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Molecular Characterization of Classical Enterotoxin Genes among *Staphylococcus aureus* Isolates from Food Handlers in Baghdad City, Iraq

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Abstract

Staphylococcus aureus (*S. aureus*) represents one of the most prevalent pathogens responsible for food poisoning because of its capacity for producing staphylococcal enterotoxin. The main goal of this investigation was to determine the presence of *S. aureus* and evaluate its enterotoxin genes in randomly selected food handlers from different restaurants and cafeterias in Baghdad City, Iraq. A total of 325 nasal swabs were collected from healthy food handlers and examined for the presence of *S. aureus* and classical enterotoxin genes. The strains were determined using confirmatory biochemical, morphological, and molecular (*nuc* gene) techniques. Five Staphylococcal enterotoxin genes, including *sea, seb, sec, sed,* and *see*, were screened using multiplex polymerase chain reaction. Out of the 325 samples, 30.15% harbored *S. aureus* pathogens that were confirmed by the existence of *nuc* gene in all isolates. 46.93% of isolates were positive for one or more classical genes. 22.44% of these isolates harbored a single enterotoxin gene. The prevalent single gene was *sea* (9.1%), followed by *seb* (6.1%), *sec* (4%) and lastly, *sed* (3%). The *see* gene was not observed in any of the isolates. 14 (14.28%), 6 (6.1%) and 4 (4%) exhibited two, three, or four genes, respectively. The *sea* + *seb* (5.1%) and *sea* + *sec* (4%) were the most frequent combinations. The current data conclusively demonstrated a high rate of enterotoxigenic *S. aureus* nasal carriage among the food handlers studied. Thus, comprehensive and continual evaluation of hygienic protocols and infection prevention plans is strongly recommended. Finally, nasal decolonization could be one of the most effective ways to minimize staphylococcal food poisoning and protect restaurant customers.

Keywords: Food borne diseases, *Staphylococcus aureus*, Nasal carriage, Enterotoxin genes, Food handlers

التوصيف الجزيئي لجينات السموم المعوية بين المكورات العنقودية الذهبية المعزولة من متداولي الأغذية في مدينة بغداد، العراق

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الخلاصة

تمثل بكتريا المكورات العنقودية الذهبية واحدة من أكثر مسببات الأمراض المسؤولة عن التسمم الغذائي بسبب قدرتها على إنتاج السموم المعوية. الهدف الرئيسي من هذه الدراسة هو عزل وتشخيص بكتريا المكورات العنقودية

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الذهبية وتقييم جينات السموم المعوية من مقدمي الأغذية الذين تم اختيارهم عشوائيا من مختلف المطاعم والكافتيريات في مدينة بغداد .تم جمع 325 مسحة أنفية لفحص وجود بكتريا المكورات العنقودية الذهبية وجينات السموم المعوية الخاصة بها. وتم تشخيص العزلات بالاعتماد على الطرق المظهرية للعزلات والفحوصات الكيموحيوية والجزيئية حيث تم استهداف الجين *nuc*. تم فحص خمسة من جينات السموم المعوية والتي تتضمن *sea, seb, sec, sed* و*see* باستخدام تفاعل البلمرة المتسلسل وقد اظهرت النتائج ان من بين 325 من المتعاملين مع الأغذية، كان 30.15% حاملين لبكتيريا المكورات العنقودية الذهبية في أنوفهم والذي تم تأكيده من خلال وجود جين *nuc* في جميع العزلات. 46.93% من هذه العزلات كانت إيجابية لواحد أو أكثر من جينات السموم المعوية. بالإضافة الى ذلك, 22.44% من العزلات الإيجابية لجينات السموم المعوية تحتوي على جين واحد من هذه السموم المعوية. كان الجين الوحيد السائد هو *sea* (9.1%)، يليه *seb* (6.1%)، ثم *sec* (4%)، وأخيرًا *sed* (3%). لم يلاحظ ظهور جين *see* في جميع العزلات. أظهر 14 (14.28%) و6 (6.1%) و4 (4%) اثنين أو ثلاثة أو أربعة جينات للسموم المعوية، على التوالي. كانت المجموعات الأكثر شيوعًا هي *sea + seb* (5.1%) و *sea + sec* (4%). أظهرت البيانات الحالية بشكل قاطع وجود نسبة عالية من المكورات العنقودية الذهبية المولدة لجينات السموم المعوية بين متداولي الأغذية الذين تمت دراستهم. وبالتالي، يوصى بشدة بإجراء تقييم شامل ومستمر لبروتوكولات النظافة وخطط الوقاية من العدوى. أخيراً, يمكن أن يكون إنهاء الاستعمار الأنفي أحد أكثر الطرق فعالية لتقليل التسمم الغذائي بالمكورات العنقودية وحماية عملاء المطاعم.

1. Introduction

Foodborne infections are a major public health concern, with approximately 600 million people worldwide becoming sick each year because of consuming contaminated food [1]. Among the reasons for foodborne diseases, bacteria play an important role, and *Staphylococcus aureus* (*S. aureus*) is one of the most predominant bacteria leading to Staphylococcal food poisoning (SFP) [2,3]. The issue is most common in developing countries, where the majority of cases is underreported or overlooked [4].

Even though *S. aureus* is non-spore forming, its opportunistic nature encourages growth at different pH levels and temperature ranges [5]. These characteristics encourage the emergence of bacteria in a variety of food products, such as unpasteurized milk, dairy products, egg products, poultry, meat products, and ready-to-eat food [6]. Following contamination, *S. aureus* grows more quickly due to inadequate hygiene procedures and storage circumstances, which enable it to attain the cell density required for enterotoxin production [7].

S. aureus is one of the most ubiquitous, versatile, and highly adaptive pathogens, leading to a wide spectrum of illnesses that range from self-limiting to life-threatening [8]. It is often found in a large percentage of healthy people. Of the general population, 20% are considered persistent carriers of this organism, whereas 60% of people are estimated to be intermittent carriers [9]. This bacterial species commonly colonizes the mucous membranes and skin of humans, especially the nasal cavity, which is the most frequent carriage site for this bacterium [10].

Food handlers are one of the most significant sources of food contamination and the transmission of SFP through food handlers is a major issue around the world [1, 12]. Nasal carriers are not only susceptible to contracting endogenous and exogenous illnesses but also play a role in the dissemination of pathogenic strains into the environment [13]. While handling food, the hands of nasal carriers represent the major vehicle for the spread of *S. aureus* [14].

Indeed, poor personal hygiene and inappropriate product handling are two major sources of food contamination. Hence, colonized and non-symptomatic carrier handlers who have enterotoxigenic *S. aureus* in their hands or on their noses can become the source of food contaminated by sneezing, respiratory secretions, or manual contact [15]. It has been previously reported that food handlers with a long period of work expertise had a lower incidence of bacterial contamination on their hands. This may be clarified by the fact that experienced food handlers have more hygiene routines than unskilled food handlers [16]. As a result, detecting symptomatic carrier food handlers is critical to preventing food contamination and food poisoning.

Staphylococcal food intoxication occurs when enough of one or more staphylococcal enterotoxins contained in the food are consumed [17]. Among these enterotoxins, members of the classical enterotoxin genes (*sea*, *seb*, *sec*, *sed*, and *see*) have been implicated in 95% of outbreaks of food-borne illnesses [18,19]. These virulence genes can survive under harsh conditions such as heating, low pH and proteolytic digestion [20]. Due to their stability, SE detection is a definite method for confirming outbreaks and strain enterotoxicity. Furthermore, consumption of food contaminated with enterotoxigenic *S. aureus* can quickly result in an outbreak of foodborne disease because of the stable features of SEs and the low dose needed to elicit symptoms [21,22]. The *nuc* gene serves as a marker, and the heat-resistant nuclease gene (*nuc*) is tightly linked to the formation of enterotoxin and can be regarded as an indicator of infection with enterotoxin-producing *S. aureus* [23]. The main clinical manifestations of SFP are vomiting, sudden onset of nausea, abdominal cramps and diarrhea. Therefore, enterotoxins pose the greatest threat and detriment to human health [24].
Currently, multiple molecular approaches have been successfully established for the identification of staphylococcal enterotoxins, including multiplex polymerase chain reaction [25]. Thereby, the current study sought to ascertain the prevalence and occurrence of *S. aureus* nasal carriage among food handlers, as well as the occurrence of enterotoxin genes in isolated strains.

Materials and Methods

2.1 *Cases of suspected S. aureus nasal carriage*

All participants, or their legal guardians, signed a written consent form. The Ethics Committee of the College of Science, Al-Nahrain University also approved the study protocol. Each Food handler was asked to fill out a self-administered questionnaire to collect information on the processing way, food preparation, hygiene precautions used and risks related to *S. aureus* nasal carriage. Furthermore, sanitary, health and socioeconomic-related data of the participants were collected.

2.2 *Sample Collection and S. aureus Isolation*

Nasal swab samples were collected from 325 healthy food handlers working in various cafeterias and restaurants (food preparation, kitchen assistants, food serving, waiters and cleaning utensils) in different localities of Baghdad province, Iraq. The study was conducted from January to July 2023.
Nasal swab samples were obtained from each person's anterior nares (left and right) using the dry cotton-wool swabs (Transwab®) in Amies medium. The swabs were rubbed over the inside of each nostril at least 5–6 times while applying even pressure and rotating the swab constantly. After that, all samples were sent to the laboratory for microbiological investigation within 2 hours. The nasal swab specimens were incubated at 37 °C for 6–12 hours and then inoculated on Blood agar (MERCK, Darmstadt, Germany) and Mannitol salt agar (MERCK, Darmstadt, Germany) and then incubated aerobically for 24–48 hours at 37 °C. Suspect *S.*

aureus colonies from both cultures were obtained and further identified by Gram-stained morphology, mannitol fermentation and biochemical characterization such as oxidase, DNase, catalase, lipase, clumping factor and coagulase test and mannitol fermentation [26,27]. The PCR of the *nuc* gene was used as a final confirmation of the presence of *S. aureus* species. All isolates were preserved in Luria-Bertani broth (LB) with 20% glycerol at -70 °C.
All *S. aureus* colonies detected by biochemical examination were verified using species-specific PCR targeting the *nuc* gene. Typical colonies of the biochemically confirmed *S. aureus* were picked from nutrient agar and cultured in 5 ml of Luria-Bertani broth (LB) for 24 h at 37°C.

The extraction of genomic DNA was carried out using a DNA extraction kit (Qiagen, USA) following the instructions provided by the manufacturer. The reaction mixtures were performed in a total volume of 25 µL containing 12.5 µL of PCR master mixes (BioNeers, USA), 1 µL of each forward and reverse primer, 2µl of extracted DNA template and nuclease-free water which was added up to 25 µL. The amplification reactions were carried out employing a Mastercycler nexus gradient thermocycler (Eppendorf, Germany) with the following conditions: Initial denaturation for 5 minutes at 94°C, then 30 cycles of denaturation for 1 minute at 95°C. This was followed by annealing for 1 minute at 54°C, extension for 30 seconds at 72°C, and final extension for 10 minutes at 72°C.

Table 1: Primer sequences, anticipated product size, and sets of multiplex PCR used.

Target	Primer sequence (5′→3′)	Amplicon size (bp)	References
nuc	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	279	[29]
sea	CCTTTGGAAACGGTTAAAACG TCTGAACCTTCCCATCAAAAAC	127	[30]
seb	TCGCATCAAACTGACAAACG GCAGGTACTCTATAAGTGCC	477	[30]
sec	CTCAAGAACTAGACATAAAAGCTAGG TCAAAATCGGATTAACATTATCC	271	[30]
sed	CTAGTTTGGTAATATCTCCTTTAAACG TTAATGCTATATCTTATAGGGTAAACATC	319	[30]
see	CAGTACCTATAGA- TAAAGTTAAAACAAGC TAACTTACCGTGGACCCTTC	178	[30]

2.3 *Multiplex PCR for Classical Enterotoxin Genes*

The amplification process of chosen classical genes, including *sec, sea, see, sed* and *seb* was accomplished using five primer combinations in one reaction mixture. The primers used in the PCR of the present work are presented in Table 1. PCR for identifying enterotoxin genes was performed in the manner outlined by Savariraj *et al*., [28]. Only isolates that tested positive for *nuc* gene were selected for classical enterotoxin genes. In brief, amplification reactions were carried out in a 25µl mixture including 12.5 µl of two X PCR master mixes (Fermentas, Germany), 50 pmol of each primer, and 2µl of DNA template, with the final volume being adjusted to 25µl via the addition of nuclease-free water. Amplification processes were carried out by using a DNA thermal cycler (Master Cycler Gradient, Germany) as follows: Initial denaturation at 95°C (10 min), followed by 30 cycles of denaturation at 95°C (1 min), annealing at 64°C (1 min), extension at 72°C (1 min) and final extension step at 72°C (10 min).
After gel electrophoresis on 2.5% agarose gel, the PCR products were stained with 1% ethidium bromide and bands were photographed using the ImageMaster VDS software (Amersham Pharmacia Biotech). A 100 bp DNA ladder (Thermo Scientific, USA) was used to

compare the sizes of the amplification products. Negative controls included nuclease-free water.

3. Results

3.1 The Prevalence of S. aureus among Samples

In the present study, the *S. aureus* strains were identified and confirmed by morphological examination, culture characteristics on Mannitol salt agar, and a biochemical reaction test. Finally, PCR assays targeting the *nuc* gene for all putative *S. aureus* isolates were also detected, and results demonstrated that all food handler strains carried the *nuc* gene region, as shown in Figure 1. Of 325 food handler samples, the isolation and identification of *S. aureus* showed that 98 (30.15%) of the total studied samples were contaminated by *S. aureus*.

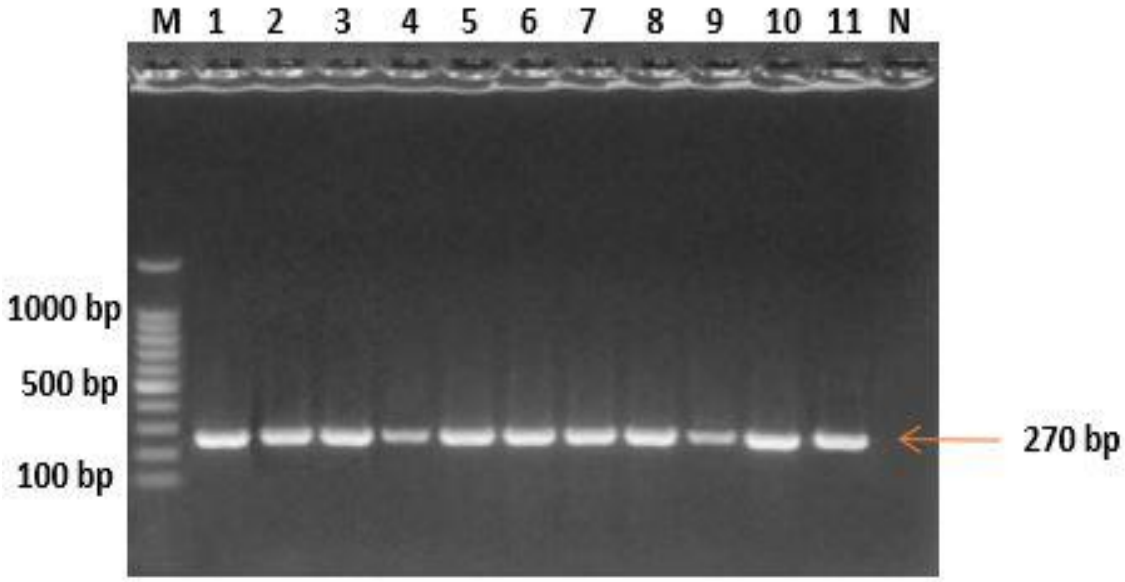

Figure 1: Electrophoresis of amplified gene. Visualization was achieved by 2.5% agarose electrophoresis and ethidium bromide staining for 1 hour at 70 V. Lane M = marker with 100 bp. Lanes 1-11 represent positive samples for *nuc* gene with amplicon size of 279bp. Lane N: Negative control.

3.2 Screening of Staphylococcal Enterotoxins in Food Handler Samples

All isolated strains from food handler samples were tested using Multiplex PCR for the presence of five enterotoxins genes, including *sea*, *seb*, *sec*, *sed* and *see*. Out of 98 *S. aureus* isolates, 46 (35.5%) were found to have one or more enterotoxin genes, as shown in Figure 2. Among the 98 *S. aureus* isolates, 22 (22.44%) carried only one enterotoxin gene. A comparison of SE gene frequency among food handler isolates showed a different distribution of these enterotoxin genes, as summarized in Table 2. However, the most prevalent SE gene among food handler isolates was *sea*, which was detected in 9.1% of the isolates, followed by *seb* (6.1%), *sec* (4%) and *sed* (3%). However, SE genes were not detected in the samples. Besides, the presence of multiple SE genes with different combinations was found among food handler positive isolates, in which 14 strains (14.28%) harbored two genes, 6 (6.1%) exhibited three genes and 4 (4%) carried four genes. Interestingly, the most frequently detected gene combinations were *sea+seb* (5.1%) and *sea+sec* (4%).

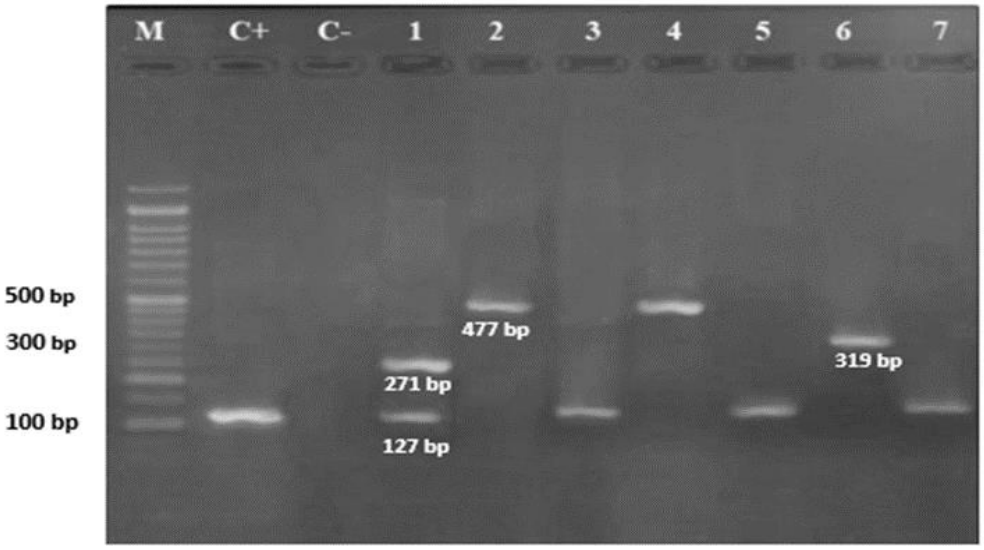

Figure 2: Multiplex PCR amplification for detection of *sea*, *seb*, *sec* and *sed* genes. Lane 1: 100 bp DNA ladder (visualized by 2.5% agarose electrophoresis and ethidium bromide staining for 1 hour at 70 V). Lane C+: Represents positive control, Lane C-: Represents Negative control. Lanes 1_7: Positive SE genes at 127 bp (*sea*), 271 bp (*sec*), 319 bp (*sed*) and 477 bp (*seb*).

Table 2: Frequency of classical enterotoxin genes in food handler isolates.

Enterotoxin	No. (%) of isolates
sea	9 (9.1%)
seb	6 (6.1%),
sec	4 (4%)
sed	3 (3%)
see	0 (0)
Sea + seb	5 (5.1)
Sea + sec	4 (4%)
Sea + sed	2 (2%)
Seb + sec	3 (3%)
sea +seb+sec	2 (2%)
sea+seb+sed	3 (3%)
seb+sec+sed	1 (1%)
sea+seb+sec+sed	2 (2%)
seb+sec +sed+see	2 (2%)
Total	**46 (46.93)**

4. Discussion

Foodborne diseases are currently recognized as one of the most serious public health issues [31]. Furthermore, they are significant causes of mortality and morbidity and thus a significant impediment to global socioeconomic development [32,33]. Several investigations conducted across the world have strongly demonstrated that the presence of enterotoxigenic *S. aureus* in both food products and food processing facilities is the main cause of staphylococcal food intoxication [34,35,36].

Food handlers could represent potentially the cause of food poisoning and foodborne disease resulting from poor personal hygiene or ingesting spoiled, toxic, or contaminated food [37]. Even though cooking can kill *S. aureus*, the enterotoxins are not eliminated and can still contaminate cooked foods due to the unhygienic behaviors of food workers who carry the bacteria in their skin, nostrils and gastrointestinal tract [38]. Therefore, nasal carriage is a significant risk agent in foodborne illness outbreaks caused by *S. aureus* and it has remained one of the most combative bacterial factors of infection [39].

The results of the present study showed that 98 (30.15%) of the food handlers were carriers of *S. aureus*. These results are remarkably similar to a study conducted by Saber *et al*. [40] in Egypt, who indicated that 30% of food handlers harbored *S. aureus*. However, this value is higher in comparison to those reported by other previous research conducted in various parts of the world, such as 20.1% identified by Fooladvand *et al*. [41] in Iran, 23.3% identified by El-Zamkan *et al*. [42] in Egypt, 23.8% detected by Osman *et al*. [43] in Lebanon, 27% identified by Beyene *et al*. [44] in Ethiopia, 8.9% detected by Alves *et al*. [45] in Portugal and 21.7% detected by Vicar *et al*. [46] in food workers in Ghana. However, our result is lower than those found by Nasrolahei *et al*. [47] and Pereira *et al*. [48], who indicated a prevalence of 65.4% and 50% isolates among food handlers in Iran and Brazil, respectively. The variation could be attributed to a variety of factors, including the geographical region, different samples, personal hygiene practices, educational attainment, cleanliness of equipment and utensils, laws in place in each country, and working environment for food handlers [49]. These findings demonstrated that food workers are largely responsible for food contamination by pathogenic microbes. Thus, the prevention of food contamination by pathogenic bacteria mostly depends on improved food handler behaviors, enhanced understanding of sanitary practices, and efficient food preparation and storage techniques [50].

According to the present study, classical enterotoxin genes were detected in 46.69% (46/98) of all isolates from samples collected from food handlers, which is higher than that of a study reported by Ahmed [51], who detected the prevalence of SE genes in 34.4% of food handler isolates. However, it was lower than those mentioned in previous studies with food handlers, which reported that the prevalence rates of the toxin-encoding gene were 50%, 70%, and 96%, respectively [52,41,48].

Considering the genes encoding enterotoxins, 22 (22.44%) out of 46 isolates of *S. aureus* were positive for at least one enterotoxin gene. The most prevalent gene detected in all isolates was *sea* (9.1%). The current findings support previously conducted studies that indicated that *sea* was the most prevalent gene among others isolated from food handlers [41,53].
The *seb* and *sec* genes were detected in 6.1% and 4% of *S. aureus* strains examined, respectively. This is in contrast to other research performed in Brazil, where a high incidence of the *sec* gene was identified in food handler samples compared to the *seb* gene [48].

The *sed* gene was identified in 3% of *S. aureus* strains in this investigation. Previous studies revealed that the *sed* gene was rarely or never detected in *S. aureus* isolates among the other enterotoxin genes [54,43]. Finally, the *see* gene was not detected in our study, which is noteworthy as the *see* gene is closely linked to human contamination [21]. This observation is in agreement with other previous findings [41,51].

In our investigation, multiple SE genotypes were also detected and 57.77% of *S. aureus* isolates contained more than one classic enterotoxin gene. Of which, 14.28% of isolates harbored two genes, and 6.1% and 4% isolates carried three and four genes, respectively. In this

context, the most frequent combinations of SE genes among food handler isolates were *sea + seb* (5.1%) and *sea + sec* (4%).

Numerous investigations have revealed the distribution of one or more enterotoxin genes, either alone or in combination with other genes. A study conducted by Udo *et al*. [55] detected that 64.7% of *S. aureus* isolates from people handling food in Kuwait restaurants carried genes for two to four staphylococcal enterotoxins. In addition, Ahmed [51] demonstrated that 9.6% of the isolates were positive for two genes, with *sea + sec* representing the most common combination (4.3%). Fooladvand *et al*. [41] revealed that 40.8% of SEs gene-positive *S. aureus* isolates contained two SEs genes, 7% carried three genes and 1.9% harbored four genes.

Considering that, most of the isolates in our study possessed genes encoding several virulence factors, suggesting that they could be the source of serious infections with *S. aureus*. Therefore, food poisoning can occur as food handlers who carry these strains contaminate food [56]. Thus, our findings confirm the present practice of evaluating workers at restaurants for *S. aureus* carriers and referring them to the appropriate health authorities for decontamination. Decontaminating food handlers harboring *S. aureus* is critical, as their contact with and possible contamination of the served and prepared food in restaurants poses a public health risk. As a result, using the Multiplex PCR approach, the danger foci can be quickly detected and potential poisoning avoided. The spread of these microorganisms must be kept under control. Correctly detecting the significant and common pathogens in the hospital is crucial for any society's health care system. It is recommended to gather information from more hospitals and cities. Evaluations of other *S. aureus* genes are also necessary.

5. Conclusions

The present study, conducted in Baghdad City, Iraq, revealed a high rate of *S. aureus* nasal carriage and enterotoxin genes among those who handle food, which implies a severe public health hazard. These findings underscore the critical need for preventative actions such as increased public awareness initiatives, consistent surveillance of food handlers for food-borne diseases, and comprehensive training on basic hygiene and health care. The regular wearing of nasal masks by restaurant staff and those who handle food, as well as routine medical examinations, could prevent the transmission of *S. aureus* strains. Lastly, the present result emphasizes the importance of implementing effective quality assurance programs in regions where restaurant food products come into close contact, as a regulatory measure for protecting the community. Future research on efficient techniques for eradicating staphylococcal nasal carriage is required to minimize the risk of infection. The findings of this study can be useful in instructing public health and mitigation actions like proper food handling procedures among food handlers, as well as strengthening future monitoring and epidemiological investigations.

Ethical consideration

The data were collected after written informed consent was obtained from all study participants, and the project was approved by the local ethical committee at Al-Nahrain University, Iraq

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