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Original Article

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* (SCC*mec*) complex. Nineteen MRSE strains were isolated from bovine milk, chickens, and dogs, and their genetic characteristics were investigated by multilocus sequence typing and SCC*mec* typing. Among the animal MRSE strains, the most frequent SCC*mec* 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. Although most of the bovine MRSE and MRSA isolates possessed SCC*mec* type IV sequences, our results suggest that the intra-species gene transfer of the SCC*mec* 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*, SCC*mec*

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* (SCC*mec*) [15]. SCC*mec* 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 SCC*mec*, 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 SCC*mec* 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 SCC*mec* should be monitored. However, only a few veterinary investigations of MRSE infections

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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 SCC*mec* 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 SCC*mec* 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 detected using a microdilution test according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [8]. Isolates with a MIC $\geq$ 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* strainswere confirmed by detection of the *mecA* gene, and PCR-based SCC*mec* 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-PCR1reaction, and the type of *ccr* complex (*ccr*AB1, *ccr*AB2, *ccr*AB3, *ccr*AB4, or *ccr*C) 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 SCC*mec* 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, pyrR, 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 chicken strains) and 54.5% (6 out of 11 canine strains), respectively.

Antimicrobial resistance of MRSE

The oxacillin MICs for the 19 MRSE isolates were1 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, cephalothin, 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 SCC*mec* types of the MRSE strains were defined by determining the *ccrAB* types and *mec* gene complex classes. Sequence analysis confirmed that 16MRSE 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 SCC*mec* 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 SCC*mec* 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 SCC*mec* type IV-MRSE and 25 SCC*mec* 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 *ccraSA1*) 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 SCC*mec* type detected in MRSE isolates was type IV, which is characterized by *ccrAB* type 2 and the class B *mec* gene complex. SCC*mec* type IV is often found in community-acquired MRSA among humans [3,10], as well as in animal MRSA [23]. SCC*mec* type IV is much smaller than that of other SCC*mec* elements, perhaps increasing its mobility and propensity for horizontal transfer to the diverse genetic backgrounds of *Staphylococcus* strains [1]. SCC*mec* 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	SCC*mec* type	MLST type	Group
								mecA	*mecR1* (MS/PB)	*mecI*	*IS1272*	class				
Chungnam	2007	Chicken	A2	7	Anus	≥128	K-AM-P-GM-E-TE	+	+/−	−	+	B	2	IV	ST57	2
Chungnam	2007	Chicken	A2	18	Anus	32	K-GM-TE	+	+/−	−	+	B	2	IV	ST57	2
Chungnam	2007	Chicken	A3	5	Anus	64	K-AM-P-GM-E-TE	+	+/−	−	+	B	2	IV	ST57	2
Chungnam	2007	Chicken	A3	12	Anus	16	AM-P	+	+/−	−	+	B	2	IV	ST57	2
Gangwon	2006	Bovine	GWHW	8	Milk	8	K-AM-P	+	−/−	−	−	NT	2	NT	ST5	2
Gangwon	2006	Bovine	GWHW	28	Milk	4	K-AM-P-GM-TE	+	−/−	−	−	NT	NT	NT	ST20	1
Gyeonggi	2006	Bovine	G1SH	58	Milk	32	AM-P-E-TE	+	+/−	−	+	B	2	IV	ST57	2
Gyeonggi	2003	Bovine	G1NL	80	Milk	1	AM-P-TE	+	+/−	−	+	B	2	IV	ST57	2
Jeonnam	2000	Bovine	JNNL	84	Milk	64	-	+	+/−	−	+	B	2	IV	ST57	2
Gyeonggi	2006	Bovine	G1HA	235	Milk	8	K-AM-P-GM-E	+	+/−	−	+	B	2	IV	ST57	2
Chungnam	2007	Bovine	CNH2	468	Milk	64	K-GM	+	+/−	−	+	B	2	IV	ST5	2
Chungnam	2007	Bovine	CNH2	479	Milk	64	K-GM	+	+/−	−	+	B	2	IV	ST5	2
Chungnam	2007	Bovine	CNH2	485	Milk	64	K-GM	+	+/−	−	+	B	2	IV	ST5	2
Chungbuk	2007	Canine	CNU	35	Ear canals	≥128	K-AM-P-GM-E-TE	+	+/−	−	+	B	2	IV	ST64	Singleton
Gyeonggi	2007	Canine	HM	11	Skin	64	K-AM-P-GM-E	+	+/−	−	+	B	2	IV	ST20	1
Gyeonggi	2007	Canine	HM	12	Nasal	1	AM-P	+	−/−	−	−	NT	NT	NT	ST20	1
Gyeonggi	2007	Canine	HM	20	Nasal	32	K-AM-P-GM-E	+	+/+	+	−	A	NT	NT	ST20	1
Seoul	2007	Canine	SNU	3	Wound	64	AM-P	+	+/−	−	+	B	2	IV	ST57	2
Seoul	2007	Canine	SNU	27	Nasal	4	K-AM-P-GM-E-TE	+	+/+	+	−	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.

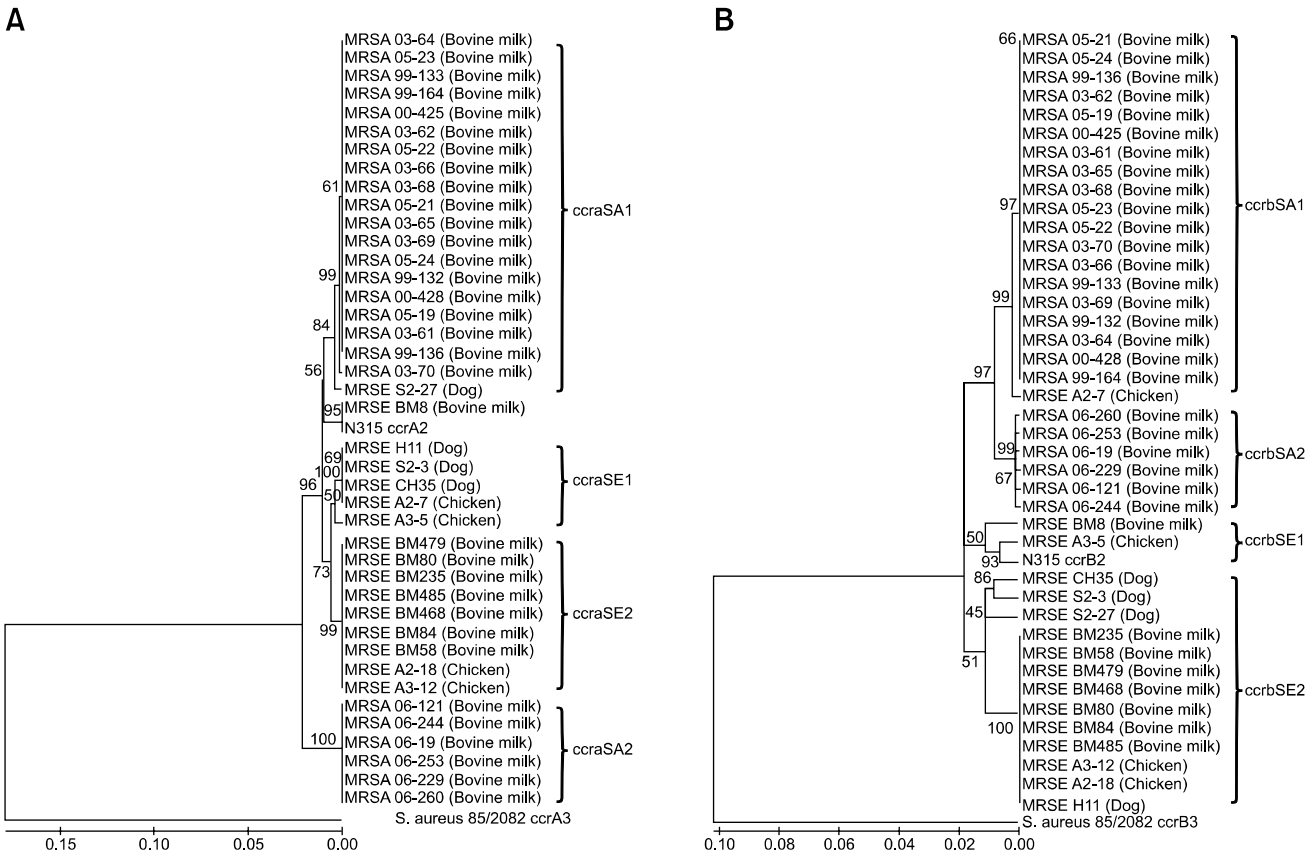

Fig. 1. Parsimony trees showing phylogenetic relationships among the *ccrA2* (A) and *ccrB2* (B) genes in MRSE and MRSA isolated from animal sources. Sequences from *Staphylococcus* (*S.*) *aureus ccrA3* (A; GenBank accession no. AB037671) and *S. aureus ccrB3* (B; GenBank accession no. AB037671) were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

implying that the same SCC*mec* 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 SCC*mec* type IV [24,31].

Because the *ccr* complex determines the site specificity of the SCC*mec* complex, the sequence similarities of *ccrA* and *ccrB* genes are important for the mobility of SCC*mec* 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 SCC*mec* 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 SCC*mec* 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 SCC*mec* complexes independently. However, it is also possible that these results reflect 1) clonal distribution of a few dominant MRSE strains and 2) intra-species SCC*mec* horizontal transfer. To confirm the source of SCC*mec* of MRSE strains of this study, more comprehensive analysis of SCC*mec* 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 SCC*mec* between *S. epidermidis* and *S. aureus* in bovine isolates has not yet been found, further investigations of the horizontal transfer and source of SCC*mec* should be performed to prevent the spread of MRSE and MRSA infection.

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