

In Vitro Antimicrobial Activities of Fusidic Acid and Retapamulin against Mupirocin- and Methicillin-Resistant *Staphylococcus aureus*

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Background: The *in vitro* activities of retapamulin and fusidic acid against clinical isolates of mupirocin-resistant and methicillin-resistant *Staphylococcus aureus* (MRSA) from Korea are not well understood. **Objective:** This study aimed to determine the activities of retapamulin and fusidic acid against clinical isolates of mupirocin-resistant MRSA. **Methods:** Clinical isolates of mupirocin-resistant MRSA were collected from two tertiary hospitals. The minimal inhibitory concentrations of mupirocin, fusidic acid, and retapamulin were determined using agar dilution method. Polymerase chain reaction was used to confirm the identity of the species and the presence of resistance genes. Pulsed-field gel electrophoresis (PFGE) patterns of chromosomal DNA were used to determine the genetic similarity of high-level mupirocin-resistant isolates. **Results:** Of the 497 MRSA isolates tested, 22 (4.4%) were mupirocin-resistant. Of these, 9 (1.8%) and 13 (2.6%) had high-level and low-level mupirocin resistance, respectively. Analysis of the PFGE patterns of the high-level mupirocin-resistant MRSA isolates identified five clusters. All 13 of the low-level mupirocin-resistant isolates were resistant to fusidic acid but susceptible to retapamulin. However, among the 9 high-level mupirocin-resistant isolates, 56% were resistant to fusidic acid, and all were susceptible to retapamulin. **Conclusion:** Retapamulin is highly active *in vi-*

tro against Korean clinical isolates of high-level mupirocin- and methicillin-resistant *Staphylococcus aureus* with different genetic backgrounds. Fusidic acid is more active against high-level mupirocin-resistant MRSA than low-level mupirocin-resistant MRSA. (Ann Dermatol 27(5) 551~556, 2015)

-Keywords-

Fusidic acid, Methicillin-resistant *Staphylococcus aureus*, Microbial sensitivity tests, Mupirocin, Retapamulin

INTRODUCTION

Topical antibiotics are commonly used to treat superficial bacterial skin infections caused by *Staphylococcus aureus* and *Streptococcus pyogenes*. Currently, the most commonly used topical antibiotics in Korea are fusidic acid and mupirocin. Staphylococci are often resistant to fusidic acid than to mupirocin. Consequently, mupirocin is used more frequently than fusidic acid. Since these drugs are classified as generic, the potential for abuse is high. The rate of resistance of methicillin-resistant *S. aureus* (MRSA) to these drugs has also increased¹⁻⁴. Therefore, new topical antibiotics are required for the treatment of *S. aureus* infections.

Retapamulin is a pleuromutilin antibiotic derived from a mushroom, *Clitopilus scyphoides*. In Korea, the drug was launched in July 2011 under the name "Altargo." It is classified as a prescription drug. Currently, the susceptibility of *S. aureus* in Korea to retapamulin is not well understood. In this study, we evaluated the prevalence of mupirocin-resistant MRSA and the epidemiologic relationship between high-level mupirocin-resistant isolates from two Korean tertiary hospitals. In addition, the susceptibility of

Received February 10, 2015, Revised March 20, 2015, Accepted for publication April 22, 2015

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the isolates to fusidic acid and retapamulin was also determined.

MATERIALS AND METHODS

Bacterial isolates

In total, 497 MRSA isolates were collected from clinical cultures from patients at two tertiary university hospitals in Korea between March 2011 and May 2012. The specimens were obtained from the skin, pus, blood, central venous catheter tip, urine, and wounds. *S. aureus* was identified using traditional methods, such as the coagulase test. Susceptibility to methicillin was determined with disk diffusion, using a 30- μ g cefoxitin disk, and an agar screen test, using 6 μ g/ml oxacillin, according to guidelines provided by the Clinical and Laboratory Standards Institute (CLSI).

According to guidelines provided by the British Society for Antimicrobial Chemotherapy (BSAC), disk diffusion using 5- μ g and 200- μ g mupirocin disks was used to determine the mupirocin resistance of MRSA. Individual 5- μ g mupirocin disks were used to distinguish isolates that were susceptible to mupirocin (>14-mm inhibition zone) from isolates with resistance (no inhibition zone). Individual 200- μ g mupirocin disks were used to distinguish isolates with high-level resistance (no inhibition zone) from isolates that were susceptible or had low-level resistance (>14-mm inhibition zone).

Detection of *femA*, *mecA*, and *mupA* genes with polymerase chain reaction

To confirm the identity of the MRSA isolates, *femA* and *mecA* genes were detected using polymerase chain reaction (PCR)-based method. In addition, to confirm the identity of the high-level mupirocin-resistant isolates, the *mupA* gene was detected using PCR-based method. For PCR, DNA was extracted using the heating method. *femA* (372 bp), *mecA* (554 bp), and *mupA* (1.6 kb) genes were amplified with the primers presented in Table 1.

Pulsed-field gel electrophoresis with high-level mupirocin-resistant MRSA

The genetic relatedness of high-level mupirocin-resistant MRSA isolates was determined with pulsed-field gel electrophoresis (PFGE) analysis. A mixture containing the *Sma*I restriction enzyme (Sib Enzyme Ltd., Novosibirsk, Russia) was employed to prepare chromosomal restriction fragments. The chromosomal restriction fragments were separated by electrophoresis using a CHEF-DR II system (Bio-Rad Laboratories Inc., Hercules, CA, USA). The electrophoresis parameters included an initial pulse of 5 s, final pulse of 40 s, 200 V, 20 h, and 12°C to 14°C.

The restriction pattern was analyzed with the GelCompar II version 4.6 software (Bio-Rad Laboratories Inc.) using the Dice coefficient. Cluster analysis of the similarity matrices was performed using the unweighted pair group method with arithmetic mean, with a tolerance of 0.80%. The similarity cutoff value was 99%.

Mupirocin, fusidic acid, and retapamulin susceptibility tests using the agar dilution method

The agar dilution method was used to determine the minimal inhibitory concentrations (MICs) according to the guidelines provided by the CLSI. *S. aureus* ATCC 25923 and *S. aureus* ATCC 43300 were used as control strains.

Serial two-fold dilutions of the antibiotics were prepared in Muller-Hinton agar (Difco Laboratories, Detroit, MI, USA). The strains were subcultured on tryptic soy agar (Difco Laboratories), suspended in tryptic soy broth (Difco Laboratories), and adjusted to the turbidity of a 0.5 McFarland standard. Next, the suspension was diluted 1:10 and inoculated on each plate using a Steer replicator. The inoculated plates were incubated at 35°C for 24 h. The lowest concentration of antibiotic that inhibited the visible growth of an organism was regarded as the MIC, and the presence of a single colony was ignored. The MIC₅₀ and MIC₉₀ were the MICs that inhibited 50% and 90% of the isolates, respectively.

Susceptibility to mupirocin and fusidic acid was deter-

Table 1. List of primers used for the amplification of the *mecA*, *femA*, and *mupA* genes from *Staphylococcus aureus*

Gene	Primer	Oligonucleotide sequence (5'-3')	Polymerase chain reaction product size
<i>femA</i>	<i>femA</i> -F	CATGATGGCGAGATTACAGG	372 bp
	<i>femA</i> -R	CGCTAAAGGTACTAACACACCGG	
<i>mecA</i>	<i>mecA</i> -F	ATGAGATTAGGCATCGTTCC	554 bp
	<i>mecA</i> -R	TGGATGACAGTACCTGAGCC	
<i>mupA</i>	<i>mupA</i> -F	CCCATGGCTTACCAGTTGA	1.6 kb
	<i>mupA</i> -R	CCATGGAGCACTATCCGAA	

mined according to the guidelines provided by the European Committee for Antimicrobial Susceptibility Testing (EUCAST) and BSAC. In the case of mupirocin, MIC thresholds of $\geq 8 \mu\text{g/ml}$ and $> 256 \mu\text{g/ml}$ indicated resistance and high resistance, respectively. In the case of fusidic acid, MIC thresholds of $\geq 2 \mu\text{g/ml}$ and $> 128 \mu\text{g/ml}$ indicated resistance and high resistance, respectively. In the case of retapamulin, MIC thresholds of $\geq 2 \mu\text{g/ml}$ and $\leq 0.5 \mu\text{g/ml}$ indicated resistance and susceptibility, respectively, according to the epidemiologic cutoff values from EUCAST.

RESULTS

Prevalence of mupirocin-resistant MRSA

Of the 497 clinical isolates of MRSA from the two tertiary hospitals, 22 (4.4%) were mupirocin-resistant. Of these, 9 (1.8%) and 13 (2.6%) had high and low levels of resistance, respectively. Using PCR-based method, the *femA* and *mecA* genes were detected in all of the mupirocin-re-

sistant MRSA isolates, and the *mupA* gene was detected in all of the high-level mupirocin-resistant MRSA isolates.

Genotyping mupirocin-resistant MRSA using PFGE patterns

Analysis of the PFGE patterns of the genomic DNA of the 9 high-level mupirocin-resistant MRSA isolates identified 5 clusters. The results indicate that the isolates are not closely related, except for the two isolates in cluster I (Fig. 1).

Susceptibility of mupirocin-resistant MRSA to fusidic acid and retapamulin

The MIC values of fusidic acid for the low-level mupirocin-resistant MRSA isolates were $\geq 128 \mu\text{g/ml}$, but the MIC and MIC₉₀ values of fusidic acid for the high-level mupirocin-resistant isolates were ≤ 0.5 to $\geq 32 \mu\text{g/ml}$ and $\geq 32 \mu\text{g/ml}$, respectively. The MIC and MIC₉₀ values of retapamulin for both the low- and high-level mupirocin-resistant MRSA isolates were $0.5 \mu\text{g/ml}$ and $0.5 \mu\text{g/ml}$, respectively.

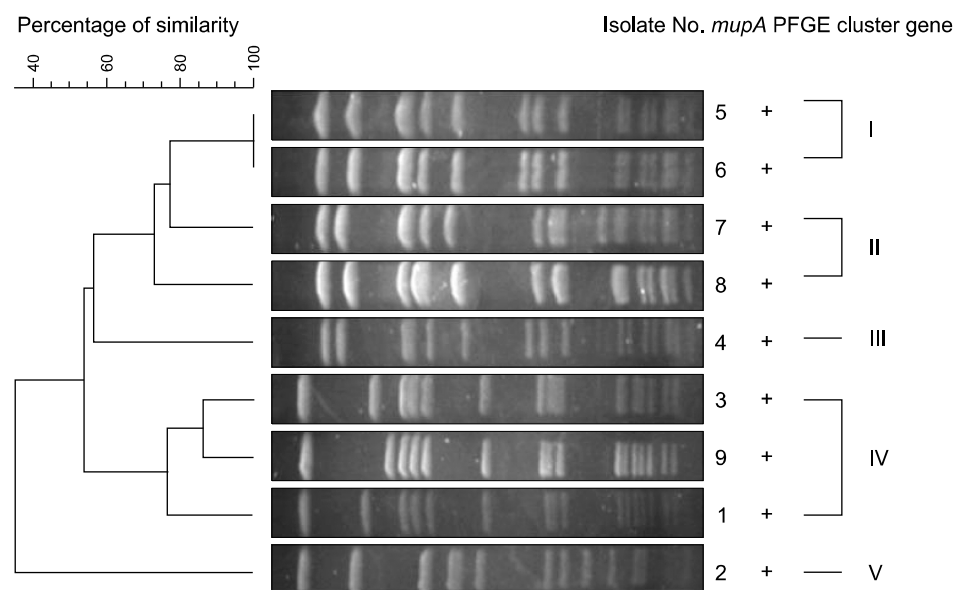


Fig. 1. The unweighted pair group method with arithmetic mean was used to assess the pulsed-field gel electrophoresis (PFGE) profiles of *Smal*-restricted chromosomal DNA from high-level mupirocin- and methicillin-resistant clinical isolates of *Staphylococcus aureus*. Five clusters were identified, indicating that the majority of the isolates are not closely related.

Table 2. Antimicrobial activity of fusidic acid and retapamulin against mupirocin- and methicillin-resistant *Staphylococcus aureus* isolates

MUP-R level (tested no.)	Antimicrobial agent	MIC ($\mu\text{g/ml}$)			Resistance (%)
		Range	MIC ₅₀	MIC ₉₀	
Low level (n = 13)	Mupirocin	64	64	64	100
	Fusidic acid	≥ 128	≥ 128	≥ 128	100
	Retapamulin	0.5	0.5	0.5	0
High level (n = 9)	Mupirocin	≥ 512	≥ 512	≥ 512	100
	Fusidic acid	≤ 0.5 to ≥ 32	2	≥ 32	55.6
	Retapamulin	0.5	0.5	0.5	0

MUP-R: mupirocin-resistance, MIC: minimal inhibitory concentration.

Analysis of the MICs showed that all 13 low-level mupirocin-resistant isolates were resistant to fusidic acid but susceptible to retapamulin. In contrast, among the 9 high-level mupirocin-resistant isolates, 5 (55.6%) were resistant to fusidic acid, and all were susceptible to retapamulin (Table 2).

DISCUSSION

Fusidic acid and mupirocin are the most widely used topical antibiotics for the treatment of superficial skin infections caused by *S. aureus* and *S. pyogenes*. Owing to the fact that they can be purchased without a doctor's prescription in Korea, the drugs can be used indiscriminately. Misuse of topical antibiotics could lead to pathogens with increased resistance to topical antibiotics. This leads to a risk of increasing the selective survival of MRSA because its resistance to these topical antibiotics is higher than that of methicillin-sensitive *S. aureus* (MSSA)^{1,5,6}.

Mupirocin, a topical antibiotic originally isolated from *Pseudomonas fluorescens*, inhibits bacterial protein synthesis by competitively binding to isoleucyl tRNA synthetase (encoded by *IleS*). It was introduced into clinical practice in 1985. Shortly after, in 1987, clinical isolates resistant to mupirocin were first reported, and the resistance rate has since then increased progressively⁷. In Korea, topical mupirocin has been in use since 1994 to eradicate staphylococcal infection in hospitals, and the use of mupirocin has increased at an alarming rate. Currently, the resistance rate of *S. aureus* to mupirocin is reported as 5% to 25.3%¹⁻⁴. Park et al.⁸ reported that 27 of 193 (14.0%) MRSA isolates in Korea were resistant to mupirocin. In our study, the prevalence of mupirocin resistance is identified to be 4.4%. The observed disparity in the resistance rate is believed to reflect the differences in the study populations, such as the study region, underlying disease, and history of antibiotic use. Mupirocin resistance in staphylococci is commonly categorized as either low-level resistance (MICs of 8~256 $\mu\text{g/ml}$) or high-level resistance (MICs >256 $\mu\text{g/ml}$). Low-level mupirocin resistance arises from point mutations in the chromosomally encoded native *IleS* gene, whereas high-level resistance is related to the acquisition of a plasmid containing the *mupA* resistance element, which possesses a modified *IleS*-2 gene¹. Low-level mupirocin-resistant strains are considered to have no clinical significance since the concentration of mupirocin in the 2% ointment (20,000 $\mu\text{g/ml}$) exceeds the MICs for the low-level mupirocin-resistant strains. Therefore, topical mupirocin can eradicate low-level mupirocin-resistant strains. In contrast, high-level mupirocin-resistant strains that cannot be eradicated by mupirocin treatment pose a serious

clinical problem. This resistance to mupirocin and other antibiotics can be transferred together by plasmids carrying various resistance genes, including *mupA*. Additionally, other bacteria can be a reservoir for the *mupA* gene. Fortunately, high-level resistant strains are observed less frequently than low-level resistant strains⁹⁻¹¹. In the present study, among 22 mupirocin-resistant isolates, 13 were low-level resistant strains and 9 were high-level resistant strains.

PFGE is one of the most prominently used methods for epidemiologic typing and also for determining the genetic relatedness of bacterial isolates. However, it requires technical expertise, long processing time, and expensive instrumental setup^{12,13}. In our present study, the PFGE patterns of chromosomal DNA were used to determine the epidemiologic molecular clonality of high-level mupirocin-resistant MRSA isolates. The results showed that all isolates, except for the two in cluster I, were unrelated. We believe that the genetically unrelated isolates independently acquired a plasmid containing the *mupA* gene. All of the unrelated, high-level mupirocin-resistant MRSA isolates were inhibited by retapamulin in our studies.

Fusidic acid inhibits bacterial protein synthesis by interfering with the translocation of elongation factor G from the ribosome. In the United Kingdom, 50% of the *S. aureus* isolates from dermatology patients were resistant to fusidic acid; on the other hand, 78% of the *S. aureus* isolates from atopic patients were resistant to fusidic acid¹⁴. This indicates that fusidic acid resistance is more prevalent than mupirocin resistance in *S. aureus*. A retrospective analysis reported that, among the 482 *S. aureus* isolates obtained from infected skin wounds in a Korean tertiary hospital between October 2009 and October 2011, 48.3% were MRSA, and 45.9% were resistant to fusidic acid⁴. Importantly, 4.8% of the *S. aureus* isolates were resistant to both fusidic acid and mupirocin⁴, demonstrating that new topical antibiotics are required to treat infections caused by mupirocin- and fusidic acid-resistant bacteria. Fusidic acid-resistant isolates are classified into two groups: low-level resistant isolates (MIC 2~32 $\mu\text{g/ml}$) and high-level resistant isolates (MIC >128 $\mu\text{g/ml}$). Low-level resistance is related to the acquisition of a plasmid containing *fusB*, which encodes a FusB family protein that protects drug target sites. High-level resistance results from point mutations in the chromosomally encoded native *fusA* gene^{15,16}. This information suggests that, while plasmid propagation could give rise to a combination of low-level fusidic acid resistance and high-level mupirocin resistance, chromosomal mutation could give rise to a combination of high-level fusidic acid resistance and low-level

mupirocin resistance. In our current study, the rate of resistance to fusidic acid was observed to be lower in high-level mupirocin-resistant isolates compared to low-level mupirocin-resistant isolates.

Retapamulin is a derivative of pleuromutilin, which is derived from *Clitopilus scyphoides*, an edible mushroom. Pleuromutilin displays a unique mode of action, which involves inhibiting protein synthesis primarily by inhibiting ribosomal activity at three sites in the following manner: selectively binding to a site on the 50S subunit of the bacterial ribosome, binding to protein L3 at site P of the ribosome, and inhibiting ribosomal peptidyl transferase activity. Multi-target sites and novel binding sites that differ from those of other antibiotics minimize the development of retapamulin resistance. Currently, there are no approved CLSI or BSAC breakpoints for retapamulin; there is only an epidemiologic cutoff value recommended by EUCAST. As per the guidelines of EUCAST and Traczewski and Brown¹⁷, MICs of ≤ 0.5 , 1, and ≥ 2 $\mu\text{g/ml}$ can be interpreted as susceptible, intermediate, and resistant, respectively. In an *in vitro* study of 664 *S. aureus* isolates from the United Kingdom, retapamulin at concentrations of ≤ 0.25 mg/L inhibited 663 (99.9%) isolates including many fusidic acid-resistant and/or highly mupirocin-resistant isolates¹⁸. Candel et al.¹⁹ reported that, in Spain, retapamulin inhibited all isolates of MSSA and MRSA that were susceptible to linezolid; however, linezolid-resistant MRSA isolates were resistant to retapamulin, with MICs over 32 mg/L. A report from the United States of America in 2013 revealed that, among 155 MRSA isolates, 2.6% were resistant to retapamulin²⁰. The test organisms included strains resistant to vancomycin, linezolid, daptomycin, and mupirocin. Thus, a small number of studies have tested the susceptibility of *S. aureus* to retapamulin. However, the susceptibility of Korean isolates of *S. aureus* to retapamulin is not known. In our study, we did not identify any mupirocin-resistant MRSA isolates that were also resistant to retapamulin.

To the best of our knowledge, this is the first report to describe the *in vitro* antimicrobial activity of retapamulin against clinical isolates of mupirocin-resistant MRSA. From the results of this study, we conclude that retapamulin could be an effective antibiotic for the treatment of mupirocin-resistant MRSA infections in Korea. The resistance rate to fusidic acid was lower in high-level mupirocin-resistant isolates than in low-level mupirocin-resistant isolates, suggesting the importance of additional studies with more isolates. Furthermore, the abuse of topical antibiotics should be avoided to forestall further increases in antibiotic resistance.

ACKNOWLEDGMENT

This paper was supported by Wonkwang University in 2015.

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