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-IS257 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-IS257; 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.
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. pseudintermedius) 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 SCCmec 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 IS257, and previously described typical mupA-IS257 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 | TATATTATGCCATGGAAGGTTGG                                     | 458 bp   | 3         |
|                       | MupA-R | AATAAAAATCGCTGGAAGATTGG                                     |          |           |
| mupB                  | MupB-F | CTAGAAGTCGATTTGGAGTAG                                      | 674 bp   | 8         |
|                       | MupB-R | AGTGTCTAAAATGATAAGACGATC                                    |          |           |
| IS257-mupA junctions | M1     | GTTTATCTTTTCTGGATTGG                                    | Variable | 7         |
| IS257-mupA junctions | MupAR  | GGCATGGGCGGAAAATCCGTGAG                                   |          |           |
| IS257-mupA junctions | 1234*  | CTCTAATTCACTGTAACGCC                                   |          |           |
| IS257-mupA junctions | 1235*  | GGGCGGATGATGAGACGATC                                   |          |           |

*To identify the IS257 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.
High-level mupirocin resistance in diseased companion animals

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

SCCmec typing for the 17 HLMR strains with the meca gene was determined by a multiplex PCR method [8].

**Fig. 1.** High-level mupirocin resistance associated with plasmid-mediated mupA-IS257 junctions. Primer annealing sites for the detection of mupA gene and adjacent insertion sequence IS257 (A). Detection of mupA and IS257 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, 17-109, 17-147, 18-325, 19-181, 19-525, 19-805, 19-850, 19-877, 19-878, and 19-902. PCR amplification across mupA-IS257 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.
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 (0.3)             |
| 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%).
High-level mupirocin resistance in diseased companion animals

Three SCCmec subtypes were identified in 7 of 17 HLMR strains with the mecA gene by multiplex PCR. SCCmec 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 SCCmec 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 IS257 was only detected in 14 staphylococci strains, except for 3 C. auriscanis strains. In contrast, the mupB gene was not detected in all HLMR strains. Three of HLMR staphylococci corresponded to mupA-IS257 spacer (ca. 1.7 kb), but the remaining 3 PCR products (ca. 0.75 kb) contained

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

| Strains No. | Bacterial species | Coagulase | Mannitol | MUP | PEN 0.25 | FOX 0.05 | ERY | TMP 0.16 | SMX 0.16 | CLI 0.25 | FUS | GEN 0.5 | CHL 32 | SYN 4 | CHH
|-------------|------------------|-----------|----------|-----|----------|---------|-----|--------|--------|--------|-----|--------|------|------|------|
| 17-1        | Staphylococcus haemolyticus | –         | –        | ≥ 256 | ≥ 2     | 8       | ≥ 16 | 4      | ≥ 512  | 2      | < 0.12 | 4    | ≥ 16   | < 4  | 5    
| 17-26       | Staphylococcus haemolyticus | –         | –        | ≥ 256 | ≥ 2     | ≥ 8     | ≥ 16 | 4      | ≥ 512  | 2      | < 0.12 | 4    | ≥ 16   | < 4  | 5    
| 17-71       | Staphylococcus epidermidis  | –         | –        | ≥ 256 | ≥ 2     | > 8     | ≥ 16 | 4      | ≥ 512  | 2      | < 0.12 | 4    | ≥ 16   | < 4  | 5    
| 17-76       | Corynebacterium auriscanis  | –         | –        | ≥ 256 | ≥ 2     | > ≥ 8   | 16   | ≥ 8    | 32     | ≥ 512  | ≥ 0.5 | ≥ 16   | < 4  | 5    
| 17-80       | Corynebacterium auriscanis  | –         | –        | ≥ 256 | ≥ 2     | ≥ 8     | ≥ 16 | < 0.25 | ≥ 32   | ≥ 512  | ≥ 0.5 | ≥ 16   | < 4  | 5    
| 17-109      | Corynebacterium auriscanis  | –         | –        | ≥ 256 | ≥ 0.5  | 4       | ≥ 8  | < 0.25 | ≥ 32   | 512    | 0.5   | ≥ 4     | < 0  | 5    
| 17-147      | Staphylococcus epidermidis  | –         | –        | ≥ 256 | 1      | < 0.25 | 4    | ≥ 8    | ≥ 32   | 128    | ≥ 0.12 | ≥ 16   | < 4  | 5    
| 18-325      | Staphylococcus warneri      | –         | +        | ≥ 256 | 2      | 0.5     | 8    | < 0.25 | ≥ 2    | 128    | ≥ 0.12 | ≥ 16   | < 4  | 1    
| 19-2        | Staphylococcus intermedius  | –         | –        | ≥ 256 | ≥ 2     | 2       | 8    | < 0.25 | ≥ 2    | 128    | ≥ 0.12 | ≥ 4    | < 0  | 5    
| 19-181      | Staphylococcus pseudintermedius | –         | –        | ≥ 256 | > 2     | ≥ 4     | ≥ 8  | ≥ 32   | 512    | ≥ 16   | ≥ 0.5  | ≥ 4     | 8    | 4    
| 19-525      | Staphylococcus haemolyticus | –         | +        | ≥ 256 | ≥ 2     | ≥ 8     | ≥ 16 | 8      | ≥ 32   | 128    | ≥ 0.12 | ≥ 4    | < 0  | 5    
| 19-805      | Staphylococcus cohnii        | –         | +        | ≥ 256 | ≥ 2     | 2       | 4    | ≥ 8    | ≥ 32   | 516    | ≥ 0.5  | ≥ 8     | < 0  | 5    
| 19-816      | Staphylococcus pseudintermedius | +         | –        | ≥ 256 | ≥ 2     | 4       | 4    | ≥ 8    | ≥ 32   | 256    | ≥ 0.5  | ≥ 8     | < 0  | 5    
| 19-850      | Staphylococcus haemolyticus | –         | –        | ≥ 256 | ≥ 2     | ≥ 8     | ≥ 8  | ≥ 32   | 512    | ≥ 16   | 0.5    | < 4     | 8    | 0    
| 19-877      | Staphylococcus pseudintermedius | +         | –        | ≥ 256 | ≥ 2     | ≥ 8     | ≥ 8  | ≥ 32   | 256    | ≥ 16   | 0.25   | < 4     | 8    | 0    
| 19-878      | Staphylococcus haemolyticus | –         | –        | ≥ 256 | ≥ 2     | ≥ 8     | ≥ 16 | 4      | ≥ 32   | 512    | 2      | ≤ 0.12 | ≥ 4  | 2    
| 19-902      | Staphylococcus epidermidis  | –         | –        | ≥ 256 | ≥ 2     | < 0.25 | 16   | ≥ 0.25 | ≥ 2    | 512    | ≥ 0.12 | ≥ 16   | < 4  | 5    

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).
High-level mupirocin resistance in diseased companion animals

**Table 4. Minimum inhibitory concentrations of mupirocin, PCR results of mupirocin resistance-associated mupA-IS257 junctions and SCCmec typing in 17 high-level mupirocin-resistant strains**

| Strains | No. | Species | Disease | Specimen | Identified bacteria | MICs of mupirocin (µg/mL) | PCR results across mupA-IS257 junctions | SCCmec typing 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 |
| 19-877  | 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.

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.

https://doi.org/10.4142/jvs.2020.21.e40
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 IS257, 6 included plasmid-mediated mupA-IS257 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-IS257 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 IS257.

In conclusion, HLMR staphylococci harboring plasmid-mediated mupA-IS257 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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