Isolation and Characterization of Staphylococci from External Auditory Meatus of Dogs with or without Otitis Externa with Special Reference to *Staphylococcus schleiferi* subsp. *coagulans* Isolates

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ABSTRACT. Staphylococci were isolated from the external auditory meatus in 14 (48.3%) of 29 dogs affected with otitis externa (OE dogs) and 28 (68.3%) of 41 dogs without OE (non-OE dogs). Twenty-two OE isolates were identified as belonging to 12 species, and 42 non-OE isolates were identified as belonging to 13 species. The predominant species found in both OE and non-OE isolates were *S. intermedius*, and *S. epidermidis*. Thirty-eight (59.4%) of 64 isolates were resistant to one or more of the 17 antimicrobial agents tested. Resistance to PCG and ABPC was most frequent. *S. schleiferi* subsp. *coagulans*, a recent etiologic agent of canine OE, was isolated from OE and non-OE dogs. All of the 5 *S. schleiferi* subsp. *coagulans* isolates showed typical characteristics. No clear difference in the extracellular enzyme or toxin profiles, nor in the PFGE patterns, was demonstrated between the OE and non-OE isolates of *S. schleiferi* subsp. *coagulans*. A new PCR primer set specific for 16S rDNA was designed to identify strains of *S. schleiferi* subsp. *coagulans*. The amplified fragment was detected in all of the 5 isolates as well as the type strain GA 211 (=JCM 7470) and a reference strain GA 11, but was not detected in any strains of the related species, *S. aureus*, *S. intermedius* and *S. hyicus*. The PCR may allow a simple, rapid and precise identification of *S. schleiferi* subsp. *coagulans*, in addition to the standard tube test for free coagulase.

KEY WORDS: canine external ear, identification, PCR, *Staphylococcus schleiferi* subsp. *coagulans*, *Staphylococcus* species.

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Otitis externa (OE) is common in dogs and is a disease of multifarious etiology. The yeast and bacterium most frequently isolated from OE dogs are *Malassezia pachydermatis* and *Staphylococcus intermedius* [2, 11, 20, 23], respectively. Staphylococci including *S. intermedius* are present in the nasal vestibulum, external auditory meatus, intestinal and urinogenital tracts, anal mucosa, and on the skin surface of dogs. Knowledge of the composition of the normal staphylococcal flora in the external auditory meatus is an important factor in determining the etiology and epidemiology of staphylococcal OE in dogs. However, the ecology of the staphylococci inhabiting the external auditory meatus of healthy dogs has not yet been studied sufficiently.

Determination of the susceptibility of *Staphylococcus* species to antimicrobial agents is of great importance in the selective use of chemotherapeutics, the evaluation of new antimicrobial agents, and in the development of drug resistance through continuous use of antimicrobial agents against field isolates. However, there is little information concerning the antimicrobial susceptibility patterns of *Staphylococcus* species isolated from OE and non-OE dogs in Japan [18].

Recently, coagulase-positive *S. schleiferi* subsp. *coagu-*

lans has also been isolated from the external auditory meatus of OE dogs [8]. Subsequently, the organism was cultured from dogs with pyoderma [1, 5, 7] and from a finger-wound infection in a human patient [21]. Recognition of the clinical importance of *S. schleiferi* subsp. *coagulans* in veterinary and human medicine is growing. To our knowledge, there are no reports on the production of extracellular enzymes and toxins of putative virulence factors of the organism. Phenotypes of *S. schleiferi* subsp. *coagulans* are closely related to those of *S. intermedius* [8]. In routine laboratory practice, *S. schleiferi* subsp. *coagulans* can be distinguished from *S. intermedius* on the basis of its acetoin production and acid production from trehalose. However, more precise, rapid and simple identification systems are necessary to determine the etiology and epidemiology of *S. schleiferi* subsp. *coagulans* infections.

The present study was conducted to determine the species distribution and susceptibility to antimicrobial agents of staphylococci isolated from the external auditory meatus of OE dogs and non-OE dogs. We also examined the production of some extracellular active substances and the genotypes of *S. schleiferi* subsp. *coagulans* strains isolated in this study. In addition, we evaluated a PCR method designed to amplify species-specific sequences in the 16S rDNA so that the strains of *S. schleiferi* subsp. *coagulans* could be identified.

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MATERIALS AND METHODS

Specimens: Samples were collected from 135 external auditory meatus of 29 OE dogs (53 ears) and 41 non-OE dogs (82 ears). The dogs tested visited one or the other of two hospitals in Hokkaido and Okayama prefectures between 2001 and 2002. The isolation of staphylococci was performed using swabs taken from the external auditory meatus of each dog.

Isolation of staphylococci: Swab specimens were directly inoculated onto heart infusion agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 7.5% NaCl, and were incubated aerobically for 48 hr at 37°C with a further two or three days incubation at room temperature (20°C to 25°C). Colony characteristics (morphology and pigment) were recorded for each different staphylococcal isolate, and one representative colony of each colony type per specimen was picked and transferred to heart infusion agar.

Identification of *Staphylococcus* species: Staphylococci were identified on the basis of colony characteristics, Gram stain and morphology, catalase reaction, colony pigmentation, and anaerobic acid production from glucose. *Staphylococcus* species were identified using the coagulase test and the ID32 Staph system (Bio Merieux, S.A., France).

Antimicrobial susceptibility tests: Antimicrobial susceptibility tests were performed with a commercially available paper disk diffusion method (Showa Yakuhin Kako Co., Ltd., Tokyo, Japan) using Sensitivity disk agar-N (Nissui). The antimicrobial agents tested were as follows: benzylpenicillin (PCG), ampicillin (ABPC), cefazolin (CEZ), cefmetazole (CMZ), imipenem (IPM), streptomycin (SM), kanamycin (KM), gentamicin (GM), erythromycin (EM), lincomycin (LCM), tetracycline (TC), doxycycline (DOXY), chloramphenicol (CP), norfloxacin (NFLX), ciprofloxacin (CPFX), ofloxacin (OFLX), and vancomycin (VCM). Detection of the *mecA* gene of antimicrobial resistant strains was carried out using the PCR method as described previously [10].

Detection of extracellular enzymes and toxins: Detection of extracellular enzymes of *S. schleiferi* subsp. *coagulans* was performed by the method of Hájek [6] and Devriese *et al.* [3, 4]. Staphylococcal enterotoxins (SEs; SEA, SEB, SEC, and SED), toxic shock syndrome toxin-1 (TSST-1), and staphylococcal exfoliative toxins (ETs; ET-A and ET-B) were detected by the reversed-passive latex agglutination (RPLA) method using SET-RPLA, TSST-RPLA, and ET-RPLA (Denka Seiken Co., Ltd., Tokyo, Japan), respectively.

Phage typing: Phage typing was performed as described previously [9] using 14 phages of the typing set for *S. intermedius* strains.

PFGE analysis: Preparation of chromosomal DNA and cleavage of genomic DNA with *Sma*I (New England BioLabs, Beverly, Mass.) were performed as described previously [17]. PFGE was performed with a 1% agarose slab gel (Seakem GTG, FMC Bioproducts, Rockland, Me.) using a CHEF-DR II system (Bio-Rad Laboratories Inc, Hercules,

Calif.) in 0.5 × Tris-borate-EDTA maintained at 14°C. The running parameters used were as follows: initial pulse time, 5 sec; final pulse time, 40 sec; voltage, 6V/cm; running time, 22 hr. The PFGE patterns were interpreted based on the criteria of Tenover *et al.* [19].

16S rDNA specific PCR for identification of *S. schleiferi* subsp. *coagulans*: The bacteria were grown on a heart infusion agar (Nissui) plate at 37°C overnight. Bacterial DNA was extracted by a standard method as described previously [10]. A pair of primers was designed to amplify the 1369 bp region of the 16S rDNA of *S. schleiferi* subsp. *coagulans* (DDBJ accession number AB009945). Primer-1 (5'-GAACGGACAAGGAGCTTGCTCCTTTGAA-3') and primer-2 (5'-GTTACAAACTCTCGTGGTGTGAA-3') correspond to the nucleotide residues 61 to 88 and 1407 to 1429, respectively. The PCR was performed in a 20- μ l reaction volume. Each reaction mixture contained 1 μ l (50 to 150 ng) of the sample DNA solution, 0.02 μ M of each primer, 0.5 units of *Taq* DNA polymerase (TaKaRa *Taq*, TaKaRa Shuzo Co., Ltd., Shiga, Japan), and 200 μ M of each d NTP (TaKaRa) in a PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, TaKaRa]. The amplification reaction was carried out in a GeneAmp PCR System 9700 (Perkin-Elmer Applied Biosystems, U.S.A.) using the following program: 1 min at 94°C, followed by 25 cycles of 1 min at 94°C, 1 min at 57°C and 2 min at 72°C. The program was completed with an additional 7-min extension step at 72°C. After the amplification, 2 μ l of the reaction mixture was analyzed by electrophoresis on a 1.5% agarose gel in Tris-acetate-EDTA buffer at 100 V for 20 min and stained with ethidium bromide. The identification of *S. schleiferi* subsp. *coagulans* was evaluated based on the size of amplification product.

RESULTS

Isolation of staphylococci: Staphylococci were isolated from the external auditory meatus in 14 (48.3%) of 29 OE dogs and 28 (68.3%) of 41 non-OE dogs (Table 1). Twenty-two OE isolates were identified as belonging to 12 species, and 42 non-OE isolates were identified as belonging to 13 species. *S. intermedius* and *S. epidermidis* were the predominant species isolated from OE and non-OE dogs.

S. schleiferi subsp. *coagulans* strains were identified by means of 16S-23S rDNA intergenic spacer PCR analysis [14], of which the length polymorphisms of *S. schleiferi* subsp. *coagulans* were the same as that of *S. schleiferi* subsp. *schleiferi* (Fig. 1), and also by means of metabolic characteristics such as the production of free coagulase with rabbit plasma, acetoin and β -hemolysin, and the lack of acid production from trehalose or maltose.

Antimicrobial susceptibility tests: The results of antimicrobial susceptibility tests for 64 staphylococcal isolates including 15 species are presented in Table 2. Thirty-eight (59.4%) isolates were resistant to one or more of the 17 antimicrobial agents tested. Resistance to PCG (56.3% of strains) and ABPC (54.7%) was the most frequent, followed

Table 1. Species distribution of staphylococci isolated from the external auditory meatus of OE^{a)} and non-OE dogs

Species	Frequency of isolation	
	OE dogs 14/29 ^{b)} (48.3%)	Non-OE dogs 28/41 (68.3%)
<i>S. intermedius</i>	4 ^{c)} (28.6%)	11 (39.3%)
<i>S. aureus</i>	1 (7.1%)	1 (3.6%)
<i>S. schleiferi</i> subsp. <i>coagulans</i>	2 (14.3%)	1
<i>S. capitis</i>	1	2 (7.1%)
<i>S. caprae</i>	1	2
<i>S. chromogenes</i>	1	1
<i>S. cohnii</i> subsp. <i>cohnii</i>	1	0
<i>S. epidermidis</i>	5 (35.7%)	7 (25.0%)
<i>S. haemolyticus</i>	1	0
<i>S. hominis</i>	0	1
<i>S. lentus</i>	0	1
<i>S. saprophyticus</i>	2	5 (17.9%)
<i>S. sciuri</i>	1	1
<i>S. warneri</i>	1	3 (10.7%)
<i>S. xylosum</i>	0	3
Unidentifiable	4 (28.6%)	8 (28.6%)

a) OE: otitis externa.

b) No. of positive dogs/No. of dogs examined.

c) No. of individuals from which *Staphylococcus* species were isolated. Fig. 1. 16S-23S rDNA intergenic spacer region patterns of *S. schleiferi* subsp. *coagulans*. Lanes: M, size markers; 1–5, isolates in this study; 6–8, reference strains (GA 11, GA 64, and GA 288) [8]; 9, the type strain GA 211 (=JCM 7470) [8]; 10, *S. schleiferi* subsp. *schleiferi* (the type strain ATCC 43808); 11, *S. aureus* (strain FDA 209P); 12, *S. intermedius* (the type strain ATCC 29663).

by KM (14.1%), EM (14.1%), NFLX (9.4%), LCM (7.8%), SM (6.3%), CPFX (3.1%), OFLX (3.1%), TC (3.1%), and GM (1.6%). Three (7.9%) and 35 (92.1%) of the resistant isolates were single and multiple resistant types, respectively. Furthermore, 38 resistant isolates were examined for the presence of the *mecA* gene. Two isolates of *S. caprae* and one isolate of *S. xylosum* carried the *mecA* gene.

Characteristics of *S. schleiferi* subsp. *coagulans*: Five isolates of *S. schleiferi* subsp. *coagulans* from OE dogs (Nos. 1 and 2) and non-OE dog (No. 3) unexceptionally produced coagulase, β -hemolysin, DNase, lipase, and protease, but failed to produce gelatinase, staphylokinase, hyaluronidase, protein A, SEs, TSST-1, and ETs (Table 3).

All of the 5 isolates were sensitive to phages of the phage set for *S. intermedius*. Three isolates showed the phage pattern 06/40/58/93, and the remaining 2 isolates showed 06/40/58 and 06/40/58/93/E74. The PFGE patterns of the 5 isolates are shown in Fig. 2. Identical PFGE patterns were shown in isolates from the left and right ears of individual dogs (Nos. 2 and 3) (Table 3). The PFGE pattern of the OE isolate in Hokkaido (No.1) was similar to that of OE isolates in Okayama (No.2), which is located geographically at a great distance from Hokkaido. In addition, OE and non-OE isolates showed similar patterns, suggesting that they were genetically closely related.

16S rDNA specific PCR for identification of *S. schleiferi* subsp. *coagulans*: The 16S rDNA of *S. schleiferi* subsp. *coagulans*, of which the PCR target fragment was made up of 1369 bp corresponding to the sequences of the gene, was detected in all of the 5 isolates of *S. schleiferi* subsp. *coagulans* as well as in the type strain GA 211 (=JCM 7470) and the reference strain GA 11 (Fig. 3). For the strains of *S. aureus* (n=5), *S. intermedius* (n=5), and *S. hyicus* (n=2) used to confirm the specificity of the PCR, the specific amplification product was not observed.

DISCUSSION

One purpose of this study was to determine the species distribution and susceptibility to antimicrobial agents of staphylococci from the external auditory meatus of OE and non-OE dogs.

Novobiocin-sensitive species were the predominant inhabitants in the external auditory meatus of the two groups of dogs. The most frequently isolated species were *S. intermedius* and *S. epidermidis*. This result agrees in gross with the study performed by Uchida *et al.* [20], who found that the commonly isolated novobiocin-sensitive species were *S. intermedius*, *S. chromogenes*, and *S. haemolyticus* in OE dogs, and *S. chromogenes* and *S. epidermidis* in non-OE dogs.

Resistance to PCG or ABPC is common in staphylococci of dog origin [12,13]. In the present study, about 60% of the 64 staphylococcal isolates were resistant to one or more of the antimicrobial agents tested. Resistance to PCG and ABPC was found in 56.3% and 54.7% of the strains, respectively. Interestingly, resistance to these antimicrobial agents was detected in 11 *Staphylococcus* species. The quinolones are approved for use in small animal medicine in Japan. The frequencies of resistance to NFLX, OPLX and CPFX were low, ranging from 3.1% to 9.4%, showing that resistance to quinolones among staphylococci is still rare in dogs. Among 3 coagulase-positive staphylococci, *S. aureus* and *S. schleiferi* subsp. *coagulans* isolates were susceptible to all of the 17 tested antimicrobials, while about 83% of *S. intermedius* isolates were resistant, and all of the *S. intermedius* isolates were resistant to PCG and ABPC. The high frequency of resistance to these antimicrobial agents has already been reported in *S. intermedius* of dog origin in the United States [13,16] and the UK [15]. Quinolone-resistant

Table 2. Antimicrobial resistance patterns of staphylococcal isolates from the external auditory meatus of OE and non-OE dogs

Species	No. of antimicrobial resistant isolates/ No. of isolates tested	Antimicrobial resistance pattern (Origin and No. of isolates)
<i>S. intermedius</i>	14/17 (82.4%)	PCG/ABPC (OE, 2; non-OE, 7), PCG/ABPC/KM(non-OE, 1), PCG/ABPC/EM/KM/SM(non-OE, 1) PCG/ABPC/EM/KM/SM/LCM(OE, 1; non-OE, 1), PCG/ABPC/EM/KM/SM/LCM/NFLX/CPLX/OFLX(non-OE, 1),
<i>S. aureus</i>	0/2	
<i>S. schleiferi</i> subsp. <i>coagulans</i>	0/5	
<i>S. capitis</i>	2/3 (66.7%)	PCG(non-OE, 1), PCG/ABPC(OE, 1)
<i>S. caprae</i>	3/3 (100%)	PCG/ABPC(non-OE, 1), PCG/ABPC/KM/NFLX(non-OE, 1) ^{a)} PCG/ABPC/NFLX/CPFX/OFLX(OE, 1) ^{a)}
<i>S. chromogenes</i>	0/2	
<i>S. cohnii</i> subsp. <i>cohnii</i>	1/1 (100%)	PCG/ABPC(OE, 1)
<i>S. epidermidis</i>	6/12 (50.0%)	PCG/ABPC(OE, 1; non-OE, 3), PCG/ABPC/KM/NFLX(non-OE, 1), EM(non-OE, 1)
<i>S. haemolyticus</i>	1/1 (100%)	PCG/ABPC/TC/KM/GM/NFLX(OE, 1)
<i>S. hominis</i>	1/1 (100%)	PCG/ABPC/EM(non-OE, 1)
<i>S. lentus</i>	0/1	
<i>S. saprophyticus</i>	3/7 (42.9%)	PCG/ABPC(non-OE, 3)
<i>S. sciuri</i>	2/2 (100%)	PCG/ABPC(OE, 1), PCG/ABPC/EM/TC/KM(non-OE, 1)
<i>S. warneri</i>	2/4 (50.0%)	PCG/ABPC/KM(OE, 1), NFLX (non-OE, 1)
<i>S. xylosus</i>	3/3 (100%)	PCG/ABPC(non-OE, 1), PCG/ABPC/EM/LCM(non-OE, 2) ^{a)}
Total	38/64 (59.4%)	

a) Positive for *mecA* gene. Isolates of methicillin-resistant *S. caprae* (2 isolates) and *S. xylosus* (1 isolate) were from OE and non-OE dogs, and non-OE dog, respectively.

Table 3. Characteristics of *S. schleiferi* subsp. *coagulans* isolates

Characteristic	OE			Non-OE	
	Hokkaido Dog No.1	Okayama Dog No.2		Hokkaido Dog No.3	
	Right ear	Left ear	Right ear	Left ear	Right ear
Coagulase for rabbit plasma	+	+	+	+	+
Enterotoxins (A, B, C, D)	—	—	—	—	—
α -hemolysin	—	—	—	—	—
β -hemolysin	+	+	+	+	+
TSST-1 toxin	—	—	—	—	—
Exfoliative toxins (ET-A, ET-B)	—	—	—	—	—
DNase	+	+	+	+	+
Gelatinase	—	—	—	—	—
Lipase	+w ^{a)}	+w	+w	+w	+w
Protease	+	+	+	+	+
Staphylokinase	—	—	—	—	—
Hyaluronidase	—	—	—	—	—
Protein A	—	—	—	—	—
Phage pattern	06/40/ 58/93	06/40/58	06/40/58/ 93/E74	06/40/ 58/93	06/40/ 58/93

a) Weak reaction.

S. intermedius was not isolated from healthy and diseased dogs from 1982 to 1999 [18], and only one of the 17 isolates in this study was quinolone-resistant. With respect to methicillin resistance, 2 isolates of *S. caprae* and one isolate of *S. xylosus* were found to be resistant to methicillin. This is the first evidence of the existence of methicillin-resistant *S. caprae* in dogs.

Another purpose of this study was to characterize *S. schleiferi* subsp. *coagulans* isolates by using biological

properties and PFGE typing and to develop a PCR method for the identification of this organism based on the 16S rDNA sequence.

The clinical significance of *S. schleiferi* subsp. *coagulans* has been fully ascertained. Igimi *et al.* [8] suggested a pathogenic role for the organism in canine OE. Recently, the organism has also been isolated from dogs affected with pyoderma [1, 5, 7] and from a finger wound infection in a human [21]. No previous studies on the production of extra-

Fig. 2. PFGE of *Sma* I-digested genomic fragments of *S. schleiferi* subsp. *coagulans* isolated from OE and non-OE dogs. Lanes: M, size markers; 1, one isolate from OE dog (No. 1) in Hokkaido; 2, two isolates from left and right ears of OE dog (No. 2) in Okayama; 3, two isolates from left and right ears of non-OE dog (No.3) in Hokkaido. See Table 3 for the origin and characteristics of isolates.

cellular enzymes and toxins of *S. schleiferi* subsp. *coagulans* isolated from dogs and a human have been conducted. In the present study, 5 isolates of *S. schleiferi* subsp. *coagulans* from OE and non-OE dogs were compared for their ability to produce a range of virulence-associated factors, which are thought to contribute to the pathogenicity of the organism. However, no clear differences in the extracellular enzyme and toxin profiles between the two groups of isolates were demonstrated. PFGE analysis also showed that both of the OE and non-OE isolates are very closely related. These results suggest that similar or identical strains of *S. schleiferi* subsp. *coagulans* found in OE dogs are distributed in non-OE dogs, though the number of strains examined in this study was too small to draw a definite conclusion. A detailed comparison of the characteristics between OE and non-OE isolates should be studied in future.

S. intermedius is a representative pathogenic bacterium that is frequently isolated from dogs with OE and pyoderma. Strains of *S. schleiferi* subsp. *coagulans* are phenotypically similar to strains of *S. intermedius*. Simple identification systems for correctly identifying strains of *S. schleiferi* subsp. *coagulans* are not currently available. The PCR primers designed in this study gave a rapid and simple identification of the organism. Though the PCR in this study cannot distinguish *S. schleiferi* subsp. *coagulans* from *S. schleiferi* subsp. *schleiferi*, as reported by Frank *et al.* [5], it is easy to differentiate these two subspecies on the basis of their tube coagulase test [8] and urease production [21]. Previously, we [22] designed a primer set for PCR specific for 16S rDNA in order to identify the strains of canine pathogenic *S. intermedius*. These two primer sets for PCR

Fig. 3. Agarose gel electrophoresis of the PCR products from strains of *S. schleiferi* subsp. *coagulans*, *S. aureus*, *S. intermedius*, and *S. hyicus*. Lanes: M, size markers; 1, the type strain GA 211 (=JCM 7470) [8]; 2, a reference strain GA 11 [8]; 3–7, dog isolates in this study; 8, strain FDA 209P; 9, NCTC 10442; 10, chicken strain; 11–12, dog strains; 13, type strain ATCC 29663; 14–17, dog strains; 18, type strain NCTC 10350; 19, chicken strain.

specific for the 16S rDNA of *S. schleiferi* subsp. *coagulans* or *S. intermedius* are useful for determining the etiology and epidemiology of the canine staphylococcal infections that spawn these coagulase-positive species.

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