Detection of Enterotoxigenic *Staphylococcus aureus* in Meat Product Sandwiches Using Multiplex PCR

Marwa Salah EL-Maghraby[1]; Mohamed Ahmed Hassan [2]; Faten Said Hassanin [2] and Nahla Ahmed Shawky[3]

[1] *Faculty of Veterinary Medicine, Menofyia University*

[2] *Department of Meat Hygiene and Control, Faculty of Veterinary Medicine, Benha University*

[3]*Department Food Hygiene, Shebin elkom Lab., Animal Health Research Institute*

A B S T R A C T

The current work was performed to detect *Staphylococcus aureus* and their enterotoxin genes as *sea, seb, sec* and *sed* among street vended meat products represented by beef burger, kofta and sausage (30 of each) using traditional method of bacteriological isolation and multiplex PCR. The results illustrated that 37 (41.11%) out of 90 meat product samples harbored *S.aureus* pathogen. Accurately, *S.aureus* enterotoxin genes were detected in 13 (35.1%) out of 37 isolates. 3 isolate for beef burger samples mostly harboured (2) *sea* and (1) *seb* and the other 6 strains was negative for classic enterotoxin genes. Meanwhile, 5 isolates for kofta samples harboured (2) *sea*, (1) *sec*, (1) *sed* and (1) *sea* & *sed* gene and the other 7 were negative for classic enterotoxin genes, but 16 isolates of sausage samples harboured (2) *sea*, (1) *seb*, (1) mixed *seb* & *sec* and (1) *mixed sea, sec & sec* genes and 11 was negative for classic enterotoxin genes. Furthermore, *sea* is more prevalent in 6 samples (16.2%) followed by *seb* in 2 samples (5.4%) and *sec, sed, sea &sed, seb &sec* and *sea &sec & sed* in one sample (2.7%). Finally, Multiplex PCR is efficient in detection of SEs in food. Contamination of meat products with such serous pathogen was discussed.

Keywords: *S. aureus, multiplex PCR.*

(http://www.bvmj.bu.edu.eg) **(BVMJ-35(1): 190-196, 2018)**

1. INTRODUCTION

Staphylococcus aureus is recognized worldwide as an important food-borne pathogen because of its ability to produce a wide range of extracellular toxin proteins and virulence factors typically resulting in sudden onset of nausea, violent vomiting, abdominal cramps and sometimes diarrhea (Rosengren et al., 2013).

Several methods have been developed for the detection of enterotoxigenic *S. aureus* strains. Conventional methods for the detection of toxin producing *S. aureus* strains are based on immunological procedures measuring the toxin in the culture supernatants of suspected *S. aureus* strains such as reverse passive latex agglutination kit (RPLA) or in contaminated food extracts or in-patient specimens such as ELISA.

Over the past 15 years there has been an important evolution in molecular approaches for the rapid detection of food borne pathogens. Modern rapid methods as

Polymerase chain reaction (PCR) have high sensitivity, specificity and reduce detection time. It offers advantages over conventional diagnostic methods (Shah et al., 2009).

It is important to recognize that this technique will identify only strains harboring the toxin genes and independent of the expression and secretion of the toxin. Also, it can provide the information required for appropriate therapy and infection control during outbreaks of *S. aureus* because it was specially developed to fit into the daily work pattern of a routine clinical laboratory (Shimaa 2016).

Therefore, the aim of this study was to investigate the occurrence of *S. aureus* and their enterotoxin genes in ready to eat meat samples collected from several street vendors in Menoufia government.

2. Materials and methods

Collection of Samples:

A total of 90 random samples of street vended meat product sandwiches represented by beef burger, kofta and sausage (30 of each) were collected from several street vendors in Menoufia government.

Preparation of samples (ICMSF, 1996):

To 25 grams of the sample, 225 ml of sterile peptone water were added and thoroughly mixed using sterile blender for 1.5 minutes, from which 10-fold serial dilutions was prepared.

Isolation and Identification of Staphylococcus aureus

One ml from each of previously prepared serial dilutions was spread over Baired Parker agar plate using a sterile bent glass spreader. The inoculated and control plates were inverted and incubated at 37°C for 48 hours. The suspected colonies of Staphylococcus aureus appear as black, shiny, circular, smooth, and convex with narrow white margin and surrounded by a clear zone extending into opaque.

Polymerase Chain Reaction (PCR)

1. Materials used for PCR:

1.1. Reagents used for agrarose gel electrophoresis:

1.1.1. Agarose powder, Biotechnology grade (Bioshop^{R}, Canda inc. lot No: OE16323).

It prepared in concentration 2% in 1× TAE buffer.

1.1.2. Tris acetate EDTA (TAE) electrophoresis buffer (50 × liquid concentration) (Bioshop^{R} , Canda inc. lot No: 9E11854).

The solution diluted to 1× by adding 1 ml stock solution to 49 ml double dist. Water to be used in the preparation of the gel or as a running buffer.

1.1.3. Ethedium bromide solution (stock solution) biotechnology grade (Bioshop ® Canda Inc, Lot No: 0A14667):

The stock solution was diluted by 25µl /200ml double distilled water and stored covered at 4ºC. It was used for staining of PCR products that electrophoreses on agarose gel to be visualized by UV light.

1.2. Gel loading buffer (6×stock solution)(Fermentas, lot No: ooo56239).

The components were dissolved in sterile double distilled water and stored covered with aluminum foil at room temperature.

1.3. DNA ladder (molecular marker):

100 bp (Fermentas, lot No: 00052518).

1.4. 5X Taq master (Fermentas):

Containing polymerase enzyme, Magnesium chloride (Mg Cl_2), Deoxy nucleotide triphosphate (dNTP) and PCR grade water.

1.5. Primer sequences of S. aureus used for *PCR identification system:*

Application of PCR for identification and characterization of virulence factors including

Enterotoxins A, B, C and D (sea, seb, sec & sed) of S. aureus was performed essentially by using primers (Pharmacia Biotech) as shown in table 1.

2. DNA Extraction using QIA amp kit (Shah et al., 2009):

After overnight culture on nutrient agar plates, one or two colonies were suspended in 20 ml of sterile distilled water, and the suspension was then heated at 100ºC for 20 minutes. Accurately, 50-200 µl of the culture were placed in Eppendorf tube and the following steps were carried out:

2.1. Equal volume from the lysate (50-200 µl) was added, addition of 20-50µl of proteinase K, then incubation at 56 ºC for 20-30 min. After incubation, 200 µl of 100% ethanol was added to the lysate.

2.2. The solution was added to the column and centrifuged at 8000 rpm for 1 min. then the filtrate was discarded.

2.3. The sediment was washed using AW1 buffer (200 µl), the column was centrifuged at 8000 rpm / 1 min, and the filtrate was discarded.

2.4. Washing was applied by using the AW2 buffer (200µl), the column was centrifuged at 8000 rpm / 1 min. and the filtrate was discarded.

2.5. The column was placed in a new clean tube then, 25-50 µl from Elution buffer was added, centrifuged at 8000 rpm/1min. The column was discarded. The filtrate was put in clean tube containing the pure genomic DNA. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

3. DNA Amplification reaction of enterotoxin genes of S. aureus (Mehrotra et al., 2000):

The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany). Ten µl of DNA sample was diluted in 990 µl of nuclease free water for PCR. The genomic DNA samples were amplified by PCR in a reaction mixture (25µl) containing 13.25 sterile dH_2O, 2.5ml 10 x buffer, 0.63ml 10mMNTPs, 1ml 25Mm $MgCl_2$, 1.25 µl primer F (20pmol/ml), 1.25 µl primer R (20pmol/ml) and fill up to 25 µl PCR grade water. Multiplex PCR was applied for demonstration of *S. aureus* enterotoxin genes (*sea, seb, sec* & *sed*). DNA amplification was performed using the following conditions: initial denaturation for 5 min at 95°C followed by 30 cycles of denaturation (94°C for 2 min), annealing (55°C for 1 min), and extension (72°C for 2 min). A final extension step (72 °C for 5 min) was performed after the completion of the cycles. Amplified products were analyzed by 3% of agarose gel electrophoresis in 1x TBE buffer stained with ethidium bromide and visualized on UV transilluminator at 254 nm. A 100 bp DNA ladder was used as a marker for PCR products.

3. RESULTS

The results illustrated that 37 (41.11%) out of 90 meat product samples harbored S.aureus pathogen. S.aureus enterotoxin genes was detected in 13 (35.1%) out of 37 isolates. Three isolates form beef burger samples mostly harboured (2) sea and (1) seb and the other 6 strains were negative for classic enterotoxin genes. Meanwhile, 5 isolates for kofta samples harboured (2) sea, (1) sec, (1) sed and (1) sea & sed gene and the other 7 were negative for classic enterotoxin genes, but 16 isolates of sausage samples harboured (2) sea, (1) seb, (1) mixed seb & sec and (1) mixed sea, sec & sec genes and 11 was negative for classic enterotoxin genes. (Table 1).

sea is more prevalent in 6 (16.2%) followed by seb 2 (5.4%) and sec, sed, sea &sed, seb &sec and sea &sec & sed in 1(2.7%). (Table 1). Multiplex PCR reaction was carried out and resolved by agarose gel electrophoresis. (Photo. 1, 2&3).

Table 1: Target genes and primers used in PCR

Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
sea (F)	5′ TTGGAAACGGTTAAAACGAA′3		
sea (R)	5′ GAACCTTCCCATCAAAAACA ′3	120	
seb (F)	5′ TCGCATCAAACTGACAAACG ′3		
seb (R)	5′ GCGGTACTCTATAAGTGCC ′3	478	
sec (F)	5′ GACATAAAAGCTAGGAATTT ′3		Rall et al. (2008)
sec (R)	5′ AAATCGGATTAACATTATCC ′3	257	
sed (F)	5′ CTAGTTTGGTAATATCTCCT ′3		
sed (R)	5′ TAATGCTATATCTTATAGGG ′3	317	

Table 2: Incidence of enterotoxin genes of *S. aureus* isolated from the examined samples of meat product sandwiches by using multiplex PCR.

Enterotoxin	Beef burger (12)		Kofta (12)		Sausage (16)		Total (37)	
	No.	%	No.	%	No.	%	No.	%
A	2	22.2	2	16.7	2	12.5	6	16.2
B	1	11.1	0	0	1	6.3	2	5.4
C	0	0	1	8.3	0	0	1	2.7
D	0	0	1	8.3	0	0	1	2.7
A & D	0	0	1	8.3	0	0	1	2.7
B & C	0	0	0	0	1	6.3	1	2.7
A, C & D	0	0	0	0	1	6.3	1	2.7
-ve strains	6	66.7	7	58.4	11	68.7	24	64.9
Total	9	100	12	100	16	100	37	100

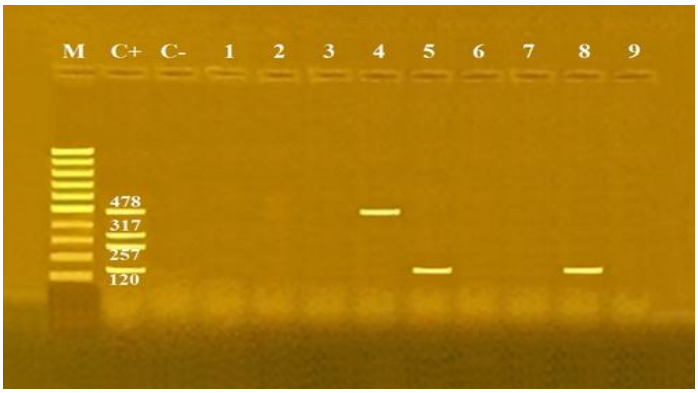

Fig.1. Agarose gel electrophoresis of multiplex PCR of sea (120 bp), seb (478 bp), sec (257 bp), and sed (317 bp) enterotoxin genes for characterization of S. aureus isolated from beef burger.

Lane M: 100 bp ladder as molecular size DNA marker.

Lane C+: Control positive for for sea, seb, sec and sed genes.

Lane C-: Control negative.

Lane 4: Positive S. aureus strain for seb gene.

Lanes 5 & 8: Positive S. aureus strains for sea gene.

Lanes 1, 2, 3, 6, 7 & 9: Negative S.aureus strains for enterotoxins .

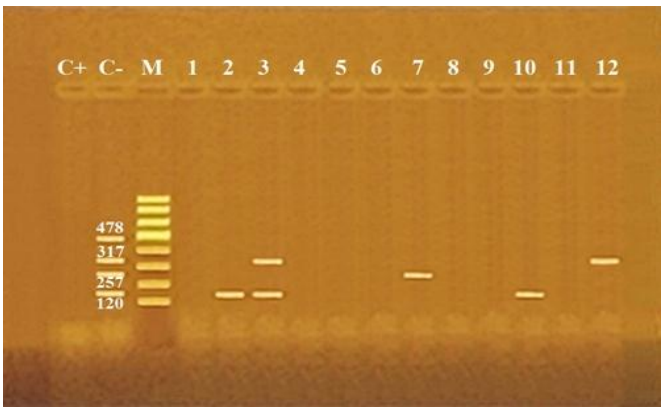

Fig.2. Agarose gel electrophoresis of multiplex PCR of sea (120 bp), seb (478 bp), sec (257 bp), and sed (317 bp) enterotoxin genes for characterization of S. aureus isolated from kofta.

Lane M: 100 bp ladder as molecular size DNA marker.

Lane C+: Control positive for for sea, seb, sec and sed genes.

Lane C-: Control negative.

Lanes 2 & 10: Positive S. aureus strains for sea gene.

Lane 7: Positive S. aureus strain for sec gene.

Lane 12: Positive S. aureus strain for sed gene.

Lane 3: Positive S. aureus strain for sea and sed genes.

Lanes 1, 4, 5, 6, 8, 9 & 11: Negative S.aureus strains for enterotoxins.

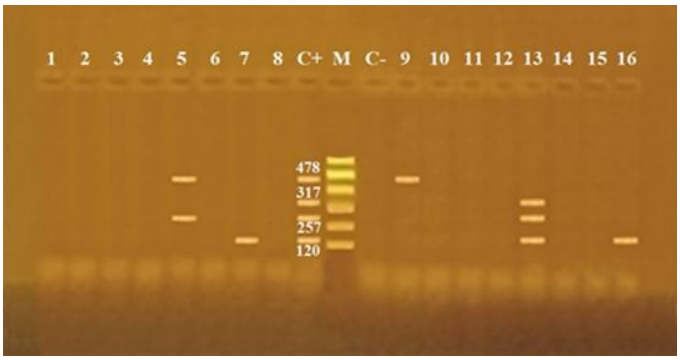

Fig.3. Agarose gel electrophoresis of multiplex PCR of sea (120 bp), seb (478 bp), sec (257 bp), and sed (317 bp) enterotoxin genes for characterization of S. aureus isolated from sausage.

Lane M: 100 bp ladder as molecular size DNA marker.

Lane C+: Control positive for for sea, seb, sec and sed genes.

Lane C-: Control negative.

Lanes 7 & 16: Positive S. aureus strains for sea gene.

Lane 9: Positive S. aureus strain for seb gene.

Lane 5: Positive S. aureus strain for seb and sec genes.

Lane 13: Positive S. aureus strain for sea, sec and sed genes.

Lanes 1, 2, 3, 4, 6, 8, 10, 11, 12, 14 & 15: Negative S.aureus for enterotoxins.

4. DISCUSSION

It was cleared that 9, 12 and 16 isolates of examined beef burger, kofta and sausage samples, respectively (37 positive samples) were subjected to multiplex PCR for detection of S. aureus classic enterotoxin genes and the results were as follow: only 3 isolates for beef burger samples mostly harboured (2) sea and (1) seb and the other 6 strains were negative for classic enterotoxin genes Meanwhile, 5 isolates for kofta samples harboured (2) sea ,(1)sec , (1)sed and (1) sea & sed gene and the other 7 were negative for classic enterotoxin genes, but 16 isolates of sausage samples harboured (2) sea, (1) seb, (1) mixed seb & sec and(1) mixed sea , sec & sec genes and 11 was negative for classic enterotoxin genes (Table, 1 and Photo. 1,2 &3). The samples were negative to SEE, this might be explained by the fact that these isolates either have not harboured see gene or they might have other types of SEs which are family of 21 serological types of heat stable enterotoxins. The obtained results are nearly similar to those reported by Morshdy et al. (2016), Ibrahim (2016) and Alshimaa and Nahed (2017) who detected staphylococcal enterotoxins by multiplex PCR technique.

Staphylococcal enterotoxin A was mostly found in meat product samples as shown in Table, 2 and Photo. 1,2 &3, and this result is dangerous, because it is considered to be the most frequently occurring staphylococcal enterotoxin responsible for causing staphylococcal enterotoxicosis. As SEA is highly resistant to proteolytic enzymes, also it was recovered from 77.8% of all food poisoning outbreaks in the United States

followed by SED (37.5%) and SEB (10%). Serious pathogens and to obtain final products with a maximum limit of safety.

5. Conclusion

This study concluded that S. aureus producing enterotoxins was isolated from beef products samples in street vended meat products sandwiches sold on the street of Menoufia governorate. Multiplex PCR could be used to detect classical types of SEs in different prepared foods. All precautions during manufacture, handling and storage of meat products should be adopted to control these serious pathogens and to obtain final products with a maximum limit of safety.

6. REFERENCES

Alshimaa, A. H.and Nahed, M. A. (2017): *Staphylococcus aureus* and their enterotoxin genes in fast food and food handlers from different food premises in Sohag City, Egypt. Int. J. Agric.Sc & Vet.Med 5(1) :2320-3730.

Ibrahim, Shimaa M. A. (2016): Detection of *Staphylococcus aureus* classic enterotoxin genes in some meat products using multiplex PCR. Ph.D Thesis (Meat Hygiene), Fac. Vet. Med., Kafrelsheikh Univ.

International commission of Microbiological Specification for Foods "ICMSF" (1996): Microorganisms in Food. I-Their Significance and methods of enumeration. 3rd Ed. Univ. of Toronto, Canada.

Mehrotra, M.; Wang, G. and Johnson, W. (2000): Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1, and methicillin resistance. J. Clin. Microbiol., 38:1032–1035.

Morshdy, A M.A.; El-Atabany,A.I.; Tharwat ,A.E.and Rezk,M.A. (2016) Toxogenic *Staphylococcus aureus* in ready to eat foods in Sharkia. 3rd International Food Safety Conference, Damanhur University.

Rall, V.; Vieira, F.; Rall, R.; Vieitis, R.; Fernandes, A.; Candeias, J.; Cardoso,K. and Araujo, J. (2008): PCR detection of staphylococcal enterotoxin genes in *Staphylococcus aureus* strains isolated from raw and pasteurized milk. Vet. Microbiol. 132: 408-413.

Rosengren, A.; Lindblad, M. and Lindqvist, R.; (2013): The effect of undissociated lactic acid on S. aureus growth and enterotoxin A production .Int. J. Food Microbiology.162: 159-166.

Shah, D., Shringi, S., Besser, T. and Call, D. (2009): Molecular detection of foodborne pathogens, Boca Raton: CRC Press, In Liu, D. (Ed). Taylor & Francis group, Florida, USA, Pp. 369-389.