Detection of virulence genes of enterotoxigenic *Staphylococcus aureus* isolated from chicken meat cuts by using PCR

Fatin S. Hassanen[1], Fahem A. Shaltout[1], Amani, M. S.[1], Maarouf, A. A. A.[2], Rasha, N. A[2]

[1]Food hygiene Dept., Faculty of Veterinary Medicine, Benha University. [2]Animal Health Research Institute, Benha Branch – Food Hygiene Dept.

A B S T R A C T

The present study was performed on 120 random samples of fresh chicken meat cuts of Drumstick, thigh and breast (40 of each) which were purchased from different supermarkets and retail chicken butchers at Kaliobia Governorate, for detection of virulence genes responsible for *Staphylococcus. aureus* enterotoxins production by using PCR.A total of 33 examined samples were positive for *S.aureus* counts represented as 13(32.5%) from drumstick samples; 11(27.5%) from thigh samples and 9(22.5%) from breast samples. The results revealed that 8 strains out of 10 random examined strains by SET- RPLA test were enterotoxigenic and classified according to type of toxin into (3A;1C,2D and 2A&C).Moreover, PCR results cleared that*; sea* gene was detected in 4 studied strains; *seb* gene in one strain and *sec*; *sed* and *see* genes were detected in 3 strains out of 10 studied strains.

Keywords: Chicken meat, enterotoxigenic *S.aureus*, virulence genes

(http://www.bvmj.bu.edu.eg) (BVMJ-33(2): 410-417, DECEMBER, 2017)

1. INTRODUCTION

Chicken meat is a nutritious, healthy food that is low in fat and cholesterol compared to other meats but an excellent source of protein. On the other hand, it is an ideal medium for bacterial growth because of high moisture content, richness in nitrogenous compounds (essential amino acids, proteins), good source of minerals, vitamins and other growth factors (Prange *et al*., 2005).

Chicken meat is a common vehicle of Staphylococci mainly *Staph. aureus* that considered as one of the most important causes of foodborne outbreaks in people (Losito et al., 2005).

Staphylococcus aureus is the most pathogenic species that is considered the third-most important cause of food-borne disease in the world (Liu et al., 2006 and Normanno et al., 2007) and has two aggravating characteristics, toxin production and antimicrobial resistance *S. aureus* produce disease when the bacteria contaminate food, produce some enzymes which are implicated with staphylococcus invasiveness and many extracellular substances some of which are heat stable enterotoxins that renders the food dangerous even though it appears normal and extensive cooking can killed the bacteria but the toxins may not be destroyed because most of them are gene based i.e. they can be carried on the plasmid (Prescott et al., 2005). *S. aureus* produces a wide variety of toxins including Staphylococcal enterotoxins (SEs; SEA to SEE, SEG to SEI, SER to SET) with demonstrated emetic activity, and Staphylococcal-like (SEl) proteins, which are not emetic in a primate model (SElL and SElQ) or have yet to be tested (SElJ, SElK, SElM to SElP, SElU, SElU2 and SElV). SEs and SEls have been traditionally subdivided into classical

(SEA to SEE) and new (SEG to SElU2) types. The enterotoxin genes are accessory genetic elements in *S. aureus*, meaning that not all strains of this organism are enterotoxin producing (María et al., 2010).
The Staphylococcal enterotoxins (SEs) are responsible for the symptoms that associated with Staphylococcal food poisoning (Lieweiyn and Cohen, 2002). The disease is characterized by rapid onset symptoms including nausea, violent vomiting, abdominal cramps and diarrhea lasting from 24 to 48 h and the complete recovery usually occurs within 1-3 days. The illness is usually self-limiting and only occasionally it is severe enough to warrant hospitalization. Moreover, *sea* is the most common enterotoxin recovered from food poisoning outbreaks (Pinchuk et al., 2010; María et al., 2010 and Shijia et al., 2016).
RPLA (Reverse Passive Latex Agglutination) can identify enterotoxins using specific antibodies for each of the enterotoxins, but cross reaction between SEA and SEE have been reported (Sergeev et al., 2004).
Several reports have described the development of polymerase chain reaction technique (PCR) protocols for the detection of SEs genes as it is not require bacterial enrichment before detection of a specific gene (Mehrotra et al., 2000 and Sharma et al., 2000). So, the present study was performed for studying virulence genes enterotoxin production of *S. aureus* strains isolated from raw chicken meat cuts samples**.**

2. MATERIAL AND METHODS

2.1. Samples collection

A grand total of 120 random samples (about 250 g for each) of fresh chicken meat cuts of Drumstick, thigh and breast (40 of each) were purchased from different supermarkets and retail chicken butchers at Kaliobia governorate. Each sample was kept in a separate sterile bag and transferred directly to lab without undue delay for bacteriological examination.

2.2. Bacteriological examination

1. Preparation of samples (APHA, 2001)
Twenty five grams of the sample were taken under aseptic condition to sterile Stomacher bag then 225 ml sterile 0.1% peptone water were added. The contents were homogenized at Stomacher (MA 106402 France, 450 to 640 strokes per minute) for 2 minutes and the mixture was allowed to stand for 5 minutes at room temperature. The contents were transferred into sterile flask and thoroughly mixed by shaking and 1 ml was transferred into separate tube each containing 9 ml sterile 0.1% peptone water, from which tenth-fold serial dilutions were prepared. The prepared samples were subjected to the following bacteriological examination:
2. Isolation and identification of suspected S. aureus (Quinn et al., 2002)
3. Detection of enterotoxins producing isolates by SET- RPLA technique (Igarashi et al., 1986).
4. Genotyping of enterotoxins virulence genes by using PCR (Sambrook et al., 1989).

3. RESULTS

The results obtained in Table (2) revealed that, 33 isolates of coagulase positive *S.aureus* were isolated from examined fresh chicken meat cuts (drumstick, thigh and breast samples represented as 13(32.5%) from drumstick samples; 11(27.5%) from thigh samples and 9(22.5%) from breast samples. Moreover, 87samples out of 120 ones were accepted, as they were free from coagulase Positive *S.aureus* isolates according to EOS (2005).
As shown in results of SET- RPLA test in table (3) 8 strains of 10 random examined strains (80.0%) were enterotoxigenic and classified according to type of toxin into 3A; 1C, 2D and 2A&C.
The PCR results showed that, *sea* was detected in 4 studied strains (40.0%); *seb* gene in one strain (10.0%) and *sec*; *sed* and *see* genes were detected in 3 strains (30.0% for each) as shown in Table (4). Regarding to enterotoxins A Fig., 1

showed that, the *sea* gene was amplified in 4 of studied *S.aureus* strains (40.0%) at 102 bp.

The *seb* gene was amplified in one of studied *S.aureus* strains (10.0%) at 164 bp (Fig. 2). Meanwhile, the *sec* gene was amplified in 3 of studied *S.aureus* strains (30.0%) at 451 bp (Fig. 3).In addition, the *sed*gene was amplified in 3 of studied *S.aureus* strains (30.0%) at 278 bp (Fig. 4). Moreover, the results of PCR for amplification of *see* gene in *S.aureus* isolates (Fig. 5) showed that, *see* gene was amplified in 3 of studied *S.aureus* strains (30.0%) at 209 bp.

Table (1): Oligonucleotide primers sequence, target genes, amplicons sizes and cycling conditions

Gene	Primer sequence (5'-3')	amplified segment (bp)	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension	Reference
Sea	GGTTATCAATGTGCGGGTGG CGGCACTTTTTTCTCTTCGG	102 bp	94°C 5 min.	94°C 30 sec.	50°C 30 sec.	72°C 30 sec.	35	72°C 7 min.	Mehrotra et al., 2000
Seb	GTATGGTGGTGTAACTGAGC CCAAATAGTGACGAGTTAGG	164 bp	94°C 5 min.	94°C 30 sec.	50°C 30 sec.	72°C 30 sec.	35	72°C 7 min.	
Sec	AGATGAAGTAGTTGATGTGTATGG CACACTTTTAGAATCAACCG	451 bp	94°C 5 min.	94°C 30 sec.	50°C 40 sec.	72°C 40 sec.	35	72°C 7 min.	
Sed	CCAATAATAGGAGAAAATAAAAG ATTGGTATTTTTTTTCGTTC	278 bp	94°C 5 min.	94°C 30 sec.	50°C 30 sec.	72°C 30 sec.	35	72°C 7 min.	
See	AGGTTTTTTCACAGGTCATCC CTTTTTTTTCTTCGGTCAATC	209 bp	94°C 5 min.	94°C 30 sec.	50°C 30 sec.	72°C 30 sec.	35	72°C 7 min.	

Table (2): Incidence of Coagulase Positive *S. aureus* in examined samples of fresh chicken meat cuts (n=40 for each sample)

Samples	No.	Positive		No. of accepted samples	No. of non-accepted samples
		No.	%*		
Drumstick	40	13	32.5	27	13
Thigh	40	11	27.5	29	11
Breast	40	9	22.5	31	9
Total	120	33	27.5	87	33

* Percentage in relation to total number of sample in each row.

Table (3): The incidence of enterotoxins production from isolated *S.aureus* using SET-RPLA test

No. of *S.aureus*	Enterotoxigenic strains		Type of enterotoxin			
	NO.	%	A	C	D	A&C
10	8	80.0	3	1	2	2

Table (4): The results of PCR amplifications of different used genes of *S.aureus*

S.aureus strains	Virulence genes					
	Sea	*Seb*	*Sec*	*Sed*		*See*
1	+	-	+	-		-
2	-	-	-	-	+	
3	-	-	-	-		-
4	+	-	+	-		-
5	-	-	-	+	-	
6	-	-	-	-		+
7	-	-	-	-		-
8	-	-	+	+		-
9	+	+	-	-	+	
10	+	-	-	+		-
Total No.	4	1	3	3		3
%	40	10	30	30		30

sea (enterotoxin A)*seb*(enterotoxin B), *sec* (enterotoxin C)*sed*(enterotoxin D), *see* (*enterotoxin E).*

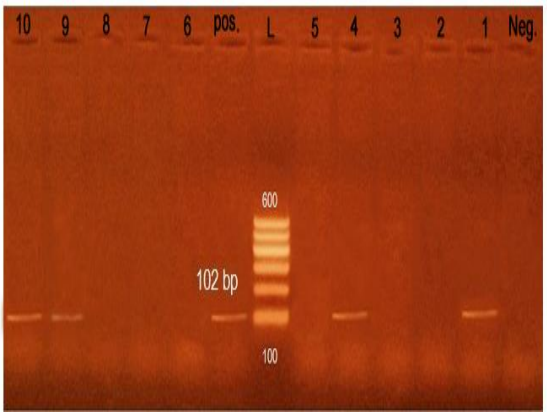

Fig (1): Agarose gel electrophoresis of enterotoxin A (*sea*) gene. Lane L: 100-600 bp DNA Ladder. Negative control (Listeria reference strain, NCINB 50007). Pos.: Positive control (*S.aureus* referencestrain at 102 bp). Lane 1; 4; 9&10: *S.aureus* (Positive)at 102bp. Lane 1-8 and 10:*S.aureus* (Negative)

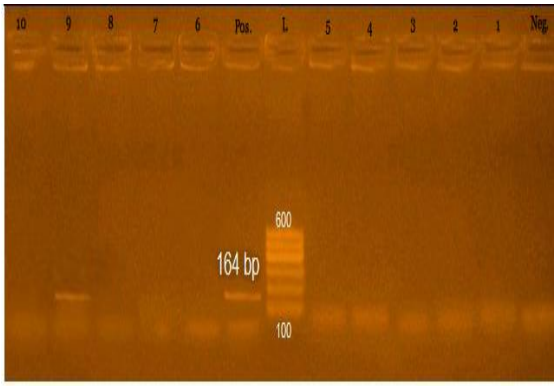

Fig (2): Agarose gel electrophoresis of enterotoxin A (*seb*) gene. Lane L: 100-600 bp DNA Ladder. Negative control (Listeria reference strain, NCINB 50007). Pos.: Positive control (*S.aureus* referencestrain at 164 bp). Lane 9: *S.aureus* (Positive) at 102bp. Lane 1-8 and 10:*S.aureus* (Negative)

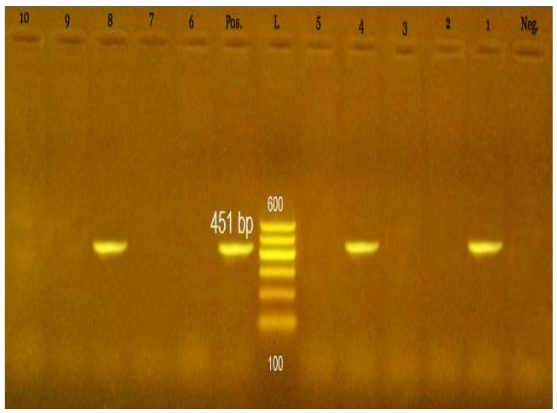

Fig (3): Agarose gel electrophoresis of enterotoxin A (*sec*) gene. Lane L: 100-600 bp DNA Ladder. Negative control (Listeria reference strain, NCINB 50007). Pos.: Positive control (*S.aureus* referencestrain at 451 bp). Lane 1; 4; 8: *S.aureus* (Positive) at 451bp. Lane 2;3;5;6;7;9 &10:S.aure*us* (Negative)

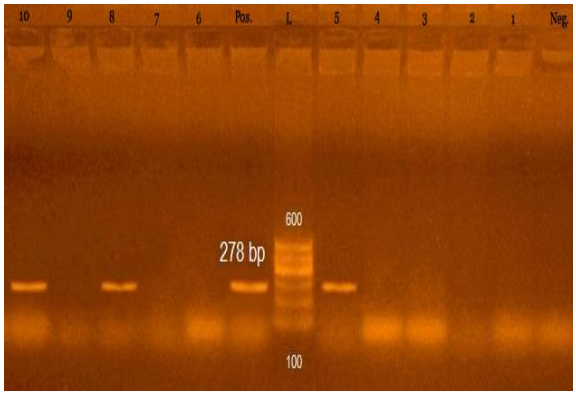

Fig (4): Agarose gel electrophoresis of enterotoxin A (*sed*) gene. Lane L: 100-600 bp DNA Ladder. Negative control (Listeria reference strain, NCINB 50007). Pos.: Positive control (*S.aureus* reference strain at 278 bp). Lane 5; 8 &10: S.aureus (Positive) at 278bp. Lane 1;2;3;4;6;7 &9:S.aureus (Negative)

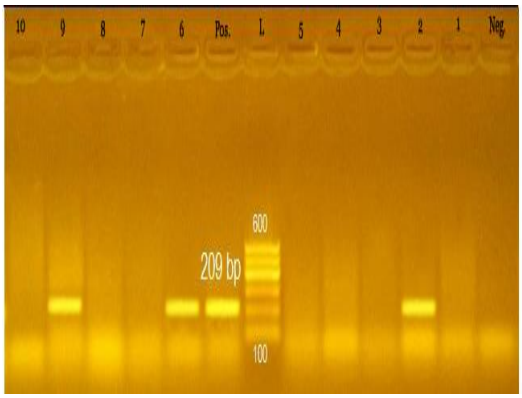

Fig (5): Agarose gel electrophoresis of enterotoxin A (*see*) gene. Lane L: 100-600 bp DNA Ladder. Negative control (Listeria reference strain, NCINB 50007). Pos.: Positive control (*S.aureus* reference strain at 209 bp). Lane 2; 6 &9: S.aureus (Positive) at 209bp. Lane 1;3;4;5;7;8 &10:S.aureus (Negative)

4. DISCUSSION

Chicken meat is a common vehicle of food borne illness, Staphylococci mainly *S. aureus* is the most important causes of food poisoning through enterotoxins (SEs) production and the presence of these organisms has relevant public health implications (Prange et al., 2005 and Bhaisare et al., 2014)

Most previous studies were interested with coagulase positive *S.aureus* strains of Staphylococci species as it is still a major cause of food poisoning due to ingestion of enterotoxin (Le Loir, 2003) and the ability to produce such enterotoxin in food is more likely when competing microorganisms were absent, resulting in symptoms of intoxication and common symptoms appear approximately 3-8 hr. after ingestion as nausea, vomiting, abdominal cramps and diarrhea. Generally, symptoms are short in duration, ‘‘approximately 24-48hrs (Sandle and Mckillip, 2004).

The results obtained in table (2) came in accordance with those obtained by Enany et al. (2013) they reported that 25% were positive for *S.aureus* Meanwhile: these results were disagreed with those of Mohamed- Ghada (2010) and Abo-Samra (2013). They cleared that; the incidence of *S.aureus* in fresh chicken meat samples was 26 (52%).

The presence of coagulase positive *S. aureus* in chicken meat and its products indicates poor hygiene of meat handlers as well as lack of sterilization of utensils and they grow without pronounced change in odour or taste in the products and producing heat stable enterotoxins which lead to food poisoning with severe diarrhoea and gastroenteritis among consumers (Le Loir, 2003andBakr*et al.*, 2004).

Staphylococcus auerus is important in relation to poultry meat hygiene because of its ability to produce enterotoxins. These enterotoxins are serologically grouped into four major classical types which are SEA, SEB, SEC and SED detected by SET-RPLA (Zouharova and Rysanek 2008) and Bendahou et al. (2009). This result nearly similar to that recorded by Afifi-Dina (2016) who mentioned that *S.aureus* strains out of 10 randomly ones examined by SET- RPLA test were enterotoxigenic and classified according to type of toxin into 3A;1 B& 1C.

The PCR results as shown in Table (4) regarding to enterotoxins A (Fig., 1) came in harmony with those of Nemati (2014); Abdalrahman et al. (2015) and Afifi-Dina (2016) who stated that enterotoxin A (*sea*) was detected in 2 studied strains only. Meanwhile, these results were disagreed with that of FeBler et al. (2011) who failed to detect *sea* gene in *S.aureus* strains isolated from poultry and poultry products. According to Fig. 2 nearly similar results were obtained by Abdalrahman et al. (2015) and Moustafa et al. (2016) who found *seb* (8.3%). Meanwhile according toFig., 3.nearly similar results were obtained by Chen et al. (2013); Madahi et al. (2014) who found that sec was 12.50% and Nemati (2014). In addition Fig4 nearly similar results were obtained by Pelisser et al. (2009); Madahi et al. (2014) 8.33% *sed* and Moustafa et al. (2016). Moreover, the results of PCR in Fig., 5 were nearly similar to those obtained by Chiang et al. (2006) and Pelisser et al. (2009) who

showed that, out of 102 COPS isolates 4 *see* were positive for *see*

CONCLUSION

The present study proved that the examined fresh chicken meat cuts were contaminated with Enterotoxigenic coagulase Positive *S.aureus* which is considered of serious public health hazard for production of various enterotoxins those resulting in food poisoning intoxication.

5. REFERENCES

Abdalrahman, L. S.; Stanley, A.; Wells, H. and Fakhr, M. K. (2015): "Isolation, virulence, and antimicrobial resistance of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin sensitive *Staphylococcus aureus* (MSSA) Strains from Oklahoma retail poultry meats". Int. J. Environ. Res. Public Health, 12: 6148-6161.

Abo-Samra, R. G. (2013): "*Staphylococcus aureus*, Salmonella species and *Listeriamonocytogenes* in locally fresh and frozen chicken meat in Dammietta city". Animal Health Research J.,1(2):1-10.

Afifi-Dina, H.M. (2016): Characterization of Methicillin-Resistant *Staphylococcus aureus* (MRSA) isolated from food products of poultry origin In Egypt. M.V. Sc. Thesis (Bacteriology, Immunology and Mycology), Fac. Vet. Med., Benha Univ.

APHA "American Public Health Association" (2001): Compendium of Methods for the Microbiological examination of Foods. 4th Ed. F.P. Downes and K. Ito(editors), APHA. Washington D.C., USA.

Baker, M. D. and Acharya, K. R. (2004): Superantigens: structure function relationships. Int. J. Med. Microbiol.,293: 529–537.

Bendahou, A.; Abid, M.; Bouteldoun, N.;Catelejine D. and Labbadi, M. (2009): Enterotoxigenic coagulase positive staphylococcus in milk and milk products, Iben and jben, in northern Morocco. J. Infected Developing Countries, 3 (3): 169-175.

Bhaisare, D.B.; Richard, T.D. and Punniamurthy, C.N. (2014): Bacterial Pathogens in Chicken Meat: Review. International J. Life Sciences Research, 2(3): 1-7.

Chen, H.; He, W.I.; Liu, Y.; Qi, I.; Zhao, C.; Zhang, F.; Li, I.F. and Wang, H. (2013): Food-animal related *S. aureus* multidrug-resistant ST9 strains with toxin genes. Foodborne Pathogen Dis., 10(9):782-788.

Chiang, Y. C.; Chang, I. T.; Lin C. W.; Yang, C. Y. and Tsen, H. Y. (2006): PCR primeres for detection of *staphylococcal* enterotoxins K,L and M; and survey of *staphylococcal* enterotoxins types in *S. aureus* isolates from food poisoning cases in Taiwan. J. Food Port,. 69(5): 1072-1079.

Egyptian Organization For Standardization 'E.O.S, 1090'' (2005): Egyptian Organization For Standardization and quality control. Egyptian Standards for frozen poultry and rabbit.

Enany, M. E.; Abd El Wanis, S. A.; EL-Gammal, A. M. and Abo Eillil, S. A. (2013): "Phenotypic and genotypic characterization of Methicillin resistant *S. aureus* (MRSA) isolated from broiler chickens of traditional poultry slaughter shops in Ismailia province". SCVMJ, XVIII (2): 189-199.

FeBler, A.T.; Kadlec, K.; Hassel, M.; Hauschild, T.; Eidam, C. ; Ehricht, R.; Monecke, S. and Schwarz, S. (2011): Characterization of Methicillin-Resistant *Staphylococcus aureus* Isolates from Food and Food Products of Poultry Origin in Germany. Appl. Environ. Microbiol. , 77 (20): 7151-7157.

Igarashi, H.; Fujikawa, H.; Shingaki, M. and Bergdoll, M. S. (1986): "Latex agglutination test for Staphylococcus

toxic Shock syndrome toxin1" J. Clinic. Microbial., 23: 516-521.

Leloir, Y.; Baron, F. and Gautier, M. (2003): "Review: *Staph. aureus* and food poisoning". J. Genetics and Molecular Research, 2 (1): 63 - 76.

Lieweiyn, M. and Cohen, J. (2002): **"**Superantigens: Microbial agents that corrupt immunity". Lancet Infect Dis **2**: 156-162.

Liu, X.; Chen, Y.; Fan, Y. and Wang, M. (2006): Foodborne diseases occurred in 2003 - report of the national foodborne diseases surveillance system, China. Chinese Journal of Hygiene Research, 35: 201-204.

Losito, P.; Vergara, A.; Muscarieli, T. and lanieri, A. (2005): **"**Antimicrobial susceptibility of environmental *Staphylococcus aureus* strains isolated from a pigeon slaughterhouse in Italy". Poultry Sci., 84(11): 1802-1807.

Madahi, H.; Rostami, F.; Rahimi, E. and Dehkordi, F. S. (2014): "Prevalence of Enterotoxigenic *Staphylococcus aureus* isolated from chicken nugget in Iran". Jundishapur J. Microbiol., 7(8):1-6.

María, Á. A.; María, C. M. and María, R. R. (2010): Food Poisoning and *Staphylococcus aureus* Enterotoxins. Toxins (Basel), 2(7): 1751–1773.

Mehrotra, M.; Wang, G. and Johnson, W. M. (2000): "Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, Exfoliative Toxins, Toxic Shock Syndrome Toxin 1 and Methicillin Resistance". J. Clinical Microbiol., 38(3): 1032-1035.

MohamedGhada, M.; Ebraheem. Lubna, M.; and ThabtManal,H. (2010): Electrophoretic analysis and Immunological characterization of staphylococcus aureus isolated from chicken meat. Assiut Vet. Med. J.,56:(127).

Moustafa, N. Y.; Abd El-Hafiz-Reham,M. and ElBahy-Engy F. (2016): Incidence of *Staphylococcus aureus* and Salmonella in Poultry Meat. Global Veterinaria, 16 (6): 570-578.

Nemati, M. (2014): "Prevalence of Enterotoxin Genes in Poultry *Staphylococcus aureus*Isolates". Bull. Env. Pharmacol. Life Sci., 3(2): 14-18.

Normanno, G.; La Salandra, G.; Dambrosio, A.; Quaglia, N.C. ; Corrente , M.; Parisi, A.; Santagada, G.; Firin, U. A.; Crisetti, E. and Celano, G. V. (2007): "Occurrence, characterization and antimicrobial resistance of enterotoxigenic *Staphylococcus aureus* isolated from meat and dairy products". International J. Food Microbiology, 115: 290-296.

Pelisser,M.R.;Klein,C.S.;Ascoli,K.R.;Zotti, T.R.,andArisil,A.C.M..(2009) :Occurance of *Staphylococcus aureus* and multiplex PCR detection of classical enterotoxin genes in cheese and meat products . Braz. J. Microbial., 40: 145-148 .

Pinchuk, I.V.; Beswick, E.J. and Reyes, V.E. (2010): Staphylococcal Enterotoxin. Toxins (Basel), 2(8): 2177–2197.

Prange, A.; Birzele, B.; Hormes J. and Modrow, H. (2005):Investigation of different human pathogenic and food contaminating bacteria and mould grown on Selenite/ Selenate and Tellurite / Tellurate by-x ray absorption spectroscopy. Food Control, 16: 713-728.

Prescott, M.; Harley, P. and Klein, D. A. (2005): "Microbiology". 6th Ed. McGraw Hill. New York U.S.A.: p. 910.

Quinn, P. J., Markey, B. K., Carter, M. E., Donnelly, W. J. C., Leonard, F. C. and Maguire, D. (2002): "Veterinary microbiology and microbial disease". Iowa State Univ. Press, Blackwell Science Ltd, chapters 26-36: 84-96.

Sambrook, J.; Fritscgh, E.F. and Mentiates. (1989): Molecular coloning. A laboratory manual. Vol !., Cold spring Harbor Laboratotry press, New York.

Sandel, M. K. and Mckillip, J. L. (2004): "Virulence and recovery of Staphylococcus aureus relevant to the food industry using improvements on traditional approaches". J. Food Control, 15:5-10.

Sergeev N.,Volokhov, D., Chizhikov V., and Rasooly A. (2004): Simultaneous analysis of multiple staphylococcal enterotoxin genes by an oligonucleotide microarrary assay. T clinMicrobiol., 42(5): 2134-43.

Sharma,NK.;Rees, C.E and Dodd, C.E. (2000):Development ofasingle-reaction multiplex PCR toxin typing assay for staphylococcus aureus strains. Appl Environ.Microbiol .,66 (4): 1347-53.

Shijia, W. ; Nuo, D. ; Huajie, G.; Liling H.; Hua Y., Wenhui, G. and Zhouping W.(2016): A review of the methods for detection of *Staphylococcus aureus* Enterotoxins. Toxins, 8:176.

Zouharova, M. and Rysanek, D. (2008): Multiplex PGR and RPLA identification of S. aureusenterotoxigenic strains from Bulk Tank milk. Zoonoses Public Health, 55(6): 313-319.