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Genomic analysis and One-Health approach to antimicrobial resistance in *Staphylococcus pseudintermedius* isolates from canine pyoderma

개의 농피증에서 분리한 *Staphylococcus pseudintermedius* 의 항생제 내성에 대한 유전체 분석과 원헬스적 접근

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ABSTRACT

Staphylococcus pseudintermedius is a member of the normal skin microbiota of dogs; however, as an opportunistic pathogen, it is also the cause of the majority of canine bacterial infections. Many classes of antimicrobial drugs are commonly used in small animal practice for the treatment of *S. pseudintermedius* infection, leading to the massive problem of rapidly emerging antimicrobial resistance. In particular, methicillin-resistant *S. pseudintermedius* (MRSP) poses a challenge

for veterinary medicine. Therefore, the aim of this thesis was to investigate clonal distribution of MRSP isolated from dogs in Korea, the emergence of multidrug-resistant *S. pseudintermedius* (MDRSP) related with pRE25-like elements, and to uncover genetic relationship of methicillin resistance between staphylococci isolated from dogs affected with pyoderma and their owners.

In chapter 1, the clonal distribution of MRSP isolates from dogs affected with pyoderma and otitis externa in Korea was determined. Sixty MRSP isolates were obtained from the lesions of dogs with canine pyoderma and otitis externa. Their genetic diversity was assessed by multilocus sequence typing (MLST), direct repeat unit (*dru*) typing, and staphylococcal cassette chromosome *mec* (SCC*mec*) typing. The major *dru* types were dt11a and dt11y. Two clonal complexes (CCs), CC568 and CC677, were revealed by MLST; this result differed from the dominant STs detected in MRSP isolates from Europe, North America, and other Asian countries. SCC*mec* type V was the major type (27/60, 45%).

In chapter 2, the emergence of multi-drug resistant *S. pseudintermedius* (MDRSP) carrying pRE25-like elements was analyzed. In total, 46 MDRSP strains were isolated from clinical cases of canine pyoderma. Their genetic characteristics were revealed by MLST, polymerase chain reaction (PCR) targeting of pRE25-like elements, and whole-genome sequencing (WGS). WGS detected that the chromosomal 22-kb pRE25-like elements contained five

antimicrobial-resistant genes (*cat*, *erm(B)*, *aphA3*, *aadK*, and *sat4*), *IS1252*, *IS256*, and a toxin-antitoxin (TA) system within copies of *IS1216*. BLASTN alignment revealed that 84% of the chromosomal 22-kb pRE25-like elements sequence is homologous (99.8% identity) to the enterococcal pRE25 plasmid sequence. PCR showed that 52% of the MDRSP isolates were positive for pRE25-like elements and were thus presumed to contain pRE25-like elements (pRE25 group). The STs of the pRE25 group were diverse, with 18 STs identified, including 12 STs first reported in Korea.

In chapter 3, the genetic relationship of methicillin resistance between staphylococci isolated from dogs affected with pyoderma and skin of dog owners was investigated by WGS. A total of 101 *Staphylococcus* strains were isolated from 31 dog-owner pairs. The clonal lineage was characterized by MLST. Genetic relatedness of the isolates from dogs and owners was first evaluated with *dru* and SCC*mec* typing, and WGS confirmed the similarity of DNA sequences and the structural composition of SCC*mec*. In one pair, *S. pseudintermedius* (dog), *S. schleiferi* (dog), and *S. epidermidis* (dog owner) isolated from a dog affected with pyoderma and the dog owner showed remarkable genetic similarity in SCC*mec* with respect to DNA sequences, *dru* type, structure composition of *ccrC* and the *mec* complex, and DR-1 in *orfX*, which is considered to be the insertion site of SCC*mec*.

In conclusion, a relatively high prevalence (41.9%, 60/143) and independent clonal characteristics compared with other countries highlight the rapid emergence of MRSP isolated from dogs in Korea. Moreover, MDRSP induced by pRE25-like elements is suspected to be widespread, and the MLST results indicated Korea as the potential geographical origin of the MDRSP lineage carrying pRE25-like elements. These results suggest that veterinary antimicrobial stewardship has not been applied consistently in the field of small animal practice in Korea, thus calling for a long-term national action plan.

The risk of SCC*mec* transfer between dogs and their owners was estimated by WGS, demonstrating remarkable genetic similarity of SCC*mec* among *S. pseudintermedius* (dog), *S. schleiferi* (dog), and S. *epidermidis* (dog owner) in one of the pairs. Therefore, a strategy countering the spread of methicillin-resistant staphylococci should be based on the cooperation of veterinary medicine and human medicine under the One-Health concept.

Keywords: *Staphylococcus pseudintermedius*, methicillin resistance, multidrug resistance, SCC*mec*, canine pyoderma

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CONTENTS

ABBREVIATIONS

aadE	aminoglycoside adenylyltransferase
aadK	aminoglycoside-6-adenylyltransferase
aphA3	aminoglycoside-3-phosphotransferase
BLAST	Basic Local Alignment Search Tool
cat	chloramphenicol acetyltransferase
ccr	cassette chromosome recombinase
CCs	clonal complexes
CLSI	Clinical and Laboratory Standards Institute
dfrG	dihydrofolate reductase
dru	direct repeat unit
dts	*dru* types
erm(B)	rRNA adenine N-6-methyltransferase
gap	glyceraldehyde-3-phosphate dehydrogenase
MDRSP	multidrug-resistant *Staphylococcus pseudintermedius*
MLST	multi-locus sequence typing
MRSA	methicillin-resistant *Staphylococcus aureus*
MRSP	methicillin-resistant *Staphylococcus pseudintermedius*
NT	non-typeable
nuc	thermonuclease
PCR	polymerase chain reaction
PSK	post-segregational killing
rec	recombinase
sat	streptothricin N-acetyltransferase
SCC*mec*	staphylococcal cassette chromosome *mec*
spa	staphylococcal protein A
ST	sequence types
TA	toxin-antitoxin
tuf	elongation factor Tu
VNTR	variable number tandem repeat
WGS	whole-genome sequencing

GENERAL INTRODUCTION

Staphylococcus intermedius was first reported in 1976 in isolates from pigeons, dogs, minks, and horses (Hájek, 1976), and is traditionally regarded as the main organism isolated from cases of canine pyoderma and otitis (Gross *et al.*, 2005). Despite a high level of phenotypic similarity, *S. pseudintermedius* was reclassified as a distinct species from *S. intermedius* in 2005 based on biochemical features and DNA-DNA hybridization results (Devriese *et al.*, 2005). *S. intermedius* and *S. pseudintermedius*, along with *Staphylococcus delphini* originally isolated from dolphins (Varaldo *et al.*, 1988), are categorized as the *Staphylococcus intermedius* group because of their phylogenetic relatedness (Bannoehr *et al.*, 2007; Sasaki *et al.*, 2007b). Further identification revealed that most of the strains isolated from dogs that were initially identified as *S. intermedius* based on phenotypic methods are actually *S. pseudintermedius* (Devriese *et al.*, 2009).

S. pseudintermedius is part of the normal microbiota of dogs, and colonizes the skin and mucocutaneous sites such as the mouth, nose, and anus (Allaker *et al.*, 1992). However, *S. pseudintermedius* can also be a major pathogen of the pyoderma and a cause of ear infections in dogs, and is the predominant cause of canine bacterial infections. *S. pseudintermedius* is rarely isolated from humans and seems to show host specificity to canine corneocytes (Simou *et al.*, 2005). However, transmission between humans and their dogs is nevertheless possible,

and *S. pseudintermedius* is considered to be a zoonotic pathogen (Guardabassi *et al.*, 2004; Tanner *et al.*, 2000).

The antimicrobial resistance of *S. pseudintermedius* has emerged rapidly in recent years, representing a huge problem for infection control in veterinary medicine because of the limited antibiotic options available for treatment. The methicillin resistance of *S. pseudintermedius* is mediated by *mecA* gene modifying penicillin-binding protein, similar to methicillin-resistant *Staphylococcus aureus* (MRSA) (Weese and van Duijkeren, 2010). Since 2000, methicillin-resistant *S. pseudintermedius* (MRSP) isolates harboring *mecA* have been reported globally across North America, Europe, and Asia, with various clonal distributions. Recent studies have revealed the increased (20–47%) prevalence of MRSP isolates collected from dogs and cats (Beck *et al.*, 2012; De Lucia *et al.*, 2011; Feng *et al.*, 2012).

The emergence of multidrug-resistant *S. pseudintermedius* (MDRSP) has also been reported in several studies, which is associated with Tn*5405*-like elements carrying up to five resistance genes (*aphA3*, *sat*, *aadE*, *erm*(B), and *dfrG*) (McCarthy *et al.*, 2015; Ruscher *et al.*, 2010). The pRE25-like enterococcal plasmid pWZ909 is known to mediate the delivery of vancomycin resistance to MRSA via a Tn*1546*-like transposon (Zhu *et al.*, 2010). However, it remains unclear whether pRE25-like elements contribute to multidrug resistance in *Staphylococcus* spp.

The potential transfer of antimicrobial resistance genes between humans and animals has received increasing attention. The One-Health approach to humans and animals is a current strategy to respond to the increased risk of the emergence of antimicrobial-resistant organisms. In particular, detection of the same resistance genes or mobile genetic elements in bacteria isolated from both humans and animals has provided insights into the mechanisms and patterns of the transfer of resistance genes in natural environments (Guardabassi *et al.*, 2004). Because most resistance genes are associated with mobile genetic elements (i.e., transposons, integrons, or plasmids), such transfer within bacterial species is not in itself a novel finding. However, clinical reports of genetic associations between isolates from humans and animals with respect to antimicrobial resistance remain scarce and are particularly rare in the context of interspecies relationships.

Accordingly, the aim of this study was to investigate the clonal distribution of MRSP isolated from dogs in Korea, the emergence of MDRSP related with pRE25-like elements, and the genetic relationship of methicillin resistance between staphylococci isolated from dogs affected with pyoderma and their owners.

CHAPTER I: Clonal distribution of methicillin-resistant *Staphylococcus pseudintermedius* isolates from canine pyoderma and otitis externa

1. Introduction

In 2005, *S. pseudintermedius* was reclassified from *S. intermedius* according to distinct biochemical features and DNA-DNA hybridization results (Devriese *et al.*, 2005) and were traditionally regarded as the main organism isolated from canine pyoderma and otitis. In dogs, *S. pseudintermedius* is part of the normal microbiota and colonizes the skin and mucocutaneous sites such as the mouth, nose, and anus (Allaker *et al.*, 1992). A more recent study further confirmed that most canine bacterial infections are caused by *S. pseudintermedius* (Devriese *et al.*, 2009).

MRSP has emerged very rapidly in recent years, posing a challenge for infection control in veterinary medicine because of the limited antibiotic options available for its treatment (Ruscher *et al.*, 2009). The methicillin resistance of *S. pseudintermedius* is mediated by *mecA* gene modifying penicillin-binding protein, similar to MRSA (Weese and van Duijkeren, 2010). Since 2000, MRSP isolates harboring *mecA* have been reported globally across North America, Europe, and

Asia, with various clonal distributions. Recent studies have revealed the increased (20–47%) prevalence of MRSP collected from dogs and cats (Beck *et al.*, 2012; De Lucia *et al.*, 2011; Feng *et al.*, 2012). The aim of this chapter is describing the clonal characteristics of MRSP isolates in Korea and comparing their molecular lineages with the dominant strains reported from other countries.

2. Material and Methods

2.1 Sample collection and identification

A total of 186 clinical isolates were collected aseptically from infection sites of 186 dogs (136 dogs, pyoderma; 50 dogs, otitis externa) from 2011 to 2015. All animals had owners visiting the Veterinary Medical Teaching Hospital of Seoul National University. Each lesion site was sampled using a cotton swab and cultivated overnight at 37°C on blood agar. All isolates were identified using the Vitek 2 system (Biomerieux, Lyon, France). Isolates identified as *S. pseudintermedius* were then subjected to molecular identification using polymerase chain reaction (PCR) targeting the *nuc* (Table 1) (Sasaki *et al.*, 2010) and *mecA* (Table 3) (Kondo *et al.*, 2007) genes. All PCR products were sequenced using an ABI PRISM 3730xl apparatus (Applied Biosystems, Foster City, CA, USA) to confirm the species identification. The sequencing reaction was performed using 2 μL of the purified PCR-amplified DNA and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following the manufacturer instructions.

2.2 Antimicrobial susceptibility testing

Susceptibility testing was performed using the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines published in 2016 (CLSI, 2016). The antibiotics examined included penicillin, oxacillin, amikacin, gentamicin, erythromycin, clindamycin, tetracycline, minocycline, ciprofloxacin, norfloxacin, trimethoprim/sulfamethoxazole, chloramphenicol, and rifampicin.

2.3 Multilocus sequence typing (MLST), *mec*-associated direct repeat unit (*dru*) typing, and staphylococcal protein A (*spa*) typing

Sequence types (STs) were determined by MLST using seven housekeeping genes (Table 2) (Solyman *et al.*, 2013). MLST sequences were compared with allele sequences in the *S. pseudintermedius* MLST database (http://pubmlst.org/spseudintermedius) to determine the allele number. The clonal relationship of STs was predicted by eBURST analysis (Feil *et al.*, 2004). New ST numbers were assigned by the database curator (vincent.perreten@vetsuisse.unibe.ch). The *dru* variable number tandem repeat (VNTR) region adjacent to *IS431* in staphylococcal cassette chromosome *mec*

(SCC*mec*) was sequenced and characterized as previously described (Goering *et al.*, 2008). The *dru* region was amplified using the primer pair 5′-GTTAGCATATTACCTCTCCTTGC-3′ and 5′-GCCGATTGTGCTTGATGAG -3′. Sequenced tandem repeats were identified (Benson, 1999) and analyzed using the *dru* database website (http://dru-typing.org), and novel *dru* types (dts) were assigned by the database curator (richardgoering@creighton.edu). A minimum spanning tree was generated using BioNumerics v7.6 (Applied Maths, Austin, TX, USA). *spa* typing was conducted as previously described (Moodley *et al.*, 2009), and *spa* genes were PCR-amplified with the primer pair SIspa F (5′-AACCTGCGCCAAGTTTCGATGAAG-3′) and SIspaR (5′-CGTGGTTTGCTTTAGCTTCTTGGC-3′). The *S. pseudintermedius spa* database was provided by Arshnee Moodley (asm@sund.ku.dk) with an agreement concerning the sharing of the database. Sequencing reactions were performed using 2 μL of purified PCR-amplified DNA and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer instructions.

2.4 Characterization of SCC*mec*

The SCC*mec* type was determined using multiplex PCR (Table 3) as reported previously (Kondo *et al.*, 2007) to detect *mecA*, identify the cassette chromosome recombinase (*ccr*) complex, and distinguish classes A, B, and C *mec*. The presence of *ccr*A5/B3 in SCC*mec* type VII-241 cassettes was screened using primers 5′-GCCAAAATTTCTTTCGAGACC-3′ and 5′-TACGTGCGAGTCGATTTGTT-3′ (Perreten *et al.*, 2010). SCC*mec* II-III was distinguished from SCC*mec* III based on absence of the cadmium resistance operon (Perreten *et al.*, 2010).

3. Results

3.1 Identification and antimicrobial susceptibility testing

Vitek 2 identification revealed *S. pseudintermedius* (n = 143, 77%) as the major species, followed by *S. schleiferi* (11%) and *S. aureus* (7%). Other *Staphylococcus* spp. identified (all n = 1) included *S. hyicus*, *S. chromogenes*, *S. sciuri*, *S. simulans*, *S. parasanguinis*, *S. haemolyticus*, and *S. hominis*. All *S. pseudintermedius* isolates were positive in PCR identification, and the sequence of the PCR product was confirmed (Fig. 1). Among the 143 *S. pseudintermedius* isolates, there were 60 MRSP strains as confirmed by PCR detection of *mecA* and resistance to penicillin and oxacillin (Table 4).

Resistance rates of MRSP to the other eight antibiotics were high (≥40%): tetracycline (86.6%, 52/60), trimethoprim-sulfamethoxazole (85%, 51/60), erythromycin (83.3%, 50/60), clindamycin (81.6%, 49/60), ciprofloxacin (60%, 36/60), norfloxacin (58.3%, 35/60), chloramphenicol (46.6%, 28/60), and gentamicin (45%, 27/60). However, the resistant rates to rifampicin (3.3%, 2/60), amikacin (1.6%, 1/60), and minocycline (1.6%, 1/60) were very low.

3.2 Molecular characteristics of MRSP

MLST revealed 39 different STs among the isolates (Fig. 3), including 21 new international STs, which were assigned as novel STs in the *S. pseudintermedius* MLST database (ST563, ST565, ST566, ST567, ST568, ST573, ST574, ST575, ST578, ST580, ST581, ST584, ST585, ST586, ST587, ST588, ST674, ST677, ST708, ST710, and ST712). There was no dominant ST; six isolates displayed the identical ST.

In *dru* typing, 14 dts were detected (dt11a, dt11av, dt11ax, dt11ca, dt11cj, dt11i, dt11p, dt11x, dt11y, dt10a, dt10dh, dt9a, dt9bo, and dt9bs), with no *dru* detected in three isolates. Three unclassified allele combinations were found and added as new types (dt11av, dt10dh, and dt9bs) to the dt database. The major types were dt11a (n = 19) and dt11y (n = 16), identified in 35 (61.4%) of the 57 isolates (Fig. 4).

spa typing characterized only 21 (35%) of the 60 isolates, including *spa* types t02 (n = 8), t05 (n = 5), t06 (n = 6), and t15 (n = 2) identified. The predominant SCC*mec* was type V (45%, 27/60) (Fig. 2). Thirty isolates were non-typeable because of a combination of SCC*mec* components that did not match any other SCC*mec* types confirmed in previous studies (Table 5). Three isolates contained only *mecA*, and no other SCC*mec* components were detected using multiplex PCR. The strain information and molecular characteristics of all 60 MRSP isolates analyzed in this study are summarized in Table 4.

4. Discussion

The overall prevalence of MRSP identified in this study (41.9%, 60/143) from isolates of infected dogs in Korea is markedly higher than those reported previously in Europe and North America (Griffeth *et al.*, 2008; Gronthal *et al.*, 2017; Hanselman *et al.*, 2008; Kania *et al.*, 2004; Maluping *et al.*, 2014). However, a high prevalence range of about 30–60% has been reported elsewhere in Asia, including in Japan, Thailand, and China (Chanchaithong *et al.*, 2014; Feng *et al.*, 2012; Kawakami *et al.*, 2010; Sasaki *et al.*, 2007a). These high prevalence ranges highlight the rapid emergence of MRSP in Asian countries. Compounding this problem, the resistance rates to other antibiotics were also high. Multidrug-resistant bacteria are a serious global concern, and virtually all of the MRSP (58/60) isolates identified in this study were defined as multidrug-resistant strains (Magiorakos *et al.*, 2012). Dissemination of multidrug-resistant MRSP (MDR-MRSP) isolates among dogs with owners could represent a common problem, limiting the ability of antibiotic treatment at veterinary clinics.

The genetic pool of MRSP in Korea revealed an independent lineage. MLST identified 21 novel STs, which accounted for 53.8% (21/39) of the total STs found in this study. No dominant STs were detected, and almost all of the STs were represented by only one or two isolates each, with only ST365 and ST584 found in multiple isolates with identical STs (n = 5 and n = 6, respectively). Thus, the MRSP population was highly diverse, and many different STs have acquired

methicillin resistance. Clonal complexes (CCs) 568 and 677 were detected in the Korean MRSP population (Fig. 3), and 40% (24/60) of the MRSP isolates carried CC568 (n = 14) and CC677 (n = 10). These CCs do not belong to any previously reported CCs predominant in Europe, North America, and Asia (Pires dos Santos *et al.*, 2016). In addition, CC568 and CC677 showed different tendencies with regard to the SCC*mec* type: non-typeable SCC*mec* was very dominant in CC568 (10/14, 71.4%), but almost all of the CC677 isolates belonged to SCC*mec* type V (9/10, 90%). The single STs were not clustered by any of the other characteristics besides CCs (i.e., source, *dru* type, *spa* type, and SCC*mec* type).

The presence of *spa* type t02 (n = 8), t05 (n = 5), t06 (n = 6), and t15 (n = 2) was confirmed. The presence of t15 is a novel finding in Asia. Nevertheless, *spa* typing was determined to be inappropriate for the molecular analysis of MRSP. Another study showed that the *spa* region of 65% of the isolates (39/60) was not amplified by PCR designed for MRSP (Moodley *et al.*, 2009), and *spa* is not always present in MRSP strains (Perreten *et al.*, 2013). Previous studies conducted in Spain and the Netherlands (Gómez-Sanz *et al.*, 2011; Laarhoven *et al.*, 2011) reported non-typeable isolates using conventional *spa* typing. Non-typeable isolates comprise ≥50% of the isolates in Asian countries, with *spa*-negative strains accounting for 62.7% (27/43) of all strains in Japan (Ishihara *et al.*, 2015), 88.2% (30/34) of strains identified in Thailand as well as Israel (Perreten *et al.*, 2013), and 78.2% (54/69) of the strains in China (Feng *et al.*, 2012). This tendency of high *spa*-negative prevalence rates has thus far only been

reported in Asian countries, indicating a potential specific molecular characteristic of Asian MRSP isolates.

The genetic diversity of MRSP isolates was further analyzed by *dru* typing. The dominant dts were dt11a (n = 19) and dt11y (n = 16), accounting for 61.4% of all isolates analyzed in this study. There were three dt11a isolates and six dt11y isolates in CC568; however, these dts were equally distributed in CC677 (dt11y = 2 and dt11a = 3). Only ST370 (CC677) had both dt11a and dt11y. Notably, dt11y was detected in 28.5% (16/56) of the Korean MRSP isolates, which has not been reported to be predominant in any region or country to date. One study of MRSP isolates from Europe, the United States, and Canada revealed that dt9a (43.4%) and dt11a (25.3%) were the most frequent types, with dt11y comprising only 0.6% (n = 6) of the total population (Kadlec *et al.*, 2015). Considering the 14.7% (5/34) prevalence of dt11y reported in Thailand and Israel (Perreten *et al.*, 2013), dt11y might be an important *dru* type in Asian MRSP clones; thus, further monitoring in Asian regions could be necessary. Moreover, although dt9a is a commonly reported international *dru* type clone, it was classified in only three isolates (0.5%) in the present study.

Two major SCC*mec* types of MRSP are disseminated in Europe (type II-III) and North America (type V). In Asia, the prevalence of these two major types differs between regions and countries. Type V is dominant in Thailand and South China (Chanchaithong *et al.*, 2014; Feng *et al.*, 2012), whereas type II-III is a major clone in Japan and North China (Ishihara *et al.*, 2015; Wang *et al.*, 2012b).

In the present study, 45% (27/60) of the Korean MRSP isolates were identified to be of type V. Therefore, with respect to SCC*mec* dissemination, Thailand and South China display a closer genetic relationship with Korea than with Japan and North China. Many isolates (50%) were non-typeable because the combination of *ccr* and *mec* complexes did not match with the conventional classification method (International Working Group on the Classification of Staphylococcal Cassette Chromosome, 2009). Genome sequencing confirmed six SCC*mec* types of MRSP: type II-III and type VII-241 (Descloux *et al.*, 2008), type V (Black *et al.*, 2009), pseudo-type 57395 (Perreten *et al.*, 2013), type IV (McCarthy *et al.*, 2015), and type AI16-SCCczrAI16-CI (Chanchaithong *et al.*, 2016). However, none of these previously confirmed types could be associated with any of the non-typeable (NT) combinations of *ccr* and *mec* in the present MRSP isolates. The group of NT SCC*mec* isolates had some common genetic characteristics of potential interest. Of the total 30 NT SCC*mec* isolates, 14 belonged to dt11y (14/30 46.6%), whereas only two dt11y isolates were detected in the SCC*mec* type V group (2/27, 7.4%); thus, dt11y was the dominant *dru* type in the NT SCC*mec* group followed by dt11a (7/30, 23.3%). The repeat unit in *dru* typing is adjacent to IS*431* in the SCC*mec* complex (Goering *et al.*, 2008). Therefore, this result supports that dt11y and NT SCC*mec* isolates in this study have a significant genetic relationship. Among the MLST CCs, CC568 was more closely associated with the NT SCC*mec* isolates (71.4%, 10/14) than CC677, which mostly (9/10, 90%) belonged to SCC*mec* type V.

Sequence typing and *dru* typing revealed the independent clonal characteristic of Korean MRSP isolates compared with isolates from other countries. The SCC*mec* type V was commonly detected in previously reported Asian MRSP clones, but the present results suggest that a new type might exist.

Table 1. Molecular identification of *Staphylococcus* spp.

Gene	Primer	Sequence (5′- 3′)	Product (bp)	Reference
nuc (*S. pseudintermedius*)	pse-F	TRGGCAGTAGGATTCGTTAA	926	Sasaki *et al*., 2010
	pse-R	CTTTTGTGCTYCMTTTTGG		
nuc (*S. schleiferi*)	sch-F	AATGGCTACAATGATAATCACTAA	526	
	sch-R	CATATCTGTCTTTCGGCGCG		
nuc (*S. aureus*)	au-F	TCGCTTGCTATGATTGTGG	359	
	au-R	GCCAATGTTCTACCATAGC		
gap	gap-F	ATGGTTTTGGTAGAATTGGTCGTTTA	~931	Ghebremedhin *et al*., 2008
	gap-R	GACATTTCGTTATCATACCAAGCTG		
tuf	tuf-F	GCCAGTTGAGGACGTATTCT	412	Heikens *et al*., 2005
	tuf-R	CCATTTCAGTACCTTCTGGTAA		

Table 2. PCR primers for multilocus sequence typing (MLST) of *S. pseudintermedius***.**

Gene	Primer	Sequence (5′- 3′)	Product (bp)	Reference
tuf	tuf-F	CAATGCCACAAACTCG	500	Solyman *et al*., 2013
	tuf-R	CAATGCCACAAACTCG		
cpn60	cpn60-F	GCGACTGTACTTGCACAAGCA	552	
	cpn60-R	AACTGCAACCGCTGTAAATG		
pta	pta-F	GTGCGTATCGTATTACCAGAAGG	570	
	pta-R	GCAGAACCTTTTGTTGAGAAGC		
purA	purA-F	GATTACTTCCAAGGTATGTTT	490	
	purA-R	TCGATAGAGTTAATAGATAAGTC		
fdh	fdh-F	TGCGATAACAGGATGTGCTT	408	
	fdh-R	CTTCTCATGATTCACCGGC		
ack	ack-F	CACCACTTCACAACCCAGCAAACT	680	
	ack-R	AACCTTCTAATACACGCGCACGCA		
sar	sar-F	GGATTTAGTCCAGTTCAAAATTT	521	
	sar-R	GAACCATTCGCCCCATGAA		

Table 3. PCR primers for characterization of SCC*mec* type.

Gene	Primer	Sequence (5′- 3′)	Product (bp)	Reference
mecA	mA1	TGCTATCCACCCTCAAACAGG	286	Kondo *et al*., 2007
	mA2	AACGTTGTAACCACCCCAAGA		
ccrA1-ccrB	α1	AACCTATATCATCAATCAGTACGT	695	
	βc	ATTGCCTTGATAATAGCCITCT		
ccrA2-ccrB	α2	TAAAGGCATCAATGCACAAACACT	937	
	βc	ATTGCCTTGATAATAGCCITCT		
ccrA3-ccrB	α3	AGCTCAAAAGCAAGCAATAGAAT	1,791	
	βc	ATTGCCTTGATAATAGCCITCT		
ccrA4-ccrB4	α4.2	GTATCAATGCACCAGAACTT	1,287	
	β4.2	TTGCGACTCTCTTGGCGTTT		
ccrC	γR	CCTTTATAGACTGGATTATTCAAAATAT	518	
	γF	CGTCTATTACAAGATGTTAAGGATAAT		
mecA-mecI	mI6	CATAACTTCCCATTCTGCAGATG	1,963	
	mA7	ATATACCAAACCCGACAACTACA		
mecA-IS1272	IS7	ATGCTTAATGATAGCATCCGAATG	2,827	
	mA7	ATATACCAAACCCGACAACTACA		
mecA-IS431	IS2	TGAGGTTATTCAGATATTTCGATGT	804	
	mA7	ATATACCAAACCCGACAACTACA		

Table 4. MRSP strain information in this study and molecular characteristics.

Strain	Origin	Source	MLST	*dru* type	*spa* type	SCC*mec*	Clonal Complex
SNU10P12	dog	pyoderma	568	11av	neg.	NT	CC568
SNU11P4	dog	pyoderma	578	11a	neg.	type V	CC568
SNU11P5	dog	pyoderma	584	11y	t05	NT	CC568
SNU12P5	dog	pyoderma	584	11y	t05	NT	CC568
SNU12P8	dog	otitis externa	584	11y	t15	NT	CC568
SNU12P9	dog	pyoderma	584	11y	t02	NT	CC568
SNU12P13	dog	pyoderma	588	11i	neg.	NT	CC568
SNU12P14	dog	otitis externa	584	11y	t15	NT	CC568
SNU13P4	dog	pyoderma	566	11a	neg.	NT	CC568
SNU13P18	dog	otitis externa	568	11y	neg.	NT	CC568
SNU14P7	dog	otitis externa	584	neg.	neg.	contained only *mecA*	CC568
SNU14P10	dog	otitis externa	566	11ca	neg.	NT	CC568
SNU15P41	dog	pyoderma	692	10a	neg.	type V	CC568
SNU15P45	dog	pyoderma	712	11a	neg.	type V	CC568
SNU10P15	dog	pyoderma	575	10dh	t06	type V	CC677
SNU10P16	dog	pyoderma	565	9bo	neg.	type V	CC677

SNU11P2	dog	pyoderma	68	11a	t06	type V	CC677
SNU11P18	dog	pyoderma	565	11y	neg.	type V	CC677
SNU12P18	dog	pyoderma	124	11av	neg.	type V	CC677
SNU13P7	dog	otitis externa	370	11a	neg.	NT	CC677
SNU13P8	dog	otitis externa	54	11a	neg.	type V	CC677
SNU13P14	dog	otitis externa	370	11y	neg.	type V	CC677
SNU14P36	dog	otitis externa	677	11ax	neg.	type V	CC677
SNU15P34	dog	pyoderma	17	11av	neg.	type V	CC677
SNU10P2	dog	pyoderma	71	9a	t02	NT	-
SNU10P3	dog	pyoderma	362	11a	neg.	type V	-
SNU10P5	dog	otitis externa	362	11a	neg.	type V	-
SNU10P6	dog	pyoderma	581	11av	neg.	NT	-
SNU10P13	dog	pyoderma	573	11y	t05	NT	-
SNU10P14	dog	pyoderma	574	11y	t05	NT	-
SNU11P6	dog	pyoderma	121	11a	t06	NT	-
SNU11P9	dog	otitis externa	123	9a	t02	NT	-
SNU11P10	dog	otitis externa	195	11a	neg.	NT	-
SNU11P11	dog	pyoderma	422	11a	t06	NT	-
SNU11P14	dog	pyoderma	365	11y	t05	NT	-

SNU11P17	dog	pyoderma	362	9bs	neg.	type V	-
SNU11P19	dog	pyoderma	369	11ax	neg.	type V	-
SNU12P2	dog	pyoderma	586	10dh	t06	type V	-
SNU12P3	dog	pyoderma	365	11y	t02	NT	-
SNU12P6	dog	pyoderma	587	11a	t02	type V	-
SNU12P7	dog	pyoderma	585	11y	t02	NT	-
SNU12P11	dog	pyoderma	365	11x	t02	NT	-
SNU12P12	dog	pyoderma	365	11y	t02	NT	-
SNU12P19	dog	pyoderma	71	9a	t06	NT	
SNU13P1	dog	otitis externa	563	11a	neg.	type V	-
SNU13P5	dog	pyoderma	567	11a	neg.	NT	-
SNU13P12	dog	pyoderma	45	neg.	neg.	type V	-
SNU14P12	dog	pyoderma	361	11a	neg.	NT	-
SNU14P13	dog	pyoderma	45	11cj	neg.	type V	-
SNU14P18	dog	otitis externa	674	11a	neg.	type V	-
SNU14P24	dog	pyoderma	585	11y	neg.	NT	-
SNU14P30	dog	otitis externa	365	11y	neg.	NT	-
SNU14P31	dog	pyoderma	581	11av	neg.	NT	-
SNU15P20	dog	pyoderma	708	11p	neg.	contained only *mecA*	-

SNU15P21	dog	pyoderma	474	neg.	neg.	contained only *mecA*	-
SNU15P32	dog	pyoderma	710	11a	neg.	type V	-
SNU15P37	dog	pyoderma	361	10a	neg.	type V	-
SNU15P39	dog	pyoderma	85	11a	neg.	type V	-
SNU15P42	dog	pyoderma	363	11ax	neg.	type V	-
SNU15P43	dog	pyoderma	563	11a	neg.	type V	-

NT, non-typeable; neg., negative.

Table 5. Non-typeable combinations of SCC*mec* components.

SCCmec component	Number of strain
mecA, ccrA2B2, ccrA3B3, ccrC, mecI, IS*431*	1
mecA, ccrA3B3, ccrC, mecI, IS*431*	2
mecA, ccrA1B1, ccrC, mecI, IS*431*	2
mecA, ccrA2B2, ccrA3B3, ccrC, IS*431*	1
mecA, ccrA1B1, ccrA3B3, ccrC, IS*431*	1
mecA, ccrA1B1, ccrC, IS*431*	9
mecA, ccrC, mecI, IS*431*	3
mecA, ccrA2B2, ccrA3B3, mecI	1
mecA, ccrA1B1, IS*431*	1
mecA, ccrA1B1, mecI	1
mecA, ccrC, mecI	4
mecA, mecI	4
Total	30

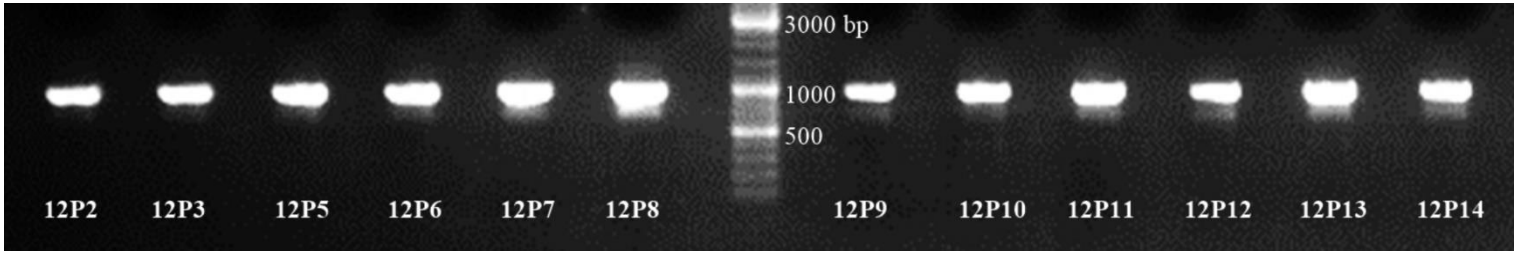

Figure 1. PCR amplification of the *nuc* gene for *S. pseudintermedius* identification.

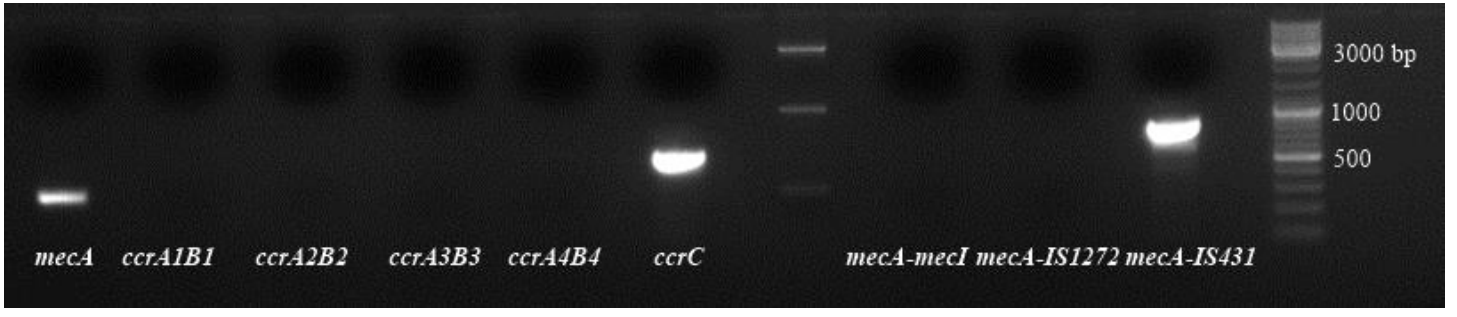

Figure 2. PCR amplification of SCC*mec* elements (11P4, SCC*mec* type V).

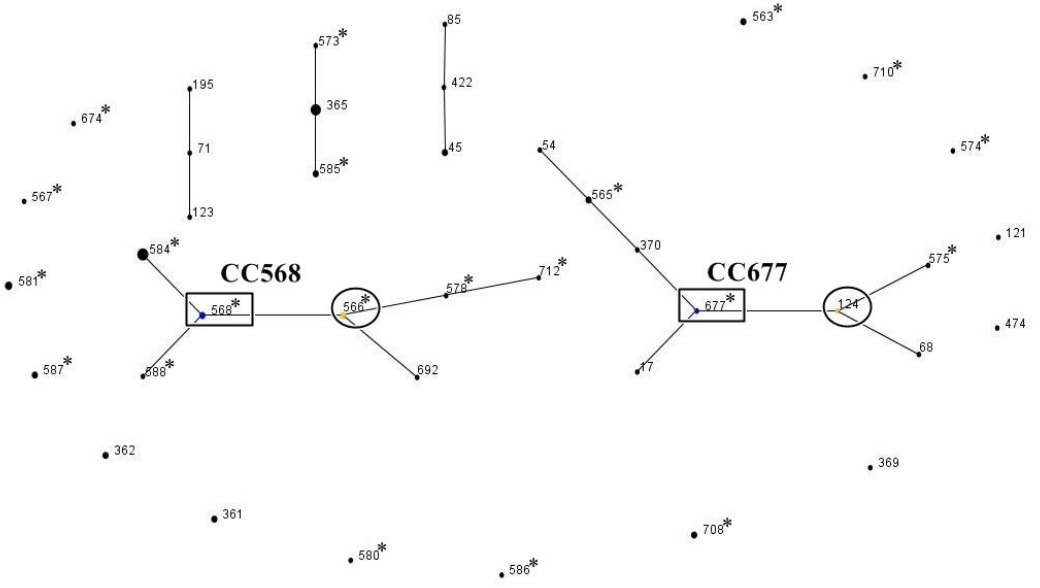

Figure 3. Clonal relationship of the MRSP sequence types displayed as a single eBURST (group definition to zero of seven shared alleles). Open squares, group founder; open circles, sub-group founder; asterisks, novel STs found in this study. The population of the MRSP strain was highly diverse, and many different STs acquired methicillin resistance. CC568 and CC677 were detected by eBURST.

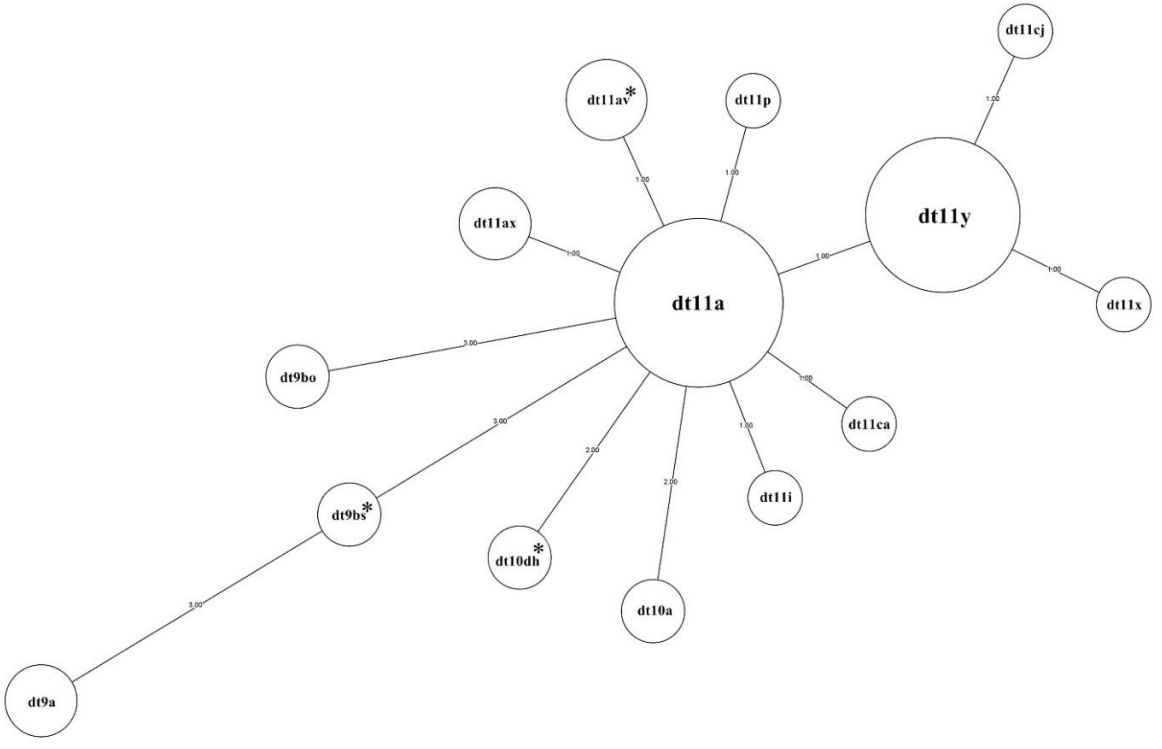

Figure 4. Minimum spanning tree of the 14 *dru* types identified in MRSP isolates (asterisks indicate novel *dru* types found in this study); dt11a and dt11y were the dominant *dru* types identified.

CHAPTER II: Emergence of a pRE25-like element from *Enterococcus* spp. in multidrug-resistant *Staphylococcus pseudintermedius* isolates from canine pyoderma

1. Introduction

Staphylococcus pseudintermedius is a major bacterial pathogen of small animals, particularly in dogs (Devriese *et al.*, 2009). Owing to the high rate of use of many classes of antimicrobial drugs in small animal practice, antimicrobial resistance has become a huge clinical challenge. Chapter I demonstrated the extremely high prevalence of multidrug resistance among MRSP isolates from dogs with canine pyoderma (96.6%, n = 60) along with remarkably high resistance rates to most of the major antibiotics, excluding beta-lactam antibiotics. The emergence of multidrug-resistant *S. pseudintermedius* (MDRSP) has been reported in several studies, which has been associated with the presence of Tn*5405*-like elements carrying up to five resistance genes (*aphA3, sat, aadE, erm*(B), and *dfrG*) (McCarthy *et al.*, 2015; Ruscher *et al.*, 2010). Tn*5405* was first reported in staphylococci (Boerlin *et al.*, 2001; Derbise *et al.*, 1996) and was then

screened in enterococci (Werner *et al.*, 2003). However, multidrug resistance pRE25-like elements have not been reported in staphylococci to date. Enterococcus pRE25 is a conjugative and mobilizing multidrug resistance plasmid from *Enterococcus faecalis* RE25 (Schwarz *et al.*, 2001). The pRE25-like enterococcal plasmid pWZ909 is known to mediate the delivery of vancomycin resistance to methicillin-resistant *S. aureus* via a Tn*1546*-like transposon (Zhu *et al.*, 2010). However, it is unclear whether pRE25-like elements contribute to multidrug resistance in *Staphylococcus*. Therefore, The goal of this study is to investigate the emergence of multidrug-resistant *S. pseudintermedius* (MDRSP) related with pRE25-like elements.

2. Material and Methods

2.1 Sample collection and antimicrobial susceptibility testing

In total, 46 MDRSP isolates from dogs with canine pyoderma were collected from samples submitted for antimicrobial susceptibility testing during 2017–2018. All dogs had owners (n = 46) and visited the Department of Dermatology, Veterinary Medical Teaching Hospital of Seoul National University. Each lesion site was sampled using a cotton swab and cultivated overnight at 37°C on blood agar. All isolates were identified using a Vitek 2 system (Biomerieux, Lyon, France) and were subjected to molecular identification using PCR targeting the *nuc* gene (Table 1) (Sasaki *et al.*, 2010). All PCR products were sequenced using an ABI PRISM 3730xl system (Applied Biosystems, Foster City, CA, USA) to confirm the species identification. The sequencing reaction was performed using 2 μL of purified PCR-amplified DNA and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following the manufacturer instructions. Susceptibility testing was performed using the disc diffusion method in accordance with the 2016 CLSI guidelines (CLSI, 2016). The antibiotics examined included penicillin, oxacillin, amikacin, gentamicin, erythromycin, clindamycin, tetracycline, doxycycline, ciprofloxacin, norfloxacin, trimethoprim/sulfamethoxazole, chloramphenicol, and rifampicin. Detection of

mecA was performed by PCR (Table 3) as reported previously (Kondo *et al.*, 2007), and resistance to penicillin and oxacillin was used as a criterion for MRSP detection (CLSI, 2016). Diagnosis of multidrug resistance followed the previously proposed definition (Magiorakos *et al.*, 2012).

2.2 Whole-genome sequencing (WGS)

Among the 46 MDRSP isolates collected in this study, four isolates (D11, D20, D21, and D26) were subjected to WGS. For PacBio RS sequencing, 8 μg of input genomic DNA was used to prepare a 20-kb library. For gDNA with a size below 17 kb, Bioanalyzer 2100 (Agilent, CA, USA) was used to determine the actual size distribution. The library insert sizes were in the optimal size range. The gDNA was sheared using g-TUBE (Covaris Inc., Woburn, MA, USA) and purified using AMPure PB magnetic beads (Beckman Coulter Inc., Brea, CA, USA) if the apparent size was greater than 40 kb. The total 10-μL library was prepared using PacBio DNA Template Prep Kit 1.0 (for 3–10 kb). SMRTbell templates were annealed using PacBio DNA/Polymerase Binding Kit P6. PacBio DNA Sequencing Kit 4.0 and 8 SMRT cells (Pacific Biosciences, CA, USA) with C4 chemistry were used for sequencing, and then 240-min movies were captured for each SMRT cell using a PacBio RS II (Pacific Biosciences, CA, USA) sequencing platform by Macrogen (Seoul, Korea).

2.3 MLST and PCR to detect pRE25-like elements

Sequence types (STs) were determined by MLST using seven housekeeping genes (Table 2) (Solyman *et al.*, 2013). MLST sequences were compared with allele sequences in the *S. pseudintermedius* MLST database (http://pubmlst.org/spseudintermedius) to determine the allele number. The clonal relationship of ST was predicted by eBURST analysis (Feil *et al.*, 2004). New ST numbers were assigned by the database curator (vincent.perreten@vetsuisse.unibe.ch).

The presence of pRE25-like elements was analyzed by PCR using three primer sets: recombinase (*rec*), 5′-GAAATATGGATATGCACGTGTC-3′ and 5′-GTACTGCGACTGAAACCG-3′; transposase *IS1252*, 5′-GAAACATCGTCTTGCCAAAG-3′ and 5′-CCAATTAGAGAATTCTTTCCCAC-3′; and transposase *IS1216*, 5′-GATTATTGTAGCCGTGGGC-3′, and 5′-CCTTTAATCGTGGTAGAGGC-3′. The PCR conditions were 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min 30 s, and a final cycle of 72°C for 10 min. All PCR products were sequenced using an ABI PRISM 3730xl system (Applied Biosystems) to confirm the accuracy of amplified target genes. The expected size of each PCR product was 551 bp (*rec*), 419 bp (*IS1252*), and 537 bp (*IS1216*). The sequencing reaction was performed using 2 μL of purified PCR-amplified DNA and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems)

following the manufacturer instructions. Plasmid DNA was screened using the NucleoBond Xtra Midi (MACHEREY-NAGEL GmbH & Co. KG, Duren, Germany) plasmid DNA extraction kit and the same PCR method.

3. Results

3.1 Antimicrobial susceptibility testing of MDRSP isolates

Among the 46 MDRSP isolates, 39 isolates were methicillin-resistant (MDR-MRSP). The resistance rates were highly variable among the antibiotics tested: penicillin 100% (46/46), oxacillin 84.7% (39/46), amikacin 2% (1/46), gentamicin 57.1% (28/46), erythromycin 69.5% (32/46), clindamycin 63% (29/46), tetracycline 93.4% (43/46), doxycycline 45.6% (21/46), ciprofloxacin 52.1% (26/46), norfloxacin 52.1% (26/46), trimethoprim/sulfamethoxazole 69.5% (32/46), chloramphenicol 52.1% (24/46), and rifampicin 0% (0/46). Therefore, high resistance rates (>40%) were observed to all antibiotics except for amikacin and rifampicin, with extremely high resistance to tetracycline detected.

3.2 Molecular characteristics of pRE25-like elements

Among the MDR-MRSP isolates, one isolate (*S. pseudintermedius* D20, ST563) was selected for pilot analysis of the WGS protocol. WGS revealed multidrug-resistant pRE25-like elements in the chromosomal DNA of *S. pseudintermedius* D20. The 22-kb pRE25-like elements found in this study contained five antimicrobial resistance genes (*cat, erm(B), aphA3, aadK,* and *sat4*), *IS1252, IS256,* and TA system within copies of *IS1216* (Fig. 6): *rec* (recombinase protein) and *IS1252* were detected beside the copy of *IS1216*; *cat* (chloramphenicol acetyltransferase) and *erm(B)* (rRNA adenine N-6-methyltransferase, erythromycin resistance) were located in adjacent regions; and *aphA3* (aminoglycoside 3'-phosphotransferase), *aadK* (aminoglycoside 6-adenylyltransferase), and *sat4* (streptothricin N-acetyltransferase) clustered together. A total of four copies of *IS1216* existed in pRE25-like elements, and the antimicrobial resistance genes were distributed in between the copies of *IS1216*. The *cat* and *erm(B)* genes were surrounded by the first and second copies of *IS1216*, whereas *aphA3, sat4,* and *aadK* were clustered consecutively between the second and third copies of *IS1216*.

3.3 Clonal analysis of MDRSP isolates by MLST and PCR analysis of pRE25-like elements

Twenty-four isolates (24/46, 52.1%) were positive for pRE25-like elements as determined by PCR targeting of *rec, IS1252,* and *IS1216* (Fig. 5). Six isolates were PCR-positive for only *rec*, which were presumed to not contain pRE255-like elements because their antibiogram did not correspond with pRE25-like elements (all six isolates were susceptible to chloramphenicol). Sixteen isolates were negative for pRE25-like elements, as determined by PCR. The PCR results of all 46 isolates in this study were negative for plasmid DNA.

The MLST results revealed 30 different STs, including 11 novel STs (ST1052, ST1093, ST1328, ST1330, ST1340, ST1341, ST1393, ST1394, ST1395, ST1396, and ST1397) that were registered in the MLST database (Fig. 7). There was no dominant ST; the maximum number of identical STs was four for ST54. Two clonal complexes (CC566 and CC1328) were detected and were almost composed of isolates positive for pRE25-like elements (except for one isolate, D24). ST585, ST365, and ST573 were clustered by the only isolate negative for pRE25-like elements, with no group founder present (Table 6).

3.4 Additional WGS to isolates positive for PCR analysis of pRE25-like elements

The three MDR-MRSP isolates (D11, D21, and D26) positive for pRE25-like elements in PCR analysis were additionally subjected to WGS. The clonal difference of these three isolates was confirmed by MLST (D11-ST1093, non-clustered single ST; D21-ST1328, CC1328; D26-ST692, CC566). WGS revealed that the three analyzed MDR-MRSP isolates had identical multidrug-resistant pRE25-like elements as those detected in D20 at the same location in their chromosomal DNA.

4. Discussion

The mobile genetic elements and resistance genes found in this study were highly related to the enterococcal multidrug-resistant plasmid pRE25. In BLASTN alignment analysis, the 50-kb enterococcal pRE25 genome (GenBank: NC_008445.1) covered 84% of the sequence of 22-kb pRE25-like elements (GenBank: MK775653) with an identity of 99.8% (Fig. 6). Compared to the plasmid pRE25, the *Enterococcus faecium* UW8175 plasmid (GenBank: CP011830.1) showed 71% sequence coverage and 99.9% identity, but the related region was identical to that of pRE25. All isolates that showed above 50% sequence coverage related to the query belonged to *Enterococcus* spp.

IS1252 and *IS256* are not derived from enterococcal pRE25. *IS256* is known to be scattered among *Staphylococcus* spp. (Dyke *et al.*, 1992). However, *IS1252* had been reported only in enterococci and streptococci until a recent study detected *IS1252* along with *IS1216*, as well as a novel phenicol-oxazolidinone-tetracycline resistance gene (*poxtA*), in a clinical MRSA isolate (Antonelli *et al.*, 2018). Copies of *IS1216* were also found to exist in multidrug-resistant pRE25-like elements in the present study. Although detection of *IS1252* and *IS1216* in staphylococci has thus far only been reported in *S. aureus* (*poxtA*) and *S. pseudintermedius* (multidrug-resistant pRE25-like elements), these mobile elements could pose a potential risk for the horizontal transfer of antimicrobial resistance within staphylococci.

Overall, 52% of the MDRSP isolates were positive for all pRE25-like elements, namely *rec*, *IS1252*, and *IS1216*, and corresponded with the phenotypic antibiogram of multidrug resistance pRE25-like elements (gentamicin resistance was intermediate in D9, D25, D26, D32, D35, and D39). Although indirect screening through PCR targeting of pRE25-like elements and the antibiogram has unavoidable limitations in terms reliability, BLASTN analysis revealed that the amplicon sequences of the mobile genetic elements targeted by PCR (*rec*, *IS1252*, and *IS1216*) matched with only those of enterococci, except for *IS1252* and *IS1216*, which matched with *poxtA* of *S. aureus*. Thus, the mobile genetic elements targeted by PCR in this study are very scarce among staphylococci, and the MDRSP isolates positive for all pRE25-like elements (*rec*, *IS1252*, and *IS1216*) could be presumed to contain the multidrug-resistant pRE25-like elements detected in this study. The MDRSP isolates presumed to contain multidrug-resistant pRE25-like elements also corresponded with the phenotypic antibiogram of multidrug-resistant pRE25-like elements. In particular, the chloramphenicol resistance was notable, as the MDRSP isolates negative for all pRE25-like elements (16/46), and those positive for only *rec* (6/46) were susceptible to chloramphenicol, whereas the MDRSP isolates positive for all pRE25-like elements (24/46) were resistant to chloramphenicol.

The prevalence rate of pRE25-like elements was much higher than expected. Approximately half of the total MDRSP isolates were suspected to contain pRE25-like elements, suggesting that pRE25-like elements are well-adapted in *S.*

pseudintermedius. Clonal analysis by MLST supported this hypothesis. The STs of isolates presumed to contain pRE25-like elements (pRE25 group) were diverse with 18 STs identified. The isolates presumed not to contain pRE25-like elements were classified as the non-pRE25 group, with 12 STs identified in this group. Thus, a total of 30 STs were identified in this study (Fig. 7). Among the 18 STs of the pRE25 group, 12 STs were first reported from Korea (Kang *et al.*, 2017; Lee *et al.*, 2018). Therefore, Korea is suspected to be the geographical origin of pRE25-like elements in the MDRSP lineage, although further international studies are needed to confirm the exact geographical origin. In addition, the clonal complex of the STs was interesting. Twenty of the 46 isolates belonged to three clusters of STs (Fig. 7) separated by the pRE25 group or non-pRE25 group. Clusters CC566 and CC1328 comprised isolates of the pRE25 group, whereas clusters ST585, ST365, and ST573 comprised isolates of the non-pRE25 group. Mixed ST clusters with harmonious coexistence of the pRE25 and non-pRE25 group were not detected. STs belonging to both pRE25 and non-pRE25 groups were also absent. This clonal distribution suggests that once pRE25-like elements flow into the genetic pool, the clonal lineage containing pRE25-like elements will rapidly dominate over others.

Cluster CC566 shared ST of founder and sub-founder with CC568 detected in chapter 1, which investigated MRSP isolates. Therefore CC566 & CC568 is a very important clonal lineage for antimicrobial-resistant *S. pseudintermedius* in Korea, and long-term surveillance for CC566 & CC568 is necessary.

Exposure to antibiotics is known to play a major role in the selection pressure of multidrug-resistant mobile genetic elements. Moreover, the toxin-antitoxin (TA) system inserted in multidrug-resistant pRE25-like elements (toxin zeta, antitoxin epsilon, and omega transcriptional regulator) may contribute to the survival of isolates containing these elements. The TA system in plasmid DNA is known to participate in stable plasmid inheritance by killing plasmid-free segregants in a process termed post-segregational killing (PSK) (Gerdes *et al.*, 1986). The exact function of the chromosomal TA system is under debate, but a recent study suggested that the chromosomal TA system acts as an anti-addiction module that protects the host bacteria against PSK mediated by their plasmid-encoded counterparts (Saavedra De Bast *et al.*, 2008). *S. pseudintermedius* is known to prefer chromosomal DNA for transferring transposon-borne resistance genes, and transfer via resistance plasmids has rarely been detected (Kadlec and Schwarz, 2012). Therefore, the resistance plasmid-free daughter cells of *S. pseudintermedius* may frequently be encountered under antibiotic pressure. The TA system inserted in pRE25-like elements of *S. pseudintermedius* could have an advantage in stable maintenance of the clonal lineage because the acquired chromosomal TA system may reduce the risk of PSK when the daughter bacteria do not inherit the resistance plasmid copy.

After the transfer of plasmids between enterococci and staphylococci was discovered (Schaberg *et al.*, 1982), the spread of vancomycin resistance by transfer from enterococci to staphylococci (Zhu *et al.*, 2010) was observed. The

multidrug resistance pRE25-like elements from enterococci were first detected in this study and are suspected to be widely spread in the veterinary environment of Korea. This circumstance could represent a pre-stage of invasion to human medicine; thus, the veterinary environment could play an important role as an intermediate in the transfer of multidrug resistance from enterococci to staphylococci. The continuous investigation is therefore required for the staphylococci commonly isolated from humans (e.g., *S. aureus* and *S. epidermidis*).

Table 6. Multidrug-resistant *S. pseudintermedius* strain information and molecular characteristics.

Strain	Origin	Source	MLST	PCR positive	Clonal Complex	Resistance phenotype
D9	dog	pyoderma	496	*rec, IS1252, IS1216*	1328	PEN-OXA-ERY-CLI-TET-CIP-NOR-SXT-CHL
D13	dog	pyoderma	121	*rec, IS1252, IS1216*	1328	PEN-OXA-GEN-ERY-CLI-TET-DOX-CIP-NOR-SXT-CHL
D21	dog	pyoderma	1328	*rec, IS1252, IS1216*	1328	PEN-OXA-GEN-ERY-CLI-TET-DOX-CIP-NOR-SXT-CHL
D28	dog	pyoderma	1328	*rec, IS1252, IS1216*	1328	PEN-OXA-GEN-ERY-CLI-TET-CIP-NOR-SXT-CHL
D32	dog	pyoderma	1394	*rec, IS1252, IS1216*	1328	PEN-OXA-ERY-CLI-TET-CIP-NOR-SXT-CHL
D45	dog	pyoderma	1386	*rec, IS1252, IS1216*	1328	PEN-GEN-ERY-CLI-TET-SXT-CHL
D23	dog	pyoderma	568	*rec, IS1252, IS1216*	566	PEN-OXA-GEN-ERY-CLI-TET-DOX-CHL
D26	dog	pyoderma	692	*rec, IS1252, IS1216*	566	PEN-OXA-ERY-CLI-TET-CHL
D38	dog	pyoderma	794	*rec, IS1252, IS1216*	566	PEN-OXA-GEN-ERY-CLI-TET-CIP-NOR-SXT-CHL
D39	dog	pyoderma	794	*rec, IS1252, IS1216*	566	PEN-OXA-CLI-TET-CIP-NOR-SXT-CHL

D43	dog	pyoderma	794	*rec, IS1252, IS1216*	566	PEN-OXA-GEN-ERY-TET-CIP-NOR-SXT-CHL
D46	dog	pyoderma	566	*rec, IS1252, IS1216*	566	PEN-OXA-GEN-ERY-CLI-TET-DOX-CHL
D24	dog	pyoderma	1340	none	566	PEN-OXA-GEN-TET-DOX-CIP-NOR-
D10	dog	pyoderma	585	none	cluster*	PEN-OXA-GEN-TET-DOX-SXT
D15	dog	pyoderma	585	none	cluster*	PEN-OXA-ERY-CLI-TET-DOX-CIP-NOR-SXT
D18	dog	pyoderma	585	none	cluster*	PEN-OXA-TET-DOX-SXT
D14	dog	pyoderma	365	none	cluster*	PEN-OXA-TET-SXT
D36	dog	pyoderma	365	none	cluster*	PEN-OXA-GEN-TET-SXT
D37	dog	pyoderma	365	none	cluster*	PEN-OXA-TET-SXT
D42	dog	pyoderma	573	none	cluster*	PEN-OXA-TET-DOX-SXT
D3	dog	pyoderma	567	*rec, IS1252, IS1216*	-	PEN-OXA-GEN-ERY-CHL
D4	dog	pyoderma	567	*rec, IS1252, IS1216*	-	PEN-OXA-GEN-ERY-TET-CHL
D7	dog	pyoderma	1052	*rec, IS1252, IS1216*	-	PEN-OXA-GEN-ERY-CLI-TET-CHL

D11	dog	pyoderma	1093	*rec*, *IS1252*, *IS1216*	-	PEN-OXA-GEN-ERY-CLI-TET-DOX-CIP-NOR-SXT-CHL
D16	dog	pyoderma	241	*rec*, *IS1252*, *IS1216*	-	PEN-GEN-ERY-CLI-CHL
D17	dog	pyoderma	45	*rec*, *IS1252*, *IS1216*	-	PEN-OXA-GEN-ERY-CLI-TET-DOX-CIP-NOR-CHL
D20	dog	pyoderma	563	*rec*, *IS1252*, *IS1216*	-	PEN-OXA-GEN-ERY-CLI-TET-DOX-CHL
D25	dog	pyoderma	1341	*rec*, *IS1252*, *IS1216*	-	PEN-ERY-CLI-TET-DOX-CIP-NOR-SXT-CHL
D35	dog	pyoderma	1396	*rec*, *IS1252*, *IS1216*	-	PEN-OXA-ERY-CLI-TET-SXT-CHL
D40	dog	pyoderma	1396	*rec*, *IS1252*, *IS1216*	-	PEN-OXA-GEN-ERY-CLI-TET-CIP-NOR-SXT-CHL
D41	dog	pyoderma	1397	*rec*, *IS1252*, *IS1216*	-	PEN-GEN-ERY-CLI-TET-SXT-CHL
D44	dog	pyoderma	45	*rec*, *IS1252*, *IS1216*	-	PEN-OXA-GEN-ERY-CLI-TET-CIP-NOR-CHL
D1	dog	pyoderma	54	none	-	PEN-OXA-TET-DOX-CIP-NOR-SXT
D2	dog	pyoderma	787	none	-	PEN-OXA-GEN-ERY-CLI-TET-DOX-SXT
D5	dog	pyoderma	787	none	-	PEN-OXA-ERY-CLI-TET-DOX-CIP-NOR-SXT
D6	dog	pyoderma	54	none	-	PEN-OXA-GEN-TET-DOX-CIP-NOR-SXT

D8	dog	pyoderma	366	none	-	PEN-OXA-GEN-TET-CIP-NOR
D12	dog	pyoderma	54	none	-	PEN-GEN-TET-DOX-
D19	dog	pyoderma	580	none	-	PEN-OXA-GEN-ERY-CLI-TET-CIP-NOR-SXT
D22	dog	pyoderma	1330	none	-	PEN-OXA-ERY-CLI-SXT
D27	dog	pyoderma	54	*rec*	-	PEN-OXA-GEN-TET-CIP-NOR-SXT
D29	dog	pyoderma	366	*rec*	-	PEN-OXA-GEN-TET-CIP-NOR
D30	dog	pyoderma	370	*rec*	-	PEN-OXA-ERY-CLI-TET-DOX-CIP-NOR-SXT
D31	dog	pyoderma	1393	*rec*	-	PEN-ERY-CLI-TET-CIP-NOR-SXT
D33	dog	pyoderma	787	*rec*	-	PEN-OXA-ERY-CLI-TET-DOX-CIP-NOR-SXT
D34	dog	pyoderma	1395	*rec*	-	PEN-AMK-ERY-TET-DOX-CIP-NOR-SXT

cluster*= STs were clustered together, but there was no group founder (Fig. 7).

PEN, penicillin; OXA, oxacillin; GEN, gentamicin; AMK, amikacin; ERY, erythromycin; CLI, clindamycin; TET, tetracycline; DOX, doxycycline; CIP, ciprofloxacin; NOR, norfloxacin; SXT, trimethoprim/sulfamethoxazole; CHL, chloramphenicol

Figure 5. PCR amplification of *rec*, *IS1252*, and *IS1216* in multi-drug resistant *S. pseudintermedius* (strain D11).

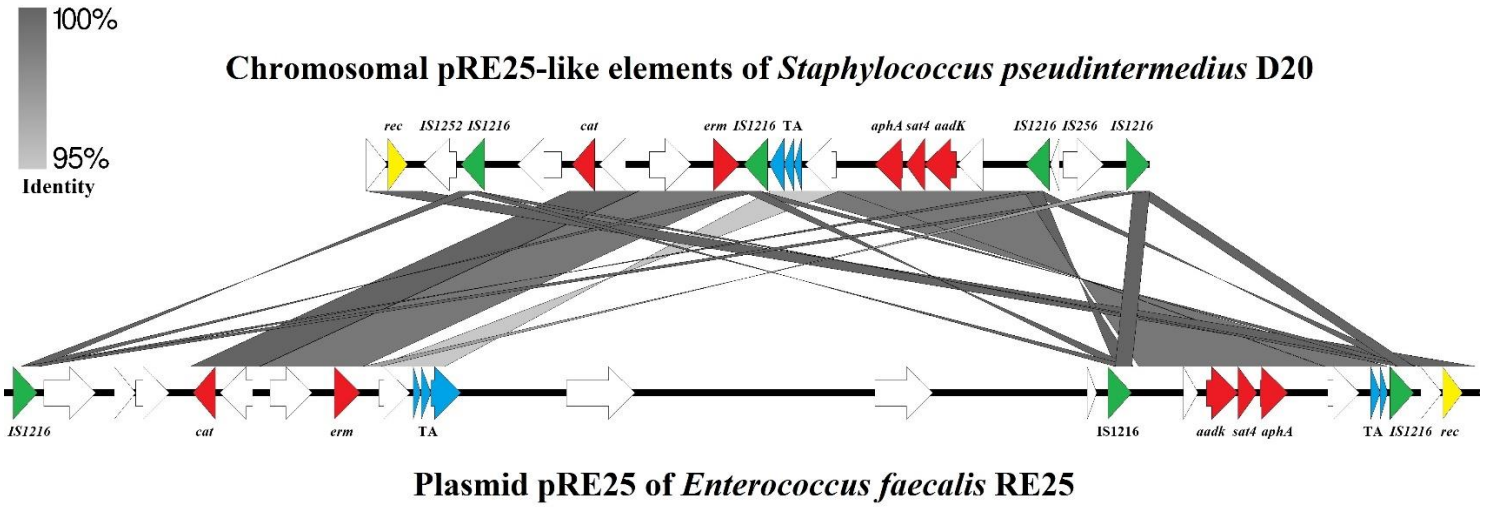

Figure 6. Comparison between chromosomal pRE25-like elements of *Staphylococcus pseudintermedius* D20 and plasmid pRE25 of *Enterococcus faecalis* RE25 is visualized by the Artemis comparison tool and Easyfig (Carver *et al.*, 2005; Sullivan *et al.*, 2011). TA, toxin-antitoxin system; *rec*, recombinase; *cat*, chloramphenicol acetyltransferase; *erm*, rRNA adenine N-6-methyltransferase; *aphA*, aminoglycoside 3'-phosphotransferase; *aadK*, aminoglycoside 6-adenylyltransferase; *sat4*, streptothricin N-acetyltransferase.

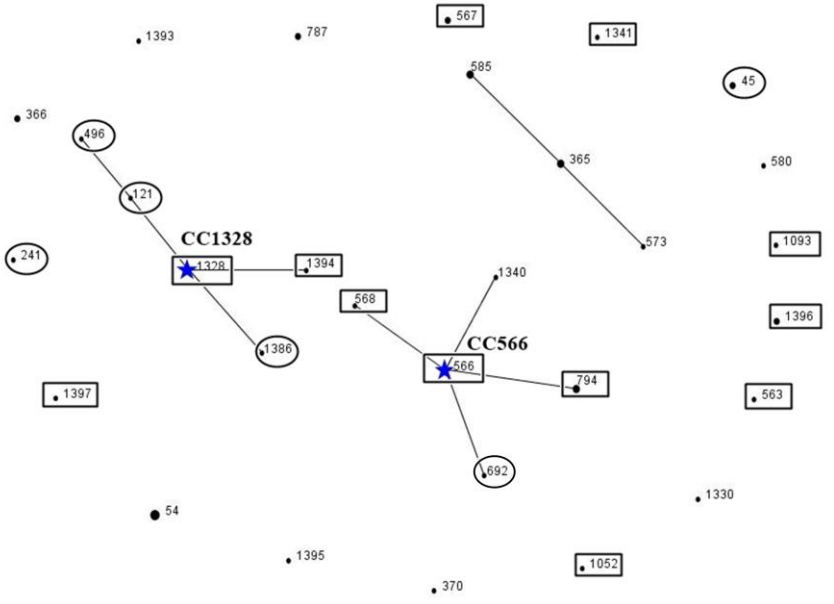

Figure 7. Clonal relationship of multidrug-resistant *Staphylococcus pseudintermedius* sequence types (STs) displayed as a single eBURST (group definition to zero of seven shared alleles). Open squares, STs presumed to contain pRE25-like elements and first reported in Korea; open circles, STs presumed to contain pRE25-like elements but first reported in other countries; asterisk, group founder

CHAPTER III: Genetic relationship of methicillin resistance between *Staphylococcus* isolates from companion dogs affected with pyoderma and dog owners

1. Introduction

The One-Health approach to human and veterinary medicine is a current strategy to respond to the increased risk of the emergence of multidrug-resistant organisms. Numerous previous studies have reported an epidemiological association among pathogens isolated from humans and animals (e.g., MRSA, vancomycin-resistant *Enterococcus faecium*) (Simjee *et al.*, 2002; Van Balen *et al.*, 2017). Dogs are the natural host of *S. pseudintermedius* as a major pathogen associated with pyoderma and otitis externa of dogs. However, various human infections of *S. pseudintermedius* have also been reported, and its zoonotic potential is generally recognized (Chuang *et al.*, 2010; Little *et al.*, 2019; Somayaji *et al.*, 2016). MRSP is also a huge problem. A molecular study revealed that MRSP strains isolated from a dog and from the anterior nares of the dog

owner were identical bacterial clones, indicating the horizontal transfer of MRSP between the dog and owner (Soedarmanto *et al.*, 2011).

Staphylococcal cassette chromosomes are a fragment of DNA that insert into the *orfX* gene and can encode antibiotic resistance and/or virulence gene (Malachowa and DeLeo, 2010). Staphylococcal cassette chromosome *mec* (SCC*mec*) is a mobile genomic island containing the *mec* gene that induces methicillin resistance, and shows ability for transfer among staphylococci species given that SCC*mec* with identical nucleotide sequences were found in different species (Miragaia, 2018). However, little is known about SCC*mec* transfer between different species of staphylococci in a clinical setting. The aim of this study is revealing the genetic relatedness of the SCC*mec* region of *Staphylococcus* isolates from dogs affected with pyoderma and dog owners.

2. Material and Methods

2.1 Sample collection, identification, and antimicrobial susceptibility testing

Thirty-one pairs of companion dogs and owners participated in this study with informed consent (Seoul National University IRB No. 1712/001-009). All dogs included in the study had owners that visited the Veterinary Medical Teaching Hospital of Seoul National University. Clinical isolates were collected from the pyoderma lesion of the dogs, and from the nasal cavity and finger of the owner using a cotton swab. The swabs were cultivated overnight at 37°C on blood agar and oxacillin resistance screening agar (Oxoid, Hampshire, UK). Obtained isolates were identified using the Vitek 2 system (Biomerieux, Lyon, France). All isolates identified as *Staphylococcus* spp. by the Vitek 2 system were then subjected to molecular identification via PCR to amplify the *Staphylococcus nuc, tuf,* and *gap* genes (Table 1) (Ghebremedhin *et al.*, 2008; Heikens *et al.*, 2005; Sasaki *et al.*, 2010), followed by PCR product sequencing using an ABI PRISM 3730xl apparatus (Applied Biosystems) to confirm the species. The sequencing reaction was performed using 2 μL of purified PCR-amplified DNA and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following the manufacturer instructions.

Susceptibility testing was performed using the disc diffusion method according to the 2016 CLSI guidelines (CLSI, 2016) to the following antibiotics: penicillin, oxacillin, cefoxitin, amikacin, gentamicin, erythromycin, clindamycin, tetracycline, doxycycline, ciprofloxacin, norfloxacin, trimethoprim/sulfamethoxazole, chloramphenicol, and rifampicin.

The owners with *S. pseudintermedius* detected in the nasal cavity were requested to come back for a second sampling 4 weeks after the initial screening to determine whether MRSP carriage was persistent or transient.

All owners participated in the study filled in a questionnaire form (Table 7) for estimating healthcare risk factors related to community-acquired MRSA (David *et al.*, 2008). Although this definition is validated for only MRSA, the same concept of distinguishing community or hospital related strains is also useful to other methicillin-resistant staphylococci.

2.2 MLST, *mec*-associated *dru* typing, and characterization of SCC*mec*

The STs of *S. pseudintermedius* were determined by MLST using primers of seven housekeeping genes (Table 2) (Solyman *et al.*, 2013). STs of MLST sequences were compared with allele sequences in the *S. pseudintermedius* MLST database (http://pubmlst.org/spseudintermedius) to determine the allele number. STs of *S. epidermidis* were also analyzed by the previously described MLST method (Table 8) (Thomas *et al.*, 2007) and compared with allele sequences in the *S. epidermidis* MLST database (http://pubmlst.org/sepidermidis). New ST numbers were assigned by the database curator (vincent.perreten@vetsuisse.unibe.ch, *S. pseudintermedius*; keith.jolley@zoo.ox.ac.uk, *S. epidermidis*). The *dru* VNTR region adjacent to IS*431* in SCC*mec* was sequenced and characterized as previously described (Goering *et al.*, 2008). The *dru* region was amplified using the primer pair 5′-GTTAGCATATTACCTCTCCTTGC-3′ and 5′-GCCGATTGTGCTTGATGAG -3′. Sequenced tandem repeats were found using the tandem repeats finder (http://tandem.bu.edu/trf) (Benson, 1999) and analyzed using the *dru* database website (http://dru-typing.org). Novel *dru* types (dts) were assigned by the curator (richardgoering@creighton.edu). The SCC*mec* type was determined using multiplex PCR (Table 3) (Kondo *et al.*, 2007) to detect *mecA*, identify the *ccr* complex, and distinguish classes A, B, and C *mec*. When a SCC*mec* type had

additional *ccr* components, the name of *ccr* genes was also recorded along with the SCC*mec* type. All PCR products were sequenced using an ABI PRISM 3730xl apparatus (Applied Biosystems) to confirm the target site. The sequencing reaction was performed using 2 μL purified PCR-amplified DNA and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following the manufacturer instructions. The presence of *ccr*A5/B3 in SCC*mec* type VII-241 cassettes was screened using primers 5′-GCCAAAATTTCTTTCGAGACC-3′ and 5′-TACGTGCGAGTCGATTTGTT-3′ (Perreten *et al.*, 2010). The presence of SCC*mec* II-III was distinguished from SCC*mec* III by detecting the absence of the cadmium resistance operon (Perreten *et al.*, 2010).

2.3 WGS

For PacBio RS sequencing, 8 μg of input genomic DNA was used for the preparation of a 20-kb library. For gDNA with a size less than 17 kb, Bioanalyzer 2100 (Agilent, CA, USA) was used to determine the actual size distribution. The library insert sizes were in the optimal size range. The gDNA was sheared with g-TUBE (Covaris, MA, USA) and purified using AMPure PB magnetic beads (Beckman Coulter, CA, USA) if the apparent size was greater than 40 kb. The gDNA concentration was measured using both a NanoDrop spectrophotometer and a Qubit fluorometer, and approximately 200 ng/μL of gDNA was run on a field-inversion gel. A 10-μL library was prepared using PacBio DNA Template Prep Kit 1.0 (for 3–10 kb). SMRTbell templates were annealed using PacBio DNA/Polymerase Binding Kit P6. PacBio DNA Sequencing Kit 4.0 and 8 SMRT cells were used for sequencing. SMRT cells (Pacific Biosciences, CA, USA) were obtained using C4 chemistry, and 240-min movies were captured for each SMRT cell using the PacBio RS II (Pacific Biosciences, CA, USA) sequencing platform. Based on the WGS data, direct repeats (DR) containing the insertion site sequence (ISS), which is considered as an integration site in the SCC*mec* region, were identified (Wang *et al.*, 2012a).

3. Results

3.1 Identification and antimicrobial susceptibility testing

The Vitek 2 system and *nuc*, *tuf*, *gap* gene sequencing revealed a total 101 *Staphylococcus* strains isolated from 31 dog-owner pairs (Table 9). Species of staphylococci isolates from the dogs were distributed as follows: *S. pseudintermedius* (n = 25), *S. schleiferi* (n = 7), *S. cohnii* (n = 1), *S. haemolyticus* (n = 1). and *S. warneri* (n = 1). A total of 35 strains were isolated from the dogs, and *S. pseudintermedius* was a major strain (n = 25). The staphylococci isolated from owners included *S. epidermidis* (n = 35), *S. pseudintermedius* (n = 22), *S. schleiferi* (n = 2), *S. warneri* (n = 1), *S. cohnii* (n = 2), *S. pasteuri* (n = 1), *S. hominis* (n = 1), *S. haemolyticus* (n = 1), and *S. saprophyticus* (n = 1). Therefore, 66 strains were isolated from owners, and *S. epidermidis* was dominant, followed by *S. pseudintermedius*.

In a total of 101 *Staphylococcus* strains, 99 strains were diagnosed as methicillin-resistant *Staphylococcus* strains by PCR detecting *mecA* gene, oxacillin (*S. pseudintermedius*, *S. schleiferi*) and cefoxitin resistance (coagulase-negative *Staphylococcus* spp.) in disc diffusion test (CLSI, 2016; Huse *et al*., 2018). In spite of using oxacillin resistance screening agar, two *S. pseudintermedius* isolates (18D2 and 18D25) were susceptible to oxacillin in the

disc diffusion test and did not carry *mecA* gene. When the *S. pseudintermedius* group isolated from dogs were compared with *S. epidermidis* group isolated from the owner, the resistance rate to clindamycin, tetracycline, chloramphenicol, and trimethoprim/sulfamethoxazole were remarkably higher than *S. epidermidis* isolates from the owner. Amikacin resistance was very scarce in both groups (≤ 2%). Rifampicin resistance was not detected in this study. Detailed resistance rates are described in Table 10.

Among the 31 owners who participated in this study, *S. pseudintermedius* was isolated from 16 owners (nasal cavity, three owners; finger, seven owners; both nasal cavity and finger, six owners). *S. schleiferi* was isolated from the finger of two owners. Nine owners had *S. pseudintermedius* in their nasal cavity, and a second sampling was performed 4 weeks after the initial screening. In five owners (5/9, 55%), *S. pseudintermedius* was isolated from the second sampling, and the ST, SCC*mec* type, *dru* type, and phenotypic antimicrobial resistance profile were identical with those of *S. pseudintermedius* isolated from the initial screening. In the second sampling, *S. epidermidis* was isolated from three owners, and the culture result in oxacillin resistance screening agar was negative in one owner.

The result of the questionnaire form (Table 7) from the dog owners showed that most of the owners did not have remarkable characteristics associated with healthcare risk factors validated for MRSA (David *et al.*, 2008). Twenty-two owners (22/31, 70.9%) checked “No” for all related questions. Six owners had a history of surgery, and one owner was hospitalized during the previous year. Two

owners had both a history of surgery and hospitalization. A questionnaire form was not appropriate for dogs because most of the dogs were castrated or spayed (26/31, 83%). Five dogs were hospitalized during the previous year, and eight dogs had a history of previous isolation of methicillin-resistant staphylococci. The result of the questionnaire form did not have a relationship with antimicrobial susceptibility testing, ST, *dru* type, and SCC*mec* type.

3.2 ST, *dru* type, and SCC*mec* type

Among the 25 *S. pseudintermedius* isolates from 31 dogs, 22 STs were detected. There was no dominant ST; the maximum number of identical STs was three for ST585. All STs found in dogs were ST45, ST54 (n = 2), ST112, ST121, ST241, ST365 (n = 2), ST366, ST367, ST496, ST563, ST567, ST568, ST580, ST585 (n = 3), ST787, ST1052, ST1093, ST1328, ST1330, ST1340, and ST1341. Six STs were novel (ST1052, ST1093, ST1328, ST1330, ST1340, and ST1341). Twelve STs were identified in the 35 *S. epidermidis* isolates from the 31 dog owners. The major STs were ST35 (n = 10) and ST5 (n = 7), and the others were ST2, ST20, ST59 (n = 4), ST81, ST130 (n = 5), ST173 (n = 2), ST192, ST213, ST476, and ST838. ST838 was a new type.

The predominant SCC*mec* was type V in the *S. pseudintermedius* isolates from dogs. Ten isolates showed SCC*mec* type V. SCC*mec* type V additionally carrying *ccrA1B6* was detected in five isolates. One isolate had *mec*A with *ccrA1B1* (18D30), and seven isolates did not carry the *ccr* gene in their SCC*mec* (18D11-2, 18D15-2, 18D17, 18D19, 18D22, 18D23, and 18D29-1). Two isolates did not have *mecA* or SCC*mec* components. Seven *S. schleiferi* isolates from the dogs showed type V (n = 5), type V with *ccrA1B1* (n = 1), and *mecA* with *ccrC* (n = 1). The *S. cohnii* isolate (18D11-1), *S. haemolyticus* isolate (18D27-2), and *S. warneri* isolate (18D31) from dogs had *mecA* with *mecI* and *ccrA1B3*; type V; only *mecA*, respectively.

Of the total 35 *S. epidermidis* isolates from dog owners, the SCC*mec* types were related to type IV or type V. Sixteen isolates showed SCC*mec* type IV. Among the SCC*mec* type IV isolates, four isolates additionally had *ccrC*. Most of the SCC*mec* type V (n = 17) detected in *S. epidermidis* isolates from dog owners had additional *ccr* genes. Only one *S. epidermidis* isolate (18H20-F2) carrying SCC*mec* type V did not have additional *ccr* genes. SCC*mec* type II and *mecA* with *ccrA2B2* was detected in one isolate each. The two *S. schleiferi* isolates (18H12-F and 18H21-F) from dog owners had SCC*mec* type V and only the *mecA* gene, respectively. The *S. warneri* (18H2-F), *S. pasteuri* (18H12-N1), *S. hominis* (18H12-N3), *S. haemolyticus* (18H14-F), and *S. saprophyticus* (18H25-F) isolates had type V; type IV; *mecA* with *mecI*; only *mecA*; and *mecA* with *mecI*, *ccrA1B3*, respectively. The two *S. cohnii* isolates (18H20-F1 and 18H27-F1) carried SCC*mec* type III with *ccr*C and SCC*mec* type II, respectively.

Eleven *dru* types (10a, 10dh, 11a, 11af, 11ah, 11ax, 11bm, 11cj, 11cv, 11v, and 11y) were found in the *S. pseudintermedius* isolates from dogs. The major *dru* type was 11a (n = 9), and four isolates were negative in PCR amplification for *dru* typing (18D2, 18D6, 18D25, and 18D30). Sixteen *dru* types (4h, 8ar, 10a, 10dl, 10dm, 10dp, 10dq, 11a, 11bn, 11cf, 11dm, 11dn, 11do, 11p, 13y, and 13z) were detected in the *S. epidermidis* isolates from dog owners. The predominant *dru* types were 10a (n = 10) and 11p (n = 9). Most of the isolates carrying *dru* type 10a showed a trend related to SCC*mec* type IV (9/10), and all isolates carrying *dru* type 11p were related to SCC*mec* type V (9/9). The *dru* types

detected in both *S. pseudintermedius* and *S. epidermidis* were 10a and 11a. All *S. epidermidis* isolates carrying *dru* type 10a had SCC*mec* type IV or IV with *ccrC* except for one isolate that had SCC*mec* type II (18H3F). However, *S pseudintermedius* isolates carrying *dru* type 10a had SCC*mec* type V or type V with *ccrA1B6* except for one isolate that had *mecA* with *IS431* (18D19).

3.3 WGS

Among the total 31 pairs of companion dogs and owners who participated in this study, 12 pairs had the same type of SCC*mec* (type V) but different *dru* types. *S. schleiferi* isolated with *S. pseudintermedius* in five pairs. One pair carrying the same SCC*mec* type V and *dru* type 11a was detected: 18D20-1 (*S. pseudintermedius,* ST563), 18D20-2 (*S. schleiferi*), and 18H20-F2 (*S. epidermidis,* ST20). These three isolates were submitted for WGS, which revealed that the SCC*mec* region of J3, *mec* complex and J2 had an average 96.8% homology within *S. pseudintermedius* (GenBank: MN580540), *S. schleiferi* (GenBank: MN580541) from the dog, and *S. epidermidis* (GenBank: MN580542) from the dog owner (Fig. 8). The SCC*mec* region of J1 was heterogeneous, whereas *S. pseudintermedius* and *S. schleiferi* had identical CRISPR-Cas genes. SCC*mec* of S. *epidermis* had a type 1 restriction-modification system at the J1 region instead of CRISPR-Cas genes. Both CRISPR-Cas genes and type 1 restriction-modification systems existed in the J1 region of *S. schleiferi*.

Except for *S. pseudintermedius* and *S. schleiferi* isolates, *S. epidermidis* (18H20-F2) and *S. warneri* (18H2-F) isolated from dog owners also carried SCC*mec* type V and *dru* type 11a. However, the pair of *S. warneri* (18H2-F) was methicillin-susceptible *S. pseudintermedius* and did not have SCC*mec* elements (18D2). Additional WGS was performed for *S. warneri* (18H2-F, GenBank: MN580543) to check the genetic relation of SCC*mec* with those of 18D20-1 (*S.*

pseudintermedius) and 18H20-F2 (*S. epidermidis*) (Fig. 9). The *mec* complex region of 18H2-F (*S. warneri)* was identical with that of 18D20-1 (*S. pseudintermedius*), but the location of *ccrC* was different. In addition, SCC*mec* of 18H2-F (*S. warneri*) had additional cadmium and arsenic resistance genes at the J2 region. These heavy metal resistance genes were not detected in the SCC*mec* of 18D20-1 (*S. pseudintermedius*), 18D20-2 (*S. schleiferi*), and 18H20-F2 (*S. epidermidis*).

Seventeen *S. epidermidis* isolates (45.7%, 17/35) had SCC*mec* type V. Among these, only 18H20-F2 showed *dru* type 11a and SCC*mec* type V, which were not related to other *ccr* genes. The other 16 *S. epidermidis* isolates had SCC*mec* type V related with *ccrA1B1* or *ccrA2B2*, and their *dru* types were 11p (n = 9), 11dn, 11do, 10dl, 10dm, 8ar, and untypeable (n = 2). The major *dru* type was 11p (n = 9), and the ST of all *S. epidermidis* isolates carrying dt11p was ST35. Among these ST35 *S. epidermidis* isolates carrying SCC*mec* type V related with *ccrA1B1* or *ccrA2B2*, dt11p. 18H13-N2 (SCC*mec* type V with *ccrA1B1*, dt11p, ST35) and 18H2-N (SCC*mec* type V with *ccrA1B1*, dt11p, ST35) were submitted for WGS as representative isolates. The WGS result showed that 18H20-F2 (dt11a) had a close genetic relationship (>90% homology) with 18H13-N2 (dt11p, GenBank: MN580544) and 18H2-N (dt11p, GenBank: MN580545) at the *mec* complex and J2 region (Fig. 10). However, 18H13-N2 (dt11p) and 18H2-N (dt11p) had one *ccrC* gene, whereas two *ccrC* genes were detected in 18H20-F2 (dt11a). As confirmed by the PCR result, 18H13-N2 (dt11p) and 18H2-N (dt11p) carried

additional *ccrA1* and *ccrB1* genes, which did not exist in SCC*mec* of 18H20-F2 (dt11a).

The SCC*mec* regions of six isolates (18D20-1, 18D20-2, 18H20-F2, 18H2-F, 18H13-N2, 18H2-N) were analyzed by WGS in this study, and 11 different direct repeats (DRs) considered as an integration site in the SCC*mec* region were detected (Table 11).

4. Discussion

Many previous studies related to the horizontal transfer of methicillin resistance focused on the transfer of the pathogen itself. In particular, the relationship of methicillin resistance between animals and humans has thus far mainly been considered by isolating human endemic MRSA strains from dogs or isolation of dog endemic MRSP strains from humans (Fabri *et al.*, 2018; van Duijkeren *et al.*, 2011). In the present study, 51.6% (16/31) of dog owners had MRSP in their nasal cavity or finger. *S. schleiferi* was isolated from a finger of two owners. When comparing the clonality of MRSP isolated from dog owners with MRSP isolated from their dog, the ST, SCC*mec* type, *dru* type, and phenotypic antimicrobial resistance profile were identical in most cases, except for four of the 16 pairs (18H16-N2, 18H16-F, 18H19-F, 18H26-F1, and 18H29-F). This result suggests that MRSP isolated from dog owners has a remarkable genetic relationship with that isolated from their dogs, and that horizontal transfer of MRSP is a current risk.

Among the 31 dog owners, nine carried *S. pseudintermedius* in their nasal cavity, and a second sampling was performed 4 weeks after the initial screening. In five owners (5/9, 55%), MRSP isolated from the second sampling showed the identical ST, SCC*mec* type, *dru* type, and phenotypic antimicrobial resistance profile to that isolated from the initial screening. This result indicates that the long-term carriage of MRSP in the skin of dog owners occurs frequently. Isolation

of MRSP in the human nasal cavity and the potential long-term carriage of MRSP in humans was previously suggested in a study focusing on small animal veterinarian practice (Paul *et al.*, 2011). However, they determined a carriage rate of 3.9% (5/128); thus, the rate of 29% (9/31) in the dog owners in this study is much higher than expected. Another study also reported MRSP cultured from 4% (2/45) of the nasal samples of dog owners (van Duijkeren *et al.*, 2011). The main reason for the high carriage rate of MRSP of dog owners in the present study is presumed to be related to the active lesion of pyoderma in their dogs. All dogs who participated in this study had an active lesion of pyoderma; thus, the frequent contact by grooming could be the main route of direct MRSP transmission between dogs and their owners. The high prevalence rate of MRSP is another potential reason. As indicated in chapter I, the prevalence rate of MRSP isolated from dogs in Korea was found to be markedly higher than the rates reported in other studies from Europe and North America.

Among the total of 31 dog-owner pairs that participated in this study, one pair (18D20-1, *S. pseudintermedius*, ST563; 18D20-2, *S. schleiferi*; 18H20-F2, *S. epidermidis*, ST20) showed a remarkable genetic relationship for SCC*mec* in WGS analysis. *S. pseudintermedius* ST563 and *S. epidermidis* ST20 were only detected in 18D20-1 and 18H20-F2, respectively. These three isolates had the identical location of *ccrC* and the *mec* complex in their SCC*mec*. In addition, the J3 and J2 regions located between *ccrC* and the *mec* complex showed an average of 96.8% homology. This result itself is not sufficient to confirm the horizontal

transfer between the dog and owner because genetic similarity is only one of the expected factors. However, considering the number of cases, the genetic similarity within *S. pseudintermedius, S. schleiferi*, and *S. epidermidis* isolated from the dog and owner represents a very rare case that has not been reported to date.

The methicillin-resistant *Staphylococcus* spp. isolates from dog owners other than *S. pseudintermedius* and *S. schleiferi* included the following species: *S. epidermidis* (n = 35), *S. schleiferi* (n = 2), *S. warneri* (n = 1), *S. cohnii* (n = 2), and one isolate each of *S. pasteuri*, *S. hominis*, *S. haemolyticus*, and *S. saprophyticus*. Among these staphylococci isolated from dog owners, SCC*mec* type V and dt11a were detected in only two isolates (18H20-F2 and 18H2-F). 18H20-F2 (*S. epidermidis*) belonged to the pair carrying SCC*mec* type V and dt11a (Fig. 8). Although the pair of 18H2-F (*S. warneri*) was a methicillin-susceptible *S. pseudintermedius* (18D2), the SCC*mec* region of 18H2-F was further checked. Comparison to the SCC*mec* of 18H20-F2 (*S. epidermidis*) showed partial homology in the *mec* complex, *ccrC*, and type 1 restriction-modification system, whereas the structural composition of the SCC*mec* element was quite different (Fig. 9). This result supports that PCR typing of SCC*mec* and *dru* is simply a screening method, and actual structural and genetic similarity should be further compared based on WGS to analyze the entire sequence of SCC*mec* as seen in the pair of 18H20-F2 (*S. epidermidis*, dog owner) and 18D20-1 (*S. pseudintermedius*, dog). Nevertheless, an interesting finding was detected

when the SCC*mec* region of 18H2-F (*S. warneri*) from the dog owner was analyzed by the BLASTN database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The first-ranked match with the highest query cover (82%) and identity (99.52%) was *S. schleiferi* strain 2317-03 isolated from the skin infection of a dog in the USA (Misic *et al.*, 2015). The next ranking matches were *S. pseudintermedius* strain NA45 isolated from a canine infection in North America (query cover, 77%; percent identity, 99.5%) (Riley *et al.*, 2016), *S. haemolyticus* JCSC1435 (query cover, 71%; percent identity, 99.%), and *S. aureus* RIVM3897 (query cover, 68%; percent identity, 99.5%). The fact that the first and second-ranked matches were *S. schleiferi* and *S. pseudintermedius* isolated from a dog indicates the SCC*mec* region of 18H2-F (*S. warneri*) isolated from the dog owner in this study also has a prominent genetic relationship with *S. schleiferi* and *S. pseudintermedius* isolated from the dog.

18H20-F2 was the only *S. epidermidis* isolate carrying SCC*mec* type V and dt11a, and the other 16 *S. epidermidis* isolates (16/35) had SCC*mec* type V related with *ccrA1B1* or *ccrA2B2*. These 16 *S. epidermidis* isolates had additional *ccr* genes and dt11a was not detected in their *dru* types (11p, n = 9; 11dn; 11do; 10dl; 10dm; 8ar; untypeable, n = 2), All of the dt11p isolates showed ST35. SCC*mec* of two representative isolates showed dt11p compared with 18H20-F2 (SCC*mec* type V, dt11a) for estimating the difference of structure and DNA sequence. If they showed only a few differences, the potential for a mutual genetic interaction between 18H20-F2 (*S. epidermidis*, dog owner) and 18D20-1 (*S.*

pseudintermedius, dog) based on genetic similarity might be weakened. The WGS results showed that SCC*mec* of 18H20-F2 (SCCmec type V, dt11a, ST20) exhibited remarkable differences with 18H13-N2 (SCC*mec* type V with *ccrA1B1*, dt11p, ST35) and 18H2-N (SCC*mec* type V with *ccrA1B1*, dt11p, ST35) (Fig. 10). They only showed similarity at the *mec* complex and J2 region, and structural differences were prominent. The location of the *mec* complex and number of *ccrC* genes also differed. In contrast, SCC*mec* of 18H13-N2 and 18H2-N had a strong relationship. The location of the *mec* complex was identical, and 80% of the entire SCC*mec* region had 99.9% identity. Likewise the 18H2-F (*S. warneri*), the SCC*mec* of 18H13-N2 and 18H2-N were analyzed in the BLASTN database, but neither *S. pseudintermedius* nor *S. schleiferi* was detected at high ranks. *S. aureus, S. epidermidis*, and *S. haemolyticus* were matched with the SCC*mec* sequences of 18H13-N2 and 18H2-N with >50% homology.

The distribution of DRs was also a notable finding. DRs are considered as an integration site in SCC*mec* and are important sequences in the epidemiology of SCC*mec* acquisition (Wang *et al.*, 2012a). The endpoint of SCC*mec* had DR-8 in all three *S. epidermidis* isolates (18H20-F2, 18H13-N2, and 18H2-N). 18H13-N2 (dt11p) and 18H2-N (dt11p) had an identical DR-4 in the middle point of SCC*mec* and had DR-11 at the start point. In contrast, 18H20-F2 (dt11a) did not have DR-4, and DR-1 was located at the start point of SCC*mec* instead of DR-11. With respect to the dog and dog owner pair (18D20-1, 18D20-2, and 18H20-F2) showing remarkable genetic relatedness, DR-1 was located in common at the

SCC*mec* start point of *S. pseudintermedius* (18D20-1, SCC*mec* type V. dt11a, dog), *S. schleiferi* (18D20-2, SCC*mec* type V. dt11a, dog), and *S. epidermidis* (18H20-F2, SCC*mec* type V. dt11a, dog owner) (Fig. 8). Because SCC*mec* always starts at *orfX* (*rlmH*, rRNA methyltransferase), which is conserved among all staphylococci, *orfX* is very important as the insertion site of SCC*mec* (Boundy *et al.*, 2013), and the DR at the start point of SCC*mec* is located in this conserved *orfX*. Therefore, the identical DR-1 at the start point of SCC*mec* of *S. pseudintermedius* (18D20-1, dog), *S. schleiferi* (18D20-2, dog), and *S. epidermidis* (18H20-F2, dog owner) could be strong evidence for an epidemiological relationship of SCC*mec* combined with the genetic and structural similarity detected in this study.

In summary, although the prevalence rate was very low (3%, 1/31), *S. pseudintermedius* (dog), *S. schleiferi* (dog), and S. *epidermidis* (dog owner) isolated from a dog and dog owner pair in this study showed remarkable genetic similarity of SCC*mec* in terms of DNA sequences, *dru* type, the structure composition of *ccrC* and the *mec* complex, and identical DR-1 in *orfX*, which is considered the insertion site of SCC*mec*. These results support the risk of SCC*mec* transfer in a veterinary clinical context between dogs and dog owners sharing a living environment.

Table 7. Questionnaire form for estimating healthcare risk factors.

Hospitalization over 48 hours in present		
Dog	Yes	No
Dog owner	Yes	No
Receipt of hemodialysis		
Dog	Yes	No
Dog owner	Yes	No
Receipt of surgery		
Dog	Yes	No
Dog owner	Yes	No
Residence in a long-term care facility, or hospitalization during the previous year		
Dog	Yes	No
Dog owner	Yes	No
The presence of an indwelling catheter or a percutaneous device at the time culture samples were obtained		
Dog	Yes	No
Dog owner	Yes	No
Previous isolation of methicillin-resistant staphylococci		
Dog	Yes	No
Dog owner	Yes	No

Table 8. PCR primers for multilocus sequence typing (MLST) of *S. epidermidis.*

Gene	Primer	Sequence (5′- 3′)	Product (bp)	Reference
arcC	arcC-F	TGTGATGAGCACGCTACCGTTAG	465	Thomas *et al*., 2007
	arcC-R	TCCAAGTAAACCCATCGGTCTG		
aroE	aroE-F	CATTGGATTACCTCTTTGTTCAGC	420	
	aroE-R	CAAGCGAAATCTGTTGGGG		
gtr	gtr-F	CAGCCAATTCTTTTATGACTTTT	438	
	gtr-R	GTGATTAAAGGTATTGATTTGAAT		
mutS	mutS-F	GATATAAGAATAAGGGTTGTGAA	412	
	mutS-R	GTAATCGTCTCAGTTATCATGTT		
pyrR	pyrR-F	GTTACTAATACTTTTGCTGTGTTT	428	
	pyrR-R	GTAGAATGTAAAGAGACTAAAATGAA		
tpi	tpi-F	ATCCAATTAGACGCTTTAGTAAC	424	
	tpi-R	TTAATGATGCGCCACCTACA		
yqiL	yqiL-F	CACGCATAGTATTAGCTGAAG	416	
	yqiL-R	CTAATGCCTTCATCTTGAGAAATAA		

Table 9. *Staphylococcus* isolates and their molecular characteristics from dogs and owners.

Dog group					Owner group				
Strain	**Identificaion**	**ST**	**SCC*mec***	***dru***	**Strain**	**Identificaion**	**ST**	**SCC*mec***	***dru***
18D1	*S. pseudintermedius*	54	V	11af	18H1-N	*S. epidermidis*	59	IV	13y
					18H1-F	*S. pseudintermedius*	54	V	11af
18D2	*S. pseudintermedius*	112	neg.	neg.	18H2-N	*S. epidermidis*	35	V, ccrA1B1	11p
					18H2-F	*S. warneri*	-	V	11a
18D3	*S. schleiferi*	-	mecA , ccrC	11bn	18H3-N	*S. epidermidis*	81	IV	10a
					18H3-F	*S. epidermidis*	-	II	10a
18D4	neg.	-	-	-	18H4-N	*S. epidermidis*	35	V, ccrA1B1, ccrA2B2	11p
					18H4-F	*S. epidermidis*	35	V, ccrA1B1, ccrA2B2	11p
18D5	*S. pseudintermedius*	567	V, ccrA1B6	11v	18H5-N	*S. epidermidis*	59	IV	10a
					18H5-F	*S. pseudintermedius*	567	V, ccrA1B6	11v
18D6	*S. pseudintermedius*	787	V, ccrA1B6	neg.	18H6-N	*S. epidermidis*	476	IV	11bn
					18H6-F	*S. epidermidis*	2	IV	10a
18D7	*S. pseudintermedius*	54	V	11a	18H7-N	*S. pseudintermedius*	54	V	11a
					18H7-F	*S. pseudintermedius*	54	V	11a
18D8	*S. pseudintermedius*	1052	V	11a	18H8-N	*S. pseudintermedius*	1052	V	11a
					18H8-F	*S. epidermidis*	59	IV	4h
18D9	*S. pseudintermedius*	366	V	11ax	18H9-N	*S. epidermidis*	5	IV	10a
					18H9-F	*S. pseudintermedius*	568	V	10a
18D10	*S. pseudintermedius*	496	V, ccrA1B6	10a	18H10-N	*S. pseudintermedius*	496	V, ccrA1B6	10a
					18H10-F	*S. pseudintermedius*	496	V, ccrA1B6	10a

18D11-1	*S. cohnii*	-	mecA, mecI, ccrA1B3	8s	18H11-N	neg.	-	-	-
18D11-2	*S. pseudintermedius*	585	mecA. mecI	11y	18H11-F	*S. pseudintermedius*	585	mecA. mecI	11y
18D12	*S. schleiferi*	-	V, ccrA1B1	11di	18H12-N1	*S. pasteuri*	-	IV	10m
					18H12-N2	*S. epidermidis*	213	V, ccrA2B2	10dl
					18H12-N3	*S. hominis*	-	mecA, mecI	11b
					18H12-F	*S. schleiferi*	-	mecA	11bn
18D13-1	*S. pseudintermedius*	1093	V, ccrA1B6	11a	18H13-N1	*S. pseudintermedius*	1093	V, ccrA1B6	11a
18D13-2	*S. schleiferi*	-	V	11a	18H13-N2	*S. epidermidis*	35	V, ccrA1B1	11p
					18H13-F	*S. pseudintermedius*	1093	V, ccrA1B6	11a
18D14	neg.	-	-	-	18H14-N	*S. epidermidis*	35	V, ccrA1B1, ccrA2B2	11p
					18H14-F	*S. haemolyticus*		mecA	8aq
18D15-1	*S. schleiferi*	-	V	11dl	18H15-N	*S. pseudintermedius*	365	mecA. mecI	11bm
18D15-2	*S. pseudintermedius*	365	mecA. mecI	11bm	18H15-F	*S. epidermidis*	192	V, ccrA2B2	10dm
18D16	*S. pseudintermedius*	121	V, ccrA1B6	11ah	18H16-N1	*S. epidermidis*	5	IV	10a
					18H16-N2	*S. pseudintermedius*	566	V	10a
					18H16-F	*S. pseudintermedius*	566	V	10a
18D17	*S. pseudintermedius*	365	mecA, mecI	11cv	18H17-N	*S. epidermidis*	5	IV	10a
					18H17-F	neg.	-	-	-
18D18	*S. pseudintermedius*	367	V	10dh	18H18-N1	*S. pseudintermedius*	367	V	10dh
					18H18-N2	*S. epidermidis*	130	IV	10a
					18H18-F	*S. pseudintermedius*	367	V	10dh
18D19	*S. pseudintermedius*	585	mecA,IS431	11a	18H19-N	*S. epidermidis*	35	V, ccrA1B1, ccrA2B2	11p
					18H19-F	*S. pseudintermedius*	585	V	11a

18D20-1	*S. pseudintermedius*	563	V	11a	18H20-N1	*S. epidermidis*	5	IV	10a
18D20-2	*S. schleiferi*	-	V	11a	18H20-F1	*S. cohnii*	-	III, ccrC	9a
					18H20-F2	*S. epidermidis*	20	V	11a
18D21	*S. schleiferi*	-	V	11a	18H21-N	*S. epidermidis*	5	IV	11dm
					18H21-F	*S. schleiferi*		V	11a
18D22	*S. pseudintermedius*	45	mecA	11cj	18H22-N	*S. epidermidis*	59	IV	11cf
					18H22-F	neg.	-	-	-
18D23	*S. pseudintermedius*	585	mecA mecI	11y	18H23-N	neg.	-	-	-
					18H23-F	*S. pseudintermedius*	585	mecA mecI	11y
18D24	*S. pseudintermedius*	580	V	11a	18H24-N	*S. epidermidis*	130	V, ccrA2B2	8ar
					18H24-F	*S. pseudintermedius*	580	V	11a
18D25	*S. pseudintermedius*	241	neg.	neg.	18H25-N	*S. epidermidis*	5	mecA, ccrA2B2	10dp
					18H25-F	*S. saprophyticus*	-	mecA,mecI,ccrA1B3	11a
18D26	*S. pseudintermedius*	1328	V	11a	18H26-N1	*S. pseudintermedius*	1328	V	11a
					18H26-N2	*S. epidermidis*	173	V, ccrA2B2	neg.
					18H26-F1	*S. pseudintermedius*	1329	mecA, mecI	11cy
					18H26-F2	*S. epidermidis*	173	V, ccrA2B2	neg.
18D27-1	*S. pseudintermedius*	1330	V	11a	18H27-N	*S. epidermidis*	838	IV, ccrC	13z
18D27-2	*S. haemolyticus*	-	V	2e	18H27-F1	*S. cohnii*	-	II	7ac
					18H27-F2	*S. epidermidis*	5	IV, ccrC	10dq
18D28	*S. schleiferi*	-	V	11a	18H28-N	*S. epidermidis*	130	V, ccrA1B1, ccrA2B2	11dn
					18H28-F	*S. epidermidis*	130	V, ccrA1B1, ccrA2B2	11do

18D29-1	*S. pseudintermedius*	568	mecA	11y	18H29-N	*S. epidermidis*	35	V, ccrA1B1, ccrA2B2	11p
18D29-2	*S. pseudintermedius*	1340	V	11a	18H29-F	*S. pseudintermedius*	568	mecA, mecI	11y
18D30	*S. pseudintermedius*	1341	mecA, ccrA1B1	neg.	18H30-N	*S. epidermidis*	35	V, ccrA1B1, ccrA2B2	11p
					18H30-F	*S. epidermidis*	35	V, ccrA1B1, ccrA2B2	11p
18D31	*S. warneri*	-	mecA	neg.	18H31-N	*S. epidermidis*	130	IV, ccrC	10a
					18H31-F	neg.	-	-	-

neg.= negative

Table 10. Antimicrobial susceptibility testing profile of *S. pseudintermedius* isolated from dogs and *S. epidermidis* isolated from owners in this study.

Antibiotics	*S. pseudintermedius* isolated from dogs (n = 25)	*S. epidermidis* isolated from owners (n = 35)
Penicillin	100%	100%
Oxacillin	92%	-
Cefoxitin	-	100%
Gentamicin	56%	60%
Amikacin	0%	2%
Erythromycin	64%	60%
Clindamycin	60%	17%
Tetracycline	84%	43%
Doxycycline	60%	40%
Ciprofloxacin	56%	29%
Norfloxacin	56%	40%
Chloramphenicol	44%	2%
Trimethoprim/sulfamethoxazole	64%	5%
Rifampicin	0%	0%

Table 11. Direct repeats (DRs) detected in this study

Direct repeats (DR)	Sequences	Species in this study	Strain
DR-1	GAGGCT**TATCATAA**AT	*S. pseudintermedius, S. schleiferi, S. epidermidis*	18D20-1, 18D20-2, 18H20-F2
DR-2	GAAAAT**TATCATAA**TA	*S. pseudintermedius, S. schleiferi*	18D20-1, 18D20-2
DR-3	GAAGCT**TATCATAA**AT	*S. pseudintermedius, S. schleiferi*	18D20-1, 18D20-2
DR-4	GAAGCA**TATCATAA**AT	*S. schleiferi, S. epidermidis*	18D20-2, 18H13-N2, 18H2-N
DR-5	GAAAGT**TATCATAA**GT	*S. schleiferi, S. epidermidis*	18D20-2, 18H13-N2
DR-6	GAGGAA**TAATAATA**GT	*S. schleiferi*	18D20-2
DR-7	GAAGCG**TATAATAA**GT	*S. epidermidis*	18H20-F2
DR-8	GAAGCG**TATCGTAA**GT	*S. epidermidis*	18H20-F2, 18H13-N2, 18H2-N
DR-9	GAAGCG**TATCACAA**AT	*S. warneri*	18H2-F
DR-10	GAAGCG**TATCATAA**GT	*S. warneri*	18H2-F
DR-11	GAAGCG**TACCACAA**AT	*S. epidermidis*	18H13-N2. 18H2-N

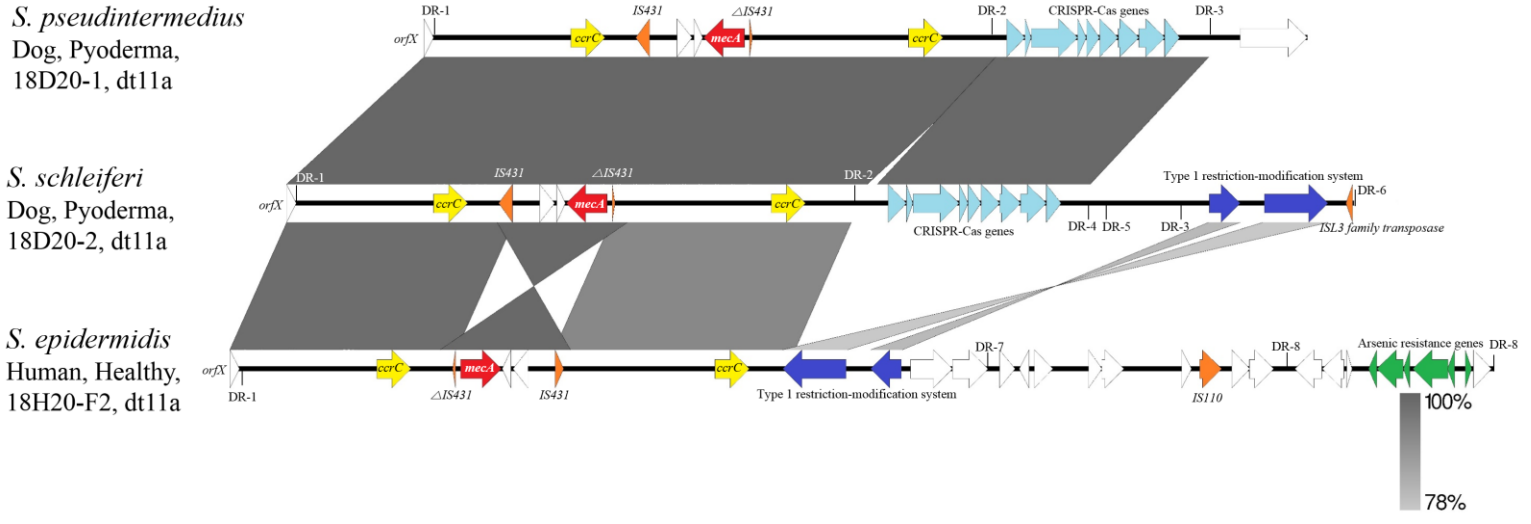

Figure 8. Genomic comparison of the SCC*mec* region in *S. pseudintermedius* (18D20-1), *S. schleiferi* (18D20-2) from the dog, and *S. epidermidis* (18H20-F2) from the dog owner.

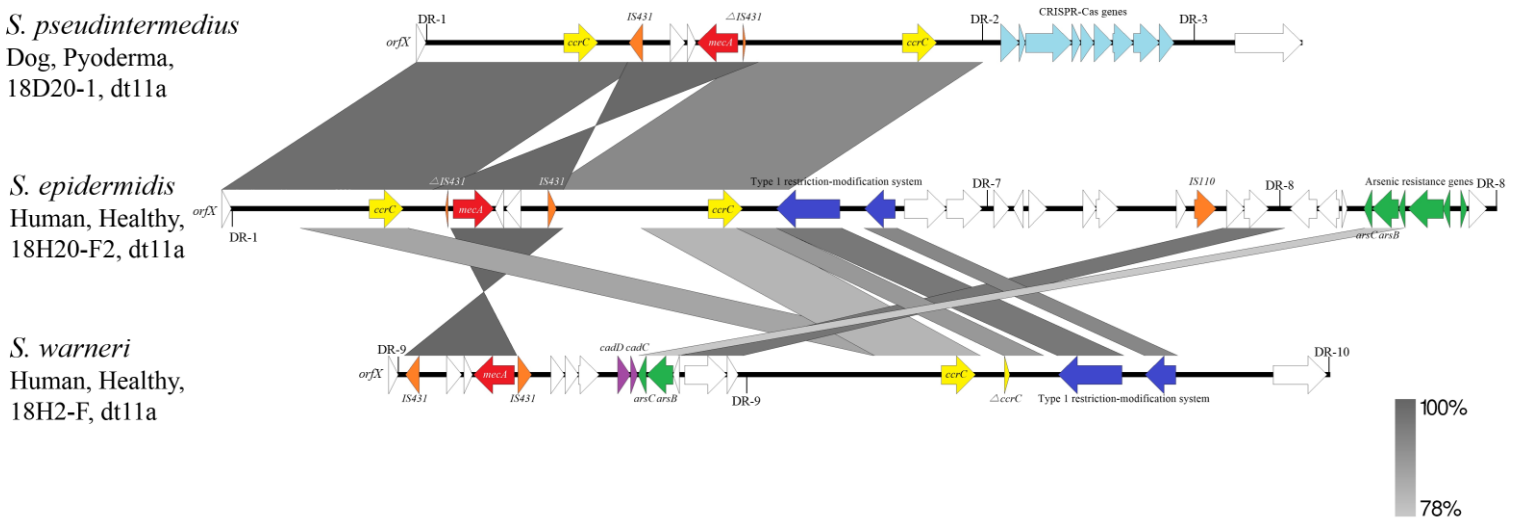

Figure 9. SCC*mec* of 18H2-F (*S. warneri*) compared genetically with 18H20-F2 (*S. epidermidis*) and 18D20-1 (*S. pseudintermedius*).

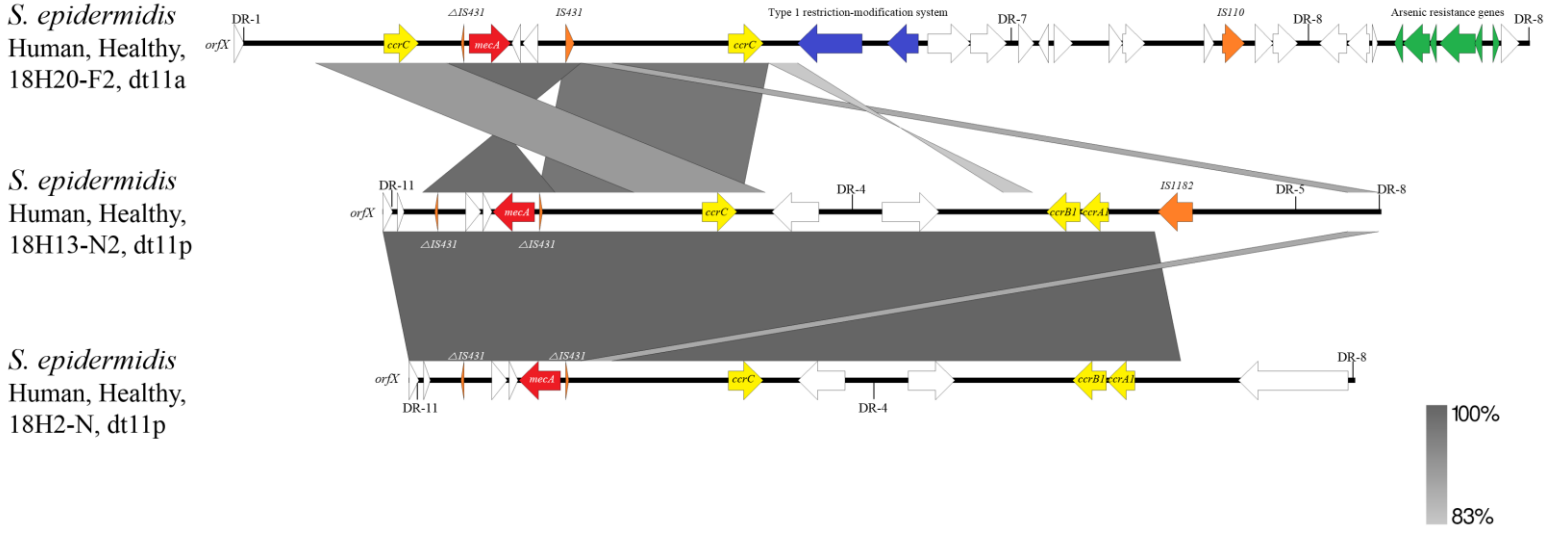

Figure 10. Genomic comparison of the SCC*mec* region in 18H20-F2, 18H13-N2, and 18H2-N

CONCLUSION

This study was designed to investigate the clonal distribution of MRSP isolated from canine pyoderma and otitis externa in Korea, the emergence of MDRSP, and the genetic relationship of methicillin resistance between staphylococci isolated from dogs affected with pyoderma and the dog owners. The main conclusions are as follows.

1. Two clonal complexes, CC568 and CC677, were identified in the clonal lineage of MRSP isolated from canine pyoderma and otitis externa in Korea, and this result differed from the dominant STs previously detected in MRSP isolates from Europe, North America, and other Asian countries. SCC*mec* type V was the major type, and *dru* types 11a and 11y were dominant.

2. Chromosomal pRE25-like elements carrying five antimicrobial-resistant genes (*cat*, *erm(B)*, *aphA3*, *aadK*, and *sat4*) were first detected in *Staphylococcus* species. BLASTN analysis revealed that the 50-kb enterococcal pRE25 genome covered 84% of the 22-kb pRE25-like elements sequence with 99.8% identity, and PCR targeting mobile

genetic elements indicated that multidrug-resistant pRE25-like elements are suspected to be widespread in *S. pseudintermedius* isolated from dogs in Korea.

3. *S. pseudintermedius* (dog), *S. schleiferi* (dog), and S. *epidermidis* (dog owner) isolated from a dog affected with pyoderma and its owner showed remarkable genetic similarity of SCC*mec* in DNA sequences, *dru* type, structure composition of *ccrC* and the *mec* complex, and identical DR-1 in *orfX*, which is considered the insertion site of SCC*mec*. These results support the risk of SCC*mec* transfer in veterinary clinical circumstances between dogs and dog owners.

The high prevalence (41.9%, 60/143) and independent clonal characteristics compared with other countries highlight the rapid emergence of MRSP isolated from dogs in Korea. Moreover, the multidrug resistance of *S. pseudintermedius* induced by pRE25-like elements is suspected to be widespread, and the MLST results indicated that Korea is the potential geographical origin of pRE25-like elements in the MDRSP lineage. Overall, these results suggest that veterinary antimicrobial stewardship has not been applied consistently in the field of small

animal practice in Korea, and thus a long-term national action plan is urgently needed.

The risk of SCC*mec* transfer between dogs and dog owners was estimated by WGS. *S. pseudintermedius* (dog), *S. schleiferi* (dog), and S. *epidermidis* (dog owner) isolated from a dog and dog owner pair showed the remarkable genetic similarity of SCC*mec*. Therefore, a strategy countering methicillin-resistant staphylococci should be based on the cooperation of veterinary medicine and human medicine under the One-Health concept.

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국 문 초 록

개의 농피증에서 분리한 *Staphylococcus pseudintermedius* 의 항생제 내성에 대한 유전체 분석과 원헬스적 접근

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Staphylococcus pseudintermedius 는 개의 정상 피부 미생물 총을 구성하며 개에서 발생하는 대부분의 세균성 감염에서 원인체로 작용한다. 소동물 수의임상에서 다양한 계열의 항생제가 *Staphylococcus pseudintermedius* 감염을 치료하기 위하여 사용되고 있으며 이와 더불어 빠르게 확산되고 있는 항생제내성이 큰 수의학적 문제로 대두되고 있다. 따라서 본 연구에서는 국내 개에서 분리한 메치실린 내성 *Staphylococcus pseudintermedius* (MRSP) 균주의 유전형과 다제내성 *Staphylococcus pseudintermedius* 의 pRE25-like element 를 분석하고 농피증에 이환된 개와 그 보호자 사이에서 분리한 메치실린 내성 포도상구균의 유전학적 연관성을 평가하였다.

제 1 장에서는 국내 개의 농피증과 외이염에서 분리한 MRSP 60 주의 유전형 분포에 대한 분석을 다좌위 서열 형별분석 (MLST), direct repeat unit (*dru*) typing 그리고 Staphylococcal Cassette Chromosome *mec*

(SCC*mec*) typing 을 통해 실시하였다. 주요 *dru* type 은 dt11a 와 dt11y 이었으며 MLST 를 통해 CC365 와 CC677 두 개의 clonal complex 가 존재하고 있음을 밝혀내었다. 이러한 유전형 분포는 유럽과 북미, 다른 아시아 국가에서 보고되는 양상과 달랐으며 우세한 SCC*mec* type 은 type V 로 전체 중 45%를 차지하였다.

제 2 장에서는 pRE25-like elements 와 관련하여 발생하는 다제내성 *Staphylococcus pseudintermedius* (MDRSP) 에 대한 연구를 실시하였다. 총 46 개의 MDRSP 균주가 개의 농피증 병변에서 분리되었으며 균주의 유전학적 특성을 밝히기 위하여 MLST, pRE25-like elements 에 대한 PCR 그리고 전장유전체분석 (WGS)을 실시하였다. WGS 를 통해 5 개의 다른 항생제 내성유전자 (*cat, erm(B), aphA3, aadK, and sat4*) 와 *IS1252, IS256*, 그리고 *IS1216* 로 구성되는 이동성 유전인자의 집합인 chromosomal 22-kb pRE25-like elements 가 포도상구균류에서 최초로 발견되었다. pRE25-like elements 염기서열의 84%는 BLASTN 데이터베이스를 통한 유전체 비교 분석상 장구균의 pRE25 플라스미드 DNA 와 99.8%의 동질성을 보였으며 이는 반코마이신내성이 장구균에서 포도상구균으로 전달된 것과 같이 항생제 다제내성의 발생 또한 장구균과 포도상구균이 밀접한 유전학적 관련성을 지니고 있을 가능성을 시사한다. pRE25-like elements 의 이동성 유전인자중 포도상구균류에서는 매우 드물지만, 장구균에서는 흔하게 발견되는 recombinase 와 insertion sequence 를 타겟으로 하는 3 종의 PCR 을 실시하였고 전체 균주 중 52%에 해당하는 균주가 3 종의 PCR 모두에 양성반응을 보임으로써 pRE25-like elements 를 보유하고 있을 가능성이 높은 균주로 확인되었다. 이 균주들에 대한 MLST 를 실시한 결과 전체 18 종의 Sequence type (ST) 이

존재하였으며 그중 13 종의 ST 가 한국에서 발견 및 보고되었던 ST 로 확인되었다.

제 3 장에서는 농피증에 이환된 개와 보호자에서 분리한 포도상구균 사이의 메치실린 내성에 대한 유전학적 연관성에 대한 연구를 진행하였다. 총 101 주의 포도상구균이 31 쌍의 개와 보호자의 비강, 손가락 피부에서 분리되었다. 메치실린 내성을 유도하는 *mecA* 유전자가 탑재되어 있는 이동성 유전인자인 SCC*mec* 유형에 대한 1 차 스크리닝으로 *dru* typing 및 PCR 을 통한 SCC*mec* typing 을 모든 균주에 실시하였고 *S. pseudintermedius* 와 *S. epidermidis* 는 유전형 분석을 위한 MLST 를 실시하였다. SCC*mec* 유형에 대한 유전학적 연관성이 높을 것으로 추정되는 후보 균주에 대해서는 WGS 를 통해 최종적인 SCC*mec* 의 비교분석을 실시하였다. 개의 농피증 병변에서 분리한 *S. pseudintermedius* 및 *S. schleiferi* 와 보호자의 손가락 피부에서 분리한 *S. epidermidis* 로 구성되는 1 쌍에서 동일한 *dru* type 11a 및 SCC*mec* type V 를 가지는 것을 확인하여 해당 균주에 대해 WGS 를 실시하였다. WGS 결과 상 SCC*mec* 구성에 핵심적인 *mec* complex 와 *ccrC* 유전자가 분포하는 지역의 염기서열이 높은 동질성 (평균 96.8%)을 가지고 있고 *mec* complex 와 *ccrC* 의 SCC*mec* 지역내 위치와 분포가 일치하며 SCC*mec* 의 시작지점인 insertion site 로써 작용하는 *orfX* 의 direct repeats (DRs) 가 본 연구에서 확인된 DR-1 으로 3 개 균주가 일치하는 결과를 얻을 수 있었다.

결론적으로, 국내 MRSP 가 높은 유병률 (41.9%, 60/143)과 해외 다른 국가와 비교하여 독립적인 유전형 분포를 가지고 있는 것, 그리고 pRE25-like elements 에 의한 다제내성 *S. pseudintermedius* 가 넓게 분포하고 있는 것으로 추정되며 이러한 균주의 ST 분포가 국내에서 발견되어 보고되었던 ST 가 다수를 차지하고 있어 외래유입이 아닌 국내발생 균주일

가능성이 높은 점을 종합하면 국내 소동물 수의임상에서 Antimicrobial Stewardship 이 적절하게 작동하지 못하고 있으며 장기적인 국가행동계획 (National Action Plan) 이 시급한 상태임을 시사하고 있다. 또한 개와 그 보호자에서 분리한 메치실린 내성 *S. pseudintermedius, S. schleiferi* 및 *S. epidermidis* 의 SCC*mec* 이 높은 유전학적 동질성을 가지는 것은 메치실린 내성의 발생 및 전파에 있어 개와 보호자 사이에서 단순히 병원체 자체의 이동뿐만 아니라 포도상구균류 간의 유전학적 상호작용 또한 존재할 가능성이 높다고 볼 수 있다. 따라서 원헬스적 개념에 따른 인의와 수의분야의 공조가 메치실린 내성 포도상구균류의 확산에 대한 성공적인 임상적 대응에 필수적일 것으로 사료된다.

주요어: *Staphylococcus pseudintermedius*, 메치실린 내성, 다제 내성, SCC*mec*, 개 농피증

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