

## **DETECTION OF ENTEROTOXIN GENES OF *STAPHYLOCOCCUS AUREUS* ISOLATES FROM RAW MILK .**

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### **ABSTRACT**

A total of 200 samples of raw milk (100 cow milk and 100 buffaloe milk) were collected from different markets in Basrah city and were analyzed for the presence of *Staphylococcus aureus* . Results indicate that this bacterium was observed in 28.5% of total samples (30% of cow milk and 27% of buffalo milk)

Enterotoxin genes (*Sea-See*) were investigated using a polymerase chain reaction (PCR). Staphylococcal enterotoxin C gene ( *Sec* ) was detected in 24.56% of the *S. aureus* isolates , while none of the *S. aureus* isolates harbouring *Sea, Seb,Sed* or *See* genes.

### **INTRODUCTION**

*Staphylococcus aureus* is an important pathogen due to the combination of toxin-mediated virulence, invasiveness and antibiotic resistance (1). This bacterium causes nosocomial infection ,as well as community acquired disease, the spectrum of *S.aureus* infection ranges from pimple and furuncles to toxic shock syndrome and sepsis (2) *S.aureus* also are important mastitis pathogens in animals(3) .Most of which depend on numerous virulence factors, on the other hand, some infection such as Staphylococcal food poisoning ,rely on one single type of virulence (SEs) factors (1).

The ability of *S. aureus* strains to produce one or more SEs in food products is linked to staphylococcal food poisoning (4). Staphylococcal food poisoning was a major concern in Public health programs worldwide (1). According to public health and food safety experts, each year millions of illnesses throughout the world can be traced to food-borne pathogens (5) and *S. aureus* considered one of the major causes of gastroenteritis resulting from consumption of contaminated food products (1 ,6).

Various methods have been developed for detecting enterotoxin production but the PCR technique offers the possibility of detecting specific gene sequences by DNA amplification ,therefore its combines all the favoured advantages and provide the ideal solution for SEs detection from various *S.aureus* isolates(7).Thus this work was design to investigate the presence of *Sea* , *Seb*, *Sec* , *Sed* and *See* enterotoxin genes in *S. aureus* isolates by high sensitive high specific technique .

## **MATERIALS AND METHODS**

### **Samples collection :**

Ten ml of milk was collected in 10 ml disposable sterile screw-cap tubes. Samples were immediately transported to the laboratory and kept at 4 °C for no more than 24 hrs before freezing. From each sample, 1.5 ml of milk was pipetted into sterile microcentrifuge tubes and centrifuged at 10,000 rpm for 5 min at room temperature. The supernatant was then discarded and the pellet was stored at -20 °C until laboratory processing. (8).

### **Laboratory diagnosis :**

The specimens were directly inoculated onto plated of mannitol salt agar ( MSA ) and incubated at 37 °C for 24 hrs. All colonies from primary cultures were purified by subculture onto MSA medium and incubated at 37 °C for 24- 48 hrs (9).Suspected *S. aureus* isolates were detected according to( 10 ).

### **Molecular detection of *Sea* to *See* genes (using multiplex PCR technique) :**

The DNA was extracted and purified according to the instructions of Promega kit ( Promega / USA ).The *SEs* genes were studied according to protocol of (7). The PCR amplification mixture (25µl)which includes 12.5 µl of green master mix ( which contains bacterially derived Taq DNA polymerase , dNTPs , MgCl<sub>2</sub> and reaction buffer at optimal concentration for efficient amplification of DNA templates by PCR ) , 2.5 µl of template DNA , 1 µl of each primers given in( Table 1) and 4µl of nuclease free water to complete the amplification mixture to 25 µl .

Table (1): Oligonucleotide primers sequences used for PCR amplification of enterotoxins (*SEs*) genes according to Sharma *et al.*( 7 )

Primer name and size	Description	Nucleotide sequence (5→3)	PCR product size (bp)
SA-U (20)	Universal forward primer	TGTATGTATGGAGGTGTAAC	
SA-A (18)	Reverse primer for <i>SEA</i>	ATTAACCGAAGGTTCTGT	270
SA-B (18)	Reverse primer for <i>SEB</i>	ATAGTGACGAGTTAGGTA	165
SA-C (20)	Reverse primer for <i>SEC</i>	AAGTACATTTTGTAAGTTCC	69
SA-D (20)	Reverse primer for <i>SED</i>	TTCGGGAAAATCACCCCTAA	306
SA-E (16)	Reverse primer for <i>SEE</i>	TTCGGGAAAATCACCCCTAA	213

The PCR tubes containing amplification mixture were transferred to preheated thermocycler and start the program as in the following in table (2).

Table (2): PCR amplification program for enterotoxins (*SEs*) genes detection according to Sharma *et al.*( 7 )

Step	Temperature (C°)	Time	NO. of cycle
Initial denaturation	94	5min	1
Denaturation	94	30s	25
Annealing	50	30s	
Extension	72	30s	
Final extension	72	2min	1

The results of PCR were performed after the amplification process. 10 µl from amplified sample was directly loaded in a 2% agarose gel containing 0.5 µl /25 ml ethidium bromide with the addition of loading buffer and DNA size marker as standard in

electrophoresis and run at 75 V for 1 hr , then the products were visualized by UV transilluminator.

**Statistical analysis :**

In order to determine the statistical significances among different variables SPSS program ( Statistical package for social sciences ) version 11 , was used . Chi – square and analysis of variance tests were applied to analyze the obtained results .

**RESULTS**

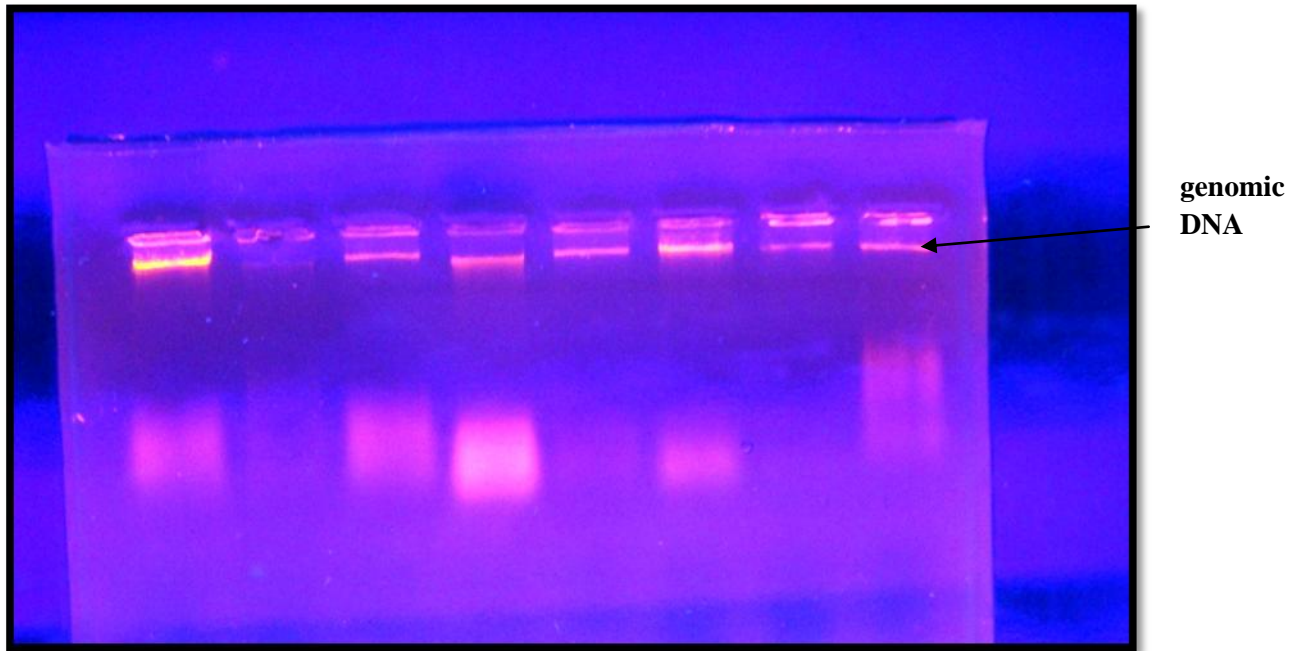
According to the present results the *S.aureus* positive isolates were 57 out of 200 tested samples ( 28.5% ) table 3 .

The PCR analysis was applied to DNA extracted from pre-conventional microbiological and serological confirmed of *S.aureus* isolates from milk samples, figure (1).

Out of 57 isolates were analyzed by PCR technique for *SEs* genes , 14 isolates (24.56%) , found to possess *Sec* enterotoxin gene , while *Sea, Seb, Sed* and *See* were not detected in raw milk sample ( table 3 ).Only the band with suspected size 69bp (in case of *Sec* gene) observed while no bands were observed in negative isolates figure (2).

Table ( 3 ) :- Number, percentage and type of *SEs* in cow and buffalo milk samples .

Type of sample	No. of samples	No. of <i>S.aureus</i> isolates	No. and type of <i>SEs</i> genes
Raw cow milk	100	30	( 9 ) C ( 30% )
Raw buffalo milk	100	27	( 5 ) C ( 18.51% )
Total	200	57	( 14 ) C ( 24.56% )
		X = 0.221 , P> 0.05	X = 1.011 , P> 0.05



Figure(1).Total genomic DNA extracted from *S.aureus* isolates using 1%agarose gel electrophoresis.

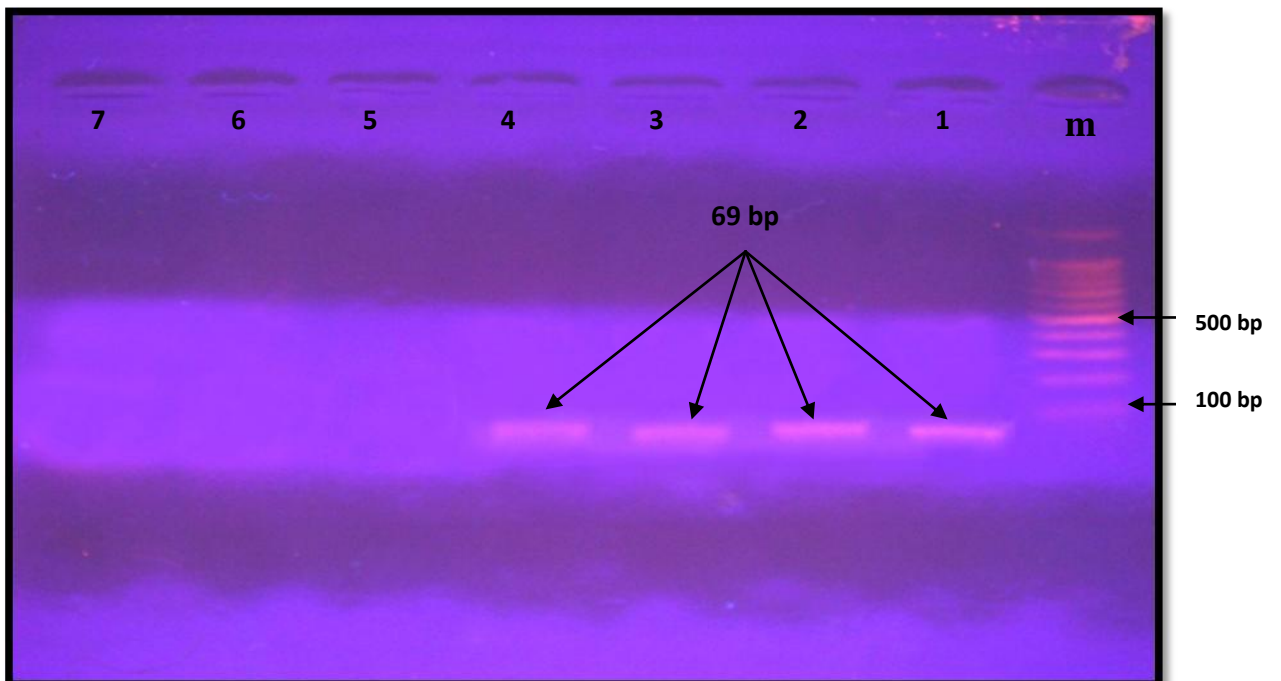


Figure (2) Electrophoresis in 2% agarose. M Lane= DNA ladder .Lane 1,2,3,4, =SEs 69 bp positive isolates. Lane 5,6 negative isolates . Lane 7= control negative

## DISCUSSION

Raw milk samples obtained from Basrah market were contaminated with *S.aureus* isolates in a percentage of ( 28.5 % ) . This type of microorganisms possess the ability to produce the enterotoxins which make the risk factor on public health .

By using the PCR technique , only *Sec* gene was detected in the *S.aureus* isolated from raw milk of cow and buffalo samples, and none of these strains harbouring *Sea*, *Seb*, *Sed* and *See* genes.

This result agreed with Sharma *et al* . (7) who found *Sec* gene in 11.1% of the *S. aureus* isolates of milk samples and none of these isolates harbouring other *SEs* genes. Also similar finding was documented by Tsegmed ( 8) Who found *Sec* gene in 19% of raw milk *S. aureus* isolates and none of these isolates produced *Sea*, *Seb*, *Sed* and *See*.

Other investigators showed that *Sec* was the most frequent type in the *S.aureus* isolated from milk and milk products of the bovine and ovine. (11, 12 ,13 ,14 ,15,16).

The highest frequency of *Sec* in bovine and ovine *S. aureus* isolates from may be occurred because Staphylococcal isolates from different animal species produce host specific SECs.( 17, 6 ). Furthermore, the SEs could be able to indicate the origin of the *S.aureus* strains because it was observed that a higher ratio of isolates from bovine produced SEC and those from human produced mainly SEA (18)

In the present study , *S. aureus* isolates showed the capacity for harboring enterotoxin in percentage of 24.56% . This result agreed with Lee Loir *et al*( 1 ) and Moon *et al*. (19) Who estimated the percentage of enterotoxigenic strains around 25% and 23.6% respectively. Nevertheless, estimation varies considerably from one food to other and from one report to another (1). The recent and earlier reports from different countries found high variability in the percentage of enterotoxigenic isolates of milk and milk products ranged from 0 to 68.4% (20, 21 , 13 , 22, 23, 16, 24 , 25, 26, 27, 28, 18).

Many authors used PCR for detection of staphylococcal enterotoxin genes and all of them found high variability in the presence of enterotoxin genes (16,24, 5,29).The significant differences in toxicity of *S.aureus* isolates from bulk milk and mastitis milk contributed to genetic variation of enterotoxin genes with reference to geographical locations.( 30,31) or might be due to differences in the reservoir in the various countries or ecological origin of strains, the sensitivity of detection methods, detected genes and number of samples, and kinds of examined samples included in these studies (24) .

The detection of *SE*-genes by PCR allows the determination of potentially enterotoxigenic *S. aureus* irrespective of whether the strain produces the toxin or not, the inability to detect the enterotoxin by immunological methods may occur due either to low-level production of enterotoxin or to mutation in the coding region or in a regulatory region (7). For this reason, PCR may be considered more sensitive than methods that determine SE production as immunological methods (32,33)

The ability of *S. aureus* isolates to produce one or more SEs in food products is linked to staphylococcal food poisoning (4). Enterotoxigenic strains of *S.aureus* have been reported to cause a number of diseases or food poisoning outbreaks in many countries because of ingestion of contaminated dairy products or milk with Staphylococcal enterotoxins (34, 15, 5, 35, 36).

In the present work 75.44% of *S. aureus* isolates were negative to the five classical enterotoxin genes .This might be explained by the fact that these isolates either have not harboured any gene of enterotoxins or thy might have other types of SEs which are family of 18 serological types of heat stable enterotoxin (37 ,35 , 28,6).

**الكشف عن جينات الذيفانات المعوية في جرثومة المكورات العنقودية الذهبية المعزولة من الحليب الخام بتقنية تفاعل البلمرة المتعدد .**

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### **الخلاصة**

تم خلال الدراسة جمع 200 عينة من الحليب الخام ( 100 من حليب الأبقار و 100 من حليب الجاموس ) من اسواق مختلفة في محافظة البصرة ، وحللت هذه العينات لغرض التحري عن وجود المكورات العنقودية الذهبية .أظهرت النتائج إن نسبة العزل لهذه الجرثومة كانت 28.5% من المجموع الكلي للعينات ( 30% من حليب الأبقار و 27% من حليب الجاموس ).

تم التحري عن وجود الجينات ( See-Sea ) المشفرة للذيفانات المعوية في جرثومة المكورات العنقودية الذهبية المعزولة من الحليب الخام باستخدام تقنية تفاعل البلمرة المتعدد . وجد إن 24.56% من جرثومة المكورات العنقودية الذهبية تمتلك ألجين المسؤول عن إنتاج الذيفان المعوي نوع C، بينما لم تظهر بقية الجينات في إي عزله.

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