Phenotypic and Genetic Antibigram of Methicillin-Resistant *Staphylococci* Isolated from Bovine Mastitis in Korea

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ABSTRACT

Staphylococcus aureus belongs to the group of major contagious mastitis pathogens, whereas the coagulase-negative staphylococci (CNS) are also capable of causing opportunistic bovine mastitis. Many of these strains are resistant to penicillin or ampicillin because of the long-term use of β -lactam antibiotics in agricultural and healthcare settings. Based on the simple and highly specific coagulase genotyping by PCR-RFLP used for discriminating among *Staph. aureus* strains, the relationship between phenotypic antibiogram and the polymorphism of coagulase gene was determined in this study. The staphylococci strains (835 *Staph. aureus* and 763 CNS) were isolated from 3,047 bovine mastitic milk samples from 153 dairy farms in 8 provinces from 1997 to 2004 in the Republic of Korea. Twenty-one (2.5%) *Staph. aureus* and 19 (2.4%) CNS strains were resistant to methicillin [oxacillin minimum inhibitory concentration (MIC) ≥ 4 $\mu\text{g/mL}$]. The *mecA* gene was also found in 13 methicillin-resistant *Staph. aureus* (MRSA) and 12 methicillin-resistant CNS (MRCNS) isolates with a significantly higher detection rate of the *mecA* gene in MRSA with high MIC (≥ 16 $\mu\text{g/mL}$) compared with those with MIC ≤ 8 $\mu\text{g/mL}$. Methicillin-resistant *Staph. aureus* and MRCNS were also more resistant to other antibiotics (ampicillin, cephalothin, kanamycin, and gentamicin) than methicillin-susceptible staphylococci. Among 10 different *coa* PCR-RFLP patterns (A to J) in 706 *Staph. aureus* strains, the main types were A (26.9%), B (17.0%), G (10.5%), and H (15.4%), with the frequent observation of the A and H types (6 and 10 isolates) in MRSA. This study indicates that major epidemic *Staph. aureus* clones may be spread between different dairy farms, and the profile of *coa* genotype can be applied for epidemiological investigations and

control of bovine mastitis, particularly one caused by MRSA with specific prevalent *coa* types.

Key words: bovine mastitis, staphylococci, methicillin resistance, genotyping

INTRODUCTION

Although *Staphylococcus aureus* is clearly the primary pathogen, CNS are also considered opportunistic pathogens that cause conditions such as skin inflammation in humans and animals (Schalm et al., 1971; Harmon et al., 1990). The increasing resistance of staphylococci to β -lactam antibiotics has become a major clinical problem (Roberson et al., 1992; Aarestrup et al., 1995). Staphylococcal isolates are frequently resistant to penicillinase-resistant penicillins (Ogawa, 1981). Organisms exhibiting this type of resistance are referred to as methicillin (oxacillin)-resistant staphylococci (MRS; Mandell et al., 1995).

Resistance to methicillin was first described for *Staph. aureus* in 1960, shortly after the introduction of the drug into clinical practice in hospitals (Jevons, 1961). Since then, methicillin-resistant *Staph. aureus* (MRSA) has gradually disseminated and began causing serious nosocomial infections worldwide in the 1970s (Locksley et al., 1982). By the mid-1990s, MRSA had become a major problem because the strains generally exhibited multiple resistance to tetracyclines, aminoglycosides, macrolides, lincosamides, and some other antimicrobial drugs, and has continued to spread through new communities (Mandell et al., 1995; Voss and Doebbeling, 1995; Ayliffe, 1997). Methicillin-resistant *Staph. aureus* has become widespread in Korea. The rate of methicillin resistance among *Staph. aureus* isolates from humans in Korea is greater than 50% (Lee et al., 2001).

The isolation of MRSA from animals was first reported in 1972 following its detection in milk from mastitic cows (Devriese and Vandamme, 1972). Since then, reports have been published on MRSA infection in domestic animals, including dogs, cats, cattle, sheep,

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chickens, rabbits, and horses (Devriese and Hommez, 1975; Hartmann et al., 1997; Pak et al., 1999; Tomlin et al., 1999; Lee, 2003; Goni et al., 2004). In recent years, the number of cases has shown an increasing trend (Rich and Roberts, 2004; O'Mahony et al., 2005; Weese et al., 2005).

Staphylococcus aureus remains one of the most significant organisms associated with clinical and subclinical bovine mastitis worldwide. It is believed that these infections respond poorly to therapy with antimicrobial agents, whether given parenterally or via the intramammary route (Sutra and Poutrel, 1990; Vintov et al., 2003). The occurrence and transmission of antimicrobial-resistant *Staph. aureus* itself or its gene has been suggested as one of the reasons for difficulty in antibiotic therapy (Goh et al., 1992; Kreiswirth et al., 1993). Therefore, the determination of susceptibility or resistance of strains to antibiotics is very important from a clinical and economic point of view. Moreover, the public health of this issue is of great importance because antibiotic therapy of infectious diseases in animals poses the risk of selection of resistant strains and introduction of these strains into the food chain (White and McDermott, 2001; Lee, 2003).

β -Lactam antibiotics are frequently used in intramammary infusion therapy. Bacterial β -lactam resistance mechanisms include production of β -lactamases and low-affinity penicillin-binding protein 2a (**PBP2a**). The latter, designated for methicillin resistance, precludes therapy with any of the currently available β -lactam antibiotics, and may predict resistance to several classes of antibiotics other than β -lactams (Odd and Maeland, 1997; CLSI, 2002).

Goh et al. (1992) successfully traced the source of a nosocomial infection in a local hospital in Vancouver, Canada, by PCR amplification of the variable region of the coagulase gene, followed by *AluI* restriction and analysis of RFLP. More recently, the observation of a coagulase gene polymorphism and the presence of certain clonal types were also found by random amplification of polymorphic DNA PCR typing of 75 isolates of *Staph. aureus* from several geographical areas in the United States (Matthews et al., 1994) and PCR-coagulase gene typing of 187 isolates, each from a different dairy farm in Denmark (Aarestrup et al., 1995). Additionally, Hookey et al. (1998, 1999) described a coagulase gene-based PCR-RFLP technique that could differentiate the major and minor epidemic MRSA strains from humans in England.

However, only limited information is available regarding the relationship between coagulase genotypes and antimicrobial resistance of *Staph. aureus* isolated from bovine mastitis. Therefore, we investigated the distribution of MRSA and methicillin-resistant CNS

(**MRCNS**), and polymorphisms of the coagulase gene by PCR-RFLP of *Staph. aureus* isolated from bovine mastitic milk samples in Korean dairy herds.

MATERIALS AND METHODS

Herd Selection and Milk Sampling

Using aseptic techniques, 14,688 milk samples were collected from individual quarters of cows from 153 herds located across the nation. Among them, 3,047 milk samples with problems of high bulk tank milk SCC (>200,000 cells/mL) were further examined for this study from August 1997 to December 2004, in Korea. Somatic cell counts were measured by using a Milko-Scan 4000 (Foss Electric Co., Hillerød, Denmark).

Strains and Identification of Staphylococci

Staphylococcal strains were isolated from milk samples in dairy herds by methods developed according to the protocols of the National Mastitis Council (Harmon et al., 1990). An aliquot of 10 μ L of each sample was spread over blood agar plates (Promed, Ansan, Korea) containing 5% washed sheep erythrocytes and incubated at 37°C for 24 h. Colonies suspected of being staphylococci were subcultured on blood agar plates, and the isolation and identification of *Staph. aureus* and CNS were performed according to the methods of Roberson et al. (1992) as follows: gram staining, coagulase test, Baird-Parker medium culture test, DNase test, Voges-Proskauer test, mannitol fermentation test, and Vitek GPI card (bioMérieux, Lyon, France). The staphylococci isolates were kept frozen (−70°C) prior to use. Reference strains used for quality control of MRSA, methicillin-susceptible *Staph. aureus* (**MSSA**), and CNS were as follows: *Staph. aureus* ATCC 43300, *Staph. aureus* ATCC 25923, and *Staphylococcus epidermidis* ATCC 12228, respectively.

Antibiotic Susceptibility Test

The MIC test for oxacillin resistance and the disk diffusion test for other antibiotics were performed and interpreted as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2002; formerly the National Committee for Clinical Laboratory Standards). The antibiotic susceptibility test was performed using the disk diffusion technique on Mueller-Hinton agar (Difco, Sparks, MD). Commercially available disks of the antimicrobial drugs (BBL, Sparks, MD) that have been used most frequently for the treatment of bovine mastitis in Korean dairy herds were selected and tested in this study on the basis of data on annual products of antibiotic used for intramammary infusion provided

by Korea Animal Health Products Association (<http://www.kahpa.or.kr>). Ampicillin, cephalosporins, gentamicin, neomycin, and norfloxacin are the most commonly used antibiotics for intramammary infusion in lactating cows in Korean dairy herds. Also, antimicrobial dry cow therapy has been routinely used in all cows in the majority of dairy herds. Dry cow therapy usually consists of cloxacillin and cephalosporins used alone or in combination with other agents such as neomycin or norfloxacin.

The plates were incubated at 30°C for methicillin (5 µg/disk) and 37°C for other antibiotics, including penicillin G (10 units/disk), ampicillin (10 µg/disk), cephalothin (30 µg/disk), kanamycin (30 µg/disk), gentamicin (10 µg/disk), amikacin (30 µg/disk), tetracycline (30 µg/disk), and erythromycin (15 µg/disk). Methicillin resistance of staphylococci was screened by disk diffusion test with an oxacillin disk on Mueller-Hinton agar and confirmed by MIC test with oxacillin. Phenotypic methicillin resistance was defined with an oxacillin MIC ≥ 4 µg/mL. Additionally, the susceptibility of methicillin-resistant staphylococci to amoxicillin-clavulanic acid (20 and 10 µg/disk) was tested to identify the β -lactamase hyper-producing strain. The antibiotic susceptibility test of these strains to vancomycin (1 µg/disk), clindamycin (2 µg/disk), and trimethoprim-sulfamethoxazole (1.25/23.75 µg/disk) was also performed. *Staphylococcus aureus* ATCC 25923 was used as a quality control reference strain.

Preparation of Whole-Cell DNA for PCR

In this study, whole cellular DNA was isolated as described by Hookey et al. (1998) with some modifications. Cells grown in 1.5 mL of brain heart infusion broth (BHI, Difco) at 37°C for 24 h were harvested, centrifuged at $16,000 \times g$ for 3 min, washed twice with 0.7 mL of Tris-EDTA buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0), and resuspended in Tris-EDTA buffer. The cells were added to 30 µL of 20% SDS (Sigma, St. Louis, MO), 5 µL of proteinase K (20 mg/mL, Promega Corp., Madison, WI), and 50 µL of lysozyme (200 µg/mL, Sigma), and were then mixed and incubated at 37°C for 90 min. After 100 µL of cetyltrimethylammonium bromide/NaCl (10% wt/vol, 0.7% NaCl, Sigma) was added with mixing and incubated at 65°C for 20 min, the mixture was centrifuged at $12,000 \times g$ for 15 min (4°C). The lysate was extracted with equal volumes of phenol:chloroform:isoamylalcohol (25:24:1, Sigma) and centrifuged at $12,000 \times g$ for 15 min (4°C). The supernatant in aqueous phase was added to 1 vol of chloroform:isoamylalcohol (24:1, Sigma) and centrifuged at $12,000 \times g$ for 15 min (4°C). The DNA was precipitated from the aqueous phase with 2 to 3 vol of isoamylalcohol

at -70°C overnight. After centrifugation, the pellet was washed with 70% ethanol, dried with a vacuum drier, and resuspended in 30 µL of Tris-EDTA buffer (Sigma). The DNA concentration was determined by UV spectrophotometry (Bio-Rad Laboratories Inc, Hercules, CA) at A_{260} , and the extract was stored at 4°C. Approximately 50 to 100 ng of DNA was taken for PCR amplification.

PCR Assay for Detection of *coa* and *mecA* Genes

The presence of the *mecA* gene encoding PBP2a of CNS was verified by a PCR assay used by Weller (1999) as follows: amplification of the *mecA* gene was performed using the primers *mec* A1 (5'-AAAATCGATGGTAAAGGT TGGC-3') and *mec* A2 (5'-AGTTCTGCAGTACCGGATTTGC-3'), yielding a PCR product of 533 bp. The 2 sets of primers for detection of the *coa* gene (encoding staphylococcal coagulase) and *mecA* gene of *Staph. aureus* by multiplex PCR assay have been described previously by Kearns et al. (1999). Primers of *mec* A1 (5'-CGGTAACATTGATCGCAACGTTCA-3') and *mec* A2 (5'-CTTTGGAACGATGCCTAATCTCAT-3') amplified a 214-bp fragment of the *mecA* gene. Primers *coa* 1 (5'-GTAGATTGGGCAATTACATTTTGAGAG-3') and *coa* 2 (5'-CGCATCAGCTTTGTTATCCCATGTA-3') amplified a 214-bp fragment of the *coa* gene. Positive and negative controls (*Staph. aureus* ATCC 43300, *Staph. aureus* ATCC 25923, and *Staph. epidermidis* ATCC 12228) were included with each run for *mecA* and *coa* gene amplification.

For multiplex PCR amplification, 1 µL of DNA sample was added to 49 µL of a PCR mixture containing 4 mM MgCl₂, 200 µM deoxynucleotide triphosphate, 15 pmol of each primer, and 1 unit of *Taq* DNA polymerase. The thermocycling conditions were as follows: predenaturation at 94°C for 1 min for 1 cycle, followed by 15 cycles of denaturation at 94°C for 30 s, annealing at 68°C for 30 s, and extension at 72°C for 30 s in a thermal cycler (PerkinElmer 2400, Warrington, UK). Thereafter, 20 further cycles of amplification were completed as above, but using an annealing temperature of 60°C. The reaction was completed with a final extension at 72°C for 2 min. Amplified products were separated by electrophoresis at 110 V for 30 min through 3.0% agarose gel and visualized under UV light.

Analysis of *coa* Gene by PCR-RFLP of *Staph. aureus*

Primers for analysis of the *coa* gene by PCR-RFLP of *Staph. aureus* were used as described by Kaida et al. (1989). The forward primer, *Coa*-1 (5'-ATAGAA ATGCTGGTACAGG-3'; MRSA 213, Accession No. X16457;

Table 1. Isolation of *Staphylococcus aureus* from mastitic milk of dairy cows from 1997 to 2004 in several provinces in Korea

Year	No. of herds with <i>Staph. aureus</i> isolated	Province					Total <i>Staph. aureus</i> isolates	
		Gyeonggi	Gangwon	Chung- cheong	Jeolla	Gyeong- sang		Total MRSA
No. of isolates (%) per year								
1997–1998	30	43 (26.1)	6 (3.6)	98 (59.4)	14 (8.5)	4 (2.4)	0 (0)	165 (100)
1999–2000	52	99 (50.8)	11 (5.6)	50 (25.6)	29 (14.9)	6 (3.1)	17 (8.7)	195 (100)
2001–2002	16	24 (68.6)	0 (0)	2 (5.7)	8 (22.9)	1 (2.9)	0 (0)	35 (100)
2003–2004	55	196 (44.5)	92 (20.9)	151 (34.3)	0 (0)	1 (0.1)	4 (0.9)	440 (100)
Total	153	362 (43.4)	109 (13.1)	301 (36.0)	51 (6.1)	12 (1.4)	21 ¹ (2.5)	835 (100)

¹21 methicillin-resistant *Staph. aureus* (MRSA) strains were isolated from 11 herds located nationwide.

1513–1531), and the reverse primer, *Coa*-2 (5'-GCTTCCGATTGTTTCG ATGC-3'; 2188–2168) were used. In this study, PCR was performed under the conditions described by Hookey et al. (1998) with some modifications: PCR was performed in a 50- μ L volume with 10 $\times$ PCR buffer (Promega) containing 100 mM Tris-HCl (pH 8.3), 50 mM KCl, 6 μ L of MgCl₂ (25 mM, Promega), 400 μ M deoxynucleoside triphosphate mixture (Promega), 2.5 units of *Taq* polymerase (Promega), 1 μ L of each primer (100 pmol/ μ L), 2.5 μ L of dimethyl sulfoxide, and 100 ng of DNA template. Finally, PCR-quality water (Sigma) was added to make a final volume of 50 μ L. Amplification of DNA was performed using the following cycling parameters: predenaturation at 94°C for 5 min for 1 cycle, and then denaturation at 94°C for 20 s, annealing at 58°C, and extension at 70°C for 15 s for 35 cycles on a thermal cycler (PerkinElmer 2400). A 7-min extension at 72°C for 15 s was included at the end of final cycle. After electrophoresis, the gel was stained with ethidium bromide and the amplicons were visualized. The PCR product (10 μ L) was incubated with 2 units of *Alu*I restriction endonucleases (Promega) for 1 h at 37°C according to the instructions of the manufacturer. The resulting fragments were electrophoresed in 2.75% agarose gel at 110 V for 1 h, stained with ethidium bromide (0.5 μ g/mL), and visualized under UV light.

Statistical Analyses

The Mantel-Haenszel χ^2 test and Fisher's exact test were used to examine differences between the observed antibiotic susceptibility percentage and the detection rate of the *mecA* gene for staphylococci with different methicillin and oxacillin susceptibilities. The statistical analyses were performed using the Analyze-it program (Analyze-it Software Ltd., Leeds, UK).

RESULTS

We isolated staphylococci from bovine mastitic milk samples from 153 dairy farms located across Korea. With confirmation by Vitek GPI card, a total of 835 *Staph. aureus* and 763 CNS strains were isolated and identified (Tables 1 and 2). When isolates with oxacillin MIC ≥ 4 μ g/mL were classified as being methicillin-resistant, 21 (2.8%) *Staph. aureus* and 19 (2.4%) CNS strains were resistant to methicillin, respectively (Tables 1 and 2). The level of oxacillin resistance of these isolates ranged between 4 and 256 μ g/mL, and many isolates (20 *Staph. aureus* and 17 CNS) were resistant to 4 to 64 μ g of oxacillin/mL in the initial culture. Among 153 dairy farms sampled over 7 yr, 21 MRSA isolates were obtained from 11 herds located in Gyeonggi, Chungcheong, Jeolla, and Gangwon provinces, but was not detected in Gyeongsang province.

By multiplex PCR, we determined the 214- and 117-bp PCR products of the *mecA* gene and the *coa* gene, respectively (Figure 1). All of the *Staph. aureus* isolates including MRSA and MSSA harbored the genes encoding staphylococcal coagulase. The *mecA* gene was found in 13 of 21 MRSA strains and 12 of 19 MRCNS strains (Table 3). Moreover, the detection rate of the *mecA* gene in MRSA with MIC ≥ 16 μ g/mL was higher than in MRSA with MIC ≤ 8 μ g/mL ($P < 0.05$). The MIC for the 8 *mecA*-negative *Staph. aureus* were all less than 32 μ g/mL. However, in the case of 19 MRCNS strains, the *mecA* gene was not detected in 2 isolates at high MIC (32 μ g/mL) and there was no significant difference in the detection rate of the *mecA* gene among MRCNS strains with different MIC.

We compared the 8 types of antibiogram of MRSA and MSSA isolates in this study (Table 4). Most isolates were resistant to penicillin and ampicillin, but susceptible to amikacin, erythromycin, gentamicin, cephalothin, kanamycin, and tetracycline. Additionally, the MIC of vancomycin for the MRSA isolates was less than

Table 2. Isolation of CNS strains from mastitic milk of dairy cows in Korea

Species	1999–2000	2003	2004	Total (MRCNS/all CNS) ¹
	No. of CNS isolates and distribution rate (%) per year			
<i>Staphylococcus simulans</i>	20 (24.7)	126 (31.5)	102 (36.1)	4/248 (1.6)
<i>Staphylococcus haemolyticus</i>	0 (0)	83 (20.7)	70 (24.8)	0/153 (0)
<i>Staphylococcus epidermidis</i>	22 (27.2)	7 (1.8)	13 (4.6)	4/42 (9.5)
<i>Staphylococcus sciuri</i>	0 (0)	124 (31.0)	19 (6.7)	3/143 (2.1)
<i>Staphylococcus xylosus</i>	8 (9.9)	18 (4.5)	39 (13.8)	1/65 (1.5)
<i>Staphylococcus saprophyticus</i>	13 (16.0)	6 (1.5)	15 (5.3)	5/34 (14.7)
<i>Staphylococcus warneri</i>	0 (0)	10 (2.5)	10 (3.5)	0/20 (0)
<i>Staphylococcus hominis</i>	7 (8.6)	6 (1.5)	7 (2.5)	1/20 (5.0)
<i>Staphylococcus intermedius</i>	11(13.6)	0 (0)	0 (0)	1/11 (9.0)
Others	0 (0)	20 (5.0)	7 (2.5)	0/27 (0)
Total	81 (100)	400 (100)	282 (100)	19/763 (2.5)

¹MRCNS = methicillin-resistant CNS.

2 µg/mL. The percentage of MRSA isolates resistant to ampicillin, cephalothin, kanamycin, gentamicin, and erythromycin was significantly higher ($P < 0.05$) than that of MSSA isolates. The MRCNS isolates were significantly more resistant ($P < 0.05$) to penicillin, ampicillin, cephalothin, kanamycin, gentamicin, and tetracycline compared with the MSSA isolates.

Among all of the MRSA isolates ($n = 21$), 15 strains were susceptible to amoxicillin-clavulanate, but all MRCNS isolates were susceptible to amoxicillin-clavulanate. The MRSA and MRCNS isolates were highly resistant to penicillin (90.5 and 100%) and ampicillin (90.5 and 89.5%), whereas all the MRSA and MRCNS isolates were susceptible to vancomycin, amikacin, and

trimethoprim-sulfamethoxazole. The overall resistance to kanamycin (47.6 and 78.9%), gentamicin (33.3 and 31.6%), tetracycline (23.8 and 57.9%), erythromycin (57.1 and 15.7%), and clindamycin (14.3 and 21.1%) was observed among the MRSA and MRCNS isolates tested, respectively. Except for 2 MRSA isolates (one had resistance to gentamicin, kanamycin, and erythromycin; the other had resistance to gentamicin, clindamycin, and erythromycin) the MRSA and MRCNS isolates were not multiresistant to more than 3 of the non- β -lactam antimicrobial agents tested.

Amplification of the 3' end of the *coa* gene and subsequent agarose gel analysis of the PCR amplification products showed a single band between 620 and 809 bp for each of the 706 *Staph. aureus* isolates. Restriction enzyme digestion by *AluI* generated 10 different coagulase gene RFLP patterns (A to J; Figure 2). The distribution of PCR-RFLP patterns of *Staph. aureus* isolated in this study according to the location of origin is summarized in Table 5.

DISCUSSION

Mastitis is the single most common reason for antimicrobial drug use in dairy herds, because antibiotic therapy is a major component and a primary tool for mastitis control in lactating and dry cows (Rajala-Schultz et al., 2004). The development of antibiotics for treatment of bacterial infection has led to improvements in health and elongation of life (Voss and Doebbeling, 1995). However, improper use of antibiotics creates problems such as the emergence of bacterial resistance to antibiotics (Saroglou et al., 1980; Chambers, 1997). One of these problems, the occurrence of methicillin resistance, has been observed frequently in recent years (Kreiswirth et al., 1993; O'Mahony et al., 2005). Therefore, infections by MRS require rapid and accurate diagnosis for

Figure 1. Detection of coagulase gene (*coa*) and *mecA* gene by multiplex PCR from methicillin-resistant *Staphylococcus aureus* (MRSA). Lane 1 = 100-bp molecular weight ladder; lanes 2 to 7 = isolated MRSA; lane 8 = *Staph. aureus* ATCC 43300 (MRSA); lane 9 = isolated methicillin-susceptible *Staph. aureus* (MSSA); lane 10 = *Staph. aureus* ATCC 25923 (MSSA).

Table 3. Characteristics of methicillin-resistant *Staphylococcus aureus* (MRSA) and CNS (MRCNS) isolated from bovine mastitis milk¹

			MIC of oxacillin (µg/mL)							Total, no. (%)
			≤4	8	16	32	64	128	256	
MRSA	No. of isolates		4	4	6	2	3	1	1	21 (100)
	<i>mecA</i> gene	Positive	1	1	5	1	3	1	1	13 (61.9)
		Negative	3	3	1	1	0	0	0	8 (38.1)
MRCNS	No. of isolates		2	4	4	3	2	1	2	19 (100)
	<i>mecA</i> gene	Positive	1	2	3	2	2	1	1	12 (63.2)
		Negative	1	2	1	1	0	0	2	7 (36.8)

¹When analyzed by Fisher's exact test, the detection rate of *mecA* gene in MRSA with MIC ≥16 µg/mL was higher than those with MIC ≤8 µg/mL ($P < 0.05$). However, there was no significant difference in the detection rate of *mecA* gene between MRCNS strains with different MIC.

elimination at an early stage, because these strains can cause severe damage to infected sites and may be widespread in the environment (Lee et al., 2004). In most routine microbiological settings, the detection of methicillin resistance among staphylococcal isolates is based on phenotypic assays such as the disk diffusion test and MIC determination. Genetic confirmation of positive findings based on detection of the *mecA* gene has also been reported (Udo et al., 1996).

In the present study, 835 *Staph. aureus* and 763 CNS were isolated from IMI in cows. *Staphylococcus simulans*, *Staphylococcus haemolyticus*, *Staphylococcus sciuri*, *Staphylococcus xylosus*, *Staph. epidermidis*, and *Staphylococcus saprophyticus* isolates were identified by using biochemical tests. These results agree somewhat with earlier studies in which *Staph. simulans*, *Staphylococcus chromogenes*, *Staph. epidermidis*, and *Staph. haemolyticus* were found to be the commonly isolated CNS species from bovine mastitis milk (Rajala-Schultz et al., 2004; Taponen et al., 2006). The CNS species have been isolated mostly from the udders of unbred, pregnant, or freshly calved heifers, the milker's hand, or the environment of farms (Schalm et al., 1971; Harmon et al., 1990; Rajala-Schultz et al., 2004).

The spread of MRSA and CNS isolates is of great concern in hospitals and other healthcare settings. About 80 to 90% of CNS isolated from patients in hospi-

tals and 30 to 40% of isolates obtained from healthy carriers in the community were MRCNS (Silva et al., 2001). The *mecA* gene encoding methicillin resistance is widely disseminated among various *Staphylococcus* species. This widespread distribution of *mecA* might be due to the horizontal transmission between CNS isolates and *Staph. aureus* (Archer and Niemeyer, 1994).

Only a few studies have reported the isolation of MRS from bovine mastitis (Kwon et al., 2005). In this study, 2.8% of *Staph. aureus* and 2.4% of CNS strains showed methicillin resistance. Of CNS species from bovine mastitis, 5 isolates of *Staphylococcus saprophyticus*, 4 isolates of *Staphylococcus simulans*, 4 isolates of *Staph. epidermidis*, 3 isolates of *Staphylococcus sciuri*, and 1 isolate each of *Staphylococcus xylosus*, *Staphylococcus intermedius*, and *Staphylococcus hominis* were found to be methicillin-resistant. This is a lower resistance rate than in the case of bacteria isolated from nosocomial infections, which showed greater than 50% methicillin resistance in Korea (Lee et al., 2001). Additionally, on most Korean dairy farms, the infected cows with *Staph. aureus* would be rapidly culled or treated early with antimicrobial dry cow therapy to prevent the transmission to healthy cows and to control the low SCC of the bulk tank milk. Korean dairy farmers also keep their animals for only a few lactations (2.4) on

Table 4. Comparison of differences in antibiotic susceptibility¹ between methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staph. aureus* (MSSA), and between resistant (MRCNS) and susceptible CNS (MSCNS) isolated from bovine mastitis milk

			Antibiotic							
Group	No. of total isolates (%)		P	AM	CF	K	GM	AN	TE	E
			No. of antibiotic-resistant isolates (%)							
<i>Staphylococcus aureus</i>	MRSA	21 (100)	19 (90.5)	19 (90.5)	4 (19.0)	10 (47.6)	7 (33.3)	0 (0)	5 (23.8)	12 (57.1)
	MSSA	602 (100)	486 (80.7)	368 (61.1)	19 (3.2)	135 (22.4)	90 (14.9)	23 (3.8)	57 (9.4)	53 (8.8)
CNS	MRCNS	19 (100)	19 (100)	17 (89.5)	15 (78.9)	15 (78.9)	6 (31.6)	0 (0)	11 (57.9)	3 (15.7)
	MSCNS	735 (100)	435 (59.2)	424 (57.7)	35 (47.6)	132 (18.0)	75 (10.2)	14 (1.9)	144 (19.6)	61 (8.3)

¹P = penicillin G 10 U; AM = ampicillin, 10 µg; CF = cephalothin, 30 µg; K = kanamycin, 30 µg; GM = gentamicin, 10 µg; AN = amikacin, 30 µg; TE = tetracycline, 30 µg; E = erythromycin, 15 µg (Becton, Dickinson and Company, Sparks, MD).

Figure 2. The coagulase gene of *Staphylococcus aureus* was amplified and digested with *AluI* restriction enzyme. Lane 1 = pGEM DNA marker (Promega Corp, Madison, WI); lanes 2 to 11 = PCR-RFLP patterns A to J, respectively.

average. It seems that MRSA were transferred from humans to cows first and divergence in MRSA may have occurred in cattle strains after transfer (Lee, 2003).

Antibiotics belonging to the macrolide (erythromycin), lincosamide (clindamycin), and streptogramin classes are widely used in the treatment of staphylococcal infections in human patients. As a result of this widespread use, the level of resistance in these bacteria is rising rapidly (Ardic et al., 2005). The important feature of MRSA is that they generally exhibit multiple resistances to tetracyclines, aminoglycosides (gentamicin), macrolides, lincosamides, and some other antimicrobial drug communities (Voss and Doebbeling, 1995; Ardic et al., 2005). In a recent report in Korea, MRSA isolates originated from a Korean hospital (Cha et al., 2005) were resistant to erythromycin (98%), tobramycin (96%), gentamicin (89%), and tetracycline (85%), and trimethprim-sulfamethoxazole (37%), whereas most of MRSA isolates were susceptible to chloramphenicol (98%) and rifampin (96%). Additionally, most of MRSA isolates belonging to ST5 (sequence type 5) from Japan (Ko et al., 2005) and the United States (Chung et al., 2004) were susceptible to trimethprim-sulfamethoxazole.

A US study (Seguin et al., 1999) suggested that veterinary hospital staff were the primary source of infection although the mode of transmission was unclear, whereas a Canadian study (Weese et al., 2005) concluded that environmental contamination played a significant role, in particular via contamination of stalls housing MRSA-positive horses. Additionally, O'Mahony et al. (2005) have shown that transmission of 2 strains of MRSA occurred in veterinary practices in

Table 5. Distribution of PCR-RFLP types of *Staphylococcus aureus* isolated from mastitic milk of dairy cows in the Republic of Korea

Province	Strain ¹	PCR-RFLP type										No. of isolates
		A	B	C	D	E	F	G	H	I	J	
Gyeonggi	MRSA	2	0	0	0	0	0	0	6	0	0	8
	MSSA	88	88	5	9	32	4	16	34	29	9	315
Chungcheong	MRSA	3	0	1	0	0	0	2	2	0	0	8
	MSSA	60	22	51	26	5	31	42	35	0	9	272
Jeolla	MRSA	0	1	0	0	0	0	0	1	0	0	2
	MSSA	15	2	4	3	1	0	13	11	0	0	49
Gangwon	MRSA	1	0	0	0	1	0	0	1	0	0	3
	MSSA	12	7	0	0	0	0	0	18	0	0	37
Gyeongsang	MRSA	0	0	0	0	0	0	0	0	0	0	0
	MSSA	8	0	1	0	0	1	1	1	0	0	12
Total	MRSA (%)	6 (28.6)	1 (4.8)	1 (4.8)	0 (0)	1 (4.8)	0 (0)	2 (9.5)	10 (47.6)	0 (0)	0 (0)	21 (100)
	MSSA (%)	184 (26.9)	119 (17.4)	61 (8.9)	38 (5.5)	38 (5.5)	36 (5.3)	72 (10.5)	99 (14.5)	29 (4.2)	9 (13.1)	685 (100)

¹MRSA = methicillin-resistant *Staph. aureus*; MSSA = methicillin-susceptible *Staph. aureus*.

Ireland and that 1 strain may have arisen from human hospitals.

In this study, the presence of the *mecA* gene was investigated by PCR and multiplex PCR methods. The positive detection rates of *mecA* in MRSA and MRCNS were 61.9 and 63.2%, respectively. Despite the standardized recommendations for the susceptibility testing of MRS given by the CLSI (2002), many of the isolates in this study that did not carry *mecA* were phenotypically resistant to methicillin according to the oxacillin agar screen test and MIC determination. These strains appeared to be β -lactamase hyper-producing strains because they remained susceptible to amoxicillin-clavulanate. The phenotypic expression of resistance can vary depending on the growth conditions (e.g., the temperature or osmolarity of the medium), making susceptibility testing of MRS by standard microbiological methods potentially difficult (Chambers, 1997). The multiplex PCR method detecting the *coa* and *mecA* genes from staphylococci isolated in this study takes approximately 1.5 h and provides a definitive answer for the presence of the *mecA* gene, whereas the phenotypic tests do not. Otherwise, strains that are phenotypically resistant to oxacillin but are *mecA* negative can be easily identified by their susceptibility to amoxicillin-clavulanate.

Antimicrobial use in both animals and humans needs to be ascertained because it must be considered when interpreting antibiogram resistogram typing results (O'Mahony et al., 2005). We performed the antibiotic susceptibility test with staphylococci isolated in this study using the 8 antimicrobial drugs used most frequently for the treatment of bovine mastitis in Korean dairy herds. Most isolates were susceptible to cephalothin, gentamicin, kanamicin, amikacin, erythromycin, and tetracycline, but were less susceptible to penicillin and ampicillin. We also compared differences in antibiotic susceptibility between methicillin-resistant and methicillin-susceptible staphylococci isolates. The percentage of MRSA and MRCNS isolates with antibiotic resistance was significantly higher than that of MSSA and methicillin-susceptible CNS strains ($P < 0.05$).

Such profiles of antibiotic resistance occur frequently in many of the MRSA isolates from other countries (Saroglou et al., 1980; Voss and Doebbeling, 1995; Hsueh et al., 1999; Melter et al., 1999; Seguin et al., 1999). However, the isolates from this study were susceptible to amikacin. In this study, the high resistance of *Staph. aureus* to penicillin (81.1%) is in agreement with results reported by Aarestrup et al. (1995). In their study, 75% of the strains isolated from bovine mastitis in Denmark were resistant to penicillin. The increasing resistance of *Staph. aureus* strains involved in bovine mastitis to penicillin might be related to improper use

of this drug in mastitis treatment and the routine use of β -lactam antibiotics for intramammary infusion during the dry period in Korean herds. Methicillin-resistant staphylococcal isolates were frequently resistant to most of the commonly used antimicrobial agents, including the aminoglycosides, macrolides, chloramphenicols, tetracyclines, and fluorquinolones (Mandell et al., 1995). Additionally, MRS strains should be considered resistant to all cephalosporins, cephems, and other β -lactams and carbapenems, regardless of the in vitro test results obtained with those agents (CLSI, 2002).

In the present study, MRSA and MRCNS isolates from bovine mastitis were resistant to ampicillin (90.5 and 89.5%), gentamicin (33.3 and 31.6%), tetracycline (23.8 and 57.9%), erythromycin (57.1 and 15.7%), and clindamycin (14.3 and 21.1%), respectively. However, the MRSA and MRCNS isolates from this study were susceptible to amikacin and trimethoprim-sulfamethoxazole. Only 2 MRSA strains showed multiresistance patterns (resistant to 3 or more antibiotics other than β -lactam) on the basis of the multiresistance definition by Coombs et al. (2004). Moreover, in our previous study, multilocus sequence typing analysis showed that MRSA isolates from bovine mastitis milk had no genetic relatedness with community-acquired MRSA, which had caused human infection in Korea (Kwon et al., 2005). Therefore, those MRSA isolates from bovine mastitis milk had newly found community-acquired MRSA characteristics although there was no evidence for the evolution of bovine milk MRSA. However, no attempt was made to investigate the carriage of MRS by owners or by uninfected animals in our study. More studies on the spread of MRSA between human and veterinary fields and the control of MRSA are needed in dairy husbandry.

A subtyping system based on the size and restriction patterns of PCR products of the 3' end of the coagulase gene (Goh et al., 1992) was used successfully to examine epidemiological relationships between Australian isolates of bovine *Staph. aureus*. Restriction analysis of PCR products of the same size using *AluI*, *CfoI*, and *HaeIII* provided a convenient method of confirmation of the PCR types (Raimundo et al., 1999). Because *Staph. aureus* strains have a variable number of short sequence repeat regions in the coagulase gene, we investigated polymorphisms of the *coa* gene in MRSA and MSSA isolated from bovine mastitic milk. A single band between 620 and 809 bp was observed for each of the 706 *Staph. aureus* isolates in this study. This is different from results of a previous study that showed a single band between 547 and 875 bp using the same method (Hookey et al., 1998). The reason for the different size in amplicon of *coa* gene may be similar to an observation

of the *coa* gene polymorphism and its distinct size in different isolates (Phonimdaeng et al., 1990).

The analysis of coagulase genes by PCR-RFLP revealed 10 distinct patterns. The main types of the coagulase genes were A (26.9%), B (17.0%), G (10.5%), and H (15.4%), and the distribution rate of the 4 main types comprised 69.8% of the isolates in this study. Most of the *Staph. aureus* isolates showed the same RFLP pattern for each farm (data not shown). Coagulase genotypes differed by geographic location, and only a few genotypes prevailed in each location. Interestingly, when 10 distinct RFLP patterns were compared among 21 MRSA and 685 MSSA isolates, different genotypes were observed. The D, F, I, and J genotypes were not found in MRSA. By contrast, the A and H genotypes (6 and 10 isolates, respectively) were frequently detected in MRSA.

Our data indicate that there are strains that are common to several herds, and major epidemic MRSA clones may be spread between different dairy farms. This corresponds to the findings of others indicating that there exist strains that are common to several herds, even in separate states (Matthews et al., 1994; Annemuller et al., 1999; Raimundo et al., 1999; Su et al., 1999). This study suggests that major epidemic MRSA clones can spread from cow to cow or between different dairy farms, and the profile of the coagulase genotype technique can be applied for epidemiological investigations of bovine mastitis. Moreover, the prudent use of antibiotics and rapid and continuous screening for resistant microorganisms should be more focused to prevent the emergence and spread of MRSA and MRCNS.

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