이학석사 학위논문

Association with Fusidic Acid Resistant Determinants of *Staphylococcus aureus* with Molecular Epidemiological types

Staphylococcus aureus의 fusidic acid 내성 유전자형과 분자역학적 형별의 연관성

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김 민 정

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이 논문을 이학석사 학위 논문으로 제출함

2020년 2월

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Abstract

Fusidic acid is an antibiotic that has been used for the treatment of skin and soft tissue infection or bone and joint infection caused by gram-positive organisms including *Staphylococcus aureus*. Fusidic acid works by interfering with bacterial protein synthesis, specifically by preventing the translocation of the elongation factor G [1)] from the ribosome. Major fusidic acid resistance determinants are an alteration of the binding site (*fusA* or *fusE=rlpF*) or acquired fusidic acid resistance (*fusB*, *fusC*, and *fusD*). The prevalence of fusidic acid resistance in methicillin-susceptible S. aureus (MSSA) and methicillin-resistant S. aureus (MRSA) were stratified by sequence type (ST) and *spa* type. The breakpoint of fusidic acid resistance was 16 μg/ml in this panel, however, fusidic acid MIC values ≥ 2 μg/ml is considered to be resistant according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints. There were two types of fusidic acid resistance, Low level- resistance (2 < MIC ≤ 32 μg/mL) and high level-resistance (MIC ≥ 64 μg/mL). In addition, The prevalence of fusidic acid resistance mechanisms was aimed to determine. Also, The distribution of fusidic acid resistance determinants was examed. From 2014 to 2018, *S. aureus* bloodstream isolates were collected in Asan Medical Center, Seoul, Republic of Korea. Isolates were examined for the presence of resistance determinants and examined for *fusA* mutation and *fusE* mutation. A total of 550 *S. aureus* (236 MRSA and 314 MSSA) isolates, the rate of fusidic acid resistance was 41% in MRSA and 37% in MSSA. Of these, 90 of 119 ST5-MRSA isolates were resistant to fusidic acid, whereas 43 of 52 ST72-MSSA were resistant to fusidic acid. substitutions in EF-G were detected, L461K alteration was predominant and only 4 isolates showed other single amino acid substitutions (V90I, N463S, P404L, and R483C). In this study, fusidic acid resistance was dominant in ST5-MRSA and ST72-MSSA with the predominance of *fusA* mutation in high resistance. Also, fusidic acid resistance was common in ST1-MSSA with the predominance of *fusC* acquisition in low resistance. In this study, ST5-MRSA- II-t2460 was a predominant clone, accounting for 43 % (39/90) of fusidic acid-resistant ST5-MRSA isolates. Secondly, ST5-MRSA- II-t9353 one of major, accounting for 13% (12/90) of fusidic acid-

resistant ST5-MRSA isolates. Among 4 fusidic acid resistance, MRSA isolates, 4 belonged to ST5-MRSA-II-t002 clones carrying *fusC* with low-level fusidic acid resistance. In this study, The prevalence of fusidic acid resistance in MSSA and MRSA stratified by ST and *spa* type was investigated. In addition, The prevalence was aimed to estimate of fusidic acid resistance determinants.

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Introduction

Widespread development of antimicrobial resistance by major bacterial pathogens has emerged in global medical interests including *S. aureus*. Antimicrobial resistance was first identified during the 1940s and 50s with penicillin resistance in *S. aureus*. In the 1970s and 1980s, MRSA rapidly appeared in major nosocomial pathogens in hospitals around the world. Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections have emerged worldwide since the 1990s, but the dominant clone varies with a geographic lesion[1)]. In USA, Panton Valentine leukocidin (PVL)-positive sequence type (ST)8 SCC*mec* type IVa (USA300) is the predominant CA-MRSA clone[1, 2)]. In Korea, Panton-Valentine leukocidin (PVL)-negative ST72 SCC*mec* type IVc is the predominant CA-MRSA clone and ST5-SCC*mec* type II is the representative hospital-associated MRSA (HA-MRSA) clone[3-5)]. The origin of the Korean CA-MRSA (ST72 SCC*mec* IV) has not yet been elucidated. Fusidic acid is an antibiotic that has been used for the treatment of skin and soft tissue infection or bone and joint infection caused by gram-positive organisms including *S. aureus*[6)]. Recently, Fusidic acid-resistant *S. aureus* has been reported in Europe, North America, Australia, New Zealand and Taiwan, and the prevalence of fusidic acid resistance ranged from 1.4% to 52.5%[7-11)]. Major fusidic acid resistance determinants are alteration of the binding site (f*usA* or *rlpF)* or acquired fusidic acid resistance (*fusB, fusC* and *fusD*)[6)]. In *staphylococci*, high-level fusidic acid resistance is usually associated with mutations in *fusA* encoding EF-G, while low-level resistance is generally caused by the horizontally transferable genes including *fusB, fusC* and *fusD*[12)]. In this study, The prevalence of fusidic acid resistance in MSSA and MRSA stratified by ST and *spa* type was investigated. In addition, The prevalence was aimed to estimate of fusidic acid resistance determinants.

Materials and Methods

1. Isolation of Bacteria

A total of 550 non-duplicated *S. aureus* (236 MRSA and 314 MSSA) clinical isolates from patients were collected from 2014 to 2018, *S. aureus* bloodstream isolates from adult patients (≥ 16 years old) were collected in Asan Medical Center, a 2,700-bed tertiary referral center in Seoul, Republic of Korea. Initial screening of fusidic acid resistance was performed by MicroScan PBC 28 panel (Simens, West Sacramento, CA). The breakpoint of fusidic acid resistance was 16 μg/ml in this panel. Genomic DNA was extracted using a Labopass genomic DNA extraction kit (Cosmogenetech®, Seoul, Korea) stored at −20 °C and prepared for PCR assays. All isolates were classified into MRSA and MSSA according to the presence or absence of the *mec* gene by PCR. The PCR conditions were as follows: 30 cycles each of denaturation at 94° C for 30 seconds, 55°C for 30 seconds, and 72°C 30 seconds and the final extension at 72°C for 5 minutes. The sequence information of each primer was as followed by table 1[13]. All isolates were grown in the Blood agar plate (BAP) at 37 . Stock cultures were stored at -80 in 20 % glycerol until tested.

2. Staphylococcal chromosomal cassette *mec (*SCC*mec)* typing

This study elucidated the complete structure of three major *mec* elements, also referred to as the staphylococcal chromosomal cassette (SCC*mec*). SCC*mec* is composed of CCR complex type and mec complex type and SCC*mec* is divided into four types. The multiplex PCR includes eight loci (A through H) selected on the basis of the previously described mec element sequences (Table 2). Genetic analysis was performed using the Muti-PCR method. Type I (34 kb) was identified in the first MRSA strain isolated in 1961 in the United Kingdom (strain NCTC10442), type II (52 kb) was identified in an MRSA strain isolated in 1982 in Japan (strain N315), and type III (66 kb) was identified in an MRSA strain isolated in 1985 in New Zealand (strain 82/2082)(14, 15). Moreover, a smaller fourth *mec* element, SCC*mec* type IV (20 to 24 kb). The PCR conditions were as follows: 30 cycles each of denaturation at 94° C for 30 seconds, 53°C for 30 seconds, and 72°C for 1minute and the final extension at 72°C for 4 minutes. The sequence information of each primer was as followed by table 2.

3. Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) is a sequence-based genotyping method[1]. MLST analyzed the base sequences of seven housekeeping genes of *Staphylococcus aureus*, carbamate kinase (*arcC*), shikimate dehydrogenes e (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferas (*pta*), triosephophate isomerase (*tpi*), acetyl coenzyme A acetyltransferase (*yqi*), and determined the MLST sequence type(ST) for each bacterial isolate using MLST database(http://www.mlst.net). The PCR conditions were as follows: 30 cycles each of denaturation at 94° C for 30 seconds, 53°C for 30 seconds, and 72°C for 1minute and the final extension at 72°C for 5 minutes. The sequence information of each primer was as followed by table 3.

4. *spa* typing

Spa typing is based upon sequence analysis of variable number tandem repeats in the gene encoding protein A (*Spa*)[(1)]. Each new base composition of the polymorphic repeat found in a strain is assigned a unique repeat code. The repeat succession for a given strain determines its *spa* type. This method is Suitable for *S. aureus* outbreak investigation worldwide. The *spa* types were assigned through the use of BioNumerics software version 7.6.2 (Applied Maths, Sint-Martens-Latem, Belgium). The PCR conditions were as follows: 30 cycles each of denaturation at 94° C for 30 seconds, 53°C for 30 seconds, and 72°C minute and the final extension at 72°C for 4 minutes. The sequence information of each primer was as followed by table 4.

5. Antibiotic Susceptibility Test and Resistance Investigation

In vitro susceptibilities to fusidic acid was evaluated in triplicate by the broth microdilution reference method as described in the Clinical and Laboratory Standards Institute (CLSI) guidelines. Fusidic acid was dissolved in the specified solvent and added to sterilized Muller-Hinton Ⅱ medium (BD, Franklin Lakes, NJ, USA) to make a mixture of the desired antibacterial agent concentration. The tested isolates were suspended in tryptic soy broth (BD, Franklin Lakes, NJ, USA) to adjust the turbidity to 0.5 McFarland standard and diluted again to 1/10. The results were read out based on the defined method after culturing at 37° C for 24

hours. Fusidic acid Minimum inhibitory concentration (MIC) values ≥ 2 μg/ml is considered to be resistant according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints. *S. aureus* ATCC 29213 was used as a reference strain. All QC results were published in CLSI documents.

6. Mutations in *fusA* and *fusE*.

All strains with fusidic acid MIC values of ≥ 2 μg/ml, which were resistant according to the EUCAST breakpoints. PCR was performed using the genomic DNA of each strain as a template, and the site corresponding to each base pair in the *fusA* gene using *fusA*-1 (F, R) *fusA*-2 (F, R) primers and in the *fusE* gene using *fusE* (F, R) primer. Amplification products of 1510, 1259 and 696 bp were produced with specific oligonucleotides designed for the detection of *fusA-1*, *fusA-2*, and *fusE*, respectively (Fig.1 and Fig.2). PCR conditions using *fusA-1,2* primers and *fusE* were as follows.: 30 cycles each of denaturation at 95° C for 1 minute, 50°C for 1 minute, and 72°C 1 minute and the final extension at 72°C for 7 minutes. The sequence information of each primer was as followed by table 5. Thereafter, the *fusA* resistance gene was confirmed after electrophoresis using 1.2% agarose gel. The nucleotide sequences and deduced amino acid sequences were compared with sequences available through the Internet using BLAST (http://www.ncbi. nlm.nih.gov/blast/).

7. Detection of fusidic acid acquired resistance.

All strains with fusidic acid MIC values of ≥ 2 μg/ml, which were resistant according to the EUCAST breakpoints. The presence of *fusB*, *fusC*, and *fusD* was tested by PCR. Amplification products of 292, 128 and 245 bp were produced with specific oligonucleotides designed for the detection of *fusB*, *fusC* , and *fusD*, respectively (Fig.3). PCR conditions were as follows: 30 cycles each of denaturation at 95° C for 1 minute, annealing (30 s; at 47°C for *fusC*, 49°C for *fusB* and *fusD*), and 72°C 1 minute and the final extension at 72°C for 7 minutes. PCR products were separated by electrophoresis on 1.2% agarose gels. sequence information of each primer was as followed by table 5. Thereafter, the *fusA* resistance gene was confirmed after electrophoresis using 1.2% agarose gel.

Table 1. Primers used for amplification of the *mecA* genes from *S. aureus*.

Gene	Primer	Primer sequence	Amplicon size(bp)
mec	*mecA*-F	5'-AAA ATC GAT GGT AAA GGT TGG C-3'	532
	mecA-R	5'-AGT TCT GCA GTA CCG GAT TTG C-3'	

Table 2. Primers used in the multiplex PCR for SCC*mec* typing

Locus	Primer	Primer sequence	Amplicon size(bp)	SCC*mec* type
A	CIF2 F2	5'-TTCGAGTTGCTGATGAAGAAGG-3'	495	I
	CIF2 R2	5'-ATTTACCACAAGGACTACCAGC-3'		
B	KDP F1	5'-AATCATCTGCCATTGGTGATGC-3'	284	II
	KDP R1	5'-CGAATGAAGTGAAAGAAAGTGG-3'		
C	MECI P2	5'-ATCAAGACTTGCATTCAGGC-3'	209	II, III
	MECI P3	5'-GCGGTTTCAATTCACTTGTC-3'		
D	DCS F2	5'-CATCCTATGATAGCTTGGTC-3'	342	I, II, IV
	DCS R1	5'-CTAAATCATAGCCATGACCG-3'		
E	RIF4 F3	5'-GTGATTGTTCGAGATATGTGG-3'	243	III
	RIF4 R9	5'-CGCTTTATCTGTATCTATCGC-3'		
F	RIF5 F10	5'-TTCTTAAGTACACGCTGAATCG-3'	414	III
	RIF5 R13	5'-GTCACAGTAATTCCATCAATGC-3'		
G	IS431 P4	5'-CAGGTCTCTTCAGATCTACG -3'	381	
	pUB110 R1	5'-GAGCCATAAACACCAATAGCC-3'		
H	IS431 P4	5'-CAGGTCTCTTCAGATCTACG-3'	303	
	pT181 R1	5'-GAAGAATGGGGAAAGCTTCAC-3'		

Table 3. Primers used in PCR for Multilocus sequence typing (MLST).

Locus	Primer	Primer sequence	Amplicon size(bp)
arc	*arc*-F	5'-TTG ATT CAC CAG CGC GTA TTG TC -3'	456
	arc-R	5'-AGG TAT CTG CTT CAA TCA GCG -3'	
aro	*aro*-F	5'-ATC GGA AAT CCT ATT TCA CAT TC -3'	456
	aro-R	5'-GGT GTT GTA TTA ATA ACG ATA TC -3'	
glp	*glp*-F	5'-CTA GGA ACT GCA ATC TTA ATC C -3'	465
	glp-R	5'-TGG TAA AAT CGC ATG TCC AAT TC -3'	
gmk	*gmk*-F	5'-ATC GTT TTA TCG GGA CCA TC -3'	429
	gmk-R	5'-TCA TTA ACT ACA ACG TAA TCG TA -3'	
pta	*pta*-F	5'-GTT AAA ATC GTA TTA CCT GAA GG -3'	474
	pta-R	5'-GAC CCT TTT GTT GAA AAG CTT AA -3'	
tpi	*tpi*-F	5'-TCG TTC ATT CTG AAC GTC GTG AA -3'	402
	tpi-R	5'-TTT GCA CCT TCT AAC AAT TGT AC -3'	
yqi	*yqi*-F	5'-CAG CAT ACA GGA CAC CTA TTG GC -3'	516
	yqi-R	5'-CGT TGA GGA ATC GAT ACT GGA AC -3'	

Tabel 4. Primers used for amplification of the *spa* genes from *S. aureus.*

Gene	Primer	Primer sequence	Amplicon size(bp)
spa	*spa*-F	5'- ACG GCA TCC TTC GGT GAG C -3'	532
	spa-R	5'- GCT TTT GCA ATG TCA TTT ACT G-3'	

Table 5. Primers used for amplification of the *fusA-E* genes from *S. aureus*.

Gene	Primer	Primer sequence	Amplicon size(bp)
fusA	*fusA1*-F	5'-ACGATGGAAGATCGTTTAGC-3'	1510
	fusA1-R	5'-TGGTCAGCTTTAGATTTTGGC-3'	
	fusA2-F	5'-AGGTACAATGACATCTGGTTC-3'	1259
	fusA2-R	5'-TCTCTCATGATAGTTTCTCACC-3'	
fusB	*fusB*-F	5'-ATTCAATCGGAAACCTATAATGATA-3'	292
	fusB-R	5'-TTATATATTTCCGATTTGATGCAAG-3'	
fusC	*fusC*-F	5'-GAT ATT GAT ATC TCG GAC TT-3'	128
	fusC-R	5'-AGT TGA CTT GAT GAA GGT AT-3'	
fusD	*fusD*-F	5'-AATTCGGTCAACGATCCC-3'	465
	fusD-R	5'-GCCATCATTGCCAGTACG-3'	
fusE	*fusE*-F	5'- TGTTGGTGGAGAAATTATCGC-3'	696
	fusE-R	5'- CCTGATAAGTTAGTACGAACACG-3'	

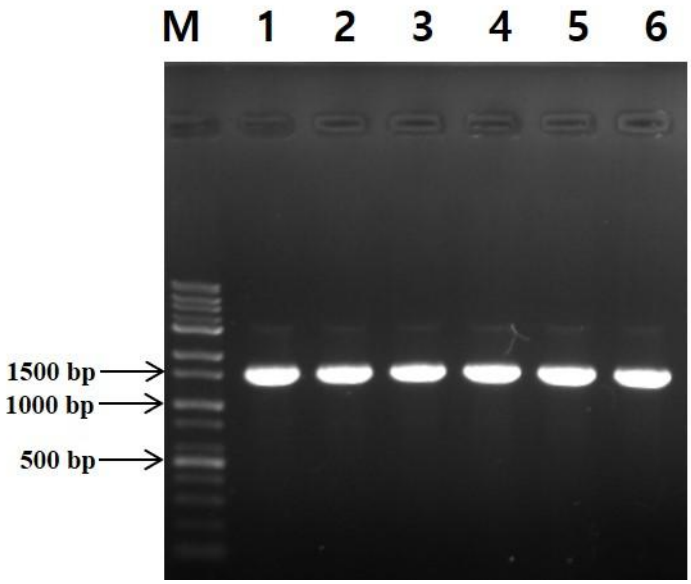

Fig.1 . PCR amplification of the *fusA1* gene from fusidic acid-resistant *S.aureus* isolates. Lane M, 100bp size marker; lane 1-6, fusidic-resistant S.aureus isolates; DNA molecular size marker (1kb bp DNA Ladder, LaboPass™)

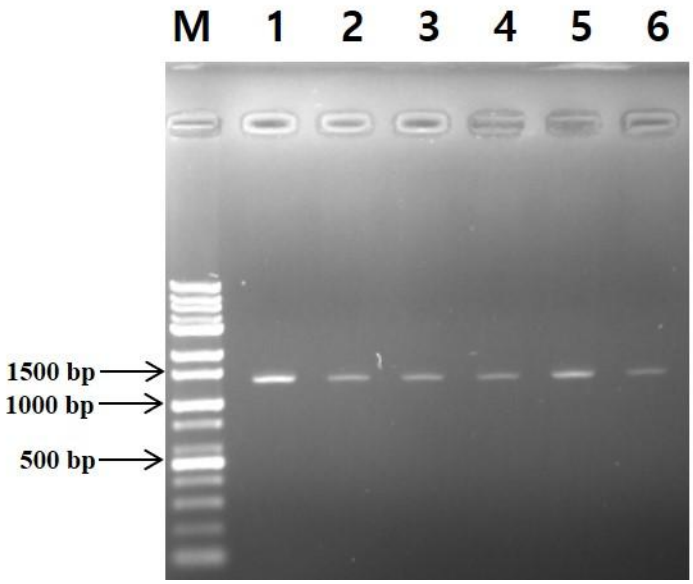

Fig.2. PCR amplification of the *fusA2* gene from fusidic acid-resistant *S.aureus* isolates. Lane M, 100bp size marker; lane 1-6, fusidic-resistant S.aureus isolates; DNA molecular size marker (1kb bp DNA Ladder, LaboPass™)

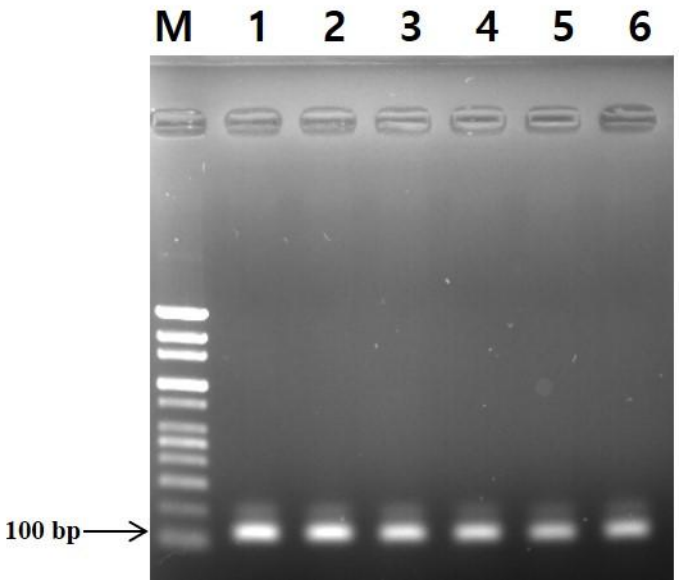

Fig.3 . PCR amplification of the *fusC* gene from fusidic acid-resistant *S.aureus* isolates. Lane M, 100bp size marker; lane 1-6, fusidic-resistant S.aureus isolates; DNA molecular size marker (100 bp DNA Ladder, LaboPass™)

Results

Fusidic acid resistance in MRSA and MSSA.

During the study period, a total of 550 S. aureus (236 MRSA and 314 MSSA) isolates were performed MIC test following CLSI broth micro-dilution (BMD) method guideline, of which 213 (39%) were fusidic acid-resistant. The dominant MRSA clones were ST72 and ST5 among *S. aureus* isolates. The dominant MSSA clones were ST1, ST5, ST6, ST8, ST15, ST72, ST97, ST188 and ST513 among *S. aureus* isolates. Conversely, of the 117 ST72-MRSA isolates, only 7 (6%) were resistant, whereas 43 (83%) of 52 ST72-MSSA were resistant ($P < 0.001$). Among 34 ST1-MSSA isolates, 32 (94%) were resistant, and among 30 ST513-MSSA isolates, 29 (97%) were resistant. (table 6).

Prevalence of fusidic acid resistance determinants in MRSA.

The present study was designed to determine the prevalence of fusidic acid resistance determinants among 97 MRSA isolates, the entire *fusA* gene was sequenced, and other fusidic acid resistance genes (*fusB*, *fusC*, and *fusD*) were detected by PCR. Amplification with primers specific for *fusB*, *fusC*, and *fusD* revealed that none of all MRSA isolates possessed either *fusB* or *fusD* but 7 MRSA isolates carried *fusC*. *FusA* mutation was the most prevalent resistance mechanism in MRSA (90/97, 93%) (table 7)[9)]

Prevalence of fusidic acid resistance determinants in MSSA.

MIC test was conducted on four types of ST1, ST5, ST72, and ST513, which were the most resistant. The present study was designed to determine the prevalence of fusidic acid resistance determinants among 109 MSSA isolates, the entire *fusA* gene was sequenced, and other fusidic acid resistance genes (*fusB*, *fusC*, and *fusD*) were detected by PCR. Of the 77 isolates with *fusA* mutation, single-amino-acid substitutions were found in 77 isolates. Amplification with primers specific for *fusB*, *fusC*, and *fusD* revealed that none of all MSSA isolates possessed either *fusB* or *fusD* but 32 MSSA isolates carried *fusC*. *FusA* mutation was the most prevalent resistance mechanism in MSSA (77/109, 71%) (table 7)[9)]

Mutations in *fusA*.

The nucleotide sequences of *fusA* from all MRSA isolates was determined. Point mutations in *fusA* were detected in 90 MRSA isolates. Of 46 MRSA-ST5, L461K (AAT → AGT) was the most prevalent mutation with 82 (98%), all with high-resistance results. only 2 (2%) isolates showed other single amino acid substitutions were N463S(AAT→AGT) with high-level resistance and V90I(GTA→ATA) with low-level resistance. Among 7 MRSA-ST72, L461K (AAT → AGT) was the most predominant mutation of 4 (57%), all with high-resistance results. only 2 (2%) isolates showed other single amino acid substitutions were N463S(AAT→AGT) with high-level resistance and V90I(GTA→ATA) with low-level resistance. Then, the nucleotide sequences of *fusA* from all MSSA isolates was determined. Point mutations in *fusA* were detected in 77 MSSA isolates. The most predominant mutation was L461K (AAT → AGT) 77 (100%) with high-level resistance, but no *fusA* mutation was detected in ST1-MSSA. In this study, none of the fusidic acid-resistant strains showed no mutation in *fusE*. (table 8-9)

Molecular characteristics of fusidic acid-resistant *S. aureus* isolates

Molecular characteristics of fusidic acid-resistant *S. aureus* isolates. The molecular characteristics of fusidic acid-resistant *S. aureus* isolates were listed. The clonal complex 5 (CC5) was the 90/119 (76%) of the isolates tested in MRSA and was 5/17 (29%) of the isolates tested in MSSA. The 16 *spa* types were identified among the 90 fusidic acid-resistant MRSA isolates, among which t2460 (43 %, 39/90) was the most prevalent *spa* type. The *spa* types for 8 isolates were not identified. Among the 90 MRSA isolates, 90 harbored SCC *mec* types II. In this study, ST5-MRSA- II-t2460 was a predominant clone, accounting for 43 % (39/90) of fusidic acid-resistant ST5-MRSA isolates (table 13). Secondly, ST5-MRSA- II-t9353 one of major, accounting for 13% (12/90) of fusidic acid-resistant ST5-MRSA isolates. Among 4 fusidic acid resistance MRSA isolates, 4 belonged to ST5-MRSA-II-t002 clones carrying *fusC* with low-level fusidic acid resistance. The clonal complex 72 (CC72) was the 7/117 (6%) of the isolates tested in MRSA and was the 43/52 (83%) of the isolates tested in MSSA. The 8 *spa* types were identified among the 43 fusidic acid-resistant MSSA isolates, among which t126 (100 %, 34/34) was the most prevalent *spa* type. The *spa* types for 1 isolate was not identified. In this study ST72-MSSA-t126, one of major, accounting for 65% (34/52) of fusidic

acid resistant ST72-MSSA isolates (table 12). The clonal complex 20 (CC20) was the 29/30 (97%) of the isolates tested. The 4 *spa* types were identified among the 29 fusidic acid resistant MSSA isolates, among which t164 (86%, 25/29) was the most prevalent *spa* type. The *spa* types for 1 isolate was not identified. The clonal complex 1 (CC1) was the 32/34 (94%) of the isolates tested. The 6 *spa* types were identified among the 32 fusidic acid resistant MSSA isolates, among which t127 (81%, 26/32) was the most prevalent *spa* type. The *spa* types for 1 isolate was not identified. Among 26 fusidic acid resistance MSSA isolates, 26 belonged to ST1-MSSA-t127 clones carrying *fusC* with low-level fusidic acid resistance.

Table 6. Proportion of fusidic acid resistant Staphylococcus aureus stratified by methicillin resistance and sequence type (ST)

Sequence Type	MRSA (%)	MSSA (%)	P value
ST5	90/119 (76)	7/28 (25)	<0.001
ST72	7/117 (6)	43/52 (83)	<0.001
ST1	0/0 (0)	28/34 (82)	N/A
ST513	0/0 (0)	29/30 (97)	N/A

Table 7. Fusidic acid resistance determinants genes of MRSA and MSSA isolates

Species	No. (%) of isolates with different FA resistance determinants				
	***fusA* mutation**	***fusB***	***fusC***	***fusD***	***fusE* mutation**
MRSA (97)	90 (93)	0 (0)	7 (7)	0 (0)	0 (0)
MSSA (109)	77 (71)	0 (0)	32 (29)	0 (0)	0 (0)
Total (206)	167 (81)	0 (0)	39 (19)	0 (0)	0 (0)

[a] number of isolates showing Fusidic acid MIC ≥ 2 μg/ml

Table 8. Distribution of fusidic acid MIC and resistance determinants among fusidic acid-resistant S. aureus isolates

Resistance determinant (n)	No. of isolates with different fusidic acid MIC (μg/ml)		
	2-16	**32-64**	**≥128**
fusA point mutation (167)	3	0	164
fusB (0)	0	0	0
fusC (39)	37	2	0
fusD (0)	0	0	0
fusE point mutation (0)	0	0	0
Total (206)	40	2	164

Table 9. *FusA* (EF-G) mutant phenotypes

Amino acid substitution	Nucleotide substitution	Number of isolates	FA[a] MIC (μg/ml)
L461K	TTA→AAA	162 (85 MRSA, 77 MSSA)	>128
N643S	AAT→AGT	1 MRSA	>128
R483C	CGT→TGT	1 MRSA	2
V90I	GTA→ATA	1 MRSA	4
P404L	CCA→CTA	1 MRSA	8

[a] FA, fusidic acid

Table 10. Prevalence of fusidic acid resistance according to the *spa* type in ST5-MRSA and ST5-MSSA isolates

ST5-MRSA		ST5-MSSA	
spa type(n)	No. (%) of isolates with FA[a] resistance	*Spa* type(n)	No. (%) of isolates with FA resistance
t2460 (40)	39 (98)	t179 (4)	0 (0)
t002 (17)	4 (24)	t688 (4)	0 (0)
t9353 (12)	12 (100)	t1560 (3)	2 (67)
t439 (3)	3(100)	t002 (2)	0 (0)
t436 (3)	3(100)	t640 (2)	0 (0)
t264 (5)	5 (100)	t126 (1)	1 (100)
t535 (3)	3 (100)	t2460 (n=1)	1 (100)
t111 (4)	0 (0)	t264 (1)	1 (100)
t17573 (3)	3 (100)	t586 (1)	0 (0)
t18239 (2)	2 (100)	t021 (1)	0 (0)
t5076 (2)	2 (100)	t062 (1)	0 (0)
t148 (2)	0 (0)	t105 (1)	0 (0)
t12703 (1)	1 (100)	t1303 (1)	0 (0)
t1560 (1)	1 (100)	t148 (1)	0 (0)
t564 (1)	1 (100)	t2302 (1)	0 (0)
t586 (1)	1 (100)	t442 (1)	0 (0)
t688 (1)	1 (100)	ND[b] (2)	0 (0)
t539 (1)	1 (100)		
t105 (1)	0 (0)		
t242 (1)	0 (0)		
t2431 (1)	0 (0)		
t324 (1)	0 (0)		
t601 (1)	0 (0)		
ND[b] (12)	8 (67)		
Total (119)	90 (76)	Total (28)	5 (18)

[a]FA, fusidic acid; [b]ND, non determined

Table 11. Prevalence of fusidic acid resistance according to the *spa* type in ST72-MRSA and ST72-MSSA isolates

ST72-MRSA		ST72-MSSA	
spa type (n)	No. (%) of isolates with FA[a] resistance	*Spa* type(n)	No. (%) of isolates with FA resistance
t324 (46)	0 (0)	t126 (34)	34 (100)
t148 (12)	0 (0)	t324 (4)	0 (0)
t664 (12)	0 (0)	t8421 (2)	2 (100)
t2431 (5)	0 (0)	t148 (2)	0 (0)
t2461 (4)	0 (0)	t1151(1)	1 (100)
t11313(2)	0 (0)	t127 (1)	1 (100)
t15957 (2)	0(0)	t18240 (1)	1 (100)
t901(2)	0 (0)	t206 (1)	1 (100)
t126 (1)	1 (100)	t2460 (1)	1 (100)
t17573 (1)	1 (100)	t4727(1)	1 (100)
t2460 (1)	1 (100)	t4359 (1)	0 (0)
t9061 (1)	1 (100)	t2453 (1)	0 (0)
t664 (1)	1 (100)	t2461 (1)	0 (0)
t008(1)	0 (0)	ND[b] (1)	1 (100)
t10205 (1)	0 (0)		
t10275 (1)	0 (0)		
t10555 (1)	0 (0)		
t4359 (1)	0 (0)		
t5440 (1)	0 (0)		
t6509 (1)	0 (0)		
t7820 (1)	0 (0)		
ND (20)	2 (10)		
Total (117)	7 (6)	Total (52)	43 (83)

[a]FA, fusidic acid; [b]ND, non determined

Table 12. Prevalence of fusidic acid resistance according to the *spa* type in ST1-MSSA and ST513-MSSA isolates

ST1-MSSA		**ST513-MSSA**	
spa typing	No. (%) of isolates with FA resistance	*spa* typing	No. (%) of isolates with FA resistance
t127 (n=26)	26 (100)	t164 (26)	25 (96)
t189 (n=2)	0 (0)	t1544 (1)	1 (100)
t12303 (n=1)	1 (100)	t3589 (1)	1 (100)
t126 (n=1)	1 (100)	t3929 (1)	1 (100)
t128 (n=1)	1 (100)	ND[b] (1)	1 (100)
t177 (n=1)	1 (100)		
t2207 (n=1)	1 (100)		
t693 (n=1)	1 (100)		
Total (n=34)	32 (94)	Total (30)	29 (97)

[a]FA, fusidic acid; [b]ND, non determine

Table 13. Molecular characteristic of fusidic acid resistant *S.aureus* isolates

CC[a] (No.)	STs[b] (No.)	spa types (No.)	MRSA (No.)	MSSA (No.)	SCCmec (No.)	*fusA mutation (No.)*	*fusB (No.)*	*fusC (No.)*	*fusD (No.)*	FA MICs (No.)
5(87)	ST5	t2460(40)	39	1	II(39)	L461K(40)	0	0	0	>128(40)
		t002(4)	4		II(4)		0	4	0	16(3);4(1)
		t9353(12)	12		II(12)	L461K(12)	0	0	0	>128(12)
		t439(3)	3		II(3)	L461K(3)	0	0	0	>128(3)
		t463(3)	3		II(3)	L461K(3)	0	0	0	>128(3)
		t264(6)	5	1	II(5)	L461K(6)	0	0	0	>128(6)
		t535(3)	3		II(3)	L461K(3)	0	0	0	>128(3)
		t539(1)	1		II(1)	V90I(1)	0	0	0	4(1)
		t17573(3)	3		II(3)	L461K(3)	0	0	0	>128(3)
		t18239(2)	2		II(2)	L461K(2)	0	0	0	>128(2)
		t5076(2)	2		II(2)	L461K(2)	0	0	0	>128(2)
		t12703(1)	1		II(1)	L461K(1)	0	0	0	>128(1)
		t1560(3)	1	2	II(1)	L461K(3)	0	0	0	>128(3)
		t564(1)	1		II(1)	L461K(1)	0	0	0	>128(1)
		t586(1)	1		II(1)	L461K(1)	0	0	0	>128(1)
		t688(1)	1		II(1)		0	1	0	16(1)
		t126(1)		1		L461K(1)	0	0	0	>128(1)
72(47)	ST72	t126(35)	1	34	IV(1)	L461K(35)	0	0	0	>128(35)
		t8421(2)		2		L461K(2)	0	0	0	>128(2)
		t1151(1)		1		L461K(1)	0	0	0	>128(1)
		t127(1)		1		L461K(1)	0	0	0	>128(1)
		t18240(1)		1		L461K(1)	0	0	0	>128(1)
		t4727(1)		1		L461K(1)	0	0	0	>128(1)
		t206 (1)		1		L461K(1)	0	0	0	>128(1)
		t2460(2)	1	1	II(1)	L461K(1)	0	0	0	>128(1)
		t17573(1)	1		II(1)	L461K(1)	0	0	0	>128(1)
		t9061(1)	1		IV(1)	R483C(1)	0	0	0	2(1)
		t664(1)	1		IV(1)	P404L(1)	0	0	0	8(1)
20(28)	ST513	t164(25)		25		L461K(25)	0	0	0	>128(26)
		t1544(1)		1		L461K(1)	0	0	0	>128(1)
		t3589(1)		1		L461K(1)	0	0	0	>128(1)
		t3929(1)		1		L461K(1)	0	0	0	>128(1)
1(32)	ST1	t127(26)		26			0	26	0	32(2);16(22);8(1);4(1)
		t12303(1)		1			0	1	0	16(1)
		t126(1)		1			0	1	0	16(1)
		t128(1)		1			0	1	0	16(1)
		t177(1)		1			0	1	0	16(1)
		t2207(1)		1			0	1	0	16(1)
		t693(1)		1			0	1	0	16(1)

a: CC, clone complex; b: ST, sequence type

Discussion

In this study, Fusidic acid resistance was common in ST5-MRSA and ST72-MSSA with a dominance of *fusA* mutation. Overall, the prevalence of Fusidic acid resistance was low (39%) in this study, which was lower than this country in Greece (52.5%), and higher than those in other European countries (1.4%-19.9%)[8] New Zealand (29%)[11], and North America (0.3%-7.0%)[7]. The possible reason for this finding is that topical fusidic acid has been very widely used as an over-the-counter medication in Korea since 1980, as Williamson et al. found that increased use of topical fusidic acid was concurrent with an increase of fusidic acid-resistant *S. aureus*[11]. In 2004, a nationwide survey in Korea identified that the rate of fusidic acid resistance in MSSA was 31.7% and that in MRSA was 14.1% [16], while our study showed that the rate of fusidic acid resistance in MSSA was 37% and that in MRSA was 41%. Therefore, The rate of fusidic acid resistance in *S.aureus* had been increased. *FusA* mutation was the most prevalent fusidic acid-resistant mechanism (81%) and acquired fusidic acid resistance was not common (29%). However, in contrast to our finding, acquired fusidic acid resistance is more common than *FusA* mutation in Europe [8, 10]. In this study, three dominant clones (t127 CC1 MSSA, t002 CC5 MRSA and t688 CC5 MRSA) habor *fusC* gene were major clones in fusidic acid resistance *S. aureus* isolates with low-level resistance, which was similar to that in New Zealand [11]. In addition, a high prevalence of *fusC* (59%) was observed in Taiwan which was mediated by novel SCC*fusC* carrying *fusC* and *speG* in fusidic acid-resistant MRSA [16]. It may be associated with a geographical difference in fusidic acid resistance mechanisms. fusidic acid resistance was common in ST5-MRSA and ST72-MSSA and rare in ST5-MSSA and ST72-MRSA. The most common clone was observed that t2460 ST5-MRSA and all were fusidic acid-resistant while t324 ST72-MRSA was the most common clone but was usually susceptible to fusidic acid. The most common clone was observed that t126 ST72-MSSA and all were fusidic acid-resistant while t179 ST5-MSSA was the most common clone but was usually susceptible to fusidic acid. Our study has several limitations to note. First, as it was a single-center study in South Korea, further multicenter study is warranted to generalize our findings. Second, Whole-genome sequencing for t324 ST72-MSSA, t324 ST72-MRSA, t2460 ST5-MSSA, and t2460 ST5-MRSA is needed for comparison of the genome sequences and

further understanding of S. aureus evolution and virulence. In conclusion, fusidic acid resistance was common in ST5-MRSA, ST1-MSSA, ST513-MSSA and ST72-MSSA, and rare in ST5-MSSA and ST72-MRSA. These results indicate that most of the strains produced high-level resistance in the L461K of the *fusA* mutation, which is an important mutation that determines the high-level resistance of fusidic acid. In addition, few *spa*-type results show that in some types, *fusC* is an important factor in determining the low-level resistance of fusidic acid.

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국문요약

연구배경: 황색포도알균(*Staphylococcus aureus*)은 지역사회와 병원 감염의 중요한 원인균으로 피부 및 연 조직 감염, 폐렴, 균혈증 등을 일으킨다. 카테터 또는 인공 기구의 사용 및 침습적 시술이 늘면서 황색포도알균에 의한 심각한 감염이 증가하고 있으며, 이들 균주들은 기존 항생제에 대해 내성을 보이는 경우가 많아 적절한 항생제 치료가 어려워지고 있다. Fusidic acid 항생제는 황색포도알균 등의 그람 양성균에 의해 발생하는 피부 및 연조직 감염 또는 뼈 및 관절 감염의 치료에 사용되는 항생제이다. Fusidic acid 항생제는 세균의 단백질 합성을 방해함으로써 특히 리보솜에서의 신장 인자 G (EF-G)의 전좌를 막아 작용한다. 주요 fusidic acid 내성 결정 인자는 결합 부위의 변화 (*fusA* 또는 *fusE* = *rlpF*) 또는 fusidic acid의 내성 획득(*fusB*, *fusC* 및 *fusD*)이 있다. 본 연구는 메티 실린 내성 황색 포도상 구균 (MRSA) 과 메티 실린 감수성 황색 포도상 구균 (MSSA)의 fusidic acid 내성의 유병률을 시퀀스 유형 (ST) 및 스파(*spa*) 유형에 따라 조사하였고 또한 fusidic acid 항생제의 내성 결정인자들의 분포도를 조사하였다. 본 연구는 항생제 내성 문제를 정확하게 파악하기 위해서는 항생제에 내성인 균주들의 다양한 타입에 따른 균주 파악과 내성에 따른 유병률을 평가하는 것을 목표로 하였다.

연구방법: 2014년부터 2018년까지의 서울아산병원에서 등록된 550명의 중복되지 않은 균혈증 환자의 혈액으로부터 236개의 MRSA 균주 그리고 314개의 MSSA 균주를 수집했다. 각 수집된 균주들은 전부 MLST sequence type 그리고 *spa* type를 확인하였고. 결정된 ST에 따라 분류된 균주들을 대상으로 European Committee on Antimicrobial Susceptibility Testing (EUCAST) 가이드라인에 근거하여 Minimum inhibitory concentration (MIC, 최소억제농도) 검사를 통하여 내성 균주들을 선별하였다. 선별된 균주들을 대상으로 내성기전을 파악하기 위하여 nucleotide 서열 및 추론 된 아미노산 서열을 BLAST (http://www.ncbi.nlm.nih.gov/blast/)를 통해 비교 분석하였다.

연구결과: 결과적으로 236개의 MRSA 균주 중에서 97개(41%) 그리고 314개의 MSSA 균주 중에서 116개(49%)가 내성 균주로 분리되었다. Fusidic acid에 내성을가진 *S. aureus* 모든 균주에서 *fusA* gene 그리고 *fusE* gne이 발견되었고,

내성 기전을 확인하기 위해서 *fusA* mutation 부위를 확인한 결과 MRSA의 균주 90개(ST5: 84, ST72: 6) 에서 L461K 변형이 우세하였으며, 단 4 개의 서로 다른 단일 아미노산 치환 (V90I, N463S, P404L 및 R483C)이 일어났다. 이때 L461K와 N463S는 고도 내성의 결과를 갖는 반면에 V90I, P404L 그리고 R483C는 저도 내성의 결과를 가졌다. 나머지 7균주는 *fusC*를 얻어 저도 내성의 결과를 가졌다. 반면에 MRSA와 다르게 MSSA에서 *fusA* mutation 부위를 확인한 결과 ST에 따른 결과가 확연하게 구분되었는데, MSSA의 107개(ST1: 32, ST5: 5, ST72: 43, ST513: 29)에서 ST5, ST72 그리고 ST513의 77개 전부 L461K 변형에 의해서 내성을 일으켰고 이들은 전부 고도 내성의 결과를 나타냈다. 하지만 ST1의 32개는 전부 *fusC*를 얻어 저도 내성의 결과를 나타냈다. 마지막으로 내성을 갖는 균주들을 대상으로 17개의 *spa* type의 결과를 확인하였고 ST5-MRSA-t2460, ST72-MSSA-t126, ST1-MSSA-t127 그리고 ST513-MSSA-t164의 결과를 나타냈다.

결론: 이 연구에서, fusidic acid 내성은 ST5-MRSA 및 ST72-MSSA에서 흔했으며 ST5-MSSA 및 ST72-MRSA에서는 드물었다. 전반적으로, Fusidic acid 내성 유병률은 이연구에서 39 %로 낮았는데, 이는 그리스보다 낮았으며 (52.5 %), 다른 유럽 국가들보다 높았다 (1.4 % -19.9 %)[8]. 뉴질랜드 (29 %)[11], 북미 (0.3 % -7.0 %)[7]. 대한민국에서의 2004 년에 MSSA 내성률은 31.7 %, MRSA 14.1 % [8] 반면, 본실험의 결과는 MSSA 내성률은 37 %이며 MRSA 41 %였다. fusidic acid 내성기전 중에서 *FusA* 돌연변이 이 가장 흔하게 나타났다(81%) 그리고 유전자 획득에 의해서 내성을 일으키는 건 흔하지 않다(29%). 그러나, 본 실험과는 달리, 유전자 획득에 의해서 내성을 일으키는 건 흔하지 않다 유럽에서 *FusA* 돌연변이보다 더 흔하다 [8, 10]. 본 발명자들은 t2460 ST5-MRSA가 가장 흔한 클론이고 전부다 내성 이었지만, t324 ST72-MRSA는 가장 흔한 클론 이었지만 대부분이 감수성을 나타내 관찰되었다. 본 발명자들은 t126 ST72-MSSA가 흔한 클론이고 전부다 내성 이었지만, 반면 t179 ST5-MSSA가 가장 흔한 클론이지만 가장 흔한 클론 이었지만 대부분이 감수성을 나타내 관찰되었다. 본연구에서 몇가지 부족한점이 있는데 첫번째로

한국에서의 단일 센터 연구 였기 때문에, 우리의 연구 결과를 일반화하기위한 추가 멀티 센터 연구가 필요하다. 두번째로는 t324 ST72-MSSA, t324 ST72-MRSA, t2460 ST5-MSSA, and t2460 ST5-MRSA의 정확한 비교를 위해서 whole genome 시퀀스 비교가 필요하다.