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Detection and characterization of potential virulence determinants in *Staphylococcus pseudintermedius* and *S. schleiferi* strains isolated from canine otitis externa in Korea

Gi Yong Lee [1], **Soo In Lee** [2], **Ji Heon Park** [1], **Sun Do Kim** [2], **Geun-Bae Kim** [2], **Soo-Jin Yang** [1,*]

[1]Department of Veterinary Microbiology, College of Veterinary Medicine and Research Institute for Veterinary Science, Seoul National University, Seoul 08826, Korea
[2]School of Bioresources and Bioscience, Chung-Ang University, Anseong 17546, Korea

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***Corresponding author:**
Soo-Jin Yang
Department of Veterinary Microbiology, College of Veterinary Medicine and Research Institute for Veterinary Science, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 08826, Korea.
Email: soojinjj@snu.ac.kr
https://orcid.org/0000-0003-3253-8190

ABSTRACT

Background: A recent increase in the occurrence of canine skin and soft tissue infections, including otitis externa and pyoderma, caused by antimicrobial-resistant *Staphylococcus pseudintermedius* and *S. schleiferi* has become a significant public and veterinary health issues.
Objective: We investigated the virulence potentials associated with the occurrence of canine otitis externa in *S. pseudintermedius* and *S. schleiferi.*
Methods: In this study, the prevalence of genes encoding leukocidins, exfoliative toxins, and staphylococcal enterotoxins (SEs) was investigated using previously characterized *S. pseudintermedius* (n = 26) and *S. schleiferi* (n = 19) isolates derived from canine otitis externa. Susceptibility to cathelicidins (K9CATH and PMAP-36) and hydrogen peroxide (H_2O_2) was also examined in both staphylococcal species.
Results: A high prevalence of genes encoding leukocidins (*lukS/F-I*, *lukS1/F1-S*, and *lukS2/F2-S*), exfoliative toxins (*siet*, *expB*, and *sset*), and SEs was identified in both *S. pseudintermedius* and *S. schleiferi* isolates. Notably, *S. pseudintermedius* isolates possessed higher number of SE genes, especially newer SE genes, than *S. schleiferi* isolates harboring *egc* clusters. Although no significant differences in susceptibility to K9CATH and H_2O_2 were observed between the two isolate groups, *S. pseudintermedius* isolates exhibited enhanced resistance to PMAP-36 compared to *S. schleiferi* isolates.
Conclusions: These findings suggest that high a prevalence of various toxin genes together with enhanced resistance to cathelicidins may contribute to the pathogenicity of *S. pseudintermedius* and *S. schleiferi* in canine cutaneous infections.

Keywords: *S. pseudintermedius*; *S. schleiferi*; canine otitis externa; virulence

INTRODUCTION

Staphylococci are frequently implicated in opportunistic skin and soft tissue infections in humans and companion animals [1]. Although *Staphylococcus pseudintermedius*, belonging to the *S. intermedius* group has been recognized as a major cause of cutaneous infections,

ORCID iDs
Gi Yong Lee
https://orcid.org/0000-0001-5308-0065
Soo In Lee
https://orcid.org/0000-0003-4558-5981
Ji Heon Park
https://orcid.org/0000-0002-5843-785X
Sun Do Kim
https://orcid.org/0000-0003-1394-3844
Geun-Bae Kim
https://orcid.org/0000-0001-8531-1104
Soo-Jin Yang
https://orcid.org/0000-0003-3253-8190

Author Contributions
Conceptualization: Lee GY, Kim GB, Yang SJ; Formal analysis: Lee GY, Yang SJ; Funding acquisition: Yang SJ; Investigation: Lee GY, Yang SJ; Methodology: Lee GY, Lee SI, Park JH, Kim SD; Project administration: Lee GY, Yang SJ; Supervision: Yang SJ; Writing - original draft: Lee GY, Yang SJ; Writing - review & editing: Kim GB, Yang SJ.

Conflict of Interest
The authors declare no conflicts of interest.

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including atopic dermatitis, pyoderma, and otitis externa [2], the recent emergence of *S. schleiferi* in canine otitis externa and pyoderma has become a significant public and veterinary health issues [3,4].

As a major canine-associated cutaneous pathogen, *S. pseudintermedius* possesses various virulence factors, including hemolysins [5], exfoliative toxins (ETs) [6-8], leukocidins [9,10], staphylococcal enterotoxins (SEs) [11], and toxic shock syndrome toxin (TSST) [12]. These virulence factors likely influence severity of skin and soft tissue infections and clinical outcomes. Recently, various *S. pseudintermedius*-specific toxins, such as *S. pseudintermedius* exfoliative toxins (SIET, ExpA, and ExpB) [6-8,13], leukocidins (LukS/F-I) [9,10], and canine SEC (SEC_{canine}) [14], have been reported. Although less common than *S. pseudintermedius*, *S. schleiferi* has become a significant canine pathogen carrying multiple potential virulence factors such as TSST and SEs [3,15].

In addition to their array of toxins, the ability of *S. pseudintermedius* and *S. schleiferi* to resist host defense cationic antimicrobial peptides (HD-CAPs) secreted by host keratinocytes and immune cells may play an important role in the pathogenicity and clinical outcomes of canine cutaneous infections [2,3,16]. Previous studies have indicated that staphylococcal isolates with higher levels of resistance to HD-CAPs tend to display increased *in vivo* virulence and carry a higher risk of severe clinical outcomes [2,3,17].

Although several studies on colonization of dogs by *S. pseudintermedius* and *S. schleiferi* have been reported in Korea [2,3,15], to the best of our knowledge, no previous study in Korea has investigated comparative virulence potential of these two major canine pathogens in terms of skin and soft tissue infections. Thus, in the current study, we investigated the carriage rates of major staphylococcal toxin genes, including ETs, leukocidins, SEs, and TSST genes, in *S. pseudintermedius* and *S. schleiferi* strains isolated from canine otitis externa. Moreover, susceptibility to two prototypical cathelicidins of canine (K9CATH) and porcine (PMAP-36) origins was determined in the *S. pseudintermedius* and *S. schleiferi* isolates. Furthermore, hydrogen peroxide (H_2O_2) resistance profiles were analyzed for the two groups of staphylococcal species.

MATERIALS AND METHODS

S. pseudintermedius and *S. schleiferi* isolates

A collection of 26 *S. pseudintermedius* and 19 *S. schleiferi* isolates were selected from recently described staphylococcal strains isolated from canine otitis externa in Korea [2,3]. All *S. pseudintermedius* and *S. schleiferi* isolates were identified by using both matrix-assisted laser desorption ionization (MALDI)-Biotyper bacterial identification system (Bruker Daltonics, Germany) and 16S rRNA sequencing (Cosmogenetech, Korea).

All isolates were grown in Mueller-Hinton broth (Difco Laboratories, USA) or tryptic soy broth (Difco Laboratories), depending on the experiment. All broth cultures were grown in Erlenmeyer flasks (< 15% of total flask volume) at 37°C with shaking at 200 rpm.

Detection of toxin genes in *S. pseudintermedius* and *S. schleiferi* isolates

Staphylococcal toxin genes in all 45 isolates were detected by polymerase chain reaction (PCR) analyses. Genes encoding ETs (*siet*, *expA, and expB*) [6,8,13] and leukocidins (*lukS/F-I*) [18] in *S. pseudintermedius* isolates were detected as previously described. For *S. schleiferi* isolates, specific primer sets were designed to detect the ET (*sset*) and leukocidins (*lukS/F-S*) based on the published genomic data of NCTC12218 (GenBank accession No. LR962863) and TSCC54 (GenBank accession No. AP014944). Specific PCR primer sets and conditions are listed in **Table 1**.

Presence of 18 different SE genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *selj*, *selk*, *sell*, *selm*, *seln*, *selo*, *selp*, *selq*, *selr*, and *selu*) and the TSST gene (*tst-1*) in the 45 staphylococcal isolates was examined using previously described multiplex PCR assays, with minor modifications [19,20]. Briefly, six separate multiplex PCR assays were employed to detect 18 SE and TSST-1 genes: SE-MIX1 (*sea*, *seb*, *sec*, *sed*, *see*), SE-MIX2 (*selr*, *seln*, *selu*), SE-MIX3 (*selj*, *seg*, *sei*), SE-MIX4 (*selq*, *sell*), SE-MIX5 (*selo*, *selm, tst-1*), and SE-MIX6 (*selk*, *selp*, *seh*). PCR amplification of the SE and TSST-1 genes was carried out with initial denaturation at 94°C for 3 min followed by 28 cycles of amplification (denaturation at 95°C for 30 sec, annealing at 53°C for 45 sec, and extension at 72°C for 45 sec) and a final extension at 72°C for 10 min. Reference genomic DNA samples from previously characterized *S. aureus* strains (MW2: *sea*, *seh*, *selk*, *selp*; COL: *seb*; N315: *sec*, *selm*, *selo*; FRI472: *sed*, *seg*, *sei*, *selj*, *seln*, *selr*, *selu*; FRI913: *see*, *sell*, *seq*, *tst-1*) were used as positive controls in each multiplex PCR reaction. Representative results of the PCR-detection are shown in **Supplementary Fig. 1**.

Table 1. Primer sets and conditions of polymerase chain reaction amplification for staphylococcal toxins for *S. pseudintermedius* and S. *schleiferi*

Name	Gene	Primer	Sequence (5′-3′)	Size (bp)	References
S. pseudintermedius	*lukS-I*	SP_lukS-F	TGTAAGCAGCAGAAAATGGGG	503	[9]
		SP_lukS-R	GCCCGATAGGACTTCTTACAA		
	lukF-I	SP_lukF-F	CCTGTCTATGCCGCTAATCAA	572	[9]
		SP_lukF-R	AGGTCATGGAAGCTATCTCGA		
	siet	SP_siet-F	ATGGAAAATTTAGCGGCATCTGG	359	[41]
		SP_siet-R	CCATTACTTTTCGCTTGTTGTGC		
	expA	SP_expa-F	CAATCATATAATGAGGAAGAAATATTAAAAAAGCAA	737	[7]
		SP_expa-R	TTCTTCTTGTAATTTAGCTCTTTTTTTCAAGTCTTC		
	expB	SP_expb-F	CGCCTGGCGTATATGCTAAA	595	This study
		SP_expb-R	AAGCCAGATCCTGAATTTCC		
		95°C for 30 sec, (95°C for 30 sec, 52°C for 45 sec, 72°C for 45 sec) X 32 cycles, and 72°C for 10 min			
S. schleiferi	*lukS1-S*	SS_lukS1-S-F	TATTGTCGCCGAACAACAAA	510	This study
		SS_lukS1-S-R	TTAACGCCCCATGCTACATT		
	lukF1-S	SS_lukF1-S-F	TGCAGATGCAGATCGATTTAATA	796	This study
		SS_lukF1-S-R	AGCAGTGTGGTTTTGCCAAT		
	lukS2-S	SS_lukS2-S-F	GCTATATAAAGCCCCGAACA	486	This study
		SS_lukS2-S-R	CTGTTGTAAGGAAAGACGGA		
	lukF2-S	SS_lukF2-S-F	ACTTTCAAGTCACGCTTTTG	651	This study
		SS_lukF2-S-R	ATAAAGTTCTCACCGGCATT		
	sset	SS_sset-F	ATGGAAAATTTAGCGGCATCTGG	243	This study
		SS_sset-R	CCATTACTTTTCGCTTGTTGTGC		
		95°C for 30 sec, (95°C for 30 sec, 53°C for 45 sec, 72°C for 45 sec) X 32 cycles, and 72°C for 10 min			
S. pseudintermedius and *S. schleiferi*	*eta*	eta-F	CTAGTGCATTTGTTATTCAAGACG	119	[42]
		eta-R	TGCATTGACACCATAGTACTTATTC		
	etb	etb-F	ACGGCTATATACATTCAATTCAATG	262	[42]
		etb-R	AAAGTTATTCATTTAATGCACTGTCTC		
	tst-1	tst-F	AAGCCCTTTGTTGCTTGCG	447	[42]
		tst-R	ATCGAACTTTGGCCCATACTTT		
		95°C for 30 sec, (95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec) X 30 cycles, and 72°C for 10 min			

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In vitro susceptibilities to K9CATH and PMAP-36

Cathelicidins play pivotal roles in host innate immune defense against bacterial pathogens during skin and soft tissue infections [21]. Two prototypical cathelicidins, K9CATH (RLKELITTGGQKIGEKIRRIGQRIKDFFKNLQPREEKS) [22] and PMAP-36 (GRFRRLRKKTRKRLKKIGKVLKWIPPIVGSIPLGCG) [23], were synthesized at GL Biochem (China) with a purity of > 95%.

To assess potential differences in susceptibility to cathelicidins between the two groups of staphylococci, *in vitro* survival assays were performed for all 26 *S. pseudintermedius* and 19 *S. schleiferi* isolates against K9CATH and PMAP-36 at pH 5.5. *In vitro* susceptibility to K9CATH and PMAP-36 was determined using a 2 h survival assay in RPMI-1640 medium (Sigma-Aldrich, USA) containing 5% Luria-Bertani broth as previously described [24]. To mimic the physiological conditions of canine otitis externa and acidic phagolysosomes [25], assay conditions were adjusted to pH 5.5 using 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer. Briefly, the assays were performed with 0.25 μg/mL of K9CATH or PMAP-36 using an initial staphylococcal inoculum of ~5×10^3 CFUs. Cathelicidin concentrations were determined based on extensive preliminary experiments. Data represent the relative percentage of surviving staphylococcal cells from cathelicidin-treated versus untreated conditions (± SD). A minimum of three independent experiments were performed for each isolate.

In vitro susceptibility to hydrogen peroxide

In vitro susceptibility to hydrogen peroxide (H_2O_2) was determined for all *S. pseudintermedius* and *S. schleiferi* isolates, as previously described [26]. Briefly, ~5×10^7 CFUs of overnight-grown *S. pseudintermedius* or *S. schleiferi* cells were incubated with 1.5% H_2O_2 at 37°C. After 2 h of incubation, 1,000 U/mL of catalase (Sigma-Aldrich) was added to remove all residual H_2O_2 in the solution. To enumerate surviving staphylococcal cells, reaction solutions were diluted ten-fold, then spread on tryptic soy agar plates. Data were presented as mean percentages of surviving cells of H_2O_2-treated versus untreated cells (± SD). Three independent experiments were performed for all staphylococcal isolates.

Statistical analysis

Two-sided Mann-Whitney *U* tests of variance with Duncan's *post hoc* correction were performed for multiple comparisons using IBM SPSS Statistics 25 software (USA). The significance threshold was set at $p < 0.05$.

RESULTS

Toxin gene profiles of staphylococcal isolates

As shown in **Table 2**, 43/45 (95.6%) staphylococcal isolates from canine otitis externa possessed leukocidin genes. Only one *S. pseudintermedius* and one *S. schleiferi* isolates tested were negative for all leukocidin genes. All 18 *S. schleiferi* isolates carrying *lukS1-S* and *lukF1-S* genes were also positive for *lukS2-S* and *lukF2-S*. Similar to the high prevalence of leukocidin genes, all 26 *S. pseudintermedius* isolates and 18/19 *S. schleiferi* isolates were positive for the ET genes *siet* and *sset*, respectively (**Table 2**). Interestingly, although 12 *S. pseudintermedius* isolates (46.2%) were positive for *expB*, none of the *S. pseudintermedius* isolates carried *expA*.

In addition to the leukocidin and ET genes, a high prevalence of SE genes was observed in *S. pseudintermedius* and *S. schleiferi* isolates (**Table 2**). At least one of the 18 SE genes was detected in

Table 2. Comparative profiles of SE, TSST, leukocidin, and exfoliative toxin genes between *S. pseudintermedius* and *S. schleiferi* isolates obtained from canine otitis externa

Toxin	No. of SE-positive strain (%)		
	S. pseudintermedius (n = 26)	*S. schleiferi* (n = 19)	Total (n = 45)
SEs			
sea	-	-	-
seb	-	-	-
sec	1 (4)	-	1 (2.2)
sed	-	-	-
see	15 (57.7)	-	15 (33.3)
seg	25 (96.2)	10 (52.6)	35 (77.8)
seh	6 (23.1)	-	6 (13.3)
sei	25 (96.2)	10 (52.6)	35 (77.8)
selj	-	-	-
selk	-	2 (11)	2 (4.4)
sell	1 (3.8)	6 (31.6)	7 (15.6)
selm	25 (96.2)	14 (73.7)	39 (86.7)
seln	24 (92.3)	-	24 (53.3)
selo	-	1 (5)	1 (2.2)
selp	8 (30.8)	-	8 (17.8)
selq	1 (3.8)	6 (31.6)	7 (15.6)
selr	-	-	-
selu	22 (84.6)	-	22 (48.9)
TSST			
tst-1	-	-	-
Leukocidins			
lukS-I	25 (96.2)	ND	25 (55.6)
lukF-I	25 (96.2)	ND	25 (55.6)
lukS1-S	ND	18 (94.7)	18 (40)
lukS2-S	ND	18 (94.7)	18 (40)
lukF1-S	ND	18 (94.7)	18 (40)
lukF2-S	ND	18 (94.7)	18 (40)
Exfoliative toxins			
siet	26 (100)	ND	26 (57.8)
expA	-	ND	-
expB	12 (46.2)	ND	12
sset	ND	18 (94.7)	18 (40)
eta	-	-	-
etb	-	-	-

SE, staphylococcal enterotoxin; TSST, toxic shock syndrome toxin; ND, not detected.

25/26 *S. pseudintermedius* (96.2%) isolates and 16/19 *S. schleiferi* (84.2%) isolates (**Tables 3** and **4**). Among the 18 screened SE and TSST genes, *selm* was most frequently detected in both *S. pseudintermedius* (25/26, 96.2%) and *S. schleiferi* (14/19, 73.7%) isolates, followed by *seg* and *sei* (96.2% in *S. pseudintermedius* and 52.6% in *S. schleiferi* for each gene). While carriage of three SE genes (*seg-sei-selm*) was identified in 96.2% (25/26) and 47.4% (9/19) of *S. pseudintermedius* and *S. schleiferi* isolates, respectively, carriage of 5 (*seg-sei-selm-seln-selu*) and 7 (*sec-seg-sei-sell-selm-seln-selu*) SE genes was detected only in *S. pseudintermedius* isolates (84.6% and 4% of the isolates, respectively) (**Tables 3** and **4**). Although high carriage rates of *see* (15/26, 57.7%), *seln* (24/26, 92.3%), and *selu* (22/26, 84.6%) were identified in *S. pseudintermedius* isolates, none of the *S. schleiferi* isolates possessed these three SE genes. One *S. pseudintermedius* (SP21) and three *S. schleiferi* (SS7, SS13, and SS14) isolates were negative for all SE genes (**Tables 3** and **4**). All *S. pseudintermedius* and *S. schleiferi* isolates were negative for TSST gene. No significant correlations were found between the prevalence of toxin genes (leukocidins, ETs, and SEs) and profiles of sequence types (MLST) among the *S. pseudintermedius* isolates (**Table 3**). Similarly, methicillin resistance due to harboring SCC*mec* V did not affect toxin gene profiles in either *S. pseudintermedius* (**Table 3**) or *S. schleiferi* (**Table 4**).

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Table 3. Profiles of genes encoding staphylococcal toxins in *S. pseudintermedius* strains isolated from canine otitis externa

Strain	MLST[a]	SCC*mec*[a]	Toxins		
			SEs	Leukocidin	Exfoliative toxin
SP1	ST568	V	*see*, *seg*, *sei*, *selm*, *seln*, *selu*	*lukS-I*, *lukF-I*	*siet*
SP2	ST551	V	*see*, *seg*, *sei*, *selm*, *seln*, *selu*	*lukS-I*, *lukF-I*	*siet*
SP3	ST568	V	*see*, *seg*, *seh*, *sei*, *selm*, *seln*, *selu*	*lukS-I*, *lukF-I*	*siet*
SP4	ST568	V	*see*, *seg*, *sei*, *selm*, *seln*, *selu*	*lukS-I*, *lukF-I*	*siet*
SP5	ST429	-	*seg*, *sei*, *selm*, *seln*, *selp*, *selu*	*lukS-I*, *lukF-I*	*siet*
SP6	ST155	-	*sec*, *seg*, *sei*, *sell*, *selm*, *seln*, *selu*	*lukS-I*, *lukF-I*	*siet*
SP7	ST155	-	*see*, *seg*, *sei*, *selm*, *seln*, *selu*	*lukS-I*, *lukF-I*	*siet*
SP8	ST580	V	*see*, *seg*, *sei*, *selm*, *seln*, *selu*	*lukS-I*, *lukF-I*	*siet*, *expB*
SP9	ST774	-	*see*, *seg*, *sei*, *selm*, *seln*, *selu*	*lukF-I*	*siet*
SP10	ST706	V	*seg*, *sei*, *selm*, *seln*, *selq*, *selu*	*lukS-I*, *lukF-I*	*siet*, *expB*
SP11	ST316	V	*see*, *seg*, *sei*, *selm*, *seln*, *selu*	*lukS-I*, *lukF-I*	*siet*
SP12	ST133	-	*see*, *seg*, *sei*, *selm*, *seln*, *selu*	*lukS-I*, *lukF-I*	*siet*, *expB*
SP13	ST17	NT	*see*, *seg*, *sei*, *selm*, *seln*, *selu*	*lukS-I*, *lukF-I*	*siet*, *expB*
SP14	ST72	V	*see*, *seg*, *sei*, *selm*, *seln*, *selu*	*lukS-I*, *lukF-I*	*siet*
SP15	ST76	-	*seg*, *sei*, *selm*, *seln*, *selu*	*lukS-I*, *lukF-I*	*siet*, *expB*
SP16	ST1	V	*see*, *seg*, *sei*, *selm*, *seln*, *selu*	*lukS-I*, *lukF-I*	*siet*, *expB*
SP17	ST566	V	-	*lukS-I*, *lukF-I*	*siet*
SP18	ST571	V	*seg*, *sei*, *selm*, *seln*, *selp*, *selu*	*lukS-I*, *lukF-I*	*siet*, *expB*
SP19	ST76	V	*see*, *seg*, *sei*, *selm*, *selp*	*lukS-I*, *lukF-I*	*siet*
SP20	NT	V	*see*, *seg*, *sei*, *selm*, *seln*, *selp*, *selu*	*lukS-I*, *lukF-I*	*siet*, *expB*
SP21	NT	-	*seg*, *sei*, *selm*, *seln*, *selu*	-	*siet*
SP22	NT	-	*seg*, *seh*, *sei*, *selm*, *seln*, *selp*, *selu*	*lukS-I*, *lukF-I*	*siet*, *expB*
SP23	NT	-	*seg*, *seh*, *sei*, *selm*, *seln*, *selu*	*lukS-I*, *lukF-I*	*siet*
SP24	ST195	-	*seg*, *seh*, *sei*, *selm*, *seln*, *selp*, *selu*	*lukS-I*, *lukF-I*	*siet*, *expB*
SP25	ST809	V	*seg*, *seh*, *sei*, *selm*, *seln*, *selp*	*lukS-I*, *lukF-I*	*siet*, *expB*
SP26	ST903	NT	*see*, *seg*, *seh* , *sei*, *selm*, *seln*, *selp*	*lukS-I*, *lukF-I*	*siet*, *expB*

SE, staphylococcal enterotoxin; TSST, toxic shock syndrome toxin; NT, nontypeable.
[a]Profiles of sequence types and SCC*mec* types were previously published [4].

Table 4. Profiles of genes encoding staphylococcal toxins in *S. schleiferi* strains isolated from canine otitis externa

Strain	SCC*mec*[a]	Toxins		
		SEs[a]	Leukocidin	Exfoliative toxin
SS1	-	*seg*, *sei*, *selk*, *sell*, *selm*, *selq*	*lukS1-S*, *lukF1-S*, *lukS2-S*, *lukF2-S*	*sset*
SS2	V	*seg*, *sei*, *sell*, *selm*, *selq*	*lukS1-S*, *lukF1-S*, *lukS2-S*, *lukF2-S*	*sset*
SS3	V	*seg*, *sei*, *sell*, *selm*, *selq*	*lukS1-S*, *lukF1-S*, *lukS2-S*, *lukF2-S*	*sset*
SS4	V	*seg*, *sei*, *sell*, *selm*, *selq*	*lukS1-S*, *lukF1-S*, *lukS2-S*, *lukF2-S*	*sset*
SS5	-	*seg*, *sei*, *sell*, *selm*, *selq*	*lukS1-S*, *lukF1-S*, *lukS2-S*, *lukF2-S*	*sset*
SS6	VII	*seg*, *sei*, *sell*, *selq*	*lukS1-S*, *lukF1-S*, *lukS2-S*, *lukF2-S*	*sset*
SS7	-	-	*lukS1-S*, *lukF1-S*, *lukS2-S*, *lukF2-S*	*sset*
SS8	-	*seg*, *sei*, *selm*	*lukS1-S*, *lukF1-S*, *lukS2-S*, *lukF2-S*	*sset*
SS9	-	*seg*, *sei*, *selm*	*lukS1-S*, *lukF1-S*, *lukS2-S*, *lukF2-S*	*sset*
SS10	V	*seg*, *sei*, *selm*	*lukS1-S*, *lukF1-S*, *lukS2-S*, *lukF2-S*	*sset*
SS11	-	*selm*	*lukS1-S*, *lukF1-S*, *lukS2-S*, *lukF2-S*	*sset*
SS12	-	*selm*	*lukS1-S*, *lukF1-S*, *lukS2-S*, *lukF2-S*	*sset*
SS13	-	-	*lukS1-S*, *lukF1-S*, *lukS2-S*, *lukF2-S*	*sset*
SS14	-	-	*lukS1-S*, *lukF1-S*, *lukS2-S*, *lukF2-S*	*sset*
SS15	-	*seg*, *sei*, *selm*	*lukS1-S*, *lukF1-S*, *lukS2-S*, *lukF2-S*	*sset*
SS16	-	*selo*	*lukS1-S*, *lukF1-S*, *lukS2-S*, *lukF2-S*	*sset*
SS17	-	*selk*, *selm*	-	-
SS18	-	*selm*	*lukS1-S*, *lukF1-S*, *lukS2-S*, *lukF2-S*	*sset*
SS19	-	*selm*	*lukS1-S*, *lukF1-S*, *lukS2-S*, *lukF2-S*	*sset*

SE, staphylococcal enterotoxin; TSST, toxic shock syndrome toxin.
[a]Profiles of SCC*mec* and SE types were previously published [6].

Susceptibilities to cathelicidins

As shown in **Fig. 1A**, no significant difference in susceptibility to K9CATH (0.25 μg/mL) was observed between *S. pseudintermedius* and *S. schleiferi* isolates ($p = 0.474$). In contrast, *S.*

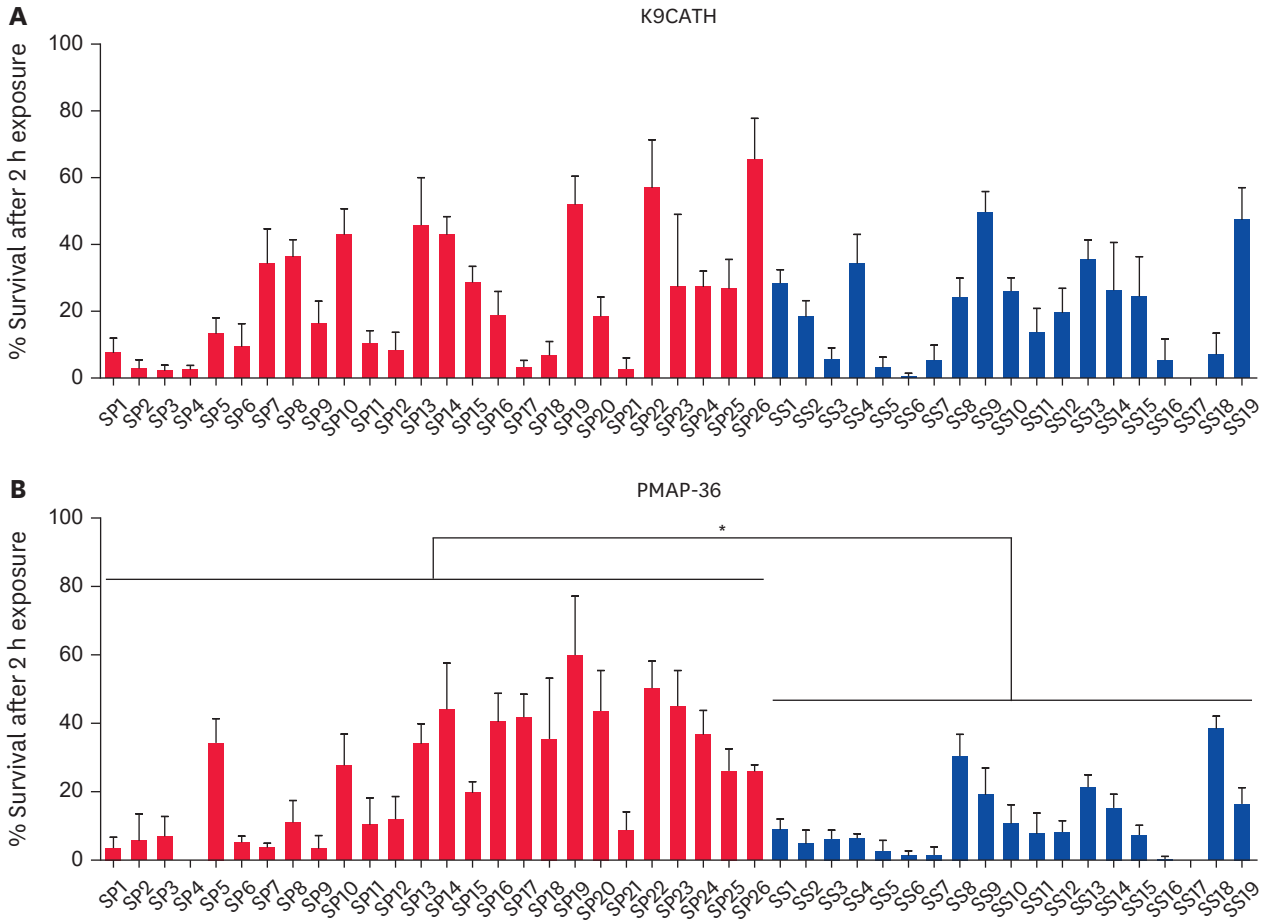

Fig. 1. *In vitro* susceptibility of *S. pseudintermedius* and *S. schleiferi* isolates to K9CATH (A) and PMAP-36 (B). The *in vitro* susceptibility assays were performed with 0.25 μg/mL of K9CATH or PMAP-36 using an initial bacterial inoculum of ~5 × 10^5 CFUs at pH5.5. Each bar represents the mean ± SD of three independent experiments on each isolate.
*$p < 0.05$.

pseudintermedius isolates displayed significantly higher resistance when treated with PMAP-36 (0.25 μg/mL) at pH 5.5 ($p = 0.012$) (**Fig. 1B**).

Susceptibility to hydrogen peroxide

As shown in **Fig. 2**, 26 *S. pseudintermedius* and 19 *S. schleiferi* isolates exhibited varying survival rates after a 2 h exposure to 1.5% H_2O_2. No significant difference in mean resistance to H_2O_2 was observed between *S. pseudintermedius* and *S. schleiferi* isolates.

DISCUSSION

Coagulase-positive *S. pseudintermedius* has frequently been implicated in various skin and soft tissue infections in dogs [5]. Although at a lower frequency than *S. pseudintermedius*, coagulase-negative *S. schleiferi* also causes a significant number of dermatological infections in dogs [4,27]. While the prevalence of *S. pseudintermedius* and *S. schleiferi* in infected dogs and their antimicrobial resistance have been actively investigated [4,5,27], few studies have focused on the virulence factors involved in the pathogenesis of these two canine-associated staphylococcal species.

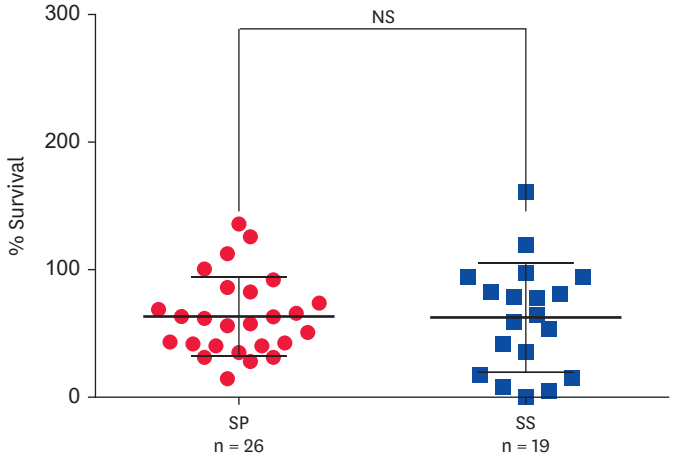

Fig. 2. *In vitro* susceptibility of *S. pseudintermedius* and *S. schleiferi* isolates to hydrogen peroxide (H_2O_2). Staphylococcal cells (~5 × 10^7 CFUs) were incubated with 1.5% H_2O_2 at 37°C for 2 h and surviving cells were counted on TSA plates. Data represent the mean ± SD of three independent experiments.
NS, not significant.

In this study, we examined 26 *S. pseudintermedius* [2] and 19 *S. schleiferi* [3] isolates collected from canine otitis externa cases in Korea to assess the virulence potential associated with canine skin and soft tissue infections.

Among the wide array of virulence determinants, pore-forming toxins, such as ETs and superantigens (SAgs), have been known to be involved in skin and soft tissue infections [28]. Similar to the Panton-Valentine leukocidin in *S. aureus*, a bi-component leukocidin from *S. intermedius*, LukS/F-I, has been well characterized [10,18]. Consistent with previous studies, which reported a high prevalence (92.3%–100%) of LukS/F-I in *S. pseudintermedius* isolates recovered from healthy dogs [5,29,30] and dogs with clinical symptoms [5,9,31], all *S. pseudintermedius* isolates in this study were positive for LukS/F-I genes, except for one *S. pseudintermedius* isolate (SP21) (**Tables 2** and **3**). Interestingly, two different types of leukocidins, LukS/F1-S and LukS/F2-S, which share amino acid sequence similarities of 54%–59% and 56%–58% with LukS/F-I of *S. pseudintermedius*, respectively, were detected in *S. schleriferi* isolates (**Tables 2** and **4**). The specific role of LukS/F1-S and LukS/F2-S in the context of canine skin and soft tissue infections need to be further characterized in the future study.

In addition to leukocidins, ETs in *S. pseudintermedius* such as SIET, ExpA, and ExpB have been proposed to be involved in the clinical outcomes of cutaneous infections. Similar to previous studies, which reported 92.3%–100% SIET [5,29-32], all 26 *S. pseudintermedius* and 18/19 *S. schleiferi* isolates in this study possessed *siet* or *sset* (**Table 2**). However, it has been demonstrated that SIET exhibits very limited enzymatic activity toward canine Dsg 1, which is expressed throughout the epidermal layer in canine skin [7]. The cytotoxic and exfoliative effects of *sset* have not yet been elucidated. Although the prevalence of ExpA and ExpB in *S. pseudintermedius* isolates was lower than that of SIET in previous studies [6,8,29-32], recent studies have revealed that both ExpA and ExpB are capable of digesting canine Dsg1 [6-8]. In this study, while none of the *S. pseudintermedius* isolates carried *expA*, 46.2% (12/26 isolates) of *S. pseudintermedius* isolates were positive for *expB* (**Table 2**), which was significantly higher than those of previous studies (7.6%–23.2%) [6,31]. These results indicate a high prevalence of genes encoding leukocidins and ETs in *S. pseudintermedius* and *S. schleiferi* isolates regardless of their genetic background and methicillin resistance.

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As shown in **Tables 3** and **4,** *S. pseudintermedius* isolates tend to have more SE genes than *S. schleiferi* isolates. However, the overall prevalences of *S. pseudintermedius* and *S. schleiferi* isolates carrying at least one SE gene were 96.2% (25/26) and 94.7% (18/19), respectively. Previous studies have reported a 48.8% - 65.7% SE gene prevalence with the highest frequency of *selq* in *S. pseudintermedius* isolates [11, 33]. In our study, the most frequently detected SE genes were *selm*, *seg*, and *sei* in both *S. pseudintermedius* and *S. schleiferi* isolates. Notably, a distinctive type of SEC among canine-associated staphylococci (SEC_{canine}) [14] was identified in one *S. pseudintermedius* isolate (SP6) (**Table 3**). DNA sequencing analysis of SEC_{canine} in the SP6 strain confirmed 100% match to the previously reported amino acid sequence of SEC_{canine}-positive *S. pseudintermedius* strain (NCBI Reference Sequence WP_130882709). The superantigenic activity of SEC_{canine} has been suggested to be involved in the clinical severity of canine skin infections [14]. The presence of multiple SE genes in the enterotoxin gene cluster (*egc* locus) was also identified in both staphylococcal species [34]. The *egc* has been described to contain the so-called new enterotoxin genes, such as *seg*, *sei*, *sem*, *sen*, *seo*, and *seu*, in *S. aureus* [34]. The *egc* is usully located on the genomic island and is incorporated into the chromosome as a prophage. Previous studies also revealed most frequent detection of the SE genes included within the *egc*, such as *seg*, *sei*, *selm*, *sen*, and *seo*, in livestock-associated *S. aureus* [35]. In this study, the *egc* clusters comprised of *seg-sei-selm* were detected in 96.1% (25/26) and 47.4% (9/19) of *S. pseudintermedius* and *S. schleiferi* isolates, respectively, suggesting horizontal transmission of *egc* clusters among different species of staphylococci. The *seg-sei-selm-seln-selu* cluster was detected only in *S. pseudintermedius* isolates (21/26, 80.8%), suggesting that *S. pseudintermedius* may have enhanced frequency of horizontal SE gene transfer compared to *S. schleiferi*.

It should be recognized that the presence of genes encoding leukocidins, ETs, and SEs in *S. pseudintermedius* and *S. schleiferi* isolates may not directly correlate with the toxin production. In addition, there is a possibility that sequence variations in SE genes may not have been detected with the PCR-based method [36]. Future studies are warranted to investigate SE gene sequence variations using whole genome sequence analyses [37] and various factors affecting expression of these toxin genes in the context of canine skin and soft tissue infections.

The ability to survive bactericidal activity of cathelicidins is important for the onset and persistence of staphylococcal skin infections [21]. It has been shown that an endogenous canine cathelicidin, K9CATH, exerts antimicrobial properties against pathogens implicated in canine skin and ear infections [2,3,16,22,38]. Although previous studies have evaluated the antimicrobial properties of K9CATH at pH 7.4 [2,3,16,22,38], bactericidal activity of K9CATH has not been examined under acidic conditions, which represent the pH range of the external ear canals in dogs with otitis externa [25,39]. Although varying degrees of viability were identified among the staphylococcal isolates at pH 5.5, there was no significant difference in K9CATH susceptibility between S. *pseudintermedius* and *S. schleiferi* isolate groups (**Fig. 1A**). However, S. *pseudintermedius* isolates displayed significantly higher resistance than *S. schleiferi* isolates when exposed to PMAP-36 (0.25 μg/mL) at pH5.5 (**Fig. 1B**). The different susceptibility profiles of K9CATH and PAMP-36 indicate that cathelicidins originating from different animal species may exert bactericidal activity against *S. pseudintermedius* and *S. schleiferi* via distinct mechanisms.

Given the impact of innate immune responses, such as oxidative bursts in the phagolysosomes of phagocytes, on the pathogenesis of staphylococci during skin and soft tissue infections [40], *in vitro* susceptibility to H_2O_2 was examined in *S. pseudintermedius* and *S. schleiferi* isolates. As shown in **Fig. 2**, no significant difference in susceptibility to H_2O_2 was observed between

the two species of staphylococci. The similar levels of resistance to H_2O_2 between the *S. pseudintermedius* and *S. schleiferi* isolates may be an example of convergent adaptation, as both isolate groups were obtained from clinical canine otitis externa, which promoted the adaptation of the isolates to various innate host immune defense of canine ear canals.

In conclusion, the results of this study provide important insights into the virulence potentials of *S. pseudintermedius* and *S. schleiferi* isolates collected from canine cutaneous infections. Our results suggest that: i) genes encoding leukocidins and ETs are highly prevalent in both *S. pseudintermedius* and *S. schleiferi* isolates obtained from canine otitis externa; ii) *S. pseudintermedius* isolates tended to have higher numbers of SE genes, especially non-classical SE genes, than *S. schleiferi* isolates by harboring *egc* clusters; iii) there are no differences in susceptibilities to K9CATH and H_2O_2 between *S. pseudintermedius* and *S. schleiferi* isolates, indicating both species have developed strategies to overcome the canine innate immune response; iv) enhanced resistance to PMAP-36 in *S. pseudintermedius* versus *S. schleiferi* indicates that *S. pseudintermedius* might have advantage to adapt to different host species.

SUPPLEMENTARY MATERIAL

Supplementary Fig. 1

Polymerase chain reaction -detection of SE, leukocidin, and exfoliative toxin genes. (A) Lanes 1-6, SE genes from reference *S. aureus* strains; lane 8, *sec* of SP6 strain; Lanes 9-12, *seg*, *sei*, *sel*, and *sem* genes of SS8 strain; and Lane 13-14; *seln* and *selu* genes of SP3 strain. (B) lanes 1-6, toxin genes of SP8 strains and lanes 7-11, toxin genes of SS4 strain. Specific primers set were designed based on public whole genome sequence data of *S. pseuditntermedius* (strain HKU10-03; GenBank accession No. CP002439 [*lukS-I* and *lukF-I*], strain ED99; GenBank accession No. CP002478 [*siet*], strain AI14; GenBank accession No. CP031604 [*expA*], and strain MS5134; GenBank accession No. AB569087 [*expB*]) and *S. schleriferi* (NCTC12218; GenBank accession No. LR962863 [*sset*], and TSCC54; GenBank accession No. AP014944 [*lukS1-S*, *lukF1-S*, *lukS2-S*, and *lukF2-S*]).

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