Prevalence of Methicillin Resistance and Panton-Valentine Leukocidin Genes in Staphylococci Isolated from Pırlak 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 belonging to 235 Pırlak sheep screened for subclinical mastitis. The species from Pırlak 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 Pırlak sheep with subclinical mastitis in Turkey.

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

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Türkiye’de Subklinik Mastitisli Pırlak Köyünlardan İzole Edilen Stafilokoklarda Metisilin Direnci ve Panton-Valentine Lökositin Genlerinin prevalansı

ÖZ
Bu çalışmada, subklinik mastitis yönünden taranan 235 Pırlak koyun ait 464 meme lobu süt örneklerinin 47 Stafilokoks türüne 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 süt örnekleri kullanıldı. Toplam 10 suşun (%21,3) mcA geni taşıdığı, bunlardan ikisinin (%4,2) ayrıca pvl genine de sahip olduğu belirlendi. mcA 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 suş (%17,0) pvl geni belirlenirken, bunlardan altısı (%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 Lökositin, 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 Kivircık 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 (Yılmaz 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 *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.

**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 500 μL sterile deionized water and the tubes were held in a 100°C water bath for 10 min. After this process, the suspension was centrifuged 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 | CAGCTCGTGTCGTAGATGTACATTGTGTCACCTTCG                 | 420          | Strommenger et al. 2003    |
| mecA   | CCTAGTAAAGCTCCGAACTAGTCCATTCGGTCCA                   | 314          | Choi et al. 2003           |
| pvl    | ATCATTAGGTAATGCTGAGATGATCCAGATGAATGTCATCAAGATGCAAAAGC | 433          | Lina et al. 1999          |

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 | mecA and 16S |
|                     |       | rDNA        | rDNA  |
| Initial denaturation| 1     | 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); sütün 1-3: mecA geni pozitif S. aureus suşları; sütün 4,5,10: mecA geni pozitif S. xylosus suşları; sütün 6: 16SrDNA negatif kontrol (steril distiller su); sütün 11,12: mecA geni pozitif S. epidermidis suşları; sütün 13: mecA geni pozitif S. lentus suşu; sütün 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.
**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 Coagulate 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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