

Study on the Methicillin-resistant Gene Distribution of Staphylococci Isolated from Dogs and Cats

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Abstract : Although, in human medicine, strains of methicillin-resistant staphylococci have become the most important causative agents of nosocomial infections, studies on the small animals are very limited. The aim of this study was to determine *mecA* gene and susceptibility to antibiotics of staphylococci strains isolated from clinically ill or healthy dogs and cats, during the period August 2002-July 2003. A total of 136 staphylococci (87 coagulase-positive and 49 coagulase-negative) were investigated for antibiotic resistance, using disk diffusion and minimum inhibitory concentration (MIC) test. The *mecA* gene was detected using the polymerase chain reaction. The isolates belonged to the species *S. aureus* (53 isolates), *S. intermedius* (34 isolates), *S. epidermidis* (26 isolates) and other coagulase-negative staphylococci (CNS, 23 isolates). Of the 136 isolates, 43 (31.6%) were *mecA*-positive and the frequency of the presence of *mecA* gene varied among the different species. All *S. aureus* strains were *mecA*-negative and were found to be susceptible, with an oxacillin MIC $\leq 1 \mu\text{g/ml}$. Five (13.6%) isolates of 36 that exhibited oxacillin resistance on the MIC testing were found to be *mecA*-negative, suggesting not all *mecA*-positive strains may be an oxacillin resistant. However, the *mecA* presence of the strains was correlated with high oxacillin resistance: 71.4% (10 isolates of 14; $P < 0.001$) for *mecA*-positive *S. intermedius* and 72.4% (21 isolates of 29; $P < 0.001$) for *mecA*-positive CNS isolates. About 69% (94 isolates of 136) showed resistance to at least one drug, and 22.8% (31 isolates) were resistant to four or more different drug classes. Resistance (36 isolates, 71.7%) to penicillin G was a common finding. This study suggest that the *mecA*-positive staphylococci are prevalent in small animals, and selection of antibiotics to treat infections caused by *mecA*-positive staphylococci may be very limited because of multi-drug resistance.

Key words : Methicillin-resistant, staphylococci, *mecA* gene

Introduction

Staphylococcal strains are frequently found in the animal hospital environments¹⁵. Since 1960s, methicillin resistance in staphylococci have been posed an important universal problem particularly in human hospital settings⁵. The infections they cause are serious and difficult to treat because they are resistant to multiple antibiotics. In addition, methicillin resistant *S. aureus* (MRSA) cause an overall increase in the incidence of nosocomial staphylococcal infections¹⁴.

Infections caused by coagulase negative staphylococci (CNS) in human beings were almost always hospital-acquired and were mainly attributed to the adverse consequences in invasive therapeutic procedures and prosthetic surgical implant devices including indwelling catheters¹², or severe underlying disease²¹. Although *Staphylococcus* species other than *S. intermedius* have rarely been associated with specific disorders in dogs and cats, some of CNS strains may be considered as potential pathogen in dogs, as they have been frequently isolated from deep pyoderma¹⁶. Furthermore, the number of *mecA*-bearing methicillin-resistant strains has been increased among CNS, including *S. epidermidis*^{6,22} and are increasingly recognized as true nosocomial pathogens in specific circumstances^{6,25}.

It has been established that MRSA strains carry *mecA* gene which encodes penicillin-binding protein 2a. This protein is responsible for methicillin resistance in staphylococci^{4,13,23,26}. Although several studies revealed that *mecA* gene is distributed widely among methicillin-susceptible *S. aureus*, the distribution of these genes and antimicrobial susceptibility of the staphylococci isolated from animal populations have not been fully established.

The first aim of this study was to examine the species distribution of staphylococci from clinical specimens and to determine antimicrobial susceptibility to methicillin and other antimicrobials among the isolates. Secondly, to determine whether or not the presence of *mecA* gene in isolates were associated with the level of antibiotic resistance, as observed for certain *S. aureus* strains¹.

Materials and Methods

Bacterial Strains, Sample Processing and Identification of Isolates

A total of 136 staphylococci isolates (116 dogs, 20 cats), that had been collected between August 2002-July 2003 from two animal clinics (Chuncheon, Kangwon-Do), were included in the study. They were isolated from clinical specimens: wounds (n = 47, 34.6%), intravenous catheters (n = 9, 6.6%), urine cultures (n = 14, 10.3%), conjunctiva (n = 27, 19.9%), anterior nares (n = 24, 17.6%), and blood cultures (n = 15,

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11%). All the specimens were inoculated in Brain Heart Infusion broth (Difco) and incubated at 37°C. If growth occurred after 24 or 48 h, Gram-stains were made and examined microscopically for organisms. Colonies with morphology compatible with *Staphylococcus* species were transferred to Tryptic Soy agar (Difco), 5% bovine blood agar and Manitol Salt agar (Merck). Staphylococci were identified on the basis of colony characteristics, Gram stain, pigment production, hemolysis on 5% bovine blood agar, catalase activity, tube coagulase test (rabbit plasma), mannitol fermentation, urease, novobiocin resistance and deoxyribonuclease test^{11,20}. Isolates of other bacterial genera were not considered in this study.

Antibiotic Resistance Phenotypes

All the staphylococci isolates were tested for susceptibility to antimicrobial agents by the disk diffusion method on Muller-Hinton agar (Difco) and incubation at 37°C, as previously described^{5,18}. Antibiotic susceptibility tests were performed against the following antibiotics: penicillin G (10 units), gentamicin (10 µg), oxacillin (5 µg), rifampin (10 µg), erythromycin (15 µg), enrofloxacin (5 µg), tetracycline (30 µg), clindamycin (2 µg), trimethoprim-sulfamethoxazole (25 µg) and vancomycin (30 µg), by employing commercial disks (BBL). After measuring the antimicrobial zone diameters the strains were categorized as susceptible or resistant to the drug, according to the instructions of the manufacturers of the antibiotic disks.

Minimum Inhibitory Concentration

Minimum inhibitory concentrations (MICs) were determined by broth microdilution with cation-supplemented Mueller-Hinton broth containing 2% NaCl. Bacteria were inoculated at a final density of about 5×10^5 CFU/ml and incubated at 35°C for 24 h before MICs were determined. The breakpoints for susceptibility and resistance of oxacillin were ≤ 2 µg/ml and ≥ 4 µg/ml, respectively.

Preparation of Chromosomal DNA and PCR Amplification

Total genomic DNA was prepared using the methods as described by Wilson²⁸ with a little modifications. The procedures were described previously in detail by the author¹⁹. The *mecA* gene was detected by PCR using a pair of primers described previously². Five µl of template DNA was added to 50 µl of the PCR reaction mixture that contained 5 µl of $10 \times$ Taq DNA polymerase buffer (500 mM KCl, 100 mM Tris-HCl, 1% Triton X-100, Promega), 6 µl of 25 mM MgCl₂, 2 µl of 12.5 mg/ml BSA, 1 µl of 10 mM dNTP (dATP, dCTP, dGTP, dTTP), 1 µl of 5 U/µl Taq polymerase (Promega), 27 µl of sterilized DW and 100 pM each of a pair of primers. After predenaturation at 94°C for 2 min, the reactions were allowed to proceed with 30 cycles of denaturation (94°C, 30 s), annealing (57°C, 40 s), and extension (72°C, 90 s). Ten of the PCR amplification product mixed with 1 µl of gel loading buffer (0.25% bromophenol blue tracking dye in 25% Ficoll) was analyzed by electrophoresis on 1.5% agarose gel containing 0.5 µg/ml ethidium bromide for 30 min at 100 V in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) and visualized with UV light. A 123-bp and 1-kbp ladder (Gibco, BRL) were used as molecular size markers.

Results

The distribution of *mecA* by different staphylococci species are shown in Table 1. Among the 136 isolates, *S. aureus* accounted for 39% (53 isolates) followed by *S. intermedius* 25% (34 isolates), *S. epidermidis* 19% (26 isolates) and other CNS 17% (23 isolates). The *mecA* gene was detected in 43 (31.6%) isolates. Detection rate was highest in *S. epidermidis* (16/26, 61.5%), followed by *S. hemolyticus* (11/18, 61.1%), *S. simulans*, (1/2, 50%), *S. intermedius* (14/34, 41.2%). All isolates of *S. aureus* were oxacillin susceptible with *mecA*-

Table 1. Detection of *mecA* gene in different species of staphylococci and their resistance level to oxacillin

Species	Presence of <i>mecA</i> gene	No. of strains*		Total	% <i>mecA</i> positive
		Susceptible	Resistant		
<i>S. aureus</i>	+	0	0	0	0
	-	53	0	53	
<i>S. intermedius</i>	+	4	10	14	41.2
	-	18	2	20	
<i>S. epidermidis</i>	+	5	11	16	61.5
	-	9	1	10	
<i>S. simulans</i>	+	0	1	1	50.0
	-	1	0	1	
<i>S. saprophyticus</i>	+	1	0	1	33.3
	-	2	0	2	
<i>S. hemolyticus</i>	+	2	9	11	61.1
	-	5	2	7	

*The breakpoints for susceptibility and resistance of oxacillin were ≤ 2 µg/ml / and ≥ 4 µg/ml, respectively.

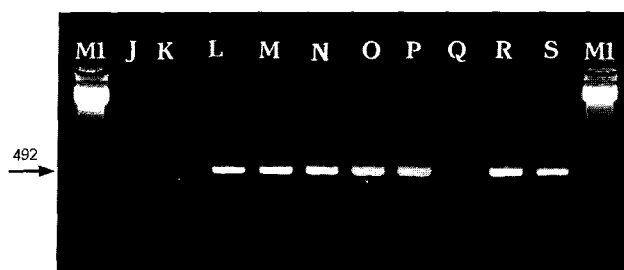


Fig 1. Amplification of *mecA* gene (533-bp) from staphylococci species. Lanes: J, K, Q for *mecA*-negative staphylococci; M, N, O, P, R, S for *mecA*-positive staphylococci; L for *mecA*-positive reference strain (ATCC 33519); M1 for 123-bp molecular size marker.

negative. Some selected isolates produced positive result in PCR for *mecA* with DNA fragment of 533-bp are shown in Fig 1.

Table 2 shows the MIC of oxacillin for the *mecA*-positive (*S. intermedius*) or -negative isolates. All *S. aureus* showed oxacillin MIC ≤ 1 $\mu\text{g/ml}$. Not all *mecA*-positive strains showed oxacillin resistant; 5 (13.6%) of 36 isolates that exhibited oxacillin resistance on the MIC testing were found to be *mecA*-negative. Of 29 *mecA*-positive CNS isolates, 8 (27.6%) were classified as susceptible (MIC, 2 $\mu\text{g/ml}$), whereas 17 (85%) of 20 *mecA*-negative CNS isolates showed oxacillin susceptible (MIC, ≤ 1 $\mu\text{g/ml}$).

The results of the antimicrobial susceptibility test for each isolates are presented in Table 3 and 4. Ninety four (69.1%) isolates of 136 showed resistance to at least one drug and 31 (22.8%) were resistant to four or more different drug classes. Resistance to penicillin G was a common finding and was observed in 36 (71.7%) of the 136 isolates. Coagulase-positive strains were more frequently resistant to the majority of the antibiotics (78.2%) than CNS (65.3%) strains. The most active antimicrobial agents against staphylococci was gentamicin, with only 33 (24.3%) isolates showing resistance to

Table 2. Oxacillin MICs for coagulase-positive (*S. intermedius*) and coagulase-negative staphylococci (CNS) strains by positive (+) or negative (-) for *mecA* in PCR

MIC ($\mu\text{g/ml}$)	<i>S. intermedius</i>		CNS*	
	<i>mecA</i> (+) (n=14)	<i>mecA</i> (-) (n=20)	<i>mecA</i> (+) (n=29)	<i>mecA</i> (-) (n=20)
32	1	0	3	0
16	2	0	6	0
8	4	2	4	0
4	3	0	8	3
2	0	1	1	0
1	1	3	0	12
0.5	1	12	1	1
<0.5	2	2	6	4

All *S. aureus* strains were susceptible, with an oxacillin MIC, ≤ 1 $\mu\text{g/ml}$.

*CNSs were *S. epidermidis*, *S. simulans*, *S. saprophyticus*, and *S. hemolyticus*.

Table 3. Resistant phenotypes of the staphylococci isolates to four or more antibiotics

Organism	No. of resistant samples to ≥ 1 antibiotics (% resistance)	Resistance pattern (no. of isolates)
<i>S. aureus</i> (n=53)	39 (73.6)	PN*, GN, RF, ER, CN (11) PN, GN, EN, TMP/SMX (3) PN, GN, TT, CN, TMP/SMX (1)
<i>S. intermedius</i> (n=34)	28 (82.4)	PN, OX, EN, TT (6) PN, GN, OX, TMP/SMX (1)
<i>S. epidermidis</i> (n=26)	14 (53.8)	PN, OX, RF, TT, TMP/SMX (3) PN, GN, ER, RF, TMP/SMX (1)
<i>S. simulans</i> (n=2)	1 (50)	PN, GN, ER, CN (1)
<i>S. saprophyticus</i> (n=3)	1 (33.3)	PN, GN, EN, CN (1)
<i>S. hemolyticus</i> (n=18)	11 (61.1)	PN, OX, ER, RF, (2) PN, OX, ER, TT, TMP/SMX (1)
Total (n=136)	94 (69.1)	31

*PN, penicillin G; GN, gentamicin; OX, oxacillin; RF, rifampin; ER, erythromycin; EN, enrofloxacin; TT, tetracycline; CN, clindamycin; TMP/SMX, trimethoprim-sulfamethoxazole.

Table 4. Susceptibility rate of coagulase-positive (COP, n=87) and coagulase-negative (CNS, n=49) staphylococci against antimicrobial groups

Chemical group*	No. of susceptible isolates		Susceptibility (%)	p-value**
	COP (%)	CNS (%)		
Penicillin	19 (21.8)	17 (34.7)	28.3	<0.05
Aminoglycoside	63 (72.4)	40 (81.6)	77.0	NS
Macrolide	64 (73.3)	38 (78.5)	75.9	NS
Quinolone	61 (69.8)	34 (70.3)	70.1	NS
Tetracycline	65 (74.6)	34 (69.4)	72.0	NS
Lincomycin	62 (70.4)	40 (78.9)	74.7	NS

*Penicillin, penicillin G; Aminoglycoside, gentamicin; Macrolide, erythromycin; Quinolone, enrofloxacin; Tetracycline, tetracycline; Lincomycin, clindamycin.

**Comparison between COP and CNS.

that drug.

Discussion and Conclusion

Because staphylococcal strains are recognized as normal microflora of the skin of human beings, cattle, dogs and cats^{7,8,15,26}, it is difficult to determine the pathogenicity of the strains isolated from a variety of clinical specimens. The coagulase-positive strains of staphylococci were the most prevalent in this study, isolated from 64% of the samples. Only 2 isolates of 49 CNS were *S. simulans* isolated from cats. Based on the literatures, this strain was the most prevalent staphylococcal species at various sites of clinically healthy cats^{7,8}.

It has been claimed that determination of methicillin resistance by susceptibility testing should be supported by *mecA* gene detection to improve identification of methicillin resistant staphylococci in the clinical laboratory^{5,17}. If applied for direct identification of *S. aureus* in clinical specimens or blood cultures, detection of the *mecA* gene will perform better than the traditional culture methods, which frequently fail even with *mecA*-negative *S. aureus*²⁰. The PCR assay has proved very useful for detection of methicillin resistant strains of *S. aureus* when the conventional methods had given equivocal results^{24,27}. Correlation between the presence of *mecA* gene and the level of resistance to oxacillin has not been fully evaluated. This study revealed that the *mecA* presence of the strains was, in general, significantly associated with high oxacillin resistance: 71.4% (10 isolates of 14; $P < 0.001$) for *mecA*-positive *S. intermedius* and 72.4% (21 isolates of 29; $P < 0.001$) for *mecA*-positive CNS isolates. However, this relationship may vary depending on the characteristics of bacterial strains, which largely caused by different resistant mechanisms between them. For example, of 14 *mecA*-positive *S. intermedius* isolates, 4 (28.6%) were classified as susceptible (Table 1) and 2 strains of *S. intermedius* which did not have the *mecA* gene were resistant to oxacillin, suggesting that different resistant mechanisms may be involved in methicillin-resistance among strains. Similar phenomena

were also observed in other studies^{17,27}. All *S. aureus* strains were *mecA*-negative and were found oxacillin susceptible simultaneously. Studies on the prevalence of *mecA* gene in small animals were very limited. A study⁹ reported that 23 of the 25 methicillin-resistant isolates and none of the methicillin-susceptible isolates possessed the *mecA* gene.

In this study, overall antibiotic resistance rate ranged 24-30%, with the exception of resistance (71.7%) to penicillin G. About 23% of the isolates showed multi-drug resistance. This resistance pattern was relatively higher than those of other studies^{15,16,25}. Interestingly, the most active antimicrobial agents against the isolates was gentamicin, represented by susceptibility of 77%: 72.4% for *S. intermedius* and 81.6% for CNS isolates. In a report¹⁹ studied in a university animal hospital, this antibiotic showed the least susceptibility of 56.3% against *S. epidermidis* among 14 antibiotics examined. Further clinical evaluation on susceptibility by regional variation among domestic animal hospital environments is required.

It is well known that methicillin resistance in staphylococci may evade detection by standard susceptibility testing, partially due to the heterogenous expression of *mecA*^{5,10,17}. In this study, 12 (27.9%; 4 *S. intermedius* and 8 CNS isolates) of 43 *mecA*-positive isolates were *mecA*-positive but oxacillin susceptible. Brakstad *et al*³ reported similar results in which some *mecA*-positive *S. aureus* strains were oxacillin resistant with high oxacillin MICs, but were negative for penicillinase production. They explained this characteristic attributed by a variety environmental and genetic regulatory factors with a negative influence on the gene expression.

In summary, *mecA* gene was distributed widely among a variety of staphylococcal species isolated from dogs and cats. Based on the MIC testing, some strains showed multi-drug resistance, and *mecA*-positive strains were more likely resistant to some selected antibiotics examined. In addition compared to CNS isolates, coagulase-positive strains tended to show a high resistance. The prospect of emerging methicillin resistant staphylococci in animal population is particularly challenging.

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개와 고양이에서 분리된 메티실린 내성 포도상구균의 내성인자 분포조사

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요약 : 인의학에서 메티실린 내성 포도상구균주는 병원감염의 주요 원인균으로 보고되고 있지만 소동물에서 이에 대한 연구는 거의 없다. 본 연구에서는 2002년 8월부터 2003년 7월까지 개와 고양이에서 분리된 136개의 포도상구균 분리주 (coagulase 양성 87주, coagulase 음성 49주)에 대하여 항생제 감수성 검사와 이들 분리주에서 메티실린 내성 유전자인 *mecA* 분포상황을 조사하였다. 136개 분리주중 43주 (31.6%)가 *mecA* 유전자를 가지고 있었고, 유전자의 분포율은 균주에 따라 상당한 차이를 보였다. 43주의 *mecA* 양성균주 중 31주 (72.1%)가 oxacillin 내성을 보여 *mecA* 양성균주가 반드시 oxacillin 내성과 일치하는 것은 아님을 시사하였다. 그러나 *mecA* 양성균주일수록 oxacillin 내성율이 높았는데 *S. intermedius*의 71.4% ($p < 0.001$), coagulase 음성균주의 경우 72.4%가 내성을 보였다 ($p < 0.001$). 분리주의 94주(69%)가 적어도 하나 이상의 항생제에 내성을 보였고 특히 31주(22.8%)는 4가지 이상의 항생제에 동시에 내성을 보였다. Penicillin 항생제에 내성율이 71.7%로 가장 높았다. 본 연구는 국내 소동물에서 *mecA* 양성균주가 존재하며, 이러한 균에 의해 유도된 감염증을 치료할 때 다제내성의 특성 때문에 항생제 선택의 폭이 매우 제한될 수 있음을 시사한다.

주요어 : Methicillin 내성, 포도상구균, *mecA* 유전자