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Determination of prevalence, virulence characterization, and antimicrobial resistance patterns of emerging methicillin- and vancomycin-resistant *Staphylococcus aureus* in frozen fish fillet and shrimp

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Abstract

Staphylococcus aureus is one of food poisoning bacteria. This study assessed the prevalence, virulence factors, and antimicrobial susceptibility profiles of *S. aureus* isolated from frozen fish and shrimp in Egypt. Ninety samples from frozen fish fillets and shrimps (45 for each) were examined for *S. aureus* prevalence using VITEK 2 compact system, followed by molecular confirmation by *nuc* gene, virulence characterization, and its resistance genes. The overall prevalence rate of *S. aureus* was 14.44% (13/90). Fish fillet had the highest mean total *S. aureus* count ($9.50 \pm 3.50 \times 10^5$ colony forming unit g^{-1}), followed by shrimp ($7.50 \pm 3.30 \times 10^5$ colony-forming unit g^{-1}), with a non-significant difference among fish fillet and shrimp. All confirmed *S. aureus* isolates were lecithinase producers and showed β-hemolysis, and coagulase positive, and confirmed molecularly *coa* gene positive. All isolates were resistant to ampicillin (100%), both erythromycin and clindamycin (69.23%), and tetracycline (61.53%), followed by vancomycin (46.15%). However, all isolates were sensitive to linezolid, tigecycline (84.70%), and trimethoprim/sulfamethoxazole (61.53%). Twelve (92.30%) phenotypically cefoxitin and oxacillin-resistant and molecularly *mec*A recovered *S. aureus* isolates were confirmed as methicillin-resistant *S. aureus*, while based on vancomycin-resistant phenotypically, and molecularly *van*A recovered *S. aureus* isolates were confirmed as vancomycin-resistant *S. aureus*. The emergence of multidrug-resistant methicillin-resistant *S. aureus* and vancomycin-resistant *S. aureus* in frozen fish fillets and shrimp indicates public health hazards, so there is a need for food safety measures alongside reliable detection methods of resistant bacteria along the food chain.

Introduction

Staphylococcus aureus may be considered an opportunistic bacterium in healthy humans and animals, which causes food poisoning and diverse infections, from skin and soft tissue infections to more life-threatening illnesses like endocarditis, septicemia, osteomyelitis, pneumonia and toxic shock syndrome.[1] The most predominant cause of food poisoning worldwide is the presence of staphylococcal infection which is related to food poisoning and has been shown to cause nausea, vomiting and diarrhea.[2] Poor hand hygiene may negatively impact food safety and spread *S. aureus* strains during production, processing and distribution.[3] Dynamic interactions between handlers, foods and contact surfaces transmit the disease in food industries. *Staphylococcus aureus* strains are usually spread to foods by handlers by improper handling and respiratory secretions.[3,4] Methicillin-resistant *S. aureus* (MRSA) is one of the twelve priority infections that pose hazards to human health, according to the World Health Organization.[1] An increase has occurred in the recognition of MRSA in food products.[5] The MRSA has been identified in fish and fish products.[6]

Staphylococcus aureus is responsible for a range of diseases through enzymes and toxins production as well as the invasion of host cells including hemolysins.[7] Numerous studies have shown a strong correlation between the hemolysins of *S. aureus* and infections in both humans and animals.[8] Coagulase, a virulence factor of *S. aureus*, initiates prothrombin, consequently promoting

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blood coagulation.[9] Lecithinase, categorized as a phospholipase, enhances bacterial pathogenicity. This enzyme typically destabilizes animal cell membranes by penetrating them, resulting in the formation of a hole that leads to cell lysis or degrading phospholipids.[10]

Antimicrobials against Gram-positive bacteria, such as beta-lactam antibiotics alone or in combination with aminoglycosides, have always been used to treat staphylococcal infections.[6] The antibiotics used resulted in an exponential rise in antibiotic resistance and the emergence of multi-drug-resistant bacteria rendered eradication challenging. It threatened its effectiveness in prevention and treatment.[9] It is possible that beta-lactam medications including penicillin, cephalosporins, carbapenem and others are ineffective against MRSA,[11] which is considered one of the nosocomial infections associated with dramatically increased morbidity and mortality.[12] For many years now, vancomycin has been the final line of defense against MRSA infections.[13] However, its overuse has led to the development of vancomycin-resistant *S. aureus* (VRSA), vancomycin-intermediate *S. aureus* and heterogeneous-vancomycin-intermediate *S. aureus*.[14]

There is a serious risk to consumers health when MRSA and VRSA exist in food since the resistance might be transmitted to the consumers.[15] The presence of *S. aureus* in fish products is generally attributed to contamination occurring during handling and processing.[16] The simultaneous use of antimicrobial agents in aquaculture, along with the resulting contamination of aquatic ecosystems, may facilitate the selection, emergence, and dissemination of antibiotic-resistant *S. aureus*.[17] There has been a limited amount of literature published on the topic of MRSA and VRSA. This study was carried out to monitor the occurrence of MRSA and VRSA as well as their phenotypic virulence factors and antimicrobial resistance patterns in fisheries products.

Materials and Methods

Collection of fish and shrimp samples. A total number of 90 frozen fish fillet and shrimp samples (45 samples for each) were collected from different fish markets at Cairo Governorate. Samples were collected in the ice box and transported to the microbiology lab as soon as possible. All procedures in the present study complied with the protocols established by the Animal Ethics Review Committee of Suez Canal University (SCU-VET-AREC-2025002), Ismailia, Egypt.

Isolation and identification of *Staphylococcus aureus*. Frozen fish fillets and shrimp samples have been processed in accordance with ISO 6888–1 and ISO 6888–2:2003 to isolate and determine *S. aureus*.[18] A 25.00 g (± 1.00 g) sample was obtained from each sample utilizing sterilized blades. Subsequently, 225 mL of 0.10% peptone water (Oxoid, Hampshire, UK) was incorporated and shaken at 300 rpm for 1 min utilizing a Seward Stomacher 400 (London, UK). Using the pouring plate method, 100 µL was inoculated onto Baird-Parker agar (Oxoid) supplemented with egg yolk tellurite emulsion (Oxoid) and incubated for 24 hr at 37.00 ˚C. Suspected colonies, identified by black colonies with a halo zone, were preserved in Tryptic soy broth with 20.00% glycerol at –20.00 ˚C for subsequent analysis. The isolates were phenotypically identified utilizing the VITEK 2 compact system (BioMérieux, Marcy l'Etoile, France).[19]

***Staphylococcus aureus* virulence factors detection.** In the coagulase test, for each strain, 500 µL of re-suspended rabbit plasma was added to a glass tube with, inoculation of 500 µL bacterial culture and the tubes were cultured for 4 hr at 35.00 ˚C.[20] Clot formation was assessed hourly. After 4 hr, the tubes were incubated for a further 20 hr at 25.00 ˚C, during which clot formation was observed.[20] Concerning the hemolytic activity of *S. aureus*, strains were streaked onto 5.00% sheep blood agar (Oxoid) and incubated at 37.00 ˚C for 24 hr to assess their hemolytic activity.[21] Regarding lecithinase activity, the Baird-Parker medium was employed to evaluate lecithinase production. A loopful of recovered strains was inoculated into media and incubated at 37.00 ˚C for 24 hr. The emergence of an opaque zone encircling the colony suggested a positive result.[22]

Antimicrobial susceptibility testing. An antimicrobial susceptibility test was done using the disk diffusion method according to Clinical and Laboratory Standards Institute,[23] guidelines against 15 antimicrobial discs (Oxoid) of 12 classes. The multiple antibiotic resistance (MAR) index was assessed according to Sandhu *et al*.[24] The examined isolates were categorized as multidrug-resistant (MDR; MDR: resistant to ≥ one antibiotic in ≥ three antimicrobial classes) as previously described by Magiorakos *et al*.[25]

Molecular confirmation and determination of *MRSA* and *VRSA* genes. Genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions employing 20.00 mg mL^{-1} lysozyme (Sigma-Aldrich, St. Louis, USA) to disrupt the bacterial cell wall. The *nuc* gene for *S. aureus* confirmation, *coa* gene for *S. aureus* coagulase, the *mec*A gene for methicillin resistance detection and the *van*A gene for vancomycin resistance detection were identified using uniplex polymerase chain reaction (Table 1).

Statistical analysis. The Chi-square test via SPSS Software (version 26.0; IBM Corp., Armonk, USA) was used to evaluate the significance between fish fillets and shrimp, and the $p < 0.05$ was considered statistically significant using R software (version 4.0.2; https://www.r-project.org/). The graphs were illustrated. Clustering was illustrated using factoextra package by R-software. The sample size was calculated using G*Power (version 3.1.9.7; Heinrich Heine Universität, Düsseldorf, Germany).

Table 1. Oligonucleotide primers' sequences used for PCR amplification of *Staphylococcus aureus* genes.

Target genes	Primer sequence (5'-3')	Amplicon size (bp)	References
nuc	F: GCGATTGATGGTGATACGGTT R: AGCCAAGCCTTGACGAACTAAAGC	270	26
coa	F: ATAGAGATGCTGGTACAGG R: GCTTCCGATTGTTCGATGC	630	27
***mec*A**	F: GTAGAAATGACTGAACGTCCGATAA R: CCAATTCCACATTGTTTC R: GGTCTAA	310	28
***van*A**	F:CATGACGTATCGGTAAAATC R: ACCGGGCAGRGTATTGAC	885	29

nuc: Nuclease, *coa*: Coagulase, *mec*A: Methicillin resistance gene, *van*A: Vancomycin resistance gene.

Results

Prevalence and phenotypic characterization of *S. aureus* in fish fillet and shrimp samples. Among the 90 samples, 13 samples were positive for *S. aureus*, resulting in a prevalence of 14.44%. Specifically, 17.77% (8/45) of fish fillets and 11.11% (5/45) of shrimps were positive, fish fillets demonstrated more positive samples for *S. aureus* (61.53%, 8/13) followed by shrimps (38.46%, 5/13). No significant difference was noted between fish fillets and shrimps (p = 0.4054; X^2 = 0.692). All positive *S. aureus* isolates were phenotypically identified utilizing the VITEK 2 compact system and confirmed molecularly by detection of the *nuc* gene that is specific to *S. aureus* isolates.

Virulence factors of recovered isolates. All examined isolates were positive for coagulase enzyme which was confirmed phenotypically by the coagulase test and detected molecularly by the *coa* gene. Concerning other virulence factors, lecithinase activity, and hemolysis, all isolates were lecithinase producers and exhibited β hemolysis on blood agar.

***Staphylococcus aureus* enumeration in fish fillet and shrimps samples.** The mean *S. aureus* counts overall was 9.50×10^5 colony forming unit (CFU) g^{-1} with a minimum value of 1.00×10^4 CFU g^{-1} in fish and a maximum value of 2.00×10^5 CFU g^{-1}. The mean *S. aureus* count overall was 7.00×10^5 CFU g^{-1} with a minimum value of 1.00×10^4 CFU g^{-1} in shrimp and a maximum value of 3.00×10^5 CFU g^{-1} in shrimp.

Antimicrobial susceptibility. The antimicrobial susceptibility pattern was carried out for 13 isolates and the results are presented in Tables 2 and 3. Of the 13 recovered *S. aureus* isolates, all were resistant to ampicillin (100%), 92.30% of the isolates were resistant to penicillins (oxacillin) and cephalosporin (cefoxitin). Also, 69.23% of the isolates were resistant to macrolides (erythromycin) and lincomycin (clindamycin), 61.53% of isolates were resistant to tetracycline (tetracycline), 46.15% of isolates were resistant to glycopeptide (vancomycin) and rifamycin (rifampicin) and 23.07% of isolates were resistant to aminoglycoside (gentamycin). However, all confirmed *S. auraus* isolates were sensitive to linezolid (linezolid) and 84.61% of isolates were sensitive to glycylcyclines (tigecycline), 38.46% of isolates were sensitive to Sulfonamide (trimethoprim/sulfamethoxazole), 61.60% of isolates were sensitive to glycopeptide (teicoplanin) and sulfonamide (trimethoprim/sulfamethoxazole), 53.84% of isolates were sensitive to fluroquinolones (moxifloxacin), rifamycin (rifampicin) and fluroquinolones (ciprofloxacin) and 46.15% of isolates were sensitive to glycopeptide (vancomycin), (Table 2 and 3). Statically, the recovered isolates of *S. aureus* showed a significant difference among the resistance of isolates for different antimicrobials (X^2 = 34.551, p = 0.001712).

Table 2. Results of antimicrobial sensitivity testing of *Staphylococcus aureus* using disk diffusion method (n = 13).

Antimicrobial classes	Antimicrobial agents	Resistant		Intermediate		Sensitive	
		No.	%	No.	%	No.	%
Penicillins	Ampicillin (10.00 µg)	13	100	-	-	-	-
	Oxacillin (1.00 µg)	12	92.30	-	-	1	7.69
Cephalosporins	Cefoxitin (30.00 µg)	12	92.30	-	-	1	7.69
Glycopeptides	Teicoplanin (30.00 µg)	1	7.69	4	30.76	8	61.53
	Vancomycin (30.00 µg)	6	46.15	1	7.69	6	46.15
Linezolids	Linezolid (30.00 µg)	-	-	-	-	13	100
Aminoglycosides	Gentamycin (10.00 µg)	3	23.07	5	38.46	5	38.46
Fluroquinolones	Ciprofloxacin (5.00 µg)	6	46.15	-	-	7	53.84
	Moxifloxacin (5.00 µg)	6	46.15	-	-	7	53.84
Lincomycins	Clindamycin (2.00 µg)	9	69.23	-	-	4	30.76
Macrolides	Erythromycin (15.00 µg)	9	69.23	-	-	4	30.76
Glycylcyclines	Tigecycline (15.00 µg)	2	15.38	-	-	11	84.61
Rifamycins	Rifampicin (5.00 µg)	6	46.15	-	-	7	53.84
Tetracyclines	Tetracycline (30.00 µg)	8	61.53	-	-	5	38.46
Sulfonamides	Trimethoprim/Sulfamethoxazole (25.00 µg)	5	38.46	-		8	61.53

Table 3. The frequency of the phenotypic multidrug-resistance among the *Staphylococcus aureus* isolates (n = 13).

No. of isolates	Phenotypic resistant	No. of classes /No. of agents	Resistant type	MARI	*S. aureus* type
1	AMP, OX, FOX, CIP, MXF, VA, SXT, RIF, E, CLI, TE	9 classes/11 agents	MDR	0.73	MRSA VRSA
1	AMP, OX, FOX, CIP, MXF, VA, SXT, RIF, E, CLI	8 classes/10 agents	MDR	0.66	MRSA VRSA
2	AMP, OX, FOX, TGC, RIF, TE, CIP, MXF, SXT	7 classes/9 agents	MDR	0.60	MRSA
1	AMP, OX, FOX, TEC, VA, RIF, E, CLI	6 classes/8 agents	MDR	0.53	MRSA VRSA
1	AMP, OX, FOX, VA, SXT, TE, E, CLI	7 classes/8 agents	MDR	0.53	MRSA VRSA
2	AMP, OX, FOX, CIP, MXF, E, CLI	5 classes/7 agents	MDR	0.46	MRSA
1	AMP, OX, FOX, GEN, VA, CLI, TE	6 classes/7 agents	MDR	0.46	MRSA VRSA
1	AMP, OX, FOX, GEN, VA, TE	5 classes/6 agents	MDR	0.40	MRSA VRSA
1	AMP, TE, GEN, CLI, E	5 classes/5 agents	MDR	0.40	-
1	AMP, OX, FOX, TE, RIF	5 classes/4 agents	MDR	0.40	MRSA
1	AMP, OX, FOX, E, CLI	5 classes/4 agents	MDR	0.40	MRSA

AMP: Ampicillin; OX: Oxacillin; FOX: Cefoxitin; CIP: Ciprofloxacin; MXF: Moxifloxacin; VA: Vancomycin; SXT: Trimethoprim-sulfamethoxazole; RIF: Rifampicin; E: Erythromycin; CLI: Clindamycin; TE: Tetracycline; TGC: Tigecycline; TEC: Teicoplanin; GEN: Gentamycin; MARI: Multi-antimicrobial resistance index; MDR: Multidrug-resistant; MRSA: Methicillin-resistant *S. aureus*; VRSA: Vancomycin-resistant *S. aureus*.

Twelve phenotypically (92.30%) cefoxitin and oxacillin-resistant recovered *S. aureus* isolates were confirmed as MRSA and confirmed molecularly by *mec*A gene, while based on vancomycin-resistant phenotypically and confirmed molecularly by *van*A gene, six (46.15%) recovered *S. aureus* isolates were confirmed as VRSA.

Of note, 100% (13/13) of the tested MRSA isolates were MDR with MAR indices over 0.40, and 100% (6/6) of the tested VRSA isolates were MDR with MAR indices over 0.40 (Table 3 and Fig. 1). The results of the MAR index for different samples are shown in Table 3. MRSA mean MAR index (0.57) was the highest followed by VRSA (0.32).

Clustering of MRSA and VRSA isolates. The MRSA isolates were categorized into five clusters (L1, L2, L3, L4, and L5) and the VRSA isolates were categorized into four clusters (L1, L2, L3, and L4) based on the distribution of antimicrobial resistance phenotypes, virulence factors, *nuc*, *coa*, *mec*A, and *van*A genes as determined by hierarchical clustering analysis (Fig. 2). Clusters L1 and L2 of MRSA comprised isolates sourced from fish samples while L4 consisted of isolates obtained from shrimp samples. Cluster L3 and L5, on the other hand, contained isolates sourced from both fish and shrimp samples. Clusters L1, L2, and L3 of VRSA comprised isolates sourced from fish samples, while L4 consisted of isolates obtained from shrimp samples. While no discernible pattern appeared in the clustering of isolates from various sources, certain isolates from distinct sources exhibited indistinguishable profiles of anti-microbial resistance phenotypes.

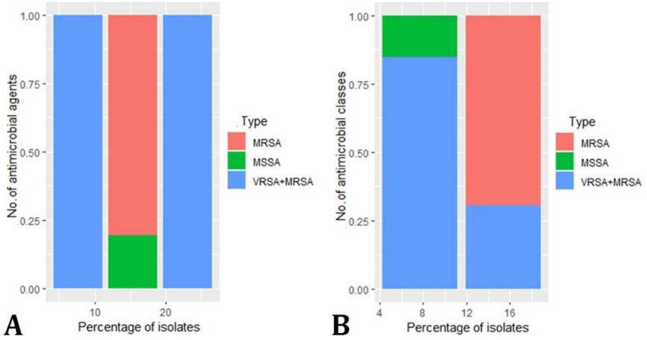

Fig. 1. The percentage of resistant isolates to **A)** The number of antimicrobial agents and **B)** The number of antimicrobial classes.

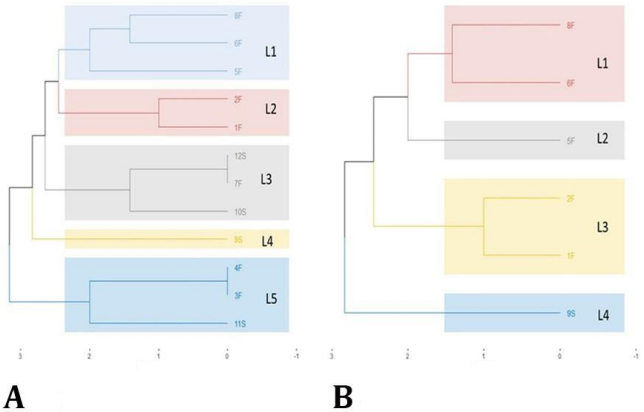

Fig. 2. Hierarchical clustering of virulence factors, *nuc*, *coa*, *mec*A, and *van*A genes in fish and seafood *Staphylococcus aureus* isolates. **A)** Methicillin-resistant *S. aureus* illustrates five clusters L1 - L5. **B)** Vancomycin-resistant *S. aureus* illustrates four clusters (L1 - L4).

Discussion

Unsanitary behaviors among fish handlers and their interaction with contaminated surfaces, including benches, tables, and unwashed knives, could compromise the quality and safety of fish and shrimp.[30] This contamination may have arisen from contaminated freezer systems or insufficient transportation of aquatic products, non-adherence of personnel to food hygiene standards, inadequate storage conditions and the accumulation of fish or shrimp, potentially resulting in contamination expanding beyond fresh products. Farm-raised fish displayed reduced contamination levels compared to improperly frozen and packaged products.[31]

The prevalence of *S. aureus* was 14.44% in this study from fish fillets and shrimps was similar to previous studies in Iran (17.00%) of fish and shrimp,[31] in Malaysia (15.00%) of shellfish at retail markets[32] higher than Islam *et al.*,[33] who noted 7.00% in raw fish in Bangladesh and lower than those studies reported in China (37.00%) of fish and shellfish,[34] and in Northwest Spain (43.50%) of fishery products.[35]

Compared to others, the different prevalences of *S. aureus* noted in this study might be attributed to processing differences and hygiene practices among symptomatic and asymptomatic fish handlers.[36] The average number of *S. aureus* in the examined samples varied according to the species, from $7.00 \pm 3.30 \times 10^5$ in shrimps to $9.50 \pm 3.50 \times 10^5$ in fish fillets that exceed the established maximum permissible limits stated by the Egyptian Organization for Standardization.[37] European Commission Regulation 1441/2007 defines process hygiene criteria that establish limits of 10^2 to 10^3 coagulase-positive Staphylococci *per* gram in chopped products of crustaceans and molluscan shellfish. Where values exceeding 1.00×10^3 CFU g^{-1} are determined, the appropriate response is to enhance production hygiene,[38] which aligns with our results.

Coagulase production is a significant phenotypic characteristic for identifying *S. aureus*.[35] The evaluation of MRSA strains indicated a complete presence of the *coa* gene, which is consistent with previous findings.[39] The isolates are confirmed by detecting the *nuc* gene.[40] Hemolysin is a critical virulence factor for *S. aureus*.[41]

Antibiotic resistance may propagate via residual antibiotics in food products, the transmission of resistant foodborne pathogens, or the consumption of resistant strains that exist in the original food microflora, resulting in the transfer of resistance to pathogenic microorganisms. In our study, 100% of the tested MRSA isolates were MDR with MAR indices over 0.40, and 100% of the tested VRSA isolates were MDR with MAR indices over 0.40.[42] Vázquez-Sánchez *et al.*, reported similar percentages (100%) in Spain. Resistance to penicillin was 100%, similar to Vázquez-Sánchez *et al*.[42] Among *S. aureus* isolates, resistance to tetracycline at 61.53% was nearly similar to a study in Egypt that revealed 60.00% tetracycline resistance among salted fish,[43] and lower than Vázquez-Sánchez *et al.*,[42] who detected 82.40% among fishery products. Our isolates were resistant to erythromycin at 69.23%, similar to Fri *et al.*,[44] who noted 67.00% in marine fish and Morshdy *et al.*,[43] who noticed 70.00% in salted fish. Our clindamycin resistance results (69.23%) nearly aligned with Fri *et al.*,[44] who noted 82.00% in marine fish.

Regarding MRSA, which is not part of the normal microbiota of fish or crustaceans, MRSA contamination may have increased due to bulk sales of the examined products and poor handling by processors or retailers. Although sellers use disposable gloves, some bacteria can adhere to retail food personnel hands, causing cross-contamination if not changed regularly.[45] This study demonstrated that a high percentage of recovered isolates were MRSA, which aligned with previous studies.[46] The rising resistance of MRSA indicates an urgent need to incorporate newer regimens to combat invasive MRSA infections.[44] The resistance of isolates to vancomycin was 46.15% higher than that of Fri *et al.*,[44] who detected 30.00% vancomycin-resistant isolates. The current scenario is worrying as vancomycin is the standard treatment for invasive MRSA infections in humans.[13] Vancomycin-resistant MRSA has been identified in camel meat, with all isolates classified as vancomycin-resistant *S. aureus* being MRSA strains.[44]

Freezing below − 18.00 ˚C is commonly employed to preserve fish and fishery products. Freezing is not naturally lethal; however, bacterial levels diminish due to the physical harm induced by ice crystals and alterations in osmotic conditions.[47] However, it can grow within a broader range of 6.50 to 46.00 ˚C.[48] At low temperatures, *S. aureus* cells may sustain damage without being inactivated.[49] Freezing below – 18.00 ˚C is frequently used to preserve fish and seafood products. Our findings demonstrated that MRSA and VRSA display enhanced survival capabilities in shrimp and fish fillets at freezing temperatures. The persistence of MRSA and VRSA in frozen shrimps and fish fillets in this study was significant as they underwent considerable handling during processing, potentially resulting in contamination with *S. aureus*. Freezing does not reduce bacterial contamination from improper handling, especially for MRSA, which can endure freezing conditions.[50] Our investigation revealed that frozen fish had a higher infection frequency than shrimp, aligned with Arfatahery.[31]

In conclusion, this study demonstrated that fish and shrimp might be a source of MRSA and VRSA. To reduce the issue of methicillin-resistant staphylococci and vancomycin-resistant staphylococci, implementing basic food safety measures, including proper hand hygiene, heat treatment and adequate food storage, is adequate and represents a health issue for human health.

Respecting effective veterinary practices and hygiene protocols that address food safety risks throughout the food chain is essential.

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Conflict of interest

The authors have no conflict of interest.

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