Journal of Microbiology, Immunology and Infection (2016) 49, 394—401

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jmii.com

ORIGINAL ARTICLE

RNA polymerase B subunit gene mutations in biofilm-embedded methicillin-resistant *Staphylococcus aureus* following rifampin treatment

Hung-Jen Tang [a,b], Chih-Cheng Lai [c], Po-Ren Hsueh [d,e], Chi-Chung Chen [f,g], Kuan-Ying Wu [f], Yi-Chung Lin [f], Chun-Cheng Zhang [a], Tzu-Chieh Weng [a], Yu-Hsin Chiu [h], Han-Siong Toh [a], Shyh-Ren Chiang [a], Wen-Liang Yu [a,f], Wen-Chien Ko [i,**], Yin-Ching Chuang [a,f,h,*]

[a] *Department of Medicine, Chi Mei Medical Center, Tainan, Taiwan*
[b] *Department of Health and Nutrition, Chia Nan University of Pharmacy and Science, Tainan, Taiwan*
[c] *Department of Intensive Care Medicine, Chi Mei Medical Center, Liou Ying, Tainan, Taiwan*
[d] *Department of Laboratory and Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan*
[e] *National Taiwan University College of Medicine, Taipei, Taiwan*
[f] *Department of Medical Research, Chi Mei Medical Center, Tainan, Taiwan*
[g] *Institute of Biotechnology, National Cheng Kung University Medical College and Hospital, Tainan, Taiwan*
[h] *Department of Internal Medicine, Chi Mei Medical Center, Liou Ying, Tainan, Taiwan*
[i] *Department of Medicine, National Cheng Kung University Medical College and Hospital, Tainan, Taiwan*

Received 12 February 2015; received in revised form 2 June 2015; accepted 30 June 2015
Available online 1 August 2015

KEYWORDS
biofilm-embedded MRSA;
mutations;

Abstract *Background/Purpose:* This study was conducted to compare the mutation rates of different *rpoB* sites and rifampin minimum inhibitory concentration (MIC) changes prior to and after rifampin therapy for biofilm-embedded methicillin-resistant *Staphylococcus aureus* (MRSA) isolates.

* Corresponding author. Yin-Ching Chuang, Department of Medical Research, Chi Mei Medical Center, Number 901 Chung-Hwa Road, Yung-Kang District, 710 Tainan, Taiwan.
** Corresponding author. Wen-Chien Ko, Department of Internal Medicine, National Cheng Kung University Hospital, Number 138, Sheng Li Road, 704 Tainan, Taiwan.
E-mail addresses: winston3415@gmail.com (W.-C. Ko), chuangkenneth@hotmail.com (Y.-C. Chuang).

http://dx.doi.org/10.1016/j.jmii.2015.06.006

rpoB gene

Methods: The screening of rifampin-resistant MRSA isolates, from the biofilm at Day 5 with or without exposure to the susceptible breakpoint concentration of rifampin recommended by the Clinical and Laboratory Standards Institute (1 mg/L), was conducted using agar plates containing rifampin. A partial fragment of RNA polymerase B subunit gene (*rpoB*), including clusters I and II, was amplified and sequenced. The rifampin MIC values and mutation frequencies at different sites of *rpoB* were measured and evaluated in rifampicin-resistant isolates.
Results: Rifampin-resistant mutants could be selected from all of 39 randomly selected rifampin-susceptible MRSA isolates in the biofilm model. The spontaneous mutation frequency ranged from 1.00×10^{-4} to 3.85×10^{-7}. Mutation at codon 481 was most commonly found at 35 (89.7%) of 39 MRSA isolates. Without rifampin induction, the MIC ranged between 0.125 mg/L and1024 mg/L and mutation sites included cluster I 464, 466, 468, 471, 474, 477, 481, 484, 486 and cluster II 519, 527, 529 with the percentage of 471 (35.9%), 477 (33.3%), 481 (53.8%), and 484 (35.9%). Conversely, with the induction of rifampin, the MIC value ranged ~256—1024 mg/L. The mutation sites that were more concentrated included 468 (17.9%), 477 (30.8%), 481 (89.7%), 484 (17.9%), and 486 (33.3%).
Conclusion: We documented high rifampin resistance induction activity when MRSA was engaged in biofilm with rifampin exposure. Monotherapy seems to be inadequate for MRSA in biofilm. There is an urgent need for developing effective combination therapies with less rifampin resistance-inducing activities for treating MRSA in biofilms.

Introduction

Biofilm-embedded methicillin-resistant *Staphylococcus aureus* (MRSA) has been a common clinical problem, and antimicrobial therapy has always been of limited success if the infected prosthesis or foreign bodies were retained.[1,2] The development of antibiotic combinations to improve the antibacterial activity against the biofilm-embedded microorganisms has been welcomed.[3—5] One of the common combinations was the rifampin-containing regimen. However, it is well known that rifampin-resistant isolates with point mutations in RNA polymerase B subunit genes (*rpoB*) were common with rifampin therapy for planktonic MRSA, with a mutation frequency of $\sim 10^{-6}$—10^{-8}.[6—8] The emergence and spread of rifampin-resistant MRSA during vancomycin—rifampin combination therapy in an intensive care unit has been reported.[9] The possibility of the emergence of rifampin-resistant mutants with *rpoB* mutation was high when rifampin was used as a component of combination therapy to treat biofilm-embedded MRSA infections.[4,10,11] The clinical setting may further induce the production of vancomycin-intermediate *S. aureus*.[12,13] These research results highlighted the risk of treatment failure with the combination of vancomycin and rifampin.

According to our recent study on rifampin-based combinations against biofilm-embedded MRSA, we found that some combinations were prone to induce rifampin-resistant mutants with high rifampin minimum inhibitory concentrations (MICs).[11] The phenomenon was more obvious for vancomycin-, teicoplanin-, or daptomycin-based combination regimens. However, the frequency of emergence of rifampin-resistant mutants and genetic profiles among biofilm-embedded MRSA is not clear. Therefore, we decided to study some of the MRSA isolates from the program Tigecycline *In vitro* Surveillance in Taiwan (TIST), and to investigate rifampin-resistant patterns and *rpoB* mutation profiles among biofilm-embedded MRSA isolates.

Methods

Isolates

One hundred MRSA isolates causing a variety of clinical infections, including central venous catheter, vascular graft, orthopedic prostheses, or ventricular shunt infection, were obtained from the TIST study at 22 hospitals from 2006 to 2010.[14] Staphylococci were identified with colonial morphology, Gram stain, and coagulase test. MRSA was further confirmed by the tube coagulase test and growth on 6 μg/mL oxacillin salt agar screen plates. Isolates were stored at −70°C in Protect Bacterial Preservers (Technical Service Consultants Limited, Heywood, UK) until use. From 78 rifampin-susceptible strains, 39 unique isolates were randomly selected for further study, and their genetic relatedness was excluded by pulse-field gel electrophoresis as previously described.[15,16]

Antibiotics

The antibiotics tested included vancomycin, rifampin, and minocycline (Sigma-Aldrich, St Louis, MO, USA), fosfomycin (Ercros, Barcelona, Spain), linezolid and tigecycline (Pfizer, New York, NY, USA), fusidic acid (Leo Pharma, Ballerup, Denmark), teicoplanin (Sanofi-Aventis, Bridgewater, NJ, USA), ciprofloxacin (Bayer, Leverkusen, Germany), and daptomycin (Cubist Pharmaceuticals, Lexington, MA, USA). The interpretation criteria for the susceptibility test and the MIC determined by the agar dilution tests were based on the recommendations of the Clinical and Laboratory

Standards Institute or the British Society for Antimicrobial Chemotherapy.[17–19] For the fosfomycin susceptibility test, glucose-6-phosphate (25 μg/mL) was added to the agar plate. The daptomycin susceptibility test was performed in Müeller–Hinton broth (Oxoid Microbiology Products, Basingstoke, UK) adjusted to 50 μg/mL of calcium as per the standard methodology. Müeller–Hinton agar (Oxoid Microbiology Products) was used for MIC determination of *S. aureus*. Inocula were prepared by suspending growth from overnight cultures in saline to a turbidity of a 0.5 McFarland standard. Inoculated plates were then incubated in ambient air at 37°C for 24 hours. *S. aureus* ATCC 29213 was included as the control strain in each run of MIC measurements.

Killing effects of antimicrobial agents in the biofilms

Biofilms of individual strains were prepared in 24-well culture plates, according to a previously described method.[14] The medium in the well was removed by aspiration, and the biofilm was treated using rifampin alone. The concentrations of rifampin were adjusted to the susceptible breakpoint concentration (SBC) recommended by the Clinical and Laboratory Standards Institute[12] (1 μg/mL). The drug-containing medium was gently aspirated after 24 hours at 37°C. The biofilm on the wells was incubated with fresh drug dilution for 5 consecutive days and sonicated by a water-table sonicator for 5 minutes. The disrupted biofilm was serially diluted and plated for viable cell counting at 37°C following overnight culture. The detection limit of the plating count was 100 CFU/mL. All tests were performed in triplicate for each experiment to ensure reproducibility.

Determination of spontaneous mutation frequency for rifampin resistance

The screening of resistant strains from the biofilm at Day 5 was performed on agar plates containing 0 μg/mL, 0.05 μg/mL, 2 μg/mL, 8 μg/mL, or 64 μg/mL rifampin. In all cases, a sample of 100 μL from the sonicator-disrupted biofilm was serially diluted and plated for viable cell counting at 37°C following overnight culture. After 24–36 hours, six colonies growing on selective plates with rifampin (0.05 μg/mL) and nonselective plates (plates without rifampin) were selected. The MICs, mutation rates, and mutation frequencies were calculated. Mutation rate was defined as the percentage of mutation isolates among the 39 isolates and calculated as the isolates number with mutation colonies divided by 39. Mutation frequency was defined as the colony counts from plates with different rifampin concentrations divided by the colony counts from the plate without rifampin. Silent mutation was defined as the nucleotide change without the corresponding amino acid substitute.

rpoB mutation detection and DNA sequencing

Genomic DNA from MRSA was purified and used as a template for polymerase chain reaction (PCR) amplification. In the present study, a 460-bp *rpoB* fragment, including clusters I and II of *rpoB*, was amplified and sequenced by primers rpoB1 and rpoB2 as described previously.[20] The DNA sequences of the region of 1318–1602 at the nucleotide positions (nt) of *rpoB*, corresponding to codons 440–534 [amino acid (aa) number], which includes the RFP resistance-determining cluster I (1384–1464 nt, 462–488 aa) and cluster II (1543–1590 nt, 515–530 aa) of *S. aureus*, were amplified by PCR with the primers rpoB-F (5′-CCG TCG TTT ACG TTC TGT AGG-3′) and rpoB-R (5′-AAA GCC GAA TTC ATT TAC ACG-3′). The PCR products were sequenced with the same primers by the dideoxy chain termination method in an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Data analyses were performed using SPSS for Windows 17.0 (SPSS Inc., Chicago, IL, USA). Because of the small sample size and the violation of the normal distribution assumption of optical density ratios, Mann–Whitney *U* test was used to compare the differences between the two groups. Kruskal–Wallis *H* test and Dunn's test were applied for multiple comparisons. Statistical significance was set to a $p < 0.05$.

Results

Among the 100 MRSA isolates from the TIST, $MIC_{50/90}$ (μg/mL) is listed in Table 1 and 78 isolates were susceptible to rifampin. All 39 randomly selected rifampin-susceptible MRSA isolates (MIC $\leq$ 0.03 μg/mL) yielded rifampin-resistant mutants in the biofilm model. The spontaneous rifampin mutation frequency (MIC $\geq$ 0.05 μg/mL) for the 39 MRSA isolates ranged from 1.00×10^{-4} to 3.85×10^{-7}, with a mean mutation frequency of 2.49×10^{-5} (Figure 1).

The results for the 39 MRSA isolates in the biofilm after they had been exposed to 1 μg/mL of rifampin (SBC) for 5 days, including the bacterial loads ($\log_{10}$ CFU/mL) in the control plate without rifampin and plates containing 0.05 μg/mL, 2 μg/mL, 8 μg/mL, or 64 μg/mL of rifampin, can be seen in Figure 2. The mutation rate was 100%, and the mutation frequency was 1 after the rifampin treatment, and all of the mutation isolates had high rifampin MICs (> 64 μg/mL).

We also checked the rifampin MICs of the biofilm-embedded MRSA mutants cocultivated with rifampin (1 μg/mL) for 5 days, which grew on 0.05 μg/mL and the *rpoB* sequences. The mutation sites in *rpoB* and the MIC changes in the 39 MRSA isolates embedded in the biofilm are shown in Table 2. Codons 468, 477, 481, 484, and 486 were common hot spots. Among the 39 MRSA isolates, all the isolates had at least one mutation site. Only one isolate (TIST 97) had two concomitant codon mutations over 468 and 482. Nine isolates had only one codon change: 481 His–Tyr (CAT–TAT, 8 isolates) and 477 Ala–Asp (GCT–GAT, 1). Twenty isolates had two codon changes, and eight isolates had three codon changes. Only one isolate possessed four mutation sites. A mutation at codon 481 was the most common and was found in 35 (89.7%) isolates, of which 31 (79.5%) had 481 His–Tyr (CAT–TAT). All 39 parent strains had low MICs (ranging between 0.015 μg/mL and 0.03 μg/mL), but all of the mutants had high MICs, >256 μg/mL (> 1024 μg/mL in 35 strains).

Table 1 The MIC of rifampin susceptible and non-susceptible methicillin-resistant *Staphylococcus aureus* isolates from Tigecycline *In vitro* Surveillance in Taiwan (TIST).

RIF non-susceptible						
N = 22	MIC range	MIC50	MIC90	S (%)	I (%)	R (%)
VA	1—2	2	2	100	0	0
TGC	0.25—1	0.5	0.5	90.9	—	9.1
MNO	0.125—8	0.25	8	77.3	22.7	0
TEC	0.5—2	2	2	100	0	0
FA	0.25—>64	0.25	8	86.4	—	13.6
LNZ	2—8	4	4	95.5	—	4.5
CIP	1—>64	>64	>64	9.1		90.9
RIF	2—>32	4	>32	0	45.5	54.5
FOS	1—>1024	>1024	>1024	45.5	0	54.5
DAP	0.125—1	0.5	1	100	—	0
RIF susceptible						
N = 78	MIC range	MIC50	MIC90	S (%)	I (%)	R (%)
VA	1—2	2	2	100	0	0
TGC	0.25—2	0.5	0.5	91	—	9
MNO	0.125—8	0.25	8	76.9	23.1	0
TEC	0.5—2	1	2	100	0	0
FA	0.25—>64	0.25	8	84.6	—	15.4
LNZ	2—8	4	4	94.9	—	5.1
CIP	0.25—>64	1	>64	56.4	2.6	41
RIF	0.016—0.5	0.016	0.03	100	0	0
FOS	1—>1024	4	16	98.7	0	1.3
DAP	0.25—1	0.25	0.5	100	—	0

CIP = ciprofloxacin; DAP = daptomycin; FA = fusidic acid; FOS, fosfomycin; LNZ = linezolid; MIC = minimum inhibitory concentration; MNO = minocycline; RIF = rifampicin; TEC = teicoplanin; TGC = tigecycline; VA, vancomycin; S = Susceptible; I = Intermediate; R = Resistant.

The sequence analyses of *rpoB* in the rifampin-resistant mutants derived from the 39 biofilm-embedded MRSA isolates are shown in Tables 2 and 3. All amino acid substitutions were found in cluster I. His481Tyr/Leu/Asp substitution was noted in 33 (84.6%) isolates (MIC, 256—1024 μg/mL), Ser486Leu in 13 isolates (33.3%; MIC, 512 μg/mL), Ala477Asp in 12 isolates (30.8%; MIC, 512 μg/mL), Gln468Lys/Leu/Arg in eight isolates (20.5%; MIC, 512—1024 μg/mL), and Arg484His in seven isolates (17.9%; MIC, 512 μg/mL). An MRSA isolate could have one to four amino acid substitutions, and an amino acid position, such as codon 481, could have one of three substitutes.

We also analyzed the mutation percentages of the *rpoB* mutation sites of the 39 MRSA isolates with or without rifampin in the biofilm and MICs (Table 3). Without rifampin induction, the MIC ranged between 0.125 μg/mL and 1024 μg/mL. The mutation sites included codons 464, 466, 468, 471, 474, 477, 481, 484, or 486 of cluster I and 519, 527, or 529 of cluster II. Among them, the percentage of different mutation sites included 471 aa-14 isolates (35.9%), 477 aa-12 isolates (30.8%), 481 aa-17 isolates (43.6%), and 484 aa-13 isolates (33.3%). Conversely, with rifampin induction, the MIC after mutation ranged between 256 μg/mL and 1024 μg/mL. The mutation sites induced by rifampin were more concentrated, including codon 468 aa-8 isolates (20.5%), 477 aa-12 isolates (30.8%), 481 aa-33 isolates (84.6%), 484 aa-7 isolates (17.9%), and 486 aa-13 isolates (33.3%). By contrast, a silent mutation site at 474 (AAC—AAT) was found in 24 (61.5%) of the 39 MRSA isolates.

Discussion

The study by Raad et al[4] showed that rifampin could initially cause a significant decline in the MRSA bacterial load in biofilm. However, after repeated daily exposure to rifampin, most of the MRSA isolates developed resistance to this antibiotic.[4] According to our previous study, the rifampin MICs of biofilm-embedded MRSA isolates significantly increased from 0.015 μg/mL to $\geq$ 4 μg/mL after 5 days of rifampin monotherapy at the SBC. By contrast, fosfomycin exposure did not lead to evident MIC changes in a similar setting.[10]

In vivo rifampin-resistant isolates emerging during combination therapy of rifampin and vancomycin have been previously reported.[21] However, the combination of vancomycin plus rifampin proved to be effective in resistance prevention in an animal model of MRSA foreign body osteomyelitis.[22] Although the results were diverse from different studies, we believe that such a combination may easily induce rifampin resistance, especially for biofilm-embedded MRSA.[11] In our MRSA biofilm study, we found a rapid increase in the rifampin MICs from $<$ 0.06 μg/mL to $>$ 64 μg/mL during rifampin monotherapy, as well as when vancomycin, teicoplanin, or daptomycin were combined with rifampin.[11] High-level rifampin-resistant isolates ($>$ 64 μg/mL) were commonly found among the above-mentioned combinations.

The average frequency of rifampin mutation of *S. aureus* without rifampin exposure was reported to be 3.2×10^{-9},[6] and rifampin-resistant mutants emerged at a frequency of $\sim 10^{-8}$ if induced by rifampin therapy.[7] Another study reported a mutation frequency of 10^{-6}—10^{-8} in planktonic MRSA isolates after exposure to rifampin at the concentration of 1/2 MIC for 10 consecutive days.[8] However, in our study the frequency of spontaneous mutation in the 39 biofilm-embedded clinical MRSA isolates without rifampin exposure, 2.49×10^{-5}, was 100—100,000 times higher than in the planktonic MRSA, highlighting the resistance-prone microenvironment of biofilm formation.

Amino acid residues 468, 477, 481, and 486 have been reported to be the common mutation sites in rifampin-resistant MRSA isolates,[15,22—24] in accordance with our results. The mutation sites of high-level rifampin-resistant isolates (MIC $>$ 128 μg/mL) were located in the published hot spots, including the Gln468Lys, Ala477Asp, His481Tyr, or Ser486Leu substitutions.[13] However, amino acid substitutions at Ala473[24] were not found in our MRSA isolates. There were no mutations in the rifampin resistance-determining cluster II (515—530 aa) among the rifampin selected mutants. A collection of additional MRSA isolates from other hospitals will be available for *rpoB* sequencing among the rifampin-resistant isolates in the future.

Our mutation sites in the biofilm-embedded MRSA isolates without rifampin exposure were similar to those

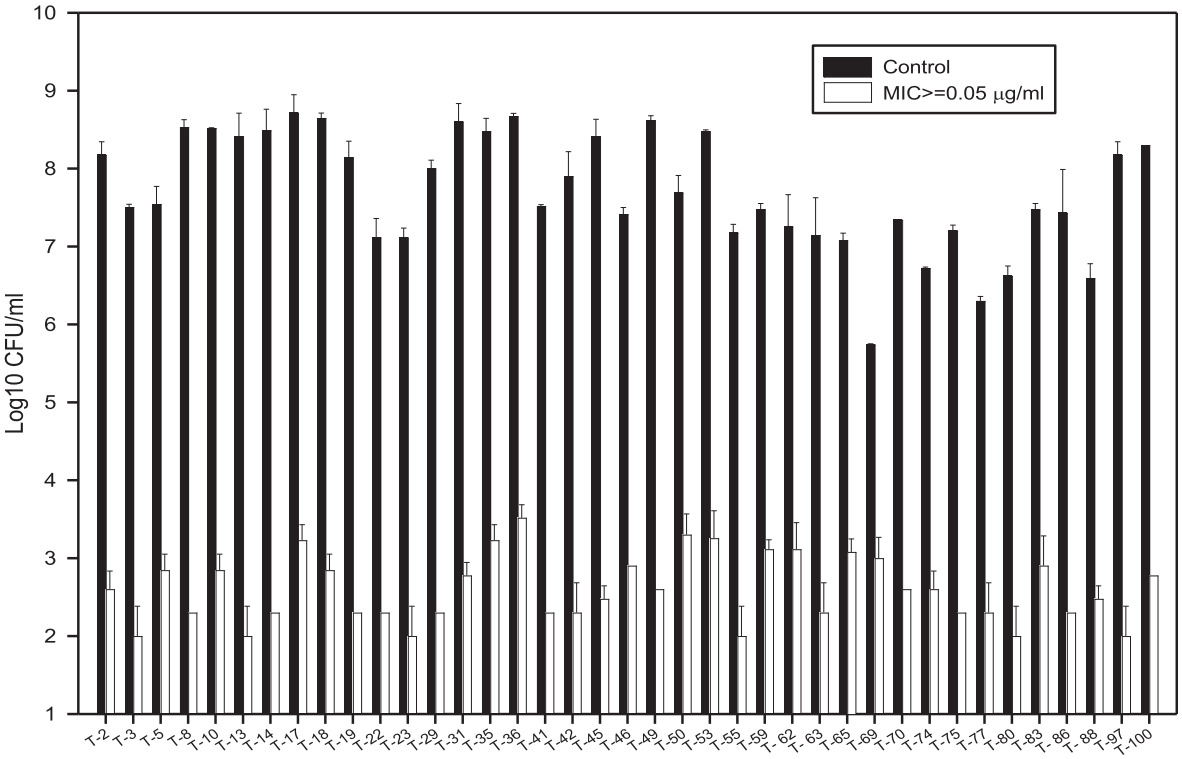

Figure 1. Bacterial load ($\log_{10}$ CFU/mL) of 39 methicillin-resistant *Staphylococcus aureus* isolates in the biofilm after 5 days without exposure to any antibiotics. The colony grew in control (black bar) and rifampicin 0.05 μg/mL containing medium (white bar). Mean mutation frequency, 2.49×10^{-5}; mutation frequency range from 1.00×10^{-4} to 3.85×10^{-7}.

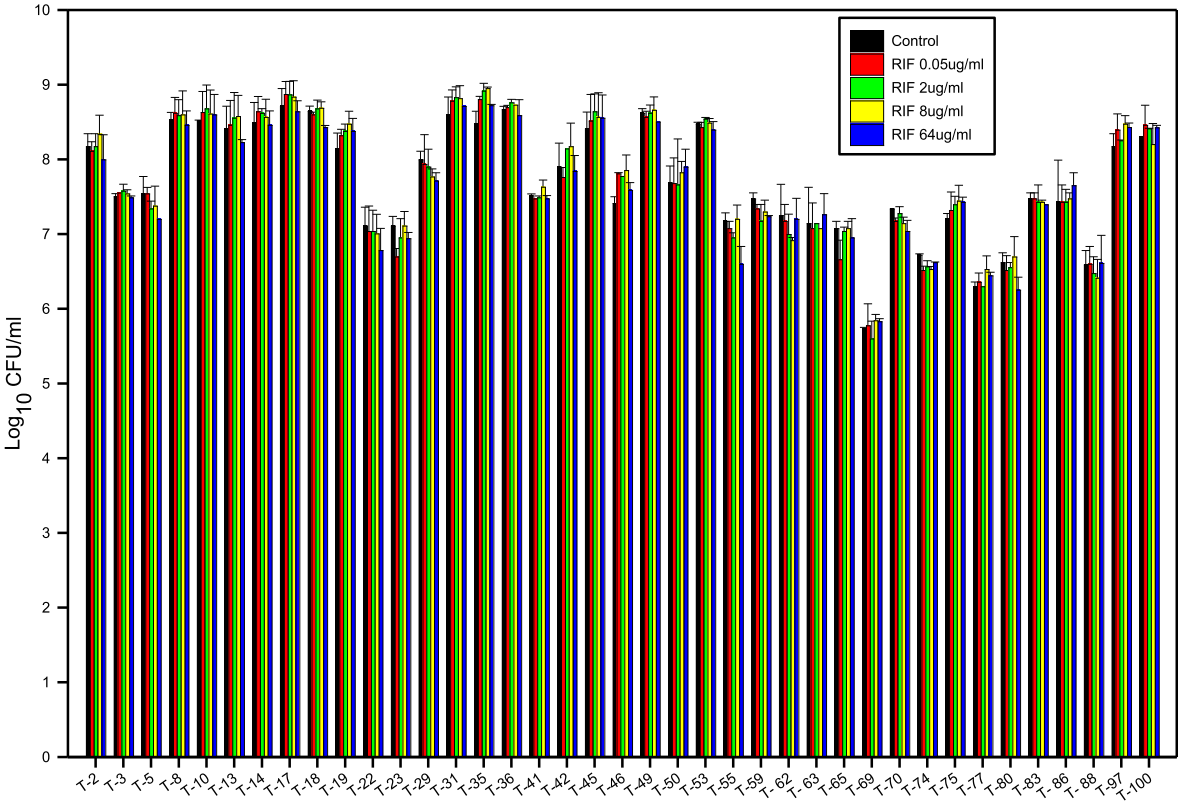

Figure 2. Bacterial load ($\log_{10}$ CFU/mL) of 39 methicillin-resistant *Staphylococcus aureus* isolates in the biofilm after 5 days of exposure to rifampicin 1 (μg/mL; susceptible breakpoint concentration). The colony grew in control (black bar) and rifampicin 0.05 μg/mL (red bar), 2 μg/mL (green bar), 8 μg/mL (yellow bar), and 64 μg/mL (blue bar) containing medium. Mean mutation rate was ~100% after the rifampicin treatment.

Table 2 Mutation sites of *rpoB* and minimum inhibitory concentrations (MICs) of 39 methicillin-resistant *Staphylococcus aureus* isolates in the biofilm cocultivated with rifampicin (1 mg/L) for 5 days.

Strain	Mutation(s) in rpoB	Total No. of biofilm	MIC of the parent strain (μg/mL)	MIC (μg/mL)
T-2	481 His→Tyr(CAT→TAT)/481 His→Leu(CAT→CTT)	1.5×10^8	0.015	1024/256
T-3	481 His→Tyr(CAT→TAT)	3.2×10^7	0.015	1024
T-5	477 Ala→Asp(GCT→GAT)	5.5×10^7	0.015	512
T-8	481 His→Tyr(CAT→TAT)/486 Ser→Leu(TCA→TTA)	1.4×10^8	0.03	1024/512
T-10	481 His→Tyr(CAT→TAT)	6.2×10^7	0.015	1024
T-13	468 Gln→Leu(CAA→CTA)/481 His→Tyr(CAT→TAT)/484 Arg→His(CGT→CAT)	2.6×10^8	0.03	512/1024/512
T-14	481 His→Tyr(CAT→TAT)/484 Arg→His(CGT→CAT)/486 Ser→Leu(TCA→TTA)	3.1×10^8	0.03	1024/512/512
T-17	481 His→Tyr(CAT→TAT)/486 Ser→Leu(TCA→TTA)	5.2×10^8	0.015	1024/512
T-18	481 His→Tyr(CAT→TAT)	7.5×10^7	0.015	1024
T-19	481 His→Tyr(CAT→TAT)/481 His→Asp(CAT→GAT)	1.4×10^8	0.03	1024/1024
T-22	481 His→Tyr(CAT→TAT)/484 Arg→His(CGT→CAT)	2.3×10^7	0.015	1024/512
T-23	477 Ala→Asp(GCT→GAT)/481 His→Tyr(CAT→TAT)	1.3×10^7	0.015	512/1024
T-29	477 Ala→Asp(GCT→GAT)/481 His→Asp(CAT→GAT)	1.0×10^8	0.03	512/1024
T-31	481 His→Tyr(CAT→TAT)	4.0×10^8	0.03	1024
T-35	481 His→Tyr(CAT→TAT)	3.0×10^8	0.015	1024
T-36	468 Gln→Leu(CAA→CTA)/481 His→Tyr(CAT→TAT)	2.0×10^8	0.015	512/1024
T-41	477 Ala→Asp(GCT→GAT)/481 His→Tyr(CAT→TAT)	1.3×10^7	0.015	512/1024
T-42	477 Ala→Asp(GCT→GAT)/481 His→Tyr(CAT→TAT)/486 Ser→Leu(TCA→TTA)	8.0×10^7	0.015	512/1024/512
T-45	481 His→Tyr(CAT→TAT)/481 His→Asp(CAT→GAT)	2.6×10^8	0.015	1024/1024
T-46	481 His→Tyr(CAT→TAT)/484 Arg→His(CGT→CAT)/486 Ser→Leu(TCA→TTA)	2.6×10^7	0.015	1024/512/512
T-49	468 Gln→Lys(CAA→AAA)/477 Ala→Asp(GCT→GAT)	1.2×10^8	0.015	1024/512
T-50	481 His→Tyr(CAT→TAT)/486 Ser→Leu(TCA→TTA)	4.9×10^7	0.015	1024/512
T-53	481 His→Tyr(CAT→TAT)/486 Ser→Leu(TCA→TTA)	4.0×10^7	0.015	1024/512
T-55	481 His→Tyr(CAT→TAT)/481His→Leu(CAT→CTT)/484 Arg→His(CGT→CAT)	1.5×10^7	0.015	1024/512/512
T-59	477 Ala→Asp(GCT→GAT)/481 His→Tyr(CAT→TAT)/481 His→Leu(CAT→CTT)/486 Ser→Leu(TCA→TTA)	3.0×10^7	0.015	512/1024/256/512
T-62	468 Gln→Leu(CAA→CTA)/477 Ala→Asp(GCT→GAT)/486 Ser→Leu(TCA→TTA)	8.0×10^7	0.015	512/512/512
T-63	468 Gln→Lys(CAA→AAA)/481 His→Tyr(CAT→TAT)	1.4×10^8	0.015	1024/1024
T-65	481 His→Tyr(CAT→TAT)/484 Arg→His(CGT→CAT)	1.2×10^7	0.015	1024/512
T-69	468 Gln→Lys(CAA→AAA)/484 Arg→His(CGT→CAT)	5.5×10^5	0.015	1024/512
T-70	477 Ala→Asp(GCT→GAT)/486 Ser→Leu(TCA→TTA)	2.2×10^7	0.015	512/512
T-74	477 Ala→Asp(GCT→GAT)/481 His→Tyr(CAT→TAT)/481 His→Leu(CAT→CTT)	5.3×10^6	0.015	512/1024/256
T-75	481 His→Tyr(CAT→TAT)	1.6×10^7	0.015	1024
T-77	481 His→Tyr(CAT→TAT)	2.0×10^6	0.015	1024
T-80	477 Ala→Asp(GCT→GAT)/481 His→Tyr(CAT→TAT)	4.2×10^6	0.015	512/1024
T-83	481 His→Tyr(CAT→TAT)	3.0×10^6	0.015	1024
T-86	468 Gln→Lys(CAA→AAA)/486 Ser→Leu(TCA→TTA)	1.7×10^8	0.015	1024/512
T-88	477 Ala→Asp(GCT→GAT)/481 His→Tyr(CAT→TAT)/486 Ser→Leu(TCA→TTA)	1.9×10^7	0.015	512/1024/512
T-97	468/482 Gln→Leu/Lys→Asp(CAA→CTA/AAA→AAT)/481 His→Asp(CAT→GAT)	1.5×10^8	0.015	1024/1024
T-100	468 Gln→Arg(TCA→TTA)/486 Ser→Leu(TCA→TTA)	2.0×10^8	0.015	512/512

reported in the literature.[23] However, the mutation sites of the rifampin-treated biofilm-embedded isolates were limited to codons 468, 477, 481, 484, and 486, which was more "localized" than those of biofilm-embedded isolates without rifampin exposure. The MICs of the rifampin-treated biofilm-embedded MRSA isolates were always higher than those of the biofilm-embedded MRSA isolates without rifampin exposure, indicating that there is a

Table 3 Mutation percentages for different mutation sites, minimum inhibitory concentrations (MICs), and mutation sites in the *rpoB* gene of 39 methicillin-resistant *Staphylococcus aureus* isolates with or without rifampicin in biofilm.

Biofilm without rifampin			Biofilm with rifampin		
Mutation site	Mutation *N* (%)	MIC	Mutation site	Mutation *N* (%)	MIC
Cluster I					
464	2 (5.1)				
Ser→Pro(TCT→CCT)	2 (5.1)	256			
466	1 (2.6)				
Leu→Ser(TTA→TCA)	1 (2.6)	<1			
468	8 (20.5)		468	8 (20.5)	
Gln→Lys(CAA→AAA)	4 (10.3)	1024	Gln→Lys(CAA→AAA)	5 (12.9)	1024
Gln→Leu(CAA→CTA)	4 (10.3)	512	Gln→Leu(CAA→CTA)	2 (5.1)	512
			Gln→Arg(TCA→TTA)	1 (2.6)	512
471	14 (35.9)				
Asp→Asn(GAC→AAC)	4 (10.3)	<1			
Asp→Glu(GAC→GAG)	1 (2.6)	<1			
Asp→Gly(GAC→GGC)	3 (7.7)	<1			
Asp→Val(GAC→GTC)	1 (2.6)	32			
Asp→Tyr(GAC→TAC)	4 (10.3)	32			
Asp→Cys(GAC→TGC)	1 (2.6)	<1			
474	1 (2.6)				
Asn→Lys(AAC→AAG)	1 (2.6)	8			
477	12 (30.8)		477	12 (30.8)	
Ala→Asp(GCT→GAT)	7 (17.9)	512	Ala→Asp(GCT→GAT)	12 (30.8)	512
Ala→Val(GCT→GTT)	6 (15.4)	2			
481	17 (43.6)		481	33 (84.6)	
His→Tyr(CAT→TAT)	13 (41.9)	1024	His→Tyr(CAT→TAT)	31 (79.5)	1024
His→Leu(CAT→CTT)	3 (7.7)	256	His→Leu(CAT→CTT)	4 (10.3)	256
His→Asp(CAT→GAT)	2 (5.1)	1024	His→Asp(CAT→GAT)	4 (10.3)	1024
His→Asn(CAT→AAT)	3 (7.7)	512			
484	13 (33.3)		484	7 (17.9)	
Arg→His(CGT→CAT)	11 (28.2)	256	Arg→His(CGT→CAT)	7 (17.9)	512
Arg→Ser(CGT→AGT)	2 (5.1)	64			
Arg→Cys(CGT→TGT)	1 (2.6)	16			
486	3 (7.7)		486	13 (33.3)	
Ser→Leu(TCA→TTA)	3 (7.7)	512	Ser→Leu(TCA→TTA)	13 (33.3)	512
			468/482	1 (2.6)	
			Gln→Leu/Lys→Asp (CAA→CTA/AAA→AAT)	1 (2.6)	1024
Cluster II					
519	1 (2.6)				
Pro→Leu(CCT→CTT)	1 (2.6)	<1			
527	3 (7.7)				
Ile→Phe(ATT→TTT)	3 (7.7)	32			
529	1 (2.6)				
Ser→Leu(TCA→TTA)	1 (2.6)	256			

greater potential for the induction of rifampin resistance in the biofilm. As for the mutation in codon 474 (AAC→AAT), which is rarely mentioned in the literature, we noted it in ~60% of our MRSA isolates. Its significance is unknown and needs further evaluation.

In conclusion, we documented a high induction potential of rifampin resistance when MRSA was engaged in the biofilm with rifampin exposure. Therefore, rifampin monotherapy is inadequate for MRSA in biofilm. Development of combination regimens with minimal rifampin resistance inducing potential for MRSA in biofilms is warranted.

Conflicts of interests

None declared

Acknowledgments

The authors acknowledge the members of the Research Laboratory of Infectious Diseases of the Chi Mei Medical Center (Tainan, Taiwan) for their assistance in the statistical analyses of these data. This study was supported by

grants from the National Science Council (NSC102-2314-13-384-009-MY2) and Chi Mei Medical Center Research Foundation (CMFHT10202, CMFHR10244).

References

1. Prince AS. Biofilms, antimicrobial resistance, and airway infection. *N Engl J Med* 2002;**347**:1110—1.
2. Edmiston Jr CE, Goheen MP, Seabrook GR, Johnson CP, Lewis BD, Brown KR, et al. Impact of selective antimicrobial agents on staphylococcal adherence to biomedical devices. *Am J Surg* 2006;**192**:344—54.
3. Wu WS. Efficacy of combination oral antimicrobial agents against biofilm-embedded methicillin-resistant Staphyococcus aureus. *J Microbiol Immunol Infect* 2013;**46**:89—95.
4. Raad I, Hanna H, Jiang Y, Dvorak T, Reitzel R, Chaiban G, et al. Comparative activities of daptomycin, linezolid, and tigecycline against catheter-related methicillin-resistant Staphylococcus bacteremic isolates embedded in biofilm. *Antimicrob Agents Chemother* 2007;**51**:1656—60.
5. Aboltins CA, Page MA, Buising KL, Jenney AW, Daffy JR, Choong PF, et al. Treatment of staphylococcal prosthetic joint infections with debridement, prosthesis retention and oral rifampicin and fusidic acid. *Clin Microbiol Infect* 2007;**13**:586—91.
6. Wichelhaus T, Schafer V, Brade V, Boddinghaus B. Differential effect of rpoB mutations on antibacterial activities of rifampicin and KRM-1648 against *Staphylococcus aureus*. *J Antimicrob Chemother* 2001;**47**:153—6.
7. O'Neill AJ, Cove JH, Chopra I. Mutation frequencies for resistance to fusidic acid and rifampicin in *Staphylococcus aureus*. *J Antimicrob Chemother* 2001;**47**:647—50.
8. Schmitz FJ, Fluit AC, Hafner D, Beeck A, Perdikouli M, Boos M, et al. Development of resistance to ciprofloxacin, rifampin, and mupirocin in methicillin-susceptible and -resistant *Staphylococcus aureus* isolates. *Antimicrob Agents Chemother* 2000;**44**:3229—31.
9. Ju O, Woolley M, Gordon D. Emergence and spread of rifampicin-resistant, methicillin-resistant *Staphylococcus aureus* during vancomycin—rifampicin combination therapy in an intensive care unit. *Eur J Clin Microbiol Infect Dis* 2006;**25**:61—2.
10. Tang HJ, Chen CC, Cheng KC, Toh HS, Su BA, Chiang SR, et al. In vitro efficacy of fosfomycin-containing regimens against methicillin-resistant *Staphylococcus aureus* in biofilms. *J Antimicrob Chemother* 2012;**67**:944—50.
11. Tang HJ, Chen CC, Cheng KC, Wu KY, Lin YC, Zhang CC, et al. In vitro efficacies and resistance profiles of rifampin-based combination regimens for biofilm-embedded methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2013;**57**:5717—20.
12. Matsuo M, Hishinuma T, Katayama Y, Cui L, Kapi M, Hiramatsu K. Mutation of RNA polymerase beta subunit (rpoB) promotes hVISA-to-VISA phenotypic conversion of strain Mu3. *Antimicrob Agents Chemother* 2011;**55**:4188—95.
13. Watanabe Y, Cui L, Katayama Y, Kozue K, Hiramatsu K. Impact of rpoB mutations on reduced vancomycin susceptibility in *Staphylococcus aureus*. *J Clin Microbiol* 2011;**49**:2680—4.
14. Hsueh PR. Tigecycline In-vitro Surveillance in Taiwan (TIST). *Int J Antimicrob Agents* 2008;**32**:S173.
15. Chung M, de Lencastre H, Matthews P, Tomasz A, Adamsson I, Aires de Sousa M, et al. Molecular typing of methicillin-resistant *Staphylococcus aureus* by pulsed-field gel electrophoresis: comparison of results obtained in a multilaboratory effort using identical protocols and MRSA strains. *Microb Drug Resist* 2000;**6**:189—98.
16. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995;**33**:2233—9.
17. Andrews JM. Determination of minimum inhibitory concentrations. *J Antimicrob Chemother* 2001;**48**:5—16.
18. Institute Clinical and Laboratory Standards. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically*, 7th ed. CLSI Document M7—A7. Wayne, PA: Institute Clinical and Laboratory Standards.
19. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing; 16th informational supplement*. M100-S16. Wayne, PA: Clinical and Laboratory Standards Institute; 2006.
20. Wichelhaus TA, Schafer V, Brade V, Boddinghaus B. Molecular characterization of rpoB mutations conferring cross-resistance to rifamycins on methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 1999;**43**:2813—6.
21. Aubry-Damon H, Soussy CJ, Courvalin P. Characterization of mutations in the rpoB gene that confer rifampin resistance in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 1998;**42**:2590—4.
22. Vergidis P, Rouse MS, Euba G, Karau MJ, Schmidt SM, Mandrekar JN, et al. Treatment with linezolid or vancomycin in combination with rifampin is effective in an animal model of methicillin-resistant *Staphylococcus aureus* foreign body osteomyelitis. *Antimicrob Agents Chemother* 2011;**55**:1182—6.
23. Wichelhaus TA, Boddinghaus B, Besier S, Schafer V, Brade V, Ludwig A. Biological cost of rifampin resistance from the perspective of *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2002;**46**:3381—5.
24. Mick V, Dominguez MA, Tubau F, Linares J, Pujol M, Martin R. Molecular characterization of resistance to Rifampicin in an emerging hospital-associated methicillin-resistant *Staphylococcus aureus* clone ST228, Spain. *BMC Microbiol* 2010;**10**:68.