

Staphylococcus aureus Enterotoxin A Gene Isolated From Raw Red Meat and Poultry in Tehran, Iran

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Background: *Staphylococcus aureus* is the most prevalent infectious agent of food materials. Enterotoxin producing types of *S. aureus* cause well-known food-borne disease. Staphylococcal Enterotoxin A (SEA) is the most important agent of gastroenteritis.

Objectives: The present study aimed to screen the raw meat samples collected from different regions of Tehran for *S. aureus* infection and type of encoding enterotoxin.

Materials and Methods: Hundred and eighty six meat samples were collected randomly from city dealers and transferred to laboratory within screw cap containers. The samples were first cultured according to the standard bacteriological methods and then *S. aureus* isolates were identified using standard bacteriological tests. The isolates were subjected to Polymerase Chain Reaction (PCR) to detect gene encoding SEA.

Results: *Staphylococcus aureus* isolated from 29 (15.6%) meat samples including beef 14.8%, raw lamb 15%, raw chicken 15.7% and raw turkey 16.6%. Using special primer sets proved that the species isolated from five samples (two raw chicken, two raw beef and one raw turkey) encoded enterotoxin A.

Conclusions: Although staphylococcal contamination within food material is more or less a routine, but detection of enterotoxin encoding species from raw meat samples is alarming for health authorities. These data highlight the importance of periodic surveillance of raw meat distributed among ordinary consumers.

Keywords: Staphylococcus Aureus; Enterotoxin; SAE; Food Poisoning; Raw Meat

1. Background

Staphylococcus aureus is one of the most commonly found pathogenic bacteria which is hard to eliminate from the human environment (1). *Staphylococcus aureus* produces a group of 21 enterotoxins, many of which are heat-resistant in foods (2, 3). Therefore, measures to prevent the growth of *S. aureus* are critical because normal temperatures used in cooking will not destroy the toxins, and foods containing staphylococcal enterotoxin usually look and taste normal (4, 5). This may be enhanced by physico-chemical factors that affect the meat quality and allow the food contaminant bacteria resist against some environmental conditions (6, 7) removed by another bio-substances (8).

Staphylococcal enterotoxin (SE) A is one of the most important gastroenteritis causing agents. In some areas, more than 50% of food poisoning (FP) is caused by

Staphylococcal Enterotoxin A (SEA) (7). The primary habitat of this microorganism is the mucosa of the nasopharynx and the skin of humans and animals (9). Despite its pathogenicity, *S. aureus* is also harbored in the nares of about 20 to 30% of healthy people, while about 60% of the population harbors the microorganism intermittently (10). Although the number of outbreaks reported annually has decreased in the last few decades, staphylococcal food poisoning is still reported as the third most prevalent cause of foodborne illness worldwide (6, 11). In several countries the foods that most frequently cause this type of food poisoning are red meat, poultry, and their products (12, 13). It has been reported in various countries that most raw fresh and frozen poultry, both chicken and turkey, are frequently contaminated with *S. aureus*. To prevent food poisoning, it is important to determine how much actual contamination with enterotoxigenic *S. aureus* in retail raw chicken meat occurs (12). Contami-

Implication for health policy/practice/research/medical education:

The present study aimed to screen the raw meat samples collected from different regions of Tehran, Iran to detect the rate of contamination with *Staphylococcus aureus* and determine the type of encoding enterotoxin.

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nated raw meat is one of the main sources of food-borne illnesses (14). The risk of the transmission of zoonotic infections is also associated with contaminated meat (15). The amount of staphylococcal enterotoxins required to establish typical symptoms of food poisoning is very low, ranging from 20 ng to 1 µg which corresponds to approximately 105 staphylococci colony-forming units per gram of food (10, 16). In humans, symptoms can occur within a few hours (1 to 6) after the ingestion of very small quantities of toxin (0.5 ng/mL) (6, 7). While staphylococci commonly occur on the skin and nasopharynx of healthy poultry, Staphylococci are among the most predominant groups during the slaughtering and processing of poultry, and they have been isolated from air samples, neck skin of chicken carcasses, and equipment and machinery surfaces. Also *S. aureus* can survive, colonize, and persist at various processing stages in plants due to the expression of various key properties, including adhesion and chlorine resistance. In a typical processing operation, after slaughtering and de-feathering, fresh chicken carcasses are eviscerated and washed. These procedures, especially de-feathering, increase the contamination by *S. aureus* (3, 17). The bacterial contamination of poultry products occurs due to its improper control that depends on various factors, such as initial level of contamination of carcasses, the duration and temperature of storage, and hygienic practices during handling (9). *Staphylococcus aureus* is frequently isolated from ground meat. Enterotoxigenic strains of *S. aureus* in ground meat can grow to a sufficient level to allow a toxic dose of enterotoxin produced prior to consumption. The initial contamination of meat occurs during slaughtering. Hygiene deficiencies cannot be compensated even by the most rigorous hygiene measures during post production process. Microbiological hygienic measures in meat production aim at protecting the consumer against pathogenic agents. To prevent contamination of meat with *S. aureus*, sources of this bacteria must be determined and known well (14). Moreover, enterotoxin genes are not distributed uniformly among different *S. aureus* strains in different areas. Genetic variation among enterotoxin genes occurs in these strains (18). The origin of SFP varies significantly among countries which are mainly due to different eating and consumption habits in those countries. Numerous studies on contamination of raw and cooked food with staphylococcal enterotoxigenic strains have been conducted in various countries.

2. Objectives

The present study aimed to evaluate the presence of *S. aureus* in red meat and poultry collected from retail stores in Tehran, Iran and to detect the presence of staphylococcal enterotoxin A gene by Polymerase Chain Reaction (PCR) method.

3. Materials and Methods

3.1. Samples

Hundred and eighty six samples of raw meats including 47 raw beef, 20 lamb, 89 chicken, and 30 turkey samples were collected randomly from retail butchereries and supermarkets of Tehran.

All samples were aseptically collected, placed in sterilized containers, and stored in a cool place to transfer to the laboratory.

3.2. Microbiological Analysis

A 25 g sample was homogenized using a meat grinder under aseptic conditions and it was added to 225 mL of sterile Buffered Peptone Water and incubated at 37°C for 24 hours in order to culture the organisms. Then, 0.1 mL of sample was plated onto Baird-Parker agar supplemented with egg yolk telluride emulsion and incubated at 37°C for 24 to 48 hours. Colonies showing characteristic phenotype of *S. aureus* (i.e, circular, black, convex and with or without light halo on BP agar) were sub-cultured on 5% sheep blood agar to isolate single colonies.

Staphylococcus aureus was identified through a characteristic hemolysis pattern on sheep blood agar, Gram staining results, catalase reaction (using 0.3% hydrogen peroxide) and coagulase tests. Confirmation was provided by PCR targeting the *S. aureus* specific *nuc* gene (*S. aureus* species specific).

3.3. Isolation of Genomic DNA

Total genomic DNA was isolated by a commercial kit (gram positive bacteria mini-prep genomic DNA extraction kit of IBRC, catalog # MBK0031) according to the supplier's instructions. lisozim and lisostafin enzymes were applied to destruct the bacterial cell wall. The presence, concentration, and purity of genomic DNA in the prepared samples were detected by measuring the absorbance at 260 and 280 nm wavelengths with an Ultraspec 3000 spectrophotometer.

3.4. PCR Primers

Primers for PCR were synthesized by TAG Copenhagen based on sequences published (18-20) for *entA* and (21) for *nuc* genes (Table 1). The BLAST was used to determine the specificity of the primer sequences further.

3.5. Polymerase-Chain Reaction (PCR)

Enterotoxin A gene was detected by PCR with the method described by Sharma et al. (20). Each polymerase chain reaction (PCR) contained 5 µL PCR Buffer 10x, 4 µL MgCl₂ 50 mM, 1 µM dNTP mix 10 mM, 1 U Taq DNA Polymerase, 10 pmol of each primer, and 1 µL DNA. The final volume

was adjusted to 50 µL by adding sterile ultra-pure water. The mixes were submitted to a program performed on a thermo cycler with an initial denaturation step at 94°C for 4 min, 35 amplification cycles each with 20 seconds at 94°C; 30 seconds at 48°C (for SAE) or 62°C (for *nuc*); 20seconds at 72°C followed by an additional extension step of 5 minutes at 72°C. Positive and negative controls were included in each PCR run. The following strains were used as positive controls in this study: ATCC 13565 or ATCC=25923 (SEA). Furthermore, all isolated strains were investigated and identified by PCR, and PCR products were visualized after electrophoresis on 2% agarose gel and the product size was estimated using a 100-bp DNA ladder.

4. Results

In the current study, 186 samples of meat materials including 47 beef, 20 lambs, 89 chickens, and 30 turkeys were collected and analyzed to detect *S. aureus*. Out of

these samples, 29 (15.6%) were identified as *S. aureus* positive using the above mentioned microbiological and biochemical methods. After DNA extraction, the samples were investigated for the presence of *nuc* gene. The 397 base pare fragment amplification of *nuc* gene shows the existence of this gene within the bacteria which determines the presence of *S. aureus*. The results showed that all 29 isolates were identified as belonging to *S. aureus*. Out of 29 confirmed isolates, 14 chickens (15.7%), 5 turkeys (16.6%), 3 lamb (15%), and 7 beef (14.8%) contained *S. aureus* (Table 2 and Figure 1).

PCR results revealed that 17.2% of *S. aureus* isolates encoded enterotoxin A. The frequency of enterotoxin prevalence in chicken, turkey and beef isolates was 14.2, 20 and 28.5%, respectively. No SEA was harbored in lamb isolates (Table 2), (Figure 1). The 270 bp fragment in PCR was related to amplification of a part of SEA gene, Figure 2 shows the result of PCR amplification of positive enterotoxin a samples.

Table 1. Nucleotide Sequence of Primers Chosen to Detect *nuc* and the Gene Encoding SEA For PCR

Gene	Sequence (5´-3´)	Size of Amplified Products (bp)
<i>nuc</i>	Forward: CTGGCATATGTATGGCAATTG	397
-	Reverse: AATGCACTTGCTTCAGGACC	-
SEA	Forward: TGTATGTATGGAGGTGTAAC	270
-	Reverse: ATTAACCGAAGGTTCTGT	-

Table 2. Detection of *Staphylococcus aureus* Encoding SEA Gene in Meat Materials by PCR

Meat Type	No. of Samples	No. of <i>S. aureus</i> Positive Samples (%)	No. of Enterotoxin A Positive Samples (%)
Chicken	89	14 (15.7)	02 (14.2)
Turkey	30	05 (16.6)	01 (20)
Lamb	20	03 (15)	00 (0)
Beef	47	07 (14.8)	02 (28.5)
Total	186	29 (15.6)	05 (17.2)

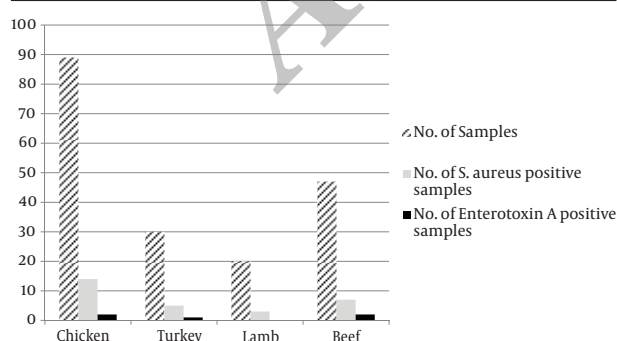
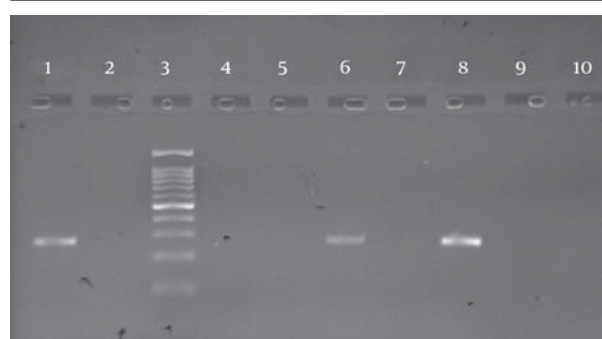


Figure 1. The Frequency of *S. aureus* and Enterotoxin a Positive Samples

Figure 2. Agarose Gel Electrophoresis Patterns of PCR Amplification



1) *S. aureus* (positive control), 2) *Staphylococcus epidermidis* (negative control), 3) 100 bp marker, 4, 5, 7, 9, 10) *S. aureus* without enterotoxin A gene, 6, 8) *S. aureus* with enterotoxin A gene

In order to determine the specificity of PCR reaction, *S. epidermidis* (negative control) and *S. aureus* (positive control) were tested as indicated in Figure 2.

5. Discussion

The obtained results were in agreement with previous reports on risk factors of food staphylococcal contamination. *Staphylococcus aureus* is a major, versatile human pathogen which colonizes the muco-cutaneous surfaces of animals and human beings. The chemotherapy of staphylococcal infections is not always satisfactory due to the widespread anti-microbial resistance (22). *Staphylococcus aureus* can easily spread throughout the meat during slaughtering, preparation, packaging, storage and handling processes. Maltreatment of meat contaminated by handling would be necessary to produce the levels of organisms encountered in outbreaks of food poisoning. Such treatment would appear to require a considerable time lapse between contamination and sales or consumption of the meat and storage of the product at room temperature or above (23). The level of contamination can substantially increase or decrease by poor or good slaughter procedures, respectively. Contamination of muscle tissue during the slaughter process may occur as a result of direct or indirect contact with e.g. faeces, skin, contaminated tools and equipment, personnel and clothing. During the process of meat production the contamination raises. The hands of workers are an important primary source of contamination of products with *S. aureus* during meat processing. Working operations in the production of meat: hide removal, evisceration, splitting of carcasses, trimming and washing of surface, and handling of carcasses all contribute to contamination of the meat (14). The bacterial contamination of meat products occurs due to its improper control which depends on various factors, such as initial level of contamination of carcasses, the duration and temperature of storage, and hygienic practices during handling (9). Potential sources of contamination with *S. aureus*, such as the nares, throat, hands and nails of food handling personnel have been discussed previously. Food contact surfaces such as grinders, knives, storage utensils, cutting blocks and saw blades may also be sources of contamination. These potential sources will be considered in further study of these establishments. The presence of *S. aureus* in foods constitutes a significant risk and can be used as an indication of cross contamination (24). Studies support the fact that *S. aureus* is found in raw meat more than cooked meat. The presence of *S. aureus* in the analyzed products is a potential health risk for consumers since the pH and aw values of these kinds of products are favorable for *S. aureus* growth (3). Raw meat and meat products are repeatedly reported to be associated with staphylococcal food poisoning worldwide, according to the Korean Food and Drug Administration, 30% of SFP incidents from 2001 to 2006 in Korea involved meat and meat products

(25). To prevent food poisoning, it is important to determine the level of contamination with enterotoxigenic *S. aureus* in retail raw meat (12). Since SEA is toxic even in low concentrations (0.6 ng/mL), detection of *S. aureus* strains which harbor SEA synthesis gene is important (26). Since foods like kebabs and hamburgers have the most contact with hands during their preparation, it is most likely that cooking temperature is not sufficient to remove the primary and secondary contamination, because the normal cooking temperatures do not destroy toxins efficiently (24). In the present study, the prevalence of *S. aureus* was 15.6%. Moreover, the presence of *S. aureus* encoding enterotoxin A among meat samples was 17.6%.

In Slovak republic, out of the 43 staphylococcal strains isolated from different foods, 15 strains (34.88%) were found to be enterotoxigenic and out of these 15 strains, seven (16.28%) contained enterotoxin A encoding gene (26). In 2011 in Marmara, Turkey, the results of a survey reported that 13.8% of the samples were contaminated with *S. aureus* which among them 62.9% were enterotoxigenic and 8.6% of them encoded SEA. The results of two studies also showed that the prevalence of SEA encoding gene in isolated strains were 15.4% and 18.8% (27). A study by Moon et al. on three groups of food revealed that the contamination of meat products with *S. aureus* was the highest 36% (28). The above results are almost similar to those of the current study. In contrast, in 2011 a survey in USA on red meat and poultry samples from five different cities reported that 47% of samples contained 77% turkey, 41% chicken and 37% beef were *S. aureus* positive (29). In China, a study on staphylococcal enterotoxin genes among goat samples showed SEA occurrence in 36.8% of the isolates (18). Out of 342 analyzed samples in Pakistan in 2009, *S. aureus* were found in 24 samples (7%) (15). In a survey conducted in 2006 to 2007 in Tehran Iran, 9.5% of the samples were positive for *S. aureus* among which 3.7% were isolated from raw and cooked meat products (30); In another study conducted in Tehran, similar results of *S. aureus* contamination were found, and the frequency of *S. aureus* isolates that harbored SEA and SEA+C genes was 8% and 9% respectively (31). The result of SEA frequency in the above mentioned study was somehow similar to that of the current study.

In assessment of SEA production, the result of a study in Japan revealed that out of 444 meat samples 65.8% were *S. aureus* positive which 17.9% produced only SEA, 2.6% A+B and 2.6% A+C enterotoxins (12). In a study in Italy, 45.2% of strains isolated from meat products were found to produce enterotoxins while 30.3% of them produced SEA (7). These variations in results could be due to geographical differences, which could be related to different environmental conditions, herd nutrition, the ecological origin, and the nature of the strains (18).

The main cause of differences in the frequency of SEA encoding strains in this study as well as other studies might be the origin of bacteria isolation which could

vary in animals, humans, infections, foods or environment (32). In the present study, the samples were collected from retail stores of Tehran which were cut in pieces and stored in refrigerators and this could increase the chance of contamination through contact with surfaces, knives or personnel's hands.

Staphylococcal enterotoxins are mostly carried on mobile genetic elements, which enable them to transfer horizontally among bacterial populations (33). The reason for the observed discrepancy is unclear. This could be due to the fact that SEA is carried by a family of temperate bacteriophages whose genomes incorporate and replicate with that of *S. aureus*; Moreover, the geographical distribution of these phages is irregular (6, 34).

It is noteworthy that the PCR is only able to demonstrate the existence of enterotoxin genes in *Staphylococcus aureus* isolates and does not prove that the production of SEs proteins occurs. To demonstrate the ability of a strain to produce sufficient amount of SEs protein to induce disease, bioassays or immunological methods to detect SEs protein must be developed. In the current study, only the presence of enterotoxin A gene was investigated, and phenotypical assessment or expression of gene was not evaluated. Using PCR method, the bacteria and its type can be recognized before any toxin production occurs (35)

As the result of the current study, the prevalence *Staphylococcus aureus* type A enterotoxigenic strains in the collected samples was identified and a potential risk factor among raw meat products in retail stores was proved. These findings suggest that further investigations seem to be essential for national health improvement.

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