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ORIGINAL RESEARCH

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

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Chih-Cheng Lai[1]
Chi-Chung Chen[2,3]
Ying-Chen Lu[3]
Yin-Ching Chuang[2,4]
Hung-Jen Tang[5,6]

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

Background: The aim of this study was to investigate the genotypic differences between different sequence type MRSA isolates, especially focusing on silent *rpoB*474 mutations and the relationship between such mutations and ciprofloxacin resistance.

Methods: Seventy-nine MRSA isolates were obtained for antibiotic susceptibility tests and molecular study.

Results: Among these isolates, we found that the MIC_{50}, MIC_{90}, and minimum inhibitory concentration (MIC) range of ciprofloxacin were much higher for the isolates without the *rpoB*474 mutation than for isolates with the *rpoB*474 mutation. A total of 87.5% of the isolates with the *rpoB*474 mutation were susceptible to ciprofloxacin, but none of the isolates without the *rpoB*474 mutation were susceptible to ciprofloxacin. For 27 MRSA isolates without rpo474 silent mutation but with *gyrA*86/126 silent mutation, all of them belonged to SCC*mec* III, and had high ciprofloxacin MIC levels. For another 44 MRSA isolates with rpo474 silent mutation but without *gyrA*86/126 silent mutation, all of them showed low ciprofloxacin MIC levels, all of them belonged to either SCC*mec* IV or V. Furthermore, MRSA ciprofloxacin resistance was found to be associated with the mutations *gyrA* S84L/*parC* S80F or *gyrA* S84L, and S85P/*parC* S80Y.

Conclusion: Most occurrences of this *rpoB*474 silent mutation were found in community acquired-MRSA (CA-MRSA) isolates with susceptibility to most antibiotics, especially for ciprofloxacin and vice versa. Thus, this mutation may help to differentiate the different microbiologic characteristics of MRSA clinical isolates.

Keywords: MRSA, silent mutation, *rpoB*474, ciprofloxacin, resistance

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.[1–3] 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, gyrA, gyrB, sulA, dfrB, erm, cfr,* and *mprF*.[4–8] 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.[9] 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*,[9] and Marty et al reported that the highly discriminatory *rpoB* species-specific PCR-RFLP analysis allows for fast and simple molecular identification

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

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of *Staphylococcus* and other bacteria.[10] 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[11] 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.[12] 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.[13] *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 *mec*A 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.[14–16] 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 *mec*A gene was performed according to the protocol described by Vannuffel et al.[17] *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.[18] 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.[19, 20] 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 ACA CC) and reverse (TAC GCG CTT CAG TAT AAC GC); (c) *parC*-forward (ACT TGA AGA TGT 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 SCC*mec* typing, MLST typing, and pulsed-field gel electrophoresis (PFGE). The SCC*mec* types were determined by the multiplex PCR strategy developed by Kondo et al.[21] The MLST was carried out as previously described.[22] The sequences of the PCR products were compared with the existing sequences available on the MLST website (http://saureus.mlst.net) for *S. aureus*.[23] DNA extraction and SmaI restriction were performed as previously described. The PFGE patterns were visually examined and interpreted according to the criteria developed by Tenover et al.[24] 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

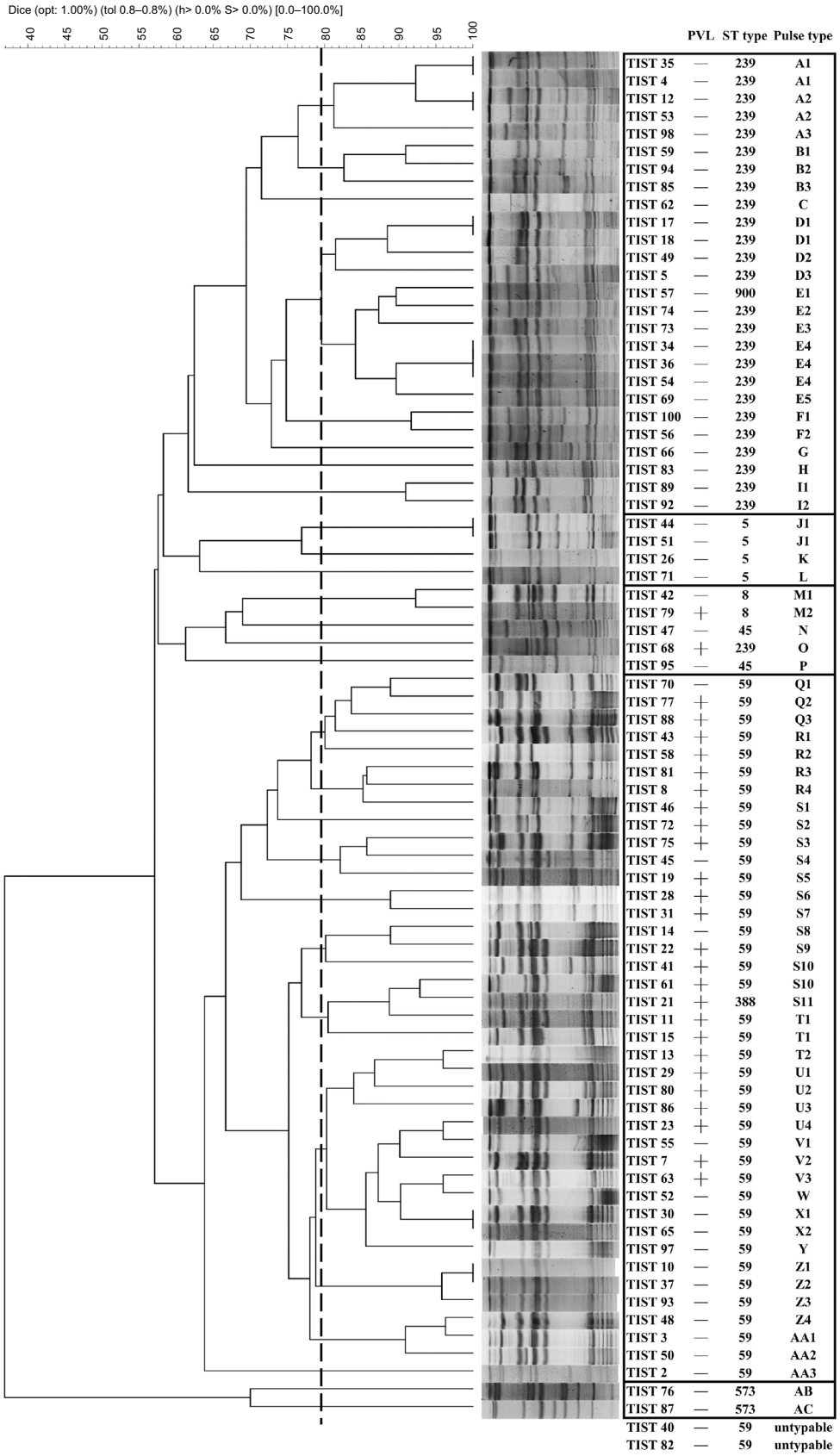

Figure 1 PFGE and molecular patterns of MRSA isolates.

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.

Table 1 The MIC values of MRSA isolates according the presence of the *rpoB*474 silent mutation

Antibiotics	No *rpoB*474 silent mutation (n=31)				*rpoB*474 silent mutation (n=48)				MIC interpretive criteria		
	MIC_{50}, mg/L	MIC_{90}, mg/L	MIC range, mg/L	Susceptibility, %	MIC_{50}, mg/L	MIC_{90}, mg/L	MIC range, mg/L	Susceptibility, %	S	I	R
OXA	> 128	> 128	4–> 128	0.0	16	128	<1–> 128	14.6	≤2		≥ 4
ERY	> 128	> 128	0.5–> 128	3.2	> 128	> 128	0.5–> 128	6.3	≤0.5	1–4	≥ 8
GEN	> 128	> 128	<1–> 128	9.7	<1	> 128	<1–> 128	50.0	≤4	8	≥ 16
CM	16	16	8–64	0.0	64	128	8–128	0.0	≤0.5	1–2	≥ 4
VA	2	2	1–2	100	1	2	1–2	100	≤2	4–8	≥ 16
TGC	0.5	1	0.25–1	83.9	0.5	0.5	0.25–1	95.8	≤0.5[a]		
MNO	8	8	0.125–8	45.2	0.25	0.5	0.125–8	95.8	≤4	8	≥ 16
TEC	2	2	0.5–2	100	1	1	0.5–2	100	≤8	16	≥ 32
FA	0.5	8	0.5–8	71.0	0.25	0.5	0.25–8	97.9	≤1[b]		
LNZ	4	4	2–4	100	4	8	2–8	89.6	≤4		≥ 8
CIP	> 64	> 64	16–> 64	0.0	0.5	2	0.25–> 64	87.5	≤1	2	≥ 4
RIF	0.016	2	0.016–> 32	80.6	0.016	2	0.016–> 32	89.6	≤1	2	≥ 4
FOS	16	32	1–> 1024	90.3	4	8	1–> 1024	95.8	≤32[b]		
DAP	0.5	1	0.125–1	100	0.25	0.5	0.25–1	100	≤1		

Notes: [a]MIC interpretive criteria were assessed according to the FDA guidelines. [b]MIC interpretive criteria were assessed according to the BSAC guidelines.
Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; MIC, minimum inhibitory concentration; OXA, oxacillin; ERY, erythromycin; GEN, gentamicin; CM, clindamycin; VA, vancomycin; TGC, tigecycline; MNO, minocycline; TEC, teicoplanin; FA, fusidic acid; LNZ, linezolid; CIP, ciprofloxacin; RIF, rifampicin; FOS, fosfomycin; DAP, daptomycin; S, susceptible; I, intermediate; R, resistant.

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 *rpoB*474 silent mutation, respectively. All isolates were susceptible to vancomycin, teicoplanin, and daptomycin. For oxacillin, erythromycin, gentamicin, tigecycline, minocycline, fusidic acid, rifampicin, and fosfomycin, the susceptibility rates of 48 *rpoB*474 silent mutation isolates were higher than those of the 31 unmutated *rpoB*474 isolates. In contrast, the susceptibility rate against linezolid of the non-*rpoB*474 silent mutation isolates was 100% and higher than *rpoB*474 silent mutation isolates susceptibility rate of 89.6%. For ciprofloxacin, we found a significant difference regarding MICs and the susceptibility rate between isolates with and without the *rpoB*474 silent mutation. The MIC_{50}, MIC_{90}, and MIC range was much higher for the isolates without the *rpoB*474 silent mutation than with the *rpoB*474 silent mutation. A total of 87.5% of the isolates with the *rpoB*474 silent mutation were susceptible to ciprofloxacin, but none of the isolates without the *rpoB*474 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 *gyrA*86/126 silent mutation (Group A), all of them belonged to SCC*mec* 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 *gyrA*86/126 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 *gyrA*86/126 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 *gyrA*86/126 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 SCC*mec* IV or V.

Table 3 summarizes the distribution of SCC*mec* types and silent mutations of *rpoB*474 and *gyrA*86/126 among all clinical isolates. For 27 SCC*mec* type III MRSA isolates, none had the *rpoB*474 silent mutation, but all had the *gyrA*86/126 silent mutation. In contrast, for most of the SCC*mec* type IV and V isolates, the *rpoB*474 silent mutation rate was 95.5% and 96.2%, respectively. None of the isolates had the *gyrA*86/126 silent mutation.

Table 2 The ciprofloxacin MIC values of MRSA isolates with respect to the *rpoB*474 and *gyrA*86/126 gene mutations

rpoB 474/ *gyrA* 86, 126 sm	Isolates	Ciprofloxacin MIC	*gyrA*	*parC*	SCC*mec*
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/ G108D	S80F	III
–/+	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/ S85P	S80F	III
–/+	TIST66	> 64	S84L	S80F	III
–/+	TIST68	64	S84L/ E88L	S80F/ E84K	III
–/+	TIST69	64	S84L	S80F	III
–/+	TIST73	> 64	S84L/ S85P	S80F	III
–/+	TIST74	> 64	S84L/ S85P	S80F	III
–/+	TIST83	> 64	S84L/ S85P	S80F	III
–/+	TIST85	> 64	S84L	S80F	III
–/+	TIST89	> 64	S84L	S80F	III
–/+	TIST92	64	S84L/ S85P	S80F	III
–/+	TIST94	> 64	S84L	S80F/S81P	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 (110sm)	S80F (77sm)	IV
Group C					
–/–	TIST26	> 64	S84L/ S85P	R61L/ S80Y/ S81P	II
–/–	TIST42	> 64	S84L	S80Y	IV
–/–	TIST47	16	S84L (110sm)	S80F (77sm)	V
–/–	TIST71	> 64	S84L	S80Y	II
Group D					
+/–	TIST2	0.25	x	x	IV
+/–	TIST3	0.5	x	x	IV
+/–	TIST7	0.25	x	x	IV
+/–	TIST8	0.25	x	x	V
+/–	TIST10	0.25	x	x	IV
+/–	TIST11	0.5	x	x	V
+/–	TIST13	0.25	x	x	V
+/–	TIST14	0.25	x	x	V
+/–	TIST15	0.5	x	x	V
+/–	TIST21	0.25	x	x	V
+/–	TIST22	0.25	x	x	V
+/–	TIST23	0.25	x	x	IV
+/–	TIST28	0.25	x	x	V
+/–	TIST29	0.25	x	x	V
+/–	TIST30	0.25	x	x	IV
+/–	TIST31	0.25	x	x	V
+/–	TIST37	1	x	x	IV
+/–	TIST40	1	x	x (77/81/ 113sm)	V
+/–	TIST41	0.5	x	x	V
+/–	TIST43	0.5	x	x	V
+/–	TIST45	1	x	x	V
+/–	TIST46	0.5	x	x	V
+/–	TIST48	0.5	x	x	IV
+/–	TIST50	1	x	x	IV
+/–	TIST52	1	x	x	IV
+/–	TIST55	1	x	x	IV
+/–	TIST58	0.5	x	x	V
+/–	TIST61	1	x	x	V
+/–	TIST63	1	x	x	IV
+/–	TIST65	0.25	x	x	IV
+/–	TIST70	0.25	x	x	V
+/–	TIST72	2	x	E84K	V
+/–	TIST75	1	x	x	IV
+/–	TIST76	1	x	S80F/ S123F	IV
+/–	TIST77	0.25	x	x	V
+/–	TIST79	0.5	x	x	IV
+/–	TIST80	0.5	x	x	V
+/–	TIST81	0.25	x	x	V
+/–	TIST82	1	x	x (77/ 81/113sm)	V
+/–	TIST86	0.25	x	x	V
+/–	TIST87	1	x	S80F/ S123F	IV
+/–	TIST88	0.25	x	x	IV
+/–	TIST93	2	x	S80F	IV
+/–	TIST97	0.25	x	x	IV

Notes: Group A : *rpoB*474 wild type/*gyrA*86 and126 mutation with high ciprofloxacin MIC. Group B: *rpoB*474 mutation/*gyrA*86 and126 wild type with high ciprofloxacin MIC. Group C: *rpoB*474 wild type/*gyrA*86 and126 wild type with high ciprofloxacin MIC. Group D: *rpoB*474 mutation/*gyrA*86 and126 wild type with low ciprofloxacin MIC.

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

Table 3 MRSA isolate SCC*mec* patterns

SCC*mec*	Numbers	No. (%) of MRSA isolates with *rpoB*474 mutations	No. (%) of MRSA isolates with *gyrA*86/126 mutation
II	4	2 (50)	0 (0)
III	27	0 (0)	27 (100)
IV	22	21 (95.5)	0 (0)
V	26	25 (96.2)	0 (0)

Notes: *rpoB*474: 474aac-aat silent mutation. *gyrA*86/126: 86att-atc/126gcg-gca silent mutation.
Abbreviation: MRSA, methicillin-resistant *Staphylococcus aureus.*

Discussion

This study had several interesting findings regarding the association between *rpoB*474 silent mutations and the different SCC*mec* and ST type MRSA isolates. Nearly all of the 48 MRSA isolates (Groups B and D) carried SCC*mec* type IV and V had the *rpoB*474 silent mutation. In contrast, none of them had the *gyrA*86/126 silent mutation and most of them do not have double mutations of *gyrA /par C* gene. Furthermore, these isolates exhibit high susceptibility to ciprofloxacin, and other antibiotics. All of these characteristics are consistent with microbiological features of community-acquired-MRSA (CA-MRSA).[25,26] Additionally, most of the CA-MRSA isolates in this study were found to be ST59, which is the major type of CA-MRSA in Taiwan.[27,28] In contrast, for the group of 31 MRSA isolates without the *rpoB*474 silent mutation (Groups A and C), all of them had the double mutations in the *gyrA* and *parC* genes, and showed high ciprofloxacin MIC levels. Among them, SCC*mec* type III compromised most of the MRSA isolates (n=27, 87.1%). These findings indicate that MRSA without the *rpoB*474 silent mutation is associated with higher resistance to ciprofloxacin, and most of these isolates should be classified as hospital-acquired MRSA (HA-MRSA) carrying SCC*mec* type III. In summary, we found the different presentations between MRSA isolates with and without *rpoB*474 silent mutation and this mutation may help differentiate the different microbiologic characteristic of MRSA clinical isolates.

In this study, MRSA ciprofloxacin resistance was found to be associated with the mutations of *gyrA* S84L/*parC* S80F or *gyrA* S84L, S85P/*parC* S80Y. This is consistent with previous findings that double mutants in the *parC* and *gyrA* genes may cause high-level resistance to ciprofloxacin with an MIC value ≥ 64 mg/dL.[29] Meanwhile, we also found a reciprocal relationship of *rpoB*474, gyrA86/126 silent mutation and *gyrA*/*parC* mutation that had never been reported before. Previous study suggested that *rpoB* mutation could be a "regulatory" mutation to decrease vancomycin susceptibility in heterogeneous vancomycin-intermediate *S. aureus* (hVISA) and VISA phenotype acquisition.[30,31] 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.[32] Pietsch 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.[32] However, *rpoB*474 and *gyrA*86/126 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 *rpoB*474, *gyrA*86/126 silent mutation and *gyrA/parC* mutation with ciprofloxacin MIC and antibiotic resistance. Most occurrences of this *rpoB*474 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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