Master of Science

Prevalence of rifampin resistance in *Staphylococcus aureus* isolates

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Prevalence of rifampin resistance in *Staphylococcus aureus* isolates

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Abstract

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Antibiotic resistance remains an important problem around the world and the frequency of *Staphylococcus aureus* (*S. aureus*) resistant to rifampin (RIF) has increased dramatically. Mutations in the rifampin resistant determining region (RRDR) of *rpoB* have been shown to confer resistance to RIF in *S. aureus*. This study aimed to investigate the prevalence of RIF resistance in clinical isolates, and to analyze mutation of *rpoB* in 1615 *S. aureus*. The samples obtained from Asan Medical Center, Seoul, Republic of Korea from 2008 to 2017. The MIC of RIF was carried out by using the broth microdilution method following standards recommended by the CLSI. The molecular typing of the isolates was performed MLST, *spa* and SCC*mec* and tested for *rpoB* mutation by PCR. Of the 843 MRSA isolates, 52(6.2%) were resistant to RIF and among 772 MSSA isolates, 5 (0.6%) were resistant to RIF ($p<0.001$). Of the 52 isolates, 51 (98.1%) were high-level RIF resistant (MIC≥8 ㎎/L) while only one (1.9%) has a low-level resistance to RIF (MIC4 ㎎/L). We identified 19 different types of mutations in the *rpoB* gene mutation analysis. Of these, single mutation (33/48, 68.8%) and multiple mutations (15/48, 31.3%) were confirmed.

The most common single mutation is A477D (17/48, 35.4%) and the most common spa is t2460 (27/52, 51.9%). We found ST5-MRSA-Ⅱ-spa t2460 (26/52, 50%) molecular type with high resistance to RIF. In conclusion, RIF R of *S. aureus* is closely associated with mutations in the *rpoB* gene and these data suggest that ST5-MRSA-II-spa t2460 confers resistance to RIF.

Keywords: *Staphylococcus aureus*, rifampin resistant, *rpoB* gene, A477D, Mutation

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Introduction

Staphylococcus aureus (*S. aureus*) is one of the most prevalent and clinically significant pathogens worldwide, which causes a variety of illnesses, ranging from minor infections of the skin to life-threatening infections with bacteremia, endocarditis, pneumonia and toxic shock syndrome[1]. Especially Multidrug-resistance and high infection rates are common features of *S. aureus* and are growing problems in hospital settings[2]. MRSA, which has been isolated from domestic clinical trials, has accounted for 60-70% since the early 1990s and more than 80% in domestic nursing hospitals[3]. From witnessing the rapid decrease in recent years of the efficacy of antibiotic treatment of common diseases due to the emergence of multi-antibiotic resistance[4]. Rifampicin resistance emerges easily in *S. aureus*, Especially in methicillin resistant Strains[5]. The prevalence of RIF-R MRSA has risen rapidly in the past few years and remains at a high resistance rate[2]. Rifampin is one of a small number of antimicrobial agents that retains activity against multidrug-resistant MRSA. Rifampin has potent anti-staphylococcal activity and is currently indicated in combination therapy for implant-associated infections, serious *S. aureus* infections, and to eradicate asymptomatic carriage of MRSA[6, 7]. Rifampin alone is very effective against Gram-positive cocci, such as *S. aureus* and has excellent bactericidal properties against staphylococci. It can also effectively eliminate adherent staphylococci on the stationary phase, where MIC is 10-100 times higher than normal[8, 9]. RIF interferes with transcription and elongation of RNA by binding to the DNA-dependent RNA polymerase[10]. Rifampicin resistance in all bacterial species has been attributed to changes in the beta subunit structure of RNA polymerase brought about by missense mutations in the *rpoB* gene[11, 12].

Thus, while it is well recognized that mutations in *rpoB* play a central role in

S. aureus antimicrobial resistance and persistence, we lack an accurate understanding of the commonly selected mutations among *S. aureus* globally and the phenotypic consequences linked to specific mutations[13]. This study aimed to investigate the prevalence of RIF resistance in clinical isolates, and to analyze mutation of *rpoB* in 1615 *S. aureus*.

Materials and Methods

1. Collection and Isolates of *Staphylococcus aureus*

The study sample consisted 1615 *S. aureus* strains obtained with blood infection from Asan Medical Center, Seoul, Republic of Korea from 2008 to 2017. The collection of *S. aureus* was performed using Blood agar plate. This sterile medium was streaked with a cotton swab and *S. aureus* incubated over-night at 37℃. This isolate was grown to screen and analyze *S. aureus.* The Strains were stored in 20% glycerol-tryptic soy broth at -80℃ (Becton Dickinson, Sparks, MD).

2. *Agr* functionality test

We used δ-hemolysin activity to determine *agr* functionality by cross-streaking vertically to RN4220 and test strain on a sheep blood agar plate (BAP). The *β*-hemolysin (Traber & Novick, 2006) produced by RN4220 enables detection of δ-hemolysin[14]. δ-hemolytic activity was presented by an enhanced area of hemolysis at the intersection streaks.

3. Antimicrobial susceptibility tests

The antimicrobial susceptibility profiles of the *S. aureus* isolates were determined by the broth microdilution method according to the Clinical and Laboratory Standard Institute (CLSI)[15]. For broth microdilution method, serial twofold dilutions were carried out in cation-adjusted Mueller-Hinton Ⅱ broth (Becton Dickinson, Sparks, MD) in microtiter plates following standard criteria[15]. MIC is determined by the broth microdilution method and each spot is inoculated with 10 ^ 6 CFU. After 16-20 hours of incubation at 37 ℃, the MIC value is considered when the bacteria do not grow at the lowest concentration of antibiotics. Reference strain American Type Culture

Collection (ATCC) 29213 was used for quality control. MICs of rifampin (Sigma, Darmstadt, Germany) were carried out by the broth microdilution method, following standard criteria[15]. Based on the CLSI guidelines, isolates were interpreted as RIF-susceptible (MIC≤1㎎/L) and RIF-resistant (MIC≥4 ㎎/L).

Molecular typing

4. Detection of *mecA* gene

The *mecA* gene sequence (532 bp) of all MRSA isolates was amplified by PCR. The amplification of *mecA* gene using *mecA1* (5 ′ AAA ATC GAT GGT AAA GGT TGG C 3′) and *mecA2* (3 ′ AGT TCT GCA GTA CCG GAT TTG C 5 ′) primers and sequence analysis. The PCR conditions were an initial denaturation at 3min, followed by 30 cycles of 94℃ for 30s, 55℃ for 30s, and 72℃ 30s and final extension at 72℃ for 4min. PCR products (10㎕) were separated by 1% agarose gel in 0.5X Tris-borate-EDTA buffer at 100V and visualized with RedSafe.

5. Multilocus sequence typing (MLST)

The MLST of isolates were conducted through amplification of internal fragments of seven housekeeping genes of *S. aureus* as described previously [16]. The fragments were amplified by using the primers (reference): carbamate kinase (arcC), shikimate dehydrogenes e (aroE), glycerol kinase (glpF), guanylate kinase (gmk), phosphate acetyltransferas (pta), triosephophate isomerase (tpi), and acetyl coenzyme A acetyltransferase (yqiL). Following purification and sequencing of these genes, allele quantification and sequence typing were assigned using a well-characterized online database (https://pubmlst.org/).

6. SCC*mec* typing

The SCC*mec* typing of MRSA isolates was performed using the multiplex

PCR method of Oliveira and de Lencastre[17]. The eight loci (A through H) and specific pairs of primers for SCC*mec* types and subtypes I, II, III and IV as described previously[18]. The multiplex PCR conditions were an initial denaturation at 4 min, followed by 30 cycles of 94℃ for 30s, 53℃ for 30s and 72℃ 1min and final extension at 72℃ for 4min. PCR products (10ul) were separated by 1.8% agarose gel in 0.5X Tris-borate-EDTA buffer at 135V and visualized with RedSafe.

7. *Spa* typing

The staphylococcus protein A (spa) variable repeat region from each MRSA isolate was amplified by simplex PCR oligonucleotide primers as previously described[17, 19]. Purified spa PCR products were sequenced and typing of *spa* was carried out using the public *spa* database web site (http://spa.ridom.de/) for all *S. aureus* isolates.

8. Detection of rifampin resistance-associated mutations by PCR

Template DNA for PCR was obtained using WizPrep gDNA Mini Kit (Seongnam, Republic of Korea). Total DNA from *S. aureus* was purified and used as a template for amplification by PCR. rifampicin resistance determining region (RRDR) of *rpoB* gene sequence of 690 bp (nucleotides 1307 to 2020), was amplified by PCR. The amplification of *rpoB* gene using RRDR1 (5 ′ TTC AAG ATA CTG AGT CTA TCA CAC C 3 ′) and *rpoB* RRDR2 (3 ′ GCA CG T GAT TCT GGT GCA GCT ATT A 5 ′) primers and sequence analysis. The amplification was carried out in 52 RIF-R and 50 RIF-S strains. Amplification was carried out as previously described[20]. 5 oligonucleotide primers rpoB1-F (5 ′ ATG GTA TTT AGC TAA AAG CGG 3 ′), rpoB1-R (3 ′ GCA CTG AAA ACA CTG AAC AA 5 ′), rpoB2-F (5 ′) ATT AGG TTT CTC AAG TGA CC 3 ′), rpoB2-R (3 ′ CCA TTA GCT GAG TTA ACG CAT 5 ′), rpoB3-F (5 ′ AAG CAG TGC CTT TGA TGA ATC C 3 ′), rpoB3-R (3 ′ CCT AAA GGT GTA ACT GAG TT 5 ′), rpoB4-F (5 ′ TTG GTG CAG AAG TAA

AAG ATG G 3 '), rpoB4-F (3 ' GGT GTA ATG TAC ATG TTG AA 5 '), rpoB5-F (5 ' AAT CTT GGT ATT CAC GTT GC 3 ') and rpoB5-R (3 ' GCT GAA TTT TAT TGA TGA TT 5 ') were identified using for mutation. The PCR products were purified and analyzed by DNA sequencing. The PCRs were performed in a DNA thermal cycler (Thermo Fisher Scientific, Waltham, United States of America). The *rpoB* PCR cycling programs consisted of an initial denaturation (4 min at 94℃) followed by 35 cycles of denaturation (30 sec at 94℃), annealing (45 sec at 53℃), and extension (45 sec at 72℃), with a final extension (3 min at 72℃). The except RRDR PCR cycling programs consisted of an initial denaturation (4 min at 94℃) followed by 35 cycles of denaturation (45 sec at 94℃), annealing (45 sec at 50℃), and extension (1 min at 72℃), with a final extension (10min at 72℃). DNA sequencing was carried out at the COSMO genetech (Seoul, Republic of Korea). The nucleotide sequences obtained were compared to the *rpoB* wild type sequence from *S. aureus* subsp. aureus (GenBank accession number: N315) using the clustalw software (https://www.genome.jp/tools-bin/clustalw).

9. Statistical analysis

The statistical distribution and clinical characteristics of the patients were compared using cross analysis and χ^2-test respectively. A P-value <0.05 was considered statistically significant. All statistical analyses were performed using the statistical package SPSS (v. 24.0) software (SPSS Inc.).

Results

1. Characteristics of study population and strains.

Among the 1615 *S. aureus* samples, 57 (0.4%) were resistant to RIF. Antibiotic susceptibility testing by the broth microdilution method revealed that 52 RIF-R *S. aureus* isolates (6.2%) were MRSA and 5 RIF-R *S. aureus* isolates (0.6%) were MSSA. Among the 57 RIF-R *S. aureus* samples, 52 (91.2) were MRSA, whereas 5 (8.8) isolates were MSSA. RIF resistance was more common in MRSA than MSSA ($P < 0.001$). Of the 57 RIF-R *S. aureus*, 44 (77.2) isolates were ST5, whereas 13 (22.8) isolates were non-ST5 ($P < 0.001$) (Table 3). Conversely, of the 1557 RIF-S *S. aureus*, 499 (32) isolates were ST5, whereas 1058 (68) isolates were non-ST5. Among 52 RIF-R cases, 37 (71.2%) were male while 15 (28.8%) were female patients with bacteremia.

Table 1. Sequence Type (ST) and resistance rate of MRSA and MSSA According to rifampin Susceptibility

MRSA (n=843)	ST5 (n=507)	ST72 (n=264)	ST254 (n=6)	ST1 (n=3)
RFP R (n=52) 6.2%	44 (84.6%, 44/52) resistance rate: 8.7%	5 (8.6%, 5/52) resistance rate: 1.9%	2 (3.4%, 2/52) resistance rate: 33.3%	1 (1.7%, 1/52) resistance rate: 33.3%
RFP S (n=791) 93.8%	463 (58.5%, 463/791)	257 (32.3%, 257/796)	4 (0.5%, 4/796)	2 (0.3%, 2/796)
MSSA (n=772)	ST72 (n=139)	ST6 (n=51)	ST101 (n=13)	ST45 (n=8)
RFP R (n=5) 0.6%	2 (33.3%, 2/6) resistance rate: 1.4%	1 (16.7%, 1/6) resistance rate: 2.0%	1 (16.7%, 1/6) resistance rate: 7.7%	1 (16.7%, 1/6) resistance rate: 12.5%
RFP S (n=767) 99.4%	137 (17.8%, 137/767)	50 (6.5%, 50/767)	12 (1.6%, 12/767)	7 (0.9%, 7/767)

Table 2. Clinical and microbiological characteristics of the rifampin-resistant MRSA isolates studied

Patient isolate	Year/month	Age(years)	Sex	Source	Site of acquisition	ST	SCC*mec*	*SPA* type
1	06-Sep-08	55	M	blood	HA	5	ⅡA	t2460
2	09-Sep-08	64	M	blood	HA	5	ⅡB	t2460
3	20-Sep-08	72	M	blood	HA	1	ⅣA	t2460
4	12-Oct-08	81	M	blood	nosocomial	5	ⅡB	t2460
5	28-Jul-09	76	M	blood	nosocomial	5	ⅡB	Unknown
6	11-Sep-09	84	M	blood	nosocomial	5	Ⅱ	t002
7	04-Oct-09	78	M	blood	nosocomial	5	ⅡB	t002
8	29-Oct-09	63	F	blood	nosocomial	5	Ⅱ	t002
9	08-Nov09	84	M	blood	nosocomial	5	ⅡB	t9353
10	08-Dec-09	64	M	blood	nosocomial	5	ⅡB	t2460
11	06-Aug-10	63	M	blood	nosocomial	5	ⅡB	t1228
12	16-Oct-10	87	F	blood	nosocomial	5	ⅡB	t2460
13	19-Dec-10	70	M	blood	nosocomial	5	ⅡB	t324
14	27-Dec-10	77	M	blood	nosocomial	5	ⅡB	t9353
15	16-Jan-11	46	M	blood	nosocomial	5	ⅡB	t2460
16	20-Jan-11	68	M	blood	HA	5	ⅡB	t2460
17	04-Feb-11	81	F	blood	nosocomial	5	ⅡB	t2460
18	08-May11	49	M	blood	nosocomial	254	ⅠC	t688
19	08-Jun-11	72	M	blood	nosocomial	5	ⅡB	t2460
20	19-Jun-11	60	M	blood	nosocomial	5	ⅡB	t2460
21	28-Jul-11	69	F	blood	nosocomial	254	ⅠC	t324
22	28-Jul-11	70	F	blood	nosocomial	5	ⅡB	t2460
23	05-Sep-11	52	M	blood	nosocomial	5	ⅡB	t2460
24	12-Oct-11	52	M	blood	nosocomial	5	ⅡB	t2460

25	07-Dec-11	69	F	blood	HA	5	ⅡB	t2460
26	08-Jan-12	63	M	blood	nosocomial	72	ⅣA	t664
27	09-Jan-12	28	M	blood	nosocomial	5	ⅡB	t2460
28	22-Mar-12	37	F	blood	nosocomial	5	ⅡB	t002
29	11-Apr-13	79	M	blood	nosocomial	5	ⅡB	t2461
30	16-Apr-13	32	F	blood	HA	72	ⅣA	Unknown
31	24-Jan-14	75	F	blood	nosocomial	5	ⅡB	t2460
32	18-May14	65	M	blood	nosocomial	72	ⅣA	t2431
33	12-Jul-14	67	M	blood	nosocomial	5	ⅡB	t2460
34	30-Aug-14	71	M	blood	nosocomial	5	ⅡB	t2460
35	20-Oct-14	65	F	blood	HA	5	ⅡB	t324
36	07-Jan-15	57	M	blood	nosocomial	5	ⅡB	t2460
37	12-Jan-15	55	F	blood	nosocomial	5	ⅡB	t9363
38	09-Mar-15	62	M	blood	nosocomial	5	ⅡB	t2460
39	24-Jul-15	22	F	blood	nosocomial	5	ⅡB	t2460
40	04-Oct-15	51	F	blood	CA	5	ⅡB	t9353
41	06-Nov15	66	M	blood	nosocomial	5	ⅡB	t2460
42	17-Dec-15	62	M	blood	nosocomial	5	ⅡB	t2460
43	19-Feb-16	40	M	blood	HA	5	ⅡB	t2460
44	01-Jun-16	69	M	blood	nosocomial	5	ⅡB	t264
45	19-Jul-16	62	M	blood	nosocomial	5	ⅡB	t2460
46	01-Aug-16	66	M	blood	nosocomial	5	ⅡB	Unknown
47	30-Aug-16	73	M	blood	HA	5	ⅡB	t2460
48	24-Nov16	52	F	blood	CA	5	ⅣA	Unknown
49	30-Dec-16	66	M	blood	CA	72	ⅣA	t148
50	15-Mar-17	48	M	blood	nosocomial	5	ⅡB	t18239
51	19-Jun-17	77	M	blood	HA	72	ⅣA	t324
52	04-Aug-17	81	F	blood	HA	5	ⅡB	t564

CA: Community-acquired, HA: Health care-associated

2. Delta-hemolysin activities genotypic characteristics of *S. aureus* isolates by rifampin

Table 3 shows the differences in genotypic characteristics between *S. aureus* isolates with RIF susceptible and resistance. The *agr* functionality test was performed with 1615 *S. aureus*. There were 14 (24.6%) functional *agr* (hemolytic strains) resistant to RIF and 835 (53.6%) *agr* (hemolytic strains) susceptible to RIF. whereas, 43 (75.4%) dysfunctional *agr* (nonhemolytic strains) rifampin resistance and 723 (46.4%) dysfunctional *agr* (nonhemolytic strains) susceptible to rifampin.

Table 3. Genotypic characteristics of *S. aureus* isolates stratified by rifampin

Genotype	No. (%) of isolates		*P* value
	Rifampin resistance	Rifampin susceptible	
n= 1615			
MRSA	52 (91.2)	791 (48.9)	<0.001
MSSA	5 (8.8)	767 (47.5)	
n= 1615			
agr fx	14 (24.6)	835 (53.6)	<0.001
agr dysfx	43 (75.4)	723 (46.4)	
n=1614			
ST5	44 (77.2)	499 (32)	<0.001
Non-ST5	13 (22.8)	1058 (68)	

$p^{*} < .1$, $p^{**} < .05$, $p^{***} < .001$

3. Molecular typing

The SCC*mec* typing, MLST and spa typing were carried out in the 52 RIF-R MRSA strains. Results are shown in Table2. Analysis of MLST showed that these 843 MRSA and 772 MSSA isolates are 507 ST5 (60.1%), 264 ST72 (31.3), 6 ST254 (0.7%), 3 ST1 (0.4%) in MRSA and 139 ST72 (18%), 51 ST6 (6.6%), 13 ST101 (1.7%), 8 ST45 (1%) in MSSA. 41 different STs were found among the MSSA isolates. Also, MLST analysis revealed that a total of 84.6% of MRSA RIF-R isolates belonged to ST5, with 5 ST72 (8.6%), 6 ST254 (3.4%), and 3 ST1 (1.7%).

Analysis of Staphylococcal chromosomal cassette *mec* (SCC*mec*) typing showed that these 52 isolates of RIF-R MRSA are 40 Ⅱ B (76.9%), 7 Ⅳ A (13.5%), 2 Ⅰ C (3.8%), 2 Ⅱ (3.8%), 2 Ⅱ A (1.9%). The SCC*mec* type Ⅱ B (76.9%) was predominant.

The results of spa typing revealed a diverse distribution among 52 RIF-R MRSA strains. The spa types of the 52 MRSA were t2460 (51.9%), t002 (7.7%), t324 (7.7%), t9353, t324, t564, t1228, t18239, t2461, t264, t664, t2461, t148, t688, t9363 and 4 spa types were left undetermined.

We found ST5-MRSAⅡ-spa t2460 (26/52, 50%) molecular type with high resistance to RIF. The remaining molecular types were identified as ST5-MRSAⅡ-t9353 (3/52, 5.8%) and ST5-MRSAⅡ-t002 (2/52, 3.8%). Among MSSA isolates, the ST72-MSSAⅠ-spa t126 (2/6, 33.3%) isolates from the persistent carrier were resistant to RIF.

Table 4. Prevalence of rifampin resistance according to the spa type in ST5-MRSA, non ST5-MRSA

	No. (%) of isolates with rifampin resistance	
spa typing	ST5-MRSA	Non ST5-MRSA
t2460 (n=27)	26 (96.3)	1 (100)
t002 (n=4)	4 (100)	0 (0)
t9353 (n=3)	3 (100)	0 (0)
t324 (n=4)	2 (50)	2 (50)
t564 (n=1)	1 (100)	0 (0)
t1228 (n=1)	1 (100)	0 (0)
t18239 (n=1)	1 (100)	0 (0)
t2461 (n=1)	1 (100)	0 (0)
t264 (n=1)	1 (100)	0 (0)
t664 (n=1)	0 (0)	1 (100)
t2461 (n=1)	0 (0)	1 (100)
t148 (n=1)	0 (0)	1 (100)
t688 (n=1)	0 (0)	1 (100)
t9363 (n=1)	1 (100)	0 (0)
Unknown (n=4)	3 (75)	1 (25)
Total (n=52)	44 (84.6)	8 (15.4)

4. Profile of *rpoB* gene mutations in Rifampin resistant isolates

Among the 52 RIF-R MRSA isolates, 51 isolates showed high-level rifampin resistance (MIC ≥8 ㎎/L) and 1 isolates showed low-level rifampin resistance (MICs 4㎎/L). Results are shown in Figure 1. Mutations were not detected in 4 of the 52 isolates resistant to RIF. Amplification of the 714bp RRDR region of *rpoB* gene processed for DNA sequence analysis. *rpoB* gene mutations were detected in 41 out of 48 (85.4%) isolates when compared with *rpoB* gene sequence of the reference strain. Out of 48, 17 (35.4%) isolates showed a mutation at codon 477 (gct to gat), 4 (8.3%) isolates exhibited a mutation at codon 481 (cat to tat). 8 RIF-resistant *S. aureus* isolates (I448L, T480K, L485I, E568K, D668E, T801A, K1166I, K1584I) did not any known RIF-resistant associated mutations throughout the *rpoB* gene. Amino acid substitution was not detected among the 50 RIF-S isolates.

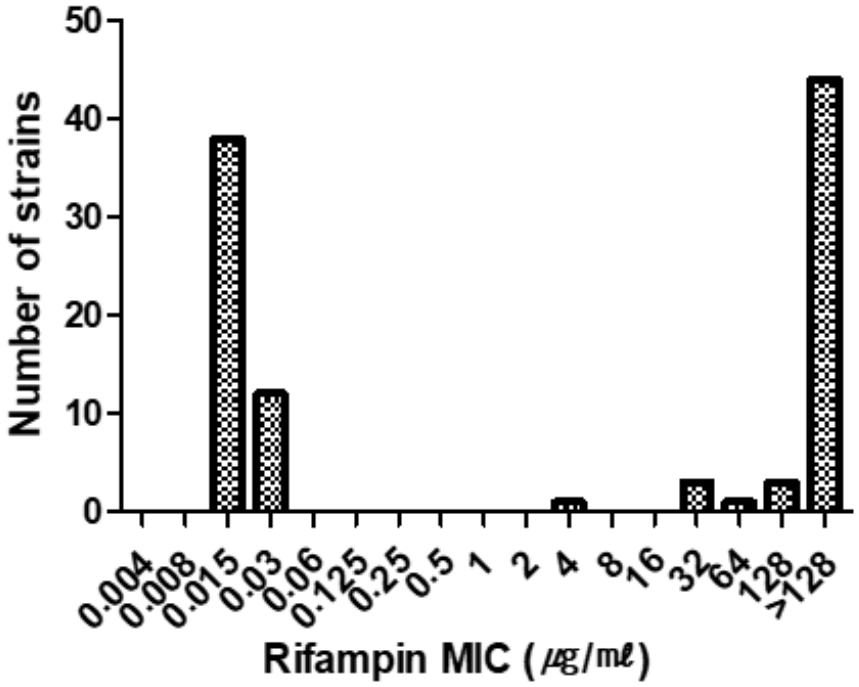

Fig 1. Distribution of RIF MICs for 102 MRSA strains isolated at Seoul Asan Medical Center between 2008 and 2017. Of the 843 MRSA isolates, 52(6.2%) were resistant to RIF and among 772 MSSA isolates, 5 (0.6%) were resistant to RIF (p <0.001). Of the 52 isolates, 51 (98.1%) were high-level RIF resistant (MIC ≥8 ㎎/L) while only one (1.9%) has a low-level resistance to RIF (MIC 4 ㎎/L).

Table 5. Correlation of mutations in the *rpoB* gene and the level of resistance to rifampin

MRSA *rpoB* mutations		Rifampicin MIC	
Nucleotide mutation	Amino acid substitution	MIC ㎍/㎖	Number of isolates
cgt/ctt	R197L	128	1
cgt/cat	R484H	>128	1
agc/aac+ tct/cct	S463N, S464P	>128, 128	2
tct/cct	S464P	32	2
tct/cct+ att/ctt	S464P, I527L	>128	1
caa/cga	Q468R	>128	1
caa/cta	Q468L	>128	2
cat/tat	H481Y	>128	4
cat/aat+ att/atg	H481N, I527M	>128	1
cat/aat+ att/atg+ gaa/aaa	H481N, I527M, E568K	>128	1
cat/aat+ tca/tta	H481N, S529L	>128	1
cat/cgt	H481R	>128	1
gct/act+ cat/aat	A477T, H481N	>128	1
tca/tta	S486L	>128	1
gct/gat	A477D	>128	17
gct/gat+ act/gct	A477D, T801A	>128	2
gac/ggc	D471G	>128	1
gac/gaa+ gct/gat	D471E, A477D	>128	1
caa/cta+ gat/gaa	Q468L, D668E	>128	1
caa/cta+ aaa/ata	Q468L, K1166I	>128	1
att/ctt+ gct/gat	I448L, A477D	>128	1
agc/aac+ tct/cct+ aaa/ata	S463N, S464P, K1584I	128	1
gaa/aaa	E568K	>128	1

cta/ata+ acg/aag	L485I, T480K	64	1
att/cat	I527H	32	1

5. Resistance pattern profile about rifampin resistant MRSA

Fig 2 show the percentage of antibiotic resistance of isolated *S. aureus*. The results indicate that 52 RIF-R MRSA. Out of these MRSA strains 100%, 94.2%, 92.3%, 94.2%, 76.9%, 73.1%, 100%, 100%, 71.1%, 0%, 7.7% were resistant for ampicillin, clindamycin, ciprofloxacin, Erythromycin, Fusidic acid, Gentamicin, Oxacillin, Penicillin, Tetracycline, Quinupristin and TMP-SMX respectively.

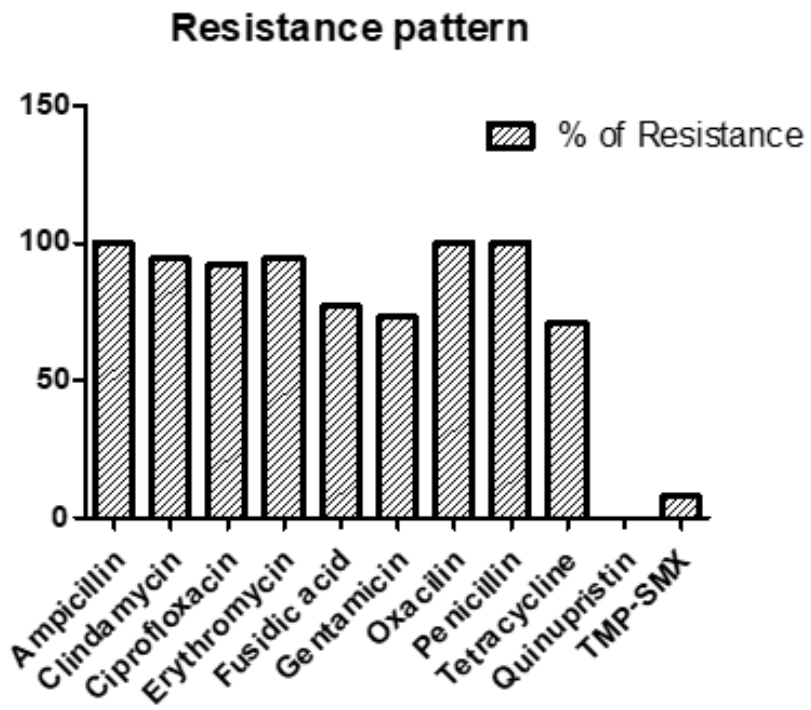

Fig 2. Percentage of antibiotic resistance in rifampin resistant MRSA

Discussion

The epidemiology of prevalent MRSA clones is changing dynamically and geographically. Previous studies had reported high prevalence of ST59 and ST338 in community-acquired infections across China while ST8 was the major clonal lineage in United States and Europe[21, 22]. We aimed to help establish the foundation for *S. aureus* molecular biology research by sequencing patterns of rifampin resistance. We were subdivided into four molecular types. Our results suggest that ST5-MRSAⅡ-spa t2460 clones with RIF resistant were clonally spread in the hospital. In particular, MRSA strains showed high resistance rates to various antibiotics[23].

Drug resistance mostly develops by mutational interplay rather than acquisition of resistant genes transfer from other bacteria[24]. Mutations in *rpoB* gene have been reported earlier in Asian countries, which are generally associated with a high-level of resistance to RIF[25]. Here, we have amplified and sequenced portions of *rpoB* from RIF-R *S. aureus* isolates. High-level rifampicin resistance may also be attributed to additional mutations within *rpoB*, as previously described[26].

Mutations conferring rifampicin resistance are confined almost exclusively to the *rpoB* gene in most microorganisms (24). In this study, sequencing of the RRDR successfully identified 46 of 52 (88.5%) of the RIF-R MRSA isolates. Except for RRDR, *rpoB* gene inside mutation was 9/52 (17.3%). 4 (7.7%) RIF-R MRSA isolates lack any mutation in the RRDR region of the *rpoB* gene, though these isolates were phenotypically resistant to RIF. This difference might persist due to genotype variations prevailing worldwide however, the presence of mutations in the *rpoB* gene other than the RRDR region could not be excluded as described by Heep et al[27]. Lack of RRDR mutations in resistant isolates might be due to the presence of other rare

rpoB mutations or another mechanism of resistance to rifampicin[27, 28]. These findings are consistent with previous reports from several countries[27-31]. Also, 4 strains (7.7%) likely possess additional mutations outside the RRDR, as they exhibited rifampin resistance levels higher than those conferred simply by the mutations that they possessed in their RRDR alleles.

Mutant H481Y is known to be the most prevalent, but these results were different from previous studies in other regions where the H481Y MRSA clone predominates. These majority of the 17 RIF-R MRSA (n = 17, 35.4%) have the amino acid substitution A477D, which is different from previous reports[22]. The transformation of A477D mutated *rpoB* into the wild-type *S. aureus* strains also makes a rifampicin-resistant phenotype, indicating that the mutations contribute to rifampicin resistance in *S. aureus*. For the A477D mutant, this substitution places a negatively charged carboxylate unit in close proximity to an existing carboxylate from H481, which lies close to the protein-DNA interface and this would increase the negative charge on this surface of the protein and destabilize the enzyme-DNA interaction due to electrostatic repulsion[32]. Even if this substitution did not face the rifampicin target region, it could induce a conformational change that indirectly prevents antibiotic binding to the target site, this determining RIF-R[33].

Mutations in *rpoB* are not only associated with resistance to RIF, but also to other antibiotics including vancomycin and daptomycin[34, 35]. In our study, ampicillin, Oxacillin and Penicillin of 52 RIF-R MRSA were 100 percent resistant. Further research is needed to understand whether cross-resistance to other antibiotics developed during prolonged RIF exposure, or whether resistance rapidly evolved during exposure to antibiotics[36].

Necessary to prevent and protect the spread of high-level rifampicin resistant *S. aureus*. In addition, Treatment of MRSA infections requires prompt elimination and the use of appropriate antibiotics. Vancomycin is still the most highly recommended antibiotic, but if treatment fails, the most researched and proven therapeutic rifampin can be considered alone or in

combination with rifampin-fusidic acid[37]. Further study needs to broaden the understanding of the development of MRSA disease and apply full genome sequencing to analyze underlying mechanisms.

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국문 요약

건강 관리에 따른 항균제 사용이 증가함에 따라, 다중 내성을 가진 황색포도알균 (*Staphylococcus aureus*)이 출현하여 전세계의 병원 및 지역사회 감염의 가장 흔한 원인이 되었다. 항생제 내성은 여전히 전세계에 중요한 문제로 남아있고 특히, rifampin (RIF)에 내성을 가진 methicillin에 내성을 갖는 황색포도알균 (methicillin-resistance *S. aureus*, MRSA)에서 쉽게 나타난다고 보고되었다. 본 연구는 임상 분리주에서 RIF 내성에 기여한 *rpoB* 유전자의 돌연변이 및 내성 유병률을 연구하고 RIF에 내성이 있는 *S. aureus* 분자 메커니즘을 분석하였다.

2008년부터 2017년까지 서울아산병원에서 분리한 총 1615 개의 *S. aureus*를 RIF 내성 유병률을 조사하고 *rpoB*의 돌연변이를 분석하기 위해 사용하였다. *mec*A, *rpoB* 유전자의 존재 여부 확인과 MLST, *spa* typing 및 SCC*mec*의 분자학적 특징을 규명하기 위해 각각의 특정 primer를 이용한 PCR 기법이 사용되었다. 유전자형 특징을 확인하기 위해 *Agr* functionality test을 하였고 항생제 감수성은 CLSI가 권장하는 표준에 따라 broth microdilution 방법을 사용하여 결정되었다. 또 실험 결과에 유의성을 판단하기 위해 통계분석을 실시하였다.

843개의 MRSA 균주 중 52개 (6.2%)가 RIF에 내성을 보였고, 772개의 MSSA 균주 중에서 5개 (0.6%)가 RIF에 내성이 있었다 ($p < 0.001$). 52 개의 균주로 감수성 테스트 결과, 51개 (98.1 %)는 높은 수준의 RIF 내성 (MIC ≥8 ㎎/L)인 반면, 단 한 개 (1.9%) 균주 만이 RIF에 대한 낮은 수준의 내성 (MIC ≤ 4㎎/L)을 가졌다. 분자학적 검사를 수행했을 때 MLST, SCC*mec*, spa typing의 대표적인 타입으로는 각각 ST5 (44/52, 84.6%), ⅡB (40/52, 76.9%), t2460 (27/52, 51.9 %)이 우세했다. 52개의 MRSA 균주와 무작위로 선별한 50개의 MSSA 균주로 *rpoB* 유전자 돌연변이를 분석한 결과 19 종류의 돌연변이를 확인하였다. 이들 중 단일 돌연변이 (33/48, 68.8%) 및 다중 돌연변이 (15/48, 31.3%)를 확인하였고 가장 흔한 단일 돌연변이는 A477D (17/48, 35.4%) 이었다. 결론적으로, *S. aureus*의 RIF 내성은 *rpoB* 유전자의 돌연변이와 밀접한 관련이 있으며, 이러한 데이터는 ST5-MRSA-II-spa t2460 (26/52, 50%)이 RIF에 대한 내성을

부여한다는 것을 시사한다.

MRSA 치료에 있어서 반코마이신은 여전히 가장 권장되는 항생제이지만 치료가 실패하면 가장 연구되고 입증된 항생제인 RIF을 고려할 수 있다. 이 논문은 혈액에서 분리된 MRSA의 분자생물학적 분석을 시행하고 rifampin에 내성을 일으키는 돌연변이를 확인하였다는데 큰 의의를 가질 수 있다. 또한 높은 수준의 RIF 내성을 갖는 *S. aureus*를 연구함으로써 감염의 확산을 예방하고 방지하는데 도움이 될 것이다. 추가 연구는 MRSA 질환의 발달에 대한 이해를 넓히고 전체 게놈 시퀀싱을 적용하여 기본 메커니즘을 분석하는 연구가 이루어져야 될 것으로 생각한다.

주요어: 황색포도알균, 리팜핀 내성, *rpoB* 유전자, A477D, 돌연변이