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Original article

Determination of antibiotic resistance profiles and biofilm production of *Staphylococcus* spp. isolated from Anatolian water buffalo milk with subclinical mastitis

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Abstract

Mastitis is one of the most crucial diseases of dairy animals. Especially subclinical mastitis (SCM) has negative impacts on of dairy economy in term of reducing milk quality and quantity also premature culling and cost of therapy. Staphylococci are important etiological agents in SCM. The aim of the study was to investigate the biofilm production and antibiotic resistance profiles of *Staphylococcus* spp. other than *S. aureus* isolated from milks of Anatolian water buffalo with subclinical mastitis. Twenty-two coagulase negative staphylococci (CNS) identified phenotypically were also identified with PCR as *Staphylococcus* spp. other than *S. aureus*. Biofilm productions were investigated both by Congo Red Agar Method and PCR. The antibiotic resistance profiles of the isolates were determined by Disc Diffusion Method and they were antibiotyped. Only three (13.6%) isolates were biofilm positive both phenotypically and genotypically. All isolates except for two were resistant against at least two antibiotics. Multidrug-resistance among the isolates was low (13.6%). Antibiotyping results showed that the similarities among the strains were between 30-100%. Genotyping of the strains revealed that a genetic heterogeneity was found among CNS isolates and their similarities were between 43% and 93%. In conclusion, CNS isolates identified as subclinical mastitis agents in buffaloes showed a high antibiotic resistance profile especially against oxacillin and vancomycin. Further studies should be conducted to investigate new mechanisms and/or genes responsible for antibiotic resistance in buffaloes.

Key words: antibiotic resistance, biofilm production, buffalo, microbiological analysis, subclinical mastitis

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Introduction

Mastitis, especially subclinical mastitis, is the biggest economic problem of dairy industry. Buffalo breeding is carried out in a few countries, including Turkey. The most important product of the buffalo, which is also raised for meat, is milk (Borghese and Mazzi 2005).

Mastitis can be caused by several bacteria species and *Staphylococcus aureus* is considered to be the prevailing mastitis pathogen (Athar 2006). However, coagulase negative species staphylococci (CNS) also infect mammary glands in which lead to persistent or subclinical mastitis. More than 10 CNS species have been isolated from mastitis, but only a few species (*Staphylococcus chromogenes*, *Staphylococcus arneri*, *Staphylococcus epidermidis*) predominate. Although CNS are not considered as important as *S. aureus* among mastitis pathogens, it is considered to be among the most common agents causing mastitis in many countries. As is generally known, mastitis caused by CNS seems to respond well to antimicrobial treatment. However, it is considered that it tends to be more resistant to antimicrobials than *S.aureus* and easily develop multiresistance (Taponen and Pyörala 2009). Mastitis has usually been treated with several commercial antibiotics and inappropriate use of these agents is one of the most important causes of antibiotic resistance. Many studies have been conducted on antibiotic resistance profiles of bacteria isolated from various disease cases as an important part of the solution to the problem of antibiotic resistance worldwide.

Prolong infections are most often associated with bacterial growth, which forms as sticky colonies surrounded by a large exopolysaccaride matrix. This structural bacterial complex is called a biofilm. Bacteria in this complex are not susceptible to phagocytosis by macrophages and resistant to some antibiotics (Raza 2013).

In this study we aimed to determine the antibiotic resistance profiles and biofilm production of CNS from subclinical mastitic Anatolian water buffalo milks.

Materials and Methods

Milk samples

This study was carried out on 36 milk samples obtained from non-pregnant, clinically healthy but subclinical mastitic animals in small family-type farms of the Anatolian water buffaloes located around the Kızılırmak delta in Samsun. After the teat ends were disinfected with cotton swabs containing 70% alcohol, and the foremilk from quarters were discarded. Subclinical mastitis was detected with California mastitis test (CMT) that was performed directly to the milk samples taken manually from each quarter using the method of Schalm et al. (1971). Without evaluating each milk sample as +1, +2, +3, directly positive samples were taken into 50 ml sealed tubes and delivered to the laboratory under cold chain.

Bacterial strains and identification

Twenty-two staphylococci were isolated from milk of Anatolian water buffaloes with subclinical mastitis. Isolates were identified as coagulase negative staphylococci by conventional test (clumping factor test) and also were differentiated from *S. aureus* using PCR. DNA extraction from colonies was done by boiling method (Vurucu et al. 2019) and DNA concentrations were adjusted to 50 ng/µl by measuring with nanodrop spectrophotometer.

PCR studies were performed according to the *Staphylococcus* spp. specific 16S rRNA gene (F:5'-AAC TCT GTT ATT AGG GAA GAA CA-3'; R: 5'-CCA CCT TCC TCC GGT TTG TCA CC-3') reported by Ciftci et al. (2009). For this aim, five microliter of the rapid extracted DNA was used as a template in a 25 µl PCR mixture containing 1XPCR buffer (50 mm KCl, 20 mM Tris HCl), 5 µl of 25 mM MgCl2, 3 µl of 10 mM deoxynucleoside triphosphate (dNTP) mix, 1 µl of 20 µM each 16S rRNA primers, and 2U of Taq DNA polymerase. The amplification of DNA was performed as follows: 94°C for 5 min of initial denaturation; 30 cycles of 94°C for 45 s, 68°C for 45 s and 72°C for 90 s; and a final extantion at 72°C for 10 min. Amplicons were loaded onto 1.5% Agarose Gel containing 1 µg/ml ethidium bromide. The 756-bp (16S rRNA) of amplified DNA fragments were seperated by agarose gel electrophoresis and visualized under UV-light.

Antibiotic susceptibility test and antibiotyping

Antibiotic susceptibilities of the isolates against 7 antibiotics [amoxycillin-clavulanic acid (20 µg+10 µg), oxacillin (5 µg), vancomycin (30 µg), cephalothin (30 µg), enrofloxacin (5 µg), tetracycline (30 µg), gentamicin (30 µg)] belonging to 5 different antibiotic classes were determined by Kirby-Bauer Disc Diffusion Tests. From the isolates, 0.1 ml of the suspension prepared at 0.5 McFarland density in FTS was taken and lawn culture was performed on MHA. Selected antibiotic discs were placed on the agar surface and incubated at 37°C for 24 hours. The resulting zone diameters were evaluated according to CLSI (2019).

The isolates were antibiotyped based on their susceptibility patterns and a dendrogram was generated

Table 1. The primers used for determination of vancomycine resistance genes.

Primers		Oligonucleotide sequences (5'-3')	Expected band sizes (bp)	Annealing temperatures dereceleri (°C)
vanR1	F	AGC GAT AAA ATA CTT ATT GTG GA	645	53
vanR2	R	CGG ATT ATC AAT GGT GTC GTT		
vanS1	F	TTGGTTATAAAATTGAAAAATAA	1155	47
vanS2	R	TTAGGACCTCCTTTTATC		
vanH1	F	ATCGGCATTACTGTTTATGGAT	943	55
vanH2	R	TCCTTTCAAAATCCAAACAGTTT		
vanA1	F	ATGAATAGAATAAAAGTTGCAATAC	1029	52
vanA2	R	CCCCTTTAACGCTAATACGAT		
vanX1	F	ATGGAAATAGGATTTACTTT	609	46
vanX2	R	TTATTTAACGGGGAAATC		
vanY1	F	ATGAAGAAGTTGTTTTTTTTA	912	47
vanY2	R	TTACCTCCTTGAATTAGTAT		
vanZ1	F	TTATCTAGAGGATTGCTAGC	454	51
vanZ2	R	AATGGGTACGGTAAACGAGC		

Table 2. Oligonucleotide sequences used in multiplex PCR and the resulting band patterns of SCC*mec* types.

Target	Primer	Sequence	PCR product (bp)
ccrA2-B	β	F: ATTGCCTTGATAATAGCCYTCT	937
	α3	R: TAAAGGCATCAATGCACAAACACT	
ccrC	ccrCF	F: CGTCTATTACAAGATGTTAAGGATAAT	518
	ccrCR	R: CCTTTATAGACTGGATTATTCAAAATAT	
IS1272	1272F1	F: GCCACTCATAACATATGGAA	415
	1272R1	R: CATCCGAGTGAAACCCAAA	
mecA–IS431	5RmecA	F: TATACCAAACCCGACAACTAC	359
	5R431	R: CGGCTACAGTGATAACATCC	

as described by Gulhan et al. (2015). This procedure was performed by means of the Pearson product moment correlation coefficient and the unweighted pair group method using arithmetic averages (UPGMA) cluster analysis. The antibiotic susceptible/resistance patterns were analyzed to obtain dendrogram with cut-off value of 70%.

Determination of *mecA*, SCC*mec* and *van* genes

The genes responsible for resistance to vancomycin (*vanA*, *vanR*, *vanS, vanH, vanX, vanY* and *vanZ*) were investigated by PCR using a protocol described previously (Dezfulian et al. 2012). The oligonucleotide primers, annealing temperatures and expected amplicon sizes for PCR were shown in Table 1. For the PCR, five microliter of the extracted DNA was used as a template in a 25 µl PCR mixture containing 1XPCR buffer (50 mm KCl, 20 mM Tris HCl), 1.5 mM MgCl2, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 40 pmol each primers, and 2U of Taq DNA polymerase. The amplification of DNA was performed as follows: 94°C for 5 min of initial denaturation; 35 cycles of 94°C for 1 min, annealing for each gene shown in Table 1 and 72°C for 2 min; and a final extantion at 72°C for 10 min. Amplicons were loaded onto 1.5% Agarose Gel containing 1 µg/ml ethidium bromide and visualized under UV-light.

To determine the *mecA* gene for phenotypically methicillin resistant strains, a PCR protocol was per-

Table 3. Antibiotic resistance profiles of *Staphylococcus* spp.

		Antibiotic discs						
		ENR	V	T	AMC	OXA	G	CEP
R	n	3	18	3	10	15	3	12
	%	13.6	81.8	13.6	45.4	68.1	13.6	54.5
I	n	1	2	2	0	0	0	3
	%	4.5	9	9	0	0	0	13.6
S	n	18	2	17	12	7	19	7
	%	81.8	9	77.2	54.5	31.8	86.3	31.8

S: sensitive, I: Intermediate resistant, R: Resistant; ENR: enrofloxacin, V: vancomycin, T: tetracycline, AMC: Amoxycillin-clavulanic acid, OXA: oxacillin, G: gentamicin, CEP: cephalothin.

formed as described by Ciftci et al. (2009). The strains giving a 320 bp band were evaluated as positive for the *mecA* gene.

To discriminate the SCC*mec* types to which *S. aureus* isolates belong a multiplex PCR protocol was used (Boye et al. 2007). The primers and expected band sizes used in this multiplex PCR protocol are given in Table 2.

Detection of biofilm formation

Biofilm formation of the isolates was determined phenotypically using Congo Red Agar (CRA) Test. After cultivation of *Staphylococcus* spp. onto Congo Red Agar (CRA) plates containing 0.8 g of Congo Red dye and 36 g of sucrose, the strains were inoculated in CRA plates and incubated at 37°C for 24-72 hours. Black colonies on Congo Red Agar were considered biofilm positive, while colorless colonies considered were negative.

The genotypic determination of slime production, the PCR targetting *icaA* and *icaD* genes responsible for biofilm formation were performed as described by Yazdani et al. (2006). For the amplifying of *icaA*, AF (5'-CCT AAC TAA CGA AAG GTA G-3') and AR (5'-AAG ATA TAG CGA TAA GTG C -3') primers and of *icaD* gene, DF (5'-AAA CGT AAG AGA GGT GG-3') and DR (5'-GGC AAT ATG ATC AAG ATA-3') primers were used. Bands of 1315 bp and 381 bp were considered positive for the *İcaA* and *İcaD* genes, respectively. Five microliters of the extracted DNA was used as a template in a 50 µl PCR mixture containing 1X PCR buffer (50 mm KCl, 20 mM Tris HCl), 5 mM $MgCl_2$, 0,2 mM of each deoxynucleoside triphosphate (dNTP), 0.4 µM each primer and 1.5 U of Taq DNA polymerase. The amplification of DNA was performed as follows: 92°C for 5 min of initial denaturation; 30 cycles of 92°C for 1 min, 49°C for 1 min and 72°C for 1 min; and a final extention at 72°C for 7 min. Amplicons were loaded onto 1.5% Agarose Gel containing 1 µg/ml ethidium bromide. The presence and molecular weight of the amplified DNA fragments were confirmed by agarose gel electrophoresis and visualized under UV-light.

Genotyping of the isolates

Coagulase negative staphylococci strains were genotyped by RAPD-PCR (Versalovic and Lupski 2002, Fındık et al. 2009) using M13 (5´-GAG GGT GGC GGT TCT-3´) primer. Grouping of the RAPD-PCR patterns was carried out using the UPGMA cluster analysis. The strains grouping coefficients of similarity of 70% for RAPD typing were applied.

Results

Identification of the isolates

A total of 22 isolates were identified as CNS conventionally. These coagulase negative isolates were also identified as staphylococci other than *S. aureus*.

Antibiotic susceptibility test and antibiotyping

All CNS strains except for two were found to be resistant to at least two antibiotics. Antibiotic susceptibility patterns are given in Table 3. Most strains (81.8%) were vancomycin resistant, followed by oxacillin with a resistance percentage of 68.2%. Multi-drug resistance among the strains was low (13.6%).

Based on these patterns, the strains were antibiotyped and a dendrogram was generated. According to the dendrogram, the similarity among the strains ranged between 30-100%. The isolates were grouped into four clusters (AA-AD) with 70% cut-off value. Cluster AD included the most of the strains (36.36%) and in this cluster the strains showed similarity between 74% and 100% (Fig. 1).

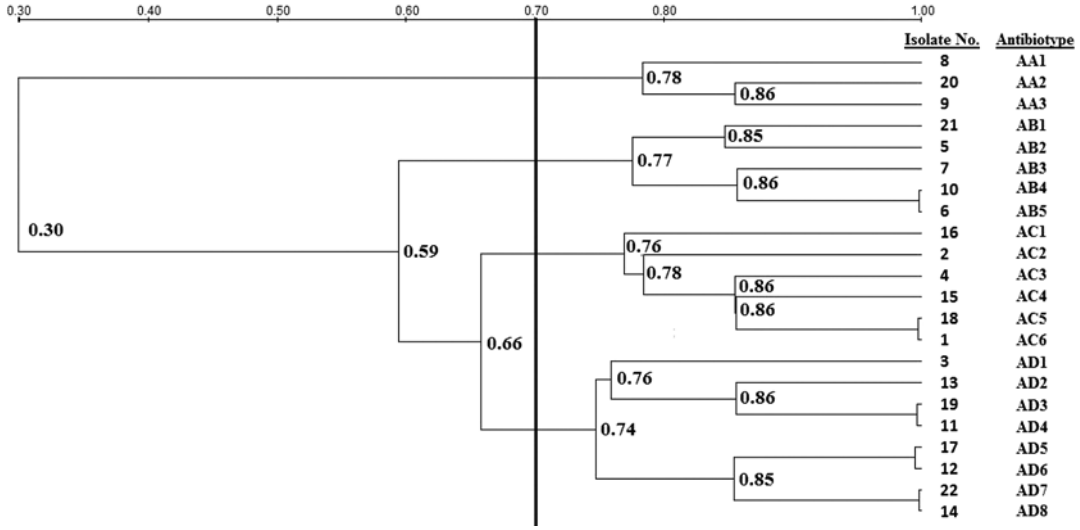

Fig 1. The antibiotyping results of *Staphylococcus* spp.

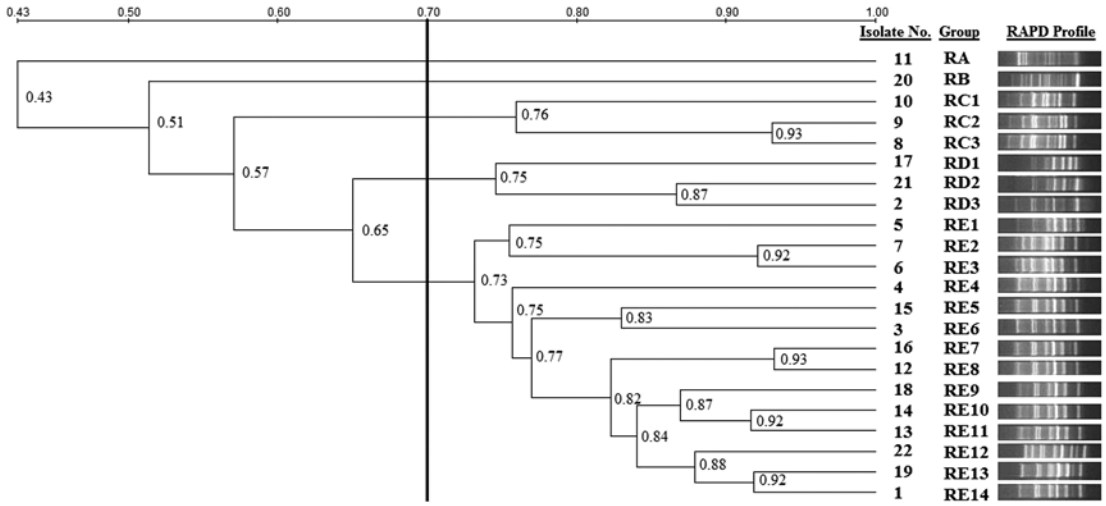

Fig 2. The genotyping results and dendrogram of *Staphylococcus* spp

Detection of *mecA*, *mecC*, SCC*mec* *vanA*, *vanB* and *vanC* genes by PCR

All isolates were found negative for *mecA,* SCC*mec* and *van* genes.

Detection of biofilm formation

Three strains (13.6%) were found to be positive on Congo Red Agar and these strains possessed *icaA* and *icaD* genes.

RAPD-PCR

According to evaluation of dendrogram generated from RAPD-PCR for 70% cut-off, the strains were grouped into 3 clusters (RC, RD and RE) and 2 unique types (RA, RB). Cluster RE included most of the strains (63.6%) and in this cluster the strains showed similarity between 73% and 93% (Fig. 2).

Discussion

Subclinical mastitis is the most important disease affecting the dairy industry. It causes high losses in the amount and quality of milk (Bradley 2002, Özenç et al. 2008, Ergun et al. 2009, Sudhan and Sharma 2010). Unlike clinical mastitis, subclinical mastitis is the problem of the herd and its detection is not as easy as clinical mastitis. Therefore, fast, accurate identification and correct struggle are essential to minimize financial losses and sustainability of herd health.

Coagulase-negative staphylococci are part of the normal flora of the skin of the teat and external orifice of the streak canal. Any factors irritating or damaging teat skin cause an increase in the number of CNS at these locations. Though CNS are not as pathogenic as the other principal mastitis pathogens such as *S. aureus* and *S. agalactiae* and infection mostly

remains subclinical, they have become most common in mastitis cases and considered as emerging mastitis pathogens. They can also cause persistent infections resulting in increased milk somatic cell count (SCC) and decreased milk quality (Pyörala and Taponen 2009). Mastitis cases caused by CNS are usually self-limiting, however, there are reports of clinical mastitis cases that often require antimicrobial treatment (Taponen et al. 2006).

Coagulase negative staphylococci, which are thought to be non-pathogenic for a long time, are now known to be responsible for important infections in both humans and animals, including mastitis. Prudent use of antibiotics plays an important role in effectively treating and controlling mastitis cases, including those caused by CNS. Therefore, there are many studies worldwide to determine and monitor antibiotic susceptibilities of bacterial strains including CNS that cause mastitis (Gentilini et al. 2002, Turutoğlu et al. 2006, Sawant et al. 2009). Most CNS were resistant against vancomycin (81.8%), oxacillin (68.2%) and cephalothin (54.5%), they were susceptible to enrofloxacin (86.4%), tetracycline (86.4%), gentamycin (86.4%), amoxycillin-clavulanic acid (54.6%). Aslantaş et al. (2014) have determined that most MR-CNS (methicillin resistant-CNS) isolates from subclinical bovine mastitis were susceptible to tetracycline (100%) and vancomycin (100%). In our study, most MR-CNS (73.3%) were also found to be resistant to vancomycin resistant. The high incidence of antimicrobial resistance, especially against oxacillin and vancomycin, among CNS isolated from buffalo milk with mastitis suggest that antibiotic susceptibility profiles should be monitored periodically.

Coagulase negative staphylococci are considered as important reservoirs of antibiotic resistance genes and associated mobile genetic elements, and there is the risk of transfer them between staphylococci (Patridge et al. 2018). They are also believed to contribute to the emergence of methicillin resistant *S. aureus* (MRSA) clones (Xu et al. 2018). The transfer of *mecA* gene, which is responsible for methicillin resistance, from CNS species to *S. aureus in vivo,* has been demonstrated (Wielders et al. 2001, Harrison et al. 2014). In this study, 15 CNS (other than *S. aureus*) were methicillin resistant phenotypically, but they had no *mecA* gene. Aslantaş et al. (2014) have reported that all isolates phenotypically resistant to oxacillin did not have the *mecA* gene, which was found in only 14.6% of the isolates. Although detection of the *mecA* gene by polymerase chain reaction (PCR) is the gold standard for the identification of oxacillin-resistant *Staphylococcus* and SCC*mec* typing by multiplex PCR permits the characterisation of the *Staphylococcus* species, the absence of *mecA* gene within resistant staphylococcal isolates was listed worldwide. It has been reported that moderate methicillin resistance was observed in isolates that lacked the *mecA* gene mutations (Hiramatsu et al. 1992) and the complete absence of five major SCC*mec* types and *mecA* genes as well as the gene product of PBP2a in phenotypically methicillin resistant staphylococci has been reported. This has been suggested to occur due to overproduction of β-lactamase. This resistance mechanism has already been proven for *S. aureus*, but it is the first time that it has been reported for CNS (Petinaki et al. 2002). Moreover, Ba et al. (2014) have mentioned specific alterations in different amino acids present in protein binding proteins cascade (PBPs 1, 2, and 3) which may be the basis of resistance.

Mobile genetic elements such as genomic islands, bacteriophages, pathogenicity islands, chromosomal cassettes, plasmids, insertion sequences and transposons play an important role in the spread of resistance and virulence in staphylococci. Among them, SCC (Staphylococcal Casette Chromosome) is a well-developed vehicle for genetic exchange of genes among staphylococcal species and carries *mecA* genes as well as other functional genes (Hanssen and Sollid 2005). Coagulase negative staphylococci are recognized as a large reservoir of SCC*mec*, however between CNS, SCC*mec* has been studied less frequently than *S. aureus* and data of SCC*mec* in CNS are relatively absent (Saber et al. 2017). The presence of SCC*mec* elements in phenotypically methicillin resistant CNS was investigated in this study. But no SCC*mec* type was detected in the strains. However, studies reporting the presence and types of SCC*mec* in CNS were available in Turkey (İnegöl and Türkyılmaz 2012, Aslantaş et al. 2014) and other countries (Ruppe et al. 2009, Chen et al. 2017).

Though resistance to vancomycin among coagulase-negative staphylococci was first reported 40 years ago (Siebert et al. 1979) the first clinically significant isolate has been reported in 1987 (Swalbe et al. 1987). Since that time, there have been many reports of clinically relevant coagulase-negative staphylococci that had diminished susceptibility to vancomycin (Sujatha and Praharaj 2012). In a study performed by Pamuk et al. (2010), vancomycin resistance has been found in 16.7% and 14.9% of CNS isolates isolated from buffalo milk and tulum cheese, respectively. Aslantaş et al. (2014) have reported that most MR-CNS isolates were also highly resistant to vancomycin (100%) as well as to erythromycin (92.3%), fusidic acid (84.6%), penicillin (76.9%), and rifampycin (61.5%), and susceptible to mupirocin (100%), tetracycline (100%), clindamycin (92.3%), and sulfamethoxazole-trimethoprim (69.2%). In this study, the resistance rate against vancomycin (81.8%) was higher than to oxacillin (68.2%). Howe-

ver, any *van* gene (*VanA, B, C and D*) was not found in the CNS strains. The exact mechanism of glycopeptide resistance between CNS is still unclear. Cell wall thickening has been reported for glycopeptide-resistant CNS (*S. epidermidis* and *S. haemolyticus*). Some glycopeptide-resistant CNS may possess an excess of glycopeptide-binding sites by virtue of the overproduction of cell wall peptidoglycan material (Becker et al. 2014). Thus, one can consider that the basic mechanisms leading to a reduced susceptibility to glycopeptides may be similar in CNS and *S. aureus* (Becker et al. 2014). The mechanism of resistance to vancomycin in the CNS strains found in this study is not van gene mediated. Although the precise genetic mechanism for vancomycin resistance in these staphylococcal strains awaits elucidation, the thickening of the cell wall may have contributed to the vancomycin resistance in the staphylococcal strains studied (Palazzo et al. 2005).

Today, many phenotypic and genotypic methods are used for bacterial typing. Antibiotyping, which is one of the phenotypic methods and based on antibiotic patterns, is also frequently used today. According to the evaluation of dendrogram based on the 70% similarity index, strains were divided into 4 different clusters (A1-A4). Similarity rates of strains in all clusters were close to each other and ranged from 74% to 86%. All strains found to be resistant to all beta lactam group antibiotics tested in the study were included in the same cluster (A2). In cluster A1, which includes 3 isolates with 78% similarity, the isolates were found to be resistant to at least 5 antibiotics. Likewise, most of the isolates with 77% similarity level (A2 cluster) were resistant to 4 antibiotics, while isolates with similarity levels of 76% (A3 cluster) and 74% (A4 cluster) were found to be resistant to 3 or less antibiotics. The isolates showing multiple antibiotic resistances were included in the A1 cluster.

Many genotyping methods have been used in polymorphism analysis including multilocus sequence typing (MLST), multilocus variable number tandem repeat analysis (MLVA), pulse field gel electrophoresis (PFGE) and PCR based methods such as random amplified polymorphism DNA (RAPD) PCR, restriction fragment length polymorphic DNA (RFLP) PCR. Among them, RAPD-PCR typing is widely used to characterize and differentiate staphylococcus isolates. It is a simple, useful and economically affordable technique (Zare et al. 2019). After genotyping of CNS strains using RAPD-PCR, variation among strains was determined. Based on 70% cut-off, strains revealed the presence of 22 RAPD types including 3 major clusters and 2 unique types. The similarity among the strains was ranged from 43% to 93%. Cluster RE included 14 strains (represented 63.6% of strains). Strains in this cluster showed similarity between 73% and 93%. There are many studies on genotyping of *S. aureus* isolates from various sources including mastitic milk using RAPD-PCR (Fitzgerald et al. 2000, Reinoso et al. 2004, Morandi et al. 2010, Zare et al. 2019). However, there are a limited number of reports about genotyping of CNS isolated from mastitic milk by RAPD-PCR. Qu et al. (2018) have investigated the genetic relationships of staphylococci isolated from mastitic cow milk using RAPD-PCR. They have grouped *Staphylococcus aureus* into 12 genotypes, of which 2 types represented 56% of isolates. *Staphylococcus chromogenes* have been clustered into 8 RAPD types, with 2 prevalent types containing 73% of isolates. A study has concentrated on *S. epidermidis* by investigating possible transmission of *S. epidermidis* from milkers to cows and the clonal diversity within unrelated bovine *S. epidermidis* has been investigated by Pulsed-field gel electrophoresis (Thorberg 2008). This study has revealed that RAPD-PCR can be used for typing CNS strains and the genetic relatedness and diversity among strains can be demonstrated.

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