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RESEARCH ARTICLE

Determination of phenotypical and genotypical characterization and antimicrobial resistance genes of *Staphylococcus aureus* isolated from milk of dairy cows with mastitis

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Mastitisli süt ineklerinden izole edilen *Staphylococcus aureus*'ların fenotipik ve genotipik karakterizasyonu ve antimikrobiyal direnç genlerinin belirlenmesi

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Öz

Amaç: Mastitis, önemli ekonomik kayıplara neden olan süt ineklerinin en yaygın hastalıklarından biridir. *Staphylococcus aureus*, subklinik mastitise neden olan penetrasyonunu ve dokuların tahribatını kolaylaştıran çeşitli virülans faktörlerine sahiptir. Bu çalışmada, *S. aureus*'un fenotipik ve genotipik özelliklerini ve antimikrobiyal duyarlılığını araştırılması amaçlanmıştır.

Gereç ve Yöntem: Mastitisli süt ineklerinden izole edilen toplam 241 *S. aureus* suşu fenotipik olarak (katalaz, koagülaz, hemoliz, DNaz, mannitol fermantasyonu ve biyofilm oluşturma) ve genotipik olarak polimeraz zincir reaksiyon tekniği (PCR) kullanılarak test edildi. Antimikrobiyal duyarlılık testi 15 farklı antibiyotik kullanılarak yapıldı.

Bulgular: İzolatlar farklı hemoliz aktivitesi göstermiştir (β %47, α %42, γ %10 ve δ %1), ancak sadece β-hemolitik suşlar CAMP reaksiyonu göstermiştir. Tüm izolatlar %7,5 NaCl içeren MSA üzerinde büyüyebilmesine rağmen, izolatların %80,5'inde mannitol fermantasyon aktivitesi gözlenmiştir. Nuc geni tüm izolatlarda tespit edildi, ancak izolatların sadece %84,2'si DNase aktivitesi göstermiştir. Kongo kırmızı agar biyofilmleri tespit etmek için bir tarama yöntemi olarak kullanılabilirken, kristal viyole boyama yöntemi daha yeterli sonuçlar verir. Sec geni, enterotoksin genleri arasında en sık rastlanan gendir (%84). MecA genini barındırdığı üç izolat tespit edildi, ancak metisiline duyarlıydı.

Öneri: Sonuç olarak, izolatların fenotipik özelliklerindeki değişim *S. aureus* izolatlatının yanlış sınıflandırılmasına yol açmakta ve moleküler yöntemlerin kullanılması ihtiyacını arttırmaktadır. *S. aureus*'un hızlı ve doğru moleküler tiplenmesi, bu bulaşıcı mikroorganizmanın prevalansını belirleyebilir ve salgın enfeksiyonları önleyebilir.m kullanımının oksidatif strese karşı koruyucu etkisi görülmüştür.

Anahtar kelimeler: *Staphylococcus aureus*, fenotipleme, genotipleme, antimikrobiyal duyarlılık

Abstract

Aim: Mastitis is one of the most common diseases of dairy cattle and causes significant economic losses. *Staphylococcus aureus* produces many virulence factors that facilitate the adhesion and penetration of damaged tissues, and thereby, cause subclinical mastitis. This study was aimed at investigating the phenotypic and genotypic characteristics and antimicrobial susceptibility of *S. aureus*..

Materials and Methods: A total of 241 *S. aureus* strains isolated from bovine mastitis cases were tested phenotypically (catalase, coagulase, haemolysis, DNase, mannitol fermentation and biofilm formation) and genotypically (by the polymerase chain reaction (PCR) technique). Antimicrobial susceptibility was tested using 15 different antibiotics.

Results: While the isolates showed different levels of haemolytic activity (β 47%, α 42%, γ 10% and δ 1%), only the β-haemolytic strains produced a positive CAMP-like reaction. Although all isolates were able to grow on MSA containing 7.5% NaCl, mannitol fermentation activity was observed in 80.5% of the isolates. The nuc gene was detected in all isolates, but only 84.2% of the isolates showed DNase activity. The Congo red agar method can be used to detect the biofilm forming capability of isolates, but the crystal violet staining method gives more reliable results. The sec gene was the most common enterotoxin genes (84%). Three isolates harboured the mecA gene, but were sensitive to methicillin.

Conclusion: Phenotypic variations among isolates result in the misclassification of *S. aureus* strains and require the use of molecular methods. The rapid and accurate molecular typing of *S. aureus* can aid in both determining the prevalence of this infectious microorganism and preventing epidemic infections.

Keywords: *Staphylococcus aureus,* phenotyping, genotyping, antimicrobial susceptibility

Introduction

Mastitis is one of the most common diseases of dairy cattle and causes significant economic losses. Mastitis is described as the inflammation of the mammary gland resulting from by bacterial infection and is manifested by local and systemic symptoms. *Staphylococcus aureus* is an important pathogenic microorganism which causes clinical and subclinical mastitis (Qayyum et al 2016). *S. aureus* produces several virulence factors that facilitate the adhesion of this bacterium and the penetration of damaged tissues (Akineden et al 2001). While virulence factors including protein A, clumping factor and collagen adhesin are associated with bacterial adhesion, biofilm formation enables microorganisms to survive in the presence of antibiotics (Delgado et al 2011). The detection of differences in the gene patterns of *S. aureus* aids in predicting the prevalence of the microorganism and fighting against infections (Kalorey et al 2007).

The procedure used for the identification of *S. aureus* is the same worldwide, and for this purpose catalase, coagulase, haemolytic activity, DNase, mannitol fermentation and the Christie, Atkins and Munch-Petersen (CAMP) reaction are tested. Although *S. aureus* growth on sheep blood agar is often characterized by complete haemolysis (β-haemolysis), non-haemolytic (γ-haemolysis) strains of *S. aureus* have also been reported (Bello and Qahtani 2005). Furthermore, while *S. aureus* differs from other staphylococci by its ability to produce deoxyribonuclease and grow in the presence of 7.5% NaCl, DNase-negative strains have been reported (Gündoğan et al 2006). *S. aureus* activates an alternative autophagic pathway by producing a pore-forming toxin referred to as α-haemolysin (Hla) (Mestre and Colombo 2012). *Rhodococcus equi* shows synergistic haemolytic activity with *S. aureus* on 5% sheep blood agar (Prescott 1991). Since the identification of *S. aureus* cannot be guaranteed with a single phenotypic test, a combination tests is being used in several developing countries for the rapid identification of this species. The combined use of mannitol fermentation, DNase, and coagulase tests has been reported to yield 100% specificity and 67% sensitivity (Kateete et al 2010). Biofilm is a bacterial defense mechanism, which can increase the ability of bacterial to withstand the effects of antimicrobial agents (Christensen et al 1982, Günaydın et al 1995, Gündoğan et al 2006). Staphylococcal protein A (*spa*), which is an IgG-binding protein localized to the cell wall of *S. aureus*, is known to be one of the virulence factors of this bacterium owing to its ability to inhibit phagocytosis by capturing IgG in an inverted orientation (Heilmann 2011). Generally, *spa* is encoded by the *spa* gene, which has 2 regions (*spa*-IgG (encodes the IgG-binding region of *spa*) and *spa-X* (the highly variable region of spa)). Molecular typing of the *spa-X* gene has been used in the genotyping of *S. aureus* (Votintseva et al 2014). Staphylococcal enterotoxins (SEs) are effective exotoxins, which are produced mainly by *S. aureus* and can cause staphylococcal food poisoning (SFP). These exotoxins are part of the most important family of pyrogenic toxin superantigens. *S. aureus* produces a variety of SEs (i.e. SEA, SEB, SEC, SED and SEE) (Argudín et al 2010). Furthermore, *S. aureus* strains show a genetic diversity in their ability to resist antimicrobial agents (penicillin, methicillin, tetracycline, gentamycin, streptomycin, erythromycin, clindamycin, etc.) (Wang et al 2015).

The aim of this study was to investigate the phenotypic (catalase, coagulase, mannitol fermentation, DNase, β-haemolysis, CAMP and biofilm formation) and genotypic (thermo-nuclease, protein A, biofilm formation and staphylococcal enterotoxins) characteristics of *S. aureus* isolates, and the presence of antimicrobial resistance genes (β-lactam (*mecA*), tetracycline (*tetK* and *tetM*), macrolides (*ermA*, *ermB* and *ermC*) and lincosamides (*linA*)) in these isolates.

Material and Methods

Samples

In this study, a total of 241 *S. aureus* isolates, which were isolated from milk samples of dairy cows with mastitis at the laboratory of the Department of Microbiology, Faculty of Veterinary Medicine, Selçuk University, were used. While 37 of the isolates were isolated between 2017-2019 (Group A), 204 were isolated between 2005-2009 under a research project (TUBITAK-TOVAG -1050245) (Group B). *R. equi* was used for the CAMP-like test, and the *S. aureus* ATCC 25923 reference strain was used as a positive control in most tests.

Phenotypic characterization

Isolates, determined to be positive for catalase and coagulase, were considered as *S. aureus*. Haemolytic activity was observed on 5% sheep blood agar (Zhang et al 2016). Mannitol fermentation was tested by inoculating the isolates onto mannitol salt agar (MSA) (Oxoid Ltd, UK) containing 7.5% NaCl (Kateete et al 2010). DNase activity was confirmed by inoculating the isolates onto DNase agar (LABM, UK), incubating the plates at 37oC for 24 hours, and assessing the results by pouring 2 ml of 1 N HCl on the agar plates (Kateete et al 2010). The CAMP test was performed on 5% sheep blood agar in the presence of *R. equi*, such that the *S. aureus* isolates were inoculated perpendicular to *R. equi* (Müller et al 1988). Biofilm formation was examined by two techniques, namely, the Congo red agar (CRA) and crystal violet staining (CVS) (Sigma Aldrich, Germany) methods. Once CRA was prepared (brain heart infusion agar (Oxoid Ltd, UK), sucrose and Congo red stain (Sigma Aldrich, Germany)) and poured into sterile plates, then the isolates were inoculated onto CRA and the plates were incubated at 37oC for 24 hours (Gündoğan et al 2006). CVS has found common use in assessing the biofilm

forming capability of bacteria. CVS enables the assessment of quantitative biofilm formation by measuring the optical density of stained bacterial films adherent to the base of plastic tissue culture plates (Stepanović et al 2007). Isolates were inoculated into tryptic soy broth and incubated at 37oC for 24 hours. After the incubation period the cultures were diluted 1:100 in tryptic soy broth (TSB) (LABM, UK) supplemented with 1% glucose (Merck, Germany), and 600 µL of each sample was distributed into 3 wells (200 µL per well) on a sterile flat-bottom 96-well polystyrene microtiter plate covered with a lid and incubated at 37oC for 24 hours. All samples were triplicate, and non-inoculated TSB supplemented with 1% glucose was used as a negative control. Later, the wells were emptied and washed 3 times with 300 µL of sterile phosphate-buffered saline (PBS pH: 7.2) (Merck, Germany). Biofilm fixation was performed by adding 200µL of Bouin's fixative (saturated aqueous picric acid solution (Merck, Germany), formalin-glacial acetic acid (Merck, Germany)) for 30 minutes, then discarding it and leaving the plate to dry at 60oC for 1 hour. To demonstrate the presence of a biofilm, bacteria were stained with 200 µL of 2% Hucker's crystal violet (Sigma Aldrich, Germany) for 15 minutes, which was later rinsed off under running tap water. Next, 150 µL of 95% ethanol was added to each well and the plate was shaken on a rotator at least 30 minutes. The optical density (OD) was measured at 570 nm (Stepanović et al 2007).

Antimicrobial susceptibility test

Antimicrobial susceptibility was tested by inoculating the isolates onto trypticase soy agar (TSA) (LABM, UK) in the presence of the following antibiotics; penicillin (P, 10 µg Bioanalyse, Turkey), oxacillin (OX, 1 µg Bioanalyse, Turkey), cefoxitin (FOX, 30 µg Bioanalyse, Turkey), methicillin (ME, 5 µg Bioanalyse, Turkey), amoxicillin (AX 2 µg Bioanalyse, Turkey), gentamicin (CN, 10 µg Bioanalyse, Turkey) , streptomycin (S, 10 µg Bioanalyse, Turkey), ciprofloxacin (CIP, 5 µg Bioanalyse, Turkey), tetracycline (TE, 30 µg Bioanalyse, Turkey), rifampicin (RA, 5 µg Bioanalyse, Turkey), vancomycin (VA, 30 µg Bioanalyse, Turkey), chloramphenicol (C, 30 µg Bioanalyse, Turkey), erythromycin (E, 15 µg Bioanalyse, Turkey), azithromycin (AZM, 15 µg Bioanalyse, Turkey) and clindamycin (DA, 2 µg Bioanalyse, Turkey) (Bauer et al 1966, Hadimli et al 2001).

Genotypic characterization

While the 16S rRNA and nuc primers were used to confirm *S. aureus* isolates, the mecA primer was used to distinguish methicillin-resistant *S. aureus* (MRSA) strains. In addition, virulence genes (i.e. biofilm (*icaA* and *icaD*), protein A (*spa-IgG* and *spa-X*) and enterotoxins (*sea, seb, sec, sed* and *see*)) and antimicrobial resistance genes (i.e. *tetK, tetM, ermA,*

Table 1. List of primers used in this study

	Gene	Sequence 5'-.....-3"	PCR*	Size of amplified products (bp)
Virulence genes	16S rRNA	F: AACTCTGTTATTAGGGAAGAACA R: CCACCTTCCTCCGGTTTGTCACC	1	756
	nuc	F: CGATTGATGGTGATACGGTT R: ACGCAAGCCTTGACGAACTAAAGC	2	279
	icaA	F: ACACTTGCTGGCGCAGTCAA R: TCTGGAACCAACATCCAACA	4	188
	icaD	F: ATGGTCAAGCCCAGACAGAG R: AGTATTTTCAATGTTTAAAGCAA	4	189
	spa-IgG	F: CACCTGCTGCAAATGCTGCG R: GGCTTGTTGTTGTCTTCCTC	DP**	390, 590, 810, 920, 970
	spa-X	F: CAAGCACCAAAAGAGGAA R: CACCAGGTTTAACGACAT		110, 220, 253, 270, 290, 315
	sea	F: GGTTATCAATGTGCGGGTGG R: CGGCACTTTTTTCTCTTCGG		102
	seb	F: GTATGGTGGTGTAACTGAGC R: CCAAATAGTGACGAGTTAGG		164
	sec	F: AGATGAAGTAGTTGATGTGTATGG R: CACACTTTTAGAATCAACCG	6	451
	sed	F: CCAATAATAGGAGAAAATAAAAG R: ATTGGTATTTTTTTTCGTTC		278
	see	F: AGGTTTTTTCACAGGTCATCC R: CTTTTTTTTCTTCGGTCAATC		209
Antimicrobial resistance genes	*mecA*	F: GTAGAAATGACTGAACGTCCGATA R: CCAATTCCACATTGTTTCGGTCTAA	3	314
	tetK	F: GTAGCGACAATAGGTAATAGT R: GTAGTGACAATAAACCTCCTA	3	360
	tetM	F: AGTGGAGCGATTACAGAA R: GTAGTGACAATAAACCTCCTA	3	158
	ermA	F: GTTCAAGAACAATCAATACAGAG R: GGATCAGGAAAAGGACATTTTAC	1	421
	ermB	F: CCGTTTACGAAATTGGAACAGGTAAAGGGC R: GAATCGAGACTTGAGTGTGC	5	359
	ermC	F: GCTAATATTGTTTAAATCGTCAATTCC R: GGATCAGGAAAAGGACATTTTAC	1	572
	linA	F: GGTGGCTGGGGGGTAGATGTATTAACTGG R: GCTTCTTTTGAAATACATGGTATTTTTCGATC	3	323

*PCR protocol number as shown in Table 2.
**Duplex protocol: 20 cycles 94°C / 60 sec, 60°C / 60 sec, 72°C / 90 sec, 20 cycles 94°C / 60 sec, 51°C / 60 sec, 72°C / 90 sec. Initial denaturation at 94°C for 5 minutes and final extension at 72°C for 5 minutes.

ermB, ermC and *linA*) were investigated. A phylogenetic tree was constructed using enterobacterial repetitive intergenic consensus-2 (ERIC-2) primer (Table 1 and Table 2).

Results

α, β, γ and δ-haemolytic activities were observed in 100 (42%), 113 (47%), 25 (10%) and 3 (1%) isolates, respectively (Table 3).

All isolates were able to grow on MSA in the presence of 7.5% NaCl, but only 194 isolates (80.5%) were able to use mannitol. Moreover, 30 (93.7%) out of 32 MRSA isolates were mannitol fermenters and only 2 isolates were mannitol-negative (Table 3).

DNase activity was observed in 203 isolates (84.2%) (Table 3). The results of the CAMP test showed that 121 out of the 241 isolates (50%) were capable of forming a synergistic haemolysis with *R. equi*. Out of the 121 CAMP-positive isolates, 113 (93%) were β-haemolytic, 5 (4%) were α-haemolytic and 3 (3%) were δ-haemolytic. All β-haemolytic isolates were CAMP-positive (Table 3). It was ascertained that 223 isolates (92.5%) were capable of forming biofilms on CRA. Furthermore, the CVS technique demonstrated that 194 isolates (80.5%) showed 3 different levels of biofilm strength. Accordingly, 42 (17%), 57 (24%) and 95 (39%) of these isolates formed weak, moderate and strong biofilms, respectively. On the other hand, 183 isolates (76%) were shown to form biofilms by both the CRA and CVS techniques (Table 3).

The antimicrobial susceptibility test demonstrated that 29 isolates (12%) were resistant to at least one of the antibiotics methicillin, oxacillin and cefoxitin. Three isolates were detected to harbour the mecA gene, but were sensitive to methicillin.

Table 2. Conventional PCR protocols

*PCR Protocol Number	Denaturation Temperature / Time	Annealing Temperature. / Time	Elongation Temperature. / Time	Number of Cycles
1	94°C / 60 sec	59°C / 60 sec	72°C / 90 sec	30
2	94°C / 60 sec	58°C / 60 sec	72°C / 90 sec	30
3	94°C / 60 sec	53°C / 60 sec	72°C / 90 sec	30
4	94°C / 60 sec	56°C / 60 sec	72°C / 90 sec	30
5	94°C / 60 sec	65°C / 60 sec	72°C / 90 sec	30
6	94°C / 60 sec	55°C / 60 sec	72°C / 90 sec	35

*All of the PCR protocols started with an initial denaturation at 94°C for 5 minutes and ended with a final extension at 72°C for 5 minutes.

Table 3. Phenotypic characterization results

Tests			Group A	Group B	All isolates
Catalase			100%	100%	100%
Coagulase			100%	100%	100%
Hemolysis	α		8%	40%	42%
	β		84%	48%	47%
	γ		8%	11%	10%
	δ		0%	1%	1%
Mannitol			82.3%	70.2%	80.5%
DNase			83.8%	84.3%	84.2%
CAMP			84%	44%	50%
Biofilm	CRA		97.2%	91.6%	92.5%
	CVS	Strong	11%	45%	39%
		Moderate	22%	24%	24%
		Weak	24%	16%	17%

Table 4. Results of the antimicrobial susceptibility test

Antibiotics	Group A			Group B			All isolates		
	R	S	I	R	S	I	R	S	I
Methicillin	9	28	0	12	192	0	21	220	0
Amoxicillin	16	21	0	126	78	0	142	99	0
Oxacillin	8	29	0	15	189	0	23	218	0
Cefoxitin	1	36	0	4	200	0	5	236	0
Penicillin	16	21	0	128	76	0	144	97	0
Gentamycin	4	33	0	44	160	0	48	193	0
Vancomycin	0	37	0	4	200	0	4	237	0
Tetracycline	6	31	0	37	167	0	43	198	0
Streptomycin	32	3	2	185	7	12	217	10	14
Erythromycin	4	33	0	39	165	0	43	198	0
Azithromycin	5	32	0	38	165	1	43	197	1
Clindamycin	2	35	0	24	176	4	26	211	4
Chloramphenicol	0	37	0	31	173	0	31	210	0
Rifampicin	0	37	0	10	194	0	10	231	0
Ciprofloxacin	0	37	0	8	196	0	8	233	0

R, Resistant; S, Sensitive; I, Intermediate

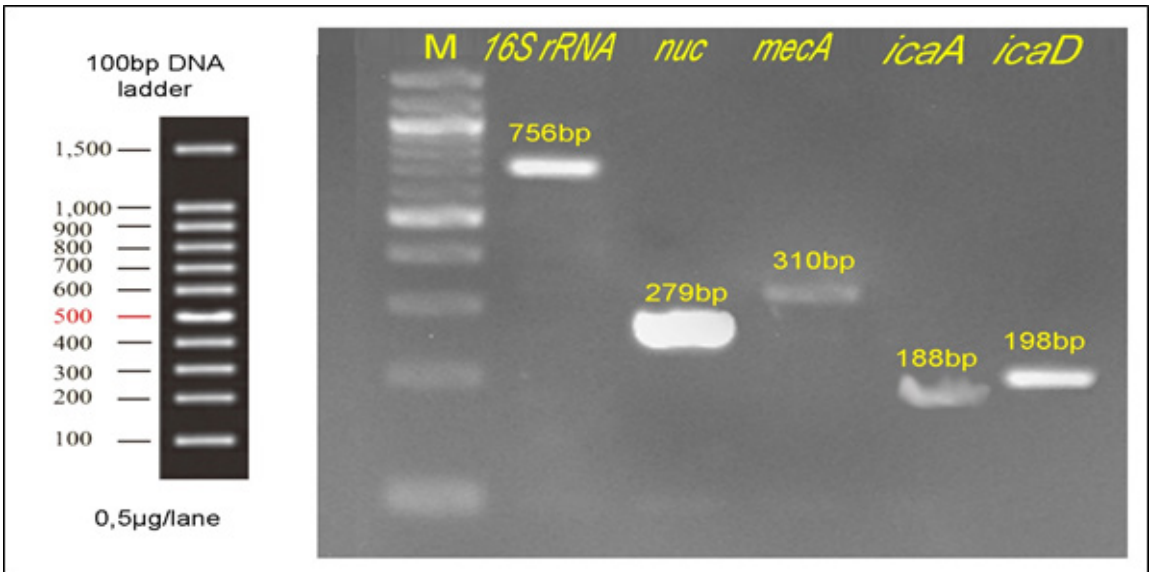

Figure 1. PCR amplification products of the 16S rRNA, *nuc, mecA, icaA* and *icaD* genes

Multidrug resistance (resistance to more than 5 antibiotics) was recorded in 60 isolates (25%). On the other hand, most of the isolates were susceptible to vancomycin (237-98%), ciprofloxacin (233-97%), rifampicin (231-96%) and clindamycin (211-87%). Furthermore, 217 isolates (90.7%) were resistant to streptomycin (Table 4).

As the 16S rRNA and nuc genes were detected in all isolates, these isolates were genetically confirmed to be *S. aureus*. The presence of the *mecA* gene was determined in 32 isolates (13.3%) (Fig. 1). The PCR analysis of the *icaA* and *icaD* genes showed that 239 isolates (99%) harboured at least one of these 2 genes (Fig 1).

The genotyping of the isolates based on the amplification of the *spa-IgG* and *spa-X* genes demonstrated 44 patterns, of which P007, P003 and P022 were observed in 31 (13%), 20 (8%) and 15 (6%) isolates, respectively (Fig 2) (Table 5).

The *sec, seb, sea, sed* and *see* genes were detected in 202 (84%), 76 (32%), 46 (19%), 21 (9%) and 3 (1%) isolates, respectively (Fig. 3).

The *tetK* gene was detected in 202 isolates (83%) and the *tetM* gene was determined in 45 isolates (19%). Moreover, all of the tetracycline resistant isolates harboured both of the *tetK* and *tetM* genes (Fig 4). The macrolide genes *ermA, ermB* and *ermC* were present in 79 (33%), 101 (42%) and 153 (63%) isolates, respectively (Fig 4). The lincosamide gene (*linA*) was detected in 200 isolates (83%). Out of these 200 isolates, 26 (13%) were resistant to clindamycin (Fig 4).

The amplification of the ERIC-2 primer divided the isolates into 143 patterns distributed in 35 clusters (Fig 5). Almost all isolates were close to each other in the phylogenetic tree, except for 4 isolates that displayed different phenotypic properties (Fig 6).

Table 5. Polymorphism of the *spa* genes.

Groups	Isolates	Percentage	Bands (bp)
P001	2	0,83	220
P002	1	0,41	220, 253
P003	20	8,30	970
P004	14	5,81	970, 320
P005	7	2,90	970, 290
P006	12	4,98	970, 253
P007	31	12,86	970, 220
P008	9	3,73	970, 220, 320
P009	6	2,49	970, 220, 290
P010	3	1,24	970, 110
P011	1	0,41	970, 110, 253, 290
P012	1	0,41	970, 110, 220
P013	1	0,41	970, 110, 220, 253
P014	13	5,39	920
P015	4	1,66	920, 290
P016	1	0,41	920, 253
P017	12	4,98	920, 220
P018	1	0,41	920, 220, 320
P019	1	0,41	920, 110
P020	1	0,41	920, 110, 253
P021	2	0,83	920, 110, 220
P022	15	6,22	810
P023	6	2,49	810, 320
P024	7	2,90	810, 290
P025	13	5,39	810, 253
P026	11	4,56	810, 220
P027	2	0,83	810, 220, 320
P028	2	0,83	810, 220, 290
P029	2	0,83	810, 220, 253
P030	1	0,41	810, 220, 253, 290
P031	11	4,56	810, 110
P032	2	0,83	810, 110, 290
P033	1	0,41	810, 110, 253
P034	4	1,66	810, 110, 220
P035	2	0,83	810, 110, 220, 253
P036	1	0,41	590
P037	4	1,66	590, 290
P038	1	0,41	590, 253
P039	1	0,41	590, 220
P040	3	1,24	590, 110, 253
P041	2	0,83	390
P042	1	0,41	390, 320
P043	5	2,07	390, 220
P044	1	0,41	390, 110

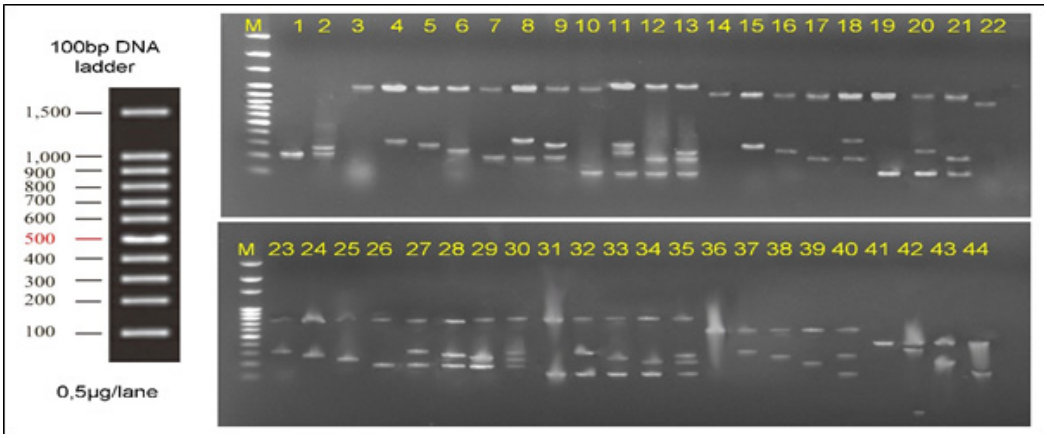

Figure 2. PCR amplification products of the protein A genes (*spa-IgG* and *spa-X*)

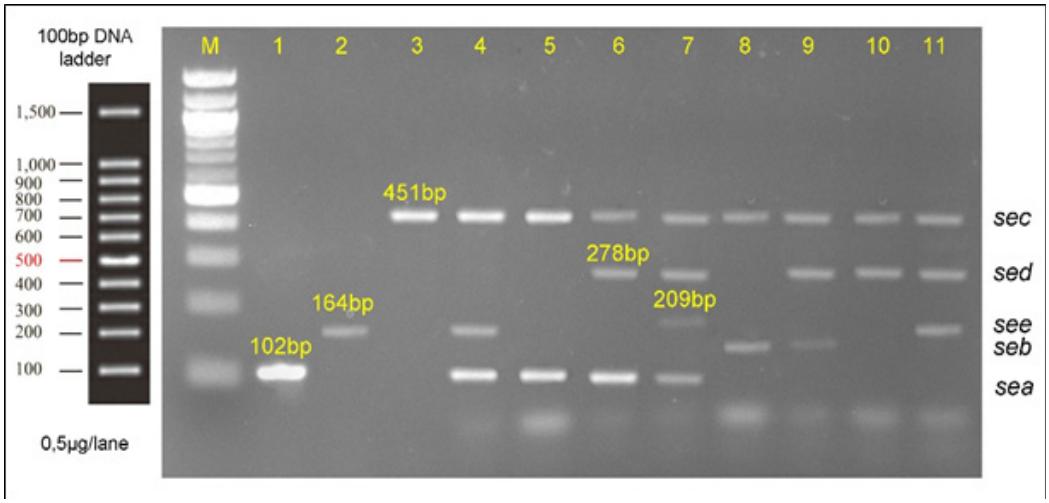

Figure 3. PCR amplification products of the staphylococcal enterotoxin genes (*sea, seb, sec, sed* and *see*)

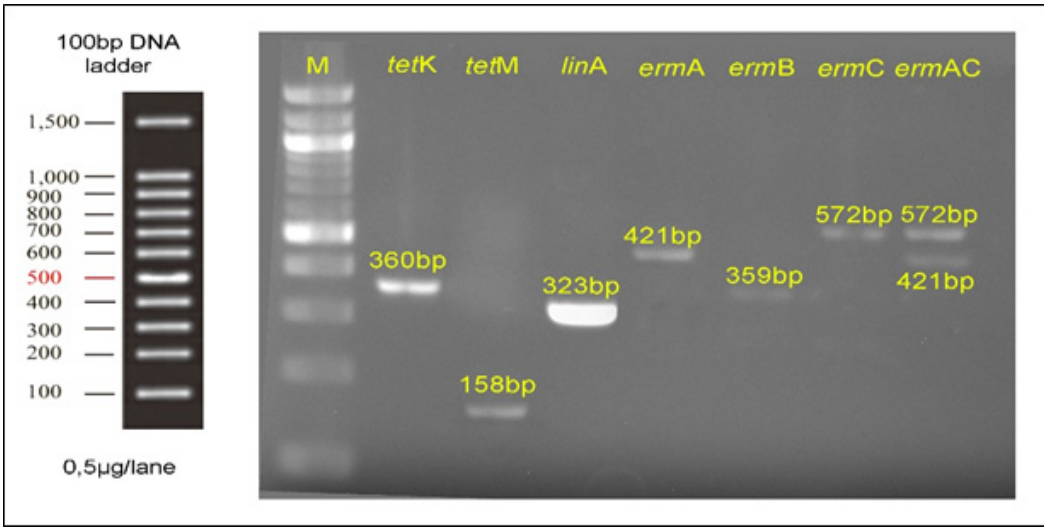

Figure 4. PCR amplification products of the *tetK, tetM, linA, ermA, ermB* and *ermC* genes

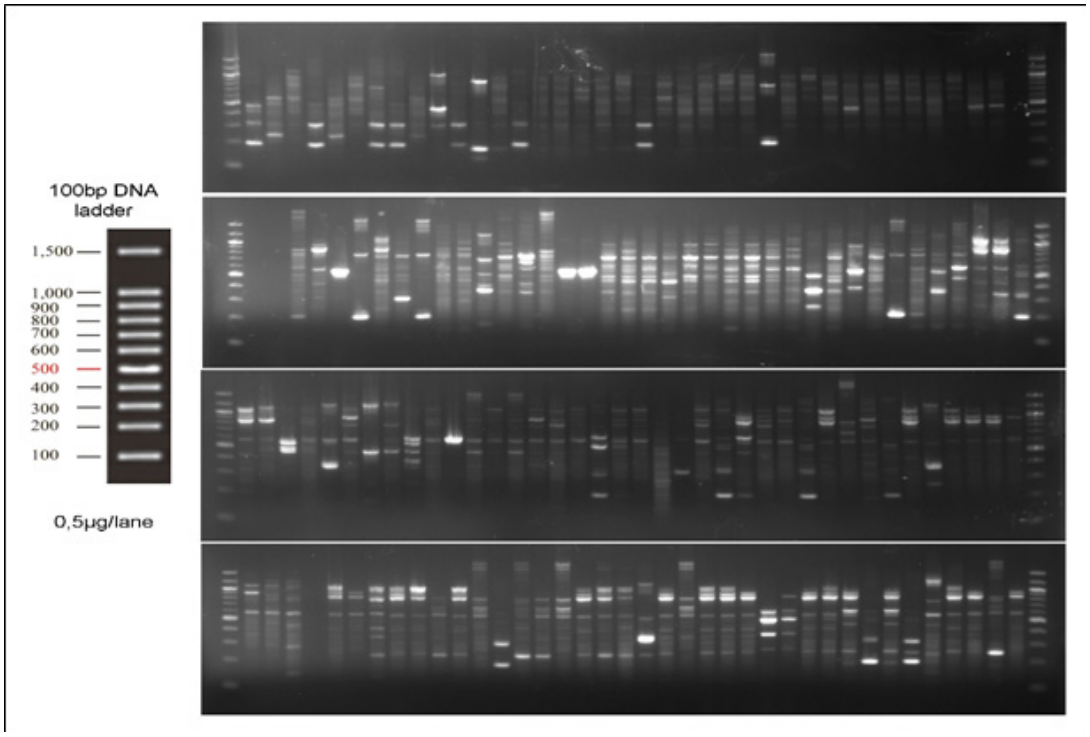

Figure 5. Genotyping of the *S. aureus* strains

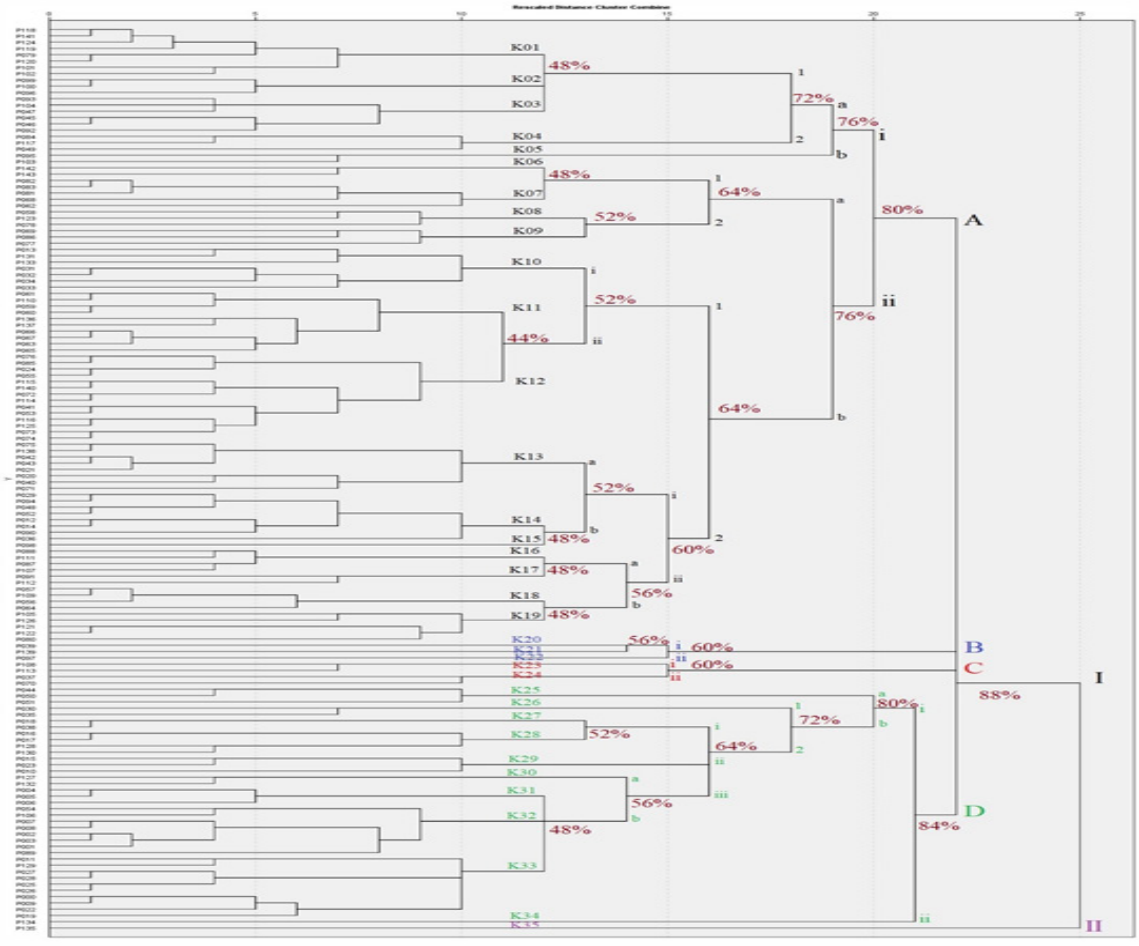

Figure 6. Phylogenetic tree of the *S. aureus* isolates

Discussion

Mastitis is described as the bacteria-induced inflammation of the mammary gland and is manifested by local and systemic symptoms. *S. aureus* is an important pathogenic microorganism that causes clinical and subclinical mastitis. It produces several virulence factors that make it easier for *S. aureus* to adhere to and penetrate damaged tissues (Qayyum et al 2016).

Almost all Staphylococcus species produce the catalase enzyme, which allows bacteria to resist intracellular and extracellular killing by hydrogen peroxide. *S. aureus* is distinguished from other Staphylococcus species by its ability to produce coagulase. Coagulase is an enzyme that converts fibrinogen to fibrin. Several studies (Normanno et al 2005, Gündoğan et al 2006, Akineden et al 2008, Arslan et al 2009, Kateete et al 2010, Aydınalp 2015) have used the catalase and coagulase tests to detect *S. aureus* in Turkey and varying levels of positive results (100%, 85% and 94%) have been reported. This variation in study results has been attributed to 2 reasons. Accordingly, although *S. aureus* is known to be catalase-positive, *S. aureus* subsp. anaerobius is catalase-negative (Dezfulian et al 2010) and not all coagulase-positive staphylococci are *S. aureus*, as S. intermedius, S. hyicus and S. delphini are also known to be coagulase positive (Normanno et al 2005). The catalase and coagulase test results obtained in the present study are highly compatible with the results of the previous research referred to above.

S. aureus produces hemolysin as an exotoxin to destroy the erythrocyte membrane either completely or partially. Several studies conducted in different parts of the world (Caroll et al 1993, Aarestrup et al 1999, Larsen et al 2002, Er et al 2005, Akineden et al 2008, Rajic 2013, Zhang et al 2016) have shown that *S. aureus* strains display different haemolytic activity. In the present study, 83.8% of the isolates in Group A showed β-haemolytic activity, while only 46.9% of the isolates in Group B were β-haemolytic. Carroll et al (1993) and Aarestrup et al (1999), reported that long-term storage may have an impact on the phenotypic properties of isolates, which explains the big difference observed between the isolates included in Group A and Group B in the present study.

MSA supplemented with 7.5% NaCl inhibits the growth of non-pathogenic staphylococci and improves the isolation of *S. aureus*. In previous studies (Kampf et al 1998, Han et al 2007, Kateete et al 2010), MSA has been used to isolate MRSA from clinical specimens. In the present study, statistical analyses showed that 30 (93.7%) out of 32 MRSA isolates were mannitol fermenters and only 2 isolates were unable to ferment mannitol. Although data available on mannitol-negative *S. aureus* is rare, few studies (Murphey and Rosenblum 1964, Tu and Palutke 1976, Smyth and Kahlmeter 2005, Shittu et al 2007) have reported the presence of mannitol-negative *S. aureus* in relation to genetic and enzymatic defects. In the present study, the 2 mannitol-negative MRSA isolates differed from the other isolates in their phenotypic and genotypic properties.

Extracellular DNase activity can enhance the prevalence of pathogenic bacteria and increase the incidence of diseases. Menzies (1977), Gündoğan et al (2006) and Kateete et al (2010), have reported DNase activity levels of 98%, 94.5% and 75%, respectively, for *S. aureus*. These results agree with the DNase activity determined for *S. aureus* in the present study. Moreover, no significant difference was observed between Group A and Group B isolates, as they produced 83.8% and 84.3% positive test results, respectively. This suggests that long-term storage has no effect on the DNase activity of *S. aureus* strains.

The interaction between β-haemolytic *S. aureus* and *R. equi* brings about synergistic haemolysis in sheep blood agar. This feature distinguishes β-haemolytic *S. aureus* from other strains. A previous study (Lo et al 2011) showed that the CAMP reaction occurred with the growth of β-haemolytic *S. aureus* isolates, but not with α-haemolytic isolates, on sheep blood agar. Another study (Akineden et al 2008) suggested that δ-haemolytic activity can be shown only on horse blood agar. In the present study, all of the β-haemolytic isolates were CAMP-positive and 3 isolates showed no haemolysis on sheep blood agar, but were CAMP-positive. Therefore, these isolates were considered to be δ-haemolytic isolates. In addition, 5 α-haemolytic isolates in Group B produced positive CAMP test results. As positive CAMP test results have not been reported for α-haemolytic *S. aureus* in any previous study, these isolates were considered to be β-haemolytic, which had lost some of their phenotypic properties due to long-term storage.

Biofilms are bacterial cells arranged in multiple layers that stick to each other as well as to surfaces. The CRA and CVS methods are the most commonly used tools for the assessment of biofilm formation. Several studies (Christensen et al 1985, Chaieb et al 2005, Sudagidan and Aydin 2009, Torlak et al 2017, Şahin and Kaleli 2018) have proven the CVS method to be adequate in detecting biofilm formation. The icaA and icaD genes were detected in 86,6% of 112 *S. aureus* isolates from subclinical bovine mastitis cases in Hatay, and 70.5% of these isolates were capable of forming biofilms (Aslantaş and Demir 2016). Another study conducted in Hatay (Tel et al 2012) showed the presence of both genes in all isolates, 91.81% of which were determined to be capable of forming biofilms by the CVS method. Torlak et al (2017) reported that PCR and CVS results for the icaA and icaD genes were compatible. In the present study, the comparison of the phenotypic and genotypic results obtained for biofilm formation revealed that the *icaA* and *icaD* genes were present in all of the strong biofilm-forming isolates and in 70% of the moderate

biofilm-forming isolates. Therefore, it was concluded that both genes are required to create a strong biofilm.

S. aureus has the ability to resist almost all antimicrobial agents. Yang et al (2018) indicated that the misclassification of MRSA isolates has been reported in previous studies conducted in China (Cui et al 2009, Wang et al 2014), since these studies have identified MRSA phenotypically based on their resistance to methicillin, oxacillin or cefoxitin with no reference to the presence of the *mecA* gene. In the present study, the *mecA* gene was detected in 32 isolates, 29 of which were identified as MRSA by the disc diffusion method. Choi et al (2003) hypothesized that resistance to β-lactam antibiotics is not consistently expressed by the *mecA* gene, as auxiliary genes such as *femA* and *mecR* can participate in the expression of the β-lactam gene. This explains the detection of 3 *mecA*-positive methicillin-susceptible *S. aureus* (MSSA) isolates in the present study. Additionally, since these 3 isolates showed a deficiency in some phenotypic properties (DNase, mannitol fermentation, β-haemolysis activity and CAMP reaction), it was considered that these isolates had undergone a mutation that led to a lack of expression of some genes. Moreover, the comparison of Group A and Group B isolates for antimicrobial susceptibility test results showed that antimicrobial resistance had increased in recent years.

Tetracycline shows bacteriostatic and bactericidal activity against *S. aureus* depending on its concentration (Heman-Ackah 1976). Kumar et al (2010) reported that the *tetK* gene is more common than the *tetM* gene. Schmitz et al (2001), reported that while the *tetK* gene was more prevalent than the *tetM* gene in MSSA isolates, the *tetM* gene was more prevalent in MRSA isolates, and all isolates that carried the *tetM* gene were resistant to tetracycline. In the present study, the *tetK* and *tetM* genes were detected in 202 (83%) and 45 (19%) isolates, respectively. Furthermore, only 43 isolates that harboured both genes were resistant to tetracycline.

Kot et al (2012) and Wang et al (2015) reported that the prevalence of the *ermC* gene among *S. aureus* strains was higher than that of the *ermA* and *ermB* genes. Sampimon (2011) hypothesized that isolates which carry all the resistance genes for specific antibiotics, may be able to survive at higher levels in the presence of high concentrations of these antibiotics, when compared to isolates carrying les of these genes. The molecular analysis of macrolide resistance genes in the present study revealed that 32 isolates carried all three of these genes and were resistant to both erythromycin and azithromycin. Kumar et al (2010) determined the absence of the *ermA* and *ermC* genes in erythromycin-resistant *S. aureus* using the multiplex-PCR technique. In the present study, the multiplex-PCR technique was performed for the 32 isolates, which carried all three of the macrolide resistance genes. However, the results revealed that while the *ermB* gene was not observed in all isolates, the *ermA* and *ermC* genes were observed alone in 8 and 13 isolates, respectively. Therefore, the multiplex-PCR technique is not recommended for the detection of macrolide resistance genes.

Previous studies (Lüthje and Schwarz 2006, Sampimon 2011) have reported that some *S. aureus* strains are sensitive to clindamycin but resistant to pirlimycin, whilst other strains are sensitive to pirlimycin but resistant to lincomycin. In the present study, the *linA* gene was detected in 200 isolates, but only 26 isolates showed resistance to clindamycin.

Several studies (Kalorey et al 2007b, Votintseva et al 2014) have reported the *spa-IgG* and *spa-X* genes to be diverse. In addition, isolates producing more than one band of *spa-X* amplicons have also been reported (Rathore et al 2012, Bhati et al 2016). In the present study, the polymorphism of the *spa* gene demonstrated that while the 790 bp band was common in 105 isolates, the 220 bp band was observed as a polymorphic band in all MRSA isolates and in 55% of the multi-drug resistant isolates (more than 5 antibiotics). Therefore, the 220 bp band may be associated with multidrug resistance genes. Future studies may reveal the role of protein A in multiple drug resistance.

All enterotoxin genes are located on mobile genetic elements, and their spread among *S. aureus* isolates can modify their ability to cause disease and contribute to the evolution of this important pathogen (Argudín et al 2010). Previous studies (Zschöck et al 2000, Basanisi et al 2016, Gandhale et al 2017) have revealed that the sec gene is more prevalent than the other enterotoxin genes. Furthermore, Rall et al (2008) suggested that the pathogenicity of *S. aureus* may be greater than thought. In the present study, the prevalence of the sec gene (84%) was found to be significantly higher than that of the other enterotoxin genes. However, 97% of the isolates in group A carried at least one enterotoxin gene. Therefore, further studies are needed to elucidate the expression of staphylococcal enterotoxin genes and to evaluate their importance in the pathogenicity of *S. aureus*.

The amplification of the ERIC-2 primer divided the isolates into 35 clusters (K1-K35) distributed within 2 groups (I and II). While 98% of the isolates fell under Group I, only 2% of the isolates fell under Group II. Isolates belonging to Group II differed from the other isolates in terms of their phenotypic and genotypic characteristics. In addition, most of the MRSA isolates were under cluster K12 of Group I, except for 4 isolates that were under cluster K35 of Group II in the phylogenetic tree. This suggested that, the isolates in Group II might have undergone a mutation or lost some of their characteristics because of prolonged storage. Moreover, all K35 isolates shared the same pattern (P043) of spa genes, which suggested a compatibility between the patterns of *spa* genes and the clusters in the phylogenetic tree.

Conclusion

The variation in the phenotypic properties (catalase, coagulase, mannitol fermentation, DNase, β-haemolysis, CAMP reaction, biofilm formation and antibiotic susceptibility) of isolates leads to the misclassification of *S. aureus* strains and requires the use of molecular methods to distinguish MRSA strains from MSSA strains. The rapid and accurate molecular typing of *S. aureus* can aid in both determining the prevalence of this infectious microorganism and preventing epidemic infections. Studying the phenotypic and genotypic characteristics of isolates may contribute to a better understanding of the epidemiology and aetiology of *S. aureus* infections.

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Conflict of Interest

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References

Aarestrup FM, Larsen H, Eriksen N, Elsberg C, et al., 1999. Frequency of α and β haemolysin in *Staphylococcus aureus* of bovine and human origin: A comparison between pheno and genotype and variation in phenotypic expression. Acta Pathol Microbiol Immunol Scand, 107(4), 425-30.

Akineden O, Annemuller C, Hassan AA, Lammler C, et al., 2001. Toxin genes and other characteristics of *Staphylococcus aureus* isolates from milk of cows with mastitis. Clin Diagn Lab Immunol, 8(5), 959-964.

Akineden Ö, Hassan AA, Schneider E, Usleber E, 2008. Enterotoxigenic properties of *Staphylococcus aureus* isolated from goats milk cheese. Int J Food Microbiol, 124(2), 211-216.

Argudín MÁ, Mendoza MC, Rodicio MR, 2010. Food poisoning and *Staphylococcus aureus* enterotoxins. Toxins, 2(7), 1751-173.

Arslan E, Celebi A, Acik L, Ucan US, 2009. Characterisation of coagulase positive Staphylococcus species isolated from bovine mastitis using protein and plasmid patterns. Turk J Vet Anim Sci, 33(6), 493-500.

Aslantaş Ö, Demir C, 2016. Investigation of the antibiotic resistance and biofilm forming ability of *Staphylococcus aureus* from subclinical bovine mastitis cases. J Dairy Sci, 99(11), 8607-8613.

Aydınalp R. 2015. Mastitisli ineklerden izole edilen *Staphylococcus aureus* suşlarının bazı virulans faktörlere [SPA (Xr ve IgG bağlanma bölgesi), COA, cLFA] göre tiplendirilmesi, Yüksek Lisans Tezi, Selçuk Üniversitesi Fen Bilimleri Enstitüsü, Konya.

Basanisi M, Nobili G, La Bella G, Russo R, et al., 2016. Molecular characterization of *Staphylococcus aureus* isolated from sheep and goat cheeses in southern Italy. Small Rumin Res, 135, 17-19.

Bauer AW, Kirby WMM, Sherris JC, Turck M, 1966. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol, 36:493-496.

Bello C, Qahtani A, 2005. Pitfalls in the routine diagnosis of *Staphylococcus aureus*. Afr J Biotechnol, 4(1), 83-86.

Bhati T, Nathawat P, Sharma SK, Yadav R, et al., 2016. Polymorphism in spa gene of *Staphylococcus aureus* from bovine subclinical mastitis. Vet World, 9(4), 421-424.

Carroll J, Cafferkey M, Coleman D, 1993. Serotype F double and triple converting phage insertionally inactivate the *Staphylococcus aureus* β-toxin determinant by a common molecular mechanism. FEMS Microbiol Lett, 106(2), 147-155.

Chaieb K, Mahdouani K, Bakhrouf A, 2005. Detection of icaA and icaD loci by polymerase chain reaction and biofilm formation by Staphylococcus epidermidis isolated from dialysate and needles in a dialysis unit. J Hosp Infect, 61(3), 225-230.

Choi SM, Kim SH, Kim HJ, Lee DG, et al., 2003. Multiplex PCR for the detection of genes encoding aminoglycoside modifying enzymes and methicillin resistance among Staphylococcus species. J Korean Med Sci, 18(5), 631-636.

Christensen GD, Simpson WA, Bisno AL, Beachey EH, 1982. Adherence of slime producing strains of Staphylococcus epidermidis to smooth surfaces. Infect Immun, 37(1), 318-326.

Christensen GD, Simpson WA, Younger J, Baddour L, et al., 1985. Adherence of coagulase negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol, 22, 996-1006.

Cui S, Li J, Hu C, Jin S, et al., 2009. Isolation and characterization of methicillin-resistant *Staphylococcus aureus* from swine and workers in China. J Antimicrob Chemother, 64(4), 680-683.

Delgado S, Garcia P, Fernandez L, Jimenez E, et al., 2011. Characterization of *Staphylococcus aureus* strains involved in human and bovine mastitis. FEMS Immunol Med Microbiol, 62(2), 225-235.

Dezfulian A, Salehian M, Amini V, Dabiri H, et al., 2010. Catalase negative *Staphylococcus aureus* isolated from a diabetic foot ulcer. Iran J Microbiol, 2(3), 165-167.

ER DS, Boechat JUD, Martins JCD, Ferreira WPB, et al., 2005. Hemolysin production by *Staphylococcus aureus* species

isolated from mastitic goat milk in Brazilian dairy herds. Small Rumin Res, 56(1-3), 271-275.

Gandhale D, Kolhe R, Nalband S, Deshpande P, et al., 2017. Molecular types and antimicrobial resistance profile of *Staphylococcus aureus* isolated from dairy cows and farm environments. Turk J Vet Anim Sci, 41(6), 713-724.

Günaydın M, Leblebicioğlu H, Saniç A, Pirinççiler M, 1995. Koagülaz negatif stafilokoklarda slime yapımı ve antibiyotik direnci ile ilişkisi. Mikrobiyol Bul, 29, 26-31.

Gündoğan N, Citak S, Turan E, 2006. Slime production, DNase activity and antibiotic resistance of *Staphylococcus aureus* isolated from raw milk, pasteurised milk and ice cream samples. Food Control, 17(5), 389-392.

Hadimli HH, Ateş M, Güler L, Kav K, et al., 2001. Mastitisli süt ineklerinden izole edilen stafilokokların β- laktamaz aktiviteleri ve antibiyotiklere duyarlılıkları. Vet Bil Derg, 17(4), 21- 25.

Hadimli HH, Erganiş O, Kav K, Sayın Z, 2005. Evaluation of a combined vaccine against staphylococcal mastitis in ewes. Bulletin Vet Res Ins Pulawy, 49(2), 165-167.

Han Z, Lautenbach E, Fishman N, Nachamkin I, 2007. Evaluation of mannitol salt agar, CHROMagar Staph aureus and CHROMagar MRSA for detection of meticillin resistant *Staphylococcus aureus* from nasal swab specimens. J Med Microbiol, 56(1), 43-46.

Heilmann C. 2011. Adhesion mechanisms of staphylococci. Adv Exp Med Bio, 715, 105-123.

Heman-Ackah SM, 1976. Comparison of tetracycline action on *Staphylococcus aureus* and Escherichia coli by microbial kinetics. Antimicrob Agents Chemother, 10(2), 223-228.

Kalorey DR, Shanmugam Y, Kurkure NV, Chousalkar KK, et al., 2007. PCR-based detection of genes encoding virulence determinants in *Staphylococcus aureus* from bovine subclinical mastitis cases. J Vet Sci, 8(2), 151-154.

Kampf G, Lecke C, Cimbal A-K, Weist K, et al., 1998. Evaluation of mannitol salt agar for detection of oxacillin resistance in *Staphylococcus aureus* by disk diffusion and agar screening. J Clin Microbiol, 36(8), 2254-2257.

Kateete DP, Kimani CN, Katabazi FA, Okeng A, et al., 2010. Identification of *Staphylococcus aureus*: DNase and Mannitol salt agar improve the efficiency of the tube coagulase test. Ann Clin Microbiol Antimicrob, 9(1), 23-29.

Kot B, Piechota M, Wolska KM, Frankowska A, et al., 2012. Phenotypic and genotypic antimicrobial resistance of staphylococci from bovine milk. Pol J Vet Sci, 15(4), 677-683.

Larsen H, Aarestrup FM, Jensen N, 2002. Geographical variation in the presence of genes encoding superantigenic exotoxins and β hemolysin among *Staphylococcus aureus* isolated from bovine mastitis in Europe and USA. Vet Microbiol, 85, 61-67.

Lo C-W, Lai Y-K, Liu Y-T, Gallo RL, et al., 2011. *Staphylococcus aureus* hijacks a skin commensal to intensify its virulence: immunization targeting β-hemolysin and CAMP factor. J Invest Dermatol, 131(2), 401-409.

Lüthje P, Schwarz S, 2006. Antimicrobial resistance of coagulase negative staphylococci from bovine subclinical mastitis with particular reference to macrolide lincosamide resistance phenotypes and genotypes. J Antimicrob Chemother, 57(5), 966-969.

Menzies RE, 1977. Comparison of coagulase, deoxyribonuclease (DNase), and heat stable nuclease tests for identification of *Staphylococcus aureus*. J Clin Pathol, 30(7), 606-608.

Mestre MB, Colombo MI, 2012. *Staphylococcus aureus* promotes autophagy by decreasing intracellular cAMP levels. Autophagy, 8(12), 1865-1867.

Müller F, Schaal KP, von Graevenitz A, Von Moos L, et al., 1988. Characterization of *Rhodococcus equi* like bacterium isolated from a wound infection in a noncompromised host. J Clin Microbiol, 26(4), 618-620.

Murphey W, Rosenblum E, 1964. Mannitol catabolism by *Staphylococcus aureus*. Arch Biochem Biophys, 107, 292-297.

Normanno G, Firinu A, Virgilio S, Mula G, et al., 2005. Coagulase positive staphylococci and *Staphylococcus aureus* in food products marketed in Italy. Int J Food Microbiol, 98(1), 73-79.

Prescott JF, 1991. *Rhodococcus equi*: An animal and human pathogen. Clin Microbiol Rev, 4(1), 20-34.

Qayyum A, Khan JA, Hussain R, Khan A, et al., 2016. Molecular characterization of *Staphylococcus aureus* isolates recovered from natural cases of subclinical mastitis in Cholistani cattle and their antibacterial susceptibility. Pak J Agric Sci, 53(4), 971-976.

Rajić Savić NS. 2013. Fenotipske i genotipske karakteristike koagulaza pozitivnih stafilokoka izolovanih iz vimena krava. Doktora Tezi, Belgrad Üniversitesi, Veteriner Fakültesi, Belgrad.

Rall VLM, Vieira FP, Rall R, Vieitis RL, et al., 2008. PCR detection of staphylococcal enterotoxin genes in *Staphylococcus aureus* strains isolated from raw and pasteurized milk. Vet Microbiol, 132(3-4), 408-413.

Rathore P, Kataria A, Khichar V, Sharma R, 2012. Polymorphism in *coa* and *spa* virulence genes in *Staphylococcus aureus* of camel skin origin. J Camel Pract Res, 19(2), 129-134.

Sampimon O, 2011. Antimicrobial susceptibility of coagulase negative staphylococci isolated from bovine milk samples. Vet Microbiol, 150, 173-79.

Schmitz F-J, Krey A, Sadurski R, Verhoef J, et al., 2001. Resistance to tetracycline and distribution of tetracycline resistance genes in European *Staphylococcus aureus* isolates. J Antimicrob Chemother, 47(2), 239-240.

Shittu A, Lin J, Morrison D, 2007. Molecular identification and characterization of mannitol negative methicillin resistant *Staphylococcus aureus*. Diagn Microbiol Infect Dis, 57(1), 93-95.

Smyth R, Kahlmeter G, 2005. Mannitol salt agar cefoxitin combination as a screening medium for methicillin resistant *Staphylococcus aureus*. J Clin Microbiol, 43(8), 3797-3799.

Stepanović S, Vuković D, Hola V, Bonaventura GD, et al., 2007. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for as-

sessment of biofilm production by staphylococci. Apmis, 115(8), 891-899.

Strommenger B, Kettlitz C, Werner G, Witte W, 2003. Multiplex PCR assay for simultaneous detection of nine clinically relevant antibiotic resistance genes in *Staphylococcus aureus*. J Clin Microbiol, 41(9), 4089-4094.

Sudagidan M, Aydin A, 2009. Screening virulence properties of staphylococci isolated from meat and meat products. Vet Med Austria / Wien Tierärztl Mschr, 96, 128-134.

Şahin R, Kaleli İ, 2018. Comparison of genotypic and phenotypic characteristics in biofilm production of *Staphylococcus aureus* isolates. Mikrobiyol Bul, 52(2), 111-112.

Tel O, Aslantaş Ö, Keskin O, Yilmaz E, et al., 2012. Investigation of the antibiotic resistance and biofilm formation of *Staphylococcus aureus* strains isolated from gangrenous mastitis of ewes. Acta Vet Hung, 60(2), 189-197.

Torlak E, Korkut E, Uncu AT, Şener Y, 2017. Biofilm formation by *Staphylococcus aureus* isolates from a dental clinic in Konya, Turkey. J Infect Public Heal, 10(6), 809-813.

Trzcinski K, Cooper BS, Hryniewicz W, Dowson CG, 2000. Expression of resistance to tetracyclines in strains of methicillin resistant *Staphylococcus aureus*. J Antimicrob Chemother, 45(6), 763-70.

Tu KK, Palutke WA, 1976. Isolation and characterization of a catalase negative strain of *Staphylococcus aureus*. J Clin Microbiol, 3(1), 77-78.

Votintseva AA, Fung R, Miller RR, Knox K, et al., 2014. Prevalence of *Staphylococcus aureus* protein A (*spa*) mutants in the community and hospitals in Oxfordshire. BMC Microbiol, 14, 63-74.

Wang D, Wang Z, Yan Z, Wu J, et al., 2015. Bovine mastitis *Staphylococcus aureus*: Antibiotic susceptibility profile, resistance genes and molecular typing of methicillin-resistant and methicillin-sensitive strains in China. Infect Genet Evol, 31: 9-16.

Wang X, Li G, Xia X, Yang B, et al., 2014. Antimicrobial susceptibility and molecular typing of methicillin-resistant *Staphylococcus aureus* in retail foods in Shaanxi, China. Foodborne Pathog Dis, 11(4), 281-286.

Yang X, Liu J, Huang Y, Meng J, et al., 2018. Prevalence, molecular characterization, and antimicrobial susceptibility of methicillin-resistant *Staphylococcus aureus* from different origins in Sichuan Province, China, 2007–2015. Foodborne Pathog Dis, 15(11), 705-710.

Zhang H, Zheng Y, Gao H, Xu P, et al., 2016. Identification and characterization of *Staphylococcus aureus* strains with an incomplete hemolytic phenotype. Front Cell Infect Microbiol, 6, 146, doi:10.3389/fcimb.2016.00146.

Zschöck M, Botzler D, Blocher S, Sommerhauser J, et al., 2000. Detection of genes for enterotoxins (ent) and toxic shock syndrome toxin-1 (tst) in mammary isolates of *Staphylococcus aureus* by polymerase chain reaction. Int Dairy J, 10(8), 569-574.

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