Prevalence of Mupirocin Resistance in *Staphylococcus pseudintermedius*

Running title: Prevalence of Mupirocin Resistance in *S. pseudintermedius*

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Abstract

In the United States, veterinary use of mupirocin is primarily limited to the treatment of canine pyoderma caused by methicillin-resistant *Staphylococcus pseudintermedius* (MRSP). In this study, only one of 581 *S. pseudintermedius* isolates tested was resistant to mupirocin and carried the high-level mupirocin resistance gene, *ileS2* on a plasmid.

Keywords

*Staphylococcus pseudintermedius*
Mupirocin resistance
PCR
*ileS2*

*Staphylococcus pseudintermedius* is the primary bacterial pathogen isolated from canine pyoderma and also causes post-surgical infections in dogs (1, 2). Methicillin resistance and
multi-drug resistance are increasing in *S. pseudintermedius*, thus limiting the options for
therapeutic treatment of canine skin infections (2). Mupirocin is a bacteriostatic antibiotic that
reversibly binds to isoleucyl tRNA synthetase to disrupt protein synthesis and is widely used to
eliminate nasal carriage of methicillin-resistant *Staphylococcus aureus* (MRSA) in human
MRSA carriers (3). Mupirocin has been used on only a limited basis in veterinary medicine but
is approved in the United States for the treatment of bacterial skin infections and superficial
pyoderma in dogs (4).

In *S. aureus*, two levels of mupirocin resistance have been identified. Low-level
mupirocin resistance occurs due to a point mutation to the chromosomal ileS gene that encodes
the native isoleucyl-tRNA synthetase. The minimum inhibitory concentration (MIC) for
mupirocin for staphylococci carrying the low-level resistance is \( \geq 8 \mu g/mL \) but \( \leq 256 \mu g/mL \)
(5). Conversely, high-level mupirocin resistance (MIC \( \geq 512 \mu g/mL \)) is usually conferred by the
plasmid-borne ileS2, although a chromosomal location of *ileS2* has been reported (5). Recently
*ileS2* plasmid-mediated mupirocin resistance was found in a mupirocin-resistant, methicillin-
susceptible *S. pseudintermedius* isolated from a dog in Croatia (6). The goal of the present study
was to determine the prevalence of mupirocin resistance in *S. pseudintermedius* isolated from
patients presented to a veterinary hospital in Texas.

In this study, 581 isolates of *S. pseudintermedius* were screened for phenotypic low-level
mupirocin resistance. Isolates were collected from veterinary patients, predominantly dogs \( (n = 446) \) but also included isolates from cats \( (n = 9) \). Some patients were cultured at multiple sites
and contributed more than one isolate, and of these 21 patients contributed more than two
isolates. The isolates included a historical collection of 403 isolates from clinical infections and
contained both methicillin-resistant *S. pseudintermedius* MRSP isolates \( (n = 153) \) and
methicillin-susceptible *S. pseudintermedius* (MSSP) (*n* = 250). The isolates from clinical infections were collected from the following anatomic sites: skin (*n* = 96), external ear canal (*n* = 31), wounds (*n* = 79), post-operative infections (*n* = 33), urine or the urinary tract (*n* = 87) and other sources (*n* = 77). Additional isolates were collected during a study of MRSP prevalence in canine patients without clinical staphylococcal infection that presented for elective orthopedic procedures. The MRSP prevalence study yielded 178 *S. pseudintermedius* isolates (13 MRSP and 165 MSSP) collected from the nares or perineum of 129 dogs.

All isolates were presumptively identified as *S. pseudintermedius* at the time of collection based on Gram-stain reaction, colony color, and biochemical tests. Tests measured the ability of the isolates to produce hemolysis on trypticase soy agar supplemented with 5% sheep blood agar (blood agar plates) (BD Diagnostic Systems, Sparks, MD), to produce coagulase, to produce catalase, and to grow on salt-mannitol agar. Isolates were also tested for resistance to polymyxin B, ability to utilize trehalose, and production of urease. At the time of initial collection, isolates were tested for antimicrobial susceptibility using commercially available systems (GPS card, VITEK, bioMérieux, Durham, NC; COMPAN1F and COMPAN2F panels, TREK Sensititre, TREK Diagnostics, Cleveland, OH) and additionally tested for methicillin resistance by oxacillin disk diffusion testing and polymerase chain reaction (PCR) for the presence of *mecA*. The *mecA* PCR used a previously published protocol with methicillin-resistant *S. aureus* (ATCC 33591) and methicillin-susceptible *S. aureus* (ATCC 29213 or ATCC 25923) as positive and negative controls respectively (7). Isolates were stored frozen in 10% glycerol at -80°C in 96-well deep well plates and later inoculated aseptically using a 96-pin replicator onto Mueller-Hinton agar (BD Diagnostic Systems) and onto Mueller-Hinton agar supplemented with 8 µg/ml mupirocin (Sigma-Aldrich, St. Louis, MO) (mupirocin plate hereafter) to screen for low-level resistance to
mupirocin. *Pseudomonas aeruginosa* (ATCC 27853) was used as a positive control for mupirocin resistance (8). The bacterial concentrations were not standardized prior to screening. Plates were incubated for 24 hrs at 37°C and then examined.

Colonies were screened by PCR for the presence of the *ileS2* gene using the previously published primers *mupA* and *mupB* to amplify a 458 bp fragment of the *ileS2* gene (9) and primers M1 and M2 to amplify a 237 bp fragment of the gene (Table 1) (10). A total reaction volume of 50 µl was used with the final concentrations of reagents as follows: 2.5 mM MgCl₂, 0.2mM dNTPs, 2.5 pmol of each primer and 2.5 U Taq polymerase per reaction (Lucigen, Middleton, WI). Three to five colonies isolated from the mupirocin plate were used for colony PCR. Reactions were run in a 2720 Thermal Cycler (Applied Biosystems, Grand Island, NY) using the settings: 95°C for 5 min, 35 cycles of 95°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec, followed by 72°C for 7 min and then held at 4°C. Negative controls included water with no template DNA and DNA from a known mupirocin-sensitive, methicillin-sensitive *S. aureus* (ATCC 29213). No positive control was available. The products were then run on a 2% agarose gel for 2 hours at 70V, visualized with GelRed® (Phenix Research, Candler, NC) and compared to a 100 bp molecular weight marker (Invitrogen, Grand Island, NY).

Plasmid purification was performed using the QIAprep Spin Miniprep® plasmid purification kit according to the manufacturer’s instructions (Qiagen, Valencia, CA). Samples were evaluated for concentration and quality using a NanoDrop® spectrometer (Thermo Scientific, Waltham, MA) prior to PCR testing. PCR was used to evaluate the IS257-*ileS2* spacer regions using a previously published molecular classification system (11). The primers IS257₅, *ileS2*-5’, *ileS2*-3’, and IS257₉ (Table 1) were used in various combinations as previously described under the conditions: 94°C for 5 min, thirty cycles of 94°C for 30 sec, 60°C for 40
PCR was performed to identify the native ileS gene using the primers ileS-F1 and ileS-R1 (Table 1) (6). Conditions for thermal cycler were: 94°C for 5 min; 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec, then 72°C for 7 min and held at 4°C.

PCR products were purified using either the QIAprep Gel Purification kit (Qiagen, Valencia, CA) or the Zymoclean Gel DNA Recovery Kit® (Zymo Research, Irvine, CA) according to the manufacturers’ protocols. Purified PCR products were then cloned into pT7Blue plasmid vector using the Novagen pT7Blue Perfectly Blunt Cloning Kit® (EMD Chemicals, Inc.; Darmstadt, Germany) following manufacturer’s protocol. Resultant plasmids containing the upstream IS257-ileS2 junction, the downstream ileS2-IS257 junction and the 945 bp fragment of the native ileS gene were submitted to the Texas A&M Gene Technologies Laboratory for sequencing. Resultant sequences were compared to sequences in GenBank JX186508, JX186509, JX186511, JX186512, JX186513, and JX186514 using MEGA5.1 software (6, 12).

Of the 581 isolates tested, only one isolate was resistant to mupirocin. The isolate, 39-045, was originally cultured from the nares of a healthy, one-year-old, castrated, male, Bernese mountain dog presenting for an orthopedic evaluation. This isolate was pan-susceptible to all antimicrobials tested using the COMPAN2F drug panel and negative for the presence of the mecA gene via PCR analysis. The prevalence of mupirocin resistance in dogs without clinical staphylococcal infections that presented for elective orthopedic procedures was 1 in 129 or 0.8%.

An additional, 194 S. pseudintermedius isolates were collected from 158 dogs with clinical infections during the same period of collection (September 22, 2010 and February 8, 2012) resulting in a total of 372 S. pseudintermedius isolates from 287 dogs. The prevalence of
mupirocin resistant *S. pseudintermedius* in dogs cultured between September 22, 2010 and February 8, 2012 was therefore 1 in 287 dogs or 0.3%.

The mupirocin-resistant isolate was analyzed for the presence of high-level mupirocin resistance by plasmid DNA isolation followed by PCR amplification of two different regions of the plasmid-borne *ileS2* gene. The presence of a 458 bp band with *mupA* and *mupB* primers and 237 bp band with M1 and M2 primers indicate that the isolate contains the *ileS2* gene (Figure 1).

To further determine structural type of the plasmid, PCR for the IS257-*ileS2* spacer regions was performed following a previously published molecular classification system (11). The fragments are similar to the amplification for structural group S2 *ileS2* plasmids found in *S. aureus* pattern II, with bands sized between 1,000-bp and 1,650-bp for primers *ileS2*-3’ & IS257_F and at between 2,000 and 3,054-bp for primers IS257_R & *ileS2*-5’ (Figure 2). This structural group is similar to the structure previously reported for the plasmid-borne *ileS2* identified in *S. pseudintermedius* isolated from a dog with pyoderma in Croatia (6). The resultant PCR products were sequenced and compared to the previously published *ileS2* sequences from *S. pseudintermedius*, JX186508 and JX186509 (6). Sequences from this study were deposited in GenBank as KJ000545, KJ000546, and KJ000547. Comparison of JX186509 with KJ000545 using MEGA5.1 software indicated 99% similarity between the two sequences. Comparison of JX186508 with KJ000546 and KJ000547 indicated 100% and 99% similarity between the sequences respectively.

To determine whether isolate 39-045 had both an *ileS* mutation and *ileS2* plasmid simultaneously, PCR amplification of the chromosomal *ileS* gene was also performed using previously published primers (6). The resultant 945-bp product was sequenced and analyzed using MEGA5.1 software and the sequence was deposited in GenBank as KJ000544. Analysis
indicated a 99% similarity between isolate 39-045 and the previously published sequences of the
*S. pseudintermedius* chromosomal ileS gene: JX186511, JX186512, JX186513, and JX186514 (6).

In summary, this study found that the prevalence of mupirocin resistance in *S. pseudintermedius*
isolated from dogs was 0.3% (1/287) or 0.8% (1/129) healthy dogs without active, clinical staphylococcal infections. While no mupirocin resistant isolates were found in our collection of isolates from dogs with clinical disease, the presence of plasmid-mediated mupirocin resistance is of concern as previous work has demonstrated that mupirocin resistance can be transmitted from one species of *Staphylococcus* to another *in vivo* (13). Increased rates of methicillin-resistance and multi-drug resistance in *S. pseudintermedius* and approval of mupirocin for use in dogs have made mupirocin an attractive alternative for topical use in canine pyoderma (2). This could result in increased mupirocin-resistance in *S. pseudintermedius* over time. Although our study found only one mupirocin-resistant *S. pseudintermedius* isolate, 36.5 percent of U.S. households own a dog (14) and there is the potential for transmission of mupirocin resistance from canine isolates of *S. pseudintermedius* to human isolates of *S. aureus* or vice versa. This could have implications for public health. For these reasons, mupirocin resistance should be monitored and carefully considered before mupirocin is used in canine patients. **Acknowledgements**

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References


Figure 1. Detection of *ileS2* using the polymerase chain reaction (PCR). Lanes 1-3 include PCR reactions amplified with primers *mupA* and *mupB* primers (9). Lanes 4-6 include PCR reactions amplified with M1 and M2 primers (10). The molecular weight marker used in lane 1 and 8 was a 100 bp DNA ladder (Invitrogen, Grand Island, NY). Template DNA used for PCR was plasmid DNA from isolate 39-045 (lanes 2 and 5) or genomic DNA from ATCC 29213 (lanes 3 and 6). Water was substituted for DNA in lanes 4 and 7.
Figure 2. Detection of the ileS2-SI257 junctions in isolate 39-045 using ileS2-5', IS257-F, ileS2-3', and IS257-R primers (11). Primer pairs for each reaction were as follows: PCR 1 - ileS2-5' and IS257F; PCR 2 - ileS2-5' and IS257R; PCR 3 - ileS2-3' and IS257F; and PCR 4 - ileS2-3' and IS257R. Lanes 2, 6, and 10 are products from PCR1; Lanes 3, 7, and 11 are products from PCR2; Lanes 4, 8, and 12 are products from PCR3; Lanes 5, 9, and 13 are products from PCR 4; and lanes 1 and 14 are a 1 kb DNA ladder (Invitrogen, Grand Island, NY). Template DNA in Lanes 2-5 is plasmid DNA from isolate 39-045. Template DNA in lanes 6-9 is genomic DNA from ATCC 29213 used as a negative control. In lanes 10-13 water was substituted for template DNA as a negative control. No positive controls were available.
### Table 1. Primers used in this study.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer</th>
<th>Sequence (From 5’ to 3’)</th>
<th>Reference</th>
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<td></td>
<td>mupB</td>
<td>AATAAAATCAGCTGGAAAGTGTTG</td>
<td>(9)</td>
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<td></td>
<td>M1</td>
<td>GTTTATCTTCTGATGCTGAG</td>
<td>(10)</td>
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<tr>
<td></td>
<td>M2</td>
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<td></td>
<td>IS257R</td>
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