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Prevalence, Methicillin Resistance and Inducible Clindamycin Resistance of *Staphylococcus aureus* Isolated from Retail Ice Cream in Mansoura, Egypt

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Abstract

This work aimed at determination of the prevalence and antimicrobial resistance (AMR) of *S. aureus* species and investigating the presence of the enterotoxins (SEA and SEB) in the obtained isolates in consumed Egyptian ice cream. Thirty ice cream samples were obtained from many ice cream shops, dairy shops, supermarkets and local markets from different localities at Mansoura city. Samples were spread on Baird Parker selective agar media for bacterial isolation. The isolates were characterized by antibiotic susceptibility testing and resistance genes. *S. aureus* was detected in 60% (18 out of 30) of ice cream samples with a minimum and maximum count of $4x10^2$ and $1.5x10^7$ CFU/g, respectively. Furthermore, 4 isolates (22%) out of the total isolates (n=18) were positive for *nuc* gene. Of these positive isolates, one isolate (25%) was positive for *mec* A and *sea* genes, while *seb* was not detected. The AMR profile of molecularly positive nuc gene *S. aureus* isolates revealed that the highest resistance was against ampicillin, cefazolin, Cefoxitin, cefotaxime, cefepime, azithromycin and amoxycillin- clavulanic acid (100%) followed by gentamicin and erythromycin (75%), and imipenem, tetracycline and clindamycin (50%). No resistance was found to sulphamethazone-trimethoprim, doxycycline, ciprofloxacin, levofloxacin, vancomycin and linezolid. Our results showed that 100% of the molecularly positive *nuc* gene isolates was methicillin resistant *Staphylococcus aureus* (MRSA) and 50% was inducible clindamycin resistant *S. aureus* (ICRSA). The MRSA and ICRSA are potential risks for health. Poor hygienic measures with ice cream manufacture may lead to contamination of ice cream with highly resistant enterotoxigenic *S. aureus*.

KEYWORDS
MRSA, sea, *seb*, *nuc*, *mec*A, ICRSA, Ice cream,Antimicrobial resistance, MAR index

INTRODUCTION

Ice cream is a nutritious frozen dairy dessert popularly consumed by all age groups specially children throughout the year particularly in summer (Samir *et al*., 2019). Ice cream has been reported to be contaminated with diverse bacteria from different sources during manufacture, processing, and handling, thus, it can act as a vehicle of food-borne diseases (Sotohy *et al*., 2022).

Staphylococcus aureus is a common food-borne pathogenic bacterium which is normally inhabitant in skin and mucous membranes and in the nasopharynx of about 20–30% of healthy people. These bacteria are able to produce heat-stable enterotoxins (Abri *et al*., 2019).

Staphylococcal food poisoning (SFP) is a worldwide food-borne illness with high occurrence, second to salmonellosis, which is caused by staphylococcal enterotoxins (SEs) (Meshref *et al*., 2019). Improperly prepared food contaminated with bacterium or its enterotoxins in sufficient concentrations ($1x10^5$) can cause SFP within few hours (Sotohy *et al*., 2022). Traditional enterotoxins A, B, C, D, and E can withstand temperatures of up to 100°C for many minutes (Ahmed *et al*., 2019).

Antimicrobial resistance (AMR) is a worldwide public health problem. The development of AMR has been linked to the wide utilization of antimicrobial drugs or with their use as growth promoters for animals. The use of antimicrobials in a period shorter than the recommended can also be a contributor to AMR (Samir *et al*., 2019).

Studies performed in the last decade reported the possibility of AMR transmission through food chains and the significance of the food-handling environment as a likely source for AMR and dissemination (Kasem *et al*., 2021).

Staphylococci frequently show multiple antimicrobial resistance patterns. Certain *S. aureus* strains show resistance to methicillin, which has been identified as methicillin resistant *Staphylococcus aureus* (MRSA). The latter is an important resistant strain with low affinity to β-lactams (Ahmed *et al*., 2019).

The presence of MRSA in milk can be because of the excessive utilization of similar antimicrobials e.g. oxacillin or penicillin in breeding the animals. In contrast, the utilization of macrolide, lincosamides and streptogramin B (MLSB) antimicrobials can increase the resistance rates of erythromycin and clindamycin in the animal due to cross-resistance. These strains can be directly transmitted from animals to the human or through consuming the dairy products (Mahdavi *et al*., 2019).

The methicillin resistance is detected clinically through detecting *mec*A gene by PCR and through detecting the resistance

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to cefoxitin. The *mec*A gene encodes for penicillin-binding protein responsible for methicillin resistance. MRSA often shows resistance to multiple antibiotics, not only to penicillin but also for other antimicrobials such as macrolides, fluoroquinolones, aminoglycosides, tetracycline, and lincosamides. MRSA can cause serious infectious diseases in humans e.g. endocarditis, pneumonia, otitis media, skin infection, osteomyelitis, septic arthritis, and soft tissue infection. Thus, the emergence of multidrug-resistant MRSA is a significant public health concern (Algammal *et al*., 2020).

MLSB antibiotics are frequently utilized in treating staphylococcal infection (both methicillin-susceptible *S. aureus* and MRSA). This extensive use of MLSB has led to an increased resistance to them particularly to clindamycin, amongst staphylococci (Lall and Sahni, 2014).

Resistance to MLSB family often results from acquisition of erythromycin resistance methylase (*erm*) genes, which encode enzymes that methylate 23S rRNA. Expression of MLS resistance may be constitutive (methylase is always produced) or inducible (methylase is produced only in presence of a macrolide inducer e.g. erythromycin and azithromycin) (Drinkovic *et al*. 2001).

Clindamycin is an effective antibiotic belonging to the MLSB family and it has a good. Among the MLSB, clindamycin is also commonest antimicrobial used for treating Staphylococcal infection. Expression of inducible clindamycin resistance (ICR) could thus limit its effectiveness (Ammar *et al*., 2016).

In contrast to constitutive resistance, the inducible resistance to clindamycin cannot be recognized by routine antimicrobial susceptibility testing; however it can be detected using the D-test (Fiebelkorn *et al*., 2003). Failure to identify inducible ICR may result in clinical failure of clindamycin (Drinkovic *et al*., 2001). On the contrary, labeling all erythromycin-resistant staphylococcal strains as clindamycin resistant would prevent clindamycin use for an infection caused by clindamycin-susceptible staphylococci (Lall and Sahni, 2014).

So, this work aimed at the determination of the prevalence and the AMR of *S. aureus* species and investigating the presence of SEA and SEB enterotoxins in the isolates from Egyptian ice cream samples.

MATERIALS AND METHODS

Sample collection

Thirty ice cream samples were obtained from many dairy shops, ice cream shops and supermarkets from different localities of Mansoura city, Egypt during the period from 1 September 2022 till 26 September 2022. The samples were transferred in a clean and dry icebox at 4°C to the Food Hygiene, Safety and Technology Department laboratory at Mansoura University to be examined.

Isolation, enumeration and identification of S. aureus

The *S. aureus* count was determined using the surface plate method. In brief, 0.1 ml of prepared dilutions of each sample was spread on Paired Barker agar media (Oxoid, Hampshire, England) combined with 5% egg yolk tellurite emulsion, and then underwent incubation at 37oC for 24-48 hours (Greenwood and Roberts, 2008). The suspected black and shiny colonies surrounded by a clear zone were picked up for further biochemical and molecular identification (MacFaddin, 2000). The prevalence rate of *S. aureus* was calculated using one *S. aureus* isolate from each sample that tested positive.

Antimicrobial susceptibility

Disk-diffusion test

This was performed using Mueller Hinton agar-based agar disk-diffusion test. Different concentrations of sensitivity disks (Oxoid, Hampshire, England) were utilized. Antibiotics utilized included Ampicillin, Cefazolin, Cefoxitin, Cefotaxime, Cefepime, Azithromycin, Amoxycillin Clavulanic Acid, Gentamicin, Erythromycin, Imipenem, Tetracycline, Clindamycin, Sulphamethazone-trimethoprim, Doxycycline, Ciprofloxacin, Levofloxacin, Vancomycin and Linezolid. Inhibition zones on each plate were measured according to the Clinical and Laboratory Standard Institute's guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2020). Multiple drug resistance (MDR) was defined as resistance to ≥3 antimicrobials (Magiorakos *et al*., 2012).

Inducible Clindamycin Resistance (D-test)

The D-test was utilized to detect the ICRSA according to the CLSI (CLSI, 2020). MRSA isolates were cultured on Muller Hinton agar until the bacterial culture reached 0.5 McFarland standards. Then, 2 µg clindamycin and 15 µg erythromycin disks were placed on the agar with 20 mm distance. The blunting of the inhibition zone of clindamycin disk around the erythromycin disk (forming D shape) was determined as ICRSA (Mahdavi *et al*. 2019).

MAR index determination

It underwent calculation as follows: Number of antimicrobial agents which showed resistance divided by the number of utilized antimicrobial agents (Sandhu *et al*., 2016).

Molecular Identification and Characterization

The primers of genes utilized for *S. aureus* characterization are demonstrated in Table 1. Overnight, broth cultures of *S. aureus* (n=18) were centrifuged at 15000 rpm for 5 min. Pellets were suspended in 100 ul *nuc*lease free water then boiled (Ahmed and

Table 1. Primers utilized for PCR.

Gene	Primer sequence (5'-3')	Size	Reference
nuc	F- GCG ATT GAT GGT GAT ACG GTT R- AGC CAA GCC TTG ACG AAC TAA AGC	279 bp	Brakstad *et al*. (1992)
mecA	F- ACT GCT ATC CAC CCT CAA AC R- CTG GTG AAG TTG TAA TCT GG	163 bp	Mehrotra et al. (2000)
sea	F- TGCAGGGAACAGCTTTAGGCAA R- GATTAATCCCCTCTGAACCTTCC	500 bp	Sallam et al. (2015)
seb	F-CCTAAACCAGATGAGTTGCACAAAGCG R- TCCTGGTGCAGGCATCATGTCATA	600 bp	Sallam et al. (2015)

Dablool, 2017) for DNA extraction prior to PCR partial amplification of *nuc*A, *mec*A, *sea* and *seb* genes. PCR was performed according to Resendiz-Nava *et al*. (2019). Amplification was done using EasyTaq PCR Super Mix (2X) [Cat. No. AS111] according to manufacturer instructions. A thermal cycler (SimpliAmpTM, Applied Biosystems, USA) was used to amplify the DNA. The cycling conditions for the primers of *nuc*, *mec*A, *sea* and *seb* genes are shown in Table 2. Amplified DNA was detected in 1% agarose gel electrophoresis using TR201 UV Transilluminator (acculab, Canada).

RESULTS

Isolation and identification of S. aureus

S. aureus was detected in 18 samples with an incidence of 60%. Out of the 60 isolates, 55 (91.67%) were positive for *S. aureus*. The minimum *S. aureus* count was 4 x 10^2 CFU/ml while the maximum count was 1.5 x 10^7 CFU/ml.

On the Baird parker agar, *S. aureus* were identified as black colonies surrounded by clear zone. Microscopically, *S. aureus* appeared as non-spore forming Gram positive cocci that formed irregular grape-like clusters. Biochemically, *S. aureus* were catalase-positive and oxidase-negative.

Molecular identification of Staphylococci

Eighteen isolates (one from each positive sample) were examined by PCR using *nuc* gene as a marker gene in *S. aureus* isolates. Four out of these 18 isolates (i.e. 22 %) were positive for *S. aureus* as shown in Figure 1.

All 4 positive *nuc* gene isolates were examined for *mec*A, *sea* and *seb* genes. Only 1 isolate was positive for both *mec*A and *sea* genes, while *seb* was not detected (Figure 2).

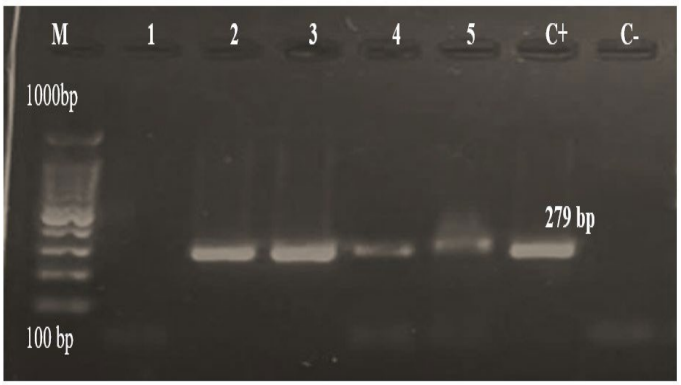

Fig. 1. Representative agarose gel electrophoresis of PCR amplicons of the marker the *nuc* gene (279 bp) in *S. aureus* isolates. Lane M: 100 bp ladder as molecular size DNA marker. Lane 2-5: positive *nuc* gene isolates. Lane C+: control positive. Lane C-: control negative.

Results of AMR

The AMR patterns of *S. aureus* against 18 antibiotic classes are shown in Table 3 and Figure 3. *S. aureus* strains exhibited

Table 2. Cycling conditions of PCR procedure.

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	Number of cycles	Final extension
nuc	94°C for 2min	94°C for 30sec	55°C for 30sec	72°C for 1min,20sec	35	72°C for 7 min
mecA		94°C for 30sec	57°C for 45sec			
sea		94°C for 30sec	58°C for 30sec			
seb		94°C for 30sec	59°C for 45sec			

Table 3. AMR of *S. aureus* isolates with positive *nuc* gene (n= 4).

Antimicrobial Family/Class Name	Antimicrobial agent	Code	S		I		R	
			No.	%	No.	%	No.	%
Aminopenicillins B-lactams	Ampicillin	AMP	0	0	-	-	4	100
1st generation cephalosporin	Cefazolin	CZ	0	0	-	-	4	100
2nd generation cephalosporin	Cefoxitin (MRSA)	FOX	0	0	-	-	4	100
3rd generation cephalosporin	Cefotaxime	CTX	0	0	-	-	4	100
4th generation cephalosporin	Cefepime	FEP	0	0	-	-	4	100
Aminoglycosides	Gentamicin	CN	0	0	1	25	3	75
Fluoroquinolone 2nd generation	Ciprofloxacin	CIP	0	0	4	100	0	0
Fluoroquinolone 3rd generation	Levofloxacin	LEV	3	75	1	25	0	0
Macrolides	Azithromycin	AZM	0	0	0	0	4	100
	Erythromycin	E	0	0	1	25	3	75
Lincosamides	Clindamycin ICR	DA	2	50	0	0	2	50
Sulfa drugs	Sulphamethazone-trimethoprim	SXT	2	50	2	50	0	0
Tetracyclines	Tetracycline	TE	0	0	2	50	2	50
	Doxycycline	DO	2	50	2	50	0	0
β-lactam combination agent	Amoxycillin-clavulanic acid	AMC	0	0	0	0	4	100
Glycopeptides	Vancomycin	VA	4	100	-	-	0	0
Carbapenems	Imipenem	IPM	2	50	-	-	2	50
Oxalidinones	Linezolid	LZD	4	100	-	-	0	0

Fig. 2. obtained band at 500bp in gel electrophoresis after PCR that target *sea* gene of *S. aureus*.

the highest resistance against ampicillin, cefazolin, cefoxitin, cefotaxime, cefepime, azithromycin and amoxicillin-clavulanic acid (100%) followed by gentamicin (75%), erythromycin (75%), imipenem (50%), tetracycline (50%), clindamycin (50%). There was no resistance to sulphamethazone-trimethoprim, doxycycline, ciprofloxacin, levofloxacin, vancomycin or linezolid. All (100%) of the molecularly positive *nuc* gene isolates (n=4) were methicillin resistant *Staphylococcus aureus* (MRSA) and 50% (2 out of 4 isolates) showed inducible clindamycin resistance as determined by the D-test (Figure 4).

The MAR index was in the range of 0.5 and 0.61 (mean = 0.55). All isolates (n=4) in our study showed MDR (MAR index > 0.2) (Table 4).

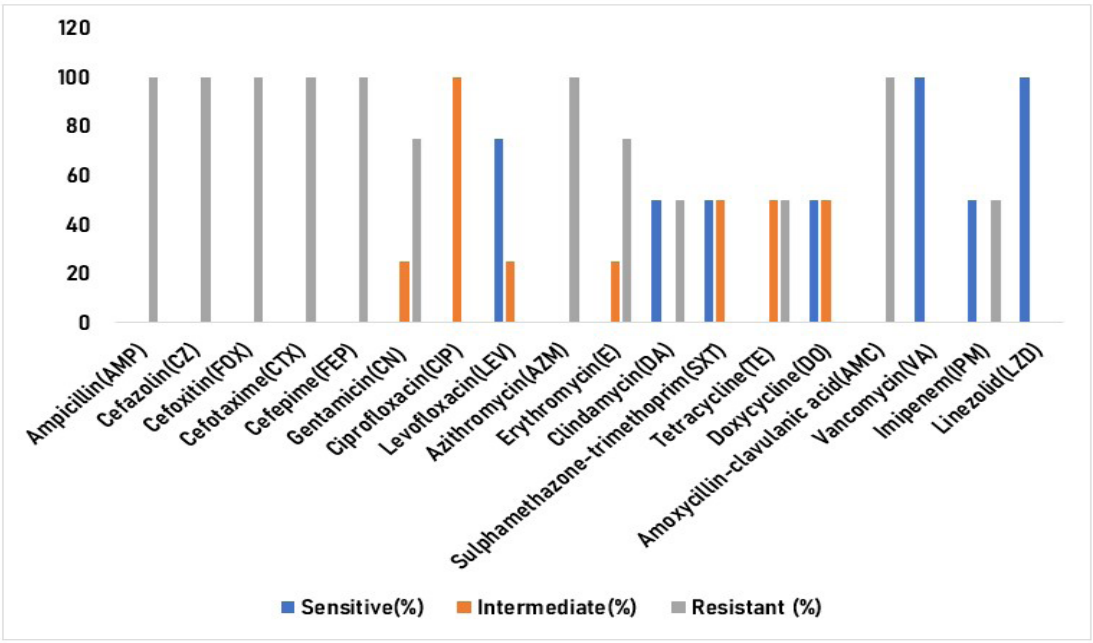

Figure 3. Antimicrobial susceptibility of isolated *S. aureus*.

Table 4. AMR Profile of *S. aureus* isolates.

Isolate No.	Antibiotics	No. of antibiotic classes	MAR Index	Type of Resistance
12	E, AZM, FOX, CTX, CPM, AMC, TE, CZ, AMP, ICR	9	0.5	MDR
17	CN, DA, AZM, FOX, CTX, CPM, AMC, CZ, AMP	9	0.5	
16	CN, E, AZM, FOX, CTX, CPM, AMC, TE, CZ, AMP, IPM, ICR	11	0.61	
26	CN, E, DA, AZM, FOX, CTX, CPM, AMC, CZ, AMP, IPM	11	0.61	

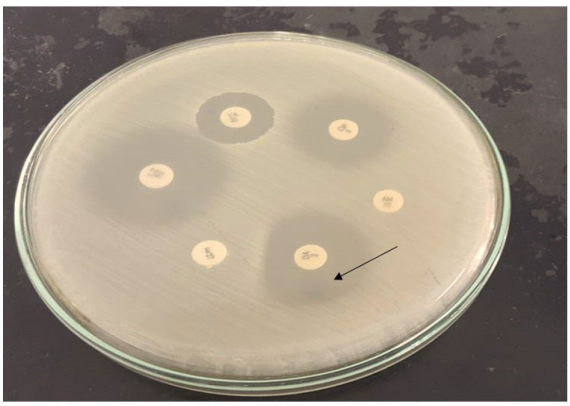

Fig. 4. Inhibition zone of different antibiotics (ampicillin, ciprofloxacin, vancomycin, linzolid, erythromycin and clindamycin) in addition to double-disk diffusion test (D test) showing erythromycin disk induction of clindamycin resistance. The inhibition zone is blunted proximal to the clindamycin disk forming a D shape (arrow).

DISCUSSION

Ice cream is a commonly consumed dairy product by all age groups, mainly children. Thus, its microbial contamination is a significant concern. The present work aimed at the identification of *S. aureus* and the detection of its AMR profile and methicillin resistance gene in ice cream samples.

Our results showed that 60% (18/30) of ice cream samples were positive for *S. aureus* with a minimum count of $4x10^2$ CFU/g and a maximum count of $1.7x10^7$CFU/g. This count exceeded the permissible limit of the Egyptian standards (ES, 2005) and was higher than the maximal limit of 100 CFU/g set by the European Economic Community food legislation for frozen milk-based products (EEC, 1992). In addition, according to the Turkish Food Codex, *S. aureus* count in ice cream should be < 10^2-10^3 CFU/g (Anonymous, 2009), which also was exceeded by our results.

These findings are nearly similar to the results recorded in many previous studies (Al-Ashmawy *et al.*, 2016; Ahmed *et al.*,

2019; Taban *et al*., 2021) but are higher than that obtained by Abo El-Makarem (2017); Abri *et al*. (2019); Kruy *et al*. (2001); Samir *et al*. (2019) and Zhang *et al*. (2022). Other reports (Kandil *et al*., 2018; Sotohy *et al*., 2022) recorded a higher *S. aureus* prevalence. This difference among studies might be because of poor hygienic measurements during ice cream manufacture.

In our work, it was found 4 out of *S. aureus* isolates (i.e. 22%) were positive *nuc* gene. It is stated that *nuc* gene has the potential for rapid confirmation for *S. aureus* isolates (Kandil *et al*., 2018; Kasem *et al*., 2021). Using the PCR, *mec*A and *sea* genes were amplified. The PCR has high sensitivity whereas conventional method has less sensitivity as there are many microorganisms give positive biochemical reaction and positive cultures but are negative by PCR (El-Nagar *et al*., 2017).

The *mec*A gene confirms MRSA identification. In this work, only 1 isolate out of 4 (25%) was positive *mec*A and this agrees Akanbi *et al*. (2017) and Zhang *et al*. (2022) who reported MRSA in 15.4% and 22.7% of isolates, respectively and disagree with Al-Ashmawy *et al*. (2016), Ramadan *et al*. (2023) and Samir *et al*. (2019) who detected *mec*A gene in 53%, 66.6% and 100% of *S. aureus* isolates, respectively. In the study by Taban *et al*. (2021), *mec*A was not detected in any of ice cream isolates.

The ingestion of SEs causes food-borne disease leading to nausea, emesis, diarrhea and abdominal cramps (El-Nagar *et al*., 2017). One report showed that SEA was the most frequently produced toxin by enterotoxigenic staphylococci, followed by SEB (Gücükoğlu *et al*., 2013). The *sea* gene was identified in 25% (1 out of 4) of isolates in our work, which was nearly similar to Younis *et al*. (2021) results but lower than that in other reports (Gücükoğlu *et al*., 2013; Al-Ashmawy *et al*., 2016; Ahmed *et al*., 2019). In contrast, Sotohy *et al*. (2022) failed to detect the *sea* gene in the examined ice cream isolates.

AMR is a global public health problem. The emergence of MDR in MRSA is associated with a failure in treating and controlling infections (Algammal *et al*., 2020; Motamedi *et al*., 2010).

In our work, resistance to ampicillin was 100%. The high resistance to β-lactams was not surprising because they are frequently utilized in in human and animals to treat infections (Gundogan *et al*., 2005; Samir *et al*., 2019).

MRSA are always resistant to multiple antimicrobials which include penicillin, methicillin, oxacillin, cefoxitin, amoxicillin-clavulanic acid, amoxicillin-sulbactam, quinolones, macrolide, cephalosporins, tetracyclines and chloramphenicol (Algammal *et al*., 2020)

All *S. aureus* isolates demonstrated resistance to cefoxitin and ampicillin (100%) in our study. This is because cefoxitin is utilized as a surrogate for *mec*A-mediated methicillin resistance according to CLSI (2020). Similar findings were reported by Ramadan *et al*. (2023) and nearly similar results were reported by Akanbi *et al*. (2017) who recorded that the resistance of *S. aureus* to cefoxitin was 76.7% and was 96.7% to ampicillin. Contrarily, Geidam *et al*. (2012) and Nam *et al*. (2011) reported that resistance rates against cefoxitin (oxacillin) were 6.2% and 28%, respectively.

The methicillin resistance can be detected by PCR-based identification of the *mec*A gene and by resistance to cefoxitin (Algammal *et al*., 2020; Ramadan *et al*., 2023). However, this work demonstrated that three *S. aureus* isolates had resistance to cefoxitin (oxacillin) and neither of them were positive for *mec*A. Thus, the 4 isolates showed methicillin resistance and only 1 isolate had *mec*A gene which agree with Akanbi *et al*. (2017) who found that only 5 isolates (out of 22 isolates) were positive for *mec*A gene. Oxacillin has been proposed as a proxy for testing susceptibility to methicillin and β-lactam antibiotics. This explains why all oxacillin-resistant isolates did not carry *mec*A (Kuehnert *et al*., 2005; Ba *et al*., 2014).

Our work showed that all isolates had sensitivity to linzolid and vancomycin with rare resistance to ciprofloxacin which agree with Ramadan *et al*. (2023). Other studies (Al-Ashmawy *et al*., 2016; Kasem *et al*., 2021) reported that vancomycin resistance was 10% and 8%, respectively. A higher resistance to ciprofloxacin (66.7%) was recorded by Akanbi *et al*. (2017).

In our study, 50% of the isolates showed susceptibility to tetracyclines. This agrees with what was recorded by Geidam *et al*. (2012) and nearly agree with Akbar and Anal (2013) who found that about 55.27% of isolates were sensitive to tetracyclines. These results disagree with other studies which revealed that *S. aureus* were resistant to tetracyclines in 65.2% (Al-Ashmawy *et al*., 2016), 77.2% (Yurdakul *et al*., 2013) and 30% (Kasem *et al*., 2021) of isolates.

The changing pattern in antimicrobial susceptibility has renewed the interest in clindamycin use. As a result, we determined the ICR among *S. aureus* isolates. Of note, about 50% of isolates showed inducible resistance to clindamycin. Expression of ICR could thus limit its effectiveness against MRSA (Ammar *et al*., 2016). Our finding agrees with a study which revealed that the maximum peak prevalence of ICR among *S. aureus* isolates recorded in the Africa was 44.0% in Egypt (Assefa, 2022).

The MDR of *S. aureus* isolates was 100% which is much higher in comparison with that recorded by Liu *et al*. (2017) and Kasem *et al*. (2021) (72.94% and 90%, respectively). This indicates the hazardous high resistance against antimicrobial agents among isolated *S. aureus* in food.

CONCLUSION

The investigated ice cream sold in Mansoura, Egypt showed contamination with *S. aureus* which may result in food poisoning. The high incidence of inducible clindamycin-resistant MRSA strains in ice cream samples in Egypt can be linked to poor hygienic measures during production and preservation of ice cream. *S. aureus* isolates also exhibited resistance to different antibiotics tested. Thus, food inspection and frequent bacteriologic surveillances by food control agencies are recommended to control the occurrence of *S. aureus* in dairy products including ice cream.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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