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RESEARCH ARTICLE

Prevalence of Methicillin Resistance and Panton-Valentine Leukocidin Genes in Staphylococci Isolated from Pirlak Sheep with Subclinical Mastitis in Turkey

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ABSTRACT

This study aimed to investigate the presence of *mecA* and *pvl* genes in 47 Staphylococci previously isolated from 464 half-udder milk samples belong to 235 Pirlak sheep screening for subclinical mastitis. The species from Pirlak sheep used in the present study included: 13 *S. aureus*, 13 *S. epidermidis*, six *S. xylosus*, five *S. chromogenes*, three *S. simulans*, three *S. hyicus*, two *S. warneri*, one *S. lentus* and one *S. saprophyticus*. A total of 10 strains (21.3%) were determined to harbour *mecA* gene, of these, two (4.2%) also contained *pvl* gene. The strains carrying *mecA* gene were found to be *S. aureus* (3/13), *S. xylosus* (3/6), *S. epidermidis* (2/13), *S. lentus* (1/1) and *S. hyicus* (1/3). The presence of *pvl* gene was determined in a total of eight strains (17.0%), six (12.8%) of these were alone. Of *pvl* positive strains, three, three, one, and one were *S. aureus*, *S. xylosus*, *S. simulans* and *S. hyicus*, respectively. To our knowledge, this is the first study showing the presence of *mecA* and *pvl* genes in the Staphylococci isolated from Pirlak sheep with subclinical mastitis in Turkey.

Keywords: Mastitis, Methicillin Resistance, Panton-Valentine Leukocidin, Sheep, Staphylococci

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Türkiye'de Subklinik Mastitisli Pırlak Koyunlardan İzole Edilen Stafilokoklarda Metisilin Direnç ve Panton-Valentine Lökosidin Genlerinin Prevalansı

ÖZ

Bu çalışmada, subklinik mastitis yönünden taranan 235 Pırlak koyuna ait 464 meme lobu süt örneğinden daha önce izole edilen 47 Stafilokok türünde *mecA* ve *pvl* genlerinin varlığının araştırılması amaçlandı. Çalışmada, Pırlak koyunlardan izole edilen 13 *S. aureus*, 13 *S. epidermidis*, altı *S. xylosus*, beş *S. chromogenes*, üç *S. simulans*, üç *S. hyicus*, iki *S. warneri*, bir *S. lentus* ve bir *S. saprophyticus* suşu kullanıldı. Toplam 10 suşun (%21,3) *mecA* geni taşıdığı, bunlardan ikisinin (%4,2) ayrıca *pvl* genine de sahip olduğu belirlendi. *mecA* geni taşıyan suşlar *S. aureus* (3/13), *S. xylosus* (3/6), *S. epidermidis* (2/13), *S. lentus* (1/1) ve *S. hyicus* (1/3) olarak bulundu. Toplam sekiz suşta (%17,0) *pvl* geni belirlenirken, bunlardan altısının (%12,8) bu geni tek başına taşıdığı tespit edildi. *pvl* pozitif suşların üçü *S. aureus*, üçü *S. xylosus*, biri *S. simulans* ve biri *S. hyicus* olarak belirlendi. Bilgimize göre, bu çalışma Türkiye'de subklinik mastitisli Pırlak koyunlardan izole edilen Stafilokoklarda *mecA* ve *pvl* genlerinin varlığını gösteren ilk çalışmadır.

Anahtar Kelimeler: Koyun, Mastitis, Metisilin Direnci, Panton-Valentine Lökosidin, Stafilokok

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INTRODUCTION

The importance of methicillin resistant Staphylococci (MRS), especially methicillin resistant *Staphylococcus aureus* (MRSA), has been emphasized in terms of public and animal health because these agents have been accepted to be humanosis and/or zoonosis pathogens (Pantosti 2012). Methicillin resistance has become an increasing urgent problem in veterinary medicine after the first report of MRSA in dairy cows with mastitis (Cuny et al. 2010, Caruso et al. 2016, Aires-de-Sousa 2017). This resistance results from the production of an alternative penicillin-binding protein (PBP2a or PBP2′) encoded by the *mecA* gene (Pinho et al. 2001). Although sufficient data on the presence of *mecA* gene in Staphylococci, particularly *S. aureus* strains, isolated from bovine mastitic milk samples have been found (Moon et al. 2007, Türkyılmaz et al. 2010, Gezgen and Seker 2016), the investigations related to the prevalence of this gene in Staphylococci isolated from sheep are limited (Vyletělová et al. 2011, Ünal and Çinar 2012, Ünal et al. 2012).

Panton-Valentine leukocidin (*PVL*) encoded by lukF-PV and lukS-PV genes is a leukocytolytic toxin causing leukocyte destruction and tissue necrosis (Yoong and Torres 2013). Although epidemiologically the *PVL* toxin has been linked to community-acquired methicillin resistant *S. aureus* infections (CA-MRSA) in the past (Lo and Wang 2011), nowadays, it has been shown that *PVL* may be found in both methicillin susceptible *S. aureus* (MSSA) and MRSA strains (Sharma-Kuinkel et al. 2012). The role of *PVL* in human Staphylococcal infections is not clear and remains an issue of contention (Sharma-Kuinkel et al. 2012). Similarly, its pathogenic role in the pathogenesis of mastitis is still controversial (Ikawaty et al. 2010, Gezgen and Seker 2016).

The Pirlak breed obtained by crossing the Daglic with the Kivircik has a feature between these two breed in terms of basic phenotypic and production performance characteristics. This mid-sized coarse wool sheep also produces meat and milk and is raised in some provinces of the Aegean, Marmara and Mediterranean regions in Turkey (Yilmaz et al. 2013). The researches on the etiology of mastitis in this particular breed are limited (Ozenc et al. 2011) and any data has not been also described on the presence of methicillin resistance and Panton-Valentine leukocidin genes in the Staphylococci isolated from Pirlak sheep. Therefore, we aimed to investigate the *mecA* and *pvl* genes in Staphylococci previously isolated from Pirlak sheep with subclinical mastitis in Afyonkarahisar province of Turkey..

MATERIALS and METHODS

Bacterial strains

A total of 47 Staphylococci isolates used in this study were previously isolated from 464 half-udder milk samples belong to 235 Pirlak sheep screening for subclinical mastitis in Afyonkarahisar, Turkey. All isolates were confirmed using Crystal™ Identification Systems Gram-Positive ID kit (Becton, Dickinson and Company, NJ, USA). The species from Pirlak sheep used in the present study included: 13 S. aureus, 13 *Staphylococcus epidermidis*, six *Staphylococcus xylosus*, five *Staphylococcus chromogenes*, three *Staphylococcus simulans*, three *Staphylococcus hyicus*, two *Staphylococcus warneri*, one *Staphylococcus lentus* and one *Staphylococcus saprophyticus*.

Detection of 16S rDNA, mecA and pvl genes by PCR

In this study, MRSA ATCC® 33591 and *PVL S. aureus* ATCC® 49775 strains were used as positive control strains and MSSA ATCC® 25923 was used as negative control strain (Oxoid Microbiology Products, Hampshire, UK). DNAs were extracted from control and all test strains using boiling method. The fresh colonies growing on Trypticase Soy Agar (Oxoid Microbiology Products, Hampshire, UK) were suspended in eppendorf tubes containing 500 µL of sterile deionized water and the tubes were held in a 100ºC of water bath for 10 min. After this process, the suspension was centifugated at 9,167 g for 5 min and the supernatant was used for PCR assay (Gezgen and Seker 2016).

Duplex PCR was used for the detection of 16S rDNA and *mecA* genes, while singleplex PCR was performed for *pvl* gene. A total of 25 µL PCR mixture included 2.5 µL 10X PCR buffer, 25 mM MgCl2, 10 mM dNTP mix, 20 µM each primers, 1U of Taq DNA polymerase, 5 µL of template DNA and deionized water. The oligonucleotide primers and PCR amplification conditions of 16S rDNA, *mecA* and *pvl* genes were shown in Table 1 and Table 2, respectively. All products were analyzed by 1.5% agarose gel electrophoresis and visualized using ethidium bromide under U.V. light.

RESULTS

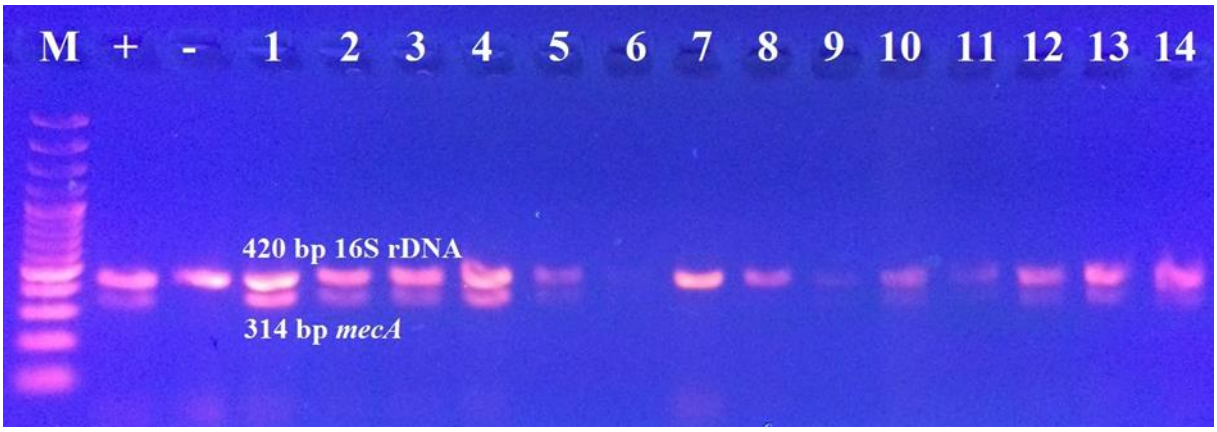
All of 47 *Staphylococcus* strains obtained from half-udder milk samples of Pirlak sheep showed 16S rDNA specific bands. A total of 10 (21.3%) strains were determined to harbour *mecA* gene, of these, two (4.2%) also contained *pvl* gene. The strains carrying *mecA* gene were found to be *S. aureus* (n=3), *S. xylosus* (n=3), *S. epidermidis* (n=2), *S. lentus* (n=1) and *S. hyicus* (n=1) (Figure 1). The presence of *pvl* gene was determined in a total of eight strains (17.0%). Of *pvl* positive strains, three, three, one, and one were S. aureus, *S. xylosus*, *S. simulans* and *S. hyicus*, respectively (Figure 2). The distribution of *mecA* and *pvl* genes was shown in Table 3.

Tablo 1. Çalışmada kullanılan oligonükleotid primerleri
Table 1. Oligonucleotide primers used in this study

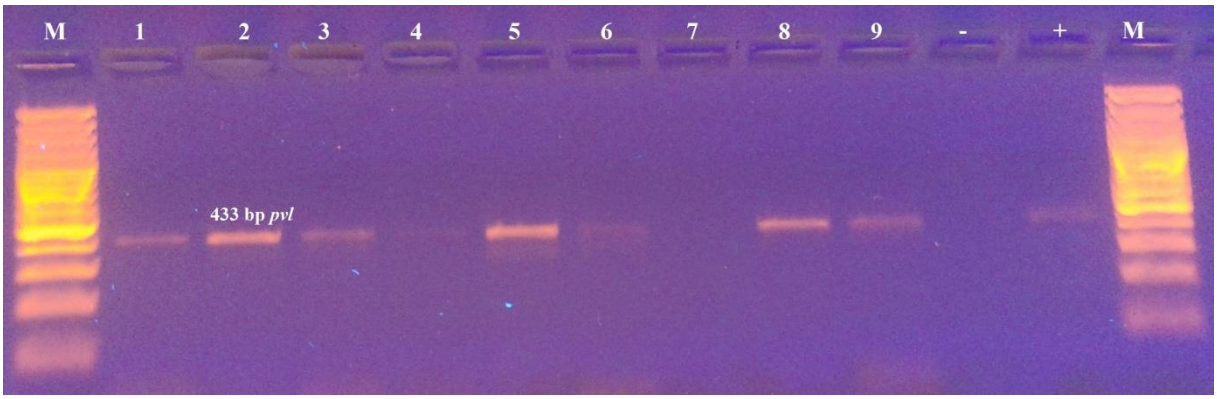
Gene	Sequence (5'→3')	Product (bp)	Reference
16S rDNA	CAGCTCGTGTCGTGAGATGT	420	Strommenger et al. 2003
	AATCATTTGTCCCACCTTCG		
mecA	CCTAGTAAAGCTCC□ GAA	314	Choi et al. 2003
	CTAGTCCATTCGGTCCA		
pvl	ATCATTAGGTAAAATGTCTGGACATGATCCA	433	Lina et al. 1999
	GCATCAASTGTATTGGATAGCAAAAGC		

Tablo 2. 16S rDNA, *mecA* ve *pvl* genleri için PZR amplifikasyon koşulları.
Table 2. PCR amplification conditions of 16S rDNA, *mecA* and *pvl* genes (Gezgen and Seker 2016)

Step	Cycle	Temperature		Time	
		mecA and 16S rDNA	*pvl*	*mecA* and 16S rDNA	*pvl*
Initial denaturation	1	95°C	95°C	5 min	5 min
Denaturation	30	95°C	94°C	2 min	30 sec
Annealing	30	54°C	62°C	1 min	30 sec
Extension	30	72°C	72°C	1 min	1 min
Final extension	1	72°C	72°C	7 min	5 min



Şekil 1. 16S rDNA ve *mecA* genlerinin dubleks PZR ile belirlenmesi. M: 100 bp DNA ladder; +: pozitif kontrol (MRSA ATCC® 33591); -: negatif kontrol (MSSA ATCC® 25923); sütun 1-3: *mecA* geni pozitif *S. aureus* suşları; sütun 4,5,10: *mecA* geni pozitif *S. xylosus* suşları; sütun 6: 16SrDNA negatif kontrol (steril distile su); sütun 11,12: *mecA* geni pozitif *S. epidermidis* suşları; sütun 13: *mecA* geni pozitif *S. lentus* suşu; sütun 14: *mecA* geni pozitif *S. hyicus* suşu
Figure 1. Detection of 16S rDNA and *mecA* genes by dublex PCR. M: 100 bp DNA ladder; +: positive control (MRSA ATCC® 33591); -: negative control (MSSA ATCC® 25923); lane 1-3: *mecA* gene positive *S. aureus* strains; lane 4,5,10: *mecA* gene positive *S. xylosus* strains; lane 6: 16SrDNA negative control (sterile distilled water); lane 11,12: *mecA* gene positive *S. epidermidis* strains; lane 13: *mecA* gene positive *S. lentus* strain; lane 14: *mecA* gene positive *S. hyicus* strain.



Şekil 2. PZR ile *pvl* geninin belirlenmesi. M: 100 bp DNA ladder; sütun 1-3: *pvl* geni pozitif *S. aureus* suşları; sütun 4-6: *pvl* geni pozitif *S. xylosus* suşları; sütun 7: *pvl* geni negatif suş; sütun 8: *pvl* geni pozitif *S. simulans* suşu; sütun 9: *pvl* geni pozitif *S. hyicus* suşu; -: negatif kontrol (steril distile su); +: pozitif kontrol (*PVL S. aureus* ATCC® 49775).
Figure 2. Detection of *pvl* gene by PCR. M: 100 bp DNA ladder; lane 1-3: *pvl* gene positive *S. aureus* strians; lane 4-6: *pvl* gene positive *S. xylosus* strains; lane 7: *pvl* gene negative strain; lane 8: *pvl* gene positive *S. simulans* strain; lane 9: *pvl* gene positive *S. hyicus* strain; -: negative control (sterile distilled water); +: positive control (*PVL S. aureus* ATCC® 49775).

Tablo 3. Subklinik mastitisli Pırlak koyunlardan izole edilen Stafilokoklarda PZR ile belirlenen *mecA* ve *pvl* genlerinin dağılımı.
Table 3. Distribution of *mecA* and *pvl* genes detected by PCR in Staphylococci isolated from Pirlak sheep with subclinical mastitis.

Species (no of tested strain)	Genes	No of isolates
S. aureus (n=13)	*mecA*	2
	pvl	2
	mecA, pvl	1
S. epidermidis (n=13)	*mecA*	2
S. xylosus (n=6)	*mecA*	2
	pvl	2
	mecA, pvl	1
S. chromogenes (n=1)	-	-
S. simulans (n=3)	*pvl*	1
S. hyicus (n=3)	*mecA*	1
	pvl	1
S. warneri (n=2)	-	-
S. lentus (n=1)	*mecA*	1
S. saprophyticus (n=1)	-	-

DISCUSSION

The present study investigated the presence of *mecA* and *pvl* genes in Staphylococci previously isolated from Pirlak sheep with subclinical mastitis.

Staphylococci are the most common etiologic agents isolated from subclinical mastitis cases in sheep (Ozenc et al. 2011, Gelasakis et al. 2015, Queiroga 2017). In recent years, methicillin resistance mediated by the *mecA* gene has been increasingly reported in Coagulase Negative Staphylococci (CNS) as well as in *S. aureus* isolated from bovine mastitic milk samples (Vyletělová et al. 2011, Ünal and Çinar 2012, Gelasakis et al. 2015). However, the reports and data related to the prevalence of this gene in Staphylococci isolated from sheep with subclinical mastitis are limited (Vyletělová et al. 2011, França et al. 2012, Ünal et al. 2012, Martins et al. 2015). Vyletělová et al.

(2011) reported that the *mecA* gene was obtained in none of *S. aureus* and *S. lentus* strains isolated from sheep with subclinical mastitis. In another study, it was emphasized that none of 37 Staphylococci strains isolated from ovine subclinical mastitic milk samples harboured the *mecA* gene (França et al. 2012). Martins et al. (2015) reported that none of 18 *S. aureus* strains obtained from 473 subclinical mastitic milk samples were carried the *mecA* gene. Similarly, Ünal et al. (2012) from Turkey emphasized that none of 21 *S. aureus* strains isolated from ewes' milk harboured the *mecA* gene. In another study from Turkey, it was reported that *mecA* positivity was found in three (7.5%) of 40 CNS strains isolated from ewes with subclinical mastitis. In the same study, two and one of *mecA* positive strains were identified to be *S. lentus* and *S. xylosus*, respectively (Ünal and Çinar 2012). In our study, *mecA* positivity was found in 10 of 47 (21.3%) Staphylococci strains isolated from Pirlak sheep with subclinical mastitis. The strains carrying *mecA* gene were determined to be *S. aureus* (3/13), *S. xylosus* (3/6), *S. epidermidis* (2/13), *S. lentus* (1/1) and *S. hyicus* (1/3). Compared the other investigations from different countries, the *mecA* positivity obtained from our study may be associated with the intensive and prolonged use of nonspecific antibiotics for the treatment of mastitis in Turkey. However, the geographical variations may be effective on the difference of *mecA* gene prevalence in the strains. These results also showed that CNS, like as S. aureus, isolated from Pirlak sheep may be potential reservoirs for *mecA* genes and this may pose a public health risk in terms of dissemination of methicillin resistant strains. It was reported that *mecA* positive CNS strains may transfer the resistance gene to *S. aureus* and other Staphylococci (Huber et al. 2011).

PVL is one of the most important and extensively investigated proteins that belong to bicomponent synergohymenotropic toxins family (Yoong and Torres 2013). Although *PVL* is frequently reported as a common virulence factor in MRSA strains, especially CA-MRSA strains, this toxin gene has also been isolated from MSSA in recent years (Strommenger et al. 2003). It has been reported these bicomponent toxins are secreted by some strains of mastitis-causing S. aureus, but data on the prevalence of leukotoxins among strains obtained from small ruminants with mastitis is limited. In Brazil, while Aires-de-Sousa et al. (2007) reported that none of the 16 Staphylococci isolates obtained from sheep harboured the *pvl* gene, Martins et al. (2015) emphasized the exotoxin *PVL* was detected in only one (5.5%) strain of 18 *mecA* negative *S. aureus* strains obtained from sheep with subclinical mastitis. In a study from Turkey, it was reported that 14 (66.6%) of the 21 *S. aureus* isolates from mastitic milk samples of small ruminants had *pvl* gene while none of the isolates harboured *mecA* gene (Ünal et al. 2012). In another study, Ünal and Çinar (2012) determined the *pvl* gene in one *S. simulans* and one *S. warneri* strain among 40 ewe CNS isolates. In the same study, the researchers emphasized none of *mecA* positive strains harboured the *pvl* gene. According to results of our study, a total of eight (17.0%) strains had the *pvl* gene, six of these were alone. The *pvl* gene positive strains were determined to be *S. aureus* (3/13), *S. xylosus* (3/6), *S. simulans* (1/3) and *S. hyicus* (1/3). (Table 3). These findings suggested that *pvl* gene may also be common in CNS isolates and may also be present in *mecA* negative strains. Rainard and Riollet (2006) reported that the neutrophil phagocytosis is a significant defense factor against bacteria causing mastitis on the mammary gland of ruminants. Although the role of *PVL* on mastitis is not clearly understood, the production of this leukotoxin may give more advantages to Staphylococci to resist host defense mechanisms and to settle in the mammary gland.

The present study revealed that CNS, like as S. aureus, isolated from Pirlak sheep could be potential reservoirs of *mecA* and *pvl* genes. This may pose a public health risk due to the horizontal transfer of these attributes of pathogenicity to commensals or pathogenic bacteria. In our study, it was also shown that the *pvl* gene could also be found in *mecA* negative strains as well as in *mecA* positive strains. Although the strains carrying both *pvl* and *mecA* genes are considered to be more pathogenic, it should not be ignored the other strains carrying these genes alone, especially CNS strains, may also be potential pathogens for human and animals.

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