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Molecular Characterization of High-Level Mupirocin Resistance in *Staphylococcus pseudintermedius*

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The genetic analysis of high-level mupirocin resistance (Hi-Mup^r) in a *Staphylococcus pseudintermedius* isolate from a dog is presented. The Hi-Mup^r *ileS2* gene flanked by a novel rearrangement of directly repeated insertion sequence IS*257* elements was located, together with the aminoglycoside resistance *aacA-aphD* determinant, on a conjugative plasmid related to the pSK41/pGO1 family plasmids.

Staphylococcus pseudintermedius is one of the most common pathogens isolated from skin and ear infections in dogs. Although both methicillin-susceptible and methicillin-resistant *S. pseudintermedius* (MSSP and MRSP, respectively) have been isolated from serious human infections (1), the emergence and spread of MRSP are a major veterinary issue, with approximately 8% of all *S. pseudintermedius* isolates from diseased dogs and cats in Croatia being methicillin resistant (1, 2).

Mupirocin is a topical antibiotic frequently used for preoperative elimination of nasal carriage of methicillin-resistant *S. aureus* (MRSA) in humans (3). High-level mupirocin resistance (Hi-Mup^r) in *S. aureus* is generally conferred by the plasmid-associated *ileS2* gene, which encodes an alternate isoleucyl-tRNA synthetase that is not bound by mupirocin (4). *ileS2* is frequently found on pSK41-like plasmids, where it is flanked by IS*257* insertion elements (5). Recently, the determination of the organization of IS*257-ileS2* spacer regions by PCR and sequencing has proven to be useful for typing *ileS2*-bearing plasmids (6).

Although mupirocin is used only sporadically in veterinary medicine, it has been recommended for treating recurrent interdigital furunculosis, callus pyoderma, and muzzle acne in dogs (7). In *S. pseudintermedius*, resistance to mupirocin has been reported occasionally, but its molecular basis has not been investigated (8, 9). The aim of the present study was to examine the occurrence of resistance to mupirocin in *S. pseudintermedius* strains isolated from infected sites of dogs and cats in Croatia and to reveal the molecular mechanism of mupirocin resistance.

In total, 106 clinical isolates previously identified as *S. pseudintermedius* by a multiplex PCR method (2, 10) were included in this study. They were obtained from 102 dogs and 4 cats in the period from April to September 2011. All isolates were associated with clinical disease and were recovered from the affected sites: skin ($n = 40$), external ear canal ($n = 39$), conjunctival sac ($n = 8$), nostrils ($n = 7$), infected wounds ($n = 6$), urine ($n = 4$), and female genital tract ($n = 2$).

MICs of mupirocin were determined by broth microdilution using doubling dilutions of mupirocin (0.03125 to 1,024 mg/liter) in Mueller-Hinton broth (Bio-Rad, France) by following the CLSI guidelines (11). The MIC of gentamicin was determined by Etest (AB bioMérieux, France).

Whole DNA was isolated using 2% Chelex-100 solution (Bio-Rad, USA) (2). The *ileS2* gene fragment was amplified and sequenced using previously described primers (12). Amplification and sequencing of IS*257-ileS2* segments were performed as described previously (6).

Primers ileS-F1 (5′-CGTGACCGTGGCGAATGGGT-3′) and ileS-R1 (5′-GTATGCGGAATGATTGGCG-3′) were designed based on sequences of two *S. pseudintermedius* strains deposited in GenBank (accession no. CP002439.1 and CP002478.1) and used for amplification and sequencing of a 956-bp *ileS* gene fragment in the single Hi-Mup^r *S. pseudintermedius* strain detected in this study, designated HR547/11, and in three mupirocin-susceptible clinical isolates selected on the basis of their sequence types (STs): HR294/11 (MSSP ST117), HR23161 (MRSP ST106), and HR1084/08 (MRSP ST71, a dominant European MRSP clone). Sequences were aligned in CLC Genomics Workbench 4.9 (CLC bio, Denmark) and investigated for the presence of mutations conferring low-level mupirocin resistance in *S. aureus* (13).

Multilocus sequence typing (MLST) was performed according to the method of Bannoehr et al. (14), and sequence types were assigned using the key table kindly provided by the curator of the *Staphylococcus intermedius* group MLST database, Vincent Perreten.

Plasmid DNA was extracted with the QIAprep spin plasmid kit (Qiagen, Hilden, Germany), with the addition of lysostaphin (Sigma Chemical Co., St. Louis, MO), and digested separately with EcoRI and HindIII. Probes for *ileS2*, *aacA-aphD*, and *traK* were generated with the PCR digoxigenin (DIG) probe synthesis kit (Roche). Digested plasmid DNA was separated on 0.8% agarose and transferred to a Hybond-N+ membrane (Amersham). Southern blots were hybridized overnight at 68°C.

The MICs of mupirocin for the 105 susceptible strains ranged from ≤0.03125 to 0.25 mg/liter, while a single mupirocin-resistant/methicillin-susceptible isolate, designated HR547/11, exhibited a Hi-Mup^r phenotype (MIC > 1,024 mg/liter). HR547/11 showed additional resistances to gentamicin (MIC = 8 mg/liter), kanamycin, tobramycin, and penicillin and belonged to ST44. It

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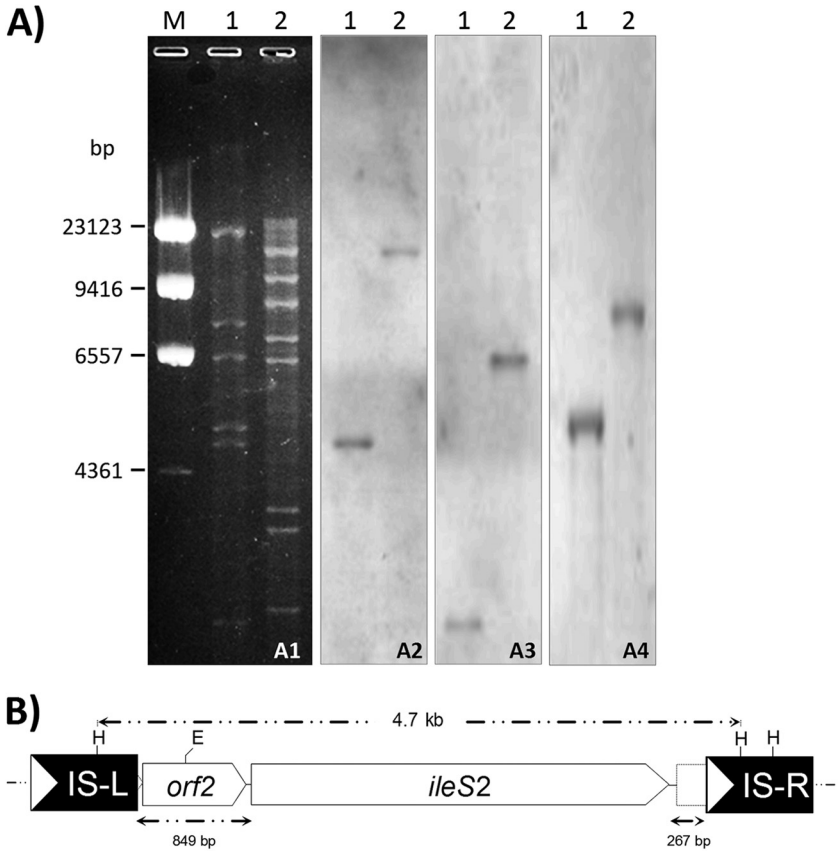

FIG 1 (A) Analysis of plasmid pKM01 by restriction endonuclease analysis and Southern hybridization. (A1) Restriction endonuclease analysis of pKM01. Lane M, lambda phage DNA digested with HindIII as a molecular size standard; lane 1, pKM01 digested with HindIII; lane 2, pKM01 digested with EcoRI. Numbers on the left correspond to the molecular sizes (in base pairs) of λ DNA HindIII restriction fragments. Southern hybridization was carried out with the *ileS2*-containing DNA as a probe (A2), an *aacA-aphD* gene fragment as a DNA probe (A3), and a *traK* gene fragment as a DNA probe (A4). The lane contents of panels A2 to A4 are the same as in panel A1. (B) Genetic organization of IS*257*s flanking the *ileS2* gene on the pKM01 plasmid, encoding Hi-Mup^r. Restriction endonuclease cleavage sites are abbreviated as follows: E, EcoRI, and H, HindIII. Upstream (IS-L) and downstream (IS-R) IS*257* elements flanking the *ileS2* gene are represented by solid boxes; the white arrows indicate the direction of IS*257* transposase transcription. The *ileS2* gene and the predicted open reading frames (ORFs) up- and downstream are represented as arrows, with the arrowhead indicating their orientation. Truncated ORFs are shown using a dotted line. The broken arrows above the diagram note the extent of the HindIII fragment hybridizing with the *ileS2* probe in restriction fragment length polymorphism (RFLP) analyses. Below are shown the sizes (in bp) of sequences corresponding to up- and downstream IS*257*-*ileS2* spacers.

was isolated from an infected surgical wound after operative treatment of the anterior cruciate ligament injury in a 6-year-old Dachshund, which was seen by the Clinic for Surgery, Orthopedics and Ophthalmology at the Faculty of Veterinary Medicine, University of Zagreb. Antimicrobial history was obtained from the owner, a nurse working in a medical center. The dog received a combination of systemic (spiramycin and metronidazole) and topical (wound treatment with povidone-iodine and hydrogen peroxide) therapy. There was no prior exposure to mupirocin. Posttreatment swabs taken after 2 weeks failed to yield pathogenic bacteria.

A specific *ileS2* gene fragment was PCR amplified from HR547/11, and its DNA sequence had 100% similarity with the respective *ileS2* gene sequences deposited in GenBank (accession no. HQ625435 to HQ625438). *ileS2* was not detected in mupirocin-susceptible *S. pseudintermedius* strains (HR294/11, HR23161, and HR1084/08; mupirocin MICs, 0.25, 0.0625, and 0.0625 mg/liter, respectively). Analysis of the plasmid DNA content of the HR547/11 strain revealed the presence of a single high-molecular-weight conjugative plasmid called pKM01. Southern blotting confirmed that *ileS2* and the *aacA-aphD* antibiotic resistance genes, as well as the conjugative-transfer-associated *traK* gene, were located on pKM01. These findings suggest that pKM01 belongs to the pSK41 family of conjugative plasmids (5, 15). Molecular analysis of IS*257*-*ileS2* spacer regions on pKM01 demonstrated that the *ileS2* gene is flanked by directly repeated IS*257*s showing an UpR849-DnR267 (upstream R849-downstream R267) rearrangement (Fig. 1).

ileS gene fragments amplified from HR547/11, HR294/11, HR23161, and HR1084/08 showed more than 99% sequence similarity between each other and with the sequences deposited in GenBank. All possessed a T-to-A silent mutation at position 1764 (T1764A) of the *ileS* gene (numbering is according to the *ileS* gene of HKU10-03, accession no. CP002439.1).

Only a few studies have reported *S. pseudintermedius* strains resistant to mupirocin, but the mechanism and the level of resistance were not investigated (8, 9). For instance, one mupirocin-resistant isolate was found among 8 MRSP strains obtained from dogs with superficial pyoderma in a study conducted in the United States, where mupirocin is licensed for use in dogs (9). The *ileS2*

gene is generally found on large staphylococcal plasmids but has also been detected rarely in the chromosome of *S. aureus* (16). It has been postulated previously that the members of the *S. intermedius* group prefer transposon-encoded resistance genes due to the large number of insertion sequences found in their chromosomal DNA (17). To investigate the location of *ileS2* in HR547/11, we performed Southern blotting experiments, which confirmed that both the *ileS2* and *aacA-aphD* genes were situated on a conjugative plasmid probably belonging to the pSK41/pGO1 family (5). To our knowledge, this is the first description of a plasmid related to the pSK41 family in *S. pseudintermedius*. Resistance genes, such as *ileS2*, are often integrated by the activities of IS*257* insertion elements (15). The *ileS2* gene of HR547/11 was also flanked by two copies of IS*257*, as previously found in mupirocin-resistant *S. aureus* (6). The IS*257-ileS2* configuration (UpR849-DnR267) found on pKM01 has not been identified previously and represents a novel finding. The possibility of the presence of mutations within the native *ileS* gene and their influence on MICs was excluded by partial sequencing and comparison with the *ileS* gene sequences of mupirocin-susceptible strains. Interestingly, compared to *S. aureus* (GenBank accession no. X74219), all examined strains had a T1764A silent mutation in the *ileS* gene sequence.

Worryingly and taking into account the limitation of having collected a single mupirocin- and gentamicin-resistant *S. pseudintermedius* isolate, such strains could become more prevalent in the future. Thus, therapy with gentamicin, kanamycin, or tobramycin, which are more commonly used in veterinary medicine than mupirocin, might favor their coselection and plasmid maintenance.

In summary, we report a detailed genetic analysis of mupirocin resistance in an *S. pseudintermedius* isolate from a dog. The identification of the conjugative plasmid pKM01, bearing *ileS2* and *aacA-aphD* resistance determinants in *S. pseudintermedius*, is worrisome and could lead to a future greater dissemination of such antibiotic resistances. Thus, veterinarians should be aware of this issue when prescribing mupirocin in pet animals. However, it will be necessary to carry out additional studies to have a complete picture of Hi-Mup^r and associated resistances, as well as its impact on the epidemiology of the global antibiotic resistance of *S. pseudintermedius*.

Nucleotide sequence accession numbers. The nucleotide sequences of the left and right IS*257-ileS2* spacer regions (accession no. JX186508 and JX186509) and a partial sequence of *ileS2* from mupirocin resistance plasmid pKM01 (accession no. JX186510) and partial sequences of the *ileS* gene from strains HR547/11, HR294/11, HR23161, and HR1084/08 (accession no. JX186511, JX186512, JX186513, and JX186514, respectively) were deposited in GenBank.

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