Staphylococcal cassette chromosome mec (SCCmec) characterization and molecular analysis for methicillin-resistant Staphylococcus aureus and novel SCCmec subtype IVg isolated from bovine milk in Korea

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Objectives: To identify the staphylococcal cassette chromosome mec (SCCmec) types of methicillin-resistant Staphylococcus aureus (MRSA) isolated from bovine milk, and examine the genetic relatedness between MRSA from bovine milk and MRSA from human isolates.

Methods: Antimicrobial susceptibility tests were performed on MRSA isolated from bovine milk. PCR and sequencing analysis were performed to determine the SCCmec type of MRSA, and to confirm their toxin carriage. Genetic relatedness among the bovine isolates and between bovine and human isolates was detected with PFGE and multilocus sequence typing (MLST).

Results: Fourteen MRSA and a silent mecA-carrying methicillin-susceptible S. aureus (smMSSA) were isolated from the milk of cows with an isolation ratio of 0.18%. SCCmec of 14 MRSA strains were designated as new subtype IVg, and one smMSSA strain was not classified. All 14 MRSA strains shared Panton-Valentine leucocidin (PVL) and staphylococcal enterotoxin D (SED), SEI and SEJ; the smMSSA strain had only PVL. All MRSA and smMSSA isolates showed no multidrug resistance and had community-acquired MRSA (CA-MRSA) characteristics. PFGE revealed that all isolates except the smMSSA belonged to the same genetic lineage, and MLST analysis showed that they had no genetic relatedness with CA-MRSA which had caused human infection in Korea.

Conclusions: MRSA isolated from bovine milk harboured a unique SCCmec subtype, and they may not be correlated with the emergence of CA-MRSA in human infection in Korea.

Keywords: MRSA, PFGE, MLST

Introduction

Since the first discovery of methicillin-resistant Staphylococcus aureus (MRSA) in 1961, only 1 year after the introduction of methicillin, MRSA has become one of the most prevalent pathogens causing nosocomial infections.1,2 MRSA produces a specific penicillin-binding protein PBP2’ (or PBP2a) that possesses reduced affinities for binding to β-lactam antibiotics resulting in β-lactam antibiotic resistance.2,3 PBP2’ is encoded by the mecA gene carried by a large mobile genetic element, staphylococcal cassette chromosome mec (SCCmec), which is integrated at the 3’ ends of orfX on the chromosomes of MRSA strains.4–6 The SCCmec element contains the mec gene complex composed of the mecA gene and its regulators, and the ccr gene complex that encodes site-specific

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recombinases, ccrA and ccrB, which are responsible for the mobility of SCCmec. 3,4 The mec gene complex has been classified into four classes: class A, mecI-mecR1-mecA-ISA431; class B, IS2722-mecR1-mecA-ISA431; class C, ISA431-mecR1-mecA-ISA431; and class D, mecR1-mecA-ISA431. 7-8 There are five allotopes of the ccr gene complex; type 1, ccrA1ccrB1; type 2, ccrA2ccrB2; type 3, ccrA3ccrB3; type 4, ccrA4ccrB4; and type 5, ccrC, which was newly found in community-acquired MRSA (CA-MRSA). SCCmec is classified into five allotopes according to the combination of the mec gene complex class and the ccr gene complex type: type I SCCmec, class B mec gene complex and type 1 ccr gene complex; type II SCCmec, class A mec gene complex and type 2 ccr gene complex; type III SCCmec, class A mec gene complex and type 3 ccr gene complex; type IV SCCmec, class B mec gene complex and type 2 ccr gene complex; and type V SCCmec, class C (subtype of class C) mec gene complex and type 5 ccr gene complex. 5,6,7,8

Recently, MRSA infections have increasingly been reported among groups of patients with no apparent connection to hospitals. 5,6,9,10 The CA-MRSA strains that cause non-hospital-associated infections are characteristically susceptible to many antibiotics, harbour type IV SCCmec, which is much smaller in size than other SCCmec elements, and contains no other antibiotic resistance genes except mecA.11,12 In contrast to CA-MRSA, the majority of hospital-acquired MRSA (HA-MRSA) strains carry one of two types of SCCmec, type II or III, and show characteristics of multidrug resistance. 4,13 The origin of CA-MRSA strains with the SCCmec type IV element remains unclear. One hypothesis is that the CA-MRSA strains might have originated from HA-MRSA strains and have undergone deletions of antibiotic resistance genes under low antibiotic selective pressure in the community. The other hypothesis is that the strains may have resulted from SCCmec transfer to the genetic backgrounds of methicillin-susceptible S. aureus (MSSA) from other staphylococci that harboured genes associated with SCCmec. 4,14 Additionally, there has been another increasing concern that human infections in the community may be caused by the MRSA from contaminated foods and livestock products including bovine milk. 16 Although MRSA strains isolated from humans have been well characterized and examined by analyses of their SCCmec complex, antimicrobial resistance patterns, PFGE profiles and multilocus sequence typing (MLST), there have been few studies about MRSA isolated from animals or livestock products and their SCCmec complex characteristics. Therefore, the aim of this study was to confirm the SCCmec type of MRSA that originated from bovine milk and compare the epidemiological relatedness among strains and also between bovine milk isolates and human isolates. In doing so, we discovered new SCCmec complex subtype IVg from bovine milk MRSA isolates and demonstrated using MLST analysis that MRSA isolated from bovine milk in Korea is not genetically correlated with other CA-MRSA of human origin.

Materials and methods

MRSA isolates

In 1999, 2000 and 2003, we examined 75,335 quarter milk samples from various provinces in Korea; 20,157 samples from 655 farms in Gyeonggi, 18,556 samples from 623 farms in Chungcheong, 12,346 samples from 476 farms in Jeonbuk, 11,460 samples from 366 farms in Jeonnam, 4,763 samples from 177 farms in Gyeongbuk, 4,028 samples from 127 farms in Gyeongnam, 3,590 samples from 121 farms in Gangwon and 435 samples from 10 farms in Jeju provinces. Milk somatic cell count was analysed with Milkscan 4000 (Foss Electric Co., Hillerød, Denmark) or with Somacount 150 (Bentley Instruments, Inc., Chaska, MN, USA) within 24 h after sampling. Among these samples, 9,055 milk samples that had >500 000 somatic cells/mL were further examined for the isolation of mastitis-causing bacteria. Milk was streaked on a 5% sheep blood agar plate (Promed, Ansan, Korea) and the colonies showing the phenotypic characteristics of S. aureus were subcultured on the Baird-Parker agar (Becton Dickinson; BD, Franklin Lakes, NJ, USA) containing 5% egg yolk tellurite (BD). S. aureus was confirmed with catalase, oxidase, DNase, TNase and VP tests. Also, 16S rRNA-specific PCR and mec gene PCR were carried out as described previously. 17,18 Representative MRSA isolates are listed in Table 1. Other mastitis-causing bacteria including coagulase-negative staphylococci, coliforms, Enterococcus spp. and Streptococcus spp. were isolated according to previously described methods. 19,20

Antimicrobial susceptibility tests

Antimicrobial susceptibility tests were performed following the guidelines of the NCCLS. 21 A disc diffusion test was performed for the screening of susceptibility to 14 antimicrobials: ampicillin, nafcillin, oxacillin, penicillin, cefalothin, imipenem, ciprofloxacin, clindamycin, chloramphenicol, erythromycin, gentamicin, tetracycline, vancomycin and trimethoprim/sulfamethoxazole (BD BBL, Sparks, MD, USA). Susceptibility, intermediate resistance and resistance were discriminated according to the manufacturer’s guidelines. MICs of oxacillin were detected with a microdilution test of the NCCLS for the S. aureus isolates. 21 S. aureus ATCC 29213 was used as a standard. Antimicrobial susceptibility tests and MIC calculations were performed in triplicate.

PCR amplification and sequencing

DNAs encompassing the entire SCCmec sequence were amplified by long-range PCR with several sets of primers (Figure 1). Primer set ccrA and cLs1 was used to cover the region from the left extremity to the ccr gene complex (L-C region) of SCCmec type IVa, and primer set ccrA and cL2b detected the L-C region of SCCmec type IVb, as described previously. 2 Primers ccrA5 and mecR8 were used for the middle part (from the region just upstream from ccrA to mecR1, called the C-M region), and two overlapping primer sets (primers is4 and mA2 and primers mA3 and cR1) were used to cover the right extremity (I-R region). After purifying the long-range PCR product with a QIAEX II gel extraction kit (Qiagen, Hilden, Germany), major open reading frames (ORFs) were amplified with specific primers using purified PCR products as templates (Table 2). Amplification of ORF CQ001, CQ002 and CQ003 was performed with an L-C region template produced by the ccrA5 and cLs1 primer set, and ORF CM001, CM002 and M001 were amplified with an L-C region template produced by the ccrA5 and cL2b primer set. For the C-M region template, ccrA2B2, 1S1272 and mecI genes were amplified. The purified I-R region amplicon was digested with restriction enzymes NotI and BstO1 to amplify the IS431. The left junction between chromosome and SCCmec was amplified with an L-C region template produced by the ccrA5 and cL2b primer set, and the right junction between SCCmec and chromosome was produced with the template amplified by the mr2 and cr4 primer set, as described previously. 4 Amplified ORF products were purified with a QIAquick gel extraction kit (Qiagen) and sequenced. Staphylococcal enterotoxins (SEs) harboured by MRSA isolates were detected according to Kwon et al. 22 and Panton-Valentine
Table 1. Characteristics of representative MRSA isolates and a silent mecA-carrying S. aureus (M03-72) isolated from bovine milk

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Province of isolation</th>
<th>Date of isolation</th>
<th>PCR and sequencing results for localization of representative genes in the mec gene complexa</th>
<th>SCCmeca complex type</th>
<th>Staphylococcal enterotoxin (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M99-132</td>
<td>Gyeonggi</td>
<td>October 1999</td>
<td>+ c mecA mecR1 (MS/PB) mecI IS1272 – cR1 + B 2 IVg e + SED, SEI, SEJ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M99-164</td>
<td>Chungcheong</td>
<td>October 1999</td>
<td>+ – mecA mecR1 (MS/PB) mecI IS1272 – cR1 + B 2 IVg + SED, SEI, SEJ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M00-425</td>
<td>Gyeonggi</td>
<td>May 2000</td>
<td>+ + mecA mecR1 (MS/PB) mecI IS1272 – cR1 + B 2 IVg + SED, SEI, SEJ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M03-68</td>
<td>Gyeonggi</td>
<td>October 2003</td>
<td>+ + mecA mecR1 (MS/PB) mecI IS1272 – cR1 + B 2 IVg + SED, SEI, SEJ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M03-72</td>
<td>Chungcheong</td>
<td>October 2003</td>
<td>+ – mecA mecR1 (MS/PB) mecI IS1272 – cR1 + B 2 IVg + SED, SEI, SEJ</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

M99-132, M00-425 and M03-68 represented 2, 1 and 7 other MRSA, respectively, isolated from animals of the respective farms in corresponding years.

aLocalizations of the essential genes in the mec gene complex were estimated by PCR and sequencing. The mecA gene and its regulator genes mecR1 [both the membrane spanning region (MS) and penicillin-binding region (PB)] and mecI are identified.
bPanton-Valentine leucocidin.
cA plus sign (+) indicates that a DNA fragment was amplified and corresponded to PVL whose sequences were analysed on the basis of type IV SCCmec carried by strain 8/6-3P (subtype IVb; DDBJ/EMBL/GenBank accession no. AB063173).
dA minus sign (–) indicates that no DNA fragment was amplified.
eSubtype IVg was designated on the basis of unique nucleotide sequences (GenBank accession no. DQ106887).
fA plus sign (+) indicates that a DNA fragment was amplified and corresponded to that sequence in type IV SCCmec.
gNot classified.

leucocidin (PVL) was also amplified with specific primers (Table 2). Sequence analyses of extracted PCR products were performed at Bionics Co., Ltd (Seoul, Korea). Sequence data were analysed using DNASTAR software (DNASTAR, Inc., Madison, WI, USA), were compared with reference sequences on the GenBank database by a BLAST search, and a phylogenetic tree of the ccr gene complex was constructed using the Jotun Hein method.

For the M03-68 isolate, the full size SCCmec complex was sequenced and the data were deposited in the GenBank (accession no. DQ106887).

**PFGE analysis**

PFGE was performed with some modifications as described previously. For preparation of sample plugs, 5 × 10⁸ cfu/mL cells were embedded in 400 µL (1.5 × 5 × 5 mm) of 1% low melting point agarose (Sigma, St Louis, MO, USA). The sample plugs were incubated in the EC buffer (10 mM of Tris, pH 7.2, 50 mM of NaCl, 0.2% of sodium deoxycholate and 0.5% of sodium lauryl sarcosine) with 50 U/mL of lysostaphin (Sigma) and 10 mg/mL of lysozyme (Sigma) for 2 h at 37°C without agitation and were further incubated overnight at 50°C with an EC buffer containing 0.1 mg/mL of proteinase K (Sigma). The plugs were washed four times with 50 mL of Tris-EDTA at 25°C for 30 min with gentle agitation and were then treated with 40 U of SmalI (Roche Molecular Biochemicals, Mannheim, Germany) at 25°C for 24 h. The digested plugs were subjected to PFGE with a 1% agarose gel (certified megabase agarose; Bio-Rad Laboratories, Hercules, CA, USA). The PFGE was performed at 14°C for 20 h in 0.5× Tris/borate/EDTA running buffer using a CHEF DR III apparatus (Bio-Rad Laboratories) with an initial pulse time of 10 s, a final pulse time of 50 s and an electric field strength of 6 V/cm. After staining with ethidium bromide, PFGE patterns were grouped according to the criteria suggested by Tenover et al.

**Southern hybridization**

Southern hybridization was carried out to confirm the existence of the chromosome-left SCCmec junction in the M03-72 isolate with the digoxigenin-based non-radioisotope system of Boehringer GmbH (Mannheim, Germany). The probe for the chromosome-left SCCmec
SCCmec characterization for *S. aureus* milk isolates

<table>
<thead>
<tr>
<th>Amplified ORF</th>
<th>Primer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>mecA</em></td>
<td>L: 5'-GTAGAAATGACTGAACGTCCGATAA-3'</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CCAACTCCATGTTTCCGCTTA-3'</td>
<td></td>
</tr>
<tr>
<td><em>mecRI</em> (MS)</td>
<td>mecRA1: 5'-GTCTCAGTTAATTCACTT-3'</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>mecRA2: 5'-GTCTCAGTTAATTCACTT-3'</td>
<td></td>
</tr>
<tr>
<td><em>mecRI</em> (PB)</td>
<td>mecRB1: 5'-AACACCGTTAATCTGCAACA-3'</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>mecRB2: 5'-GATTAAATTTTGTCGAATGCCC-3'</td>
<td></td>
</tr>
<tr>
<td><em>mecI</em></td>
<td>mecI1: 5'-ATTGCCGAAAAGCACACACA-3'</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>mecI2: 5'-GACTGTTGTCTCGCTCTT-3'</td>
<td></td>
</tr>
<tr>
<td><em>mecRI</em> (MS), IS1272 IS43I</td>
<td>5'-GGACAACCTTAAGCCAGGTA-3' with mecRA1 primer</td>
<td>45, this study</td>
</tr>
<tr>
<td></td>
<td>L: 5'-GAATATGCCCCATTTTGTGA-3'</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td>R: 5'-AATGTATGCTCTCTGCCATC-3'</td>
<td></td>
</tr>
<tr>
<td><em>ccr</em> gene (<em>ccrA2B2</em>)</td>
<td>α3: 5'-TAAAGGATCAATGACACAAAAC-3'</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>β3: 5'-ATTGGCTCTTATGAAATAGCCTCT-3'</td>
<td></td>
</tr>
<tr>
<td>CM001, CM002, M001</td>
<td>M001-R: 5'-GCAATAGCCACATCTAAATGAA-3' with cl2b primer</td>
<td>2, this study</td>
</tr>
<tr>
<td>CQ001, Q001, CQ002</td>
<td>CQ002-L: 5'-AGGCATTCCAAAGGACAG-3' with cl2s1 primer</td>
<td>2, this study</td>
</tr>
<tr>
<td>Left chromosome-SCCmec junction</td>
<td>attb4-L: 5'-ATGAACGTTGGATTATATGTGC-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>attb4-R: 5'-GTGAAGTGTGAGGAGAAG-3'</td>
<td></td>
</tr>
<tr>
<td>Right chromosome-SCCmec junction</td>
<td>mR8: 5'-ATGGAAACGCTGCGAGGCTAACT-3'</td>
<td>5,6</td>
</tr>
<tr>
<td></td>
<td>cr2: 5'-AAACGACATGAAATACCATC-3'</td>
<td></td>
</tr>
<tr>
<td>Panton-Valentine leucocidin</td>
<td>L: 5'-GAGACTATTTTGTGCCAGAC-3'</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CCTTGCAAGGCCCTTA-3'</td>
<td></td>
</tr>
</tbody>
</table>

junction was generated by PCR amplification of DNA from M99-132 with primers att4b-L and att4b-R, and all procedures were based on the manufacturer’s manual.

**MLST**

MLST was performed as described by Enright et al.\(^{26}\) Alleles at the seven loci, *arcC, aroE, glpF, gmk, pta, tpi* and *yqiL*, were assigned by comparing the sequences at each locus with those of the known alleles in the *S. aureus* MLST database. The allele numbers at each of the seven loci define the allelic profile of each isolate and an allelic profile is defined as a sequence type (ST). To determine the group of each ST on the basis of the MLST database, the eBURST program was used.\(^{27}\)

Grouping was carried out using an analysis panel that selects six minimum numbers of identical loci out of seven loci for group definition and three minimum single locus variant contents for subgroup definition.

**Results**

**Isolated MRSA strains from bovine milk**

Nine thousand and fifty-five milk samples that had over 500,000 somatic cells/mL, were selected out of 75,335 of quarter milk samples that were collected in 1999, 2000 and 2003, and a total of 15 presumptive MRSA isolates, which carried the *mecA* gene, were identified with an isolation ratio of 0.18% (Table 1). Among them, M99-132, M99-133 and M99-136 were isolated from a farm in 1999, and M00-425 and M00-428 were isolated from the same farm in 2000. M03-61, M03-62, M03-64, M03-65, M03-66, M03-68, M03-69 and M03-70 were identified from another farm in 2003. The strains isolated from one farm shared common phenotypic and genotypic characteristics. Therefore, M99-132, M00-425 and M03-68 are listed in Table 1 as the representative isolates for the corresponding farms. Although we screened milk samples from eight different provinces from all over Korea, presumptive MRSA were identified only in Gyeonggi and Chungcheong provinces. Other mastitis-causing bacteria including *Enterococcus* spp., *S. aureus*, coliforms, coagulase-negative staphylococci and *Streptococcus* spp. were also identified with isolation ratios of 4.21, 2.74, 1.52, 1.24 and 0.52%, respectively.

**Antibiotic susceptibility patterns**

The antibiotic susceptibility and MICs of oxacillin of the 15 presumptive MRSA isolates were confirmed. All isolates from 1999 to 2000, and M03-62, M03-64 and M03-66 were only resistant to β-lactam (penicillin, nafcillin, ampicillin and oxacillin) and aminoglycoside antibiotics (gentamicin). M03-61, M03-65, M03-68 and M03-69 isolates showed additional resistance or intermediate resistance to ciprofloxacin. M03-70 showed resistance to β-lactam only. The MICs of oxacillin of these 14 isolates were determined as being in the range of 16–64 mg/L. Therefore, these 14 isolates were regarded as MRSA. One isolate, M03-72, which was only resistant to ampicillin and penicillin but not to oxacillin in both disc diffusion and microdilution tests, was regarded as MSSA. However, we designated M03-72 as a silent *mecA*-carrying MSSA (smMSSA) due to its carriage of the *mecA* gene. According to the antibiogram, there were no isolates that showed multiresistance patterns (resistant to three or more antibiotics other than β-lactam), on the basis of the multiresistance definition by Coombs et al.\(^{28}\)

**SCCmec characteristics**

All 14 MRSA isolates harboured SCCmec type IV composed of the class B *mec* gene complex and the type 2 *ccr* gene complex (Table 1). SCCmec subtypes IVa–IVf have been determined by their unique sequences in the type IV SCCmec complex.\(^{2,29,30}\)
In those studies, MRSA CA05 (subtype IVa) and 8/6-3P (subtype IVb) encode three unique ORFs in their L-C regions (CQ001, Q001 and CQ002, and CM001, CM002 and M001, respectively) and MR108 (subtype IVc) had Tn4001 bracketed by a pair of IS431 copies.2,29 SCCmec subtypes IVe and IVf had unique sequences in the downstream region of IS431mec.30 In this study, the designation of subtype IVg to the elements of bovine milk MRSA isolates was also assigned based on the unique nucleotide sequences at the L-C regions. The whole L-C region of subtype IVg, represented as that of M03-68 (accession no. DQ106887), was amplified by the primer sets of SCCmec subtype IVb. However, it revealed unique sequences composed of five ORFs. Among them, PK01 and PK03 showed 23 and 56% identities with hypothetical proteins of Listeria monocytogenes, respectively. PK02 had 62.1% identity with hypothetical protein SAS0036 of S. aureus. PK04 and PK05 showed 96 and 100% identities with the hypothetical proteins SA0064 and SA0059 (N031), respectively, which were carried by pre-MRSA strain N315 (DDBJ/EMBL/GenBank accession nos D86934 and NC002745). Therefore, this new SCCmec element was designated as subtype IVg.

The smMSSA isolate, M03-72, had only the mecA gene and did not have the other additional genes of the SCCmec complex. Its PCR product amplified with M001-R and cL2b primers corresponded to SA1388, which encodes conserved hypothetical protein, and to SA1043, which encodes aspartate transcarbamoylase chain A, of N315, with 88 and 99% similarity, respectively.

The ccrA2B2 gene complex was detected in the 14 MRSA isolates, and their sequence substitutions were compared with those of CA05, 8/6-3P, MR108 and N315 strains (Figure 2). ccrA2B2 gene sequences of the bovine milk isolates, except M99-133 and M00-428, were the same and were more homologous with ccrA2B2 of N315 (98.7% similarity) than with those of CA05 and 8/6-3P (97.1% similarity) or MR108 (98.5% similarity). M99-133 had the most variant ccrA2B2 gene sequences among the bovine milk isolates, resulting in 97.9% similarity with other bovine milk MRSA isolates. Its ccrA2B2 gene sequences revealed 96.6, 95.0 and 96.4% similarity with those of N315, CA05 (or 8/6-3P) and MR108, respectively. M00-428 had 99.8% sequence similarity with other bovine milk isolates.

**Boundaries of SCCmec**

Nucleotide sequences around the left and right boundaries of the SCCmec of the 14 MRSA isolates and one smMSSA (M03-72) isolate were compared with those of N315, CA05 and 8/6-3P strains (Figure 3). A chromosome-left SCCmec junction of M03-72 was not detected in PCR, although the existence of chromosome-left SCCmec junction was confirmed via Southern hybridization (data not shown). All other sequences of the 15 isolates were exactly matched and showed 96, 84 and 92% similarity with those of N315, CA05 and 8/6-3P, respectively. Sequence variations of these four representative strains, M99-132, N315, CA05 and 8/6-3P, were found only in the chromosome-left SCCmec junction, whereas sequences in the chromosome-right SCCmec junction showed 100% similarity.

**PFGE analysis**

Isolates giving PFGE patterns with fewer than four fragment differences are considered to be the same strain, and isolates that show seven or more band differences are considered unrelated strains as
SCCmec characterization for *S. aureus* milk isolates

![Diagram](image_url)

Figure 3. Chromosome-SCCmec junction sequences of *S. aureus* isolated from bovine milk. All 14 MRSA isolates (type IVg) and the silent meca-carrying MSSA (smMSSA) isolate whose chromosome-left SCCmec junction was not detected by PCR, showed exact sequence homologies and the sequences are represented by that of M99-132. The nucleotide sequences around the left (a) and right (b) boundaries were aligned with those of CA05 (type IVa; DDBJ/EMBL/GenBank accession no. AB063172), 8/6-3P (type IVb; AB063173) and N315 (type II; D86934). The dotted arrow indicates inverted repeats of IR-L and IR-R at both extremities of SCCmec elements. The solid arrow indicates direct repeats of DRscc-L and DRscc-R. The nucleotide sequences of four representative strains, which were different from each other, are underlined with a solid line.

determined by Tenover et al.25 In this study, all MRSA isolates showed no more than one band difference among them, whereas the smMSSA had over seven fragment differences (data not shown). Therefore, the 14 MRSA strains were regarded as being the same strain and the smMSSA was considered unrelated to the lineage of the 14 MRSA.

**MLST analysis**

All MRSA isolates, which had SCCmec type IVg, showed ST5 with an allelic profile of 1-4-1-4-1-1-10. A non-classified smMSSA isolate, M03-72, had an allelic profile of 3-35-48-19-20-26-39 and was revealed as ST580. ST5 is included in group 1 of a total of 27 groups and singletons in the MLST database. However, M03-72 did not match any of the pre-existing groups and it was determined to be a singleton on the basis of BURST analysis.

**Discussion**

Fourteen MRSA strains and an smMSSA from the milk of cows that were suspected to be infected with mastitis were isolated in 1999, 2000 and 2003. They showed CA-MRSA characteristics on the basis of SCCmec complex analysis and antimicrobial susceptibility tests. The 14 MRSA isolates encoded the same SE genes, SED, SEI and SEJ (Table 1), and all 15 isolates shared PVL, which is known as being carried by most CA-MRSA strains rather than by HA-MRSA strains, although there was no probability of co-acquisition with the SCCmec complex.33 Fey et al.12 reported that CA-MRSA poses a serious health risk because of its ability to produce superantigenic toxin. However, Sousa and de Lencastre suggested no relatedness between toxin carriage and SCCmec type IV, as shown in this study. We detected the SEs and PVL genes from other SCCmec type II and III isolates of human origin and found that they had different types of SEs and PVL (data not shown). Therefore, the high toxin carriage rate and the same toxin-harbouring tendency of bovine milk isolates in this study seem to be due to their clonal relatedness. According to PFGE analysis, the 14 MRSA isolates were considered as being the same strain. These MRSA isolates seemed to have originated from the same genetic lineage and then spread nationwide, resulting in the most prevalent MRSA strain in milk from cows with mastitis in Korea. All 15 isolates were isolated from two provinces, Gyeonggi and Chungcheong, which are adjacent to each other geographically, although meca non-carrying MSSA were isolated from all provinces (data not shown). Since MRSA has been introduced as a mastitis-causing pathogen to the dairy farms in Gyeonggi or Chungcheong province, it might expand into the neighbouring area and then spread to other areas. Therefore, a method of MRSA detection and prevention should be established in Korean dairy farms. All the MRSA isolates in this study showed ST5, which was included in group 1 according to the BURST analysis. ST5 includes various isolates that display SCCmec types from type I to IV.32,33 Among them, two pandemic MRSA clones, the New York/Japan clone (ST5-MRSA-SCCmec type II) and the paediatric clone (ST5-MRSA-SCCmec type VI) were determined.33,34 The pandemic paediatric clone, which showed SCCmec type IV and an antimicrobial resistance pattern similar to our bovine milk isolates, was isolated from 1990 to 1998 at various paediatric settings in Argentina, Colombia, Japan, Poland, Portugal and the USA.34,35 Because ST5 and group I include many MSSA isolates, the pandemic clone and the New York/Japan clone were recognized as having evolved independently through insertion into the MSSA genetic backgrounds of SCCmec types II and IV, respectively.32,33 Nevertheless, it was not clear whether the CA-MRSA strains were nosocomial strains that had ‘escaped’ from the human hospital environments or whether these strains represented a new acquisition of SCCmec DNA.14 The location at which these bovine milk isolates with CA-MRSA characteristics emerged in Korea is also curious. There are three hypotheses that can explain this. First,
these ST5-MRSA-SCCmec type IV bovine milk isolates may have developed from ST5-MSSA, regardless of other HA-MRSA or CA-MRSA, which appears to be an archaic ancestor of ST5 circulating worldwide as presented by Monkolrattanothai et al. It is noted that the relatively small size of SCCmec type IV may result in increased mobility and, therefore, may have a greater propensity for horizontal transfer to the diverse genetic backgrounds of S. aureus. This is consistent with the number of STs; SCCmec type IV was associated with a comparatively large number of STs implying that the same SCCmec complex might be inserted into diverse genetic backgrounds horizontally. The second hypothesis is that these ST5-MRSA-SCCmec type IV bovine milk isolates may have originated from a pandemic paediatric clone that had been introduced from foreign countries via human travel or animal import. Paediatric clones have been identified in Japan and the USA; Japan is geographically close to Korea and, recently, both countries have experienced increased cultural exchanges with Korea. The list of Holstein cow-importing countries, where some paediatric clones have been identified, also supports this idea. The third hypothesis is that ST5-MRSA-SCCmec type IV bovine milk isolates may be derived from the ST5-MRSA-SCCmec type II of HA-MRSA strains. In Korea, ST5-MRSA-SCCmec type II (group 1) and ST239-MRSA-SCCmec type III (group 3) are the predominant genotypes of HA-MRSA. Comparison of spa types of ST5-MRSA-SCCmec type II and ST239-MRSA-SCCmec type III suggests that ST5-MRSA-SCCmec type II strains have been spreading for a relatively long time and that ST239-MRSA-SCCmec type III was recently introduced to Korea from another country. Some SCCmec type IV MRSA were also isolated from neonates and primary care obstetric clinics, but their ST types were ST1, ST493 and single locus variants of ST1. All these SCCmec type IV isolates of human origin were included in group 5. Therefore, the ST5-MRSA-SCCmec type II genotype strains are probably a prototype of ST5-MRSA-IV strains.

Several reports based on epidemiological research and molecular approaches have suggested that CA-MRSA escaped from human hospitals. Multiresistant SCCmec type IV strains isolated from human hospitals also support this hypothesis. Until now, ST5-MRSA-SCCmec type II but not ST5-MRSA-SCCmec type IV isolates have been reported by human hospitals in Korea and the bovine milk isolates with ST5-MRSA-SCCmec type IV in this study showed more sequence homologies with SCCmec type II than with SCCmec type IV strains in terms of ccr genes, ORFs in the L-C region, and chromosome-SCCmec junctional sequences (Figures 2 and 3). Therefore, ST5-MRSA-SCCmec type IV bovine milk isolates may be derived from the ST5-MRSA-SCCmec type II of HA-MRSA strains if the third hypothesis is true. After introduction into dairy husbandries, ST5-MRSA-SCCmec type II strains might lose other antibiotic resistance genes such as Tn554 and pUB110 because of low antibiotic selective pressure in the community.

It is theoretically possible that the ST5-MRSA-SCCmec type II of HA-MRSA strains has emerged from ST5-MRSA-SCCmec type IV bovine milk isolates. However, this hypothesis is not realistic because bovine milk MRSA strains were identified with a very low isolation rate (0.18%) compared with the recent high isolation rates (60–80%) of HA-MRSA in Korea. What is important is that methicillin and oxacillin have not been used as therapeutic agents or feed additives in cow farm management in Korea. Antibiotics were used less frequently in cattle breeding than in any other livestock husbandry with just 6% annual usage in 2001. Other β-lactam antibiotics, ampicillin and penicillin, were also infrequently used on cow farms, corresponding to 14.2% of the total annual β-lactam antibiotic usage of livestock husbandries in 2001. These tendencies were maintained before and after 2001, according to the Korea Animal Health Products Association. Although we have examined MRSA from various sources of livestock products and abattoir samples from January 2000 to August 2003, MRSA strains were identified from them (data not shown). Therefore, it is strange that MRSA were isolated from the milk of cows that were treated with relatively smaller amounts of antibiotics. This discrepancy may be due to the chronic state of mastitis by S. aureus infection; it is difficult to cure and easily recurs. Therefore, it is possible that a relatively large amount of antibiotics was administered to the infected cows to treat the S. aureus-causing mastitis, and that may be the reason for the emergence of MRSA in the bovine milk. However, if so, it still does not explain why SCCmec type IV, rather than other multiresistant SCCmec types, is carried by MRSA isolates that originated in bovine milk.

Although there was no authenticating evidence for the evolution of bovine milk MRSA, it seemed clear that they had no genetic relatedness with human infection by CA-MRSA in Korea. One study reported a high genetic relatedness between MRSA isolates that originated from bovine milk and human isolates in Korea on the basis of random amplified polymorphic DNA, which suggested a high possibility of community infection of humans caused by MRSA originating from bovine milk. Further research within our study, using more reliable molecular analysis methods like PFGE, MLST analysis and the characterization of SCCmec, revealed that this possibility was unlikely, however. Still, more studies on the spread of MRSA between human and veterinary fields and the control management of MRSA in dairy husbandry should be carried out continuously due to the finding of SCCmec type IVg and the smMSSA strain. The existence of novel SCCmec subtype IVg strains suggests the possibility of the emergence of another type of MRSA strain. The smMSSA strain may be a new prototype of MRSA after the acquisition of other virulence genes, or may be an origin of mecA-transferring S. aureus. Therefore, the prudent use of antibiotics and rapid and continuous screening for resistant microorganisms should be more focused to prevent the emergence and spread of new types of MRSA.

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