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Molecular identification of *Staphylococcus aureus*-related enterotoxin genes in cheese samples

Zahira Abdljabbar Al-Zuhairi[1], Esraa Taher Muslim[1], Orooba Meteab Faja[1]*, Ziad M. Alkhozai[2] and Basima Jasim Mohammed[1]

[1]*Collage of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah City, Iraq*
[2]*Collage of Biotechnology, University of Al-Qadisiyah, Al-Diwaniyah City, Iraq*

ABSTRACT

Background: Dairy products are considered some important sources of various nutritional compounds; however, pathogenic bacterial growth is a critical destructive factor to these products leading to consumer health and system financial crises.

Aim: The current study was carried out to identify if there is any presence of *Staphylococcus aureus*-related enterotoxin genes in cheese samples.

Methods: The research included the collection of 35 samples. The samples passed through conventional cultivation processes and a PCR method to detect the presence of *icaA*, *sea*, *hla*, and *fnbA* enterotoxin genes in these samples.

Results: The conventional identification revealed the growth of *S. aureus* from the cheese samples. The PCR findings recorded the presence of the *icaA*, *sea*, *hla*, and *fnbA* in 31 (88.5%), 27 (77%), 19 (54%), and 12 (34%), respectively, of cheese samples. The sequencing revealed close similarities with global isolates, which reached up to 98.5% of identity.

Conclusion: The current results indicate the presence of enterotoxin genes of *S. aureus* in high rates in the dairy products examined, which reveals critical problems of food safety due to the possible presence of enterotoxins in consumer dairy products.

Keywords: Enterotoxin genes, Food-borne diseases, Food safety, *Staphylococcus aureus*.

Introduction

The incidence of foodborne diseases has increased over time, posing a significant global human health concern, with an estimated annual occurrence of over 600 million individuals. Foodborne microorganisms, such as *Staphylococcus aureus*, cause numerous diseases and pose a danger to people health and economic stability due to their detrimental impact on health. Such microorganisms sometimes are present in dairy products and are considered important sources of food-borne diseases. Various pathogenic bacteria have the ability to infect food items during production, processing, preservation, and transportation before they are consumed. Foodborne infections are responsible for causing extensive gastrointestinal diseases, which have been predicted to result in a substantial economic and health impact (Martinović *et al*., 2016; Carstens *et al*., 2019; Ling *et al*., 2019; Faour-Klingbeil and Todd, 2020). Children under the age of five account for over 30% of deaths caused by foodborne illnesses, as reported by the WHO. Foodborne bacteria can lead to a range of symptoms, ranging from mild to severe, including diarrhea and potentially debilitating illnesses including meningitis. Young children are especially susceptible to foodborne infections due to their less robust immune systems. Additionally, they may have an increased propensity to engage in behaviors such as placing their hands in their mouths or consuming food that has been compromised with germs (Scallan Walter *et al*., 2020; Qiu *et al*., 2021; Van Puyvelde *et al*., 2021; Belina *et al*., 2021; Prata *et al*., 2021; Akter *et al*., 2021).

Approximately 25% of the US population suffers from foodborne diseases annually, despite the fact that American cuisine is considered one of the cleanest providers of food globally. Several studies have discovered that the occurrence and importance of various foodborne illnesses are influenced by the interactions between disease-causing microbes, humans, food, and the surrounding environment. Foodborne disorders are caused by harmful microbes, including bacteria, viruses, fungi, and parasites. Among these, bacterial microbes are the most frequent causes and can exhibit various traits and activities (Malachowa and DeLeo, 2010; Hoffmann *et al*., 2021; Ishaq *et al*., 2021; Jahan *et al*., 2021; Saravanan *et al*., 2021; Zarkani and Schikora, 2021; Ge *et al*., 2022). Several bacteria, including *Clostridium botulinum* and *C. perfringens*, have the ability to generate spores and

***Corresponding Author:** Orooba Meteab Faja. Department of Public Health, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah City, Iraq. Email: *orooba.faja@qu.edu.iq*

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have exceptional heat resistance. For instance, *S. aureus* and *C. botulinum* are microorganisms that may generate heat-resistant toxins. Typically, they exhibit mesophilic characteristics, indicating that their optimal temperature range for growth is between 20°C and 45°C. In addition, certain bacteria like *Listeria monocytogenes*, which are responsible for causing foodborne illnesses, have the ability to last under refrigerated conditions or temperatures below 10°C. These infections can still be transferred to humans (Elbehiry *et al*., 2017; Söderqvist *et al*., 2017; Schirone *et al*., 2017).

Staphylococcus aureus enterotoxins exhibit resistance to high temperatures, degrading enzymes, destroying agents, and a broad spectrum of pH levels. Therefore, it remains intact in the gastrointestinal tract, where it can bypass the stomach, and target the intestinal cells. As *S. aureus* thrives in many harsh environments, it has the potential to contaminate livestock products throughout processing and preparation (Argudín *et al*., 2010). Dairy products are considered some important sources of various nutritional compounds; however, pathogenic bacterial growth is a critical destructive factor to these products leading to consumer health and system financial crises. The current study was carried out to identify if there is any presence of *S. aureus*-related enterotoxin genes in cheese samples.

Materials and Methods

Samples

The research included the collection of 35 samples, which were divided equally into cheese samples. *Staphylococcus aureus* was isolated from cow milk cheese purchased from a local market located in Al-Diwaniyah City, Iraq, using the microbiological procedures outlined in the Ministry of Food and Drug Safety's published guidelines (2018). The samples from cheese were diluted by a factor of ten in a solution of 0.1% peptone water prior to their inoculation onto agar plates nutrient agar, blood agar (BA), and brain heart infusion agar utilizing the pour plate technique. The plates were then incubated at a temperature of 37°C for a duration of 24 hours. Subsequently, the colonies were sub-cultivated to get a culture that was free from impurities. The isolates were preserved in the event that the microorganisms were required to be identified. The strains were determined based on their Gram staining, morphological features of colonies, biochemical features (including sugar fermentation), as well as catalase and coagulase analyses. The isolates were confirmed using a 16S rRNA gene-originated PCR test. This investigation involved the testing of 70 isolates of *S. aureus* recovered from 35 cheese samples.

Detection of enterotoxin-based genes

DNA extraction

The isolation of genomic DNA from bacterial isolates (SAD6, SAD7, SAD8, SAD9, and SAD10) was performed using the methodology outlined in the Wizard Genomic DNA Purification Kit, Promega. To perform the RNA lysis, 3 µl of RNase solution was used. The mixture was subjected to incubation at a temperature of 37°C for 15 minutes. In the case of protein precipitation, 200 µl of Protein Precipitation Solution was introduced into the cell lysate. Subsequently thoroughly blended by the vortex. Subsequently, the sample was subjected to incubation at a low temperature of –30°C. Subsequently, the sample was subjected to centrifugation at a speed of 13,000 revolutions per minute for a duration of 5 minutes. The DNA was NanoDrop-estimated.

PCR for bacterial genotyping

The PCR depended on the *16S rRNA* gene as a target by utilizing the primer set; 27F: AGAGTTTGATCATGGCTCAG and 1492R: GGTTACCTTGTTACGACTT (Jarraud *et al*., 2002). The PCR reaction of 50 µl included 10–100 ng DNA template, 20 mM Tris-HCl (pH >8), 50 mM KCl, 1.5 mM MgCl2, 2 µl each DNTP, 0.4 µM per primer, and 2.5 U DNA polymerase. The PCR thermal was: 94°C elongated for 120 seconds for a primary denaturation, 35 repeats of a main denaturing step, an annealing process, and a main-extension program at 94°C elongated for 60 seconds, 55°C elongated for 90 seconds, and 72°C elongated for the 60 seconds, respectively, and 72°C elongated for 180 seconds of a final-extension step. Then, the purified PCR products were sent to Sanger-method-based sequencing in Korea. The resulting data

Table 1. Primers used in the study.

Target and size (bp)	Nucleotides orders	Source
hla (209)	F: CTGATTACTATCCAAGAAATTCGATTG R: CTTTCCAGCCTACTTTTTTATCAGT	(Jarraud e*t al*., 2002)
fnbA (1279)	F: GTGAAGTTTTAGAAGGTGGAAAGATTAG R: GCTCTTGTAAGACCATTTTTCTTCAC	(Xu *et al*., 2015)
icaA (770)	F: CCTAACTAACGAAAGGTAG R: AAGATATAGCGATAAGTGC	(Vasudevan *et al*., 2003)
sea (120)	F: GAAAAAAGTCTGAATTGCAGGGAACA R: CAAATAAATCGTAATTAACCGAAGGTTC	(Mashouf *et al*., 2015)

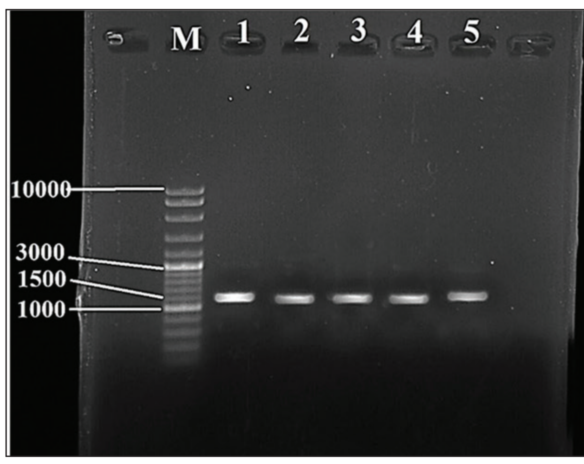

Fig. 1. Image of *16S rRNA*-based PCR-1%-agarose gel electrophoresis of nine isolates of *Staphylococcus aureus* of cheese samples. M: ladder; Lane 1 to 5: SAD6, SAD7, SAD8, SAD9, and SAD10, respectively.

were processed and analyzed using NCBI websites and MEGA X software for the alignment and phylogenetic tree building.

PCR for the enterotoxin genes

The samples were passed through a conventional PCR method to detect the presence of *icaA*, *sea*, *hla*, and *fnbA* enterotoxin genes in the samples. The Accupower PCR PreMix kit used was purchased from (Bioneer, Daejeon, Korea). The thermal conditions were 95°C for 3–5 minutes for the first step. In 30–35 cycles, 95°C for 30 seconds for the denaturation, 52°C and 57°C for 30–45 seconds for the annealing, and 72°C for 1 minute for the extension, and 72°C for 5–10 minutes for the final extension. The primers are listed in Table 1.

Ethical approval

The study has been approved by the ethics committee of The College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah City, Iraq.

Results

The cultivation and conventional identification revealed the growth of *S. aureus* from the cheese samples. For the genotyping, the PCR amplified at 1,500 bp (Fig. 1). The PCR findings recorded the presence of the *icaA*, *sea*, *hla*, and *fnbA* in 31 (88.5%), 27 (77%), 19 (54%), and 12 (34%), respectively, of cheese samples (Fig. 2). The amplification of these genes was successfully recorded in cheese samples.

The sequencing of the isolates revealed close relationships of the current isolates with isolates deposited previously in the GeneBank by researchers from around the world (Table 2 and Fig. 3).

Discussion

Over 20 enterotoxin and enterotoxin-like coding genes have been identified as playing a substantial role in various phases of host colonization, gastrointestinal

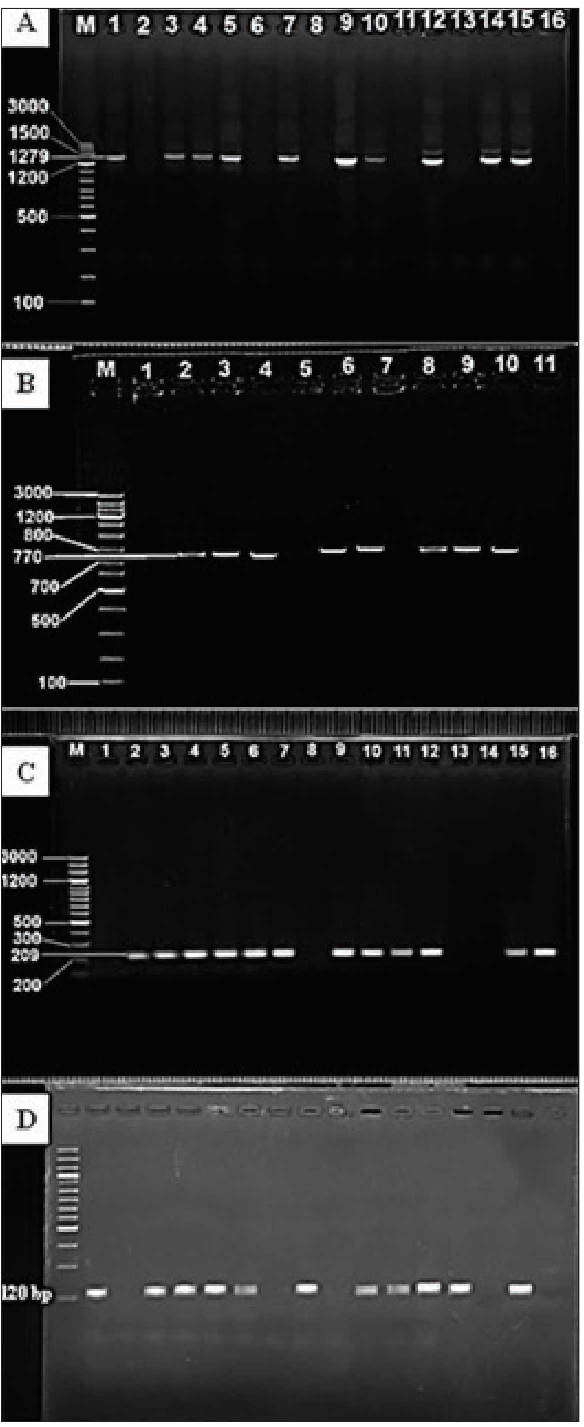

Fig. 2. Image of agarose gel electrophoresis based on the PCR-directed amplification of *Staphylococcus aureus* enterotoxin genes from cheese. (A) *fnbA* gene; (1,3,4,5,7,9,10,12,14,15): Positive PCR samples. (B) *icaA* gene; (2,3,4,6,8,9,10): Positive PCR samples (C) *A hla*; (2,3,4,5,6,7,9,10,11,12,15,16): Positive PCR samples. (D) *sea* gene; (1,3,4,5,8,10,11,12,13,15): Positive PCR samples. M: 100bp ladder.

infections, the invasion of skin and mucus, and host defense systems. Food has a crucial role in the transmission and dissemination of antibiotic resistance genes. *Staphylococcus aureus* harbors pathogenicity genes on movable chromosomal components, including plasmids and staphylococcal pathogenic islands. These components have the ability to transmit horizontally across different strains. Enterotoxins from *S. aureus* in cheese can overcome body defense mechanisms, such as resistance to breakdown by digestion enzymes and acids, heat resistance, and encouraging the production of high amounts of cytokines (Malachowa and DeLeo, 2010; Wu *et al*., 2011; Alibayov *et al*., 2014).

Table 2. Current and GeneBank-based comparison of *Staphylococcus aureus* isolates sequences of cheese samples.

Strain code	Accession number	Similarity %
SAD6	PP321295	97.58
SAD7	PP321296	96.68
SAD8	PP321297	97.34
SAD9	PP321298	98.50
SAD10	PP321299	95.34

Mashouf *et al* (2015) found that *S. aureus* strains with enterotoxigenic properties most commonly exhibited Staphylococcal Enterotoxin A. Furthermore, the seg gene was the prevailing enterotoxin among their newly discovered enterotoxin genes. The sea gene was identified in 25.5% (*n* = 25), and it was also found to exist alongside with the seg gene in 14.3% (*n* = 14) of cases. Our analysis identified the presence of the strain exclusively in one sample isolated from cheese. This outcome was congruent with the study conducted by Alibayov *et al*. (2014) In the same way, Cramton *et al*. (1999) noted that even if *S. aureus* strains include the *ica* locus, they may still be unable to generate biofilm in a laboratory setting due to the fact that biofilm development on non-living surfaces is greatly influenced by the specific circumstances under which the bacteria are grown. A study conducted by Arciola

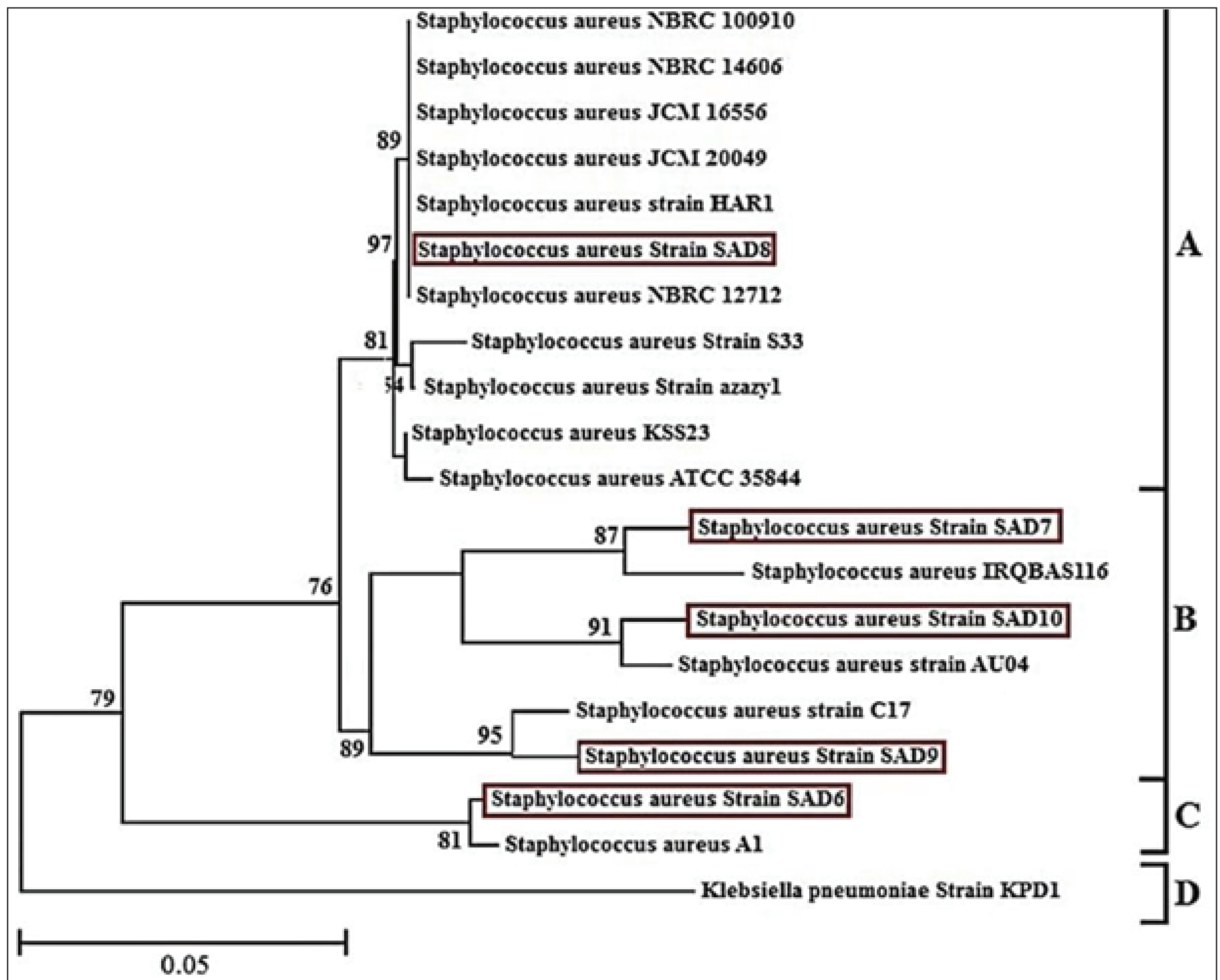

Fig. 3. Phylogenetic tree based on the *16S rRNA* gene for cheese-sample-isolated *Staphylococcus aureus*.

et al. (2001 a,b) revealed that red forms of slime-positive *S. aureus* were shown to be deficient in the *icaA* and *icaD*. They proposed that the altered physical characteristics may be linked to the complete removal of the *ica* locus.

The study conducted by Fooladi *et al*. (2010) revealed that around 32% of the dairy products examined were found to be contaminated. Unconventional milking practices, such as utilizing unclean milking equipment or bare hands, can also transmit *S. aureus*. The majority of the isolates examined in this investigation included the sea gene, with the sed gene being present in subsequent frequency. Additional researchers have proposed the prevalence of the sea (Imani Fooladi *et al*., 2010; Kamarehei *et al*., 2013; Seyoum *et al*., 2016). The findings obtained from the current study revealed that many enterotoxin genes can coexist inside a single isolate. This is necessary to reduce the hazard of *S. aureus* contamination in foods and improve health services, applying other tracks of controlling at all costumes of food processing. Pelisser *et al*. (2009) informed the needed number of live bacteria at least 106 colony-forming units per gram (CFU/g) of *S. aureus* for producing of enterotoxins (Argudín *et al*., 2010). We have to bear this issue in mind while we are facing with food hygiene issues and we have to use many food preservative techniques in order to control bacterial growth in foods. To investigate the grouping of the isolates of the present study, they were compared with the results of other global and Iraqi isolates which published in PubMed. Travelling efforts of humans transport many bacteria from one country to another and import of animals and migrating birds which many all the time to different areas around the world influences the evolution process for many bacteria physiology and leads to different genomic differences of isolates (Jarraud *et al*., 2002; Aguiar *et al*., 2024; Aleksic *et al*., 2024).

The current findings tell us that the genomes of enterotoxins genes of *S. aureus* found in a high rate of the dairy products samples which tested in the current study, showing some critical situations about food safety and surely consumers are threatened with the enterotoxins possible presence in the dairy products which they are consuming.

Acknowledgments

None.

Conflict of interest

No conflict of interest is presented in the current study.

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Data availability

The datasets used and/ or analyzed during the current study are available from the corresponding author (Orooba Meteab Faja) on reasonable request.

Authors' contributions

All authors participated equally in this work.

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