The aim of this study was to determine the antibiotic resistance of coagulase-negative staphylococci (CNS) isolated from raw buffalo milk, tulum cheese and clotted cream in Afyonkarahisar province of Western Turkey. A total of 210 samples were examined between February 2008 and August 2009; of the samples examined 70 were raw buffalo milk, 70 tulum cheeses, and 70 clotted creams. Thirty four isolates (16.2%) obtained from 210 samples were detected as CNS. Of these isolates 12 (35.3%) were recovered from raw buffalo milk, 14 (41.2%) from tulum cheese and 8 (23.5%) from clotted cream. Kirby-Bauer disc diffusion method was used to detect the antibiotic resistance of CNS isolated from samples according to the standards of Clinical Laboratory Standards Institute. Erythromycin was found to be the least effective antibiotic as 79.4% of CNS isolates recovered from raw buffalo milk, tulum cheese and clotted cream samples were resistant to this drug, followed by amikacin (50%), cefalotine (44.1%), penicillin (38.2%), neomycin (35.3%), streptomycin (35.3%), gentamicin (32.4%) and oxacillin (29.4%).

**Key Words**
Antimicrobial resistance
Buffalo milk
Cheese
Clotted cream
Coagulase-negative staphylococci

**SUMMARY**

Manda sütü ve bazı süt ürünleriinden elde edilen koagulaz negatif stafilokokların antibiotik dirençliliği

Bu araştırmaın amacı, Türkiye’nin Batı illerinden olan Afyonkarahisar’da kaymak, tulum peyniri ve manda sıtından izole edilen koagulaz negatif stafilokokları antibiyotik direncini belirlememesidir. Şubat 2008 ile Ağustos 2009 döneminde 70’i tulum peyniri, 70’i kaymak, 70’i manda sıtı olmak üzere toplam 210 örnek incelenmiştir. Incelenen 210 örnek 34’üden (%16.2) koagulaz negatif stafilokok izolatı edilmiştir. Bu izolatların 12’si (%35.3) manda sıtından, 14’ü (%41.2) tulum peynirindenden, 8’i (%23.5) kaymant izole edilmiştir. Diğer ise izole edilen koagulaz negatif stafilokokların antibiyotik dirençliliğini belirlemek için CLSI’nin (Clinical Laboratory Standards Institute) önerdiği Kirby-Bauer Disk Difüzyon metodu kullanılar. Manda sıtı, tulum peyniri ve kaymak örneklerinden elde edilen izolatların %79,4’üne az etkili antibiyotiklerin eritrosmisin olduğu bulundu. Bunu %50 ile amikasin, %44.1 ile sefalotin, %38.2 ile penisalin, %35.3 ile streptomisin, %32.4 ile gentamisin ve %29.4 ile oksasiller takip etti.
INTRODUCTION

Coagulase-negative staphylococci were isolated from different milk products and raw buffalo milk and screened for their antibiotic resistance.1,3 CNS are emerging as important mastitis pathogens and can be the cause of substantial economic losses.4,5 Animals used for food and animal products may serve as a reservoir for resistant bacteria that may be transmitted to human beings. Worldwide concern over the emergence of resistant bacteria in domestic animals and its influence on human medicine is mostly linked to the use of antibiotics as growth promoters, even though some data may be controversial.6

*Staphylococcus* spp. are common commensals on the skin and mucous membranes of humans and warm-blooded animals. Most species are non-pathogenic and may help prevent colonization of the skin by other potential pathogens.7 They are also isolated from a wide range of foodstuffs such as meat, cheese and milk, and from environmental sources such as soil, sand, air and water. Some species of *Staphylococcus* such as *S. xylosus, S. carnosus* and *S. equorum* are used for flavor and aroma formation during the ripening of fermented foods in the food industry.8

Coagulase-negative *Staphylococcus* (CNS) spp. have traditionally been considered to be non-pathogen for a long time. However, this view is changing, because there has been increasing evidence that some CNS species have become predominant pathogens isolated from human and animals in several countries.4,9-11 It has been reported that some CNS species can present a medical risk. For example *S. saprophyticus*, which may contribute to aroma formation in sausage12 and help prevent off-flavor during sausage ripening,13 is an ubiquitous species and is also involved in acute urinary tract infections in young adult women.10 In recent years, they have also been isolated from animals with subclinical and occasional clinical mastitis and their importance on mastitis has been emphasized in many countries.4,9,14-16 CNS are thought to be a reservoir for resistance genes amplified through antibiotic selection that occurs when antibiotics administered to animals achieve low concentrations on the skin.17 Resistant bacteria on animals’ bodies such as *S. xylosus, S. equorum, S. capras, S. sciuri* and *S. capitis* may contaminate milk or meat and be subsequently isolated from fermented foods made from raw products. The resistance genes might in some instances be transferred from Staphylococci of animal origin to *Staphylococcus* that cause infections in humans. Thus, antimicrobial treatment may be ineffective for several Staphylococcal infections.8 In human medicine, antimicrobial multi-resistance is frequently encountered and methicillin-resistant *S. aureus* (MRSA)18,19 and methicillin-resistant CNS (MR-CNS)11,20 strains are among the most threatening bacteria involved in nosocomial infections. MRSA as well as multi-resistant *S. aureus* strains and MR-CNS are also reported in veterinary medicine.4,21 Although penicillinase or β-lactamase resistant penicillins such as methicillin and oxacillin are not used in veterinary medicine in Turkey except for cloxacillin, methicillin-resistant *Staphylococci* have been important because of their resistance to all other beta-lactam antibiotics.22,23

Afyonkarahisar is located in the western region of Turkey and is becoming significant as a breeding area of water buffaloes and dairy cows owing to suitable conditions. The milk and milk products such as tulum cheese and clotted cream obtained from these animals are the sought-after Turkish products. Tulum cheese is one of the most popular semi-hard cheeses in Turkey, and is produced from raw sheep, goat or cow milk. In Turkey, although there were several investigations on the antibiotic resistance or susceptibility of CNS isolated from mastitic milk samples,16,24 the studies on the isolation and antibiotic resistance of CNS isolated from milk products are limited.25 For this reason, in this study it was aimed to detect the antibiotic resistance of CNS isolated from raw buffalo milk, tulum cheese and clotted cream in Afyonkarahisar.

MATERIALS and METHODS

Collection of Samples

A total of 210 samples were examined between February 2008 and August 2009 in Afyonkarahisar, Turkey. Of the samples examined 70 were raw buffalo milk, 70 tulum cheeses, and 70 clotted creams. Raw buffalo milk samples were obtained from various private milk producers. The other samples were obtained from public bazaars in various regions of Afyonkarahisar. Ten samples of each specimens were collected every month. The samples of each specimens were collected every month. The samples were aseptically transported to laboratory under <10°C.
Isolation of CNS from Samples

Ten grams of each sample were added to 90 mL peptone water and homogenized. An inoculation from each of the dilutions was streaked onto Baird-Parker agar (Oxoid CM 0275) supplemented with egg yolk telluride emulsion (Oxoid SR 0054) and plates were incubated at 37°C for 24 – 48 hours. The moist, smooth, spherical, convex, well-defined contours and grey-black colonies were counted and expressed in CFU/mL. The five typical colonies were subcultured in blood agar containing 7% sheep blood and the plates were aerobically incubated at 37°C for 24 h. After the incubation, Gram staining, catalase test, glucose oxidation fermentation test and tube coagulase test with rabbit plasma were performed for colonies growing on blood agar. Gram positive, catalase positive and coagulase negative isolates were identified as CNS.

Antimicrobial Susceptibility Test

Kirby-Bauer disc diffusion method was used to detect the antibiotic resistance of CNS isolated from samples according to the standards of Clinical Laboratory Standards Institute (CLSI). Pure colonies from the blood agar, incubated 37°C for 18 h, were suspended in 2 mL sterile saline to a density approximately equal to McFarland opacity No. 0.5. A dry cotton wool swab was placed into the suspension and excess liquid was expressed against the inside of the tube. The bacterial suspension was then inoculated onto Mueller-Hinton agar (Oxoid CM0337) with the swab in such a way that the whole surface of the agar was covered. The plates were incubated at 37°C for 24 h. The results were recorded by measuring the diameter of the zone of inhibition according to the interpretive standards of CLSI. The following 14 antimicrobial agents were tested: erythromycin (15 µg), neomycin (10 µg), gentamicin (10 µg), tetracycline (10 µg), cefotaxime (30 µg), amikacin (30 µg), cefalotin (30 µg), penicillin (10 µg), oxacillin (1 µg), vancomycin (30 µg), streptomycin (10 µg), ampicillin (10 µg), chloramphenicol (30 µg) and clindamycin (2 µg). Beta lactamase activities of isolates were also examined by using nitrocefin test sticks (Oxoid BR 66 A) as described by the manufacturer.

RESULTS

In the present study, a total of 34 isolates (16.2%) obtained from 210 samples were detected as CNS. Of these isolates 12 (35.3%) were recovered from raw buffalo milk, 14 (41.2%) from tulum cheese, and 8 (23.5%) from clotted cream. A number of isolates recovered from samples and the counts of CNS in the examined food are shown in Table 1.

Thirty four CNS isolates were assayed for antimicrobial resistance using standard disc diffusion test and interpreted via CLSI guidelines. CNS isolates recovered from raw buffalo milk samples showed the highest in vitro resistance rate to erythromycin (75%), followed by neomycin (50%), amikacin (41.7%), cefalotin (33.3%) and oxacillin (33.3%). Similarly, erythromycin was found to be the least effective antibiotic as 92.9% of CNS isolates recovered from tulum cheeses were resistant to this drug, followed by amikacin (64.3%), cefalotin (64.3%), penicillin (64.3%), and streptomycin (50%). The highest in vitro resistance rate for 8 CNS isolates recovered from clotted creams was detected against erythromycin (62.5%), followed by neomycin (37.5%), amikacin (37.5%), gentamicin (37.5%) and streptomycin (37.5%). None of the 8 CNS isolates recovered from clotted creams showed resistance to vancomycin and ampicillin.

The antibiotic resistance rates of CNS isolated from raw buffalo milk, tulum cheese and clotted cream are detailed in Table 2. None of the isolates recovered from samples showed beta lactamase activity.

Table 1. CNS count in the examined food samples

<table>
<thead>
<tr>
<th>Samples</th>
<th>No. of samples</th>
<th>No. of isolates (%)</th>
<th>CNS count (cfu/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw buffalo milk</td>
<td>70</td>
<td>12 (33.3)</td>
<td>1.7x10⁴</td>
</tr>
<tr>
<td>Tulum cheese</td>
<td>70</td>
<td>14 (41.2)</td>
<td>2.2x10⁴</td>
</tr>
<tr>
<td>Clotted cream</td>
<td>70</td>
<td>8 (23.5)</td>
<td>1.0x10⁴</td>
</tr>
<tr>
<td>Total</td>
<td>210</td>
<td>34 (16.2)</td>
<td>1.3x10⁴</td>
</tr>
</tbody>
</table>
This study investigated the antibiotic resistance of CNS isolated from raw buffalo milk, tulum cheese and clotted cream in Afyonkarahisar. The studies related to isolation of CNS have been focused on mastitic milk samples due to an increase in udder infections caused by CNS recently. The literature often refers to CNS as minor mastitis pathogens, which suggest that CNS are non-pathogenic or mildly pathogenic. However, CNS are frequently isolated as a significant cause of subclinical mastitis and they cause the increase in the somatic cell counts of infected quarters. CNS are also known to cause clinical mastitis and persistent IMI (Intramammary Infections) that can last for several months during lactation in the absence of intervention.

The different isolation rates were reported on CNS species related with mastitis in various countries. Gentilini et al., reported that isolation rate of CNS from 900 bovine milk samples was 13.6% in Argentina. In a study from Germany, 35% of quarters with subclinical mastitis harbored CNS. In Tennesse, USA, the average proportion of CNS infections in high SCC herds was 28%, and herd prevalence ranged from 12% to 41%. The highest prevalence of intramammary infections (IMI) with CNS was reported in Finland, where CNS were isolated from 50% of the quarters positive for bacterial growth in a nationwide survey. In Turkey, the isolation rates of CNS were reported as 20.6% from buffalo milk samples and 20% from bovine milk samples. Despite several investigations on the isolation of CNS from mastitic milk samples, the reported studies on the isolation of CNS from milk products are limited. It was reported by Cunha et al., that a total of 88 different food samples were analyzed and 22.7% were positive for CNS in Brazil. They also reported that isolation rates of CNS from milk and white cheese samples were 10% and 5%, respectively. In a study from Turkey, the isolation rates of CNS were reported as 76.2% from milk and 78.8% from cream samples. In the present study, a total of 34 isolates (16.2%) obtained from 210 samples were detected as CNS. Of these isolates 12 (35.3%) were recovered from raw buffalo milk, 14 (41.2%) from tulum cheese, and 8 (23.5%) from clotted cream. The regional variations and number samples analyzed may be the reason for inconsistent isolation rates.

Several susceptibility or resistance studies have been conducted on CNS isolated from mastitic milk samples. Generally, it was reported that CNS isolates used in these studies showed a high level of resistance to penicillin, erythromycin, ampicillin, cloxacillin and oxacillin. However, the investigations on the antibiotic susceptibility or resistance of CNS isolated from milk products are limited. Some studies showed that Staphylococci from cheese, mostly identified as S. xylosus strains, harbored resistance to chloramphenicol, tetracycline, erythromycin and, in lower proportions, to gentamycin, penicillin, lincomycin and kanamycin. A study carried out to the antibiotic resistance of CNS associated with food and used in starter cultures, emphasized that resistance to lincomycin, penicillin, fusidic acid, oxacillin, ampicillin and tetracycline were predominant.

In our study, erythromycin was found to be the least effective antibiotic as 79.4% of CNS isolates recovered from raw buffalo milk, tulum cheese and clotted cream samples were resistant to this drug, followed by amikacin (50%), cefalotin (44.1%), penicillin (38.2%), neomycin (35.3%), streptomycin (25.3%).

### DISCUSSION

**Table 2. Results of CNS isolates obtained from food samples**

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Buffalo milk (n=12 isolates)</th>
<th>Tulum cheese (n=14 isolates)</th>
<th>Clotted cream (n=8 isolates)</th>
<th>Total (n=34 isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td><strong>Erythromycin (15µg)</strong></td>
<td>3</td>
<td>9</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td><strong>Neomycin (10µg)</strong></td>
<td>6</td>
<td>50</td>
<td>11</td>
<td>78</td>
</tr>
<tr>
<td><strong>Gentamicin (10µg)</strong></td>
<td>10</td>
<td>83</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td><strong>Tetracycline (10µg)</strong></td>
<td>11</td>
<td>91</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td><strong>Cefotaxime (30µg)</strong></td>
<td>11</td>
<td>91</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td><strong>Amikacin (30µg)</strong></td>
<td>8</td>
<td>68</td>
<td>6</td>
<td>41</td>
</tr>
<tr>
<td><strong>Cefalotin (30µg)</strong></td>
<td>7</td>
<td>58</td>
<td>5</td>
<td>41</td>
</tr>
<tr>
<td><strong>Penicillin (10µg)</strong></td>
<td>10</td>
<td>83</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td><strong>Oxacillin (1µg)</strong></td>
<td>8</td>
<td>66</td>
<td>4</td>
<td>33</td>
</tr>
<tr>
<td><strong>Vancomycin (30µg)</strong></td>
<td>10</td>
<td>83</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td><strong>Streptomycin (10µg)</strong></td>
<td>10</td>
<td>83</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td><strong>Ampicillin (10µg)</strong></td>
<td>10</td>
<td>83</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td><strong>Clindamycin (2µg)</strong></td>
<td>10</td>
<td>83</td>
<td>2</td>
<td>16</td>
</tr>
</tbody>
</table>

**S**: Susceptible; **R**: Resistant
cin (35.3%), gentamicin (32.4%) and oxacillin (29.4%). These results were in agreement with previous studies, especially, in terms of resistance to erythromycin, penicillin, gentamicin and oxacillin. However, the rate of resistance to some antimicrobials showed inconsistency with other similar studies. This may be linked to many factors such as the differences in animal production systems and national policies for the use of antimicrobial drugs in each country. In the present study, the resistance to oxacillin was also remarkable. Although penicillinase or β-lactamase resistant penicillins such as methicillin and oxacillin are not used in veterinary medicine in our country except for cloxacillin, methicillin-resistant Staphylococci have been important because of their resistance to all other beta-lactam antibiotics.22,23 Some investigations on CNS isolated from different food products showed that some CNS strains produce enterotoxin.39,3 According to our knowledge, no CNS isolated from milk or dairy products have ever been involved in food poisoning or human pathology cases after ingestion of dairy products. Nevertheless, a possible clinical significance of some species should not be disregarded in patients with depressed immune systems, long, severe hospital treatments, or in the presence of an indwelling catheter or foreign materials. Some researchers also emphasized that resistance genes might in some instances transfer from Staphylococci of animal origin to Staphylococci that cause infections in humans. Thus, CNS could represent a natural reservoir for antibiotic resistance genes.17,37,40 The demand on milk and milk products has increased in our region recently owing to suitable conditions for breeding buffalo and dairy cows. Considering the antibiotic resistance and enterotoxin production of some CNS strains isolated from food samples, their importance should not be disregarded for human, especially immunocompromised patients.

**KAYNAKLAR**


