Characterization of Methicillin-Resistant *Staphylococcus pseudintermedius* Isolated from Dogs in Veterinary Hospitals in Korea

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ABSTRACT

Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) has emerged as an important health threat in companion animals, with potential for transmission to humans. However, little is known about MRSP circulating in Korea. Accordingly, in this study, we determined the prevalence, antimicrobial resistance profile` and clonal distribution of MRSP in Korea. Forty of 57 (70.17%) swab samples collected from dogs at five veterinary hospitals were positive for *S. pseudintermedius* (SP), and 21 dogs (36.84%) were carrying MRSP strains. Overall, 26 of 50 isolates (52.0%) were MRSP.

Antimicrobial susceptibility tests against 18 antimicrobial agents (13 classes) revealed that all MRSP were multi-drug resistant isolates showing resistance to a wide range of antimicrobial classes. Compared to susceptible isolates, MRSP isolates showed higher resistance rates to most antimicrobials, including oxacillin (100%), cefazolin (96.2%), cefoxitin (96.2%), cefotaxime (100%), and trimethoprim-sulfamethoxazole (100%). Multilocus sequence typing revealed 27 distinct sequence types without a predominant type, including 22 novel types and 3 novel clonal complexes, indicating independently evolving lineages in Korea. Future studies on clones that will evolve to be a major clonal lineage in Korea could give us an important insight regarding the key factors related to the widespread of selected clones in the world.

INTRODUCTION

Staphylococcus pseudintermedius (SP) is an opportunistic pathogen of the skin associated with post-operative infections in companion animals, and is often considered a potential

Table 1. *Primers used in this study.*

Target	Primers	Sequence (5′→3′)	Size (bp)	Reference
S. pseudintermedius	*pse-F2*	F: TRGGCAGTAGGATTCGTTAA	926	(8)
	pse-R5	R: CTTTTGTGCTYCMTTTTGG		
mecA	*MRS1-F*	F: TAGAAATGACTG ACGTCCG	153	(9)
	MRS2-R	R: TTGCGATCAATGTTACCGTAG		
tuf	*tur-F*	F: CAATGCCACAAACTCG	500	(14)
	tur-R	R: GCTTCAGCGTAGTCTA		
cpn60	*cpn60-F*	F: GCGACTGTACTTGCACAAGCA	552	(14)
	cpn60-R	R: AACTGCAACCGCTGTAAATG		
pta	*pta-F*	F: GTGCGTATCGTATTACCAGAAGG	570	(14)
	pta-R	R: GCAGAACCTTTTGTTGAGAAGC		
purA	*purA-F*	F: GATTACTTCCAAGGTATGTTT	490	(14)
	purA-R	R: TCGATAGAGTTAATAGATAAGTC		
fdh	*fdh-F*	F: TGCGATAACAGGATGTGCTT	408	(14)
	fdh-R	R: CTTCTCATGATTCACCGGC		
ack	*ack-F*	F: CACCACTTCACAACCCAGCAAACT	680	(14)
	ack-R	R: AACCTTCTAATACACGCGCACGCA		
sar	*sar-F*	F: GGATTTAGTCCAGTTCAAAATTT	521	(14)
	sar-R	R: GAACCATTCGCCCCATGAA		

zoonotic pathogen.[1,2] With increased antimicrobial resistance, methicillin-resistant *S. pseudintermedius* (MRSP) has emerged as an important opportunistic pathogens represent a serious health concern for companion animals in veterinary medicine.[3] Moreover, MRSP has been reported to cause human infections, and many lines of evidence suggest that it poses a significant human health risk.[4,5] Since methicillin resistance makes it difficult to control the infection and kill the pathogen effectively, several studies have been performed to understand the resistance dissemination mechanism. *In vitro*, *Staphylococcus epidermidis* can act as a reservoir of antimicrobial resistance genes transferred to *Staphylococcus aureus* via horizontal gene transfer.[6] Thus, there is concern of the potential horizontal transmission of the resistant *mecA* gene of MRSP to susceptible strains of other Staphylococcus species or its potential transfer from a companion animal to the human host. In such cases, companion animals could be considered an important reservoir for the dissemination of methicillin resistance to humans. Although MRSP isolates from other countries have been analyzed in great depth from various perspectives, including antimicrobial resistance genes, susceptibility, and multilocus sequence typing (MLST), there is relatively sparse information available about the status of MRSP isolates circulating in Korea. Therefore, in this study, we investigated the prevalence, antimicrobial resistance profile, and clonal distribution of MRSP in dogs in Korea.

MATERIALS AND METHODS

Isolation and Identification of SP

All protocols and procedures were approved by the institutional review board at the Seoul National University (IRB No. 1208/001-004). Swab samples were collected from the external auditory meatus, medial canthus, interdigital cleft, nasal cavity, anus, and

skin of 57 dogs who visited three local and two veterinary teaching hospitals in Korea in 2012 and 2016. Samples were stored in individual collection tubes with Amies transport medium (Yu-Han Lab Tech, Seoul, Korea), and transported to our laboratory on the same day of collection. The swab was directly streaked on 5% sheep blood agar plates and incubated at 37°C for 18–24 hr. Presumptive *staphylococci*-like colonies were selected based on the colony morphology, and confirmed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF)[7]. The identification of SP candidates was then further confirmed by polymerase chain reaction (PCR) using a set of SP-specific primers (Table 1).[8]

Detection of MRSP

MRSP was determined by detection of the staphylococcal methicillin-resistant determinant gene (*mecA*) by PCR using gene-specific primers (Table 1)[9] in all SP isolates identified as described above. Since oxacillin or cefoxitin is commonly used as a surrogate for the determination of methicillin resistance,[10] phenotypic resistance to methicillin was also determined by an oxacillin susceptibility test according to the CLSI guideline.[11]

Antimicrobial Susceptibility Test

The antimicrobial susceptibility of the isolates was tested by the standard disk diffusion method according to the CLSI guideline.[11] A total of 18 antimicrobial agents (13 different classes) that are commonly used in veterinary medicine in Korea were included in the test:

- penicillin (10 units)
- oxacillin (1 μg)
- cefazolin (30 μg)
- cefoxitin (30 μg)
- cefotaxime (30 μg)
- vancomycin (30 μg)
- gentamicin (10 μg)
- amikacin (30 μg)
- quinupristin-dalfopristin (15 μg)
- rifampin (5 μg)
- chloramphenicol (30 μg)
- trimethoprim-sulfamethoxazole (1.25 μg, 23.75 μg)
- ciprofloxacin (5 μg)
- minocycline (30 μg)
- tetracycline (30 μg)
- linezolid (30 μg)
- erythromycin (15 μg), and
- chloramphenicol (30 μg)

(BBL, Sensi-Disc Susceptibility Test Discs; Becton Dickinson, MD, USA).

Escherichia coli ATCC 25922 was included as a reference strain for quality control. Multi-drug resistance (MDR) was defined as resistance to ≥3 classes of antimicrobials.[12,13]

MLST

MLST analysis was performed as described previously[14] using seven different housekeeping genes (ack, cpn160, fdh, pta, purA, sar, turf) (Table 1). The sequence type (ST) was determined using the PubMLST database (http://pubmlst.org/speudintermedius). New STs were assigned by submitting the sequence data of new types to the database curator (Vincent.perreten@vetsuisse.unibe.ch). The clonal relatedness of MRSP was analyzed by eBURST using the database of MLST of MRSP in Korea and those from this study.[15,16]

RESULTS

Isolation and Identification of SP Isolates

Among the 57 dogs, 40 (70.17%) were found to be contaminated, colonized, or infected with SP, and 21 of these dogs (36.84%) were MRSP-positive. Overall, a total of 50 SP strains were isolated, 26 of which (52.0%) tested positive for MRSP.

Antimicrobial Resistance

The results of antimicrobial susceptibility testing of all SP isolates are presented in Table 2. Overall, the SP isolates showed high resistant rates to penicillin (96.0%), trimethoprim-sulfamethoxazole (74.0%), tetracycline (68.0%), and erythromycin (64.0%), whereas no resistance was detected against vancomycin, amikacin, or rifampin

Table 2. *Antimicrobial susceptibility of 59 isolates of S. pseudintermedius*

Class	Antimicrobials	Number of isolates resistant to antimicrobials (%)		
		Total	MSSP	MRSP
Penicillin	**P**	48 (96.0)	22 (91.7)	26 (100.0)
	OX	26 (52.0)	0 (0.0)	26 (100.0)
Cephems	**CZ**	30 (60.0)	5 (20.8)	25 (96.2)
	FOX	34 (68.0)	9 (37.5)	25 (96.2)
	CTX	30 (60.0)	4 (16.7)	26 (100.0)
Glycopeptides	**VA**	0 (0.0)	0 (0.0)	0 (0.0)
Aminoglycosides	**GM**	11 (22.0)	4 (16.7)	7 (26.9)
	AN	0 (0.0)	0 (0.0)	0 (0.0)
Streptogramins	**SYN**	1 (2.0)	0 (0.0)	1 (3.8)
Ansamycins	**RA**	0 (0.0)	0 (0.0)	0 (0.0)
Phenicol	**C**	11 (22.0)	4 (16.7)	7 (26.9)
Folate pathway inhibitor	**SXT**	37 (74.0)	11 (45.8)	26 (100.0)
Fluoroquinolones	**CIP**	22 (44.0)	8 (33.3)	14 (53.8)
Tetracyclines	**MI**	1 (2.0)	1 (4.2)	0 (0.0)
	TE	34 (68.0)	15 (62.5)	19 (73.1)
Oxazolidinones	**LZD**	2 (4.0)	1 (4.2)	1 (3.8)
Macrolides	**E**	32 (64.0)	15 (62.5)	17 (65.4)
Licosamides	**CC**	17 (34.0)	7 (29.2)	10 (38.5)

MSSP, methicillin-susceptible Staphylococcus pseudintermedius; MRSP, methicillin-resistant Staphylococcus pseudintermedius; P, penicillin; OX, oxacillin; CZ, cefazolin; FOX, cefoxitin; CTX, cefotaxime; VA, vancomycin; GM, gentamicin; AN, amikacin; SYN, quinupristin-daptopristin; RA, rifampin; C, chloramphenicol; SXT, trimethoprim-sulfamethoxazole; CIP, ciprofloxacin; MI, minocycline; TE, tetracycline; LZD, linezolid; E, erythromycin; CC, clindamycin.

in any SP isolate. Among the 50 SP isolates, 45 (90.0%) were determined to show MDR. Compared to the methicillin-susceptible isolates, the MRSP isolates generally showed higher resistance rates to most antimicrobials, including oxacillin (100%), cefazolin (96.2%), cefoxitin (96.2%), cefotaxime (100%), and trimethoprim-sulfamethoxazole (100%). In addition, all of the MRSP isolates exhibited MDR. The average number of antimicrobials to which the isolates were resistant was higher in the MRSP group (5.92) than in the susceptible group (4.16) (Table 3).

MLST

The PCR products of seven housekeeping genes (ack, cpn60, fdh, pta, purA, sar, tuf) for each SP isolate were sequenced to determine the STs (Table 4). MLST analysis revealed 27 distinct STs, including 22 novel STs newly identified in this study and five previously reported STs [ST585 (n = 6), ST568 (n = 1), ST362 (n = 4), ST690 (n = 1), and ST709 (n = 1)] from 44 SP isolates. The STs of six SP isolates were not determined (Table 4). There was no dominant ST found. However, multiple isolates of same STs (ST585 and ST362) were detected. Three of the six ST585 isolates were obtained from three dogs living in the same house, and showed an identical antimicrobial resistance profile. Although all of the

Table 3. *Distribution of S. pseudintermedius according to the number of antimicrobial class resistances.*

Group	No. of drug class	No. of isolates (%)		
		MSSP (%)	MRSP (%)	Total (%)
Non- MDR	**0**	0 (0.0)	0 (0.0)	**0 (0.0)**
	1	3 (12.5)	0 (0.0)	**3 (6.0)**
	2	2 (8.3)	0 (0.0)	**2 (4.0)**
MDR	**3**	6 (25.0)	1 (3.8)	**7 (14.0)**
	4	1 (4.2)	4 (15.4)	**5 (10.0)**
	5	6 (25.0)	3 (11.5)	**9 (18.0)**
	6	3 (12.5)	8 (30.8)	**11 (22.2)**
	7	2 (8.3)	8 (30.8)	**10 (20.0)**
	8	0 (0.0)	2 (7.7)	**2 (4.0)**
	9	1 (4.2)	0 (0.0)	**1 (2.0)**
No. of MDR (%)		19 (79.2)	26(100)	**45 (90.0)**
Total No. of isolates (%)		24	26	**50**
Average No. of drug class resistances		**4.16**	**5.92**	**5.08**

MSSP, methicillin-susceptible Staphylococcus pseudintermedius; MRSP, methicillin-resistant Staphylococcus pseudintermedius

ST585 isolates were collected from the same university teaching hospital in 2012, ST362 isolates were from different places in different years (two isolates in 2012 and two isolates in 2016).

To analyze the clonal relationships of MRSP STs in Korea, all available Korean MLST data, including those from this study, were clustered using eBURST analysis. The analysis identified three clonal complexes (CC) that were categorized by their putative founders as CC362, CC371, and CC788 (Fig. 1). Most STs identified in this study belonged to CC362 or CC788. CC362 contained ST362, ST789, ST790, and ST791, whereas CC788 contained ST568, ST585, ST788, ST792, ST793, and ST795. Correlation with the antimicrobial resistance profile revealed that multiple isolates of a same ST (ST362 or ST585) showed various resistance patterns based on the place and year of their isolation. In general, the isolates belonging to CC362 showed resistance to more number of antimicrobial classes than those belonging to CC788, and there was a notable difference in resistance to fluoroquinolones (ciprofloxacin) between the two CCs (Table 4).

DISCUSSION

In this study, the overall prevalence of MRSP colonization or contamination was 36.84% (21/57 dogs), which is similar to that reported in Japan (29.82%) (17), but much higher than the prevalence rates reported in other countries (2% and 3.1%).[18,19] Previous studies reported a prevalence of MRSP among SP isolates in Korea to be 17.3–29.3% during 2008–2010,20-22 which is much lower than the prevalence rates reported in a very recent study (41.9%)[16] and this study (52.0%), indicating that the prevalence of MRSP has significantly increased in Korea over the past few years. Similarly, the rapid increase of MRSP prevalence in dogs has been reported in several Asian countries posing a serious health concern in veterinary clinics.[23-25]

Table 4. *Genetic and phenotypic characterization of MRSP and MSSP isolates*

Group	No. of drug class resistances	Resistance phenotype[a]	Years	Isolation place[b]	Source	No of strains	ST type
MRSP	8	RRSRSSSRRRSRR	2012	A	Interdigital cleft	1	787*
	8	RRSSSSRRRRSRR	2016	D	Skin (Pyoderma lesion)	1	NDc
	7	RRSSSSRRRRSRS	2012	B	Interdigital cleft	1	ND
	7	RRSSSSSRRRSRR	2012	B	Medial canthus	1	362
	7	RRSSSSSRRRSRR	2012	B	External auditory meatus	1	790*
	7	RRSSSSSRRRSRR	2012	B	External auditory meatus	1	791*
	7	RRSSSSSRRRSRR	2016	D	Skin (Pyoderma lesion)	1	ND
	7	RRSSSSSRRRRSS	2016	D	Skin (Pyoderma lesion)	1	794*
	7	RRSSSSRRRSSRR	2016	D	Skin	1	795*
	7	RRSSSSSRRRSRR	2016	D	Skin	1	362
	6	RRSRRSSRSRSSS	2012	B	Interdigital cleft	1	585
	6	RRSRSSRRSSSRS	2012	C	Anus	1	568
	6	RRSRSSSRSSSRR	2012	B	External auditory meatus	1	788*
	6	RRSSSSSRRRSRS	2012	E	Medial canthus	1	789*
	6	RRSSSSSRRSSRR	2012	B	Anus	1	793*
	6	RRSSSSSRRRSRS	2012/2016	B, D	Interdigital cleft, Skin (Pyoderma lesion)	2	362
	6	RRSSSSSRRRSSRS	2016	D	Skin	1	ND
	5	RRSRSSSRSRSSS	2012	B	Medial canthus, External auditory meatus	3	585
	4	RRSSSSSRSRSSS	2012	B	Interdigital cleft	1	792*
	4	RRSSSSSRSRSSS	2012	B	External auditory meatus	1	585
	4	RRSSSSSRSRSSS	2016	D	Skin (Pyoderma lesion)	1	ND
	4	RRSSSSSRSRSSS	2016	D	Skin	1	ND
	3	RRSSSSSRSSSSS	2012	D	External auditory meatus	1	585

	9	RRSRSSRRRRSRR	2012	B	Medial canthus	1	775*
	7	RRSSSSRRSRSRR	2016	D	Skin	1	785*
	7	RRSSSSSRRRSRR	2016	D	Skin	1	709
	6	RRSSSSRRSRSRS	2012	D	Medial canthus	1	774*
	6	RRSSSSSRSRSRR	2012	B	External auditory meatus	1	783*
	6	RRSSSSSRRRSRS	2016	D	Skin	1	786*
	5	RRSSSSSSRRSRS	2012	B	Interdigital cleft	1	776*
	5	RRSRSSSSRRSSS	2012	B	Medial canthus	1	777*
	5	SRSSSSSRRRSRS	2012	B	Medial canthus	1	690
	5	RSSSSSSRSRSRR	2012	B	Interdigital cleft	1	783*
	5	RSSSSSRRSSSRR	2012	B	Interdigital cleft	1	784*
	5	RSSSSSSRRRSRS	2016	D	Skin	1	786*
MSSP	4	RSSSSSSRSSSRR	2012	B	Medial canthus	1	783*
	3	SSSRSSSSRRSSS	2012	B	External auditory meatus	1	777*
	3	RRSSSSSSSRSSS	2012	A	Medial canthus, External auditory meatus	1	779*
	3	RSSSSSSSSRSRS	2012	A	External auditory meatus	1	780*
	3	RRSSSSSSSSSRS	2012	A	Interdigital cleft	1	780*
	3	RSSSSSSSSRRSS	2012	A	Medial canthus	1	781*
	3	RRSRSSSSSSSSS	2012	A	Interdigital cleft	1	782*
	2	RRSSSSSSSSSSS	2012	A	Interdigital cleft	1	778*
	2	RSSSSSSSSSSRS	2012	A	Interdigital cleft	1	780*
	1	RSSSSSSSSSSSS	2012	A	Interdigital cleft	1	779*
	1	RSSSSSSSSSSSS	2012	A	Interdigital cleft	1	781*
	1	RSSSSSSSSSSSS	2012	A	Anus	1	782*

MRSP isolates are frequently associated with resistance to a wide range of antimicrobial agents.[26] Consistent with this finding, all MRSP were determined as MDR in this study. Methicillin resistance due to penicillin-binding protein encoded by *mecA* primarily confers resistance to several β-lactam antimicrobial agents.[27] However, the gene is often associated with resistance to other classes of antimicrobials.[26,28] Compared with methicillin-susceptible isolates in this study, the MRSP isolates showed higher resistance to β-lactam antimicrobial agents such as penicillin, oxacillin, cefazolin, cefoxitin, and cefotaxime, as well as to non- β-lactam antimicrobial agents such as trimethoprim-sulfamethoxazole, tetracycline, and erythromycin. Moreover, the resistance rate to six or more antimicrobial classes was more than twice as high among the MRSP isolates than that for the susceptible isolates, and MRSP isolates were resistant to more antimicrobials on an average. A recent study suggested that the increased antimicrobial resistance of MRSP is closely related with the acquisition of multiple resistance genes.[29] These resistance genes may have been co-selected by the use of numerous classes of antimicrobials in veterinary medicine.[29]

The recent increase of MRSP prevalence worldwide has been associated with the clonal spread of a few dominant STs. ST71 and ST68 were the predominant types in North America and several European countries.[26,30-32] In North China, ST71 was widely spread.[33] However, the present study suggests no predominant STs, but rather a considerable diversity in STs among the MRSP isolates in Korea, with several new STs identified. This finding is consistent with that reported in a recent study by Kang et al.[16] With accumulated MLST data of MRSP in Korea, the current study identified the emergence of three lineage clusters and many nonclonal (singletons) MRSPs. Two clonal lineages (CCs 371 and 788) were re-named from those in Kang's study (CCs 677 and 568, respectively) due to change of the founder STs with added MLST data of newly identified STs in this study. CC362 was a newly detected clonal lineage in this study. All the three CCs do not belong to dominant CCs spreading worldwide,34 indicating independent development of clonal lineages of MRSP in Korea.[16]

Although not dominant, multiple isolates of the same STs (ST585 and ST362) were obtained in this study. ST585 isolates were found from a same place only in 2012, whereas ST362 isolates were from multiple places in both 2012 and 2016. Regarding the antibiogram profile, the best difference between the two STs was found in the resistance to ciprofloxacin (fluoroquinolone). ST362 and its clonal lineage (CC362) exhibited antimicrobial resistance to ciprofloxacin (fluoroquinolone), which was not detected in ST585 and CC788. Acquisition of new antimicrobial resistance genes could be an important factor for successful dissemination of major clonal lineages. Almost all isolates of CC71 and CC45, which have been known to be the most successful MRSP clones worldwide,[29] showed resistance to fluoroquinolones.

It is currently unclear whether this resistance characteristic was the key factor for the successful establishment of those clones. Future studies to determine if ST362 with its clonal lineage could be successfully disseminated across Korea might give us an answer to this question.

CONCLUSION

In conclusion, there has been a rapid emergence and clonal spread of MRSP in veterinary medicine worldwide. However, although the prevalence of MRSP has rapidly increased in the past few years, no major clonal spread has been detected yet in Korea. Most MRSP isolates had new STs. The identified clonal lineages were different from the major CCs in other countries. The ST and lineage information obtained in this study, with continued surveillance to track which clonal set is going to successfully evolve in the future, should be informative to understand the major key factors for the dominant spread of selective clonal populations of MRSP across the world.

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