Acta Scientiae Veterinariae

ISSN: 1678-0345

ActaSciVet@ufrgs.br

Universidade Federal do Rio Grande do Sul

Brasil

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Acta Scientiae Veterinariae, vol. 44, 2016, pp. 1-9

Universidade Federal do Rio Grande do Sul

Porto Alegre, Brasil

Available in: http://www.redalyc.org/articulo.oa?id=289043697024

Acta Scientiae Veterinariae, 2016. 44: 1373.

RESEARCH ARTICLE
Pub. 1373

ISSN 1679-9216

Investigation of Methicillin Resistance and Panton-Valentine Leukocidin in Staphylococci Isolated from Bovine Mastitis*

Cansu Gezgen[1] & Esra Seker[2]

ABSTRACT

***Background*:** Mastitis, which is inflammation of the mammary gland, is among the most important diseases in dairy herds resulting in reductions of milk yield and milk quality. Although several groups of microorganisms have been reported as etiological agents of mastitis, Staphylococci are the most frequently isolated bacteria from bovine mastitic milk samples. The aim of this study was to isolate the *Staphylococcus* species from bovine mastitis, investigate the *mecA* ve *pvl* genes in isolated species by PCR and determine the antibiotic resistance of methicillin resistant strains to some antibiotics commonly used in veterinary field.

***Materials, Methods & Results*:** In the present study, 972 half-udder milk samples (n = 757 CMT positive, n = 215 CMT negative) were used from 251 lactating cows from 34 different enterprises located center town and villages of Ödemiş, İzmir. Ten microliters of each milk sample was inoculated onto Columbia blood agar, containing 7% of sheep blood and incubated under aerobic conditions for 24-48 h at 37°C. The certain identification of *Staphylococcus* isolates was achieved using Crystal™ Identification Systems Gram-Positive ID kit. Bacterial DNAs were extracted from all strains using boiling method and strains were screened for the presence of 16S rDNA, *mecA* and *pvl* genes by PCR. The antimicrobial resistance of MRS species was determined by using disc diffusion method. A total of 182 (18.72%) *Staphylococcus* strains were isolated from 972 half-udder milk samples. Of 182 *Staphylococcus* strains, 137 (75.27%) and 45 (24.73%) were detected as CPS and CNS, respectively. Among the 11 different *Staphylococcus* species, *S. intermedius* (42.30%) was the most common species isolated, followed by *S. aureus* (32.97%) and *S. saprophyticus* (10.99%). The *mecA* positivity was found in only 4 (2.2%) *S. intermedius* strains, while *pvl* toxin gene was determined in none of the strains. Four MR *S. intermedius* strains were resistant to oxacillin and cefoxitin. The resistance was also found to erythromycin (50%), rifampicin (25%), gentamicin (25%), tetracycline (25%) and trimethoprim/sulfamethoxazole (25%) in the isolates.

***Discussion*:** In this study, *S. intermedius* had the highest isolation rate and this finding was considered remarkable. Generally, in mastitis diagnostics all CPS isolates are classified as *S. aureus*. In our study, the certain identification of all CPS may explain the high isolation rate of *S. intermedius*. The sampling method may also be the reason of higher isolation rate for *S. intermedius* in accordance with the most common ones causing mastitis. All of *mecA* positive strains were *S. intermedius* and this was the another remarkable finding of our study. Because similar result was seen in only one study from Korea, while the investigation finding related to MR *S. intermedius* was not determined in Turkey. However, the *mecA* positivity found in our study was lower than the other author's isolation rate. The difference between the sample size, geographical variations and diversity in strains may be the causes of this discrepancy. It was investigated *pvl* toxin gene in 182 *Staphylococcus* strains by PCR and found this gene in none of strains. According to this finding, it was considered that *pvl* gene may have not an efficient role in the pathogenesis of mastitis in terms of sampled animals and sampling area. Antibiotic resistance of 4 MR *S. intermedius* strains against various antibiotics commonly used in Turkey was investigated. Of methicillin resistant strains, 2, 1 and 1 were resistant to 3, 6 and 5 antibiotics, respectively. It was considered that geographical differences, number of tested isolates and diversity in MR strains may be effective on the antibiotic resistance levels. This is the first study showing the presence of *mecA* gene in *S. intermedius* strains isolated from bovine mastitic milk samples in Turkey.

Keywords: antimicrobial resistance, mastitis, methicillin resistance, Panton-Valentine leukocidin, *Staphylococcus* spp.

Received: 7 February 2016 Accepted: 8 June 2016 Published: 30 June 2016

*Article based on a Thesis submitted by the senior author in partial fulfillment of requirements for the Master's Degree.

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INTRODUCTION

Mastitis, which is defined as an inflammatory reaction of mammary gland, continues to be prominent problem affecting the animal and public health in dairy industry [2,10,12]. Among several groups of microorganisms playing role in the etiology of mastitis, Staphylococci are the most frequently isolated bacteria from bovine mastitic milk samples [9,21,24,32]. Methicillin resistant Staphylococci (MRS) have been considered as an emerging problem in veterinary medicine in recent years [22,29,33]. Methicillin resistance is mediated by the mecA gene located on the chromosome of MRS. MRS are often resistant to antimicrobials both all β-lactams and other than β-lactams are widely used in both human and veterinary medicine [11,25]. Panton-Valentine leukocidin (PVL) is a cytotoxin believed by many researchers to be responsible for the severe symptoms of *Staphylococcus aureus* infections [17,18]. However, the pathogenic role of PVL in the pathogenesis of mastitis is still controversial [15,34,38].

In Turkey, the studies investigating the presence of *mecA* and *pvl* toxin genes in Staphylococci isolated from mastitic milk samples are limited [7,34]. Therefore, this study was aimed to isolate the *Staphylococcus* species from bovine mastitis, investigate the *mecA* ve *pvl* genes in isolated species by Polymerase Chain Reaction (PCR) and determine the antibiotic resistance of methicillin resistant strains to some antibiotics commonly used in veterinary field. To our knowledge, this is the first study investigating the presence of *mecA* and *pvl* genes in the Staphylococci isolated from quarter milk samples in İzmir province of Turkey.

MATERIALS AND METHODS

Milk samples and bacteriological identification

A total of 972 half-udder milk samples were collected from 251 lactating cows on 34 different private enterprises in the center town and villages of Ödemiş, located in the İzmir province of Turkey. No antibiotics had been applied to the animals in the previous three months. Firstly, California mastitis test (CMT) was applied for each mammary quarter according to the method described by Schalm *et al*. [28] and scores were evaluated as +1, +2, +3 and negative. Before sampling for bacteriologic examination, the teat ends were cleaned by 70% alcohol and dried. The first streams of foremilk were discharged, and then 10 mL of milk was collected aseptically from each udder half into sterile vials. Samples were immediately transported to the laboratory in a cool box on ice. Ten μL of each milk sample was inoculated onto Columbia blood agar[1], containing 7% of sheep blood and incubated under aerobic conditions for 24-48 h at 37°C. The infection status of milk samples were determined according to the procedures recommended by the National Mastitis Council [23]. Intramammary infection was defined when ≥ 500 cfu/mL colonies were found. Each different colony was examined macroscopically (colony morphology, haemolysis, pigment production) and microscopically (Gram staining). Then oxidase, catalase, slide and tube coagulase and anaerobic fermentations of glucose and mannitol tests were performed to suspected colonies [13]. The certain identification of Staphylococcus isolates was achieved using Crystal™ Identification Systems Gram-Positive ID kit[2]. In all tests, methicillin resistant *S. aureus* (MRSA) ATCC® 33591 and methicillin sensitive *S. aureus* (MSSA) ATCC® 25923 were used as control strains[1]. All isolates identified were stored at -20°C in brain heart infusion broth containing 15% glycerol until further analysis.

Detection of 16S rDNA, mecA and pvl genes in Staphylococcus *spp. by PCR*

DNA was extracted from the positive controls[1] (MRSA ATCC® 33591 and PVL *S. aureus* ATCC® 49775), negative control[1] (MSSA ATCC® 25923) and all test strains using boiling method. For this purpose, all strains were inoculated onto Trypticase Soy Agar[1]. After the incubation, fresh colonies were suspended in 500 μL of DEPC-treated water (DNase-RNase free). The suspension was held in a 100ºC of water bath for 10 min. After centrifugation at 9,167 *g* for 5 min, the supernatant containing bacterial DNA was used as a template for subsequent PCR mixture [39].

The oligonucleotide primers described by Strommenger *et al*. [30], Choi *et al*. [4] and Lina *et al*. [17] were used for the detection of 16S rDNA (*Staphylococcus* spp. specific), *mecA* (methicillin resistance specific) and *pvl* (Panton-Valentine toxin specific) genes, respectively. For the detection of a 420 bp 16S rDNA gene forward 5'-CAGCTCGTGTCGTGAGATGT-3' and reverse 5'-AATCATTTGTCCCACCTTCG-3' primers, a 314 bp *mecA* gene forward 5'-CCTAGTAAAGCTCCGGAA-3' and

reverse 5'-CTAGTCCATTCGGTCCA-3' primers were used. Amplification of a 433 bp *pvl* gene was performed using the forward 5'-ATCATTAGGTAAAATGTCTG-GACATGATCCA-3' and reverse 5'-GCATCAAST-GTATTGGATAGCAAAAGC-3' primers. Five µL of the extracted DNA were used as a template in a 25 µL PCR mixture containing 10X PCR buffer, 25 mM MgCl2, 10 mM dNTP mix, 20 µM each primers, 1U of Taq DNA polymerase and deionized water. The PCR amplification conditions of 16S rDNA and *mecA* genes consisted an initial denaturation step at 95°C for 5 min, and 30 cycles of 95°C for 2 min, 54°C for 1 min, 72°C for 1 min and a final step 72°C for 7 min. The amplification cycles of *pvl* gene were programmed as 5 min at 95°C for initial denaturation; 30 cycles, 30 s at 94°C, 30 s at 62°C, 1 min at 72°C; and 5 min final extension step at 72°C. All products were analyzed by 1.5% agarose gel electrophoresis and visualized using ethidium bromide under U.V. light. Molecular size markers[3] (100-bp DNA ladder) were included in each agarose gel.

Antimicrobial Susceptibility Test

The antimicrobial resistance of MRS species was determined by using Kirby-Bauer disc diffusion method test on Mueller Hinton agar[1], according to the guidelines of the Clinical and Laboratory Standards Institute [6]. For this purpose, amoxicillin+clavulanic acid (30 µg), enrofloxacin (5 µg), cefoxitin (30 µg), oxacillin (1 µg), cephalothin (30 µg), rifampicin (5 µg), gentamicin (10 µg), erythromycin (15 µg), tetracycline (30 µg) and trimethoprim/sulfamethoxazole (25 µg) antibiotic discs[1] were used. While the plates including oxacillin and cefoxitin discs were incubated at 35°C for 24 h, the other plates were incubated at 37°C for 18 h (CLSI, 2013). MRSA ATCC® 33591 and MSSA ATCC 25923® were used as positive and negative quality control strains1, respectively.

RESULTS

Isolation and identification findings

A total of 182 (18.72%) *Staphylococcus* spp. were identified from 972 half-udder milk samples (757 CMT positive, 215 CMT negative) belong to 251 lactating cows by using Crystal™ Identification Systems Gram-Positive ID kit. It was determined that 120 (47.8%) of 251 cows were infected with the least one *Staphyloccus* species. Of 182 *Staphylococcus* strains, 137 (75.27%) were found as coagulase-positive Staphylococci (CPS) and 45 (24.73%) as coagulase-negative Staphylococci (CNS). A total of 11 different species were identified in this study. The most frequently isolated species were *Staphylococcus intermedius* (42.30%), followed by *S. aureus* (32.97%) and *Staphylococcus saprophyticus* (10.99%). The identified species and their isolation rates were shown in Table 1. Also, 122 (16.11%) and 60 (27.90%) Staphylococcus species were isolated from 757 CMT positive and 215 CMT negative milk samples, respectively. The distribution of species according to CMT scores was shown in Table 2.

Table 1. Identified *Staphylococcus* species from dairy cow quarter milk samples and their isolation rates.

Species	n	%
S. aureus	60	32,97
S. intermedius	77	42,30
S. saprophyticus	20	10,99
S. haemolyticus	11	6,04
S. simulans	5	2,75
S. capitis	3	1,65
S. epidermidis	2	1,10
S. kloosii	1	0,55
S. sciuri	1	0,55
S. schleiferi subsp. *schleiferi*	1	0,55
S. auricularis	1	0,55
Total	182	100

Table 2. Distribution of *Staphylococcus* species isolated from dairy cow quarter milk samples according to CMT scores.

Species (n = 182)	CMT score			
	+1 (n)	+2 (n)	+3 (n)	Negative (n)
S. aureus	15	27	9	9
S. intermedius	18	21	8	30
S. saprophyticus	1	6	1	12
S. haemolyticus	4	1	1	5
S. simulans	1	2	2	-
S. capitis	1	-	-	2
S. epidermidis	-	2	-	-
S. kloosii	1	-	-	-
S. sciuri	1	-	-	-
S. scleiferi subsp. *scleiferi*	-	-	-	1
S. auricularis	-	-	-	1
Total	42 (23.07%)	59 (32.42%)	21 (11.54%)	60 (32.97%)

PCR findings

16S rDNA specific bands were seen in all of 182 *Staphylococcus* isolates obtained from half-udder milk samples, 4 (2.2%) of these harboured the *mecA* gene. It was also detected all of *mecA* positive strains were *S. intermedius*. Thus, the *mecA* positivity was found to be 5.19% in 77 *S. intermedius* isolates. Two of methicillin resistant *S. intermedius* strains were obtained from the same enterprises. In our study, none of the isolates had *pvl* toxin gene. Amplification of 16S rDNA (420 bp) and *mecA* (314 bp) genes in *S. intermedius* strains was shown in Figure 1.

Antimicrobial susceptibility test

According to Kirby-Bauer disc diffusion test results, all of methicillin resistant *S. intermedius* strains were resistant to oxacillin and cefoxitin, while they were sensitive to amoxicillin+clavulanic acid and cephalothin. The resistance to erythromycin (50%), rifampicin (25%), gentamicin (25%), tetracycline (25%) and trimethoprim/sulfamethoxazole (25%) was also found in the *mecA* positive *S. intermedius* strains. Antibiotic resistance of MR *S. intermedius* strains was shown in Table 3.

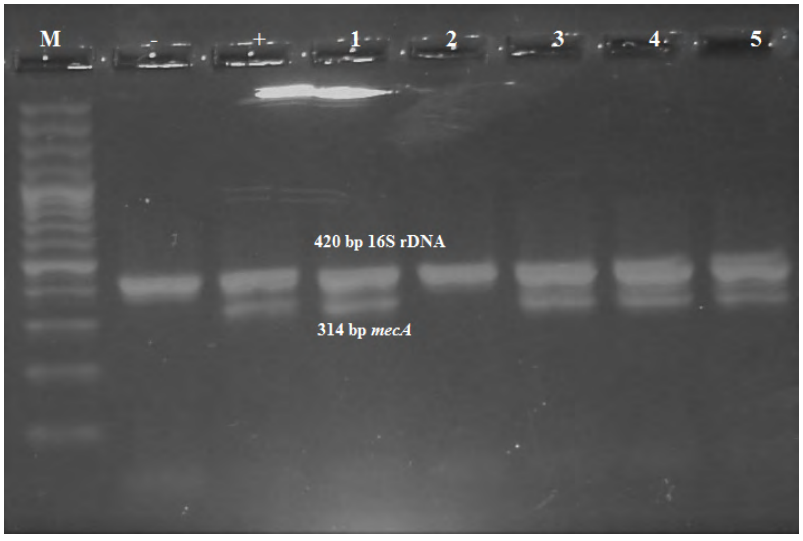

Figure 1. Detection of 16S rDNA and *mecA* genes by mPCR. M: 100 bp DNA ladder; -: negative control (MSSA ATCC® 25923); +: positive control (MRSA ATCC® 33591); lane 1,3-5: *mecA* gene positive *S. intermedius* strains; lane 2: *mecA* gene negative *Staphylococcus* isolate.

Table 3. Antibiotic resistance of 4 MR *S. intermedius* strains.

Antibiotic	*mecA* positive *S. intermedius* (n = 4)					
	S		I		R	
	n	%	n	%	n	%
Amoxicillin+clavulanic acid (30 µg)	4	100	-	0	-	0
Enrofloxacin (5 µg)	3	75	1	25	-	0
Cephalothin (30 µg)	4	100	-	0	-	0
Rifampicin (5 µg)	3	75	-	0	1	25
Gentamicin (10 µg)	3	75	-	0	1	25
Erythromycin (5 µg)	1	25	1	25	2	50
Tetracycline (30 µg)	3	75	-	0	1	25
Trimethoprim/sulfamethoxazole (25 µg)	3	75	-	0	1	25
Oxacillin (1 µg)	-	0	-	0	4	100
Cefoxitin (30 µg)	-	0	-	0	4	100

S: Sensitive; I: Intermediate; R: Resistant.

DISCUSSION

The present study investigated the presence of *mecA* and *pvl* genes in *Staphylococcus* spp. isolated from bovine mastitic milk samples by PCR and the antibiotic resistance of methicillin resistant strains to antibiotics commonly used in veterinary field.

Staphylococci are the most common bacterial agents isolated from bovine with mastitis. However, the distribution of species obtained from the studies related to the etiology of Staphylococcal mastitis have shown the variation [14,24,36]. Vishnupriya *et al*. [36] reported that 22 and 74 of 96 *Staphylococcus* spp. isolated from 158 CMT positive milk samples were CPS and CNS, respectively. In the same study, the most frequently isolated species were found to be *S. aureus* (n = 20) and *Staphylococcus epidermidis* (n = 17). In a study, it was reported that 113 (71.5%) *Staphylococcus* spp. were isolated from 158 CMT positive milk samples, of these 5 and 108 were found to be *S. aureus* and CNS, respectively. It was also emphasized *Staphylococcus haemolyticus* and *Staphylococcus chromogenes* were the most frequently isolated species among the CNS [14]. Pehlivanoğlu & Yardımcı [24] from Turkey reported the highest isolation rate belonged to *S. aureus* (n = 65) and *S. intermedius* (n = 17) among the 100 *Staphylococcus* strains isolated from CMT positive half-udder milk samples. In our study, of 182 *Staphylococcus* isolates obtained from 972 mammary quarter milk samples, 137 (75.27%) were determined to be CPS and 45 (24.73%) to be CNS. Among the 11 different *Staphylococcus* species, *S. intermedius* (n = 77; 42.30%) was the most common species isolated, followed by *S. aureus* (n = 60; 32.97%) and *S. saprophyticus* (n = 20; 10.99%) (Table 1). Compared with the other investigation results [14,24,36], while the similarity in terms of isolated species was found, the differences were detected in the isolation rates of species. In the present study, the high isolation rate of *S. intermedius* was remarkable for us. Taponen [31] has emphasized that in mastitis diagnostics all CPS isolates are usually classified as *S. aureus* and all other isolates as CNS. In our study, the identification of all CPS was achieved by using identification kit. This certain identification may explain the high isolation rate of *S. intermedius*. Unlike other studies, we also made the bacterial isolation from both CMT positive and CMT negative milk samples. It was considered this sampling method may be the reason of higher isolation rate for *S. intermedius* in accordance with the most common ones causing mastitis. In the present study, *S. saprophyticus* was the predominant CNS species and this was another remarkable finding of our study. Taponen [31] has reported that *S. saprophyticus* may be found in the cows' environment. However, some researchers emphasized that the increase was determined in the isolation rate of this agent from animals with mastitis [3,37].

The California mastitis test has been accepted as a quick and simple screening test to predict somatic cell count from individual quarters or composite milk. However, microbiological growth has been shown in CMT negative mammary quarters as well as CMT positive ones [2]. Bhutto *et al.* [2] detected the bacteriological growth in the 57.6% of CMT negative half-udder milk samples, while bacteriological isolation from CMT negative milk samples was found to be 30.1% in another study [1]. We collected 972 half-udder milk samples, of which was 757 CMT positive and 215 CMT negative. The isolation rate of Staphylococcal agents from CMT negative half-udder milk samples was found to be 32.97%, while this rate was 16.11% in CMT positive ones (Table 2). According to some authors, the sensitivity and specificity of CMT are unsteady and also sensitivity of CMT may be low for different pathogens [26,27].

Methicillin resistance is mediated by the *mecA* gene located on the chromosome of MRS [25]. Various researchers have reported the different *mecA* prevalence in Staphylococci, especially *S. aureus*, isolated from bovine mastitic milk samples [5,7,8,19,22,24]. In a study from Germany, 15 (12.39%) of 121 CNS isolates were determined to be *mecA* positive [8]. Moon *et al*. [22] found the *mecA* positivity in 13 and 12 of 835 *S. aureus* and 763 CNS strains, respectively, while the *mecA* positivity was found to be 9.2% (n = 15) in 163 *S. aureus* strains in another study [19]. Similarly, the investigation results related to the presence of *mecA* in Staphylococci are variable in Turkey. In a study, methicillin resistance was found in 20 of 100 *Staphylococcus* strains [24], while Erdem & Türkyılmaz [7] determined *mecA* positivity in only two of *S. aureus* strains isolated from 145 mastitic milk samples. In another investigation, only 4 (6.7%) of 59 *S. aureus* isolates were found to be MRSA [5]. In our study, 16S rDNA specific bands were detected in all of 182 *Staphylococcus* isolates obtained from half-udder milk samples, 4 (2.2%) of these harboured the *mecA* gene. All of *mecA* positive strains were *S. intermedius* and this result was remarkable for us. When the comprehensive literature investigation was made, the report on MR *S. intermedius* was seen in only one research from Korea. In this study, Moon *et al*. [22] reported that only one of 20 MRS strains was *S. intermedius*. In Turkey, the investigation finding related to MR *S. intermedius* was not determined. It was considered that our different result may be associated with certain identification of *S. intermedius*, while CPS isolates are usually classified as *S. aureus* in several reports [31]. The *mecA* positivity found in our study was lower than the prevalence of *mecA* gene reported by other authors. The difference between the sample size, geographical variations and diversity in strains may be the causes of this discrepancy. It was also considered the specific and controlled antibiotic usage for the treatment of mastitis in sampling area may be effective on this result. When we conferred with veterinarians working in sampling area, they emphasized that the suitable, controlled and synergical effective antibiotics are usually used according to antibiotic susceptibility test results in Ödemiş.

One of the several virulence factors that are produced by *S. aureus* is PVL and may contribute to its pathogenicity. Generally, PVL toxin causing pneumonia and necrotizing dermatitis are more widely in human population [17,18]. The *pvl* gene encoding this toxin has been reported in 0-56% of *S. aureus* strains isolated from animals with mastitis [15,33,34,38]. Zecconi *et al*. [38] determined the *pvl* gene positivity as 56% in *S. aureus* strains. In another study, the *pvl* gene was found in none of 76 *S. aureus* strains isolated bovine mastitic milk samples [15]. In Turkey, Ünal [34] reported the prevalence of *pvl* toxin gene to be 6.6% in *S. aureus* isolates, while this gene was determined in none of 16 MRSA strains by Türkyılmaz *et al*. [33]. We investigated *pvl* toxin gene in 182 *Staphylococcus* strains by PCR and found this gene in none of strains. According to this finding, it was considered that *pvl* gene may have not an efficient role in the pathogenesis of mastitis in terms of sampled animals and sampling area.

Methicillin resistant Staphylococci species have been recognized to be resistant to antimicrobials both all β-lactams and other than β-lactams are widely used in both human and veterinary medicine. Various researchers have emphasized that this problem causes an increase in the risk of treatment failure and cost for antimicrobial therapy and hospitalization, while the range of therapeutic options decrease [20,25]. In several studies related to the antibiotic resistance in MRSA strains isolated from animals with mastitis, the multiple antibiotic resistance have usually emphasized in strains [16,33,35]. We investigated the antibiotic resistance of 4 MR *S. intermedius* strains against various antibiotics commonly used in Turkey. All of MR *S. intermedius* strains were resistant to oxacillin and cefoxitin, while

they were sensitive to amoxicillin+clavulanic acid and cephalothin. Also, the resistance to erythromycin (50%), rifampicin (25%), gentamicin (25%), tetracycline (25%) and trimethoprim/sulfamethoxazole (25%) was found in the *mecA* positive *S. intermedius* strains. Similarly other author's reports, of methicillin resistant strains, 2, 1 and 1 were resistant to 3, 6 and 5 antibiotics, respectively. We considered that geographical differences, number of tested isolates and diversity in MR strains may be effective on the antibiotic resistance levels.

CONCLUSION

In the present study, the presence of *mecA* gene in *S. intermedius* strains isolated from bovine quarter milk samples was reported for the first time in Turkey. It was shown that *S. intermedius* may have an efficient role in the etiology of bovine mastitis, as well as *S. aureus*. This result showed the importance of certain bacterial identification in the mastitis diagnostic. In our study, the *mecA* positivity was lower than the prevalence of *mecA* gene reported by other authors. Nevertheless, there is a need for awareness, appropriate intervention and control measures because of the zoonosis or humanosis importance of methicillin resistant strains. Also, specific and synergical effective antibiotics should be used for the treatment of mastitis to prevent an increasing resistance problem to antimicrobials all over the world.

MANUFACTURERS

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***Funding*.** This study was financially supported by the Afyon Kocatepe University Scientific Research Projects Coordination Unit (Grand number 14.SAG.BIL.03).

***Declaration of Interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content of the paper.

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