Characterization of Methicillin-Resistant *Staphylococcus aureus* Isolated from Dogs in Korea

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ABSTRACT. Twelve strains of the methicillin-resistant *Staphylococcus aureus* (MRSA) recovered from hospitalized dogs were analyzed for *in vitro* antimicrobial susceptibility and virulence, and were genetically characterized by pulsed-field gel electrophoresis (PFGE). Antibiotic susceptibility test showed that nearly all isolates were resistant to β -lactam antibiotics tested and all the strains were fully susceptible to glycopeptides. There were no inhibitory activities among the aminoglycosides. The 50% lethal dose (LD₅₀) was determined by intraperitoneal injection of cell suspensions and estimated by the Spearman-Kärber method. The mouse lethality of MRSA and methicillin-susceptible *S. aureus* (MSSA) was not significantly different in both normal and cyclophosphamide-treated mice ($p > 0.05$), indicating that they were equally virulent. There was a great difference in the incidence of toxin production between the MRSA and MSSA group; 83.3% (10 of 12) of the MRSA and 14.3% (1 of 7) of the MSSA were toxin producers. The predominant types produced by MRSA was B. All the MRSA strains were capsular type 5 producers, while of 7 MSSA strains, four were type 5, one for type 8, and two were nontypeable. Based on the PFGE analysis, the 12 MRSA isolates generated 9 to 11 fragments in the size range of <48.5 to 630.5 kb, and yielded 6 different patterns. The results indicated that production of toxin and capsule type do not play a role in the pathogenicity to mouse and PFGE is a valuable tool for the characterization of MRSA. This report is the first such cases in the veterinary literature in Korea and may indicate the frequent emergence of MRSA in veterinary clinic hereafter.—**KEY WORDS:** antibiotic susceptibility, canine, methicillin-resistant *Staphylococcus aureus*, pathogenicity, pulsed-field gel electrophoresis.

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Since first isolation of methicillin-resistant *Staphylococcus aureus* (MRSA) in 1972 [8], several reports from animal-linked population have been documented [4, 5, 14, 29, 32]. This indicates that infections caused by this organism in animals could become more frequent. Historically, in Korea, MRSA was not detected until early 1970s. Since then, MRSA from humans was began to be reported, and epidemics of MRSA became a clinically significant problem in hospital environment.

Normally, an MRSA infection leads to a carrier state in healthy persons, whereas it causes severe morbidity and mortality in hospitalized patients [2, 3, 18]. This implies that MRSA is an unusual opportunistic pathogen, which displays features of a virulent pathogen. *S. aureus* causes two main types of infection [30]; cutaneous or mucosal infections, and septicemic infections which are generally associated with visceral (abscesses, endocarditis, lung infections) or bone (osteomyelitis) infections. In animals, *S. aureus* is mainly involved in intramammary infections of lactating cows [27] and superficial pyoderma in dogs [7].

Isolates of MRSA are usually resistant to multiple antibiotics, including β -lactam, aminoglycosides, macrolides, lincosamides, chloramphenicol, and more recently, fluoroquinolones [21, 22, 24] and are a major cause of nosocomial infections throughout the world. The multiple antimicrobial resistance of these isolates makes them responsible for high mortality rates in immunocompromised patients. Furthermore, it is likely that the pathogenesis of

MRSA disease is multifactorial, involving a complex host/pathogen relationship; extracellular hemolysins, enzymes, or toxins.

The present study was undertaken to identify presence of the organism in domestic dogs and to describe antibiotic susceptibility pattern and pathogenicity. Finally, MRSA isolates were epidemiologically differentiated by genomic DNA fingerprinting using pulsed-field gel electrophoresis (PFGE).

MATERIALS AND METHODS

Bacteria isolation and biochemical test: *S. aureus* suspected colonies on blood agar plates were selected and underwent standard biochemical test as described by Quinn *et al.* [26]. Methicillin resistance was confirmed by the agar screening test, using Mueller-Hinton agar containing 4% NaCl and 6 μ g/ml oxacillin and reading after 24 hr incubation at 35°C. These isolates were further identified by the polymerase chain reaction method using the *mecA* gene, which is specific for MRSA [38]. Twelve MRSA and seven methicillin-susceptible *S. aureus* (MSSA) were isolated from dogs hospitalized with underlying diseases over an eight-month period of 1998.

Antibiotic susceptibility test: Antimicrobial agents used for susceptibility testing were purchased from a commercial sources (Sigma, see Table 1). The susceptibility of the isolate to the various antimicrobial agents was determined

in triplicate by using a macrobroth dilution method in cation-supplemented Mueller-Hinton broth plus 2% NaCl at a final inoculum of 5×10^5 CFU/ml from an overnight growth of the organism. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of antimicrobial that inhibited the visible growth of the test organism in 24 hr of incubation at 35°C. MIC₅₀ values represent the MICs of the antibiotics, which inhibited at least 50% of strains. Susceptibility to each antimicrobial was defined using the breakpoint categories of the NCCLS [23] or manufacture's recommendations.

Capsule typing: The capsule typing of each isolate was done by the method described by Lee *et al.* [20].

LD₅₀ determination: Five groups of ten mice per decreasing twofold bacterial doses (range 10^8 – 10^9) were injected intraperitoneally with 0.5 ml of isolates suspended with PBS. After 15 days of observation, 50% lethal dose (LD₅₀) of each bacterial strain was calculated. For immunosuppression, for days before challenge with *S. aureus* mice were injected intraperitoneally with cyclophosphamide (Sigma) at a dose of 250 mg/kg of mouse weight. The next dose was periodically given 2 days after the first.

Enterotoxin typing: Enterotoxins and toxic shock syndrome toxin-1 (TSST-1) were detected by the reversed-passive latex agglutination (RPLA), with commercial kit tests (SET-RPLA and TST-RPLA, Denka Seiken Co., Ltd., Tokyo, Japan).

DNA Preparation, digestion, and PFGE: The preparation of chromosomal DNA of MRSA isolates and fragmentation of their genomic DNA with *Sma*I (New England BioLabs, Beverly, Mass.) were performed as described [11, 31]. Electrophoresis was performed with a CHEF-DR II system (Bio-Rad Laboratories) as previously described [31]. PFGE was carried out as follows: initial pulse = 5 sec, final pulse = 40 sec, voltage = 6 V/cm, temperature = 14°C, time = 22 hr. The lambda ladder DNA concatemers (New England BioLabs) for determining the size of the largest *Sma*I digest fragments were used as molecular size standards. Interpretation of PFGE patterns was performed according to the guidelines of other researchers [1, 35].

Statistical analysis: Fifteen-day LD₅₀ and 95% confidence interval were determined by the method of Spearman-Kärber [9]. Probit analysis was done to compare differences between two lethality curves. A value of $p < 0.05$ was considered statistically significant. All analyses used PC-SAS (release 6.04; SAS institute, Cary, NC).

RESULTS AND DISCUSSION

Antibiotic susceptibility: The MIC values of 12 isolates are shown in Table 1. Mupirocin and glycopeptides inhibited all the isolates tested, followed by rifampicin (58.3%), novobiocin (50%) and trimethoprim-sulfamethoxazole (TMP/SMZ) (50%). Aminoglycosides, β -lactam, and quinolones had little or no activity against these bacteria. Of the other agents, chloramphenicol and

minocycline showed moderate inhibitory effect of 41.7% each. In terms of MICs, mupirocin was one of the most active drug together with glycopeptides. All isolates showed resistant to imipenem which has never been used in our hospital, indicating a high cross-resistant between β -lactam antibiotics. Therefore, the result of this study emphasizes the multiply resistant nature of the MRSA strains.

In Korea, since the introduction of quinolones into veterinary medicine in early 1990, the incidence of ciprofloxacin and enrofloxacin (Baytril®) has rarely been studied in spite of unrestricted use in local clinics. In this study, the development of ciprofloxacin resistance of *S. aureus* accompanied by high-level cross-resistance to enrofloxacin as well as increased resistance to other classes of antimicrobials. Resistance of rifampicin and TMP/SMZ in *S. aureus* from animals has also not been documented in Korean veterinary literature. It is surprising to note that in the present study these two antibiotics showed only moderate inhibitory effect on MRSA, ranging 50–58.3%. This may indicate that the widespread use of ciprofloxacin alone or in combination with rifampin, for therapy of other susceptible organisms will continue to be a major challenge in veterinary medicine, as described by others studying in

Table 1. Range of MICs of the antimicrobials against 12 MRSA isolates

Antimicrobial agent	MIC, $\mu\text{g/ml}$		Percent susceptible
	Range	50% ^{a)}	
β-lactam			
Ampicillin	8–32	32	16.7
Cefamandole	8–64	32	0
Cefazolin	32–>128	64	0
Cefmetazole	16–>64	>64	8.3
Cefoperazone	>64	>64	0
Imipenem	>16	>16	0
Aminoglycoside			
Amikacin	16–>64	32	8.3
Gentamicin	16–>128	64	0
Tobramycin	>32	>32	0
Glycopeptide			
Teicoplanin	0.5–2	<0.5	100
Vancomycin	0.25–1	<0.25	100
Quinolone			
Ciprofloxacin	16–>32	16	0
Enrofloxacin	32–256	64	0
Chloramphenicol	8–64	16	41.7
Minocycline	0.5–64	8	41.7
Mupirocin	0.25–2	0.25	100
Novobiocin	0.25–4	1	50.0
Rifampicin	<0.06–>4	0.25	58.3
TMP/SMZ ^{b)}	1–>8	4	50.0

a) 50% stands for MICs for 50% of isolates tested.

b) TMP/SMZ: trimethoprim/sulfamethoxazole.

Breakpoints for resistance were (in $\mu\text{g/ml}$): ampicillin, cefamandole, cefazolin, amikacin, teicoplanin, vancomycin, and chloramphenicol, ≥ 32 ; cefmetazole and cefoperazone, ≥ 64 ; gentamicin, tobramycin, and TMP/SMZ, ≥ 8 ; imipenem, minocycline, and mupirocin, ≥ 16 ; ciprofloxacin and rifampicin, ≥ 4 ; enrofloxacin, ≥ 0.5 ; and novobiocin, ≥ 2 .

Table 2. Comparison of toxin type of the isolates and their LD₅₀ values against mice

Toxin type	MRSA		MSSA	
	No. of isolates	Log LD ₅₀	No. of isolates	Log LD ₅₀
Non toxigenic	2	8.40 ± 0.05 ^{a)} (7.81 ± 0.09) ^{b)}	6	8.23 ± 0.05 (7.79 ± 0.07)
B	6	8.28 ± 0.09 (7.97 ± 0.11)	1	8.14 (7.97)
C + TSST-1	3	8.20 ± 0.06 (7.67 ± 0.09)	0	N.D. ^{c)}
TSST-1	1	8.32 (7.72)	0	N.D.
Total	12	8.29 ± 0.07 (7.79 ± 0.10)	7	8.18 ± 0.05 (7.88 ± 0.08)

a) LD₅₀ values expressed as means ± SD against normal mice. b) LD₅₀ values against cyclophosphamide-treated mice. c) Not done.

humans [19, 34]. The present data might be of some clinical relevance, when alternative compounds are needed for clinicians to initiate specific, effective chemotherapy in dogs with MRSA infection. Particularly, mupirocin, one of the most active drug may be clearly one of the few options for this purpose.

Lethality of MRSA and MSSA in mice: Overall, the mean LD₅₀ (log 10 CFU) of the 12 MRSA strains in normal mice was 0.11 higher than that of the 7 MSSA strains, whereas in cyclophosphamide-treated mice, that of MRSA was 0.09 lower than that in MSSA (Table 2). Pretreatment of mice with cyclophosphamide decreased the mean LD₅₀ for MRSA strains more than that for MSSA strains. However, so far as the values of LD₅₀ in both normal and cyclophosphamide-treated mice were concerned, there were no differences in the virulence between the MRSA and MSSA strains ($p > 0.05$).

The difference in virulence among MR- and MSSA strains has still been controversial. Some researchers reported [6] that there was a significant difference in the virulence between the two strains. On the other hand, other researchers [10] proclaimed that differences in the virulence were not observed. Peacock *et al.* [24] found that MRSA after intraperitoneal injections was not significantly different from those for three MSSA strains tested. In his intravenous injection model, on the contrary, some MSSA strains showed more virulent than MRSA, others did not. The discrepancy of these studies as to the virulence may originate from the difference in phenotypic characteristics of strains used, experimental conditions such as inoculation dose or route of inoculation, or alternatively some other virulence-related factors not considering in this study such as α -hemolysin and exoprotein [16]. Further studies involving these factors will be required to fully define the virulence of MRSA.

Capsule type and toxin production: The capsular polysaccharide has gained considerable attention as virulence factors for some strains of staphylococci [33]. Of eleven types of capsule, type 5 and 8 have been reported the most prevalent in human beings [25]. However, study on the capsule and toxin type of the isolates recovered from companion animals has never been reported. In this study twelve MRSA isolates were found to produce capsular type 5. Of 7 MSSA strains tested, four were type 5, one was

type 8, and two were nontypeable. 83.3% (10/12) of MRSA isolates produced one or more toxins, with the most predominant enterotoxin B (60%). Of these, four strains (40%) produced TSST-1 and three were co-producers of enterotoxin C and TSST-1. Of the 7 MSSA strains tested, only one strain (14.3%) was of enterotoxin B producer (Table 2). MRSA strains showed the patterns of multiple enterotoxin producers (40%), as reported others in human cases [15, 17]. There was no correlation in pathogenicity on mouse, regardless of toxin and capsule types.

Chromosomal DNA profiles by PFGE: The importance of typing *S. aureus* strains for epidemiological reasons is generally acknowledged; however the choice of the right typing method is often difficult because of inherent theoretical and technical limitations [13, 37]. Of these methods, PFGE have been used extensively to distinguish different strains of *S. aureus* and other staphylococci [12, 31]. The genomic DNA subtyping of *Sma*I digests yielded well-resolved patterns of at least 9 or 11 fragments of 48.5–630.5 kb in 12 MRSA isolates (Fig. 1). The 12 isolates produced 6 different patterns. Six isolates in lanes 1 through 6 gave the same PFGE pattern which was composed of at least 9 distinct fragments of the following sizes: 605 kb, 388 kb, 325 kb, 268 kb, 177 kb, 129 kb, 110 kb, 75 kb, and 60 kb. Two isolates in lanes 8 and 10 had the same PFGE pattern composed of 11 distinct *Sma*I restriction fragments (582 kb, 500 kb, 325 kb, 272 kb, 216 kb, 129 kb, 114 kb, 110 kb, 75 kb, 60 kb, and 54 kb). The isolate in lane 7 missed the fragments of 500 kb and 114 kb, but showed additional 2 fragments of 453 kb and 156 kb, compared with lanes 8 and 10. The remaining 3 isolates (lanes 9, 11, and 12) have unique restriction patterns of 9 to 11 fragments. This result reaffirmed the usefulness of PFGE for strain differentiation.

Epidemiology of MRSA: During the study period three putative episodes of MRSA were suspected, in which 6 isolates for the first episode, 4 for the second, and 2 for the third were obtained (Table 3). In April 1998 an MRSA-colonized dog with distemper, transferred from a local animal clinic, apparently was the index case (Fig. 1, lane 1) preceding subsequent five cases (lanes 2–6) of MRSA infections in 2-week period. Because no attempt was made to obtain environmental specimens from the local clinic, the obvious evidence of inter-hospital transmission was not

Fig. 1. PFGE of *Sma*I-digested genomic fragments of 12 MRSA isolates from hospitalized dogs (Lanes 1–12). M indicates the lambda ladder DNA concatemers as molecular size marker (kb).

Table 3. Characteristics of the 12 MRSA isolates included in the study

Isolates no.	Date of culture	Underlying disorder	Specimen
1	Apr. 1998	Distemper	Conjunctiva
2	Apr. 1998	Intestinal intussusception	Anterior nare
3	Apr. 1998	Chronic renal failure	Anterior nare
4	Apr. 1998	Parvovirus infection	Catheter
5	Apr. 1998	Pneumothorax	Catheter
6	Apr. 1998	Chronic renal failure	Catheter
7	Jul. 1998	Intestinal foreign body	Postoperative wound infection
8	Jul. 1998	Bronchitis	Anterior nare
9	Jul. 1998	Diabetes mellitus	Anterior nare
10	Jul. 1998	Intestinal intussusception	Anterior nare
11	Oct. 1998	Pneumonia	Anterior nare
12	Oct. 1998	Recurrent pyoderma	Skin lesion

identified. Three-month after the first episode a poodle with intestinal obstruction was transferred, and he showed culture positive from the wound site after surgery (lane 7). During his stay, another three dogs (lanes 8–10) showed culture positive. The remaining two MRSA were isolated from those that were hospitalized in October with pneumonia (lane 11) and recurrent pyoderma (lane 12), respectively.

With these results, we demonstrated the presence of several different strains of MRSA in our hospital, and genomic DNA fingerprinting by PFGE could be powerful methods for differentiation of the MRSA-associated infections. Unlike in human medicine [28, 36] little information is available about the route of transmission in veterinary medicine. To understand whole spectrum of

epidemiology of the MRSA in dogs, it is necessary to study on the relationships between dogs and their human contacts.

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