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Antimicrobial Resistance of Methicillin-Resistant Staphylococci Isolates from Dog Ears in Korea

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Abstract : Methicillin-resistant staphylococci (MRS), which are often multi-drug resistant (MDR), are important pathogens in both human and veterinary healthcare. The purpose of this study was to characterize the antimicrobial resistance of MRS isolated from dog ears in Korea. From 827 dog ears, staphylococci were cultured from both ears with otitis externa (n = 161, 41.0%) and healthy ears (n = 135, 31.1%). The prevalence of coagulase-positive staphylococci (CoPS) in ears with otitis externa (58.4%) was significantly higher ($p < 0.05$) than in healthy ears (28.2%), while the prevalence of coagulase-negative staphylococci (CoNS) in healthy ears (74.8%) was higher ($P < 0.05$) than in ears with otitis externa (41.6%). Forty-six (35.1%) and 74 (44.8%) CoPS and CoNS isolates, respectively, were determined to be MRS. Antimicrobial resistance in MRS was most frequently observed for penicillin (76.7%), ampicillin (61.7%), kanamycin (61.7%), erythromycin (47.5%), tetracycline (47.5%), and trimethoprim/sulfamethoxazole (46.7%). Overall, the MDR isolates were resistant to significantly more ($P < 0.05$) antimicrobial agents tested than methicillin-sensitive staphylococci in this study. These results provide therapeutic guidelines for the treatment of otitis externa in dogs from Korean veterinary hospitals, and the significant associated health concern to companion animals and their human contacts.

Key words **:** Methicillin-resistant staphylococci, multi-drug resistant, dog ears.

Introduction

Staphylococcus species are present on the skin and mucosal membranes, and cause a variety of opportunistic infections in humans and animals. These species can be divided into coagulase-positive staphylococci (CoPS) and coagulase-negative staphylococci (CoNS). CoPS cause more severe skin and ear infections, including otitis externa, compared to CoNS (24). Although CoNS are typically not considered pathogenic, they have emerged as important reservoirs of antimicrobial resistance genes (23).

Otitis externa is a frequent problem in dogs, and *Staphylococcus* spp. are the most prevalent bacterial species in both dog ears with otitis externa (22) and healthy dog ears (29). Otitis externa is initially treated empirically, where the antimicrobial agent used for treatment is chosen based on the organisms observed upon cytological examination. However, the continued emergence of antimicrobial resistance is the primary concern when treating staphylococcal infections. Methicillin-resistant staphylococci (MRS) are important pathogens in both human and veterinary healthcare, and are often multi-drug resistant (MDR), which limits therapeutic options (26). MRS are a result of the acquisition of an altered penicillin-binding protein, PBP2a, which is encoded by *mecA* and catalyzes the synthesis of the bacterial cell wall in the presence of otherwise inhibitory concentrations of beta-lactam (1,15). The transfer of resistance among dogs and other domestic animals is possible, and these carrier animals are reported to act as reservoirs for MRS that can spread to other animals, as well as humans (18). Currently, methicillin-resistant *S. aureus* and CoNS are frequently observed and cause serious public health problems (12). The purpose of this study was to characterize the antimicrobial resistance of MRS isolated from dog ears in Korea.

Materials and Methods

Sample collection

Staphylococci were collected from 10 veterinary hospitals in 2016. In total, 827 client-owned dogs from a variety of breeds were assigned to 1 of 2 groups, where 434 dogs were in the cohort with healthy ears and no otitis externa and 393 dogs were in the group that had otitis externa. Following routine clinical examination, both ear canals of the dogs were aseptically swabbed prior to confirmation of otitis externa by otoscopic examination. Samples were transported to the bacteriology laboratory at ambient temperature and processed within 1 h of collection.

Bacterial isolation and identification

Swabs from both ears from each dog were inoculated into tryptic soy broth (TSB; Difco, USA) containing 10% NaCl and these inoculated cultures were incubated at 37°C for 18-24 h. Subsequently, one loopful of each TSB inoculate was

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Table 1. Oligonucleotide primers used for species identification of staphylococci from dog ears

Identification	Primer name	Sequence (5' to 3')	Amplicon size (bp)	Reference
Staphylococus spp.	Sta-F	GGCCGTGTTGAACGTGGTCAAATCA	370	(20)
	Sta-R	TIACCATTTCAGTACCTTCTGGTAA		
S. xylosus	S-xy-F	AACGCGCAACGTGATAAAATTAATG	539	(20)
	S-xy-R	AACGCGCAACAGCAATTACG		
S. saprophyticus	S-sap-F	ACGGGCGTCCACAAAATCAATAGGA	221	(20)
	S-sap-R	TCAAAAAGTTTTCTAAAAAATTTAC		
S. capitis	S-cap-F	ACTACGCCTATGATTATTGC	525	(13)
	S-cap-R	GAYGCTTCTTTACCATAGGG		
S. caprae	S-rae-F	TTGTTCTWGCACTYATTGCG	1,227	(13)
	S-rae-R	TTTTATAGAACAGGGTCGAC		
S. epidermidis	S-epi-F	TTGTAAACCATTCTGGACCG	251	(13)
	S-epi-R	ATGCGTGAGATACTTCTTCG		
S. hominis	S-hom-F	TACAGGGCCATTTAAAGACG	177	(13)
	S-hom-R	GTTTCTGGTGTATCAACACC		
S. haemolyticus	S-hae-F	GGTCGCTTAGTCGGAACAAT	271	(25)
	S-hae-R	CACGAGCAATCTCATCACCT		
S. pseudintermedius	S-pse-F	TAGGCAGTAGGATTCGTTAA	926	(24)
	S-pse-R	CTTTTGTGCTTCCTTTTGG		
S. schleiferi	S-sch-F	AATGGCTACAATGATAATCACTAA	526	(24)
	S-sch-R	CATATCTGTCTTTCGGCGCG		
S. delphini group A	S-dea-F	TGAAGGCATATTGTAGAACAA	661	(24)
	S-dea-R	CGRTACTTTTCGTTAGGTCG		
S. delphini group B	S-deb-F	GGAAGRTTCGTTTTTCCTAGAC	1,135	(24)
	S-deb-R	TATGCGATTCAAGAACTGA		
S. intermedius	S-in-F	CAATGGAGATGGCCCTTTTA	125	(2)
	S-in-R	AGCGTACACGTTCATCTTG		
S. aureus	clfA-F	GCAAAATCCAGCACAACAGGAAACGA	638	(16)
	clfA-R	CTTGATCTCCAGCCATAATTGGTGG		

streaked onto Baird-Parker agar plates (Oxoid, Basingstoke, UK) supplemented with an egg yolk-tellurite emulsion (Oxoid). After incubating for 48 h at 37°C, two staphylococcal-like colonies were transferred from these plates to blood agar containing 5% sheep blood. Staphylococci were identified based on colony morphology and standard techniques, including Gram staining, tests for catalase and coagulase activity, and phenotype when grown on mannitol (bioTRADING, the Netherlands) (3). Isolate identities were confirmed at the genus level by PCR (Table 1). *S. shleiferi* was identified and confirmed to the subspecies level based on a coagulase test using rabbit serum (24). If two isolates from the same sample appeared to be the same species with the same pattern of antimicrobial susceptibility, then one isolate was randomly chosen to be included in this study.

Antimicrobial susceptibility tests

Antimicrobial resistance of the staphylococci isolates was evaluated using the disc diffusion test with antimicrobial discs (BD, Biosciences) for 25 antibiotics. These antibiotics were penicillin (10 unit), ampicillin (10 μg), amoxicillin-clavulanic acid (20/10 μg), cefazolin (30 μg), cephalothin (30 μg), cefuroxime (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), cefepime (30 μg), cefoxitin (30 μg), vancomycin (30 μg), teicoplanin (30 μg), gentamicin (10 μg), amikacin (30 μg), kanamycin (30 μg), erythromycin (15 μg), tetracycline (30 μg), doxycycline (30 μg), ciprofloxacin (5 μg), norfloxacin (10 μg), nitrofurantoin (300 μg), clindamycin 2 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), chloramphenicol (30 μg), and rifampin (5 μg). To identify MRS, the minimal inhibitory concentration of oxacillin (Oxoid) was determined using the agar dilution method. The results were interpreted according to Clinical and Laboratory Standards Institute guidelines (7). MDR was defined as acquired non-susceptibility to at least 1 agent in 3 or more antimicrobial categories. The reference strains *S. aureus* ATCC 25922 and *S. intermedius* ATCC 29663 were used for quality control when determining minimum inhibitory concentration and zone diameter.

Detection of *mecA*

To confirm MRS, the presence of *mec*A was detected by PCR using the primers mecA-F (5'-TCCAGATTACAACT-

Table 2. Prevalence of staphylococci and frequency of methicillin-resistant staphylococci from dog ears

Discrimination	Group		Frequency of MRS* (%)	Frequency of *mecA* gene (%)
	Otitis externa	Healthy ear		
No. of dogs tested	393	434	-	-
No. of staphylococci positive dogs (%)	105 (26.7)	87 (20.0)	-	-
No. of staphylococci isolated (%)	161 (41.0)	135 (31.1)	120 (40.5)	45 (15.2)
Coagluase positive Staphylococci (%)	94 (58.4)[a]	37 (28.2)	46 (35.1)	7 (5.3)
S. aureus	0 (0.0)	0 (0.0)	-	-
S. pseudintermedius	11 (6.8)	9 (6.7)	9 (45.0)	3 (15.0)
S. intermedius	8 (4.9)	12 (8.9)	8 (40.0)	2 (10.0)
S. schleiferi coagulans	11 (6.8)	2 (1.5)	0 (0.0)	0 (0.0)
S. delphini group A	0 (0.0)	0 (0.0)	-	-
S. delphini group B	0 (0.0)	0 (0.0)	-	-
Other Staphylococci	62 (38.5)	14 (10.4)	29 (38.2)	2 (2.6)
Coagluase negative Staphylococci (%)	67 (41.6)	98 (74.8)[a]	74 (44.8)	38 (23.0)
S. epidermidis	17 (10.6)	40 (30.5)	23 (40.4)	14 (24.6)
S. saprophyticus	4 (2.5)	9 (6.9)	11 (84.6)	7 (53.8)
S. capitis	2 (1.2)	7 (5.3)	1 (11.1)	0 (0.0)
S. schleiferi schleiferi	7 (4.3)	1 (0.8)	2 (25.0)	0 (0.0)
S. haemolyticus	2 (1.2)	3 (2.3)	4 (80.0)	4 (80.0)
S. hominis	1 (0.6)	1 (0.8)	1 (50.0)	1 (50.0)
S. caprae	0 (0.0)	0 (0.0)	-	-
Other staphylococci	34 (21.1)	37 (28.2)	32 (45.1)	12 (16.9)

*MRS, methicillin-resistant staphylococci.
[a]There were a significantly higher ($P < 0.05$) between otitis externa and healthy ear.

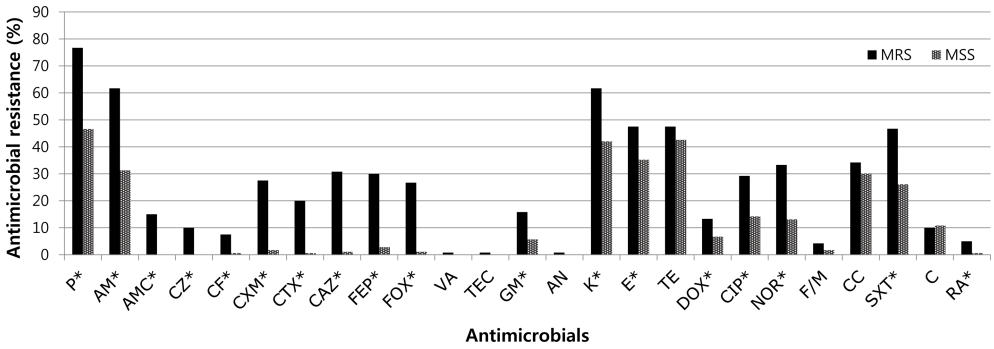

Fig 1. Comparison of antimicrobial resistance between methicillin-resistant staphylococci (MRS) and methicillin-sensitive staphylococci (MSS) against 25 different antimicrobials included 13 different antimicrobial classes by disk diffusion method. MRS, methicillin-resistant staphylococci; MSS, methicillin-sensitive staphylococci; P, penicillin; AM, ampicillin; AMC, amoxicillin-clavulanic acid; CZ, cefazolin; CF, cephalothin; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; FOX, cefoxitin; VA, vancomycin; TEC, teicoplanin; GM, gentamicin; AN, amikacin; K, kanamycin; E, erythromycin; TE, tetracycline; DOX, doxycycline; CIP, ciprofloxacin; NOR, norfloxacin; F/M, nitrofurantoin; CC, clindamycin; SXT, trimethoprim sulfamethoxazole; C, chloramphenicol; RA, rifampin.
*There were significantly differences ($P < 0.05$) between MRS and MSS.

TCACCAGG-3') and mecA-R (5'-CCACTTCATATCTTGT-AACG -3') as described previously (21).

Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences version 18.0 (SPSS; IBM, Korea). The chi-square ($\chi 2$) test was used for categorical variables, while Fisher's exact test was used to identify statistically significant differences in the number of antimicrobial classes between MRS and methicillin-sensitive staphylococci (MSS). $P < 0.05$ was considered statistically significant.

Results

The prevalence and distribution of staphylococci isolated from dog ears are shown in Table 2. Of 827 dogs, 192 (23.2%) were positive for staphylococci, and 296 (39.8%) staphylococci were cultured from the dog ears, including those with ears with otitis externa (n = 161, 41.0%) and healthy (n = 135, 31.1%). The prevalence of CoPS in the ears with otitis externa (58.4%) was significantly higher ($p < 0.05$) than in healthy ears (28.2%). Meanwhile, CoNS were more frequently isolated ($P < 0.05$) from healthy ears (74.8%) than

Table 3. Distribution of multi-drug resistant staphylococci from dog ears

No. of antimicrobial classes shown resistance	Otitis externa		Healthy ears		Total	
	MRS* (n = 59)	MSS** (n = 102)	MRS (n = 61)	MSS (n = 74)	MRS (n = 120)	MSS (n=176)
3	3 (5.1)	8 (7.8)	8 (13.1)	6 (8.1)	11 (9.2)	14 (8.0)
4	2 (3.4)	8 (7.8)	13 (21.3)	3 (4.1)	15 (12.5)	11 (6.3)
5	7 (11.9)	10 (9.8)	12 (19.7)	7 (9.5)	19 (15.8)	17 (9.7)
6	6 (10.2)	11 (10.8)	8 (13.1)	9 (12.2)	14 (11.7)	20 (11.4)
7	6 (10.2)	6 (5.9)	4 (6.6)	3 (4.1)	10 (8.3)	9 (5.1)
8	10 (16.9)	3 (2.9)	3 (4.9)	0 (0)	13 (10.8)	3 (1.7)
9	6 (10.2)	0 (0)	3 (4.9)	1 (1.4)	9 (7.5)	1 (0.6)
10	6 (10.2)	1 (1.0)	0 (0)	0 (0)	6 (5.0)	1 (0.6)
11	1 (1.7)	0 (0)	1 (1.6)	0 (0)	2 (1.7)	0 (0.0)
Total[a]	47 (79.7)	47 (46.1)	52 (85.2)	29 (39.2)	99 (82.5)	76 (43.2)

Multi-drug resistance was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories.
*MRS, methicillin-resistant staphylococci.
**MSS, methicillin- sensitive staphylococci.
[a]There were significantly difference ($P < 0.05$) between MRS and MSS.

those with otitis externa (41.6%). *S. pseudointermedius* was detected at a similar frequency in ears with otitis externa (n = 11, 6.8%) and healthy ears (n = 9, 6.7%). *S. epidermidis* was the primary staphylococci isolated from healthy ears (n = 40, 30.5%). Forty-six (35.1%) and 74 (44.8%) MRS isolates were confirmed to be CoPS and CoNS, respectively. However, *mecA* was detected in only 45 MDR isolates, where 7 were CoPS and 38 were CoNS.

A comparison of the antimicrobial resistance in the 120 MRS and 176 MSS isolates is presented in Fig 1. MRS strains most frequently displayed antimicrobial resistance to P (76.7%), Am (61.7%), K (61.7%), E (47.5%), TE (47.5%), and SXT (46.7%). The proportion of MRS isolates resistant to antimicrobials tested in this study was significantly higher ($P < 0.05$) than the proportion of MSS isolates, with the exception of for antimicrobials VA, TEC, AN, TE, F/M, CC, and C.

One hundred and seventy-five isolates (59.1%) were MDR against 3 or more antimicrobial classes (Table 3). Although the MDR population did not differ significantly between the isolates from ears with otitis externa and healthy ears, there was significantly more ($P < 0.05$) antimicrobial resistance in MRS isolates than MSS.

Discussion

Ear disease is one of the most common conditions in dogs, and staphylococci are among the most common isolates that may cause canine otitis externa (3,29). This study profiled the distribution and antimicrobial resistance of staphylococci isolated from dog ears at veterinary hospitals. In a previous study, Moon et al. (19) reported that the prevalence of staphylococci isolated from dog ears in Korea was 35.5%, which is similar to the prevalence observed in our study (35.8%). In this study, staphylococci were isolated from 31.1% of healthy ears. However, the proportion of staphylococci isolates from ears with otitis externa (41.0%) was significantly higher ($P < 0.05$) than that from healthy ears, as described by other authors (17,22).

CoPS are generally regarded as more pathogenic than CoNS (29). Similar to previous studies (18,28), CoPS were more prevalent than CoNS in dogs with otitis externa. By contrast, the prevalence of CoNS was higher than CoPS in healthy dog ears, which is consistent with previously published findings (26,27).

The prevalence of MRS (40.5%) in dog ears was higher (18.2%) than noted in a previous study in Korea (19). Moon et al. (19) reported the rate of MRS isolated from dogs, cats, and hospital staff and environments was 36.9%, while Jang et al. (14) reported MRS were isolated from 27.3% of dogs in veterinary hospitals. Although methicillin-resistant *S. aureus* was not found in this study, the number of MRS isolates obtained is likely an underestimation of the significance of the associated health concern to companion animals and their human contacts.

All 45 *mecA*-positive staphylococci isolates showed resistance to OX based on CLSI guidelines. Interestingly, 75 isolates lacking *mecA* were phenotypically resistant to OX. Eckholm et al. (10) and Schmidt et al. (26) also reported on some staphylococci strains with phenotypic methicillin resistance in the absence of *mecA*. In this study, these *mecA*-negative staphylococci were perhaps beta-lactamase hyper-producing isolates because the majority (89.3%, data not shown) remaining susceptible to AMC (28). Other alternative mechanisms associated with acquisition of modified PBPs are also a possibility (11).

The most prevalent MRS species isolated from dog ears was *S. epidermidis*. Kern et al. (15) reported methicillin-resistant *S. epidermidis* is the predominant methicillin-resistant CoNS species found in humans, and methicillin-resistant *S. epidermidis* can circulate between humans and animals. Moreover, the prevalence of *mecA*-positive CoPS (5.3%) was lower than *mecA*-positive CoNS (23.0%), as described in another study (6). The majority of *S. saprophyticus* and *S. haemolyticus* isolates were *mecA*-positive in this study, where the high prevalence of *mecA*-positive *S. haemolyticus* has been previously observed in companion animals (8,23). The findings of this study increase our understanding of the spread of antimicrobial resistance between animals and humans.

The resistance to the antimicrobials tested in this study was

significantly higher ($P < 0.05$) in MRS than MSS, and MRS isolates were more commonly MDR than MSS isolates. The cross-resistance most frequently found in MDR-MRS isolates was to penicillin, ampicillin, cephalosporins, kanamycin, and erythromycin. These resistance profiles are similar to those observed in other studies in several countries, including Korea (5,6,14,28). In many countries, the first-line antimicrobials used to treat otitis externa are β-lactams and fluoroquinolones. However, β-lactam antibiotics should not be used for MRS infections, irrespective of the susceptibility report (7). Furthermore, although resistance to cephalosporins was relatively less common in this study, most MDR-MRS strains were resistant to cephalosporins. In addition, except for β-lactams, the resistance to AMC, VA, TEC, GM, AN, DOX, F/N, C and RA was less frequent than for fluoroquinolones. Therefore, these antibiotics may be potential treatment options for otitis externa in Korea. Similar studies have reported that amoxicillin-clavulanic acid, gentamicin, and rifampin are effective in dogs (4,9,29). It is expected that the results of the present study will benefit the therapeutic guidelines for the treatment of otitis externa in dogs from Korean veterinary hospitals. In addition, as the prevalence of MRS and MDR has increased over time, monitoring and molecular epidemiologic studies should be regularly performed.

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