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Identification of bacteria isolated from dairy goats with subclinical mastitis and investigation of methicillin and vancomycin resistant *Staphylococcus aureus* strains

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Summary: The objective of this study was to determine methicillin and vancomycin resistance phenotypically by agar disc diffusion method (ADDM) and minimal inhibitory concentration (MIC) test and genotypically by polymerase chain reaction (PCR) in *Staphylococcus aureus* strains isolated from goat milk. A total of 466 milk samples were collected from 233 goats in herds with mastitis problems in Burdur province of Turkey. The microorganisms were isolated from 122 (26.18%) of goat milk samples and among these microorganisms 53 (42.06%) were coagulase negative staphylococci (CNS), 43 (34.23%) were *Staphylococcus aureus*, 16 (12.08%) were *Escherichia coli*, 10 (7.94%) were *Candida* spp. and 4 (3.17%) were *Brucella melitensis*. Seven of *S. aureus* isolates were determined resistant to methicillin by ADDM and five of these isolates were found resistant to methicillin by MIC. *mec*A and *van*A genes can not be determined in *S. aureus* isolates by PCR. *Staphylococcus aureus* isolates were found to be susceptible to vancomycin by ADDM and MIC test. In conclusion, *S. aureus* and CNS are found to be the most isolated species from goat milk in Burdur province. In addition to that, the absence of *mec*A and *van*A genes in the *S. aureus* isolated from goat milk showed that goat milk does not play a significant role in the spreading of MRSA.

Keywords: Goat, methicillin, *S. aureus*, vancomycin.

Subklinik mastitisli keçilerden izole edilen bakterilerin identifikasyonu ve metisilin ve vankomisin dirençli *Staphylococcus aureus* suşlarının araştırılması

Özet: Bu çalışmanın amacı, keçi sütlerinden izole edilen *Staphylococcus aureus* izolatlarında fenotipik ve genotipik metisilin ve vankomisin direncinin agar disk difüzyon (ADDM), minimal inhibitory konsantrasyon (MİK) ve polimeraz zincir reaksiyonu (PZR) metodları ile belirlenmesidir. Bu amaçla, Burdur ilinde mastitis problemi yaşanan sürülerde bulunan 233 adet keçiden 466 adet süt örneği toplandı. Keçi sütlerinin 122 (%26.18)'sinden mikroorganizma izole edildi. İzole edilen mikroorganizmaların 53 (%42.06)'ü koagülaz negatif stafilokok (KNS), 43 (%34.23)'ü *Staphylococcus aureus*, 16 (%12.08)'sı *Escherichia coli*, 10 (%7.94)'u *Candida* spp. ve 4 (%3.17)'ü ise *Brucella melitensis* olarak belirlendi. *Staphylococcus aureus* izolatlarının 7'si ADDM ile metisiline dirençli bulunurken, bu izolatların 5'i MİK ile fenotipik olarak metisiline dirençli bulundu. *Staphylococcus aureus* izolatlarında *mec*A ve *van*A genleri PZR ile belirlenemedi. *Staphylococcus aureus* izolatları ADDM ve MİK ile vankomisine duyarlı bulundu. Sonuç olarak *S. aureus* ve KNS'nin Burdur ilinde keçi sütlerinden en sık izole edilen bakteriler olduğu belirlendi. Bununla birlikte, keçi sütlerinden izole edilen *S. aureus* suşlarında *mec*A ve *van*A genlerinin saptanamaması, MRSA'nın yayılmasında keçi sütlerinin önemli bir rol oynamadığını göstermiştir.

Anahtar sözcükler: Keçi, metisilin, *S. aureus*, vankomisin.

Introduction

Staphylococcus aureus (S. aureus) causes clinical and subclinical mastitis in farm animals (4,15) and food borne infection in human due to the contamination of goat, sheep milk for traditional caprine and ovine milk products are not subjected to pasteurization (23). Although several infectious agents have been isolated from goat mastitis (4, 19, 21, 36, 45), *S. aureus* is the most important mastitis pathogen, due to economical loses and decrease in milk production of dairy goat in worldwide (21). β-lactam antibiotics were generally preferred for the treatment of *S. aureus* infections in humans and animals (15, 29, 36). But, methicillin resistance *S. aureus* strains with use of β-lactamase resistant penicillins have started to attract attention all over the world and are responsible for hospital infections (9).

Methicillin resistance in staphylococci is mediated by *mec*A gene, which encodes a penicillin binding protein 2a (PBP-2a). This gene leads to reduce affinities to β-lactam antibiotics and present in all of methicillin resistant

staphylococci (17). Methicillin resistance *S. aureus* (MRSA) is the most important pathogen isolated from human nosocomial infections and MRSA infections of humans are increasing substantially in worldwide that is not only related to the resistance to β-lactam antibiotics, but also related with resistance to other antibiotics (aminoglycosides, macrolides and quinolones etc.) (3, 22, 25, 43). Penisillinase resistant penicillins are not used in veterinary medicine except cloxacilline performed intramammary in cattle. In recent years, MRSA has been isolated from various animals and animal products such as milk and cheese (2, 17, 39, 43). But, the studies about the presence of MRSA in goat milk and milk products has limited. MRSA has been detected from individual milk sample, bulk tank milk of goats and nasal swab of farm personnel (39). Similarly, Cartimiglia et al., (16) reported that MRSA was determined in bulk tank milk from dairy goat farms in Northern Italy. Chu et al., (12) announced that eleven MRSA isolates were identified from four goat farms. Altough MRSA was detected in cattle milk and milk products, it was not reported in goat milk and milk products in studies except for one study in Turkey. Aras et al., (2)'s study is the first report of MRSA isolation from goat mastitis in Turkey. In this study, two MRSA strains were isolated from mastitis and identified as MRSA by PCR (2). In methicillin resistant strains, chromosomal genes that are different from the *mec*A gene and which are necessary for expression of resistance are identified and these genes are named as fem factors (9). It is reported that *fem*A gene is only a feature of *S. aureus* strains and not found in other staphylococci (9).

Vancomycin has been a final choice antibiotic used for the treatment of MRSA infections in humans. Vancomycin resistance in enterococci is encoded by five *van* genes. Noble et al. (30) showed that the *van* genes have been found in *S. aureus* strains and methicillin resistant strains can acquire these genes. Hiramatsu et al., (20) reported the first MRSA with reduced susceptibility to vancomycin in Japan in 1996. Subsequently, vancomycin intermediate *S. aureus* (VISA) isolates have been reported in worldwide (31, 35, 37, 41). After a short time, in 2002, vancomycin resistant *S. aureus* (VRSA) was identified in Michigan (35). In all of these cases, patients had been treated with vancomycin or teicoplanin within six months (32). In studies (10, 35, 42), it was reported that VRSA isolates recovered from humans can carry *mec*A and *van*A genes. *In vivo* and *in vitro* transmission of *van*A genes from *Enterococcus faecalis* to *S. aureus*, VRSA can also be seen in animals, if MRSA and VRE are in the same animal (30). Although vancomycin resistance was reported by phenotypical tests in farm animals (1, 6), the presence of *van* genes have not yet been showed in livestock (1, 6, 8, 31, 41).

In this study, we aimed to identify microorganisms causing goat mastitis and to determine methicillin and vancomycin resistance in *S. aureus* isolates recovered from goat milk samples.

Materials and Methods

***Sampling*:** Four hundred sixty six milk samples were collected from 233 goats in herds with subclinical mastitis in five villages (Kurna, Düver, Güneyyayla, Kayaaltı and Kökez) of Burdur province of Turkey. Sampling was done in enterprises that had mastitis problem and samples were collected from the animals suspected of subclinical mastitis. The teats were cleaned by using 70% alcohol. After, the first few streams of foremilk were discarded, the milk samples were aseptically collected into sterile tubes.

***Bacterial isolates*:** Each milk samples were inoculated onto blood agar base (Oxoid, CM0055, UK) added 5% sheep blood and then the plates were incubated at 37°C for 18-24 h, aerobically. The microorganisms were identified by conventional microbiological procedures such as colony morphology, Gram staining, haemolysis, catalase, coagulase, DNAse, Voges-Proskauer, acetoin test, carbonhydrate fermentation tests etc. (47). The presence of *fem*A genes in the isolates identified as *S. aureus* using phenotypic tests were investigated by PCR and these isolates were confirmed as *S. aureus. Staphylococcus aureus* isolates were stored at -20°C in brain hearth infusion broth (Oxoid, CM1135, UK) containing 15% (v/v) glycerol.

***Antimicrobial susceptibility tests*:** The phenotypic resistance of the *S. aureus* strains to oxacillin was determined by ADDM and MIC test. *S. aureus* strains were cultured in blood agar with 5% sheep blood for 18-24 h at 37°C and strains were suspended in tryptic soy broth (TSB) (Oxoid, CM0129, UK) for McFarland Standard No. 0.5. The broth was cultured onto Mueller Hinton agar (Oxoid, CM0337, UK) with 2% NaCl for oxacillin resistance and onto Mueller Hinton agar for vancomycin resistance. The oxacillin (1 µg, Oxoid, UK) and vancomycin disc (30 µg, Oxoid, UK) were added and incubated for 24 h at 37°C. Inhibition zone diameters were evaluated according to Clinical and Laboratory Standards Institute (CLSI) (14).

The MIC for oxacillin (Sigma Chemical Co., St. Louis, MO, USA) were determined using a broth macrodilution method according to CLSI (9). *Staphylococcus aureus* isolates were tested into Mueller-Hinton broth (Oxoid, CM0405, UK) supplemented with 2% NaCl containing oxacillin in concentrations ranging from 0.5 to 256 µg/ml. MIC test for vancomycin was performed with Mueller Hinton broth containing vancomycin concentrations ranging from 0.5 to 256 µg/ml. The tubes containing oxacillin and vancomycin

were incubated at 35°C for 24 h and the MIC was defined as the lowest concentration of antibiotics that prevented the visible growth. The isolates were evaluated as susceptible (S), intermediate (I) and resistant (R) according to the MIC breakpoints of CLSI (14). In addition to the test organisms, MICs of the following control strains were also tested: *S. aureus* 27R (methicillin resistant), *S. aureus* 25923 (methicillin susceptible) and *Enterococcus faecalis* (*E. faecalis*) poultry isolate (vancomycin resistant).

β-lactamase hyperproduction cause borderline resistance in *mec*A negative isolates. For determination of overproduction of staphylococcal β-lactamase, ADDT was performed with amoxycillin clavulanic-acid (30µg, Oxoid, UK) disc.

***DNA* extraction**: *S. aureus* isolates and the control strains were inoculated into brain hearth infusion broth and incubated at 37°C for 24 h. Then the culture was centrifuged in 12000 rpm for 10 min. Bacterial pellet was resuspended in 200 µl of phosphate buffer solution (PBS) and centrifuged in 12000 rpm for 10 min again. The supernatant was transferred mini centrifuge tube. DNA samples were kept at -20°C until use.

***Detection of mec*A, *fem*A *and van*A genes:** Primers, target genes, PCR product sizes and references used for PCR protocols are presented in Table 1. PCR amplifications for each of *mec*A, *fem*A and *van*A genes were performed in a volume of 25 µl by using primers in Table 1. The reaction mixture of *mec*A, *fem*A and *van*A contained 5 µl template DNA, 12.5 µl 2×PCR Mastermix (Applied Biosystem, Roche, USA) and 1 µl each primers (100 pmol). PCR assay for *fem*A was preformed as follows: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C 45 s, annealing at 54°C for 45 s and extention at 72°C for 45 s. Finally, a 5 min extention period at 72°C was carried out (28). The amplification of *mec*A gene was performed as previously described by Ardic et al., (3). PCR assay for *van*A amplification; reaction was performed with the following program: an initial denaturation at 95°C for 10 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and polymerisation at 72°C for 30 s, and a final extension at 72°C for 10 min (13). *Staphylococcus aureus* 27R strain and an *E. faecalis* poultry isolate were used as positive control for *mec*A and *van*A genes, respectively. *S. aureus* 25923 was used as positive control strain for *fem*A gene and negative control strain for *mec*A gene.

PCR products were electrophoresed (Scie-Plas, HU10, UK) in 1.5% agarose gel stained with 0.5 µg/ml ethidium bromid at 100 V for 45 min and bands were imaged (EDAS 290, Eastman Kodak Company, Rochester, NY, USA) under UV light (UV-transilluminator, CLP, USA).

Results

In this study, all of 466 milk samples were collected from 233 goats with subclinical mastitis. Bacterial cultures were positive in 122 (26.18%) of 466 milk samples and 126 microorganisms were isolated from the samples. The isolated microorganisms were 53 (42.06%) CNS, 43 (34.23%) *S. aureus*, 16 (12.08%) *E. coli*, 10 (7.94%) *Candida* spp. and 4 (3.17%) *B. melitensis*.

Seven *S. aureus* isolates were found to be oxacillin resistant by ADDM. However, five of these isolates were found to be resistant to oxacillin by MIC test. MIC values for oxacillin were >256 µg/ml in 1 isolate, >128 µg/ml in 2 isolates and >32 µg/ml in 2 isolates. β-lactamase hyperproducers may cause to borderline resistance in *mec*A negative isolates. Thus, the overproduction of staphylococcal β-lactamase was determined with the susceptibility tests to amoxycillin-clavulanic acid (30 µg, Oxoid) in phenotypically MRSA isolates and five isolates were found to be susceptible to this antibiotic.

All of the *S. aureus* isolates (100%) were found susceptible to vancomycin by ADDM and MIC values. Forty three isolates were investigated for and vancomycin resistance genes (*mec*A and *van*A). According to the PCR results, all of the isolates were positive for *fem*A genes. However, *mec*A and *van*A genes were not detected in *S. aureus* isolates.

Table 1. Primers, target gene, PCR product size and references used in the study.
Tablo 1. Çalışmada kullanılan primerler, hedef gen, PZR ürün büyüklüğü ve referanslar.

Target gene	Sequences (5'-3')	Amplicon size (bp)	Reference
*mec*A	5'-CCT AGT AAA GCT CCG GAA-3' 5'-CTA GTC CAT TCG GTC C-3'	314	Choi et al. (11); Ardic et al. (3)
*fem*A	5'-AAAAAAGCACATAACAAGCG-3' 5'-GATAAAGAAGAAACCAGCAG-3'	132	Mehrotra et al. (28)
*van*A	5'-CAT GAA TAG AAT AAA AGTTGCAATA-3' 5'-CCCCTTTAACGCTAATACGATCAA-3'	1030	Clark et al. (13)

Discussion and Conclusion

Mastitis is an important problem in dairy goat herds, due to difficulties in treatment and control. Several researchers reported that CNS was isolated from dairy goats with subclinical and clinical mastitis and the isolation rate was changed between 40%-88.5% (4, 15, 18, 19, 21, 36, 45). In this study, the milk samples were collected from dairy goat herds with mastitis problem. CNS was determined to be the most frequently isolated bacterium (42.06%) from the goat milk. These results confirmed that CNS is the most commonly reported agent in subclinical mastitis of goats (4, 15, 19, 36, 45). However, Bergonier et al., (5) stated that CNS was isolated from subclinical mastitis and *S. aureus* was isolated from clinical mastitis. But, *S. aureus* was the second bacterial agent (34.23%) isolated from goats with subclinical mastitis in this study. This result was found to be higher than the results reported by other studies (4, 15, 18, 19, 21, 36, 45). In this study, *E. coli*, *Candida* spp. and *B. melitensis* were isolated from goat milks, too. The researchers were reported that different microorganisms may cause goat mastitis (18, 19, 21, 27). Mastitis agents may vary depending on the conditions of care in goat farms and the area in which the study is performed.

Treatment of mastitis has been generally made by using antibiotics (7, 15, 36). The use of antibiotics randomly without antibiotic susceptibility test leads to resistance to antibiotics. MRSA isolates with multidrug resistance are generally isolated from human, but these agents have been isolated from several animal species in recent years, too (2, 17, 24, 33). In this study, phenotypic and genotypic methicillin and vancomycin resistance were investigated in *S. aureus* isolates by ADDM, broth macrodilution method and PCR. Phenotypic methicillin resistance was detected in only seven isolates by ADDM. Five of these isolates were determined as methicillin resistant by broth dilution method. MIC values were changed between 32 µg/ml and 256 µg/ml in the isolates. But, *mec*A gene was not detected in these isolates. We thought that these isolates may be shown borderline resistance due to overproduction of β-lactamase. Borderline isolates that do not contain *mec*A gene that have been reported in several studies (9, 22, 25). The hyperproduction of β-lactamase in staphylococci can be determined using β-lactamase inhibitors, such as clavulanate and sulbactam. Thus, we tested the oxacillin resistant isolates by ADDM and macro dilution test with amoxycillin clavulanic-acid disc to determine overproduction of β-lactamase. The isolates were found susceptible to amoxycillin clavulanic-acid. These results support the researchers who reported that the over production of staphylococcal β-lactamase can leads to phenotypic methicillin resistance (9, 22, 25).

*mec*A is a gene encoding resistance to methicillin and PCR is a gold standard method in molecular diagnosis of methicillin resistance (9). Some researchers (38, 45) did not detect *mec*A gene in *S. aureus* isolates from goat milk. However, Aras et al. (2) reported that 2 out of 42 *S. aureus* isolated from goat with clinical mastitis were identified as MRSA by disc diffusion test in Turkey. Also, they were determined the presence of *mec*A gene in *S. aureus* isolates by PCR. According to the study results, the researchers suggested that MRSA might be causative agent for goat mastitis represent a major concern for public health. But, our results support the researchers (2, 38, 45) who reported that goat milk does not play a significant role in the spreading of MRSA and this situation does not represent a great public health concern.

*fem*A gene is a marker used for genotypic identification at the species level of *S. aureus* isolates (3, 9). In this study, the *fem*A gene was investigated in the isolates and the control strains, and all of them were found positive for *fem*A. MRSA is the most important pathogen isolated from human nosocomial infections in recent years, and MRSA has been isolated from various animals (2, 17, 39, 43), too. The treatment of MRSA infections in humans has been made by vancomycin being final choice (9, 20, 31). Presence of VRSA has been investigated by phenotypic methods in goat and cattle milk and phenotypic vancomycin resistance were not determined in *S. aureus* isolates (26, 45, 46). On the other hand, Umaru et al., (44) reported that phenotypic VRSA was isolated from fresh and fermented milk in Nigeria. But, in this study, the presence of *van*A gene which is regarded as the gold standard was not investigated by PCR. Similarly, Bhattacharyya et al. (6) reported VRSA and VISA strains in goat milk. But, the presence of *van*A could not be determined in this study. Although several genes encoding vancomycin resistance have been determined in enterococci, *van*A gene is generally detected in VRSA isolates (13). Until today, VRSA isolates including *van* genes encoding vancomycin resistance were not determined in farm animals (6, 13, 26, 34, 40, 45, 46). Although MRSA was generally reported in mastitis cases of farm animals (2, 12, 16, 17, 22, 43), VRSA has not been determined in animals, yet. In this study, MRSA and VRSA were not detected in goat herds with mastitis problems, too.

In summary, this study shows that *S. aureus* and CNS are found to be the most isolated species from goat milk in Burdur province. However, the absence of *mec*A and *van*A genes in the *S. aureus* isolated from goat milk showed that goat milk does not play a significant role in the spreading of MRSA.

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