Antimicrobial resistance and genetic characterization of coagulase-negative staphylococci from bovine mastitis milk samples in Korea

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ABSTRACT

Coagulase-negative staphylococci (CNS) are one of the most common bovine mastitis pathogens found worldwide. In this study, we investigated the prevalence and distribution of CNS species in mastitis milk samples and further characterized the methicillin-resistant (MR) CNS. A total of 311 CNS were isolated from 3,692 quarter milk samples from 1,373 dairy cattle at 81 farms between 2013 and 2017. Further evaluation of the CNS isolates revealed 14 CNS species among the samples and 3 predominant species—namely, *Staphylococcus chromogenes*, *Staphylococcus simulans*, and *Staphylococcus epidermidis*. Resistance was higher in *S. epidermidis* than in other CNS species except for resistance against oxacillin in *Staphylococcus sciuri*. Resistance to β -lactams was the most common in all CNS species (8.4% in ampicillin, 21.2% in oxacillin, and 13.5% in penicillin). Conversely, only minimal resistance to cephalothin, ceftiofur, and pirlimycin/novobiocin was found. Twenty-one isolates from 4 species were *mecA*-carrying MRCNS strains, including 18 *S. epidermidis* and 1 each of *S. sciuri*, *Staphylococcus equorum*, and *Staphylococcus hominis*. The majority of the *mecA*-carrying MRCNS isolates were produced in the biofilm. Furthermore, multidrug-resistant sequence type 179 isolate produced the strongest biofilm. Seven genotypes were detected in the 18 MR *S. epidermidis* strains, the most predominant of which persisted on a farm for 2 yr. Our findings for the antimicrobial susceptibility profiles and genotypic characterization of the MRCNS isolates could provide valuable information for controlling the spread of resistance and the selection of appropriate antimicrobial therapies for mastitis in the future. Further, strategic antibiotic use for mastitis treatment and hygienic management practices aimed at

the prevention of the growth of resistant bacteria are urgently needed on dairy farms.

Key words: coagulase-negative staphylococci, antimicrobial resistance, methicillin resistance, molecular typing, mastitis in dairy cattle

INTRODUCTION

Mastitis is the most important disease in the dairy industry today because it is responsible for direct and indirect losses (Feßler et al., 2010) via increases in the number of somatic cells and decreases in both the quality and production of milk. Mastitis results from IMI caused by various bacterial species (Botrel et al., 2010). Distribution of the pathogens that cause IMI varies among different countries and herds. One of the common causes of IMI is CNS, which are the microorganisms most commonly observed in cases of subclinical mastitis (Pyörälä and Taponen, 2009; Mørk et al., 2012; Moser et al., 2013).

Antimicrobial therapy is generally used for mastitis control. Because CNS are known to be more resistant to antibiotics than *Staphylococcus aureus*, CNS outbreaks require close monitoring (Taponen and Pyörälä, 2009). Currently, β -lactam antimicrobials and macrolides are used in the treatment of CNS infections. However, some studies have reported an increase in CNS resistance to β -lactam antibiotics, particularly penicillin and ampicillin, which is a worldwide trend (Botrel et al., 2010; Persson et al., 2011).

Penicillinase-producing staphylococci have been increasing in both humans and animals (Chambers, 2001; Qian et al., 2015). Thus, penicillinase-stable β -lactams were widely used as a viable treatment for these resistant bacteria for many years (Qian et al., 2015). However, by the 1990s, methicillin-resistant *S. aureus* and methicillin-resistant CNS (MRCNS) had emerged and quickly became serious nosocomial infections worldwide (Chambers, 2001; Qian et al., 2015). Furthermore, these resistant bacteria have been reported as an emerging

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problem in veterinary medicine, including bovine mastitis (Moon et al., 2007; Feßler et al., 2010; Sampimon et al., 2011). Specifically, methicillin-resistant *Staphylococcus epidermidis* (MRSE) was detected in bovine mastitis samples from Germany and Finland (Feßler et al., 2010; Gindonis et al., 2013).

In Korea, several studies were conducted on the etiology and antimicrobial resistance of major mastitis pathogens such as *Escherichia coli*, *S. aureus*, and CNS (Moon et al., 2007; Nam et al., 2010; Tark et al., 2017). Moreover, a study based on the distribution of CNS species and their antimicrobial resistance was conducted from 2003 to 2008 (Nam et al., 2010); however, recent information on this subject is still somewhat limited. Thus, the aims of this study were to investigate the species distribution and antimicrobial resistance of recently isolated CNS and to analyze the molecular genetic characteristics of MRCNS.

MATERIALS AND METHODS

Collection of Quarter Milk Samples

From January 2013 to June 2017, a total of 3,692 quarter milk samples from 1,373 lactating cows on 81 dairy farms nationwide were examined at the Mastitis Diagnostic Laboratory in the Animal and Plant Quarantine Agency (Gimcheon, Korea; Table 1). The milk samples used in this study were collected from lactating cows in herds with suspected mastitis as part of a routine diagnostic sampling submitted for determination of etiology of mastitis and antimicrobial susceptibility of the isolates. The 81 dairy farms sampled were located in the following provinces: Gyeonggi (n = 24), Chungnam (n = 7), Chungbuk (n = 23), Gyeongbuk (n = 15), Jeonnam (n = 1), Jeju (n = 1), Gangwon (n = 1), and unknown (n = 9). Among them, 70 farms were sampled only once in the 5-yr study period, whereas multiple sampling visits were conducted at the remaining 11 farms. During the study period, 501 to 1,059 milk samples were collected at the sample level, and 187 to 345 were collected at the farm level per year. Sampling methods adhered to the standard recommendations by NMC (1999).

Bacterial Isolation and Identification

Somatic cell count values for the quarter milk samples were measured using a Fossmatic System 4000 (Foss Electric, Hillerød, Denmark) within 1 d of collection. The milk samples with SCC ≥200,000 cells/mL were examined for the presence of mastitis pathogens. The proportion of samples with an SCC >200,000 cells/mL

was 38.9% (1,436/3,692 samples). Milk sample cultures were performed according to recommendations by NMC (1999). Briefly, milk samples (10 µL) were streaked onto blood agar plates (Komed, Gyeonggi, Korea). Suspected colonies were identified using MALDI-TOF MS (Biomerieux, Marcy L'Etoile, France). The CNS isolates were then stored at -80°C until use.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of the CNS isolates was determined using the broth microdilution method according to CLSI (2012) guideline M100-S22 using Sensititer mastitis plates (CMV1AMAF, Trek Diagnostics, Cleveland, OH) containing antibiotics, including ampicillin, cephalothin, ceftiofur, erythromycin, penicillin, oxacillin, penicillin/novobiocin, pirlimycin, tetracycline, and sulfadimethoxine.

Because the concentration range (2–4 µg/mL) of oxacillin in the CMV1AMAF panel did not include the breakpoint (0.5 µg/mL), we additionally evaluated minimal inhibition concentrations by broth microdilution testing using oxacillin (Sigma-Aldrich, St. Louis, MO) at a range of 0.064 to 16 µg/mL according to CLSI (2012) guideline M100-S22. All *mecA*-positive CNS isolates were tested for antimicrobial susceptibility using EUST plates (Sensititer, Trek Diagnostics) to investigate resistance to various antimicrobials, including gentamicin, kanamycin, streptomycin, rifampin, ciprofloxacin, vancomycin, linezolid, trimethoprim, clindamycin, chloramphenicol, mupirocin, quinupristin/dalfopristin, fusidate, and tiamulin. *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212 were used as quality control strains. Minimal inhibition concentration values were interpreted according to CLSI (2012) guideline M100-S22, CLSI (2017b) guideline M100-S27, and CLSI (2017a) guideline VET 06. Multidrug resistance was defined as resistance to 3 or more antimicrobial subclasses.

Table 1. Quarter milk samples collected from dairy farms from 2013 to 2017

Year	No. of farms	No. of cattle	No. of milk samples	No. of samples where SCC ≥200,000 cells/mL
2013	23	238	599	195
2014	13	331	798	230
2015	16	272	1,059	447
2016	14	345	735	360
2017	15	187	501	204
Total	81	1,373	3,692	1,436

Detection of *mec* Genes and *Staphylococcal* Cassette Chromosome *mec* Typing

Presumptive MRCNS colonies were identified by detecting the *mecA* (Mason et al., 2001) and *mecC* (Stegger et al., 2012) genes by PCR. The staphylococcal cassette chromosome *mec* typing of the MRCNS isolates was performed using a multiplex PCR method as described previously (Boye et al., 2007).

Pulsed-Field Gel Electrophoresis and Multilocus Sequence Typing

The MRCNS isolates were further analyzed using pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). Genomic DNA for PFGE was prepared following the procedure reported by McDougal et al. (2003). The PFGE of *Sma*I-digested DNA was performed using a CHEF-Mapper apparatus (Bio-Rad, Hercules, CA). Electrophoresis was performed using 1% SeaKem Gold agarose at a voltage of 6 V/cm for 21 h at 14°C in 0.5× Tris-borate-EDTA buffer (Bioneer, Daejeon, Korea) with pulse time increased from 5 s (initial switch time) to 40 s (final switch time). Similarities of the restriction fragment length polymorphisms were analyzed using Bionumerics software (version 4.0; Applied Maths, Sint-Martens-Latem, Belgium), which produced a dendrogram. Clustering was carried out using the unweighted-pair group method with arithmetic averages based on the Dice similarity index.

The MLST was carried out according to the method described by Thomas et al. (2007) for *S. epidermidis* and Zhang et al. (2013) for *Staphylococcus hominis*. Primers specific for 7 housekeeping genes (*arcC*, *aroE*, *gtr*, *mutS*, *pyrR*, *tpi*, and *yqiL*) for *S. epidermidis* and 6 genes (*arcC*, *gtr*, *glpK*, *pta*, *tpi*, and *tuf*) for *S. hominis* were used for PCR amplification and sequencing (Macrogen, Seoul, Korea). Allele numbers and sequence types were assigned using the MLST website (<http://www.mlst.net>).

Biofilm Production Testing

Biofilm formation was determined using 96-well microtiter plates (SPL, Pocheon, Korea) as described by Felipe et al. (2017). Briefly, a culture of each isolate grown overnight was adjusted to 10^6 cfu/mL. A volume of the suspension (150 µL) was transferred to 3 wells of the microtiter plate and incubated at 37°C for 24 h. Following incubation, wells were washed 3 times with PBS, air dried, and stained with 0.1% crystal violet solution for 30 min at room temperature. Then, the mi-

croter plates were washed with distilled water and air dried, and the adherent biofilm was dissolved in 95% ethanol for 5 min. Biofilm formation was measured at 490 nm in an ELISA reader set (Biotek, Winooski, VT) and expressed in optical density (OD) values. Isolates showing the ability to produce a biofilm were classified as weak [$OD_{negative\ control\ (NC)} < OD \leq 2 \times OD_{NC}$], moderate ($2 \times OD_{NC} < OD \leq 4 \times OD_{NC}$), or strong ($OD > 4 \times OD_{NC}$). Biofilm production for each isolate was determined via 3 separate experiments, and each independent experiment included triplicates for each isolate. Absorbance values greater than $\pm 4 \times$ the standard deviation were rejected.

Statistical Analysis

Quantitative variables were the prevalence of CNS in bovine mastitis milk samples. Qualitative variables were the species groups between 3 major CNS (*Staphylococcus chromogenes*, *Staphylococcus simulans*, and *S. epidermidis*) and other species of CNS from 2013 to 2017. Differences between CNS groups were compared using Student's *t*-test where appropriate for quantitative variables and using Levene's test where appropriate for qualitative variables. A *P*-value of < 0.05 was considered significant. Rex software (version 3.0.3; RexSoft Inc., Seoul, Korea) was used for all analyses.

RESULTS

Prevalence of CNS

A total of 311 CNS were isolated from 1,436 quarter milk samples between 2013 and 2017. Of these, 2 (0.6%) could not be identified at the species level, and the remaining 309 (99.3%) were identified as belonging to 14 different *Staphylococcus* species using MALDI-TOF (Table 2). The predominant species were *S. chromogenes* (34.4%) followed by *S. simulans* (20.3%) and *S. epidermidis* (18.6%). The sum of the 3 most common CNS species accounted for 73.3% of the total isolates found during this study period. No significant differences were found in the distribution of the 3 predominant species of CNS by year except for *Staphylococcus saprophyticus* (15.4%) compared with *S. epidermidis* (7.7%) in 2014 (Table 2).

Antimicrobial Resistance of CNS Isolates

Antimicrobial susceptibility testing for the 311 CNS isolates showed the highest resistance rate against oxacillin (21.2%), followed by penicillin (13.5%), tetracycline

Table 2. Distribution of CNS species isolated from bovine mastitis milk samples from 2013 to 2017

Species	No. (%) of isolates					Total
	2013	2014	2015	2016	2017	
<i>S. chromogenes</i>	14 (25.0)	14 (35.9)	28 (34.6)	35 (34.7)	16 (47.1)	107 (34.4)
<i>S. simulans</i>	9 (16.1)	11 (28.2)	14 (17.3)	23 (22.8)	6 (17.6)	63 (20.3)
<i>S. epidermidis</i>	11 (19.6)	3 (7.7)	19 (23.5)	17 (16.8)	8 (23.5)	58 (18.6)
<i>S. xylosus</i>	9 (16.1)	1 (2.6)	6 (7.4)	5 (5.0)	2 (5.9)	23 (7.4)
<i>S. haemolyticus</i>	3 (5.4)	1 (2.6)	7 (8.6)	7 (6.9)	1 (2.9)	19 (6.1)
<i>S. sciuri</i>	3 (5.4)	0 (0)	1 (1.2)	11 (10.9)	0 (0)	15 (4.8)
<i>S. saprophyticus</i>	1 (1.8)	6 (15.4)	0 (0)	0 (0)	0 (0)	7 (2.3)
<i>S. hominis</i>	1 (1.8)	1 (2.6)	2 (2.5)	1 (1.0)	1 (2.9)	6 (1.9)
<i>S. warneri</i>	1 (1.8)	1 (2.6)	1 (1.2)	0 (0)	0 (0)	3 (1.0)
<i>S. capitis</i>	0 (0)	0 (0)	1 (1.2)	1 (1.0)	0 (0)	2 (0.6)
<i>S. equorum</i>	1 (1.8)	0 (0)	0 (0)	1 (1.0)	0 (0)	2 (0.6)
<i>S. auricularis</i>	0 (0)	1 (2.6)	1 (1.2)	0 (0)	0 (0)	2 (0.6)
<i>S. cohnii</i>	1 (1.8)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.3)
<i>S. vitulinus</i>	1 (1.8)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.3)
Unidentified	1 (1.8)	0 (0)	1 (1.2)	0 (0)	0 (0)	2 (0.6)
Total	56 (100)	39 (100)	81 (100)	101 (100)	34 (100)	311 (100)

(10.3%), ampicillin (8.4%), and pirlimycin (6.8%; Table 3; Supplemental Table S1, <https://doi.org/10.3168/jds.2019-17028>). No resistance to cephalothin was found in this study. Antimicrobial resistance rates varied by CNS species. Resistance of *S. epidermidis* was higher than that of other CNS species; resistance rates of *S. epidermidis* to oxacillin, tetracycline, penicillin, ampicillin, pirlimycin, and erythromycin were 32.8, 25.9, 24.1, 17.2, 17.2, and 13.8%, respectively. Resistance rates for *S. simulans* were minimal, with less than 10.0% resistance to all of the antimicrobials tested—specifically, erythromycin (3.2%), pirlimycin (4.8%), and tetracycline (7.9%). Further, no resistance of *S. simulans* to ampicillin, ceftiofur, cephalothin, oxacillin, penicillin, or sulfadimethoxine was found. Multidrug resistance was found in *S. epidermidis* (5.2%), *S. chromogenes* (2.8%), and other CNS species (7.7%; Table 3; Supplemental Table S1, <https://doi.org/10.3168/jds.2019-17028>). A total of 112 of the 311 isolates (36.0%) were resistant to 1 or more antimicrobials, and the remaining isolates were susceptible to all of the antimicrobials used in this study. Twenty-four resistance patterns were observed in 1 or more of the resistant CNS isolates. The most common combination was resistance to β -lactams and tetracycline (2.6%; Supplemental Table S2, <https://doi.org/10.3168/jds.2019-17028>).

Molecular Characterization of MRCNS

Oxacillin resistance attributed to the *mecA* gene was present in 31.8% (21/66) of the oxacillin-resistant isolates. None of the methicillin-resistant isolates were positive for the *mecC* gene. A total of 21 *mecA*-positive isolates were detected on 7 farms located in 3 provinces.

The 21 *mecA*-positive CNS comprised 18 *S. epidermidis* and 1 each of *Staphylococcus sciuri*, *Staphylococcus equorum*, and *S. hominis*. Two staphylococcal cassette chromosome *mec* types (type I, 4.8%; type IV, 71.4%) were identified using multiplex PCR. The remaining 5 (23.8%) *mecA*-positive isolates were not classified using this method. A total of 18 MRSE showed 3 sequence types (**ST**) (ST59, ST179, and ST640), 5 PFGE types (P1–P5), and 2 staphylococcal cassette chromosome *mec* types (I and IV). Combining these methods produced a total of 7 different clones with 1 predominant clone (P2-IV-ST59, 38.9%). Distribution of molecular clones varied according to the farms. The predominant types found on each farm were P3-IV-ST59 ($n = 4$) on farm A and P2-IV-ST59 ($n = 7$) on farm B. Of note, 2 predominant clones found on farm B were persistent on that farm because 1 cow (no. 360) carried MRSE for 2 yr. All 16 ST59 MRSE isolates were resistant to fewer than 2 antibiotics, whereas the ST179 MRSE isolate was resistant to 8 antimicrobials. Although the majority (76.2%) of MRCNS isolates were weak producers of biofilm, a persistent clone (P2-IV-ST59), which presented in the quarter milk samples of the same cow for 2 yr, was a moderate producer. Furthermore, 1 MRSE ST 179 strain was discovered to be a strong producer (Table 4; Supplemental Figure S1, <https://doi.org/10.3168/jds.2019-17028>).

DISCUSSION

In the present study, we found a high prevalence of *S. chromogenes* and high antimicrobial resistance of *S. epidermidis* in dairy milk. Furthermore, *mecA*-carrying *S. epidermidis* produced biofilms and was persistent on

Table 3. Antimicrobial resistance of CNS isolates from bovine mastitis milk samples

Species (no. of isolates)	Antimicrobial resistance rate (no. of resistant isolates)										Multidrug resistance
	Ampicillin	Ceftiofur	Cephalexin	Erythromycin	Oxacillin	Penicillin	Penicillin/novobiocin	Pirlimycin	Sulfadimethoxine	Tetracycline	
<i>S. chromogenes</i> (n = 107)	9.3 (10)	0 (0)	0 (0)	1.9 (2)	12.1 (13)	16.8 (18)	0 (0)	4.7 (5)	0 (0)	3.7 (4)	2.8 (3)
<i>S. simulans</i> (n = 63)	0 (0)	0 (0)	0 (0)	3.2 (2)	0 (0)	0 (0)	0 (0)	4.8 (3)	0 (0)	7.9 (5)	0 (0)
<i>S. epidermidis</i> (n = 58)	17.2 (10)	0 (0)	0 (0)	13.8 (8)	32.8 (19)	24.1 (14)	0 (0)	17.2 (10)	0 (0)	25.9 (15)	5.2 (3)
<i>S. xyloso</i> (n = 23)	0 (0)	4.3 (1)	0 (0)	4.3 (1)	26.1 (6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
<i>S. haemolyticus</i> (n = 19)	10.5 (2)	0	0 (0)	0 (0)	10.5 (2)	15.8 (3)	0 (0)	0 (0)	5.3 (1)	0 (0)	0 (0)
<i>S. sciuri</i> (n = 15)	6.7 (1)	6.7 (1)	0 (0)	0 (0)	93.3 (14)	6.7 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Other (n = 26)	11.5 (3)	3.8 (1)	0 (0)	3.8 (1)	46.2 (12)	23.1 (6)	3.8 (1)	11.5 (3)	0 (0)	34.6 (9)	7.7 (2)
Total (n = 311)	8.4 (26)	1.0 (3)	0 (0)	4.5 (14)	21.2 (66)	13.5 (42)	0.3 (1)	6.8 (21)	0.3 (1)	10.3 (32)	2.6 (8)

dairy farms. Coagulase-negative staphylococci are considered one of the major pathogens commonly isolated from subclinical mastitis infections throughout the world (Taponen and Pyörälä, 2009; Botrel et al., 2010). In this study, a total of 311 (21.7%) CNS were isolated from 1,436 quarter milk samples with SCC $\geq$ 200,000 cells/mL. A study in the Czech Republic found that 53.5% of quarter milk samples with subclinical bovine mastitis harbored CNS (Cervinkova et al., 2013). In Germany, the ratio of CNS infection was also high for SCC quarter foremilk samples at 17.2% (Schwarz et al., 2010). Studies conducted in the United States found that 35.4% of IMI in dairy heifers were due to CNS (Piepers et al., 2010).

A variety of CNS species were detected in bovine mastitis milk samples in the present study. Three predominant CNS species (*S. chromogenes*, *S. simulans*, and *S. epidermidis*) accounted for 73.3% of the total CNS isolates. Our finding that *S. chromogenes* (34.4%) was the most predominant species was in agreement with findings reported in other countries. In Switzerland, *S. chromogenes* (24.0%) and *S. epidermidis* (22.0%) were the most common CNS isolates causing mastitis (Frey et al., 2013). In the Netherlands, 17 CNS species were isolated from mastitis samples; *S. chromogenes* (35.8%), *S. epidermidis* (9.4%), and *Staphylococcus xyloso* (8.8%) were the most common CNS found (Sampimon et al., 2011). Species-level epidemiological studies conducted in European dairy herds have also shown that *S. chromogenes* is a more specific udder pathogen than the other CNS and tends to cause persistent IMI (Supré et al., 2011).

However, there was a very large difference in the distribution of CNS species between the present study and a previous study (Nam et al., 2010) performed by our team 8 yr ago. *Staphylococcus chromogenes* and *S. simulans* were the most predominant species in the present and previous studies (Moon et al., 2007; Nam et al., 2010), respectively. Furthermore, *S. chromogenes* was not detected in our previous study, possibly due to differences in the identification methods used, sampling schedule, and mastitis control strategies (Piessens et al., 2011). In the present study, CNS isolates were identified using MALDI-TOF, whereas a Vitek2 system (bioMérieux, Hazelwood, MO) was used in the previous study. It is known that commercial phenotypic methods have limited accuracy when identifying CNS of veterinary origin, presumably due to the limited number of veterinary isolates incorporated into the database (Zadoks and Watts, 2009). Although information about the age of sampled cows was not available in both studies, another presumed reason might be the age of sampled cows. *Staphylococcus chromogenes*, the most predominant species in this study, is a major CNS species in

Table 4. Characterization of 21 *mecA* carrying methicillin-resistant CNS isolated from bovine mastitis milk samples

Isolate	Isolation date ¹	Farm ID	Cattle ID	Province	Species	MIC of OXA ² (µg/mL)	Biofilm production	SCC _{mec} ³	ST ⁴	Pulsotype	Non-β-lactam antimicrobial resistance ⁵
13-A-297-4	2013.5	A	297	Chungnam	<i>S. epidermidis</i>	1	Weak	IV	ST59	P3	TET, TMP
13-A-360-2	2013.5	A	A360	Chungnam	<i>S. epidermidis</i>	0.5	Weak	IV	ST59	P3	TET, TMP
13-A-385-3	2013.5	A	385	Chungnam	<i>S. epidermidis</i>	0.5	Weak	IV	ST59	P3	TET, TMP
13-A-399-2	2013.5	A	399	Chungnam	<i>S. epidermidis</i>	0.5	Weak	IV	ST59	P3	TET, TMP
15-B-360-3-3	2015.4	B	B360	Gyeonggi	<i>S. epidermidis</i>	0.5	Moderate	IV	ST59	P2	KAN, TET
15-C-9569-2	2015.4	C	9569	Gyeonggi	<i>S. epidermidis</i>	4	Strong	IV	ST179	P4	CLI, ERY, FUS, GEN, KAN, PRL, TET, TMP
15-G-285-1	2015.5	G	285	Gyeonggi	<i>S. epidermidis</i>	0.5	Weak	I	ST640	P5	FUS, TMP
16-B-518-1	2016.8	B	518	Gyeonggi	<i>S. epidermidis</i>	0.5	Weak	IV	ST59	P2	KAN, TET
16-B-508-3	2016.8	B	508	Gyeonggi	<i>S. epidermidis</i>	0.5	Moderate	NT ⁶	ST59	P1	KAN
16-B-518-1	2016.9	B	518	Gyeonggi	<i>S. epidermidis</i>	0.5	Weak	NT	ST59	P2	KAN
16-B-516-3	2016.10	B	516	Gyeonggi	<i>S. epidermidis</i>	0.5	Weak	IV	ST59	P1	KAN
16-B-548-2	2016.11	B	548	Gyeonggi	<i>S. epidermidis</i>	1	Weak	IV	ST59	P1	KAN, TET
17-B-501-3	2017.1	B	501	Gyeonggi	<i>S. epidermidis</i>	0.5	Weak	IV	ST59	P2	KAN
17-B-360-1	2017.5	B	B360	Gyeonggi	<i>S. epidermidis</i>	0.5	Weak	IV	ST59	P1	KAN, TET
17-B-360-2	2017.5	B	B360	Gyeonggi	<i>S. epidermidis</i>	0.5	Moderate	IV	ST59	P2	KAN, TET
17-B-511-3	2017.5	B	511	Gyeonggi	<i>S. epidermidis</i>	0.5	Weak	IV	ST59	P2	KAN
17-B-511-2	2017.5	B	511	Gyeonggi	<i>S. epidermidis</i>	0.5	Weak	IV	ST59	P2	KAN
17-B-562-2	2017.5	B	562	Gyeonggi	<i>S. epidermidis</i>	0.5	Weak	IV	ST59	P2	KAN
13-F-20	2013.1	F	20	Chungbuk	<i>S. sciuri</i>	4	Moderate	NT	ND ⁷	ND	FUS, KAN
13-E-14477-1	2013.9	E	14477	Chungbuk	<i>S. equorum</i>	1	Weak	NT	ND	ND	CHL, CLI, FUS, P/N, PRL, TMP, ERY, FUS, MUP, PRL
14-D-132-4	2014.4	D	132	Gyeonggi	<i>S. hominis</i>	0.5	Weak	NT	ST15	ND	

¹Date format is year.month.
²Minimum inhibitory concentration of oxacillin.
³Staphylococcal cassette chromosome *mec*.
⁴Sequence type.
⁵TET = tetracycline; TMP = trimethoprim; KAN = kanamycin; CLI = clindamycin; ERY = erythromycin; FUS = fusidate; GEN = gentamicin; PRL = pirlinycin; CHL = chloramphenicol; P/N = penicillin/novobiocin; MUP = mupirocin.
⁶Non-typeable.
⁷Not determined.

precalving heifers and primiparous cows (Trinidad et al., 1990; Taponen et al., 2006; Rajala-Schultz et al., 2009), whereas *S. simulans*, the most predominant species in our previous study, was mostly isolated from cows that generally became infected with CNS during later lactation (Gröhn et al., 2004; Taponen et al., 2007). In addition, control strategies for infected udders on dairy farms, such as type of germicide used for teat dipping and antimicrobials used for treatment, could affect the distribution of CNS species (Hogan et al., 1987). Other currently unknown herd-level factors or environmental conditions also influence the establishment of staphylococcal species in dairy herds.

Antimicrobial treatment has been used as a successful strategy for controlling mastitis caused by CNS species (Feßler et al., 2010). β -Lactams, macrolides, and lincosamides were the antibiotics recommended for the treatment of mastitis caused by CNS species (Pyörälä and Taponen, 2009; Frey et al., 2013; Tark et al., 2017). In the present study, the most common resistance found in all of the CNS species was that against the β -lactams (8.4% in ampicillin, 21.2% in oxacillin, and 13.5% in penicillin). These resistance results were similar to those from 2 previous studies (Frey et al., 2013; Moser et al., 2013). Rate of resistance to penicillin in CNS was 36.4% in Norway (NORM-VET, 2005), 25.0% in Denmark (DANMAP, 2001), 53.0% in the Netherlands (MARAN, 2008), and 23.3% in Sweden (Persson et al., 2011).

The rate of resistance to β -lactams in this study was lower than that in previous studies from Korea (Moon et al., 2007; Nam et al., 2010). Moon et al. (2007) showed that the rates of resistance to penicillin and ampicillin for CNS isolated from 1997 to 2004 were 60.2 and 58.4%, respectively. In addition, Nam et al. (2010) showed that the rates of resistance to 2 antimicrobials were 52.9 and 45.0%, respectively, for CNS isolated from 2003 to 2008. Furthermore, we found very large differences in antimicrobial resistance at the species level between this study and a previous study by our team in some CNS species, such as *S. xylosus* and *S. sciuri*. Comparison of the susceptibility patterns from the present study with those of other studies must be made cautiously because of differences in antimicrobial exposure, susceptibility testing methods, and milk samples used (Erskine et al., 2002). Rate of resistance to most antimicrobials in 2 species was much lower than that in our previous study (Nam et al., 2010), presumably due to a decrease in the sale of antimicrobials to Korean livestock farms, although information on antimicrobial usage was not available for the farms we sampled in this study (APQA, 2017). In addition, according to national statistics, the proportion of dairy farms with SCC of

>200,000 cells/mL of bulk milk dramatically decreased in Korea from more than 68% in 2003 to around 40% in 2017 (MAFRA-KDC, 2018). This may have reduced the necessity of antimicrobials due to reduced mastitis on dairy farms and could lead to a decrease in resistance. Another reason might be the difference in the methods for susceptibility assay between broth microdilution and disc diffusion methods. Furthermore, the change in predominant clones that were susceptible to antimicrobials could be another reason (Chen et al., 2014). Thus, further studies on the molecular typing of isolates and differences in susceptibility testing methods are needed to elucidate the dramatic decrease in the resistance of some CNS species.

In our study, the *mecA* gene was not detected by PCR in 68.2% of MRCNS isolates. This may be attributed to the fact that mechanisms other than expression of the *mecA* gene can cause methicillin resistance (Massidda et al., 1996, 2006; Zhang et al., 2009; Fowoyo and Ogunbanwo, 2017). Interestingly, about 95.5% of the MRCNS strains in this study showed a borderline minimal inhibition concentration range of 0.5 to 1 μ g/mL. The borderline methicillin resistance might be due to their plasmid determinants including overexpression of *blaZ* β -lactamase or encoding of resistance to cadmium or other gene products (Massidda, 1996, 2006). Another possible reason is that primers used in this study could not detect *mecA* alleles due to the diversity of MRCNS *mecA* alleles from animal sources (Zhang et al., 2009).

The *mecA* gene was identified predominantly in *S. epidermidis* (85.7%) in this study. The occurrence of *mecA*-positive *S. epidermidis* from bovine milk samples has been previously reported in Korea (Moon et al., 2007; Park et al., 2013), Switzerland (Frey et al., 2013), Finland (Gindonis et al., 2013), and Germany (Feßler et al., 2010). Moreover, another study found that methicillin-resistant staphylococci could be transmitted from animals to the humans caring for them (Thorberg et al., 2006). For example, the same genotype of *S. epidermidis* was found in isolates from a milkers' skin as well as milk samples in Denmark (Thorberg et al., 2006). Interestingly, ST59, one of the predominant types in this study, was also reported from veterinary staff in animal hospitals in Korea (Moon et al., 2012). In addition, this was one of the dominant types isolated from the community and hospital environment in China (Du et al., 2013) and has also been reported in catheter-related bacteremia and even from healthy volunteers in Belgium (Cherifi et al., 2013).

By the combination of MLST, PFGE, and staphylococcal cassette chromosome *mec* typing results, identical clones were observed in isolates from each farm. For

example, ST59-P2-IV, ST59-P1-IV, and ST59-P3-IV clones were isolated from different cows on the same 2 farms (farm B and farm A). This might suggest that transmission of MRSE isolates occurred within farms.

Furthermore, a single cow (no. 360) on farm B carried an identical clone of MRSE (ST59-P2-IV) for 2 yr. Persistence of *S. epidermidis* infections has also been reported in Sweden (Thorberg et al., 2009) and Norway (Mørk et al., 2012). Biofilm formation by CNS could have contributed to persistence in this cow by promoting and sustaining these microbes on external and internal udder tissues (Sahal and Bilkay, 2014; Felipe et al., 2017). Thus, biofilm production could be an important factor involved in the establishment and maintenance of CNS that cause IMI (Sahal and Bilkay, 2014; Felipe et al., 2017). Moreover, the MRSE ST179 isolate produced the strongest biofilm, which was resistant to multiple antimicrobials. Multidrug-resistant *S. epidermidis* (Du et al., 2013; Sahal and Bilkay, 2014) and *S. aureus* (Cha et al., 2013) from human clinical isolates also showed strong biofilm formation ability. Although a strong biofilm producer clone with multiple drug resistance was detected in only 1 isolate among the *mecA* carrying MRSE, further studies are needed to investigate biofilm production in relation to antimicrobial-resistant CNS isolates including *S. epidermidis* to elucidate the relationship among antimicrobial resistance, biofilm production, and persistence of IMI.

CONCLUSIONS

The CNS isolates exhibited higher antimicrobial resistance rates in this study than in previous studies reported from other countries. Resistance was highest for the β -lactam antimicrobials that were frequently used for gram-positive mastitis treatment at the dairy farms. Further, identical PFGE and ST of *mecA*-positive MRSE with biofilm formation ability were found to persist on the same farm for 3 yr, which suggested that transmission and persistence might be occurring at the farm level. Although the prevalence of MRSE isolates with either multiple resistance or a strong biofilm ability was relatively low in this study, a more extensive examination of other CNS isolates is important to improve mastitis control strategies within Korea. In addition, the presence of human-associated MRSE in dairy cows is of great concern because transmission to humans is inevitable. Thus, hygienic management programs designed to reduce the incidence of mastitis combined with strategic antimicrobial use plans for dairy farms are required immediately to reduce future emergence of resistant bacteria.

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