A THESIS FOR THE DEGREE OF MASTER

Identification of fusidic acid resistance in clinical isolates of *Staphylococcus pseudintermedius* from dogs

개에서 분리한 *Staphylococcus pseudintermedius* 균주들에서의 fusidic acid에 대한 내성 발생 및 내성 발생 기전

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Identification of fusidic acid resistance in clinical isolates of *Staphylococcus pseudintermedius* from dogs

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Abstract

Fusidic acid is a topical steroidal antibiotic that is generally used to treat skin infections in both human and veterinary medicine. Although genetic research and geographic distribution of resistance to the drug in *Staphylococcus aureus* have already been established, only limited data are available regarding the incidence of fusidic acid resistance in animal-derived bacterial strains.

The objective of this study was to evaluate the occurrence and mechanism of fusidic acid resistance in clinical isolates of *Staphylococcus pseudintermedius* from dogs.

A total of 52 clinical isolates of *S. pseudintermedius* were obtained from dogs with pyoderma and otitis between 2017 and 2018 in veterinary teaching hospital of Seoul National University. Fusidic acid resistance was determined by disc diffusion method and MIC value using E test. Isolates showing fusidic acid resistance were performed PCR to detect presence of *fusA, fusB, fusC,* and *fusD.* To detect *fusA* mutation, entire *fusA* gene were further sequenced.

Among 52 clinical isolates, 14 isolates including 1 MSSP and 13 MRSP strains were resistant to fusidic acid. All fusidic acid resistant strains were identified as low level resistance. Among fusidic acid resistant strains, point mutations of *fusA* were confirmed in 11 isolates and amino acid substitutions were detected in 5 isolates at 6 different positions. *fusC gene* was detected in 7 isolates and there was no *fusB* and *fusD*.

This study demonstrated the occurrence and mechanism of fusidic acid resistance in clinical *S. pseudintermedius* from dogs. Continuous monitoring for fusidic acid resistance should be recommended and strategic approach to control antibiotic resistance will be needed in small animal practice.

Key words: Canine, fusidic acid resistance, methicillin resistance, *Staphylococcus pseudintermedius*, *fusA*

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1. Introduction

Staphylococcus pseudintermedius is a major bacterial species associated with canine pyoderma and otitis.[1] With the emergence of meticillin-resistant *S. pseudintermedius*,[2] the recent increase in antimicrobial resistance and the limited treatment options for bacterial infections have attracted attention.[1, 3] Fusidic acid is a steroidal antibiotic derived from the fungus Fusidium coccineum[4] that is used topically for skin infections and systemically for bone and joint infections caused by Gram-positive bacteria, mainly of the *Staphylococcus* genera.[5,6] After preventing the release of elongation factor G (EF-G) from ribosomes, fusidic acid blocks protein synthesis.[7,8] Topical fusidic acid is broadly used in human medicine as well as veterinary medicine. It is approved in Europe, Australia, Canada and Asian countries, including South Korea, Japan, and Thailand, while not yet in the United States.[9] In Korea, fusidic acid is licensed for both topical and systemic use and topical preparations are available for purchase without a doctor's prescription. Fusidic acid resistance in *S. aureus* is relatively higher in Korea than in other Asian countries.[6]

Resistance to fusidic acid can be categorized into two types: low level resistance with minimum inhibitory concentrations (MICs) of 2–32 μg/ml

and high level resistance with MICs > 128 μg/ml.[10] Fusidic acid resistance is generally caused by two major genetic mechanisms. One mechanism involves a change in the drug target position mediated by point mutations associated with amino acid substitution in *fusA* encoding EF-G.[11, 12] Various amino acid substitutions in the protein product of *fusA* have been reported in fusidic acid resistant *S. aureus*, some of which result in high level resistance.[12, 13] Domain III (amino acids 404–483) is the most common location of these mutations while mutations in domain I (amino acids1–280) and V (amino acids 606–693) have also been reported.[8] The second mechanism is protection of the drug target site via the acquisition of fusB family genes *(fusB, fusC,* and *fusD*) that encode cytoplasmic proteins.[14, 15]

Given that fusidic acid has been used in human medicine for more than six decades, genetic research and the geographic distribution of resistance to this drug in *S. aureus* have already been globally established.[16] Despite the broad use of fusidic acid for veterinary purposes, only limited data are available regarding the incidence of fusidic acid resistance in animal derived bacterial strains.[17,18,19] Although fusidic acid resistance in *S. pseudintermedius* has been reported in European countries and the United States, there is limited data in Asia.[19] The purpose of this study was to evaluate the occurrence and mechanisms of fusidic acid resistance in clinical

S. pseudintermedius isolates from dogs.

2. Materials and Methods

2.1. Bacterial isolates

From January 2017 to December 2018, fifty-two *S. pseudintermedius* isolates were identified by the Seoul National University Veterinary Teaching Hospital in Korea. The isolates were collected from client-owned dogs with pyoderma (36 of 52, 69%) or otitis externa (16 of 52, 31%),which were referred cases from local veterinary clinics. After cytological examination confirming the presence of coccoid bacteria, the samples were collected from skin lesions or ear exudate using sterile cotton swabs and transported to the laboratory in Amies transport medium (Yuhan Labtech, Seoul, Korea). Each isolate was identified by colony type and morphology, Gram staining and biochemical testing using the VITEK 2 system (BioMerieux; Hazelwood, MO, USA). PCR amplification of the thermonuclease gene was performed to identify Staphylococcus species.[20]

2.2. Antimicrobial susceptibility testing

Resistance to fusidic acid was initially evaluated using the disk diffusion method with a 10μg fusidic acid disk; according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines for human

isolates, an inhibition zone of less than 24 mm in diameter indicates fusidic acid resistance.[21] Susceptibility of isolates to other antimicrobial drugs, such as penicillin, oxacillin, gentamicin, amikacin, ciprofloxacin, ofloxacin, tetracycline, clindamycin, erythromycin, trimethoprim–sulfamethoxazole and mupirocin was testedaccording to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[22] Meticillin resistance in *S. pseudintermedius* was confirmed using the oxacillin disk diffusion method according to CLSI guidelines,[22] and the presence of *mecA* was confirmed using PCR amplification.[23] The MICs of the isolates showing resistance to fusidic acid in the disk diffusion method were tested using the E–test (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions. Briefly, E test strips were placed on Muller–Hinton agar with inocula of bacterial suspensions with a McFarland turbidity of 0.5. After incubation for 18–24 h at 37°C, the MICs were determined as the concentration at which bacterial growth was inhibited by 80%. MICs of ≥ 2 μg/ml indicated resistance to fusidic acid using the EUCAST resistance breakpoint.[21]

2.3. Detection of fusidic acid resistance-related genes (fusA, fusB, fusC,and fusD)

All *S. pseudintermedius* isolates displaying zone diametersof < 24 mm and MICs ≥ 2 μg/mlwere tested using PCR for the presence of *fusA*,*fusB*,

*fusC*and *fusD*. Genomic DNA was extracted using InstaGene Matrix (Bio-Rad, Hercules, CA, USA). The PCR conditions and oligonucleotides used in this study are listed in Table 1,[20, 23, 24] and target band products were visualized using agarose gel electrophoresis.

DNA sequencing of fusA was performed using an ABI Prism 3730XL analyzer and a BigDye® Terminator v3.1 Cycle Sequencing Kit. The nucleotide seqences were amplified and compared with the published sequences of *fusA* from *S. peudintermedius* HKU 10-03 (GeneBank accession number: CP002439.1) based on BLAST (http://www.ncbi.nlm.nih,gov/blast/).

3. Results

3.1. Bacterial strains and antibiotics susceptibility testing

The phenotypic results of fusidic acid-resistant isolates are listed in Table 2. Among the 52 clinical isolates, 40 were meticillin-resistant. Fusidic acid resistance was detected in 14 isolates, 13 of which were also meticillin-resistant. All fusidic acid-resistant isolates had MICs of 2–16 μg/ml, indicating low level resistance.

3.2. Identification of fusidic acid resistance-related genes

Among the 14 fusidic acid resistant isolates *fusA, fusB, fusC,* and *fusD* were carried by 11, 0, 7 and 0 isolates, respectively. Chromosomal *fusA* was amplified and compared with the previously reported sequence of *S. pseudintermedius* HKU10-03. Point mutations, including silent mutations and amino substitutions were detected in all 11 *fusA* positive isolates and amino acid substitutions were detected in five isolates. Six different amino acid substitutions were identified at six different positions. Single amino acid substitution was found in three isolates, whereas two isolates had double amino acid substitutions. A substitution of valine with isoleucine at position 90 (V90I) was observed in two isolates; substitutions of alanine

with valine at position 376 (A376V), proline with leucine at position 404 (P404L), isoleucine with threonine at position 461 (I461T), isoleucine with tyrosine at position 61 (I61Y) and threonine with serine at position 62 (T62S) were each detected once. Domain I was the most common site of amino acid substitutions, followed by domains III and II. PCR using primers for *fusB*, *fusC,* and *fusD* illustrated that none of the fusidic acid-resistant strains harboured *fusB* or *fusD*, whereas *fusC* was detected in seven isolates. However, three fusidic acid resistant isolates (FU17-2, FU17-5 and FU18-2) did not show known resistance mechanisms, which were point mutations associated with amino acid substitution or harbouring of fusB family genes.

4. Discussion

In the present study, 40 of 52 *S. pseudintermedius* isolates were resistant to meticillin and 14 were resistant to fusidic acid. In accordance with a prior report of the prevalence of fusidic acid resistance in *S. aureus*,[8] we have documented that fusidic acid resistance in more common in meticillin-resistant isolates.

Fusidic acid resistance has been reported since the drug was introduced in the 1960s and the incidence of resistance to the drug has continuously increased.[11] The prevalence of fusidic acid resistance in *S. aureus* varies by geographic distribution from 0.3% to 52.5%.[16,24,25] In Korea, among 482*S.aureus* isolates collected from skin wounds in a Korean tertiary hospital between 2009 and 2011, 48.3% were meticillin-resistant and 45.9% were fusidic acid resistant.[26] Notably, 4.8% were resistant to both fusidic acid and mupirocin.[26] In this study conducted in the Seoul National University Veterinary Teaching Hospital in Korea, the prevalence of fusidic acid resistance in *S. pseudintermedius* was 27%. Because fusidic acid is commonly used for treating canine skin infections, the prevalence of resistance should be monitored in order to inform judicious empirical use; especially because a positive correlation between fusidic acid use and increased resistance in *S. aureus* in human medicine has been

demonstrated.[27]

This study identified *fusA* point mutations associated with amino substitution and carriage of *fusC* as the predominant mechanisms of fusidic acid resistance. Compared with a previous study that reported that *fusA* polymorphisms in *S. aureus* occurred most commonly in domain III,[8] our results indicated that amino acid substitutions in *S. pseudintermedius*most frequently occurred in domain I, followed by domains III and II. In addition, amino acid substitutions from valine to isoleucine at codon 90 (V90I) and from alanine to valine at codon 376 (A376V) have been reported in *S. pseudintermedius* and *S. aureus*,[19, 28] and from proline to lysine at codon 404 (P404L) in *S. aureus*.[24] The I461T mutation has been reported previously in *S. pseudintermedius*,[19] but the I61Y and T62S substitutions are reported for the first time in the study reported here.

A previous report on *S. aureus* found that high level fusidic acid resistance is caused by amino acid changes including L461K and H457Y of *fusA*, whereas low level resistance arose from the acquisition of *fusB, fusC,* and *fusD*, which is referred to as acquired resistance.[28] One study also reported the amino acid substitutions of *fusA* in *S. pseudintermedius* isolates showing high level resistance.[19] However, in the present study, we investigated *fusA* mutationsin all resistant isolates and found no high level

resistance *fusA* mutation; only those associated with relatively low level resistance to fusidic acid. This implies that low level resistance can also arise from amino acid substitutions in *fusA*. Furthermore, multiple silent mutations were found in our *fusA*-positive isolates, but the role of these mutations remains to be identified. Despite several efforts to amplify *fusA* it was not identified in the isolates (FU17-5, FU18-3 and FU18-7). This could be because of one or more mutations have occurred at the site of attachment of the designed primer.

Among the acquired resistance mechanisms, *fusC* was previously detected in *S. pseudintermedius* and was associated with low level resistance.[12,19,28] Similarly, *fusC* was the predominant resistance mechanism found in our study and all isolates harbouring *fusC* showed low level resistance. The presence of the plasmid mediated resistance of fusB family genes indicated possible horizontal as well as vertical movement of genetic elements. Genetic transfer of antimicrobial resistance between humans and dogs has been documented in Staphylococcus spp.,[29,30]and implies that monitoring for fusidic acid resistance in veterinary medicine is also important for public health.

In one isolate, the combination of *fusA* point mutations associated with amino acid substitutions and harbouring of *fusC* were detected in the same

strain and this is a novel finding in *S. pseudintermedius*. The MIC value of this strain was 12 μg/mL and there was no significant increase in MIC compared with other isolates having a single resistance mechanism. The lack of an increase in the MICs in strains with multiple resistance mechanisms suggested that the combination of resistance mechanisms provides no distinct benefit to survival and antibiotic resistance in the bacterium; consequently, these combinations were rarely observed.[14, 31] Moreover, another study revealed the presence of *fusC* in strains susceptible to fusidic acid.[19] Therefore, further research is needed on *fus* gene screening among fusidic acid susceptible isolates. The absence of a known resistance mechanism in three of our resistant isolates suggests the existence of other resistance mechanisms. Therefore, further studies are required to identify other possible resistance mechanisms such as the *rplF* mutation (also known as the *fusE* mutation) which has been rarely reported in *S. aureus* and has not yet been reported in *S. pseudintermedius*.[13,19,25]

All 14 fusidic acid resistant isolates in this study showed low level resistance. Even so, a previous study on skin concentration after administration of fusidic acid in canine models revealed that the concentrations in the skin are about 1,000 times higher than the break point of the EUCAST, based on its systemic use.[32] This indicates that skin concentrations after using fusidic acid may easily overcome resistant

isolates expressing low level resistance. Considering our findings, there is a need for practical criteria for antimicrobial susceptibility testing, which can be applied clinically to topical antimicrobial preparations.

Limitations of this study include the small sample size and lack of *fus* gene screening of fusidic acid susceptible isolates. Since this study was conducted in a veterinary teaching hospital in Korea, most of bacterial isolates were obtained from cases with chronic or recurrent infection. In order to estimate the prevalence of fusidic acid resistance among *S. pseudintermedius* isolates from the general population of dogs in Korea, a wider epidemiological study is needed.

5. Conclusions

In conclusion, this study has demonstrated that fusidic acid resistance is a common phenomenon among canine *S. pseudintermedius* isolates in a Korean veterinary teaching hospital and has defined some of the genetic mechanisms of resistance. These findings suggests that more extensive epidemiological studies are needed to define the prevalence of fusidic acid resistance in staphylococci carried by the general canine population because the drug is commonly used in both human and veterinary healthcare with implications of drug resistance for both canine and human health.

Table 1. Oligonucleotides used as primers for PCR and sequencing analysis in this study

Primer name	Sequence (5'→3')	Product size (bp)	Target gene	Reference
nucSP-F	TRG GCA GTA GGA TTC GTT AA	926	*nuc*	20
nucSP-R	CTT TTG TGC TYC MTT TTG G			
mecA-F	GTA GAA ATG ACT GAA CGT CCG ATA A	310	*mecA*	23
mecA-R	CCA ATT CCA CAT TGT TTC GGT CTA A			
fusA-1F	CTC GTA AYA TCG GTA TCA TG	2002	*fusA*	24
fusA-1R	GCA TAG TGA TCG AAG TAC			
fusB-1F	TCA TAT AGA TGA CGA TAT TG	496	*fusB*	
fusB-1R	ACA ATG AAT GCT ATC TCG AC			
fusC-1F	GAT ATT GAT ATC TCG GAC TT	128	*fusC*	
fusC-1R	AGT TGA CTT GAT GAA GGT AT			
fusD-1F	TGC TTA TAA TTC GGT CAA CG	525	*fusD*	
fusD-1R	TGG TTA CAT AAT GTG CTA TC			
fusA_seq1	TAA GGG TCA GTC ATA ACT TT	Sequencing only	-	
fusA_seq2	TTC AAA AAC AAA GGT GTT CA		-	
fusA_seq3	ATG TAT TCA CGA GGA AC		-	

Table2. Characterization of fusidic acid-resistant *Staphylococcus pseudintermedius*

Isolate	Sampling date	Source	Methicillin-resistant	Fusidic acid resistance		Resistant mechanism	Mutation in *fusA*		Co-resistant antibiotics
				Disk diffusion diameter (mm)	MIC (μg/ml)		Amino acid substitution (domain)	Silent mutation	
FU17-1	2017	Skin	MRSP	14	6	*fusA*	V90I (domain Ⅰ)	+	P, OX, CN, TE, SXT
FU17-2	2017	Skin	MRSP	8	8	-	-	+	P, OX, CN, TE, CIP, OFX
FU17-3	2017	Skin	MSSP	10	8	*fusA*	A376V/ P404L (domain Ⅱ, Ⅲ)	+	P, CN, TE, CIP, OFX, DA, ERY, SXT
FU17-4	2017	Skin	MRSP	16	4	*fusA*	I461T (domain Ⅲ)	+	P, OX, CN, TE, CIP, OFX, DA, SXT
FU17-5	2017	Skin	MRSP	10	2	-			P, OX, CN, TE, SXT
FU18-2	2018	Ear	MRSP	8	6	-	-	+	P, OX, CN, TE, CIP, OFX
FU18-3	2018	Skin	MRSP	9	8	*fusC*			P, OX, CN, TE, CIP, OFX, SXT
FU18-4	2018	Skin	MRSP	10	8	*fusC*	-	+	P, OX, CN, TE, CIP, OFX, SXT
FU18-5	2018	Skin	MRSP	10	8	*fusC*	-	+	P, OX, CN, TE, CIP, OFX, SXT
FU18-6	2018	Skin	MRSP	16	4	*fusA*	V90I (domain Ⅰ)	+	P, OX, CN, TE, CIP, OFX, ERY, SXT, MUP
FU18-7	2018	Ear	MRSP	9	12	*fusC*			P, OX, CN,TE, CIP, OFX, ERY, SXT
FU18-8	2018	Skin	MRSP	11	16	*fusC*	-	+	P, OX, CN, TE, CIP, OFX, DA, ERY, SXT
FU18-9	2018	Skin	MRSP	10	8	*fusC*	-	+	P, OX, CN, TE, CIP, OFX, DA, ERY, SXT
FU18-10	2018	Ear	MRSP	11	12	*fusA*, *fusC*	I61Y/ T62S (domain Ⅰ)	+	P, OX, CN, TE, CIP, OFX, DA, ERY, SXT

P, penicillin; OX, oxacillin; CN, gentamicin; AK, amikacin; TE, tetracycline; CIP, ciprofloxacin; OFX, ofloxacin; DA, clindamycin; ERY, erythromycin; SXT, trimethoprim sulfamexothazole; MUP, mupirocin

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국문초록

개에서 분리한 *Staphylococcus pseudintermedius* 균주들에서의 fusidic acid에 대한 내성 발생 및 내성 발생 기전

지도교수: 황철용

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Fusidic acid는 인의 및 수의학 분야에서 피부 질환을 치료하는 국소 항생제로 널리 사용되어 왔다. 사람의 피부에서 분리한 *Staphylococcus aureus*에서는 fusidic acid 내성과 관련된 발생율 및 내성 발생 기전이 널리 연구되어 있다. 수의학 분야에서도 국소 항생제로서 현재 다양하게 사용되고 있으나 내성 발생에 대한 연구가 부족한 실정이다. 그렇기에 본 학위논문은 2017년에서

2018년에 개의 피부 및 귀에서 분리한 *Staphylococcus pseudintermedius* 균주들에서의 fusidic acid에 대한 내성 발생 및 내성 발생 기전을 연구함에 있다.

2017년과 2018년 사이에 농피증과 외이염이 있는 개의 피부와 귀에서 분리된 52개의 *S. pseudintermedius* 균주들을 대상으로 하였으며 디스크 확산법 (Disc diffusion test) 및 E-test를 이용하여 내성을 확인하였다. 내성이 확인된 균들에 대해 이미 연구가 진행된*S. aureus*에서의 fusidic acid 내성과 관련된 유전자인 *fusA, fusB, fusC* 그리고 *fusD*의 유무를 중합 효소 연쇄 반응 (Polymerase chain reaction)를 통해서 확인하였으며 *fusA*유전자는 추가적으로 유전자배열을 확인하여 점 돌연변이 여부 및 그에 따른 아미노산 변화를 확인했다.

전체 52개의 *S. pseudintermedus* 균주들에서 14개 (27%)가 fusidic acid에 내성이 있는 것으로 확인 되었으며 이 중 한 개는 메치실린에 내성이 없고 나머지 13개는 메치실린에 내성이 있는 것으로 확인되었다. Fusidic acid에 대한 내성이 확인된 모든 균주들의 MIC 범위는 2-16 μg/mL 사이로 낮은 정도의 내성이 있는 것으로 확인되었다. 이중에서 *fusA*의 점 돌연변이는 11개의 균주들에서 확인 되었고 아미노산 변화는 5개에서 6개소의 다른 부위

에서 확인되었다. *fusB*와 *fusD*유전자는 확인되지 않았고 *fusC*유전자만 7개의 균주들에서 확인되었다.

이 결과는 소동물 임상에서 2017년에서 2018년 사이에 피부 및 귀에서 분리된 *S. pseudintermedius* 균들에서의 fusidic acid 내성율이 14% 정도이며 이와 관련하여 내성획득은 *fusA*유전자의 점 돌연변이로 인한 아미노산 변화 그리고 *fusC*유전자의 획득이 주요하다는 사실을 말해주고 있다. 이는 소동물 임상에서 항생제 내성을 줄이기 위한 전략적인 접근의 중요성을 시사하고 있다.

주요어: 개; fusidic acid 내성; 메치실린 내성; 포도상 구균; *fusA*

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