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GENOTYPING OF STAPHYLOCOCCUS AUREUS ISOLATED FROM SOME READY TO EAT CHICKEN PRODUCTS

By

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ABSTRACT

Hundred samples of chicken products (nuggets, shawarma, chicken burger and chicken fillet, Twenty-five samples from each n= 25) were collected aseptically from different restaurant at Giza governorate. They were examined microbiologically for isolation of *S. aureus* using recommended selective media as well as the recommended tests. the mean value of coagulase positive *S.aureus* in nuggets and chicken fillet were $0.4 \times 10^2 \pm 0.001 \times 10^2$and $0.5 \times 10^2 \pm 0.002 \times 10^2$ CFU/g respectively. However, the mean values of *S.aureus* of shawarma and chicken burger were $9 \times 10^2 \pm 0.01 \times 10^2$ and $2.1 \times 10^2 \pm 0.007 \times 10^2$ CFU/g. From the accepted percent of examined ready to eat nuggets, shawarma, chicken burger and chicken fillet were 92, 80, 84 and 88 % respectively. While the rejected percent of examined ready to eat nuggets, shawarma, chicken burger and chicken fillet were 8, 20, 16band 12 % respectively. *S. aureus* isolates were confirmed by PCR. Staphylococcal enterotoxin A was detected in 7% of the total examined samples and produces one or more toxins simultaneously. The identified isolates were screened for their capability of producing some virulence factors namely: hemolysin, licithinase and proteinse enzymes using well agar diffusion assay as zone diameters (mm) on blood agar, egg yolk agar and casein agar plates.

INTRODUCTION

Staphylococcal food poisoning (SFP) is an intoxication that results from the consumption of foods containing sufficient amounts of one (or more) preformed enterotoxin **(Dinges *et al.*,2000 and Le Loir, 2003*)*.** Symptoms of SFP have a rapid onset (2-8 h), and include nausea, violent vomiting, and abdominal cramping, with or without diarrhea **(Tranter, 1990 and Balaban and Rasooly, 2000).** The disease is usually self-limiting and typically resolves within 24 - 48 h after onset. Occasionally it can be severe enough to warrant hospitalization, particularly when infants, elderly or debilitated people are concerned **(Murray, 2005).**

Staphylococcal enterotoxin (SEA) is one of the most important gastroenteritis causing agents. In some areas, more than 50% of food poisoning (FP) is caused by Staphylococcal Enterotoxin A (SEA) (**Di *Giannatale et al.*, 2011)**. The primary habitat of this microorganism is the mucosa of the nasopharynx and the skin of humans and animals **(da Silva *et al.,* 2010).** Despite its pathogenicity, ***S. aureus*** is also harbored in the nares of about 20 to 30% of healthy people, while about 60% of the population harbors the microorganism intermittently **(Normanno *et al.,* 2007).** Although the number of outbreaks reported annually has decreased in the last few decades, ***staphylococcal*** food poisoning is still reported as the third most prevalent cause of foodborne illness worldwide **(Zhang *et al.,* 1998 and Le Loir *et al.,* 2003).** In several countries the foods that most frequently cause this type of food poisoning are poultry, and their products **(Wieneke *et al.,* 1993 and Kitai *et al.,* 2005).** To prevent food poisoning, it is important to determine how much actual contamination with enterotoxigenic *S. aureus* in retail raw chicken meat occurs **(Kitai *et al.,* 2005).** Extracellular proteins of pathogenic bacteria are main contributors of pathogenesis and are indisputably involved in bacterial virulence. These proteins have a range of biological functions ranging from host cell toxicity to more suitable alterations of the host cell for the benefit of the invader **(Wooldridge, 2009)**. The virulence factors of pathogenic bacteria are divided into several groups on the basis of the mechanism of virulence and function. Of the important ones are secretary proteins such as toxins and enzymes **(Wu *et al.*, 2008).** PCR-based techniques are used increasingly in food-microbiology research as they are well developed and when applied as culture confirmation tests, they are reliable, fast and sensitive. PCR methods offer a sensitive and specific detection of pathogens and can discriminate virulent bacteria from avirulent members of the same species as well **(Olsen, 2000)**. In the last 10 years, many authors have proposed the use of PCR for the detection of food-borne pathogens to replace the time-consuming culture-based classical techniques **(Miethke *et al.,* 1992 and Gravet *et al.,* 1999**). They are rapid, easy to handle, sensitive and specific and therefore constitute very valuable tools for routine applications.

Aim of the work:

This study was planned to evaluate the ***Staphylococcus aureus*** in some chicken products (nuggets, shawarma, chicken burger and chicken fillet) as follow:

1- *S.aureus* count, identification and confirmation using PCR

2- Detection of *S. aureus* entero- toxins using SET-RPLA and PCR.

MATERIAL AND METHODS

Collection of samples:

A total of hundred samples of chicken products (nuggets, shawarma, chicken burger and chicken fillet, Twenty-five samples from each n= 25) were collected aseptically from different restaurants in Giza governorate, placed in sterilized containers, and stored in a cool place to transfer to the laboratory.

1. *S.aureus* count, isolation, identification and confirmation:

According to **FDA (2001):**

1.1. Preparation of sample homogenate:

Twenty-five grams of samples were homogenized using a meat grinder under aseptic condition and it was added to 225 mL of sterile Buffered Peptone Water then, dilutions 10^2 and 10^3 were obtained. Homogenates were incubated at 37°Cfor 24 hours in order to culture the organisms in microbiological examination.

1.2. *S. aureus* count:

One mL from each sterile dilution was plated onto Baird-Parker agar supplemented with egg yolk tellurite emulsion (as 0.3, 0.3 and 0, 4 ml) and incubated at 37°C for 24 to 48 hours. Presumptive colonies showing characteristic phenotype of *S. aureus* (i.e., circular, black, convex and with or without light halo on BP agar) count number of colonies on triplicate plates represented by colonies giving positive coagulase test and multiply by the sample dilution factor. Report this number as number of S. aureus (CFU)/g of tested food.

1.3. Confirmation of *S. aureus* by coagulase test:

Test of coagulase was performed (***S. aureus*** coagulase +ve) using kits **(Oxoid, 1990),** and then further identification was made by using biochemical tests. According to FDA 2001.

1.4. Confirmation by PCR:

Targeting the *S. aureus* specific nuc gene (*S.aureus* species specific) for positive samples only.

2. Detection of Staphylococcal enterotoxins:

Using SET-RPLA (reversed passive latex agglutination) test which may be used to detect staphylococcal enterotoxins in a wide variety of foods and to give a semi-quantitative result. The test may also be used to demonstrate enterotoxin production in isolates of S. aureus grown in culture. The SET-RPLA test kit is based upon the reports by **Oda *et al.* (1979) and Shingaki *et al.* (1981).**

3. **Determination of virulence factors:**

Hemolysis(Beta), lecithinase and proteinase (Casein) producing enzymes were determined using agar well diffusion assay according to **Reinheimer *et al.*(1990) and Misra and Kuila (1992).**Wells in blood agar, egg yolk agar and casein agar plates were filled with 40ul aliquots of filter-sterilized (0.45µm pore size) bacterial cultures filtrates. Plates were incubated at 37°C C for 24 hours.

3.1. Molecular Biology Studies:

3.1.1. Determination of cellular and extracellular protein patterns using SDS-PAGE technique according to laemmli (1970) and LKB Application note (1977).

The examined bacterial strains were grown in 50ml Tryptone Soya Broth (TSB) at 30°C for 24 hours. Bacterial cells were harvested by centrifugation and the filtrates were separated for further extraction of extracellular proteins. Bacterial pellets were washed twice using sterile bi-distilled water. The bacterial pellets were sonicated, re-suspended in sterile distilled water and centrifuged. The precipitated cellular proteins were then separated and re-suspended in phosphate buffer pH 7. The supernatants were separated by centrifugation and concentrated100 times using 70% saturated $(NH_4)_2$. SO_4.The precipitated extracellular proteins were re-suspended in phosphate buffer pH 7. 100µl of each cellular and extracellular protein preparations were mixed with 50µl of treatment buffer separately and boiled in a water bath for 5 minutes then injected into the wells of the prepared polyacrylamide gel. The molecular weights of separated proteins were determined by electrophoresis compared with marker proteins having molecular weights ranging between 14 to 116 kDa after staining with commasie blue. The molecular weights of separated proteins were determined by electrophoresis compared with marker proteins having molecular weights ranging between 14 to 116 kDa after staining with commasie blue.

3.1.2. DNA extraction for multiplex- PCR:

DNA templates of the tested bacterial cultures were prepared from 4-hours cultures grown in TSB at 30ºC separately according to the method described by **Ngamwongsatit *et al.* (2008).**

3.1.3. Specific multiplex PCR amplification conditions for Staphylococcus aureus enterotoxins Genes sea, seb, sec, sed and see **(according to Pinto *et al.* 2005).**

PCR amplifications were conducted in a solution containing 1XPCR buffer (10mM Tris–HCl, pH 8.8; 1.5mM MgCl2,50 mM KCl,0.1% Triton X-100), 100 mM of each dNTP,1 mM of each primer and 0.5U of thermostable DNA polymerase and 5 µl of DNA template, in a final

volume of 50µl. Amplification conditions were: 5 min at 94ºC, 35 cycles of 30s at 94ºC, 45 s at the corresponding annealing temperature and 45 s at 72ºC and a final extension of 10 min at 72ºC. PCR products (15 microliter) were electrophoresed through 2% agarose gel in TAE buffer (40mM Tris - acetate, pH 8.0; 1mM Na2EDTA). Amplicons sizes were estimated using 100bp DNA ladder (Amersham, USA) run on the same gel.

The oligonucleotides used in this work, their sequences, target positions and size of amplification fragments are summarized in the following tabulation according to **(Pinto *et al.,* 2005)**.

Oligonucleotides used for multiplex PCR amplification of *S. aureus*enterotoxins genes.

Target gene	Primer	Size in bp	Primer sequence(5'-3')	T ºC	Location Within genes	Poductsizein bp
sea	**SEA-3**	**21**	**CCTTTGGAAACGGTTAAAACG**	**60**	**487–507**	**127**
	SEA-4	**22**	**TCTGAACCTTCCCATCAAAAAC**	**62**	**592–613**	
seb	**SEB-1**	**20**	**TCGCATCAAACTGACAAACG**	**58**	**634 - 653**	**478**
	SEB-2	**20**	**GCAGGTACTCTATAAGTGCC**	**60**	**1088 - 1110**	
sec	**SEC-5**	**21**	**GAACTAGACATAAAAGCTAGG**	**58**	**670 - 690**	**244**
	SEC-6	**20**	**CATTCTTTGTTGTAAGGTGG**	**56**	**913 -894**	
sed	**SED-1**	**20**	**CTAGTTTGGTAATATCTCCT**	**54**	**354 -373**	**317**
	SED-2	**20**	**TAATGCTATATCTTATAGGG**	**52**	**652 - 671**	
see	**SEE-1**	**20**	**TAGATAAAGTTAAAAAACAAGC**	**54**	**491-510**	**170**
	SEE-2	**20**	**TAACTTACCGTGGACCCTTC**	**60**	**640 - 659**	

Reactions were carried out with the following cycling conditions: initial denaturation at 95ºC for 5 min, followed by30 cycles of 94ºC for 45 sec, annealing at 54&56ºC for 1 min in case of *hbl*C and at 58ºC in case of *cyt*K, elongation at 72ºC for 2 min and final extension at72ºC for 5 min. Amplicons were separated on 1.5% agarose gel and sizes were estimated using 100bp DNA ladder (Amersham, USA) run on the same gel. Primers used in this study were synthesized by Metabion International AG, Lena-Christ-Strasse 44/I, Deutschland.

RESULTS AND DISCUSSION

Contaminated food is a real threat to human welfare. Food-borne diseases are mainly caused by pathogenic bacteria which are either transmitted to humans from the animal reservoir or which contaminate the food process line. *S. aureus* currently attracted increasing attention due to its capability of producing a range of enterotoxins and tissue degrading enzymes **(Lund and Granum, 1997, Klotz *et al.,* 2003, Do Carmo *et al.,* 2004 and Schoeni and Wong, 2005)**. From the results obtained in (Table 1) it is showed that, the mean value of coagulase positive *Staphylococcus aureus* of nuggets and chicken fillet were $0.4 \times 10^2 \pm 0.001 \times 10^2$ and $0.5 \times 10^2 \pm 0.002 \times 10^2$ CFU/g respectively which were exceeded the permissible limit recommended by **ES 3493 (2005)**. Nearly similar results obtained by **Margaret *et al.* (2009).** However, the mean values of ***S.aureus*** of shawarma and chicken burger were $9 \times 10^2 \pm 0.01 \times 10^2$ and $2.1 \times 10^2 \pm 0.007 \times 10^2$CFU/g which were exceeded the permissible limit recommended by **ES 3493(2005)**. The prevalence of *S. aureus* in many poultry products indicating that consumers are at potential risk of *S. aureus* colonization and subsequent infection. Presence of pathogens in food products imposes potential hazard for consumers and causes grave economic loss and loss in human productivity via food-borne disease. Symptoms of SFD include nausea, vomiting, and abdominal cramps with or without diarrhea. Preventive measures include safe food handling and processing practice, maintaining cold chain, adequate cleaning and disinfection of equipment, prevention of cross-contamination in home and kitchen, and prevention of contamination from farm to fork should be applied. **(Kadariya *et al.,* 2014).** From the results obtained in (Table 2) it is mentioned that, the accepted percent of examined ready to eat nuggets, shawarma, chicken burger and chicken fillet were 92, 80, 84 and 88 % respectively. While the rejected percent of examined ready to eat nuggets, shawarma, chicken burger and chicken fillet were 8, 20, 16 and 12 % respectively. So, the chicken nuggets samples are the least contaminated (Table 3) detecting staphylococcal enterotoxin types using SET-RPLA (reversed passive latex agglutination) test. Enterotoxin A was detected in a high number of chick products (seven samples) in comparison with other types of enterotoxins. Enterotoxin E and C was detected in three and two chicken product samples respectively, while each Enterotoxin B and D was detected in one chicken product sample. The total percent of enterotoxin A was detected in 7% from total examined chicken products. However enterotoxins types B, C, D and E were detected as 1, 2, 1 and 3 % from total examined chicken products respectively, as mentioned in (Table 4) and Fig. (1).

The results in (Table 5**)** it was clear that, the *S.aureus* produces one or more toxins simultaneously. Classically, SEs have been divided into five major serological types **(SEA, SEB, SEC, SED, and SEE**) on the basis of their antigenic properties **(Su and Lee Wong, 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 **(Bergdoll, 1989)** Staphylococcal food poisoning (SFP), a form of enteritis, is intoxication rather than a disease resulting from ingestion of food contaminated with preformed staphylococcal enterotoxins **(Bergdoll *et al.* 1974).** Symptoms of SFP usually occur within 1- 6 hrs. After the food intake, are characterized by nausea, vomiting, abdominal cramps and diarrhea. These symptoms usually subside in 1-3 days but the patient remains sick for 7-10 days due to the result of toxic shock **(Jett *et al.,* 1994 and Do Carmo *et al.,* 2004).** Regarding the enterotoxin genotype, previous studies on *S. aureus* proved that enterotoxin PCR determinations are in a high agreement (97-100 %) with the toxin production as defined by Immunoassays **(McLauchlin *et al.,* 2000, Fueyo *et al.,* 2001and Letertre *et al.,* 2003).** (Table 5) cleared that, the Enterotoxin genotyping of tested strains revealed presence of ***sed*** gene in both strains S.aureus (S1 and S3) and ***sea*** gene in strain **(***S1*) *S. aureus* strains were chosen as the most potent strains concerning their virulence factors. The identified isolates were screened for their capability of producing some virulence factors namely: hemolysin, licithinase and protease enzymes using well agar diffusion assay as zone diameters (mm) on blood agar, egg yolk agar and casein agar plates. **Pinto *et al.* (2005)** found a total of 40 (30%) *S. aureus* food isolates positive for ***SEe*** genes. Among that, the ***SEc*** genotype was the most frequent (22 strains, 20 % of total *SE* positive strains) and ***SEa*** the second more frequent (14 strains, 13%), which is in accordance with the results obtained by **(Fueyo *et al*., 2001)** which mentioned that in Spain, *S. aureus* isolates from nasal carriers and handled foods contained ***SEs*** (21.2%), TSST-1 (3.7%) and combinations of these toxins (3.7%). Data on enterotoxin genotype confirmed the grouping of strains nuc PCR positive and ***se*** PCR positive in *S. aureus* clusters. (Table 6) cleared that, the differences in toxin types depend upon the origins of staphylococcal food poisoning which differed widely among countries. This may be due to differences in the consumption and food habits in each of the countries. The ***SEa*** gene is present in higher percent (7%) than other enterotoxin genes from chicken meat products. The lowest percent is 1% of genes ***SEb*** and ***SEd*** genes

respectively which isolated from chicken products. The differences in the distributions of enterotoxin types in the populations strengthen the probability that some pathogenic *S.aureus* strains may returned to the different environmental, types of examined samples and geographical distributions. Enterotoxin genotyping of tested strains revealed presence of ***SEd*** gene in both strains S. aureus (S1 and S3) and ***SEa*** gene in strain *S1* only. Data on enterotoxin genotype confirmed the grouping of strains nuc PCR positive and ***se*** PCR positive in *S. aureus* clusters. On the basis of these results, we suggest amplification of enterotoxin genes as target genes using multiplex PCR test as a rapid and valuable technique that can be applied directly to single colonies growing on selective plates for a rapid, accurate and unequivocal identification of S. aureus. It could be implemented as an alternative to phenotypic and immunology-based tests in the routine food microbiological analysis **(Klotz *et.al,* 2003).**

Table (1): *Staphylococcus aureus* count (CFU/g) of examined ready to eat chicken products (nuggets, shawarma, chicken burger and chicken fillet), n=25 samples of each product.

Count (CFU/g) / Samples	Minimum	Maximum	Mean	±SE
nuggets	<10	4×10^2	0.4×10^2	$\pm 0.001 \times 10^2$
shawarma	<10	6×10^3	9×10^2	$\pm 0.01 \times 10^2$
chicken burger	<10	3×10^3	2.1×10^2	$\pm 0.007 \times 10^2$
chicken fillet	<10	3×10^2	0.5×10^2	$\pm 0.002 \times 10^2$

N.B. <10 means negative plates.

Table (2): Percentage of accepted and rejected examined ready to eat chicken products (nuggets, shawarma, chicken burger and chicken fillet) Due to *Staphylococcus aureus* count (CFU/g) :(total samples 100, 25 samples from each product).

Samples	Accepted		Rejected	
	No	%	No	%
nuggets	23	92	2	8
shawarma	20	80	5	20
chicken burger	21	84	4	16
chicken fillet	22	88	3	12

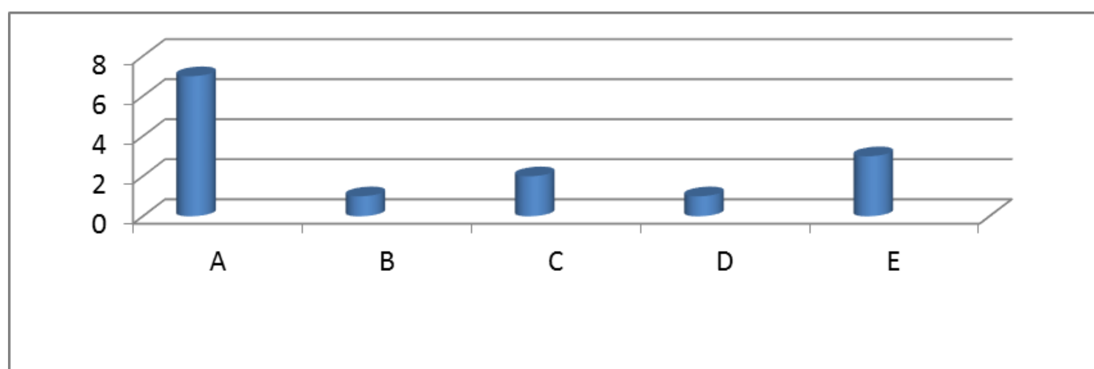
Table(3): Staphylococcal enterotoxins using SET-RPLA (reversed passive latex agglutination) test.

Samples	No. of examined samples	No. of S. aureus +ve Samples	Types of Enterotoxin strains				
			A	B	C	D	E
Nuggets	25	2	1	-	-	-	1
shawarma	25	5	3	-	1	-	1
Chicken burger	25	4	2	1	-	1	-
chicken fillet	25	3	1		1		1
Total	100	14	7	1	2	1	3

Table (4): Percent of enterotoxin types in chicken products using SET-RPLA (reversed passive latex agglutination) test.

Enterotoxin type									
A		B		C		D		E	
No	%	No	%	No	%	No	%	No	%
7	7	1	1	2	2	1	1	3	3

Percent



Type of enterotoxin

Fig. (1): Percent of enterotoxin types in chicken products positive samples (n=100):

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Table (5): Test for virulence factors of toxic bacterial isolates.

Code	Identification	Zone in (mm)		
		Lecithinase	Haemolysin (Beta)	Proteinase (Casein)
S	*S. aureus*	30	33	28
S1	*S. aureus*	21	30	23
S3	*S. aureus*	34	23	24
S3	*S. aureus*	31	28	31

Table (6): Enterotoxin genes types by coagulase positive staphylococcus aureus isolates in examined chicken product samples.

Types of samples	N0. Of isolates	Sea		Seb		Sec		Sed		See	
		No	%	No	%	No	%	No	%	No	%
Nuggets	2	1	1	-	-	-	-	-	-	1	1
shawarma	5	3	3	-	-	1	1	-	-	1	1
Chicken burger	4	2	2	1	1	-	-	1	1	-	-
chicken fillet	3	1	1	-	-	1	1	-	-	1	1
Total	14	7	7	1	1	2	2	1	1	3	3

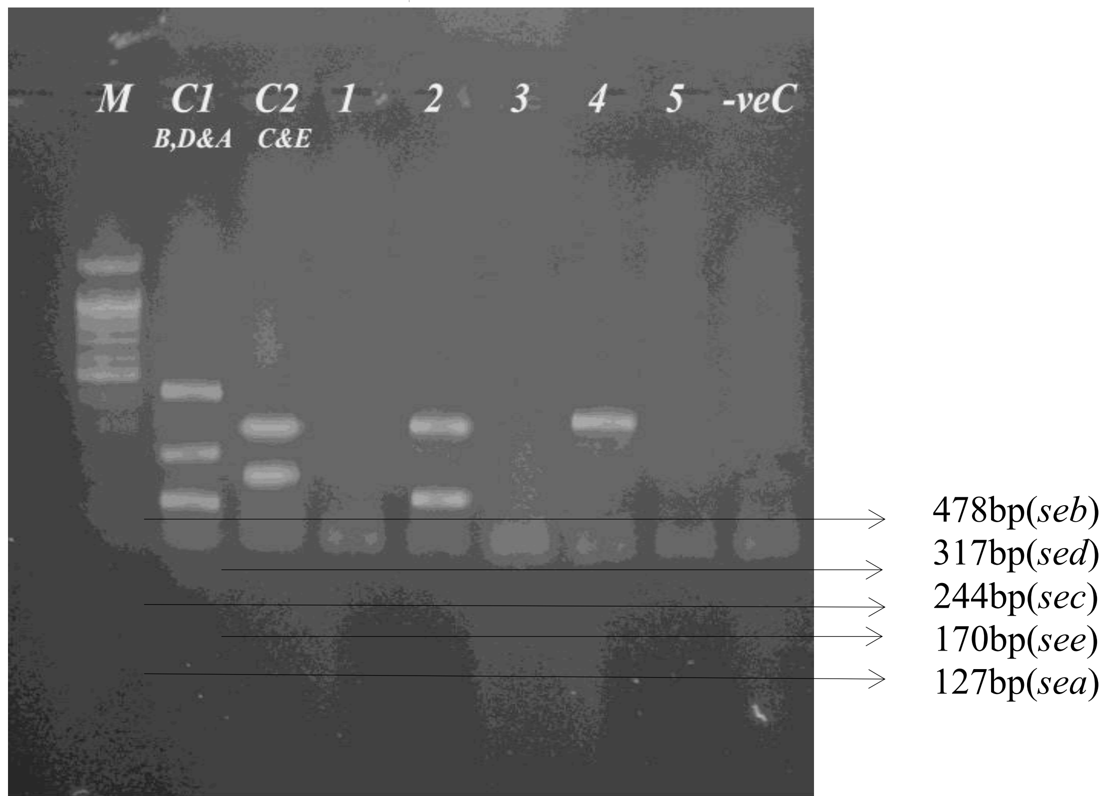

Fig. (2): Agarose gel showing the PCR amplicons resulting from amplification of enterotoxins Genes *Sea, seb, sec, sed and see* using their specific primers. Lane M is 100bp DNA ladder marker; lanes 1, 2, 3and4 DNA amplicons of S. aureus S, S1, S2&S3 respectively. The gel reveals presence of enterotoxins.

Conclusion and recommendations:

Proper storage of food is an important part of reducing the risk of food poisoning. Foods must be stored in the refrigerator and eaten within a short period of time. The microbes on our food that can cause poisoning are usually temperature controlled by heating (cooking) and/or chilling (refrigerating) the food. While reusing the refrigerated food, it should be heated it properly if need to be restored or eaten after sometime. The quantity of bacteria also rises due to bacterial multiplication. Though bacteria are limited to the foods but given the chance to grow they can easily spread around the kitchen - via our hands, chopping boards, cloths, knives and other utensils and even through fomites also. They may cross-contaminate other foods - especially cooked and ready-to-eat foods. Good kitchen and personal hygiene practices as well cooked foods properly are important to help control the consumption of contaminated foods and hence preventing food poisoning. Considerable research effort is still

required for better understanding of the interactions between *S. aureus* and the food matrix and of the mechanisms of SE production in foodstuffs. These studies should lead to better control and a subsequent reduction of staphylococcal food poisoning outbreaks. On the basis of PCR results, we suggest amplification of enterotoxin genes as target genes using multiplex PCR test as a rapid and valuable technique that can be applied directly to for a rapid, accurate and unequivocal identification of *S. aureus***.** It could be implemented as an alternative to phenotypic and immunology-based tests in the routine food microbiological analysis.

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التصنيف الجينى للميكروب المكور العنقودي الذهبى المعزول من بعض منتجات الدواجن المعدة للأستهلاك

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الملخص العربى

تم جمع مائة عينة من منتجات الدجاج (ناجتس،الشاورما،برغرالدجاج ودجاج فيليه بواقع خمسة وعشرون عينة من كل نوع) من مطاعم مختلفة في محافظة الجيزة. تم فحص هذه العينات ميكروبيولوجيا لعزل الميكروب المكور العنقودي الذهبي باستخدام الطرق المرجعيه. وكان متوسط عدالميكروب المكور العنقودي الذهبي الايجابي لتجمع البلازما في عينات الناجتس ودجاج فيليه $0.4 \times 10^{2} \pm 0.001 \times 10$ & $0.5 \times 10^{2} \pm 0.002 \times 10^{2}$ خلية / جم من العينه على التوالي والتي كانت في الحدود المسموح بها طبقا للمواصفه القياسيه المصريه رقم 3493 لسنة 2005 وكان متوسط عد الميكروب المكور العنقودي الذهبي الايجابي لتجمع البلازما في عينات الشاورما وبرغر الدجاج $9 \times 10^{2} \pm 0.01 \times 10^{2}$ و $2.1 \times 10^{2} \pm 0.007 \times 10^{2}$ خلية / جرام من العينه علي التوالي والتي كانت اعلي من الحدود المسموح بها طبقا للمواصفه القياسيه المصريه رقم 3493 لسنة 2005. ،وكانت النسبة المقبولة من عينات الناجتس، الشاورما، برغرالدجاج وفيليه الدجاج 92، 80، 84 و 88٪ على التوالي. في حين كانت نسبة رفض عينات الناجتس، الشاورما، برغرالدجاج ودجاج فيليه 8، 20، 16&12 %على التوالي. لذلك فإن عينات الناجتس ذات جودة عاليه أكثر من المنتجات الأخرى. تم الكشف عن السم نوعه A في 7% من اجمالي العينات المفحوصه وكانت المكورات العنقودية الذهبية تنتج واحد أو أكثر من السموم في وقت واحد. وتم تقسيمها إلى خمسة أنواع (*SEA*،*SEB*،*SEC*،*SED*، *SEE*) على أساس الخصائص الأنتيجينيه. ،وكان نوع*SEA* هو السم المعوي الأكثر شيوعا ويسبب التسمم الغذائي. تم فحص المعزولات التي تم تحديدها لقدرتها على إنتاج جينات الضراوه وهي انزيمات اللثيثينيز والهيموليسين والأنزيم البروتيني باستخدام طريقة الانتشار وقياس القطر بالمليمترات على أجار الدم، اجار صفار البيض وأطباق اجارالكازين.