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Investigation of the prevalence of enterotoxigenic *Staphylococcus aureus* isolates in hamburgers and kebabs in Mashhad and the SE gene profile of the isolates

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ABSTRACT

The study aimed to investigate the frequency of enterotoxin-producing *Staphylococcus aureus* isolates in hamburgers and meat bites in Mashhad and the frequency of the gene. In this practical descriptive research, the number of 175 meat samples including 70 hamburger samples, 70 kebab bite samples, and 35 handmade hamburger samples in a non-repetitive and cluster sampling method, from the brand various commercial products were collected from the supply centers in Mashhad from April to June 2023. After culturing the samples in a specific medium and isolating the isolates suspected *of Staphylococcus aureus*, the bacteria were identified using morphological and biochemical characteristics. Then they were confirmed by using a specific primer and polymerase chain reaction (PCR). Using PCR, the frequency of enterotoxin-producing genes was detected and the antibiotic resistance of *Staphylococcus aureus* isolates was checked by disc diffusion method in food samples. Among the 175 meat products tested, 28 hamburger samples (16%), 12 kebab samples (7%), and 15 handmade hamburger samples (about 9%) were pathogenic bacteria. were infected with *Staphylococcus aureus*. Compared to kebab samples, hamburger samples showed higher contamination, and more of them were reported as *Staphylococcus aureus* positive. *nuc* gene was detected in all bacteria identified by biochemical method. The presence of the sea gene was detected in 45 samples of tested meat products (25.71% of all samples). *sec* and see genes were found in 4 (2.28%) and 6 (4.08%) food samples, respectively. Genes encoding used and sed were not found in any of the studied samples. In addition, many isolates showed high resistance to tetracycline, clindamycin, and oxacillin; some were resistant to more than one antibiotic. In this study, the results obtained from the SEA-F and SEA-R blast analyses revealed that the sequences had more than 98% agreement with the overlapping results.

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1- Introduction

Food safety is a significant public health concern worldwide. *Staphylococcus aureus* (*S. aureus*) is indeed considered one of the most important foodborne pathogens worldwide. This bacterium is a gram-positive (stain purple by Gram stain), non-spore-forming, nonmotile, coagulase-producing facultative anaerobes that is cocci-shaped and tend to be arranged in clusters [1,2].

S. aureus as a dangerous pathogen produces a variety of virulence factors that contribute to its ability to cause food poisoning: Staphylococcal enterotoxins (SEs), exfoliative toxins (ETs) A and B (ETA and ETB), cytolysins (leukocidins and hemolysins), toxic shock syndrome (TSS), enzymes (coagulase, hyaluronidase, staphylokinase, lipase, and nuclease), antigens: (capsule and adhesins), and so on. To synthesize SEs, the required critical density of the coagulase-positive *S. aureus* strain should be higher than 10^5 colony-forming units (CFU) per gram or milliliter. Staphylococcal enterotoxins are globular proteins with a molecular weight of 22-29 kDa and belong to the large family of pyrogenic toxin superantigens (PTSAgs). These toxins are considered to be one of the most potent food toxins; their very low concentrations (20 ng to 1 µg) cause symptoms such as fever, vomiting, nausea, abdominal pain and diarrhea [2,3]. Staphylococcal enterotoxins are generally divided into two main groups: Classical enterotoxins: These toxins all exhibit emetic activity and are the most commonly identified toxins (more than 75%) in staphylococcal food poisoning (SFP) outbreaks worldwide, and they include SEA, SEB, SEC, SED, and SEE. Non-classical (newer) enterotoxins: Non-classical SEs are new enterotoxins that have been isolated from outbreaks of SFP infections in 5% of cases. This group includes SEG–SElY and beyond. *S. aureus* is frequently isolated from environmental samples as well as from the skin, nostrils and respiratory system of animals and humans. However, various foods such as milk and dairy products, raw meat and meat products, bakery products, salads, eggs and egg-based foods, ready-to-eat foods and seafood, etc., are commonly involved in staphylococcal food poisoning [1,4]. Staphylococcal food poisoning (SFP) is an intoxication that results from the consumption of foods comprising sufficient quantities of one or more preformed enterotoxin. Contaminated food is a potential carrier of enterotoxin-producing *S. aureus* strains. This contamination may occur in the following different ways: food-contact surfaces, food producers and distributors, raw materials, tools, equipment, and utensils, air and dust, *etc*. Food handlers carrying enterotoxin producing S. aureus in their noses or on their hands are regarded as the main source of food contamination via manual contact or through respiratory secretions. In fact, *S. aureus* is a common commensal bacterium of the skin and mucosal membranes of humans and most mammals. Raw meat and meat products have been identified as major reservoirs of *S. aureus* contamination [2,4]. Proper heat treatment often eliminates vegetative forms of *S. aureus*. However, staphylococcal enterotoxins are highly resistant to thermal treatments and digestion by proteolytic enzymes, even though the vegetative bacterial cells can be inactivated by heat. Therefore, identification of enterotoxin producing staphylococci, as well as assessing other microbial risk factors associated with raw and pre-processed meat products, is of critical importance for food safety. Additionally, risk assessment and microbial monitoring play a significant role in ensuring the quality of meat products [2,4]. In this regard, several studies have been conducted. Komodromos et al. (2022) investigated the prevalence, genetic diversity, and infectious characteristics of *S. aureus* and methicillin-resistant *S. aureus* (MRSA) from the incoming meat and the meat products, the environment, and the workers' nasal cavities, in two meat-processing establishments in northern Greece. *S. aureus* was isolated from 13.8% of the 160 samples examined, while only one sample (0.6%) was found to be contaminated with MRSA carrying the *mecA* gene. The evaluation of the

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antimicrobial susceptibility of the isolates showed their low antimicrobial resistance. A resistance rate to penicillin antibiotics (68.2%), amoxicillin/clavulanic acid (36.4%) and tetracycline (18.2%) was found. 31.8% of the isolates were sensitive to all antimicrobial compounds examined [5]. In the study was designed by Mohammed, *et al.*, (2021) *S. aureus* was detected in 86% of the tested samples, 90% in beef burger and 82% in hot dog sandwiches. Out of the 106 confirmed coagulase-positive strains, approximately 13% were enterotoxin producing strains and about 44 of them carried the *mecA* gene, indicating their methicillin resistance [6]. Savariraj, *et al.*, (2021) studied the prevalence and enterotoxin gene profiles of *S. aureus* isolated from retail chicken meat in Chennai, India. Among the 120 meat samples collected, 66.67% (80/120) of the samples were positive for the presence of *S. aureus*. The profile of enterotoxin coding genes of the isolates showed the presence of 9 genes (*sea-sej*) that 50.52% (42/80) of the isolates carrying one or more genes coding for these toxins. The seb gene was detected in many isolates, followed by *seg*, *sei*, *sec*, *sed*, and *sej* either alone or in combination. None of these isolates harbored *sea*, *see*, and *seh* [7].

Generally, it is extremely difficult to detect S. aureus and Staphylococcal enterotoxins (SEs) in food. The polymerase chain reaction (PCR) is one of the most efficient and rapid method for identification of many foodborne pathogens, such as *S. aureus* strains and its synthetic enterotoxins. Therefore, according to the above-mentioned, the aim of present study was to investigate enterotoxigenic *S. aureus* isolated from hamburgers and kabab loghmeh and the SE gene profile in Mashhad.

2- Materials and Methods

2.1 Materials

Peptone water, Brad-Parker agar (BPA), menthol salt agar (MSA), nutrient agar (NA) and egg yolk were purchased from Ibresco company (Iran). DNase test agar and agarose were prepared from Condalab company (Spain). Mueller Hinton agar (MHA), glycerol and hydrochloric acid (HCL) were bought from Merk company (Germany). Ladder DNA marker, primers, master Mix and DNA green viewer were purchased from Sinaclon (Iran). DNA loading dye and TBE (Tris/Borate/EDTA) buffer were obtained from Parstous company (Iran). Gram differential staining kit was bought from Labtron company (Iran). Catalase reagent, lyophilised rabbit plasma and immersion oil were prepared from Bahar Afshan company (Iran). Potassium tellurite was purchased from Quelab (Canada).

2.2 Methods

2.2.1 Preparation of Microbial Culture Media

Buffered peptone water (BPW) was applied to prepare the initial suspension and decimal dilutions. Brad-Parker agar was used for selective isolation and enumeration of coagulase-positive *S. aureus*. After the end of autoclaving and cooling of the culture medium to approximately 45 ^{o}C, 10 mL of potassium tellurite solution and 50 mL of egg yolk were added to the culture medium using a sterile pipette. Additionally, mannitol salt agar (MSA) selective-differential culture medium was used to confirm the presence of *S. aureus* bacteria. The susceptibility of *S. aureus* to antibiotic agents was also tested using Mueller-Hinton agar (MHA) culture medium. Nutrient agar (NA) non-selective and nutritious culture medium was used for the growth and proliferation of this bacterium [8].

2.2.2 Preparation of 0.5 McFarland Standard

McFarland standards were used as the reference in order to adjust the turbidity of the bacterial suspension. After culturing *S. aureus* on the surface of NA culture medium, using a sterile loop, a single colony of the bacterium was

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removed and suspend in a tube containing physiological serum. McFarland turbidity standards were used to standardize the approximate number of bacteria in a liquid suspension by visually comparison of turbidity levels between the test suspension and the McFarland standard. In order to determine the proper density of standard turbidity, the amount of light absorption was measured using a spectrophotometer. A 0.5 McFarland standard was prepared by mixing 0.05 mL of 1.175% barium chloride dihydrate ($BaCl_2.2H_2O$), with 9.95 mL of 1% sulfuric acid (H_2SO_4). After shaking well, the turbidity of prepared solution was compared with a white background with thick black horizontal lines. The resulting mixture was placed in a foil-covered screw-cap tube and kept at room temperature (25 °C) [9].

2.2.3 Ethical Considerations

In the present investigation, all the information received from the food factories producing kababs and hamburgers was confidential. No fees were received from the food manufacturers. The ethics code with ID IR.IAU.MSHD.REC.1402.072 was obtained from the Research Ethics Committee.

2.2.4 Sample Collection and Preparation

In this descriptive research, a total of 175 meat samples including 70 hamburger, 70 kabab loghmeh and 35 handmade hamburger samples in a non-repetitive manner from commercial brands and different meat percentages (30, 60, 70 and 90%) were collected by cluster method during the month from April to June from different areas of Mashhad. Next, meat samples in the vicinity of ice packs, transferred to the Microbiology Laboratory of Islamic Azad University of Mashhad. First, 10 gr of each sample was transferred under sterile conditions by sterile tweezers to tubes containing 90 mL of buffered peptone water and mixed well for 5 min to form a homogeneous suspension. Then, 100 µL of the homogenized mixture was cultured on the surface of Brad-Parker agar culture medium. Finally, the inoculated plates were incubated at 37 °C for 24-48 hours [1].

2.2.5 Revival of Standard Bacterial Culture

In order to control the results obtained from this research, a lyophilized ampoules of standard strain, *S. aureus* ATCC 33591, was purchased from Iranian Research Organization for Science and Technology (IROST). First, to activate the lyophilized bacteria, the head of ampoule was broken according to the producer's instructions under sterile conditions. Using a sterile Pasteur pipette, about 0.4 mL of nutrient broth culture medium was added to the ampoule and mixed well. Afterward, the prepared suspension was inoculated in tubes containing buffered peptone water and next, the bacterial suspension cultured on Bird-Parker agar. The samples were incubated at 37 °C for 24-48 hours [10].

2.2.6 Morphological and Biochemical Characterization of *S. aureus* Isolates

The presumptive *S. aureus* isolates were identified by different morphological and biochemical tests. All experimental protocols were approved by the Institute of Standards & Industrial Research of Iran (ISIRI): NO: 6806-1 (Microbiology of food and animal feeding stuffs - Enumeration of coagulase - Positive Staphylococci (*Staphylococcus aureus* and species) technique using Baird-Parker agar medium, 2006) [11]; NO: 2304 (Raw frozen hamburger- Specifications, 2007) [12].

2.2.6.1 Morphological Test

In order to investigate the macroscopic morphological characteristics, first, 10 gr of the homogenized sample was completely mixed with 90 mL of buffered peptone water to create a homogeneous suspension. Later, 100 µL of the prepared suspension was cultured on the

[DOI: 10.22034/FSCT.21.157.157]

surface of Bird-Parker agar medium and the plates incubated at 37 °C for 24-48 hours. The grown colonies were examined and classified into two main groups: Defined colonies: They appeared as shiny, gray-black, convex colonies (with a diameter of 1-1.5 mm after 2 hours of incubation and a diameter of 1.5-2.5 mm meters after incubation for 48 hours). Some strains of *S. aureus* produce both clear and opaque zones; Undefined colonies: They appeared as gray-shiny black colonies, with no visible zones surrounding the colonies [13].

2.2.6.2 Biochemical Tests

Defined colonies of *S. aureus* used to perform biochemical tests (catalase, coagulase, DNase and mannitol fermentation) [13].

2.2.6.2.1 Catalase Test

The catalase test was used to distinguish staphylococci (catalase-positive) from streptococci (catalase-negative). Hydrogen peroxide (H_2O_2) is one of the final products of the oxidative metabolism of aerobic and facultative anaerobic bacteria. H_2O_2 accumulation in bacterial cells can damage biomolecules and stimulates cell death. However, bacterial catalase enzyme breaks the released H_2O_2 into water and oxygen molecules. Consequently, a simple test to demonstrate if bacteria produce catalase is to add H_2O_2 to bacteria. In order to perform this test, a drop of H_2O_2 was poured on the surface of a clean slide and a colony of pure bacterial culture was added to it using a sterile swab. This reaction was evidenced by the rapid formation of bubbles showing a positive test [13].

2.2.6.2.2 Deoxyribonuclease (DNase) Test

The deoxyribonuclease is an important diagnostic test for identification of *S. aureus* strains. For this purpose, a pure colony of *S. aureus* was cultured on the surface of DNase agar medium and after 24 hr of incubation at 37°C, the formation of a clear zone around the colony was checked. A clear zone surrounding bacter ial growth indicated a positive reaction [13].

2.2.6.2.3 Coagulase Test

Coagulase is a protein enzyme produced by some *S. aureus* strains that enables the conversion of fibrinogen to fibrin. This test was done by adding 5 drops of an overnight broth culture of the test organism to l mL of human or rabbit plasma diluted l:5 in physiological serum. The tubes are incubated at 37 °C for 4 hr in water bath and inspected hourly for clot formation by leaning the tube. Clot will float in the fluid or the whole plasma converts into gel [13].

2.2.6.2.4 Mannitol Fermentation

Unlike coagulase-negative staphylococci, *S. aureus* is capable of fermenting mannitol. Fot this purpose, *S. aureus* was cultured on the surface of Mannitol salt agar (MSA) medium and then the plates were incubated for 24 hr at 37 °C. The formation of yellow colonies indicated the fermentation of mannitol and the confirmation of *S. aureus* [13].

2.2.7 DNA Extraction by Boiling Method

First, a pure colony of *S. aureus* was dissolved in 1 mL of nutrient broth medium and the suspension was centrifuged at 3000 rpm for 2 min. After discarding the supernatant, the pellet was redissolved in 500 µL of distilled water, and the mixture was recentrifuged at 3000 rpm for 2 min. This step was repeated three times to completely isolate the bacterial cells from the culture medium. Next, 200 µL of distilled water was added to the microtube containing the bacterial culture and the microtube was placed in a hot water bath with a temperature of 95 °C until the bacterial cells were lysed, the proteins

[DOI: 10.22034/FSCT.21.157.157]

were denatured and also, the genetic material was released. After that, the microtubes were centrifuged at 10000 rpm for 10 min. At the end, the supernatant containing the extracted DNA was kept in a -20 °C freezer [14].

2.2.8 Determination of the Quantity and Quality of DNA by a NanoDrop Spectrophotometer

The quantity and quality of the extracted DNA was measured using a NanoDrop spectrophotometer. The ratio of absorbance at 260 and 280 nm was used to assess the purity of DNA. The 260/280 absorption ratio of 1.8-2 considered as evidence of absence of protein contamination and purity of DNA [15].

2.2.9 Confirmation of the Identity *of S. aureus* Isolates using *nuc* Gene Specific Primer

All *S. aureus* isolates were confirmed by PCR reaction using the specific primer of the heat-resistant nuclease (*nuc*) gene (Table 1). For this purpose, primers based on previous studies were used [16]. Specificity of the primers for the desired sequence was blasted and checked at the NCBI site. Primers was synthesized by SinaClon and The thermal cycler was used for the amplification for bacterial gene according to a specific time and temperature schedule. The amount and volume of materials used in *nuc* gene PCR is shown in Table 2. Gene amplification was performed by thermocycler Kyratec in Korea and according to the temperature- time program in 35 cycles (Table 1). The reference strain, *S. aureus* ATCC 33591, was used as a positive control.

Table 1. PCR reaction conditions used for *nuc* gene amplification to identify *S. aureus* isolates.

Step	Temperature (ºC)	Time (min)	Cycle No.
Initial denaturation	94	5	
Denaturation	94	45	35
Annealing	58	45	
Extension	72	48	
Final extension	72	5	

2.2.10 Electrophoresis of PCR Products

After PCR and gene amplification, gel electrophoresis was used to determine molecular weight and separate charged DNA molecules. First, 0.8 gr of agarose powder was mixed with 60 ml of 1x TBE solution and heated until the agarose powder was fully dissolved and a clear solution was appeared. After cooling the solution and reaching its temperature to approximately 50 °C, 3 µL of 0.5% Green-weaver dye was added to the solution. Then, the mixture was poured into the gel casting tray and the combs of the well were placed on the gel. After gel solidification, the combs were carefully removed and the gel was placed in the electrophoresis chamber, which was already filled with TBE buffer solution. Next, 3 µL of the PCR product was mixed with 1 µL of loading buffer and poured into the gel

[DOI: 10.22034/FSCT.21.157.157]

wells, and 3 µL of DNA Ladder Size marker was added to the middle well of the column. In order to trace the PCR products on the gel, after the end of the electrophoresis time, the gel was placed in the gel documentation system and imaging of the gel was done [1, 17].

2.2.11 Rapid Detection of *S. aureus* in Food Samples Using *nuc* Gene Specific Primers

In this study, *S. aureus* was detected by PCR using specific primers based on the *nuc* gene. For this purpose, 10 g of homogenized hamburger samples were added to 90 mL of Brain heart infusion (BHI) broth medium. The samples were incubated at 30 °C for 18 hours. Next, the samples were centrifuged at 12,000 rpm for 20 min. After discarding the supernatant, the pellets were mixed with 1 mL of sterile distilled water and placed in a hot water bath with a temperature of 100 °C for 10 min. Then, the samples were centrifuged at 3300 rpm for 5 min, and the supernatants were kept at -20 °C freezer. After direct extraction of food samples by boiling method, the required materials for PCR were prepared (Table 2) [16].

Table 2. Different volumes of ingredients used in *nuc* gene PCR to identify *S. aureus* isolates.

Ingredients	Initial concentration	Volume(µL)
DNA template	-	2
Upstream primer	10 Pmol/µL	1
Downstream primer	10 Pmol/µL	1
PCR master mix	2 X	12.5
Double distilled water	-	8.5
-	-	25

2.2.12 Determination of Antimicrobial Resistance by Disk Diffusion Method

The disk diffusion method was used to assess the antibiotic susceptibility of *S. aureus* against different antibiotics (Table 3) [2]. After adjusting the pH of the Mueller-Hinton agar medium in the range of 2.7-4.7, the plates were incubated at 37 °C for 24 hours. To prepare standard microbial suspension, overnight culture of bacteria was used. An amount of the colony was transferred to a tube containing 2 mL of sterile physiological serum, and after mixing, the turbidity of the bacterial suspension was compared with the 0.5 McFarland standard. Antibiotic discs were placed on the surface of MHI medium that has been inoculated to form the bacterial lawn. After incubation at 37 °C for 24 hours, the aura diameter of growth inhibition zone of bacterial growth was measured using a ruler and compared with the standard table. Disc diffusion test was performed for each of the isolates.

Table 3. The antibiotic discs used for antimicrobial susceptibility of *S. aureus* isolates.

[DOI: 10.22034/FSCT.21.157.157]

Antibiotic disc	Abbreviation	Antibiotic concentration g)(μ
Gentamicin	GM	10
Oxacillin	OX	1
Tetracycline	TE	30
Clindamycin	CC	2
Chloramphenicol	C	30
Trimethoprim	TMP	5
Erythromycin	E	15

2.2.13 Detection of Enterotoxigenic Isolates of *S. aureus* by PCR Method

In order to identify enterotoxin encoding genes in *S. aureus* isolates, PCR method was used. The genes studied in this investigation included *sea*, *seb*, *sec*, *see* and *sed* (Table 4).

Table 4. Characteristics of specific primers used in PCR reaction.

Target gene	PCR product length (bp)	Oligonucleotide sequence (5´-3´)
nuc	279	GCGATTGATGGTGATACGGTT
sea	127	AGCCAAGCCTTGACGAACTAAAGC
seb	477	CCTTTGGAAACGGTTAAAACG
sec	451	TCTGAACCTTCCCATCAAAAAC
sed	278	TCGCATCAAACTGACAAACG
see	209	AGGTACTCTATAAGTGCCTGCCT

2.2.14 Synthesis of Specific Primers

First, the sequence of *sea*, *seb*, *sec*, *see* and *sed* genes was obtained from the database of the National Center for Biotechnology Information (NCBI) and appropriate primers were selected to identification of the target genes. Then, in order to check the specificity and overlapping of the sequences, the primers were blasted in the NCBI database. To synthesize the primers, the target sequences were sent to SinaClon company. Table 4 shows the characteristics of the primers used in PCR. Synthetic primers were received in a lyophilized state, and dissolved in distilled water under sterile conditions according to the manufacturer's instructions. After that, the prepared solutions were diluted with distilled water at a ratio of 1:10.

[DOI: 10.22034/FSCT.21.157.157]

2.2.15 Optimization of the PCR Method for Detection Exotoxin Encoding Genes in *S. aureus* Isolates

For this purpose, color master mix of SinaClon company was used. Experiments were repeated several times with positive and negative control samples to obtain the best conditions for PCR of each gene. The reaction materials were added in a microtube under sterile conditions and in a container of water and ice (Table 5). The temperature-time-conditions of PCR used for the amplification of *sea, seb, sec, sed* and *see* genes are presented in Table 6.

Table 5. Different volumes of ingredients used in the amplification of enterotoxin genes in *S. aureus* isolates.

Ingredients	Initial concentration	Volume (µL)
DNA template	-	2
Upstream primer	10 Pmol/µL	1
Downstream primer	10 Pmol/µL	1
PCR master mix	2 X	12.5
Double distilled water	-	8.5
-	-	25

2.2.16 *sea* Gene Sequencing

In order to confirm the *sea* gene in *S. aureus* isolates, the PCR products of two isolates were sequenced by Sanger method using the desired primers in Central Jihad University Laboratory of Mashhad, and then checked by Snap Gene V5.3.1 software. The sequences were compared and blasted with the nucleotide information of *S. aureus* sequenced based on the *sea* gene available on the NCBI database. Moreover, in this study, the PCR products of two isolates that had the gene encoding for enterotoxin A were also blasted.

2.3 Data Analysis

In order to determine the antibiotic susceptibility, the disk diffusion method was repeated three times and the mean diameter of the corresponding inhibition zone was calculated. Excel software was used to plot the graphs. Additionally, NCBI database was also used for sequencing analysis.

Table 6. PCR reaction conditions used for *sea, seb, sec and sed* and *see* genes amplification to identify *S. aureus* isolates.

[DOI: 10.22034/FSCT.21.157.157]

Sea gene			
Step	(ºC) Temperature	Time(min)	Cycle No.
Initial denaturation	94	4	35
Denaturation	94	1	
Annealing	53	1	
Extension	72	1	
Final extension	72	10	
Seb gene			
Step	(ºC) Temperature	Time(min)	Cycle No.
Initial denaturation	94	4	35
Denaturation	94	1	
Annealing	55	1	
Extension	72	1	
Final extension	72	5	
Sec gene			
Step	(ºC) Temperature	Time(min)	Cycle No.
Initial denaturation	94	4	35
Denaturation	94	1	
Annealing	51	1	
Extension	72	1	
Final extension	72	5	
sed and *see* genes			
Step	(ºC) Temperature	Time(min)	Cycle No.

[DOI: 10.22034/FSCT.21.157.157]

Initial denaturation	94	4	35
Denaturation	**94**	**1**	
Annealing	50	1	
Extension	72	2	
Final extension	72	10	

3- Results and Discussion

3.1 Prevalence of *S. aureus* Isolated from Meat Samples

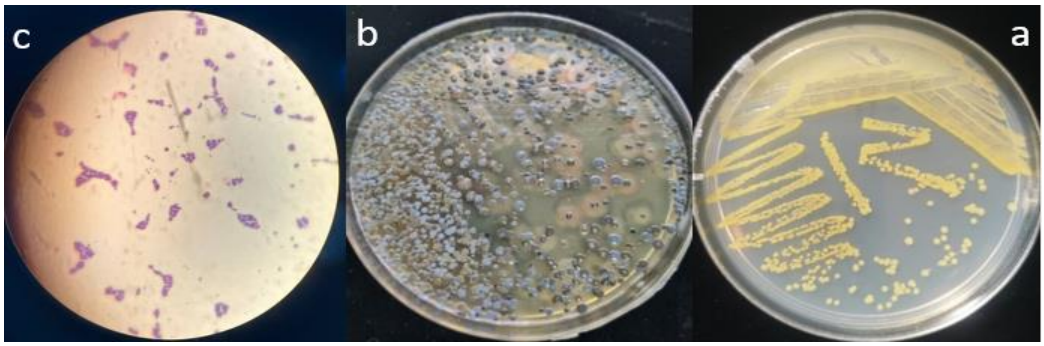
Several studies have shown that *S. aureus* frequently isolated from various foods, such as milk and dairy products, meat products, and traditional cream sweets [18]. The results obtained from the microbial and biochemical tests showed that among 175 samples, 28 hamburgers (16%), 12 kabab loghmehs (7%), and 15 handmade hamburgers (approximately 9%) were contaminated with *S. aureus*, and then, out of 175 samples, 55 (32%) were positive for *S. aureus*. In another study, 387 food samples were randomly collected from different areas of Baghdad. Morphological investigations and microbial tests performed on the samples led to the identification of 112 staphylococcal isolates. The results of biochemical and molecular analysis exhibited that among the isolated strains, only 49 were *S. aureus*. The highest prevalence of *S. aureus* isolates was found in ready-to-eat (RTE) or cooked foods (42.8%) followed by salads and appetizers (24.5%), meat products (14.3%), dairy products (10.2%) and cakes (8.2%), While, none of the chicken meat products were contaminated with *S. aureus* [1].

3.2 Morphological and Biochemical Identification of *S. aureus*

In order to characterization of *S. aureus*, the following morphological and biochemical tests were performed:

3.2.1 Characteristics of *S. aureus* on Nutrient Agar and Brad-Parker Agar Media and Gram Staining

In nutrient agar, the colony morphology was found to be golden yellow circular (Figure 1-a). In Baird-Parker agar, *S. aureus* isolates produce dark gray to black, shiny, convex colonies with entire margins and clear zones (Figure 1-b). Also, in the gram staining test, *S. aureus* colonies were all identified as gram positive cocci in clusters (Figure 1-c).



[DOI: 10.22034/FSCT.21.157.157]

Figure 1. *S. aureus* colonies on nutrient (a) and Baird-Parker (b) agar culture medium and gram staining of one of the isolates of *S. aureus* (gram-positive, magnification × 100) (c).

3.2.2 Biochemical Characterization of *S. aureus*

For this purpose, catalase, deoxyribonuclease (DNase), coagulase and mannitol fermentation tests were performed. The catalase test is used to distinguish staphylococci (catalase-positive) from streptococci (catalase-negative). Hydrogen peroxide (H_2O_2) is an important oxidizing molecule in the oxidative metabolism of aerobic and facultative anaerobic bacteria. Hydrogen peroxide is responsible for certain bactericidal activities observed in biological systems, such as growth inhibition of one bacterial species by another, and killing of attacking microorganisms by activated phagocytic cells. However, bacterial catalase enzyme decomposes H_2O_2 into water and oxygen. Performing this test was accompanied by the formation of oxygen gas bubbles, which clearly indicated a catalase positive result. (Figure 2-$a_{1,2}$).

In order to determine the ability of *S. aureus* to hydrolyze DNA and utilize it as carbon and energy source for its growth and reproduction, the deoxyribonuclease test was performed. The formation of a clear zone surrounding the growth line of *S. aureus* on the surface of DNase test agar showed production of DNase enzyme. It should be noted that all the isolates were DNase positive (Figure 2-$b_{1,2}$). The coagulase test was also used to distinguish between *S. aureus* (coagulase positive) and coagulase negative staphylococci. Coagulase is a plasma-clotting protein enzyme that binds to prothrombin and converts fibrinogen (soluble) to fibrin (insoluble). The results obtained from this test confirmed that the *S. aureus* isolates were positive for coagulase (Figure 2-$c_{1,2}$). It was also found that *S. aureus* was able to ferment mannitol and produced yellow colonies when colonized on the surface of a mannitol salt agar (MSA) medium. Many species of coagulase-negative staphylococci and micrococci that are unable to ferment mannitol are observed as small red colonies on the surface of MSA medium (Figure 2-$d_{1,2}$).

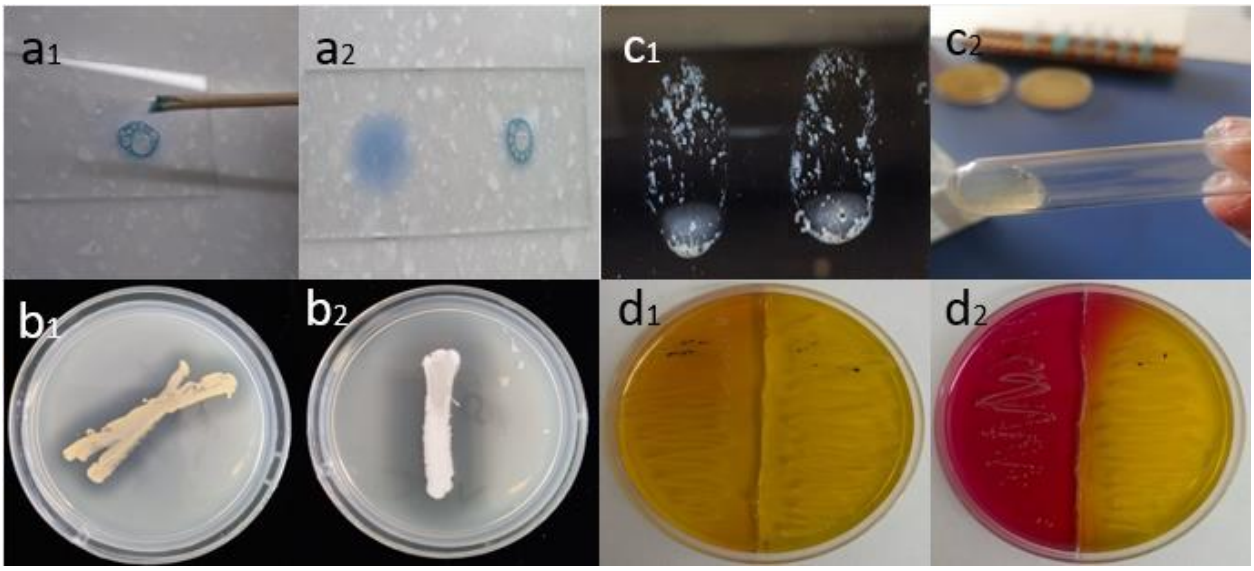


Figure 2. In the catalase test, the bubbles resulting from production of oxygen gas clearly indicate a catalase positive result ($a_{1,2}$), In the deoxyribonuclease (DNase) test, if *S. aureus* produce DNase enzymes, in sufficient quantity to hydrolyse the DNA, then clear zones are seen around the colonies ($b_{1,2}$), The coagulase test distinguishes *S. aureus* from other staphylococci, c_1: The slide test, c_2:

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The tube test and On mannitol salt agar (MSA), only pathogenic *S. aureus* produces small colonies surrounded by yellow zones, while other coagulase-negative staphylococci produce small pink or red colonies with no colour change to the medium ($d_{1,2}$).

3.3 Counting Bacterial Colonies of *S. aureus*

According to standard methods of Iranian national standards No. 6806-1, the number of colonies on the plate containing 10 gr of food culture, ranging from 1 to 1000, was considered +1 [11]. Those that ranged from 10^3 to 10^4 were reported as +2, and those that had more than 10^4 were considered as +3. Among the 55 isolates identified in the present study, 44, 6, and 5 isolates were classified as +3, +2, and +1, respectively (Table 7).

Table 7. The results of counting *S. aureus* colonies.

Colony No.	Isolate No.
1+	5
2+	6
3+	44

3.4 Results of DNA Quality and Quantity Determination

Table 8 shows the quantitative results obtained from the NanoDrop spectrophotometer of some *S. aureus* isolates.

Table 8. Quantitative estimation of DNA concentration by NanoDrop Spectrophotometer.

Position	260 (nm, raw)	280 (nm, raw)	320 (nm, raw)	260 (nm)	280 (nm)	260/280	L/µµg
A3	0.58	0.055	0.047	0.002	0.003	0.594	0.002
B2	0.067	0.055	0.045	0.013	0.006	2.339	0.013
B3	0.073	0.065	0.055	0.010	0.005	1.827	0.010
C2	0.091	0.078	0.062	0.020	0.011	1.789	0.020
C3	0.079	0.070	0.055	0.014	0.010	1.469	0.014
D2	0.132	0.111	0.087	0.036	0.019	1.900	0.036
D3	0.083	0.079	0.073	0.00	0.001	0.571	0.00

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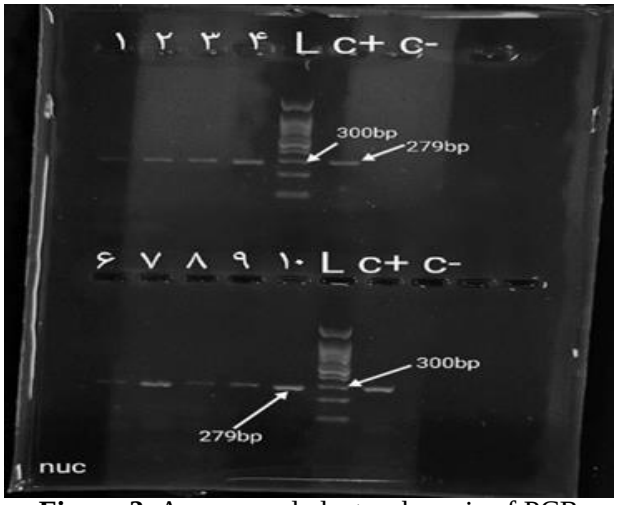

Figure 3. Agarose gel electrophoresis of PCR product using primers for detection of the *nuc* gene of *S. aureus*. L: Ladder, C^+: Positive control, C^-: Negative control

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3.5 Results of Identification of *S. aureus* Isolates By *nuc* Specific Primers

For this purpose, a 100 base pair (bp) marker was used. The PCR product of *nuc* gene encoding thermonuclease enzyme was a fragment equal to 279 bp. After several repetitions, the optimal temperature for PCR of this primer was obtained with temperatures of 60-59-58-57-56 °C by gradient method at 58 °C. Among a total of 175 food samples, the *nuc* gene was detected in 55 meat products (28 hamburgers, 12 kabab loghmehs, and 15 handmade hamburgers, 31.5% of all samples) (Figure 3).

3.6 Determination of Antimicrobial Resistance of *S. aureus*

In Table 9, the results of the antibiotic susceptibility of *S. aureus* isolated (n = 55) from hamburger and kabab samples against different antibiotics are presented. *S. aureus* displayed the highest resistance to both tetracycline and clindamycin and lowest against gentamicin antibiotic. According to the findings of this research, 52 isolates of *S. aureus* (94.5%) were found to be low resistance to gentamicin, only one isolate (1.8%) was semi-susceptible, and 2 isolates (3.6%) were resistant. Out of 55 examined isolates, 37 isolates (57%) were resistant to oxacillin. Furthermore, 40 isolates of *S. aureus* (72%) showed high resistance to tetracycline and clindamycin antibiotics. Regarding the antibiotic effect of chloramphenicol, it can be mentioned that 37 isolates (67.2%) were susceptible and 18 isolates (32.8%) were resistant.

Only 13 isolates (23.7%) exhibited resistance toward the inhibitory activity of trimethoprim. Examination the antibiotic effect of erythromycin on the isolates also revealed that 14 isolates (25.4%) were susceptible, 2 isolates (3.6%) were semi- susceptible, and 39 isolates (70.9%) were resistant (Figure 4). Multidrug resistance (MDR) is defined as resistance to three or more antibiotic classes which is increasing worldwide. It can be concluded that among all isolates, 40 isolates (72%) showed resistance against both tetracycline and clindamycin. Besides, 39 isolates (70.9%) were resistant to erythromycin, and 37 isolates (57%) showed resistance to oxacillin.

In a similar study, the prevalence, antibiotic susceptibility and ability to produce enterotoxin

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of *S. aureus* strains isolated from retail raw meats in Turkey were investigated. Based on the findings of the research, it was found that the prevalence of *S. aureus* in the screened samples was found to be 21.23%. In addition, SE gene regions were identified in 65.62% of strains. The predominant SEs detected in the isolates were *sea*, *sed*, and *seb*. Many staphylococcal isolates displayed resistance to kanamycin, telithromycin, penicillin G, streptomycin, erythromycin, cloxacillin, ampicillin, pristinamycin, nalidixic acid, azithromycin, and ciprofloxacin, and MDR was detected in 96.87% of isolates [2].

3.7 Results of Rapid Detection of *S. aureus* in Food Samples by *nuc* Specific Primers

Five food samples, those colony counts were +3 and +4 in culture, were directly extracted and amplified by PCR. Electrophoresis of PCR products of *S. aureus* isolates were carried out by identification of the *nuc* gene in 5 food samples (2 hamburgers, 2 kabab loghmehs and 1 handmade hamburger) (Figure 5).

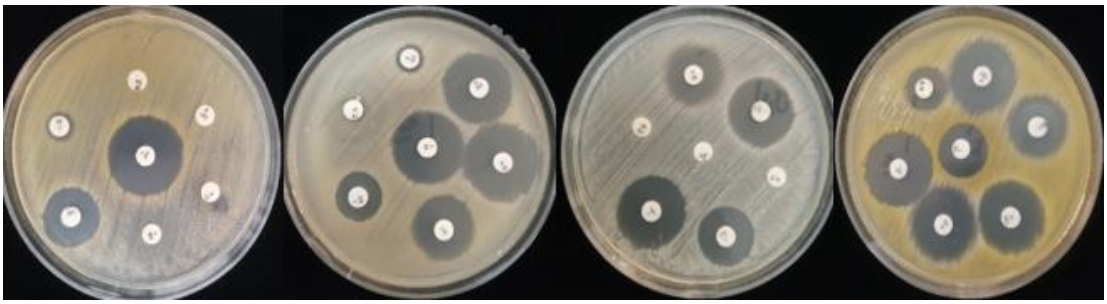

Figure 4. Antibiogram test results of some *S. aureus* isolates.

3.8 Results of Tracking *seb*, *sed*, *sea*, *sec* and *see* Genes using PCR

The *seb* and *sed* genes were not found in any of the studied meat samples. The presence of *sea* gene was detected in 45 samples of the tested meat products (25.71% of all collected samples). The PCR products of *S. aureus* isolates to detect the *sea* gene were a fragment with a length of 127 bp (Figure 6-a). A DNA ladder was used to determine the molecular weight and accuracy of detect genes. Of the 55 isolates studied, 4 isolates were positive for *sec* gene (2.28% of all collected samples). The length of PCR products of *sec* gene was equal to 451 bp (Figure 6-b). PCR products of *see* gene formed a band in 209 bp region. Out of 55 isolates, the presence of *see* gene was detected in 6 isolates (4.08% of all collected samples) (Figure 6-c).

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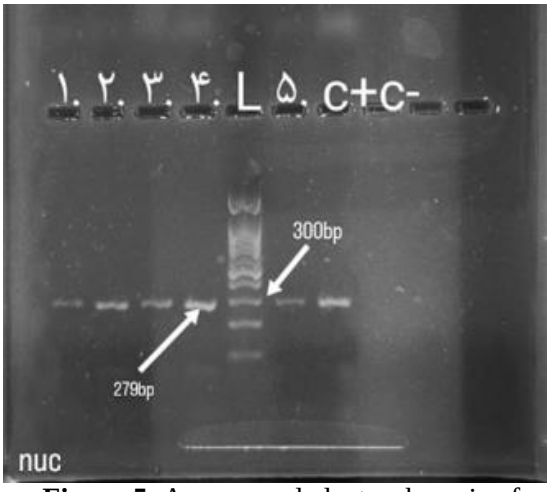

Figure 5. Agarose gel electrophoresis of PCR product using primers for detection of the *nuc* gene of *S. aureus* by direct extraction. L: Ladder, C^+: Positive control, C^-: Negative control.

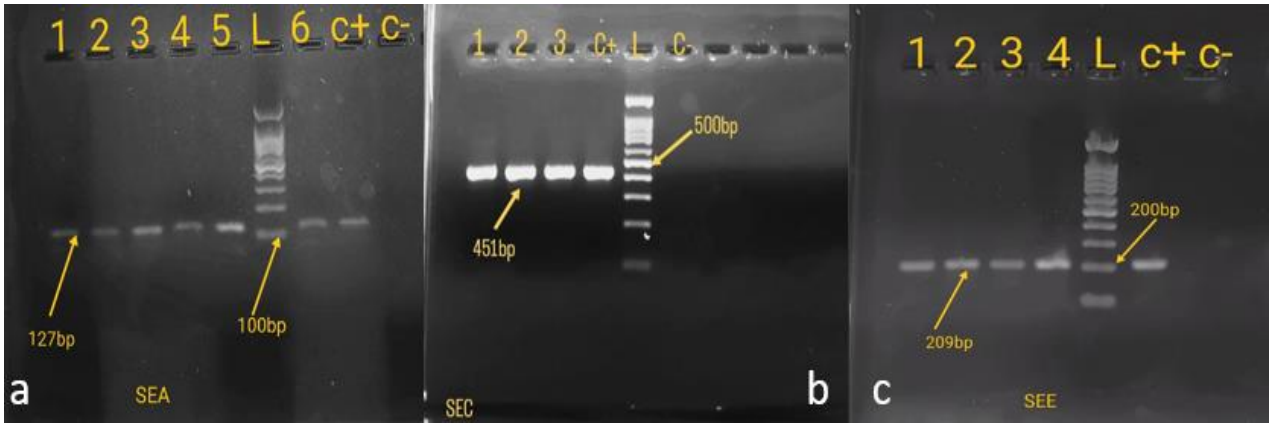

Figure 6. Agarose gel electrophoresis of PCR product using primers for detection of the *sea* (a), *sec* (b) *and see* (c) genes of *S. aureus*. L: Ladder, C^+: Positive control, C^-: Negative control.

3.9 Identification of *S. aureus* Isolates by 16S rRNA Gene Sequencing

The results of the SEA-D4 and SEA-A4 BLAST analysis revealed that the sequences overlapped with more than 98% matching results. Then, the results were entered into GenBank, and in this database, the of gene, protein, and their sequence indicated that the sequence was enterotoxin A. In all BLAST analysis, only the results related to enterotoxin A gene was displayed. Similarly, genomic DNA was successfully purified from different isolates of *S. aureus* without cleavage or contamination of the DNA. The findings of the study showed that the 16S rDNA genes of different isolates with a size of approximately 1525 bp were successfully amplified. The 16S rDNA sequence data obtained from the GenBank database confirmed that all the isolated strains (100%) were identified as *S. aureus* strains and the similarity of the BLAST analysis was in the range of 96-99% [19].

Also, in the present study, the protein sequence was transferred to NCBI protein BLAST to confirm, and the result of the BLAST again showed that the sequence was related to enterotoxin A with 100% match. Consequently, all the results obtained from the BLAST were only related to enterotoxin A gene and BLAST analysis revealed no similarity to other proteins.

[DOI: 10.22034/FSCT.21.157.157]

Each peak represents a single nucleotide in the DNA sequence, and each nucleotide has a different colour: Black (G), Blue (C), Red (T) and Green (A). In the blast of SEA-A4 and SEA-D4 isolates, only three nucleotide mismatches were observed, which are marked with blue, yellow and red colors. It was also placed in two regions of nucleotides A and G and in the other sequence of nucleotides T and C (Table 10).

Table 10. Sequence of SEA-A4 and SEA-D4 isolates.

Sequence SEA-A4	CCATAATAAGCACCATACAAGTCTACTTTTTTCCCTTTATATTTATCAACAA TATCCTTTGAATCAAAATCTACTAATAAATCGTTATACCACGGATGACCTG TGAAAAA
Gen Bank: CP053353.1 staphylococcal enterotoxin type A	CCATAATAAGCACCATACAAGTCTACTTTTTTCCCTTTATATTTATCAACAA TATCCTTTGAATCAAAATCTACTAATAAATCGTTATACCACGAATGATCTG TAAAAAA
Sequence SEA-D4	TGATAACCATAATAAGCACCATACAAGTCTACTTTTTTCCCTTTATATTTAT CAACAATATCCTTTGAATCAAAATCTACTAATAAATCGTTATACCACGGAT GACCTGTGAAAA
Gen Bank: CP053639.1 staphylococcal enterotoxin type A	TGATAACCATAATAAGCACCATACAAGTCTACTTTTTTCCCTTTATATTTAT CAACAATATCCTTTGAATCAAAATCTACTAATAAATCGTTATACCACGAAT GATCTGTAAAAA

4-Conclusion

The results of this study showed that of the total samples (175 meat products), 28 hamburgers (16%), 12 kabab loghmehs (7%), and 15 handmade hamburgers (9%) were contaminated with *S. aureus*. The *nuc* gene was detected in 55 meat products (28 hamburgers, 12 kabab loghmehs, and 15 handmade hamburgers, and a total of 31.5% of samples). In 45 samples of examined meat products (8.81%), the presence of *sea* gene was discovered. The *sec* and *see* genes were found in 4 (2.7%) and 6 (11%) food samples, respectively. In addition, the *seb* and *sed* genes were not detected in any of the tested samples. Also, the results of the SEA-F and SEA-R BLAST analyzes indicated that the sequences had more than 98% matching with the overlapping results. The results obtained from the protein sequence in NCBI protein BLAST with 100% match exhibited that the sequence was related to enterotoxin A. Furthermore, the results of antibiotic susceptibility revealed a high resistance of *S. aureus* to the test antibiotics. Therefore, according to the obtained results, it can be noticed that the presence of Staphylococcus aureus carrying virulence factors, as thermostable nuclease and enterotoxins, should be considered as a potential risk factor for food safety.

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مقاله علمی_پژوهشی

بررسی فراوانی ایزوله‌های *استافیلوکوکوس اورئوس* مولد انتروتوکسین در همبرگر و کباب لقمه در مشهد و پروفایل ژن SE

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چکیده

هدف از این مطالعه بررسی فراوانی ایزوله‌های *استافیلوکوکوس اورئوس* مولد انتروتوکسین در همبرگر و گوشت لقمه در مشهد و فراوانی ژن SE بود. بدین منظور تعداد ۱۷۵ نمونه‌ی گوشتی با روش نمونه‌برداری خوشه‌ای، از برندهای تجاری مختلف طی فروردین تا خرداد ۱۴۰۲ از مراکز عرضه در سطح مشهد جمع‌آوری شد. پس‌از کشت نمونه‌ها در محیط کشت‌های اختصاصی و جداسازی ایزوله‌های مشکوک به *استافیلوکوکوس اورئوس*، باکتری‌ها با استفاده از ویژگی‌های مورفولوژیکی و بیوشیمیایی شناسایی شدند و سپس، با استفاده از پرایمرهای اختصاصی و واکنش زنجیره پلیمراز (PCR) تأیید هویت شدند. فراوانی ژن‌های مولد انتروتوکسین نیز ردیابی گردید و مقاومت آنتی‌بیوتیکی ایزوله‌های *استافیلوکوکوس اورئوس* با روش دیسک دیفیوژن بررسی شد. نتایج نشان داد که از ۱۷۵ فراورده‌ی گوشتی، ۲۸ نمونه‌ی همبرگر (۱۶ درصد)، ۱۲ نمونه‌ی کباب لقمه (۷ درصد) و ۱۵ نمونه‌ی همبرگر دستی (۹ درصد) به باکتری *استافیلوکوکوس اورئوس* آلوده بودند. در مقایسه با نمونه‌های کباب لقمه، نمونه‌های همبرگر آلودگی بیشتری داشتند و به‌عنوان *استافیلوکوکوس اورئوس* مثبت گزارش شدند. ژن *nuc* در تمامی باکتری‌های شناسایی‌شده با روش‌های بیوشیمیایی ردیابی گردید. حضور ژن *sea*، *sec* و *see* به ترتیب در ۴۵ (۲۵/۷۱ درصد)، ۴ (۲/۲۸ درصد) و ۶ (۴/۰۸ درصد) نمونه تشخیص داده شد. در هیچ‌یک از نمونه‌های غذایی، ژن‌های کدکننده‌ی *seb* و *sed* یافت نشد. به‌علاوه، بسیاری از ایزوله‌ها مقاومت بالایی در برابر آنتی‌بیوتیک‌های تتراسایکلین، کلیندامایسین و اگزاسیلین نشان دادند. همچنین براساس نتایج به دست‌آمده از آنالیزهای بلاست SEA-F و SEA-R مشخص شد که توالی‌ها دارای بیش‌از ۹۸ درصد تطابق با نتایج هم‌پوشانی بودند.

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