

Profiles of Enterotoxin Genes and Antimicrobial Resistance in *Staphylococcus pseudintermedius* Strains Isolated from Livestock and Companion Animals

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ABSTRACT - *Staphylococcus pseudintermedius* is an opportunistic pathogen in dogs and is recognized as a zoonotic pathogen causing public health concern. Although canine-associated *S. pseudintermedius* has mainly been recognized for its antimicrobial resistance and ability to cause skin infections in dogs, information on antimicrobial resistance profiles and enterotoxigenicity of *S. pseudintermedius* in livestock is very limited. In this study, we investigated the prevalence of 18 different staphylococcal enterotoxin (SE) genes and toxic shock syndrome toxin gene (*tst-1*) in *S. pseudintermedius* strains isolated from dogs, pigs, and beef cattle. Moreover, antimicrobial resistance profiles of the strains were determined along with the presence of *mecA* and SCC*mec* types. Except for one bovine isolate, all *S. pseudintermedius* isolates from dogs and pigs were resistant to multiple drugs (≥ 4 different drugs). Four out of six canine isolates were methicillin resistant and carried SCC*mec* type V. In addition, 11 different SE genes (*seb*, *sec*, *see*, *seg*, *sei*, *sej*, *sel*, *seo*, *sep*, *seq*, and *seu*) and *tst-1* were identified in *S. pseudintermedius* isolates from dogs, pigs, and beef cattle. Most *S. pseudintermedius* isolates (83%) harbored multiple SE genes, and *sel* (42%) and *sep* (42%) were most frequently detected in the isolates. Our results suggested that *S. pseudintermedius* isolates from livestock and companion animals may serve as a reservoir for SE genes and antimicrobial resistance.

Key words : *Staphylococcus pseudintermedius*, Antimicrobial resistance, Staphylococcal enterotoxins (SEs)

Staphylococcus pseudintermedius is an opportunistic pathogen, causing pyoderma, atopic dermatitis, otitis externa, skin and soft tissue infections in domestic animals^{1,2}. Although *S. pseudintermedius* has most frequently been isolated from the nares, mouth, and skin regions of healthy dogs and cats as well as from dogs and cats with skin infections^{2,4}, the carriage of *S. pseudintermedius* in other animals such as birds, horses, and goats has recently been reported^{1,5,6}. Pilla *et al.* (2013) also reported the occurrence of *S. pseudintermedius* in bovine mastitis⁷. Since these animal-associated staphylococci can be transmitted by direct/indirect contacts with the animals or through consumption of foods of animal origin, *S. pseudintermedius* has raised food safety concerns over the past decades⁸. More recently, increased number of zoonotic infections has been reported in humans as antimicrobial resistance in *S. pseudintermedius* increases worldwide⁹⁻¹². Methicillin-resistant *S. pseudintermedius* (MRSP) and

multidrug-resistant (MDR) MRSP are of particular concern because β -lactam antibiotics are still the first choice of treatment for staphylococcal infections¹³⁻¹⁵.

There are more than 20 different staphylococcal enterotoxins (SEs) that are functionally related and have similarities in sequences^{16,17}. These SEs are known to be associated with food poisoning and toxic shock syndrome in humans¹⁶⁻¹⁸. Although the SEs have been identified in at least several species of staphylococci, *S. aureus* has most frequently been reported to be enterotoxigenic¹⁶⁻²⁰. There are only few studies so far that described the presence of SE genes and toxic shock syndrome toxin gene (*tst-1*) in *S. pseudintermedius*, especially in antimicrobial resistant *S. pseudintermedius* strains from livestock and companion animals^{7,21-23}. Thus, in this study, we determined and compared the occurrence of SE genes and *tst-1* in *S. pseudintermedius* strains isolated from dogs, pigs, and beef cattle. In addition, antimicrobial resistance profiles in the *S. pseudintermedius* strains were examined. Furthermore, the presence of *mecA* and staphylococcal cassette chromosome *mec* (SCC*mec*) types of *mecA*-positive *S. pseudintermedius* strains were determined along with their antimicrobial resistance profiles.

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Table 1. The characteristics of *S. pseudintermedius* strains isolated from dogs, pigs and beef cattle

Strain	Origin	Antimicrobial resistance profiles ¹⁾	<i>mecA</i>	SCC <i>mec</i>	OXA MICs (µg/mL) ²⁾
SP-34 ³⁾	Dog	AMP-CHL-ENR-ERY-KAN-OXA-SXT-TET	+	V	2
SP-35		AMP-CHL-ERY-GEN-KAN-SXT-TET	-		0.25
SP-36 ³⁾		AMP-CHL-ERY-KAN-OXA-RIF-SXT-TET	+	V	>256
SP-37 ³⁾		AMP-CHL-ERY-GEN-KAN-OXA-SXT-TET	+	V	1.5
SP-38 ³⁾		AMP-CHL-ENR-KAN-OXA-SXT-TET	+	V	0.75
SP-39		AMP-CHL-ERY-GEN-KAN-SXT-TET	-		0.25
SP-242	Pig	ENR-ERY-KAN-SXT	-		0.125
SP-273		ENR-ERY-KAN-SXT	-		0.125
SP-111		ENR-ERY-KAN-SXT	-		0.25
SP-281		ENR-ERY-KAN-SXT	-		0.125
SP-362		CHL-ENR-ERY-KAN-SXT	-		0.125
SP-471	Cow	-			1

¹⁾ AMP, ampicillin; CHL, chloramphenicol; ENR, enrofloxacin; ERY, erythromycin; GEN, gentamicin; KAN, kanamycin; OXA, oxacillin; RIF, rifampin; SXT, sulfamethoxazole-trimethoprim; TET, tetracycline

²⁾ Oxacillin minimum inhibitory concentrations (MICs) indicate ≥ 0.5 µg/mL; methicillin-resistant *S. pseudintermedius* (MRSP) (OXA MICs ≥ 0.5 µg/mL) are by shading

³⁾ indicates MRSP

Materials and methods

Bacterial isolation and identification

The 12 *S. pseudintermedius* isolates used in this study are listed in Table 1. A total of 401 swab samples were collected from dogs (n = 42), pigs (n = 190), and beef cattle (n = 169) between 2017 and 2018. Six canine-associated *S. pseudintermedius* strains were isolated from ear canals of dogs attending three different tertiary veterinary hospitals in Seoul, Seongnam, and Yongin. Five swine-associated *S. pseudintermedius* strains and one bovine-associated *S. pseudintermedius* strain were isolated from nasal swab samples from finishing pigs and beef cattle, respectively.

Each swab sample was inoculated onto Baird Parker Agar (BPA; Difco Laboratories) and incubated at 37°C for 24-48 h. All putative staphylococcal colonies were selected and subcultured on BPA for identification. All *S. pseudintermedius* strains were identified using the Vitek 2 system (bioMérieux, Marcy-l'Étoile, France) and 16S rRNA sequencing method as described previously²⁴⁾. In addition, the sequence of *tuf* gene was analyzed using a set of specific primers (Forward, 5'-GCCAGTTGAGGACGTATTCT-3'; Reverse, 5'-CCATTTTCAGTACCTTCTGGTAA-3') to confirm *S. pseudintermedius* isolates²⁵⁾.

Antimicrobial susceptibility test

Susceptibilities to antimicrobial agents were determined using

the disk diffusion methods according to the 2019 Clinical and Laboratory Standards Institute (CLSI) guidelines²⁶⁾. The antimicrobial agents used were ampicillin (AMP, 10 µg), chloramphenicol (CHL, 30 µg), enrofloxacin (ENR, 5 µg), erythromycin (ERY, 15 µg), gentamicin (GEN, 30 µg), kanamycin (KAN, 30 µg), oxacillin (OXA, 1 µg), rifampin (RIF, 5 µg), sulfamethoxazole-trimethoprim (SXT, 23.73-1.25 µg) and tetracycline (30 µg) (BD Difco, Detroit, MI, USA). The minimum inhibitory concentrations (MICs) to oxacillin were determined by using micro-broth dilution method²⁶⁾.

Detection of antimicrobial resistant genes

Total genomic DNA samples were prepared from *S. pseudintermedius* as described previously²⁷⁾. The presence of *mecA* gene was screened in all *S. pseudintermedius* strains, and SCC*mec* types were determined on *mecA*-positive *S. pseudintermedius* strains using the multiplex PCR method as described before²⁷⁾.

Detection of staphylococcal enterotoxin (SE) genes

A total of 19 different SE genes were detected in the 12 *S. pseudintermedius* strains as previously described^{16,18)}. Briefly, the carriage of 5 classical SE genes (*sea*, *seb*, *sec*, *sed*, and *see*) and 13 newer SE genes (*seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq*, *ser*, and *seu*) was examined by eight sets of multiplex PCR assays. Primers used for amplification of SE genes and their expected sizes are

Table 2. Primer sequences used for SEs, *tst-1*, and *mecA* genes PCR amplification

Genes	Primer	Oligonucleotide sequence (5'-3')	Size (bp)	PCR set	Reference
<i>sea</i>	F	CAGCATACTATATTGTTTAAAGGC	400	1-1	(18)
	R	CCTCTGAACCTTCCCATC			
<i>seb</i>	F	GTATGGTGGTGTAACCTGAGCA	351	1-2	(18)
	R	TCAATCTTCACATCTTTAGAATCA			
<i>sec</i>	F	CTCAAGAACTAGACATAAAAAGCTAGG	271	1-2	(39)
	R	TCAAAAATCGGATTAACATTATCC			
<i>sed</i>	F	CTAGTTTGGTAATATCTCCTTTAAACG	319	1-1	(39)
	R	TTAATGCTATATCTTATAGGGTAAACATC			
<i>see</i>	F	CAGTACCTATAGATAAAGTTAAAACAAGC	178	1-2	(39)
	R	TAACCTACCGTGGACCCTTC			
<i>seg</i>	F	AAGTAGACATTTTTGGCGTTCC	287	2-1	(40)
	R	AGAACCATCAAACCTCGTATAGC			
<i>seh</i>	F	GTCTATATGGAGGTACAACACT	213	2-2	(40)
	R	GACCTTTACTTATTTGCTGTC			
<i>sei</i>	F	GGTGATATTGGTGTAGGTAAC	454	2-1	(40)
	R	ATCCATATTCTTTGCCTTTACCAG			
<i>sej</i>	F	ATAGCATCAGAACTGTTGTTCCG	152	2-1	(40)
	R	CTTTCTGAATTTTACCACCAAAGG			
<i>sek</i>	F	TAGGTGTCTCTAATAATGCCA	293	3-2	(40)
	R	TAGATATTCGTTAGTAGCTG			
<i>sel</i>	F	TAACGGCGATGTAGGTCCAGG	383	4-1	(40)
	R	CATCTATTTCTTGTGCGGTAAC			
<i>sem</i>	F	GGATAATTCGACAGTAACAG	379	3-2	(40)
	R	TCCTGCATTAATCCAGAAC			
<i>sen</i>	F	CATCATGCTTATACGGAGGAG	301	4-2	(18)
	R	CCCCTGAACCTTTTACGTT			
<i>seo</i>	F	TGTGTAAGAAGTCAAGTGTAG	214	3-2	(40)
	R	TCTTTAGAAATCGCTGATGA			
<i>sep</i>	F	TGATTTATTAGTAGACCTTGG	381	2-2	(40)
	R	ATAACCAACCGAATCACCAG			
<i>seq</i>	F	TCAAGGAGTTAGTTCTGGAAATT	251	4-1	(18)
	R	GCTTACCATTGACCCAGAGA			
<i>ser</i>	F	GGATAAAGCGGTAATAGCAG	166	4-1	(40)
	R	GTATTCCAAACACATCTAAC			
<i>seu</i>	F	ATCAGAAACAAACATTAAGCCCA	301	4-2	(18)
	R	TGACCATTTCTTCGATAAACTTTAT			
<i>tst-1</i>	F	AAGCCCTTTGTTGCTTGCG	447	3-1	(39)
	R	ATCGAACTTTGGCCATACTTT			
<i>mecA</i>	F	TGCTATCCACCCTCAAACAGG	286		(27)
	R	AACGTTGTAACCACCCCAAGA			

shown in Table 2. The multiplex PCR assays were carried out with eight different sets of mixtures using the following conditions: an initial denaturation at 95°C for 3 min; 30 cycles of denaturing at 95°C for 30s, annealing at 53°C for 45s, extension at 72°C for 40s; and a final elongation at 72°C for 10 min. In addition, a singular PCR reaction for toxic shock syndrome toxin-1 (*tst-1*) gene was conducted as described before¹⁸. Mixture of genomic DNAs from

reference *S. aureus* strains were used for positive controls for each PCR assay (COL: *seb*; FRI472: *sed*, *seg*, *sej*, *sel*, *sem*, *sen*, *seo*, *ser*, *seu*; FRI913: *sea*, *sec*, *see*, *sek*, *selq*, *tst1*; and MW2: *seh*; N135: *sei*, *sep*)¹⁸.

Results and discussion

Although coagulase-positive *S. pseudintermedius* has

most frequently been isolated from both healthy dogs and dogs with skin infections²⁻⁴), the colonization of *S. pseudintermedius* has also been observed in the other small animals and farm animals^{1,5,6}). As shown in Table 1, prevalence of *S. pseudintermedius* in different animal species varied from 0.6 to 14.3%. Six, five, and one *S. pseudintermedius* strains were isolated from dogs (14.3%), pigs (3%), and beef cattle (0.6%), respectively. To the best of our knowledge, this is the first study to report swine-associated *S. pseudintermedius* strains in Korea, particularly MDR *S. pseudintermedius* strains.

As shown in Table 1, antimicrobial susceptibility assays revealed different antimicrobial resistance patterns among *S. pseudintermedius* isolates depending on origins of isolation. Interestingly, the six *S. pseudintermedius* isolates from dogs exhibited higher levels of MDR phenotype than other animal isolates. Of note, methicillin resistance was observed only in 4 canine-associated *S. pseudintermedius* isolates. These 4 MRSP isolates harbored SCCmec type V for methicillin resistance. The bovine isolate (SP-471) was susceptible to all the 11 antimicrobial agents tested, including OXA. In contrast to the SP-471, a previous study reported isolation of bovine-associated *S. pseudintermedius* from subclinical dairy cow mastitis with MDR phenotype, especially methicillin resistance⁷). Similar to the *S. pseudintermedius* isolates from dogs, five swine-associated *S. pseudintermedius* isolates exhibited MDR phenotype. Except for the single bovine isolate, all other *S. pseudintermedius* isolates from dogs and pigs displayed resistance to ENR, ERY, and SXT. These high levels of resistance to non- β -lactam antibiotic agents in *S. pseudintermedius* isolates from animals have also been described in previous studies^{1,2,28}). In addition, in line with the previous studies, which reported >57% of CHL resistance^{14,28-30}), 7 out of 12 *S. pseudintermedius* isolates (58.3%) were resistant to CHL. Although methicillin resistance and highest level of MDR phenotype was observed only in canine-associated *S. pseudintermedius* isolates, continued monitoring of antimicrobial resistance in *S. pseudintermedius* isolates are necessary in major companion animals and farm animals.

Staphylococcal food poisoning cases acquired by eating enterotoxin-contaminated food are the one of the most commonly reported types of foodborne diseases worldwide¹⁷). The frequent incidence of food poisoning by staphylococci is in part due to the fact that staphylococci, such as *S. aureus*, can grow over a wide range of hosts and environments³¹). Although the majority of studies related to SEs have been associated with *S. aureus*, several groups have reported that *S. pseudintermedius* strains isolated from dogs produce SEs. Aside from dogs, very few information is available for *S. pseudintermedius* isolates from other

animals, especially food-producing animals. As shown in Table 3, 11 different SE genes (*seb*, *sec*, *see*, *seg*, *sei*, *sej*, *sel*, *seo*, *sep*, *seq*, and *seu*) and *tst-1* were identified in *S. pseudintermedius* strains isolated from dogs, pigs, and beef cattle. Except for one isolate (SP-281), all *S. pseudintermedius* isolates harbored at least one of the 19 SE genes. Although the bovine isolate (SP-471) were susceptible to all antimicrobial agents tested, SP-471 carried highest number of SE genes (9 SE genes) among the 12 *S. pseudintermedius* isolates. It has been reported that the most common SEs are SEA and SEB in staphylococci-related food poisoning^{16,17,19}). Previous studies also reported that canine-associated *S. pseudintermedius* isolates most frequently carry *sec*^{21-23,32}). However, none of the 12 *S. pseudintermedius* isolates carried *sea* and only 2/12 (16.7%) *S. pseudintermedius* isolates were positive for *seb*. In addition, only 2/6 canine-associated *S. pseudintermedius* isolates were positive for *sec*. Although these *sec* genes were not sequenced for further analysis, it has been reported that canine-associated *S. pseudintermedius* often produce SEC variant (SEC_{canine})³³). In addition to the SEC_{canine}, several antigenic variants of SEC (i.e. SEC_{bovine}, SEC_{ovine}, SEC1-3) have also been reported^{21,32}). Interestingly, *see* and *seq* genes were detected only in canine isolates, indicating that these genes might be host specific. Structure of SEE is similar to SEA and has been reported in some cases of food poisoning^{17,34}). Tanabe *et al.* (2013) also reported presence of *seq* in *S. pseudintermedius* strains isolated from dogs²³). Among the 12 SE genes detected, *sel* and *sep* were each identified in 5 *S. pseudintermedius* isolates, resulting in highest prevalence rate (41.7%) among the 19 different SE genes. Previously, SEL was detected in a pathogenicity island of bovine mastitis *S. aureus* (SaPIbov) isolate exhibiting a variety of biological activities including superantigenic, pyrogenic, and endotoxigenic activity in a rabbit model³⁵). SEP was also characterized in a MRSA strain isolated from a human bacteremia case³⁶). In addition to SEs, toxic shock syndrome toxin (TSST-1), encoded by *tst-1*, has been well studied as a non-specific T-cells activator and inducer of fatal toxic shock syndrome, although TSST-1 lacks emetic activity^{16,17,37}). While all 6 *S. pseudintermedius* isolates from dogs were negative for *tst-1*, four isolates from pigs and one isolate from beef cattle were found to be positive for *tst-1* gene. In a previous study, Hu *et al.* (2008) showed that *sec*, *seg*, *sei*, *sel*, *sem*, *seo*, and *tst-1* genes were frequently associated with SCCmec type I and type II³⁸). As shown in Table 3, only the four MRSP isolates which carries SCCmec type V had *see* or *seq* genes. These results indicate that prevalence of SE genes and *tst-1* among *S. pseudintermedius* isolates may be associated with host factors in different animals and environmental factors.

Table 3. The prevalence of SEs and *tst-1* genes from *S. pseudintermedius*

Strain (No. of isolates)	Origin	Number of SE genes																			
		<i>sea</i>	<i>seb</i>	<i>sec</i>	<i>sed</i>	<i>see</i>	<i>seg</i>	<i>seh</i>	<i>sei</i>	<i>sej</i>	<i>sek</i>	<i>sel</i>	<i>sem</i>	<i>sen</i>	<i>seo</i>	<i>sep</i>	<i>seq</i>	<i>ser</i>	<i>seu</i>	<i>tst-1</i>	
SP-34						1									1						
SP-35						1											1				
SP-36	Dog			1								1									
SP-37						1									1	1					
SP-38																	1				
SP-39					1				1			1									
SP-242				1						1										1	
SP-273								1												1	
SP-111	Pig		1	1			1			1		1			1				1	1	
SP-281																					
SP-362										1	1	1			1	1					
SP-471	Cow		1				1		1	1	1			1	1				1	1	
Total			2	4		3	3		3	4		5			2	5	3			2	4

In conclusion, our results indicate that *S. pseudintermedius* strains isolated from livestock and companion animals became resistant to multiple antimicrobial agents used to treat infections in humans. Although methicillin resistance was observed only in canine isolates, all the pig isolates were resistant to at least 4 different antimicrobial agents. In addition to antimicrobial resistance, most *S. pseudintermedius* isolates carried multiple SE genes and *tst-1* that can cause food-producing in humans. Our results also indicated that *S. pseudintermedius* isolates from livestock and companion animals may serve as a reservoir for staphylococcal enterotoxin genes.

국문요약

*Staphylococcus pseudintermedius*는 개에서 기회감염을 유발하는 병원체이며, 공중보건학적으로도 주요한 인수공통 병원체이다. 개에서 분리된 *S. pseudintermedius* 균주들은 주로 항생제 내성 및 개에서 피부 감염을 유발하는 주요 원인균으로 연구되어 왔지만, 가축에서 분리된 *S. pseudintermedius* 균주들의 항생제 내성 및 장내 독소 생성에 대한 정보는 매우 제한적이다. 본 연구에서는 개, 돼지, 육우에서 분리된 *S. pseudintermedius* 균주들에서 18가지의 장내 독소 (staphylococcal enterotoxin; SE) 유전자와 toxic shock syndrome toxin 유전자(*tst-1*)의 분포양상을 조사하였다. 또한, *S. pseudintermedius* 균주들의 항생제 내성 양상과 더불어 *mecA* 유전자 및 SCCmec type 또한 확인하였다. 육우에서 분리한 하나의 균주를 제외한 모든 개와 돼지 분리주들이 4개 이상의 항생제에 내성을 보였으며, 개에

서 분리된 6개의 균주 중 4개의 *S. pseudintermedius* 균주들이 메티실린 내성과 더불어 SCCmec V를 가진 것으로 확인되었다. 총 11개의 SE 유전자들 (*seb*, *sec*, *see*, *seg*, *sei*, *sej*, *sel*, *seo*, *sep*, *seq*, *seu*) 및 *tst-1*가 개, 돼지 및 육우로부터 분리된 *S. pseudintermedius* 균주들에서 확인되었으며, 대부분의 분리주들 (83%)에서 2개 이상의 SE 유전자들이 확인되었고, 그 중 *sel* (42%) 및 *sep* (42%)가 가장 빈번하게 검출되었다.

본 연구를 통하여 반려견에서 뿐만 아니라 주요 가축에서 존재하는 *S. pseudintermedius* 균주들에서 높은 항생제 내성 양상을 확인 하였으며, 항생제 내성과 더불어 여러 staphylococcal enterotoxin 및 *tst-1* 유전자들을 전파할 가능성을 확인 하였다.

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