

Molecular Identification of Enterotoxin Genes in *Staphylococcus aureus* Isolated from Milk and Dairy Products in Hilla City

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ABSTRACT

Background: *Staphylococcus aureus* is a major foodborne pathogen responsible for staphylococcal food poisoning (SFP), primarily due to its production of enterotoxins. Contaminated milk and dairy products serve as potential reservoirs for enterotoxigenic *S. aureus*, posing a significant public health risk. **Objective:** This study aimed to molecularly detect enterotoxin genes in *S. aureus* isolates obtained from milk and dairy products in Hilla, Iraq, and assess their prevalence about food safety concerns. **Methods:** A total of 29 *S. aureus* isolates were collected from raw milk and milk-derived products in Hilla between September and November 2023. The isolates were confirmed using PCR amplification of the 16S rRNA gene. The presence of enterotoxin genes (sea and sec) was detected using molecular techniques. **Results:** All 29 isolates (100%) tested positive for the sea (enterotoxin A) gene, confirming the widespread presence of enterotoxigenic *S. aureus* in milk samples. Additionally, the sec (enterotoxin C) gene was detected in 19 isolates (65.5%), indicating a high prevalence of multiple enterotoxins. The findings highlight raw milk as a significant reservoir of *S. aureus* strains capable of producing enterotoxins, which may contribute to foodborne illness outbreaks. **Conclusion:** The high prevalence of enterotoxin-producing *S. aureus* in raw milk and dairy products in Hilla underscores the need for stringent food safety measures, routine microbiological surveillance, and effective processing techniques to mitigate the risk of SFP. Further studies are recommended to assess antibiotic resistance patterns and the potential for toxin production in clinical cases.

Keywords: Food Poisoning, *Staphylococcus Aureus*, Enterotoxins, PCR, 16S rRNA

INTRODUCTION

One of the most common opportunistic bacterial infections that infect humans, *S. aureus*, causes significant morbidity and death worldwide [1]. Clinicians, particularly those involved in infectious diseases, can discuss the pathogen's prevalence, complexity, and potentially disastrous character when it comes to invasive infections. The long evolutionary history of *S. aureus* as a multi-host opportunistic pathogen belies its common association with human colonisation and infection worldwide. Between 20% and 30% of people regularly have *S. aureus* in their nasal cavities [2, 3]. Several investigations have been conducted to identify the relationship between the prospective pathogen and the host microbiota, highlighting the importance of various commensals in recognising *S. aureus* colonisation [4]. Although colonisation is safe and poses no health risk, it is a fundamental factor in enhancing various infections that result from the colonising strain [5]. The infections can range from soft tissue and mild skin infections to serious, invasive conditions such as osteomyelitis and septic arthritis, bacteraemia or septicaemia, pneumonia, and endocarditis [1]. Acute, recurrent, or chronic and persistent infections represent the most common features associated with *S. aureus* infections. It is essential to comprehend the complex pathophysiological relationships between *S. aureus* and its hosts for developing effective treatments and clinical responses to these infections [6]. Different food poisoning epidemics are caused by *S. aureus*, a Gram-positive bacterium, since staphylococcal enterotoxins (SEs) generated by *S. aureus* are considered the causative agent [7]. Additionally, several virulence factors, including immune-modulatory toxins, enterotoxins, proteases, hemolysins, leukocidins, and exfoliative toxins, can be produced by *S. aureus* [8]. Toxic-shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxins (SEs) are

popularly referred to as "superantigens" [9]. One of the most prevalence virulence variables belongs to a large family of toxins that are structurally related such as staphylococcal enterotoxins (SEs). Furthermore, these variables can promote T lymphocyte proliferation, encourage the release of cytokines, and result in cell death [9]. Similar to toxins, bacteriophages, pathogenicity islets, and plasmids can interestingly transport genes encoding [10]. As it was discussed in previous investigations, a toxigenic process introduced with a class of toxins named SEs in staphylococcal food poisoning [11]. Vomiting and diarrhoea represent the main gastrointestinal symptoms associated with the toxins above [12]. Almost 27 kinds of SEs were recognised and identified as single-chain proteins with molecular weights ranging from 19 to 29 kD. The effects of SEs are largely due to two harmful activities: one is neurotoxic, stimulating the vagus nerve and the brain's emetic centre to induce vomiting reflexes, and the other is superantigenic, causing high fever and severe T lymphocyte activation [13]. Most cases of SFP are caused by consuming food that has been contaminated at various stages, including harvesting, processing, shipping, storage, cooking, or handling. Additionally, inadequate chilling procedures may also contribute to the development of staphylococcal bacteria and the production of toxins [14]. Enterotoxigenic *S. aureus*-infected animals are another important source of contamination [15]. This may happen in raw milk, cheese, and raw and processed meats [11, 16]. Since SEs are thermostable, they require prolonged boiling or autoclaving to gradually reduce their effectiveness, even if the organism is inactivated and cannot be eliminated from foodstuffs [15, 17]. Several techniques can be used to detect SEs, including immunoassays, immunodiffusion, radioimmunoassay, latex agglutination [18], and

double gel diffusion assays. Nevertheless, the scope of SEs detected by commercially available diagnostic kits is not comprehensive [19, 20]. Experiments have recently been conducted to identify the presence of *S. aureus* enterotoxin genes. PCR and LAMP assays may be used to directly test food samples for the presence of bacteria, as shown by Goto et al. and Yin et al. [21, 22]. Alternatively, these tests can be conducted after a culture stage to enhance the bacterial concentration. Although SFP outbreaks have occurred worldwide, there is a limited availability of comprehensive incidence numbers, with reports only available from a few subregions [23]. Data accessibility is greatly limited in low- and middle-income countries owing to many issues, including a scarcity of regular monitoring methods and a substantial percentage of afflicted persons who do not seek medical care. This lack of medical treatment hinders the availability of clinical samples [24]. In Japan, a single incident in the summer of 2000 resulted in nearly 14,000 cases of illness and one death. This was caused by consuming pasteurized products that contained small amounts of SEs [25]. This incident highlights the potential extent of SFP outbreaks.

MATERIAL AND METHODS

Bacterial Isolation and Identification

Between September and December 2023, 107 *S. aureus* isolates were obtained from milk and dairy product samples collected from various markets in Hilla, Iraq. In the next step. Routine microbiological techniques identified all isolates. The swabs were introduced into nutritional agar plates and placed in an incubator with oxygen at a temperature of 37°C for 24 to 48 hours. The obtained colonies were determined to be *S. aureus* via conventional laboratory identification methods, including analysis of their colonial shape, Gramme stain, coagulase test, and growth on mannitol salt agar (MSA) [26].

DNA Extraction

Place up to 10 bacterial cells in a 1.5 ml microcentrifuge tube. For one minute, spin at 14,000–16,000 x g and then remove the

supernatant. In a 15 mL centrifuge tube, add 200 µL of Gramme+ Buffer per sample, which is the correct amount. In the 15 ml centrifuge tube, dissolve the Lysozyme completely by adding 0.8 mg/200 µl of Gramme+ Buffer and mixing well.

Use a pipette or vortex to resuspend the pellet after adding 200 µL of Gramme+ Buffer to the sample in the 1.5 mL microcentrifuge tube, ensuring that Lysozyme is incorporated. Incubate for thirty minutes at 37°C. Replace the incubation tube every 10 minutes. By vortexing, incorporate 20 µl of Proteinase K, ensuring that ddH₂O has also been added. At minimum, incubate at 60°C for ten minutes. Turn the incubation tube over every three minutes. Continue to step two, lysis.

Geneaid Biotech Ltd., Taiwan provided the DNA extraction kit. The concentration and integrity of genomic DNA in the prepared samples were assessed using a Nanodrop instrument to measure absorbance at 260 and 280 nm.

Conventional PCR Amplification for the Detection the 16S rRNA and Diferent Staphylococcal Enterotoxins

Using the primers listed in Table 1, the 16S rRNA gene and the staphylococcal enterotoxin (SEA, SEC) genes were examined. 12.5 µL of PCR Master Mix, 1 µL (10 µM) of primers, 2 µL of template DNA, and up to 25 µL of nuclease-free water were used in each PCR reaction. For every PCR reaction, the first step was an initial denaturation at 94 °C for 5 minutes.

Then, there were 35 cycles of denaturation at 94 °C for 30 seconds, annealing at the temperature specified in Table 1, and finally, extension at 72 °C for 45 seconds. Ultimately, each PCR reaction was terminated after five minutes at 72 °C with a final extension. After separating the 100 bp plus DNA ladder and the resulting PCR products on a 1.5% agarose gel, red save was applied. A UV transilluminator (UV-TM-25-230 v) was used to capture photographs of the produced amplification bands.

Table 1: The Primers Employed in the Current Investigation for Detecting 16S rRNA and Staphylococcal Enterotoxin Genes

Primer Name	Primer Sequence	Annealing Temp. (°C)	Size (bp)	References
16S rRNA	AGAGTTTGTATCTGGCTCAG			
F	AAGGAGGTGATCCAGCCGCA	55	1500	[27]
R				
sec	GACATAAAAGCTAGGAATTT			
F	AAA ACGATCAATTTTACAGC	57	257	[28]
R	TCGGATTAACATTATCCA			
sea				
F	TGCATGTTTTCAGAGTTAATC	57	544	[29]
R				

Results

S. aureus is a prevalent constituent of the indigenous microbiota found in the nasal passages and epidermis of humans [30]. Inducing severe necrotic lesions, abscesses, food poisoning, and bacteremia, it is regarded as a potential pathogen due to the adverse effects it may cause.

The findings demonstrate that 29 *S. aureus* isolates were recovered from 32 milk samples and recognised using the 16S rRNA gene. According to genotype, all 29 isolates (Fig. 1) had the staphylococcal

product of the 16S rRNA gene (1500 bp), which has an impact on both human and animal health [31]. The results presented in (Fig.2) show that all of 29 samples of *S.aureus* positive for sea gene (*S.aureus* enterotoxin A).

The staphylococcal product of the sea gene (544 base pairs) was identified cytotypically in each of the 29 isolates (Fig. 2). Out of the 29 samples, the findings indicate that 19 of them included *S. aureus* isolates that tested positive for the sec gene, specifically the *S. aureus* enterotoxin C gene. Genotypically, the sec gene product, which is 257 base pairs in length, was discovered in all 19 isolates (Fig. 3).



FIGURE 1: Agarose Gel electrophoresis of PCR Products Encoding the 16SrRNA Gene Isolated from *S. Aureus*. Lane L: A Size Marker for 100 bp DNA, *S. Aureus* Produced a Positive Result in Lanes 1-15, but a Negative Result in Lane 16.

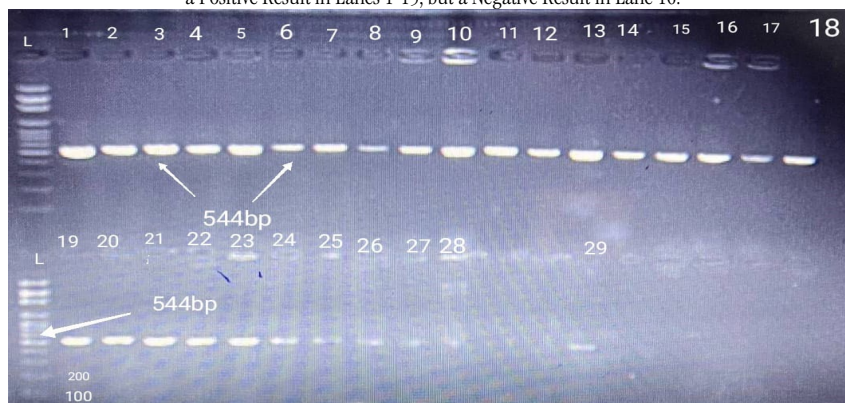


FIGURE 2: Electrophoresis of Sea Gene PCR Products from *S. Aureus* Isolates on an Agarose Gel.

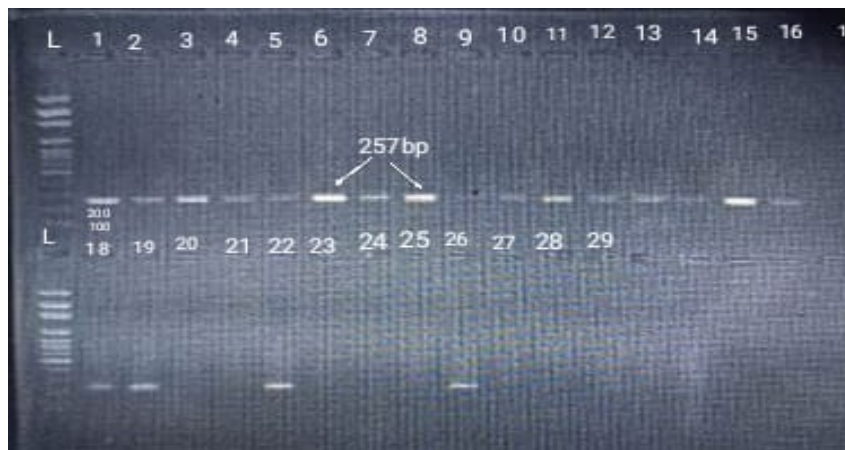


FIGURE 3: Electrophoresis of Sec Gene PCR Products from *S. Aureus* Isolates on an Agarose Gel.

Discussion

The commensal pathogen *S. aureus* affects humans and animals, causing serious illnesses that have a detrimental financial impact on the farming sector, despite *S. aureus* being resistant to every antibiotic ever discovered [32]. *S. aureus* in general and multidrug-resistant (MDR) strains were observed in bovine milk samples in the present investigation. Food handlers are considered the primary source of food contamination, as their hands and nasal passages are often contaminated with enterotoxigenic *S. aureus*. Due to regional variations in food consumption patterns, the distribution of the

classical SE gene varies among nations. The link between food contaminated with *S. aureus* and food handlers was validated by the PCR results of the recovered *S. aureus* [33, 34]. Globally, foodborne outbreaks linked to milk and dairy products have led to hospitalisations and deaths [35]. One important bacteria that causes toxin-mediated food poisoning is *Staphylococcus aureus*. *S. aureus* 29 samples were detected in this research from among the 107 raw milk and dairy products samples that were analysed. This was done using morphological, cultural, biochemical, and molecular features. This research bears resemblance to a recent study conducted in Sharkia Governorate, Egypt [36]. However, recent studies done in China [37],

Algeria [38], and Turkey [39] have found greater prevalence rates of *S. aureus*. Biochemical and morphological standards are used to identify all *S. aureus* isolates.

Moreover, the isolate's identification was confirmed by conducting a PCR experiment targeting the staphylococcal 16S rRNA gene. The obtained results agree with the investigation findings of Al-Alak and Qassim [40]. Enterotoxins are considered superantigens because they can induce polyclonal T lymphocytes, suppress the livestock's immune system, and contribute to the development of chronic intramammary infections [41]. According to the agarose gel electrophoresis results, it was found that sea was the most common type of *S. aureus* enterotoxin

in all isolates, representing 29 (100%), followed by sec, which accounted for 19 (65.5%). The current outcomes agree with an investigation done by Diab et al. in El-Behira Province, Egypt [42]. Another finding revealed that all isolates were effective, which agrees with a study conducted by Taher et al., although the results varied with the sec gene, representing 12/12 (100%) [43].

Moreover, the gene's frequency level was considerably higher than those displayed in earlier investigations by Mansour et al. [44]. Thus, the variety in findings is attributed to several essential variables, such as the origin, location, size, and sensitivity of the methods employed for identification.

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