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Genetic Characterization of Multi Drug Resistant *Staphylococcus* Spp. in Raw Milk and Karish cheese

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ABSTRACT

Multi drug resistant (MDR) *Staphylococcus* spp. particularly the emerging Methicillin-resistant *S. aureus* (MRSA) strains are of serious zoonotic public health concern. The scope of this study was to analyze the incidence of *Staphylococcus* spp. with a focus on *S. aureus* and MRSA strains and evaluate their staphylococcal enterotoxins (SE) and resistance genes. A total of 330 samples of raw milk from animals (cows, buffaloes, sheep and goat) and Karish cheese (as a major risk for in dairy industry) were screened and examined. The prevalence ratio of *S. aureus* in raw milk (from examined farm animals or milk smallholders) and in cheese was 38.5% (127/330) however, non-S. *aureus* (NSA) was identified in 16.7% (55/330). Multi Drug Resistant (MDR) pattern of *S. aureus* isolates was exhibited with identification of MRSA strains in (44.9%). Penicillin showed the highest resistance level (55.1%) however, gentamycin was the most sensitive one. *S. aureus* strains were confirmed with the presence of *nuc* gene in 100% with PCR tool however, PCR for SE genes declared that *seb* was prevalent in 85.7% followed by *sed* and *sea* genes in (64.3% and 28.5%), respectively. Furthermore, β-lactam resistance (*blaZ* and *mecA*) genes were found in 100% and 50%, respectively. This data suggested that monitoring and surveillance plans with strict control measures in dairy farms should be applied to lessen the spread of MDR *Staph* spp. especially MRSA strains. Also, awareness of milk smallholders and farm workers must be raised to ensure the quality of raw milk for safe public health concern..

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INTRODUCTION

Staphylococcus species is ubiquitous organisms persist normally in the air, soil, water, milk and milking equipment in dairy animal farms **(Saka and Terzi Gulel 2018)**. *S. aureus* is one of major pathogenic food-borne bacteria according to the records of WHO organization **(Mahfoozi et al. 2019)**.

Raw milk is an extremely common and genuine risk to the dairy industry in which *S. aureus* could be a potential pathogen that are responsible for intramammary infections in dairy animals leading to mastitis and huge economic losses **(De Silva et al. 2016)**. Although the milk possesses a high nutritive value and rich protein, vitamins, and minerals; it also had been regarded as favorable medium for viability and growth of various types of food borne organisms intensifying the public health risks in both human and animals **(Pandey et al. 2014)**. *Staph aureus* organisms were detected previously in raw milk, cheese, ice cream, clotted cream, and butter **(Saka and Terzi Gulel 2018)**.

The virulence and pathogencity of *S. aureus* could emerge from its capability to produce a set of potent intracellular proteins (toxins) that called Staphylococcal Enterotoxins or (SE). They could intern contaminate the milk either in raw form or in other artisanal form of dairy products **(Gebremedhin et al. 2022; Lee et al. 2016)**. Staphylococcal Enterotoxins are heat-stable, single-chain proteins, of low molecular weight (27-31 kDa). These toxins could resist the action of denaturing agents and a wide range of pH **(Özdemir and Keyvan 2016)**. Also, they couldn't be degraded with the proteolytic enzymes (pepsin, trypsin, and chymotrypsin) so can be active even after ingestion inside the digestive tract passing through the stomach and attacking the intestinal cells **(Mahfoozi et al. 2019).** Hence, SE producing strains of *S. aureus* could grow in variable extreme conditions and easily contaminate the dairy products during stages of processing or preparation constituting great potential risks of food poisoning **(Wang et al. 2017).**

The scientists identified about twenty-three serologically distinct SE and Sel (Staphylococcal Enterotoxin like) proteins **(Benkerroum 2018; Rong et al. 2017)**. Both SE and Sel toxins were found to be intended for major outbreaks of staphylococcal food-borne **(Umeda et al. 2017)**. It is recorded that 95% of food poisoning outbreaks with *Staph species*; were linked with the main five classical biotypes (A, B, C, D, and E) of *S. aureus* while 5% only could be happened due to the new biotypes **(Tang et al. 2011)**. Polymerase Chain Reaction (PCR) is an efficient, rapid, accurate and reliable screening assay for the detection of SE toxins in raw milk samples that might be contaminated with *S. aureus* organisms **(Avila-Novoa et al. 2018).**

MRSA are emerging serious group of *S. aureus* species that mainly associated with several cases of human hospital and community-acquired infections **(Gopal and Divya 2017)**. MRSA are one of the life-threatening contagions which is truly notable in various types of contaminated milk and dairy products resulting in community health anxiety **(Alghizzi and Shami 2021)**. Moreover, MRSA was reported in many studies in bovine milk and dairy animals especially with mastitis that permit MRSA group to be readily transferable to humans in contact **(Basanisi et al. 2017; Caruso et al. 2016; Liu et al. 2022)**. On the contrary, little knowledge had been reported about the prevalence of MRSA in other animals (e.g., sheep, goats, camels), although the consumption of milk from different animal species is more and more common.

Unfortunately, in the veterinary field, antibiotics had been utilized for both therapeutic and sub-therapeutic purposes to promote growth and improve feed efficiency in animals **(Van Soest et al. 2016)**. This practice led to phenomena which is known as antimicrobial resistance (AMR). AMR of *S. aureus* strains had been exponentially growing and developing in the last decade all over the world. It is associated mainly with an indiscriminate usage of antimicrobials by healthcare providers, untrained practitioners, or medication consumers **(Pekana et al. 2017).** So, no susceptibility of *S. aureus* strains against penicillin and tetra-

cycline drugs for example was documented due to regular use in the treatment in dairy farms that could produce new antibiotic resistant strains and the growing AMR phenomenon **(Gebremedhin et al. 2022).** Nowadays, there is an increased public and scientific concern regarding extensive use of antimicrobials to limit the emergence and dissemination of multiple antibiotic resistant zoonotic bacterial pathogens **(Bueno et al. 2018; El-Fateh et al. 2020).**

The aim of this study was to estimate the prevalence of *S. aureus* spp. particularly MRSA strains in raw milk and Karish cheese and detect their staphylococcal count with a focus on their phenotypic and genotypic antibiotic resistance traits along with PCR analysis of staphylococcal enterotoxins (SE) in these isolates trying to evaluate the potential risks from contaminated milk and products in human and public health concerns.

MATERIALS and METHODS

1-Study design and sample Collection:

A total of 330 samples were used in this study. Two hundred and fourty raw milk samples (60 raw bulk tank milk samples were obtained from cows and 60 from buffaloes in different dairy farms which depend mainly on traditional methods of hand milking technique, 120 raw unpasteurized milk samples from milk smallholders), 30 ewe's milk samples, 30 ewe's goat milk and 30 homemade Karish cheeses were collected and examined. The samples were gathered from different districts at Ismailia Governorate, Egypt. This study was performed during the period from October 2021 to July 2023.

In a farm, the bulk tank milk had been collected after milking all dairy animals then mixed in a large container in a specific milk collection area. The samples were taken before anyone enter the farm for keeping a good hygiene in the dairy farms at the milk collection site. To make representative samples of bulk milk either at farms or from smallholder sources; samples should be thoroughly homogenized and mixed before taking the representative sample. Also, all samples were collected aseptically in sterile plastic tubes and stored at 4°C to be further examined.

However, Karish cheese samples were homemade synthesized. About one hundred grams of Karish cheese of each sample was collected in sterile separate containers and maintained at 4°C to be further examined. All samples were taken under sterile conditions and transported without any delay to AHRI (Animal Health Research Institute) bacteriological laboratory to be bacteriological and PCR examined.

2- Enumeration of *Staphylococci* and *S. aureus* in raw samples of milk and Karish cheese:

Total *Staphylococci* and *S. aureus* count was performed according to **(IS0, 2003: 6888–1:1999 +A1)** guidelines.

3- Bacterial isolation of *Staphylococci* spp. in raw milk and Karish cheese:

The isolation and identification of *Staphylococci* spp. and *S. aureus* was done according to **(IS0, 2003: 6888–1:1999 +A1)**. The produced typical colonies of *Staphylococci* spp on Mannitol salt and Baird parker agar media plates were picked up to be purified on trypticase soya agar and re-incubated for 24 h at 37° C (**TSA, Oxoid Ltd., Hampshire, England**). All identified Staphylococci colonies were stained with Gram stain and biochemically identified via (catalase, oxidase, coagulase test and mannitol fermentation tests) **(Wakabayashi et al. 2018)**. Moreover, other coagulase negative *Staphylococci* (CNS) isolates were tested using the Integral system stafilococchi kit (Liofilchem). The purified Staph colonies were preserved at −80 °C in 30% glycerol for further studies.

4- Antibiogram pattern of the recovered *S. aureus* isolates:

All biochemically confirmed *S. aureus* isolates in this study were tested for antimicrobial sensitivity and resistance patterns with the Kirby–Bauer disk diffusion method and the inhibition zones were measured and interpreted as sensitive (S), intermediate (I), and resistant (R) according to the guidelines of the Clinical and

Laboratory Standards Institute; CLSI, 2020 **(Weinstein and Lewis 2020).** The following antimicrobial agents of different antibiotic classes were used: Penicillin, Norfloxacin, Oxacillin, Oxytetracycline, Chloramphenicol, Amoxicillin, Trimethoprim-sulfamethoxazole, Azithromycin, Linezolid, Doxycycline, Ciprofloxacin, Fosfomycin, Cefotaxime and Gentamycin. *S. aureus* isolates that were found to be resistant to ≥ 3 antibiotic classes were considered multidrug-resistant (MDR) strains.

5- Genotypic investigation of virulent and SE associated genes of *S. aureus* DNA isolation:

Genomic DNA extraction method for the selected *S. aureus* isolates For this purpose, overnight incubated cultures of the recovered *S. aureus* on Brain Heart Infusion broth **(BHI, Oxoid**) were prepared using 2 ml of freshly BHI broth with 0.1 ml of *S. aureus* tested cultures. The PCR sample suspensions were incubated then with 20 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After that, 2 ml of 100% ethanol was added to the lysate. The samples were then centrifuged at 5.000 rpm for 10 minutes and the supernatants were discarded. Washing of bacterial pellets twice was performed with 1 ml of saline solution, centrifuged again and resuspended in 180 µl Tris EDTA buffer (Sigma) containing 18 µl of lysostaphin (0.5 U/µl, Sigma, L7386) then incubated at 37°C for 1 hour **(Akineden et al. 2008).** This protocol of DNA extraction was done according to QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with some modifications of AHRI, from the manufacturer's recommendations.

Amplification cycling and analysis of PCR products:

In the current study, *S. aureus* reference strain (that was kindly provided by AHRI, Dokki, Giza and confirmed as *S. aureus*) used as positive control. Firstly, the selected strains of *S. aureus* were confirmed with detection of nuc identification genes using specific primers. Also, specific primer sets were used to detect (SE) staphylococcal enterotoxin (*sea*, *seb*, *sec*, *sed*, *see*) genes **(Johnson et al. 1991; Monday and Bohach 1999).** The amplification cycling conditions of each gene, primers sequences, target genes, oligonucleotide primers (that were supplied from **Metabion; Germany)** and amplicon sizes were tabulated in **(Table A)**. The PCR reaction was performed in an Applied biosystem 2720 thermal cycler. Then, separation of PCR products was done by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. After that, for gel analysis, PCR reaction products were loaded in each gel slot and the fragment sizes were determined with the use of a gene ruler 100 bp ladder (Fermentas, thermofisher, Germany). Finally, the gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software **(Keyvan and Özdemir 2016).**

Table A. Primers sequences, target genes, amplicon sizes and cycling conditions of *S. aureus* isolates

Target gene	Primers sequences	Amplified segment (bp)	Primary Denaturation ˚C/min	Amplification (30-35 cycles) Secondary denaturation ˚C/Sec	Annealing ˚C/Sec	Extension ˚C/Sec	Final extension ˚C/ min	Reference
Nuc	F: ATA GGG ATG GCT ATC AGT AAT GT R: GAC CTG AAT CAG CGT TGT CTT C	624	94/5	94/30	55/30	72/40	72/10	(Lem et al. 2001)
Sea	F: GGT TAT CAA TGT GCG GGT GG R: CGG CAC TTT TTT CTC TTC GG	102	94/5	94/30	55/30	72/40	72/10	
Seb	F: GTATGGTGGTGTAACTGAGC R: CCAAATAGTGACGAG-TTAGG	164	94/5	94/30.	57/30	72/30	72/7	(Mehrotra et al. 2000)
Sec	F: AGA TGA AGT AGT TGA TGT GTA TGG R: CAC ACT TTT AGA ATC AAC CG	541	94/5	94/30	57/30	72/30	72/7	
Sed	F: CCAATAATAGGAGAAAA-TAAAAG R: ATTGGTATTTTTTTTCGTTC	278	94/5	94/30	57/30	72/30	72/7	
See	F: AGG TTT TTT CAC AGG TCA TCC R: CTT TTT TTT CTT CGG TCA ATC	209	94/5	94/30	57/30	72/30	72/7	
mecA	F: TCCAGATTACAACTTCAC-CAGG R: CCACTTCATATCTTGTAACG	162	95/5	95/60	54/60	72/60	72/7	(Oliveira et al. 2016)
blaZ	F: TACAACTGTAATATCGGAGGG R: R: CATTACACTCTT-GGCGGTTTC	861	95/5	95/60	54/60	72/60	72/7	

RESULTS:

Total counts for Staphylococci and *S. aureus* spp. in raw milk and cheese samples:

As mentioned in table (1), the examination of raw milk samples from buffalo and cow in dairy farms and from milk smallholders (n=60) for total staphylococci count confirmed the presence of *Staphylococcus* spp. in different ratios. The mean value for *Staphylococcus* spp. count was calculated as $9.5x10^2 \pm 2.5x10^2$, $3x10^4 \pm 0.2x10^4$ for cows, $5.6x10^2 \pm 1.7x10^2$, $4.9x10^5 \pm 5.7x10^4$ for buffaloes from dairy farm and milk smallholders, respectively. Meanwhile the mean value for ewes, ewe's goat raw milk was $2.5x10^2 \pm 1\ x10^2$ and $1.2x10^2 \pm 0.2x10^2$, respectively while for Karish cheese, it was $7.5x10^2 \pm 0.4x10^2$.

Table 1. Statistical analytical results of Staphylococcal counts in different types of raw milk and Karish cheese

Samples		No. of samples	Positive samples No.	Positive samples %	Min.	Max.	Mean	±S.E
Raw bulk tank milk from animal farms	Cow	60	18	30	7x10	$5.3x10^4$	$9.5x10^2$	$2.5x10^2$
	Bufflo	60	12	20	4x10	$6x10^3$	$5.6x10^2$	$1.7x10^2$
Raw milk from smallholders	Cow	60	37	61.7	$2x10^3$	$4x10^5$	$3x10^4$	$0.2x10^4$
	bufflo	60	29	48.3	5x10	$8.5x10^6$	$4.9x10^5$	$5.7x10^4$
Raw ewes' milk		30	12	40	3x10	$1.6x10^4$	$2.5x10^2$	$1\ x10^2$
Raw ewe's goat milk		30	10	33.3	4x10	$2x10^3$	$1.2x10^2$	$0.2x10^2$
Homemade Karish cheese		30	9	30	$5x10^2$	$4x10^3$	$7.5x10^2$	$0.4x10^2$

N.B. Statistical (Min, Max and mean ±SE) was carried for positive samples only.

The obtained results in Table (2) revealed that the raw milk from smallholders have the highest staph counts than raw bulk tank milk from animal farms. Meanwhile, the highest distribution 60%, 42% and 89% of the ewe's, goat milk and Karish cheese were approximately similar in frequency distribution of staphylococcal counts.

Table 2. Frequency distribution of staphylococcal counts in different types of raw milk and Karish cheese

Frequency level	Raw bulk tank milk from animal farms				Raw milk from smallholders				Ewes sheep		Ewes Goat		Karish cheese	
	Cow		buffalo		Cow		buffalo							
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
>10-10^2	5	27.8	2	16.5	-	-	1	3.5	3	25	3	30	-	-
10^2-10^3	10	55.6	8	67	-	-	2	6.5	5	42	6	60	8	89
10^3-10^4	3	16.6	2	16.5	10	27	2	6.5	2	16.5	1	10	1	11
10^4-10^5	-	-	-	-	23	62	19	65.5	2	16.5	-	-	-	-
10^5-10^6	-	-	-	-	4	11	2	7	-	-	-	-	-	-
10^6-10^7	-	-	-	-	-	-	3	10.5	-	-	-	-	-	-
Total	18	100	12	100	37	100	29	100	12	100	10	100	9	100

Incidence and distribution of *S. aureus* and other *Staph* spp. in raw milk and cheese samples:

A total of 38.5% (127/330) were positive for coagulase positive *S. aureus* as illustrated in table (3). Statistically, non-significant differences were observed between the different sources for *S. aureus* prevalence ($p>0.05$). The total incidence rate of *S. aureus* spp. was 40 % (96/240) in cow and buffalo's raw milk, either its source was dairy farms or milk smallholders. In addition, *S. aureus* (CPS) was also isolated in 40% and 33.4 % in raw milk samples from ewes of sheep and goat origin, respectively. Also, 30% (9/30) of all examined homemade Karish cheese in this study were contaminated with *S. aureus* spp. Moreover, the totally other identified *Staph* species were 55/330 (16.7%) as shown in figure (1) and considered unfit for human consumption as the parameters mentioned in Egyptian Standards "**ES 1008-4/2005**".

Table 3. Prevalence rate of *S. aureus* and other *Staph* spp. isolates from raw milk and Karish cheese.

S. aureus spp. and origin of sample (n= number of samples)		*S. aureus* (CPS)	Other staph species (NAS)
Raw bulk tank milk from animal farms	Cows (n=60)	18/60 (30%)	10/60
	Buffaloes (n=60)	12/60 (20%)	9/60
Raw bulk milk from milk smallholders	Cows (n=60)	37/60 (61.7%)	8/60
	Buffaloes (n=60)	29/60 (48.4%)	11/60
Total raw milk from both cow and buffalo's animals (n=240)		96/240 (40%)	38/240 (15.8%)
Raw ewes' milk	Sheep (n=30)	12/30 (40%)	5/11
Raw ewe's goat milk	Goat (n=30)	10/30 (33.4%)	6/12
Total raw milk samples from all animals (n=300)		118/300 (39.3%)	49/300 (16.3%)
Homemade Karish cheese	Cheese (n=30)	9/30 (30%)	6/15 (40%)
Total	N= 330	127/330 (38.5%)	55/330 (16.7%)

CPS= Coagulase Positive *S. aureus* NSA= Non-Staph aureus

Biochemical confirmation of the recovered *S. aureus* isolates:

All the suspected *S. aureus* isolates that were yielded in this study from different sample sources gave positive reactions for catalase, Voges Proskauer and coagulase tests. Also, they fermented glucose, lactose, sucrose, maltose and mannitol with acid production, but they were negative for oxidase test. *Staph* species other than *S. aureus* (NSA) were also biochemically identified as illustrated in Figure (1). In the same context, this figure showed significant differences ($p<0.001$) between the other microbial species in different milk sources, maximizing for *S. epidermidis* (10%).

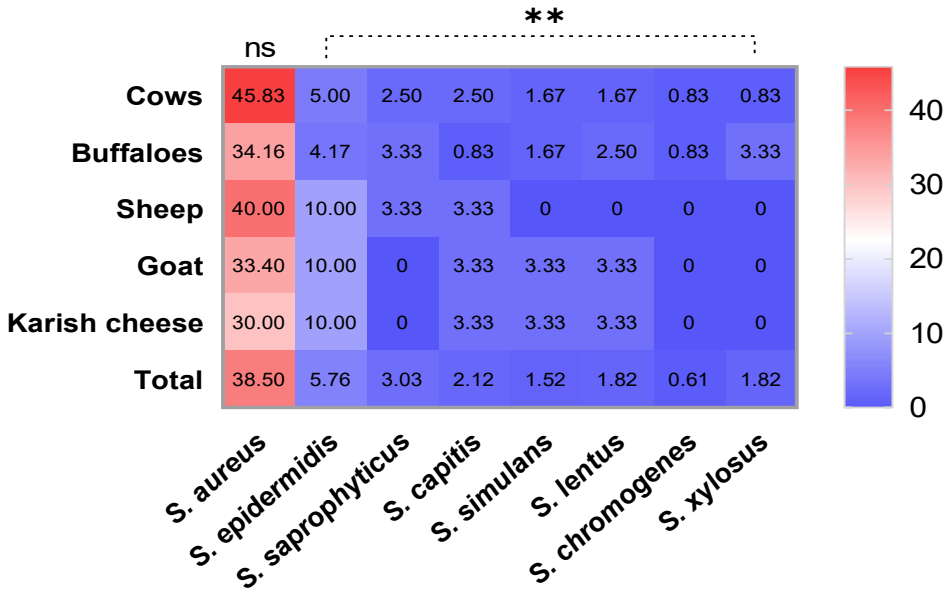

Figure 1. Prevalence rate of *S. aureus* and other *Staph* spp. isolates from raw milk and Karish cheese samples.

Antibiogram sensitivity results of *S. aureus* isolates:

Most of the tested *S. aureus* isolates in this study were multidrug resistant strains (MDR) which means that they were resistant to three or more antimicrobial agents of different antibiotic classes. Individual resistance profiles were demonstrated in **Table (4)**. The highest antibiotic resistance of *S. aureus* isolates was observed for penicillin (55.1%) in addition, other antibiotics of different classes included norfloxacin, oxacillin, oxytetracycline, chloramphenicol and amoxycillin showed a resistance range (40.2% - 51.2%) and to a lesser extent to linezolid (25.9%), doxycycline (23.6%) and ciprofloxacin (22%). Meanwhile, the highest sensitivity rates were observed in *S. aureus* isolates for gentamycin, cefotaxime and Fosfomycin. Significant associations were observed between antimicrobial resistance potency of different antibiotic and the animal sources of the recovered strain ($p<0.001$; **Figure** 2).

Moreover, according to the resistance pattern that was indicated by oxacillin resistance, MRSA (methicillin resistant *S. aureus*) species were also detected in 44.9% in all samples in this study. MRSA was isolated in 26/55 (47.3%), 18/41 (43.9%), 6/12 (50%) and 4/10 (40%) from cows, buffaloes, sheep and goat species as well as from 3/9 (33.3%) of examined cheese samples (**Figure** 2).

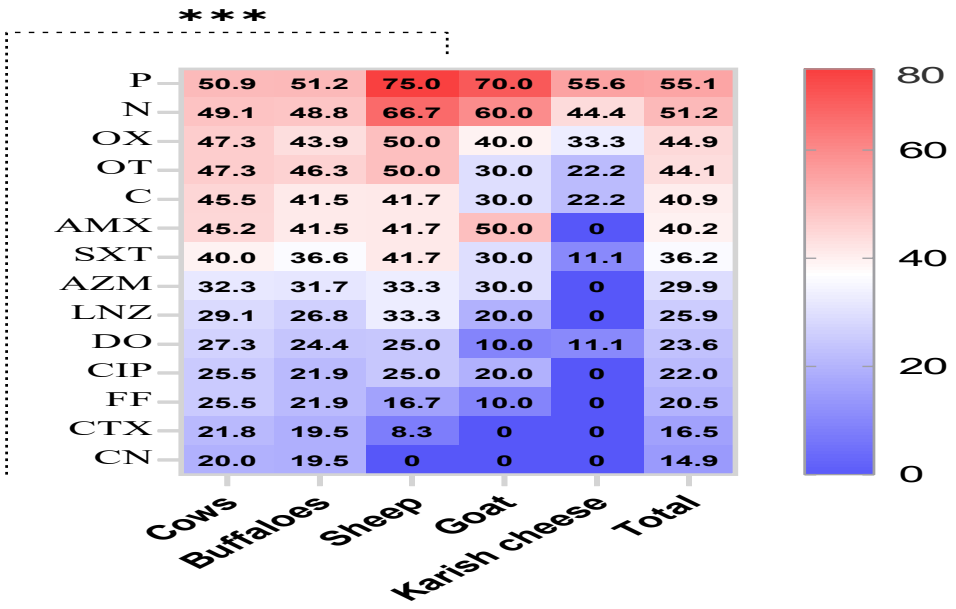

Figure 2. Antibiogram sensitivity and resistance profile patterns of the recovered *S. aureus* isolates

Table 4. Antibiogram sensitivity and resistance profile patterns of the recovered *S. aureus* isolates

Antibiotics (Type/Conc)	Abb.	Antibiotic group	recovered isolates from Cows (55)		Buffaloes (41)		Sheep (12)		Goat (10)		Karish cheese (9)		Total (127)	
			R	%	*R*	%	*R*	%	*R*	%	*R*	%	*R*	%
Penicillin (10 µg)	P	Penicillin	28/55	50.9%	21/41	51.2%	9/12	75%	7/10	70%	5/9	55.6%	70/127	55.1%
Norfloxacin (10 µg)	N	Fluoroquinolne	27/55	49.1%	20/41	48.8%	8/12	66.7%	6/10	60%	4/9	44.4%	65/127	51.2%
Oxacillin (5 µg)	OX	Penicillin	26/55	47.3%	18/41	43.9%	6/12	50%	4/10	40%	3/9	33.3%	57/127	44.9%
Oxytetracycline (30 µg)	OT	Tetracycline	26/55	47.3%	19/41	46.3%	6/12	50%	3/10	30%	2/9	22.2%	56/127	44.1%
Chloramphenicol (30 µg)	C	Chloramphenicol	25/55	45.5%	17/41	41.5%	5/12	41.7%	3/10	30%	2/9	22.2%	52/127	40.9%
Amoxicillin (25 µg)	AMX	Aminopenicillin	24/55	45.2%	17/41	41.5%	5/12	41.7%	5/10	50%	--	--	51/127	40.2%
Trimethoprim sulfamethoxazole (25 µg)	SXT	Sulfonamides	22/55	40%	15/41	36.6%	5/12	41.7%	3/10	30%	1/9	11.1%	46/127	36.2%
AZithromycin (30 µg)	AZM	Macrolides	18/55	32.3%	13/41	31.7%	4/12	33.3%	3/10	30%	--	--	38/127	29.9%
Linezolid (30 µg)	LNZ	Oxazolidinones	16/55	29.1%	11/41	26.8%	4/12	33.3%	2/10	20%	--	--	33/127	25.9%
Doxycycline (30 µg)	DO	Tetracycline	15/55	27.3%	10/41	24.4%	3/12	25%	1/10	10%	1/9	11.1%	30/127	23.6%
Ciprofloxacin (5 µg)	CIP	Fluoroquinolone	14/55	25.5%	9/41	21.9%	3/12	25%	2/10	20%	--	--	28/127	22%
Fosfomycin (10 µg)	FF	Phosphonic acid	14/55	25.5%	9/41	21.9%	2/12	16.7%	1/10	10%	--	--	26/127	20.5%
Cefotaxime (30 µg)	CTX	3rd generation cephalosporin	12/55	21.8%	8/41	19.5%	1/12	8.3%	-	--	--	--	21/127	16.5%
Gentamycin (10 ug)	CN	Aminoglycosides	11/55	20%	8/41	19.5%	0	--	--	--	--	--	19/127	14.9%

Genotypic profile of enterotoxigenic and β-lactam resistance genes of *S. aureus* isolates:

Among the selected fourteen *S. aureus* isolates from raw milk samples of cows, buffaloes, sheep, goat and cheese samples as demonstrated in Figure (3); *nuc* identification gene was identified in all isolates (100%) while, the incidence of SE (staphylococcal enterotoxins) virulence gens were analyzed in varying degrees. PCR results showed that *seb* gene was the most prevalent gene in 12/14 (85.7%) of all isolates followed by sed which was detected in 9/14 (64.3%) then sea was found in 28.5% (4/14) only; meanwhile *sea* and *seg* staphylococcal enterotoxins genes weren't detected at all. For β-lactam resistance genes; PCR detected blaZ gene in all tested isolates (100%) however, mecA (methicillin or oxacillin resistant gene) was detected in 50% of *S. aureus* isolates. The phenotypic and genotypic profiles for the selected examined isolates from different origins were also discussed in detail in table (5).

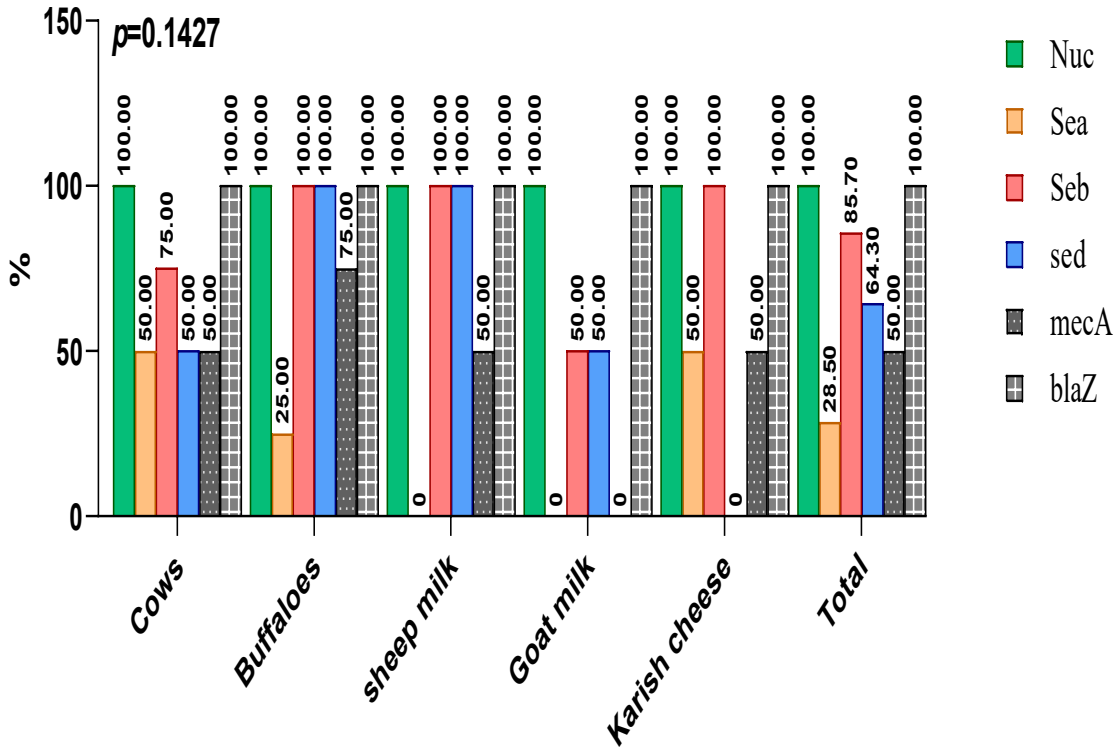

Figure 3. Distribution of enterotoxigenic (SE) and β-lactam resistance genes of *S. aureus*

Table 5. phenotypic and genotypic resistance profile of some *S. aureus* isolates:

ID of isolate	Strain source	Phenotypic resistance profile	β-lactam resistance profile
CF1	Raw cow milk	P, AMX, OX, C, N, SXT	blaZ, mecA
BF3	Raw buffalo milk	P, N, OX, LNZ, AZM, FF, OT	blaZ, mecA
CF5	Raw cow milk	P, C, OX, AMX, SXT, DO	blaZ,
BF9	Raw buffalo milk	P, N, OT, SXT, DO, CIP, LNZ	blaZ
BS1	Raw buffalo milk	P, C, N, P, SXT, LNZ, CN, OX	blaZ. mecA
S5	Raw sheep milk	P, N, OX, AMX, CN, LNZ	blaZ, mecA
BS14	Raw buffalo milk	P, SXT, CN, FF, C, OX	blaZ
CS8	Raw cow milk	P, AZM, CIP, CN, LNZ	blaZ
S4	Sheep milk	P, C, LNZ, DO, CN, AMX	blaZ
G3	Goat milk	P, OX, OT, DO, FF, CN, SXT	blaZ, mecA
CS14	Raw cow milk	P, N, C, OT, LNZ, AMX	blaZ
G4	Goat milk	P, N, C, OX, AMX, LNZ, DO, FF	blaZ, mecA
K5	Karish cheese	P, CTX, OX, OT, DO	blaZ, mecA
K8	Karish cheese	P, C, N, OT, CIP, AZM	blaZ

CF: Raw bulk tank cow milk in farm, **BF:** Raw bulk tank buffalo milk in farm, **CS:** Raw cow milk from smallholder, **BS:** Raw buffaloe milk from smallholder, S: ewes' sheep raw milk, G: ewes' goat raw milk, K: Karish cheese

DISCUSSION:

Staph aureus is an emergent pathogen of high transmission and zoonotic risk. In several reports, it had been widely isolated from raw milk and dairy products samples **(Stapels et al. 2014)**. The contaminated milk of dairy animals with *S. aureus* shed frequently such pathogens that could lead to grave public human health issues especially contagious food poisoning **(Oliveira et al. 2022).**

It is documented that there was a proportional relation between the amount of toxin to be sufficient to cause foodborne diseases and *Staphylococcus* populations when it exceeds 10^5 CFU ml^{-1} **(Pereira et al. 2018)**.

The results that were reported in table (1) in this study was lower than that recorded by **(Meshref et al. 2019)** who recorded that the average count of *S. aureus* in raw cow and buffalo milk was ($1.62\times10^8 \pm 9.5\times10^7$, 7.88×10^7 5.19×10^7 CFU/ml, 8.68×10^7 2.61×10^7), respectively. In table (2), the high percentage of *S. aureus* counts in raw milk which was obtained by milk handler indicated the poor hygienic quality under which such milk was produced. Also, higher frequency of *Staph* spp. in Karish cheese could be attributed to bad preparation technique of raw milk (without any heat treatment), contaminated utensils, uneducated persons and improper storage. They could be considered that these samples were unfit for human consumption according to the parameters that were mentioned in the Egyptian Standards "**ES 1008-4/2005**".

In the current study, the incidence of *S. aureus* was 38.5% (127/330) in all collected raw milk and Karish cheese samples (Table 3). *Staph aureus* isolates were confirmed as coagulase positive (CPS) in this study meanwhile, NAS strains were reported in Figure (1) of which some exhibited coagulase negative activity (CNS). This group of CNS could constitute serious threat for both animal and

human health since CNS were associated with several cases of subacute and chronic mastitis in dairy animals **(Makkia et al. 2022).**

This data was consistent with recent studies in Portugal and Egypt by **(Oliveira et al. 2022; Sadat et al. 2022)** in which *S. aureus* was reported in 41.1% (288/700) and 40% (136/340), respectively in raw cow's milk samples. The latter study declared that *S. aureus* was isolated from cow, buffalo, sheep and goat`s raw milk with 44 (36.7%), 65 (46.4%), 12 (30%) and 15 (37.5%), respectively. Identical isolation rate *S. aureus* (43.1%) was recorded in previous studies **(Kou et al. 2021; Traversa et al. 2015).** Moreover, **(Saka and Terzi Gulel 2018)** isolated *S. aureus* spp. in 30% of milk and 34% of cheese samples. Moreover, **(Meshref et al. 2019)** detected *S. aureus* in 13/25 of raw cow's milk, 16/25 of raw buffalo's milk and 34/50 of Karish cheese samples.

A little bit higher rate of *S. aureus* (43.1%) was stated in 62/144 milk samples **(Kou et al. 2021).** Moreover, high level of raw milk contamination with *S. aureus* spp. was recorded in many studies where it was discovered in 76.2% **(Wang et al. 2022).** Also, In Portugal, 53% of raw milk samples (that were collected from bulk cooling tanks) was contaminated with CPS *S. aureus* **(Oliveira et al. 2022)**. In addition, all seventy-five raw milk samples were positive for *Staphylococci* **(Alnakip et al. 2023**). Also, 100% (4/4) of raw cow milk, 16/20 (80%) of goat raw milk samples and 40% of cheese samples were contaminated with *S. aureus spp* **(Alghizzi and Shami 2021).**

The discrepancies in the prevalence ratio *Staphylococcus spp*. in dairy animals could be attributed to the fact that mammary gland of these animals are the main reservoir for those pathogens especially in animals with clinical or subclinical mastitis. So, it could negatively affect the quality of raw milk and produce high levels of milk contamination. In addition, bad milk storage conditions accompanied by high environmental temperature in raw milk permitted the multiplication and growth of *S. aureus* evoking their enterotoxin **(Wang et al. 2022)**. Furthermore, **(Alnakip et al. 2023)** mentioned that due to inappropriate hygienic measures particularly extensive contamination of hand personnel during cheese making or milking, inadequate thermal treatment or poor sanitation during various stages of preparation, storage, distribution and production of Karish cheese and other artisanal dairy products; varieties of food poisoning pathogens especially staphylococci spp. could be growing and might be implicated in food poisoning and gastroenteritis disorders among consumers.

Antimicrobial resistance phenomenon (AMR) in diversified sorts of bacteria is a problem of concern. AMR had been arising from an extensive use of antibiotics in food animal production, decreasing the effectiveness of different antibiotic classes for the treatment of infections in both humans and animals, particularly β-lactam antibiotics, which are the most frequently used antibiotics in the treatment of animal diseases **(Pitkälä et al. 2007)**. The risk of the emergence of novel and more resistant bacteria could increase when these antibiotics had been used in poor nations, particularly Egypt, in subtherapeutic doses to promote growth and prevent sickness **(Santy-Tomlinson 2018).**

In the current study, the recovered *S. aureus* isolates exhibited MDR traits; (i.e., they were resistant to three or more antimicrobial agents of different antibiotic classes). A wide range of resistance (14.9% - 55.1%) were displayed against multiple antibiotics of different classes as shown in figure (2) and table (4).

MRSA *Staphylococcus* species had been reported as the primary cause of hospital and community-acquired infections **(Gopal and Divya 2017)**. Globally, World Health Organization (WHO) considered MRSA as one of the three most serious difficult infectious diseases in the world **(Becker and Wardenburg 2015).** In the present study, MRSA was detected in all yielded *S. aureus* isolates from raw milk and Karish cheese in a ratio of 44.9%. Figure (2) demonstrated that MRSA was found in cows, buffoloes, sheep, goat species and Karish cheese in variant degrees. Identical results of MRSA strains that were yielded in 81/200

(40.5%) and 64/150 (42.7%) of cattle and buffalo milk samples, respectively in different localities in Egypt were recorded **(Selim et al. 2022)**. Also, also fifty-one MRSA strains were recovered from total seventy recovered *S. aureus* isolates in raw milk and cheese; of which 34 were identified in raw milk samples and 17 from cheese samples **(Alghizzi and Shami 2021).**

Conforming results of MDR *S. aureus* isolates from raw cow, buffalo, sheep and goat milk samples were confirmed with **(Abo-Shama 2014)** against varieties of antibiotics (penicillin, ampicillin, oxacillin, amoxicilin/ clavulanic acid, erythromycin and chloramphenicol). Also, high resistances against penicillin, ampicillin trimethoprim sulfamethoxazole drugs and moderate resistances to ciprofloxacin, erythromycin, clindamycin, tetracycline, chloramphenicol were recorded in MRSA strains **(Sadat et al. 2022)** however, they showed high sensitivity against gentamycin. In addition, **(Kou et al. 2021)** stated that *S. aureus* isolates were penicillin, oxacillin and erythromycin resistant strains in percentages of (72.6%, 37.1% and 32.3%), respectively. In the same line, **(Liu et al. 2022)** stated that 72.2% of *S. aureus* isolates were MDR strains with highest resistance rate against penicillin (50%), tetracycline (41.7%) and gentamicin drugs (36.1%). Many studies discussed MDR of *S. aureus* isolates from raw milk **(Katreen et al. 2018; Oliveira et al. 2022).** Moreover, the sensitivity of *S. aureus* isolates to linezolid and ciprofloxacin were in accordance with **(Selim et al. 2022)**

MRSA are defined as the Staph isolates that recorded resistance to oxacillin or methicillin drugs with antimicrobial sensitivity testing methods. Corresponding results of oxacillin resistance were stated by **(Alghizzi and Shami 2021)** in which *S. aureus* isolates were resistant to oxacillin (66.7%). Several studies also had focused on the prevalence of MRSA strains isolated from raw milk and relevant products **(Cuiping Shi et al. 2021; Sadat et al. 2022 and Wang et al. 2022)**.

It is obvious that human epidemics due to staphylococcal food poisoning (SFP) might be due to the consumption of contaminated milk or dairy products with SE producing *S. aureus* strains (**Umeda et al. 2017; Wakabayashi et al. 2018).** Numerous studies had highlighted the potential hazard of SE toxins **(Dinges et al. 2000; Sadat et al. 2022).** PCR assay played a primary role in the detection of SE enterotoxins hence, in the current investigation, SE genes of *S. aureus* isolates from raw milk of different animals and from cheese samples were studied. PCR analysis as shown in figure (3) detected that *seb* gene was the most prevalent in all tested isolates in (85.7%) followed by *sed* and *sea* genes that were found in (64.3% and 28.5%) however, *sec* and *see* genes hadn't been detected at all.

Corresponding results of **(Alghizzi and Shami 2021)** who documented that most classical SE toxins (*sea*, *seb*, *sec*, *sed*, *see*) were identified in raw milk samples of different animals in 29/33 (87.9%) more than from cheese samples in [4/33 (12.1%)]. Also, **(Meshref et al. 2019)** confirmed the presence of *seb* genes in 40% of all tested *S. aureus* strains from milk and yogurt but it was found less (20%) in both Karish cheese and ice-cream samples. Moreover, the distribution of SE enterotoxin genes of *S. aureus* in China was found somewhat like to current data **(Chao et al. 2015)**. Furthermore, relevant studies determined the presence of *sea* and *sec* genes in raw milk samples with varying degrees **(Cavicchioli et al. 2015; Kou et al. 2021 and Xing et al. 2016**). On opposing to our data of low detection rate of *sea* gene, *sea* was the most encountered gene in MRSA isolates from raw milk followed by *seb* and *sec* genes **(Sadat et al. 2022)**. The different prevalence rates of the SE enterotoxigenic genes might be attributed to differences in the geographical regions and sample sources **(Zhao et al. 2021).**

The development of β-lactam antibiotic resistance among *S. aureus* especially MRSA had been clarified via carrying *mecA* gene **(Yang et al. 2016).** In this study, MDR *S. aureus* strains showed resistance against penicillin and oxacillin (β-lactam) resistance genes using PCR tool. Also, the correlation between

the phenotypic and genotypic resistance patterns of the selected isolates from different sources in this study were illustrated in table (5). The penicillin resistant (*blaZ*) gene was found in 100% of *S. aureus* isolates however, mecA (oxacillin resistant gene) was detected in 50% of *S. aureus* isolates. *mecA* gene was also found in previous studies in MRSA isolates from raw milk (80%) and from cheese (20%) samples and 60% of these positive *mecA* isolates were resistant to oxacillin **(Alghizzi and Shami 2021; Osman et al. 2017).**

Because of widely using of β-lactam drugs in treatment of bovine mastitis; they are the drugs of choice in *S. aureus* infections in human **(Thongratsakul et al. 2020),** β-lactam resistance was extremely engendered. In the same line, blaZ resistant gene was discovered in 69.2% raw milk with **(Liu et al. 2022)** however, it was detected in 25.8% of *S. aureus* isolates from milk samples in China. **(Kou et al. 2021)** and in 32.7% (37/113) of *S. aureus* isolates from raw milk **(Sadat et al. 2022).** This data suggested that β-lactam drugs should be limited use in treatment of animals especially food producing animals along with implementation of strict hygienic measures in dairy farms for safe public health.

CONCLUSION

To sum up, this inclusive data could present a great knowledge about the possibility of spreading and transmission of *Staphylococcus* spp which carried SE toxin genes that had a virulence potential and potential health risks. A high level of contamination with *S. aureus* and MRSA species (via oxacillin resistance) was also detected in examined raw milk and Karish homemade cheese in rural areas of developing countries. Consequently, proper hygiene practices at milking and during handling and milk transportation are necessitated. Also, the hygienic awareness of farm milkers and milk small holders should be raised to prevent the spread and cloning of such virulent toxigenic pathogens to humans through the food chain. The milk should be refrigerated directly after milking to prevent it from being held at unsafe temperature to avoid SE toxins of *S. aureus* to be released. An alarming light towards MDR *S. aureus* isolates and MRSA strains in raw milk and products in this study should promote a periodically monitoring programs for antibiotics use to lessen the risks for both animal and human public health and inspection visits for dairy herds should be done to assure that workers and milkers had conformed with standards.

Conflict of interest: There is no conflict of interest.

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