

Methicillin and aminoglycoside resistance in *Staphylococcus aureus* isolates from bovine mastitis and sequence analysis of their *mecA* genes

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Abstract Although methicillin-resistant *Staphylococcus aureus* (MRSA) were generally isolated from human beings; these agents were recently isolated from various animal species. It has been shown that MRSA isolates are not only resistant to beta-lactam antibiotics, but can also be resistant to the other commonly used antibiotics. In this study, 18 phenotypic methicillin resistant *S. aureus* isolates from bovine mastitis cases were analyzed by PCR for the presence of *mecA* gene encoding methicillin resistance and *aac(6')/aph(2'')*, *aph(3')-IIIa* and *ant(4')-Ia* genes encoding aminoglycoside resistance. Out of 18 *S. aureus* isolates (oxacillin MICs, ≥ 4 $\mu\text{g/ml}$), 3 were positive for *mecA* gene. Only one from 3 *mecA* positive isolates was positive for genes encoding aminoglycoside-modifying enzymes and this isolate carried *aac(6')/aph(2'')* in combination with *aph(3')-IIIa* gene. The *aph(3')-IIIa* gene was detected in 3 isolates. These three isolates carrying the aminoglycoside-modifying enzyme genes were resistant to gentamicin, kanamycin and neomycin. The *mecA* gene of 3 MRSA isolates was sequenced. All three *mecA* genes of these isolates were identical to that found in human MRSA strains, except a one-base substitution at nucleotide position 757. From the data presented in this study, it can be concluded that MRSA isolated from bovine mastitis may be originated from human beings, but further studies are needed to investigate the possibility of zoonotic transfer of MRSA.

Keywords Bovine mastitis · *Staphylococcus aureus* · Methicillin resistance · Aminoglycoside resistance

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Introduction

Staphylococcus aureus is a major pathogen of cow's mammary gland which can become especially serious if induced by strains resistant to antimicrobial drugs (Devriese et al. 1972; Watts and Salmon 1997). Methicillin (oxacillin)-resistant *S. aureus* (MRSA) strains are among the most threatening bacteria involved in nosocomial infections (Unal et al. 1992; Vannuffel et al. 1998; McBryde et al. 2004). However, in veterinary medicine, MRSA as well as multi-resistant *S. aureus* strains are reported occasionally (Sequin et al. 1999; Lee 2003; Van Duijkeren et al. 2004; Malik et al. 2006; Weese et al. 2006). Resistance to methicillin is attributable to multiple mechanisms, but the most important mechanism is the production of a specific penicillin-binding protein 2a (PBP-2a), that has a reduced binding affinity for beta-lactamase resistant penicillins and for all other beta lactam compounds (Chambers 1997). The PBP-2a is encoded by the chromosomal *mecA* gene and has very high levels of homology in MRSA (Bignardi et al. 1996; Fluit et al. 2001). A different chromosomal gene, *femA*, which works together with *mecA* gene, is necessary for the expression of the methicillin resistance in *S. aureus* (Unal et al. 1992; Chambers 1997). The *femA* gene is not found in other *Staphylococcus* species and it appears to be a unique feature of *S. aureus* (Unal et al. 1992; Vannuffel et al. 1998).

MRSA strains are not only resistant to beta-lactam antibiotics, but can also be resistant to a wide range of antibiotics, including aminoglycosides (Shaw et al. 1993; Schmitz et al. 1999; Choi et al. 2003; Klingenberg et al. 2004; Ardic et al. 2006). The most widespread mechanism of aminoglycoside resistance is the modification of the drug by plasmid- or transposon-encoded aminoglycoside-modifying enzymes (AMEs) (Shaw et al. 1993; Schmitz et al. 1999). The most prevalent AMEs among MRSA strains are aminoglycoside-6'-N-acetyltransferase/2''-O-phosphoryltransferase [AAC(6')/APH(2'')] encoded by the *aac(6')/aph(2'')* gene; aminoglycoside-3'-O-phosphoryltransferase III [APH(3')-III] encoded by *aph(3')-IIIa* gene; and aminoglycoside-4'-O-nucleotidyltransferase I [ANT(4')-I] encoded by *ant(4')-Ia* gene (Shaw et al. 1993; Schmitz et al. 1999; Fluit et al. 2001). The number of studies carried out on genotypic resistance, in contrast to phenotypic resistance, to aminoglycosides of MRSA isolated from animals is very limited (Goni et al. 2004; Schnellmann et al. 2006).

Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates have been also identified from bovine mastitis (Kaszanyitzky et al. 2003, 2007; Lee 2003; Van Duijkeren et al. 2004; Kwon et al. 2005; Sareyyupoglu et al. 2006; Moon et al. 2007) and the epidemiological relatedness among the bovine and human MRSA isolates has been detected by several researchers (Lee 2003; Kwon et al. 2005; Kaszanyitzky et al. 2007). However, none of these studies on MRSA have compared the *mecA* genes from bovine mastitis isolates with human MRSA strains. The objectives of this study were: (i) to investigate the presence of *mecA* gene encoding methicillin resistance along with the *femA* gene specific for *S. aureus*; and *aac(6')/aph(2'')*, *aph(3')-IIIa* and *ant(4')-Ia* genes encoding aminoglycoside resistance in phenotypically methicillin resistant *S. aureus* isolates from bovine mastitis; (ii) to compare the *mecA* genes of *S. aureus* isolated from bovine mastitis with that of human MRSA strains by sequence analysis.

Materials and methods

Bacterial strains

Eighteen *S. aureus* isolates phenotypically resistant to oxacillin a disk diffusion method were selected from strain collection of the Microbiology Department of Mehmet Akif Ersoy

University, Faculty of Veterinary Medicine. These strains were isolated from bovine mastitic milk samples in Burdur province of Turkey during the years 2002–2004. *S. aureus* strains were identified by conventional methods, including Gram staining, colony morphology, haemolysis, and tests for catalase, clumping factor, coagulase, DNase, acetoin and anaerobic fermentation of mannitol (Winn et al. 2006). In PCR and antimicrobial susceptibility testing, *mecA*-positive *S. aureus* 27R (Hacettepe University, Faculty of Medicine, Microbiology and Clinical Microbiology Department, Ankara, Turkey), *mecA*-negative *S. aureus* ATCC 25923 and *aac(6′)/aph(2′′)*-positive *Enterococcus faecalis* ATCC 29212 (Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Microbiology Department, Burdur, Turkey) were used as control strains. All isolates were stored at -20°C in trypticase soy broth containing 10% glycerol. Prior to testing, all isolates were serially cultured twice on Columbia blood agar base (Oxoid Ltd, Hampshire, England) containing 5% sheep blood and incubated for 24 h at 37°C under aerobic conditions.

Antibiotic susceptibility testing and determination of minimal inhibitory concentration (MIC)

The phenotypic resistance to oxacillin and aminoglycosides of all isolates after propagated onto Columbia blood agar was determined. Antibiotic susceptibility testing was performed by disk diffusion method on Mueller-Hinton agar (Oxoid) according to the Clinical Laboratory Standards Institute (formerly National Committee of Clinical Laboratory Standards, NCCLS 1997) standards. Ten colonies from Columbia blood agar base containing 5% sheep blood incubated at 37°C for 18 h were suspended in sterile saline to a density approximately equal to McFarland Opacity Standard No. 0.5. The bacterial suspension was inoculated onto Mueller-Hinton agar with the swab to cover the whole surface of the agar. The antibiotic disks were dispensed on the surface of the media and the plates were incubated aerobically at 37°C for 18 h. The media containing 2% NaCl, in which susceptibility to oxacillin was tested, were incubated at 35°C for 24 h. The antibiotic disks included were oxacillin (1 μg , Oxoid), gentamicin (10 μg , Oxoid), neomycin (30 μg , Oxoid) and kanamycin (30 μg , Oxoid). Additionally, the susceptibility of *S. aureus* isolates to amoxicillin/clavulanic acid (20/10 μg , Oxoid) was tested to identify the β -lactamase-producing isolates. The results were recorded as susceptible, intermediate susceptible or resistant by measurement of the inhibition zone diameter according to the interpretive standards of NCCLS (1997).

The phenotypic resistance to oxacillin of the *S. aureus* isolates was confirmed by MIC test. The MICs of oxacillin, gentamicin, neomycin and kanamycin (Sigma Chemical Co., St. Louis, MO, USA) were determined using a broth macrodilution method according to British Society for Antimicrobial Chemotherapy (BSAC 2008). Organisms were tested into Mueller-Hinton broth containing each drug tested with concentrations ranging from 0.5 to 256 $\mu\text{g}/\text{ml}$. The Mueller-Hinton broth supplemented with 2% NaCl was used for determining the oxacillin MIC value. Tubes containing oxacillin were incubated at 35°C for 24 h; other tubes were incubated at 37°C for 18 h. The MIC was defined as the lowest concentration of antibiotics that prevented visible growth (Andrews 2001; BSAC 2008). Isolates were categorized as susceptible (S) and resistant (R) according to the MIC breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2008) and BSAC (2008); oxacillin $S \leq 2$ $\mu\text{g}/\text{ml}$ and $R \geq 4$ $\mu\text{g}/\text{ml}$, gentamicin and neomycin $S \leq 4$ $\mu\text{g}/\text{ml}$ and $R \geq 8$ $\mu\text{g}/\text{ml}$, and kanamycin $S \leq 8$ $\mu\text{g}/\text{ml}$ and $R \geq 16$ $\mu\text{g}/\text{ml}$. In addition to the test organisms, the following quality control strains in antibiotic susceptibility tests and MICs were also tested: *S. aureus* 27R, *S. aureus* ATCC 25923 and *E. faecalis* ATCC 29212. Antimicrobial susceptibility tests and MIC calculations were performed in duplicate.

DNA extraction from culture samples

Single colonies of isolates were inoculated into brain heart infusion (BHI, Oxoid) broth and incubated at 37°C for 24 h. BHI cultures (approximately 10⁹ bacteria per ml) were pelleted by centrifugation (3000×g for 5 min). Bacterial pellet was resuspended in 200 µl of PBS and was added 5 µL of lysozyme (Sigma; 10 mg/ml in 10 mM Tris-HCl, pH 8.0). After gently mixing, the suspension was incubated for 15 min at 37°C. DNA was purified from *S. aureus* isolates using a DNA extraction kit (Roche high pure PCR template preparation kit, Roche Diagnostics GmbH, Mannheim, Germany) in accordance with the manufacturer's recommendations. Briefly, the sample was lysed by incubating at 70°C 10 min in the presence of 200 µl of binding buffer and 40 µl of proteinase K enzyme. To extract the DNA, 100 µl of 100% isopropanol was added and mixed by vortexing. The total solution was transferred to a spin column. The column was washed to remove any residual contaminants, and the bound DNA was concentrated with elution buffer. Isolated DNA samples were kept at -20°C until use.

PCR

Primers for *mecA*, *femA*, *aac(6')/aph(2'')*, *aph(3')-IIIa*, *ant(4')-Ia*, and 16 S rRNA were chosen from published sequences and PCRs were carried out as described previously (Table 1). Primers specific for conserved regions of the staphylococcal 16 S rRNA were used as additional internal controls. Deoxyribonucleotide triphosphate (dNTP), *Taq* DNA polymerase enzyme and buffers used in PCR mixture were supplied by the manufacturer (Vivantis Ltd., Westbourne, UK). After amplification PCR products (10 µl) were electrophoresed in 1.5% agarose gel at 100 V for 45 min, stained with ethidium bromide (0.5 µg/ml) and photographed under UV light (Edas 290, Eastman Kodak Company, Rochester, NY, USA). The PCR analyses of all isolates were performed in duplicate. The control organisms (*S. aureus* 27R, *S. aureus* ATCC 25923 and *E. faecalis* ATCC 29212) were also included in PCR assays.

Table 1 Primer sequences used in PCR and the expected sizes of the products

Target genes	Primer sequence (5' – 3')	Size (bp)	References
<i>mecA</i>	Forward-CCTAGTAAAGCTCCGGAA	314	Choi et al. 2003
	Reverse-CTAGTCCATTCGGTCCA		
<i>femA</i>	Forward-CTTACTTACTGCTGTACCTG	684	Ardic et al. 2006
	Reverse-ATCTCGCTTGTGTGTGC		
<i>aac(6')/aph(2'')</i>	Forward-GAAGTACGCAGAAGAGA	491	Choi et al. 2003
	Reverse-ACATGGCAAGCTCTAGGA		
<i>aph(3')-IIIa</i>	Forward-GGCTAAAATGAGAATATCACCGG	523	Klingenberg et al. 2004
	Reverse-CTTTAAAAAATCATAACAGCTCGCG		
<i>ant(4')-Ia</i>	Forward-CAAAGTCTAAATCGGTAGAAGCC	294	Klingenberg et al. 2004
	Reverse-GGAAAGTTGACCAGACATTACGAACT		
16 S rRNA	Forward-CAGCTCGTGTGCTGAGATGT	420	Ardic et al. 2006
	Reverse-AATCATTTGTCCACCTT CG		

Sequencing of the *mecA* gene

The nucleotide sequence of the *mecA* gene was determined for 3 *mecA*-positive *S. aureus* isolates HM1, HM4 and HM5, respectively. The set of primers used for sequencing were listed in Table 2. The assay was performed in a final volume of 50 μ l reaction mixture consisted of 5 μ l template DNA, 10 μ l 10X PCR buffer plus Mg, 1 μ l dNTP (0.8 μ M), 0.5 μ l *Taq* DNA polymerase (2.5U), 0.5 μ l each of the forward and reverse primers (100pmol), and 32.5 μ l ddH₂O. The amplification was carried out in a thermal cycler (CLP, ATC401, USA) under the following conditions: DNA denaturation step of 5 min at 95°C; 40 cycles with a 45 s denaturation step at 94°C, a 45 s annealing step at 54°C, 1 min extension at 72°C; and a final 7 min extension step at 72°C. Amplification products were analyzed by electrophoresis in 1.5% agarose gels containing 0.5 μ g of ethidium bromide per ml, and visualized under UV light. The PCR products of the *mecA* gene were purified using a PCR clean-up kit (Roche) according to the manufacturer's instructions. Sequence analyses of extracted PCR products were performed at Metis Biotechnology (Macunkoy, Ankara, Turkey). DNA sequences were analyzed using CLC Main Workbench 4 (CLCbio, Denmark) software and were compared with reference sequences on the GenBank database using BLAST (www.ncbi.nlm.nih.gov) and a phylogenetic tree of *mecA* gene was performed using the Neighbor Joining method.

Nucleotide sequence accession numbers

Nucleotide sequences of *mecA* gene of *S. aureus* HM1, HM4 and HM5 isolates were entered in the GenBank (<http://www.ncbi.nlm.nih.gov>) under accession numbers EU790488; EU790489 and EU790490, respectively.

Results

Phenotypic resistance to oxacillin and aminoglycosides

The aminoglycoside resistance by disk diffusion method of the 18 phenotypic methicillin resistant *S. aureus* isolates is shown in Table 3. The phenotypic oxacillin and aminoglyco-

Table 2 The set of primers used for PCR amplification and sequencing of *mecA* gene

Name	Primer sequence (5' to 3')	Nucleotide positions	PCR product
MC1	Forward-GTCGGGTTTGGTATATATTT	45–834	790 bp
	Reverse-ATAAAGATGATGCAGTTATTGG		
MC2	Forward-ATTGGGGTCATAGCGTCAT	375–857	481 bp
	Reverse-TAAAAAGGGACTCGAAAACT		
MC3	Forward-TATTAGGTTATGTTGGTCCCA	757–1135	377 bp
	Reverse-TGTATGGCATGAGTAACAAAG		
MC4	Forward-ATGGCAAAGATATTCAACTAACTA	958–1324	367 bp
	Reverse-AATCTTGGGGTGGTTACA		
MEC	Forward-AAAATCGATGGTAAAGGTTGGC	1275–1808	534 bp
	Reverse-GCAAATCCGGTACTGCAGAACT		
MEC2	Forward-AACGCACCTCACTTATTC	1632–1988	357 bp
	Reverse-ATGAGAACGGTAATAAAAA		

Table 3 Phenotypic and genotypic oxacillin and aminoglycoside resistance in 18 *S. aureus* isolates from bovine mastitis

Isolates	PCR				Disk diffusion test				MIC($\mu\text{g/ml}$)			
	<i>mecA</i>	<i>aac(6')/aph(2'')</i>	<i>aph(3')-IIIa</i>	<i>ant(4')-Ia</i>	OX ^a	GM ^b	NM ^c	KN ^d	OX	GM	NM	KN
1	+	-	-	-	R ^e	S ^f	S	S	64	0.5	4	8
2	-	-	+	-	R	R	R	R	128	>256	>256	>256
3	-	-	-	-	R	R	R	R	64	32	>256	>256
4	+	-	-	-	R	S	I ^g	I	64	0.5	4	4
5	+	+	+	-	R	R	R	R	>256	>256	>256	>256
6	-	-	-	-	R	R	R	R	64	64	>256	>256
7	-	-	-	-	R	S	S	R	16	0.5	0.5	16
8	-	-	-	-	R	R	R	R	16	8	32	32
9	-	-	-	-	R	R	R	R	128	32	>256	64
10	-	-	-	-	R	S	I	I	4	1	4	4
11	-	-	-	-	R	S	I	I	8	4	8	8
12	-	-	-	-	R	S	R	R	4	0.5	16	16
13	-	-	-	-	R	S	S	R	16	2	0.5	16
14	-	-	+	-	R	R	R	R	64	64	>256	>256
15	-	-	-	-	R	S	S	R	4	0.5	4	16
16	-	-	-	-	R	R	R	R	16	16	128	16
17	-	-	-	-	R	R	R	R	128	128	256	256
18	-	-	-	-	R	S	S	S	4	0.5	0.5	4
<i>S. aureus</i> 27R	+	-	-	-	R	S	S	S	>256	1	2	4
<i>S. aureus</i> ATCC 25923	-	-	-	-	S	S	S	I	1	2	4	8
<i>E. faecalis</i> ATCC 29212	NT ^h	+	-	-	NT	R	R	R	NT	64	256	128

^a Oxacillin, ^b gentamicin, ^c neomycin, ^d kanamycin, ^e resistant, ^f sensitive, ^g intermediate and ^h not tested

side resistance was confirmed by determining the MICs test for all *S. aureus* isolates. The oxacillin MICs for the isolates ranged from >4 $\mu\text{g/ml}$ to >256 $\mu\text{g/ml}$. Only 4 isolates which were susceptible to amoxicillin/clavulanic acid had borderline resistance to oxacillin (MIC, 4 $\mu\text{g/ml}$). Nine isolates were resistant to all aminoglycosides tested. However, out of 18 isolates tested, 9 to gentamicin (MIC, ≥ 8 $\mu\text{g/ml}$ - >256 $\mu\text{g/ml}$), 13 to kanamycin (MIC, ≥ 16 $\mu\text{g/ml}$ - >256 $\mu\text{g/ml}$) and 11 to neomycin (MIC, ≥ 8 $\mu\text{g/ml}$ - >256 $\mu\text{g/ml}$) were found to be resistant. Antibiotic resistance patterns of isolates are shown in Table 3.

Detection of *mecA*, *aac(6')/aph(2'')*, *aph(3')-IIIa* and *ant(4')-Ia* genes

The PCR correctly determined the presence or absence of the genes of interest in all of the reference strains. In all of 18 *S. aureus* isolates, *femA* gene and 16 S rRNA were detected by PCR. Of these isolates, 3 (nos. 1, 4 and 5) were found to be *mecA* positive by PCR and these isolates were referred as HM1, HM4 and HM5, respectively. Only 3 of the isolates were carrying AME gene, while the remaining 15 isolates were negative for AME genes. Isolate number 5 had *aac(6')/aph(2'')* gene. The *aph(3')-IIIa* gene was detected in 3 isolates

(nos. 2, 5 and 14). Only one from 3 *mecA* positive isolates was positive for AME gene and this isolate carried *aac(6')/aph(2'')* in combination with *aph(3')-IIIa* gene. None of the isolates was positive for *ant(4')-Ia* gene. Three isolates carried the *aac(6')/aph(2'')* and *aph(3')-IIIa* genes were resistant to gentamicin, kanamycin and neomycin. Among the 18 isolates, 3, 6, 8, 9, 16 and 17 were determined to be resistant to all aminoglycosides tested, but these isolates were negative for AME genes (Tables 3 and 4).

Nucleotide sequencing

The *mecA* gene of three MRSA isolates (HM1, HM4 and HM5) were subjected to DNA sequencing. The sequence of *mecA* genes of these isolates were identical to reference sequence in the human MRSA strains (GenBank accession numbers: NC002745, NC003923, NC002952, AP009324, AB425824 and BA000017). One of the three isolates (HM1 isolate) had a guanine to adenine substitution, and remaining two isolates (HM4 and HM5) had an adenine to guanine substitution at nucleotide 757. Sequencing results obtained from the three isolates and analysis with Neighbor Joining method revealed 100% homology to the reference sequence in the six human MRSA strains. Phylogenetic tree of *mecA* gene sequences were shown in Fig. 1.

Discussion

Treatment and control of the mastitis caused by *S. aureus* is rather difficult in veterinary practice and may result in failure. The presence of *S. aureus* isolates resistant to antibiotics is indicated to be important factor influencing the treatment process (Devriese et al. 1972; Watts and Salmon 1997). Although MRSA exhibiting multiple resistance were generally isolated from human, these agents have recently been isolated from various animal species (Seguin et al. 1999; Lee 2003; Van Duijkeren et al. 2004, Malik et al. 2006; Schnellmann et al. 2006; Weese et al. 2006). In this study, 18 phenotypical MRSA isolates from bovine mastitis were analyzed by PCR for the presence of *mecA* gene described as a molecular marker of methicillin resistance. The *mecA* gene was detected only in 3 of 18 *S. aureus* isolates. This finding supports that the *mecA*-positive *S. aureus* can be isolated from bovine mastitis as previously reported (Devriese et al. 1972; Lee 2003; Kwon et al. 2005; Lee 2006; Sareyyupoglu et al. 2006; Moon et al. 2007).

Table 4 The presence of *mecA* and AME genes in *S. aureus* isolates and phenotypic aminoglycoside resistance

PCR results	No. of isolates	Gentamicin MIC ≥ 8 $\mu\text{g/ml}$	Neomycin MIC ≥ 8 $\mu\text{g/ml}$	Kanamycin MIC ≥ 16 $\mu\text{g/ml}$
<i>mecA</i> ⁺	3	1 (%33)	1 (%33)	1 (%33)
<i>mecA</i> ⁻	15	8 (%53)	10 (%67)	12 (%80)
AME ⁺	3	3 (%100)	3 (%100)	3 (%100)
AME ⁻	15	6 (%40)	8 (%53)	10 (%67)
<i>mecA</i> ⁺ /AME ⁺	1	1 (%100)	1 (%100)	1 (%100)
<i>mecA</i> ⁺ /AME ⁻	2	0 (%0)	0 (%0)	0 (%0)
<i>mecA</i> ⁻ /AME ⁺	2	2 (%100)	2 (%100)	2 (%100)
<i>mecA</i> ⁻ /AME ⁻	13	6 (%46)	8 (%62)	10 (%77)

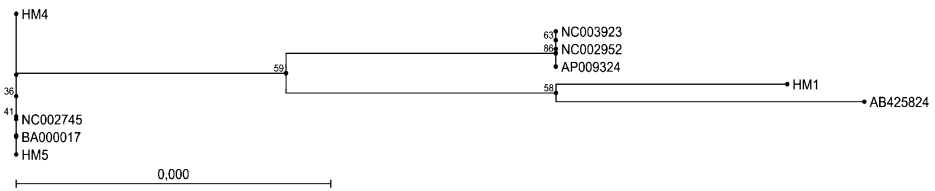


Fig. 1 Phylogenetic tree of *mecA* gene sequences. Nucleotide sequences of 3 *mecA* genes from MRSA (HM1, HM4 and HM5) isolated from bovine mastitis were compared with *S. aureus* N315 (GenBank/NC002745), MW2 (GenBank/NC003923), 252 (GenBank/NC002952), Mu3 (GenBank/AP009324), JCSC6670 (GenBank/AB425824) and Mu50 (GenBank/BA000017) strains. DNA sequences were analyzed using CLC Main Workbench 4, and the phylogenetic tree of *mecA* gene was designated using the Neighbor Joining method. Accession numbers of *mecA* gene sequences of HM1, HM4 and HM5 isolates are EU790488; EU790489 and EU790490, respectively

The *femA* (Unal et al. 1992; Vannuffell et al. 1998; Ardic et al. 2006) and 16 S rRNA (Geha et al. 1994; Kaszanyitzky et al. 2003; Van Duijkeren et al. 2004; Ardic et al. 2006) genes have been reported as valuable tools for the identification of MRSA. In our study, the *femA* gene and 16 S rRNA region were used for genotypic confirmation and for detection at the genus level of *S. aureus* isolates, respectively. These targets were also determined by PCR in all isolates.

Fifteen *S. aureus* isolates phenotypically resistant to methicillin in this study did not carry *mecA* gene, which is in agreement with the other studies that reported the genotypic profiles of the phenotypic MRSA (Bignardi et al. 1996; Kaszanyitzky et al. 2003; Lee et al. 2004; Schnellmann et al. 2006; Moon et al. 2007). Therefore, we did not find any correlation between phenotypic and genotypic methicillin resistance of the 18 *S. aureus* isolates analyzed. The oxacillin MICs for the *mecA*-positive MRSA isolates ranged from 64 µg/ml to greater than 256 µg/ml, and the MICs for *mecA*-negative MRSA were also determined as being in the range of 4–128 µg/ml. The absence of *mecA* in *S. aureus* strains whose oxacillin MIC is >4 µg/ml has been also reported by others (Murakami et al. 1991; Bignardi et al. 1996; Moon et al. 2007). Several researchers (Chambers 1997; Murakami et al. 1991) have been suggested that staphylococci resistant to oxacillin which did not carry the *mecA* gene have resistance mechanisms other than the production of PBP-2a. In the presented study, *mecA*-negative 4 isolates having borderline resistance to oxacillin (MIC, 4 µg/ml) was susceptible to amoxicillin/clavulanic acid after repeated disk diffusion testing, but the oxacillin MICs as high as 64 or 128 µg/ml were determined in some *S. aureus* isolates which lack *mecA* and these isolates were resistant to amoxicillin/clavulanic acid. The studies conducted in the same province where our isolates were obtained have been reported that the rate of the β-lactamase-producing *S. aureus* isolates from bovine mastitis were 65% and 56% (Turutoglu et al. 2002, 2006). Therefore, we think, besides the overproduction of β-lactamase, the other mechanisms such as production of a novel methicillinase, alteration of PBP subtypes and growth conditions may be involved in the expression of methicillin resistance (Geha et al. 1994; Bignardi et al. 1996; Chambers 1997; Choi et al. 2003). It has been also reported that the *mecA* gene was lost in 36 (14.4%) of 250 MRSA after 2 years of storage at -80°C (Van Griethuysen et al. 2005), and in 42 (22%) of 192 MRSA stored as lyophilized cultures (Hürlimann-Dalel et al. 1992). In the present study, 11 (61%) of 18 phenotypic MRSA isolates did not carry the *mecA* gene, and also they were not β-lactamase producer. This result may be due to storage method as reported by Hürlimann-Dalel et al. (1992) and Van Griethuysen et al. (2005), since all phenotypic MRSA used in the present study were isolated during the years 2002–2004 and were stored at -20°C.

It has been shown that many human MRSA isolates produce AMEs and thus are animoglycoside resistant (Shaw et al. 1993; Schmitz et al. 1999; Choi et al. 2003;

Klingenberg et al. 2004; Ardic et al. 2006). There are only few studies carried out on AMEs or AMEs encoded genes of animal MRSA isolates (Goni et al. 2004; Schnellmann et al. 2006). In this work, we looked at aminoglycoside resistance by identifying the corresponding genes in the phenotypic MRSA isolates from bovine mastitis. We detected that 3 isolates carrying AME genes and were 100% resistant to all aminoglycosides tested. The *aph(3')-IIIa* gene is the most prevalent gene, and the isolates carrying this gene presented high level of resistance to gentamicin, kanamycin and neomycin. This result is contrast to the findings of the other researchers who reported that *aac(6')/aph(2'')* gene is much higher than that of the other two AME genes, *aph(3')-IIIa* and *ant(4')-Ia* in *S. aureus* isolated from humans (Shaw et al. 1993; Schmitz et al. 1999; Choi et al. 2003; Klingenberg et al. 2004; Ardic et al. 2006) and animals (Goni et al. 2004; Schnellmann et al. 2006). This conflicting finding can be attributed to the differences among the isolates of *S. aureus* in different geographical regions where the studies focused on. Choi et al. (2003) have stated that 50% of the *mecA*-positive isolates carried *aac(6')/aph(2'')* gene in combination either *ant(4')-Ia* and/or *aph(3')-IIIa* gene and these isolates was highly resistant to aminoglycosides. In this study, only one from 3 *mecA*-positive isolates carried *aac(6')/aph(2'')* in combination with *aph(3')-IIIa* gene and this isolate (*S. aureus* HM5) presented high level of resistance to gentamicin, kanamycin and neomycin (MIC, >256 µg/ml). Fifteen isolates that were not detected to have any AME genes were determined to be resistant to gentamicin (40%), neomycin (53%) and kanamycin (67%). The reason for the discrepancy between PCR and MIC in these isolates is not clear; it is possible that the other mechanisms such as a loss of permeability, ribosomal alteration and a mutation of the AME genes may mediate resistance (Fluit et al. 2001; Ida et al. 2001; Choi et al. 2003). This is probably due to the uncontrolled use of aminoglycosides in the treatment of animals as described by Goni et al. (2004) and Schnellmann et al. (2006). Also, the antimicrobial agents in Turkey is widely used in cattle herds for the treatment of udder infections and the various infections therefore a high rate of resistance to these antibiotics can be expected (Turutoglu et al. 2002, 2006).

There are increasing reports on MRSA infection or colonization in animals and their zoonotic potential (Seguin et al. 1999; Lee 2003; Van Duijkeren et al. 2004, Malik et al. 2006; Schnellmann et al. 2006; Weese et al. 2006; Kaszanyitzky et al. 2007). However, a few veterinary reports have compared the *mecA* genes from animal isolates with human MRSA strains (Malik et al. 2006; Schnellmann et al. 2006). In this study, nucleotide sequence analyses showed that the *mecA* genes from bovine mastitis MRSA (HM1, HM4 and HM5) isolates were closely related to human MRSA strains. Same results were also stated about the *mecA* genes from dogs, cats (Malik et al. 2006) and horses (Schnellmann et al. 2006). Based on the results from our study and the other studies (Seguin et al. 1999; Van Duijkeren et al. 2004; Malik et al. 2006), we thought that these isolates may originally come from humans, considering that the methicillin resistance among human *S. aureus* isolates is common. However, in the recent years, it has been suggested that humans in close contact with MRSA infected or colonized animals may be infected (Weese et al. 2006; Kaszanyitzky et al. 2007) or human infections may originated by consumption of food-stuff made from infected animals (Lee 2003). In the present study, due to lack of information about the MRSA colonization status of the owners of animals infected with MRSA, the route of transmission of these isolates remains unanswered. Recently, DNA sequence-based methods for typing *S. aureus* such as multilocus sequence typing (MLST) and *spa* typing have been developed and are widely used to establish clonal relationships between strains and to compare the geographical locations of MRSA clones (Kwon et al. 2005; Kaszanyitzky et al. 2007; Witte et al. 2007; Denis et al. 2009). In this study, MRSA isolates were not typed by sequence-based methods. Therefore, our data did not enable the classification of isolates into particular clonal lineages.

It has been reported that a one-base substitution at nucleotide position 1449 of *mecA* gene results in a methionine to isoleucine and this amino acid substitution may be associated with a change in the function of PBP2a resulting in oxacillin susceptibility (Bressler et al. 2005). The nucleotide sequences of the *mecA* gene of MRSA isolates used in this study were identical to the reference sequences on the GenBank. We only found a guanine to adenine substitution in one isolate (HM1), and an adenine to guanine substitution in two isolates (HM4 and HM5) at nucleotide 757. However, a one-base substitution at nucleotide position 757 did not result in an amino acid substitution, and none of these isolates were expressed below the breakpoint (oxacillin MIC, $\geq 64 \mu\text{g/ml}$). A previous study has noted that a point mutation or a deletion in the *mecA* gene of MRSA isolates did not alter the expression of methicillin resistance (Kobayashi et al. 1998).

Our results indicate that the PCR is a specific, rapid and reliable assay for the detection of MRSA isolates and their resistance features, and that *mecA* positive *S. aureus* can be isolated from bovine mastitis cases, and that MRSA isolates from bovine mastitis may come from humans. Therefore, more studies on the epidemiological relatedness among bovine milk and human MRSA isolates should be conducted continuously. Periodic surveillance for antimicrobial resistance patterns of MRSA isolated from dairy cows with mastitis would be an important measure in detecting emergence and spreading of resistance.

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