Enterotoxin Producing Ability And Antimicrobial Susceptibility Of Coagulase-Negative Staphylococci Isolated From Goat Milk, Cheese And Salted Yoghurt In Turkey

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Abstract: The aim of this study was to determine enterotoxin producing ability and antimicrobial susceptibility of coagulase negative staphylococci (CNS) in goats' bulk milk, cheese and salted yoghurt. CNS strains were identified by using GP card in VITEK 2 system. The presence of enterotoxins was determined by enzyme immunoassay test by using RIDASCREEN test kit. Antibiotic susceptibility in CNS strains was detected by using AST-P640 card in VITEK 2 system. A total of 100 CNS strains were isolated in 22 (55%) bulk milk samples and in 23 (57.5%) cheese samples. Staphylococcus spp. could not be isolated from salted yoghurt samples. The most encountered species were S. caprae (51.9%), S. chromogenes (11.5%) and S. xylosus (9.6%) from milk samples and S. saprophyticus (60.4%), S. xylosus (12.5%) and S. haemolyticus (8.3%) from cheese samples. Four CNS strains (4%) isolated from samples were capable of producing enterotoxin. While all isolates were resistant to at least one antibiotic, 74% of CNS strains showed resistance to two or more antibiotics. Enterotoxin production ability and high antibiotic resistance of the CNS strains isolated from goat bulk milk and cheese can lead to a risk for public health.

Keywords: Antimicrobial susceptibility, Coagulase-negative staphylococci, Enterotoxin, Goat milk.

1 INTRODUCTION

Staphylococci can be found in humans, animals and environmental sources such as soil, air and water and isolated from various foodstuffs including fermented meat and cheese [1], [2]. Enterotoxigenic staphylococci can be carried by humans, animals, kitchen utensils, pests, and the foods. However, the food handlers play important role in contamination of foods with staphylococci [3] and in the epidemiology of staphylococcal food poisoning outbreaks [2], [4], [5]. The staphylococcal contamination of foods can occur at different stages from the manufacture to the consumption [6]. The most contaminated foods with enterotoxigenic staphylococci included frequently foods of animal origin such as meat, poultry or their products, fish, shellfish, salads and milk products such as milk, cream, butter and cheeses [4], [5], [7]. In general, these bacteria can contaminate also meat and dairy products if the raw materials are not pasteurized, or if recontamination of foodstuffs occurs after heat treatment [8]. It is reported by De Luca et al. [6] that Staphylococcus aureus was isolated from foods mostly during the hot months, while the other most common species S. epidermidis, S. hominis, S. xylosus were found mostly in the period between October-March. Some coagulase negative staphylococci (CNS) species such as S. xylosus and S. carnosus are considered as food-grade by some fermented meat and milk products. However, their presence or use in foods is a source of concern for human health due to their increasing clinical significance [9], because they are opportunistic pathogens in certain clinical situations in human and animals [1], [10]. Staphylococcal food poisoning is one of the most common bacterial food poisonings in the world [5], [7] and result from absorption of enterotoxins (SEs) produced by enterotoxigenic staphylococci, especially from Staphylococcus aureus in the foods consumed by human [5], [11], [12]. It is reported that milk and dairy products are also among the foodstuffs frequently involved in staphylococcal food poisoning [13]. Staphylococcal enterotoxins are produced not only by the coagulase positive staphylococci (CPS) but also by some CNS species [5], [14]. However, a food poisoning outbreak caused by only CNS strains was not reported so far [1], [9]. The inappropriate and / or excessive use of antimicrobial agents cause a considerable concern in public health due to the development of multiresistant strains [15]. Foods are an important factor for the spread of antibiotic resistance. Such spread of antimicrobial resistance can occur by means of antibiotics residues in foods, through the carriage of resistant foodborne bacteria or through the ingestion of resistant microflora strains of food and resistance transfer to pathogens [12]. CNS species tend to be more resistant to various antimicrobial agents than S. aureus and they can acquire easily ability of multiresistance [16]. It is even reported that CNS strains in contaminated foods are frequently resistant to one or more antibiotics [9], [17], and they could be also reservoirs of antibiotic resistance gene spreading [9]. Multiple antibiotic resistant S. aureus and CNS species such as S. xylosus, S. lentus, S. caprae, S. epidermidis, and S. haemolyticus have been reported earlier in foodstuffs [8]. It was previously reported that the widespread distribution of methicillin resistant CNS species and genetic transfer between CNS and S. aureus are a source of considerable concern [10]. That the goat milk represents 2% of the total milk production (18.223.712 tons) in Turkey was reported [18]. Goat milk is generally used for

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the production of traditional dairy products such as various cheese types and salted yoghurt in Turkey. Salted yoghurt, also called as "winter yoghurt", has a long shelf-life and is manufactured by boiling the yoghurt and adding salt. CNS are mainly the predominant causative agent in subclinical mastitis in goats [19]. There is a risk of using milk obtained from animals infected with subclinical mastitis in manufacture of dairy products. Also, it was stated that goats are an important reservoir for staphylococci [20]. Therefore, it is need the determination of presence, enterotoxin producing ability and antibiotic susceptibility of CNS in goats' dairy products in order to ensure food safety and protect consumer health. To our knowledge, there is no study conducted on this subject in Turkey. The objective of this study was to determine the presence, enterotoxin producing ability and antibiotic susceptibility of CNS isolated from goats' bulk milk, cheese and salted yoghurt manufactured from goat milk.

2 MATERIALS AND METHODS

2.1 Sampling

The study was carried out using a total of 100 samples obtained from different dairy plants and local retail markets in Hatay, Turkey, of which 40 samples were raw goats' bulk milk, 40 non-ripened white cheese and 20 salted yoghurt manufactured from goat milk. The samples (approx. 200 mL or g) were transported to the laboratory in sterile plastic bags under refrigerated conditions and then the microbiological analysis of the samples was undertaken no later than two hours after arrival.

2.2 Isolation and identification of CNS

10 g of each sample was taken in aseptic conditions and homogenized with 90 mL sterile peptone water using a Lab-Blender 400 Stomacher (Interscience, France) for approximately 2 min. Decimal dilutions were prepared from this homogenate. Each dilution was plated on the Baird Parker Agar in duplicate and incubated for 18-24 h at 37 °C. All typical black gray, bright, convex colonies growing on Baird Parker Agar were transferred to Brain Heart Infusion Broth and inkubated for 18-24 h at 37 °C. Then, Gram staining, catalase, oxidase activity and the coagulase test was performed [21]. Gram positive, catalase positive, oxidase negative and coagulase negative colonies were identified by using Gram positive (GP) identification card (BioMerieux GP 21342, France) in VITEK 2 automated system.

2.3 Determination of enterotoxin

5 g of each solution enriched in Brain Heart Infusion Broth was centrifuged at 5000 rpm for 15 min and filtered through sterile filters (0.2 µm, Sartorius CE-0297). 100 µl of each extracts was used for the analysis of staphylococcal enterotoxin. The presence of staphylococcal enterotoxins was determined by enzyme immunoassay test by using RIDASCREEN SET A, B, C, D, E (R-Biopharm AG, R.4101, Germany) according to the manufacturer's instructions.

2.4 Antibiotic susceptibility tests of CNS

3 ml of sterile saline (%0.45-0.50 NaCl, pH 4.5-7.0) was transferred to a transparent plastic system-specific test tube (12x75 mm) and pure colonies growing on Blood Agar after enrichment in Brain Heart Infusion Broth were transported through a loop to the tubes. Thus, it was obtained a homogeneous bacterial suspension equivalent to a 0.5 McFarland density. Antibiotic susceptibility in staphylococci strains was detected by using AST-P640 card (BioMerieux, AST-P640, 418579, France) in VITEK 2 automated system and evaluated their susceptibilities to 17 antibiotics according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2016.

3 RESULTS AND DISCUSSION

The distributions of the staphylococci strains identified from the samples were given in Table 1. 52 CNS strains were isolated from 22 of 40 (55%) goats' raw milk samples and 48 CNS strains from 23 of 40 (57.5%) goat cheese samples. Staphylococci could not be isolated from salted samples. Totally, 14 species of CNS were identified from a total of 80 milk and cheese samples. More than one species or strains were isolated from some samples in the present study. The most commonly isolated CNS strains were S. caprae (51.9% of the strains from 35% of the samples), S. chromogenes (11.5% of the strains from 12.5% of the samples) and S. xylosus (9.6% of the strains from 10% of the samples) in the milk samples and S. saprophyticus (60.4% of the strains from 40% of the samples), S. xylosus (12.5% of the strains from 10% of the samples) and S. haemolyticus (8.3% of the strains from 5% of the samples) in cheese samples (Table 1).

Table 1: *The distributions of the CNS species isolated from the goat milk and cheese samples*

CNS species isolated	Milk samples (n= 40)				Cheese samples (n= 40)				Total			
	No. of samples	% of samples	No. of strains	% of strains	No. of samples	% of samples	No. of strains	% of strains	No. of samples	% of samples	No. of strains	% of strains
S. Xylosus	4	10	5	9.6	4	10	6	12.5	8	10	11	11
S. chromogenes	5	12.5	6	11.5	-	-	-	-	5	6.3	6	6
S. haemolyticus	2	5	4	7.7	2	5	4	8.3	4	5	8	8
S. epidermidis	1	2.5	1	1.9	-	-	-	-	1	1.3	1	1
S. Caprae	14	35	27	51.9	2	5	2	4.2	16	20	29	29
S. saprophyticus	1	2.5	2	3.8	16	40	29	60.4	17	21.3	31	31
S. Capitis	1	2.5	1	1.9	-	-	-	-	1	1.3	1	1
S. Sciuri	1	2.5	1	1.9	1	2.5	1	2.1	2	2.5	2	2
S. Warneri	1	2.5	1	1.9	-	-	-	-	1	1.3	1	1
S. Hominis	2	5	2	3.8	-	-	-	-	2	2.5	2	2
S. Simulans	1	2.5	1	1.9	1	2.5	1	2.1	2	2.5	2	2
S. Cohnii	1	2.5	1	1.9	-	-	-	-	1	1.3	1	1
S. Carnosus	-	-	-	-	2	5	2	4.2	2	2.5	2	2
S. Equorum	-	-	-	-	3	7.5	3	6.3	3	3.8	3	3
Total	-	-	52	100	-	-	48	100	-	-	100	100

Table 2: *The distributions of enterotoxigenic CNS strains and the types of enterotoxins*

		Raw milk samples (n=40)	Cheese samples (n=40)	Total	SE-producing CNS species
No. of CNS positive samples (%)		22 (55%)	23 (57.5%)	45 (56.3%)	
No. of CNS strains (%)		52	48	100 (100%)	
No. of E-CNS* positive samples (%)		3 (7.5%)	1 (2.5%)	4 (5%)	
No. of enterotoxigenic CNS strains (%)		3 (5.8%)	1 (2.1%)	4 (4%)	
Type of enterotoxins	A	1	-	1 (1%)	S. chromogenes
	B	-	1	1 (1%)	S. sciuri
	C	1	-	1 (1%)	S. hominis
	D	1	-	1 (1%)	S. chromogenes

**Enterotoxigenic CNS.*

Table 3: *The distributions of the resistant CNS strains according to antimicrobial agents*

Antibiotics	Milk samples		Cheese samples		Total	
	No. of resistant strain	%	No. of resistant strain	%	No. of resistant strain	%
Oxazolidine	-		2	4.1	2	2
Penicillin	-		-		-	
Ampicillin	-		-		-	
Oxacillin	3	5.7	11	22.9	14	14
Gentamicin	-		-		-	
Ciprofloxacin	1	1.9	1	2.0	2	2
Erythromycin	8	15.3	5	10.4	13	13
Clindamycin	6	11.5	2	4.1	8	8
Linezolid	-		2	4.1	2	2
Daptomycin	-		1	2.0	1	1
Teicoplanin	2	3.8	-		2	2
Vancomycin	-		-		-	
Tetracycline	9	17.3	6	12.5	15	15
Tigecycline	-		-		-	
Fosfomycin	51	98	42	87.5	93	93
Fucidic acid	35	67.3	43	89.5	78	78
Trimethoprim /Sulfamethoxazole	-		-		-	

Table 4: *The distributions of multiple antimicrobial resistant CNS strains*

Antibiotic Numbers	Milk Samples		Cheese Samples		Total	
	No. of resistant strains	%	No. of resistant strains	%	No. of resistant strains	%
1	17	32.6	9	18.7	26	26
2	20	38.4	20	41.6	40	40
3	7	13.4	13	27.0	20	20
4	6	11.5	5	10.4	11	11
6	1	1.9	-	-	1	1
7	1	1.9	1	2.0	2	2
Total CNS Strains	52	100	48	100	100	100

A total of 4 (4%) strains of CNS were capable of enterotoxin producing, of which three were isolated from the goat milk samples and one from one cheese sample (Table 2). All CNS strains isolated from the goats' milk and the cheese samples were susceptible to ampicillin, penicillin, gentamicin, vancomycin, tigecycline and trimetoprim / sulfamethoxazole, but resistant to at least one of the remaining eleven antibiotics. The highest resistance in the CNS strains was detected to fosfomycin and fusidic acid (93% and 78%), respectively. Antibiotic resistances of the CNS strains were given in Table 3. All of the CNS strains showed resistance to at least one antibiotic, while 74% of the strains were resistant to two or more antibiotics (Table 4). Staphylococcal foodborne poisoning following ingestion of food contaminated with SEs is one of the most important food-borne illnesses [7]. Although no cases reported associated with CNS in food poisoning or human pathology resulting from consumption of foods [1], there are many studies reporting that some CNS strains isolated in foods are enterotoxin producers [14], [17], [22], [23] and the ability of CNS to produce enterotoxin in food poisoning cannot be excluded [14], [22], [24]. In a study, CNS strains were isolated from 11 different types of cheese samples (n = 72) produced from cow, goat and sheep milk [25]. The author found 17 (23.6%) cheese samples to be positive for CNS. The isolated CNS strains were belong to 8 species consisting of S. saprophyticus (35.3%), S. epidermidis (17.6%), S. hominis (11.8%), S. haemolyticus (11.8%), S. xylosus (5.9%), S. vitulinis (5.9%), S. lentus (5.9%), and S. warneri (5.9%). In the present study, CNS strains consisting of 8 species were isolated from 57.5% of the goat cheese samples. The most ecountered CNS strains in the cheese samples were S. saprophyticus (60.4%), S. xylosus (12.5%) and S. haemolyticus (8.3%) (Table 1). The contamination rate of the cheese samples with CNS in our study is higher than the result reported by Kürekci [25]. The reason for this difference may be the difference between the cheese types manufactured by different production procedures and cheeses manufactured from milk obtained from animals in different species such as cow, goat and sheep in the study mentioned above. Valle et al. [20] also reported that goats are an important reservoir for staphylococci. The high prevalence of CNS in goats' milk and cheese obtained from our study is supportive to the findings of the authors mentioned. In another study [6], the most commonly ecountered species of CNS in 135 various cheese samples were S. epidermidis (14.8% of the strains from 45.9% of the samples), S. hominis (19.5% of the strains from 45.2% of the samples), S. xylosus (19.2% of the strains from 44.4% of the samples) and S. cohnii (16.3% of the strains from 37.8% of the samples). While the most common species of CNS in cheese samples vary in comparison to the results of De Luca et al. [6], the prevalence of the CNS determined in the present study is comparable. However, Ruaro et al. [26] determined S. equorum (12%), S. lentus (12%), S. simulans (12%), S. sciuri (10%), and S. xylosus (9%) as the most ecountered CNS species in raw milk and cheeses produced in North Italy. It is reported that S. xylosus and S. equorum were the two main species encountered most frequently in some fermented foods such as cheese and dry sausage, whereas S. epidermidis and S. saprophyticus were clinical and food-related isolates [9]. The 142 CNS strains isolated from the hands of the healthy food handlers in a study of Udo et al. [22] consisted of 41 strains of S. hominis (28.8%), 36 S. warneri (25.3%), 17 S. saprophyticus (11.9%), 17 S. xylosus (11.9%), 12 S. schleiferi (8.4%), 6 each of S. epidermidis and S. haemolyticus (4.2%), 2 S. cohnii (1.4%) and one each of S. capitis, S. intermedius and S. lentus. These differences in the results may be due to the samples with different sources of contamination, animal species, and applied measures of milking and production hygiene. Staphylococcus spp. could not be isolated from salted yoghurt samples manufactured from goat milk in the present study. This result may be accounted for by the acidity of yoghurt, heat treatment applied during production and the salt added in yoghurt. It has been reported that yoghurt received the least attention among dairy products due to its high acidity and milk pasteurization which are effective barriers to the growth of pathogens [27]. That some strains of CNS such as S. chromogenes, S. haemolyticus, S. warneri, S. epidermidis, S. caprae, S. xylosus, S. sciuri, S. saprophyticus, and S. lentus are enterotoxin producers is reported [6], [20]. In a study by Valle et al. [20] on enterotoxin production by staphylococci isolated from skin, nasal mucosa and milk samples obtained from healthy goats were found that 74.3% of CPS and 22% of CNS strains were enterotoxigenic. Enterotoxin A, B and C were detected in milk samples obtained from 17 of 133 healthy goats in mentioned study. The authors stated that the most commonly detected SE is enterotoxin C produced as alone by S. chromogenes or combined with other SEs produced by S. chromogenes, S. caprae, S. xylosus, S. sciuri, S. saprophyticus, S.lentus, S. warneri, S. epidermidis, and S. haemolyticus. In another study Vernozy-Rozand et al. [23] was detected that 5.3% of 187 CNS strains isolated from goats' milk and cheese samples as enterotoxigenic. Among the CNS species, S. simulans, S. equorum, S. capitis, S. lentus and S. xylosus produced enterotoxin E in mentioned study. In a study by Udo et al. [22], 8% of the CNS from the hands of the healthy food handlers produced SE. Çepoğlu et al. [28] determined also that one of 85 CNS strains isolated from the hands of food handlers in different restaurants was SE producer. In the present study, 4% of isolated CNS strains were capable of producing toxin (Table 2). This result found in our study is similar to the result by Vernozy-Rozand et al. [23], higher than that reported by Çepoğlu et al. [28], but lower than those obtained by Valle et al. [20] and Udo et al. [22] mentioned above. In the present study, two strains of S. chromogenes and one S. hominis strains isolated from three milk samples produced enterotoxin A, D and C, respectively. Also, S. sciuri strain isolated from cheese sample was capable of producing enterotoxin B in our study. In contrast to the results of Valle et al. [20], enterotoxin E could not be detected from the milk and cheese samples in the present study. However, CNS isolated from 121 different foodstuffs including goats' cheeses produced none of SEs in another study [29]. The differences in these results may be due to the ability of CNS to produce SE or different sources of the samples from which CNS strains were isolated. That the most encountered SEs involved in staphylococcal food poisoning are enterotoxin A and D among strains from human sources is reported [4], [7], which were detected also in our study. Udo et al. [22] reported that CNS species producing SEs can co-exist with enterotoxigenic S. aureus and it is possible that enterotoxigenic CNS contribute to food poisoning. Therefore, the results obtained from our study indicate that the goats' dairy products contaminated with CNS may possess a potential risk for consumer health, if they contain enough enterotoxin. Cold storage of foods and education of consumer, food handler and processor are essential in regard of staphylococcal food poisoning prevention [11]. However, it is emphasized that the overall incidence of safety hazards was low in food-related CNS, although the significant strain to strain variations were observed in foodstuffs including dairy products [9], [26]. Also, it is ambigous that CNS isolated from milk or dairy products have been clearly implicated in food poisoning [1], [14]. Antibiotic resistance in staphylococci occurs through plasmids which can move from one species to another [30]. Therefore, this capability of staphylococci and the use of antibiotics in the treatment of animals may result in the increase in resistant strains to antimicrobial agents among bacteria [31]. In recent years, the multiresistant bacteria resulting from increasing inappropriate and / or excessive use of antimicrobial agents cause a concern in public health in the world [15]. All of the CNS strains isolated in the present study were found to be susceptible to ampicillin, penicillin, gentamicin, vancomycin, tigecycline and trimetoprim / sulfamethoxazole, but to be resistant to at least one of the remaining eleven antibiotics. The highest resistance in CNS strains identified from milk and cheese samples in our study was detected against fosfomycin and fusidic acid (93% and 78%), respectively (Table 3). The CNS strains isolated from cheese samples in the study of Kürekci [25] also were resistant to at least one antibiotic, which is in agreement with our result. In mentioned study, while all CNS strains were found to be susceptible to vancomycin, streptomycin, linezolid, and gentamycin, the resistance in CNS strains was found for penicillin (76.5%), erythromycin (35.3%), tetracycline (29.4%), and trimethoprim-sulfamethoxazole (17.6%). Udo et al. [22] reported that all CNS (142 strains) isolated from the hands of healthy food handlers in Kuwait were susceptible to vancomycin, teicoplanin, rifampicin, gentamicin, streptomycin and ciprofloxacin, but 21.1% of strains were resistant to tetracycline, 7.0% to methicillin, 6.3% to chloramphenical, 4.2% to trimethoprim, 3.5% to erythromycin, 2.1% to mupirocin. Our results obtained for gentamicin, vancomycin, tetracycline, trimethoprim, erythromycin and teicoplanin are comparable with the results reported by Udo et al. [22]. Çepoğlu et al. [28] reported that CNS strains isolated from the hands of food handlers were susceptible to vancomycin, methicillin and gentamicin, which is in agreement with our result. Even et al. [9] evaluated the antibiotic resistance in four food-related CNS species (S. epidermidis, S. saprophyticus, S. xylosus and S. equorum) isolated from various environments including cheese and dry fermented sausages. In their study, a similar result about resistance to erythromycin was found, but resistance to tetracyclin in all CNS species and resistance over 30% to penicillin in S. epidermidis and S. xylosus species were determined in contrast to our results. The differences in resistance to antibiotics may be due to the use frequency of antibiotics in the treatment of animals, animal species, sources of the CNS isolation and the ability of CNS strains to transfer of antibiotic resistance determinants. All of the CNS strains were resistant to at least one antibiotic and 74 of the 100 CNS strains isolated from milk and cheese samples showed resistance to two or more antibiotics according to the obtained results in the present study (Table 4). Moura et al. [15] found also that 64.6% of CPS and 76.5% of CNS isolated from ready-to-eat sashimi, a traditional Japanese speciality, were resistant to two or more antibiotic agents, which is similar to our results. Also, rate of multi-resistant CNS strains to three or more antimicrobial agents was found to be 18.3% in study of Udo et al. [22]. However, all 77 CNS strains isolated from raw milk and cheese was found to be negative for multiple antibiotic resistances in the study by Ruaro et al. [26].

4 CONCLUSION

In conclusion, the goat bulk milk and cheese manufactured from goat milk examined in this study poses a potential risk for consumer health due to the enterotoxigenic CNS and / or multiresistance ability of CNS strains, because some traditional cheeses are manufactured from goat milk without heat treatment. Therefore, it is necessary to improve the hygienic efficiency in milking conditions and manufacture of dairy products and cold chain measures after the milk pasteurization process in order to prevent the staphylococcal contamination and ensure food safety. Also, food workers should be trained to improve their knowledge and practices regarding food safety. Additionally, the widespread use of antimicrobial agents should be avoided to prevent the spread of antibiotic resistance in CNS. Although it is emphasized that the overall incidence of safety hazards was low in food-related CNS [9], enterotoxigenic CNS species should not be ignored in suspected cases of staphylococcal food poisoning.

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