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High-level mupirocin resistance in Gram-positive bacteria isolated from diseased companion animals

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Conflict of Interest
The authors declare no conflicts of interest.

ABSTRACT

The purpose of this study was to investigate the high-level mupirocin resistance (HLMR) in Gram-positive bacteria isolated from companion animals. A total of 931 clinical specimens were collected from diseased pets. The detection of mupirocin-resistant bacteria and plasmid-mediated mupirocin resistance genes were evaluated by antimicrobial susceptibility tests, polymerase chain reactions, and sequencing analysis. Four-hundred and six (43.6%) bacteria were isolated and 17 (4.2%), including 14 staphylococci and 3 *Corynebacterium* were high-level mupirocin-resistant (MICs, ≥ 1,024 ug/mL) harboring *mupA*. Six staphylococci of HLMR strains had plasmid-mediated *mupA*-IS*257* flanking regions. The results show that HLMR bacteria could spread in veterinary medicine in the near future.

Keywords: Diseased pets; staphylococci; high-level mupirocin resistance; *mupA*-IS*257*; spread

INTRODUCTION

Following the introduction of mupirocin antibiotics in clinical medicine in 1895, mupirocin-resistant *Staphylococcus aureus* was first reported in 1987 [1]. Since then, topical mupirocin has been widely used to decolonize methicillin-resistant *Staphylococcus aureus* (MRSA) in the nasal cavity and to treat skin or soft tissue infections. Recently, mupirocin resistance has been reported in *Staphylococcus pseudintermedius*, which is most frequently isolated from pets, and is also emerging as methicillin-resistant and multidrug-resistant bacteria [2]. Moreover, methicillin-resistant *S. aureus* and *Staphylococcus haemolyticus* have been found as mupirocin-resistant bacteria in dogs and cats [3]. The low-level resistance to mupirocin (minimum inhibitory concentration [MIC] values, ≥ 8 to 256 ug/mL) is involved in mutations in the chromosomal *ileS* gene encoding isoleucyl-tRNA synthetase. However, high-level mupirocin resistance (HLMR, MIC, ≥ 1,024 ug/mL) is associated with a conjugative plasmid harboring *ileS2* (*mupA*). In veterinary hospitals of South Korea, mupirocin is frequently used for the treatment of pets with skin diseases, such as otitis externa, superficial pyoderma, and acne. However, for HLMR, where this resistance occurs, what the causative organism is, and how it acquires resistance to other antibiotics is unknown. Therefore, the goal of this study was to investigate the HLMR in Gram-positive bacteria isolated from diseased companion animals.

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Author Contributions

Conceptualization: Oh JY; Investigation: Sum S, Oh JY; Resources: Oh JY, Sum S; Writing-original draft: Oh JY; Writing - review & editing: Park HM.

MATERIALS AND METHODS

Sampling and species identification

A total of 931 clinical samples were collected from diseased companion animals between June 2017 and September 2019 at 160 veterinary hospitals nationwide. The distribution of collected samples was as follows: ear (n = 341, 36.6%), urine (n = 175, 18.8%), skin (n = 133, 14.3%), nasal cavity (n = 115, 12.4%), blood (n = 47, 5.0%), genitalia (n = 43, 4.6%), feces (n = 40, 4.3%), eye (n = 20, 2.1%), oral cavity (n = 5, 0.5%), and other (n = 12, 1.3%). All Gram-positive bacteria were identified by the mass spectrometry microbial identification system (VITEK MS, bioMérieux, France). However, the *S. intermedius* group was classified into two genospecies (*S. intermedius* and *S. pseudintermeidus*) using polymerase chain reaction (PCR) [4].

Phenotypic identification

For the mupirocin-resistant bacteria, a hemolytic test, catalase test (hydrogen peroxide solution 3%, Sigma-Aldrich, USA), coagulase test (BD BBL Rabbit Coagulase Plasma, USA), and mannitol fermentation were evaluated on colonies grown in blood agar (Synergy Innovation, Korea) and mannitol salt agar (Beckton, Dickinson and Company, USA), respectively.

Antimicrobial susceptibility testing

The antimicrobial susceptibility tests were performed according to the Clinical and Laboratory Standards Institute guideline [5]. Initially, a disk diffusion assay was performed with 200 ug of mupirocin disk (Oxoid, Ltd., Basingstoke, England) to select the mupirocin-resistant bacteria from total isolates. The MICs were determined by the Sensititre standard susceptibility MIC plate EUST (TREK Diagnostic Systems, Thermo Fisher Scientific, UK). In addition, the agar dilution method for mupirocin (Sigma-Aldrich) in the range of 256 to 1,024 ug/ml and chlorhexidine (Merck KGaA, Darmstadt, Germany) in the range of 0.125 to 512 ug/mL was performed in Mueller-Hinton medium (Beckton, Dickinson and Company).

DNA isolation

For the detection of mupirocin resistance genes and SCC*mec* typing, total genomic DNA was extracted using a bacteria DNA purification kit (LaboPass, Cosmogenetech Co., Korea).

Detection of mupirocin resistance genes

The presence of the plasmid-mediated *mupA* gene, its flanking regions insertion sequence IS*257*, and previously described typical *mupA*-IS*257* junctions were detected by PCRs with primer sets (**Table 1**) (**Fig. 1A**) [3,6]. The base sequences of each PCR product was analyzed in the nucleotide BLAST program (http://blast.ncbi.nlm.nih.gov). In addition, the *mupB* gene was detected using a previously described method [7].

Table 1. Oligonucleotide primers used for PCR

Target	Primer	Sequence (5′ to 3′)	Amplicon	Reference
mupA	MupA-F	TATATTATGCGATGGAAGGTTGG	458 bp	3
	MupA-R	AATAAAATCAGCTGGAAAGTGTTG		
mupB	MupB-F	CTAGAAGTCGATTTTGGAGTAG	674 bp	8
	MupB-R	AGTGTCTAAAATGATAAGACGATC		
IS*257*-*mupA* junctions	M1	GTTTATCTTTCTGATGCTGAG	Variable	7
	MupAR	CTCTAATTCAACTGGTAAGCC		
	1234*	GGCATGGCGAAAATCCGTAG		
	1235*	TGGCGTATTGATGAGACGTACATC		

*To identify the IS*257* region in the high-level mupirocin resistance strains, the 429 bp of PCR product was amplified with primer set, 1234 and 1235.
PCR, polymerase chain reaction.

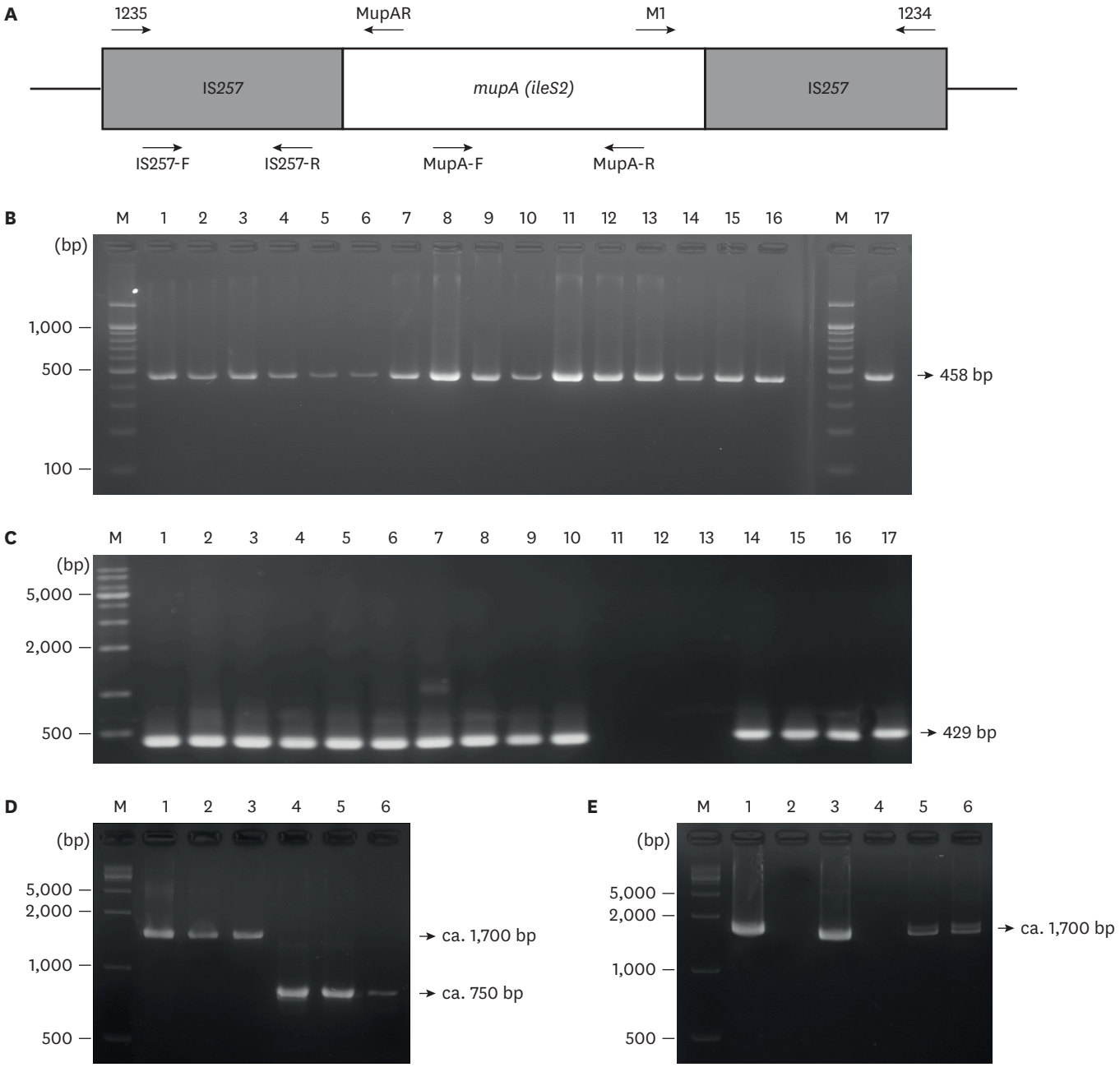

Fig. 1. High-level mupirocin resistance associated with plasmid-mediated *mupA*-IS*257* junctions. Primer annealing sites for the detection of *mupA* gene and adjacent insertion sequence IS*257* (A). Detection of *mupA* and IS*257* using simplex PCR (B and C). Lane M of Figure A and B, 100 bp size marker (ELPIS BIO, Korea) and 1 kb size marker (ELPIS BIO), respectively; lanes 1 to 17, 17-1, 17-26, 17-71, 17-76, 17-80, 17-109, 17-147, 18-325, 19-181, 19-525, 19-805, 19-816, 19-850, 19-877, 19-878, and 19-902. PCR amplification across *mupA*-IS*257* junctions (D and E). Lane M, 1 kb size marker (ELPIS BIO); Lane 1, 19-525 (*Staphylococcus haemolyticus*); Lane 2, 19-805 (*Staphylococcus cohnii*); Lane 3, 19-816 (*Staphylococcus pseudintermedius*); Lane 4, 19-850 (*S. haemolyticus*); Lane 5, 19-877 (*S. pseudintermedius*); Lane 6, 19-878 (*S. haemolyticus*).
PCR, polymerase chain reaction.

Staphylococcal cassette chromosome *mec* (SCC*mec*) typing

SCC*mec* typing for the 17 HLMR strains with the *mecA* gene was determined by a multiplex PCR method [8].

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RESULTS

Detection of mupirocin-resistant bacteria

Four hundred and six (43.6%) Gram-positive bacteria were isolated from the total samples (**Table 2**). Of the total strains, 17 (4.2%) isolated from 16 diseased dogs and 1 cat, were resistant to mupirocin (MICs, ≥ 256 ug/mL) (**Table 3**). Of these mupirocin-resistant bacteria, 5 *S. haemolyticus* were isolated from the skin and ears, followed by 3 *S. pseudintermedius* from the nasal cavity and ears, 3 *C. auriscanis* from the ear, genitalia, and nasal cavity, 3 *S. epidermidis* from the skin and 1 *S. intermedius* from the ears, 1 *S. warneri* from the urine, and 1 *S. cohnii* from the nasal cavity (**Tables 3** and **4**). Seventeen mupirocin-resistant bacteria were catalase-positive. Except for *C. auriscanis* and *S. epidermidis* strains, the remaining 11 HLMR strains were hemolytic. Two *S. pseudintermedius* were coagulase positive. Mannitol fermentation was positive in 3 *staphylococcal* species (*S. warneri*, *S. haemolyticus*, and *S. cohnii*).

Antimicrobial susceptibilities

The HLMR strains were resistant to penicillin (100%, 17/17), followed by ciprofloxacin 70.6%, cefoxitin 70.6%, erythromycin and trimethoprim 58.8%, sulfamethoxazole and tetracycline 52.9%, clindamycin and fusidate 35.3%, gentamicin 29.4%, chloramphenicol 17.6%, and quinupristin-dalfopristin 5.9% (**Table 3**). Resistance to vancomycin has not been observed in this study (data not shown). As a result, mupirocin-resistant strains were identified as multidrug-resistant bacteria, which are resistant to more than four antibiotic classes. Three *S. epidermidis* and one *S. intermedius* were 1,024 ug/mL and the remaining 13 isolates were ≥1,024 ug/mL in the MICs for mupirocin. Chlorhexidine MICs for the 14 high-

Table 2. Distribution of Gram-positive bacteria isolated from 931 clinical specimens of diseased companion animals

Gram-positive bacteria	No. (%) of isolates
Staphylococcus pseudintermedius	275 (29.1)*
Staphylococcus intermedius	51 (5.5)
Staphylococcus haemolyticus	5 (0.5)
Staphylococcus aureus	3 (0.3)
Staphylococcus schleiferi	3 (0.3)
Staphylococcus epidermidis	3 (0.2)
Staphylococcus nepalensis	2 (0.2)
Staphylococcus agalactiae	1 (0.1)
Staphylococcus sciuri	1 (0.1)
Staphylococcus lentus	1 (0.1)
Staphylococcus warneri	1 (0.1)
Staphylococcus cohnii	1 (0.1)
Streptococcus canis	2 (0.2)
Streptococcus mitis	1 (0.1)
Micrococcus luteus	4 (0.4)
Enterococcus faecalis	35 (3.8)
Enterococcus faecium	7 (0.8)
Enterococcus gallinarum	1 (0.1)
Aerococcus viridans	1 (0.1)
Bacillus cereus	2 (0.2)
Bacillus megaterium	1 (0.1)
Bacillus flexus	1 (0.1)
Corynebacterium auriscanis	4 (0.4)
Propionibacterium acnes	1 (0.1)
Clostridium perfringens	1 (0.1)
No. (%) of total	406 (43.6)

**S. pseudintermedius* was the most commonly isolated (29.1%, 275/931). This species was most frequent in the ear canal (14.3%, 133/931), followed by skin 74 (7.9%), nasal cavity 35 (3.8%), urine 19 (2.0%), eyes 2 (0.2%), oral cavity 2 (0.2%), genitalia 2 (0.2%), feces 1 (0.1%), and other 7 (0.8%).

Table 3. Phenotypes and minimum inhibitory concentrations of 17 high-level mupirocin-resistant strains

Strains No.	Bacterial species	Phenotypes*		Antimicrobial and antiseptic agent/MIC breakpoints (ug/mL)												
		Coagulase	Mannitol	MUP/≥ 256	PEN/≥ 0.25	CIP/≥ 4	FOX/≥ 8	ERY/≥ 8	TMP/≥ 16	SMX/≥ 512	TET/≥ 16	CLI/≥ 4	FUS/≥ 4	GEN/≥ 16	CHL/≥ 32	SYN/≥ 4	CHH
17-1	*Staphylococcus haemolyticus*	–	–	≥ 256	≥ 2	8	≥ 16	4	8	≥ 512	2	< 0.12	≥ 4	≥ 16	< 4	< 0.5	2
17-26	*Staphylococcus haemolyticus*	–	–	≥ 256	≥ 2	≥ 8	≥ 16	≥ 8	4	≥ 512	≥ 16	≥ 4	< 0.5	≥ 16	8	1	16
17-71	*Staphylococcus epidermidis*	–	–	≥ 256	> 2	8	8	≥ 8	< 2	256	< 0.5	< 0.12	≥ 4	4	< 4	< 0.5	4
17-76	*Corynebacterium auriscanis*	–	–	≥ 256	≥ 2	4	16	≥ 8	≥ 32	≥ 512	8	≥ 4	< 0.5	16	< 4	< 0.5	-
17-80	*Corynebacterium auriscanis*	–	–	≥ 256	≥ 2	≥ 8	16	< 0.25	≥ 32	≥ 512	< 0.5	< 0.12	< 0.5	16	32	< 0.5	-
17-109	*Corynebacterium auriscanis*	–	–	≥ 256	0.5	4	8	< 0.25	≥ 32	512	1	0.5	< 0.5	4	8	< 0.5	-
17-147	*Staphylococcus epidermidis*	–	–	≥ 256	1	< 0.25	4	≥ 8	≥ 32	128	≥ 16	< 0.12	≥ 4	16	< 4	< 0.5	2
18-325	*Staphylococcus warneri*	–	+	≥ 256	2	0.5	8	< 0.25	< 2	128	< 0.5	< 0.12	≥ 4	≥ 16	< 4	1	8
19-2	*Staphylococcus intermedius*	–	–	≥ 256	≥ 2	2	8	< 0.25	< 2	128	> 16	< 0.12	≥ 4	4	< 4	< 0.5	1
19-181	*Staphylococcus pseudintermedius*	–	–	≥ 256	> 2	1	4	≥ 8	≥ 32	≥ 512	> 16	≥ 4	< 0.5	8	8	4	2
19-525	*Staphylococcus haemolyticus*	–	+	≥ 256	≥ 2	≥ 8	≥ 16	≥ 8	8	128	1	< 0.12	≥ 4	8	< 4	< 0.5	8
19-805	*Staphylococcus cohnii*	–	+	≥ 256	≥ 2	4	4	≥ 8	≥ 32	256	≥ 16	≥ 4	< 0.5	8	64	< 0.5	1
19-816	*Staphylococcus pseudintermedius*	+	–	≥ 256	≥ 2	4	4	≥ 8	≥ 32	256	≥ 16	≥ 4	< 0.5	8	64	< 0.5	2
19-850	*Staphylococcus haemolyticus*	–	–	≥ 256	≥ 2	≥ 8	8	≥ 8	≥ 32	512	≥ 16	0.5	< 0.5	4	8	< 0.5	2
19-877	*Staphylococcus pseudintermedius*	+	–	≥ 256	≥ 2	≥ 8	4	≥ 8	≥ 32	256	≥ 16	0.25	< 0.5	8	8	< 0.5	2
19-878	*Staphylococcus haemolyticus*	–	–	≥ 256	≥ 2	≥ 8	≥ 16	4	≥ 32	≥ 512	2	≥ 4	< 0.5	≥ 16	< 4	2	4
19-902	*Staphylococcus epidermidis*	–	–	≥ 256	≥ 2	< 0.25	16	< 0.25	< 2	512	≥ 16	< 0.12	< 0.5	≥ 16	< 4	< 0.5	2
No. (%) of resistant strains				17 (100)	17 (100)	13 (70.6)	12 (70.6)	10 (58.8)	10 (58.8)	9 (52.9)	9 (52.9)	6 (35.3)	6 (35.3)	5 (29.4)	3 (17.6)	1 (5.9)	NA†

MUP, mupirocin; PEN, penicillin; CIP, ciprofloxacin; FOX, cefoxitin; ERY, erythromycin; TMP, trimethoprim; SMX, sulfamethoxazole; TET, tetracycline; CLI, clindamycin; FUS, fusidate; GEN, gentamicin; CHL, chloramphenicol; SYN, quinupristin-dalfopristin; CHH, chlorhexidine.
*Coagulase and mannitol fermentation: positive (+), negative (–); †NA, not available, because the breakpoint has not yet been established in the Clinical and Laboratory Standards Institute.

level staphylococci strains, except 3 *C. auriscanis* strains, were ≤16 ug/mL (range, 1 to 16 ug/mL; geometric mean of MIC, 4 ug/mL).

SCC*mec* subtype

Three SCC*mec* subtypes were identified in 7 of 17 HLMR strains with the *mecA* gene by multiplex PCR. SCC*mec* subtype III was found in three staphylococci (*S. epidermidis* 17-71, *S. haemolyticus* 19-850, and *S. pseudintermedius* 19-877), subtype I was identified in *S. cohnii* 19-805 and *S. epidermidis* 19-902, and subtype IVa was identified in *S. warneri* 18-325 and *S. intermedius* 19-2, respectively (**Table 4**). No SCC*mec* subtypes for the remaining 10 strains were observed.

Plasmid-mediated mupirocin resistance

The presence of the *mupA* (*ileS2*) gene was detected in the 17 HLMR strains, but IS*257* was only detected in 14 staphylococci strains, except for 3 *C. auriscanis* (**Fig. 1B and C**). However, the *mupB* gene was not detected in all HLMR strains. Three of HLMR staphylococci corresponded to *mupA*-IS*257* spacer (ca. 1.7 kb), but the remaining 3 PCR products (ca. 0.75 kb) contained

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Table 4. Minimum inhibitory concentrations of mupirocin, PCR results of mupirocin resistance-associated *mupA*-IS*257* junctions and SCC*mec* typing in 17 high-level mupirocin-resistant strains

Strains No.	Species	Disease	Specimen	Identified bacteria	MICs of mupirocin (ug/mL)	PCR results across *mupA*-IS*257* junctions				SCC*mec* typing	
						mupA	IS*257*	1235+MupAR	M1+1234	*mecA* gene	Subtype
17-1	Dog	External otitis	Ear canal	*Staphylococcus haemolyticus*	≥ 1,024	+	+	ND	ND	+	NT
17-26	Dog	Pyoderma	Skin	*Staphylococcus haemolyticus*	≥ 1,024	+	+	ND	ND	+	NT
17-71	Dog	Balanoposthitis	Genitalia	*Staphylococcus epidermidis*	1,024	+	+	ND	ND	+	III
17-76	Dog	External otitis	Ear canal	*Corynebacterium auriscanis*	≥ 1,024	+	–	ND	ND	+	NT
17-80	Dog	External otitis	Ear canal	*Corynebacterium auriscanis*	≥ 1,024	+	–	ND	ND	+	NT
17-109	Dog	External otitis	Ear canal	*Corynebacterium auriscanis*	≥ 1,024	+	–	ND	ND	+	NT
17-147	Dog	Chronic bronchitis	Nasal cavity	*Staphylococcus epidermidis*	1,024	+	+	ND	ND	+	NT
18-325	Dog	Cystitis	Urine	*Staphylococcus warneri*	≥ 1,024	+	+	ND	ND	+	IVa
19-2	Dog	Conjunctivitis	Eye	*Staphylococcus intermedius*	1,024	+	+	ND	ND	+	IVa
19-181	Cat	Bronchitis	Nasal cavity	*Staphylococcus pseudintermedius*	≥ 1,024	+	+	ND	ND	+	NT
19-525	Dog	External otitis	Ear canal	*Staphylococcus haemolyticus*	≥ 1,024	+	+	ca. 1.7 kb	ca. 1.7 kb	+	NT
19-805	Dog	Pneumonia	Nasal cavity	*Staphylococcus cohnii*	≥ 1,024	+	+	ND	ca. 1.7 kb	+	I
19-816	Dog	External otitis	Ear canal	*Staphylococcus pseudintermedius*	≥ 1,024	+	+	ca. 1.7 kb	ca. 1.7 kb	+	NT
19-850	Dog	External otitis	Ear canal	*Staphylococcus haemolyticus*	≥ 1,024	+	+	ND	ca. 0.75 kb	+	III
19-877	Dog	External otitis	Ear canal	*Staphylococcus pseudintermedius*	≥ 1,024	+	+	ca. 1.7 kb	ca. 0.75 kb	+	III
19-878	Dog	External otitis	Ear canal	*Staphylococcus haemolyticus*	≥ 1,024	+	+	ca. 1.7 kb	ca. 0.75 kb	+	NT
19-902	Dog	Dermatopathy	Skin	*Staphylococcus epidermidis*	1,024	+	+	ND	ND	+	I

PCR, polymerase chain reaction; MIC, minimum inhibitory concentration; ND, not determined; NT, not-typeable strain by multiplex PCR assay.

the *mupA* and the end region of IS*257* with two-thirds cut by PCR using a M1/1234 primer set and sequencing analysis (**Fig. 1D**). From the PCR results of the opposite junction IS*257*-*mupA*, approximately 1.7 kb of PCR products were amplified in 4 strains by PCR using MupAR/1235 primers (**Fig. 1E**). It was confirmed that IS*257* and *mupA* were connected. In addition, all HLMR strains with the *mecA* gene (MICs, 4 to ≥ 16 ug/mL) were resistant to cefoxitin (data now shown).

DISCUSSION

Mupirocin has been used worldwide as a topical antibiotic for the treatment of human skin diseases. Since the recent approval of the use of mupirocin in dogs, monitoring for high-level mupirocin-resistant bacteria has been studied. In the United States, mupirocin is limited to treating canine pyoderma. Recently the plasmid-mediated mupirocin resistance gene, *mupA* (*ileS2*) was detected in one of 581 *S. pseudintermedius* strains isolated from canine pyoderma patients in 2014 [3]. In Croatia, plasmids carrying *mupA* and the aminoglycoside resistance gene have been identified in high-level mupirocin-resistant *S. pseudintermedius* isolated from canine pyoderma patients in 2013 [9]. In Poland, 3 mupirocin-resistant staphylococci (*S. aureus*, *S. pseudintermedius*, and *S. haemolyticus*) have been reported in dogs and cats in 2019 [2]. Interestingly, mupirocin-resistant *S. pseudintermedius* strains from dogs in the United States and Poland were simultaneously resistant to methicillin. In South Korea, there have been few studies on mupirocin-resistant bacteria in companion animals until recently, when it was found that 1 (0.9%) out of 110 *S. pseudintermedius* isolates from canine pyoderma were identified as HLMR in 2018 [10]. Other countries examined the presence of HLMR in *S. pseudintermedius* isolated from canine pyoderma patients, but the HLMR bacteria in the current study were isolated from various clinical specimens. *S. haemolyticus*, *C. auriscanis* and *S. pseudintermedius* isolated from the ear canal were predominant among HLMR strains. Three *S. epidermidis* were also isolated for the first time in the skin, genitalia and nasal cavity. As a result, the HLMR bacteria found in various lesions of pets could predict the transmission of those clones or the possibility of transfer of plasmids carrying *mupA* between bacteria.

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Mupirocin has been used in human medicine in South Korea since 1994. Owing to topical antibiotics that can be purchased without a doctor's prescription, it has been reported that misuse of these antibiotics has led to an increase in bacteria such as MRSA, which are resistant to those antibiotics [11]. Additionally, six staphylococci of the 17 HLMR strains were also resistant to fusidate (fusidic acid). This topical antibiotic, like mupirocin, is also widely used for skin infected with *S. aureus* or *Streptococcus pyogenes* in human [11]. According to the Ministry of Food, Agriculture, Forestry and Livestock Food Statistics in 2015, the number of domestic pets was over 9 million, with more than 5 million (28.1%) of the total households having pets [12]. As the demand for companion animals increases, more antibiotics will be used, leading to the emergence of more resistant bacteria. Accordingly, to control the emergence of these resistant bacteria in veterinary practice, accurate identification of the causative organism and antimicrobial susceptibility testing should be essential.

Of the 14 HLMR strains detected in both *mupA* and IS*257*, 6 included plasmid-mediated *mupA*-IS*257* junctions, but some showed incomplete or truncated spacer regions in contrast to the typical plasmids previously described [13]. Recently, plasmid-mediated HLMR isolated from pets showed structures with an open reading frame or novel gene rearranged into the *mupA*-IS*257* junction [3,9], but no newly rearranged genes were found in the present study. It is also known that the plasmid-mediated *mupA* gene is present in chromosomal DNA [14], which may require further investigation of HLMR *C. auriscanis* strains that lack IS*257*.

In conclusion, HLMR staphylococci harboring plasmid-mediated *mupA*-IS*257* junctions have emerged in diseased companion animals in South Korea. Further work is needed to identify their epidemiological associations by analyzing the transmissible plasmids in HLMR strains to prevent their dissemination in the veterinary field.

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