Antimicrobial Susceptibility and Methicillin Resistance in *Staphylococcus pseudintermedius* and *Staphylococcus schleiferi* subsp. *coagulans* Isolated from Dogs with Pyoderma in Japan

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ABSTRACT. To understand species distribution, trends of antimicrobial susceptibility and prevalence of methicillin resistance in canine staphylococci in Japan, 190 coagulase-positive staphylococci (CoPS) were isolated from dogs with pyoderma in 2 Japanese veterinary referral hospitals. Using a multiplex polymerase chain reaction (M-PCR) method, two CoPS species were identified: 170 *Staphylococcus pseudintermedius* (89.5%) and 20 *S. schleiferi* subsp. *coagulans* isolates (10.5%). In these isolates, susceptibility to 7 antimicrobial agents was determined. Overall, the levels of susceptibility to cefalexin (CEX), amoxicillin/clavulanic acid (CVA/AMPC), minocycline (MINO), ofloxacin (OFLX), norfloxacin (NFLX), lincomycin (LCM) and clindamycin (CLDM) in *S. pseudintermedius* isolates were 38.2, 52.4, 34.7, 31.2, 34.1, 1.2 and 11.2%, respectively. In *S. schleiferi* subsp. *coagulans* isolates, 55% demonstrated susceptibility to CEX, 80% to CVA/AMPC, 70% to MINO, 45% to OFLX or NFLX and 30% to CLDM. None of *S. schleiferi* subsp. *coagulans* isolates was susceptible to LCM. To determine the prevalence of methicillin-resistant strains, we used a PCR method, which enabled detection of the fragment of *mecA* gene in 66.5% (113 of 170) in *S. pseudintermedius* and 30.0% (6 of 20) in *S. schleiferi* subsp. *coagulans* isolates. The frequencies of susceptibility to CEX, CVA/AMPC, OFLX, NFLX and CLDM were significantly lower in methicillin-resistant CoPS than in methicillin-susceptible CoPS isolates. These data suggest a high level of methicillin resistance in staphylococci isolated from dogs with pyoderma in Japan.

KEY WORDS: antimicrobial susceptibility, canine pyoderma, methicillin-resistant staphylococci, minocycline, *Staphylococcus* species.

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Pyoderma is a common infectious skin disorder in dogs, and most of these cases are caused by staphylococcal strains [15]. Among them, several coagulase-positive staphylococcal (CoPS) species such as *Staphylococcus intermedius*, *S. schleiferi* and *S. aureus* have been isolated from the skin in canine pyoderma [3, 12, 22]. In particular, *S. intermedius* has been recognized to constitute normal skin flora and to occasionally cause a variety of infections such as pyoderma in dogs [4, 24]. However, recent reports indicated that phenotypically identified *S. intermedius* strains could include not only true *S. intermedius* strains but also novel CoPS species such as *S. pseudintermedius* and *S. delphini*, and in fact, most of them were identified as *S. pseudintermedius* strains, not *S. intermedius* [1, 31].

The treatment of canine pyoderma usually involves antimicrobial therapy. Above all, cefalexin has been recommended as the first choice in canine pyoderma therapy [25]. However, previous studies showed that multidrug-resistant and methicillin-resistant staphylococci are present in increasing frequencies in dogs [16, 22, 23, 30, 41]. These strains cause therapeutic failure in veterinary practices and, potentially, zoonotic problems [38]. However, there are few

recent publications concerning antimicrobial susceptibility and methicillin resistance in CoPS isolated from canine pyoderma in Japan.

The purpose of this study was to determine the frequency of antimicrobial susceptibility and prevalence of methicillin resistance in staphylococcal isolates, and to demonstrate the antimicrobials that are available to treat methicillin-resistant CoPS isolates from dogs with pyoderma in Japan.

MATERIALS AND METHODS

Bacterial isolates: Bacterial isolates from lesional skin of 186 clinical cases diagnosed with canine pyoderma were collected between June 2007 and April 2009 in ASC Dermatology Service (Tokyo, Japan) and Tokyo University of Agriculture and Technology, Animal Medical Center (Tokyo, Japan). A sterile BBL CultureSwab (Becton, Dickinson and Co., Franklin Lakes, NJ, U.S.A.) was used to sample from the skin. All swabs were transported to our laboratory, following placement in Stuart's transport medium (Becton, Dickinson and Co.). The methods to identify CoPS isolates were performed as previously described [10, 14] with partially modification. Swabs were inoculated by streaking onto mannitol salt agar plates (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). A single representative colony of each sample was isolated and pure cultured on

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nutrient agar plate (Nissui Pharmaceutical Co., Ltd.). Gram-positive cocci were tested with 3% hydrogen peroxide (Kanto Chemical Co., Inc., Tokyo, Japan) for catalase activity. Catalase-positive cocci were mixed with rabbit plasma (Kohjin Bio Co., Ltd., Saitama, Japan) and incubated overnight to test for coagulase production. Coagulase-positive isolates were stored in Microbank® (Pro-Lab Diagnostics Inc., Richmond Hill, ON, Canada) at -80°C until use.

Antimicrobial susceptibility testing: Antimicrobial susceptibility tests were performed using seven antibiotics commonly selected in Japanese veterinary dermatological practice: cefalexin (CEX), amoxicillin/clavulanic acid (CVA/AMPC), minocycline (MINO), ofloxacin (OFLX), norfloxacin (NFLX), lincomycin (LCM) and clindamycin (CLDM). All specimens were tested by the disc diffusion method, and classified as susceptible, intermediate or resistant following the Clinical and Laboratory Standards Institute (CLSI) guidelines [5].

DNA extraction: DNA extracts were obtained using QuickGene Mini80 system (Fujifilm Co., Tokyo, Japan) and QuickGene DNA Tissue Kit S (Fujifilm Co.) according to the manufacturer's recommendations.

Species identification using multiplex-polymerase chain reaction (M-PCR) method: M-PCR method for the identification of CoPS species was used in the present study as previously described [32]. DNA fragments were analyzed by electrophoresis in 0.5× TBE buffer on a 1.5% agarose gel stained with 0.5 µg/ml ethidium bromide. To evaluate the sensitivity and specificity, we performed the M-PCR method against type strains of six CoPS species and *S. epidermidis*, a coagulase-negative staphylococcal species, as a negative control (listed in Table 1). We used these type strains obtained from Gifu Type Culture Collection in the Graduate School of Medicine, Department of Microbiology Regeneration and Advanced Medical Science, Gifu University (Gifu, Japan).

Determination of methicillin resistance by detection of *mecA* gene: Primers for *mecA* PCR, mA1 (5' TGCTATC-CACCCTCAAACAGG 3') and mA2 (5' AACGTTG-TAACCACCCCAAGA 3') were used in the present study as previously described [27]. The PCR amplifications consisted of pre-denaturing (94°C for 2 min), and 30 cycles of denaturation (94°C for 1 min), annealing (60°C for 1 min)

and extension (72°C for 1 min), followed by a final extension (2 min). Electrophoretic analysis of PCR products (286 bp) was performed as described above. Strains that were *mecA*-positive or -negative were determined as methicillin-resistant (MR) or methicillin-susceptible (MS) strains, respectively.

Statistical analysis: Fisher's exact test was used to compare the prevalence of susceptibility to each antimicrobial between MR and MSCoPS strains. Differences were considered significant for $P < 0.05$.

RESULTS

Isolation and species identification of coagulase-positive staphylococci: One hundred and ninety CoPS were isolated from lesional skin of 186 dogs with pyoderma. Two CoPS species were identified by M-PCR in this study: 170 of *S. pseudintermedius* (89.5%) and 20 of *S. schleiferi* subsp. *coagulans* (10.5%). Other CoPS species such as *S. intermedius* and *S. aureus* were not isolated.

Antimicrobial susceptibility tests: The sensitivities of *S. pseudintermedius* and *S. schleiferi* subsp. *coagulans* isolates to individual antimicrobials are shown in Table 2. Less than 50% of *S. pseudintermedius* strains were susceptible to CEX, MINO, OFLX, NFLX, LCM and CLDM (1.2–38.2%). Sensitivity to CVA/AMPC was the highest (52.4%). The frequencies of susceptibility to CEX, CVA/AMPC and MINO among the *S. schleiferi* subsp. *coagulans* strains were 55, 80 and 70%, while 45, 45 and 30% of strains showed sensitivity to OFLX, NFLX and CLDM, respectively. None of the strains was found to be susceptible to LCM.

Prevalence of methicillin-resistant (MR) strains: The levels of prevalence of MR *S. pseudintermedius* (MRSP) and MR *S. schleiferi* subsp. *coagulans* (MRSS) were 66.5% (113/170) and 30.0% (6/20), respectively.

Difference in susceptibility between methicillin-resistant (MR) and methicillin-susceptible (MS) staphylococcal isolates: The proportions of strains susceptible to individual antimicrobials between MR and MSCoPS strains are listed in Table 3. The frequencies of susceptibility to CEX, CVA/AMPC, OFLX, NFLX and CLDM in MRCoPS strains were significantly lower than those of MSCoPS strains ($P < 0.01$). One of the 71 MSCoPS strains and one of the 119 MRCoPS strains were susceptible to LCM, and there was no significant difference in the susceptibility rates to MINO and LCM between MR and MSCoPS strains.

DISCUSSION

In the present study, *Staphylococcus pseudintermedius* was identified in canine pyoderma in 89.5% of total isolates. Since first reported [8], *S. pseudintermedius* has been thought to be a staphylococcal species of commensal flora and a major cause of skin infection including pyoderma [1, 31]. The results of this study support the assertion that *S. pseudintermedius* may be a major pathogen of canine pyoderma.

Table 1. Gifu Type Culture collection numbers of staphylococcal type strains used as quality controls in multiplex-PCR

Coagulase-positive staphylococci
<i>Staphylococcus intermedius</i> GTC266
<i>Staphylococcus aureus</i> GTC286
<i>Staphylococcus hyicus</i> GTC305
<i>Staphylococcus delphini</i> GTC476
<i>Staphylococcus pseudintermedius</i> GTC2867
<i>Staphylococcus schleiferi</i> subsp. <i>coagulans</i> GTC12628

Coagulase-negative staphylococci
<i>Staphylococcus epidermidis</i> GTC289

Table 2. Antimicrobial susceptibility (%) of CoPS isolated from canine pyoderma

Antimicrobials	<i>S. pseudintermedius</i> n=170			<i>S. schleiferi</i> subsp. <i>coagulans</i> n=20			Total n=190		
	S	I	R	S	I	R	S	I	R
Cefalexin	65 (38.2)	6 (3.5)	99 (58.2)	11 (55.0)	0 (0)	9 (45.0)	76 (40.0)	6 (3.2)	108 (56.8)
Amoxicillin/clavulanic acid	89 (52.4)	ND	81 (47.6)	16 (80.0)	ND	4 (20.0)	105 (55.3)	ND	85 (44.7)
Minocycline	59 (34.7)	65 (38.2)	46 (27.1)	14 (70.0)	6 (30.0)	0 (0)	73 (38.4)	71 (37.4)	46 (24.2)
Ofloxacin	53 (31.2)	10 (5.9)	107 (62.9)	9 (45.0)	2 (10.0)	9 (45.0)	62 (32.6)	11 (5.8)	117 (61.6)
Norfloxacin	58 (34.1)	8 (4.7)	104 (61.2)	9 (45.0)	2 (10.0)	9 (45.0)	67 (35.3)	9 (4.7)	114 (60.0)
Lincomycin	2 (1.2)	10 (5.9)	158 (92.9)	0 (0)	6 (30.0)	14 (70.0)	2 (1.1)	15 (7.9)	173 (91.1)
Clindamycin	19 (11.2)	30 (17.6)	121 (71.2)	6 (30.0)	12 (60.0)	2 (10.0)	25 (13.2)	41 (21.6)	124 (65.3)

S: susceptible; I: intermediate; R: resistant; ND: not determined. S, I, and R were classified according to Clinical and Laboratory Standards Institute guidelines.

Table 3. Antimicrobial susceptibility of methicillin-susceptible and -resistant staphylococci isolated from dogs with pyoderma

Antimicrobials	Number (%) of susceptible strains					
	MSSP (n=57)	MRSP (n=113)	MSSS (n=14)	MRSS (n=6)	MSCoPS* (n=71)	MRCoPS* (n=119)
Cefalexin	42 (73.7)	23 (20.4)	11 (78.6)	0 (0)	53 (74.6) ^a	23 (19.3) ^a
Amoxicillin/clavulanic acid	54 (94.7)	35 (31.0)	14 (100)	2 (33.3)	68 (95.8) ^a	37 (31.1) ^a
Minocycline	20 (35.1)	39 (34.5)	11 (78.6)	3 (50.0)	32 (45.1)	42 (35.3)
Ofloxacin	35 (61.4)	18 (15.9)	8 (57.1)	1 (16.7)	43 (60.6) ^a	19 (16.0) ^a
Norfloxacin	39 (68.4)	19 (16.8)	8 (57.1)	1 (16.7)	47 (66.2) ^a	20 (16.8) ^a
Lincomycin	1 (1.8)	1 (0.9)	0 (0)	0 (0)	1 (1.4)	1 (0.8)
Clindamycin	10 (17.5)	9 (8.0)	6 (42.9)	0 (0)	16 (22.5) ^b	9 (7.6) ^b

MSSP: methicillin-susceptible *S. pseudintermedius*. MRSP: methicillin-resistant *S. pseudintermedius*. MSSS: methicillin-susceptible *S. schleiferi* subsp. *coagulans*. MRSS: methicillin-resistant *S. schleiferi* subsp. *coagulans*. * MSCoPS and MRCoPS are equal to MSSP plus MSSS and MRSP plus MRSS, respectively. a) Different superscripts within columns indicate statistically significant difference ($P < 0.0001$). b) Different superscripts within columns indicate statistically significant difference ($P < 0.01$).

S. schleiferi subsp. *coagulans* were isolated with a frequency of 10.5% from the lesional skin of dogs in the present study. Although the organism was first isolated from external auditory meatus with canine otitis externa [19], recent studies reported that the organism has also been isolated from dogs with pyoderma [3, 12, 18]. Our results also suggest that this organism is the most important pathogen in canine pyoderma after *S. pseudintermedius*.

The frequency of methicillin-resistant *S. pseudintermedius* (MRSP) isolation in this study was markedly higher than that previously observed by other researchers who reported the existence of MRSP isolated from the skin of healthy and affected dogs [16, 29, 30]. All bacterial specimens in this study were collected from dogs in a veterinary teaching hospital and a veterinary dermatology clinic. Therefore, many of the samples were possibly isolated from cases of recurrent pyoderma. Furthermore, some of these cases may have received antimicrobial treatment in the past, although the details remain unclear. Consequently, owing to selective pressure, staphylococcal isolates in this study may have showed a higher than usual rate of methicillin resistance. The prevalence of MR *S. aureus* (MRSA) strains in human hospitals was found to be 66.8% in Japan [2]. These results demonstrated a high prevalence of MR strains in humans similar to that we identified in dogs.

The rates of antimicrobial susceptibility to CEX, CVA/

AMPC, OFLX and NFLX in MRCoPS isolates in this study were significantly lower than those in MSCoPS isolates. MR staphylococci produce an altered penicillin-binding protein (PBP 2' or PBP 2a) coded by the *mecA* gene, that has extremely lower affinity for binding β -lactams [17, 37]. Therefore, the PBP 2' makes bacteria resistant to all β -lactam antibiotics. In contrast, quinolone resistance in staphylococci is caused by mutations in the *gyrA*, *gyrB*, *griA* or *griB* genes coding DNA gyrase and topoisomerase, that are essential for bacterial DNA synthesis [20, 35]. There are some reports that MRSA isolates obtained from human hosts had mutations in these genes and these isolates were resistant against old generation quinolones such as NFLX or ciprofloxacin [6, 26, 33]. Similarly, Descloix *et al.* reported that all MRSP isolates from diseased dogs also had mutations in these genes together with resistance to enrofloxacin [7]. These reports and our results suggest that it is important to confirm the susceptibility to quinolones for appropriate use in treatment of MRCoPS infections in dogs.

In this study, there was no significant difference in the susceptibility to MINO between MR and MSCoPS isolates. Two mechanisms of resistance to tetracyclines have been identified in staphylococci: active drug efflux mechanism resulting from the acquisition of *tetK* and *tetL* genes, and ribosomal protections mediated by *tetM* or *tetO* genes [11, 21, 28]. In addition, it was also reported that *tetM*- or *tetK*-

and *tetM*-positive MRSA isolates had high levels of resistance to MINO [36]. However, several recent publications have reported that *in vitro* susceptibility of MINO against MRSA isolates from human hosts was significantly high as compared to other antimicrobials such as CLDM, CVA/AMPC or levofloxacin [9, 34, 40]. In fact, our data also indicate that the proportion of MRCoPS strains resistant to MINO was the lowest among those for seven antimicrobial agents (29.4% of all MRCoPS isolates, data not shown). In addition, the proportion of MRCoPS isolates that were classified with intermediate susceptibility to MINO accounted for 35.3% (42/119) of the total CoPS isolates in our study (data not shown). The efficacy of MINO against MRCoPS may be revealed by determination of their minimum inhibitory concentrations and identification of the *tet* genes in addition to our results.

The prevalence of susceptibility to LCM and CLDM was extremely low in both MR and MSCoPS isolates in our study. Similarly, Futagawa-Saito *et al.* indicated comparably low levels of susceptibility to LCM in staphylococci isolated from healthy (8/44) and diseased (36/44) dogs in Japan [13]. However, the incidence of susceptibility to LCM and CLDM reported by Vanni *et al.* was quite high in *Staphylococcus* spp., which were isolated from healthy (255/310) and diseased (55/310) dogs in Italy between 2006 and 2007 [39]. These investigations and our results suggest that the levels of susceptibility to LCM in staphylococcal isolates from diseased dogs are possibly lower than those from healthy dogs and, at least in Japan, there is a low frequency of susceptibility to lincosamides such as LCM and CLDM in staphylococcal isolates from diseased dogs, and Japanese veterinary clinicians should not expect therapeutic efficacy of lincosamides against canine pyoderma.

In conclusion, our results indicate a high level of resistance of CoPS isolates to the antimicrobials usually used for treatment against staphylococcal infections. Moreover, a high level of MRCoPS strains seems to be increasingly common in cases of canine pyoderma. However, there are significant differences in antimicrobial susceptibilities between staphylococcal species, and between MSCoPS and MRCoPS strains, especially for β -lactams and quinolones. This suggests the importance of performing antimicrobial susceptibility testing and confirming the presence of methicillin resistance among staphylococcal isolates from canine pyoderma.

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