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Identification and Antimicrobial Susceptibility of *Staphylococcus aureus* and Coagulase Negative Staphylococci from Bovine Mastitis in the Aydın Region of Turkey

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Abstract: Three hundred bovine milk samples with mastitis were used. Eighty-five (28.33%) strains were identified as *Staphylococcus aureus* whereas 60 (20.00%) strains were isolated as coagulase negative staphylococcus. Bacteriological cultures of coagulase negative staphylococcus were identified as 20 (33.33%) strains of *S. hyicus*, 16 (26.66%) strains of *S. chromogenes*, 9 (15.00%) strains of *S. epidermidis*, 5 (8.33%) strains of *S. haemolyticus*, 4 (6.66%) strains of *S. sciuri*, 3 (5.00%) strains of *S. lentis* and 3 (5.00%) strains of *S. cohnii* subsp. *cohnii*. The antibiotic susceptibility test results showed that *Staphylococcus aureus* strains were susceptible to ciprofloxacin (100.00%), neomycin + bacitracin + tetracycline (100.00%), kanamycin (85.00%), cefquinome (85.00%) and amoxycillin + clavulanic acid (84.00%), while they were resistant to penicillin (95.00%) and oxacillin (60.00%). Beside this, it has been found that the coagulase negative staphylococcus strains were susceptible to cefquinome (100.00%) and neomycin + bacitracin + tetracycline (100.00%), but resistant to penicillin (90.00%) and oxacillin (73.00%).

Key Words: Mastitis, *Staphylococcus aureus*, coagulase negative staphylococcus identification, antimicrobial susceptibility testing

Türkiye'nin Aydın Yöresindeki Sığır Mastitislerinden *Staphylococcus aureus* ve Koagulaz Negatif Stafilokok'ların İdentifikasyonu ve Antibiyotik Duyarlılığı

Özet: Bu çalışmada toplam 300 adet mastitisli sığır süt örneği kullanıldı. Örneklerin 85 (% 28,33)'i *Staphylococcus aureus* olarak tanımlanırken, 60 (% 20,00)'i koagulaz negatif stafilokok olarak izole edildi. Koagulaz negatif stafilokokların bakteriyolojik kültürleri, 20 suş (% 33,33) *S. hyicus*, 16 suş (% 26,66) *S. chromogenes*, 9 suş (% 15,00) *S. epidermidis*, 5 suş (% 8,33) *S. haemolyticus*, 4 suş (% 6,66) *S. sciuri*, 3 suş (% 5,00) *S. lentis* ve 3 suş (% 5,00) *S. cohnii* subsp. *cohnii* olarak tanımlanmıştır. Antibiyotik duyarlılık testi sonuçları, *Staphylococcus aureus* suşlarının ciprofloksasin (% 100,00), neomisin+basitrasin+tetrasiklin (% 100,00), kanamisin (% 85,00), cefquinom (% 85,00) ve amoksisiline+klavulanik asit (% 84,00)'e duyarlı ve penisilin (% 95,00) ile oksasilin (%60,00)'e dirençli olduğunu göstermektedir. Bununla beraber, koagulaz negatif stafilokok suşlarının cefquinom (% 100,00) ile neomisin+basitrasin+tetrasiklin (% 100,00)'e duyarlı ve penisilin (% 90,00) ile oksasilin (% 73,00)'e dirençli olduğu bulundu.

Anahtar Sözcükler: Mastitis, *Staphylococcus aureus*, koagulaz negatif stafilokok, identifikasyon, antibiyotik duyarlılık testi

Introduction

The epidemiology of bovine intramammary infections (IMIs) has been characterized worldwide by an increase in the prevalence of Staphylococci. The Staphylococci are the predominant pathogen in subclinical and chronic bovine mastitis. Staphylococci are present as major mastitis pathogens in the dairy industry worldwide (1).

The observed increase in *Staphylococcus aureus* IMIs in many countries could be explained by some common features such as the increased number of bovine per herd as a consequence of the introduction of bovine from other herds and it is very difficult to cure once established in lactating bovine. With *Staphylococcus aureus* mastitis in dairy bovine the resulting damage to developing

mammary tissue can reduce milk production and cause the bovine to fail in reaching her maximum milk production potential. *Staphylococcus aureus* has been demonstrated to rapidly adapt to the changes in environment, management, mastitis control schemes, and obviously to the host (2).

Recently, *Staphylococcus aureus* IMIs have generally been characterized by a long persistence and relatively low frequency of clinical cases (3). *Staphylococcus aureus* is characterized by a variety of pathogenic factors that allow them to damage mammary gland tissues, to escape or impair immune defenses and to neutralize natural or administered antimicrobials (4).

Beside this, coagulase negative staphylococci are increasing in importance as causes of bovine IMIs throughout the world in recent years. Coagulase negative staphylococci have been isolated from milk samples collected from cows with clinical and subclinical mastitis in several countries (5).

Coagulase negative staphylococcus has been previously considered a minor pathogen of bovine mastitis; however, many studies have recently shown the importance of coagulase negative staphylococcus infection in the bovine mammary gland. Several studies indicated that coagulase negative staphylococcus is the most frequently recovered isolate from mastitis samples, especially in first lactation and unbred heifers (6). One study showed that coagulase negative staphylococcus infections caused an 8.7% loss in milk production from a 305-day milk yield total (7).

The virulence factors of staphylococci have been studied most extensively. *Staphylococcus aureus* strains isolated from bovine mastitis have been shown to express alpha, beta, gamma, and delta toxins, leukocidins, enterotoxin, and coagulase (8). Coagulase negative staphylococcus strains also produce several toxins and enzymes that could contribute to virulence, such as hemolysin, leucocidin, lipase, proteases, and DNase. Many coagulase negative staphylococcus strains isolated from mastitis samples have higher protease, DNase, and lecithinase activity than that of coagulase negative staphylococcus from normal cows (9). However, the roles of these enzymes on the pathogenesis of coagulase negative staphylococcus remain unclear.

The literature abounds with information on in vitro antibiotic susceptibility of *Staphylococcus aureus* isolates

from mastitis cases, and many more data on this subject can be found in the yearly reports of veterinary diagnostic laboratories (10). The susceptibility of coagulase negative staphylococcus associated with intramammary infection has been investigated less intensively, although antibiotics can be used successfully in the treatment of these infections in the preparturient period of heifers and multiparous cows and during lactation (11).

Elimination of this organism from dairy herds requires treatment of infected mammary glands with antimicrobial agents and aggressive culling of refractory animals. Several factors impact the effectiveness of antimicrobial therapy against *Staphylococcus aureus* and coagulase negative staphylococcus, including antimicrobial resistance, scar tissue barriers, and sequestering of bacterial cells in polymorphonuclear leukocytes (12). Of these factors, antimicrobial resistance can be most easily circumvented by selecting antimicrobial agents that are not affected by the specific resistance mechanism. β -lactam antimicrobial agents, such as the penicillins and cephalosporins, are often used to treat bovine IMIs (13).

The purpose of this study was to isolate and to identify *Staphylococcus aureus* and coagulase negative staphylococcus strains from bovine IMIs and to determine the antimicrobial susceptibilities of these strains.

Materials and Methods

Bovine Mastitic Milk Samples

Three hundred bovine mastitis milk samples from the mastitic udders determined positive by California Mastitis Test (CMT) (14) were brought to the laboratories of Microbiology Department, Faculty of Veterinary Medicine, Adnan Menderes University, between 1999 and 2002. The milk samples in tubes were transferred in insulated boxes.

Isolation of *Staphylococcus aureus* and coagulase negative staphylococcus

The milk samples were plated out on blood agar plates (Oxoid) with 5% defibrinated ovine blood and incubated at 37 °C for 24-48 h. After incubation, suspected colonies were stained by Gram staining technique and were examined using light microscope. Gram positive cocci were further identified by biochemical tests.

Identification of *Staphylococcus aureus* and coagulase negative staphylococcus

Identification of *Staphylococcus aureus* and coagulase negative staphylococcus was carried out on the basis of colony morphology, Gram staining and biochemical tests. Biochemical characteristics of the isolates were determined by using catalase, hemolysis on blood agar, coagulase, nitrate reduction, DNase agar, clumping factor, arginine dihydrolase, urease and novobiocin resistance tests. In addition fermentation tests, such as production of acid from sucrose, maltose, D-mannitol, D-trehalose, and raffinose were also carried out (15,16).

Antimicrobial Susceptibility of *Staphylococcus aureus* and coagulase negative staphylococcus

The antibiotic susceptibility tests for *Staphylococcus aureus* and coagulase negative staphylococcus strains isolated from mastitis milk samples were carried out by disc diffusion as described by Kirby-Bauer (17), using multidiscs (Oxoid) of amoxicillin + clavulanic acid (20 µg + 10 µg), kanamycin (30 µg), penicillin (10 U), erythromycine (15 µg), ciprofloxacin (5 µg), oxacillin (1 µg), oxytetracycline (30 µg), trimethoprim + sulphamethoxazole (1.25 µg + 23.75 µg), cefquinome (10 µg) and neomycin + bacitracin + tetracycline (30 µg + 10 µg + 30 µg) (Mast Diagnostics). Each isolate [10^5 CFU/ml in 0.1 ml as determined by Kirby-Bauer (17)] was first poured on Mueller Hinton Agar. Then multidiscs were placed on the MH agar plates and then the plates were incubated at 37 °C for 24 h.

Results

A total of 145 (48.33%) samples of 300 bovine mastitis milk samples were positive for Staphylococci.

Eighty-five (28.33%) strains were identified as *Staphylococcus aureus* and 60 (20.00%) strains were isolated as coagulase negative staphylococcus.

Coagulase negative staphylococci strains were identified as 20 (33.33%) strains of *S. hyicus*, 16 (26.66%) strains of *S. chromogenes*, 9 (15.00%) strains of *S. epidermidis*, 5 (8.33%) strains of *S. haemolyticus*, 4 (6.66%) strains of *S. sciuri*, 3 (5.00%) strains of *S. lentis* and 3 (5.00%) strains of *S. cohnii* subsp. *cohnii* (Table 1). The most frequently isolated species was *S. hyicus*, with an isolation rate of 33.53%.

The antibiotic susceptibility test results showed that all *Staphylococcus aureus* and coagulase negative staphylococcus strains were susceptible to neomycin + bacitracin + tetracycline and resistant to penicillin and oxacillin.

The results of the antibiotic susceptibility tests are given in Tables 2 and 3.

Table 1. CNS biotypes isolated in the study.

CNS Biotype	Number of CNS strains	Biotype percentage (%)
<i>S. hyicus</i>	20	33.33
<i>S. chromogenes</i>	16	26.66
<i>S. epidermidis</i>	9	15.00
<i>S. haemolyticus</i>	5	8.33
<i>S. sciuri</i>	4	6.66
<i>S. lentis</i>	3	5.00
<i>S. cohnii</i> subsp. <i>cohnii</i>	3	5.00

Discussion

The *Staphylococcus aureus* is primary pathogen in bovine IMIs. The high prevalence of *Staphylococcus aureus* may be attributed to the wide distribution of the organism inside the mammary gland and in the skin of the teats and udder (12). The Staphylococci have adapted to survive in the udder. They usually establish mastitis

Table 2. Susceptibilities of *Staphylococcus aureus* to various types of antibiotics

Antibiotic Discs	AMC			K			P			E			CIP			OX			OT			SXT			CEQ			NBT		
	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
<i>S. aureus</i>	14	-	71	-	13	72	81	-	4	7	17	60	-	-	85	51	-	34	-	34	51	17	21	47	13	-	72	-	-	85
Total percentage (%)	16	-	84	-	15	85	95	-	5	8	20	72	-	-	100	60	-	40	-	40	60	20	25	55	15	-	85	-	-	100

Table 3. Susceptibility of coagulase negative staphylococcus isolated in the study to various types of antibiotics.

Antibiotic Discs	AMC			K			P			E			CIP			OX			OT			SXT			CEQ			NBT		
	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
<i>S. hyicus</i>	4	-	16	-	5	15	20	-	-	2	2	16	-	-	20	17	-	3	-	4	16	5	2	13	-	-	20	-	-	20
<i>S. chromogenes</i>	4	-	12	-	1	15	16	-	-	4	4	8	-	4	12	11	-	5	-	2	14	4	-	12	-	-	16	-	-	16
<i>S. epidermidis</i>	2	-	7	-	3	6	7	-	2	2	1	6	-	5	4	7	-	2	-	-	9	-	2	7	-	-	9	-	-	9
<i>S. haemolyticus</i>	1	-	4	-	1	4	5	-	-	-	3	2	-	1	4	5	-	-	-	-	5	1	-	4	-	-	5	-	-	5
<i>S. sciuri</i>	-	-	4	-	1	3	2	-	2	-	-	4	-	-	4	4	-	-	-	-	4	2	-	2	-	-	4	-	-	4
<i>S. lentis</i>	-	-	3	-	1	2	2	-	1	-	-	3	-	2	1	-	-	3	-	-	3	-	-	3	-	-	3	-	-	3
<i>S. cohnii</i> subsp. <i>cohnii</i>	1	-	2	-	-	3	2	-	1	-	-	3	-	-	3	-	-	3	-	-	3	-	-	3	-	-	3	-	-	3
Total percentage (%)	20	-	80	-	20	80	90	-	10	13	17	70	-	20	80	73	-	27	-	10	90	20	7	73	-	-	100	-	-	100

AMC : Amoxicillin + Clavulanic acid
 (Amoxicillin 20 µg) (Clavulanic acid 10 µg)
 K : Kanamycin (30 µg)
 P : Penicillin (10 IU)
 E : Erythromycine (15 µg)
 CIP : Ciprofloxacin (5 µg)
 OX : Oxacillin
 OT : Oxytetracyclin (30 µg)
 SXT : Trimethoprim + Sulphamethoxazole (1.25 µg + 23.75 µg)
 CEQ : Cefquinome (10 µg)
 NBT : Neomycin + Bacitracin + Tetracycline (30 µg + 10 µg + 30 µg)

infections and are shed in the milk, which serves as a source of infection for the healthy bovine (18). Radostits et al. (18) asserted that *Staphylococcus aureus* is well adapted to survive in the udder and usually establishes a mild subclinical infection over a long duration.

The role of coagulase negative staphylococcus as a cause of bovine mastitis is not completely clear. Coagulase negative staphylococcus infections are associated with damage to milk secretory tissue of the mammary gland by increased connective tissue stroma, moderate increases in milk and significant production decreases (7).

In general, coagulase negative staphylococci had previously been known to be less pathogenic than the most of other organisms associated with bovine IMIs. However, the coagulase negative staphylococcus is associated with clinical mastitis in heifers. There are indications that *S. hyicus* is more pathogenic than the other species belonging to coagulase negative staphylococci (6), although other reports (19) on bovine mastitis indicate that there are no differences in pathogenicity among different coagulase negative staphylococcus species. However, *S. simulans* and *S. hyicus* were the most frequent isolates of coagulase negative staphylococcus in one study (6), and *S.*

chromogenes and *S. hyicus* were the most frequent isolates in another (20). Several studies (20,21) have identified *S. chromogenes* as the most common species associated with subclinical IMIs in heifers.

In Turkey, there are a lot of studies connected with mastitis. Kaya et al. (22) reported that *Staphylococcus aureus* was isolated from clinic and subclinic mastitic milk samples with an isolation rate of 39.40%. Isolation *Staphylococcus aureus* from bovine IMIs in the Aydin region has been previously reported (23,24). Erganiş et al. (25) isolated 26 out of 55 strains of *Staphylococcus aureus* and 28 out of 55 strains of coagulase negative staphylococcus in the Konya region of Turkey. But there has been no isolation of coagulase negative staphylococcus in the Aydin region to date.

In this study, *Staphylococcus aureus* was the causative bacteria isolated from 28.33% of the 300 milk samples. Some degree of similarity was observed between the results of our study and previous ones (12,22,24). Beside this, in our study, *S. hyicus* 20 (33.33%), *S. chromogenes* 16 (26.66%), *S. epidermidis* 9 (15.00%), *S. haemolyticus* 5 (8.33%), *S. sciuri* 4 (6.66%), *S. lentis* 3 (5.00%) and *S. cohnii* subsp. *cohnii* 3 (5.00%) were also identified.

Some studies worldwide have reported the susceptibility of *Staphylococcus aureus* and coagulase negative staphylococcus to antimicrobial agents from bovine IMIs. In most of these studies, a frequent occurrence of penicillin resistance has been found, whereas there has only been a limited occurrence of resistance to other antimicrobial agents (26). Akar et al. (23) isolated 133 *Staphylococcus aureus* strains in the Aydın region of Turkey, which were found to be resistant to penicillin with a resistant rate of 72.10%. Penicillin-resistance found in our study is in agreement with those which reported coagulase negative staphylococcus in Finland 37.2% (27) and Denmark 36.1% (28).

Major differences in the occurrence of penicillin resistance have also been observed between countries. Thus, previous reports have, as also observed in this study, found very high frequencies of penicillin resistance in the USA, England and Ireland, whereas the occurrence of penicillin resistance in the Scandinavian countries (Denmark, Norway and Sweden) is low (29).

Watts and Yancey (30) have suggested that antimicrobial susceptibility tests with clinical efficacy might be possible for mastitis pathogens that are confined primarily to milk, but it might be difficult for pathogens that invade tissue. The same researchers supported the concept that there is an agreement between results of antimicrobial susceptibility tests and clinical efficacy in short-term (<2 wk duration) *Staphylococcus aureus* IMIs, but not in chronic *Staphylococcus aureus* IMIs, was 70%.

In conclusion, this study showed that *Staphylococcus aureus* could be the predominant organism to cause bovine IMIs in the Aydın region of Turkey. But coagulase negative staphylococcus is emerging as important minor mastitis pathogens and can be the cause of substantial economic losses. The high resistance to penicillin found in this study emphasizes the importance of the identification of coagulase negative staphylococcus when an IMI is present. However, antimicrobial susceptibility patterns should further be identified for *Staphylococcus aureus* and coagulase negative staphylococcus.

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