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Staphylococcus aureus and Salted Fish: Prevalence, Antibiogram, and Detection of Enterotoxin-coding Genes

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Abstract

This study was taken to investigate the prevalence of *Staphylococcus aureus* (*S. aureus*) in four types of salted fish, namely salted sardine, fesiekh, sahlia, and salted herrings that retailed in Zagazig city, Egypt. The antimicrobial susceptibility testing of the recovered *S. aureus* isolates was examined. Moreover, PCR was used for the detection of the coding genes of *S. aureus*-enterotoxins (SE) including *Sea*, *Seb*, *Sec*, and *Sed*. The obtained results of the current investigation revealed isolation of *S. aureus* from the examined salted sardine, fesiekh, sahlia, and salted herrings at 15%, 40%, 30%, and 15%, respectively. Fesiekh had significantly ($p< 0.05$) the highest total *S. aureus* count (3.17±0.13 $\log_{10}$ cfu/g), followed by sahlia (3.08±0.13 $\log_{10}$ cfu/g), sardine (2.33±0.07 $\log_{10}$ cfu/mL), and salted herrings (2.30±0.11 $\log_{10}$ cfu/g), respectively. Besides, 10%, 35%, 20%, and 5% of the examined salted sardine, fesiekh, sahlia, and salted herrings, respectively exceeded Egyptian limits of *S. aureus*. The recovered *S. aureus* isolates showed clear multidrug resistance profiling. PCR testing of selected *S. aureus* isolates for harboring Staphylococcal enterotoxin-coding genes revealed *Sea*, and *Seb* were not detected in any examined isolate. However, *Sec* was detected in 3 *S. aureus* isolates recovered from fesiekh, and in 2 isolates that recovered from sahlia. *Sed* was only detected in 2 isolates that recovered from fesiekh. Therefore, strict hygienic measures should be adopted during handling, and processing of salted fish.

KEYWORDS
S. aureus, salted fish, sardine, fesiekh, salted herrings, antimicrobial resistance

INTRODUCTION

Fish is recognized as a necessary source of essential amino acids and high-quality protein, polyunsaturated fatty acids, vitamins, and minerals including calcium and phosphorus. In Egypt, there is a significant lack of red meat that has a significant effect on the food security, therefore, fish represents an alternative source for animal-derived protein, particularly with the relatively lower price compared to chicken and other meat sources. (Morshdy *et al*., 2013, 2019).

It has long been known that salting is an effective way to preserve fish and create novel fish products. In Egypt, the creation of traditional fish items that are used as a significant source of protein as well as being consumed on special occasions is also associated with the salting of fish. Sahlia, which is made from salted keeled mullet, fesiekh, which is made from salted mullet, and salted herrings that are smoked after salting are examples of locally produced salted fish in Egypt (Abbas *et al*., 2022). Fish can be preserved for a long time by being salted, which also enhances the fish's microbiological quality.

Microbial contamination of salted fish is controlled by the hygienic practices followed starting from catching of the fish, storage, and further processing. The sources of the microbial contamination of salted fish mainly include the operator (hands, hair, and clothes), the use of contaminated raw materials, storage containers, and equipment (Aberle *et al*., 2001; Hafez *et al*., 2018). Therefore, there is a large need for continuous monitoring of the microbial quality of the retailed salted fish in Egypt.

Throughout the world, *Staphylococcus aureus* (*S. aureus*) is a major contributor to foodborne illnesses, particularly foodborne intoxications (Darwish *et al*., 2022). In just the United States, it is responsible for roughly 241,000 illnesses annually. Human *S. aureus*-enterotoxin poisoning symptoms include vomiting, nausea, abdominal cramps, and diarrhoea. These symptoms typically appear within a few hours of consuming infected food (Hennekinne *et al*., 2012). *S. aureus* and staphylococcal enterotoxins (SEs) were isolated and detected salted fish such as fesiekh, molouha, and sardine retailed in Cairo (Ezzeldeen *et al*., 2011), and Alexandria (Elkassas, and Mousa, 2021). Globally, *S. aureus*, and its enterotoxins were detected in salted carp, and salted mullet collected from Iranian markets (Basti *et al*., 2006), and from Hout-Kasef, a traditional salted fermented fish product of salted mullet retailed in Jazan region, Saudi Arabia (Gassem, 2019).

Intensive use of antimicrobials was recently recorded in cultured tilapia, and catfish in Egypt, which gives rise for the development of antimicrobial resistant pathogens such as *S. aureus*

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(Morshdy *et al*., 2022). However, antimicrobial sensitivity testing of the recovered *S. aureus* isolates from salted fish has received less attention.

In this study, *S. aureus* prevalence rates in salted sardines, fesiekh, sahlia, and salted herrings sold in Zagazig, Egypt, were examined. Additionally, the disc diffusion assay was used to check the recovered *S. aureus* for antibiotic resistance. Additionally, PCR was used to identify the coding genes for *S. aureus*-enterotoxins such as *Sea*, *Seb*, *Sec*, and *Sed*.

MATERIALS AND METHODS

Collection of samples

Eighty random samples of salted fish, including 20 each of salted herrings, sardines, fesiekh, and sahlia, were randomly selected from retail establishments selling salted fish in Zagazig, Egypt. The samples were quickly transported cooled to the lab for bacteriological analysis.

Sample preparation

According to APHA (2001), the obtained samples were prepared for bacteriological analysis. Briefly stated, 90 mL of 1% sterile peptone water (Oxoid CM9, UK) was combined with 10 grams from each collected sample, blended for 3 minutes at 3000 rpm, and then allowed to stand for 15 minutes at room temperature before ten-fold serial dilutions were prepared.

Isolation and identification of S. aureus

According to APHA (2001), *S. aureus* isolation and identification procedures were carried out. In a nutshell, surface spreading technique was used to culture 0.1 mL of each generated dilution over a Baird Parker agar (Oxoid, UK) plate enriched with egg yolk emulsion. Plates were kept inverted for 48 hours while being incubated at 37°C. *S. aureus* colonies are round, smooth, convex, shiny, black, with a tiny white boundary, and they are encircled by a clear zone that extends into the opaque medium. Five potential *S. aureus* colonies were purified on nutrient agar slopes for additional biochemical analysis. *S. aureus* colonies were identified using morphology, biochemistry, and serology. *S. aureus* isolates were found to be positive for catalase, coagulase, hemolysis, and displayed yellow colonies surrounded by halo zones in the mannitol test during biochemical analysis.

Antimicrobial susceptibility of the recovered S. aureus isolates

Using the disc diffusion method, the antimicrobial susceptibility of the recovered *S. aureus* isolates was evaluated. We purchased antimicrobial discs from Oxoid Limited in Hampshire, United Kingdom. *S. aureus* was tested for antimicrobial sensitivity using nutrient agar plates as a culture medium. The Clinical and Laboratory Standards Institute (CLSI) criteria were used (Wayne, 2013). Additionally, Singh *et al*. (2010)'s algorithm was used to calculate the Multiple Antibiotic Resistance (MAR) index for each tested *S. aureus* isolate as follows:

Antibiotics tested / total number of resistances = MAR index

Ampicillin (10 g; AM), cephalothin (30 g; CET), chloramphenicol (30 g; C), ciprofloxacin (5 g; CIP), enrofloxacin (5 g; ENR), erythromycin (15 g; E); gentamicin (10 g; GEN); kanamycin (30 g; K); nalidixic acid (30 g; NA); neomycin (30 µg; N), oxacillin (1 µg; OX), oxytetracycline (30 µg; OXY), penicillin (10 IU; P), and trimethoprim/sulfamethoxazole (25 µg; SXT).

Molecular identification of Staphylococcal enterotoxins

Using a genomic DNA extraction kit and following the manufacturer's instructions, bacterial DNA was extracted from the isolated *S. aureus* strains that had been cultivated and identified (Alliance Global, Dubai, UAE). Table 1 lists the primer pairs that were acquired from Metabion International, Gmbh, Germany, for the *S. aureus* enterotoxin genes *Sea*, *Seb*, *Sec*, and *Sed*.

According to Darwish *et al*. (2018), PCR amplification procedures were carried out utilizing a uniplex PCR strategy on a Thermal Cycler (Master cycler, Eppendorf, Germany). The PCR cycling conditions began with an initial denaturation at 95°C for 1 min, then 35 cycles with each cycle comprising of denaturation at 95°C for 15 sec, annealing at 50°C for 30 sec, and extension at 72°C for 1 min. The process concluded with a holding phase at 4°C after the last extension stage at 72°C for 7 min. Amplified PCR products were performed on 1.5% agarose gel electrophoresis in 1x Tris Borate EDTA buffer and stained with ethidium bromide, followed by visualization on a UV transilluminator (AppliChem, GmbH, Germany). A DNA marker called DNA Ladder (100 bp, Qiagen, GmbH) was employed.

Statistical analysis

The counts of *S. aureus* were changed into base-10 logarithms of cfu/g. The one-way ANOVA procedure of SPSS v.23 (SPSS Inc., Chicago, Illinois, The USA) was used to analyze the data. Tukey's multiple comparison tests were performed to find differences in *S. aureus* counts in the samples under study that were statistically significant. A p value of 0.05 is regarded as significant. Data were expressed as means±SE.

RESULTS AND DISCUSSION

Staphylococcus aureus is a significant foodborne pathogen that causes numerous nosocomial infections worldwide and many cases of foodborne intoxications that occur via produc-

Table 1. Oligonucleotide primer sequences used in the study.

Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
Sea (F)	5′ TTGGAAACGGTTAAAACGAA′3	120	Rall *et al.* (2008)
Sea (R)	5′ GAACCTTCCCATCAAAAACA ′3		
Seb (F)	5′ TCGCATCAAACTGACAAACG ′3	478	
Seb (R)	5′ GCGGTACTCTATAAGTGCC ′3		
Sec (F)	5′ GACATAAAAGCTAGGAATTT ′3	257	
Sec (R)	5′ AAATCGGATTAACATTATCC ′3		
Sed (F)	5′ CTAGTTTGGTAATATCTCCT ′3	317	
Sed (R)	5′ TAATGCTATATCTTATAGGG ′3		

tion of heat-stable enterotoxins such as *Sea*, *Seb*, *Sec*, and *Sed* (Darwish *et al*., 2022). Herein, *S. aureus* was isolated from retailed salted fish including fesiekh, sahlia, salted sardine, and herrings at variable rates. Fesiekh had the highest prevalence rate of *S. aureus* at 40%, followed by sahlia at 30%, salted sardine at 15%, and herrings at 15%, respectively (Fig. 1). In parallel, fesiekh had significantly ($P < 0.05$) the highest total *S. aureus* count (3.17±0.13 $\log_{10}$ cfu/g), followed by sahlia (3.08±0.13 $\log_{10}$ cfu/g), salted sardine (2.334±0.07 $\log_{10}$ cfu/mL), and herrings (2.30±0.11 $\log_{10}$ cfu/g), respectively (Fig. 2). Comparing the recorded *S. aureus* counts in the present study with the established maximum permissible limits set (2 $\log_{10}$ cfu/g) by Egypt Organization for Standardization (EOS, 2005) revealed that 35%, 20%,10%, and 5% of the examined fesiekh, sahlia, salted sardine, and herrings exceeded that limit, respectively (Fig. 3).

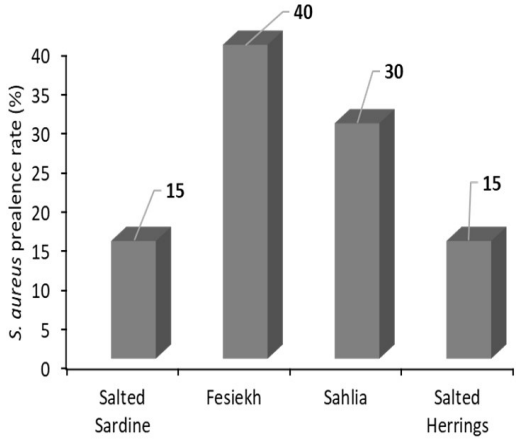

Fig. 1. Prevalence rate (%) of *S. aureus* in the examined salted sardine, fesiekh, sahlia, and salted herrings (n = 20/each).

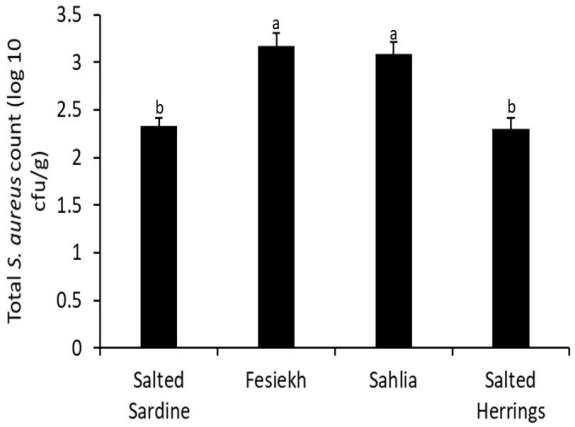

Fig. 2. Total *S. aureus* count ($\log_{10}$ cfu/g) in the examined salted sardine, fesiekh, sahlia, and salted herrings (n= 20/each). Columns carrying different letter (a, b, c) are significantly different at $p < 0.05$.

In agreement with the recorded results of the current investigation Ezzeldeen *et al*. (2011) could isolate *S. aureus* from salted fish including fesiekh, and molouha retailed in Cairo, Egypt. Furthermore, Elkassas *et al*. (2021) isolated *S. aureus* at 30% (salted sardine), 20% (fesiekh), and 10% (molouha) with mean values of 3.05±1.73, 3.45±2.14 and 2.64±1.39 $\log_{10}$ cfu/g, respectively. Globally, Basti *et al*. (2006) isolated *S. aureus* at levels higher than 5 $\log_{10}$ cfu/g in salted mullet (55%), and smoked herrings (10%) in Iran. Besides, *S. aureus* was isolated at 4% from smoked mackerel, and at 3% from salted sardine retailed in Greece (Sergelidis *et al*., 2014). Moreover, Gassem (2019) isolated *S. aureus* at 2.71–3.85 $\log_{10}$ cfu/g from salted mullet in Saudi Arabia.

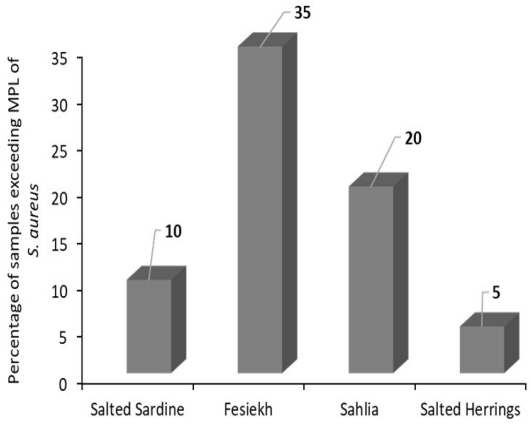

Fig. 3. Percentages of samples exceeding maximum permissible limits of *S. aureus* (2 $\log_{10}$ cfu/g) in the examined salted sardine, fesiekh, sahlia, and salted herrings (n= 20/each).

Food handlers' skin, hair, and nails may contain staphylococci (Darwish *et al*., 2022). These sources may help to clarify how *S. aureus* was isolated from salted fish sold in stores for the purpose of this investigation. The method of making fesiekh, which depends on the fermentation of raw fish, may have contributed to its high contamination rate. As a result, the initial microbial load of the raw mullet used in fesiekh preparation regulates the contamination of salted fish in environments with minimal hygiene standards (Amin and Ahmed, 2020).

S. aureus isolation rates varied among studies, which may be related to variable sanitary procedures used in the production of salted fish, differences in salt levels, dirty tools and equipment, or worker-caused contamination from poor personal hygiene (Al-Khusaibi, 2019).

Fish farming and aquaculture are increasing worldwide to fulfill the shortage in the red meat. Such activities are accompanied with the use of antimicrobials for the purpose of the prevention and control of bacterial fish diseases. However, the abuse of antimicrobials in the aquaculture might lead to the development of antimicrobial resistance (Alsayeqh *et al*., 2021). Therefore, the recovered *S. aureus* isolates were subjected to antimicrobial sensitivity testing. Interestingly, all tested isolates (100%) were resistant to at least two of the tested antimicrobials. *S. aureus* isolates were resistant to the tested antimicrobials at the following rates: gentamicin (85%), kanamycin (80%), erythromycin (70%), nalidixic acid (65%), oxytetracycline (60%), neomycin (55%), oxacillin (50%), ampicillin (30%), penicillin (30%), cephalothin (25%), trimethoprim/sulfamethoxazole (20%), ciprofloxacin (15%), enrofloxacin (15%), and chloramphenicol (10%), respectively (Fig. 4). The calculated MAR index for the recovered *S. aureus* isolates ranged between 0.142 to 0.928 with an average of 0.435 (Table 2). Similarly, *S. aureus* isolated from salted fish in Egypt had antimicrobial resistance to oxytetracycline, penicillin, and erythromycin at 41%, 30.5%, and 25% (Ezzeldeen *et al*., 2011). Furthermore, Vázquez-Sánchez *et al*. (2012) reported that all *S. aureus* isolated from salted fish were resistant to penicillin, chloramphenicol, and ciprofloxacin, and most to tetracycline (82.4%) in Spain. The uncontrolled use of antimicrobials in the intensive fish production without a proper veterinary supervision led to development of multidrug resistance among foodborne pathogens (Darwish *et al*., 2013).

Table 2. Antimicrobial resistance profiles of the recovered *S. aureus* isolates in the present study.

	Isolate	Resistance profile	MAR value	*Sea*	*Seb*	*Sec*	*Sed*
Fesiekh	*S. aureus 1*	AM, CET, C, CIP, E, GEN, K, NA, N, OX, OXY, P, SXT	0.928	-	-	+	-
Fesiekh	*S. aureus 2*	AM, CET, C, CIP, E, GEN, K, NA, N, OX, OXY, P, SXT	0.928	-	-	+	-
Fesiekh	*S. aureus 3*	CET, CIP, ENR, E, GEN, K, NA, N, OX, OXY	0.714	-	-	+	-
Fesiekh	*S. aureus 4*	CET, GEN, K, NA, N, OX, OXY, P, SXT	0.643	-	-	-	-
Fesiekh	*S. aureus 5*	CET, GEN, K, NA, N, OX, OXY, P, SXT	0.643	-	-	-	+
Fesiekh	*S. aureus 6*	ENR, E, GEN, K, NA, N, OX, OXY	0.571	-	-	-	+
Fesiekh	*S. aureus 7*	ENR, E, GEN, K, NA, N, OX, OXY	0.571	-	-	-	-
Fesiekh	*S. aureus 8*	E, GEN, K, NA, N, OX, OXY	0.5	-	-	-	-
Sahlia	*S. aureus 9*	E, GEN, K, NA, N, OX, OXY	0.5	-	-	+	-
Sahlia	*S. aureus 10*	E, GEN, K, NA, N, OX	0.428	-	-	+	-
Sahlia	*S. aureus 11*	E, GEN, K, NA, N	0.357	-	-	-	-
Sahlia	*S. aureus 12*	E, GEN, K, NA	0.285	-	-	-	-
Sahlia	*S. aureus 13*	E, GEN, K, NA	0.285	-	-	-	-
Sahlia	*S. aureus 14*	E, GEN, K	0.214	-	-	-	-
Sardine	*S. aureus 15*	AM, P, OXY	0.214	-	-	-	-
Sardine	*S. aureus 16*	E, GEN, K	0.214	-	-	-	-
Sardine	*S. aureus 17*	E, GEN	0.143	-	-	-	-
Herrings	*S. aureus 18*	AM, P, OXY	0.214	-	-	-	-
Herrings	*S. aureus 19*	AM, GEN, K	0.214	-	-	-	-
Herrings	*S. aureus 20*	AM, OXY	0.143	-	-	-	-
		MAR (Average)	0.436	-	-	-	-

AM: ampicillin, CET: cephalothin, C: chloramphenicol, CIP: ciprofloxacin, ENR: enrofloxacin, E: erythromycin, G: gentamicin, K: kanamycin, NA: nalidixic acid, N: neomycin, OX: oxacillin, OXY: oxytetracycline, P: penicillin, and SXT: trimethoprim/sulfamethoxazole

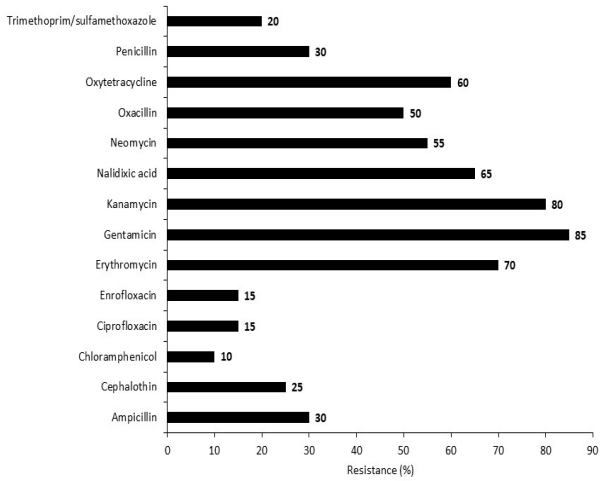

Fig. 4. Antimicrobial resistance rates (%) of the recovered *S. aureus* isolates from the examined salted sardine, fesiekh, sahlia, and salted herrings.

PCR testing of the recovered *S. aureus* isolates for harboring Staphylococcal enterotoxin-coding genes revealed that *Sea*, and *Seb* were not detected in any examined isolate. However, *Sec* was detected in 3 *S. aureus* isolates recovered from fesiekh, and in 2 isolates that recovered from sahlia. *Sed* was only detected in 2 isolates that recovered from fesiekh (Table 2). In support of our results Simon and Sanjeev (2007) reported that *Sec*, followed by *Sea* were the dominant SEs in fishery products retailed in India. Detection of enterotoxins in the identified *S. aureus* isolates in the present study agrees with Vázquez-Sánchez *et al.* (2012) who detected *Sea* in 88% of the *S. aureus* isolated from salted fish in Spain.

In many cases of food poisoning epidemics around the world, *S. aureus* is to blame. For instance, in the US in 2012, the Centers for Disease Control and Prevention (CDC) reported an incident of food poisoning caused by *S. aureus* in a military unit (CDC, 2013). Additionally, according to the European Food Safety Association, *S. aureus* was connected to 293 incidences of food poisoning in Europe in 2011 (EFSA, 2011).

CONCLUSION

The current study's findings showed that *S. aureus* contamination of retailed salted fish occurred at varying rates and occasionally exceeded the defined Egyptian limits. The recovered *S. aureus* isolates also exhibited striking multidrug resistance and a strong tolerance to antibiotics. Additionally, certain *S. aureus* isolates found in fesiekh and sahlia included the genes for *S. aureus* enterotoxins. As a result, strict hygiene guidelines should be followed when preparing and processing salted seafood.

CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

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