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ORIGINAL RESEARCH

Detection of staphylococcal enterotoxin, methicillin-resistant and Panton–Valentine leukocidin genes in coagulase-negative staphylococci isolated from cows and ewes with subclinical mastitis

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Abstract Coagulase-negative staphylococci (CNS) are the most prevalent mastitis pathogens. However, virulence characteristics of CNS have not been well determined. The presence of genes for enterotoxins (*sea-sej*), toxic shock syndrome toxin-1 (*tst*), the exfoliative toxins (*eta*, *etb*), Panton–Valentine leukocidin (*pvl*) and *mec*A of CNS species isolated from cows and ewes with subclinical mastitis was investigated in this study. A total of 121 CNS (81 cows, 40 ewes) representing 18 different *Staphylococci* species were examined by PCR, and 38.1% (33 cows and 13 ewes) of CNS isolates had one or more *se* genes. The difference between percentages for SE toxin genes of CNS strains isolated from cows (40.7%) and ewes (32.5%) was not statistically significant ($P>0.05$; $\chi^2=0.380$). It was found that *S. simulans* isolates had the highest prevalent *se* genes. Furthermore, the most common SE gene types was *seh-sej*. In this study, none of the isolates harbored the toxic shock syndrome toxin gene (*tsst*) and the exfoliative toxin genes (*eta*, *etb*). Five cow (6.17%) and three ewe CNS (7.5%) isolates had *mec*A gene. Three cow (3.7%) and two ewe CNS (5.0%) isolates had *pvl* gene. In conclusion, the present study showed that CNS species isolated from cows and ewes could serve as potential reservoir of *se*, *mec*A, and *pvl* genes.

Keywords Coagulase-negative staphylococci · Cows · Enterotoxins · Ewes · *mec*A · Panton–Valentine leukocidin

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Introduction

Staphylococci are the main aetiological agents isolated from mastitis in cows and sheep (Bergonier et al. 2003; Unal and Yildirim 2010). Although coagulase-negative staphylococci (CNS) are less pathogenic than *S. aureus*, CNS have become the most common mastitis pathogens in many countries. Several studies have recently shown that CNS species were one of the important cause of mastitis in ruminants (Ergün et al. 2009; Pyörala and Taponen 2009; Unal and Yildirim 2010; Kunz et al. 2011; Unal et al. 2011).

Many strains of *S. aureus* produce one or more exotoxins, which may contribute to the pathogenesis of mastitis in ruminants. These toxins including staphylococcal enterotoxins (SEs), staphylococcal enterotoxinlike toxins (several SEs were designated as SElike (SEl) since they do not lack emetic properties or their emetic activities have not been tested in primates), toxic shock syndrome toxin-1 (TSST-1), and exfoliative toxins (ETA, ETB) are responsible for staphylococcal food poisoning and other types of infections in humans and animals (Lina et al. 2004). Five different types of classical SEs (SEA through SEE), and new types of SEs (SEG, SEH, SEI, SElJ, SElK, SElL, SElM, SElN, SElO, SElP, SElR, SES, SET, SElU and SElV) have been found in *S. aureus* (these proteins are called superantigens (SAgs) (Vimercati et al. 2006; Nemati et al. 2008; Maslankova et al. 2009; De Oliveira Calsolari et al. 2011; Park et al. 2011). Compared to *S. aureus*, only few studies were carried out on the prevalence of staphylococcal exotoxin genes in CNS isolated from cows and sheep with subclinical mastitis (Nemati et al. 2008; Park et al. 2011).

Methicillin-resistant staphylococci are a major concern to public and animal health. The prevalence of methicillin resistance is higher in CNS than in *S. aureus*. Methicillin resistant CNS species (MR-CNS) have the *mec*A gene which may have horizontally transferred among

staphylococci. Furthermore, *mec*A-positive CNS may act as potential donors for the rise in new MRSA clones (Garza-Gonzalez et al. 2010; Huber et al. 2011). Panton–Valentine leukocidin (PVL) is a cytotoxin that causes leukocyte destruction and tissue necrosis. PVL toxin has been associated with community-acquired methicillin-resistant *S. aurues* strains (CA-MRSA). Thus, some researches (Rankin et al. 2005; Giudice et al. 2009) have studied on the presence of *pvl* gene in methicillin-resistant *S. aurues* (MRSA) and methicillin susceptibility *S. aureus* (MSSA). However, whether CNS strains which were isolated from cows and sheep with subclinic mastitis produce PVL toxins is not clearly known.

The dairy industry and public health would benefit from more research on characterization of virulence and methicillin resistance of CNS species isolated from cows and sheep with clinic and subclinic mastitis. The aim of this sudy was to investigate the presence of genes for enterotoxins (*sea-sej*), toxic shock syndrome toxin-1 (*tst*), the exfoliative toxins (*eta*, *etb*), Panton–Valentine leukocidin (*pvl*) and *mec*A of CNS strains isolated from cows and sheep with subclinical mastitis.

Materials and methods

Bacterial strains

A total of 121 CNS isolates (81 cows and 40 ewes), isolated between 2009 and 2011 at the microbiology laboratory of Veterinary Faculty of Kirikkale University, were inclueded in the study. These strains were isolated from milk samples of subclinical mastitis cows and ewes. Ten microliters of each milk samples was inoculated onto 5% sheep blood agar plates (Merck, Germany) and incubated aerobically for 48 h at 37°C. The infection status of milk samples were determinated according to the procedures recommended by the National Mastitis Council (1999). Intramammary infection was defined when ≥500 cfu/ml colonies were found. The colonies were identified on the basis of Gram staining, morphology, hemolysis characteristics, catalase, oxidase, rabbit-plasma-tube coagulase as CNS and coagulase positive staphylococci (CPS). Then, identification of CNS isolates were made using a Crystal Gram-Positive Kit (Becton Dickinson, USA). The species of CNS from cows and ewes used in the present study included: 33 *S. haemolyticus* (27 cows, six ewes), 26 *S. simulans* (19 cows, seven ewes), 12 *S. capitis* (eight cows, four ewes), nine *S. hominis* (nine cows), seven *S. lugdunensis* (three cows, four ewes), seven *S. xylosus* (three cows, four ewes), six *S. epidermidis* (three cows, three ewes), four *S. schleiferi* (two cows, two ewes), four *S. warneri* (four ewes), three *S. lentus* (one cow, two ewes), two *S.saprophyticus* (one cow, one ewe), one *S. auricularis* (one ewe), two *S. equorum* (two cows), one *S. caprae* (one ewe), one *S. cohnii* ssp. *cohnii* (one cow), one *S. hyicus* (one cow), one *S. saccharolyticus* (one cow) and one *S. vitulus* (one ewe). All isolates identified were stored at −20°C in brain heart infusion broth (Merck, Germany) containing 15% glycerol until further analysis.

Genomic DNA putrification and PCR

DNA of CNS strains were purified for PCR analysis. Briefly, CNS isolates were cultured in Mueller-Hinton broth overnigth at 37°C and then collected by centrifugation at 5,000 rpm for 10 min. The cell pellet was washed in 1 ml of TE buffer (10 mM Tris–HCl pH 8.0; 1 mM EDTA). Fifty microliters of lysostaphin (100 μg/ml) was added to the pellet, incubated for 10 min at 37°C and, subsequently, treated with 50 μl proteinase K (100 μg/ml) for 10 min at 37°C. For inactivation of proteinase K, the suspension was heated for 10 min at 100°C. Isolated DNA samples were kept at −20°C until further use (Unal et al. 1992).

Base sequences of primers for staphylococcal enterotoxin genes (*sea-sej*), the toxic shock syndrome toxin gene (*tsst*), the exfoliative toxin genes (*eta*, *etb*), the *mec*A gene and the Panton–Valentine leukocidin gene (*pvl*) were used as described by previous literature (Johnson et al. 1991; Lina et al. 1999; Monday and Bohach 1999; Choi et al. 2003; Løvseth et al. 2004). These primers are specific oligonucleotide primers for staphylococcal toxin and *mec*A genes (Johnson et al. 1991; Monday and Bohach 1999; Choi et al. 2003).

All primers used were listed in Table 1. PCR mixture used in this study was as follows: 5 μl of DNA was added to 45 μl of PCR mixture of 1× Taq polymerase buffer, 4 mM $MgCl_2$, 2 U Taq polimerase, 400 μM each deoxynucleoside triphosphat, 300 nM of each primers. Amplification cycles were programmed as 5 min at 95°C for initial denaturation; 30 cycle, 1 min at 94°C, 30 s 55°C for *mec*A gene, 30 s at 62°C for exfoliative toxin genes (*eta*, *etb*) and Panton–Valentine leukocidin gene (*pvl*), 1 min extension at 72°C; and 5 min final extension step at 72°C.

Staphylococcal enterotoxin genes were detected by multiplex PCR; some of the PCR products were similar in size and the bands were, therefore, found to be difficult to differentiate. To avoid this complication, ten primers were individed into two groups. Primers for *sed*, *see*, *seg*, *sei*, and *tsst* were combined in reaction mixture 1, and primers for *sea*, *seb-sec*, *sec*, *seh* and *sej* were combined in reaction mixture 2. We used the protocol of Løvseth et al. (2004) for m-PCR amplification: 5 μl of DNA was added to 45 μl of PCR mixture of 1× Taq polymerase buffer, 4 mM $MgCl_2$, 2 U Taq polimerase, 400 μM each deoxynucleoside triphosphat, 300 nM each primers. DNA was amplified on thermocycler (TC-PRO/Boeco) by initial denaturation for 10 min 95°C followed by 30 cycles of 95°C for 1 min, 64°C for 45 s, 72°C for 1 min, and final extention at 72°C for 1 min.

Table 1 Staphylococcal toxin-specific, staphylococcal methicilllin-resistant and Panton–Valentine leukocidin primers used in this study

Gene	Sequence (5′–3′)[a]	References	Product (bp)
sea	GCA GGG AAC AGC TTT AGG C GTT CTG TAG AAG TAT GAA ACA CG	Monday and Bohach (1999)	520
seb-sec	ACA TGT AAT TTT GAT ATT CGC ACT G TGC AGG CAT CAT GTC ATA CCA	Løvseth et al. (2004)	667
sec	CTT GTA TGT ATG GAG GAA TAA CAA TGC AGG CAT CAT ATC ATA CCA	Monday and Bohach (1999)	284
sed	GTG GTG AAA TAG ATA GGA CTG C ATA TGA AGG TGC TCT GTG G	Monday and Bohach (1999)	385
see	TAC CAA TTA ACT TGT GGA TAG AC CTC TTT GCA CCT TAC CGC	Monday and Bohach (1999)	171
seg	CGT CTC CAC CTG TTG AAG G CCA AGT GAT TGT CTA TTG TCG	Monday and Bohach (1999)	328
seh	CAA CTG CTG ATT TAG CTC AG GTC GAA TGA GTA ATC TCT AGG	Monday and Bohach (1999)	359
sei	CAA CTC GAA TTT TCA ACA GGT ACC CAG GCA GTC CAT CTC CTG	Monday and Bohach (1999)	466
sej	CAT CAG AAC TGT TGT TCC GCT AG CTG AAT TTT ACC ATC AAA GGT AC	Monday and Bohach (1999)	142
Tst	GCT TGC GAC AAC TGC TAC AG TGG ATC CGT CAT TCA TTG TTA T	Løvseth et al. (2004)	559
eta	CTA GTG CAT TTG TTA TTC AA TGC ATT GAC ACC ATA GTA CT	Johnson et al. (1991)	119
etb	ACG GCT ATA TAC ATT CAA TT TCC ATC GAT AAT ATA CCT AA	Johnson et al. (1991)	200
pvl	ATCATTAGGTAAAATGTCTGGACATGATCCA GCATCAASTGTATTGGATAGCAAAAGC	Lina et al. (1999)	433
mecA	FCCTAGTAAAGCTCCGGAA CTAGTCCATTCGGTCCA	Choi et al. (2003)	314

[a] Primer sequences are given in the 5′➔3′ direction. For each primer pair, the sequence of the 5′ primer is provided first (above), folloved by that of the 3′ primer (below)

All PCR products were separated by electrophoresis of 10 μl of reaction product in a 1.5% agarose gel (1× Tris–EDTA buffer at 100 V for 100 min) and visualized on a UV transilluminator (Ingenius, Syngene Bio Imaging). Product sizes were determined by comparison with 100-bplike marker (Vivantis, NL0401). *S. aureus* reference strains: D4508 (*sea*, *seh*), FRI913 (*see*), RIMD 31092 (*seb*, *seg*, *sei*), NTCC9393 (*sej*, *sed*, *seg*, *sei*), FRI137 (*sec*, *seh*), ATCC 49775 (*pvl*), *S. aureus* 27R (*mec*A) used in this study.

The difference between percentages for SE toxin genes of CNS strains isolated from cows and ewes were assessed by χ^2 test. The SPSS package program (Anonymous 1993) was used for statistical analysis.

Results

Of the 121 (81 cows, 40 ewes) CNS milk sample isolates tested, 46 CNS (38.1%) isolates (33 cows, 13 ewes) were detected to have one or more SE genes (Table 2). The difference between percentages for SE toxin genes of CNS strains isolated from cows (40.7%) and ewes (32.5%) was not statistically significant ($P>0.05$; $\chi^2=0.380$). The SE genes were widely distributed among 18 CNS species of 15 different CNS species investigated in the present study. No SE genes were detected *S. caprae*, *S. cohnii* ssp. *cohnii* and *S. vitulus* isolates.

A total of four different SE gene types were detected among the 46 SE gene-positive isolates (Table 3). The most common SE gene type was *seh-sej*, which was present in 63.0% (29/46), while 26% of isolates had *seh* gene (12/46), only two isolates had *sec-sej* and two isolates had *sec* gene. One isolate had *seg-sei* gene. Most of the SE gene-positive isolates had newly described SE genes, and four isolates had only classical SE gene (*sec*). Furthermore, it was found that *S. simulans* isolates had the highest prevalent *se* genes, 13 of 26 (11 cows, two ewes) *S. simulans* isolates had *se* genes, followed by 11 of 33 (10 cows and one ewes) *S. haemolyticus*, and eight of 12 (five cows and three ewes) *S. capitis*.

In this study, none of the isolates harbored the toxic shock syndrome toxin gene (*tsst*), the exfoliative toxin genes (*eta*, *etb*). Five cow CNS (6.17%) and three ewe CNS

Table 2 *se* genes positive coagulase–negative staphylococci species isolated from cows' and ewes' subclinical mastitis

CNS species	Cows *n*	Ewes *n*	SE cows toxin genes *n*	SE ewes toxin genes *n*	Total isolates *n*	Total *se* toxin genes *n*
S. auricularis	–	1	–	1	1	1
S. capitis	8	4	5	3	12	8
S. caprae	–	1	–	–	–	–
S. cohnii ssp. *cohnii*	1	–	–	–	1	–
S. epidermidis	3	3	1	–	6	1
S. equorum	2	–	1	–	2	1
S. haemolyticus	27	6	10	1	33	11
S. hominis	9	–	1	–	9	1
S. hyicus	1	–	1	–	1	1
S. lentus	1	2	–	1	3	1
S. lugdunensis	3	4	–	1	7	1
S. saccharolyticus	1	–	1	–	1	1
S. saprophyticus	1	1	1	–	2	1
S. schleiferi	2	2	1	1	4	2
S. simulans	19	7	11	2	26	13
S. vitulus	–	1	–	–	1	–
S. warneri	–	4	–	2	4	2
S. xylosus	3	4	–	1	7	1
Toplam	81	40	33 (40.7%)[a]	13 (32.5%)[a]	121	46

[a] The difference between two percentages for SE toxin genes of CNS strains isolated from cows and ewes was not significant ($P>0.05$; $\chi^2=0.380$)

(7.5%) isolates had *mec*A gene. Three cow CNS (3.7%) and two ewe CNS (5.0%) isolates had *pvl* gene. Two *S. haemolyticus* isolates (cow origin), two *S. lentus* isolates (ewes origin), two *S. xylosus* isolates (one cow origin, one ewe origin), one *S. epidermidis* and one *S. schleiferi* (cows isolates) are harboring *mec*A gene. None of these strains which had *mec*A harbored *pvl* gene.

Discussion

S. aureus is one of the most prevalent pathogens causing subclinical and clinical mastitis in cows and sheep, and also causing food poisinig in human. In the past, CNS species were accepted as the minor pathogens for those of infections. However, recently, the importance and prevalence of CNS harboring toxin and antibiotic resistance genes have been recognized as a potential for diseaes in human and animals (Rall et al. 2010; De Oliveira Calsolari et al. 2011; Park et al. 2011; Zhang et al. 2011).

The result obtained from the present study as one or more SE genes observed in CNS strains (38.0%) from cows and ewes with subclinical mastitis were consistent with the results which were 31.2% from bovine intramammary infections (Park et al. 2011) and 45.4% from newborns (De Oliveira Calsolari et al. 2011) but higher than the result of 26.2% from cheese (Rall et al. 2010). Moreover, genes encoding SEs in CNS from human and animal samples were not detected in two studies (Becker et al. 2001; Nemati et al. 2008). Becker et al. (2001) conducted a study in a total of 461 CNS isolates of 18 different (sub)species derived from clinical specimens in humans, and the second study by Nemati et al. (2008) was caried out in 102 CNS isolated from cases of subclinical and clinical bovine mastitis. The results of these two studies may be due to their study genomic DNA preperad by a boiling method used in PCR technique, not using lyostaphin and lysosime which result in a better yield and quality of DNA (Park et al. 2011).

The current study demonstrated the *seh-sej* ($n=29$) was the most common *se* gene, while only four isolates harbored the *sec* gene. This result was not consistent with the finding of Park et al. (2011) who reported that the most prevalent combination of *se* gene types were *seb*, *seln* and *selq*. However, the present study was similar to Park et al. (2011) in terms of detecting for few CNS isolates harboring the *sec* gene. On the other hand, some studies reported that SEC was the most prevalent classical SEs in CNS isolates regardless of the origin of isolates (Kuroishi et al. 2003; Da Cunha et al. 2007; De Oliveira Calsolari et al. 2011). De Oliveira Calsolari et al. (2011) showed that *sec-1* gene was found to be the most frequent (67.4%), followed by *sea* (28.6%), *tst* gene (26.5%) and *seb* gene with only one isolate of 49 toxigenic CNS isolated from human newborns. Rall et al. (2010) carried out the study on 65 CNS strains isolated from foods and analyzed only classical enterotoxin genes (*sea*, *seb*, *sec*, *sed*). They found that *sea* (18.5%) had

Table 3 Combination of genes observed in *ses* gene, *pvl* gene and *mec*A gene positive coagulase–negative staphylococci species isolated from cows and ewes subclinical mastitis

Species	Combinations of genes	No of isolates	
		Cows	Sheep
S. auricularis (*n*=1)	*seh*		1
S. capitis (*n*=8)	*seh*	1	1
	seh sej	3	2
	sec sej	1	
S. epidermidis (*n*=2)	*seh sej*	1	
	*mec*A	1	
S. equorum (*n*=1)	*sec*	1	
S. haemolyticus (*n*=13)	*seh sej*	5	1
	seh	4	
	*seh sej mec*A	1	
	*mec*A	1	
	pvl	1	
S. hominis (*n*=1)	*seh sej*	1	
S. hyicus (*n*=1)	*seg sei*	1	
S. lentus (*n*=2)	*sec sej mec*A		1
	*mec*A		1
S. lugdunensis (*n*=1)	*seh*		1
S. saccharolyticus (*n*=1)	*seh sej*	1	
S. saprophyticus (*n*=1)	*seh sej*	1	
S. schleiferi (*n*=3)	*seh*	1	
	seh sej		1
	*mec*A	1	
S. simulans (*n*=15)	*seh sej*	8	2
	seh	2	
	seh sej pvl	1	
	pvl	1	1
S. warneri (*n*=2)	*seh sej*		1
	sec pvl		1
S. xylosus (*n*=3)	*seh*		1
	*mec*A	1	1

the highest frequency, followed by *sec* in three and *seb* in two strains.

Methicillin-resistant staphylococci are still a considerable problem in public and animal health. *mec*A harboring staphylococci are considered resistant to penicilin, penems, carbapenems and cephalosporins making it difficult to treat if associated with infections (Chambers 1997). *mecA* gene positive CNS strains are a potential reservoir of this resistant gene, and may transfer the resistance gene to *S. aureus* and other staphylococci (Garza-Gonzalez et al. 2010; Huber et al. 2011). In our study, *mec*A gene were determined in five cow CNS (6.17%) and three ewe CNS isolates (7.5%). Two *S. haemolyticus* isolates (cow origin), two *S. lentus* isolates (ewe origin), two *S. xylosus* isolates (one cow origin, one ewe origin), one *S. epidermidis* and one *S. schleiferi* (cow isolates) had *mec*A gene. Similar results were also reported by Feßler et al. (2010) for 121 CNS strains isolated from cows' milk samples. Feßler et al. (2010) detected 15 (12.4%) *mec*A genes, and these MR-CNS comprised eight *S. epidermidis*, five *S. haemolyticus*, one *S. saprophyticus* and one *S. capitis*. On the other hand, Huber et al. (2011) found a higher MR-CNS prevalence (on average 48.3%) in samples from livestock, chicken carcasses, bulk tank milk, minced meat, and contact persons with livestock than the prevalence of present study. Also, Moodley and Guardabassi (2009) detected a higher MR-CNS prevalence (69%) isolated from horses, human, wash area, door handle, stall or restraint, surgical and medical equipment, keyboard or phone, treatment area, and radio than the result of our study. So, futher molecular studies on *mec*A harboring CNS species isolated from mastitis in dairy ruminants must be done to decrease the high MR-CNS prevalence on human and animals.

The exotoxin PVL produced by *S. aureus* is an important virulence factor in necrotizing diseases in humans (Lina et al. 1999). Löffler et al. (2010) showed that PVL caused rapid activation and cell death in human and rabbit neutrophils but not in murine or simian cells. Results of Nolte et al. (2005) stressed that *S. aureus* strains susceptible to methicillin but positive for *pvl* gene may be more widely distrubuted in human population. PVL of *S. aureus* isolated from cows were investigated in previous studies, and *pvl* gene was identified in 0–50% of the isolates (Zecconi et al. 2006; Aires-de-Sousa et al. 2007). However, the carriage of *pvl* gene by CNS species in animal and human is limited literature in the world. According to the results of the present study, three cow (3.7%) and two ewe CNS isolates (5.0%) had *pvl* gene, while all strains were susceptibile to methicillin. Three *S. simulans* (two cow origin, and one ewe origin), one *S. haemolyticus* (cow origin), one *S. warneri* (ewe origin).

Conclusion

In conclusion, the present study showed that CNS species isolated from cows and ewes could serve as potential reservoir of *se*, *mec*A, and *pvl* genes. Cows' and ewes' milk are of public health risk due to potent staphylococcal food poisoning and dissemination of methicillin-resistant strains. CNS species isolated from cows and ewes with subclinical mastitis are important pathogens with significant toxigenic potential, and futher studies on this subject must be done.

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