The Journal of Microbiology (2010) Vol. 48, No. 3, pp. 267-274
DOI 10.1007/s12275-010-9373-9

Antimicrobial Susceptibilities of Coagulase-Negative Staphylococci (CNS) and Streptococci from Bovine Subclinical Mastitis Cases

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(Received December 2, 2009 / Accepted January 18, 2010)

The prevalence and antimicrobial susceptibilities of Staphylococci and Streptococci were assessed from subclinical mastitis cases. One hundred Coagulase-Negative Staphylococci (CNS) and 34 Streptoccocci were identified. The most frequently isolated species were *Staphylococcus haemolyticus* (27%) and *Staphylococcus simulans* (24%). Susceptible CNS species revealed the highest resistance to penicillin G (58%), ampicillin (48%), neomycin (20%), and oleandomycin (14%). CNS methicillin resistance rates within 82 isolates were 21.95% and 1.22% by disk diffusion and PCR methods, respectively. These results suggested the disk diffusion method was more prone to yield false positives. Partial sequencing of the 16S rRNA region from the *mecA* carrying isolate (*S. haemolyticus*) was homologous with *S. haemolyticus* sequences/accessions obtained from GenBank. However, the *mecA* gene sequence from this isolate was more closely allied with the *S. aureus mecA* gene of human origins. Identical sequence data was acquired from the National Center for Biotechnology Information (NCBI) database, suggesting horizontal gene transfer between the two species. CNS β-lactamase activity within 81 isolates was 29.63%. The most frequently isolated *Streptococcus* species were *S. uberis* (52%) and *S. agalactiae* (15%). Oleandomycin was the least effective antimicrobial agent on these isolates with 59% susceptibility. Results indicated that CNS and Streptococci exhibited various antimicrobial resistance responses. Consequently, isolation and identification of udder pathogens in herds suffering from subclinical agents is essential to select the most effective antimicrobial agent. Moreover, multiple resistance features of methicillin resistant (MR) isolates should be considered during antimicrobial susceptibility tests.

***Keywords*:** coagulase negative staphylococci, bovine mastitis, methicillin resistance, streptococci

Staphylococci and Streptococci are considered causative mastitis pathogens in both clinical and subclinical cases; and *Staphylococcus aureus* is the most important pathogen among *Staphylococcus* species. However recently in both subclinical and clinical mastitis cases throughout the world, increased attention has been paid to Coagulase-Negative Staphylococci (CNS) (Moon *et al*., 2007; Sawant *et al*., 2009). CNS has been shown to increase somatic cell count (SCC) in milk and cause milk production loss and mammary tissue damage (Chaffer *et al.*, 1998). Previous research also indicated that CNS are capable of persisting in udders for longer periods of time (Aarestrup *et al*., 1999). Although CNS based mastitis infections respond well to most antimicrobial agents, some CNS caused cases are left untreated. In addition, the degree of pathology among CNS isolates differs. Mandell *et al*. (1995) indicated that some CNS isolates are resistant to penicillinase-resistant penicillins and were termed methicillin resistant (MR) Staphylococci. Furthermore, a low-affinity penicillin-binding protein 2a (PBP2a) encoded by the *mecA* gene is responsible for methicillin resistance (Hartman and Tomasz, 1984). Suzuki *et al.* (1992) determined the *mecA* gene sequence is widely distributed in all MR strains of *S. aureus* and CNS. *S. aureus* and CNS resistance to methicilline has been a major problem during the last 30 years. However, the rate of methicillin resistance among Staphylococci of bovine origins is lower than that of human origins (Gentilini *et al.*, 2002; Moon *et al.*, 2007). The resistance mechanism can also contribute to additional defenses for β-lactams and other types of antimicrobial agents (CLSI, 2002; formerly National Committee for Clinical Laboratory Standards). β-Lactamases serve as an additional bacterial resistance mechanism against β-lactam antimicrobials. Methicillin resistance can be detected using either phenotypic or genotypic methods, but Moon *et al.* (2007) reported a lack of correlation between these approaches. Streptococci are also major bovine mastitis pathogens. Denamiel *et al*. (2005) indicated that mastitis causative Streptococci are usually susceptible to penicillin G (100%), erythromycin (72.4%), and clindamycin (74.5%). Previous CNS identification methods relied on phenotypic and biochemical tests. However, none of the methods provides CNS identification success. Current molecular biology based CNS identification methods are not fully developed. For example, PCR based techniques targeting internal transcribed spacer regions, including 16S rRNA, *sodA*, *tuf*, and real-time9 PCR provide promise for future molecular based CNS identification protocols (Boerlin *et al.*, 2003; Heikens *et al.*, 2005).

The aims of this study were to determine the prevalence and antimicrobial resistance of Staphylococci and Streptococci, and the distribution of methicillin resistance among CNS isolates from subclinical mastitis cases. In addition, MR CNS isolates were detailed based on partial sequence analysis of *mecA* and 16S rRNA genes.

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Materials and Methods

Bacterial strains

All cows exhibiting a subclinical bovine mastitis case were subjected to a bacterial examination (Staphylococci=100; Streptococci=34). Subclinical cases were determined based on three parameters: California Mastitis Test; no clinical signs of diseased cows; and the SCC of milk. Milk samples (n=221) belonging to different udder quarters were aseptically collected into 50 ml sterile tubes from a dairy-farming district, Ministry of Agriculture in Kahramanmaras Province, Turkey. Subsequently, 0.01 ml of each milk sample was plated onto Blood agar (Difco, USA) plates containing 5% sheep blood and incubated at 37°C for 2 days. Suspected single colonies of Staphylococci and Streptococci were selected and preliminary strain identifications were performed based on conventional methods, including colony morphology, haemolysis, Gram staining, and catalase and coagulase (Bacti Coagulase; Merck, Germany) tests. All identified Staphylococci and Streptococci were stored at -80°C using Todd-Hewitt (Merck) and Tryptic Soy Broths (Merck), respectively.

Identification of Staphylococci and Streptococci

Each individual Staphylococci and Streptococci were identified by both conventional and the VITEK GPI card system (bioMérieux, France) methods. Thawed strains were grown on Colombia agar (Merck) containing 5% sheep blood at 37°C for 16 to 24 h, then re-plated and grown again before testing. Bacterial suspensions were prepared by emulsifying the isolates in 0.45% saline to the equivalent of a 0.5 McFarland turbidity standard with a VITEK 2 instrument (version 4.01; DensiChek, bioMérieux).

Antimicrobial susceptibility test

An antimicrobial susceptibility test was performed using the agar disk diffusion method on Mueller-Hinton agar (Merck) as described by CLSI (2002). Mueller-Hinton agar was supplemented with 5% sheep blood during Streptococci antimicrobial susceptibility testing. The plates were incubated at 37°C, except for oxacillin at 35°C, for 24 h. Antimicrobial agent concentrations on each disk (Bioanalyse, Turkey) were as follows: chloramphenicol (30 μg), neomycin (30 μg), penicillin G (10 U), streptomycin (10 μg/disk), oleandomycin (15 μg), kanamycin (30 μg), erythromycin (15 μg), tetracycline (30 μg), clindamycin (2 μg), ampicillin (10 μg), and oxacillin (1 μg). Oxacillin was used for detection of methicillin resistance. The isolates were categorized as susceptible, intermediate, and resistant based on the interpretative criteria of CLSI (2002). To determine the breakpoint value of neomycin, the BSAC standard was applied (Andrews, 2008). To determine the breakpoint value of streptomycin, a criteria developed by CLSI (1997) was applied. Breakpoint values for erythromycin were developed by CLSI and applied to oleandomycin based on Soussy *et al.* (1994). *S. aureus* ATCC 25923 was used as a reference strain. All Staphylococci were tested for β-lactamase production using nitrocefin sticks (Oxoid BR66A).

Multiple antibiotic resistance index

The multiple antibiotic resistance (MAR) index is defined as *a*/*b* where *a* represents the number of antibiotics to which the strain was resistant, and *b* represents the number of antibiotics to which the strain was exposed (Moschetti *et al*., 1997).

DNA extraction

Whole cellular DNA was prepared for PCR amplifications using the method of Bell *et al.* (1998) with some modifications. The following briefly summarizes the protocol. After growth of the isolates at 37°C in 4 ml of Nutrient broth, 1 ml of isolate was transferred into a sterile eppendorf tube and centrifuged at 10,000 rpm for 2 min. The supernatant was removed and the resulting pellet was washed with 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and re-centrifuged. The supernatant was again removed and the pellet washed with 200 μl of TE buffer; the suspension was incubated at 95°C for 20 min. Following the incubation, a final centrifugation at 4°C was performed for 2 min. The supernatant was collected and maintained at -20°C until PCR amplification. For PCR amplifications, 5 μl of this preparation was used as template DNA.

PCR

Methicillin resistance was detected by amplification of a 313-bp fragment from the *mecA* gene using primers Mec449F (5′-AAACTAC GGTAACATTGATCGCAAC-3′) and Mec761R (5′-CTTGTACCCA TTTTGATCCATTTG-3′) (Kohner *et al*., 1999). Each PCR reaction was performed in a 50 μl total reaction mixture containing 5 μl of target DNA, 200 μM deoxynucleotide triphosphates (dNTP), 40 pmol of each primer, 5 μl of 10× PCR buffer (500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.0), and 1 unit of Taq DNA Polymerase (MBI Fermentas). PCR parameters were as follows: an initial denaturation step at 94°C for 2 min; 30 cycles at 94°C for 30 sec for denaturation, 62°C for 30 sec for annealing, and 72°C for 1 min for polymerization; and a final extension at 72°C for 2 min. Reactions were performed in an Eppendorf Mastercycler® Gradient Thermal Cycler (Germany). The identification of *mecA* positive *S. haemolyticus* was verified by amplifying the DNA of the isolate with the following Staphylococcal 16S rRNA gene specific primers: 16S 387F (5′-CGAAAGCCTGACG GAGCAAC-3′) and 16S 914R (5′-AACCTTGCGGTCGTACTCCC-5′), which produced a 500 bp fragment (Kohner *et al.*, 1999). The same PCR conditions as the *mecA* gene were used for 16S rRNA gene amplifications, except the amount of each primer was decreased to 20 pmol in the final concentration. PCR products were separated by electrophoresis on 1% agarose gels and visualized using a gel documentation system (Vilber Lourmat, France).

DNA sequencing

PCR products (*mecA* and 16S rRNA genes) were cleaned and sequenced for both strands with the same primers used for PCR (Refgen Biotech. Ltd., Turkey). *mecA* and 16S rRNA gene sequences were deposited to the NCBI database with the respective accession numbers FJ654655 and FJ654656. Nucleotide sequences were compared using the BLAST search tool (http://www.ncbi.nlm.nih.gov/Blast.cgi).

Results

CNS (n=100) were isolated from 69 of 221 milk samples. A screening of each individual sample showed the following: a single *Staphylococcus* was present in 37 samples; two different Staphylococci were present in 19 samples; four Staphylococci were present in one sample; and Staphylococci and Streptococci were present in 12 milk samples. Fourteen of 221 milk samples contained either one or two *Streptococcus* species.

The distribution of CNS antimicrobial susceptibilities is presented in Table 1. The antimicrobial agents were tested at various concentrations and the highest resistance among CNS was observed for penicillin (58%), ampicillin (48%), neomycin (20%), and oleandomycin (14%). Based on the susceptibilities

Table 1. Distribution of various CNS antibiotic susceptibilities (n=100)

Antibiotic[b]	Concentration (μg/disk)	Breakpoint value (mm)[a]			CNS distribution (%)[a]		
		R	I	S	R	I	S
CHL	30	≤12	13-17	≥18	1	3	96
NEO	30	≤16	-	≥17	20	0	80
PEN	10 U	≤28	-	≥29	58	0	42
STR	10	≤11	12-14	≥15	1	19	80
OLE	15	≤13	14-22	≥23	14	48	38
KAN	30	≤13	14-17	≥18	9	25	66
ERY	15	≤13	14-22	≥23	2	18	80
TET	30	≤14	15-18	≥19	7	7	86
CLI	2	≤14	15-20	≥21	8	6	86
AMP	10	≤28	-	≥29	48	0	52

[a] R, resistant; I, intermediate; S, susceptible
[b] CHL, chloramphenicol; NEO, neomycin; PEN, penicillin G; STR, streptomycin; OLE, oleandomycin; KAN, kanamycin; ERY, erythromycin; TET, tetracycline; CLI, clindamycin; AMP, ampicillin

of CNS, the most effective antimicrobial agents were chloramphenicol (96%), tetracycline (86%), and clindamycin (86%). The distribution of antimicrobial susceptibilities of each CNS species identified in the samples is presented in Table 2. Although neomycin resistance was quite high among CNS isolates, various resistance levels were detected for each species. For instance, neomycin resistance for *S. haemolyticus* and *S. sciuri* was 88% and 0%, respectively. However, differences in other antimicrobial agents such as chloramphenicol and streptomycin among each species were not as notable as penicillin resistance.

There are two well documented mechanisms responsible for *β*-lactam resistance in CNS. One is accomplished by the presence of *β*-lactamase activity and another one is presence of *mecA* gene product in the case of penicillinase-resistant antibiotics. The results detected 24 out of 81 CNS isolates (29.26%) as *β*-lactamase producers (data not shown). Phenotypic and genotypic methicillin resistance rates among CNS isolates were 21.95% and 1.22%, respectively. Among phenotypic MR isolates, only two were *β*-lactamase producers (data not shown). A single *mecA* positive *S. haemolyticus* was detected among the CNS isolates. Figure 1 shows an approximately 313 bp PCR product representing the *mecA* gene. Nucleotide alignment results for the *mecA* and 16S rRNA gene are presented in Tables 3 and 4, respectively. A partial sequence (213 bp in length) of the *mecA* gene derived from the *mecA* carrying isolate was identical to 60 sequences from methicillin resistance related sequences identified in GenBank. The genes confirmed in GenBank were determined to be related to *S. aureus* methicillin resistance genes. In addition, a partial sequence of 436 bp from the 16S rRNA gene was identical to that of a few *S. haemolyticus* isolates, a single *S. croceolyticus*, *S. hominis* isolates, and other Staphylococci obtained from environmentally related origins (e.g., airplane cabin air, artistic heritage, squalene biodegradation, and coral surface mucus). When a shorter region of the same 16S rRNA gene (89 bp in length) region was analyzed, the majority of homologous sequences in GenBank were *S. haemolyticus* isolates, including those of bovine mastitis origins. Table 5 provides the distribution of various antimicrobial susceptibilities of Streptococci. Antimicrobial agents were tested at various concentrations and the lowest susceptibility among Streptococci was observed for oleandomycin (59%). The distribution of antimicrobial susceptibilities for each *Streptococcus* species identified in the study is provided in Table 6. Similar to CNS data, antimicrobial resistance patterns in *Streptococcus* differed among species e.g. streptomycin susceptibility for *S. uberis* and *S. agalactiae* was 94% and 28%, respectively.

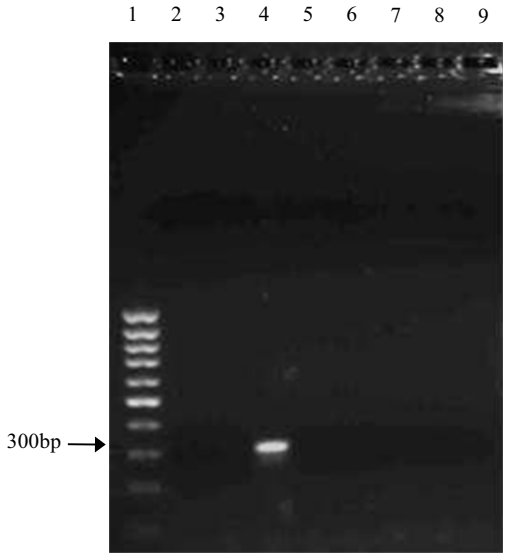

Fig. 1. Detection of *mecA* by PCR from CNS isolates. Lanes: 1, 100 bp marker; 2 to 8, PCR products from 7 CNS isolates (*S. haemolyticus*, *S. sciuri*, *S. haemolyticus*, *S. epidermidis*, *S. haemolyticus*, *S. simulans*, *S. haemolyticus*); 9, negative PCR control without DNA. Only one isolate (*S. haemolyticus*) in lane 4 exhibits the *mecA* gene product.

Table 2. Distribution of various antibiotic susceptibilities of each identified CNS species

Species	Number of isolate (%)	Antibiotic[a]									
		CHL	NEO	PEN	STR	OLE	KAN	ERY	TET	CLI	AMP
		Number (%) of susceptible CNS									
S. haemolyticus	27 (27)	26 (96)	22 (88)	6 (22)	19 (70)	8 (30)	17 (63)	26 (96)	25 (93)	24 (89)	14 (52)
S. simulans	24 (24)	23 (96)	20 (83)	8 (33)	19 (79)	9 (38)	15 (63)	17 (71)	23 (96)	24 (100)	13 (54)
S. auricularis	14 (14)	13 (93)	10 (71)	10 (71)	12 (86)	7 (50)	8 (57)	9 (64)	8 (57)	9 (64)	7 (50)
S. hominis	12 (12)	12 (100)	9 (75)	6 (50)	9 (75)	2 (17)	7 (58)	10 (83)	10 (83)	10 (83)	5 (42)
S. warneri	8 (8)	8 (100)	7 (88)	4 (50)	6 (75)	4 (50)	6 (75)	7 (88)	7 (88)	8 (100)	6 (75)
S. capitis	6 (6)	6 (100)	6 (100)	3 (50)	6 (100)	4 (67)	6 (100)	5 (83)	5 (83)	4 (67)	3 (50)
S. cohnii	3 (3)	3 (100)	2 (67)	2 (67)	3 (100)	2 (67)	2 (67)	1 (33)	2 (67)	2 (67)	0 (0)
S. xylosus	3 (3)	3 (100)	3 (100)	2 (67)	3 (100)	2 (67)	3 (100)	2 (67)	3 (100)	3 (100)	3 (100)
S. epidermidis	2 (2)	1 (50)	1 (50)	0 (0)	2 (100)	0 (0)	2 (100)	2 (100)	2 (100)	1 (50)	0 (0)
S. sciuri	1 (1)	1 (100)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	1 (100)

[a] CHL, chloramphenicol; NEO, neomycin; PEN, penicillin G; STR, streptomycin; OLE, oleandomycin; KAN, kanamycin; ERY, erythromycin; TET, tetracycline; CLI, clindamycin; AMP, ampicillin

Discussion

Recently, CNS have been identified as a group of pathogens increasing the incidence of bovine mastitis. In our study, 100 CNS were isolated from 69 of 221 milk samples obtained from subclinical cases. The CNS isolation rate was 31.22%, a rate higher (13.6%) than that obtained in Argentine samples (Gentilini *et al.*, 2002). Former studies have treated CNS as one monotypic species group, and identification of the specific one or more CNS species that cause bovine mastitis has received little attention (Salmon *et al.*, 1998; Taponen *et al.*, 2006; Moon *et al.*, 2007). In the present study, *S. haemolyticus* (27%) and *S. simulans* (24%) were the most frequently isolated species causing mastitis in subclinical cases. Moon *et al.* (2007) reported a variable distribution of CNS species isolated from mastitic milk over a three-year period. Moreover, *S. simulans* and *S. haemolyticus* were the most frequently isolated species in a one year period, results congruent with our study. Kirkan *et al.* (2005) showed that *S. hyicus* (33.3%) and *S. chromogenes* (26.7%) were the most frequently isolated species in mastitis cases from Aydín Province, Turkey. Sawant *et al.* (2009) most recently reported that *S. chromogenes*, *S. epidermidis*, and *S. hyicus* were the most predominant species isolated from bovine milk.

Identification of veterinary pathogens by conventional methods is tedious and time-consuming. Therefore, commercial phenotypic identification systems (API Staph ID32 and Vitek) are widely used by clinical microbiologists due to their convenience and cost-effectiveness. However, many of these systems include only a limited number of veterinary strains in

Table 3. Nucleotide alignment results of the *mecA* gene (19 of 60 identical sequences are represented)

Accession number	Organism	Sequence	Basepair (% Maximum identity)
AB437289.1	*S. haemolyticus* strain: 91B250	cassette chromosome *mec*, partial sequence	
AB373032.1	*S. aureus*	type 5C1 staphylococci	
AM943017.1	*S. aureus*	SCC*mec* IV element	
EU929082.1	*S. pseudintermedius*	PBP	
EU929079.1	*S. pseudintermedius*	PBP	
EU333401.1	*S. aureus*	PBP	
AB353125.1	*S. aureus*	type T staphylococcal cassette chromosome *mec*	
AM292304.1	*S. aureus*	SCC*mec* ZH47 mobile element	
AB266532.1	*S. aureus*	type-IV.3 (IVc) st	
AB236888.1	*S. aureus*	*mecA* gene for PBP2	213 (100)
AY786579.1	*S. aureus*	mutant PBP2a (*mecA*) gene	
AP006716.1	*S. haemolyticus* JCSC1435	Complete genome	
AM048803.2	*S. kloosii*	*mecA* gene; PBP2	
AM048802.2	*S. vitulinus*	*mecA* gene; PBP2	
Y13096.1	*S. sciuri* strain K8	*mecA* gene	
AB121219.1	*S. aureus*	type-V staphylococci	
X52592.1	*S. epidermidis*	*mecA* gene for PBP2	
AB033763.2	*S. aureus*	type-I staphylococci	
D86934.2	*S. aureus*	type-II staphylococci	

Table 4. Nucleotide alignment results of partial nucleotide sequences of the 16S rRNA region of *S. haemolyticus*

Accession number	Organism	Sequence	Basepair (% Maximum identity)
EU379304.1	*S. haemolyticus* strain 6R-J-5	16S rRNA gene, partial sequence	436 (100)
EU263109.1	*S.* sp. DFVB6		
EF188282.1	*S.* sp. GYZ 16S		
DQ530529.1	*S.* sp. V2mt2		
AY748913.1	*S.* sp. NIPHL090904/B4		
AF202011.1	Human oral bacterium C20		
AY953148.1	*S. croceolyticus*		
AJ458194.1	*S.* sp. 2Asq67		
EU379296.1	*S. hominis* strain 6J-4b		
DQ837034.1	*S.* sp. f50-6		
FJ394023.1	*S. haemolyticus* strain SW-1/RSH 16	16S rRNA gene, partial sequence	89 (100)
EU250486.1	*S. haemolyticus* isolate BM-MRI 17		
EU250484.1	*S. haemolyticus* isolate BM-MRI 15		
EU071616.1	*S. haemolyticus* strain EHFS1_AU1Ha		
EU071615.1	*S. haemolyticus* strain EHFS1_S12Hd		

their data base, which limits the identification accuracy of the system due to the wide range of veterinary pathogens. Recently, the VITEK 2 Gram-positive (GP) identification card (bio-Mérieux) was redesigned for increasing identification accuracy. The system has now been successively applied in isolation and identification of CNS from bovine mastitis cases (Moon *et al*., 2007). Ten different CNS species were identified in the present study using this system.

In many countries, antimicrobial susceptibilities have been primarily investigated using the disk diffusion method due to its low cost and ease of application. However, the minimal inhibitory concentration (MIC) approach has resulted in reserves of data on antimicrobial resistance. Gianneechini *et al.* (2002) reported high correlation in results between the two methods. The result of the disk diffusion method and MIC (from this and other studies) demonstrated that resistance to penicillin G was highest on CNS isolates from different geographic regions of the world, including Turkey. However, resistance to penicillin G between CNS isolates varied. For example, penicillin resistance among CNS isolates in the present study was higher (58%) than that in Uruguay (22%; Gianneechini *et al*., 2002), Argentina (27.6%; Gentilini *et al*., 2002), Finland (37.2%; Myllys *et al*., 1998), and Greece (17.1%; Fthenakis, 1998). However, the penicillin resistance rate in this study was similar to that found in the USA (57%; Owens *et al*., 1997) and Korea (60.2%; Moon *et al*., 2007). The difference in penicillin resistance rates among countries could be related to mastitis control management, where some countries strictly regulate the use of antimicrobial agents. Moreover, penicillin resistance among CNS isolates was respectively 90% and 62.5% in Aydín and Burdur regions of Turkey (Kirkan *et al*., 2005; Turutoglu *et al*., 2006). Penicillin and ampicillin are used commonly worldwide for mastitis treatment. Therefore, high levels of penicillin and ampicillin resistance between CNS were expected in the present study (Table 1). Previously, susceptibility of CNS against other antimicrobial agents was investigated. Erythromycin resistance (2%) reported here was lower than studies conducted in Greece (6.1%; Fthenakis, 1998) and Korea (8.4%; Moon *et al*., 2007), but higher than in Uruguay (0%; Gianneechini *et al.*,

Table 5. Distribution of various Streptococci antibiotic susceptibilities (n=34)

Antibiotic[b]	Concentration (μg/disk)	Breakpoint value (mm)[a]			Streptococci distribution (%)[a]		
		R	I	S	R	I	S
CHL	30	≤17	18-20	≥21	9	0	91
NEO	30	≤16	-	≥17	0	0	100
PEN	10 U	≤19	20-27	≥28	6	9	85
STR	10	≤11	12-14	≥15	6	3	91
OLE	15	≤15	16-20	≥21	3	38	59
KAN	30	≤13	14-17	≥18	0	0	100
ERY	15	≤15	16-20	≥21	3	3	94
TET	30	≤18	19-22	≥23	0	6	94
CLI	2	≤14	15-20	≥21	12	3	85
AMP	10	≤18	19-25	≥26	12	12	76

[a] R, resistant; I, intermediate; S, susceptible
[b] CHL, chloramphenicol; NEO, neomycin; PEN, penicillin G; STR, streptomycin; OLE, oleandomycin; KAN, kanamycin; ERY, erythromycin; TET, tetracycline; CLI, clindamycin; AMP, ampicillin

Table 6. Distribution of various antibiotic susceptibilities of each identified *Streptococcus* species

Species	Number of isolate (%)	Antibiotic[a]									
		CHL	NEO	PEN	STR	OLE	KAN	ERY	TET	CLI	AMP
		Number (%) of susceptible *Streptococcus*									
S. uberis	18 (52)	16 (89)	18 (100)	17 (94)	17 (94)	10 (56)	18 (100)	16 (89)	17 (94)	15 (83)	14 (78)
S. agalactiae	5 (15)	5 (100)	5 (100)	4 (80)	5 (28)	2 (40)	5 (100)	5 (100)	4 (80)	3 (60)	5 (100)
S. acidominimus	3 (9)	3 (100)	3 (100)	3 (100)	3 (17)	2 (67)	3 (100)	3 (100)	3 (100)	3 (100)	2 (67)
S. salivarius	2 (6)	2 (100)	2 (100)	0 (0)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	1 (50)
S. bovis	2 (6)	1 (50)	2 (100)	2 (100)	2 (100)	1 (50)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)
S. faecalis	1 (3)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0 (0)
S. intermedius	1 (3)	1 (100)	1 (100)	1 (100)	0 (0)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
S. constellatus	1 (3)	1 (100)	1 (100)	1 (100)	1 (100)	0 (0)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
S. anginosus	1 (3)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0 (0)

[a] CHL, chloramphenicol; NEO, neomycin; PEN, penicillin G; STR, streptomycin; OLE, oleandomycin; KAN, kanamycin; ERY, erythromycin; TET, tetracycline; CLI, clindamycin; AMP, ampicillin

2002); tetracycline resistance (7%) was lower than in Korea (20.6%; Moon *et al.*, 2007). However, in Uruguay (Gianneechini *et al.*, 2002) oxytetracycline resistance (13.9%) was higher than that detected in our study. In addition, clindamycin resistance was higher than reported in Argentina (0%; Gentilini *et al.*, 2002) and Greece (6%; Fthenakis, 1998). Differences in antimicrobial resistance observed for macrolides, aminoglycosides and quinone groups have several plausible explanations. A primary reason for the lack of congruence might be the limitations in mastitis detection and level of herd management to determine subclinical and clinical mastitis (Ruegg and Reinemann, 2002). Antimicrobial resistance might be lower in isolates obtained from subclinical rather than clinical mastitis cases, since most of the subclinical cases have been left untreated. However, Taponen *et al.* (2006) detected no difference in the total number of CNS species isolated from subclinical and clinical cases. Another factor responsible for differences in antimicrobial resistance might be related to the proportion of each CNS species in a heterogeneous group. For example, *S. epidermidis* is considered a major CNS species in clinical cases and a separate species group for accurate methicillin resistance detection (Tenover *et al.*, 1999). Furthermore, our study was consistent with a previous study by Salmon *et al.* (1998) where higher MIC_{90} values for penicillin were detected in *S. xylosus* and *S. epidermidis*, with values of 33% and 100% in our study. However, penicillin resistance patterns between *S. warneri* and *S. capitis* were not consistent with our current results.

Presence of β-lactamase activity is one of the most important factors for resistance to β-lactams, therefore we assessed β-lactamase activities between CNS isolates. In former studies, β-lactamase activities from mastitis cases ranged from 34.1% to 83.6% (Thornsberry *et al*., 1997; Myllys *et al.*, 1998; Taponen *et al.*, 2006); and in our work 81 CNS isolates showed 29.26% β-lactamase activity consistent with Gianneechini *et al.* (2002) and Taponen *et al.* (2006). In addition, penicillin resistance among CNS β-lactamase producers was 75% in the present study, which was lower than isolates evaluated from Argentina (Gentilini *et al.*, 2002).

The presence of PBP2a, the *mecA* gene product, is considered another factor responsible for β-lactams resistance. The *mecA* gene is located on a mobile genetic element designated Staphylococcal cassette chromosome *mec* (SCC*mec*), which contains the *mec* gene complex (the *mecA* gene and its regulators) and the *ccr* gene complex, encoding site-specific recombinases responsible for the mobility of *SCCmec* (Katayama *et al*., 2000). SCC*mec* typing is essential for understanding the molecular epidemiology of MR *Staphylococcus* strains. The SCC*mec* elements are classified into Types I-VI based on the *mec* and *ccr* gene complex characteristics and further distinguished into subtypes according to their junkyard DNA regions. Genotypic methicillin resistance rate between CNS in our study (1.22%) was comparable to isolates from Korea (1.57%) and Argentina (0.8%). However, a better correlation was found between genotypic and phenotypic methicillin resistance by Gentilini *et al.* (2002) and Moon *et al*. (2007) when compared to the results of our study. Turutoglu *et al.* (2006) detected a 22.8% phenotypic methicillin resistance rate for CNS isolates from Burdur region of Turkey. Studies continue to report a lack of correlation between methods using CNS isolates from clinical specimens and Staphylococci isolates from bovine mastitis (York *et al.*, 1996; Moon *et al.*, 2007). Although problems remain, laboratory modifications have been suggested to overcome the limitations and include increasing inoculum volume, incubation time, medium sodium chloride concentration, and a decrease in incubation temperature. Therefore, any correlation obtained between the two methods should be reconsidered and addressed for each CNS species.

A single *S. haemolyticus* isolate was found to be both phenotypically and genotypically MR (Fig. 1). This was unexpected because a *mecA* positive *S. haemolyticus* isolate was not reported from a bovine mastitis case. Therefore, part of the *mecA* gene region from this isolate was amplified by PCR and the resulting amplicon was sequenced. The results indicated that the *mecA* partial sequence of this isolate was grouped with other Staphylococcal *mecA* regions. However, most of the groups consisted of methicillin resistant related regions of *S. aureus* rather than methicillin resistant regions of other CNS species (Table 3). These results indicated the possibility of a *mecA* conserved region for *S. aureus* and *S. haemolyticus* or horizontal gene transfer of *mecA* between the two species. A recent study performed on clinical isolates showed evidence for horizontal SCC*mec* transfer from a MR *S. haemolyticus* to a methicillin susceptible *S. aureus* strain

ST45 (Berglund and Soderquist, 2008). Hanssen *et al.* (2004) suggested the more frequent occurrence of SCC*mec* in MR CNS rather than *S. aureus* was the result of methicillin resistance transfer to *S. aureus* from CNS reservoirs. Following comparisons of MR CNS isolates from bovine mastitis cases with isolates from clinical cases, high levels of methicillin resistance was detected among clinical isolates of *S. aureus* relative to CNS. Furthermore, methicillin resistance among CNS was rarely found in animal cases. Therefore, isolation and characterization of high numbers of MR CNS isolates from mastitis might be the key to elucidate methicillin resistance mechanisms in detail for cases in other animals. Moreover, the application of simple DNA extraction methods for CNS allows the rapid detection of the *mecA* gene. The technique used in this study was quick, easy, and economical.

Phenotypically, 18 MR CNS of the 82 tested for MR were distributed as follows: *S. auricularis* (8), *S. hominis* (3), *S. simulans* (2), *S. capitis* (2), *S. haemolyticus* (2), and *S. cohnii* (1). *S. warneri*, *S. sciuri*, and *S. xylosus* isolates did not test positive for MR. Previous data indicated that MR *S. aureus* and CNS were more resistant to other antimicrobial agents (ampicillin, cephalothin, kanamycin, and gentamicin) than methicillin-susceptible Staphylococci (Voss and Doebbeling, 1995). Ten of the 18 MR isolates had MAR values above 0.4. Although the isolates exhibited high MAR values, resistance of the isolates to penicillin and chloromphenicol was 38.9% and 5.55%, respectively. On the other hand, resistance to clindamycin was 44.4%, which was higher than that observed by Moon *et al.* (2007). Our data determined the single *mecA* positive isolate had quite a high MAR index value (0.6) for ten tested antimicrobial agents, including chloramphenicol, neomycin, penicillin, streptomycin, kanamycin, and ampicillin. This isolate reflected the high MAR index feature of MR *Staphylococcus*. Consistent with our results, Turutoglu *et al.* (2009) found a single MR *S. aureus* isolate obtained from bovine milk was resistant to neomycin, gentamycin, and kanamycin.

16S rRNA gene sequencing has been successfully used to identify many bacterial species, including some Staphylococci (Boerlin *et al.*, 2003). However, the rate of evolution of the 16S rRNA gene might be insufficient to discriminate closely related species (Heikens *et al.*, 2005). However, this gene remains widely used to identify Staphylococcal species. In our study, a partial sequence of 16S rRNA from *S. haemolyticus* was aligned with other Staphylococcal sequences from GenBank, and the primary sequences were homologous to *S. haemolyticus*.

Similar to CNS studies of bovine mastitis cases, research addressing antimicrobial resistance patterns of Streptococci is limited in Turkey. In this present study, kanamycin and neomycin were the most effective and oleandomycin was the least effective antimicrobial agent for all *Streptococcus* isolates. Although penicillin G was highly effective (85%) on these isolates, its effect was not as great as observed for Argentine isolates (100%; Denamiel *et al*., 2005). We detected low resistance (6%) to penicillin among *S. uberis*, results incongruent with reports from other studies where a complete lack of resistance was observed (Gianneechini *et al.*, 2002; Denamiel *et al.*, 2005). The susceptibility among *S. uberis* isolates for penicillin (94%) and erythromycin (89%) were high in our study. However Rossittto *et al.* (2002) detected lower susceptibility among *S. uberis* isolates for penicillin (50.4%) and erythromycin (51.9%) by applying MIC. In the present study, similar to results observed in *S. uberis*, other *Streptococcus* species exhibited different susceptibility patterns to penicillin and other antimicrobial agents consistent with the results of Rossitto *et al.* (2002).

Conclusion

Our present study showed evidence for lower genotypic methicillin resistance among isolated CNS mastitis cases, congruent with reports from other studies. The results further indicated that the partial *mecA* gene sequence of the *S. haemolyticus* isolate had a sequence homologous to the *S. aureus* accession deposited in GenBank. The most likely hypothesis is horizontal gene transfer of methicillin resistance between the two species. Therefore, more research is required to investigate detailed methicillin resistance related genes in CNS of mastitis origins. Although tetracycline, neomycin, chloromphenicol and clindamycin were the most effective antimicrobial agents in both CNS and Streptococci, species of both heterogeneous groups had variable antimicrobial resistant rates. Thus, bacterial agents of mastitis origins should be identified at the species rather than genus level for effective antimicrobial agent selection.

Acknowledgements

Authors thank Kahramanmaras Sutcu Imam University Research Fund (2005/4-17) and The Turkish Scientific Research and Technical Council (TUBITAK) for financial support.

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