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RESEARCH ARTICLE

Antimicrobial Susceptibility, Presence of Resistance Genes and Biofilm Formation in Coagulase Negative Staphlococci Isolated from Subclinical Sheep Mastitis [1]

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Summary

In this study, coagulase negative staphylococci (CoNS) (n = 70) isolated from subclinical sheep mastitis were screened for minimum inhibitory concentration (MIC) to antimicrobials used commonly in veterinary field in Turkey. In addition, plasmid profiling and biofilm production of CoNS isolates was investigated. All isolates were found to be susceptible to amoxycillin-clavulanic acid, cephalothin, gentamicin, enrofloxacin and oxacillin. The highest resistance was observed in 42.9% (n = 30) of the isolates against the beta-lactam antibiotics, penicillin and ampicillin. All beta-lactam resistant isolates produced beta-lactamase and carried *bla*Z. Tetracycline resistance was observed in 11.4% (n = 8) of the isolates, either alone or in combination with beta-lactams and macrolides. Of the tetracycline resistant 8 isolates, 5 carried the *tet*K gene, one carried the *tet*M and 2 isolates carried both genes together. Erythromycin resistance was observed in 5.7% of the isolates; *msr*A was detected alone (one isolate) or in combination with *mph*C (one isolate) and *erm*C (one isolate). *erm*A was observed only in one isolate. Most of the strains showed only a single plasmid band in size of 19.3 kb, but some had 2 to 3 plasmids ranging from >19.3 kb to 0.9 kb. Out of 70 CoNS isolates, 28 (40%) were identified as biofilm producer by Congo red agar (CRA) method, and 30 (42.9%) were positive for both *ica*A and *ica*D genes, which are known to be responsible for biofilm formation in CoNS.

Keywords: *Antimicrobial resistance, Biofilm production, Coagulase negative staphylococci, Sheep, Subclinical mastitis*

Subklinik Koyun Mastitislerinden İzole Edilen Koagulaz Negatif Stafilokok Suşlarının Antibiyotik Duyarlılıkları, Direnç Genlerinin Varlığı ve Biyofilm Sentezi

Özet

Bu çalışmada, subklinik koyun mastitislerinden izole edilen koagulaz negatif stafilokokların (KNS) (n = 70) veteriner hekimlik alanında yaygın olarak kullanılan antimikrobiyallere minimal inhibitor konsantrasyonları (MİK), plazmid profilleri ve biyofilm üretimi araştırıldı. Tüm izolatlar amoksisilin-klavulanik asid, cefalotin, gentamisin, enrofloksasin ve oksasiline duyarlı bulundu. En yüksek direnç izolatların %42.9'unda (n = 30) beta-laktam antibiyotiklere (penisilin ve ampisilin) karşı gözlendi. Beta-laktam antibiyotiklere dirençli izolatların tamamı beta-laktamaz sentezlediği ve *bla*Z genini taşıdığı belirlendi. Tetrasiklin direnci tek veya beta-laktam antibiyotikler ve makrolidlerle kombine olarak izolatların %11.4'ünde (n = 8) gözlendi. Tetrasiklin dirençli izolatların 5'inde *tet*K, birinde *tet*M ve ikisinde de her iki geni birlikte bulundu. Eritromisin direnci izolatların %5.7'sinde (n = 4) bulundu. *msr*A, *mph*C, *erm*A birer izolatta, *erm*C ise msrA geni ile kombine olarak bir izolatta tespit edildi. İzolatların büyük kısmı 19.3 kb büyüklüğünde tek plasmid gösterirken, bazı izolatlar büyüklüğü >19.3 kb ile 0.9 kb arasında değişen 2-3 plazmid taşıdığı belirlendi. Yetmiş KNS izolatından 28'i (%40) Kongo Red Agar (KRA) metodu ile biyofilm sentezi ve 30'unun da (%42.9) biyofilm sentezinden sorumlu olan *ica*A ve *ica*D genleri yönünden pozitif olduğu saptandı.

Anahtar sözcükler: *Antimikrobiyel direnç, Biyofilm sentezi, Koagulaz negatif stafilokok, Koyun, Subklinik mastitis*

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INTRODUCTION

Mastitis is recognised as one of the most important diseases affecting dairy sheep industry resulting in substantial economic losses worldwide due to reduced milk production, quality, and weak lambs [1,2]. Although a variety of microorganisms can be isolated from mastitis cases, staphylococci are the most frequent ones encountered in intramammal infections (IMI) of sheep. While *Staphylococcus aureus* is the more frequently isolated from clinical mastitis cases [3], coagulase negative staphylococci (CoNS) are the predominant microorganism in subclinical sheep mastitis [4-6]. CoNS are often the cause of persistent subclinic mastitis in dairy cattle and sheep [1]. Currently, CoNS consist of more than 30 species [7], *Staphylococcus epidermidis*, *Staphylococcus xylosus*, *Staphylococcus chromogenes* and *Staphylococcus simulans* being the most commonly isolated CoNS species in persistent subclinical IMI of sheep [2,4]. The presence of different CoNS species could be attributable to certain practices for controlling mastitis such as protocol and type of disinfectant used for teat dipping and dry-off treatments [1].

Antimicrobials play an important role not only in eliminating any existing IMI infection but also in preventing new infections at dry (dry-off) period [8,9]. Owing to the important role of antimicrobials in mastitis control programs, the determination of antimicrobial susceptibilities of mastitis pathogens is necessary for therapy and monitoring the spread of resistant strains among populations [10,11]. Also, antimicrobial resistant bacteria of animal origin can be transfered to humans via food chain and can pose public health problems [12]. Taponen and Pyorälä [7] have recently reported that CoNS tends to be more resistant than *S. aureus* and easily develop multiresistance. Studies from different parts of the world have showed that antimicrobial resistance in CoNS isolates of mastitis origin is on the rise, causing treatment failures. Also, higher percentage of penicillin resistance for CoNS isolated from bovine mastitis was reported 49.6% [13] and 62.5% [14] in Turkey. The ongoing rise in the antimicrobial resistance in CoNS highlight the importance of prudent antibiotic use in the control of mastitis and continuous surveillance of resistance levels in CoNS isolated from IMIs.

Biofilm production has been increasingly accepted to play an important role in the pathogenesis of staphylococcal mastitis [15-17]. Biofilm is an exopolisaccharide matrix formed around bacterial cells [18]. Biofilm forming ability of staphylococci helps in adherence and colonization of the organism on mammary gland epitelium, and survival in hostile environments within host. Exopolisaccharide matrix surrounding bacterial colonies protects bacteria from phagocytosis and high concentrations of antimicrobials, and allows the persistence of the infection [17,19-21].

The objectives of this study were to determine the antibiotic susceptibility profiles of CoNS strains from subclinical sheep mastitis to nine antibiotics used commonly in veterinary field in Turkey, to screen the distribution of genetic determinants of antibiotic resistance, and to investigate the biofilm forming ability and occurence of the *ica*A and *ica*D genes.

MATERIAL and METHODS

Bacterial Strains

A total of 70 CoNS isolates were used in this study. These strains were previously isolated and identified from milk samples of sheep with subclinical ewe mastitis in Hatay province of Turkey [4].

Antibiotic Susceptibility Testing

Minimum inhibitory concentrations (MIC) of 9 antimicrobial agents (penicilin G, ampicillin, amoxycillin-clavulanic acid, tetracyclin, gentamicin, cephalothin, enrofloxacin, oxacillin, and erythromycin) were determined using microbroth dilution susceptibility method in accordance with instructions in M31-A2 of the Clinical Laboratory Standards Institute [22]. The MIC test was performed using cation-adjusted Mueller-Hinton broth (MHB) (Merck, Germany) containing each drug in concentrations ranging from 0.06 to 256 µg/ml. The MHB supplemented with 2% NaCl was used for determining the oxacillin MIC value. Plates containing oxacillin were incubated at 35°C for 24 h, while other plates were incubated at 37°C for 18 h. The MIC level was defined as the lowest concentration of antimicrobial agents that inhibited visible growth.

ß-lactamase Production

Beta-lactamase sticks (Oxoid, UK, Code no: BR0066) impregnated with nitrocefin, a chromogenic cephalosporin, were used for the detection of beta-lactamase production by CoNS isolates. Development of a pink/red colour was accepted as positive reaction, while no colour change was regarded as negative reaction.

Biofilm

Biofilm formation was detected by cultivation on Congo Red agar (CRA) as described by Freeman et al.[23]. Strains producing black colonies with a rough, dry and crystalline consistency were considered biofilm producers. Strains producing red colonies with rough, dry and crystalline consistency or smooth colonies were classified as non-biofilm producers.

DNA Isolation and PCR Amplification of Resistance Genes

Genomic DNA was extracted from CoNS isolates as reported previously by Hesselbarth and Schwarz [24]. PCR amplification of resistance genes in CoNS isolates was

performed using primers described by Lüthje and Schwarz [25], Choi et al.[26], Strommenger et al.[27], Martineau et al.[28], Jensen et al.[29], Vesterholm-Nielsen et al.[30], and a primer specific to 16S rDNA described by Strommenger et al.[27]. Also, primers for the detection of intercelluler adhesion genes, *ica*A and *ica*D, were used in this study as previously described [18] *(Table 1)*.

Plasmid Profiling

Plasmid DNA was prepared according to the method described by Anderson and McKay [31]. Plasmid DNA was electrophoresed in 0.8% agarose gel in TAE buffer for 3 h at 60 V. At the end of electrophoresis, the gel without ethidium bromide was stained in the same buffer containing ethidium bromide (0.5 µg/mL). The gels were then viewed with a transilluminator and the images were captured by a video-camera as TIFF files.

RESULTS

Antimicrobial Susceptibility

The data on the antimicrobial susceptibility of 70 CoNS strains are summarised in *Table 1*. A total of 30 (42.9%) CoNS isolates were resistant to penicillin and ampicillin, 2 of which were also resistant to tetracycline. While resistance to tetracycline was found in 8 (11.4%) strains, only 4 (5.6%) strains exhibited resistance to erytromycin. CoNS isolates that were phenotypically resistant to penicillin and ampicillin were also beta-lactamase producers by nitrocefin test. No resistance was detected for amoxycillin-clavulanic acid, gentamicin, cephalothin, enrofloxacin, or oxacillin.

Prevalence of Antimicrobial Resistance Genes

All penicillin (≥0.25 µg/ml) and ampicillin resistant CoNS

Table 1. *Oligonucleotide primers used in this study*
Tablo 1. *Çalışmada kullanılan primerler*

Gene	Sequence (5'→3')	Size (bp)	References
aac(6')/aph(2")	GAA GTA CGC AGA AGA GA	491	Choi et al.[26]
	ACA TGG CAA GCT CTA GGA		
aph(3')-IIIa	AAA TAC CGC TGC GTA	242	
	CAT ACT CTT CCG AGC AA		
ant(4')-Ia	AAT CGG TAG AAG CCC AA	135	
	GCA CCT GCC ATT GCT A		
mecA	CCT AGT AAA GCT CCG GAA	314	
	CTA GTC CAT TCG GTC CA		
tetK	TAT TTT GGC TTT GTA TTC TTT CAT	360	Strommenger et al.[27]
	GCT ATA CCT GTT CCC TCT GAT AA		
tetM	AGT TTT AGC TCA TGT TGA TG	158	
	TCC GAC TAT TTA GAC GAC GG		
ermA	TCA AAG CCT GTC GGA ATT GG	440	Jensen et al.[29]
	AAG CGG TAA ACC CCT CTG AG		
ermB	CAT TTA ACG ACG AAA CTG GC	424	
	GGA ACA TCT GTG GTA TGG CG		
ermC	ATC TTT GAA ATC GGC TCA GG	275	
	CAA ACC CGT ATT CCA CGA TT		
msrA	TCC AAT CAT TGC ACA AAA TC	230	Martineau et al.[28]
	AAA CGT CAC GCA TGT CTT CA		
mphC	GAGACTACCAAGAAGACCTGACG	722	
	CATACG CCG ATT CTC CTGAT		
blaZ	TAC AAC TGT AAT ATC GGA GGG	517	Vesterholm-Nielsen et al.[30]
	CAT TAC ACT CTT GGC GGT TTC		
16S rRNA	CAG CTC GTG TCG TGA GAT GT	420	Strommenger et al.[27]
	AAT CAT TTG TCC CAC CTT CG		
icaA	CCT AAC TAACGA AAG GTA G	1315	Vasudevan et al.[18]
	AAG ATATAG CGATAA GTG C		
icaD	AAA CGT AAG AGA GGT GG	381	
	GGC AAT ATG ATCAAG ATA C		

(≥0.5 µg/ml) strains carried the *bla*Z that encode β-lactamase *(Fig. 1)*. Of the 4 erythromycin (≥8 µg/ml) resistant isolates, three carried *msr*A gene. This gene occured alone (n=1) or in combination with *mph*C (n=1), and *erm*C (n=1). *erm*A was observed in only one isolate *(Fig. 2)*. Of the 8 tetracycline resistant isolates (≥16 µg/ml), while five carried the *tet*K gene alone, 2 strains carried both *tet*K and *tet*M, and one harbored *tet*M alone *(Fig. 3)*. None of the CoNS strains contained the genes *mec*A, *aac(6')/aph(2")*, *aph(3')-IIIa*, *ant(4')-Ia,* and *erm*B genes.

Plasmid Analysis

Plasmids were detected in 69 (98.6%) of CoNS strains tested in this study. Only one CoNS isolate did not yield any plasmid of detectable size using the methods described in materials and methods. Plasmid profiles of CoNS strains are shown in *Fig. 4*. Size of plasmids varied from >19.3 kb to 0.9 kb. Seven different plasmid sizes (>19.3, 19.3, 7.7, 4.2, 2.3, 1.8, 1.6, 1.4, 0.9) were identified across the 69 isolates. Most of the strains showed only single plasmid band with size of 19.3 kb, but the rest of the strains had 2 to 3 plasmids ranging from >19.3 kb to 0.9 kb. The most common plasmid of 19.3 kb was detected in all strains. While 52 (74.3%) of CoNS strains contained single plasmid, 17 (24.3%) strains had multiple plasmids. We also attempted to determine whether there was a correlation between antimicrobial resistance and plasmid profiles of the CoNS isolates. As can be seen from *Table 2*, the 19.3 kb size plasmid was detected in every resistant isolate regardles of the resistance profile, whereas the plasmids were less commonly associated with a particular resistance phenotype. The single CoNS isolate without any detectable plasmid did not show resistance to any antimicrobials tested in this study. The observation that almost all of the CoNS isolates had the 19.3 kb plasmid, in addition to possessing other less commonly found ones, indicates that the presence of these plasmids alone was not sufficient to confer antimicrobial resistance seen in the CoNS strains tested in our study. Therefore, there is a possibility that the resistance confering genes were present on different plasmids which were gone undetected in this study and/or that the resistance traits were associated with the chromosome in the antimicrobial resistant CoNS. Although the suspect resistance-incurring genes were detected in every resistant CoNS isolate tested, the PCR was done with total genomic DNA as template in this study, and thus, it is impossible to know definitively the exact location (chromosome or plasmid) of these genes.

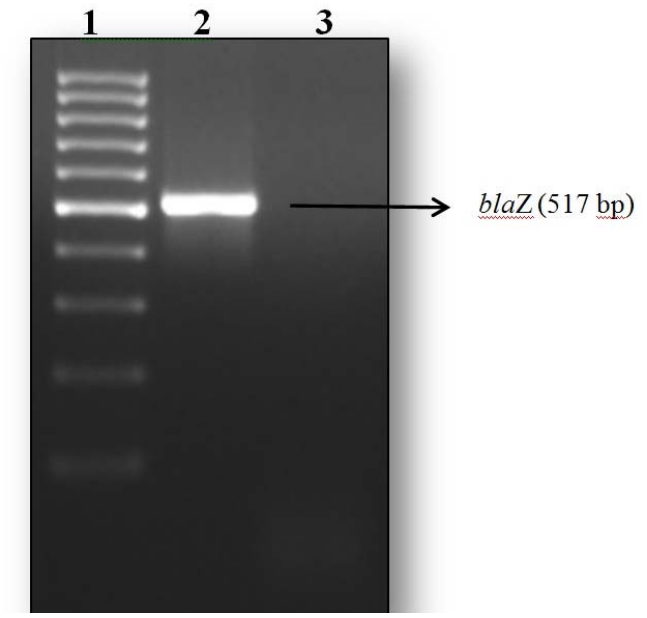

Fig 1. Agarose gel electrophoresis of *bla*Z gene. Lane 1 = 100 bp DNA marker (Fermentas) Lane 2 = *bla*Z, Lane 3 = negative control

Şekil 1. *bla*Z geninin agaroz jel elektroforez görüntüsü. Kuyucuk 1 = 100 bp DNA marker (Fermentas), Kuyucuk 2 = *bla*Z, Kuyucuk 3 = Negatif kontrol

Biofilm

Of the 70 CoNS isolates included in this study, 28 (40%)

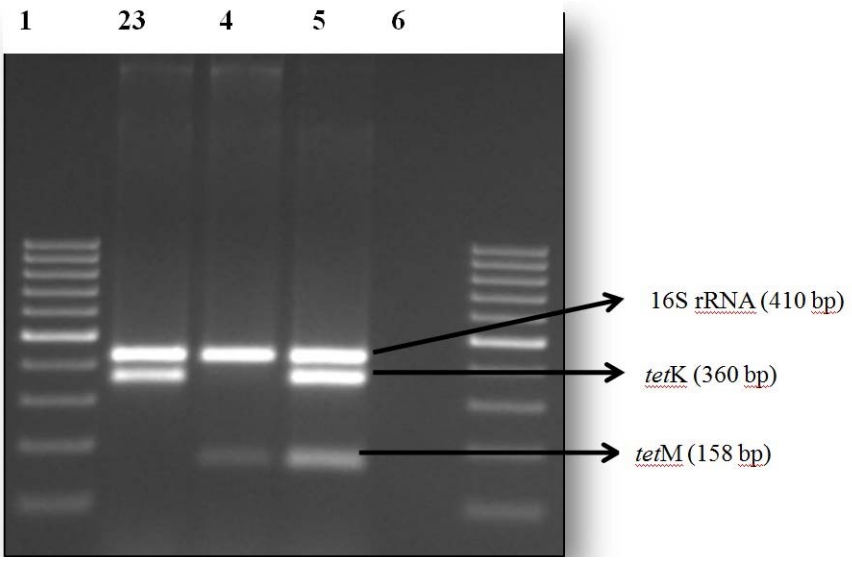

Fig 2. Agarose gel electrophoresis of macrolide resistance genes. Lane 1-7 = 100 bp molecular weight marker (100 bp), Lane 2 = *mph*C, Lane 3 = *erm*A, Lane 4 = *erm*C, Lane 5 = *msr*A, Lane 6 = Negative control

Şekil 2. Makrolid direnç genlerinin agaroz jel elektroforezi. Kuyucuk 1-7 = 100 bp moleküler marker (Fermentas), Kuyucuk 2 = *mph*C, Kuyucuk 3 = *erm*A, Kuyucuk 4 = *erm*C, Kuyucuk 5 = *msr*A, Kuyucuk 6 = Negatif kontrol

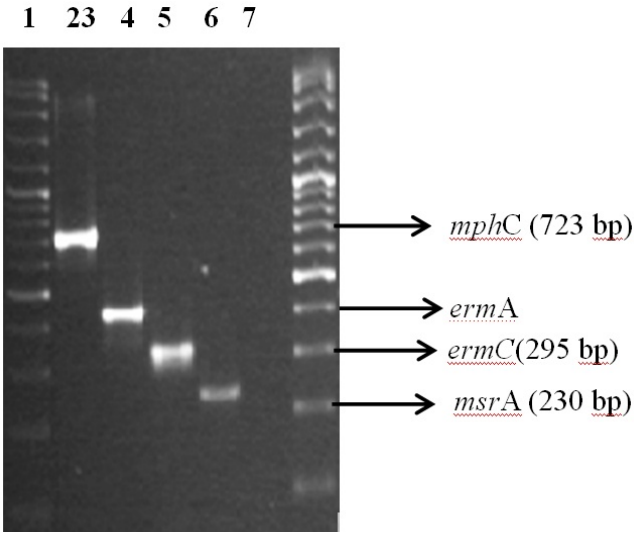

Fig 3. Agarose gel electrophoresis of *tet*K and *tet*M genes. Lane 1-6 = 100 bp molecular marker (Fermentas), Lane 2 = 16S rRNA ve *tet*K, Lane 3 = 16S rRNA and *tet*M, Lane 4 = 16S rRNA, *tet*K and *tet*M, Lane 5 = Negative control

Şekil 3. 16S rRNA, *tet*K ve *tet*M genlerinin agaroz jel elektroforez görüntüsü. Kuyucuk 1-6: 100 bp moleküler marker (Fermentas). Kuyucuk 2 = 16S rRNA ve *tet*K, Kuyucuk 3 = 16S rRNA ve *tet*M, Kuyucuk 4 = 16S rRNA, *tet*K ve *tet*M, Kuyucuk 5 = Negatif kontrol

were biofilm producers by CRA method, and 42 (60%) were found to carry both both *ica*A and *ica*D genes *(Fig. 5)*. Fourteen (20%) isolates were negative by CRA method despite their biofilm positive genotype.

DISCUSSION

ß-lactam antibiotics are among the most widely used antimicrobials for the control and treatment of mastitis. Higher resistance rates to ß-lactam antibiotics have been reported among *Staphylococcus* spp. from mastitis cases in different countries when compared with resistance rates to other antimicrobial agents [9,32,33]. In accordance with previous studies, highest resistance frequency was also observed against ß-lactam antibiotics, penicillin and ampicillin (42.8%) in the present study. However, some authors emphasized species specific differences in susceptibility patterns among CoNS isolated from mastitis cases [8,9]. Higher resistance rates against ß-lactam antibiotics were also reported by authors studied on bovine mastitis in different geographical regions of Turkey [13,14,33,34]. The high rate of resistance against ß-lactam antibiotics detected in

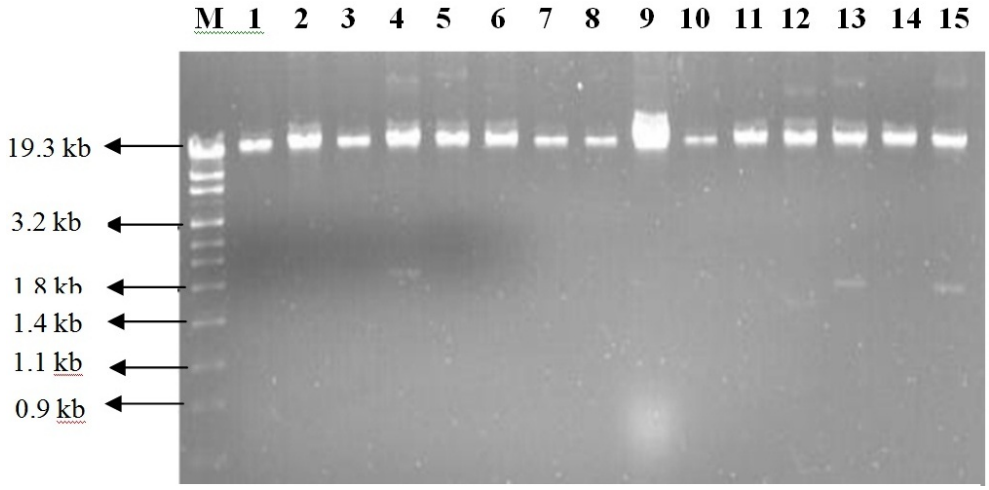

Fig 4. Agarose gel electrophoresis of plasmid patterns of representative CoNS isolates. Lane M= pUC mix marker (Fermentas), Lane 1-15 = CoNS isolates of sheep subclinical mastitis

Şekil 4. Temsili KNS izolatlarının plasmid agaroz jel elektroforezis görüntüsü. Kuyucuk 1 = pUC miks marker (Fermentas), Kuyucuk 1-15 = KNS izolatlar

Table 2. *Distribution of MIC values for the 70 CoNS strains from subclinical sheep mastitis to antimicrobials commonly used in veterinary medicine in Turkey*
Tablo 2. *Türkiye'de subklinik koyun mastitislerinden izole edilen 70 KNS suşuna karşı veteriner hekimliğinde yaygın kullanılan antimikrobiyallerin minimum inhibitor konsantrasyon değerlerinin dağılımları*

Antimicrobial Agent(s)	No. of Isolates with MIC (mg/L)														Resistant	
	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	no	%
Penicillin	38	-	1	1	-	2	1	1	1	2	6	5	11	-	30	42.9
Ampicillin	35	-	4	1	1	-	2	2	3	3	3	7	8	1	30	42.9
Amoxillin - Clavulanic Acid	26	-	11	5	5	15	8	-	-	-	-	-	-	-	-	-
Cephalothin	2	-	23	26	18	1	-	-	-	-	-	-	-	-	-	-
Gentamicin	38	-	19	8	4	-	-	64	-	-	-	-	-	-	-	-
Erythromycin	10	1	49	5	1	-	-	-	-	-	-	1	2	1	4	5.7
Tetracycline	23	-	37	1	1	-	-	-	-	-	2	2	4	-	8	11.4
Enrofloxacin	4	-	51	12	1	2	-	-	-	-	-	-	-	-	-	-
Oaxcillin + 2% NaCl	10	-	26	34	-	-	-	-	-	-	-	-	-	-	-	-

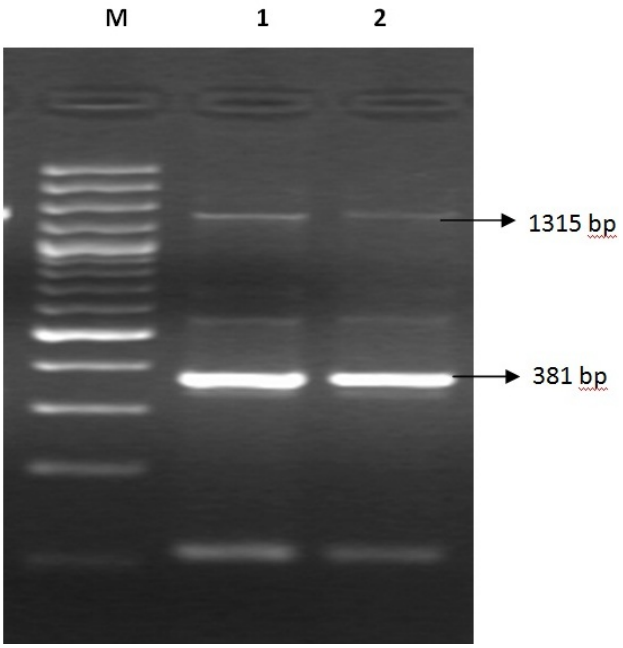

Fig 5. Amplification of *ica*A and *ica*D genes of CoNS strains of sheep subclinical mastitis. Lane M = DNA molecular weight marker (Fermentas). Lane1-2 = amplification of *ica*A and *ica*D

Şekil 5. Subklinikal koyun mastitislerinden izole edilen KNS suşlarında *ica*A ve *ica*D genlerinin amplifikasyonu. Kuyucuk 1 = 100 bp moleküler marker, Kuyucuk 1-2 = *ica*A ve *ica*D

Table 3. *Correlation among resistance phenotype, genotype and plasmid profiles*
Tablo 3. *Fenotip, genotip ve plasmid profilleri arasındaki ilişki*

Resistance Phenotype	No of Isolates	Antibiotic Resistance Genes	Plasmid DNA (kb)
P, AMP	19	*blaZ*	19.3
P, AMP, TE	2	*blaZ*, *tet*K	19.3
P, AMP	4	*blaZ*	>19.3, 19.3
P, AMP, E	1	*blaZ*, *msrA*	>19.3, 19.3, 7.7
P, AMP	1	*blaZ*	>19.3, 19.3, 2.3
P, AMP	1	*blaZ*	>19.3, 19.3, 1.8
P, AMP	1	*blaZ*	>19.3, 19.3, 4.2, 1.6, 0.9
P, AMP	1	*blaZ*	19.3, 1.4
TE, E	1	*tet*(K), *ermA*	19.3
TE	2	tet(K), tet(M)	19.3
E	1	*msr*A, *mph*(C)	>19.3, 19.3
E	1	*ermB*, *msr*A	19.3
TE	1	*tet*(M)	19.3
TE	2	*tet*K	19.3

this study could be explained by common and uncontrolled use of these group antimicrobials.

Staphylococci develop resistance to ß-lactam antibiotics with two mechanism: the production of ß-lactamases encoded by the *blaZ* gene and by the production of altered form of penicillin binding protein, PBP2α, encoded by the *mec*A gene [35]. All penicillin and ampicillin resistant CoNS strains were also ß-lactamase producers and carried *blaZ* gene in this study.

The presence of *mec*A mediated methicillin resistance has been reported in different species of staphylococci by Zhang et al.[35] from various animal species and by Sawant et al.[9] from bovine milk. Based on results of the present study, none of CoNS strains exhibited methicillin resistance both at phenotypic and genotypic level. MIC values of CoNS strains to oxacillin were between 0.03-0.125 µg/ml.

Tetracyclines are another group antimicrobials widely used in humans and animals for therapy and prevention of bacterial infections. As a consequence of misuse and overuse of tetracyclines, occurrence of tetracycline resistance in bacteria is very high. Following ß-lactam resistance, second highest resistance rate was detected against tetracycline in this study, although it was relatively low (11.4%). The occurence of tetracycline resistant genes were reported among *S. aureus* isolated from mastitic milk. Schwarz et al.[36] reported tetracyclin resistance 11.5% (n:9) of 78 *S. aureus* from subclinical mastitis, of which *tet*K in 6 isolates, *tet*L in 2 isolates, and *tet*L-*tet*M in one isolate were detected. While *tet*K located on plasmid mediates resistance by active efflux mechanism, *tet*M located in chromosome confers resistance by ribosomal protection [37]. Of the 8 tetracycline resistant isolates found in this investigation, *tet*K gene alone was present in 5 isolates, *tet*M alone was detected in only 1 isolate, and both genes were detected together in 2 isolates.

When erythromycin resistance genes were examined in the 4 resistant isolates found in our study (5.7%), *msr*A gene was detected either alone (one isolate) or in combination with *mph*C (one isolate) and *erm*B (one isolates). *erm*A alone was observed in one isolate. In accordance with these findings, Lüthje and Schwarz [25] also reported macrolide and lincosamide (ML) resistance and the presence of the associated genes in various CoNS isolates from bovine subclinical mastitis cases in Germany.

Carriage of antibiotic resistance genes on plasmids can readily lead to the transfer of resistance from one staphylococcal species to another via horizontal gene transfer and may result in the spread of resistant strains and persistent subclinical mastitis caused by multi-drug resistant staphylococci [5]. Although most of CoNS isolates harbored plasmids of various size, no apparent relationship between carriage of plasmids and antimicrobial resistance were observed in this study.

The ability of staphylococci to bind to inert or living surfaces is associated with the production of biofilm [38]. It has been shown that bacteria growing in biofilm are highly resistant to antimicrobial agents compared with non-biofilm producing bacteria due to the decreased diffusion of antimicrobials inside biofilms [21]. High frequency rate

of *ica*A and *ica*D genes were reported in *S. aureus* strains from bovine mastitis earlier studies [16,18]. In this study, 28 isolates (40%) were detected as biofilm producers by CRA method, while 42 isolates (60%) were positive for both *ica*A and *ica*D. This disagreement between phenotypic and genotypic results could possibly be explained by point mutations in the locus that negatively influence biofilm formation [38]. Similar observations have also been reported previously by Vasudevan et al.[18] and Dhanawade et al.[16].

The results from our study demonstrate that CoNS isolates from sheep subclinical mastitis are frequently resistant to ß-lactam antibiotics. Resistance rates to tetracycline and erythromycin were relatively low, and no resistance was observed against the other antimicrobials tested. These findings suggest that antimicrobials should be used cautiously to prevent the emergence and the spread of resistant bacteria. Therefore, routine isolation of mastitis pathogens and antimicrobial susceptibility testing are essential to achive effective therapy.

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REFERENCESS

1. Contreras A, Sierra D, Sànches A, Corrales JC, Marco JC, Paape MJ, Gonzalo C: Mastitis in small ruminants. *Small Rumin Res*, 68, 145-153, 2007.

2. Bergonier D, de Crémoux R, Rupp R, Lagriffoul G, Berthelot X: Mastitis of dairy small ruminants. *Vet Res*, 34, 689-716, 2003.

3. Mørk T, Waage S, Tollersrud T, Kvitle B, Sviland S: Clinical mastitis in ewes: Bacteriology epidemiology and clinical features. *Acta Vet Scand*, 49, 23-30, 2007.

4. Ergün Y, Aslantaş Ö, Doğruer G, Kireçci E, Sarıbay MK, Ateş CT, Ülkü A, Demir C: Prevalence and etiology of subclinical mastitis in Awassi dairy ewes in southern Turkey. *Turk J Vet Anim Sci*, 33, 477-483, 2009.

5. Burriel AR: Isolation of coagulase-negative staphylococci from the milk and environment of sheep. *J Dairy Res*, 65, 139-142, 1998.

6. Burriel AR: Resistance of coagulase-negative staphylococci isolated from sheep to various antimicrobial agents. *Res Vet Sci*, 63, 189-190, 1997.

7. Taponen S, Pyorälä S: Coagulase-negative staphylococci as cause of bovine mastitis-not so different from *Staphylococcus aureus. Vet Microbiol*, 134, 29-36, 2009.

8. Rajala-Schultz PJ, Torres AH, Degraves FJ, Gebreyes WA, Patchanee P: Antimicrobial resistance and genotypic characterization of coagulase-negative staphylococci over the dry period. *Vet Microbiol,* 134, 55-64, 2009.

9. Sawant AA, Gillespie BE, Oliver SP: Antimicrobial susceptibility of coagulase-negative Staphylococcus species isolated from bovine milk. *Vet Microbiol*, 134, 73-81, 2009.

10. Bengtsson B, Unnerstad HE, Ekman T, Artursson K, Nilsson-Ost M, Waller KP: Antimicrobial susceptibility of udder pathogens from cases of acute clinical mastitis in dairy cows. *Vet Microbiol,* 136, 142-149, 2009.

11. Gentilini E, Denamiel G, Betancor A, Rebuelto M, Rodriguez Fermepin M, De Torrest RA: Antimicrobial susceptibility of coagulase-negative staphylococci isolated from bovine mastitis in Argentina. *J Dairy Sci*, 85, 1913-1917, 2002.

12. Skovgaard N: New trends in emerging pathogens. *Int J Food Microbiol,* 120, 216-219, 2007.

13. Turutoglu H, Ercelik S, Ozturk D: Antibiotic resistance of *Staphylococcus aureus* and coagulase-negative staphylococci isolated from bovine mastitis. *Bull Vet Inst Pulawy,* 50, 41-45, 2006.

14. Turutoglu H, Mudul S, Pehlivanoglu F: Antibiotic susceptibility and β-lactamase prevalence for staphylococci isolated from bovine mastitic milk samples. *Acta Vet-Beograd,* 52 337-344, 2002.

15. Cucarella C, Solano C, Valle J, Amorena B, Lasa I, Penadés JR: Bap a *Staphylococcus aureus* surface protein involved in biofilm formation. *J Bacteriol,* 183, 2888-2896, 2001.

16. Dhanawade NB, Kalorey DR, Srinivasan R, Barbuddhe SB, Kurkure NV: Detection of intercellular adhesion genes and biofilm production in *Staphylococcus aureus* isolated from bovine subclinical mastitis. *Vet Res Commun*, 34, 81-89, 2010.

17. Melchior MB, Vaarkamp H, Fink-Gremmels J: Biofilms A role in recurrent mastitis infections? *Review Vet J*, 171, 398-407, 2006.

18. Vasudevan P, Nair MK, Annamalai T, Venkitanarayanan KS: Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. *Vet Microbiol*, 92, 179-185, 2003.

19. Oliveira M, Bexiga R, Nunes SF, Carneiro C, Cavaco LM, Bernardo F, Vilela CL: Biofilm-forming ability profiling of *Staphylococcus aureus* and *Staphylococcus epidermidis* mastitis isolates. *Vet Microbiol*, 118, 133-140, 2006.

20. Cucarella C, Tormo MA, Úbeda C, Trotonda MP, Monzón M, Peris C, Amorena B, Lasa I, José R, Penadés JR: Role of biofilm-associated protein bap in the pathogenesis of bovine *Staphylococcus aureus. Infect Immun*, 72, 2177-2185, 2004.

21. Melchior MB, Fink-Gremmels J, Gaastra W: Comparative assessment of the antimicrobial susceptibility of *Staphylococcus aureus* isolates from bovine mastitis in biofilm versus planktonic culture. *J Vet Med B*, 53, 326-332, 2006.

22. Clinical and Laboratory Standards Institute: Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals-Third ed., Approved Standard M31-A3, CLSI, Wayne PA, USA, 2008.

23. Freeman DJ, Falkiner FR, Keane CT: New method for detecting slime production by coagulase negative staphylococci. *J Clin Pathol*, 42, 872-874, 1989.

24. Hesselbarth J, Schwarz S: Comparative ribotyping of *Staphylococcus intermedius* from dogs pigeons horses and mink. *Vet Microbiol,* 45 11-17, 1995.

25. Lüthje P, Schwarz S: Antimicrobial resistance of coagulase-negative staphylococci from bovine subclinical mastitis with particular reference to macrolide-lincosamide resistance phenotypes and genotypes. *J Antimicrob Chemother*, 57, 966-969, 2006.

26. Choi SM, Kim SH, Kim HJ, Lee DG, Choi JH, Yoo JH, Kang JH, ShinWS, Kang MW: Multiplex PCR for the detection of genes encoding aminoglycoside modifying enzymes and methicillin resistance among Staphylococcus species. *J Korean Med Sci*, 18, 631-636, 2003.

27. Strommenger B, Kettlitz C, Werner G, Witte W: Multiplex PCR assay for simultaneous detection of nine clinically relevant antibiotic resistance genes in *Staphylococcus aureus. J Clin Microbiol*, 41, 4089-4094, 2003.

28. Martineau F, Picard FJ, Lansac N, Ménard C, Roy PH, Ouellette M, Bergeron MG: Correlation between the resistance genotype determined by multiplex PCR assays and the antibiotic susceptibility patterns of *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Antimicrob Agents Chemother*, 44, 231-238, 2000.

29. Jensen LB, Frimodt-Møller N, Aarestrup FM, Presence of erm gene classes in gram-positive bacteria of animal and human origin in Denmark. *FEMS Microbiol Lett,* 170, 151-158, 1999.

30. Vesterholm-Nielsen M, Olhom-Larsen M, Elmerdahl-Olsen J, Moller-Aarestrup F: Occurrence of the *bla*Z gene in penicillin resistant *Staphylococcus aureus* isolated from bovine mastitis in Denmark. *Acta Vet Scand,* 40, 279-286, 1999.

31. Anderson DG, McKay LL: Simple and rapid method for isolating large plasmid DNA from lactic Streptococci. *Appl Environ Microbiol,* 46, 549-552, 1983.

32. Fuda CC, Fisher JF, Mobashery S: Beta-lactam resistance in *Staphylococcus aureus* the adaptive resistance of a plastic genome. *Cell Mol Life Sci,* 62, 2617-2633, 2005.

33. Hadimli HH, Ateş M, Güler L, Kav K, Öncel T: Mastitisli süt ineklerinden izole edilen stafilokokların ß-laktamaz aktiviteleri ve antibiyotiklere duyarlılıkları. *Vet Bil Derg*, 17, 21-25, 2001.

34. Güler L, Ok U, Gündüz K, Gülcü Y, Hadimli HH: Antimicrobial susceptibility and coagulase gene typing of *Staphylococcus aureus* isolated from bovine clinical mastitis cases in Turkey. *J Dairy Sci,* 88, 3149-3154, 2005.

35. Zhang YS, Agidi SJT, LeJeune JT: Diversity of staphylococcal cassette chromosome in coagulase-negative staphylococci from animal sources. *J Appl Microbiol*, 107, 1375-1383, 2009.

36. Schwarz S, Roberts MC, Werckenthin C, Pang Y, Lange C: Tetracycline resistance in Staphylococcus spp from domestic animals. *Vet Microbiol,* 63, 217-227, 1998.

37. Fluit ADC, Visser MR, Schmitz F: Molecular detection of antimicrobial resistance. *Clin Microbiol Rev*, 14, 836-871, 2001.

38. Cramton SE, Gerke C, Schnell NF, Nichols WW, Götz F: The intercellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation. *Infect Immun,* 67, 5427-5433, 1999.