Dissimilarity of $ccrAB$ gene sequences between methicillin-resistant *Staphylococcus epidermidis* and methicillin-resistant *Staphylococcus aureus* among bovine isolates in Korea

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The sequences of the $ccrAB$ genes from bovine-, canine- and chicken-originating methicillin-resistant *Staphylococcus (S.) epidermidis* (MRSE) and bovine methicillin-resistant *Staphylococcus (S.) aureus* (MRSA) were compared to investigate the frequency of intra-species horizontal transfer of the staphylococcal cassette chromosome mec (SCCmec) complex. Nineteen MRSE strains were isolated from bovine milk, chickens, and dogs, and their genetic characteristics were investigated by multilocus sequence typing and SCCmec typing. Among the animal MRSE strains, the most frequent SCCmec type was type IV, which consisted of the type B mec complex and $ccrAB$ type 2. The $ccrA2$ and $ccrB2$ genes were sequenced from the bovine, chicken and canine MRSE strains and compared with those of the bovine MRSA strains. The sequences generally clustered as MRSA and MRSE groups, regardless of the animal source. Additionally, no bovine MRSE sequence was associated with the bovine MRSA groups. Among most of the bovine MRSE and MRSA isolates possessed SCCmec type IV sequences, our results suggest that the intra-species gene transfer of the SCCmec complex between bovine *S. aureus* and bovine *S. epidermidis* strains is not a frequent event.

**Keywords:** $ccrAB$, methicillin-resistant *Staphylococcus aureus*, methicillin-resistant *Staphylococcus epidermidis*, SCCmec

**Introduction**

Methicillin-resistant staphylococci, including *Staphylococcus (S.) epidermidis* (MRSE), have emerged as important pathogens that can cause serious infections in humans and animals [5,30]. Most MRSE strains have also been shown to be resistant to other antimicrobial agents [20], causing concern from a clinical veterinary perspective by threatening to narrow the spectrum of antimicrobial therapeutic choices. Moreover, from a zoonotic point of view, animal reservoirs of MRSE can be considered a great threat to human healthcare. Therefore, effective detection and control of MRSE associated with mastitis in dairy cows is essential.

The methicillin resistance of staphylococci is mediated by the $mecA$ gene, which is carried by a mobile genetic element known as the staphylococcal cassette chromosome mec (SCCmec) [15]. SCCmec is composed of the mec complex containing the $mecA$ gene and its regulator genes, as well as the cassette chromosome recombinase ($ccr$) complex, which encodes site-specific recombinase genes such as $ccrA$ and $ccrB$ [14,17]. These recombinase genes are responsible for the mobility of SCCmec, as they mediate its site-specific insertion and excision [14,17].

Several studies have shown that methicillin-susceptible *Staphylococcus (S.) aureus* can be converted to the pathogen, methicillin-resistant *S. aureus* (MRSA), through the transfer of SCCmec from another methicillin-resistant *Staphylococcus* species such as MRSE [2,11,12,33]. To reduce the spread of methicillin-resistant staphylococcal infections among dairy herds, the distribution of MRSE and transfer of SCCmec should be monitored. However, only a few veterinary investigations of MRSE infections...
have been published to date [4,25,27]. Here, we identified the prevalence of MRSE among various animal sources, including bovine milk, and compared their ccrAB gene sequences to those of bovine MRSA strains to assess the frequency of intra-species horizontal gene transfer of the SCCmec complex.

Materials and Methods

Bacterial strains
All utilized S. epidermidis strains were isolated from April 2006 to February 2007. Bovine strains were isolated from Quarter milk samples with over 500,000 somatic cells/mL collected from 61 dairy farms in South Korea. Milk somatic cell counts were analyzed with a Somacount 150 (Bentley Instruments, USA) within 24 h of sampling. Chicken strains were isolated from fecal swabs collected from three broiler farms in Gyeonggi and Chungnam. Canine strains were isolated from patients in two veterinary medical teaching hospitals (Chungbuk, Seoul) and one private referral animal clinic (Gyeonggi).

S. epidermidis isolates were identified using the schemes developed by Koneman et al. [19] and confirmed using a Vitek II (BioMérieux, France) and PCR with species-specific Serp0107 primers. These primers targeted the gene for a putative transcriptional regulator that is uniquely present in S. epidermidis [24]. Among 857 milk samples showing over 500,000 somatic cell/mL, 21 S. epidermidis strains were isolated. Additionally, 190 fecal swab samples were collected from chickens, from which ten S. epidermidis strains were isolated. Moreover, 175 swab samples from horizontal ear canals (n = 49), nasal mucosa (n = 45), anuses (n = 33), skin (n = 28), urine (n = 12), wound regions (n = 6), and eyes (n = 2) were collected from 35 canine outpatients, and 11 S. epidermidis strains were isolated.

For ccrAB comparison, 25 bovine MRSA strains were used in this study. These strains were previously isolated from bovine milk by the above-described method, and had been characterized as SCCmec type IV in our laboratory from 1999 to 2006 [22,23,28].

Antimicrobial susceptibility tests
The minimum inhibitory concentration (MIC) to oxacillin (Sigma-Aldrich, USA) was determined using a microdilution test according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [8]. Isolates with a MIC ≥ 0.5 μg/mL were classified as oxacillin-resistant strains. A disc diffusion test was also performed according to the guidelines of the CLSI and used to test the susceptibility of these oxacillin-resistant isolates to ten other antimicrobials [8]: amikacin, amoxicillin, ampicillin, cephalothin, erythromycin, gentamicin, kanamycin, penicillin, tetracycline, and vancomycin (BD BBL, USA). For quality control, S. aureus ATCC 25923, Escherichia coli ATCC 25922 and Enterococcus faecalis ATCC 29212 were used as reference strains.

DNA preparation and polymerase chain reaction (PCR)
S. epidermidis isolates were grown overnight on 5% sheep blood agar plates (Promed, Korea) at 37°C. Chromosomal DNA was extracted using the DNeasy tissue kit (Qiagen, Germany) according to the manufacturer’s instructions with one modification: the cell-lysis step was performed with 50 U/mL lysostaphin (Sigma-Aldrich).

MRSEs from the oxacillin-resistant S. epidermidis strains were confirmed by detection of the mecA gene, and PCR-based SCCmec typing was carried out with multiplex PCR designed by Kondo et al. [18]. Briefly, two multiplex PCR strategies were used. The mec class (A, B, or C) was identified by the M-PCR1 reaction, and the type of ccr complex (ccrAB1, ccrAB2, ccrAB3, ccrAB4, or ccrC) was determined by the M-PCR2 reaction. DNA amplification was carried out in a Mastercycler gradient (Eppendorf, Germany) using Ex Taq DNA polymerase (Takara Bio, Japan).

Sequencing and analysis
The ccrA2 and ccrB2 genes of SCCmec type IV MRSE and MRSA strains were amplified with the specific primer sets described by Hanssen et al. [11], and the PCR products were sequenced by Bionics (Korea). Sequence data were compared by the ClustalV method using the DNAstar software (USA). A phylogenetic tree of the ccr genes was constructed by the neighbor-joining method using the Mega 3.1 software [21]. The topologies of the phylogenetic trees were evaluated by bootstrap analyses with 1,000 replicates, which yielded confidence intervals for each node on the phylogenetic trees. The following reference sequences were used in the phylogenetic analyses: ccrA2 and ccrB2 from S. aureus N315 (GenBank accession no. D86934), and ccrA3 and ccrB3 from S. aureus 85/2082 (GenBank accession no. AB037671) [6].

Multilocus sequence typing (MLST) analysis
MLST analysis of the MRSE isolates was performed as described by Thomas et al. [32]. Alleles at seven loci (arcC, aroE, gtr, mutS, pyrH, tpi, and yqiL) were assigned by comparing the sequences with those of the known alleles in the S. epidermidis MLST database [16]. The allele numbers at each of the seven loci were used to define the allelic profile of each isolate, and each allelic profile was taken as a different sequence type (ST). To group the STs and identify the patterns of evolutionary descent, data from the isolates were analyzed using the eBURST program available on the MLST website [16]. Grouping
was carried out using the most stringent definition, which defined a group as having members that all shared identical alleles at more than six loci with at least one other member of the group.

Results

Identification of MRSE from animal sources
A total of 42 *S. epidermidis* strains were isolated and the isolation rates of *S. epidermidis* were 2.5%, 5.3% and 6.2% among samples collected from bovine milk, chicken and dogs, respectively. Among the 42 *S. epidermidis*, 19 strains (45.2%) were identified as MRSE based on their resistance to oxacillin and their possession of the *mecA* gene (Table 1). According to the origin, the MRSE detection rates were 42.9% (9 out of 21 bovine strains), 40% (4 out of 10 canine strains) and 54.5% (6 out of 11 canine strains), respectively.

Antimicrobial resistance of MRSE
The oxacillin MICs for the 19 MRSE isolates were 1 μg/mL or more. The animal MRSE isolates were highly resistant to ampicillin (73.7%) and penicillin (73.7%). Resistance to kanamycin (68.4%), erythromycin (42.1%), gentamicin (63.2%), and tetracycline (42.1%) was also observed (Table 1). All MRSE isolates were susceptible to amikacin, amoxicillin, cephlothin, and vancomycin. Nine of the MRSE isolates (47.4%) showed multidrug resistance patterns (2 bovine strains, 22.2%; 3 chicken strains, 75.0%; 4 canine strains, 66.7%) of resistance to three or more antimicrobials other than β-lactams (Table 1).

Identification of *ccrAB* genes and *mec* gene complexes
The *ccrAB* types of the MRSE strains were determined by determining the *ccrAB* types and *mec* gene complex classes. Sequence analysis confirmed that 16 MRSE isolates were *ccrAB* type 2, and the majority (14/19, 73.7%) of these harbored class B *mec* gene complexes (Table 1). One canine MRSE strain possessed SCCmec type II, and four MRSE strains (10.5%, 2 bovine and 2 canine strains) could not be typed.

MLST analysis
The MRSE strains isolated from different animal origins (bovine milk, chickens, and dogs) showed different MLST patterns, although some belonged to the common MLST type, ST57. While all four of the MRSE isolates from chickens were type ST57, three MLST types (ST5, 20, and 57) and four MLST types (ST2, 20, 57, and 64) were identified in MRSE isolates from bovine milk and dogs, respectively.

Four of the five MLST types identified in the animal MRSE isolates could be divided into two groups using the eBURST: group 1 included ST2 and ST20; group 2 contained ST5 and ST57; and ST64 existed as a singleton. Most of the MRSE strains isolated from bovine milk and chickens belonged to group 2, while those isolated from dogs were part of group 1.

*ccrA2* and *ccrB2* sequence alignment
As the most prevalent type of SCCmec was type IV, the *ccrAB* sequences of those strains were selected for comparison to the bovine MRSA sequences. The *ccrA2* and *ccrB2* sequences of 16 SCCmec type IV-MRSE and 25 SCCmec type IV-bovine MRSA strains were aligned and phylogenetic trees were constructed (Fig. 1). The sequence homology of *ccrA2* and *ccrB2* genes between the MRSE and MRSA was found to be 95 ~ 100% and 95.4 ~ 100%, respectively.

The tree for *ccrA2* showed that MRSA and MRSE belong to separate groups, except for MRSE S2 ~ 27 (belongs to *ccrSA1*) and MRSE BM8 (does not belong to any group) (Fig. 1A). Similarly, the tree for *ccrB2* showed that MRSA and MRSE belong to separate groups except for MRSE A2 ~ 7 (belongs to *ccrbSA1*) and N315 (belongs to *ccrbSE1*) (Fig. 1B).

Discussion
Although the prevalence of *S. epidermidis* was not high (2.5%) in bovine milk samples, we found high methicillin-resistance rates (40%, 42.9% and 54.5%) among the isolates, regardless of origin. Moreover, almost half of the MRSE isolates (47.4%) showed multidrug resistance patterns. The high detection rates of MR-CNS were also reported in previous studies. In Norway, 70 ~ 80% of CNS isolated from hospitals was methicillin-resistant, while the rate of MRSA was low [11]. Among animals, a 65.2% *mecA* detection rate was observed in isolates from sheep, goats, pigs, and chicken samples [35]. In Japan, MR-CNS was isolated from healthy horses with a 27% detection rate [34]. The high prevalence of *mecA* among CNS strains suggests that they may serve as a reservoir for antibiotic-resistant genes that can be transferred to other Gram-positive organisms, including *S. aureus* [26]. In addition, the multidrug resistant MRSEs pose a public health threat because they make treatment of infections difficult [36].

The most frequent SCCmec type detected in MRSE isolates was type IV, which is characterized by *ccrAB* type 2 and the class B *mec* gene complex. SCCmec type IV is often found in community-acquired MRSA among humans [3,10], as well as in animal MRSA [23]. SCCmec type IV is much smaller than that of other SCCmec elements, perhaps increasing its mobility and propensity for horizontal transfer to the diverse genetic backgrounds of *Staphylococcus* strains [1]. SCCmec type IV has been associated with a comparatively large number of STs,
Table 1. Genetic and phenotypic characteristics of MRSE isolates from animal sources

| Region   | Year | Origin | Farm or animal hospital ID | Animal ID | Isolation site | MIC (mg/L) oxacillin | Antimicrobial resistant patterns except oxacillin | PCR results for localization of representative genes in mec gene complex* | Ccr type | SCCmec type | MLST type | Group |
|----------|------|--------|-----------------------------|-----------|----------------|-----------------------|------------------------------------------------|-----------------------------------------------|----------|-------------|-----------|-------|
| Chungnam | 2007 | Chicken | A2                          | 7         | Anus           | ≥128                  | + +/– – + B 2 IV ST57 2 | mecA mecR1 (MS/PB) mecI IS1272 class | Ccr type | SCCmec type | MLST type | Group |
| Chungnam | 2007 | Chicken | A2                          | 18        | Anus           | 32                    | + +/– – + B 2 IV ST57 2 | | | | | |
| Chungnam | 2007 | Chicken | A3                          | 5         | Anus           | 64                    | + +/– – + B 2 IV ST57 2 | | | | | |
| Chungnam | 2007 | Chicken | A3                          | 12        | Anus           | 16                    | + +/– – + B 2 IV ST57 2 | | | | | |
| Gangwon  | 2006 | Bovine | GWHW                        | 8         | Milk           | 8                     | + +/– – – NT 2 NT ST5 2 | | | | | |
| Gangwon  | 2006 | Bovine | GWHW                        | 28        | Milk           | 4                     | + +/– – – NT NT NT ST20 1 | | | | | |
| Gyeonggi | 2006 | Bovine | G1SH                        | 58        | Milk           | 32                    | + +/– – + B 2 IV ST57 2 | | | | | |
| Gyeonggi | 2003 | Bovine | G1NL                        | 80        | Milk           | 1                     | + +/– – + B 2 IV ST57 2 | | | | | |
| Jeonnam  | 2000 | Bovine | JNNL                        | 84        | Milk           | 64                    | + +/– – + B 2 IV ST57 2 | | | | | |
| Gyeonggi | 2006 | Bovine | G1HA                        | 235       | Milk           | 8                     | + +/– – + B 2 IV ST57 2 | | | | | |
| Chungnam | 2007 | Bovine | CNH2                        | 468       | Milk           | 64                    | + +/– – + B 2 IV ST5 2 | | | | | |
| Chungnam | 2007 | Bovine | CNH2                        | 479       | Milk           | 64                    | + +/– – + B 2 IV ST5 2 | | | | | |
| Chungnam | 2007 | Bovine | CNH2                        | 485       | Milk           | 64                    | + +/– – + B 2 IV ST5 2 | | | | | |
| Chungbuk | 2007 | Canine | CNU                         | 35        | Ear canals     | ≥128                  | + +/– – + B 2 IV ST64 Singleton | | | | | |
| Gyeonggi | 2007 | Canine | HM                          | 11        | Skin           | 64                    | + +/– – + B 2 IV ST20 1 | | | | | |
| Gyeonggi | 2007 | Canine | HM                          | 12        | Nasal          | 1                     | + +/– – – NT NT NT ST20 1 | | | | | |
| Gyeonggi | 2007 | Canine | HM                          | 20        | Nasal          | 32                    | + +/– – – A NT NT ST20 1 | | | | | |
| Seoul    | 2007 | Canine | SNU                         | 3         | Wound          | 64                    | + +/– – + B 2 IV ST57 2 | | | | | |
| Seoul    | 2007 | Canine | SNU                         | 27        | Nasal          | 4                     | + +/– – + A 2 II ST2 1 | | | | | |

*Localization of the essential genes in the mec gene complex was estimated by PCR and sequencing. The mecA gene and its regulator genes, mecR1 [both the membrane spanning region (MS) and the penicillin-binding region (PB)] and mecI were identified. K: kanamycin, AM: ampicillin, P: penicillin, GM: gentamicin, E: erythromycin, TE: tetracycline, NT: not typable.
Comparing ccr genes of bovine MRSA and MRSE

Fig. 1. Parsimony trees showing phylogenetic relationships among the ccrA2 (A) and ccrB2 (B) genes in MRSE and MRSA isolate derived from animal sources. Sequences from Staphylococcus (S.) aureus ccrA3 (A; GenBank accession no. AB037671) and S. aureus ccrB3 (B; GenBank accession no. AB037671) were used as outgroups.

implying that the same SCCmec complex might be horizontally inserted into diverse genetic backgrounds [9]. Consistent with these previous reports, we found that four MLST types (ST 5, 20, 57 and 64) were associated with SCCmec type IV [24,31].

Because the ccr complex determines the site specificity of the SCCmec complex, the sequence similarities of ccrA and ccrB genes are important for the mobility of SCCmec between different strains or species [7,13,29]. In our ccrAB gene sequence analysis, we observed the relative distance between bovine MRSA strains and bovine, canine, and chicken MRSE strains. The ccrAB2 alleles were generally conserved among their own species, while most of the MRSE sequences, including the bovine sequences, clustered together separate from bovine MRSA. Only a few MRSE strains showed ccrAB sequences similar to those of the bovine MRSA sequences. Surprisingly, the few MRSE strains that resembled bovine MRSA originated from canines or chickens, not from bovine sources. These findings indicate that the bovine MRSA strains might not acquire their SCCmec sequences from bovine MRSE, but rather from other sources. Moreover, the distinct clustering of the MRSA and MRSE groups may suggest that the horizontal transfer of SCCmec between S. epidermidis and S. aureus might not be a frequent event.

There were ccr genes of MRSE strains that were clustered in the same group even though they originated from different regions and years. However, few MRSE strains showed 100% homology, despite their relative closeness to each other when compared with other MRSA strains, implying that those MRSE strains obtained their SCCmec complexes independently. However, it is also possible that these results reflect 1) clonal distribution of a few dominant MRSE strains and 2) intra-species SCCmec horizontal transfer. To confirm the source of SCCmec of MRSE strains of this study, more comprehensive analysis of SCCmec complex and genetic comparison with methicillin-susceptible S. epidermidis strains isolated together should be conducted using tools such as sequencing and pulsed field gel electrophoresis.

In sum, our investigations revealed a high methicillin-resistance rate among animal-originating MRSE strains, indicating that animals infected with MRSE could be reservoirs for human infection through contact with animals or food products. Since direct evidence of the
horizontal transfer of SCCmec between \textit{S. epidermidis} and \textit{S. aureus} in bovine isolates has not yet been found, further investigations of the horizontal transfer and source of SCCmec should be performed to prevent the spread of MRSE and MRSA infection.

Acknowledgments

This study was supported by the Korea Institute of Planning and Evaluation for Technology in Ministry of Agriculture, Food and Rural Affairs (IPET 311001-03-1-HD120) and by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2013R1A1A3008391). Additional support was provided by the Research Institute of Veterinary Science, Department of Veterinary Microbiology, College of Veterinary Medicine, and the BK21 Program for Veterinary Science, Seoul National University, Seoul, Korea.

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Comparing ccr genes of bovine MRSA and MRSE

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