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Molecular Typing and Drug Resistance Patterns of *Staphylococcus aureus* Isolated From Raw Beef and Chicken Meat Samples

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ABSTRACT

Background: *Staphylococcus aureus* is one of the most important food-borne pathogens. The objective of this study was to determine the prevalence, molecular types and drug resistance pattern of *S. aureus* isolated from retail meat in Tabriz city.

Materials & Methods: 60 raw meat samples (chicken and beef) were taken from different markets and were inoculated in selective Mueller Hinton broth media supplemented with 10% NaCl. Identification of *S. aureus* isolates was performed using conventional biochemical tests. Susceptibility to different antibiotics and genotypes of isolates were determined by disc diffusion and *spa* typing methods respectively.

Results: Fifteen *S. aureus* strains were isolated from 60 different meat samples which belonged to *spa* types t14870, t3802, t1814, t491, t386, t3424 and *spa* type t14870 with the frequency of 33.3% was the most prevalent genotype among *S. aureus* isolates. *spa* types of three isolates were not found in Ridom Spa Server data base and were considered as novel types. About 46.6% of isolates were resistant to more than one antibiotic and 13.3% of isolates were identified as methicillin resistant *S. aureus* (MRSA). Tigecycline, imipenem and ceftaroline were found to be the most effective agents against *S. aureus* isolates.

Conclusion: Oure results revealed a 25% contamination rate with *S. aureus*. Most of the molecular types of isolates were found to be linked to human infections. High rate of antibiotic resistance was observed among the isolates which poses a great threat to public health.

Keywords: *Staphylococcus aureus,* MRSA, *spa* typing, Meat, Antibiotic resistance

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Introduction

Staphylococcus aureus is one of the most important foodborne pathogens and the most common causes of food poisoning **(1)**. This bacterium is known in many countries as the third leading cause of foodborne illnesses after *Salmonella* and *Vibrio parahaemolyticus* **(2)**. Milk, dairy products and meat are some of the foods associated with staphylococcal food poisoning [1]. This bacterium multiplies quickly at room temperature and secretes its heat-resistant enterotoxins, causing food poisoning following consumption of foods contaminated with these toxins. *S. aureus* is also a cause of various diseases in humans such as skin and soft tissue infections, bacteremia and pneumonia and is a serious problem in hospitals and the food industry **(3)**. The pathogenicity of

S. aureus is mediated by the bacterial specific structure and extracellular secretions such as various toxins. In recent decades, the widespread use of antibiotics has led to the emergence of multidrug-resistant (MDR) bacterial strains. *S. aureus* has a high adaptive capacity to varying environmental conditions and quickly becomes resistant to virtually all antibiotics **(4)**. Recently, MDR strains of *S. aureus* have been frequently reported from food poisoning outbreaks and isolated from various food products **(3, 5, 6)**. In particular, isolation of methicillin-resistant *S. aureus* (MRSA) from meat products raises concerns that these contaminated meats may be a means of transmitting MRSA to human communities **(7)**. The term livestock-associated MRSA (LA-MRSA) is used to differentiate methicillin-resistant *S. aureus* of human origin (acquired from hospital or community) from those isolated from livestock. LA-MRSA strains have the potential to cause disease in humans and often show multidrug resistance profiles **(8)**. Genotyping of microbial strains is important to understand how bacteria spread, to find a possible source of infection, and to identify the dominant types. There are several molecular methods for typing of *S. aureus* and MRSA strains. These methods include DNA fingerprinting by PFGE, SCC *mec* typing and sequencing-based methods such as *spa*-typing and MLST **(3, 9)**. In *spa* typing, the polymorphism of x-region of the *spa* gene (encoding surface protein A) is examined by PCR and sequencing. Because x-region has high degrees of polymorphism, it can be used in genotyping studies. The discriminatory power of *spa* typing method is lower than PFGE and higher than MLST. This method is more cost-effective than methods such as MLST that require sequencing of at least 7 genes, or the PFGE method **(10, 11).**

Since meat and meat products are known as important reservoirs of *S. aureus* and have been involved in various outbreaks, the aim of this study was to investigate the contamination rate of meat samples collected from different parts of Tabriz city with *S. aureus* and to determine the drug resistance pattern and genotypes of obtained isolates.

Materials and Methods

Isolation of *S. aureus* from meat samples

Raw beef and chicken samples were collected from various meat shops in Tabriz from June 2019 to January 2020. For sampling, 10 grams of meat sample was taken and placed in sterile tubes containing Mueller-Hinton broth supplemented with 10% NaCl. The tubes were transferred to the laboratory at cold temperature and placed in an incubator at 37°C for 24 hours. Then, different dilutions were prepared and 10 to 20 µL of each dilution was transferred to mannitol salt agar medium and placed at 37 ° C for 24 hours. Colonies with yellow halo on mannitol salt agar medium were selected and after purification on nutrient agar medium were subjected for identification by microscopic observation and conventional biochemical methods (catalase, coagulase and DNase tests).

Antimicrobial Susceptibility Testing

For this purpose, disk diffusion was performed by Kirby Bauer method and using paper disks containing the following antibiotics: ampicillin, ceftaroline, imipenem, levofloxacin, ciprofloxacin, sulfamethoxazole-trimethoprim (BBL Sensi-Disc ™, MD, BBL) and tigecycline (Mast Co, Merseyside, UK). Interpretation of disk diffusion results was performed according to the Clinical & Laboratory Standards Institute (CLSI) **(12)**. Interpretation of the results for tigecycline was performed using FDA guidelines, according to which bacteria with an inhibition zone diameter of 19 mm and more were considered susceptible to tigecycline.

Identification of Methicillin-Resistant Strains of *Staphylococcus aureus*

Two phenotypic and genotypic methods were used to identify MRSA strains. In the phenotypic method, the susceptibility of the studied isolates to cefoxitin 30 µg (BBL Sensi-Disc ™, Becton – Dickinson, Sparks, MD) was evaluated by disk diffusion method. Strains with an inhibition zone diameter of 21 mm or less were considered as cefoxitin resistant and categorized as MRSA. In the genotypic method, detection of *mecA* and *mecC* genes was performed by PCR method using primers listed in Table 1.

Determination of Molecular types of *S. aureus* Isolates by *spa* Typing Method

DNA extraction was performed by boiling method as follows; a loop full of bacterial colonies grown on the nutrient agar medium was dissolved in 950 µL of PBS buffer. The tubes were centrifuged for 10 minutes at 7000 rpm. The precipitate was dissolved in 200µl of sterile TE buffer (1x) and boiled for 10 minutes. After centrifugation at 13,000 rpm for 20 minutes, the supernatant was transferred to another container and 1:10 dilution of supernatant was used as DNA template in PCR reaction (https: // www. eurl-ar.eu/CustomerData/Files/Folders/21-protocols/278_mcr-multiplex-pcr-protocol-v2-oct16.pdf).

To amplify the *spa* gene, PCR was performed in a final volume of 50 µL containing 25 µL of Taq DNA Polymerase Master Mix Red solution (Ampliqon, Denmark), 2.8 µL of each of the reverse and forward primers (Table 1), 17.4 µL of distilled water, 2 µL of template DNA and according to the following program:

One cycle at 95°C for 10 minutes (First denaturation), 30 cycles including 1-95°C for 30 seconds (Denaturation), 2 -58 °C for 45 seconds (Annealing), 3-72°C for 45 seconds (Extension), and final extension at 72°C for 10 minutes. The sequences of PCR products were determined by Codon company and analyzed by ChromasPro software. Isolates were assigned to

particular *spa* types using the *spa* typing website ((http://www.spaserver.ridom.de).

Table 1. Nucleotide sequences of primers used in PCR reaction

Primer name	Sequence (5' to 3')	Size of product (bp)	Reference
MecA-F MecA-R	TGGCTCAGGTACTGCTATCCAC AGTTCTGCAGTACCGGATTTGC	777	This study
MecC-F MecC-R	GAAAAAAAGGCTTAGAACGCCTC TGGCTCCTAATGCTAATGCAATG	594	This study
spa-1113f spa-1514r	TAAAGACGATCCTTCGGTGAGC CAGCAGTAGTGCCGTTTGCTT	Variable	[11]

Results

Determination of the Frequency and Drug Susceptibility of *S. aureus* Isolated from Meat Samples

A total of 60 raw meat samples (chicken (18 samples) and beef (42 samples)) were collected from meat markets in Tabriz during the study period. Fifteen isolates (25%) were obtained from these samples which were identified as *S. aureus* being observed as Gram-positive cocci with grape-like cluster arrangement under microscopic examination and being positive for catalase, coagulase and DNase tests. The contamination rates in chicken and beef samples were 27.7% and 23.8%, respectively. All isolates were evaluated for multi-drug resistance phenotype, the results of which are shown in Table 2. According to drug susceptibility testing results, all isolates (100%) were susceptible to imipenem, tigecycline and ceftaroline. The observed resistance rate to ampicillin, cefoxitin, quinolones and sulfamethoxazole-trimethoprim were 100%, 13.3%, 33.3% and 20%, respectively.

Identification of Methicillin-resistant *S. aureus* Isolates

MRSA isolates were identified by disk diffusion (cefoxitin disk) and PCR methods (detection of *mecA/C* gene). Among 15 *S. aureus* isolates obtained from meat samples, two were resistant to cefoxitin (with halo diameters of 17 and 19 mm) and harbored *mecA* gene. The *mecC* gene was not detected in any of the isolates.

Determination of Molecular Types of *S. aureus* Isolates by *spa* Typing Method

For all isolates identified as *S. aureus* by phenotypic methods, PCR for *spa* gene was performed using specific primers. Types t14870 and t3802 were the most abundant *spa* types observed in five (33.3%) and two (13.3%) isolates respectively. *spa* types of three isolates were not detected in the database and were considered as new types. Also, in terms of distribution of molecular types among different meat samples, t14870, which was the most common *spa* type was found in 40% and 30% of chicken and beef isolates, respectively. While multidrug resistance phenotype was observed in three of five isolates belonging to t14870 type (60%), the strains belonging to t3802 type (the second most common type) were associated with single drug resistance phenotype. Methicillin-resistant strains also belonged to *spa* types t1814 and t386, which were isolated from beef and chicken samples, respectively (Table 2).

Table 2. Genotype and drug susceptibility pattern of *Staphylococcus aureus* isolated from meat samples

Isolate	*spa* type	Type of meat sample	Antimicrobial resistance profile
SA1	t14870	Chicken	AM, CIP, LVX
SA2	New type	Beef	AM
SA3	t3802	Chicken	AM
SA4	t1814	Beef	AM, FOX
SA5	t14870	Chicken	AM, CIP, LVX,SXT
SA6	t14870	Beef	AM, CIP, LVX,SXT
SA7	t491	Beef	AM
SA8	New type	Chicken	AM
SA9	t3802	Beef	AM

Isolate	*spa* type	Type of meat sample	Antimicrobial resistance profile
SA10	New type	Beef	AM
SA11	t386	Chicken	AM,FOX
SA12	t14870	Beef	AM
SA13	Non typeable	Beef	AM
SA14	t3424	Beef	AM, CIP, LVX
SA15	t14870	Beef	AM, CIP, LVX,SXT

CIP, ciprofloxacin; LVX, levofloxacin; SXT, trimethoprim/sulphamethoxazole; AM, ampicillin; FOX, cefoxitin;

Discussion

Improper use of human antibiotics in agriculture as a growth promoter or as a prophylactic agent with a dose lower than the treatment dose causes selective pressure on the bacterial populations living in the intestines of animals and the development of resistance. These resistant bacteria can be transmitted directly or indirectly to humans through animal products and cause disease in humans, or they can be a repository for the transmission of antibiotic resistance genes to human pathogenic bacteria **(13, 14)**.

There are evidences supporting the transmission of extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* clones, from livestock to human being presumably through the food chains **(15)**. Numerous studies have described the colonization of various animals with *S. aureus*, and methicillin resistant isolates have also been reported from food producing animals **(16)**. In 2017, the World Health Organization recognized MRSA as one of the 12 families of bacteria that pose a serious threat to human health **(17)**. In the present study, a 25% contamination rate with *S. aureus* (27.7% chicken, 23.8% beef) was observed among meat samples collected from different parts of Tabriz city. The rate of contamination observed in this study was similar to the results of Ge *et al.*, who reported a *S. aureus* contamination rate of 27.9% in meat samples studied in the United States **(18)**. This rate of contamination is also lower than that reported by Tang *et al.*, who described *S. aureus* contamination rate of 68% in meat samples from Denmark **(19)**.

In the present study, 46.6% of the isolates were resistant to more than one antibiotic. Imipenem, tigecycline, and Ceftaroline fosamil were the most effective agents against *S. aureus* isolated from meat samples. In contrast, 100, 20 and 33% of isolates were resistant to ampicillin, sulfamethoxazole-trimethoprim and quinolones respectively. This amount of resistance observed against quinolones, as one of the most important antibiotics used for the treatment of upper respiratory and genitourinary tract infections, can be attributed to the widespread use of these antibiotics in farm animals.

In a study performed by Wu *et al.*, who studied 1,850 raw meat samples and meat products from 39 cities in China, 35% of the samples were found to be contaminated with *S. aureus*. Only 1.26% of *S. aureus* isolates obtained from meat samples were sensitive to all 26 tested antibiotics, 94.6% were non-susceptible to more than 3 antibiotics and 12% of isolates showed resistance to more than 10 antibiotics (6). Xing *et al.*, reported that 98.4% and 58.6% of the studied *S. aureus* were resistant to more than one and three antibiotics respectively **(20)**.

We found methicillin-resistant bacteria in 10 and 20% of the isolates obtained from beef and chicken samples, respectively. Isolates SA4 and SA11 (13.3%) belonging to *spa* types t1814 and t386 were classified as MRSA. Resistance to methicillin in these two strains was confirmed by both phenotypic and genotypic methods.

The frequency of MRSA observed in this study was higher than the values reported by Wu *et al.*, in which 7.14% of *S. aureus* strains isolated from meat samples were identified as MRSA **(6)**.

The prevalence of MRSA in meat samples varies in different geographical regions and rates of 1.9% in the United States, 0.5% in Korea, 13% in Denmark and 24.8% in Canada have been reported **(18, 19, 21, 22)**.

The source of microbial contamination of meat can be endogenous originating from the animal microbiota or it can be exogenous, which is related to environmental pollutants and people involved in processing and transporting meat from slaughterhouses to meat markets. Using *spa* typing technique, type t14870 with a frequency of 33.3% was identified as the predominant *spa* type in *S. aureus* isolates obtained from meat samples being observed in 40% and 30% of chicken and beef isolates, respectively. In three of the five isolates belonging to this type (60%) the multidrug resistance phenotype was observed, so that 80% of quinolones resistant isolates and all isolates resistant to sulfamethoxazole-trimethoprim belonged to type t14870. This type is one of the rare types in the world and there are few studies reporting detection of this genotype in human samples **(23)**. Also, *spa* types t3802,

t1814, t491 and t386 that were identified among the studied samples are common human types **(24-26)**. Identification of common *spa* types of human infections among isolates obtained from meat samples in this study indicates that these contaminants are probably of human origin and therefore have the potential to be pathogenic in human. Also, 3 isolates characterized with new *spa* types that were not found in the Ridom *spa* Server database and were reported for the first time in the world. Drug susceptibility testing in these isolates revealed the single drug resistance phenotype (ampicillin resistance).

The high genetic diversity observed among the studied strains indicates that the clonal expansion was not occurred and the contaminating bacteria may have originated from various sources. Wu *et al.* reported ST1-t127 and ST7-t091as the two dominant *spa* types in 10.7% and 10.6% of *S. aureus* isolates obtained from meat samples, respectively **(6)**. Narvaez *et al.* examined the prevalence of MRSA in meat samples from three pork factories in Canada. According to their results, most LA-MRSA isolates belonged to *spa* types t034 and t011. A 10% resistance rate to tigecycline was observed and less than 3% of isolates were resistant to daptomycin, gentamicin and trimethoprim-sulfamethoxazole **(22)**.

Conclusion

Overall, the results of this study showed a 25% contamination rate with *S. aureus* in raw meat samples and most of the identified molecular types were linked with human infections. Identification of MRSA as an important human pathogen, in meat samples is a serious threat to food safety as there is always a potential for these resistant isolates to easily spread across the country via food chain or direct contact. Reducing the agricultural use of important medical antibiotics such as quinolones and other families of antimicrobials in the farm animals can contribute to reduced resistance to these antibiotics. Therefore, proper control should be done on the consumption of antibiotics in food animals and food hygiene in different stages of their preparation (animal husbandry, slaughterhouse, packaging, etc.) to prevent the emergence and dissemination of drug resistant bacteria.

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Conflict of Interest

Authors declared no conflict of interests.