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Determination of SCC*mec* types in methicillin resistant staphylococci isolated from cows and farm workers*

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Summary: This study was conducted to determine staphylococcal cassette chromosome *mec* (SCC*mec*) types of methicillin resistant staphylococci (MRS) isolated from cattle and farm workers. A total of 145 subclinic mastitic bovine milk and 91 nasal swab samples (56 from cows and 35 from farm workers) were studied for presence of MRS. Methicillin resistance was detected by cefoxitin disc diffusion test. Bacterial identification and methicillin resistance was confirmed by 16S rRNA sequencing and *mec*A PCR, respectively. SCC*mec* types of these isolates were determined by multiplex PCR targeting for *mec* and *ccr* genes, associated IS element and plasmids. Among 236 total samples, 181 staphylococci, 59 of which were found as methicillin resistant were isolated. Fifty nine methicillin resistant isolates originated from 37 (62.7%) were bovine and 22 (37.3%) were human. Ten *Staphylococcus* species [*S. haemolyticus* (18), *S. epidermidis* (17), *S. aureus* (12), *S. xylosus* (4), *S. cohnii* (3), *S. equorum* (1), *S. sciuri* (1), *S. succinus* (1), *S. pseudointermedius* (1) and *S. saprophyticus* (1)] among 59 MRS were identified by 16S rRNA sequence analysis. SCC*mec* types of 2, 38, 18, and 1 MRS isolates were II, III, IV, and V, respectively. A total of 40 isolates including type II and III were hospital acquired whereas 19 isolates including type IV and V were community acquired. Almost all *S. haemolyticus* and *S. aureus* isolates carried hospital acquired type SCC*mec*. The similarity of SCC*mec* types between human and cattle isolates suggest evidence of transmission from animals to humans, or vice versa. Further studies are needed to establish clonal relationship of MRS isolated from bovine and farm workers with advanced molecular techniques.

Key words: *Staphylococcus*, methicillin, SCC*mec*, cattle, human.

Sığırlardan ve sektör çalışanlarından izole edilen metisilin dirençli stafilokoklarda SCC*mec* tiplerinin belirlenmesi

Özet: Bu çalışma sığır ve çiftlik çalışanlarından izole edilen metisilin dirençli stafilokokların (MRS) stafilokokal kaset kromozom *mec* (SCC*mec*) tiplerini belirlemek amacıyla gerçekleştirildi. Toplam 145 subklinik mastitisli inek sütü ve 91 burun sıvap örneği (56 inekten ve 35 çiftlik çalışanından) MRS varlığı yönünden incelendi. Metisilin direnci sefoksitin disk difüzyon yöntemi ile belirlendi. Bakteriyel identifikasyon ve metisilin direnci sırasıyla 16S rRNA sekansı ve *mec*A PCR ile doğrulandı. Bu izolatların SCC*mec* tipleri *mec* ve *ccr*, IS elementi ve plazmidler için multipleks PCR ile incelendi. Bu 236 örnekten 181 stafilokok izole edilirken bunların 59'u metisilin dirençli olarak belirlendi. Elli dokuz izolatın 37 (%62.7)'si sığır ve 22 (%37.3)'si insan orjinliydi. 16S rRNA sekans analizi ile incelenen 59 MRS içinde 10 tür [*S. haemolyticus* (18), *S. epidermidis* (17), *S. aureus* (12), *S. xylosus* (4), *S. cohnii* (3), *S. equorum* (1), *S. sciuri* (1), *S. succinus* (1), *S. pseudointermedius* (1), *S. saprophyticus* (1)] identifiye edildi. SCC*mec* tip II, III, IV ve V sırasıyla 2, 38, 18 ve 1 MRS izolatında belirlendi. SCC*mec* Tip II ve III'ü taşıyan 40 izolat hastane kökenli, SCC*mec* Tip IV ve V'i içeren 19 izolat toplum kökenli olarak tespit edildi. Hemen tüm *S. haemolyticus* ve *S. aureus* izolatlarının hastane kökenli SCC*mec* tiplerini taşıdığı belirlendi. Sığır ve insan stafilokok izolatlarının taşıdığı SCC*mec* tipleri arasındaki benzerlik hayvanlardan insanlara ya da tam tersi bir bulaşma olabileceğini düşündürmektedir. Sığır ve çiftlik çalışanlarından izole edilen MRS'lar arasındaki klonal ilişkinin belirlenebilmesi için ileri moleküler teknikler kullanılarak gerçekleştirilecek olan çalışmaların yapılmasına ihtiyaç duyulmaktadır.

Anahtar sözcükler: *Staphylococcus*, metisilin, SCC*mec*, sığır, insan.

Introduction

Methicillin, a semisynthetic penicillin that is poorly hydrolyzed by penicillinase, was first used clinically in 1960. Only one year later, *Staphylococcus aureus* isolates that showed resistance to methicillin were reported (8). Since then, methicillin resistance in staphylococci has appeared in countries worldwide in humans and continues to be one of the most common hospital-acquired pathogens (18, 19, 20).

It has been shown that methicillin susceptible *S. aureus* (MSSA) can become methicillin resistant *S. aureus* (MRSA) by the acquisition of a staphylococcal

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cassette chromosome *mec* (SCC*mec*) element carrying the *mec*A gene, which is responsible for methicillin resistance by the production of an altered penicillin binding protein (PBP2a) (6). PBP2a is an enzyme involved in cell wall peptidoglycan synthesis. Unlike PBPs of *S. aureus*, PBP2a does not bind to β-lactam antibiotics with high affinity (5, 19).

SCC*mec* elements, unique genomic islands that are found in staphylococcal species, have two essential components, the *ccr* gene complex and the *mec* gene complex (5, 16). The *ccr* gene complex is composed of the *ccr* genes and several of which have unknown functions. The *mec* gene complex is composed of *mec*A. Eight different SCC*mec* elements have been classified under MRSA according to the combination of the *mec* gene complex class and the *ccr* gene complex type (5). The SCC*mec* types I, II, and III are found predominantely in hospital acquired methicillin resistant staphylococci (HA MRS) isolates. The SCC*mec* types II and III are responsible for the multiple non β lactam antimicrobial resistance. The SCC*mec* type IV and V are typically found in community acquired methicillin resistant staphylococci (CA MRS) strains and lack other multidrug resistance genes (25).

Molecular typing techniques have been used with increasing frequency in studies on epidemiology of methicillin resistant staphylococci (MRS), and also for a better understanding of the evolutionary relationships among MRSA clones (17). The structure of SCC*mec* can be determined with a number of PCR based methods, such as *mec*A specific multiplex PCR by Oliveira et al. (17), where different loci on SCC*mec* type I and IV are detected. The SCC*mec* typing may provide evidence for the independent deviation of HA MRSA and CA MRSA clones. This data is epidemiologically important for infection control (17).

Staphylococci play a role as nasocomial pathogens in human medicine, as bovine mastitis pathogens in veterinary medicine (5, 18). Coagulase negative staphylococci (CoNS) have been recognized as bacteria of minor clinical importance; however, they may now be considered one of the factors that determines the generation of new MRS isolates (5, 7). Nowadays, MRS nasal carriage has become a serious problem in farm animals (5, 9) as well as in humans (18). There is limited study in farm animals about the molecular epidemiology of MRSA (23, 27, 29). Also, as far as we know, there is no data on SCC*mec* typing on methicillin resistant CoNS (MRCoNS) isolates of animal sources in Turkey. The aim of this study was to detect SCC*mec* types of MRS isolated from cattle and farm workers by PCR.

Materials and Methods

Material: A total of 145 subclinical mastitic milk obtained from 129 dairy cattle in 15 farms and 91 nasal swab samples (56 from cows and 35 from farm workers) were studied for the presence of MRS.

Sampling: Milk samples were taken as described previously (1). Nasal samples were taken for sampling from the medial septum area of both nostrils by gently rubbing mucosa approximately for 5 s with a cotton-tipped swab moistened with sterile water. Nasal swabs were transported to the laboratory in the day of sampling in Amies transport medium in cold chain.

Isolation and phenotypic characterization of staphylococci: The swabs were immediately suspended in 5 ml Tryptone Soya Broth (TSB, Oxoid) containing 7.5% sodium chloride and incubated for 48 h 37°C for selective enrichment of staphylococci. Enrichment cultures and milk samples were then streaked on to Mannitol Salt Agar (MSA, Oxoid) to isolate of staphylococci. The staphylococcal isolates were identified morphologically and biochemically by standard laboratory procedures. For discrimination of coagulase positive stahylococci (CoPS) from CoNS, the coagulase test was performed (15).

Identification of MRSA isolates: Staphylococci were tested for methicillin resistance using disc diffusion method with cefoxitin discs (30 µg; Oxoid). Zone radius were read after incubation at 37°C for 24 h. Isolates with zone diameter less than 19 mm for *S. aureus* and 24 mm for CoNS were considered as methicillin resistant (2), and studied further.

DNA extraction: DNA extraction was performed as described previously (23).

16S rRNA gene amplification for sequence analysis: 16S rRNA gene amplification and sequence analysis were performed as described previously (10). Thus, a large, 1600 bp fragment encoding 16S rRNA gene was amplified and subjected to sequence analysis for species discrimination.

PCR: A MRSA specific PCR assay was performed to discriminate the MRSA from other staphylococci and to genotypically determine methicillin resistance. For the detection of 16S 20 and 1390 universal rRNA, primers 5'-AGA GTT TGA TCC TGG CTC AG-3' and 5'-GAC GGG CGG TGT GTA CAA -3'were used as the forward and the reverse primer, respectively (21, 22). For the detection of *nuc* (*S. aureus* specific) gene, 5'-GCG ATT GAT GGT GAT ACG GTT-3' was used as forward primer and 5'-AGC CAA GCC TTG ACG AAC TAA AGC-3' was used as the reverse primer (12). For the detection of *mec*A (methicillin resistance) gene 5'-TCCAGATTACAACTTCACCAGG-3' was used as forward primer and 5'-CCACTTCATATCTTGTAACG-3' was used as reverse primer (17). Five microliters of extracted DNA was used as a template in a 45 µl PCR mixture containing 1xPCR buffer (50 mm KCl, 20 mM Tris HCl), 4 µl of 25 mM $MgCl_2$, 2 µl of 10 mM deoxynucleoside triphosphate (dNTP) mix, 1.2 µl of 100

pmol each 16S rRNA, *nuc* and *mec*A primers, and 3.6 µl of Taq DNA polymerase (5U). The amplification of DNA was performed as follows: 94ºC for 10 min of initial denaturation; 35 cycles of 94ºC for 1 min, 50ºC for 1 min and 72ºC for 1 min; and a final extension at 72ºC for 10 min. Visualization of PCR products was performed on 1.5% agarose gel stained with ethidium bromide. *Streptococcus* spp. (negative control), *S. aureus* N315 (positive control) were used as control isolates.

SCC*mec typing:* SCC*mec* typing of the isolates to Types I-IV and Type V was performed using the methods described by Oliveira and Lencastre (17) and Zhang et al. (27), respectively. *S. aureus* N315 strain was used as the positive control.

Results

Among 236 samples, 181 staphylococci of which 59 were resistant to methicillin. These MRS isolates were obtained from 12 from out of 15 farms. Methicillin resistance was detected in 24.4% (22/90), 26.8% (15/56) and 62.8% (22/35) of milk, bovine nasal and human nasal isolates, respectively.

Identification of isolates was done by sequencing a 1371 bp size PCR product by using universal 16S rRNA primers. Ten different *Staphylococcus* species was identified after sequence analysis (Table 1). *S. haemolyticus* (30.5%) and *S. epidermidis* (28.8%) were the two most commonly isolated species, followed by *S. aureus* (20.3%). The distribution of all staphylococci to the samples was given in Fig 1.

All 59 isolates phenotypically identified as MRS were subjected to a multiplex PCR for confirmation of MRSA isolates. Bacterial DNA control was done by 16S rRNA primers. Fragments of expected sizes were 1371, 279 and 162 bp for the 16S rRNA, *nuc* and *mec*A genes, respectively (Figure 2). All the 59 MRS isolates identified phenotypically were found to possess both 16S rRNA and *mec* genes, while 12 isolates also possessed the *nuc* gene. Phenotypically methicillin resistant 47 CoNS isolates were found as negative for *nuc* gene while all MRSA isolates were found to posses these 3 genes.

Four SCC*mec* type from 59 MRS isolate were determined using multiplex PCR. Of these 59 materials originated from 37 (62.7%) samples were animal and 22 (37.3%) were human. SCC*mec* Type II was found in 2, Type III in 38, Type IV in 18. A single bovine *S. epidermidis* isolate was also found to carry a type V SCC*mec* element (Figure 3). While 40 isolates including type II and III were defined as HA MRS, 19 isolates including type IV and V were CA MRS. From bovine nasal swabs 6 were found as CA MRS while, 9 were HA MRS. From human nasal swabs 9 were found as CA MRS, while 13 were HA MRS. From 22 bovine milk isolates 4 were found as CA MRS, while 18 were HA MRS. Distribution of SCC*mec* elements among MRS are presented in Table 1.

Table 1. Distrubition of SCC*mec* elements among MRS based on *Staphylococcus* species
Tablo 1. MRS izolatları arasında SCC*mec* elemanlarının *Staphylococcus* türlerine göre dağılımı

Species	Number (%)	SCC*mec* Types=Isolate number			
		II	III	IV	V
S. haemolyticus	18 (30.50)	0	16	2	0
S. epidermidis	17 (28.80)	1	7	8	1
S. aureus	12 (20.30)	1	9	2	0
S. xylosus	4 (6.70)	0	3	1	0
S. cohnii	3 (5.10)	0	1	2	0
S. equorum	1 (1.72)	0	1	0	0
S. sciuri	1 (1.72)	0	0	1	0
S. succinus	1 (1.72)	0	0	1	0
S. pseudointermedius	1 (1.72)	0	0	1	0
S. saprophyticus	1 (1.72)	0	1	0	0
Total	59 (100.0)	2	38	18	1

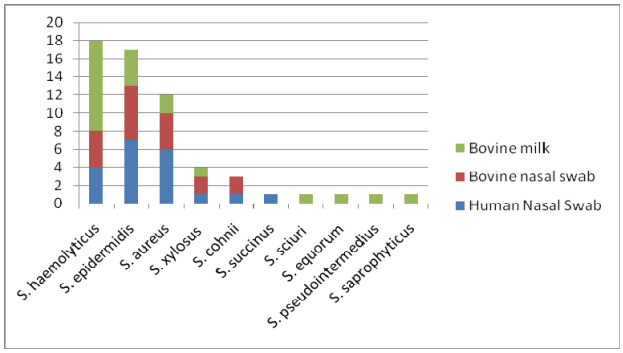

Figure 1. The distribution of staphylococci to the samples
Şekil 1. Stafilokok türlerine göre materyallerin dağılımı

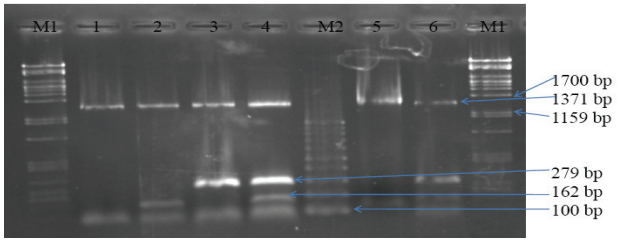

Figure 2. MRSA specific PCR. M**1:** Marker (Lambda phage DNA restricted with *Pst*I enzyme) 1: *Staphylococcus* spp. (1371 bp) 2: MRS isolate (1371 bp and 162 bp) 3: Methicillin sensitive *S. aureus* isolate (1371 bp and 279 bp) 4: MRSA isolate (1371 bp, 279 bp and 162 bp) M2: Marker (100 bp DNA ladder) 5: Negative Control (*Streptecoccus* spp., 1371 bp) 6: Positive Control (*S. aureus* N315 strain)
Şekil 2. MRSA spesifik PCR. M1: Marker (*Pst*I enzimi ile kesilmiş lambda faj DNA'sı) 1: *Staphylococcus* spp. (1371 bp) 2: Metisilin dirençli stafilokok izolatı (1371 bp ve 162 bp) 3: Metisilin duyarlı *S. aureus* suşu (1371 bp and 279 bp) 4: MRSA isolate (1371 bp, 279 bp ve 162 bp) M2: Marker (100 bp DNA ladder) 5: Negatif Kontrol (*Streptecoccus* spp., 1371 bp) 6: Pozitif Kontrol (*S. aureus* N315 suşu)

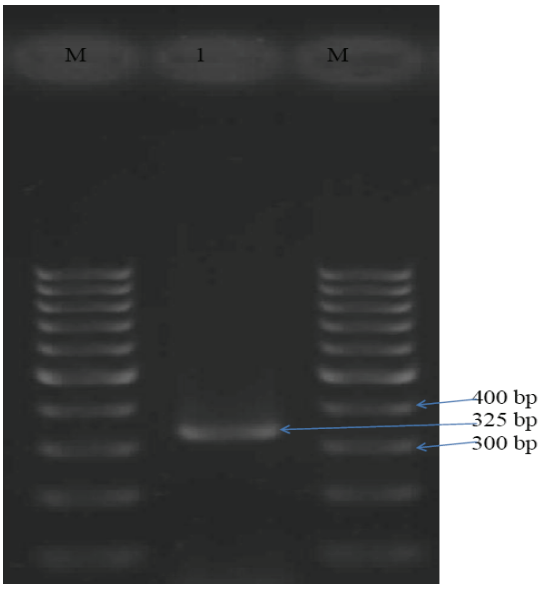

Figure 3. SCC*mec* type V **M:** Marker**:** 100 bp DNA ladder, **1:** SCC*mec* type V (325 bp)
Şekil 3. SCC*mec* tip V **M:** Marker**:** 100 bp DNA ladder, **1:** SCC*mec* tip V (325 bp)

Discussion and Conclusion

Nowadays methicillin resistance in farm animals has gained particular attention from public health authorities. Infected animals can pass MRS to humans who are in close contact with them. Infections in humans caused by livestock associated MRS have been reported (9, 26).

Different isolation rates (5.8-60.0%) for MRSA were reported in many phenotypic studies held in different years in Turkey (4, 10, 11, 13, 24). Performing only phenotypic tests has previously been shown to lead to false positive or false negative results (14). There is only one study in Turkey performed by Türkyılmaz et al. (23) in which phenotypic and genotypic investigation of MRSA isolates isolated from mastitic dairy cattle were investigated and the reported isolation rate was 17.2%. Only one study performed by Kaynarca and Türkyılmaz (10) in which methicillin resistance of CoNS was phenotypically investigated and 10 MRCoNS were isolated.

While the SCC*mec* typing of MRSA from bovine mastitis have been published (23, 27) little information is available on MRCoNS from dairy cattle in the world (3). In the present study, *S. epidermidis* and *S. haemolyticus* represented the most frequent species observed among the MRCoNS as were in the study by Febler et al. (3). Similar results were obtained from human patients (20). SCC*mec* typing identifying mainly type IV elements among *S. epidermidis* isolates from humans (20) and animal origin (29) have been reported.

MRSA colonize most frequently in the anterior nares of the nose and cause serious infections all over the world. In Turkey, MRSA colonization rate was reported very high in some medical centers making MRSA a serious problem (18). In this study, the rate of nasal carriage of MRS among farm workers found to be very high. Nasal carriage rate among cattle was only half of the carriage rate among farm worker which may indicate importance of MRS transfer from human to cattle.

Transmission of MRSA between humans and animals has previously been reported (9, 23). Among farm workers, bovine milk and bovine nasal isolates HA MRS (40 isolate) type was higher than CA MRS (19 isolate) type. This situation was especially more important among MRS isolated from bovine. The similarity of SCC*mec* types between human and cattle isolates suggest evidence of transmission from farm workers to bovine, or vice versa. Further studies are needed to establish clonal relationship of MRS from bovine and farm workers with advanced molecular techniques (Pulsed Field Gel Electrophoresis, *Staphylococcal Protein* A analysis, Multi Locus Sequence Typing). The result of these studies may shed light on the clonality and transmission of resistance strain between human and animal.

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