

Molecular Characterization and Genotyping Study of Food Poisoning *Staphylococcus aureus*, Isolated from Raw Milk and Milk Products in Al-Diwaniyah, Iraq

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Staphylococcus aureus still resembles one of the primary causes of food poisoning induced specifically by milk and its products. Recent studies from neighbor countries of Iraq showed the presence of this bacterium in the milk of cattle, sheep, and goat. The current study aimed to genetically identify *S. aureus* that was cultivated from 16 raw milk, 20 cheese, and 13 cream samples. The results show positive isolation of the bacterium in 6 (37.5%), 8 (40%), and 3(23%) samples respectively. Then, the isolates were subjected to multiplex polymerase chain reaction (mPCR) technique targeting enterotoxin genes, *sea*, *seb*, and *seg*. Interestingly, the milk samples showed amplification of these genes in 3 (33.3%), 1 (16.6%), and 4 (66.6%) isolates respectively. the cheese results revealed amplification of the genes in 4 (50%), 2 (25%), and 5 (62.5%) isolates respectively. Moreover, the cream indicated amplification of *seg* gene only in 1 (33.3%) isolate. Finally and to genotype the bacterium, the accessory gene regulator (*agr*) was employed to detect the bacterium nucleotide polymorphism. The results place the isolated *S. aureus* in different genotype groups but mostly in Group I and Group III. The current study results determine *S. aureus* as a pathogenic organism that thrives the milk and its products and might be responsible for many cases of food poisoning in the city.

Keywords: *Staphylococcus aureus*, food poisoning, dairy products, genotyping.

Food poisoning caused by ingestion of contaminated dairy products with pathogenic bacteria such as *staphylococcus aureus* is considered to be one of the major concerns that affect people life because milk and its products are important ingredients of everyday-people food such as raw milk, cheese, and cream. These products are used in food industries and that is what could make the problem of food poisoning even worse¹. Results from a recent study by² have shown the high presence of this bacterium in the

raw milk in Egypt. The presence of *S. aureus* in raw milk or its products could occur due to accidental contamination by handlers³. In the same regards subclinical mastitis is considered as a source of *S. aureus* that induces food poisoning⁴. In a study from Egypt by⁵ have detected that *S. aureus* isolates were found in 40% of the bovine mastitis tested samples that most of them are resistant to wide range of antibacterial drugs. The problem of antibiotic resistance enhances transforming into subclinical cases and causes passing of *S. aureus* and or its enterotoxins through milk to consumers^{6,7}. Diagnosis and classification of the bacterium need advanced tools and methods to help controlling the problem of food poisoning, in which genetic procedures could add major

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differences to the field due to their high specificity and sensitivity^{8,9}. *S. aureus* secretes various toxins that are responsible of food poisoning in humans such as SEA that belongs to SEs group of toxins^{10,11}. Using these enterotoxin genes to genetically diagnose and genotype strains of *S. aureus* is substantial to improve food industries and control food poisoning caused by contaminated milk and its products. To the best of my knowledge, there have been no studies conducted using these genetic tools to classify strains of *S. aureus* present in raw milk and its products in Iraq and more specifically in Al-Diwaniyah city, ~180Km south to Baghdad, and that is why this study was performed.

MATERIALS AND METHODS

Sample collection

A total number of 49 raw milk and milk product samples (16 raw milk, 20 cheese, and 13 cream) were collected from different local market in Al-Diwaniyah city, Iraq. Samples were placed into clean containers and immediately transferred to a laboratory in the College of Veterinary Medicine, University of Al-Qadisiyah, Diwaniyah, Iraq. The samples were then stored in 4 C until bacterial cultivation.

Bacterial cultivation

For primary enrichment, the samples were inoculated into Brain Heart Infusion Broth (BHIB) and incubated at 37°C overnight. After that, the bacterial growth was inoculated into mannitol salt agar (MSA) and incubated at 37°C overnight to purely isolate *Staphylococcus aureus*.

Bacterial genomic DNA extraction

Bacterial genomic DNA was extracted from *Staphylococcus aureus* isolates by using Presto™ Mini gDNA Bacteria Kit (Geneaid, USA). Briefly, 1ml of overnight bacterial growth on BHIB was placed into 1.5ml microcentrifuge tubes and centrifuged at 10000 rpm for 1 minute. The supernatant was then discarded, and the bacterial cells pellets were used to extract the DNA according to the manufacturer protocol. Finally, the extracted gDNA was checked for quantity and quality using NanoDrop spectrophotometer. The DNA was stored in -20 C to perform other assays later.

Molecular virulence characterization

Characterization of *Staphylococcus*

aureus isolates were determined by detection of some enterotoxin genes (*sea*, *seb*, and *seg*) using mPCR technique. This technique protocol was followed according to¹². The primers were designed using NCBI database and primers 3 plus and deposited in GenBank as *sea*: GQ859135.1, *seb*: AY852244.1, and *seg*: AF064773.1. The primers were purchased from Bioneer Company, South Korea.

Then, mPCR Master mix was prepared using AccuPower® Multiplex PCR PreMix kit (Bioneer Company, South Korea). The process was performed following the manufacturer's instructions.

After that, the mastermix components mentioned above were added to other principle components such as the DNA polymerase, dNTPs, and Mg⁺⁺ that are required to perform the reaction. The reaction was generated in a thermocycler (Mygene Bioneer, Korea) using the following conditions: initial denaturation temperature of 95 °C for 5 min which was followed by 30 cycles of denaturation 95 °C for 30 Sec, annealing at 60 °C for 30 Sec, and extension at 72 °C for 1min. Then, the final extension at 72 °C for 5 min was done. The PCR products were examined by electrophoresis using 1% agarose gel that was stained with ethidium bromide to be visualized under UV transilluminator.

Agr-system based Genotyping

Genotyping of the isolated *Staphylococcus aureus* was generated using *agr* gene to detect locus nucleotide polymorphism. PCR was employed to fulfill this goal and then to sort out these isolate into certain groups of I, II, III, IV. The process was carried out according to¹³, and the primers from the same reference were followed to purchase from Bioneer Company, South Korea.

Then, PCR Master mix was prepared using AccuPower® PCR PreMix (Bioneer, South Korea). The manufacturer instructions were followed to generate the mix.

Later, the mix was added to other components that are needed to perform the PCR reaction using a thermocycler (Mygene, Bioneer, South Korea). The following conditions were used: initial denaturation temperature of 95 °C for 5 min and followed by 30 cycles of denaturation 95 °C for 30 Sec, annealing 60 °C for 30 Sec, and extension 72 °C for 1min. The final extension was

at 72 °C for 5 min. The PCR products were tested by electrophoresis using a 1% agarose gel which had been stained with ethidium bromide to be visualized under UV transilluminator.

RESULTS

Bacterial cultivation

The cultivation of the bacteria showed that out of 16 raw milk, 20 cheese, and 13 cream samples were positive for *S. aureus* in 6 (37.5%), 8 (40%), and 3 (23%) samples respectively.

MPCR-enterotoxin genes

The *S. aureus* isolates were subjected to mPCR technique to target the enterotoxin genes, *sea*, *seb*, and *seg*. Interestingly, the milk showed amplification of these genes in 3 (33.3%), 1 (16.6%), and 4 (66.6%) isolates respectively. The Cheese revealed amplification of the genes in 4 (50%), 2 (25%), and 5 (62.5%) isolates respectively. Finally, the cream indicated amplification of *seg* only in 1 (33.3%) isolate as shown in figure 1.

Genotyping based on *agr* system

Table 1 reveals the results of the *agr*-system based genotyping in details. The raw milk

isolates were genotyped as 3 in Group I, 2 in Group III, and 1 in Group IV. While the cheese isolates were grouped as 4 in Group I, 1 in Group II, 2 in Group III, and 1 in Group IV. For the cream, the isolates were genetically classified as 2 in Group I, and 1 in Group III. Figure 2 shows these results of the *agr* locus nucleotide polymorphism.

DISCUSSION

Food poisoning induced by the ingestion of contaminated milk or its products with poisonous amount of *S. aureus* and or its enterotoxin is valued as a big obstacle facing food industries and public health^{11,14}. In the current study, isolation of *S. aureus* from milk, cheese, and cream indicates that this bacterium and its enterotoxin might play a major role in food poisoning in Al-Diwaniya city, where the samples were collected from. In a recent study in India by¹⁵, *S. aureus* isolates were relatively as low as 12% of the collected cow milk samples. While in the current study, *S. aureus* isolates were higher, 37.5%, in the milk samples. In Greece,¹⁶ have found that *S. aureus* isolates were 40% of the milk samples which is close to what

Table 1. Shows the groups of the isolates based on the *agr* system genotyping

Total percent	Group I	Group II	Group III	Group IV
Milk	3/6 (50%)	0/6 (0%)	2/6 (33.3%)	1/6 (16.6%)
Cheese	4/8 (50%)	1/8 (12.5%)	2/8 (25%)	1/8 (12.5%)
Cream	2/3 (66.6%)	0/3 (0%)	1/3 (33.3%)	0/3 (0%)
Total percent	9/17(52.9%)	1/17 (5.7%)	5/17(29.4%)	2/17(12%)



Fig. 1. Shows amplification of enterotoxin genes, *sea*, *seb*, and *seg* at product sizes of 504, 272, and 150bp respectively. Ladder is 1000-50bp

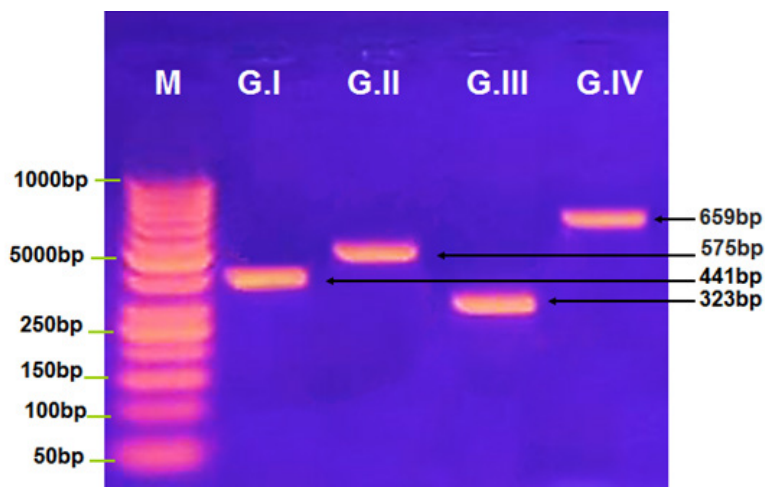


Fig. 2. Shows amplification of *agr* locus nucleotide polymorphism. Ladder is 1000-50bp

the present study has recovered. Cultivation of bacteria from cheese indicated higher presence of *S. aureus*, 87%, in Serbia¹⁷ than that in the current study, 40%. About 5.6% of *S. aureus* had been recovered from cream samples collected from markets in Iran¹⁸ which is less than that in the present study, 23%. The risk factor of increasing the incidence of food poisoning comes from the milk products such as cheese and cream. These products could get contaminated from handlers and factory workers¹⁹. The authors mentioned the isolation of the bacterium from workers in a restaurant, Italy, where cases of food poisoning had happened. Moreover, the places where these products such as cheese are processed might introduce contamination by *S. aureus* and cause food poisoning as was shown by²⁰.

In the current study, the mPCR results bring the attention that *S. aureus* isolates were from the virulent strains. The enterotoxin genes, *sea*, *seb*, and *seg* used for this purpose give high evidence that milk and its products are responsible for many cases of food poisoning in Al-Diwaniyah city. In the present study, Milk isolates of *S. aureus* contained all the three enterotoxin genes with highest percentage of *seg* gene 66.6%. However,²¹ found that *sea* gene was the dominant one in milk isolates in China. In contrast, stool samples that were collected from hospital near to Al-Diwaniyah city showed high frequency of *seg* gene 65%²². This increases the awareness that the strains isolated

from milk in the current study might have had a link with those isolated from the stool samples that mentioned above. Cheese isolates showed high percentages of *sea* and *seg* genes 50% and 62.5% respectively. While (23,24) have recognized high frequencies of *seh* and *seb* genes respectively.

The present genotyping procedure using the *agr* system has placed the *S. aureus* isolates mostly in Group I and Group III. However, *S. aureus* strains isolated from bovine mastitis in Brazil were detected to be more frequent in Group II²⁵. Relatively similar to the current study results, all strains isolated from bovine mastitis in some regions in China were also genotyped to be in Group I²⁶. Like the present findings, studies from Iran have shown that most of the strains were classified into Group I and III^{27,28}.

CONCLUSION

The current study highlights the problem of the presence of *S. aureus* in the raw milk, cheese, and cream in Al-Diwaniyah city, Iraq. The study indicates that the isolates were enterotoxin-secreted strains that were proved by the high occurrence of *seg* gene in these strains. The genotyping study using the *agr* system classified the strains mostly in Group I and Group III. Further future studies are required to test and find proper drugs that target these genes and or their products to control food poisoning in the city, the country, and the world.

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