Antibiogram and Coagulase Diversity in Staphylococcal Enterotoxin-Producing *Staphylococcus aureus* from Bovine Mastitis

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ABSTRACT

We investigated antibiogram and coagulase gene diversity in staphylococcal enterotoxin (StE)-producing *Staphylococcus aureus* isolated from raw milk samples of cows infected with mastitis from 140 dairy farms in Korea between 1997 and 2004. Of the 696 *Staph. aureus* isolates collected in this study, 164 isolates (23.6%) produced one or more staphylococcal enterotoxins (A to D), and 19 isolates (2.7%) were methicillin-resistant. The percentage of StE-producing *Staph. aureus* (SES) isolates resistant to methicillin, kanamycin, neomycin, amikacin, and tetracycline was greater than that of non-SES. Ten coagulase genotype patterns were observed, including 4 main types comprising I (25.4%), II (13.9%), VII (13.2%), and VIII (17.8%). More than 4 *Staph. aureus* types were isolated from each of 82 dairy farms in different geographic locations, and only 1 coagulase genotype pattern was observed in 39 of the herds (47.6%). There was no significant correlation between coagulase genotypes harbored by *Staph. aureus* and their specific StE type. The percentage of isolates producing major StE types (A, B, AC, and ABCD) and being resistant to cephalothin and methicillin was greater among the *Staph. aureus* isolates with the 4 predominant coagulase genotypes (I, II, VII, and VIII) than among the isolates harboring the 6 rare coagulase types (III, IV, V, VI, IX, and X). Based on coagulase gene polymorphisms, our data indicate that a broad distribution of identical or closely related enterotoxin-producing *Staph. aureus* strains seem to contribute to bovine mastitis in the Republic of Korea.

Key words: bovine mastitis, *Staphylococcus aureus*, enterotoxin, coagulase polymorphism

INTRODUCTION

Bovine mastitis is a major disease that affects the dairy industry, and *Staphylococcus aureus* is one of the

most frequently isolated pathogens from both subclinical and chronic infections (Watts, 1988; Bramley, 1992). Some *Staph. aureus* isolates from bovine milk carry different staphylococcal enterotoxins (StE) or toxic shock syndrome toxin-1 (Kenny et al., 1993; Matsunaga et al., 1993). These toxins are responsible for food poisoning outbreaks and toxigenic syndrome in humans respectively (Llewellyn and Cohen, 2002); they may also contribute to the persistence of *Staph. aureus* in bovine mammary glands and increased udder pathogenicity (Ferens et al., 1998).

Several reports (Larsen et al., 2000; Stephan et al., 2001; da Silva et al., 2005) have noted that the production of StE in *Staph. aureus* isolated from bovine mastitis may be determined by environmental and management factors in each geographical area. This genetic variability in StE production contributed to the emergence of distinct epidemiological profiles that were dependent on predominant strains within a herd. It indicates the necessity to identify such strains or subtypes before applying specific measures of mastitis control (Larsen et al., 2000; Stephan et al., 2001; da Silva et al., 2005). In addition, the staphylococcal enterotoxin C (SEC)-producing strains have been isolated frequently from bovine mastitis in northeast Switzerland (Stephan et al., 2001), Brazil (da Silva et al., 2005), and Japan (Katsuda et al., 2005). Recently, the occurrence of new types of StE (SEG to SER and SEU) has been reported, but the relationship between these new StE and bovine mastitis has not been established (Katsuda et al., 2005).

Many molecular epidemiological studies have already been conducted on enterotoxigenic *Staph. aureus* isolated from bovine milk, food, and humans (Tsen and Chen, 1992; Cremonesi et al., 2005; Boerema et al., 2006). Polymerase chain reaction and PCR-RFLP analysis of the 3' end of the gene encoding staphylococcal coagulase (*coa*) have been proposed as methods for typing *Staph. aureus* isolates for epidemiological study. It was previously described that varying numbers (3 to 9) of 81-bp tandem repeats in the *coa* gene determined sequence analysis (Lange et al., 1999; Scherrer et al.,

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In the last few years, the results of epidemiologic research based on the polymorphism of the *coa* gene in different countries indicate that a few *Staph. aureus* subtypes are responsible for most cases of bovine mastitis, and that these clones have a broad geographic distribution (Fitzgerald et al., 1997; Annemüller et al., 1999; Lange et al., 1999; da Silva and da Silva, 2006). Despite this detailed genetic characterization of StE-producing *Staph. aureus* (SES) in other countries, little is currently known of SES isolates from bovine milk in the Republic of Korea. An understanding of this epidemiological relationship is also necessary for the design of more effective mastitis control programs against enterotoxigenic *Staph. aureus*. Based on the previous experience in epidemiological investigation by PCR-RFLP analysis of the *coa* gene of *Staph. aureus* isolated from bovine mastitis in our study (Moon et al., 2003, 2007; Lim et al., 2004), we characterized subtypes and antibiograms of SES isolates derived from bovine mastitis milk collected from different provinces of the Republic of Korea by PCR-based subtyping of the *coa* gene.

Milk Sampling

Isolation and Identification of *Staph. aureus* and Enterotoxin Production

Staphylococcus aureus was isolated from milk samples in dairy herds according to the protocols of the National Mastitis Council (Harmon et al., 1990). An aliquot of 10 µL from each sample was spread over blood agar plates (Bacto-Agar, Difco, Detroit, MI) containing 5% washed sheep erythrocytes and incubated at 37°C for 24 h. Colonies suspected of being staphylococci

Table 1. Distribution of staphylococcal enterotoxin (StE)-producing or methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from mastitic milk of dairy cows from 1997 to 2004 in the Republic of Korea¹

Interval	Region												Total		Total MRSA		Total StE-producing isolates ²	
	Gyeonggi		Gangwon		Chung cheong		Jeolla		Gyeong sang		Total (all isolates)		Total MRSA		Total StE-producing isolates ²			
	Herds	Isolates	Herds	Isolates	Herds	Isolates	Herds	Isolates	Herds	Isolates	Herds	Isolates	Herds	Isolates	Herds	Isolates		
1997-1998	9	56	1	6	11	99	3	14	1	4	25	179	0	0	19	62		
1999-2000	22	89	6	11	13	47	8	26	3	6	52	179	10	17	28	57		
2001-2002	10	24	0	0	5	16	4	8	1	1	20	49	0	0	11	11		
2003-2004	20	135	11	80	11	73	0	0	1	1	43	289	1	2	25	34		
Total	61	304	18	97	40	235	15	48	6	12	140	696	11	19	83	164		

¹Among the total herds examined at this study, only the number of herds with the isolation of *Staph. aureus* isolates is indicated here.

²Only the production of StE from A to D (SEA to SED) was examined using reverse passive latex agglutination (SET-RPLA kit, Oxoid, Ltd., Basingstoke, UK) according to the kit manufacturer's instructions.

were subcultured on blood agar plates, and isolation and identification of *Staph. aureus* was performed by the method 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). Isolates of *Staph. aureus* were kept refrigerated (-70°C) for this study. The production of StE was examined using a staphylococcal enterotoxin A, B, C, D detection by reverse passive latex agglutination) according to the instructions of the kit manufacturer (Oxoid Ltd., Basingstoke, UK).

Antibiotic Susceptibility Test

The MIC test for methicillin (oxacillin) resistance and disk diffusion test on Müller-Hinton agar (Difco, Sparks, MD) 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). Commercially available (BBL, Sparks, MD) disks of antimicrobial drugs that are used frequently for the treatment of mastitis in lactating and dry cows in Korean dairy herds were tested in this study. The plates were incubated at 30°C for oxacillin (1 $\mu\text{g}/\text{disk}$) and 37°C for other antibiotics, including penicillin G (10 units/disk), ampicillin (10 $\mu\text{g}/\text{disk}$), cephalothin (30 $\mu\text{g}/\text{disk}$), amikacin (30 $\mu\text{g}/\text{disk}$), gentamicin (10 $\mu\text{g}/\text{disk}$), neomycin (30 $\mu\text{g}/\text{disk}$), kanamycin (30 $\mu\text{g}/\text{disk}$), and tetracycline (30 $\mu\text{g}/\text{disk}$). Methicillin resistance of staphylococci was screened by disk diffusion test with an oxacillin disk on Muller-Hinton agar and confirmed by MIC test with oxacillin. Phenotypic methicillin resistance was defined with an oxacillin MIC ≥ 4 $\mu\text{g}/\text{mL}$. *Staphylococcus aureus* ATCC 25923 was purchased from the American Type Culture Collection (Manassas, VA) and used as a quality control standard. Zones of growth inhibition were evaluated according to CLSI (2002).

Coagulase Gene Typing

DNA Extraction for Amplification. 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 (TE) buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0), and resuspended in TE buffer. To the cells were added 30 μL of 20% SDS (Sigma, St. Louis, MO), 5 μL of proteinase K (20 mg/mL, Promega Corp., Madison, WI), 50 μL of lysozyme (200 $\mu\text{g}/\text{mL}$, Sigma), and the solutions were then mixed

and incubated at 37°C for 90 min. To this mixture was added 100 μL of CTAB/NaCl [10% cetyltrimethylammonium bromide (wt/vol), 0.7% NaCl; Sigma]. The mixture was then incubated at 65°C for 20 min and 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 volume 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 volumes of isoamylalcohol at -70°C overnight. After centrifugation, the pellet was washed with 70% ethanol, dried with a vacuum drier, and resuspended with 30 μL of TE buffer (Sigma). The DNA concentration was determined by UV spectrophotometry (Bio-Rad Laboratories Inc., Hercules, CA) at 260 nm, and the extract was stored at 4°C . Approximately 50 to 100 ng of DNA was taken for PCR amplification.

Analysis of Coagulase Gene by PCR-RFLP of *Staph. aureus*. Primers for analysis of the *coa* gene of *Staph. aureus* by PCR-RFLP were constructed as described by Kaida et al. (1989). The forward primer, *Coa*-1 (5'-ATAGAAATGCTGGTACAGG-3'; MRSA 213; Accession No. X16457; 1,513 to 1,531), and the reverse primer, *Coa*-2 (5'-GCTTCCGATTGTTTCGATGC-3'; 2,188 to 2,168), were used. The numbers in parentheses refer to the positions on the *coa* gene sequence. In this study, PCR was performed as described by Hookey et al. (1998) with some modifications. In brief, PCR was performed in a 50- μL volume with $10\times$ PCR buffer (Promega) containing 100 mM Tris-HCl (pH 8.3), 50 mM KCl, 6 μL of MgCl_2 (25 mM, Promega), 400 μM dNTP mixture (Promega), 2.5 U of *Taq* polymerase (Promega), and 1 μL of each primer (100 pmol/ μL), 2.5 μL of dimethyl sulfoxide, and 100 ng of DNA template. Finally, PCR-grade water (Sigma) was added to a final volume of 50 μL . Amplification of DNA was performed using the following cycling parameters: predenaturation at 94°C for 5 min for 1 cycle, followed by denaturation at 94°C for 20 s, annealing at 58°C for 15 s, and extension at 70°C for 15 s for 35 cycles on a thermal cycler (model 2400, Perkin-Elmer Life and Analytical Sciences, Boston, MA). A 7-min extension step at 72°C was included at the end of the final cycle. After electrophoresis, the gel was stained with ethidium bromide and visualized by UV light. Each PCR product (10 μL) was incubated with 2 units of *Alu*I restriction endonucleases (Promega) for 1 h at 37°C according to the manufacturer's protocol. The resulting fragments were electrophoresed on a 2.75% agarose gel at 110 V for 1 h, stained with ethidium bromide (0.5 $\mu\text{g}/\text{mL}$), and visualized under UV light.

Table 2. Distribution of toxin production of staphylococcal enterotoxin (StE)-producing *Staphylococcus aureus* and PCR-RFLP genotypes of the coagulase gene of *Staph. aureus* isolated from milk of mastitic dairy cows

StE production ¹		No. of isolates	PCR-RFLP types (coagulase genotype)									
			I	II	III	IV	V	VI	VII	VIII	IX	X
StE-producing	A	73	11	5	11	1	2	4	9	23	1	6
	B	17	2	4	0	1	2	0	7	1	0	0
	C	8	4	1	0	0	0	1	0	0	2	0
	D	2	0	1	0	0	0	0	0	1	0	0
	AB	2	0	0	0	0	0	0	1	1	0	0
	AC	13	1	8	0	0	0	0	0	3	1	0
	AD	4	3	1	0	0	0	0	0	0	0	0
	BC	3	2	0	0	0	0	0	0	0	1	0
	CD	1	0	0	0	0	1	0	0	0	0	0
	ABC	4	1	0	0	0	0	1	0	2	0	0
	ACD	4	0	0	0	0	0	0	0	2	2	0
	BCD	6	0	3	0	1	0	1	1	0	0	0
	ABCD	27	4	5	2	1	0	5	3	6	1	0
	Subtotal	164	28	28	13	4	5	12	21	39	8	6
Non-StE-producing			(17) ²	(17)	(8)	(2)	(3)	(7)	(13)	(24)	(5)	(4)
		532	149	69	12	47	54	13	71	85	29	3
			(28) ²	(13)	(2)	(9)	(10)	(2)	(13)	(16)	(5)	(1)

¹Only production of StE from A to D (SEA to SED) was examined using reverse passive latex agglutination (SET-RPLA kit, Oxoid, Ltd., Basingstoke, UK) according to the kit manufacturer's instructions.

²Values in parentheses indicate the percentage of *Staph. aureus* isolates with each PCR-RFLP type of coagulase gene among StE-producing or nonproducing *Staph. aureus* isolates.

Statistical Analyses

The Mantel-Haenszel χ^2 test and Fisher's exact test were used to examine differences in the observed antibiotic susceptibility percentage between *Staph. aureus* isolates with various StE productivity and PCR-RFLP types of *coa* gene, and differences in the frequency of major StE types (A, B, AC, and ABCD) production between *Staph. aureus* isolates with the polymorphism of *coa* gene. The statistical analyses were performed using the Analyze-it program (Analyze-it Software Ltd., Leeds, UK).

RESULTS

According to phenotypic and biochemical properties, all of the 696 isolates obtained in this study were identified as *Staph. aureus*. Of these isolates, 164 (23.6%) were found to be positive for production of one or more toxins using a reverse passive latex agglutination kit (Tables 1 and 2). Of the 164 enterotoxin-producing *Staph. aureus* isolates, 100 isolates (61.0%) were involved in production of only one type of StE (SEA, 73 isolates; SEB, 17 isolates; SEC, 8 isolates; SED, 2 isolates). The remaining 64 isolates produced more than one toxin.

The resistance profiles of SES and non-SES isolates to 9 antimicrobial agents were compared. Most isolates were resistant to penicillin and ampicillin, but were susceptible to amikacin, gentamicin, cephalotin, kana-

mycin, and tetracyclin (Table 3). Of 696 *Staph. aureus* isolates obtained from 140 dairy farms between 1997 and 2004, 19 isolates (2.7%) were methicillin-resistant *Staph. aureus* (MRSA). Of the 19 MRSA isolates, 13 isolates (68.4%) produced one or more StE. Although there are differences in antibiotic resistance patterns shown by different types of SES isolates, the percentage of SES isolates resistant to oxacillin (7.9 vs. 1.1%), kanamycin (43.9 vs. 22.7%), neomycin (30.5 vs. 12.8%), amikacin (26.2 vs. 10.7%), and tetracycline (32.3 vs. 19.2%) was greater ($P < 0.05$) compared with non-SES isolates.

For *coa* genotyping, all 696 isolates were subjected to PCR amplification of the 3' end of the *coa* gene and subsequent agarose gel analysis of PCR products, which showed a single band ranging between 620 and 809 bp for each *Staph. aureus* isolate (Figure 1, panel A). Restriction enzyme digestion (by *AluI*) of the PCR products generated 10 different *coa* gene RFLP patterns (I to X). The number of fragments produced upon *AluI* digestion varied from 1 to 4, and their sizes varied from 126 to 670 bp (Figure 1, panel B). These genotypes were observed among *Staph. aureus* isolates from different geographic locations, but a few genotypes tended to prevail at each location. The main genotypes were I (25.4%), II (13.9%), VII (13.2%), and VIII (17.8%), and the distribution rates of the 4 main types made up 70.3% of the isolates (Table 2). Among the 82 dairy farms containing more than 4 isolated *Staph. aureus* strains per herd in different geographic locations, *Staph. aureus* strains with only 1 *coa* gene RFLP pat-

Table 3. Difference in antibiogram between nonstaphylococcal enterotoxin and staphylococcal enterotoxin (StE)-producing *Staphylococcus aureus*

			No. of antibiotic-resistant isolates ¹ (%)									
			No. Of isolates	OX	P	AM	CF	K	GM	N	AN	TE
StE-producing	A	73	4	49	50	4	32	19	21	19	21	
	ABCD	27	1	16	16	1	10	14	16	14	10	
	B	17	1	14	15	1	5	2	3	1	5	
	AC	13	1	8	6	1	4	1	0	2	3	
	Other	31	6	23	20	2	6	11	9	6	7	
	Subtotal	164	13 ²	112	108	9	72 ²	48	50 ²	43 ²	54 ²	
			(8)	(69)	(66)	(6)	(44)	(29)	(30)	(26)	(33)	
Non-StE-producing		532	6 ²	398	396	18	123 ²	123	68 ²	57 ²	102 ²	
			(1)	(75)	(74)	(3)	(23)	(23)	(13)	(11)	(19)	
Total		696	19	510	504	27	195	195	118	100	156	
			(3)	(73)	(72)	(4)	(28)	(28)	(17)	(14)	(22)	

¹OX = oxacillin, 1 µg; P = penicillin G, 10 U; AM = ampicillin, 10 µg; CF = cephalothin, 30 µg; K = kanamycin, 30 µg; GM = gentamicin, 10 µg; N = neomycin, 30 µg; AN = amikacin, 30 µg; TE = tetracycline, 30 µg. Methicillin resistance of staphylococci was screened by disk diffusion test with an oxacillin disk on Muller-Hinton agar (Difco, Sparks, MD) and confirmed by MIC test with oxacillin. Phenotypic methicillin resistance was defined with an oxacillin MIC ≥4 µg/mL.

²The percentage of StE-producing *Staph. aureus* isolates resistant to oxacillin, kanamycin, neomycin, amikacin, and tetracycline was greater ($P < 0.05$) than that of non-StE-producing isolates.

tern were isolated from 39 herds (47.6%), whereas *Staph. aureus* strains with 2, 3, or 4 or more *coa* genotypes were isolated from 23 (28.0%), 15 (18.3%), and 5 (6.1%) herds, respectively.

When 10 distinct *coa* genotype patterns were compared between 532 non-SES and 164 SES isolates, the differences in *coa* genotype carried by specific toxin type was not observed (Table 2). However, the percentage of isolates producing major StE types (A, B, AC, and ABCD) was greater among the *Staph. aureus* isolates with the 4 predominant genotypes (I, II, VII, and VIII) than among the isolates harboring the 6 rare coagulase types (III, IV, V, VI, IX, and X). Additionally, the *Staph. aureus* isolates carrying predominant *coa* genotypes were more resistant ($P < 0.05$) to cephalothin compared with the isolates having rare *coa* types; all the MRSA isolates had the 4 predominant *coa* genotypes (Table 4).

DISCUSSION

Milk and other dairy products have been reported to be frequently associated with *Staph. aureus* enterotoxin food poisoning. It has been supposed that the milk of infected animals may constitute the main source of enterotoxigenic *Staph. aureus* of animal origin (Bryan, 1983; Bergdoll, 1989; Gilmour and Harvey, 1990). Because of the importance of these toxins for public health and food safety, efficient screening for the prevalence of enterotoxigenic strains in bovine mastitis is necessary. The production of enterotoxins SEA to SEE by *Staph. aureus* field isolates from mastitis animals has been investigated in several studies (Bautista et al., 1988;

Kenny et al., 1993; Matsunaga et al., 1993; Zschöck et al., 2004).

In this study, the prevalence rate of enterotoxin-producing *Staph. aureus* from bovine mastitis was 23.6% when randomly selected field strains were examined. One hundred isolates (61.0%) possessed only 1 type of toxin, with SEA (42.9%) being the most frequent toxin type; the remaining 64 isolates (39.0%) participated in production of more than 1 toxin. The predominance of SEA contradicts with reports from countries such as Switzerland, Brazil, and Japan, where SEC-producing *Staph. aureus* were frequently isolated (Stephan et al., 2001; da Silva et al., 2005; Katsuda et al., 2005). Various reports have indicated that SEA is mainly from humans, whereas SEC is from animals (Bergdoll, 1989; Orden et al., 1992). In contrast, a review by Genigeorgis (1989) concluded that there is no predominant type of StE, apart from the isolates from food involved in staphylococcal gastroenteritis, where SEA is the main type of StE recovered (Genigeorgis, 1989). Further epidemiological study is required to clarify the dominant genotype and toxin type of *Staph. aureus* in the Republic of Korea by more extensive characterization of a large number of isolates from different geographical areas. As demonstrated in various studies (Su and Wong, 1995; Munson et al., 1998; Zhang et al., 1998), StE-producing strains have been widely isolated from mastitis. Recently, the occurrence of new types of StE has been reported (Zschöck et al., 2005), but none of the newly described enterotoxins (SEG, SEH, SEI, and SEJ) were investigated in this study.

The emergence of antibacterial resistance among pathogens that affect animal health is of growing con-

Figure 1. Polymerase chain reaction-RFLP patterns. The coagulase (*coa*) gene of *Staphylococcus aureus* was amplified (A) and digested with *AluI* restriction endonucleases (Promega Corp, Madison, WI) (B). Lane 1 = 100-bp molecular weight ladder (A) or pGEM DNA marker (B) (Promega); Lanes 2 to 11 = RFLP patterns I to X of the *coa* gene (B).

cern in veterinary medicine. Antimicrobial-resistant pathogens in animals have been incriminated as a potential health risk for humans from possible transmission as foodborne pathogens. Particularly, mastitis is the single greatest cause of antibacterial use on dairy farms (White and McDermott, 2001; Erskine et al., 2002). *Staphylococcus aureus* remains one of the most significant organisms associated with bovine mastitis

worldwide. It is believed that infections with *Staph. aureus* respond poorly to therapy with antimicrobial agents, whether given parenterally or via the intramammary route (Vintov et al., 2003). The occurrence and transmission of antimicrobial-resistant *Staph. aureus* itself or its gene(s) related to antibiotic resistance have been suggested as one of the reasons for the lack of response to therapy (Goh et al., 1992; Kreiswirth

Table 4. Comparison of antibiograms of *Staphylococcus aureus* with different coagulase genotypes isolated from bovine mastitis milk

Coagulase genotypes (I to X)		No. of isolates	No. of antibiotic-resistant isolates ¹ (%)								
			OX	P	AM	CF	K	GM	N	AN	TE
Predominant types	I	177	6	144	142	6	48	38	18	22	24
	VIII	124	10	104	103	8	36	34	17	15	46
	II	97	1	47	47	6	28	41	13	11	23
	VII	92	2	63	62	5	22	25	26	23	18
	Subtotal	490	19 ²	358	354	25 ²	134	138	74	71	111
			(4)	(73)	(72)	(5)	(27)	(28)	(15)	(15)	(23)
Rare types	V	59	0	41	40	0	12	11	10	5	13
	IV	51	0	42	43	0	18	17	18	3	9
	IX	37	0	34	30	0	4	4	0	1	1
	VI	25	0	11	12	1	14	13	12	13	18
	III	25	0	16	16	0	8	6	4	5	4
	X	9	0	8	9	1	5	4	0	2	0
	Subtotal	206	0 ²	152	150	2 ²	61	55	44	29	45
			(0)	(74)	(73)	(1)	(30)	(27)	(21)	(14)	(22)
Total		696	19	510	504	27	195	193	118	100	156
			(3)	(73)	(72)	(4)	(28)	(28)	(17)	(14)	(22)

¹OX = oxacillin, 1 µg; P = penicillin G, 10 U; AM = ampicillin, 10 µg; CF = cephalothin, 30 µg; K = kanamycin, 30 µg; GM = gentamicin, 10 µg; N = neomycin, 30 µg; AN = amikacin, 30 µg; TE = tetracycline, 30 µg. Methicillin resistance of staphylococci was screened by disk diffusion test with an oxacillin disk on Muller-Hinton agar (Difco, Sparks, MD) and confirmed by MIC test with oxacillin. Phenotypic methicillin resistance was defined with an oxacillin MIC ≥4 µg/mL.

²The *Staph. aureus* isolates harboring the predominant coagulase genotypes (I, VIII, II, and VII) were more resistant ($P < 0.05$) to oxacillin and cephalothin compared with the isolates having rare coagulase types (V, IV, IX, VI, III, and X).

et al., 1993). Therefore, establishing an antibiogram of strains is very important from the clinical and economic points of view.

Several studies conducted across the world have reported the susceptibility of *Staph. aureus* from bovine mastitis to antimicrobial agents. In most of those studies, the frequent occurrence of penicillin resistance has been observed, whereas only a few instances of resistance to other antimicrobial agents have been reported (Stephan et al., 2001; Werckenthin et al., 2001; Erskine et al., 2002; Vintov et al., 2003). The low percentage of *Staph. aureus* strains resistant to other antimicrobial agents except penicillin and ampicillin was confirmed in this study. Major differences in the occurrence of penicillin resistance have been observed between countries (Aarestrup and Jensen, 1998). Thus, a previous report by Vintov et al. (2003) found high frequencies of penicillin resistance in Ireland (71.4%), England (67.3%), and the United States (50%), whereas low frequencies were found in Scandinavian countries, including Denmark (18.7%), Norway (2%), and Sweden (28.5%).

The percentage of penicillin-resistant *Staph. aureus* isolated in this study was greater compared with studies performed in the United States and other countries (Aarestrup and Jensen, 1998; Erskine et al., 2002; Vintov et al., 2003). This might be related to the routine veterinary use of cloxacillin as dry cow treatment and ampicillin as lactating cow treatment against mastitis

in Korea (Moon et al., 2005, 2007). The percentage of SES isolates resistant to oxacillin, kanamycin, neomycin, amikacin, and tetracycline was greater ($P < 0.05$) than that of non-SES isolates. These findings support the suggestion that the SEC of *Staph. aureus* can escape or efficiently inhibit the immune response during infection and continue to survive in the host. The depressed host immune status may result in persistence of *Staph. aureus* in the mammary gland, degeneration into chronic mastitis status, and difficulty in treatment because of high antibiotic resistance characteristic of SES (Ferens et al., 1998).

One of the criteria for confirmation of staphylococcal food poisoning is whether the recovered isolates have enterotoxigenicity. In contrast, non-StE-producing *Staph. aureus* strains are not considered etiologic organisms for food poisoning (Chiou et al., 2000). Additionally, molecular typing of staphylococcal isolates can provide useful information about their epidemiological relatedness for the confirmation of staphylococcal foodborne outbreaks. Numerous methods for *Staph. aureus* typing have been described (Goh et al., 1992; Chiou et al., 2000). Among these, a subtyping system based on the size and restriction patterns of PCR products of the 3' end of the *coa* gene (Goh et al., 1992) was successfully used to examine epidemiological relationships among Australian isolates of bovine *Staph. aureus*.

Of the 10 different RFLP patterns observed in this study, the main types were I (25.4%), II (13.9%), VII (13.2%), and VIII (17.8%), and the distribution of the 4 main types accounted for 70.3% of the isolates. This agrees with results of a study conducted by Aarestrup et al. (1995) in which 187 *Staph. aureus* bovine isolates were grouped into 15 subtypes based on PCR-RFLP analysis of the *coa* gene. In contrast, a study conducted by Su et al. (1999) demonstrated that the profiles of *coa* genotype determined by PCR-RFLP differed among geographic location from the Czech Republic, France, the Republic of Korea, and the United Kingdom, and only a few genotypes prevailed in each location. These findings supported the conclusions of other studies (Aarestrup et al., 1995; Fitzgerald et al., 1997; Annemüller et al., 1999; Raimundo et al., 1999) in that the limited number of *Staph. aureus* isolates with dominant *coa* genotypes occurred in various herds and countries. This difference could reflect the more intensive farming practices used in the Republic of Korea compared with those in Australia, resulting in the smaller number of genotypes in circulating strains.

Sixty-two (75.6%) *Staph. aureus* isolates from 82 herds in different locations showed only 1 or 2 *coa* gene RFLP patterns in this study. Our results support previous findings that only a few *coa* gene variants predominated, although mastitis in Germany, Switzerland, and Northeast Japan was caused by *Staph. aureus* strains that had many variants of *coa* gene (Annemüller et al., 1999; Stephan et al., 2001; Katsuda et al., 2005). Although the difference in genotype by specific toxin type was not shown, the frequency of major StE types (A, B, AC, ABCD) was greater ($P < 0.05$) in the *Staph. aureus* isolates with the 4 predominant genotypes (I, II, VII, and VIII) compared with the isolates having the 6 rare coagulase types (III, IV, V, VI, IX, and X). This is different from the data published by Katsuda et al. (2005), which indicated that all of 173 isolates with the *coa* genotype A or B possessed one or more toxin genes, but no toxin gene could be detected in isolates with *coa* genotype D, except in 3 isolates. This result is in agreement with the findings of previous studies, which showed that a few *Staph. aureus* clones had broad geographic distribution in different countries (Fitzgerald et al., 1997; Annemüller et al., 1999; Lange et al., 1999).

According to Su et al. (1999), the predominant *coa* genotypes were more resistant to neutrophil activities than those with the rare genotype, which indicates that specific features in the former might help them overcome host defense mechanisms. In this study, *Staph. aureus* isolates harboring the predominant *coa* genotypes (I, II, VII, and VIII) were more resistant ($P < 0.05$) to oxacillin and cephalothin compared with the isolates having rare *coa* types (III, IV, V, VI, IX, and

X). Our results demonstrate that the patterns of antimicrobial resistance did not determine the distribution of *coa* genotypes but may be caused by the diverse selective pressure from the different usage of antibiotics on each farm. Further studies are needed to determine the common characteristics of the predominant strains. In summary, this study indicates that a broad distribution of identical or closely related enterotoxin-producing or methicillin-resistant *Staph. aureus* strains contributes to the bovine mastitis problem in the Republic of Korea.

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