Kafkas Univ Vet Fak Derg
17 (Suppl A): S55-S60, 2011
DOI:10.9775/kvfd.2010.3233

RESEARCH ARTICLE

Presence of *Staphylococcus aureus* and Staphylococcal Enterotoxins in Different Foods

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Makale Kodu (Article Code): KVFD-2010-3233

Summary

Staphylococcal food poisoning is one of the most prevalent causes of gastroenteritis throughout the world. This study has three steps. In the first step of the study, different food matrices were analyzed for the presence of *Staphylococcus aureus*. In the second step, types of staphylococcal enterotoxins (SET) from these foods were analyzed. Lastly, antibiotic resistance of *S. aureus* isolated was determined using conventional and PCR methods. For this purpose, 300 different food samples were evaluated for contamination with *S. aureus.* From these samples 221 *S. aureus* strains were isolated. The same strains were evaluated for antibiotic resistance and existence of mecA gene. All strains were at least resistant to 2 antibiotics, but no MRSA strain was detected. Staphylococcal Enterotoxins (SE) were determined with VIDAS and ELISA from the foods of concern. The results of the analysis held with Vidas SET2 showed 72 (24%) of 300 samples and with ELISA these enterotoxins showed difference in distribution.

Keywords: *S. aureus, Enterotoxins, BP-RPF, MecA,*

Farklı Gıdalarda *Staphylococcus aureus* ve Stafilokokal Enterotoksinlerin Varlığı

Özet

Staphylococcal enterotoksinler tüm dünyada gıda intoksikasyonlarında öncü etmenler arasında yer almaktadır. Bu çalışma üç aşamada gerçekleştirilmiştir. İlk aşamasında 300 faklı gıda örneğinde *Staphylococcus aureus* varlığı iki faklı agarda incelenmiş, takip eden aşamada aynı gıdalarda enterotoksinlerin varlığı araştırılmıştır. Son olarak elde edilen kültürlerin antibiyotik dirençlilikleri ölçülmüş ve Metsisiline dirençli *S. aureus* (MRSA) varlığı mecA PCR ile aranmıştır. Toplam 221 adet *S. aureus* suşu elde edilmiştir. İlgili suşlar antibiyotik direnci yönünden incelendiğinde her suşun en az iki antibiyotiğe dirençli olduğunu ancak MRSA'nın incelediğimiz örneklerde bulunmadığı ortaya konulmuştur. Örneklerde enterotoksinler için VIDAS ile tarama yapılıp ELISA ile toksinler tiplendirilmiştir. Vidas SET2 ile yapılan enterotoksin analizinde 300 örneğin 72sinin (%24) SE içerdiği ve ELISA ile bu enterotoksinlerin dağılımının farklı olduğu saptanmıştır.

Anahtar sözcükler: *S. aureus, Enterotoksin, BP-RPF, Mec A*

INTRODUCTION

Staphylococcal food poisoning is one of the most prevalent causes of gastroenteritis throughout the world [1]. Staphylococcal gastroenteritis is caused by the ingestion of food that contains one or more staphylococcocal enterotoxins (SEs), which are produced only by staphylococci. Although enterotoxin production is generally believed to be associated with coagulase and thermonuclease positive *S. aureus*, many species of staphylococci that produce neither coagulase nor thermonuclease are also known to produce enterotoxins [2].

The significant rises in the antibiotic resistance of pathogenic bacteria have brought a 12% increase in antibiotic resistant bacteria borne infections over the last 5

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years [3]. The significant antibiotic resistance of enterococci in foods sold in retail markets of Türkiye was reported [4]. Like VRE, Hospital infections caused by methicillin resistant *S. aureus* (MRSA) is difficult to treat and have been capturing public attention [3]. In 2003, 60% of *S. aureus* infections were caused by MRSA. Although MRSA has a severe clinical significance, its antibiotic resistance has no influence on staphylococcal food poisoning [5].

This study aims to determine the incidence of *S. aureus*, distribution of SEs, and the antibiotic resistance of *S. aureus* isolated from different food matrices that are sold in retail markets of Turkey. This study also aimed to determine types of SEs in these food matrices.

MATERIAL and METHODS

A total of 300 food samples were analyzed, consisting of 50 beef, 50 minced beef meat, 50 turkey meat, 50 chicken meat, 25 Turkish white cheese, 25 cheddar cheese, 25 cream cheese, and 25 bottled pasteurized milk samples. All samples were purchased from local supermarkets in Ankara. The study consists of 3 steps. In the first step, different food samples (n=300) were analyzed for existence of *S. aureus*. The second step was undertaken to determine the SE's. The third step was held to show the antibiotic resistances both with disk diffusion and *mec*A-PCR methods.

Isolation of S. aureus

S. aureus strains were isolated by using two different media. Initially, isolation of *S. aureus* was performed by using the ISO method 6888-1:1999/amd.1:2003. For solid samples 10 g of sample was placed in a sterile plastic bag, 90 ml of Ringer solution was added (BR0052, Oxoid, England) and homogenized by using a stomacher (Lab-Bender, Spain). Milk samples were not diluted and directly plated. All homogenized samples were serially diluted in 9 ml of Ringer solution and each dilution was spread on Baird Parker Agar (BP, CM 0275, Oxoid, England) plates. Likewise, from the same serial dilutions 0.1ml was spread on Baird Parker Rabbit Plasma Fibrinogen Agar (BP-RFP, Biomérieux, France). All petri dishes were incubated at 37°C for 48 h. All colonies on BP-RFP with opaque halo were accepted as coagulase positive staphylococci. Typical coagulase positive staphylococci colonies on BP agar are black or grey, shining and convex (1 mm to 1.5 mm in diameter after incubation for 24 h and 1.5 mm to 2.5 mm in diameter after incubation for 48 h) and surrounded by a clear zone.

Identification of S. aureus

From each BP agar petri dish, 2 typical colonies were chosen and coagulase test was conducted for confirmation. For this purpose, colonies were transferred into Brain Heart Infusion Broth (BHI, CM0225, Oxoid) and incubated at 37°C for 24 h. Aseptically 0.1 ml of each culture was added to 0.3 ml rabbit plasma (Bactident Coagulase, RP, 1.13306, Merck, USA) in sterile hemolysis tubes and were incubated at 37°C for 4 h. Clot formation and gel formation were accepted as positive.

All colonies from BP and BP-RFP were identified by using the API-Staph system (Biomérieux, France). After API confirmation of *S. aureus* strains, all strains were streaked on TSA slants and were stored at 4°C until the completion of all analyses.

Detection of SE's

Following the microbiological analysis, all food samples were evaluated for SE. Detection of SEs was achieved by using Vidas (Biomérieux, France) SET 2 and detection method applied in accordance with the instructions of the manufacturer. For this purpose, 25 g or 25 ml from the sample of concern was homogenized with extraction buffer which is provided by the manufacturer. The homogenized samples were incubated at 22°C for 15 min and centrifuged at 4500 rpm for 15 min at 22°C. Supernatants were transferred into tubes from a syringe containing a plug of absorbent cotton and pH values were adjusted between 7.5-8.0 and 500 µl of samples were loaded in Vidas SET 2 stripes and results were read in 80 min.

Determination of SE Types

SE types including A, B, C, D and E were determined with commercial ELISA test kit (Ridascreen, R4101, Darmstadt, Germany). The kit consisted of 96 wells including positive controls. Analyses for all different food matrices were held as indicated in the instructions.

Antibiotic Resistance

Antibiograms for each of the staphylococci isolates were determined with the disc diffusion method antimicrobial susceptibility testing system according to the National Committee for Clinical Laboratory Standards [6]. All strains were inoculated in BHI broth and incubated at 37°C. 100 µl of these overnight cultures were streak on Mueller Hinton Agar (MHA, CM0337, Oxoid, England) and antibiotic discs were applied on the surface using an antibiotic disc dispenser (ST8090, Oxoid, England). The antimicrobials and concentrations were as follows: 30 µg amoxicillin (AMC, CT223B, Oxoid, England), 50 µg furazolidone (FR, CT022B, Oxoid, England), 10 µg lincomycin (MY, CT123B, Oxoid, England), 10 IU penicillin G (P, CT043B, Oxoid, England), 25 µg trimethoprim/sulphamethaxole (SXT, CT052B, Oxoid, England), 30 µg tetracycline (TE, CT054B, Oxoid, England), 10 IU bacitracin (B, CT005B, Oxoid, England), 5 µg methicillin (MET, CT029B, Oxoid, England), 10 µg ampicillin (AMP, CT003B, Oxoid, England), 25 µg sulphamethoxazole (RL, CT051B, Oxoid, England), 300 IU polymyxin B (PB, CT044B, Oxoid, England), 30 µg oxytetracycline (OT, CT041B, Oxoid, England), 5 µg oxacillin (OX, CT0040B, Oxoid, England), 5 µg novobiocin (NV, CT037B, Oxoid, England), 10 µg of gentamycin (CN, CT024B, Oxoid, England),

15 µg erythromycin (E, CT020B, Oxoid, England), 10 µg meropenem (MEM, CT774B, Oxoid, England). MHA dishes were incubated at 37°C and evaluated after 24h of inoculation. All zones were measured using a ruler. *S. aureus* (ATCC 25923, Oxoid, England), MRSA (ATCC 43300, Oxoid, England) and *E. coli* (ATCC 8739, Oxoid, England) strains were used as quality control organisms for antimicrobial resistance analysis. The plates were removed and read manually for growth to score the MIC determinations using the CLSI guidelines for determining breakpoints.

mecA PCR

All strains were inoculated in BHI broth and an overnight culture of each strain was centrifuged at 6.000 rpm (Eppendorf, Germany) and treated with 2µl of lysostaphin (10 mg/ml) (Sigma, Italy) for 1 h at 37°C [7]. DNA template was extracted using the DNeasy Extraction Kit (QIAGEN GmbH, Germany). These templates were used in PCR protocol for the detection of the *mec*A gene using a primers pair and protocol [8]. All PCR products were visualized under UV transillumination (Metis, Turkey) following electrophoresis on 1.5% agarose gel stained with ethidium bromide and using the Gene Ruler ™ 100 bp DNA Ladder (MBI Fermentas, Milano, Italy) as a reference standard.

mecA$_1$ 5′ AAAATCGATGGTAAAGGTTGGC 3′
mecA$_2$ 5′ AGTTCTGCAGTACCGGATTTGC 3′

Statistical Analyses

Statistical analyses were performed using a commercial statistical analysis software (SPSS, v11.5, Ref. No: 9024147).

RESULTS

This study was undertaken to determine the prevalence, enterotoxin types and antibiotic resistance of *S. aureus* in different food types. A total of 300 different food samples were evaluated for coagulase positive staphylococci contamination. 112 (37.3%) of all samples were found to be contaminated with coagulase positive staphylococci. From these 112 coagulase positive staphylococci contaminated samples, there were 16 (5.4%) that were coagulase negative in the tube test but coagulase positive on BP-RFP agar. Contamination rate in beef, minced beef, turkey meat, chicken meat, Turkish white cheese, cheddar cheese, cream cheese, pasteurized milk was 36%, 70%, 48%, 52%, 20%, 12%, 0% and 4%, respectively. The percentages of samples that were coagulase negative in the tube test but coagulase positive on BP-RFP agar were 12% for minced beef, 8% for Turkish white cheese, 6% for turkey meat, and 4% for beef, chicken meat and cheddar cheese.

From 112 samples, a total of 224 coagulase positive strains (1-3 from each sample) were isolated and identified using the API-Staph System. Non *S. aureus* strains were identified in 3 (1.33%) of 224, 2 (0.89%) of which were identified as *S.simulans* and 1 (0.44%) of which was *S. hyicus*. A sum of 221 *S.aureus* strains were isolated from all food matrices. Arithmetic mean of *S. aureus* contamination among all samples was 4.5 log$_{10}$ cfu/g-ml. The incidence of *S. aureus* in different food samples is shown in *Fig. 1*.

SE analysis using Vidas SET2 from food showed that 8 of 50 (16%) beef samples showed SE distribution of 7 SEA, 5 SEC2 and 2 SED according to the ELISA test. On the other hand, 32 of 50 (64%) minced meat samples showed SE distribution of 32 SEA, 2 SEC2. In 18 of 50 (36%) turkey meat samples 13 SEB and 8 SED enterotoxins were detected, and in 14 of 50 (28%) chicken meat samples 14 SEC2 and 6 SED enterotoxins were found. In cheese samples, 1 of 25 (4%) Turkish white cheese samples were found contaminated with SEB and likewise 1 of 25 cheddar cheese samples contained SEA enterotoxin. Levels of SE in different food samples are shown in *Fig. 2*.

The results of the antimicrobial testing showed a higher resistance against PB (98%), and the lowest resistance to MET (1%). Resistance to other antibiotics were as follows: P (82%), RL (74%), NV (44%), FR (38%), B, CN and E (24%), OX (22%), ENR (18%), SXT and AMC (16%), TE and MY (14%), OT (12%), MEM, MEZ, AMP (2%). The antibiotic resistance percentages in strains are shown in *Fig. 3*.

The resistant strains with origin of food were as follows: 22 of 36 (61.11%) beef originated *S. aureus* strains were at least resistant to 3 antibiotics. 18 of 70 (25.71%) minced meat originated *S. aureus* strains were at least resistant to 2 antibiotics. 13 of 48 (27.08%) turkey meat samples were

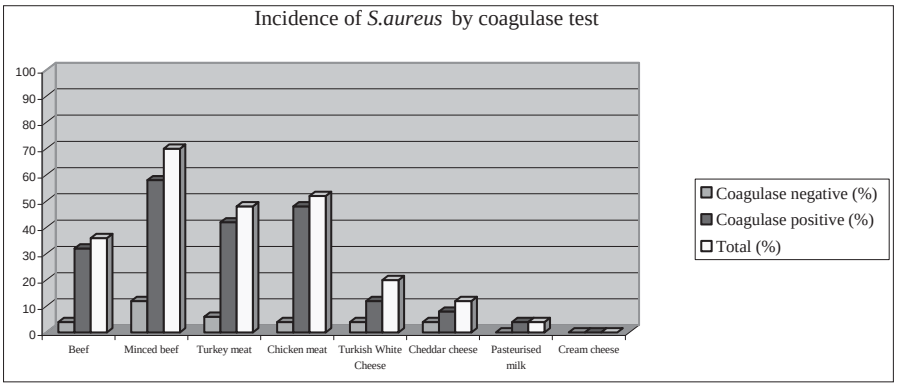

Fig 1. *S. aureus* contamination levels in different food types with respect to coagulase test

Şekil 1. Koagulaz test sonuçlarına gore farklı gıdalardaki *S. aureus* kontaminasyon seviyeleri

contaminated with *S. aureus* and strains of concern were at least resistant to 1 antibiotic. For the strains isolated from chicken meat, 20 of 52 (38.46%) were at least resistant to 4 antibiotics. For *S. aureus* strains isolated from Turkish white cheese, 9 of 10 (90%) strains were at least resistant to 1 antibiotic. 1 of 6 (16.66%) *S. aureus* strain originated from cheddar cheese was resistant to 2 antibiotics. 2 of 2 (100%) pasteurized milk originated *S. aureus* was resistant to 1 antibiotic.

The relation between types of food and the levels of bacterial contamination and antibiotic resistances showed no statistical significance. Maximum antibiotic resistance in *S. aureus* strains by food type is shown in *Fig. 4*.

The results of *mec*A PCR assay showed no MRSA existence in any of the 300 food samples. All results of the study are summarized in *Table 1*.

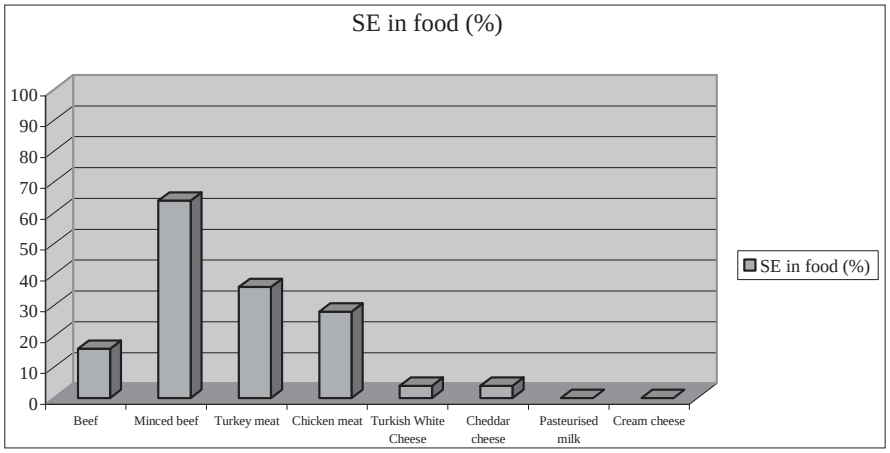

Fig 2. Contamination levels of SE in different food types

Şekil 2. Farklı gıdalardaki SE bulunma seviyeleri

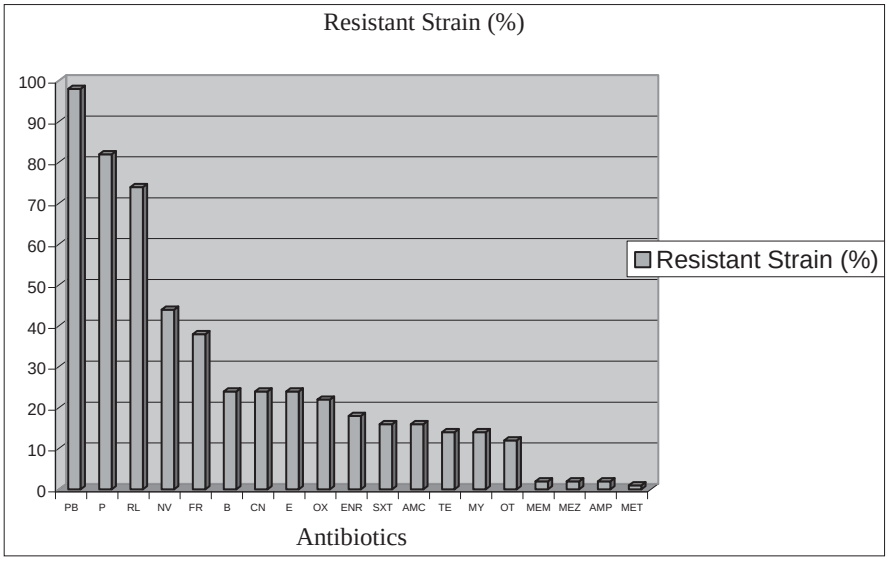

Fig 3. The antibiotic resistance of *S. aureus* strains isolated from different food types

Şekil 3. Farklı gıdalardan elde eilen *S.aureus* suşlarının antibiyotik dirençlilikleri

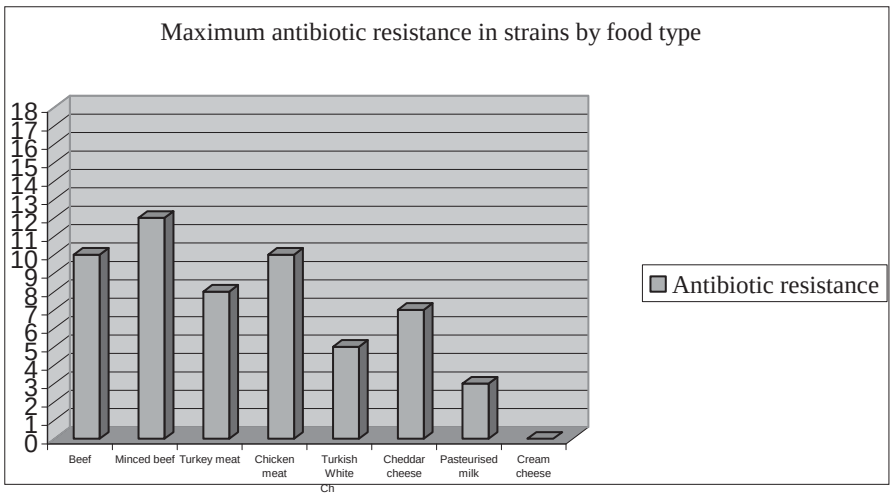

Fig 4. Maximum antibiotic resistance in *S. aureus* strains by food type

Şekil 4. *S.aureus* suşlarında maksimum antibiyotik dirençliliğinin gıda tipine gore değişimi

Table 1. *The results obtained from this study*
Tablo 1. *Çalışmadan elde edilen sonuçlar*

Type of Sample	Incidence of Coagulase Negative *Staphylococcus aureus* (%)	Incidence of Coagulase Positive *Staphylococcus aureus* (%)	Numbers of *Staphylococcus aureus* Contaminated Foods	Mean Contamination (cfu/g or cfu/ml)	SE in Food (%)	Antibiotic Resistance in Strains	*mec*A Gene PCR Positive Strains
Beef	4	32	18/50	$4.2x10^4$	16	10 of 18	0
Minced Beef	12	58	33/50	$1.3x10^5$	64	12 of 18	0
Turkey Meat	6	42	22/50	$6.2x10^3$	36	8 of 18	0
Chicken Meat	4	48	21/50	$5.9x10^4$	28	10 of 18	0
Turkish White Cheese	4	12	10/25	$1.1x10^3$	4	5 of 18	0
Cheddar Cheese	4	8	6/25	$5.0x10^2$	4	7 of 18	0
Pasteurised Milk	0	4	2/25	$1.5x10^3$	0	3 of 18	0
Cream Cheese	0	0	0/25	0	0	0	0
Total	5.33	32	112/300				

DISCUSSION

The overall *S. aureus* contamination was 37.3% (112 of 300 samples). Sixteen (5.4%) of the strains isolated from the contaminated samples were coagulase negative in the tube test but coagulase positive on BP-RFP agar. In a study [9], it is shown that some *S. aureus* strains may be coagulase negative. In our study, we also detected tube coagulase negative *S. aureus* strains. This is thought to be related with the coagulase synthesis strength of the strain.

In a study held by Kılıç [10], 52 turkey meat samples were analyzed for micrococcus/staphylococcus contamination and levels were: $1.0x10^2$ cuf/g at minimum, $4.3x10^6$ cfu/g at maximum and $6.3x10^2$ cfu/g on average. The 13 of 241 isolates (5.3%) were found to be coagulase positive. Four of the coagulase positive isolates were enterotoxigenic and 2 had only C type, 1 had only B type and 1 had both B and C type enterotoxin production ability. In another study held in Japan, a total of 444 samples of raw chicken meat (thighs, breasts, wings, livers, gizzards, hearts and ovaries) that were retailed in 145 different supermarkets in 47 prefectures in Japan were examined for contamination with *S. aureus* in association with its enterotoxigenicity. *S. aureus* was isolated from 292 (65.8%) of the samples, and from 131 of the 145 supermarkets [11]. In a study, 880 different foods were analyzed and 552 coagulase positive *S. aureus* strains were isolated, 269 of which were reported to be enterotoxigenic [12]. Most of these strains were SEA producers. Another study held in poultry carcasses indicates that most frequent enterotoxins were identified as SED and SEA [13]. The results of these two studies are in parallel with the findings of our study. In an experimental study on artificial contamination in a local food including raw meat, held by Erol et al., no SEA formation by the SEA producing strain in 24 h was recorded [14]. The results of this study are different to our results, which may be due to artificial contamination levels and conditions. Various examples of staphylococcal food poisoning are described in the literature. In one case, cheese was involved in an outbreak because it had been made from milk contaminated after pasteurization and before inoculation with lactic starter culture. In this particular case, the starter culture did not grow properly, resulting in a fermentation accident that allowed the *S. aureus* strain to develop and produce SE [15]. Another study was performed in Minas Gerais, Brazil, where 54 (43%) of 127 *S. aureus* isolates from bovine mastitis were found to be SE producers [16]. Although milk and milk products are frequently contaminated with *S. aureus,* dairy products are rarely involved in staphylococcal food poisoning because the critical cell density of $>10^5$ cfu/g^{-1} is usually not reached [17].

SE contamination in cheese samples were significantly less than meat samples ($P<0.001$). This may be due to starter culture uses. This feature has been particularly well studied in fermented food products. Genigeorgis demonstrated that the higher the concentration of competing microorganisms in milk, the lower the rate of *S. aureus* growth and SE production [18]. Competition with lactic acid bacteria has been reported in other research on cheese [19-21]. There are many studies held for both antimicrobial resistances of *S. aureus*. Results of these studies of concern show parallelism with our study [22-27]. In this study, no MRSA strain was detected by using PCR. However in some studies, MRSA existence in different food types was reported [26,27]. The difference in our results may be due to our low number of samples. In the future, a greater number of samples must be analyzed for MRSA existence.

The results of *mec*A PCR assay showed one false positive in a strain isolated from minced meat, which was repeated and gave a negative result. However, in some studies *mec*A PCR was reported to be determinative for MRSA isolated from food samples [26,27]. In another study Çepoğlu et al.[28] demonstrated high antibiotic resistance from hand, nasal cavity samples food handlers. To connect with the study the source of staphylococci must be detected which in further analysis enlighten the sources of resistance.

The results obtained from this study show that a significant multiple antibiotic resistance can be recorded in different *S. aureus* strains isolated from different food matrices. Massive use of antibiotics may cause occurrence of resistant strains like MRSA in hospitals, but there was no MRSA contamination in food samples of this study. In this study, we also found that BP-RFP agar was significantly more effective (P=0.001) than conventional BP for *S. aureus* isolation. Another point to emphasize with the results obtained from this study is that all SE analyses were held with VIDAS, which is less time consuming and more user friendly, and SE results showed that *S. aureus* strains act differently in food matrices, which shows the need for a different study with all genomic details.

ACKNOWLEDGEMENT

The authors would like to thank Afsin BUYUKBAS (MSc., MBA) and Aylin GRAVES for their support and help in translation. We are also grateful to Dr. Oliver SCHNEIDER for his kind interests in our study.

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