The clinical significance of silent mutations with respect to ciprofloxacin resistance in MRSA

Chih-Cheng Lai1
Chi-Chung Chen2,3
Ying-Chen Lu3
Yin-Ching Chuang2,4
Hung-Jen Tang5,6

1Department of Intensive Care Medicine, Chi Mei Medical Center, Liouying, Tainan, Taiwan; 2Department of Medical Research, Chi Mei Medical Center, Tainan, Taiwan; 3Department of Food Science, National Chiayi University, Chiayi, Taiwan; 4Department of Internal Medicine, Chi Mei Medical Center, Liouying, Tainan, Taiwan; 5Department of Health and Nutrition, Chia-Nan University of Pharmacy and Science, Tainan, Taiwan; 6Department of Medicine, Chi Mei Medical Center, Tainan, Taiwan

Correspondence: Hung-Jen Tang, Department of Medicine, Chi Mei Medical Center, No.901, Zhonghua Rd., Yongkang Dist., Tainan City 710, Taiwan. Tel +886 6 2812811 ext 52606 Fax +886 6 2832057 Email 8409d1@gmail.com

Introduction

The emergence of methicillin-resistant Staphylococcus aureus (MRSA) infections has become a substantial treatment challenge in hospital-associated settings and in community settings around the world.1–4 In addition to methicillin, MRSA can develop antimicrobial resistance against several different antibiotic classes, including β-lactams, quinolones, trimethoprim-sulfamethoxazole, erythromycin, clindamycin, linezolid, and daptomycin, through various resistance gene mutations, including, blaZ, mecA, parC, parE, gyrA, gyrB, sulA, dfrB, erm, cfr, and mprF.3,4 There is no exception for the rpoB gene, which encodes the β-subunit of the bacterial RNA polymerase. The mutation of this gene following rifampicin therapy can often lead to the emergence of rifampicin resistance.5 Therefore, rifampicin resistance can be detected by sequencing the rpoB gene. Hellmark et al demonstrated that rpoB sequencing could be an accurate method of species identification in staphylococci,6 and Marty et al reported that the highly discriminatory rpoB species-specific PCR-RFLP analysis allows for fast and simple molecular identification...
of *Staphylococcus* and other bacteria. This finding suggests that the *rpoB* gene may have the potential role as one of the core gene candidates for phylogenetic analyses and bacterial identification. Our recent study on the *rpoB* gene found that a point mutation in codon 474 (AAC → AAT) located in cluster I region was present in 60% of our clinical MRSA isolates, and this mutation was not associated with any induction of rifampicin resistance and was even associated with lower antibiotic resistance rates, especially for ciprofloxacin. Therefore, this study was conducted to investigate the genotypic differences among different sequence type MRSA isolates, especially focusing on silent mutations and the relationship between such mutations and ciprofloxacin resistance.

**Materials and methods**

**Clinical isolates**

Seventy-nine MRSA isolates were obtained from the Tigecycline In-vitro Surveillance in Taiwan (TIST) study at 22 hospitals from 2006 to 2010. *Staphylococci* were identified by colony morphology, Gram stain, and coagulase test results. MRSA was further confirmed by the tube coagulase test and growth on 6 μg/mL oxacillin salt agar screening plates. The *mecA* gene was confirmed using a PCR method. Isolates were stored at −70°C in Protect Bacterial Preservers (Technical Service Consultants Limited, Heywood, UK) until use.

**Antibiotic susceptibility test**

Antibiotics tested included chloramphenicol, erythromycin, gentamicin, minocycline, oxacillin, rifampin, and vancomycin (Sigma-Aldrich Co., St Louis, MO, USA), fosfomycin (Ercros, Barcelona, Spain), linezolid and tigecycline (Pfizer, Inc., New York, NY, USA), fusidic acid (Leo Pharma, Ballerup, Denmark), teicoplanin (Sanofi-Aventis, Bridgewater, NJ, USA), ciprofloxacin (Bayer AG, Leverkusen, Germany), daptomycin (Merck & Co., Inc., Kenilworth, NJ, USA). Interpretation criteria for the susceptibility test and the minimum inhibitory concentration (MIC) determined by the agar dilution tests were based on the recommendations of the Clinical and Laboratory Standards Institute (CLSI) or the British Society for Antimicrobial Chemotherapy. For the fosfomycin susceptibility test, glucose-6-phosphate (25 mg/L) was added to the agar plate. The daptomycin susceptibility test was performed in Mueller–Hinton broth adjusted to 50 mg/L of calcium per standard methodology. Mueller–Hinton agar (Thermo Fisher Scientific, Waltham, MA, USA) was employed for *S. aureus* MIC determination.

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 h. *S. aureus* ATCC 29213 was included as the control strain in each of the MIC measurements.

**Determination of the *mecA*, PVL, *rpoB*, *gyrA* and *parC* gene mutation**

PCR for the *mecA* gene was performed according to the protocol described by Vannuffel et al. *S. aureus* ATCC BAA-1707, USA400 was used as the positive control. A 433-bp nucleotide fragment located in the lukS-PV and lukF-PV operons was amplified by PCR using the primers and conditions described by Lina et al. The primer sequence was lukPV-forward (ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A) and reverse (GCA TCA AAT GTA TTG GAT AGC AAA AGC). The PCR method was used to amplify these genes including *rpoB*, *gyrA*, and *parC* with gene mutations using primers and previously described cycling conditions. The following primers were used: (a) *rpoB*-forward (CCG TCG TTT ACG TTC TGT AGG) and reverse (AAA GCC GAA TTC ATT TAC ACG); (b) *gyrA*-forward (AAT GAA CAA GGT ATG TAC TCC) and reverse (TAC CGG CTT CAG TAT AAC GC); (c) *parC*-forward (ACT TGA AGA TGG TTT AGG TGA T) and reverse (TTA GGA AAT CTT GAT GGC AA). Template DNA for PCR was prepared using InstaGene™ Matrix as recommended by the manufacturer (Bio-Rad Laboratories Inc., Hercules, CA, USA). After amplification, PCR products were purified from excess primers and nucleotides using a QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) and sequenced with the same primers by the dideoxy chain termination method in an ABI PRISM 3730 sequence analyzer (Thermo Fisher Scientific, Waltham, MA, USA).

**Molecular typing methods**

All isolates were analyzed by SCCmec typing, MLST typing, and pulsed-field gel electrophoresis (PFGE). The SCCmec types were determined by the multiplex PCR strategy developed by Kondo et al. The MLST was carried out as previously described. The sequences of the PCR products were compared with the existing sequences available on the MLST website (http://saureus.mlst.net) for *S. aureus*. DNA extraction and Smal restriction were performed as previously described. The PFGE patterns were visually examined and interpreted according to the criteria developed by Tenover et al. The similarities of PFGE profiles of each strain were compared using a Dice coefficient at 1.0% of tolerance and 0.8% of optimization.

**Results**

The PFGE patterns of all MRSA isolates were shown in Figure 1. Two isolates did not yield an interpretable result with PFGE analysis because of technical problems associated
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Figure 1 PFGE and molecular patterns of MRSA isolates.

Abbreviations: PFGE, pulsed-field gel electrophoresis; MRSA, methicillin-resistant Staphylococcus aureus; opt, optimization; tol, tolerance; PVL, Panton–Valentine leucocidin; ST, sequence type; TIST, Tigecycline In-vitro Surveillance in Taiwan.
with degradation of the genomic DNA. This may lead to only very faint bands, degraded bands, or no banding patterns. Only 26 (32.9\%) MRSA isolates were Panton–Valentine leukocidin (PVL) positive. The most common sequence type (ST) was ST59 (n=41, 51.8\%), followed by ST239 (n=26, 32.9\%), ST5 (n=4, 5.0\%), ST8 (n=2, 2.5\%), ST45 (n=2, 2.5\%), ST573 (n=2, 2.5\%), ST388 (n=1, 1.3\%), and ST900 (n=1, 1.3\%).

Table 1 shows the MICs and the susceptibility of 48 and 31 MRSA isolates with and without the rpoB474 silent mutation, respectively. All isolates were susceptible to vancomycin, teicoplanin, and daptoycin. For oxacillin, erythromycin, gentamicin, tigecycline, minocycline, fusidic acid, rifampicin, and fosfomycin, the susceptibility rates of vancomycin, teicoplanin, and daptomycin. For oxacillin, we found a significant difference regarding MICs and the susceptibility rate between isolates with and without the rpoB474 silent mutation. The MIC50, MIC90, and MIC range was much higher for the isolates without the rpoB474 silent mutation than with the rpoB474 silent mutation. A total of 87.5\% of the isolates with the rpoB474 silent mutation were susceptible to ciprofloxacin, but none of the isolates without the rpoB474 silent mutation were susceptible to ciprofloxacin.

Table 2 shows the association between the ciprofloxacin MIC and the gene mutations. For 27 MRSA isolates without rpo474 silent mutation but with gyrA86/126 silent mutation (Group A), all of them belonged to SCCmec III and had the double mutations in the gyrA and parC genes, such as gyrA S84L/parC S80F or gyrA S84L. Moreover, all of Group A MRSA isolates showed high ciprofloxacin MIC levels. For four MRSA isolates with rpo474 silent mutation but without gyrA86/126 silent mutation (Group B), all of these presented with high ciprofloxacin MIC level, but only three of them had double mutations in the gyrA and parC genes. For four MRSA isolates without rpo474 and gyrA86/126 silent mutation (Group C), all of them had double mutations in the gyrA and parC genes, and high ciprofloxacin MIC levels. For 44 MRSA isolates with rpo474 silent mutation but without gyrA86/126 silent mutation (Group D), all of them showed low ciprofloxacin MIC level, but none of them had double mutations in the gyrA and parC genes. Among group D, MRSA isolates belonged to either SCCmec IV or V.

Table 3 summarizes the distribution of SCCmec types and silent mutations of rpoB474 and gyrA86/126 among all clinical isolates. For 27 SCCmec type III MRSA isolates, none had the rpoB474 silent mutation, but all had the gyrA86/126 silent mutation. In contrast, for most of the SCCmec type IV and V isolates, the rpoB474 silent mutation rate was 95.5\% and 96.2\%, respectively. None of the isolates had the gyrA86/126 silent mutation.
Table 2 The ciprofloxacin MIC values of MRSA isolates with respect to the \textit{rpoB}474/\textit{gyrA}86/126 gene mutations

| rpoB 474/ gyrA 86, 126 sm | Isolates | Ciprofloxacin MIC | gyrA | parC | SCCmec |
|---------------------------|----------|-------------------|------|------|--------|
| **Group A**               |          |                   |      |      |        |
| +/−                        | TIST4    | > 64              | S84L | S80F | III    |
| +/−                        | TIST5    | > 64              | S84L | S80F | III    |
| +/−                        | TIST12   | > 64              | S84L | S80F | III    |
| +/−                        | TIST17   | > 64              | S84L | S80F | III    |
| +/−                        | TIST18   | > 64              | S84L | S80F | III    |
| +/−                        | TIST34   | > 64              | S84L | S80F | III    |
| +/−                        | TIST35   | > 64              | S84L | S80F | III    |
| +/−                        | TIST36   | > 64              | S84L/| S80F | III/G108D |
| +/−                        | TIST49   | > 64              | S84L | S80F | III    |
| +/−                        | TIST53   | > 64              | S84L | S80F | III    |
| +/−                        | TIST54   | > 64              | S84L | S80F | III    |
| +/−                        | TIST56   | > 64              | S84L | S80F | III    |
| +/−                        | TIST57   | 32                | S84L | S80F | III    |
| +/−                        | TIST59   | > 64              | S84L | S80F | III    |
| +/−                        | TIST62   | > 64              | S84L/| S80F | IIIB   |
| +/−                        | TIST66   | > 64              | S84L | S80F | III    |
| +/−                        | TIST68   | 64                | S84L/| S80F/| III    |
| +/−                        | TIST69   | 64                | S84L | S80F | III    |
| +/−                        | TIST73   | > 64              | S84L | S80F | III    |
| +/−                        | TIST74   | > 64              | S84L | S80F | III    |
| +/−                        | TIST83   | > 64              | S84L | S80F | III    |
| +/−                        | TIST85   | > 64              | S84L | S80F | III    |
| +/−                        | TIST89   | > 64              | S84L | S80F | III    |
| +/−                        | TIST92   | 64                | S84L | S80F | III    |
| +/−                        | TIST94   | > 64              | S84L | S80F | III    |
| +/−                        | TIST98   | > 64              | S84L | S80F | III    |
| +/−                        | TIST100  | > 64              | S84L | S80F | III    |
| **Group B**               |          |                   |      |      |        |
| +/−                        | TIST19   | 32                | x     | x     | V      |
| +/−                        | TIST44   | > 64              | S84L | S80Y | II     |
| +/−                        | TIST51   | > 64              | S84L | S80Y | II     |
| +/−                        | TIST95   | 16                | S84L | S80F | IV (110sm) (77sm) |
| **Group C**               |          |                   |      |      |        |
| +/−                        | TIST26   | > 64              | S84L/| S80F | III    |
| +/−                        | TIST42   | > 64              | S84L | S80Y | IV     |
| +/−                        | TIST47   | 16                | S84L | S80F | V      |
| +/−                        | TIST71   | > 64              | S84L | S80Y | II     |
| **Group D**               |          |                   |      |      |        |
| +/−                        | TIST2    | 0.25              | x     | x     | IV     |
| +/−                        | TIST3    | 0.5               | x     | x     | IV     |

**Notes:** Group A: rpoB474 wild type/gyrA86 and 126 mutation with high ciprofloxacin MIC. Group B: rpoB474 mutation/gyrA86 and 126 wild type with high ciprofloxacin MIC. Group C: rpoB474 wild type/gyrA86 and 126 wild type with high ciprofloxacin MIC. Group D: rpoB474 mutation/gyrA86 and 126 wild type with low ciprofloxacin MIC.

**Abbreviations:** MRSA, methicillin-resistant Staphylococcus aureus; MIC, minimum inhibitory concentration; TIST, Tigecycline In-vitro Surveillance in Taiwan.

(Continued)
susceptibility in heterogeneous vancomycin-intermediate S.
*aureus* (hVISA) and VISA phenotype acquisition. In addition,
the role of *rpoB* mutation in the ciprofloxacin resistance
has been demonstrated in the study of *Escherichia coli* with
ciprofloxacin-selected *rpoB* mutations. Pletsch et al found
that the mutations in RNA polymerase can be served as novel
contributors to the evolution of resistance to ciprofloxacin and
also significantly increase the expression of mdtK, encoding
a multidrug efflux transporter. However, *rpoB474* and
gyrA86/126 mutations in this study are silent mutations. In
contrast to true mutation, they just have nucleotide change but
do not result in new amino acid substitute. Therefore, whether
the finding is incidental or significant among MRSA isolates
remains unclear. Further study is warranted to investigate
possible mechanisms.

### Conclusion

We found the phenomenon about the relationship between
*rpoB474*, gyrA86/126 silent mutation and gyrA/par C
mutation with ciprofloxacin MIC and antibiotic resistance. Most
occurrences of this *rpoB474* silent mutation were found in
CA-MRSA isolates with susceptibility to most antibiotics,
especially for ciprofloxacin. In contrast, most of MRSA
isolates without this mutation are HA-MRSA with high
resistance to ciprofloxacin.

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### Disclosure

The authors report no conflicts of interest in this work.

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