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Detection of enterotoxin genes of *Staphylococcus aureus* isolated from herring type fish and its methicillin resistance

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Abstract
The prevalence of methiciline-resistance *staphylococcus aureus* (MRSA) was investigated in one hundred samples of smoked fish (herring fish) collected from different supermarkets in Kafr El-Sheikh governorate. *Staphylococcus aureus* was isolated from ten samples (10%). Only 6 isolates (60%) showed phenotypic resistance to methicillin using oxacillin antibiotic disc. The *mec* A gene which encoded a penicillin-binding protein causing resistance to all B- lactam antibiotics was detected by PCR in all the six MRSA isolates. It could be also observed that, all MRSA isolates harbor the staphylococcal enterotoxins genes (*sea, seb, sec*). It could be concluded that presence of MRSA in smoked fish considered as a potential health risk for consumers.

Key words: *MRSA, mec A gene, staphylococcal enterotoxins genes, smoked fish (hering fish).*

Introduction

Fish is a perishable food material that deteriorates soon after harvest at high ambient temperature **(Aberoumand, 2010)**, therefore it need immediate preservation which may be obtained by freezing, salting, sundrying, oven-drying, fermentation and smoking **(Asiedu *et al.*, 2002).**

Smoked fish is highly desirable because of its enhanced flavor, texture and protection of smoking against different deteriorations **(Sowumi, 2007)**. *Staph. aureus* is considered as one of the leading causes of food borne disease in human **(Zhang *et al*., 1998).**

The emergence of *Staph. aureus* as a serious pathogen is owing to its intrinsic virulence and its capability of adaptation to different environmental conditions and also development of resistance to almost any new antimicrobials **(WaldVogel, 2000).**

Infections caused by MRSA are of global concern **(Morgan, 2008 and Wendlandt *et al*., 2013)** where the pathogen is recognized as zoonotic causing serious human health concern. Contamination of food with this pathogen is often related to improper handling, storage conditions as well as inadequate hygienic measures and post production microbial contamination **(Ray, 2004).**

MRSA has spread worldwide in the second half of the 20 th century specifically in 1961, the year in which methicillin was markted **(Jevons, 1961)** and it is now endemic in health care facilities in all industrialized countries **(Kobayashi *et al*., 2015).**

Most of nosocomial *Staph.aureus* infections are caused by MRSA strains **(Pereira *et al*., 2009)** which recognized as the cause of high morbidity and mortality all over the world **(Ho *et al*., 2008).**

The presence of MRSA in food of animal origin, namely meat and milk has been reported **(Pexara *et al*., 2013 and Wendlandt *et al*., 2013).** While recently, it has been detected in cultured fish **(Atyah *et al*., 2010).**
The heat stable enterotoxins are the most notable virulence factors associated with *Staph. aureus* that cause food poisoning outbreaks **(Martin *et al*., 2003** and **Kerouanton *et al*., 2007).** Staphylococcal enterotoxins (SEs) function not only as potent gastrointestinal toxins but also as super antigens that stimulate non-specific T-cell proliferation, there is a high correlation between these two separate functions, as the loss of super antigen activity (due to genetic mutation) results in loss of enterotoxic activity as well **(Harris *et al*., 1993).**

SEs have been divided into five major serological types (SEA, SEB, SEC, SED and SEE) on the basis of their antigenic properties **(Su and Wongh, 1997)**. SEA is the most common enterotoxin recovered from food poisoning outbreaks **(Balaban and Rasooly, 2000)** and it is known that 59% of staphylococcal food poisoning outbreaks are caused by SEA to SEE **(Bergdol, 1989).**

Previous studies on *Staph.aureus* proved that enterotoxin gene PCR determinations are in a high agreement (97-100%) with the toxin production as defined by immunoassays **(Fueyo *et al*., 2001**; **Letertre *et al*., 2003).** Over the last few years, multiple PCR assays and multiplex PCR assays which detect gene sequences for SEs **(Becker *et al*., 1998**; **Schmitz *et al*., 1998** and **Sharma *et al*., 2000).** The aim of this study was to determine the prevelance of MRSA in smoked fish and its related enterotoxins which involved in staphylococcal food poisoning.

Materials and Methods

1-Collection of samples:

A total of 100 samples of unpacked smoked herring fish were randomly collected from Kafr EL-Sheikh Governorate shops and supermarkets with different sanitary condition and transferred to the laboratory in ice box under complete aseptic condition for bacteriological analysis.

2-Preparation of samples:

Five gm of each sample were taken under aseptic condition to sterile homogenizer flask containing 45 ml of sterile pepton water (0.1%). The mixture was allowed to stand for10 minutes at room temperature according to **(ISO/IEC, 1999).**

3-Isolation and identification of *Staph. aureus*:

One ml of supernatant was added to 5ml of enrichment Trypticase soya broth (TSB) (Oxoid) with 6.5 % NaCL and incubated aerobically at 37^0C for 18-24 hour then a loopful of enrichment broth was streaked onto Baird-Parker agar plates (BP) (Oxoid) and incubated at 37^0C for 24-48h. All plates were examined visually for typical colony types and morphological characteristics. Convex, black, shiny colonies with narrow white magrin surrounded by clear zone were regarded as *Staph. aureus.* These colonies were confirmed by conducting gram staining, coagulase, catalase, DNAse and mannitol fermentation test and other biochemical tests **(FDA, 2001).**

4-Antibiotic Resistance Test:

The methicillin-resistance phenotype of staphylococci was determined by standard disc diffusion method on Mueller-Hinton agar (Hi-Media) using oxacillin (1 μg) . The plates were incubated at 37°C for 24 h. A total of 10 isolates were included in the test. The results of antibiotic susceptibility test were interpreted as per guidelines of CLSI for *Staph. aureus* **(CLSI, 2008).**

5-Polymerase chain reaction (PCR) for detection of methicillin resistant gene and *staphylococcus* enterotoxins gene saccording to (Sambrook *et al*., 1989):

5.1 Genomic DNA extraction:

Genomic DNA from *staphylococci strains* (6 isolates) which proved to be MRSA by disc

diffusion method were extracted following the manufacturer instruction of QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

5.2 Oligonucleotide Primer:

Primers used were supplied from **Metabion (Germany)** and listed in Table(1).

5.3 DNA amplification:

Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. Master Mix was applied using Emerald Amp GT PCR master mix (Takara). The reaction was performed in an Applied biosystem 2720 thermal cycler as shown in Table (1).

Table (1). Oligonucleotides sequences and thermal profiles of PCR assays:

Target Gene / Primer sequence (5´-3´)	**Amplicon size**	**Thermal profile**	**Reference**
Sea : **F:** 5' TTGGAAACGGTTAAAACGAA3' **R:** 5' GAACCTTCCCATCAAAAACA3'	120 bp	95^{0}C for 5 min; 94^{0}C for 2 min for 30 cycle; 55^{0}C for 1 min; 72^{0}C for 2 min and 72^{0}C for 5 min	**Johnson *et al.* (1991)**
Seb: **F** :5':TCGCATCAAACTGACAAACG3' **R:** 5' GCAGGTACTCTATAAGTGCC3'	478 bp	95^{0}C for 5 min; 94^{0}C for 2 min for 30 cycle; 55^{0}C for 1 min; 72^{0}C for 2 min and 72^{0}C for 5 min	**Johnson *et al.* (1991)**
Sec: **F**:5'GACATAAAAGCTAGGAATTT3' **R:**5'AAATCGGATTAACATTATCC3'	257bp	95^{0}C for 5 min; 94^{0}C for 2 min for 30 cycle; 55^{0}C for 1 min; 72^{0}C for 2 min and 72^{0}C for 5 min	**Johnson *et al.* (1991*)***
***mec*A:** **F**: 5'-GTA GAAATG ACT GAA CGT CCG ATA A-3' **R:** 5'-CCAATT CCA CAT TGT TTC GGT CTA A-3'	310 bp	95℃for 3 min ; 94℃ for 1 min for 33 cycle; 53℃for 30 sec ; 72^{0}C for 1 min and72^{0}C for 6 min	**Frebourg *et al.*(2000)**

5.4-Analysis of the PCR Products:

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using 5V/cm. For gel analysis, 15 µl of the PCR products was loaded in each gel slot. A gene ruler100 bp DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was visualized and photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Results

1-Prevalence of *staph. aureus* isolated from smoked herring fish samples. (n=100)

Staph. aureus was isolated from ten samples out of the examined 100 herring fish samples with an isolation percent of 10%.

2- Incidence of methicillin resistant *Staph. aureus* (MRSA) from *Staph. aureus* isolates (n=10)

The 10 isolates of *Staph. aureus* were examined for methicillin resistance phenotype via antibiotic sensitivity test using oxacillin(1µg). Only 6 isolates from the ten examined *Staph.aureus* strains were methicillin resistant (MRSA)(60%).

3-Detection of *mec* A gene using PCR

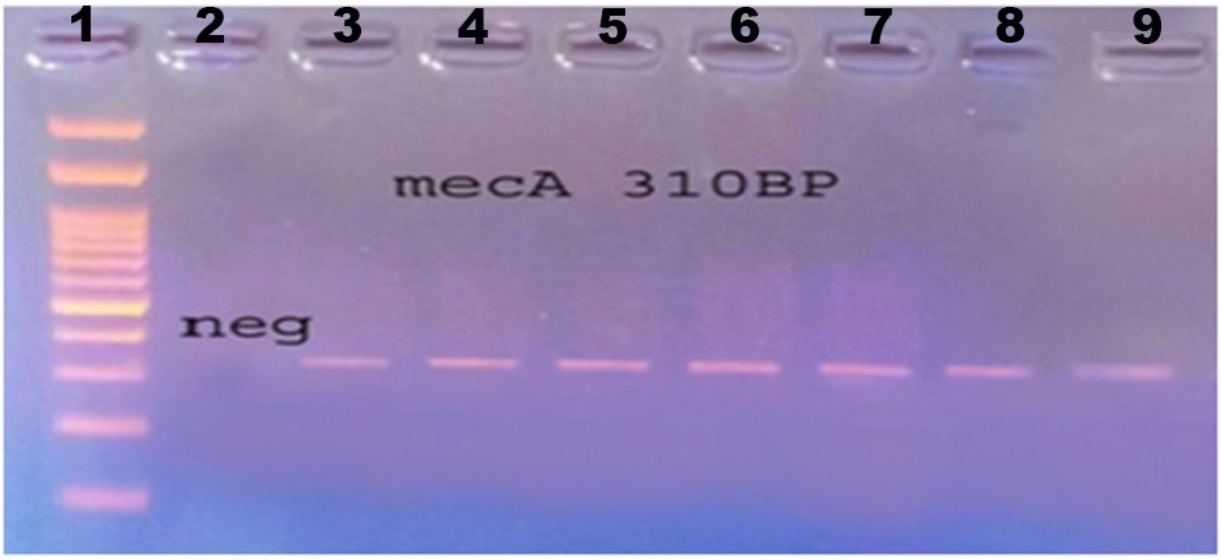

Photo (1): PCR result for ***mec*A** gene from the right to the left.
Lane (1): 100 bp ladder (QIAGEN, GmbH)
Lane (2): Negative control
Lane (3 to 8): Positive ***mec*A** gene result at 310 bp.
Lane(9): Positive control (310 bp).

4-Detection of *sec* gene using PCR:

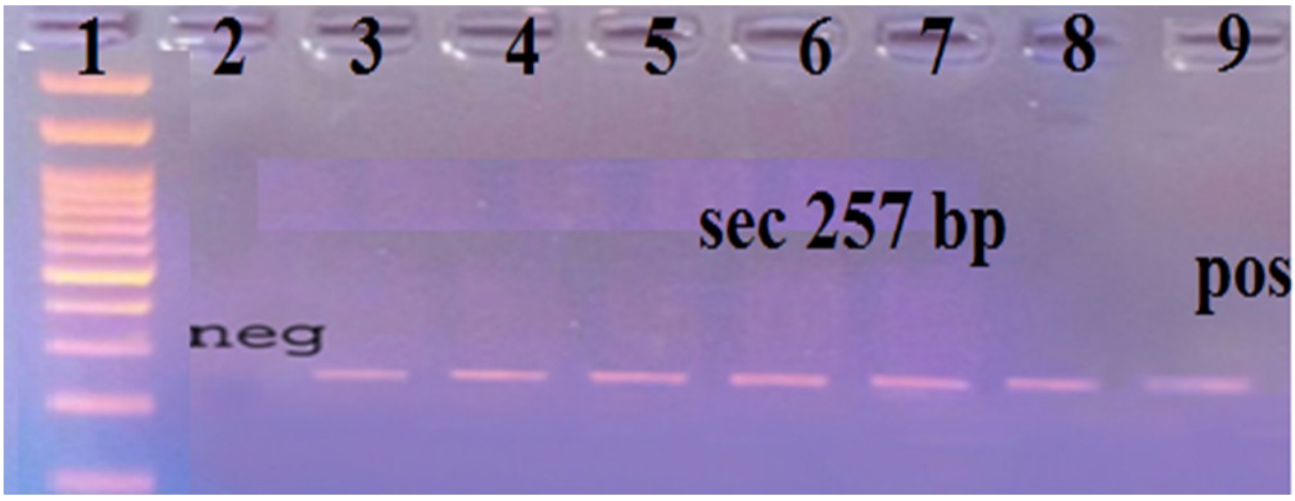

Photo (2): PCR result for ***sec*** gene**,** from the right to the left.
Lane (1): 100 bp ladder (QIAGEN, GmbH)
Lane (2): Negative control
Lane (3-8): Positive for ***sec*** gene result at 257 bp.
Lane (9): Positive control (257 bp).

5-Detection of *seb* gene using PCR

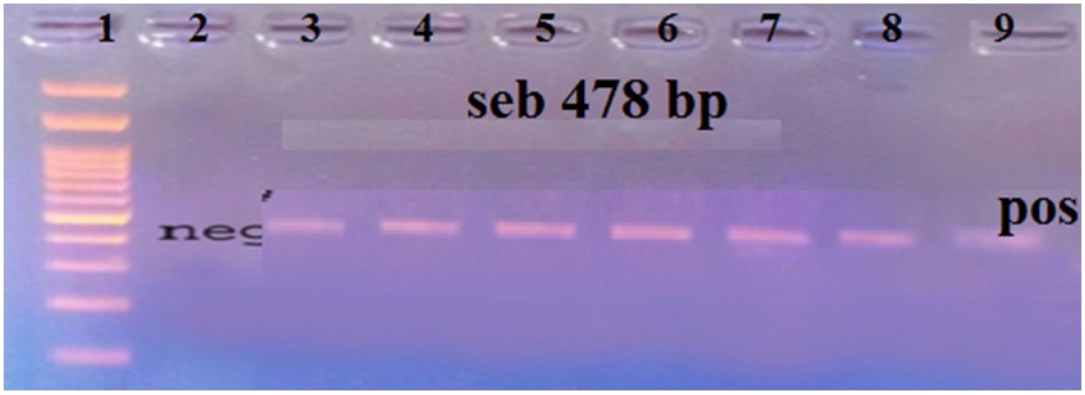

Photo (3): PCR result for ***seb*** gene,
Lane1: 100bp ladder (QIAGEN, GmbH)
Lane (2: Negative control
Lane (3-8): Positive for ***seb*** gene result at 478 bp.
Lane (9): Positive control (478 bp).

6-Detection of *sea* gene using PCR:

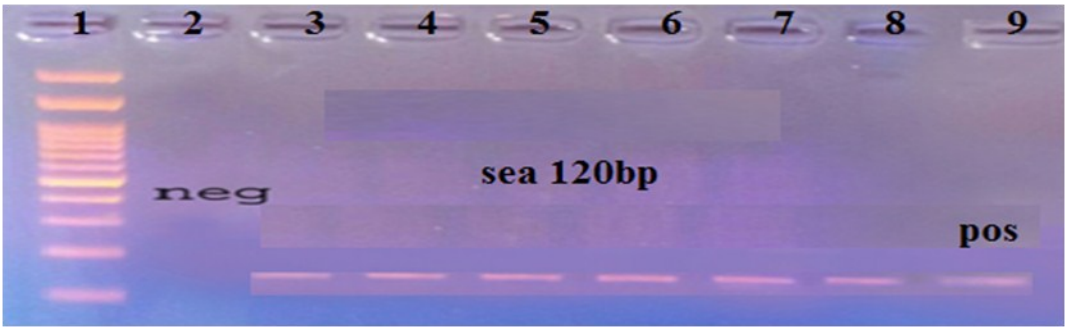

Photo (4): PCR result for ***sea*** gene, from the right to the left.
Lane (1): 100bp ladder (QIAGEN, GmbH)
Lane (2): Negative control
Lane (3-8): Positive for ***sea*** gene result at 120 bp.
Lane (9): Positive control (120 bp).

Discussion

Up to 70% of the total fish catch in the developing countries are smoked **(Ward, 1995).**

However, smoking may present some potential health issues to smoked food consumers. The most important food borne pathogen is *Staph. aureus*. Their presence is due to contamination of fish during capture, unhygienic handling and processing **Simonand Sanjeev, (2007).** The present results declared that, the incidence of *Staph.aureus* was 10% in the examined smoked herring fish samples.

The present results of isolation of *Staph. aureus* was nearly similar with that of **Shafik *et al.*, (2017)** who identified 3(12%) *Staph. aureus* in 25 coated smoked fish and 10 (20%) in 50 examined uncoated smoked herring fish. **Adelaja *et al.* (2013)** isolated *Staph. aureus* at a rate of (5% and 10%) in smoked fish from Oyan Lake and Ogun water side in Ogun state Nigeria.

Lower results of isolated *Staph. aureus* rate were detected by **Sergelidis *et al.* (2014)** who isolated *Staph. aureus* with 7%. Higher results of isolation was reported by those of **Edris *et al.* (2017); Saito *et al.* (2011)** and **Vázquez-**

Sanchez *et al.* (2012) who isolated *Staph. aureus* from herring fish with 80%, 16.5% and 26%, respectively.

Methicilin-resistant *Staph. aureus* (MRSA) are being increasingly found outside clinical settings **Reberio *et al.*, (2007).** MRSA have been found in food animal **Lee, (2003).** Recently, MRSA have been found in fishery products **Beleneva, (2011).** Although there is currently no evidence that eating food contaminated with MRSA may lead to an increased risk of human becoming carries or infected with this bacteria **EFSA, (2010),** it is important to take some preventive control measures.

Today, MRSA strains have become resistant to most common antibiotics. Therefore, treatment of infections in human and animals caused by MRSA is quite difficult **Livermore, (2000).**

The present results indicated that 6 out of 10 isolated *Staph. aureus* were detected as MRSA. Nearly similar finding was reported by **Atyah *et al.*, (2010)** who revealed that 98 out of 198 *Staph. aureus* isolates were reported as MRSA.

Lower findings were reported by **Grema *et al.* (2015)** who detected MRSA at 7 out of 33 examined *Staph. aureus* positive isolates (2.1%) and **Sergelidis *et al.* (2014)** who detected two MRSA from the seven isolates of *Staph. aureus* (28.5%). **Hammad *et al.*, (2012)** who identified five MRSA strains out of 175 *Staph. aureus* isolates from retail ready to eat raw fish in Japan. On the other hand **Noor Uddin *et al.*, (2013)** and **Vázquez-Sanchez *et al.*, (2012)** didn't identify any MRSA *Staph. aureus* from frozen fish and fishery products, respectively.

Most strains of *Staph. aureus* now showed methicillin resistance which is mediated by the *mec* A gene which is located on a foreign, mobile DNA element called staphylococcal cassette chromosome mec (SCC mec) **Arslan and Ozdemir (2017).**
As shown in photo (1), *mec* A gene was detected in all examined isolates and these results are in agreement with those of antibiotic sensitivity testing. These findings were similar with that of **Sergelidis *et al.*, (2014)** who detected that all isolates harbor the *mec* A gene. Lower findings were reported by **Kumar *et al.*, (2016)** who amplified the *mec* A gene in 11 out of 16 isolates while **Vázquez-Sanchez *et al.*, (2012)** didn't detect any PCR product for *mec* A gene.

Staphylococcal food poisoning is usually self limiting and resolves within 24 to 48 h after onset. So most cases not reported to health care services. For this reason, the actual incidence of staphylococcal food poisoning is known to be higher than reported **Smyth *et al.*, (2004).**

Staphylococcal enterotoxins (SEs) are resistant to proteolytic enzymes and also heat stable **Mossong *et al.*, (2015)**. So their presence indicated a significant food safety risk **Omeo *et al.*, (2005)**. For determination of SEs, the set RPLA is the most commonly used method. It detects a protein only if it is expressed in vitro and depend on toxin production. Recently, PCR assays have been developed to identify specific gene sequences for SEs **(Becker *et al.*, 1998** and **Sharma *et al.*, 2000).**

As shown in photos 2, 3 and 4 all of examined isolates were able to produce enterotoxins as all genes (*Sea*, *Seb* and *Sec*) amplified a PCR product which was disagreed with that of **Sergilidis *et al.*, (2014)** who revealed that non of the isolates carried enterotoxin genes. **Hammad *et al.*, (2012)** detected genes encoding SEs (*Seb and Sed*) in 14.2% of *Staph. aureus* isolates and nearly similar findings were reported by **Vázquez-Sánchez *et al.* (2012)** who indicated that 91% of *Staph. aureus* isolates carried enterotoxin genes.

SE genes were detected in a lower proportion among isolates from fishery products in other geographical regions **(Normanno *et al.*, 2005; Oh *et al.*, 2007** and **Simon and Sanjeev, 2007).**

Conclusion and Recommendations

Staphylococcus aureus can easily contaminate ready to eat fish. This contamination is usually associated with high presence of virulence genes (SE genes) and antibiotic resistance genes especially MRSA.

SEs are involved in 5% of food poisoning outbreaks but this percentage is certainly under estimation due to poor analytical performance in detection of SEs in food remnants. Therefore, the PCR assay can be used as an accurate, safe and fast technique for detection of *Staph. aureus* virulence genes and antibiotic resistance genes in smoked herring fish.

The result of this study revealed high level of MRSA among the isolates of *Staph. aureus*, this was important as it may lead to transfere of resistance between aquatic animals and humans through consumption or handling. So the world organization of aquatic animal health recommended the continuous monitoring of antimicrobial resistance in microorganisms associated with aquatic animals.

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