

## RESEARCH ARTICLE

### Methicillin-resistant *Staphylococcus aureus* in milk from dairy cows with chronic mastitis

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### Kronik mastitli inek sütlerinde metisiline dirençli *Staphylococcus aureus*

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#### Öz

**Amaç:** Bu çalışmanın amaçları: i) sürekli tekrarlayan mastitli inek sütlerinden koagulaz pozitif stafilocok ve koagulaz negatif stafilocokları izole etmek, ii) izolatları *Staphylococcus* spp., *Staphylococcus aureus* ve metisiline dirençli *Staphylococcus aureus* yönünden multipleks PCR yöntemi ile doğrulamak ve iii) disk difüzyon yöntemi ile izolatların antimikrobiyel duyarlılığını belirlemektir.

**Gereç ve Yöntem:** Bu kapsamda, Hatay ilinde sürekli tekrarlayan klinik mastit sorunu yaşayan süt ineklerinden toplam 52 adet süt örneği alındı. Koagulaz pozitif stafilocok ve koagulaz negatif stafilocokların izolasyon ve identifikasyonu klasik kültür tekniği ile yapıldı. Koagulaz pozitif stafilocok ve koagulaz negatif stafilocok olarak saptanan izolatların hepsi *16S rRNA*, *nuc* ve *mecA* gen sekansları baz alınarak, sırasıyla *Staphylococcus* spp., *S. aureus* ve metisiline dirençli *S. aureus* yönünden multipleks PCR yöntemi ile doğrulandı. Ayrıca izolatların antimikrobiyel duyarlılığı araştırıldı.

**Bulgular:** Süt örneklerinin % 48.0'inde pozitif sonuçlar elde edildi. Pozitif örneklerden toplam 56 adet stafilocok izolatı (22 adet koagulaz pozitif stafilocok ve 34 adet koagulaz negatif stafilocok) elde edildi. Koagulaz pozitif stafilocok izolatlarının 20'si (% 90.9) *S. aureus* olarak doğrulandı. *S. aureus* olarak doğrulanan izolatlarının 18'inde (% 90) *mecA* geni tespit edildi. Ayrıca, bu çalışmada izolatlarda disk difüzyon yöntemiyle, eritromisin (% 66), tetrasiklin (% 62.5), ampisilin (% 60.7), penisilin (% 60.7), sefoksitin (% 50), oksasilin (% 46.4) ve kloramfenikole (% 5.3) karşı direnç belirlendi.

**Öneri:** Çalışmada, kronik tekrarlayan mastitli inek sütlerinde antibiyotiklere dirençli stafilocokların ve özellikle metisiline dirençli *S. aureus*'un saptanması, bu bakterilerin süt ile birlikte gıda zinciri boyunca yayılma olasılığı açısından dikkate alınmalıdır.

**Anahtar kelimeler:** Koagulaz, mastit, *mecA*, süt, *Staphylococcus*

#### Abstract

**Aim:** The aims of this study were: i) to isolate coagulase positive *Staphylococcus* (CPS) and coagulase-negative *Staphylococcus* (CNS) in milk from dairy cows with chronic recurrent mastitis, ii) to verify isolates in terms of *Staphylococcus* spp., *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) by multiplex PCR, and iii) to determine the antimicrobial susceptibility of the isolates by disk diffusion method.

**Materials and Methods:** A total of 52 milk samples were collected from the dairy cows with chronic recurrent mastitis in Hatay province. Isolation and identification of CPS and CNS were detected by using classical culture method. All CPS and CNS isolates were investigated for antimicrobial susceptibility and also, were confirmed as *Staphylococcus* spp., *S. aureus* and MRSA by multiplex PCR based on *16S rRNA*, *nuc*, and *mecA* gene sequences, respectively.

**Results:** Positive results were found in 48.0% of the milk samples. A total of 56 *Staphylococcus* spp. isolates (22 CPS and 34 CNS) were obtained from the positive samples. Twenty (90.9%) of the CPS isolates were detected as *S. aureus*. The *mecA* gene was detected in 18 (90%) of the *S. aureus* isolates. By disk diffusion method, resistance to erythromycin (66%), tetracycline (62.5%), ampicillin (60.7%), penicillin (60.7%), cefoxitin (50%), oxacillin (46.4%), and chloramphenicol (5.3%) was also determined in this study.

**Conclusion:** In the study, the detection of antibiotic resistant *Staphylococcus* strains and especially MRSA in milk from dairy cows with chronic recurrent mastitis should be taken into account for potential spread through the dairy food chain.

**Keywords:** Coagulase, mastitis, *mecA*, milk, *Staphylococcus*

## Introduction

Mastitis is one of the most common diseases that reduces milk production and causes serious economic losses in dairy cattle. Bovine mastitis is generally classified as follows: “subclinical mastitis,” when infected cows had no clinically symptoms and normal milk appearance; “clinical mastitis,” when infected cows had clinically symptoms and visible abnormalities in the milk and the udder (Costa et al 2000, Sawant et al 2009, Persson et al 2011).

Staphylococci are the bacteria most commonly isolated from bovine mastitis. Depending on the coagulase ability, staphylococci are divided into two groups known as coagulase-positive *Staphylococcus* (CPS) and coagulase-negative *Staphylococcus* (CNS), which is important for considering the diagnosis and treatment of mastitis. Although some strains of *Staphylococcus aureus* are coagulase-negative, *S. aureus* generally has coagulase activity and causes clinical and subclinical mastitis. However, CNS are usually isolated from subclinical mastitis in dairy cattle (Martins and Cunha 2007, Taponen and Pyörälä 2009). Antimicrobials are commonly used both for the treatment and control of mastitis in dairy farms. One of the most important problems arising from inappropriate and random use of antimicrobials on dairy farms is the increasing antimicrobial resistance among pathogenic bacteria. Also, transfer of antimicrobial resistance genes between staphylococcal species could lead to chronic recurrent mastitis and in this case it may be difficult to treat mastitis (Costa et al 2000, Virdis et al 2010, Sampimon et al 2011, Ergün et al 2012, Zdolec et al 2016). So, multiple antibiotic resistant strains, and especially methicillin resistant *S. aureus* (MRSA) which is recognized as important nosocomial pathogen, can be spread through the dairy food chain and may pose a risk for human health.

Therefore, this study was conducted to investigate the presence of coagulase-positive *Staphylococcus* (CPS) and coagulase-negative *Staphylococcus* (CNS) in milk from dairy cows with chronic recurrent mastitis and to detect methicillin-resistant *Staphylococcus aureus* (MRSA) by PCR.

## Materials and Methods

In this study, a total of 52 milk samples were obtained from dairy cows suffering from chronic mastitis problem in Hatay province. At the sampling stage, milk samples were taken according to the last one-year farm data, infirmary records, and recurrent California Mastitis Test (CMT) positivity on the same quarter. Milk samples were aseptically collected for microbiological analysis according to the standard protocol of International Dairy Federation (IDF, 1985). Samples were transported on ice to the laboratory and analyzed on the same day.

For microbiological analysis, 10 mL of each milk sample was taken into a sterile plastic bag. Ninety mL of sterile 0.1% peptone water was added onto the sample and then the mixture was homogenized. Decimal dilutions were prepared with using this homogenate and peptone water prepared in 9 mL of sterile tubes. As a positive control, *S. aureus* ATCC 43300 (methicillin-resistant strain) was used in the study. Inoculations were done according to spreading plate technique on Baird Parker Agar with Egg Yolk Tellurite Emulsion (BP, Oxoid, CM0275; Egg Yolk Tellurite-Emulsion, Oxoid, SR0054) and incubated for 24-48 h at 37°C in aerobic conditions. At the end of the incubation, up to five typical and/or atypical colonies were selected. The selected colonies were stored in glycerinated Brain Heart Infusion (BHI) broths at -20°C for further analysis.

For coagulase test, frozen colonies were suspended in tubes containing 4-5 mL of BHI broth (Oxoid, CM0225). Broth cultures were incubated for 18-24 h at 37°C in aerobic conditions. Tube coagulase test was performed with using lyophilized-EDTA rabbit plasma (Lyophilized Coagulase Rabbit Plasma with EDTA, Oxoid, R21060). As a result of the coagulase test, positively detected colonies were recorded as coagulase positive *Staphylococcus*, while negative ones were recorded as coagulase negative *Staphylococcus* (Bennett and Lancette 1998).

For DNA extraction from the isolates, commercially available bacterial DNA extraction kit (Nucleic Acid Extraction Kit, GF-1, Vivantis, Malaysia) was used and extracted DNAs were stored at -20°C. All coagulase positive and coagulase negative isolates were confirmed as *Staphylococcus*, *S. aureus* and MRSA by multiplex PCR based on *16S rRNA*, *nuc*, and *mecA* gene sequences, respectively. Specific primers (Ella Biotech GmbH, Martinsried, Germany) described by Brakstad et al. (1992), Mehrotra et al (2000), Monday and Bohach (1999) were used in this study. For the PCR, ready-to-use PCR Master Mix (PCR Master Mix, 2X, Promega, USA) was used and reaction mix was prepared in a total volume of 25 µL contained 0.6 µL of each primer. Template DNA was added to the mix as 4 µL. Multiplex PCR amplification (Mehrotra et al 2000, Keyvan and Özdemir 2016) was carried out under these reaction conditions: pre-denaturation for 4 min at 94°C, 35 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 57.5°C, extension for 40 sec at 72°C, and then, additional extension for 10 min at 70°C. Amplicons were run at 100 V for 50 min with 1.5% agarose gel. Genes-specific DNA bands were evaluated under UV light on a gel imaging device (UVP, Upland, USA).

In the study, also, disk diffusion test was used for determination of antimicrobial susceptibility of all coagulase positive and coagulase negative isolates. The antibiotic discs were selected in line with the recommendation of Clinical and Laboratory Standards Institute (CLSI, 2015) and included

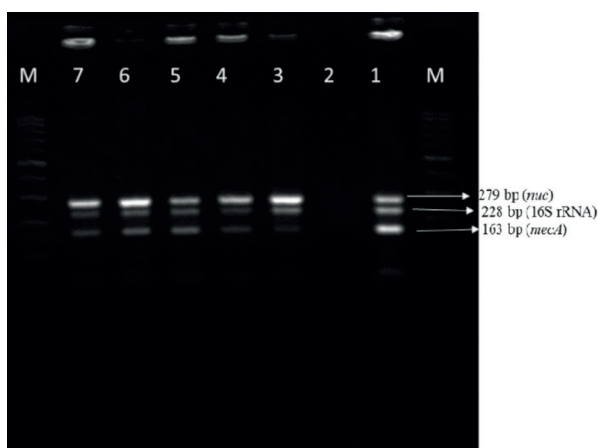


Figure 1. Electrophoresis image of the isolates [M: 100 bp DNA marker; 1: Positive control (*S. aureus* ATCC 43300), 2: Negative control, 3-7: *mecA* positive isolates (MRSA)].

penicillin, oxacillin, tetracycline, gentamicin, ampicillin, vancomycin, ceftiofur, chloramphenicol, erythromycin, and ciprofloxacin. For disk diffusion test, the cryopreserved isolates were enriched in tubes containing 4-5 mL BHI broths. Then, broth cultures were incubated at 37°C until the turbidity of broth culture reached to the 0.5 McFarland standards. After setting the turbidity of broth culture, steril cotton swab was dipped into broth, drained on the wall of tube, and the inoculum was streaked on Mueller-Hinton agar (Oxoid, CM0337). The antibiotic discs were placed on the agar surface in such a way that the distance between the centers of antibiotic discs no closer than 24 mm and a maximum of 10 disks on a 150-mm plate. Then, the plates were incubated at 37°C for 24 h. At the end of the incubation, the diameter of the inhibition zones around the discs was evaluated according to CLSI (2015).

## Results

During the study period, a total of 52 milk samples obtained from the dairy cows with chronic recurrent mastitis in Hatay province were investigated. Positive results were found in 48.0% of the milk samples, while 51.9% of them were microbiologically negative. From the 25 positive samples, a total of 56 *Staphylococcus* spp. isolates (22 CPS and 34 CNS) were obtained. When all CPS and CNS isolates were tested for *S. aureus* and MRSA by multiplex PCR, 20 (90.9%) of the CPS isolates were detected as *S. aureus* and *mecA* gene was detected in 18 (90%) of them. In this study, coagulase-negative *S. aureus* was not found and also, *mecA* gene was not detected in any CNS isolates.

When the overall antimicrobial susceptibility profiles of the isolates (CPS and CNS) were evaluated, resistance to erythromycin (66%), tetracycline (62.5%), ampicillin (60.7%), penicillin (60.7%), ceftiofur (50%), oxacillin (46.4%), and chloramphenicol (5.3%) was detected. All CPS and CNS were susceptible (100%) to vancomycin, gentamicin and ciprofloxacin. Twenty-two (64.7%) CNS isolates were resistant to one or more antimicrobials. Fifteen (44.1%) of the CNS isolates showed multidrug resistance (i.e., resistant to 2 or more antimicrobials). All CPS isolates were resistant to six tested antimicrobials at the same time. The antimicrobial susceptibility of the isolates are shown in Table 1.

## Discussion

*S. aureus* and CNS were the mainly isolated bacteria from dairy cows with mastitis (Pitkälä et al 2004, Tenhagen et al 2006) and microbiologically negative findings were also reported in some researches (Kalmus et al 2011, Persson et al

Table 1. Antimicrobial susceptibility of coagulase-negative *Staphylococcus* (CNS) (n=34) and coagulase-positive *Staphylococcus* (CPS) (n=22) isolates.

Antibiotics (Concentration)	CNS		CPS		Total (CNS+CPS)	
	R	S	R	S	R	S
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Vancomycin (30 µg)	0	34 (100)	0	22 (100)	0	56 (100)
Oxacillin (1 µg)	4 (11.76)	30 (88.24)	22 (100)	0	26 (46.43)	30 (53.57)
Chloramphenicol (30 µg)	3 (8.82)	31 (91.18)	0	22 (100)	3 (5.36)	53 (94.64)
Tetracycline (30 µg)	13 (38.24)	21 (61.76)	22 (100)	0	35 (62.5)	21 (37.5)
Erythromycin (15 µg)	15 (44.12)	19 (55.88)	22 (100)	0	37 (66.07)	19 (33.93)
Ceftiofur (30 µg)	6 (17.65)	28 (82.35)	22 (100)	0	28 (50)	28 (50)
Gentamicin (10 µg)	0	34 (100)	0	22 (100)	0	56 (100)
Ciprofloxacin (5 µg)	0	34 (100)	0	22 (100)	0	56 (100)
Ampicillin (10 µg)	12 (35.29)	22 (64.71)	22 (100)	0	34 (60.71)	22 (39.29)
Penicillin (10 I.U.)	12 (35.29)	22 (64.71)	22 (100)	0	34 (60.71)	22 (39.29)

n: number of isolates, R: resistant, S: susceptible.

2011), similar to this study. Possible reasons for the negative findings may be that the bacterial level is below the detection limit of the cultural method.

In this study, antimicrobial resistance was very high among the isolates, unlike the study conducted in Sweden (Persson et al 2011). While penicillin and ampicillin resistance is more prevalent in *S. aureus* and CNS in other studies (Costa et al 2000, Pitkälä et al 2004, Tenhagen et al 2006, Kalmus et al 2011, Persson et al 2011, Ergün et al 2012), Huber et al (2011) and Zdolec et al (2016) detected higher erythromycin and tetracycline resistance in staphylococci isolates besides beta-lactam resistance, similar to this study. This may be due to the use of these antibiotics in an uncontrolled and unconscious manner.

In Italy, Viridis et al (2010) found that CNS isolated from milk samples of goats with subclinical mastitis showed more frequently resistance to ampicillin and kanamycin, unlike this study and Frey et al (2013). Frey et al (2013) determined both multidrug resistance and *mecA* gene in CNS from bovine mastitis milk, while Viridis et al (2010) and Persson et al (2011) didn't detect *mecA* gene in their isolates. In this study, the highest susceptibility against vancomycin, gentamicin, and ciprofloxacin was similar with Costa et al (2000), Persson et al (2011), Kalmus et al (2011), Ergün et al (2012).

Sawant et al (2009), Huber et al (2011), Sampimon et al (2011), and Waller et al (2011) reported that multidrug resistance and methicillin resistance gene *mecA* is now available in CNS strains. In the present study, *mecA* gene was not detected in any CNS, although some CNS isolates were phenotypically resistant to both oxacillin and cefoxitin. As defined by Sampimon et al (2011), phenotypic and genotypic antimicrobial resistance profiles of CNS can show difference.

### Conclusion

As a result, 48.0% of the milk samples from the dairy cows with chronic recurrent mastitis were microbiologically positive for CPS and CNS. MRSA was detected in 7 (13.4%) of the samples. All isolates were resistant to 70% of the tested antimicrobials. Therefore, such studies should be repeated regularly to better define the antimicrobial susceptibility of mastitis pathogens, which is very important for effective treatment of the disease. To prevent the spread of MRSA through the dairy food chain, milk from chronic recurrent mastitic cows should not be used as mixed with milk obtained from healthy cows.

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