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Original Article
Özgün Araştırma

Investigation of *mecA*, *vanA* and *pvl* genes in *Staphylococcus aureus* strains isolated from bovine mastitis in smallholder dairy farms

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Abstract: This study aimed to isolate the *Staphylococcus aureus* from bovine mastitis, investigate the presence of *mecA, vanA,* and *pvl* genes in isolated strains, and determine the resistance of *S. aureus* strains to some antibiotics commonly used in the veterinary field. In the study, 602 mammary quarter milk samples from 170 lactating cows in 40 different small-scale dairy farms in the central towns and villages of Afyonkarahisar were used. After CMT scoring, milk samples were collected aseptically from each mammary quarter. In the study, a total of 23 (3.8%) *S. aureus* strains were isolated from 602 mammary quarter milk samples by using a commercial identification kit. The *mecA*, *vanA*, and *pvl* genes were not detected in any of the strains. The phenotypic resistance of all *S. aureus* strains to 12 antibiotics was determined by using the Kirby-Bauer disc diffusion method. The highest resistance in strains was against penicillin G (52.2%), followed by oxacillin (21.7%), erythromycin (21.7%), and amoxicillin+clavulanic acid (17.4%). In conclusion, it was noted that *S. aureus* is not a prevalent pathogen in the etiology of bovine mastitis for small-scale farms where sampling was done.

Keywords: Mastitis, methicillin resistance, Panton-Valentine leukocidin, *S. aureus,* vancomycin resistance

Küçük ölçekli süt işletmelerinde sığır mastitislerinden izole edilen *Staphylococcus aureus* suşlarında *mecA*, *vanA* ve *pvl* genlerinin araştırılması

Özet: Bu çalışmada sığır mastitislerinden *Staphylococcus aureus*'un izolasyonu, izole edilen suşlarda *mecA, vanA* ve *pvl* genlerinin varlığının araştırılması ve *S. aureus* suşlarının veteriner sahada yaygın olarak kullanılan bazı antibiyotiklere dirençliliklerinin belirlenmesi amaçlandı. Çalışmada, Afyonkarahisar'ın merkez kasaba ve köylerindeki 40 farklı küçük ölçekli süt işletmesinde bulunan 170 laktasyondaki ineğe ait 602 meme lobu süt örneği kullanıldı. CMT skorlamasının ardından, her bir meme lobundan aseptik olarak süt örnekleri toplandı. Çalışmada, ticari identifikasyon kiti kullanılarak 602 meme lobu süt örneğinden toplam 23 (%3,8) *S. aureus* suşu izole edildi. Suşların hiçbirinde *mecA*, *vanA* ve *pvl* genleri belirlenmedi. Tüm *S. aureus* suşlarının 12 antibiyotiğe karşı fenotipik dirençlilikleri Kirby-Bauer disk difüzyon yöntemi kullanılarak belirlendi. Suşlardaki en yüksek direnç oranı penisilin G'ye (%52,2) karşı iken, bunu oksasilin (%21,7), eritromisin (%21,7) ve amoksisilin+klavulanik aside (%17,4) karşı direnç oranları izledi. Sonuç olarak, *S. aureus*'un örneklemenin yapıldığı küçük ölçekli işletmeler için sığır mastitislerinin etiyolojisinde yaygın bir patojen olmadığı belirlendi.

Anahtar kelimeler: Mastitis, metisilin direnci, Panton-Valentine lökosidin, *S. aureus*, vankomisin direnci

Introduction

Mastitis is a multifactorial disease that affects the quality and quantity of milk and causes great economic losses in the dairy industry. Although many microorganisms are effective in the etiology of mastitis, *Staphylococcus aureus* (*S. aureus*) has been reported to be a common agent among bacterial mastitis pathogens by several researchers (Tenhagen et al. 2006; Igbinosa et al. 2016; Guimarães et al. 2017; Shrestha et al. 2021). Beta-lactam group antibiotics are commonly preferred in the treatment and prevention of mastitis. Methicillin resistance in *S. aureus* is based on the synthesis of penicillin-binding protein 2a (PBP2a), a new penicillin-binding protein (PBP) with a low affinity for β-lactam group antibiotics. This resistance encoded by the *mecA* gene is considered an indicator of general resistance to β-lactams (Igbinosa et al. 2016; Foster 2017; Kakoullis et al. 2021). Vancomycin is a glycopeptide antibiotic frequently preferred for the treatment of methicillin-resistant *S. aureus* (MRSA) infections in humans. After the first MRSA strain with reduced susceptibility to vancomycin was reported from Japan in 1997 (Hiramatsu et al. 1997), vancomycin-re-

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sistant *S. aureus* (VRSA) strains were reported from hospitalized patients in many countries (Sievert et al. 2008; Hasan et al. 2016; Cong et al. 2020; Wu et al. 2021). However, the reports available on the detection of VRSA by using molecular methods in *S. aureus* strains isolated from animals, particularly animals with mastitis, are limited (Pehlivanoğlu and Yardımcı 2012; Bhattacharyya et al. 2016).

In various studies on the epidemiology and pathogenesis of MRSA infections, it is emphasized that Panton-Valentine leukocidin (PVL) toxin encoded by the *lukS*-PV and *lukF*-PV genes is a potential virulence factor for community-acquired methicillin-resistant *S. aureus* (CA-MRSA) infections (Lina et al. 1999; Papanikolaou et al. 2018; Darboe et al. 2019; Amin et al. 2020; Dülger et al. 2020). Recently, with the increase in the isolation of MRSA strains from animals, an increase in studies investigating the presence of PVL toxin in these strains isolated from animals is also noteworthy (Pajić et al. 2014; Gezgen and Seker 2016; Hoque et al. 2018; Algammal et al. 2020).

Although studies investigating the prevalence of *mecA* and partially *pvl* genes in mastitis-causing *S. aureus* strains from different countries have been reported, investigations that genotypically determine vancomycin-resistance in these strains are limited. We aimed to isolate the *S. aureus* from bovine mastitis in smallholder dairy farms, investigate the presence of *mecA*, *vanA* and, *pvl* genes in isolated species and determine the antibiotic resistance of strains to some antibiotics commonly used in the veterinary field.

Materials and Methods

Milk samples and S. aureus identification: A total of 602 mammary quarter milk samples were collected from 170 lactating cows on 40 different smallholder dairy farms located in the central towns and villages of Afyonkarahisar. No antibiotics had been applied to the animals in the previous three months. Before the sampling, the California mastitis test (CMT) was applied for each mammary quarter according to the method described by Schalm et al. (1971). Afterward, the teat ends were cleaned with 70% alcohol, dried and the first streams of foremilk were discharged. Ten milliliters of milk were collected aseptically from each quarter into sterile tubes and immediately transported to the laboratory in a cool box on ice. Ten µL 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 intramammary infection levels for milk samples (≥500 cfu/mL) were determined according to the method of the National Mastitis Council (Hogan 1999). After each different colony was examined macroscopically (colony morphology, hemolysis, pigment production) and microscopically (Gram staining), and then oxidase, catalase, slide and tube coagulase, and anaerobic fermentation of glucose and mannitol tests were performed for suspected colonies (Holt et al. 2000). The certain identification of isolates was achieved using Crystal™ Identification Systems Gram-Positive ID kit (Becton, Dickinson and Company, NJ, USA). In all tests, methicillin-resistant *S. aureus* (MRSA) ATCC® 33591 and methicillin-sensitive *S. aureus* (MSSA) ATCC® 25923 were used as control strains (Oxoid Microbiology Products, Hampshire, UK).

Detection of 16S rDNA, mecA, vanA, and pvl genes in S. aureus strains by PCR: MRSA ATCC® 33591, *S. aureus* ATCC 49775 (*pvl* gene positive), *E. faecium* ATCC 51559 (*vanA* gene positive), and MSSA ATCC® 25923 were used as control strains (Oxoid Microbiology Products, Hampshire, UK) for PCR analysis. DNA extractions of all control strains except for *E. faecium* ATCC 51559 and test strains were performed using the boiling method (Gezgen and Seker 2016). DNA was extracted from *E. faecium* ATCC 51559 using a genomic DNA purification kit as described by the manufacturer (Thermo Scientific, Lithuania). For the detection of 16S rDNA, *mecA*, *vanA*, and *pvl* genes, the primers described by Strommenger et al. (2003), Choi et al. (2003), Dutka-Malen et al. (1995), and Lina et al. (1999) were used, respectively. While the PCR protocols previously described by Gezgen and Seker (2016) for 16S rDNA, *mecA*, and *pvl* genes were used, the protocol recommended by Dutka-Malen et al. (1995) for the *vanA* gene was applied.

Antibiotic Susceptibility Test: The antibiotic resistances of all strains determined to be *S. aureus* for 12 antimicrobial agents were tested using the Kirby-Bauer disc diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2007, 2012, 2013, 2016, 2017). The tested antibiotics were amoxicillin/clavulanic acid (30µg), enrofloxacin (5µg), cefoxitin (30µg), oxacillin (1µg), cephalothin (30µg), rifampicin (5µg), gentamicin (10µg), erythromycin (15µg), penicillin G (10U), tetracycline (30µg), trimethoprim/sulfamethoxazole (25µg), and vancomycin (30µg). Positive (MRSA ATCC® 33591) and negative (MSSA ATCC® 25923) control strains were used for all applications.

Results

Isolation and identification findings: A total of 23 (3.8%) *S. aureus* strains were identified from 602 mammary quarter milk samples (355 CMT positive, 181 CMT negative, and 66 CMT suspected) belong to 170 cows by using Crystal™ Identification Systems Gram-Positive ID kit. The distribution of *S. aureus* strains according to CMT scores is shown in Table 1.

Detection of 16S rDNA, mecA, vanA, and pvl genes: According to PCR results, while all of the 23 *S. aureus* strains identified by commercial identification kit harbored the 16S rDNA gene, none of the strains carried the *mecA* gene (Figure 1). The *vanA* and *pvl* genes were also determined in none of the strains.

Table 1. The distribution of *S. aureus* strains according to CMT scores.

CMT Score (n)	*S. aureus* (n=23)	
	n	%
+1 (163)	3	1.8
+2 (146)	13	8.9
+3 (46)	4	8.7
Negative (181)	3	1.6
Suspected (66)	-	-

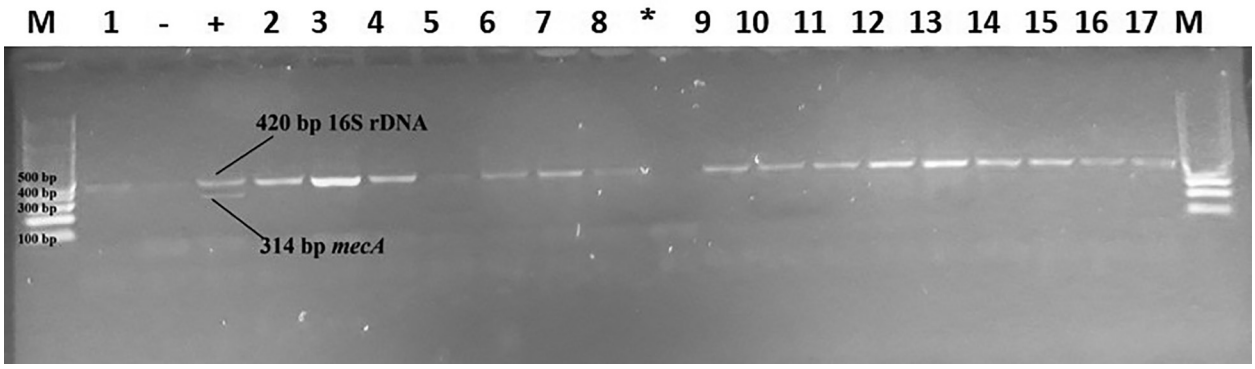

Figure 1. PCR findings. M: DNA ladder (100 bp); -: *mecA* negative control strain (MSSA ATCC® 25923); +: *mecA* positive control strain (MRSA ATCC® 33591); *: sterile distilled water; lanes 1-17: 16S rDNA positive, *mecA* negative *S. aureus* test strains

Antibiotic susceptibility test findings: In tested strains, the highest resistance rate was against penicillin G (52.2%; n=12), followed by oxacillin (21.7%; n=5), erythromycin (21.7%; n=5), and amoxicillin/clavulanic acid (17.4%; n=4). It was determined that the isolates showed a sensitivity of 95.7% against enrofloxacin and gentamicin (Table 2).

Table 2. The antibiotic resistances of *S. aureus* strains.

Antibiotic	*S. aureus* (n=23)					
	R		I		S	
	n	%	n	%	n	%
Amoxicillin/clavulanic acid (30µg)	4	17.4	-	0	19	82.6
Enrofloxacin (5µg)	1	4.3	-	0	22	95.7
Cephalothin (30µg)	3	13.0	1	4.3	19	82.7
Rifampicin (5µg)	3	13.0	-	0	20	87.0
Gentamicin (10µg)	1	4.3	-	0	22	95.7
Erythromycin (5µg)	5	21.7	12	52.2	6	26.1
Penicillin G (10U)	12	52.2	-	0	11	47.8
Tetracycline (30µg)	2	8.7	1	4.3	20	87.0
Trimethoprim/sulfamethoxazole (25µg)	3	13.0	-	0	20	87.0
Vancomycin (30µg)	3	13.0	-	0	20	87.0
Oxacillin (1µg)	5	21.7	-	0	18	78.3
Cefoxitin (30µg)	2	8.7	2	8.7	19	82.6

R: Resistant; I: Intermediate; S: Susceptible

Discussion and Conclusion

The present study investigated the role of *S. aureus* on bovine mastitis in small-scale dairy farms and the presence of *mecA*, *vanA*, and *pvl* genes in the isolated strains.

The importance of *S. aureus* has been emphasized to be the most common pathogen in the etiology of mastitis caused by *Staphylococcus* species by several researchers, but the isolation data of this agent obtained from CMT positive bovine mastitic milk samples have shown a wide variation (between 2.6% and 52.2%) according to countries (Gezgen and Seker 2016; Igbinosa et al. 2016; Guimarães et al. 2017; Hoque et al. 2018; Algammal et al. 2020; Balemi et al. 2021; Shrestha et al. 2021). In our study, a total of 23 (3.8%) *S. aureus* strains were isolated from 602 mammary quarter milk samples belonging to 170 cows. Compared with the other researcher's results, the low isolation rate of *S. aureus* was remarkable for us. It was thought that environmental and management conditions, dairy farm type, the number of samples, isolation methods, and geographical variations may be effective on this discrepancy. Also, when we conferred with the veterinarians working in the sampling area and animal owners, they emphasized that mastitis vaccines for *S. aureus* have been included in the field applications for a long time. It was concluded that this application may also explain the low isolation rate obtained in this study. The CMT is known as an economical test that allows the approximate estimation of the somatic cell count (SCC) in milk for the diagnosis of subclinical mastitis. Although it is accepted as a general opinion that animals with a positive reaction to CMT should be approached with suspicion, it is emphasized that CMT negative animals should not be considered free from mastitis agents (Bhutto et al. 2012; Gezgen and Seker 2016). In the present study, unlike other studies, we isolated the *S. aureus* from both CMT positive (5.6%) and CMT negative (1.6%) milk samples. However, the isolation rate of *S. aureus* from CMT positive and negative mammary quarters was lower than the isolation rates reported in similar studies. The fact that this study focused only on *S. aureus* isolation, while other studies included both *S. aureus* and other Staphylococci, may be effective on this difference. In addition, the low number of strains obtained from our study may be another reason affecting this difference.

In various studies, the prevalence of *mecA* gene in *S. aureus* strains isolated from cows with mastitis has been reported to be between 0% and % 47 (Pu et al. 2014; Wang et al. 2015; Gezgen and Seker 2016; Hoque et al. 2018; Algammal et al. 2020; Shrestha et al. 2021). In the present study, the *mecA* gene was determined in none of the 23 *S. aureus* strains. It was considered this result may be associated with the low number of isolated strains in our study. In this study, phenotypic resistance to methicillin was also investigated by Kirby-Bauer disc diffusion test using oxacillin and cefoxitin antibiotic discs. The resistance to oxacillin and cefoxitin was found to be 21.7% (n=5) and 8.7% (n=2), respectively. However, it was remarkable that no *mecA* gene was found in any of these phenotypically resistant strains. This finding supported the view that phenotypic resistance alone is not sufficient to determine methicillin resistance and the phenotypically methicillin-resistant strains should be also confirmed genotypically for the *mecA* gene (Wang et al. 2015).

While studies on the prevalence of VRSA in humans are widespread, investigations on the detection of vancomycin resistance genes by using molecular methods in *S. aureus* strains isolated from animals, particularly animals with mastitis, are limited (Pehlivanoğlu and Yardımcı 2012; Bhattacharyya et al. 2016). Bhattacharyya et al. (2016) found phenotypic resistance to vancomycin in seven of 274 *S. aureus* strains isolated from the milk of sheep and cows with mastitis in West Bengal, India, but the *vanA* resistance gene was detected in none of the strains. Pehlivanoğlu and Yardımcı (2012) from Turkey reported the *vanA* gene was not found in any of the 65 *S. aureus* strains isolated from bovine mastitic milk samples. Similarly, in our study, while 23 *S. aureus* strains were phenotypically resistant to vancomycin, none of these strains harbored the *vanA* gene. As suggested in the current resistance criteria of CLSI (2017), it was thought that phenotypic resistance determined in strains may not always be evidence of the presence of vancomycin resistance in the strain, and therefore, phenotypic resistance should be confirmed by the presence of vancomycin resistance genes.

The prevalence of the *pvl* gene encoding the Panton-Valentine leukocidin toxin has been reported to vary between 0% and 56% in *S. aureus* isolates isolated from cows with mastitis (Zecconi et al. 2006; Türkyılmaz et al. 2010; Pajić et al. 2014; Gezgen and Seker 2016; Hoque et al. 2018; Algammal et al. 2020). Similar to findings of researchers from Turkey (Türkyılmaz et al. 2010; Gezgen and Seker 2016), none of 23 *S. aureus* strains harbored the *pvl* gene in the present study. Some authors emphasized the presence of *pvl* toxin gene are generally related to MRSA strains and this gene is more

prevalent in human clinical *S. aureus* strains (Lina et al. 1999; Darboe et al. 2019; Amin et al. 2020). This result obtained in our study may be related to the low number of isolated strains and the absence of *mecA* gene in the strains. In addition, it can be thought that PVL may not play an active role in the pathogenesis of mastitis in terms of the sampling area and sampled animals in this study.

Bacterial mastitis is among the diseases in which antibiotics are most frequently used in the veterinary field. Therefore, studies investigating the antibiotic resistance profiles of *S. aureus* isolated from the milk of animals with mastitis are quite common. Although antibiotic resistance rates obtained from these studies have shown differences, it is reported the most common phenotypic resistance profiles in the strains are against penicillin G (Tel et al. 2012; Szweda et al. 2014; Wang et al. 2015), ampicillin (Szweda et al. 2014; Shrestha et al. 2021), trimethoprim+sulfamethoxazole (Wang et al., 2015), erythromycin (Wang et al., 2015; Shrestha et al. 2021), clindamycin (Tel et al. 2012; Wang et al. 2015), and gentamicin (Wang et al. 2015; Elias et al. 2020; Shrestha et al. 2021). We investigated the antibiotic resistance of 23 *S. aureus* strains using the Kirby-Bauer disc diffusion test. According to test results, the highest resistance rate was against penicillin G (52.2%; n=12), followed by erythromycin (21.7%; n=5), oxacillin (21.7%; n=5), and amoxicillin/clavulanic acid (17.4%; n=4) (Table 2). However, the *mecA* gene was not determined in the strains showing phenotypic resistance to oxacillin and cefoxitin. When compared with the data of other researchers, it was thought that the discrepancy in resistance rates may be related to the number of strains tested, differences in the origin of the strains, and regional differences in the preference of antibiotics used in the treatment of mastitis.

As a result, it was noted that *S. aureus* was not a dominant pathogen in the etiology of bovine mastitis for the small-scale dairy farms sampled in Afyonkarahisar. In the emergence of this result, it was thought that the inclusion of mastitis vaccines against *S. aureus* among ruminant vaccinations in recent years may be effective. In the present study, the isolation of *S. aureus* was achieved from both CMT positive and CMT negative quarter milk samples. In this context, it was thought that performing microbiological identification as well as CMT applications in the diagnosis of subclinical mastitis cases may give more accurate results. Considering that there is a positive relationship between the use of non-specific antibiotics and the increase in resistance in bacteria, it is important to select antibiotics according to the agent identification and antibiotic sensitivity test results. However, it should not be ignored that phenotypic resistance alone is not sufficient to determine resistance to methicillin and vancomycin.

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Conflict of Interest: The authors declared that there is no conflict of interest.

Ethical Statement: This study is not subject to the permission of HADYEK in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

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