A THESIS FOR THE DEGREE OF MASTER

Low prevalence of mupirocin resistance in *Staphylococcus pseudintermedius* isolates from canine pyoderma

개의 피부 농피증에서 분리한 *Staphylococcus pseudintermedius*균에서 mupirocin 내성의 낮은 유병률

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Low prevalence of mupirocin resistance in *Staphylococcus pseudintermedius* isolates from canine pyoderma

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ABSTRACT

Mupirocin is used as a topical antibiotic against staphylococci, including methicillin-resistant *Staphylococcus pseudintermedius* (MRSP). The recent emergence of resistance to mupirocin is a major concern in many countries. This study investigated the prevalence and genotype of mupirocin-resistant *Staphylococcus pseudintermedius* isolated from pet dogs with pyoderma. A total of 110 clinical isolates of *S. pseudintermedius* were collected from dogs with

pyoderma (n = 110) between July 2010 and September 2016. All animals were client-owned dogs visiting the Veterinary Medical Teaching Hospital of Seoul National University. Low- and high-level mupirocin resistance were evaluated with the broth microdilution and disk diffusion tests. Mupirocin resistant *S. pseudintermedius* was confirmed by genetic analysis of the *ileS-2* and naïve *ileS* gene. MRSP and methicillin-susceptible *S. pseudintermedius* were detected in 69 and 41 dogs, respectively. One MRSP strain was resistant to mupirocin and contained the high-level mupirocin resistance gene *ileS-2*. There were no low-level mupirocin-resistant isolates. Mupirocin is a useful topical antibiotic for *S. pseudintermdius*, but clinical MRSP isolate that have not been previously exposed to mupirocin exhibited the high-level mupirocin resistance in phenotype and genotype. Therefore, continuous monitoring for mupirocin resistance is important in small animal practice.

Keywords : Mupirocin, Antimicrobial susceptibility, *Staphylococcus pseudintermedius*, MRSP, Dog

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CONTENTS

Introduction

Mupirocin (pseudomonic acid A) is a topical bacteriostatic antibiotic derived from *Pseudomonas fluorescens*. [1-5] It competitively binds to the active site of bacterial isoleucyl t-RNA synthetase (IRS) encoded by chromosomal *ileS* gene and thereby inhibits protein synthesis. This drug is highly active against Gram-positive pathogens, especially staphylococci and streptococci. [1] In humans, mupirocin can eradicate nasal *Staphylococcus aureus* infection. [3, 6] Mupirocin resistance in *S. aureus* is classified into two types: low-level mupirocin resistance (Low-Mu^r) is caused by a point mutation in the naïve chromosomal *ileS* gene, whereas high-level mupirocin resistance (High-Mu^r) is caused by the plasmid-encoded *ileS* gene (known as *ileS-2*), which encodes a novel synthetase inducing mupirocin resistant. [7, 8] In terms of minimal inhibitory concentration (MIC), ≤ 4 mg/l is considered susceptible to mupirocin, whereas 8–256 and ≥ 512 mg/l are considered as Low-Mu^r and High-Mu^r, respectively. [9]

The genus *Staphylococcus* comprises Gram-positive bacteria that are normal skin flora in humans and animals. [10, 11] They are typically opportunistic pathogens; *Staphylococcus pseudintermedius* is the primary cause of canine pyoderma. [10, 12, 13] The recent emergence of methicillin-resistant *S. pseudintermedius* (MRSP) is a major concern in veterinary medicine. [14-18] MRSP has a *mecA* gene encoding an altered penicillin binding protein (PBP2a) that confers resistance to β-lactam antibiotics. [16, 19] Since MRSP is resistant

to most multiple drugs, systemic antimicrobial treatment may have limited efficacy; topical medication is considered as a good alternative. [7]

Current information on the prevalence and genotype of mupirocin-resistant *S. pseudintermedius* isolates in companion animals is lacking. [7, 13, 20] So, this issue was addressed in the present study using isolates from pet dogs with pyoderma.

Materials and Methods

1. Sample collection and identification

From July 2010 to September 2016, a total of 110 *S. pseudintermedius* isolates were collected from pet dogs (n = 110) with pyoderma. Dogs showing clinical signs of pyoderma (papules, pustules, erosions, ulcerations, crusts, epidermal collarettes) were selected at first. Impression smear and cotton swabbing for bacterial culture were performed from the dog's skin lesions. If the presence of cocci from typical skin lesion was demonstrated in impression smear and if bacterial pathogens were confirmed as *S. pseudintermedius* by bacterial culture, those dogs are included in this study. Cotton swabs were cultured on to Blood Agar Plates (Hangang, Gyeonggi, Korea) and incubated for 24 hours at 37℃. *S. pseudintermedius* was identified by colony morphology, Gram staining, and with the VITEK II system (bioMérieux, Hazelwood, MO, USA). After identified by the VITEK II system, *S. pseudintermedius* isolates were finally confirmed by PCR amplification of the *nuc* gene. [21] As a result, the 110 cases were confirmed as pyoderma infected with *S. pseudintermedius* (Table 1). Methicillin resistance was confirmed by the disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines and PCR amplification of the *mecA* gene. [22, 23]

Table 1. Description of dogs

Population characteristics	Number(%) of dogs
Sex	
Female	14 (12.7)
Male	10 (9.1)
Spayed female	30 (27.3)
Castrated male	56 (50.9)
Breed	
Maltese	24 (21.8)
Cocker spaniel	20 (18.2)
Shih tzu	14 (12.7)
Yorkshire terrier	11 (10.0)
Poodle	6 (5.5)
Schnauzer	4 (3.6)
Pomeranian	3 (2.7)
Golden retriever	3 (2.7)
Dachshund	3 (2.7)
Others	22 (20.0)
Mupirocin history	
Yes	20 (18.2)
No	90 (78.8)

2. *In vitro* mupirocin susceptibility testing

2.1. Disk diffusion testing

The CLSI M100-S26 recommends using the 200-μg mupirocin disk for disk diffusion method to screen High-Mu^r. [3, 23] On the other hand, a previous study showed that High- and Low-Mu^r could be distinguished using a two-disk diffusion method in *S. aureus*. [24] No zone around 5- and 200-μg disks indicates that the isolate is High-Mu^r, which is similar to CLSI criteria; no zone around the 5-μg disk accompanied by any zone around the 200-μg disk is associated with Low-Mu^r; and any zone around both disks indicates mupirocin susceptibility. [25] In this work, the two-disk diffusion method was used to simultaneously screen for high- and low-level resistance. Mueller-Hinton agar (Hangang, Gyeonggi, Korea) were swabbed in three directions with 0.5 McFarland inocula and 6mm disks containing 5 or 200-μg mupirocin (Oxoid, Basingstoke, UK) were applied. After 24 h of incubation at 37℃, zone diameters for mupirocin were read with transmitted light.

2.2. Broth microdilution testing

The MIC of mupirocin—which is the gold standard for mupirocin resistance—was determined with the broth microdilution test and interpreted according to CLSI guidelines. [23] Mupirocin powder (Sigma-Aldrich, Gyeonggi, Korea) was serially diluted in Mueller Hinton II Broth (Cation-Adjusted) to concentrations ranging from 512 to 0.25 μg/ml.

3. *In vitro* other antimicrobial susceptibility testing

Susceptibility to other antibiotics (penicillin (10 units), oxacillin (1 μg), amikacin (30 μg), gentamicin (10 μg), erythromycin (15 μg), clindamycin (2 μg), tetracycline (30 μg), minocycline (30 μg), ciprofloxacin (5 μg), norfloxacin (10 μg), trimethoprim-sulfamethoxazole (1.25/23.75 μg), chloramphenicol (30 μg), and rifampicin (5 μg)) was evaluated with the disk diffusion method according to CLSI criteria. [23]

4. PCR amplification of plasmid-encoded *ileS-2* gene

NucleoBond Xtra Midi (MACHEREY-NAGEL GmbH & Co. KG, Duren, Germany) was used to extract plasmid DNA from isolates displaying mupirocin resistant in antimicrobial susceptibility testing. The primers mupA (5'-TATATTATGCGATG GAAGGTTGG-3') and mupB (5'-AATAAAATCAGCTGGAAAGTGTTG-3') were used to amplify a 458-bp fragment of the plasmid-encoded *ileS-2* gene. [26] All PCR products were sequenced by using an ABI PRISM 3730xl apparatus (Applied Biosystems, CA, USA) to confirm amplication site.

5. Sequencing of chromosomal *ileS* gene

Genomic DNA was extracted with the InstaGene Matrix (Bio-Rad, Hercules, CA, USA). The chromosomal *ileS* gene from isolates displaying mupirocin resistant in antimicrobial susceptibility testing was sequenced by primer walking method to detect point mutations. Primers were designed to amplify the entire coding sequence of the *ileS* gene and they are shown in Table 2. The 2748-bp *ileS* gene sequence from *S. pseudintermedius* reference strain HKU10-03 (GenBank accession no.: 29755748) was used as a reference. PCR conditions were as follows: 95℃ for 5 min; 35 cycles of 95℃ for 30 s, 60℃ for 30 s, and 72℃ for 1 min; 72℃ for 7 min; and holding at 4℃. PCR products were purified using a Multiscreen filter plate (Millipore, Billerica, MA, USA) and sequenced using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) followed by analysis using Lasergene SeqMan software (DNASTAR, Madison, WI, USA).

Table 2. Primers designed to amplify the entire coding sequence of the *ileS* gene

No.	Primer name	Sequence	PCR band size (bp)
1	ileS_1F	TATCACTGATTCGCCGTGTC	931
	ileS_1R	TTCGGCGATAATGTAACGTG	
2	ileS_2F	TACCACGACAAACGTTCAGC	883
	ileS_2R	TGTTAGAGCCGTGTTGTTCG	
3	ileS_3F	CGAATGGGTCATCTCTCGTC	957
	ileS_3R	CAAACTTCTTCTGCGGTATGC	
4	ileS_4F	CAACAGACCGAATTGCTGAA	1116
	ileS_4R	CTCACCCAACGTCATTTGTG	
5	ileS_5F	AGGTGGCCCATTTGAAGGTA	609
	ileS_5R	CCACGATATTGGTCACTGCC	

Results

Of the 110 *S. pseudintermedius* isolates, 41 were classified as methicillin-susceptible *S. pseudintermedius* (MSSP) and 69 as MRSP. Data for all isolates on in vitro susceptibility to mupirocin and other antibiotics are presented in Table 3. Identical results were obtained for mupirocin resistance using the two-disk diffusion and broth microdilution tests in all 110 isolates; only one MRSP strain (0.9%)—i.e., 15P18—was highly resistant to mupirocin. There weren't any low-level mupirocin resistant isolates founded. The mupirocin resistance results are summarized in Table 4. The MIC of mupirocin for isolate 15P18 was ≥ 512 μg/ml and the diameter of clear zones around the 5- and 200-μg disks was 6 mm. PCR amplification of the plasmid *ileS-2* gene yielded a single band of approximately 450 bp in isolate 15P18, indicating the presence of the High-Mu^{r} gene *ileS-2* (Figure 1). The sequence of PCR product showed 99% similarity to the *ileS-2* gene of *S. aureus* (GeneBank accession number: NG_048008) in match analysis using NCBI BLAST.

Table 3. Antimicrobial resistance of *Staphylococcus pseudintermedius* clinical isolates

Antimicrobial agent (disk content in μg unless otherwise noted)	**No. (%) of resistant strains**
Penicillin (10 U)	106 (96.4)
Oxacillin (1)	73 (66.4)
Amikacin (30)	0 (0.0)
Gentamicin (10)	48 (43.6)
Erythromycin (15)	80 (72.7)
Clindamycin (2)	79 (71.8)
Tetracycline (30)	96 (87.3)
Minocycline (30)	0 (0.0)
Ciprofloxacin (5)	52 (47.3)
Norfloxacin (10)	52 (47.3)
T-sulfa (1.25/23.75)	82 (74.5)
Chloramphenicol (30)	38 (34.5)
Rifampicin (5)	2 (1.8)
Mupirocin (200)	1 (0.9)

Table 4. Classification of mupirocin resistance results in MRSP and MSSP isolates based on phenotypic analysis

Strains		Phenotype					
		5- and 200-μg disk diffusion			MIC (mg/l)		
Oxacillin resistance	No. of isolates	Susceptible	Low-Mu^r	High-Mu^r	≤ 4	8–256	≥ 512
MRSP	69	68	0	1	68	0	1
MSSP	41	41	0	0	41	0	0

MIC: minimum inhibitor concentration; MRSP: methicillin-resistant *S. pseudintermedius*; MSSP: methicillin-susceptible *S. pseudintermedius*

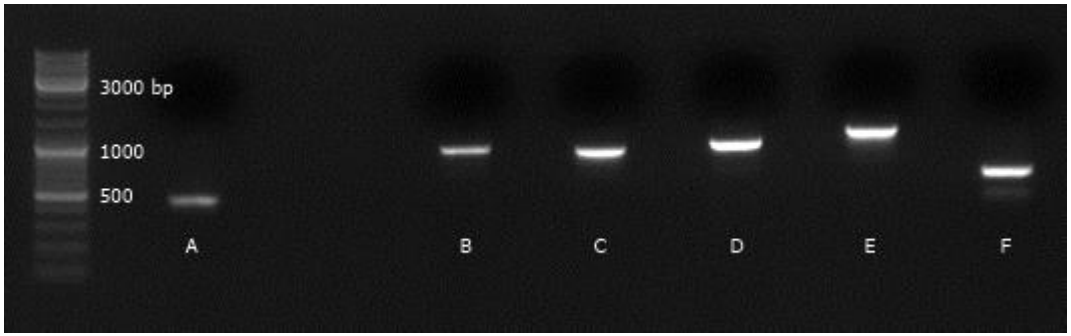

Figure 1. PCR detection of the plasmid-encoded *ileS-2* gene and DNA fragment of the chromosomal *ileS* gene in isolate 15P18.

A: *ileS-2* gene (458 bp), **B–F:** DNA fragment of the *ileS* gene; **B:** *ileS*_1 (931 bp), **C:** *ileS*_2 (883 bp), **D:** *ileS*_3 (957 bp), **E:** *ileS*_4 (1116 bp), **F:** *ileS*_5 (609 bp).

Chromosomal *ileS* gene fragments amplified from isolate 15P18 contained one missense point mutation and six silent mutations comparing with the published sequence of *ileS* gene (*ileS* gene of *Staphylococcus pseudintermedius* HKU10-03, GeneBank accession no. CP002439.1) (Figure 2). The C-to-T transversion at position 2603 (C2603T) causing an amino acid change at codon 868 from alanine to valine (A868V) was detected. In High-Mu^r clinical isolate (15P18), there was no valine-to-phenylalanine mutations at residues 588 (V588F) or 631 (V631F), which are known to the most common mutations that result in Low-Mu^r. [27, 28] The V588F and V631F mutations in isoleucyl t-RNA synthetase (IRS) affect Rossman fold where mupirocin binds. [29]

The high-level resistance isolate (15P18) was obtained from the skin swab of a 10-year-old spayed female cocker spaniel with pyoderma. The skin infection was treated with systemic antibiotics (cephalexin and ciprofloxacin) and topical chlorhexidine gluconate shampoo. This dog had not been previously administered mupirocin. The antibiotic susceptibility tests showed methicillin resistance as well as multidrug resistance (MDR) [30] and susceptibility to amikacin and rifampicin.

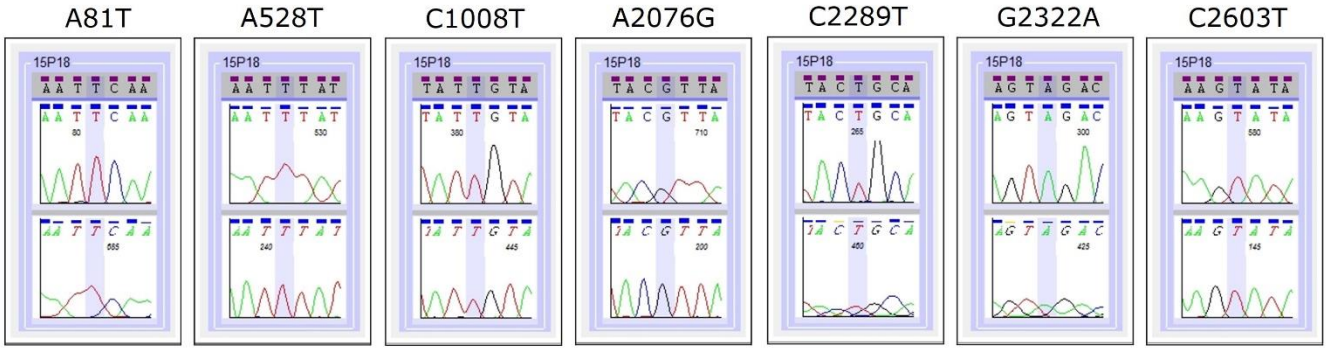

Figure 2. Point mutations in the *ileS* gene region of isolate 15P18.

Comparision of *ileS* sequences of isolate 15P18 with *Staphylococcus pseudintermedius* HKU10-03 (GenBank ID: 29755748) revealed 7 variants; A81T, A528T, C1008T, A2076G, C2289T, G2322A, and C2603T.

Discussion

Topical mupirocin ointment first became available in 1985, [31] and is now the most widely prescribed topical antibiotic for treatment of infection caused by MRSA. [32] However, mupirocin resistance was detected in the UK in 1987 [33] and since then, the resistance rate has been increasing in many countries as mupirocin usage has spread. [4, 34] Low- and high-Mu^r rates were 13.5% and 3.1%, respectively, in Germany [35] and 2.1% and 3.1%, respectively, in Belgium. [36] The prevalence of Low-Mu^r and High-Mu^r has increased from 0.9% to 8.5% and from 8.2% to 9.7%, respectively, in India [37, 38] and from 6.4% to 10.0% and from 1.6% to 7.0%, respectively, in Canada. [39]

In Korea, topical mupirocin ointment has been used for the management of staphylococcal infection in human hospitals since 1994; [34] mupirocin resistance was not detected until 1999 in a human hospital. [40] However, studies carried out in 2003 and 2012 revealed that High-Mu^r and Low-Mu^r *S. aureus* prevalence increased from 4.7% to 5.7% and from 0 to 8.3%, respectively. [34, 41] In this study, the prevalence rate of High-Mu^r *S. pseudintermedius* was 0.9%, which was lower than prevalence rate of High-Mu^r *S. aureus* in above studies but this low rate of resistance could increase with continued use of mupirocin ointment in dogs.

To date, High-Mu^r *S. pseudintermedius* isolated from dogs has only been reported in three countries (Croatia, U.S., and Turkey). [7, 13, 20] These isolates were obtained from normal nares, a post-operative infection site, and

skin infection site. There have been no studies on Low-Mu^r *S. pseudintermedius*. In the present study, High-Mu^r *S. pseudintermedius* was identified in one dog with pyoderma that was infected with MRSP. As in the previous studies, the resistance rate was < 1%. Using the two-disk diffusion method, there was one High-Mu^r and no Low-Mu^r isolate observed, which was supported by the MIC results. Therefore, the two-disk diffusion method used for *S. aureus* is also considered effective for *S. pseudintermedius*.

In previous studies about Low-Mu^r *S. aureus*, point mutations affecting the Rossman fold of isoleucyl t-RNA synthetase (IRS) (V588F and V631F) have been detected. [27, 28] In the present study, the point mutations of *ileS* gene (inducing Low-Mu^r) in High-Mu^r isolate (15P18) were investigated by sequencing chromosomal *ileS* gene. The sequencing result revealed the amino acid change at one site (A868V). This A868V mutation is considered not essential for Low-Mu^r because this residue is far from the site accommodating mupirocin in protein structure. However, a previous study found that the G1762T (V588F) mutation in chromosomal *ileS* gene was also present in a High-Mu^r isolate, [28] and this implying coexistence of high- and low-level resistance. Therefore, further genomic studies about Low-Mu^r in *S. pseudintermedius* is needed.

The plasmid-encoded *ileS-2* gene—which corresponds to High-Mu^r—is located in a mobile genetic element, allowing horizontal movement of the gene between plasmids as well as vertical inheritance. The possibility of transmission of antimicrobial resistance between dogs and their owners has already been suggested for *S. aureus* and *S. pseudintermedius*. [42-44] Thus

studies on mupirocin resistance in veterinary medicine have important implications for public health.

Unlike *S. aureus*, [44, 46] the major mechanism of transmission of antimicrobial resistance genes in *S. pseudintermedius* appears to be via transposons, which exist within chromosomal DNA rather than in plasmids. [12, 47] Since High-Mu^{r} is conferred by the plasmid-encoded *ileS-2* gene, it is presumed that mupirocin resistance rate is lower in *S. pseudintermedius* than in *S. aureus*. However, in this study High-Mu^{r} MRSP isolate was derived from a dog without prior exposure to mupirocin ointment, suggesting that MRSP may have acquired mupirocin resistance via horizontal transfer of the plasmid.

The increasing prevalence of MDR-MRSP in companion animals is a global concern. [48] However, the incidence of mupirocin resistance in MRSP is currently very low, and mupirocin ointment remains an effective treatment option for canine pyoderma with MDR-MRSP infection.

Although only one High Mu^{r}-MRSP strain was detected in this study, a clinical MRSP isolate without previous exposure to mupirocin can exhibit the High-Mu^{r} phenotype and genotype. The fact that the High-Mu^{r} gene (*ileS-2*) in plasmid DNA could be horizontally transferred underscores the importance of continuous monitoring for mupirocin resistance in small animal practice.

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국문 초록

개의 피부 농피증에서 분리한 *Staphylococcus pseudintermedius*균에서 mupirocin 내성의 낮은 유병률

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Mupirocin은 메티실린 저항성 *Staphylococcus pseudintermedius* (methicillin-resistant *Staphylococcus pseudintermedius,* MRSP)를 포함한 Staphylococci 균에 효과적으로 작용하는 국소 항생제이다. 그러나 mupirocin 항생제 사용의 증가에 따라 내성 균주가 발생하기 시작했고 이는 전세계적으로 주요 관심사가 되었다. Mupirocin 내

성은 2가지로 나눌 수 있다; 일반적으로 고도내성 (high-level mupirocin resistance)은 새로운 synthetase를 코딩하는 *ileS-2* 유전자를 세균의 plasmid내에 획득함으로써 나타내는 것이며, 저도내성 (low-level mupirocin resistance)은 chromosome의 *ileS* 유전자의 점돌연변이 (point mutation)에 의해 발생한다.

본 연구는 개의 피부 농피증에서 분리한 *S. pseudintermedius* 균에서 mupirocin 내성의 유병률을 확인하고, 해당 개체에 대해 유전적으로 분석하고자 실시하였다.

총 110개의 *S. pseudintermedius* 균주 (n = 110)를 사용하여 메티실린 내성 여부를 확인하였고, 2-disk diffusion test와 microdilution test를 이용하여 표현형 상으로 mupirocin에 대한 고도내성 혹은 저도내성을 평가하였다. 표현형 상 mupirocin 내성을 보이는 균주에 대해서 *ileS-2* 유전자의 유무를 확인하였고, *ileS* 유전자의 염기서열을 분석하여 변이 여부를 확인하였다. 그 결과, 총 69개의 MRSP 균주와 41개의 메티실린 감수성 *S. pseudintermedius* (methicillin-susceptible *S. pseudintermedius*, MSSP) 균주로 구분되었고, 이 중 단 하나의 MRSP 균주 (0.9%)에서 mupirocin에 대한 고도내성이 확인되었다; 2-disk diffusion test 결과, 5와 200 μg 디스크 주변에 생성된 발육 저지대의 직경은 6mm였으며, microdilution

test로 측정한 minimum inhibitory concentration (MIC)는 ≥ 512 μg/ml 였다. 이 균주에서 고도내성을 나타내는 내성유전자인 *ileS-2*가 확인되었다. 본 연구에서 저도내성 균주는 확인되지 않았다.

Mupirocin은 MRSP로 인한 개의 피부 농피증 치료에 아직까지 임상적으로 유효한 치료 옵션이라 할 수 있다. 그러나 본 연구에서 비록 1개이기는 하나 mupirocin에 대한 고도내성 균주가 확인된 바, 향후 mupirocin 내성율에 대해 지속적인 관찰이 필요하다는 것을 시사하고 있다.

주요단어 : Mupirocin, 항균제 감수성, *Staphylococcus pseudintermedius*, MRSP, 개

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