatment of mastitis and are responsible for approximately 95% of all milk antibiotic residue detected in milk. The *S. aureus* strains produce penicillinase and penicillin acylase enzymes forming penicilloic acid and 6-aminopenicillanic acid "inactive forms of penicillin" (Kumar *et al.*. 2019, Reis *et al.* 2020, Sambyal and Singh, 2021).

Results obtained in Sohag, Egypt by Garas (2019) showed that 72.7% of *S. aureus* strains were resistant to penicillin, and 18.18% were intermediate resistant against erythromycin. Similar results were obtained by Diab *et al*. (2021), who recorded that *S. aureus* exhibited phenotypic resistance against gentamycin. Higher vancomycin resistance 42.6% was reported by Umaru *et al*. (2014) in *S. aureus* isolated from raw milk. In contrast, Abdeen *et al*. (2021) found that *S. aureus* exhibited phenotypic resistance against chloramphenicol, and were sensitive to vancomycin and gentamicin. Also, in Nigeria, Shittu *et al*. (2011) found that all *S. aureus* isolates were sensitive to vancomycin. Furthermore, Garas (2019) noticed that all *S. aureus* isolates (100%) showed high sensitivity to vancomycin.

The obtained results illustrated the existence of MDR-*S. aureus.* This result agreed with Mbindyo *et al*. (2021), who found that MDR was observed in 29.67% of *S. aureus*. A higher rate of MDR-*S. aureus* was detected by Awad *et al*. (2017), Dai *et al.* (2019), and Garas (2019), who recorded that 83.3, 75, and 50 % of the isolated *S. aureus* strains were resistant to three or more antibiotic classes.

Table 5 and Fig. (1) showed that the molecular identification of CPSA revealed that all tested strains were confirmed as *S. aureus* using the *23S rRNA* gene. This result is in agreement with those obtained by Abd-Elaal *et al.* (2022) who found that all biochemically identified *S.aureus* strains were encoding the *23S rRNA.*

Table 6 and Figs (2-4) showed that eight *S. aureus* strains were encoding ARGs for β-lactams, four ARGs for aminoglycosides, and one ARG for vancomycin. Similarly, Ashraf *et al.* (2023) in Egypt, found that bla_Z gene was detected in 75% of *S. aureus* isolates. While, Mbindyo *et al*. (2021), and Liu *et al.* (2022) reported that the β-lactamases bla_Z gene was the most common gene found in *S. aureus* at percentages of 97, and 100%, respectively. A lower rate was detected in Iran by Hassani *et al*. (2022) who found bla_Z in *S. aureus* isolates at a rate of 12%. On the other hand, Liu *et al.* (2022) in China and Ashraf *et al.* (2023) in Egypt, found that the

aac (6') aph (2'') gene was detected in 33.3% for each, respectively.

Antibiotic residues in milk

Antibiotic residues were detected in the milk samples by using a seven-plate bioassay system, and the detected antibiotics were quantitatively confirmed using RP-HPLC. The findings reported in Tables 7 and 8, and Fig. 5 showed that the positive samples for antibiotic residues were 16.7% (20/120). The oxytetracycline residues were detected in 5% (2/40) dairy farm samples and 20% (4/20) in each farmer's house and street vendor samples, but could not be detected in samples from dairy shops. Amoxicillin residues were only detected in dairy farm samples (5%). Gentamicin residues were found in 7.5% (3/40), 20% (4/20), and 2.5% (1/40) of dairy farms, farmers' houses, and dairy shops, respectively, but could not be detected in street vendors' samples.

Comparable results were postulated in Algeria by Ammi *et al*. (2019), and in Iraq by Almashhadany (2021), who found that 12.6, and 12% of raw milk samples were positive for antibiotic residues, respectively. Lower results were published by Movassagh and Karami (2011) in Iran, who found antibiotic residues in 5.33% of milk samples. Higher rates of antibiotic residues in milk were detected, in Algeria by Layada *et al*. (2016) and in Nigeria by Olatoye *et al*. (2016), who reported that 25.3 and 40.8% were positive for antibiotic residues in raw milk samples. In Iran, Al-Zuheir (2012) and Alipour *et al*. (2014) detected that residues in milk samples were above the MRLs in 18.7 and 19.4% of milk samples, respectively.

Similarly, in Romania, Pogurschi *et al.* (2015) claimed more samples were positive for tetracyclines residues 25.7% than for β-lactam residues 5.7%. On the contrary, in India, Kumarswamy *et al.* (2018) found that 2.42 and 1.82% of raw milk samples were positive for β-lactam and tetracycline residues, respectively. In Benin, 83.9 and 16.5% of milk samples were positive for β-lactam and tetracycline antibiotic residues, respectively (Mensah *et al.* 2014).

A higher rate of β-lactam residues, in Iran Ghanavi *et al.* (2013), Algeria Ammi *et al*. (2019), and Mimoune *et al*. (2021) indicated that 23.8, 19.37 and 26.32%, of raw milk samples, were positive for the β-lactam residues, respectively. Whereas, a higher rate of oxytetracycline residues was detected by Abbasi *et al*. (2011), and Abo EL-Makarem *et al*. (2020), who revealed that oxytetracycline was detected in 57.1, and 30% of milk samples.

As indicated in Table 8 and Fig. 5, the measured concentrations ranged from 37.2 to 201.9 µg/l for oxytetracycline, 5.9 to 10 µg/l for amoxicillin, and 1.02 to 108.03 µg/l for gentamicin, in which the maximum levels exceeded MRL values (EU, 2010) for each of oxytetracycline and gentamicin, while the minimum detected concentration of amoxicillin was higher than the MRL.

This result agrees with those obtained by Al-Shaalan *et al*. (2022) who found that 10% of samples positive for oxytetracycline residues were above the MRL. On the other hand, oxytetracycline was detected below the MRL levels by Nirala *et al.* (2017), Abo EL-Makarem *et al*. (2020), Zhang *et al*. (2020), and Caminada *et al*. (2021), who revealed that the mean value of oxytetracycline concentration in the examined raw milk samples was >0.1, 97.9, 26.9, and 61.29 µg/kg.

On the contrary, the obtained levels of amoxicillin residues in raw milk were lower than those detected by Khanal *et al.* (2018), Zhang *et al*. (2020), and Caminada *et al*. (2020), with amoxicillin detection levels of 68-802, 3, and 124 µg/l. Whereas, the results of gentamicin residues were higher than those obtained by Caminada *et al*. (2020), who found gentamicin residues at rates of 0.55, and 0.6% in pasteurized and raw milk samples, respectively. Zhang *et al.* (2020) detected gentamicin residues in 0.55% of the

pasteurized milk samples with maximum residue levels of 63.5 µg/kg.

Correlation between antibiotic residues and ARGs of *S. aureus*

According to the sample source, antibiotic residues were detected in 17.5% (7/40), 40% (8/20), 2.5% (1/40), and 20% (4/20) while ARGs of *S. aureus* were found in 7.5% (3/40), 15% (3/20), 2.5% (1/40) and 5% (1/20) of dairy farms, and farmers house, dairy shops, and street vendors tested samples, respectively, with an overall residues incidence of 16.7% (20/120) compared to 6.7 (8/120) *S. aureus* ARGs (Table 9). In addition, data in Table 10 revealed that *S. aureus* could not be detected in samples with oxytetracycline and amoxicillin residues. Whereas 4/8 milk samples containing gentamycin residues were contaminated with *S. aureus* encoding gentamycin-resistant genes. On the other hand, *S. aureus* encoding *blaZ* and *vanA* genes was detected, while neither penicillin nor vancomycin was found in the samples.

In contrast to the results of Qamar *et al.* (2023), who detected tetracycline-resistant strains in 20/22 (90.1%) tested milk samples, and 17 tested positive for the *tet* gene. Similarly, Zeina *et al*. (2013) found gentamicin residues in experimentally treated cows, and all *S. aureus* isolated showed 100% resistance to gentamicin, compared to a lower resistance in milk from non-treated cows. Whereas the vancomycin-resistance gene was detected in one *S. aureus* isolate while there was no vancomycin residue in examined milk samples. Likewise, Muzammil *et al.* (2023) demonstrated that out of a total of 248 *S. aureus* isolates from mastitic milk samples, the phenotypic and genotypic prevalence of vancomycin-resistant S. aureus (VRSA) was estimated to be 17.74% and 10.89%, respectively. Also, Chieffi *et al.* (2023) investigated the occurrence of VRSA in milk at a rate of 2.2%.

CONCLUSION

The current study indicated that there is no correlation between the detection of the antibiotic residues of oxytetracycline, gentamicin, penicillin, and vancomycin in milk samples and the presence of resistant *S. aureus* strains. In addition, the detection of a resistant strain phenotypically does not necessitate the presence of ARGs genotypically. Importance of surveillance and control of antibiotic application in the dairy industry through current legislation and raising the producers' awareness.

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بقايا المضادات الحيوية وجينات المقاومة المقابلة لها من المكورات العنقودية الذهبية في الحليب الخام

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أدى استخدام المضادات الحيوية بشكل مكثف إلى ظهور تحديات خطيرة تهدد صحة الإنسان في العصر الحالي، منها بقايا المضادات الحيوية والبكتيريا المقاومة للمضادات الحيوية. تهدف هذه الدراسة إلى استكشاف العلاقة بين بقايا المضادات الحيوية والجينات المقاومة للمضادات الحيوية للمكورات العنقودية الذهبية (*S. aureus*). تم جمع ١٢٠ عينة من الحليب الخام بشكل عشوائي من مصادر مختلفة، بما في ذلك مزارع الألبان (٤٠)، منازل المزارعين (٢٠)، محلات الألبان (٤٠)، والباعة المتجولين (٢٠). تم تحليل العينات للتعرف على المكورات العنقودية الذهبية وبقايا المضادات الحيوية. وتبين أن ١٥٪ (١٢٠/١٨) من العينات كانت إيجابية للمكورات العنقودية الذهبية. وأظهرت النتائج أن ٩٤,٤٪ من العزلات كانت مقاومة للبنسلين، و٥,٦٪ مقاومة لتريميثوبريم، و٢٢,٢٪ مقاومة للجنتاميسين والأوفلوكساسين والإريثروميسين، بينما كان ٢٧,٨٪ مقاومة للفانكومايسين. وأظهرت نتائج فحص جينات ٢٣ S rRNA أن جميع العزلات كانت *S. aureus*. وتم اكتشاف وجود جينات المقاومة لمضادات الميكروبات (ARGs) لبيتا لاكتام) جين (blaZ ، وأمينوجليكوزيدات (aac (6') ((aph (2"، وفانكومايسين (vanA) في عزلات المكورات العنقودية الذهبية. كما تبين أن عينات الحليب التي تم فحصها بواسطة HPLC تحتوي على بقايا من أوكسي تتراسيكلين وأموكسيسيلين وجنتاميسين بنسب تتجاوز الحدود المسموح بها (EU-MRL)، حيث كانت نسبتها ٨,٣٪ و١,٧٪ و٦,٧٪ على التوالي، مع وجود مستويات قصوى تجاوزت الحدود المسموح بها. وتم اكتشاف وجود ARGs ل *S. aureus* متعددة المقاومة في ٤٠٪ (٢٠/٨) من عينات الحليب الملوثة ببقايا المضادات الحيوية.