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Phenotypic and Genotypic Characterization of Methicillin, Vancomycin, and Erythromycin-resistant *Staphylococcus aureus* Isolated from Milk and Dairy Products

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ABSTRACT

Detailed information on the resistance patterns of *Staphylococcus aureus* (*S. aureus*) in milk and cheese is strongly required to facilitate risk assessment analysis in case of food poisoning context and to improve therapeutic approaches used in dairy farms. The present study aimed to perform phenotypic and genotypic antimicrobial characterizations of methicillin, vancomycin, and erythromycin-resistant *S. aureus* isolated from milk and dairy products through screening *mecA*, *vanA*, and *ermC* using molecular PCR amplification technology. Moreover, the association between each genotypic and its related antibiotic resistance phenotypic features within the isolated *S. aureus* strains were analyzed. Moreover, the current study aimed to study MRSA's ability to form biofilms. Out of 226 milk and dairy product samples collected from different retailers in Giza Governorate, 69.5% of the samples were positive for the presence of *S. aureus*. The isolation rate of *S. aureus* strains from cattle milk, sheep milk, white cheese, flamenco, and mesh samples were 79.7%, 76.5%, 56.0%, 40.0%, and 94.7%, respectively. Multidrug-resistant *S. aureus* (MDR) was detected in 51% of all isolated *S. aureus* strains. All tested *S. aureus* strains were sensitive to trimethoprim-sulfamethoxazole, linezolid, ciprofloxacin, and gentamycin. However, their resistance rates against penicillin, oxacillin, vancomycin, erythromycin, tetracycline, clindamycin and chloramphenicol were 62.4%, 65.0%, 44.6%, 45.9%, 21.0%, 14.0%, and 2.5%, respectively. Of the isolated *S. aureus* strains, 72.6%, 40.1%, and 48.4% were carriers for *mecA*, *vanA*, and *ermC* genes and the amplified products were at 310, 1030, and 295 bp, respectively. Methicillin-resistant *S. aureus* isolates were detected in 47.1% of all isolated *S. aureus* strains. The results indicated that 35.0% of the tested *S. aureus* strains were genotypic *vanA* gene carriers and phenotypic resistant to vancomycin representing vancomycin-resistant *S. aureus* strains. Moreover, 42.7% of all isolated *S. aureus* strains were carriers for *ermC* gene and were phenotypic resistant to erythromycin representing erythromycin-resistant *S. aureus*. The presence of *mecA*, *vanA*, and *ermC* genes in *S. aureus* was statistically associated with their related phenotypic resistance patterns against both penicillin and oxacillin, vancomycin, and erythromycin, respectively. Moreover, along with an increase in the frequency of *mecA*, *vanA*, and *ermC* genes, their phenotypic antibiotic resistance patterns sharply increased with an odd ratio >1. Of MRSA isolates, 6.8% indicated weak biofilm-formation ability, while 93.2% exhibit no biofilm-forming ability.

Keywords: Erythromycin-Resistant, *mecA*, Methicillin-Resistant, *Staphylococcus aureus*, Vancomycin-Resistant.

INTRODUCTION

Staphylococcus aureus is a facultative anaerobic gram-positive bacterium, and it is considered one of the most predominant causes of worldwide foodborne poisoning (Cai et al., 2021). Consumption of milk and dairy products contaminated with *S. aureus* represents a public-health risk. Dairy products can provide favorable conditions for *S. aureus* propagation particularly if they are made from raw milk (Zayda et al., 2020). Contamination of dairy products by *S. aureus* can occur through the infected animal's mammary glands, mucosal surfaces, and skin. This contamination could also occur through contaminated milking equipment, workers' or milkers' hands, and also through the contaminated environment (Spanu et al., 2012).

Staphylococcus aureus bacterium has a strong ability to resist a wide range of antimicrobial agents. Multidrug-resistant *S. aureus* is a worldwide alarming challenge for public health as it is associated with high morbidity and mortality. The use of penicillin and methicillin in the mid-20th century demonstrated adequacy against *S. aureus*. However, *S. aureus* rapidly acquired resistance against these antibiotics through the production of exopolysaccharides, beta-lactamase, or penicillinase, and also due to harboring different virulence genes coding for antibiotic resistance (Rola et al., 2015) with induction of a new high drug-resistant phenotypic *S. aureus* (Cai et al., 2021). One of the most challenged antibiotic resistance *S. aureus* is methicillin-resistant *S. aureus* (MRSA) which has been spread worldwide and become a life-threatening infection associated with bacteremia, osteomyelitis, pyomyositis, or necrotizing pneumonia (Zayda et al., 2020). Methicillin-resistant *S. aureus* has been reported in milk and dairy products globally (Cai et al., 2021). Rapid and accurate detection of multidrug-resistant *S. aureus* using molecular techniques as PCR for the detection of genes related to resistance against different antibiotics has long been known as a confirmed effective

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diagnostic and useful method to prosper in infection control policy and antimicrobial therapy effectiveness (Zayda et al., 2020). On the other hand, *S. aureus* can form biofilms on different surfaces. Biofilm formation in *S. aureus* can increase antibiotic resistance criteria and the difficulty of sanitation processes (Angelidis et al., 2020).

The present study aimed to perform phenotypic and genotypic antimicrobial characterizations of methicillin, vancomycin, and erythromycin-resistant *S. aureus* isolated from milk and dairy products through screening of *mecA*, *vanA*, and *ermC* using molecular PCR amplification technology. The current study aimed also to analyze the association between each genotypic and its related antibiotic resistance phenotypic features within the isolated *S. aureus* strains. Moreover, the current study aimed to study MRSA's ability to form biofilms. As far as the researchers are concerned, this study can be one of the first studies on the comparison between phenotypic and genotypic characterizations of methicillin, vancomycin, and erythromycin-resistant *S. aureus* isolated from milk and dairy products in Egypt, in addition to studying MRSA's ability to form biofilms.

MATERIALS AND METHODS

Ethical approval

The present study does not include any *in-vivo* experiments on living animals.

Sampling

A total of 226 samples including 64 cattle milk, 34 sheep milk, 50 white cheese, 40 flamenco, and 38 mesh (mesh is the name of a traditional Egyptian cheese that is made by fermenting salty cheese for several months or years) samples were collected from different retailers in Giza Governorate. The samples were collected and transferred in an insulated icebox to the department of Microbiology and Immunology, Veterinary Division, National Research Centre, Cairo, Giza, Egypt, where they were investigated immediately for the presence of *S. aureus*.

Culture of *Staphylococcus aureus*

Isolation and identification of *S. aureus* from milk and dairy products were performed as described by Omara et al. (2016).

Phenotypic antibiotic resistance profile of the isolated *Staphylococcus aureus*

Antimicrobial resistance profiles of *S. aureus* strains were achieved by Kirby Bauer disk diffusion method based on the National Committee for Clinical Laboratory Standards (CLSI, 2013). This experiment was done using muller hinton agar (Oxoid, UK) and the following antimicrobial impregnated disks (Oxoid, UK), including erythromycin (15 µg), clindamycin (15 µg), oxacillin (1 µg), penicillin G (10 IU), trimethoprim-sulfamethoxazole (25 µg), linezolid (30 µg), tetracycline (30 µg), vancomycin (30 µg), rifampin (5 µg), chloramphenicol (30 µg), ciprofloxacin (30 µg), and gentamycin (10 µg). After inoculation of the antimicrobial impregnated disks in the cultured muller hinton agar plates, the plates were then incubated overnight at 37°C and the zone sizes of inhibition were measured in millimeters and interpreted according to CLSI (2013) guidelines.

Genotypic antibiotic resistance features

Genomic DNA extraction

Genomic bacterial DNA extraction was achieved using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) according to the manufacturer's recommendations with some modifications. Briefly, *S. aureus* isolates were cultivated on nutrient agar base (Oxoid, UK) containing 5% defibrinated sheep blood, followed by incubation at 37°C for 24 hours. The colonies were then collected in micro-centrifuge tubes with the aid of 1 ml saline, then 200 µl of that saline were incubated with both 10 µl of proteinase K and 200 µl lysis buffer for 10 minutes at 56°C. Then, 200 µl of 100% absolute ethanol was added to the previously formed lysate followed by washing and centrifugation according to the manufacturer's recommendations. The DNAs were eluted with 100 µl of elution buffer provided in the kit and all extracted DNA were then stored at -20°C.

Molecular detection of mecA, vanA, and ermC genes by PCR

Staphylococcus aureus strains were investigated using polymerase chain reaction (PCR) for the detection of antibiotic resistance genes, including (i) methicillin resistance *mecA* gene which is a determinant of methicillin resistance in *S. aureus*, (ii) glycopeptide resistance *vanA* gene which is a determinant for the vancomycin resistance in *S. aureus*, and (iii) *ermC* gene which is a determinant for the erythromycin resistance in *S. aureus* (Cai et al., 2021). The PCR amplification was achieved in a 25 µl full reaction volume containing 1 µl of both forward and reverse primers of 20 pmol concentration, 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 6 µl of DNA template, and 4.5 µl of water. After mixing the components of the PCR tube, the solution was then overlaid with 40 µl mineral oil to avoid evaporation then the tubes were placed in an applied biosystem 2720 thermal cycler (PTC100Mil Research, USA). The primers (Metabion, Germany) sequences, amplification cycling condition, and amplified sizes of PCR products are presented in Table 1.

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Table 1. Nucleotide sequences, amplification steps, and amplified PCR products size of *Staphylococcus aureus* gene-specific oligonucleotide primers

Target gene	Primer name	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
					Secondary denaturation	Annealing	Extension		
mecA	mecA1 F	GTAGAAATGACTGAACGTCCG ATAA	310	94˚C 5 minutes	94˚C 45 seconds	50˚C 45 sec.	72˚C 45 seconds	72˚C 10 minutes	(Campos-Peña et al., 2014)
	mecA2 R	CCAATTCCACATTGTTTCGGT CTA A							
vanA	vanA F	CATGAATAGAATAAAAGTTGCAATA	1030	94˚C 5 minutes	94˚C 30 seconds	55˚C 45 sec.	72˚C 1 minute	72˚C 10 minutes	(George et al., 2021)
	vanA R	CCCCTTTAACGCTAATACGATCAA							
ermC	ermC1 F	ATCTTTGAAATCGGCTCAGG	295	94˚C 5 minutes	94˚C 30 seconds	51˚C 30 seconds	72˚C 30 seconds	72˚C 7 minutes	(Schlegelova et al. (2008)
	ermC2 R	CAAACCCGTATTCCACGATT							

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Agarose gel electrophoresis for PCR product analysis

PCR products were fragmented by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) stained with ethidium bromide in 1X tris-borate-EDTA buffer at room temperature using gradients of 5 V/cm. For gel analysis, 20 µl of the uniplex PCR products were loaded in each gel slot. To determine the amplified fragment size, 100 bp DNA ladder and 100 bp plus DNA Ladders (Qiagen, Germany, GmbH) were used. The gel photos were captured by a gel documentation system (Alpha Innotech, Biometra, Italy) and the output data were examined via automatic computer software (Automatic Image Capture Software, ProteinSimple formerly Cell Biosciences, USA).

Quantitative evaluation of biofilm formation

Assessment of bacterial biofilm formation was performed as described by Wang et al. (2010). According to OD value, OD < 0.0112, 0.0112 ≤ OD < 0.0224, 0.0224 ≤ OD < 0.0448, and OD ≥ 0.0448 biofilm formations were considered as negative, weak, moderate, and strong biofilm-forming strains respectively.

Statistical analysis

The statistical analysis of the association between each genotypic and its associated phenotypic antibiotic resistance patterns in each *S. aureus* strain was analyzed and interpreted. Qualitative data were statistically represented in terms of frequency and percentage. Comparison between different groups in the present study was done using the chi-square test with the odds ratio. The probability measure p-value ≤ 0.05 is considered significant. All statistical calculations were done using SPSS (Statistical Package for Social Science) version 25.

RESULTS

Isolation and identification of *Staphylococcus aureus*

From the results presented in Figure 1, it is clearly shown that, out of the examined 226 milk and dairy products' samples, 157 (69.5%, 157/226) were positive for the presence of *S. aureus.* The isolation rates of *S. aureus* strains from cattle milk, sheep milk, white cheese, flamenco, and mesh samples were as follows; 79.7% (51), 76.5% (26), 56.0% (28), 40.0% (16), and 94.7% (36) respectively.

Phenotypic antibiotic resistance profile of the isolated *Staphylococcus aureus*

Table 2 shows the phenotypic antibiotic resistance features of the isolated *S. aureus* isolates within each category of samples (cattle milk, sheep milk, white cheese, flamenco, and mesh). Percent of *S. aureus* resistance to the number of antibiotics within each category of samples is presented in Figure 2. Notably, 22.3% (35), 16.6% (26), and 51.0% (80) of all isolated *S. aureus* strains indicated resistance to one, two, and more than two (MDR) antimicrobial agents respectively. Multidrug-resistant *S. aureus* isolates were detected in 80.4% (41), 57.7% (15), 64.3% (18), 18.8% (3), and 8.3% (3) of the *S. aureus* strains isolated from cattle milk, sheep milk, white cheese, flamenco, and mesh respectively.

Genotypic antibiotic resistance features of the isolated *Staphylococcus aureus*

Genotypic detection of *mecA*, *vanA*, and *ermC* genes within *S. aureus* isolates is presented in Table 3. Notably, 72.6% (114), 40.1 % (63), and 48.4% (76) of *S. aureus* isolates were carriers for *mecA*, *vanA*, and *ermC* genes respectively, and the amplified products were at 310, 1030, and 295 bp respectively as presented in Figures 3-5.

Statistical association between genotypic and phenotypic antibiotics resistance features of the isolated *Staphylococcus aureus*

Genotypic and its related phenotypic antibiotics resistance features of *S. aureus* within each category of samples (cattle milk, sheep milk, white cheese, flamenco, and mesh) are presented in Table 3 and Figures 6-9. The statistical analysis of the association between each genotypic and its related phenotypic antibiotics resistance features within all isolated *S. aureus* isolates is presented in Table 4.

Biofilm forming ability of MRSA isolates

The biofilm formation assay revealed that 6.8% (5) of MRSA isolates exhibit weak biofilm-forming ability while 93.2% (69) exhibit no biofilm-forming ability.

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Table 2. Phenotypic antibiotic resistance features of *Staphylococcus aureus* strains within each category of samples (cattle milk, sheep milk, white cheese, flamenco, and mesh)

Antimicrobial	Antimicrobial susceptibility of the tested *Staphylococcus aureus* isolated from milk and cheese																			
	Tested *Staphylococcus aureus* isolated from milk								Tested *Staphylococcus aureus* isolated from cheese										Total R	
	Cattle milk (no=51)			Sheep milk (no =26)			Total milk resistance (no=77)		White cheese (no=28)			Flamenco (no=16)			Mesh (no=36)			Total Cheese Resistance (no=80)		(no=157)	
	S	I	R	S	I	R	No.	%	S	I	R	S	I	R	S	I	R	No.	%	No.	%
Penicillin	3	0	48	6	0	20	68	88.3	12	0	16	11	0	5	27	0	9	30	34.1	98	62.4
Oxacillin	11	0	40	11	0	15	55	71.4	5	0	23	12	0	4	16	0	20	47	53.4	102	65.0
Vancomycin	38	0	13	8	0	18	31	40.3	5	0	23	13	0	3	23	0	13	39	44.3	70	44.6
Erythromycin	8	0	43	8	0	18	61	79.2	17	0	11	16	0	0	36	0	0	11	12.5	72	45.9
Clindamycin	42	0	9	23	0	3	12	15.6	16	12	0	7	4	5	29	2	5	10	11.4	22	14.0
Trimethoprim-Sulfamethoxazole	51	0	0	26	0	0	0	0.0	28	0	0	16	0	0	36	0	0	0	0.0	0	0.0
Linezolid	51	0	0	26	0	0	0	0.0	28	0	0	16	0	0	36	0	0	0	0.0	0	0.0
Tetracycline	23	8	20	7	6	13	33	42.9	28	0	0	16	0	0	36	0	0	0	0.0	33	21.0
Rifampin	43	0	8	23	3	0	8	10.4	28	0	0	11	5	0	36	0	0	0	0.0	8	5.1
Chloramphenicol	50	0	1	23	0	3	4	5.2	28	0	0	16	0	0	36	0	0	0	0.0	4	2.5
Ciprofloxacin	51	0	0	26	0	0	0	0.0	28	0	0	16	0	0	36	0	0	0	0.0	0	0.0
Gentamycin	51	0	0	26	0	0	0	0.0	28	0	0	12	4	0	36	0	0	0	0.0	0	0.0

S= sensitive, I= intermediate, R=resistance

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Table 3. Genotypic and its related phenotypic antibiotics resistance features of *Staphylococcus aureus* within each category of samples

Samples	No of *S. aureus* isolates	*mecA* gene and Penicillin and/or Oxacillin resistance												*vanA* gene and Vancomycin resistance						*ermC* gene and Erythromycin resistance					
		mecA gene		Penicillin		Oxacillin		*mecA* and Penicillin		*mecA* and Oxacillin		*mecA and* Penicillin and Oxacillin		*vanA* gene		Vancomycin		*vanA* and Vancomycin		*ermC* gene		Erythromycin		*ermC* and Erythromycin	
		No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
Cattle milk	51	46	90.2	48	94.1	40	78.4	45	88.2	39	76.5	39	76.5	10	19.6	13	25.5	8	15.7	45	88.2	43	84.3	42	82.4
Sheep milk	26	20	76.9	20	76.9	15	57.7	17	65.4	12	46.2	12	46.2	18	69.2	18	69.2	15	57.7	20	76.9	18	69.2	15	57.7
Total milk	77	66	85.7	68	88.3	55	71.4	62	80.5	51	66.2	51	66.2	28	36.4	31	40.3	23	29.9	65	84.4	61	79.2	57	74.0
White cheese	28	23	82.1	16	57.1	23	82.1	15	53.6	20	71.4	14	50.0	21	75.0	23	82.1	21	75.0	11	39.3	11	39.3	10	35.7
Flamenco	16	5	31.3	5	31.3	4	25.0	3	18.8	2	12.5	2	12.5	3	18.8	3	18.8	1	6.3	0	0.0	0	0.0	0	0.0
Mesh	36	20	55.6	9	25.0	20	55.6	7	19.4	19	52.8	7	19.4	11	30.6	13	36.1	10	27.8	0	0.0	0	0.0	0	0.0
Total cheese	80	51	63.8	30	37.5	51	63.8	27	33.8	46	57.5	26	32.5	40	50.0	45	56.3	39	48.8	15	18.8	15	18.8	14	17.5
Total sample	157	114	72.6	98	62.4	102	65.0	87	55.4	92	58.6	74	47.1	63	40.1	70	44.6	55	35.0	76	48.4	72	45.9	67	42.7

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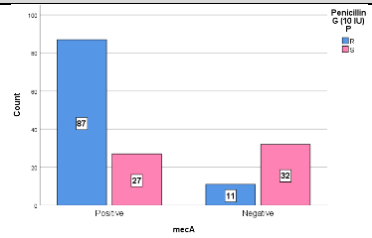
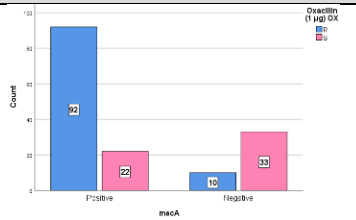
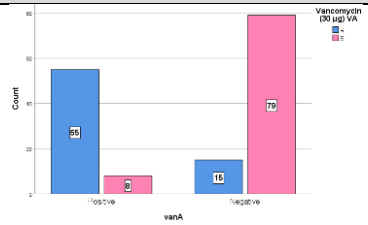
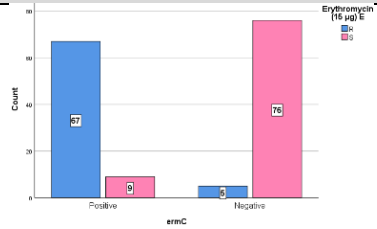
Table 4. The association between each genotypic and its related phenotypic antibiotics resistance features within all *Staphylococcus aureus* isolated from milk and dairy products

mecA	Penicillin G (10 1U)			Pearson Chi-Square	df	p-value	Cramer's V	OR for *mecA* (+ / -) (95% CI)	Bar chart
		R	S						
114 +ve	N	87	27	34.26	1	0.000	0.467	9.374	
	%	76.316%	23.684%						
43 -ve	N	11	32						
	%	25.581%	74.419%						

mecA	Oxacillin (1 µg)			Pearson Chi-Square	df	p-value	Cramer's V	OR for *mecA* (+ / -) (95% CI)	Bar chart
		R	S						
114 +ve	N	92	22	45.27	1	0.000	0.537	13.800	
	%	80.702%	19.298%						
43 -ve	N	10	33						
	%	23.256%	76.744%						

vanA	Vancomycin (30 µg)			Pearson Chi-Square	df	p-value	Cramer's V	OR for *vanA* (+ / -) (95% CI)	Bar chart
		R	S						
63 +ve	N	55	8	77.71	1	0.000	0.704	36.208	
	%	87.302%	12.698%						
94 -ve	N	15	79						
	%	15.957%	84.042%						

ermC	Erythromycin (15 µg)			Pearson Chi-Square	df	p-value	Cramer's V	OR for *ermC* (+ / -) (95% CI)	Bar chart
		R	S						
76 +ve	N	67	9	106.15	1	0.000	0.822	113.156	
	%	87.302%	12.698%						
81 -ve	N	5	76						
	%	6.173%	93.827%						

$p \leq 0.05$ is considered statistically significant, OR: Odd ratio, R: Resistant, S: Sensetive, df: Degree of freedom, + / -: Presence/ absence, 95% CI: 95% Confidence Interval

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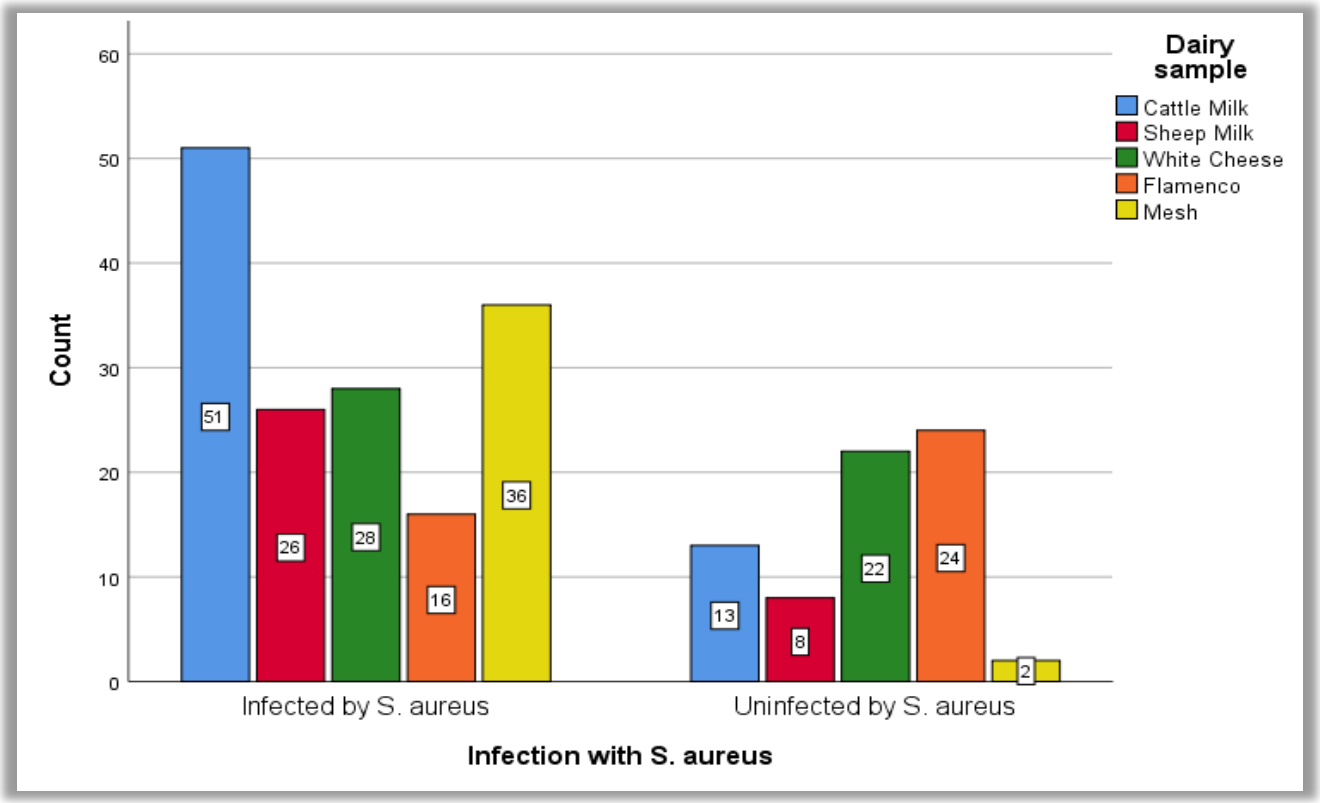

Figure 1. Contamination status of dairy samples by *Staphylococcus aureus*. The contamination rates caused by *S. aureus* within cattle milk, sheep milk, white cheese, flamenco, and mesh samples were 79.7% (51), 76.5% (26), 56.0% (28), 40.0% (16), and 94.7% (36) respectively.

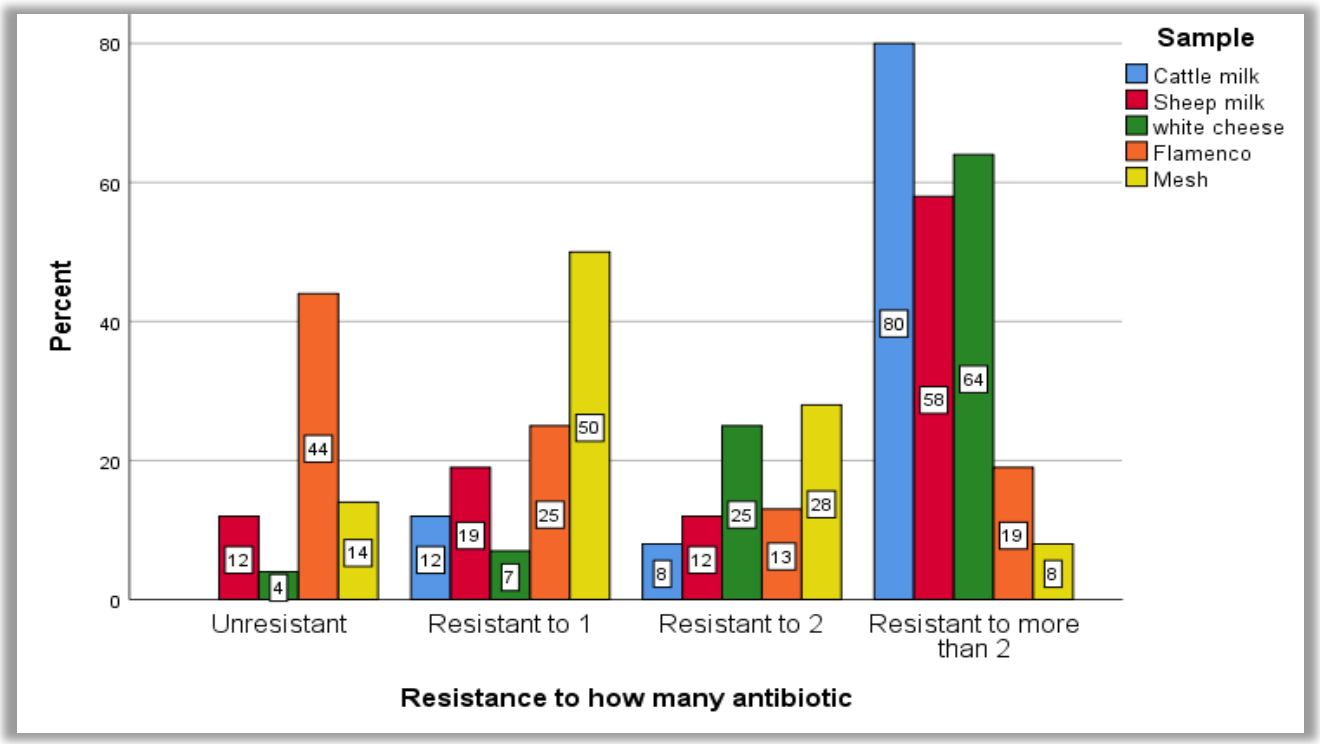

Figure 2. Percent of *Staphylococcus aureus* resistance to how many antibiotics within each category of sample. Multidrug-resistant *S. aureus* isolates were detected in 80.4% (41), 57.7% (15), 64.3% (18), 18.8% (3), and 8.3% (3) of the *S. aureus* isolated from cattle milk, sheep milk, white cheese, flamenco, and mesh respectively.

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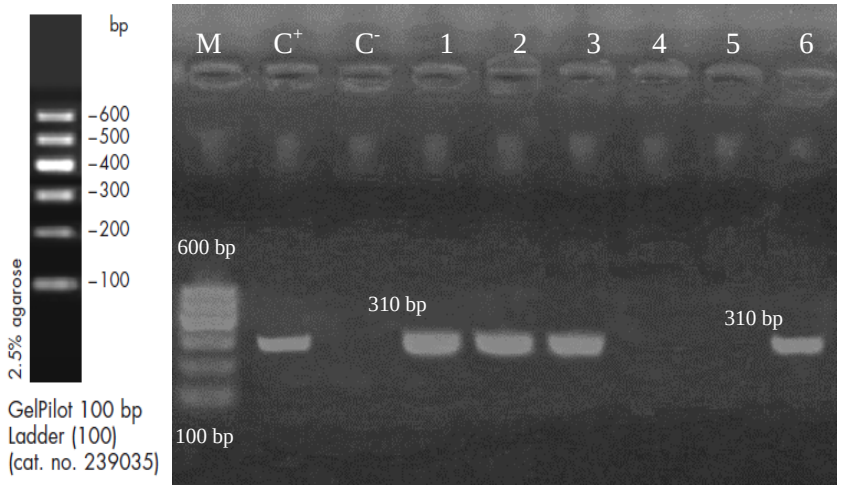

Figure 3. PCR amplified products of *mecA* gene at 310 bp among *Staphylococcus aureus* isolated from cattle milk. Lanes (M): QIAGEN GelPilot DNA 100 bp ladder; lanes (C^+): positive control *Staphylococcus aureus* (*mecA* positive); lanes (C^-): negative control *Staphylococcus aureus* (*mecA* negative); lanes (1-6): *Staphylococcus aureus* isolates from cattle milk; lane 1,2,3,6: positive samples of *mecA* gene (310 bp); lane 4,5: negative samples of *mecA* gene.

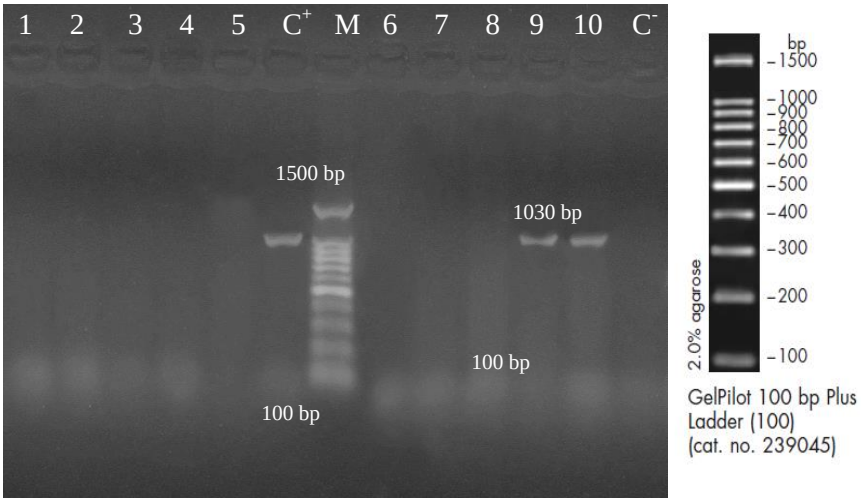

Figure 4. PCR amplified products of *vanA* gene at 1030 bp among *Staphylococcus aureus* isolated from dairy products. Lanes (M): QIAGEN GelPilot DNA 100 bp plus ladder; lanes (C^+): positive control *Staphylococcus aureus* (*vanA* positive); lanes (C^-): negative control *Staphylococcus aureus* (*vanA* negative); lanes (1-10): *Staphylococcus aureus* isolates isolated from dairy products; lane 9,10: positive samples of *vanA* gene (1030 bp); lane 1,2,3,4,5,6,7,8: negative samples of *vanA* gene.

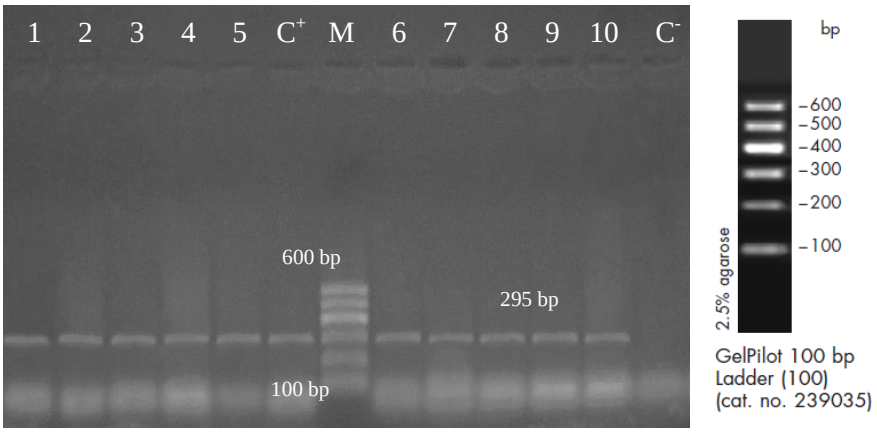

Figure 5. PCR amplified products of *ermC* gene at 295 bp among *Staphylococcus aureus* isolated from cattle milk. Lanes (M): QIAGEN GelPilot DNA 100 bp ladder; lanes (C^+): positive control *Staphylococcus aureus* (*ermC* positive); lanes (C^-): negative control *Staphylococcus aureus* (*ermC* negative); lanes (1-10): *Staphylococcus aureus* isolates isolated from cattle milk; lane (1-10): positive samples of *ermC* gene (295 bp).

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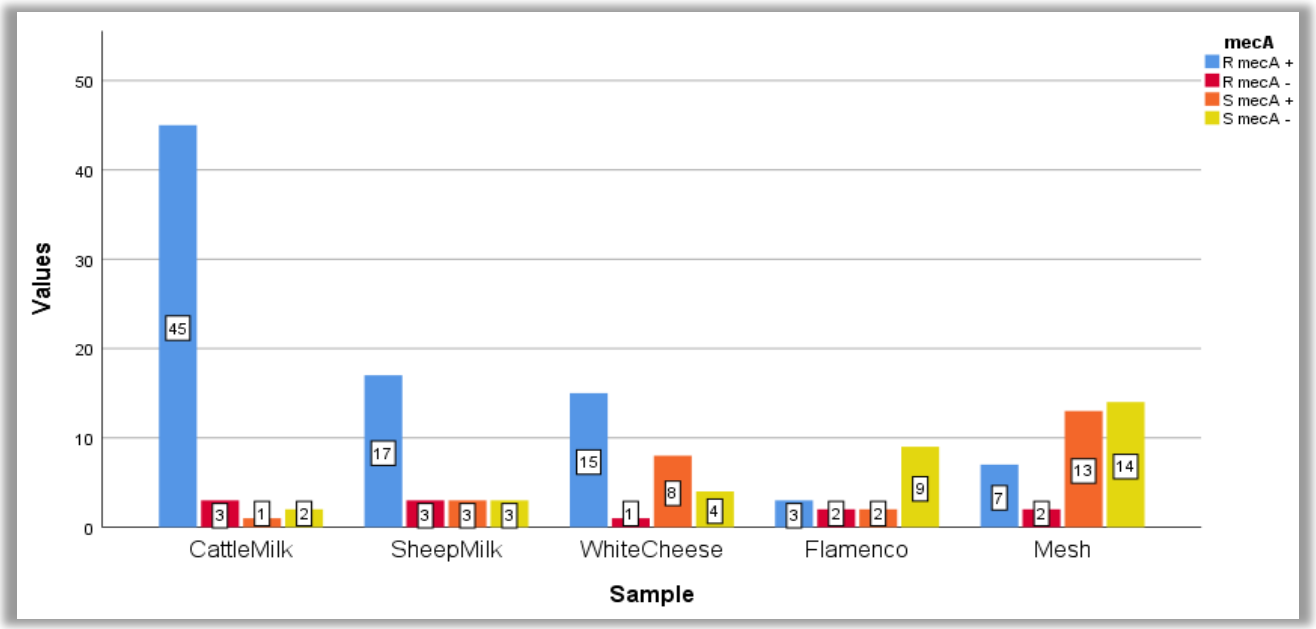

Figure 6. Presence (+) or absence (-) of *mecA* gene versus penicillin G (10 IU) resistance (R) or sensitivity (S) in *Staphylococcus aureus* isolated from each category of sample (cattle milk, sheep milk, white cheese, flamenco, and mesh). The presence of *mecA* gene in *Staphylococcus aureus* is statistically associated with its encoding phenotypic resistance patterns against penicillin. Along with an increase in the frequency of *mecA* gene, the resistance rate to penicillin sharply increased.

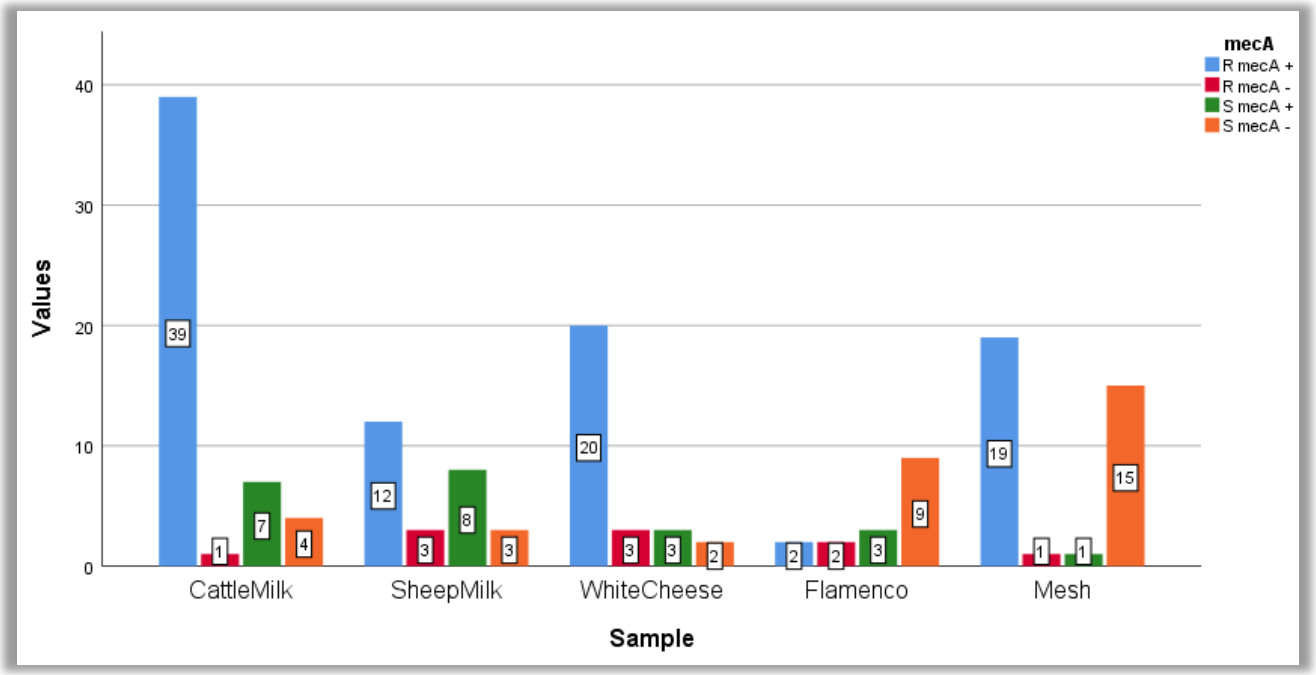

Figure 7. Presence (+) or absence (-) of *mecA* gene versus oxacillin (1 µg) resistance (R) or sensitivity (S) in *Staphylococcus aureus* isolated from each category of the sample (cattle milk, sheep milk, white cheese, flamenco, and mesh). The presence of *mecA* gene in *Staphylococcus aureus* is statistically associated with its encoding phenotypic resistance patterns against oxacillin. Along with an increase in the frequency of *mecA* gene, the resistance rate to oxacillin sharply increased.

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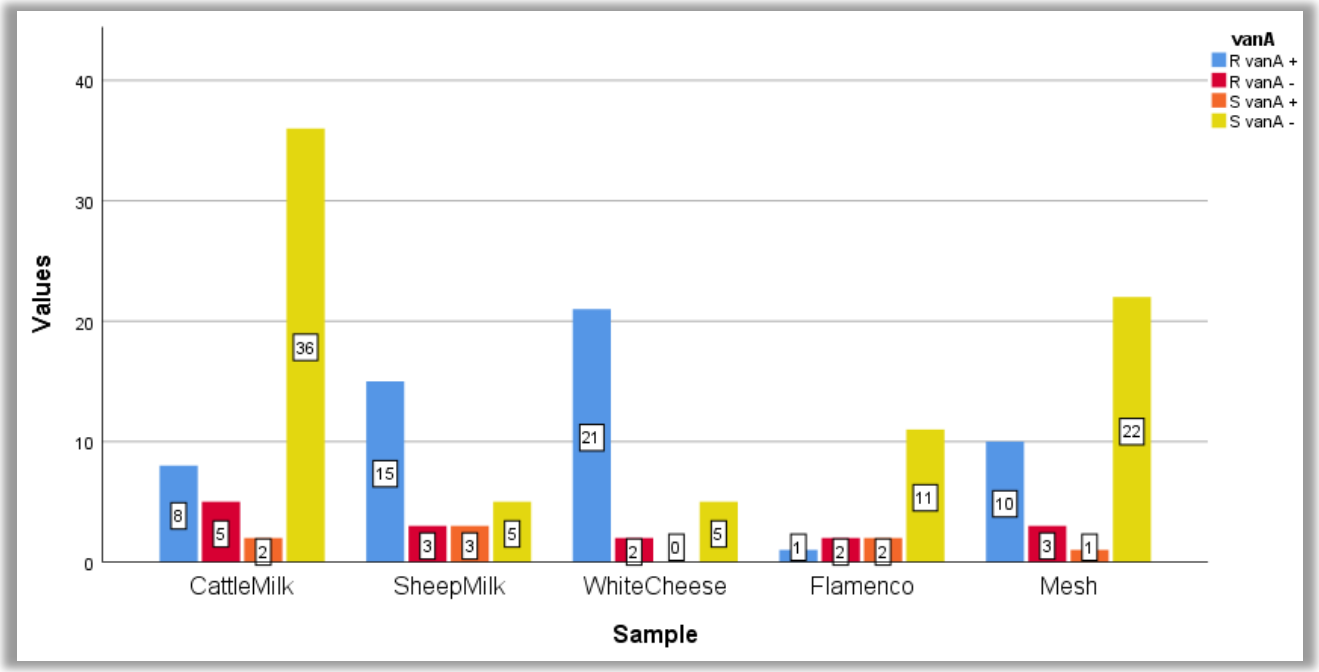

Figure 8. Presence (+) or absence (-) of *vanA* gene versus vancomycin (30 µg) resistance (R) or sensitivity (S) in *Staphylococcus aureus* isolated from each category of the sample (cattle milk, sheep milk, white cheese, flamenco, and mesh). The presence of *vanA* gene in *Staphylococcus aureus* is statistically associated with its encoding phenotypic resistance patterns against vancomycin. Along with an increase in the frequency of *vanA* gene, the resistance rate to vancomycin sharply increased.

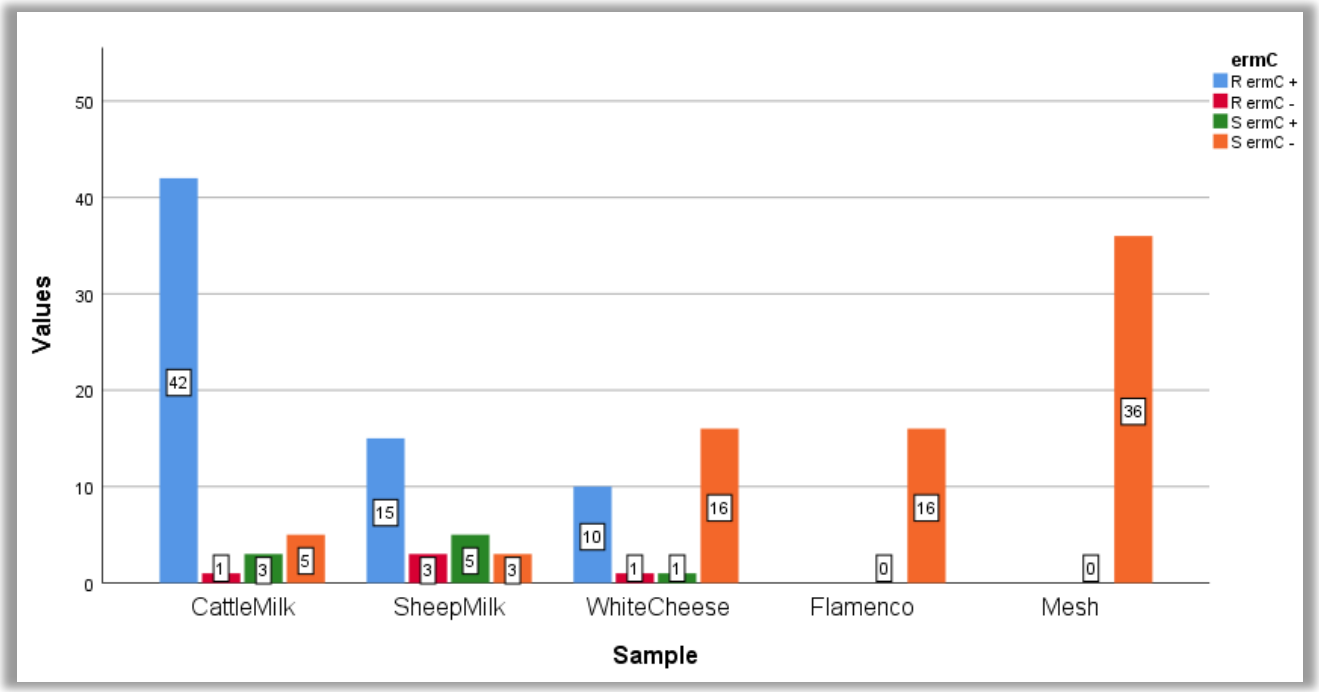

Figure 9. Presence (+) or absence (-) of *ermC* gene versus erythromycin (15 µg) resistance (R) or sensitivity (S) in *Staphylococcus aureus* isolated from each category of the sample (cattle milk, sheep milk, white cheese, flamenco, and mesh). The presence of *ermC* gene in *Staphylococcus aureus* is statistically associated with its encoding phenotypic resistance patterns against erythromycin. Along with an increase in the frequency of *ermC* gene, the resistance rate to erythromycin sharply increased.

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DISCUSSION

Staphylococcus aureus is considered a dangerous human pathogen that causes a wide range of diseases varying from mild to life-threatening illnesses. This bacterium is also considered one of the main causes of food intoxication in human beings (Zayda et al., 2020). In the current study, 69.5% out of the examined 226 milk and dairy samples were positive for the presence of *S. aureus*. Contamination of dairy products with *S. aureus* was also studied in many previous research articles. For example, in a previous study, out of the examined 144 retail raw milk samples collected from different regions in China, 43.1% were contaminated by *S. aureus* with the highest isolation rate of 61.7% from cow milk (Kou et al., 2021) which is considered a high incidence rate as that recorded in the current study. Moreover, 29 % of the examined milk samples were positive for *S. aureus* (Angelidis et al., 2020) which was lower than the obtained results of the present study. Also, 16.6% of *S. aureus* contaminations were previously recorded in the examined milk samples (Regasa et al., 2019) which also was lower than the reported value of the current investigation. Further, 14% of the examined cheese samples which were collected from Altay, Yining, and Tacheng, were positive for the presence of *S. aureus* (Cai et al., 2021) which is lower than the current study. Contamination of dairy products with *S. aureus* can occur during the production process of dairy products through using raw milk for fermentation and cheese production in an unsanitary working environment in dairy farms. The longer the production line, the higher the prevalence of *S. aureus* contamination in cheese products. Another important element is the hygiene level of the workers. Most retailers do not necessitate sterile packaging standards of cheese but they rather have them be dried in a natural environment. This inevitably leads to an increased probability of *S. aureus* prevalence rates (Cai et al., 2021). The above observations advise that an effective sanitary barrier is imperative for cheese production. The current study illustrates that there is room for improvement in these aspects so it is advisable to establish new ways to improve better hygiene practices in the production and processing of dairy products. In addition, it is imperative to conduct professional training for workers in each step of milk collection and dairy products production.

On the other hand, *S. aureus* bacterium is famous for its resistance against a wide range of antibiotics (Cheung et al., 2021). The existence of antibiotic-resistant *S. aureus* is a substantial issue that threatens human life (Frieri et al., 2017). In the current study, 22.3%, 16.6%, and 51.0% of all isolated *S. aureus* strains indicated resistance to one, two, and more than two (MDR) antimicrobial agents. The antimicrobial resistance patterns were also studied in many previous research articles. For example, 36.3%, 46.6%, and 12.8%, *S. aureus* strains isolated from milk and dairy products showed resistance to one, two, and more than two antimicrobial agents respectively which slightly vary from the present investigation (Jamali et al., 2015). Furthermore, 80.6% of *S. aureus* strains isolated from retail raw milk samples in northern Xinjiang, China, were resistant to at least one antibiotic, while 46.8% were resistant to three or more antibiotics which are nearly similar to the current investigation (Kou et al., 2021). Multidrug-resistant *S. aureus* was also detected in 15.4%, 14.3%, and 8.1% of *S. aureus* strains isolated from cow milk, cheese, and sheep milk respectively (Jamali et al., 2015). Moreover, MDR *S. aureus* was also detected in 27.4% of *S. aureus* strains isolated from cheese (Cai et al., 2021).

In the current study, clindamycin and chloramphenicol resistance in all isolated *S. aureus* strains were 14.0% and 2.5% respectively. Mostly like the present investigation, *S. aureus* strains isolated from 2650 milk and dairy products' samples, including cow milk, sheep milk, and cheese showed 11.3% clindamycin and 3.7% chloramphenicol resistance (Jamali et al., 2015). Moreover, 0.5% chloramphenicol resistance was detected among *S. aureus* strains isolated from raw milk samples (Rola et al., 2015). However, 24.2% of *S. aureus* strains isolated from cheese samples showed clindamycin resistance (Cai et al., 2021) which was higher than that of the current study. On the other hand, the high resistance level of *S. aureus* has been previously reported against methicillin, tetracycline, gentamicin, kanamycin, and streptomycin (Jamali et al., 2015). In the current study, the tetracycline resistance in *S. aureus* strains was 21.0%. In previous studies, 56.1%, 6.3%, and 0.9% of *S. aureus* strains showed tetracycline resistance in surveys done by Jamali et al. (2015), Rola et al. (2015), and Antonios et al. (2015) respectively. Data presented in the current study revealed that all tested *S. aureus* strains were sensitive to trimethoprim-sulfamethoxazole, linezolid, ciprofloxacin, and gentamycin. Consistent with the current study, Jamali et al. (2015) indicated that all of the isolated *S. aureus* strains from 2650 samples of milk and dairy products including cow milk, sheep milk, and cheese were susceptible to trimethoprim-sulfamethoxazole and ciprofloxacin.

In the present study, the resistance against penicillin and oxacillin in *S. aureus* strains was 62.4% and 65.0% respectively. The observed resistance to penicillin and oxacillin in *S. aureus* strains represents an alarming indicator for the presence of phenotypic methicillin-resistant *S. aureus* (MRSA) which is considered a public health hazard. In a previous study, 95.3% of the isolated *S. aureus* strains showed penicillin resistance which is greater than the current study (Beyene et al., 2017). Also, 72.6% of the isolated *S. aureus* strains from milk samples showed penicillin resistance which is greater than the current study (Kou et al., 2021). However, penicillin resistance was 15.5% in another study (Rola et al., 2015) which is lower than the current study. In agreement with the present study, phenotypic MRSA revealed resistance to both penicillin and oxacillin (Chajęcka-Wierzchowska et al., 2015; Regasa et al., 2019). Oxacillin-resistant *S. aureus* was also identified previously as MRSA (Jamali et al., 2015).

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Notably, livestock and their products are possible reservoirs for MDR phenotypic *S. aureus* due to the extensive utilization of antibacterial agents during livestock production (Zayda et al., 2020). In veterinary practice, *S. aureus* can easily acquire resistance to antibiotics due to extensive utilization of antibacterial agents either to control bacterial infections or to speed up animal growth rate (Frieri et al., 2017). It is actually difficult to compare the results of the current study with other studies on the prevalence of resistance characteristics of *S. aureus* in milk and dairy products due to the scarcity of research regarding these issues. However, the difference between different studies on the prevalence rate of antibiotic resistance *S. aureus* in milk and dairy products is primarily caused by the differences in the country, animal species, livestock breeding system, and sanitary conditions in the milking environment.

The isolated *S. aureus* strains were screened for the presence of *mecA*, *vanA*, and *ermC* genes. In the current study, 72.6 % of the isolated *S. aureus* strains were carriers for *mecA* gene. The presence of *mecA* gene confirms the presence of MRSA as previously mentioned by Zhang et al. (2016). Many *S. aureus* strains can be converted to MRSA due to *mecA* gene acquisition (Zhang et al., 2016). The presence of *mecA* gene revealed in turn phenotypic methicillin resistance in all genotypic MRSA strains as previously mentioned by Chajęcka-Wierzchowska et al. (2015). Moreover, Zayda et al. (2020) reported that 100% of the phenotypic oxacillin-resistant MRSA isolates were genotypic *mecA* positive. Zhang et al. (2016) reported that when only the isolates were both *mecA* positive and cefoxitin resistant, they can be then classified as MRSA strains. In another study, more than 63% of *S. aureus* isolates were carriers of *mecA* gene, and so are considered MRSA strains (Houri et al., 2020). Although 7 *S. aureus* strains showed resistance to oxacillin (0.25 μg ml^{-1}), they cannot be characterized as MRSA strains because they were *mecA* negative (Antonios et al., 2015). Therefore, a combination of genotypic and phenotypic characterizations is necessary for the confirmation of the presence of MRSA to avoid false positive or false negative MRSA confirmation (Zhang et al., 2016). In the present study, detection of MRSA isolates was done using both phenotypic and genotypic confirmation; i.e if the isolate is both penicillin and oxacillin resistant in addition to its acquisition of *mecA* gene, it is then identified as MRSA strain. Accordingly, it was indicated that 47.1% of all tested *S. aureus* strains were characterized as MRSA strains. The MRSA is also noticed by many other research articles with high incidence (Houri et al., 2020). For example, in a previous study, 21% of *S. aureus* strains isolated from cheese samples were identified as MRSA strains (Cai et al., 2021). In addition, 51.6% of the isolated *S. aureus* strains from milk samples were identified as MRSA strains (Kou et al., 2021) which is nearly similar to the present study. However, 5.4% of MRSA levels were noticed in the buffalo bulk tank milk in Italy which is lower than the present study (Giovanni et al., 2020). Further, 100% of oxacillin resistant *S. aureus* strains isolated from milk and cheese samples, were mecA gene positive confirming the presence of MRSA isolates (Zayda et al., 2020). MRSA was detected in nine *S. aureus* isolates which were carriers for *mecA* gene (Angelidis et al., 2020). Further, 2% of MRSA strains were isolated from milk and cheese (Jamali et al., 2015) which is less than the current study. Antimicrobials from five major classes, including β-lactams, aminoglycosides, tetracyclines, sulphonamides, and macrolides are the most commonly used antimicrobials in veterinary practice to control bacterial diseases in herds. However, inappropriate use of these antimicrobials for a long time has been considered to be responsible for the gradual development of resistance in *S. aureus* (Cai et al., 2021). β-lactams are the most widely used antimicrobials for treating dairy farms' infections. This may be the reason for such a high incidence rate of MRSA among dairy products (Jamali et al., 2015; Rola et al., 2015). The β-lactam resistance feature in MRSA is mainly based on two mechanisms; the first is the production of β-lactamases; an extracellular enzyme which is synthesized when *staphylococci* are exposed to the β-lactam (Panchal et al., 2020). The second is via the production of a low-affinity penicillin-binding protein 2a (PBP2a) which is controlled by *mecA* gene which codes for the PBP2a protein. *mecA* gene mediates resistance to all classes of the β-lactam antibiotics, including methicillin, oxacillin, and cephalosporins (Panchal et al., 2020). On the other hand, Chajęcka-Wierzchowska et al. (2015) reported that MRSA was phenotypic resistant to all β-lactam antibiotics, penicillins, amino-penicillins, isoxazolyl-penicillins.

In the present study, 40.1 % of the isolated *S. aureus* strains were carriers for *vanA* gene while 44.6% of them were phenotypic vancomycin-resistant. Of the phenotypic vancomycin resistance *S. aureus* strains, 78.6% were genotypic *vanA* gene carriers representing VRSA strains however the remaining 21.4% were *vanA* negative. Frieri et al. (2017) documented that the horizontal transferable silenced *vanA* can escape detection and revert into resistance during vancomycin therapy which is considered a new challenge. In a previous study, 2 of the isolated *S. aureus* strains from cow milk were vancomycin-resistant (Kou et al., 2021). Inconsistent with the present study, all of the previously isolated *S. aureus* strains from cheese and milk were sensitive to vancomycin (Castro et al., 2020; Giovanni et al., 2020).

In the current study, 48.4 % of the isolated *S. aureus* strains were carriers for *ermC* gene. In case of erythromycin resistance *S. aureus*, 67 (93.1%, 67/72) strains were carriers for *ermC* gene. However, 5 (6.9%, 5/72) were not. Erythromycin-resistant *S. aureus* was also noticed in many previous research articles. For example, Antonios et al. (2015) reported 2.8% erythromycin-resistant *S. aureus* strains isolated from Greek ovine milk. Rola et al. (2015) reported 3.4 % erythromycin-resistant *S. aureus* strains isolated from milk which was lower than that of the current investigation. Moreover, 27.4% of the isolated *S. aureus* strains from cheese samples showed resistance to erythromycin (Cai et al., 2021). Furthermore, 32.3% of the isolated *S. aureus* isolated from milk samples showed resistance to erythromycin (Kou

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et al., 2021) which was nearly similar to the current investigation. The phenotypic genotypic erythromycin resistance in *S. aureus* was also discussed in many previous research articles. For example, 7.9% of the isolated *S. aureus* strains from 2650 milk, cheese, and dairy product samples showed phenotypic erythromycin resistance profiles, while the *ermC* gene was detected in only 69.2% of these isolates (Jamali et al., 2015). Further, *S. aureus* isolated from sheep milk cheese were not carriers for *ermC* genes and were susceptible to erythromycin (Spanu et al., 2012). Previous studies have indicated that the methylase gene of macrolide *ermC* is responsible for erythromycin resistance among *S. aureus* isolates (Munita and Arias, 2016; Houri et al., 2020).

The statistical analysis of the current study revealed statistically significant results and so the alternative hypothesis that there is a significant association between each genotypic and its phenotypic features will be accepted. In other words, the presence of *mecA* gene in *S. aureus* was statistically associated with its encoding phenotypic resistance patterns against both penicillin and oxacillin. Moreover, along with an increase in the frequency of *mecA* gene, the resistance rates to both penicillin and oxacillin sharply increased. Previous studies indicated a perfect correlation between data obtained by conventional antimicrobial susceptibility patterns and PCR-based methods (Ray, 2017; Houri et al., 2020). Although there were 11 and 10 *S. aureus* strains in the current study which were phenotypically resistant to both penicillin and oxacillin respectively, they were *mecA* negative. The *mecA*-negative phenotypic-positive MRSA strains have been reported in many previous research articles. For example, 6 *S. aureus* strains (10.3%) were identified as phenotypic MRSA although they were *mecA*-negative (Zhang et al., 2016). The presence of *mecA*-negative phenotypic-positive MRSA strains in bovine milk samples was also reported in China (Wang et al., 2014). In the current study, the presence of *vanA* and *ermC* genes in *S. aureus* was statistically associated with their encoding phenotypic antibiotic resistance patterns against vancomycin and erythromycin respectively. Furthermore, along with an increase in the frequency of *vanA* and *ermC* genes, there was a sharp increase in the resistance rates against vancomycin and erythromycin respectively. Although there were 8 and 9 *S. aureus* strains in the current study which were genotypic positive for the *vanA* and *ermC* genes respectively, they were phenotypic sensitive to vancomycin and erythromycin respectively. Spanu et al. (2012) previously reported that all the examined *S. aureus* strains did not carry *vanA* gene and were susceptible to vancomycin.

On the other hand, biofilm formation assay in the current study revealed that 6.8% of MRSA isolates exhibit weak biofilm-forming ability while the remaining 93.2% exhibit no biofilm-forming ability. Notably, biofilms attached to organic and inorganic surfaces protect MRSA from antimicrobial agents, such as disinfectants or antibiotics. So, eradicating these biofilms becomes very problematic which will elevate the hazard of MRSA cross-contamination in dairy farms. Milk and dairy products contaminated with strong biofilm-forming MRSA can be considered additional risks in terms of food safety and public health hazards (Angelidis et al., 2020).

CONCLUSION

The presence of MDR *S. aureus* especially methicillin, vancomycin, and erythromycin-resistant *S. aureus* isolates in milk and dairy products poses a potential threat to public health and is of great concern in terms of animals' health. This presence highlights the need for continuous monitoring of hygiene in the dairy production chain. The presence of *mecA, vanA,* and *ermC* genes within the isolated *S. aureus* is statistically associated with their encoding phenotypic resistance patterns against both penicillin and oxacillin, vancomycin, and erythromycin respectively. Along with an increase in the frequency of *mecA, vanA,* and *ermC* genes, their phenotypic antibiotic resistance patterns sharply increased with OR >1. It is recommended to use PCR for continuous monitoring of genotypic resistance *mecA, vanA,* and *ermC* genes in addition to studying their phenotypic resistance patterns in the cases associated with *S. aureus* infection as a routine monitoring in dairy farms. Reasonable restricted use of antimicrobials is recommended in dairy farms in addition to the application of biosecurity measures and hygienic husbandry practices.

DECLARATIONS

Shimaa Tawfeeq Omara proposed the idea, design, and coordination of the current study. She performed phenotypic and genotypic identification of the isolates, performed the statistical analysis, writing, and drafting of the manuscript. Ashraf Samir Hakim and Magdy Ali Bakry participated in the collection of samples and characterization of the isolates.

Competing interests

The authors have declared that they had no competing interests.

Ethical consideration

Ethical issue including plagiarism, consent to public misconduct, data fabrication and⁄ or falsification, double publication and⁄or submission, redundancy has been checked by the authors.

Authors' contribution

All authors contributed equally to the present study.

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