

Isolation and molecular detection of enterotoxigenic *Staphylococcus aureus* from raw milk of cows

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Abstract

Staphylococcus aureus is a microbe associated with human's food poisoning. It is caused by isolates producing different heat stable enterotoxins which act as one of the most spread worldwide gastroenteritis causes. The goal of the current study was to assess the incidence of *S. aureus* (enterotoxigenic) in the raw milk samples of cows which were collected from various places of Baghdad city. Isolation and identification of *S. aureus* were done by conventional laboratory method and the diagnoses were confirmed by using molecular method RT-PCR to detect the specific gene of *S. aureus* nuc gene. In addition, we investigate the occurrence of some of Staphylococcal enterotoxins genes such as SEA, SEB, SEC, SED, and SEE. We found that 12/50 (24%) of the isolates were *S. aureus* and these isolates carried one or more of the enterotoxin genes such as enterotoxin type SEC 12/12 (100) followed by enterotoxin type SEA 7/12 (58.33%) and only 1/12 (8.33%) was type SED, while type SEB and SEE were negative 0%. Some of the isolates have genes that coded for two enterotoxins patterns such as SEA and SEC 7/12 (58.33%) and SEC and SED 1/12 (8.33%). Fewer of the isolates expressed only one genotype of enterotoxin gene like SEC 4/12 (33.3%). This study has proven that there was a high chance of occurrence for the enterotoxigenic *S. aureus* at the raw milk of cows in Baghdad city.

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Introduction

At the contamination of the raw milk, *Staphylococcus aureus* considered the most common contaminant microorganism incriminated in the staphylococcal food poisoning (1), that milk and the products derived from it may present a suitable environment for proliferating *S. aureus* and their enterotoxin as well, thus, passing pathogens to consumers (2). Furthermore, Staphylococcal foodborne poisoning might result from ingesting food (either milk or minced meat) containing Staphylococcal Enterotoxins SEs (3). These enterotoxins classical antigenic-based classification includes 5 classical types which are Staphylococcal Enterotoxin type A (SEA), B (SEB), C (SEC), D (SED) and E (SEE). Recently, modern SEs types which are: SEG, SEH, SEI, SEJ, SEK, SEL, SEM, SEN, SEO, SEP, SEQ, SER, and SEU, were mentioned by (4). However, SEs are small

proteins with molecular weight of 26.900 - 29.600 KD (5), they are capable of resist proteolytic enzymes resulting in keeping their action in the gastro-intestinal tract. Moreover, SEs are capable of resisting heat (6), leading to retaining the biological activity even beyond pasteurization, for an example, keeping the activity of the Staphylococcal enterotoxin A (SEA) may reach 30 min at 121°C (7). These strains' SEA production properties would be strain-specific (8). Milk can be contaminated with pathogenic microorganisms carrying virulent genes with varying levels of activity (9). Many virulence factors could be produced by every strain of *S. aureus*, such as foodborne intoxication which produces enterotoxins (SEA to SEE) and enterotoxins (SEG to SEQ) (10). On other hand, commercial serological kits cannot detect all of the serotypes and are limited only in serotypes (A-B). Therefore, molecular techniques such as real-time polymerase chain reaction (RT-PCR) are extremely

satisfied for detecting such genes of enterotoxins of *Staphylococcus aureus* (10).

According to the mentioned facts, the current work studied the presence of some enterotoxin genes such as (SEA, SEB, SEC, SED and SEE) in *Staphylococcus aureus* isolated from raw milk of cows and diagnosed with the aid of (RT-PCR) through detecting of NUC gene, which is specific for this bacterium (11-13).

Materials and methods

50 samples of raw milk were obtained from lactating cows with some clinical signs of mastitis such as redness, hardness, or swelling of mammary glands; and changing of milk appearance. These cows were mostly crossbreed Holstein type (3-6 years old) found in different places of Baghdad city, especially in Abu Ghraib District, during the period from January to February 2021 and examined in microbiology laboratories, College of Veterinary Medicine, University of Baghdad. Directly from manually milked individual animals, after cleaning of the teats using 75% alcohol. Initially, milk streams were discarded then 10 ml of each sample of milk was collected in sterile screw-cap containers and transported immediately to the laboratory for analysis.

Traditional bacterial examinations

Each sample was cultured directly onto Blood Agar for cultivation and examination of hemolytic reaction (14). A microscopic examination was done for the bacteria after staining with Gram's stain. These bacteria were also sub cultured onto MSA (Mannitol Salt Agar) as well, for isolation and identification of *Staphylococcus aureus*. The implanted samples were placed in the incubator at 37°C for 24 hours and then the bacteria were tested for some biochemical reactions. Conventionally, the isolates were identified using specific tests such as (Coagulase, Catalase, Phosphatase, Gelatinase, and DNase) (15).

Molecular detection using RT- PCR

DNA genome was extracted from the bacterial using the protocol of ABIopure Extraction (ABIopure™ Total DNA, USA), G. Tag qPCR Master Mix, Quantifluor dsDNA System (Promega, USA) while the primers, which were appropriate for this work, were from (Macrogen, Korea) as shown in (Table 1). These primers used in order to target the gene sequence specific for detection of *S. aureus* (*Nuc* gene) and enterotoxin genes such as (SEA, SEB, SEC, SED, and SEE). The specificity of these primers were verified according to a previous work (11). Run started on (Mic qPCR Cycler, Bio Molecular System, Australia) version 2.10.0. The concentration of extracted DNA was detected, using Quantus Fluorometer, in order to evaluate the quality of

samples for downstream applications. For 1 µl of DNA, 199 µl of diluted Quantifluor Dye was mixed. After 5min incubation at room temperature, DNA concentration values were detected. The primers, obtained by Macrogen Company in a lyophilized form, were dissolved in a nuclease free water to give a final concentration of 100pmol/µl as a stock solution. A working solution of these primers was prepared by adding 10µl of primer stock solution (stored at freezer -20°C) to 90µl of nuclease free water to obtain a working primer solution of 10pmol/µl. Each RT-PCR assay was performed in a total volume of 10µl of the mixture (9 µl of Master mix per tube and add 1µl of Template). Master mix consisted of the forward, reverse primers and template DNA. Thermal cycling was performed at 95°C for 5 minutes as (Initial Denaturation) and 40 cycles as follows: 95°C for 15 s as (Denaturation) and 50, 53 OR 60°C for 30s as (Annealing). The extension was at 72°C for 30s.

Table 1: Primers of enterotoxin genes of *S. aureus* according to Çigdem *et al.* (11)

Primer Name / Sequence	°C
SeA F/CATTGCCCTAACGTGG	60
SeA R/ATCCCCTCTGAACCTTCCCATC	
Seb F/TCGCATCAAACCTGACAAACG	53
Seb R/GCAGGTACTCTATAAGTGCCTGC	
Sec F/CTCAAGAAGTAGACATAAAAGCTAGG	53
Sec R/TCAAAATCGGATTAACATTATCC	
Sed F/GTGGTGAAATAGATAGGACTGC	60
Sed R/ATATGAAGGTGCTCTGTGG	
See F/TACCAATTAACCTTGTGGATAGAC	53
See R/CTCTTTGCACCTTACCGC	
Nuc F/TGAAGTCAAATAAATCGCTTGC	50
Nuc R/CCCTTTTCCACTAATTCCTTATTGT	

Results

The results of isolation and identification of *S. aureus* by using routine laboratory diagnostic tests showed that, out of 50 samples of raw milk of cows, 22/50 (44%) of the isolates were positive growth for *Staphylococcus spp.* and only 12/50 (24%) of the isolates were diagnosed as *S. aureus*. These isolates were confirmed by using RT-PCR when the specific gene for *S. aureus* (*NUC* gene) was detected in all isolates 12/12 (100%) (Figure 1). This study has proven that there was a high level of incidence of (*S. aureus*) isolates in the samples and all these bacteria were enterotoxigenic when were examined genetically by detecting the presence of enterotoxin genes using (RT-PCR). The most frequent gene was of enterotoxin type SEC 12/12 (100%) (Figure 2), followed by enterotoxin type SEA 7/12(58.33%) (Figure 3), and only 1/12 (8.33%) was found of type SED (Figure 4), while type SEB and SEE were negative 0% during detection. Two enterotoxins genes patterns carried by some isolates coding for SEA and SEC 7/12 (58.33%) and SEC and SED

1/12 (8.33%), while fewer of the isolates expressed only one genotype of enterotoxin gene like SEC 4/12 (33.3%).

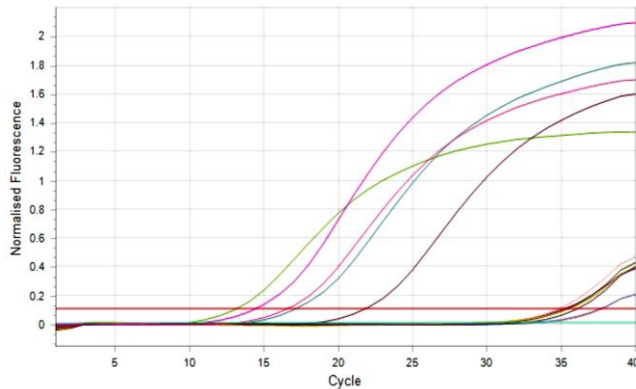


Figure 1: Cycling target (Nuc) amplification curve, quantification cycle (Cq): (13.14 - 37.71).

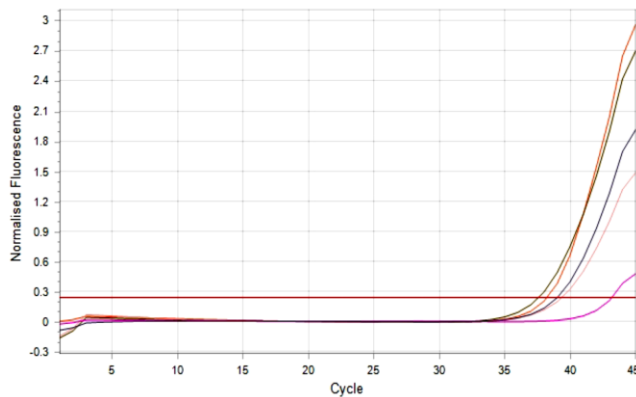


Figure 2: Cycling target (SeA) amplification curve, quantification cycle (Cq): 37.56-43.18.

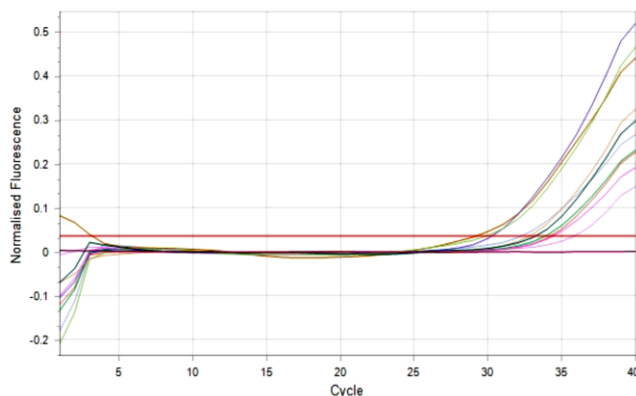


Figure 3: Cycling target (Sec) amplification curve, quantification cycle (Cq): (7.47- 35.83).

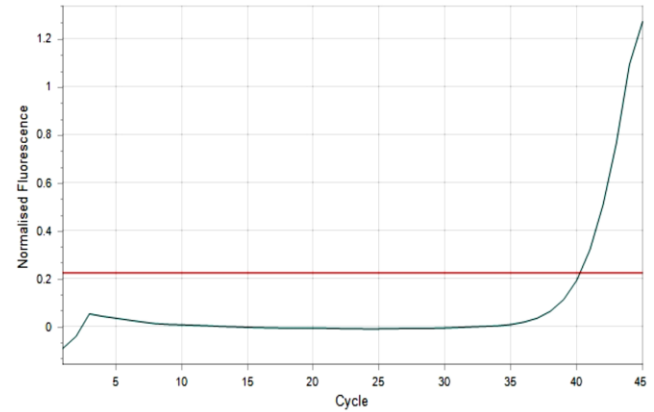


Figure 4: Cycling target (Sed) amplification curve, quantification cycle (Cq): 40.23.

Discussion

S. aureus represents one of the most famous microorganisms found in raw milk, that milk offers a suitable medium for this bacterium growth especially when decreasing of hygienic measures as well as of cooling services occurred (16). Milk contamination could be internal through infected animals' secretion, or external due to infected persons because of the commensalism the human population and *S. aureus* bacteria that approximately 50% of the people are *S. aureus* carriers, or might cause through the environment such as water, air, soil or dust (17).

These microorganisms produce enterotoxins resulting in an important issue for public health (18,19). However, there were many staphylococcal food poisoning outbreaks occurred in some countries such as in United Kingdom (20) and one recorded in Iraq (21). Our study has focused light on the high occurrence of enterotoxigenic *S. aureus* presence in raw milk of cows in Baghdad city. Out of 50 samples of the raw milk were investigated for the presence of enterotoxigenic *S. aureus*, using conventional and molecular methods.

During the processing of dairy product, such procedures are required to reduce bacterial contamination (22). We found that only 12/50 (24%) were diagnosed as *S. aureus*. The level of incidence was higher than that found by one study of Hasan and Hoshyar (23) and lower when compared with other previous works such as Hayfaa *et al.* (24), Mansour *et al.* (25), Khudor *et al.* (26).

These rates were different because of the source of milk and its being collected from infected cows. During this work, milk samples were obtained from crossbreed Holstein cows, 3-6 years old, mostly were found in Abu Ghraib District of Baghdad city. We found that all of our isolates were positive in producing the classic enterotoxins genes, such as: enterotoxin type SEC 12/12 (100%), the occurrence level of this gene was much higher than that found by other research

of Mansour *et al.* (25) who found that SEs genes with SEE seems to be the most frequent gene.

With regard to the type SEB and SEE, they were negative detections 0% during our study. Few of the isolates produced enterotoxin type SEA 7/12 (58.33%) and only 1/12 (8.33%) was type SED. Some of these isolates had two enterotoxins' genes patterns, such as SEA and SEC 7/12 (58.33%) and SEC and SED 1/12 (8.33%), while fewer of the isolates expressed only one genotype of enterotoxin gene as SEC 4/12 (33.3%). These were the only genotyping pattern observed in our work. Future studies could be done about the occurrence of newly discovered SE gene caused by new enterotoxigenic strains for *S. aureus* (27).

Conclusion

This study has proven that there was a high chance of occurrence for the enterotoxigenic *S. aureus* at the raw milk of cows in Baghdad city.

Acknowledgments

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Conflict of interest

No conflict.

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بكتيريا المكورات العنقودية الذهبية بالطريقة المخبرية التقليدية وتم التأكد من التشخيص باستخدام تفاعل السلسلة المتبلعمة الحقيقي عن طريق الكشف عن جين *nuc* الخاص بالمكورات العنقودية الذهبية. إضافة إلى ذلك، تم فحص نسبة وجود بعض الجينات الخاصة بالذيفانات المعوية للمكورات العنقودية الذهبية مثل جين *zifan* المعوي أ، جين *zifan* المعوي ب، جين *zifan* المعوي ج، جين *zifan* المعوي د، وجين *zifan* المعوي ي. وجدنا أن ٥٠/١٢ (٢٤%) من العزلات كانت موجبة للمكورات العنقودية الذهبية، وان كل هذه العزلات كانت تحمل واحد أو أكثر من جينات الذيفانات المعوية، مثل نوع *zifan* المعوي ج ١٢/١٢ (١٠٠%) تليها نوع *zifan* المعوي أ ١٢/٧ (٥٨,٣٣%) و فقط ١٢/١ (٨,٣٣%) من النوع د، بينما النوع ب و ي كانا سالبين ٠%. تحتوي بعض العزلات على جينات ترمز إلى نمطين من الذيفانات المعوية مثل *zifan* المعوي أ و ج ١٢/٧ (٥٨,٣٣%) و ج و د ١٢/١ (٨,٣٣%)، بينما عير عدد أقل من العزلات عن نمط وراثي واحد فقط من الجين المعوي ج ١٢/٤ (٣٣,٣%). أثبتت هذه الدراسة أن هناك فرصة كبيرة لحدوث سموم معوية للمكورات العنقودية الذهبية في الحليب الخام للأبقار في مدينة بغداد.

العزل والتشخيص الجزيئي للمكورات العنقودية الذهبية المنتجة للذيفان المعوي من حليب البقر الخام

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الخلاصة

المكورة العنقودية الذهبية هي ميكروب مرتبط بالتسمم الغذائي للإنسان بسبب إنتاجها للذيفانات المعوية المختلفة الثابتة للحرارة والتي تعمل كواحدة من أكثر أسباب التهاب المعدة والأمعاء انتشاراً في جميع أنحاء العالم. إن الهدف من الدراسة الحالية هو تقييم نسبة حدوث المكورات العنقودية الذهبية المنتجة للذيفان المعوي في الحليب الخام للأبقار الذي تم جمعه من أماكن مختلفة في مدينة بغداد. تم عزل وتحديد