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Isolation and characterisation of *Staphylococcus aureus* strains isolated from beef, sheep and chicken meat*

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Summary: The objective of this study was to evaluate the occurrence, enterotoxigenic properties and antibiotic resistance of *Staphylococcus aureus* in beef, sheep and chicken meat. For this purpose, 225 meat samples were obtained from different supermarkets in Ankara. Firstly, these samples were analysed phenotypically for coagulase-positive staphylococci and *S. aureus*. Then phenotypically determined *S. aureus* isolates were confirmed through PCR assay and investigated by multiplex PCR for enterotoxin genes and *mecA* gene. According to the analyses, 14.6%, 30.6% and 45.3% of the beef, sheep and chicken meat samples respectively were found to be contaminated with *S. aureus*. Eighty eight (77.1%) out of 114 *S. aureus* isolates were determined as enterotoxigenic. Also, isolates contained newly described enterotoxin like genes. These results indicated that new types enterotoxin genes could be effective in establishing food intoxication. In this study, isolates were found resistant to one or more antibiotics. None of the *S. aureus* isolates contain *mecA* gene but some of them were identified as resistant to a group of antibiotics like oxacillin and cefoxitin, which are used in determining methicillin resistance. For public health and hygienic meat production HACCP and GMP systems should be implemented effectively.

Keywords: Enterotoxin, meat, *mecA*, PCR, *Staphylococcus aureus*.

Sığır, koyun ve piliç etlerinden *Staphylococcus aureus*'un izolasyon ve karakterizasyonu

Özet: Bu çalışma sığır, koyun ve piliç etlerinde *Staphylococcus aureus*'un bulunuşu, enterotoksijenik özellikleri ve antibiyotik dirençliliklerini saptamak amacıyla yapılmıştır. Bu amaçla, Ankara'daki farklı süpermarketlerden 225 adet et örneği alınmıştır. Alınan örnekler öncelikle koagulaz-pozitif stafilokoklar ve *S. aureus* yönünden analiz edilmiştir. Fenotipik testler sonucu *S. aureus* olarak belirlenen suşlar PCR tekniği ile doğrulanarak, multipleks PCR tekniği kullanılarak enterotoksin genleri ve *mecA* geni yönünden analiz edilmiştir. Analiz sonuçlarına göre sığır, koyun ve piliç eti örnekleri *S. aureus* ile sırasıyla % 14.6, % 30.6 ve % 45.3 düzeyinde kontamine bulunmuştur. İzole edilen 114 adet *S. aureus* izolatından 88'inin (% 77.1) enterotoksijenik özellikte olduğu tespit edilmiştir. İzolatların yeni tanımlanan enterotoksin genlerine sahip oldukları belirlenmiştir. Sonuçlar, yeni tanımlanan enterotoksin genlerinin gıda intoksikasyonlarında önemli olabileceğini ortaya koymaktadır. Bu çalışmada izolatlar bir veya daha fazla antibiyotiğe dirençli bulunmuştur. *S. aureus* izolatlarının hiçbirinde *mecA* geni saptanmamasına karşın, bazı izolatlar metisilin duyarlılığının belirlenmesinde kullanılan, oxacillin ve cefoxitine dirençli bulunmuştur. Halk sağlığının korunması ve hijyenik et üretimi için HACCP ve GMP sistemlerinin etkin uygulanması önemlidir.

Anahtar sözcükler: Enterotoksin, et, *mecA*, PCR, *Staphylococcus aureus.*

Introduction

Staphylococcus aureus food poisoning is an intoxication caused by the ingestion of food containing staphylococcal enterotoxins (SEs), and is one of the most common food borne diseases in the world. The primary habitat of *S. aureus* is the nasal passage of humans and the skin and hair of warm blooded animals (12). This bacterium produces a variety of extracellular products. Many of these, including the staphylococcal enterotoxins, are virulence factors, which have been implicated in humans and animal diseases. Heat stability is one of the most important properties of SEs with regard to food safety (15). So far, 20 serologically distinct SEs have been identified. SEA, SEB, SEC, SED, SEE represent classical types, while SEG, SEH, SEI and SEJ are newly described enterotoxins called SE-like toxins (SEl) because of the absence of emetic activity (14, 16, 32).

There have also been several investigations into the growth of *S. aureus* and the production of enterotoxins in contaminated meat and meat products (24, 26, 29). Contamination of foods by *S. aureus* may occur directly from infected food-producing animals or may result from

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poor hygiene during the production process or during retail and storage of foods, since humans may carry the microorganisms (31). Besides, antimicrobial resistance is a major public health concern worldwide, due to the persistent circulation of resistant strains of bacteria in the environment and the possible contamination of food. *S. aureus* has been reported to frequently show multiple antimicrobial resistance patterns (10, 18, 32).

The aims of the present study were: i) to evaluate the occurrence of *S. aureus* in beef, sheep and chicken meat obtained in Ankara; ii) to characterise the isolated strains based on their enterotoxigenic properties and *mecA* gene; iii) to undertake antibiotic susceptibility tests.

Materials and Methods

Sample collection: Meat samples were collected monthly from randomly selected supermarkets in Ankara. Each month, 25 samples were collected and in total 225 samples of beef (n=75), sheep (n=75), and chicken meat (n=75) were analysed.

Isolation and identification of S. aureus: Isolation and identification of *S. aureus* were performed according to the EN ISO 6888-1 standard procedure of the International Organization for Standardization (7) using Baird-Parker Agar (Merck, 1.03785) supplemented with Egg Yolk Tellurite Emulsion (Merck, 1.05406). The plates were incubated under aerobic conditions at 35ºC for 24-48 h. If present, 5 egg yolk reaction-positive and 5 egg yolk reaction-negative colonies were chosen from each sample for further identification. All suspect colonies were confirmed by the coagulase test on EDTA coagulase plasma (Merck 1.13306) within 24 h at 37ºC. Then, coagulase positive colonies were analysed with Gram-staining, catalase reaction, DNase, beta hemolysis tests, clumping factor (Staphylase test, Oxoid, DR0595) and anaerobic utilisation of mannitol. According to these results, positive colonies were evaluated phenotypically as *S. aureus* (7).

DNA extraction: Phenotypically determined *S. aureus* isolates were enriched in Brain Heart Infusion Broth (Oxoid, CM0225) at 37ºC for 18-24 h, centrifuged at 10,000 xg (1 min), resuspended in 180µl TE buffer containing 7µl of lysostaphin (Sigma, L7386) and incubated 1 h at 37ºC (1). Then, DNA was extracted with DNeasy Blood&Tissue-Kit (Qiagen, 69506) according to the manufacturer instructions.

Confirmation of S. aureus isolates with PCR: Phenotypically determined *S. aureus* isolates were confirmed using PCR assay. Sequences have been published by Brakstad et al. (4) for *nuc* gene and by Monday and Bohach (21) for *16S rRNA* gene. After optimisation of PCR, amplification were performed using a thermal cycler (Eppendorf, Mastercycler) with initial denaturation step for 4 min at 94ºC, 35 cycles of 94ºC for 30 s, 57.5ºC for 30 s, 72ºC for 30 s; and a final elongation at 72ºC for 10 min (the same PCR conditions were used for detection of *mecA* and *tst* gene). PCR reaction mixtures were performed in a total volume of 25 µl, containing 0.3 µl *nuc* gene primer 1 and primer 2, 0.3µl *16S rRNA* gene primer 1 and primer 2, 0.5 µl (10 mM) dNTP, 1.5µl (25mM) $MgCl_2$, 2.5µl 10xPCR buffer, 0.2µl Taq DNA polymerase and 16.6µl aqua dest. Finally 2.5µl DNA template was added to each reaction tube. PCR products were resolved by agarose gel electrophoresis and visualised on a UV transilluminator.

Multiplex PCR for detection of Staphylococcal enterotoxin genes: Nucleotid sequences have been published by Mehrotra et al. (20) for *sea, seb, sec, sed, see, tst* and *mecA* gene, by Monday and Bohach (21) for *sej* and Jarraud et al. (8) for *seg, seh* and *sei* gene. We modified the multiplex PCR method as 3 sets to avoid incorrect primer annealing for the detection of enterotoxin genes of the isolates. Sets were designed to amplify as set 1: *seb, see, seh, sei;* set 2: *sec, sej, sed* and set 3: *sea, seg* genes (multiplex PCR conditions for set 1, initial denaturation step for 4 min at 94°C, 35 cycles of 94ºC for 30 s, 55ºC for 30 s, 72ºC for 30 s and a final elongation at 72ºC for 10 min and for set 2 and set 3 included the same constituents as in set 1 except for set 2 annealing at 55ºC and set 3 annealing at 64.6ºC). PCR reaction mixtures were performed in a total volume of 30µl, containing 0.3µl enterotoxin genes primer 1 and primer 2, 0.5 µl (10 mM) dNTP, 3.0µl (25mM) $MgCl_2$, 3.0µl 10xPCR buffer, 0.2µl Taq DNA polymerase and 20.0µl aqua dest. Finally 2.5 µl DNA template was added to each reaction tube. PCR was performed by using thermal cycler (Eppendorf, Mastercycler). PCR products were resolved by agarose gel electrophoresis and visualised on a UV transilluminator. The primer sequences and anticipated sizes of PCR products were shown in Table 1.

Antibiotic susceptibility testing: Antibiotic susceptibility testing of *S. aureus* isolates was performed on Mueller-Hinton Agar (Oxoid, CM0337) by the disk diffusion method in accordance with Clinical Laboratory Standards Institute guidelines (33). The antimicrobial agents tested included cefoxitin (30µg), tetracycline (30µg), gentamycin (10µg), oxacillin (1µg), chloramphenicol (30µg), vancomycin (30µg), ampicillin (10µg), sulphamethoxazole-trimethoprim (25µg), and erythromycin (15µg).

Reference strains: S. aureus reference strains producing *sea, seb, sec, sed, see, seg/sei, seh, sej, tst* were kindly provided by Dr. Ömer Akineden (Dairy Sciences, Institute of Veterinary Food Sciences, Justus-Liebig- University Giessen, Germany). *S. aureus* reference strains (ATCC 25923, ATCC 43300) were used from the department's strain collection.

Table 1. Primers sequences and anticipated sizes of PCR products used in present study.
Tablo 1. Çalışmada kullanılan primer sekansları ve PCR ürünlerinin beklenen aralığı.

Gene	Primer Sekansı (5'-3')	(bp)	References
nuc	1-GCGATTGATGGTGATACGGTT 2-AGCCAAGCCTTGACGAACTAAAGC	270	10
16S rRNA	1-GTAGGTGGCAAGCGTTATCC 2-CGCACATCAGCGTCAG	228	21
tst	1-ATGGCAGCATCAGCTTGATA 2-TTTCCAATAACCACCCGTTT	350	9
sea	1-GGTTATCAATGTGCGGGTGG 2-CGGCACTTTTTTCTCTTCGG	102	20
seg	1-AATTATGTGAATGCTCAACCCGATC 2-CTTATATGGAACAAAAGGTACTAGTTC	642	8
seb	1-GTATGGTGGTGTAACTGAGC 2-CCAAATAGTGACGAGTTAGG	164	20
see	1-AGGTTTTTTCACAGGTCATCC 2-CTTTTTTTTCTTCGGTCAATC	209	20
seh	1-CAATCACATCATATGCGAAAGCAG 2-CATCTACCCAAACATTAGCACC	376	8
sei	1-CTCAAGGTGATATTGGTGTAGG 2-AAAAAACTTACAGGCAGTCCATCTC	577	8
sec	1-AGATGAAGTAGTTGATGTGTATGG 2-CACACTTTTAGAATCAACCG	451	20
sej	1-CATCAGAACTGTTGTTCCGCTAG 2-CTGAATTTTACCATCAAAGGTAC	142	21
sed	1-CCAATAATAGGAGAAAATAAAAG 2-ATTGGT ATT TTT TTT CGT TC	278	20
mecA	1-ACTGCTATCCACCCTCAAAC 2-CTGGTGAAGTTGTAATCTGG	163	20

Table 2. Occurrence of coagulase-positive staphylococci and *S. aureus* in beef, sheep and chicken meat.
Tablo 2. Sığır, koyun ve piliç etlerinde koagulaz-pozitif stafilokoklar ve *S. aureus*'un bulunuşu.

Sample	Coagulase-positive staphylococci	*S. aureus*
	Number of positive samples/number analyzed (positive%)	Number of positive samples/number analyzed (positive%)
Beef	13/75 (17.3)	11/75 (14.6)
Sheep	26/75 (34.6)	23/75 (30.6)
Chicken	41/75 (54.6)	34/75 (45.3)

Results

In this study, within 146 coagulase-positive staphylococci (CPS) isolates, 114 of them were identified using classic culture methods as *S. aureus*. All of these *S. aureus* isolates were also confirmed by PCR assay (Figure 1). CPS were found in beef, sheep and chicken meat at levels of 17.3%, 34.6%, 54.6%, respectively. The prevalence of *S. aureus* observed in this study was found in beef, sheep and chicken meat at levels of 14.6%, 30.6%, 45.3%, respectively (Table 2).

Out of 114 analysed *S. aureus* strains, 88 (77.1%) were found to be enterotoxigenic (Table 3). Of the isolated enterotoxigenic strains 54 (47.3%) contained *sea*, 10 (8.7%) contained *seh*, 8 (7.0%) contained *sed+sej*, 8 (7.0%) contained *tst*, 4 (3.5%) contained *sei*, 2 (1.7%) contained *sea+seh* and 2 (1.7%) contained *sec+seg+sei+tst* type enterotoxin genes (Figures 2 and 3) (Data not shown for set 3, *mecA*, *tst* gene). However, all *S. aureus* strains were negative for *seb* and *see* gene.

Table 3. Enterotoxigenic properties of *S. aureus* isolates.
Tablo 3. *S. aureus* izolatlarının enterotoksijenik özellikleri.

Enterotoxin gene profiles	Number of isolates (%)
Sea	54 (47.3)
seh	10 (8.7)
sed+sej	8 (7.0)
tst	8 (7.0)
sei	4 (3.5)
sea+seh	2 (1.7)
sec+seg+sei+tst	2 (1.7)
Total	88 (77.1)

n=114

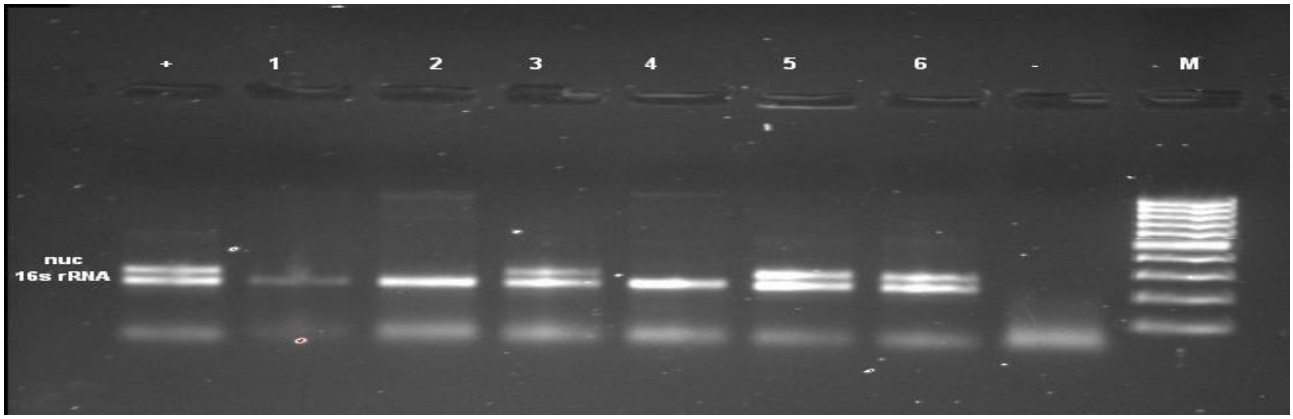

Figure 1. Confirmation of *S. aureus* isolates with *nuc* and *16S rRNA* genes. M: marker; +: positive controls; - and 1: negative control; 2, 3, 4, 5, 6: *16S rRNA* gene positive samples; 3, 5, 6: *nuc* gene positive samples.
Şekil 1. *S. aureus* izolatlarının *nuc* ve *16S rRNA* genleri ile doğrulanması. M: marker; +: pozitif kontrol; - ve 1: negatif kontrol; 2, 3, 4, 5, 6: *16S rRNA* geni pozitif örnekler; 3, 5, 6: *nuc* geni pozitif örnekler.

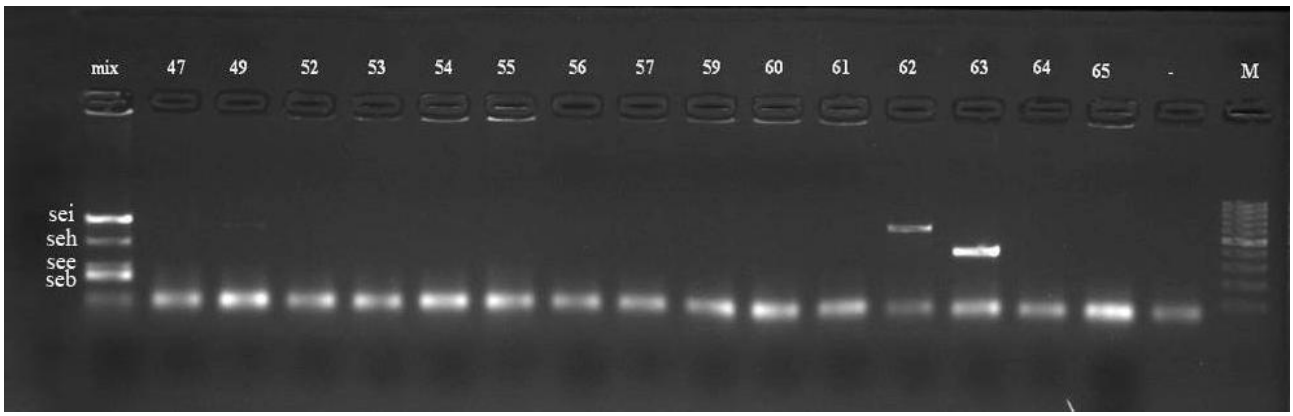

Figure 2. Agarose gel electrophoresis of Set 1 (*seb, see, seh, sei*) PCR products amplified with the multiplex PCR method. M: marker, Mix: DNA mixture of positive controls; -: Negative control; 62: *sei* positive sample; 63: *seh* positive sample.
Şekil 2. Multipleks PCR tekniği ile amplifikasyonu yapılan Set 1'deki (*seb, see, seh, sei*) PCR ürünlerin agaroz jel elektroforozis görüntüsü. M: marker, Miks: pozitif kontrollerin DNA miksi; -: negatif kontrol; 62: *sei* pozitif örnek; 63: *seh* pozitif örnek.

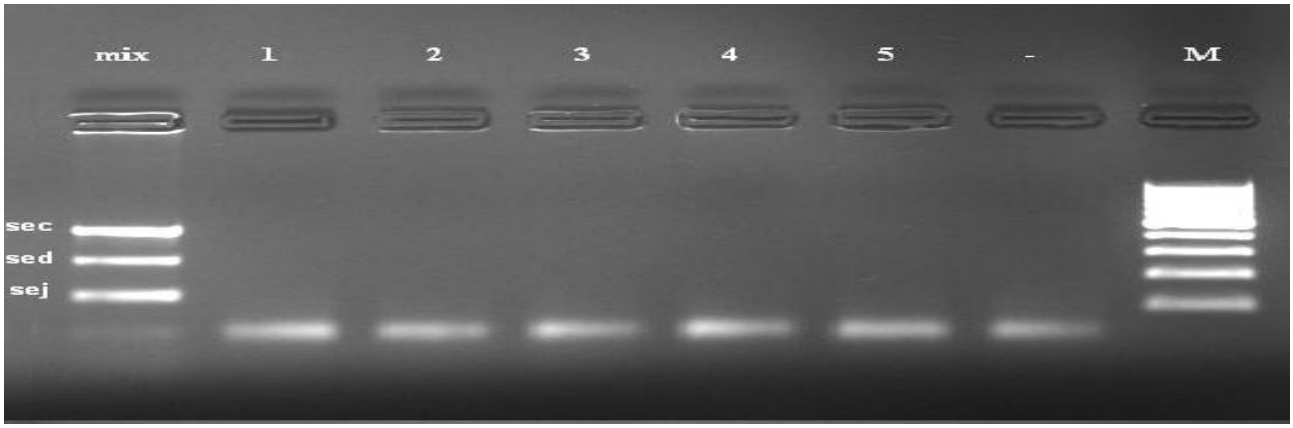

Figure 3. Agarose gel electrophoresis of Set 2 (*sec, sed, sej*) PCR products amplified with the multiplex PCR method. M: marker, Mix: DNA mixture of positive controls; -: Negative control; 1, 2, 3, 4, 5: *sec, sed* and *sej* gene negative samples.
Şekil 3. Multipleks PCR tekniği ile amplifikasyonu yapılan Set 2'deki (*sec, sed, sej*) PCR ürünlerin agaroz jel elektroforozis görüntüsü. M: marker, Miks: pozitif kontrollerin DNA miksi; -: negatif kontrol; 1, 2, 3, 4, 5: *sec, sed*, ve *sej* geni negatif örnekler.

In this study, according to the disc diffusion test results, *S. aureus* isolates indicated high levels of resistance to ampicillin (70.1%), tetracycline (47.3%), erythromycin (33.3%). In addition *S. aureus* isolates were resistant to cefoxitin (22.8%), oxacillin (15.7%), gentamicin (12.2%), chloramphenicol (5.2%), sulphamethoxazole-trimethoprim (3.5%). All of the isolates were sensitive to vancomycin (Table 4).

Table 4. Antimicrobial resistance of *S. aureus* isolates.
Tablo 4. *S. aureus* izolatlarının antimikrobiyal dirençliliği.

Antibiotics	Number of resistant isolates (%)
Amphicillin	80 (70.1)
Tetracycline	54 (47.3)
Erythromycin	38 (33.3)
Cefoxitin	26 (22.8)
Oxacillin	18 (15.7)
Gentamycin	14 (12.2)
Chloramphenicol	6 (5.2)
Sulphamethoxazole-trimethoprim	4 (3.5)
Vancomycin	0 (100)

n=114

Discussion and Conclusion

Occurrence of coagulase-positive staphylococci and S. aureus: In other comparative studies similar results were presented by Lim et al. (17) and Hanson et al. (6). Lim et al. (17) reported that, 9.7% of a total of 890 beef samples were contaminted with *S. aureus*. In addition, Hanson et al. (6) found that in the United States 6.9% of 29 beef samples were contaminated with *S. aureus*. However, our results are lower than those obtained by Kelman et al. (10) who reported that 29% of ground beef samples were contaminated with *S. aureus*. Pu et al. (29) reported that 20% of beef and beef products were contaminated with *S. aureus*. In this study 26 (34.6%) of the 75 sheep meat samples were found coagulase-positive staphylococci. Other studies have reported coagulase-positive staphylococci at levels of 24.1%, 23.4% and 70% for sheep carcasses (13, 27, 28). In this study, *S. aureus* was isolated from 34 (45.3%) of 75 chicken meat samples. These results are similar to the 43.3% incidence reported by Lim et al. (17), but lower than the 65.8% value reported by Kitai et al. (11). In addition, Hanson et al. (6) found that in the United States, 17.8% of 45 chicken meat samples were contaminated with *S. aureus*. Other studies have reported levels of *S. aureus* at 23.8% and 22.5% for raw meat samples (3, 26).

S. aureus is generally found in the natural flora of animal and human skin and the human nasal passage. In addition, slaughterhouse hygiene, slaughtering techniques, storage, transport and general rules of hygiene are important factors in staphylococal contamination of beef, sheep and chicken meat. However, sampling procedures (cotton swabbing, sponging, excising), sampling sites, sampling time and sampling at different slaughter process stages can also affect the results (5).

Enterotoxigenic properties of S. aureus isolates: Our results support previous research conclusions on the enterotoxin properties of *S. aureus* isolates (2, 24, 25, 30). In the present study, we observed that enterotoxigenic strains were relatively common among *S. aureus* isolates. In addition to the classical enterotoxin genes we also found SEl. This is at variance with other studies, which report that differences of the enterotoxigenic properties of *S. aureus* isolates are natural. Possible reasons for this situation arise from sample types, source of contaminations, bacteriologic culture methods, toxin production and detection methods.

All 114 *S. aureus* strains analysed by PCR were negative for the *mecA* gene. Nitzsche et al. (23) similarly found that *S. aureus* isolates didn't have *mecA* gene. However, several investigations reported a few MRSA isolates (3, 11, 15). Among the *S. aureus* isolates studied, various strains were found to be resistant to oxacillin and cefoxitin with disc diffusion tests but none of the isolates contained *mecA* gene by multiplex PCR. These results indicate that isolates may be a heteroresistant phenotype or carry amino acid substitutions in the transpeptidase domain of PBP2 which are responsible for the increased resistance (19, 22).

Antimicrobial resistance of S. aureus isolates: Antibiotic resistant *S. aureus* can be transmitted by food, including contaminated meat (6, 10, 23, 25, 26). Also, investigators indicated that food was a good way to transmit antibiotic resistance to humans. In our study the *S. aureus* strains were found highly resistant to ampicillin and tetracycline. This is not surprising, because amphicillin and tetracyline are broad spectrum antibiotics that are commonly used for the treatment of infections in humans and animals. In comparisone with other studies, differences in the antibiotic resistance may arise from animal population, the inappropriate use of antibiotics for each infection and the use of antibiotics as a growth factor in animal feeding and to promote animal growth.

In this study, we found that *S. aureus* isolates from beef, sheep and chicken meat samples have heterogeneous enterotoxigenic properties and newly described enterotoxin genes. The results indicated that new types enterotoxin genes could be effective in food intoxication. Also, isolates were found resistant to one or more antibiotics. None of the *S. aureus* isolates contained *mecA* gene but some of them were identified as resistant to a group of antibiotics like oxacillin and cefoxitin which are used in determining methicillin resistance. Thus, for public health and hygienic meat production HACCP and GMP systems should be implemented effectively.

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