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Molecular Typing and Antimicrobial Susceptibility of *Staphylococcus aureus* Strains Isolated from Raw Milk, Cheese, Minced Meat, and Chicken Meat Samples

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Abstract

The objectives of this study were: i) to detect the presence of *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) in raw milk, cheese, beef minced meat, and chicken meat samples; ii) to evaluate the antimicrobial susceptibility of the isolates; and iii) to determine clonal relation among the isolates by using pulsed-field gel electrophoresis (PFGE) method. Therefore, a total of 160 food samples were randomly collected between August 2014 and May 2015 in Hatay province, located in the southern Turkey. Twenty (12.5%) of the samples were found to be contaminated with *S. aureus*. A total of 40 isolates from the 20 positive samples were confirmed to be *S. aureus* by multiplex PCR based on 16S rRNA and *nuc* gene. The *mec A* gene was not detected in any of the *S. aureus* strains. In the present study, 39 out of 40 (97.5%) isolates were found to be resistant to one or more antibiotics. All of isolates were susceptible to gentamicin, oxacillin, and vancomycin. The highest resistance rate was detected in penicillin (95%) and ampicillin (92.5%), followed by tetracycline (30%), erythromycin (20%), ciprofloxacin (12.5%). Nine major patterns were determined by PFGE. In 6 of these patterns, thirty-six strains (90%) had identical PFGE profiles.

Keywords *Staphylococcus aureus*, antimicrobial susceptibility, *nuc,* raw milk, minced meat

Introduction

Staphylococcus aureus is an important pathogen that causes food poisoning and nosocomial infections. Humans and animals are the main source of *S. aureus.* In humans, this bacterium can be normally present on the skin and in the oral and nasal mucosa. Thus, the colonized people are an important source for spreading staphylococci to others and to food. Also, colonization of animals with this bacterium can pose a potential risk for public health because it may result in contamination of milk and dairy products. *S. aureus* is an important cause of mastitis and this infectious disease is a very serious problem for dairy industry (Crago *et al.*, 2012; Gundogan *et al.*, 2005; Normanno *et al.*, 2007; Peles *et al.*, 2007).

S. aureus is an osmotolerant bacterium and can survive at low water activities for extended periods. However, *S. aureus* has a weakly competitive ability. Hence, this bacterium does not grow very well in food if there are low initial concentrations of staphylococci. Also, growth of this bacterium is easily suppressed by other

microorganisms (especially lactic acid bacteria) in mixed cultures. However, *S. aureus* produces heat resistant enterotoxins, that cause food poisoning when growing in foods (Can and Çelik, 2012; Ertas *et al.*, 2010; Jay *et al.*, 2005; Pereira *et al.*, 2009; Seo and Bohach, 2007; Tang *et al.*, 2015).

Many researches have been conducted to investigate the presence of *S. aureus* in different foods. Milk, dairy products (cheese, ice cream), meats (beef, pork, turkey, chicken), eggs and egg products, and aquatic products are usually described as contaminated with *S. aureus* (Can and Çelik, 2012; Crago *et al.*, 2012; Gücükoğlu *et al.*, 2012; Gücükoğlu *et al.*, 2013; Gündoğan *et al.*, 2006; Hanson *et al.*, 2011; Hu *et al.*, 2013; Normanno *et al.*, 2007; Pereira *et al.*, 2009; Tang *et al.*, 2015).

Because antibiotics are widely used in both clinical and agricultural fields, some foodborne pathogens display resistance to more than one antibiotic called 'multidrug resistance'. Antibiotic resistance is usually genetically based, and *S. aureus* can gain resistance by mutation or conjugation (de Boer *et al.*, 2009; McEntire and Montville, 2007; Pesavento *et al.*, 2007; Smith and Jarvis, 1999; Tang *et al.*, 2015).

Methicillin-resistant *S. aureus* (MRSA) has become a widely recognized cause of nosocomial infections worldwide. Although MRSA has a clinical importance due to its antibiotic resistance, the isolation of MRSA from foods has been reported. While the antimicrobial susceptibility profiles of clinical *S. aureus* isolates are well described, limited information about the antimicrobial susceptibility of foodborne strains of *S. aureus* is available in Turkey. The aims of this study were to evaluate the presence of *S. aureus* and MRSA in various food samples, and to determine the antimicrobial susceptibility and clonal relationships of the isolates.

Materials and Methods

Sample collection

A total of 160 samples were randomly collected between August 2014 and May 2015 in Hatay province, located in the southern Turkey. Samples including 50 raw milk (20 cows', 15 goats', and 15 ewes' bulk milk), 50 traditional cheeses (25 Carra, 25 Surk), 30 chicken meat (wing and drumsticks with skin), and 30 beef minced meat were transported to the laboratory with ice packs for analysis.

Isolation of coagulase-positive staphylococci (CPS)

Briefly, ten gram of each sample was added to 90 mL of 0.1% sterile peptone water (Oxoid, England), and homogenised using a stomacher (Bagmixer400, Interscience, France) for 2 min. Serial decimal dilutions were prepared from the mixture and 0.1 mL of appropriate dilutions were spread on Baird Parker agar (Oxoid) supplemented with Egg Yolk Tellurite Emulsion (Oxoid). Plates were incubated at 37°C for 24-48 h for enumaration of staphylococci. After the incubation period, up to five typical (grey/black colonies with precipitation zones) and/or atypical (black colonies with no zones) presumptive colonies were taken. These colonies were stored at -20°C in cryovials for coagulase activity, PCR analysis, and antimicrobial susceptibility test. A tube coagulase test was applied to these colonies using rabbit blood plasma with EDTA (Oxoid). Isolates indicated coagulation were considered CPS (Bennett and Lancette, 1998).

Bacterial strains

S. aureus ATCC 25923 was used as a positive control for targeting 16S rRNA and *nuc* gene. *S. aureus* ATCC 43300 was used as a positive control for *mecA* gene.

PCR analysis

All coagulase positive isolates were identified by species-specific PCR. In this study, multiplex PCR assay was based on the detection of 16S rRNA (*Staphylococcus* genus specific), and *nuc* gene (*S. aureus* species specific). Methicillin resistance was also determined by testing for the presence of *mecA* gene. Specific primer pairs described by Brakstad *et al.* (1992), Mehrotra *et al.* (2000), Monday and Bohach (1999) were used. Primers (Ella Biotech GmbH, Germany) used in the study are shown in Table 1.

DNA extraction

DNA was extracted from the overnight Brain Heart Infusion broth (Oxoid) cultures by using a Bacterial DNA Extraction kit (Nucleic Acid Extraction Kit, Malaysia) according to the manufacturer's protocol.

PCR amplification

A multiplex PCR assay was performed in a reaction mixture of 25 μL final volume containing 1×PCR buffer (Thermoscientific, Lithuania), 1.5 mM MgCI$_2$ (Thermoscientific), 200 μM of each dNTPs (EURx, Poland), 1 U *Taq* DNA polymerase (Thermoscientific), 0.12 μM of each primer, and 2 μL template DNA. Amplification conditions were performed as follows: initial denaturation at 94°C for 4 min and then 35 cycles of denaturation at 94°C for 30 s,

Table 1. Primers used in this study for the detection of the genes

Primer	Oligo nucleotide sequence (5'-3')	Product size (bp)	Reference
16S rRNA-F 16S rRNA-R	GTA GGT GGC AAG CGT TAT CC CGC ACA TCA GCG TCA G	228	Monday and Bohach (1999)
nuc-F *nuc*-R	GCG ATT GAT GGT GAT ACG GTT AGC CAA GCC TTG ACG AAC TAA AGC	279	Brakstad *et al.* (1992)
mecA-F *mecA*-R	ACT GCT ATC CAC CCT CAA AC CTG GTG AAG TTG TAA TCT GG	163	Mehrotra *et al.* (2000)

annealing at 57.5°C for 30 s, and extension at 72°C for 40 s. Final extension cycle was performed at 70°C for 10 min (Keyvan and Özdemir, 2016; Mehrotra *et al.*, 2000). Amplification products were detected by agarose gel (1.5%) electrophoresis carried out at 100 V for 50 min (CS-300V, England). The gels were stained with ethidium bromide and visualised under a UV transilluminator (UVP, USA).

Antimicrobial susceptibility test

The *S. aureus* isolates were tested for antimicrobial susceptibility by the disc agar diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2013). The antibiotic discs were selected in line with the recommendation of CLSI and included penicillin (10 IU/disc), oxacillin (1 μg/disc), tetracycline (30 μg/disc), gentamicin (10 μg/disc), ampicillin (10 μg/disc), vancomycin (30 μg/disc), cefoxitin (30 μg/disc), chloramphenicol (30 μg/disc), erythromycin (15 μg/disc), ciprofloxacin (5 μg/disc), and rifampin (5 μg/disc). The isolates were classified as susceptible, intermediate resistant, and resistant.

Molecular typing of *S. aureus* by PFGE

Mulvey *et al.* (2001)'s protocol was used for the PFGE method. The dendogram analysis was made with Bionumerics program (Applied Maths, Inc., Belgium, 6.01 version). Tenover *et al.* (1995)'s criteria were used for detecting the clonal relation among the *S. aureus* strains.

Results and Discussion

A total of 160 food samples were examined for the presence of *S. aureus* and MRSA using multiplex PCR analysis. Twenty (12.5%) of the samples were found to be contaminated with *S. aureus*. A total of 40 isolates from the 20 positive samples were confirmed to be *S. aureus* by multiplex PCR based on 16S rRNA and *nuc* gene. Among them 25 were from raw milk, 4 were from Carra cheese,

and 11 were from chicken meat samples. The *mecA* gene was not detected in any of the *S. aureus* strains.

When we examined the studies conducted in order to determine the presence of *S. aureus* in various foods in Turkey, the contamination levels with *S. aureus* of the analysed food samples have been observed to be 53.3% by Gundogan *et al.* (2005), 61.1% by Gündoğan *et al.* (2006), 57.3% by Ertas *et al.* (2010), and 52.4% by Gücükoglu *et al.* (2012), which are quite higher than our results. Nevertheless, Can and Çelik (2012) detected *S. aureus* in only 6% of the cheese samples they had analysed, and this rate is lower than the rate we had found in our study.

Moreover, in this context, when the studies conducted in other countries are considered, it is observed that the contamination levels are different from each other, and *S. aureus* is usually isolated from foods which are rather rich in proteins. Presence of *S. aureus* was reported to be 12.8% in meat and dairy samples by Normanno *et al.* (2007), 15.6% in various food samples by Hu *et al.* (2013), and 16.4% in raw meat samples by Hanson *et al.* (2011), and these levels are close to the contamination level (12.5%) we had found in the present study. Unlike the aforementioned findings, the contamination levels with *S. aureus* have been found to be 31.9% in tank milk samples by Muehlherr *et al.* (2003), 23.8% in raw meat by Pesavento *et al.* (2007), and 85.1% in different foods in China by Tang *et al.* (2015). However, *S. aureus* was isolated from the 10.5% of the food samples held responsible for the foodborne infections by Crago *et al.* (2012) in Canada. The differences with the results of contamination levels with *S. aureus* may result from the fact that some of the foods tested in our study are traditional and have different production techniques. In addition to this, it could be also linked to the differences in personal hygiene, milking hygiene and slaughter hygiene through the food chain.

The antibiotic susceptibility profile of the isolates to 11 different antibiotics was tested; all isolates were suscepti-

Table 2. Results of antimicrobial susceptibility tests of *S. aureus* isolates (n=40)

Antibiotic	Resistance							
	Raw milk (25)		Carra cheese (4)		Chicken meat (11)		Total (40)	
	n	(%)	n	(%)	n	(%)	n	(%)
Chloramphenicol	0	0	0	0	1	9.09	1	2.50
Ampicillin	25	100	4	100	8	72.72	37	92.50
Tetracycline	7	28.00	0	0	5	45.45	12	30.00
Cefoxitin	0	0	0	0	1	9.09	1	2.50
Gentamicin	0	0	0	0	0	0	0	0
Erythromycin	4	16.00	0	0	4	36.36	8	20.00
Oxacillin	0	0	0	0	0	0	0	0
Vancomycin	0	0	0	0	0	0	0	0
Penicillin	25	100	4	100	9	81.81	38	95.00
Rifampin	0	0	0	0	0	0	0	0
Ciprofloxacin	0	0	0	0	5	45.45	5	12.50

ble to gentamicin, oxacillin, and vancomycin. The highest resistance rate was detected in penicillin (95%) and ampicillin (92.5%), followed by tetracycline (30%), erythromycin (20%), ciprofloxacin (12.5%). The lowest rate of resistance was found to be against chloramphenicol (2.5%), and cefoxitin (2.5%) (Table 2).

In this study, 39 out of 40 (97.5%) isolates were found to be resistant to one or more antibiotics. Only one isolate from chicken meat was susceptible to all the tested antibiotics. All isolates from raw milk and Carra cheese were resistant to penicillin and ampicillin.

Among the isolates collected from chicken meat, 9.0% (1/11) of them were resistant to chloramphenicol, 72.7% (8/11) were resistant to ampicillin, 45.4% (5/11) were resistant to tetracycline, 9.0% (1/11) were resistant to cefoxitin, 36.3% (4/11) were resistant to erythromycin, 81.8% (9/11) were resistant to penicillin, and 27.2% (3/11) were resistant to ciprofloxacin. In addition, two isolates (18.1%) were found to be intermediately resistant to ciprofloxacin. In this study, isolates from chicken meat were more resistant to antibiotics than the isolates collected from raw milk and Carra cheese. This difference may be due to the widely use of antibiotics for therapeutic and prophylactic purposes at poultry farms in Turkey.

When the results of antibiotic sensitivity test are examined, higher resistance rates were found against beta-lactam antibiotics, penicillin and ampicillin among the isolates from raw milk and Carra cheese. Since beta-lactams have an important place in the treatment of mastitis in Turkey, this could be explained by the extensive and uncontrolled use of these group antibiotics in agriculture. However, no isolate was found to be resistant to methicillin in this study. Apart from the penicillin resistance, Gun-

dogan *et al.* (2005), and Gündoğan *et al.* (2006) found that bacitracin and methicillin resistance was prevalent among the isolates they had obtained. The sensitivity rates of vancomycin, oxacillin, gentamicin, and rifampin we had detected in the present study were in compliance with the rates reported in some other studies (Can and Çelik, 2012; Gundogan *et al.*, 2005; Gündoğan *et al.*, 2006; Hanson *et al.*, 2011; Hu *et al.*, 2013; Normanno *et al.*, 2007; Pesavento *et al.*, 2007).

In contrast, 38% of the isolates were found to be resistant to oxacillin in a study conducted in Portugal (Pereira *et al.*, 2009). Peles *et al.* (2007) found the *S. aureus* strains isolated from the milk of mastitic cows, and the bulk tank milk to be sensitive to methicillin, cefoxitin, tetracycline and erythromycin, while penicillin resistance was found to be prevalent among the isolates in a similar manner. The fact that the isolates were found to be highly resistant (83.3%) to one or more of the antibiotics tested in a study carried out by Can and Çelik (2012) shows similarity with
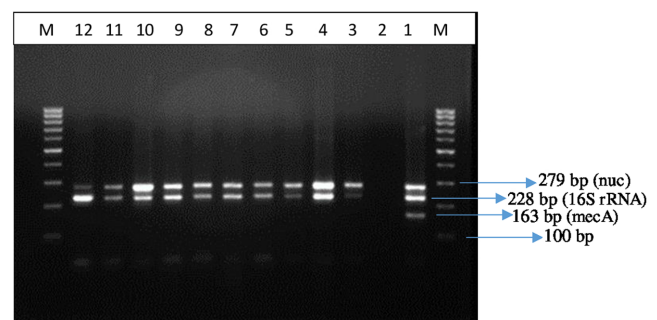

Fig. 1. Identification of *S. aureus* isolates by multiplex PCR. Lane M, 100 bp DNA marker (Bioatlas, Estonia); lane 1, positive control (*S. aureus* ATCC 43300); lane 2, negative control (H₂O); lanes 3-12, positive isolates for 16S rRNA and *nuc* genes.

Code of isolate	Source of isolate	Sampling date
C24Atyp1	Carra cheese	03.01.2015
C24Atyp2	Carra cheese	03.01.2015
C24Atyp3	Carra cheese	03.01.2015
C24Atyp4	Carra cheese	03.01.2015
7ETyp2	Eyes. raw milk	26.11.2014
7BTyp3	Eyes. raw milk	26.11.2014
7BTyp5	Eyes. raw milk	26.11.2014
10BTyp3	Eyes. raw milk	26.11.2014
15BAtyp1	Eyes. raw milk	26.11.2014
9BTyp2	Eyes. raw milk	26.11.2014
9BTyp3	Eyes. raw milk	26.11.2014
9BTyp4	Eyes. raw milk	26.11.2014
12BTyp1	Eyes. raw milk	26.11.2014
24XAtyp1	Comu. raw milk	23.08.2014
24XAtyp2	Comu. raw milk	23.08.2014
24XAtyp3	Comu. raw milk	23.08.2014
24XAtyp4	Comu. raw milk	23.08.2014
54KTyp1	Comu. raw milk	08.09.2014
54KTyp2	Comu. raw milk	08.09.2014
54KTyp3	Comu. raw milk	08.09.2014
39XAtyp1	Comu. raw milk	23.08.2014
39XAtyp2	Comu. raw milk	23.08.2014
53XTyp1	Comu. raw milk	08.09.2014
53XTyp3	Comu. raw milk	08.09.2014
53XTyp4	Comu. raw milk	08.09.2014
41XTyp2	Comu. raw milk	23.08.2014
41XTyp2	Comu. raw milk	23.08.2014
41XTyp3	Comu. raw milk	23.08.2014
42XTyp2	Comu. raw milk	08.09.2014
P17Typ1	Chicken meat	20.03.2015
P17Typ3	Chicken meat	20.03.2015
P14Atyp1	Chicken meat	20.03.2015
P14Atyp2	Chicken meat	20.03.2015
P14Atyp3	Chicken meat	20.03.2015
P1Typ2	Chicken meat	20.03.2015
P11Typ1	Chicken meat	20.03.2015
P3KAtyp3	Chicken meat	20.03.2015
P16Typ1	Chicken meat	20.03.2015
P21Atyp2	Chicken meat	20.03.2015
P22Typ4	Chicken meat	20.03.2015

Fig. 2. Dendogram of PFGE patterns of the 40 foodborne *S. aureus* strains.

the findings of our study. Unlike our study, some researchers found MRSA in raw meat, chicken meat, raw milk, dairy and soy products (Can and Çelik, 2012; de Boer *et al.*, 2009; Gundogan *et al.*, 2005; Gündoğan *et al.*, 2006; Gücüköğlu *et al.*, 2013; Hanson *et al.*, 2011; Pereira *et al.*, 2009; Tang *et al.*, 2015).

In this study, dendogram of PFGE patterns of *S. aureus* strains was also performed. *Sma*I restriction enzyme was used in PFGE method. Nine major patterns were determined in 40 strains. In 3 of the patterns there was a strain closely related to the main cluster. In 6 of the patterns, the number of strains changed between 2-16 and thirty-six strains (90%) had identical PFGE profiles. Two major groups were detected when the similarity coefficient was evaluated as 85% and above. The first group was consisted of 4 strains with a similarity rate of 88%, and the second group was consisted of 36 strains with a similarity rate of 90% (Fig. 2).

Conclusion

These results point out that *S. aureus* strains resistant to antibiotics may be not only nosocomial borne, but also present in food. Although our study is a limited research about the occurence of *S. aureus* in different foods, these analysed foods are usually consumed by local people in this area. As a result, we think the fact that resistance rates against tested antibiotics among the isolates were found to be high, and the multidrug-resistant *S. aureus* strains were also found is an important problem that should be overemphasised in terms of public health and food safety.

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