Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Antibiotic Susceptibility of Coagulase-Negative Staphylococci Isolated from Bovine Subclinical Mastitis in Turkey

Beytullah Kenar*, Yahya Kuyucuoğlu and Esra Şeker

Department of Microbiology, Faculty of Veterinary Medicine, Afyon Kocatepe University, ANS Campus, 03200, Afyonkarahisar, Turkey
*Corresponding author: bkenar@aku.edu.tr

ARTICLE HISTORY

Received: September 30, 2011
Revised: November 27, 2011
Accepted: December 25, 2011

Key words:
Antimicrobial susceptibility
Bovine mastitis
Coagulase negative
Slime production
Staphylococcus (CNS)

ABSTRACT

A total of 572 California Mastitis Test (CMT) positive milk samples were collected from 423 lactating cows on 18 private farms in the Middle Western Anatolia. Coagulase–negative staphylococci colonies and CNS species identification was performed based on conventional biochemical techniques and using the API Staph test. Slime production was detected by Congo Red Agar (CRA) method. The antibiotic susceptibility was determined according to the National Committee for Clinical Laboratory Standards guidelines (NCCLS). A total of 67 (11.7%) coagulase-negative staphylococci (CNS) were isolated from CMT positive milk samples. In total, 11 CNS species: *S. epidermidis* (n=18), *S. simulans* (n=14), *S. warneri* (n=10), *S. hominis* (n=5), *S. chromogenes* (n=4), *S. caprae* (n=4), *S. xylosus* (n=3), *S. haemolyticus* (n=3), *S. hyicus* (n=3), *S. cohnii* (n=2), and *S. capitis* (n=1) were identified. The most commonly identified CNS species were *Staphylococcus epidermidis* (26.8%) and *Staphylococcus simulans* (20.8%) followed by *Staphylococcus warneri* (14.9%). Out of 67 CNS isolates, slime production was found in 37 (55.2%) CNS strains. CNS isolates were the most resistance to trimethoprim+sulphamethoxazole (76.2%), erythromycin (73.2%), oxacillin and ampicillin (70.2%) followed by penicillin (58.3%), gentamicin (53.8%), tetracycline (52.3%), vancomycin (51.8%), ciprofloxacin (26.9%), cefoxitim (23.9%), and cephalothin (13.5%). These results indicate that CNS species are resistant at high rates to the beta-lactam antibiotics which are intensively used in the prevention and treatment of mastitis without any antibiotic susceptibility test in the Middle Western of Turkey.

To Cite This Article: Kenar B, Y Kuyucuoğlu and E Şeker, 2012. Antibiotic susceptibility of coagulase-negative staphylococci isolated from bovine subclinical mastitis in Turkey. Pak Vet J, 32(3): 390-393.

INTRODUCTION

Bovine mastitis is a multi-factorial disease. It imposes serious economic losses for the farmers and the dairy industry (Pitkälä *et al*., 2004; Pyörälä and Taponen, 2009; Thorberg *et al*., 2009). Coagulase-negative staphylococci (CNS) have traditionally been considered as minor pathogens. Their importance has increased and they have become the predominant pathogens isolated from subclinical mastitis in several countries (Kırkan *et al*., 2005; Taponen *et al*., 2007; Waller *et al*., 2011). In terms of clinical aspects CNS mastitis generally remains subclinical or displays only mild symptoms (Pyörälä and Taponen, 2009). In addition, multi-antimicrobial resistance was often seen in CNS (Pyörälä and Taponen, 2009). Slime production and biofilm-associated bacteria show an innate resistance to antibiotics, disinfectants and clearance by host defense mechanisms (Melchior *et al*., 2006).

Couto *et al.* (2001) and Thorberg *et al*. (2009) showed that the conventional biochemical analysis for the determination of *staphylococcus* at the species level appears to be expensive, laborious and time consuming although it is considered as gold standard for the identification of staphylococcus species, moreover the results indicated by Matthews *et al.* (1990) showed that agreements of Vitek and API systems with conventional methods were 44.6% and 80.8%, although additional tests were also required for final identification.

A variety of CNS species has been isolated from mastitis. *S. chromogenes, S. simulans* and *S. hyicus* have been reported most often, but many other species are also frequently mentioned. Identification of CNS species in

different studies usually rests on use of commercial identification kits based on biochemical profiles (Taponen *et al*., 2006).

The aim of the present study was to identify and determine the antibiotic susceptibility and slime production of different CNS species isolated from subclinical bovine mastitis.

MATERIALS AND METHODS

Isolation and identification: A total of 572 California Mastitis Test (CMT) positive milk samples were collected from 423 lactating cows on 18 private farms in the Middle Western Anatolia. Individual mammary quarter milk samples were aseptically collected into sterile vials just before milking. The samples were transported in a cool box at 4ºC to the laboratory. The CMT and bacteriological analyses were initiated within 24 h after sampling. For the isolation and identification of CNS, ten micro liters of each milk was streaked onto blood agar plates supplemented with 5% sheep blood (Merck, Germany) and all plates were incubated at 37°C for 24 to 48 h. Grown colonies were analyzed based on colony morphology, Gram-staining, coagulase test and susceptibility to novobiocin (5 µg) to distinguish CNS colonies and CNS species further identification was performed based on conventional biochemical techniques described by Holt *et al*., (1994) and using the API Staph test kit (BioMerieux, France) as described by the manufacturer. In addition, CNS species were distinguished from *Staphylococcus aureus* using a Slidex test (Slidex Staph-Plus, bioMerieux, France).

Slime production: Congo Red Agar (CRA) method developed by Freeman *et al*., (1989) was used for the detection of slime production. The medium was composed of Brain Heart Infusion Agar (BHIA) 37 g/l, sucrose 50 g/l, agar 10 g/l, and Congo Red 0,8 g/l. The Congo Red stain was prepared as a concentrated aqueous solution and sterilized at 121°C for 15 min. Then it was added to the agar cooled to 55°C. Plates were inoculated and incubated aerobically at 37°C for 24 h. A positive result was indicated by black colonies with a dry crystalline consistency, while pink colonies were slime negative.

Antibiotic susceptibility test: Using the disc diffusion method on Mueller Hinton Agar (Oxoid) the antibiotic susceptibility test was carried out and interpreted by the consideration of the National Committee for Clinical Laboratory Standards guidelines–NCCLS. (2002). Commercially available antimicrobial sensitivity discs (Oxoid) were used: penicillin (10 IU), ampicillin (10 µg), gentamicin (10 µg), vancomycin (30 µg), trimethoprim +sulfamethoxazole (1.25µg+23.75µg), cephalothin (30 µg), oxacillin (1 µg), cefoxitim (30 µg), ciprofloxacin (5 µg), tetracycline (30 µg), and erythromycin (15 µg). *Staphylococcus aureus* ATCC 25923 was used as control strain.

Statistical analysis: Chi-square ($\chi 2$) test was employed to analysis the relationship between the different CNS species and the slime production. The significance level was set at $P<0.05$.

RESULTS

A total of 67 (11.7%) were isolated coagulase - negative staphylococci from 572 CMT positive milk samples taken from 423 lactating cows. The CNS species identified are presented in Table 1.

Table 1: Species of coagulase-negative staphylococci isolated fromsub-clinical mastitis milk samples and their slime production.

Bacterial spp.	Number of isolated bacteria (%)	(%) Slime (+)	Slime (-)
S. epidermidis	18 (26.8)	6 (33.3)	12 (66.6)
S. simulans	14 (20.8)	6 (42.8)	8 (57.1)
S. warneri	10 (14.9)	8 (80.0)	2 (20.0)
S. hominis	5(7.4)	4 (80.0)	1 (20.0)
S. chromogenes	4 (5.9)	4 (100)	0
S. caprae	4 (5.9)	3 (75.0)	1 (25.0)
S. xylosus	3 (4.4)	1 (33.3)	2 (66.6)
S. haemolyticus	3 (4.4)	1 (33.3)	2 (66.6)
S. hyicus	3 (4.4)	3 (100)	0
S. cohnii	2 (2.9)	0	2 (100)
S. capitis	1 (1.4)	1 (100)	0
Total	67 (100)	37 (55.2)	30 (44.8)

In total, 11 CNS species recovered were *Staphylococcus epidermidis* (26.8%) and *Staphylococcus simulans* (20.8%), the most frequently identified, followed by *Staphylococcus warneri* (14.9%), *Staphylococcus chromogenes* (13.4%), and *Staphylococcus hyicus* (10.4%). A small percentage of S*taphylococcus hyicus* (4.4%), *Staphylococcus cohnii* (2.9%), and *Staphylococcus capitis* (1.4%) were also isolated. The results of slime production by different coagulase negative staphylococci species were given in Table 1. Slime production was found in 37 (55.2%) CNS strains, whereas 30 (44.8%) strains were not slime producers.

The result of antibiotic susceptibility tests of 67 CNS isolates against to penicillin, gentamicin, vancomycin, trimethoprim+sulphamethoxazole, cephalothin, ampicillin, tetracycline, oxacillin, cefoxitim, erythromycin, and ciprofloxacin are shown in Table 2. In general, the antibiotic susceptibility test of the CNS species showed the highest rates of susceptibility against cephalothin (86.5%), cefoxitim (76.1%), and ciprofloxacin (73.1%), followed by vancomycin (58.2%), tetracycline (47.7%), gentamicin (46.2%), penicillin (41.7%), ampicillin and oxacillin (29.8%), erythromycin (26.8%), and trimethoprim+sulphamethoxazole (23.8%) whereas CNS isolates were the most resistance to trimethoprim +sulphamethoxazole (76,2%), erythromycin (73,2%), oxacillin and ampicillin (70.2%) followed by penicillin (58.3%), gentamicin (53.8%), tetracycline (52.3%), vancomycin (51.8%), ciprofloxacin (26.9%), cefoxitim (23.9%), and cephalothin (13.5%). Considering individual CNS species, *S. xylosus* was %100 resistance to P and STX, *S. haemolyticus* to P, STX and AMP, *S. cohnii* to STX, AMP, and *S. capitis* to P, GM, VA, AMP and OX.

DISCUSSION

CNS in cows has often been considered as minor udder pathogens, causing relatively less udder health problems. However, CNS infections may cause substantial herd problems due to high prevalence of sub-clinical and/or clinical mastitis (Wilson et al., 1997). The proportion of CNS among bacteria isolated from milk

Table 2: Susceptibility of bovine coagulase-negative staphylococci isolates to various antibiotics

Microorganisms	Number of susceptible isolates (%)											
CNS Strains	Total	P	GM	VA	SXT	CF	AMP	TE	OX	CTX	E	CIP
S. epidermidis	18	9 (50.0)	12 (66.6)	11 (61.1)	5 (27.7)	16 (88.8)	6 (33.3)	8 (44.4)	5 (27.7)	16 (88.8)	5 (27.7)	17 (94.4)
S. simulans	14	7 (50.0)	6 (42.8)	6 (42.8)	2 (14.2)	11 (78.5)	3 (27.2)	9 (64.2)	3(27.2)	12 (85.7)	3 (21.4)	11 (78.5)
S. warneri	10	2 (20.0)	1 (10.0)	7 (70.0)	2 (20.0)	8 (80.0)	4 (40.0)	4 (40.0)	2 (20.0)	7 (70.0)	3 (30.0)	7 (70.0)
S. hominis	5	1 (20.0)	2 (40.0)	3 (60.0)	1 (20.0)	4 (80.0)	3 (60.0)	2 (40.0)	1 (20.0)	4 (80.0)	2 (40.0)	3 (60.0)
S. chromogenes	4	2 (50.0)	1 (25.0)	2 (50.0)	2 (50.0)	4 (100.0)	1 (25.0)	1 (25.0)	2 (50.0)	3 (75.0)	2 (50.0)	3 (75.0)
S. caprae	4	3 (75.0)	3 (75.0)	3 (75.0)	2 (50.0)	4 (100.0)	1 (25.0)	1 (25.0)	2 (50.0)	2 (50.0)	3 (75.0)	2 (50.0)
S. xylosus	3	0	2 (66.6)	2 (66.6)	0	3 (100.0)	2 (66.6)	2 (66.6)	1 (33.3)	2 (66.6)	1 (33.3)	2 (66.6)
S. haemolyticus	3	0	1 (33.3)	2 (66.6)	0	3 (100.0)	0	1 (33.3)	2 (66.6)	2 (66.6)	2 (66.6)	3 (100.0)
S. hyicus	3	3 (100)	2 (66.6)	2 (66.6)	1 (33.3)	3 (100.0)	0	1 (33.3)	1 (33.3)	3 (100.0)	1 (33.3)	2 (66.6)
S. cohnii	2	1 (50)	1 (50)	1 (50)	0	1 (50)	0	2 (100.0)	1 (50.0)	1 (50)	1 (50)	1 (50)
S. capitis	1	0	0	0	1 (100.0)	1 (100.0)	0	1 (100.0)	0	1 (100.0)	1 (100.0)	1 (100.0)
Total	67	28 (41.7)	31 (46.2)	39 (58.2)	16 (23.8)	58 (86.5)	20 (29.8)	32 (47.7)	20 (29.8)	51 (76.1)	18 (26.8)	49 (73.1)

P: penicillin; GM: gentamicin; VA: vancomycin; SXT: trimethoprim+sulphamethoxazole; CF: cephalothin; AMP: ampicillin; TE: tetracycline OX: oxacillin; CTX: cefoxitim; E: erythromycin; CIP: ciprofloxacin.

samples from clinical mastitis is very low in many countries (Pyörälä and Taponen, 2009; Taponen *et al*., 2006; Bradley *et al*., 2007). Overall, CNS were more common in subclinical mastitis than in clinical cases of mastitis, which was in agreement with earlier studies on either clinical or subclinical mastitis (Pyörälä and Taponen, 2009).

CNS species of *S. chromogenes*, *S. epidermidis*, *S. haemolyticus, S. hyicus, S. simulans*, and *S. xylosus* have been exclusively isolated from bovine mastitis (Matthews *et al*., 1990; Jarp, 1991; Aarestrup *et al*., 1995; Taponen *et al*., 2006; Turkyilmaz and Kaya, 2006). Waller *et al*., (2011) recently showed that the most common strain isolated in subclinical mastitis cases was *S. epidermidis* followed by *S. chromogenes, S. simulans* and *S. haemolyticus*. Furthermore, in the same study *S. epidermidis* was significantly more prevalent in subclinical mastitis than in clinical mastitis. Likewise, predominantly identified CNS species in this study was *S. epidermidis* which is supported by the findings of Waller *et al*. (2011). The results recorded by Taponen *et al*. (2006) about the prevalences of *S. chromogenes* and *S. simulans* in clinical and subclinical cases showed partial agreement with the findings of present study where the *S. simulans* and *S. chromogenes* were only prevelant in subclinical mastitis cases.

In Turkey, many studies have shown the importance of clinical and subclinical bovine mastitis. Kırkan *et al*. (2005) reported that 20 (33.33%) coagulase negative staphylococci were identified as *S. hyicus*, 16 (26.66%) as *S. chromogenes*, 9 (15.00%) as *S. epidermidis*, 5 (8.33%) as *S. haemolyticus*, 4 (6.66%) as *S. sciuri*, 3 (5.00%) as *S. lentis* and 3 (5.00%) as *S. cohnii* subsp. *cohnii*. In the study of Thorberg *et al*. (2009), *S. epidermidis* and *S. chromogenes* were the species mainly found in cows with subclinical mastitis.

In this study, the most commonly identified CNS species were *S. epidermidis* (26.8%) and *S. simulans* (20.8%) followed by *S. warneri* (14.9%), *S. chromogenes* (13.4%), and *S. hyicus* (10.4%). Much lower numbers of *S. hyicus* (4.4%), *S. cohnii* (2.9%) and *S. capitis* (1.4%) were also isolated.

Slime production plays an important role in the pathogenesis of staphylococci (Melchior *et al*., 2006). Arslan and Ozkardes (2007) reported that using Congo Red Agar method, 58 (31%) staphylococcal isolates were slime positive whereas 129 (69%) were slime negative. In this study out of 67 CNS isolates, 37 (55.2%) were slime positive whereas 30 (44.8%) were not slime producers. *S. epidermidis*, *S. simulans*, *S. warneri*, *S. hominis, S. caprae, S. xylosus*, *S. haemolyticus*, and *S. cohnii* were the slime producers except *S. chromogenes, S. hyicus* and *S. capitis* (Table 1).

In general, the antibiotic susceptibility test of the CNS species showed the highest rates of susceptibility against cephalothin (86.5%), cefoxitim (76.1%), ciprofloxacin (73.1%), and vancomycin (58.2%), followed by tetracycline (47.7%), gentamicin (46.2%), penicillin (41.7%), ampicillin (29.8%), oxacillin (29.8%), erythromycin (26.8%), and trimethoprim+ sulphamethoxazole (23.8%) in another word, CNS isolates were the most resistance to trimethoprim +sulphamethoxazole (76.2%), erythromycin (73.2%), oxacillin (70.2%), ampicillin (70.2%), and penicillin (58.3%), followed by gentamicin (53.8%), tetracycline (52.3%), vancomycin (51.8%), ciprofloxacin (26.9%), cefoxitim (23.9%), and cephalothin (13.5%). These results showed some similarities with previous studies but higher rates of resistance to the same antibiotics were also reported (Kırkan *et al.*, 2005; Arshad *et al*., 2006; Turutoglu *et al.*, 2006; Ebrahimi *et al*., 2007; Virdis *et al.*, 2010). Considering the results based on the most isolated individual species in this study, penicillin was effective on *S. epidermidis* and *S. simulans* but not on *S. warneri* and *S. hominis*. In addition to the most sensitive species to penicillin were *S. hyicus* and *S. caprae*. Moreover, no *S. xylosus, S. haemolyticus,* and *S. capitis* strains showed susceptibility to penicillin and ampicillin in this study. The differences of antibiotic susceptibility between individual species may arise from several reasons. For instance, the geographical variations in resistance profiles of CNS species have a considerable impact on antimicrobial prescription. For example, the rate of penicillin resistance (58.3%) observed in this study is much higher than those reported in other countries such as Korea (52.9%), Switzerland (31%), Finland (32%), and the USA (22.1%) (Pitkälä *et al*., 2004; Rajala-Schultz *et al*., 2009). Likewise, a high frequency of slime production and antibiotic resistance has been determined for *S. epidermidis* strains comparing to non-producing isolates (Arciola *et al*., 2005; Arslan and Ozkardes, 2007). Therefore, because of the differences occur in the efficacy of antibiotics against individual CNS species, the identification of bacterial agents has been recommended along with the antibiotic susceptibility test (Turutoglu *et al*., 2006).

The present study showed the antimicrobial susceptibilities and slime production of CNS species isolated from CMT positive bovine milk samples collected from lactating cows in the Middle Western of Turkey. In this study, relatively high numbers of different individual CNS species from CMT positive bovine milk samples have been isolated and identified in the Middle Western of Turkey. These results also indicate that CNS species are resistant at high rates to the beta-lactam antibiotics which are intensively used in the prevention and treatment of mastitis without any antibiogram test.

REFERENCES

Aarestrup FM, HC Wegener, T Rosdahl and NE Jensen, 1995. Staphylococcal and other bacterial species associated with intra-mammary infections in Danish dairy herds. Acta Vet Scand, 36: 475-487.

Arciola CR, D Campoccia, S Gamberini, ME Donati, V Pirini, L Visai, P Speziale and L Montanaro, 2005. Antibiotic resistance in exopolysaccharide-forming Staphylococcus epidermidis clinical isolates from orthopaedic implant infections. Biomaterials, 26: 6530-6535.

Arshad M, G Muhammad, M Sıddıque, M Ashraf and HA Khan, 2006. Staphylococcal mastitis in bovines and some properties of staphylococcal isolates. Pak Vet J, 26: 20-22.

Arslan S and F Ozkardes, 2007. Slime production and antibiotic susceptibility in staphylococci isolated from clinical samples. Mem Inst Oswaldo Cruz, 102: 29-33.

Bradley AJ, KA Leach, JE Bren, LE Gren and J Gren, 2007. Survey of the incidence and etiology of mastitis on dairy farms in England and Wales. Vet Rec, 160: 253-257.

Couto I, S Pereira, M Miragaia, SS Sanches and H Lencastre, 2001. Identification of clinical staphylococcal isolates from humans by internal transcribed spacer PCR. J Clin Microbiol, 39: 3099-3103.

Ebrahimi A, KHP Kheirabadi and F Nikookhah, 2007. Antimicrobial susceptibility of environmental bobine mastitis pathogens in West Central Iran. Pak J Biol Sci, 10: 3014-3016.

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

Holt JG, NR Krieg, PHA Sneath, JT Stanley and TS Williams, 1994. Bergeys Manual of Determinative Bacteriology, 9th Edition. William and Wilkins, Baltimore, Maryland, USA, pp: 532.

Jarp J, 1991. Classification of coagulase-negative staphylococci isolated from bovine clinical and subclinical mastitis. Vet Microbiol, 27: 151-158.

Kirkan S, EO Goksoy and O Kaya, 2005. Identification and Antimicrobial Susceptibility of *Staphylococcus aureus* and coagulase negative staphylococci from bovine mastitis in the Aydin region of Turkey. Turk J Vet Anim Sci, 29: 791-796.

Matthews KR, RJ Harmon and BA Smith, 1990. Protective effect of *Staphylococcus chromogenes* infection against *Staphylococcus aureus* infection in the lactating bovine mammary gland. J Dairy Sci, 73: 3457-3462.

Melchior MB, H Vaarkamp and J Fink-Gremmels, 2006. Biofilms: A role in recurrent mastitis. Vet J, 171: 398-407.

Pitkälä A, M Haveri, S Pyorälä, M Myllys and T Honkanen-Buzalski, 2004. Bovine mastitis in Finland 2001 prevalence, distribution of bacteria, and antimicrobial resistance. Dairy Sci, 87: 2433-2441.

Pyörälä S and S Taponen, 2009. Coagulase-negative staphylococci emerging mastitis pathogens. Vet Microbiol, 134: 3-8.

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

Taponen S, J Koort, J Bjorkroth, H Saloniemi and S Pyorala, 2007. Bovine intra-mammary infections caused by coagulase-negative staphylococci may persist throughout lactation according to amplified fragment length polymorphism-based an*a*lysis. J Dairy Sci, 90: 3301-3307.

Taponen S, H Simojoki, M Haveri, HD Larsen and S Pyorala, 2006. Clinical characteristics and persistence of bovine mastitis caused by different species of coagulase-negative staphylococci identified with API or AFLP. Vet Med, 115: 199-207.

Thorberg BM, EL Dnielsson-Tham, U Emanuelson and P Waller, 2009. Bovine subclinical mastitis caused by different types of coagulase negative staphylococci. J Diary Sci, 92: 4962-4970.

Turkyilmaz S and O Kaya, 2006**.** Determination of some virulence factors in *Staphylococcus* spp. isolated from various clinical samples. Turk J Vet Anim Sci, 30: 127-132.

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

Virdis S, C Scarano, F Cossu, V Spanu, C Spanu and EPL De Santis, 2010. Antibiotic resistance in *Staphyloccoccus aureus* and coagulase negative staphylocci isolated from goats with subclinical mastitis, Vet Med Int, 2010: 517060 (doi: 10.4061/2010/517060).

Waller KP, A Aspa, A Nyman, Y Persson and UG Andersson, 2011. CNS species and antimicrobial resistance in clinical and subclinical bovine mastitis Vet Microbiol, 152: 112-116.

Wilson DJ, N Ruben and H Gonzalez, 1997. Bovine mastitis pathogens in New York and Pennsylvania: Prevalence and effects on somatic cell count and milk production. J Dairy Sci, 80: 2592-2598.