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RESEARCH ARTICLE

Molecular Typing of Methicillin Resistant *Staphylococcus aureus* Strains Isolated from Cows and Farm Workers [1] [2] [3]

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Summary

The aims of this study were to isolation and identification of methicillin resistant *Staphylococcus aureus* (MRSA) from mastitic cow milk and nasal swabs of cows and related workers, investigation presence of Panton-Valentine leucocidin (PVL) genes and molecular characteristics of these isolates. One hundred forty five mastitic milk samples were obtained from of 56 cows and nasal swabs were collected from these cows and 34 farm workers. MRSA isolates were recovered by pulsed field gel electrophoresis (PFGE), staphylococcal chromosomal cassette (SCC) *mec* typing, staphylococcal protein A (*spa*) typing, multilocus sequence typing (MLST). A total of 12 MRSA were identified. All strains were PVL genes negative. PFGE analysis of all 12 MRSA isolates produced four distinct pulsotypes (PT) designated as PT A-D. The PT A (7 strains) detected sequence type (ST) were ST 239, *spa* type t030 and SCC*mec* III except one. The PT B (3 strains) were ST1294, t459 and SCC*mec* III, the PT C (1 strain) was ST1940, t660, SCC*mec* III detected, the PT D (1 strain) was ST737, t542, SCC*mec* II. Consequently, it can be said that t030 and ST239 was the most common *spa* and MLST types which isolated from cow milk and nasal swabs and nasal swabs from workers of these farms in Aydın region, Turkey.

Keywords: *MRSA, PFGE, SCCmec, spa, MLST*

Sığırlardan ve Sektör Çalışanlarından İzole Edilen Metisilin Dirençli Stafilokokların Moleküler Tiplendirmesi

Özet

Bu çalışma mastitisli sığır sütleri ile bu sığırlar ve çiftlik çalışanlarının nazal sıvaplarından metisilin dirençli *Staphylococcus aureus* (MRSA) izolasyon ve identifikasyonu, izolatların moleküler özelliklerinin ve Panton–Valentine Leucocidin (PVL) genlerinin varlığının incelenmesi amaçlandı. Elli altı sığırdan 145 mastitisli süt ile 56 sığır ve 34 çiftlik çalışanına ait nazal sıvaplar alındı. MRSA izolatlarının moleküler özellikleri itilmiş alan jel elektroforezisi (PFGE), stafilokokal protein A (*spa*), çok lokuslu dizi tiplendirme (MLST) ve stafilokokal kromozom kaset *mec* (SCC*mec*) tiplendirmesi ortaya konuldu. Tüm suşlar PVL genleri bakımından negatif idi. Toplam 12 MRSA identifikasyonu yapıldı. PFGE analizi sonucunda 12 MRSA izolatında pulsotip A-D olamak üzere dört pulsotip belirlendi. Pulsotip A olan suşların (n=7) sekans tipi (ST) tipi ST 239, *spa* tipi t030 olarak bulunurken biri hariç hepsinin SCC*mec* III taşıdığı tespit edildi. Pulsotip B (n=3) ST1294, t459 ve SCC*mec* Tip III; pulsotip C (n=1) ST1940, t660, SCC*mec* Tip III ve pulsotip D (n=1) ST737, t542, SCC*mec* tip II olarak belirlendi. Çalışma sonucunda, Aydın yöresinde sığır süt, insan ve sığır burun sıvaplarından izole edilmiş olan en yaygın *spa* tipi t030, MLST tipinin ise ST 239 olduğu belirlendi.

Anahtar sözcükler: *MRSA, PFGE, SCCmec, spa, MLST*

INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) is a significant human pathogen that is also an emerging concern in veterinary medicine. It is present in an extensive range of animal species both as a cause of infection and

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in healthy carriers [1]. Methicillin resistance is caused by a modified penicillin binding protein PBP2a, which has a low affinity for all beta lactam antimicrobials, encoded by the *mec*A gene. Most of the methicillin resistant isolates show heterogeneous resistance therefore detection of the presence of *mec*A gene by PCR is accepted as "gold standard" and genotyping of MRSA is important in order to determine outbreaks [2].

Molecular typing techniques have been used with rising frequency in studies on epidemiology of methicillin resistant staphylococci (MRS), and also for a better understanding of the evolutionary relations among MRSA clones. The most important techniques used to investigate the molecular epidemiology of *S. aureus* are pulsed field gel electrophoresis (PFGE), multilocus sequence typing (MLST), *S. aureus* protein A (*spa*) and SCC*mec* typing [3]. The organization of SCC*mec* can be determined with a number of PCR based methods, such as *mec*A specific multiplex PCR by Oliveira and Lencastre. The SCC*mec* typing may supply to determination of hospital associated MRSA (HA MRSA) and community associated MRSA (CA MRSA) clones [4]. *spa* typing uses the DNA sequencing of a polymorphic 24 bp and *S. aureus* specific *spa* gene for discrimination of *S. aureus* [5]. The *S. aureus* MLST scheme uses the allelic profile of 7 housekeeping genes to establish the strain type [6].

MRSA infections in humans are an important healthcare problem and a serious economic burden. Several local studies showed an MRSA prevalence of 12-90% in humans [7-9]. In contrast to human isolates, few genotypic studies have reported that MRSA strains isolated from cow milks in Turkey [10-12]. Minimal information is available about MRSA colonization of healthy cattle [1]. It reported that this ratio is varied from 0 to 28% in different countries [13,14]. Clearly, more study of healthy cattle is required [1]. The aims of this study were to isolation and identification of MRSA from mastitic cow milk and nasal swabs of cows and related workers, investigation presence of Panton–Valentine leucocidin (PVL) genes and molecular characteristics using PFGE, SCC*mec*, *spa* and MLST typing of these isolates.

MATERIAL and METHODS

The Ethic Committee on Research Animal Care at Adnan Menderes University of Aydin, Turkey approved all procedures in this study (No: ADÜ-HADYEK-2010/97).

Bacterial Strains

The strains were isolated from nasal swabs of 34 farm workers, 145 mastitic milk and nasal swabs of these 56 cows obtained from 15 farms.

Diagnosis of Mastitis

The diagnosis of mastitis was done by veterinary practitioners, milk samples and cow nasal swabs were taken these cows. Clinical mastitis was diagnosed by changes in the udder and milk compositions. Changes in the udder included pain, swelling, warmth and abnormal appearance (blood tinged milk, watery secretions, clots, pus) of milk. Cows that did not have clinical mastitis were subjected to further investigation for subclinical mastitis by using CMT. The procedures and interpretations were performed previously [15]. For taken milk samples, teat ends were cleaned using 70% alcohol moistened swabs and allowed to dry. After discarding the first few streams, 2-5 ml of the milk samples were collected into sterile 5 ml glass flasks.

Nasal Swaps

Human nasal swaps were taken by a nurse. 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.

Identification of MRSA Strains

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. Gram positive, catalase positive, coagulase positive, oxidase negative, bacitracin resistant, hemolytic, acetoin production positive, β galactosidase negative, mannitol-fermenting, and polymyxin B resistant staphylococci were identified as *S. aureus* [15].

S. aureus strains were tested for methicillin resistance using disc diffusion method outlined by the CLSI (2006). For this cefoxitin, discs (30 µg; Oxoid) were used. Zone sizes were read after incubation at 37°C for 24 h. Strains with zone sizes less than 19 mm were considered as methicillin resistant and studied further.

DNA Extraction

For genomic DNA from individual pure cultures of *E. coli* isolates were extracted with InstaGeneTM DNA extraction kit (Bio-Rad) according to the manufacturer's instructions.

Detection of the mecA, nuc and PVL Genes

In our study *S. aureus* specific thermonuclease and methicillin resistance genes were defined by presence of *nuc* and *mec*A genes, respectively. The oligonucleotide

primers described by and Kim et al.[17] and Oliveira and Lencastre [4] were used. Presence of PVL toxin genes are studied using method and primers described by previously [18].

Molecular Characterization of the MRSA Isolates

SCC*mec* types of the isolates were determined using methods and primers described by Oliveira and de Lencastre [4]. For the detection of *mec*A and SCC*mec* typing methicillin susceptible (*S. aureus* ATCC 29213) and methicillin resistanat (*S. aureus* HPV107, *S. aureus* N315, *S. aureus* HUSA304, *S. aureus* GRE14) reference strains used as negative and positive control in PCR, respectively. Visualization of PCR products was performed on 2% agarose gel stained with ethidium bromide.

PFGE was performed as previously described [19]. The SmaI digested DNA fragments were separated using CHEF DR III in 1% agarose gel for an initial time of 5 s and a final time of 25 s; at 14°C and during 18 h at 6.0 V with an angle of 120°. MLST and *spa* typing and analysis were performed as previously described in http://www.mlst.net and http://www.spaserver.ridom.de, respectively.

RESULTS

Isolation and Identification

From 235 (90 nasal swab, 145 mastitic milk samples) materials, 181 staphylococci were isolated and of these 92 were identified as *S. aureus* and 12 were detected as MRSA. Of these 12 materials originated from 6 (50%) were animal (2 bovine milk, 4 bovine nasal swab) and 6 (50%) were human (nasal swab). All MRSA strains were positive for *mec*A and *nuc* gene and but PVL gene negative. Fragments of expected sizes were 162 and 279 bp for the *mec*A and *nuc* genes, respectively (*Fig. 1* and *Fig. 2*). These MRSA strains were studied further.

Molecular Typing

Four SCC*mec* type from 12 MRSA isolate were determined using multiplex PCR. SCC*mec* Type II was found in 1, Type III in 10, Type IV in 1 *(Fig. 3)*. For this, one of the materials found as CA MRSA, while 11 were HA MRSA. The analysis of pulsed-field gel electrophoresis (PFGE) profiles revealed presence of 4 clusters *(Fig. 4)*. The first pulsotype (PT) A (7 isolates) clone strains were SCC*mec*

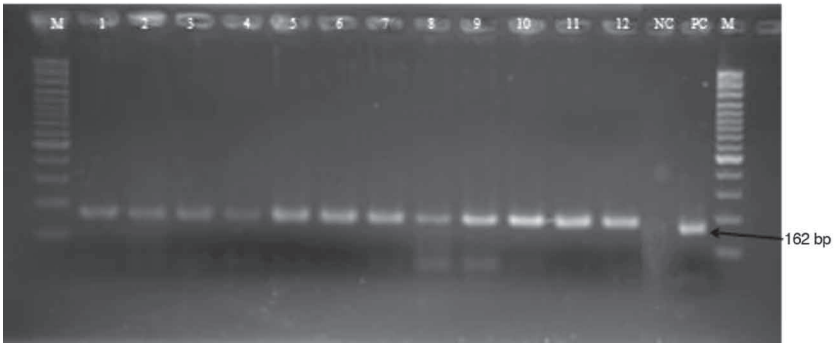
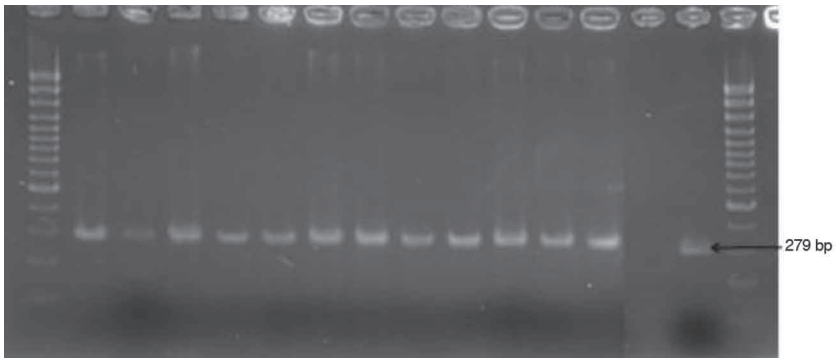


Fig 1. *mec* PCR M: Marker (100 bp DNA ladder) 1-12: *S. aureus* field isolates PC: Positive Control (*S. aureus* N315 strain, 162 bp positive) NC: Negative Control (without DNA master mix)

Şekil 1. *mec* PCR M: Marker (100 bp DNA ladder) 1-12: *S. aureus* saha izolatları PC: Pozitif Kontrol (*S. aureus* N315 suşu, 162 bp pozitif) NC: Negatif Kontrol (DNA'sız master miks)

Fig 2. *nuc* PCR M: Marker (100 bp DNA ladder) 1-12: *S. aureus* field isolates PC: Positive Control (*S. aureus* N315 strain, 279 bp positive) NC: Negative Control (without DNA master mix)

Şekil 2. *nuc* PCR M: Marker (100 bp DNA ladder) 1-12: *S. aureus* saha izolatları PC: Pozitif Kontrol (*S. aureus* N315 strain, 279 bp pozitif) NC: Negatif Kontrol (DNA'sız master mix)



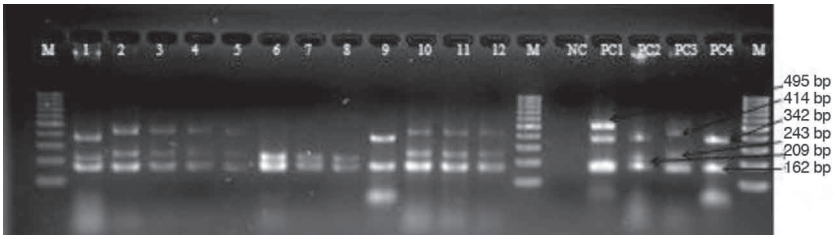


Fig 3. SCC*mec* types of isolates M: Marker (100 bp DNA ladder) 1-12: *S. aureus* field isolates PC: Positive Controls (PC1: *S. aureus* HPV107, PC2: *S. aureus* N315, PC3: *S. aureus* HUSA304, PC4: *S. aureus* GRE14) NC: Negative Control (*S. aureus* ATCC 29213)

Şekil 3. İzolatların SCC*mec* tipleri M: Marker (100 bp DNA ladder) 1-12: *S. aureus* İzolatları PC: Pozitif Kontroller (PC1: *S. aureus* HPV107, PC2: *S. aureus* N315, PC3: *S. aureus* HUSA304, PC4: *S. aureus* GRE14) NC: Negatif Kontrol (*S. aureus* ATCC 29213)

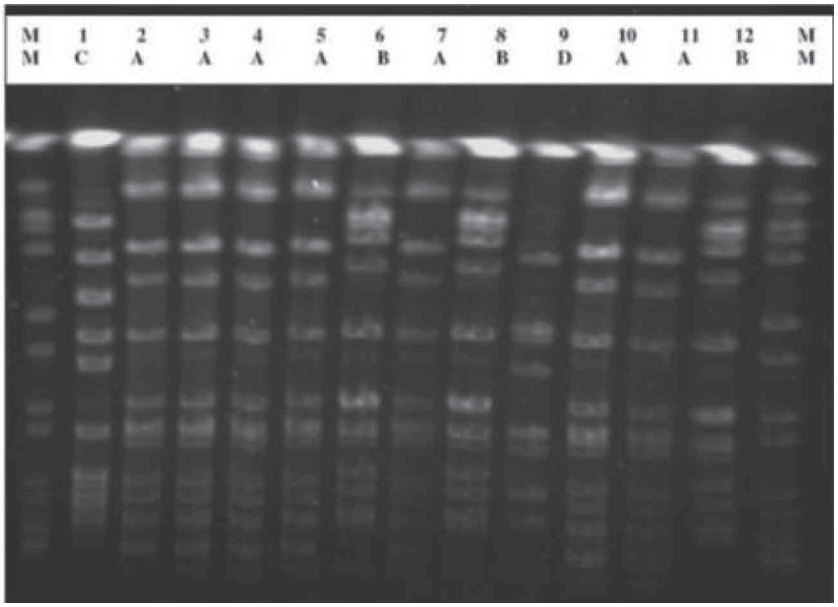


Fig 4. Pulsed-field gel electrophoresis analysis of 12 methicillin-resistant *S. aureus* strains.

PFGE profiles revealed presence of 4 clusters. M: Marker (*S. aureus* NCTC 8325 strain)

1: Pulsotype C; 2-5, 7, 10, 11: Pulsotype A; 6, 8, 12: Pulsotype B; 9: Pulsotype D

Şekil 4. On iki metisilin dirençli *S. aureus* suşunun İtilmiş Alan Jel Elektroforezisi. PFGE profilleri, 4 grup varlığını ortaya koydu. M: Marker (*S. aureus* NCTC 8325 suşu)

1: Pulsotip C; 2-5, 7, 10, 11: Pulsotip A; 6, 8, 12: Pulsotip B; 9: Pulsotip D

Table 1. *PFGE profiles, SCCmec, MLST and spa types of isolated 12 MRSA strains*
Tablo 1. *İzole edilen 12 MRSA suşunun , SCCmec, MLST ve spa tipleri ile PRGE profilleri*

Strain	Farm	Material	PFGE (PT)	SCC*mec* type	MLST (ST)	*Spa* (t)
1	1	HNS (FW)	D	II	737	542
2	2	BM	A	III	239	030
3	7	BNS	B	III	1294	459
4	7	BNS	B	III	1294	459
5	10	BM	A	III	239	030
6	10	BNS	A	III	239	030
7	11	BNS	B	III	1294	459
8	11	HNS (FW)	C	III	1940	660
9	12	HNS (FW)	A	IV	239	030
10		HNS (VP)	A	III	239	030
11		HNS (VP)	A	III	239	030
12		HNS (VP)	A	III	239	030

HNS: *Human nasal swab,* ***FW:*** *Farm Worker,* ***VP:*** *Veterinary Physician,* ***BNS:*** *Bovine Nasal Swab,* ***BM:*** *Bovine Milk*

type III (except one strain, the 9. strain SCC*mec* type IV), *spa*-types t030 (allelic profile 15-12-16-02-24-24) and MLST-types ST239 (allelic profile 15-12-16-02-24-24). The PT B (3 isolates) clone strains were type III with SCC*mec* typing, *spa*-types t459 (allelic profile 15-12-16-02-24) and MLST-types ST1294 (2-3-1-1-4-4-115). The third PT (C, 1 isolate) was SCC*mec* type III, *spa*-types t660 (allelic profile 11-12-41-20-17-12-12-17) and MLST-types ST1940 (allelic profile 213-1-4-1-5-5-4). The last, PT D (1 isolate), was SCC*mec* type II, *spa*-types t542 (allelic profile 26-23-13-23-13-23-31-05-17-25-17-25-16-28) and MLST-types ST737 (allelic profile 7-6-1-70-8-8-6). Distribution of SCC*mec*, *spa*, MLST types and PFGE profiles of 12 MRSA isolates is shown in *Table 1*.

DISCUSSION

MRSA is a growing problem in humans and animals [1,3]. While PFGE is accepted as a golden standard for determination of clonal relations between strains, SCC*mec* is used to detect the strains whether originated from hospital or community acquired. Recently, DNA sequence based methods for typing *S. aureus* such as MLST and *spa* typing have been developed and are widely used to establish clonal relationships between strains and to compare the geographical locations of MRSA clones [3,6]. In this study, the investigation of 12 MRSA strains with using PFGE, SCC*mec*, MLST, and *spa* typing methods which have been isolated from dairy cattle and farm

workers was aimed.

The description "methicillin-resistant" was first used in 1961 [20] in humans then the first MRSA strain in animals was reported at 1972 from dairy cattle with mastitis [21]. Since that time, MRSA has emerged as a significant problem worldwide. The number of cases was increased in the following years [1]. Vanderhaeghan et al.[21] reported that the share of MRSA in milk samples with clinic and subclinical mastitis was 10% in Belgium, while this share had been given as 17.2% by Turkyılmaz et al.[12] for Turkey. It reported that this ratio is varied from 0 to 60% in Turkey according to the limited number of studies in which methicillin resistance has been investigated with phenotypic methods in cow milk samples with mastitis [12]. It also claimed that it was not enough for determining the *mec*A gene only with phenotypic disc method. For that reason, results have to be confirmed with molecular methods against the probability of false positive and negative results [23].

The most common SCC*mec, spa* and MLST types show some differences according to the countries. For example, SCC*mec* Type I is common in Croatia [24] and Swiss [25]; Type II is common in Japan and Korea; Type III is common in Saudi Arabia, Singapore, China, Thailand, India [26], and also in Turkey [27]; Type IV is especially spread out in Spain [28], Greece [29]. As in humans, the most common SCC*mec* type in Turkey was Type III amongst *S. aureus* strains isolated from mastitic cow milks [11]. In this study, as parallel to above findings, the most common SCC*mec* type was found as Type III (83.3%) in both human and animals. It thought that there were a lot of hospital originated strains in animals and animals could have been infected with these strains because of poor hygienic conditions in these places.

In this study, just in humans in our country, it determined that the most common *spa* type was t030 (58.3%). t030 was isolated from strains which had been originated from human and animals between 1998 and 2011 years in Turkey, France, Iran, China, Swiss, South Africa, Check Republic, Norway, Spain, Denmark, Sweden, Lebanon, Romania, Croatia, Cyprus, Bulgaria, and France. t459 isolated from human originated samples collected between 2005 and 2011 years in Germany, Denmark, and China; t542 isolated from human originated samples collected between 2006 and 2010 years in Denmark and Germany; t660 isolated from human originated samples collected between 2007 and 2011 years in France, Holland, Spain, Sweden and Lebanon *(www.ridom.de/spa-server/)*.

MLST analysis revealed that the most common sequence type was ST239 (58.3%). This sequence type was also the most common sequence type for human in Turkey. Sequence types (ST737, ST1294, and ST1940) determined in this study had never found before in animals *(www.ridom.de/spa-server/)*. This can be aroused from there was no MLST typing before for *S. aureus* strains isolated from animals and these strains might have been originated from humans.

MRSA can be transmitted between people and animals during close contact [30]. It is certain that animals are a source of human MRSA infection in some circumstances, humans may also serve as sources of infection in animals. Some groups of individuals who work closely with animals, such as veterinarians, have high MRSA colonization rates. In our study, strains had been divided to four pulsotype. A total of 6 strains which consisted from 2 cow milk samples, 1 nasal cow nasal swaps and 3 nasal swaps taken from veterinarians who had treated these animals revealed that MRSA has clonal contagious traits. In this study, it was thought that the results of PFGE, MLST and *spa* typing methods support each other. Although it reported that animals can be a reservoir for MRSA which causes serious infections in humans [30], recent studies show that MRSA infections may be originated from humans [12,31]. In the study, the same pulsotype, MLST and *spa* typing results between two samples which had been taken from veterinarians and dairy cattle thought that veterinarians have important role in transferring of the strains to animals, and also thought that these infections may be originated from humans.

Consequently, it determined that the most common *spa* type and MLST type isolated from cow milk, cow nasal swap and human nasal swap samples in Aydın region were t030 and ST239, respectively. It recommended that a multipurpose epidemiologic study based on MLST and *spa* typing methods should be designed to compare MRSA isolates in animals in Turkey with isolates in the World.

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