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Antibiotic sensitivity patterns and molecular detection of Enterotoxin genes in *Staphylococcus aureus* isolated from frozen muscle foods

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ABSTRACT

Stapylococcus aureus is considered to be the most common pathogen in commonly consumed and improperly stored frozen foods. Hence, this study investigated the antibiotic sensitivity patterns and the occurrence of enterotoxin genes (*sea* and *seb*) in *S. aureus* isolated from frozen poultry meat of chicken, turkey and fish, namely *Trachurus trachurus, Scomber scrombus* and *Merluccious merluccious*. A total of 500 samples of raw meat from chicken (n = 130), turkey (n =130), and different fish (n =240) were randomly purchased from various markets and shopping malls in Akure between September 2020 and December 2021. The antibiotic sensitivity of isolated *S. aureus* was determined by disk diffusion method. *S. aureus* was analyzed for enterotoxin genes by multiplex polymerase chain reaction (PCR). The highest staphylococcal count (7.70×10^5 CFU/g) was observed in *T. trachurus.* One hundred and twenty five *S. aureus* were isolated from meat and fish with the highest occurrence (40.80%) in turkey meat. *S. aureus* is highly resistant (33.3 to 100%) to the tested antibiotics. The PCR assay, respectively, showed *sea* and *seb* coded for staphylococcal enterotoxin A and B. The presence of staphylococcal enterotoxin A and B in frozen muscle foods could be attributed to unhygienic conditions during processing and storage. The occurrence of antibiotic resistant and enterotoxigenic *S. aureus* in muscle foods could pose a serious threat to people when consumed. Hence, urgent attention is required to avert the incidences of staphylococcal food poisoning after consuming frozen meat and fish.

Introduction

Frozen meat, fish, poultry and sea foods are important source of proteins in diets and mostly consumed by people of all social strata (Laskowski et al., 2018). Tissues from healthy animals are sterile, but many factors can influence their microbial contamination during slaughtering. Microbial contamination is possible through the water, air, soil, from the workers andthe equipment involved and it often leads to spoilage if not properly handled, processed and preserved (Alegbeleye et al., 2018). The most common method to preserve muscle foods is by chilling and freezing, while super-chilling is also an attractive technique of preserving muscle foods to maintain their freshness, enhancing the shelf-life during storage, transportation and at retail (Banerjee and Maheswarappa, 2019). Freezing preserves muscle foods for extended periods by suppressing the growth and multiplication of microorganisms that could cause food spoilage and foodborne illnesses (Archer, 2004; Zhan et al., 2018). In frozen products, some microorganisms are killed, while others might only be sub-lethally damaged and can recover upon thawing if storage is above -10 °C. Gram negative bacteria are more susceptible to freezing injury than Gram positive

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bacteria. Hence, it supports the occurrence of *Staphylococcus* species in frozen meats and fish (Ogofure and Igbinosa, 2021).

S. aureus is a Gram-positive bacteria and facultative anaerobe, which can live as a commensal organism on the skin as well as mucosal membranes. *S. aureus* recovers and survives sub-lethalinjuries from freezing with cell membrane synthesis-related proteins, oxidative stress resistance-related proteins, and metabolism-related proteins with their virulence factors exhibit distinct expression patterns during resuscitation (Suo et al., 2018). The resuscitation mechanisms adopted by *S. aureus* together with their multiple antibiotic resistance pose a threat to food safety and are now of public health concern.

The multiple resistance of *S. aureus* to all β -lactam antibiotics has been linked to the presence of an enzyme; β-lactamase and the *mecA* gene that encode penicillin-binding protein (PBP2a), which allows the synthesis of the cell wall even at lethal concentrations of β –lactam antibiotics (Atanassova et al., 2017). *S. aureus* produces a variety of enzymes, toxins (cytotoxins, exotoxins, and exfoliative) with multiple virulence factors encoded by phages, plasmids, and pathogenicity, which has the ability to bind the major histocompatibility complex proteins of their host's immune system (Tam and Torres, 2019). Staphylococcal food poisoning is an intoxication that occurrs after consuming improperly prepared foods contaminated with *S. aureus* enterotoxins as a result of unhygienic practices including improper handling of cooked or processed muscles foods, followed by their storage conditions (Argudín et al., 2010). *S. aureus* is a major cause of food poisoning induced by heat resistant enterotoxin and is one of the leading causes of foodborne illnesses (Varshney et al., 2009; Pinchuk et al., 2010). *S. aureus* strains produce a large variety of enterotoxins A, B, C, and D that have been frequently detected in poultry meat products (Pepe et al., 2006; Akkaya et al., 2014). Enterotoxin *S. aureus* is considered to be the most common pathogen causing outbreaks of food poisoning, characterized by symptoms associated with gastroenteritis, including vomiting, nausea, abdominal pain, cramps, diarrhea and causing significant morbidity (Ortega et al., 2010). The contamination of foods with pathogenic microorganisms with reoccurrence of foodborne diseases face significant challenges in modern healthcare services and have substantial negative impacts on economy. Enterotoxins are primarily responsible for foodborne illnesses and morbidity. Hence, frequent screening of staphylococcal enterotoxins (SE) in commonly consumed foods like animal muscle foods, poultry foods, and dairy products is paramount since they are foods of proteins source. This study, therefore, investigated the antibiotics sensitivity patterns and occurrence of enterotoxins *S. aureus* in selected frozen poultry meat (frozen chicken and turkey) and fish like *Scomber scrombus*, *Trachurus trachurus* and *Merluccious merluccious* vended in Akure metropolis.

Materials and methods

Collection of meat and fish

Samples of frozen meat of chicken (*Gallus gallus domesticus*) = 130, turkey (*Meleagris gallopavo domesticus*) = 130, and different fish species, namely, horse mackerel (*Trachurus trachurus*) = 80, mackerel (*Scomber scrombrus*) = 80, and hake (*Merluccius merluccius*) = 80, were randomly purchased at different vendors in Akure. Samples were collected in ice packs and transported to Postgraduate Research Laboratory, Department of Microbiology, The Federal University of Technology, Akure, for microbiological analysis.

Isolation and identification of Staphylococcus aureus

One gram of tissue from each sample was crushed in sterile crucible and serially diluted to ten-fold dilutions, each dilution (10^4) was plated on mannitol salt agar using pour plate method and incubated at 37 ^oC for 24 hours. The colonies were counted using colony counter (TT-20, Techmel and Techmel, USA). The colony was sub-cultured to obtain pure isolate. The morphological, Gram's reaction and biochemical tests like catalase, coagulase, oxidase, methyl red, Voges Proskauer, and sugar fermentation were carried out according to methods of Olutiola et al. (2000) and Cheesbrough (2006). The identity of isolates was determined based on the biochemical reactions using Krieg et al. (2010).

Antibiotic susceptibility of Staphylococcus

The modified Kirby-Bauer method was used to determine the susceptibility of obtained isolates to some antibiotics as described by The Clinical and Laboratory Standards Institute (CLSI, 2017). The inoculum size in nutrient broth (18 h old culture) was adjusted to 0.5 McFarland turbidity standards to 1.3×10^5 CFU/ml. Thereafter, 0.1 ml of the suspension was transferred to prepared Mueller-Hinton agar and spread with a sterilized glass spreader. The surface of the agar was allowed to dry, antibiotic discs were placed at the equidistance of the plate by sterile forceps and aseptically placed on top of agar plate. The plates were incubated at 37 ^oC for 18 hours. After incubation, a clear zone of no growth in the immediate

vicinity of antibiotic disk was measured and recorded as diameter of zone of inhibition in millimeter (mm), interpreted using CLSI interpretative chat as resistance, intermediate and susceptible (CLSI, 2017).

Genomic DNA extraction and PCR test for staphylococcal enterotoxin genes

S. aureus isolates were further examined for the presence of enterotoxin genes using specific primer in a multiplex PCR assay. DNA of *S. aureus* was extracted with PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, USA) according to manufacturer's instructions. The primers used for the detection of SE genes are listed in Table 1 (Johnson et al., 1991). For polymerase chain reaction amplification, the reaction mixture contained: 2.5 µl 10 X PCR buffer (Invitrogen), 1 µl DNA, 1 µl of primer F (10 pmol), 1 µl of primer R (10 pmol), 0.5 µl dNTP (10 mM, Invitrogen), 1.5 µl $MgCl_2$ (Invitrogen), 0.5 U Taq DNA polymerase (Invitrogen) and final volume was adjusted to 25 µl by adding sterile ultrapure water. DNA amplification was performed in a thermal cycler with initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. The amplified PCR products were electrophoresed in a 1.5 % agarose gel (Sigma–Aldrich) containing 0.5 mg/ml ethidium bromide, TBE buffer (0.09 M Tris–HCl, 0.09 M boric acid, 2 mM EDTA, pH 8.3) for 30 min at 100 V and visualized under UV trans-illumination.

Table 1. Primers used for the detection of *Staphylococcus aureus* enterotoxin genes

Gene	Primers	Sequences5'-3'	Gene location	Size (bp) of PCR product
sea	SEA-F	ttggaaacggttaaaacgaa	490-509	
	SEA-R	gaaccttcccatcaaaaaca	591-610	120
seb	SEB-F	tcgcatcaaactgacaaacg	634-653	
	SEB-R	gcaggtactctataagtgcc	1091-1110	478

Source: (Johnson et al., 1991)

Data analysis

Data was statistically analyzed using SPSS version 20, was separated using Duncan's New Multiple Range test and significant difference will be value at $p \leq 0.05$.

Results and discussion

Table 2 shows total Staphylococcal count ($\times 10^5$ CFU/g) of frozen muscle foods. The staphylococcal count of 0.70 to 7.50 $\times$ 10^5 CFU/g were obtained for meat from chicken, 1.50 to 7.40 $\times$ 10^5 CFU/g, 2.10 to 7.70 $\times 10^5$ CFU/g, 3.30 to 5.10 $\times$ 10^5 CFU/g and 1.00 to 6.50 $\times 10^5$ CFU/g were recorded for turkey meat, *T. trachurus, S. scombrus* and *M. merluccious*, respectively. Findings of Bodunde et al. (2019) reported Staphylococcus count of 1.70×10^4 to 6.0×10^5 CFU/g for different muscle foods like beef (*Bos taurus*), chicken (*Gallus gallus domesticus*), turkey (*Meleagris gallopavo*), pork (*Sus scrofa domesticus*), chevon (*Capra aegagrus hircus*), mackerel (*Scomber scombrus*), horse mackerel (*Trachurus trachurus*), herrings (*Clupea pallasii*), blue whiting (*Merluccius merluccius*), and croaker (*Micropogonias undulatus*). The higher *S. aureus* count in meat and fish is a result of improper hygienic practices at the point of handling by slaughter personnel during meat production. Findings of Thwala et al. (2021) reported that a total of 2853 samples containing beef, pork, goat meat, camel meat, sheep/lamb from different African countries were highly contaminated with higher load of *S. aureus*. Species of *Staphylococcus* like S. *aureus, S. epidermidis, S. xylosus, . sciuri, S. warneri, S. saprophyticus, S. schleiferi* and *S. auricularis* were identified from ready-to-eat fish products (Sergelidis et al. 2014). Humans and animals such as cattle, pigs, chickens, turkey, horses, and sheep can be colonised by *S. aureus* on their skin and in their nares (Lozano et al., 2016). The frequency of *Staphylococci* in muscle foods (meat and fish) is revealing how commensal bacteria from human skin and mucosal surfaces contributed to the contamination during different stages of processing; slaughtering, transportation, chopping, storage and persons involved in the marketing (Wu et al., 2018). Table 3 reveals the occurrence of *S. aureus* in selected frozen muscle foods, meat from chicken, turkey and fish. Frozen meat of turkey has got the highest occurrence of *S. aureus* 40.80%, followed by meat of chicken with 30.40%, but *S. scombrus* has got the least occurrence of 2.40%. Findings of Ogofure and Igbinosa (2021) showed that beef had the highest frequency of *S. aureus* contamination (46.7%), followed by chicken (40.0%) and fish (30.0%). Wu *et al*. (2018) also reported 35% of *S. aureus* from 1,850 samples

of frozen meat and meat products sold in 39 cities and provincial capitals of China. Savariraj *et al.* (2021) isolated 66.67% of *S. aureus* from 120 chicken meat marketed in retail outlets of Chennai, India.

Higher prevalence of *S. aureus* (64%) has been reported from frozen meat and fresh meat from Karbala province in Iraq (Namir et al., 2017). Likewise, Oranusi et al. (2014); Ogidi et al. (2016); and Bodunde et al. (2019) reported the prevalence of *S. aureus* in muscle foods and ready-to- eat foods examined in Nigeria. The prevalence of *S. aureus* could be attributed to poor hygienic practice of personnel, equipment in slaughterhouses, salesmen and women.Table 4 shows the resistance percentage of *S. aureus* isolates to rifampin, gentamycin, ampicillin, nitrofurantoin, amoxicillin, oxacillin, fluoroquinolones, streptomycin, vancomycin, erythromycin and trimethoprim/sulfamethoxazole. The resistance percent ranged from 33.3 to 100%. Findings of Parvin et al. (2021) revealed the highest resistance percentage of 73.9% to 100% for *S. aureus* isolated from frozen chicken meat against cefoxitin, nalidixic acid, ampicillin and oxacillin, colistin, amoxicillin-clavulanic acid and amoxicillin, penicillin-G and cloxacillin, oxytetracycline, and cefixime. Findings of Wang et al. (2017) revealed a total of 1,150 *S. aureus* isolated from 27,000 retail foods with 97.6% of *S. aureus* dispalyed resistant to at least one antimicrobial compound like penicillin, oxacillin, cefoxitin, vancomycin, daptomycin, erythromycin, gentamicin, tetracycline, ciprofloxacin, clindamycin, trimethoprim-sulfamethoxazole, chloramphenicol, linezolid and 57.5% of these were multi drug resistant. *S. aureus* isolated from frozen meat and fish were highly resistant to some of the commercially available antibiotics (Ogofure and Igbinosa, 2021). The researchers reported the antibiotic resistance profile of *S. aureus* with high resistance to erythromycin (94%), amoxicillin/clavulanic acid (87.5%) and trime-thoprim-sulfamethoxazole (81%). Findings of Wu et al. (2018) revealed that *S. aureus* isolated from 1,850 retail meat and meat products displayed resistance of 11.0% to 85.4% to ampicillin, penicillin, erythromycin, tetracycline, kanamycin, telithromycin, clindamycin, streptomycin, norfloxacin, gentamicin, fusidic acid, ciprofloxacin, chloramphenicol, amoxycillin/clavulanic acid. *S. aureus* isolated from Oklahoma retail chicken and turkey meats displayed multiple resistance (5.4 to 94.6%) to ampicillin, tetracycline, penicillin, doxycycline, oxacillin, azithromycin, erythromycin, vancomycin, ciprofloxacin, cefoxitin, gentamicin, kanamycin, clindamycin, rifampin, trimethoprim/sulfamethazole, chloramphenicol with 12 Methicillin-Resistant *S. aureus* (MRSA) showed 100% resistance to ampicillin, penicillin, cefoxitin with 2% NaCl, oxacillin with 2% NaCl, azithromycin, and erythromycin (Abdalrahman et al., 2015). The use of medically important antibiotics in livestock production is tremendously contributing to multiple antibiotic resistance and pathogenicity of *S. aureus* (Park and Ronholm, 2021). The ability of various pathogenic bacteria to withstand antibiotic residues in muscle foods contributed to reoccurrence emergency of foodborne diseases, which have become a matter of food security worldwide (Kumar et al., 2020). Figure 1 shows agarose gel electrophoresis of the PCR product of *sea* gene in selected bacteria isolates (120 bp). Gel image indicates a positive amplification with the presence of *sea* gene in all 5 isolates loading arrangement (1-5 represent different strains of *S. aureus)*. Figure 2 shows agarose gel electrophoresis of the PCR product of *seb* gene in two *S. aureus* (Band size approximately 478 bp). Table 5 revealed the occurrence of enterotoxin producing *S. aureus*. The use of DNA hybridization and PCR approaches are reliable biological methods to detect staphylococcal enterotoxins using gene-specific nucleotide sequences as probes (Wu et al., 2016). In this study there was a low prevalence of enterotoxin producing *S. aureus* isolated from meats from chicken, turkey and fish. Findings of Ali (2014) and Arslan and Özdemir (2017) revealed the presence of enterotoxin genes in *S. aureus* isolated from fish. *S. aureus* 18/31 (58.1%) isolated from raw lamb and beef meat were positive for the presence of at least one or more SE genes, while no SE genes were found in strains isolated from cooked meat (Haghi et al., 2021). Findings of Şanlıbaba (2022) indicated *sea* and *seb* in *S. aureus* isolated from retail raw beef, sheep, and lamb meat in Turkey, which were directly connected to the contamination arose from human and animal origins. Findings of Rodríguez-Lázaro et al. (2017) examined 868 animal-derived food items; diverse meat from antelope, duck, guinea pig, pork, rodents, turkey, dairy products and eggs that were confiscated from the non- European Union passengers or illegally sold in an open market near an European Union border. The researchers revealed the presence of MRSA isolates with 73% tested positive for one or more enterotoxin genes A, B, C, D, G, H, I, and J. Kitai el. (2005) revealed that 78 enterotoxigenic *S. aureus* isolated from raw chicken meat; thighs, breasts, wings, livers, gizzards, hearts and ovaries belong to human biotype and poultry. SEs producing *S. aureus* are widely distributed in animals and humans and are frequently contaminating muscle foods leading to make Staphylococcal food poison, thereby causing a serious threat to consumers when bacteria multiply and release toxins in uncooked or inadequately cooked foods (Grispoldi et al., 2021).

Table 2. Total Staphylococcal count ($\times 10^5$ CFU/g) of frozen muscle foods vended in Akure

Locations	Points of collection	Chicken (130)	Turkey (130)	*T. trachurus* (80)	*S. scombrus* (80)	*M. merluccious* (80)
Kings market	A	5.40±0.40 [b]	0.00	2.20±0.10 [d]	4.20±0.31 [b]	6.40±0.29 [a]
	B	3.20±0.16 [d]	7.50±0.11 [a]	0.00	0.00	0.00
	C	1.40±0.02 [f]	4.70±0.93 [b]	0.00	0.00	0.00
	D	3.00±0.01 [d]	3.30±0.03 [c]	7.70±0.55 [a]	0.00	0.00
	E	0.00	1.50±0.11 [e]	2.50±0.71 [d]	0.00	0.00
FUTA South Gate	A	4.30±0.01 [c]	3.10±0.06 [c]	2.20±0.10 [d]	0.00	2.40±0.02 [c]
	B	3.80±0.11 [c]	0.00	7.70±0.12 [a]	0.00	1.30±0.00 [d]
	C	7.50±0.93 [a]	1.70±0.86 [e]	4.35±0.01 [c]	3.30±0.01 [c]	0.00
	D	3.30±0.05 [d]	2.40±0.02 [d]	5.40 ±0.32 [b]	0.00	0.00
Araromi Market	A	0.00	7.40±0.07 [a]	4.40±0.18 [c]	0.00	1.00±0.00 [d]
	B	4.90±0.01 [c]	1.80±0.01 [e]	2.10±0.08 [d]	0.00	3.20±0.44 [b]
	C	5.70±0.36 [b]	3.30±0.11 [c]	0.00	0.00	0.00
Isinkan Market	A	3.90±0.27 [c]	0.00	0.00	0.00	0.00
	B	0.70±0.00 [i]	5.50±0.50 [b]	7.50±0.06 [a]	5.10 ±0.02 [a]	0.00
	C	2.10±0.00 [e]	3.10±0.30 [c]	5.10±0.13 [b]	0.00	6.50±0.32 [a]
Shopping Malls		0.00	2.00±0.07 [e]	0.00	0.00	0.00

Values are mean of replicates (n=3). Values carrying the same superscript at the column are not significantly different at $p < 0.05$.

Table 3. Occurrence (%) of *Staphylococcus aureus* in examined frozen muscle foods

Locations	Chicken (130)	Turkey (130)	*T. trachurus* (80)	*S. scombrus* (80)	*M. merluccious* (80)
King's market	8	13	5	1	6
FUTA South Gate	15	8	3	1	0
Araromi Market	8	15	4	0	3
Isinkan Market	7	11	8	1	4
Shopping malls	0	4	0	0	0
n	38	51	20	3	13
% occurrence	30.40	40.80	16.00	2.40	10.40

n= number of *Staphylococcus aureus* isolates from meat and fish

Table 4. Resistance profile (%) of *S. aureus* isolated from selected frozen muscle Foods in Akure

Antibiotics	Chicken n=38	Turkey n=51	*T. trachurus* n=20	*S. scombrus* n=3	*M. merluccious* n=13
Rifampin	20(52.60)	33(64.70)	13(65.00)	1(33.30)	7(53.80)
Gentamycin	28(73.70)	40(78.40)	10(50.00)	0	6(46.20)
Ampicillin	16(42.10	20(39.20)	8(40.00)	0	5(38.50)
Nitrofurantoin	25(65.80)	31(60.80)	11(55.00)	0	8(61.50)
Amoxicillin	16(40.10)	42(82.40)	17(85.00)	2(66.70)	7(53.80)
Oxacillin	30(78.90)	37(72.50)	10(50.00)	3(100)	4(30.70)
Fluoroquinolones	18(47.40)	32(62.75)	8(40.00)	3(100)	4(30.70)
Streptomycin	20(52.60)	29(56.90)	15(75.00)	0	8(61.50)
Vancomycin	14(36.80)	18(35.30)	5(25.00)	1(33.30)	4(30.70)
Erythromycin	21(55.30)	30(58.80)	14(70.00)	2(66.70)	6(46.20)
Trimethoprim/sulfamethoxazole	18(47.40)	28(54.90)	8(40.00)	3(100)	6(46.20)

n= number of *S. aureus* isolates. Values in bracket represent % resistance to antibiotics.

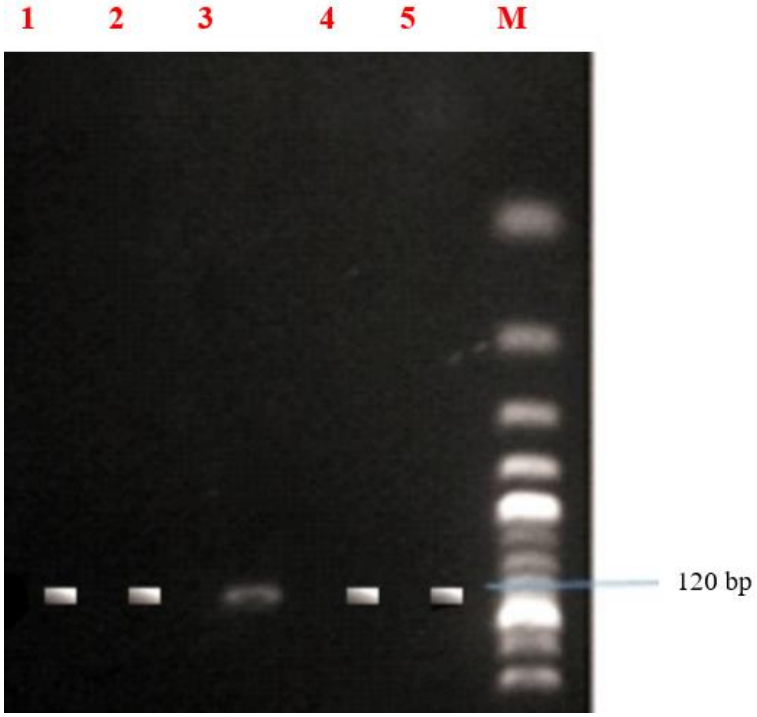

Figure 1. PCR amplification for the detection of *sea* gene in *S. aureus*. Lane M: 100 bp marker. Lanes 1-5 indicate *sea* gene (120 bp).

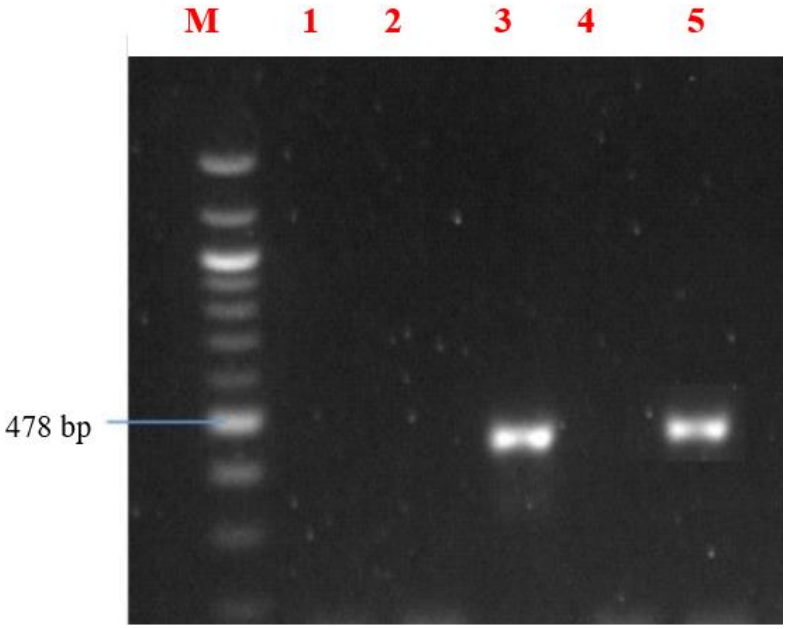

Figure 2. PCR amplification for the detection of *seb* gene in *S. aureus*. Lane M: 100 bp marker. Lanes 3 and 5 indicate *seb* gene (478 bp).

Table 5. Occurrence and antibiotic profile of enterotoxin producing *S. aureus* isolated from frozen meat and fish

Sample location	Muscle foods	Antibiotic resistance profile	Staphylococcal genes
Kings' market	Turkey	GEN, AMP, STR, VAN, AMX, OXA, FLU, ERY	*sea*
FUTA South Gate	Turkey	RFM, GEN, AMP, VAN, AMX, OXA, FLU	*sea*
Kings' market	Chicken	RFM, AMP, VAN, AMX, VAN, FLU, ERY, TMS	*sea*
FUTA South Gate	Chicken	GEN, AMP, VAN, AMX, NIT, OXA, FLU, ERY	*sea*
Isinkan Market	Chicken	RFM, VAN, AMX, STR, VAN, FLU, ERY, TMS	*sea*
Araromi Market	Chicken	RFM, AMP, VAN, AMX, NIT, OXA, VAN, FLU, ERY, TMS	*seb*
FUTA South Gate	*T. trachurus*	GEN, AMP, STR, VAN, NIT, AMX, OXA, FLU, ERY	*seb*

RFM: Rifampin, GEN: Gentamycin, AMP: Ampicillin, NIT: Nitrofurantoin, AMX: Amoxicillin OXA: Oxacillin, FLU: Fluoroquinolones, STR: Streptomycin, VAN: Vancomycin, ERY: Erythromycin, TMS: Trimethoprim/sulfamethoxazole

Conclusions

The isolation and identification of *S. aureus* from selected muscle foods could be due to various types of contamination during processing steps and poor personnel hygiene of the food handlers. The low prevalence of enterotoxin producing *S. aureus* from frozen meat and fish can still be considered as a potential source of foodborne diseases. In order to safeguard public health, public enlightenment should be sustained to raise awareness on the proper cooking, packaging and storage of poultry products. The illegal importation of poultry products should be curbed by the creation of right policies and implementation of existing laws. The elimination of plethora pathogenic bacteria from frozen foods should be emphasized. With the increase in the prevalence of antibiotic resistant bacteria in frozen foods, public health awareness is essential as a preventative measure to control the use of antibiotics in livestock production.

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