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Research Article

METHICILLIN AND VANCOMYCIN RESISTANCE PROFILES OF *STAHPYLOCOCCUS AUREUS* ISOLATED FROM BOVINE COWS MASTITIC MILK SAMPLES

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ABSTRACT. *S. aureus* may cause public health problems due to its contamination with animal products such as milk and raw milk products, called Milk-borne Disease (MBD). In this study, it was aimed to determine the prevalence of *S. aureus* and the presence of methicillin and vancomycin resistance in *S. aureus* isolates in dairy cows' milk samples in different region of Balıkesir province, which is have high cow milk production in Türkiye. One hundred seven cow milk samples collected from various private dairy farms by veterinarians and sent to the laboratory for microbiological examination for mastitis infection in Balıkesir province between September 2021 and May 2022 were examined in this study. For the isolation and identification of *S. aureus*, milk samples were first shaken slowly for homogenization and then inoculated to and were incubated at 37°C for up to 48 hours. Methicillin and vancomycin resistance profiles of *S. aureus* (n:15) isolates was investigated and evaluated phenotypically according to European Committee of Antimicrobial Susceptability Testing (EUCAST) standards. Methicillin resistance in *S. aureus* isolates was also investigated by PCR in terms of *mecA* and *mecC* genes. Methicillin resistance was determined phenotypically in 5 of 15 (33.3%) *S. aureus* isolates by disc diffusion test according to EUCAST procedure, while Vancomycin resistance was not detected in all isolated *S. aureus* strains by MIC E-test. By PCR method, *mecC* gene was detected in all MRSA strains (n:5), while *mecA* gene was not detected. It was thought that the isolates determined to be MRSA phenotypically should also be analyzed in terms of the *mecC* gene and the detection of the *mecC* gene in all MRSA isolates emphasized the importance of this situation.

Keywords: *Cow, mastitis, MRSA, PCR, VRSA.*

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is one of the important bacteria, which causes of subclinical, clinical and chronic infectious bovine mastitis all over the world and serious economic losses in dairy farms due to its effect on milk production, milk quality and bulk tank somatic cell count (SCC) [1-5]. *S. aureus* may also cause public health problems due to its contamination with animal products such as milk and raw milk products, called Milk-borne Disease (MBD) [6-9]. Methicillin resistant *Staphylococcus aureus* (MRSA) causes nasocomial infections, hospital-acquired illness, endocarditis and haemolytic pneumonia in humans [3,10]. Control of *S. aureus* infections is difficult due to its multi-drug resistance, which facilitates *S. aureus* infiltration into the host's immune system [4].

Moreover, the World Health Organization (WHO) has listed MRSA as a "priority pathogen" [11].

MRSA exhibits resistance against beta-lactam antibiotics [7,12,13] andarises from bacterial acquisition of a mobile genetic component known as Staphylococcal Cassette Chromosomal mec (SCCmec). This element harbors the *mecA* and *mecC* genes, encoding low-affinity penicillin-binding protein 2a (PBP2a), thereby imparting resistance to beta-lactam antibiotics [7,9,12,14,15].

The growing emergence and dissemination of multidrug-resistant zoonotic pathogens have raised significant alarms regarding the extensive utilization of antimicrobial agents. Bacteria resistant to antibiotics fail to react to standard antibiotic therapy, leading to prolonged illness durations. The development of resistance in *S. aureus* against antimicrobial agents is anticipated to complicate infection treatment further [16].

MRSA are divided into three groups according to their SCCmec type: hospital acquired (HA-MRSA), community acquired (CA-MRSA) and livestock acquired (LA-MRSA) [12,14]. The European Food Safety Authority (EFSA) has reported the role of animal origin in food as possible sources of MRSA [14]. The high rate of MRSA contamination on dairy farms is reported to be a result of the excessive and ineffective use of antibiotics in the treatment of animals. It has been reported that the spread of these bacteria can be caused not only by sanitation management during milking, but also by milk expressed from the udder or by farmers' hands during the milking process [7].

In the 1990s, it was suggested that the sensitivity of *S. aureus* to vancomycin had changed. In May 1996, the first infection with Vancomycin-Intermediate *S. aureus* (VISA) was reported in a patient in Japan and also, Vancomycin-Resistant *S. aureus* (VRSA; n: 14) infections were reported in patients in the United States in May 2015 [17,18]. Researchers reported that the *van*A vancomycin resistance gene, which is found in vancomycin-resistant enterococci (VRE), is present in all VRSAs. VRSA is thought to arise from specific precursor organisms, such as MRSA, which contains a pSK41-type plasmid, and VRE, which contains *vanA* encoded on a plasmid such as Inc18 [17,19].

Recently, European Committee of Antimicrobial Susceptability Testing (EUCAST) was identified the methods of methicillin and vancomycin resistance. This methods including disc diffusion, MIC Gradient E-tests and polymerase chain reaction as genotypic method [20-25].

The objective of this research was to assess the frequency of *S. aureus* occurrence and investigate the occurrence of methicillin and vancomycin resistance by disc diffusion, E-test and polymerase chain reaction (PCR) that isolated from dairy cow milk samples in different regions of Balıkesir province, a prominent region for cow milk production in Türkiye.

MATERIALS AND METHODS

Sampling

In the study, 107 cow milk samples collected from various private dairy farms, which has at least five milking cow, by veterinarians and sent to the university laboratory for microbiological examination for mastitis infection in different region of Balıkesir province between September 2021 and May 2022. Sampled cows udder's were showed at least one of the symptoms of redness, swelling, tenderness and pain. Milk samples (5-10 ml) were taken in sterile sample containers after teat dipping and the first milk was

discarded to free cup to avoid contamination in accordance with microbiological examination. The samples were transported to the laboratory by the veterinarians who took the samples while maintaining cold chain conditions. Samples that would not be analyzed immediately were frozen and stored at (-20°C) [26].

Isolation and Identification

Milk samples underwent gentle shaking for homogenization before being introduced onto Rabbit Plasma Fibrinogen-Baired-Parker (RPF-BP) Agar (Oxoid-UK). The plates were placed in an incubator set at 37°C for up to 48 hours [27].

Gram staining, catalase, and microscopic and macroscopic morphology on RPF-BP Agar were done and evaluated on the colonies that grew after incubation and isolates were identified as [26,27]. The isolates determined as *S. aureus* were put the beads and were stored in cryotubes at (-20°C).

Antibiotic Susceptibility Tests

Methicillin and vancomycin resistance profiles of *S. aureus* isolates was investigated and evaluated phenotypically according to EUCAST standards [20,21]. The isolated *S. aureus* strains were grown as pure colonies on RPF-BP agar (Oxoid, UK) in 37°C for 24 hours. Inoculum was prepared and applied from pure *S. aureus* colonies according to EUCAST procedure [20]. Methicillin resistance was investigated by disc diffusion method using cefoxitin (30 μg disk, Oxoid, UK). Vancomycin resistance was investigated by E-test using Vancomycin strips (Liofilchem, Italy) and Brian-Hearth Infusion agar (Merck, Germany). *S. aureus* strains with <22 mm zone diameter was recorded as Methicillin-resistant and >MIC 2 value was recorded as Vancomycin-resistant [20,21].

Genotypic Identification of Methicillin-Resistance

For to investigate Methicillin-resistance by PCR, DNA was purified from the 15 *S. aureus* isolates with DNA extraction kit (GeneJET Genomic DNA Purification kit MAN0012663, purify genomic DNA from gram-positive bacteria procedure, Thermo, USA) and using lysis buffer according to manufacturer procedure.

Methicillin resistance genes, *mecA* and *mecC*, were investigated by PCR method by used previously described primers and amplification conditions (Table 1) [22-25]. For *mecA* and *mecC* genes, PCR mix was conducted with a total volume of 50 μl for each genes using with 30 μl Taq polymerase Master Mix (Ampliqon, Denmark), 0.4 μl forward and 0.4 μl reverse primers, 17.2 μl PCR water (DNAase and RNAase free water) and contained 2 μl of DNA [22-25].

For *mecA* gene the amplification process began with an initial denaturation step at 94°C lasting 5 minutes. This was followed by 35 cycles of amplification: denaturation occurred at 94°C for 2 minutes, annealing took place at 57°C for 2 minutes, and extension occurred at 72°C for 1 minute each cycle. The process concluded with a final extension step at 72°C for 7 minutes [25].

The *mecC* gene was amplified under the following conditions: initial denaturation at 94°C for 15 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 59°C for 1 minute, and extension at 72°C for 1 minute, with a final elongation step at 72°C for 10 minutes [24].

PCR products were subjected to electrophoresis on a 1.5% agarose gel (Prona, USA) containing Novel juice dye (Thermo Scientific, USA) and a DNA molecular weight

marker (Gene Ruler 100bp DNA Ladder plus, Thermo Scientific, USA). Gel imaging was performed using the EBOX CX5 TS EDGE system from Vilber.

MRSA NCTC 12493 (*mecA*) and MRSA NCTC 13552 (*mecC*) were used as a reference strains, which was obtained from Ministry of Health, General Directorate of Public Health, Microbiology Reference Laboratory. DNA free PCR mix was used as negative control in PCR.

Table 1. *Primer sequences, target genes and references for mecA and mecC genes.*

Primers	Sequences	Target genes	Base pairs	References
GMECAR-1	5'-ACTGCTATCCACCCTCAAAC-3'	*mecA*	163	Mehrotra M. *et al.* [25]
GMECAR-2	5'-CTGGTGAAGTTGTAATCTGG-3'			
Primer-F	5′ -GAA AAA AAG GCT TAG AAC GCC TC-3′	*mecC*	138	Garcı´a-Alvarez *et al.* [22] Garcı´a-Garrote F. *et al.* [23] Doğan et al. [24]
Primer-R	5′ GAA GAT CTT TTC CGT TTT CAG C-3			

RESULTS AND DISCUSSION

A total of 15 (14.01%) *S. aureus* isolated and identified from 107 cow milk samples (Fig. 1). Methicillin resistance was determined phenotypically in 5 of 15 (33.3%) *S. aureus* isolates by disc diffusion test according to EUCAST procedure, while Vancomycin resistance was not detected in all isolated *S. aureus* strains by MIC E-test (Fig. 2, Fig. 3).

By PCR method, *mecC* gene was detected in all MRSA strains (n:5; 100%) (Fig. 4), while *mecA* gene was not detected.

Table 2. *Isolation and PCR results of milk samples.*

Isolated *S. aureus* strains from cow milks (from 107 milk samples)	Phenotypic MRSA positive *S. aureus* strains	Phenotypic (E-test) Vancomycin resistant *S. aureus* strains	*mecA* gene positive *S. aureus* strains	*mecC* gene positive *S. aureus* strains
15 (14.01%)	5 (33.3%)	-	-	5

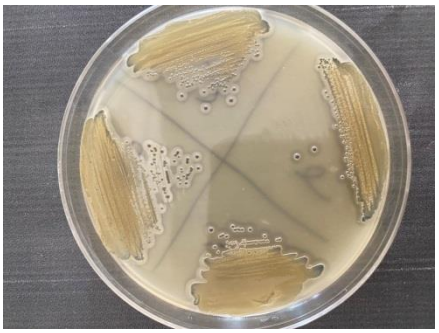

Fig. 1. *Isolated S. aureus strains on RPF-BP agar.*

Fig. 2. *Methicillin resistance in isolated S. aureus strain by disc diffusion test*

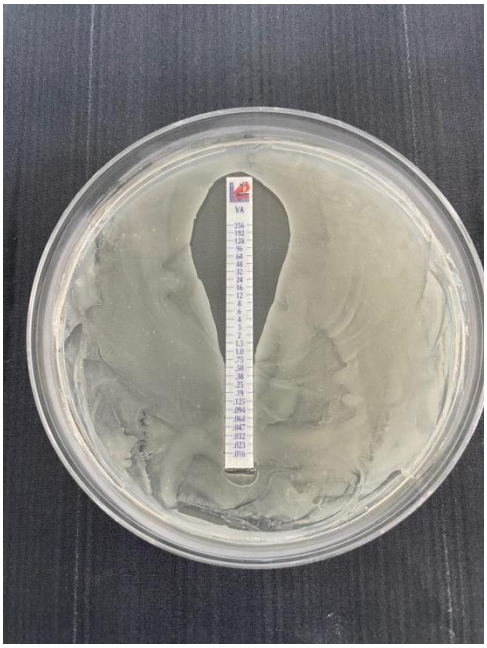

Fig. 3. *E-Test for Vancomycin in isolated S. aureus strain.*

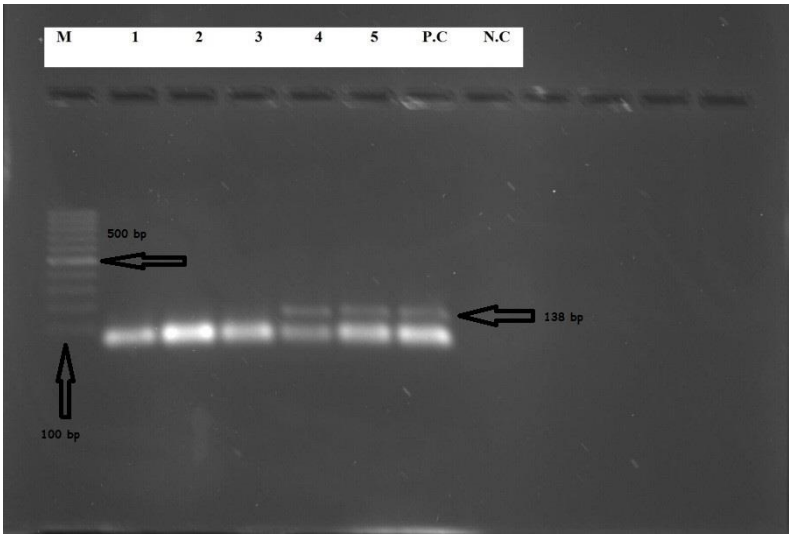

Fig. 4. *PCR image of mecC gene in isolated S. aureus strains.*

M: Marker; Line 1,2,3: mecC negative; Line 4,5: mecC positive; P.C: Positive Control; N.C: Negative Control (DNA free PCR mix)

Mastitis is the most important disease in dairy cow breeding that affects the animals and the breeder the most in terms of productivity and economy. Clinical mastitis presents with signs such as udder swelling, redness, or hardness, decreased milk quality, and the presence of flakes, clots, or pus in the milk. In contrast, subclinical mastitis lacks these overt symptoms, complicating its diagnosis and treatment [3].

In addition to being rich in terms of agriculture and animal husbandry in Türkiye, Balıkesir province is at the top in terms of dairy cow breeding. Therefore, mastitis carries an epidemiologically important disease potential in Balıkesir province in terms of preventive medicine, diagnosis and treatment.

Antimicrobial treatment plays a pivotal role in managing bovine mastitis. However, the extensive and unregulated use of antimicrobials for mastitis has exerted selective pressure on *S. aureus*, leading to the emergence of multidrug-resistant MRSA strains. Consequently, this situation has severely restricted the therapeutic choices available to veterinary professionals and clinicians alike [3].

MRSA is increasingly reported as a new problem in veterinary medicine and the emergence of MRSA causes serious zoonotic diseases [30]. Additionally, presence of MRSA in bovine mastitis is a possible risk and a source of infection for people, especially veterinarians and livestock workers [13]. With all this information, MRSA can also be evaluated with the One Health approach [6].

Risk factors for carriage of *mecC* MRSA in humans are contact with animals and the presence of an underlying disease [28]. According to the EFSA 2019-2020 report, MRSA was found to be 7.7% in 366 cow's milk samples [28].

Especially, in Balıkesir province, researchers were previously studied MRSA in milk samples. Tavşanlı and Cibik [12] reported that detected MRSA in 6 (6.3%) of 95 *S. aureus* strains isolated from 725 milk samples with subclinical mastitis, which they examined for microbiological examination for mastitis in the province of Balıkesir.

Ektik *et al.* [29] reported that they detected MRSA in 3 of 26 coagulase-positive *Staphylococcus* strains isolated from 175 cow bulk tank milk and dairy products' samples,

which they examined for microbiological examination for mastitis in the province of Balıkesir.

Also, they and reported that one of these isolates carried the *mecA* gene. Büyükcangaz *et. al.* [30] reported that they isolated 151 *S. aureus* from 480 milk samples with subclinical mastitis collected from cows in 6 different cities, including Balıkesir province between 2010 and 2012, and 62 of them were found to be resistant to cephoxitin by disc diffusion test. Also, they reported that they detected the *mecA* gene in 24 of 151 *S. aureus* isolates with their PCR test.

Aslantaş *et al.* [31] reported that they isolated 112 *S. aureus* from milk samples between 2008 and 2010 and they diagnosed MRSA by both detecting *mecA* gene in PCR and disc diffusion in 5 of these isolates.

Çiftçi *et al.* [32] reported that 59 strains were detected as *S. aureus* by both conventional tests and PCR isolated from bovine subclinic mastitic milk samples, and 13 of them were found to be methicillin resistant and 4 (30.7%) were positive for *mecA* gene.

Türüoğlu *et. al.* [33] reported that they detected *mecA* gene in 3 of 18 MRSA strains isolated from milk samples with cow mastitis between 2002-2004.

MRSA was detected by both disc diffusion and PCR in 5 of 15 *S. aureus* strains isolated in this study. Considering the cited studies in Balıkesir [12,29,30], it was determined that the presence of MRSA is still present in mastitis infections of dairy cows in different regions of Balıkesir province. Unlike the other -studies [29-33] the *mecC* gene was detected in all MRSA isolates in the PCR test in this study. This situation was found to be similar with Degaim *et. al.* [34] and Sakmanoğlu *et. al*. [35] studies. Degaim *et al.* [34] reported in their study that they found that the *mecC* gene was more important than the *mecA* gene in recognizing MRSA and pointed out that its frequency was gradually increasing in the south of Iraq. Sakmanoğlu *et al.* [35] reported in their study that they detected an unexpectedly high prevalence of *mecC* MRSA in cows with mastitis.

As seen in the results of Ektik *et al.* [29], Büyükcangaz *et. al.* [30] and Çiftçi *et al.* [32]'s studies, they could not detect genotypic resistance genes in some isolates that were found to be phenotypically resistant in the PCR test performed only for the detection of the *mecA* gene. For this reason, it was thought that the isolates determined to be MRSA phenotypically should also be analyzed in terms of the *mecC* gene.

VRSA has not yet been reported in Europe and is is rarely reported all over the world today [20]. Similarly, in this study, vancomycin resistance was not detected in all *S. aureus* isolates as a result of MIC evaluation.

CONCLUSION

As a conclusion, the presence of both *S. aureus* and MRSA was detected in this study, as can be seen in the findings of other cited studies [12,29,30] in cow milk with mastitis in Balıkesir province. Moreover, in this study, it was determined that the rate of *mecC*-MRSA was high. In this study, it was thought that the detection of the *mecC* gene in all MRSA isolates emphasized the importance of this situation. Considering that the absence of VRSA in isolated *S. aureus* strains is similar to the findings in the world, but antibiotic resistance is spreading rapidly nowadays, it was thought that vancomycin resistance should be monitored periodically in *S. aureus* isolates from an epidemiological point of view.

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

Authorship Contributions. Concept: O.B., Design: O.B., Data Collection or Processing: O.B., Analysis or Interpretation: O.B., Literature Search: O.B., Writing: O.B.

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