ANTIMICROBIAL RESISTANCE

Mechanisms of 4-quinolone resistance in quinolone-resistant and methicillin-resistant *Staphylococcus aureus* isolates from Japan and China

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Summary. Ninety-two and 33 methicillin-resistant *Staphylococcus aureus* (MRSA) strains were isolated in Japan and China respectively. They were categorised as ofloxacin-susceptible (MIC < 12.5 mg/L), moderately (MIC 12.5–25 mg/L) or highly (MIC ≥ 50 mg/L) ofloxacin-resistant. 4-Quinolone concentrations required to inhibit purified DNA gyrase from the moderately and highly quinolone-resistant MRSA were at least 20 times higher than those required to inhibit the equivalent enzyme from quinolone-susceptible strains. Reconstitution assays demonstrated that the 4-quinolone-resistant MRSA had a mutation in subunit A of DNA gyrase. A portion of the *gyrA* gene from amino acids codons 40–115 was sequenced. Four moderately resistant and seven highly resistant MRSA contained a Ser → Leu substitution at amino acid 84; one moderately and one highly resistant MRSA and one moderately resistant methicillin-susceptible *S. aureus* (MSSA) strain contained a Glu → Lys substitution at amino acid 88. Eight MRSA, including one quinolone-susceptible strain and one MSSA contained a silent mutation at amino acid 86. Uptake of ofloxacin in moderately resistant strains was almost the same in the presence or absence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), whereas in highly resistant strains, uptake increased when CCCP was added. Restriction fragment length analysis of the *norA* gene with the restriction endonuclease *Sfi*I showed a mutation of nucleotide position 1085 in all MRSA strains tested except for one highly quinolone-resistant strain. Thus the mechanisms of 4-quinolone-resistance in these MRSA isolates involved alterations in both DNA gyrase and antimicrobial uptake and efflux.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections pose serious therapeutic difficulties, since only a few effective therapeutic agents are available. Although recently synthesised fluorinated 4-quinolone antibacterial agents are active against these strains, the increase in the clinical use of 4-quinolones has led to the widespread emergence of 4-quinolone-resistant strains.¹ Three possible mechanisms of resistance to 4-quinolones have been described: (1) alteration of DNA gyrase (*gyrA*), (2) existence of an efflux system (*norA*) and alterations in the *cfx-ofx* locus. Previous workers have purified DNA gyrases from 4-quinolone-resistant MRSA and reported both alterations of subunit A of the enzyme as well as the accumulation of 4-quinolones in MRSA.^{2,3} DNA gyrase is a tetrameric protein

consisting of two A and B subunits encoded by the *gyrA* and *gyrB* genes, respectively. Several point mutations in *gyrA* that confer high-level fluoroquinolone resistance have been described.^{4–7} The membrane-associated active efflux pump (*norA*) has also been reported as a mechanism of resistance,^{8–10} and the *cfx-ofx* locus reported by Trucksis *et al.*¹¹ conferred low-level resistance (4–8-fold increase in MIC) to 4-quinolones.

Clinical isolates of MRSA from Japan and China were examined for 4-quinolone susceptibility along with the inhibitory action of these drugs on the supercoiling activities of DNA gyrases from the isolates and their uptake of ofloxacin. Genetic studies were also performed to identify the mechanism(s) of resistance to quinolones.

Table 1. Antimicrobial activities against *S. aureus* FDA 209-P, 890405 and MRSA isolates

Strain no.	MIC (mg/L)													
	OFLX	LVFX	DU-6859a	DV-7751a	CPFX	SPFX	TFLX	NFLX	NA	DMPPC	TC	MINO	KM	GM
FDA 209-P	0.39	0.2	0.006	0.025	0.1	0.05	0.025	0.2	50	3.13	0.2	0.1	0.2	0.05
87-53*	0.39	0.2	0.025	0.05	0.39	0.05	0.025	1.56	25	> 800	100	6.25	> 800	50
87-20*	12.5	6.25	0.39	1.56	25	6.25	6.25	100	> 800	> 800	> 100	6.25	> 800	12.5
87-21*	25	12.5	0.78	1.56	50	3.13	6.25	200	400	> 800	100	6.25	> 800	> 100
89-33*	12.5	6.25	0.78	1.56	25	6.25	3.13	100	800	> 800	100	6.25	> 800	100
89-36*	12.5	6.25	0.39	0.78	12.5	3.13	3.13	50	800	> 800	100	0.2	> 800	100
90-37*	50	12.5	0.78	1.56	50	6.25	25	100	200	> 800	100	12.5	> 800	100
890405†	25	12.5	0.78	1.56	50	1.56	3.13	200	800	1.56	100	25	> 800	100
890664†	100	12.5	3.13	6.25	800	12.5	25	> 200	400	50	0.39	0.2	800	12.5
890669†	100	50	6.25	6.25	800	12.5	25	> 200	400	800	0.39	0.2	100	0.39
890670†	100	50	3.13	6.25	400	12.5	25	> 200	400	800	0.39	0.2	100	0.39
890952†	100	50	3.13	6.25	400	12.5	12.5	> 200	400	> 800	0.39	0.2	100	0.39
890958†	50	25	3.13	3.13	800	6.25	6.25	> 200	400	800	0.39	0.2	100	0.39
891185†	200	100	12.5	12.5	> 800	12.5	> 100	> 200	400	> 800	3.13	0.78	200	0.39
900165†	100	50	3.13	6.25	400	12.5	12.5	> 200	400	> 800	0.39	0.2	100	0.39

OFLX, ofloxacin; LVFX, levofloxacin; CPFX, ciprofloxacin; SPFX, sparfloxacin; TFLX, tosufloracin; NFLX, norfloxacin; NA, nalidixic acid; DMPPC, methicillin; TC, tetracycline; MINO, minocycline; KM, kanamycin; GM, gentamicin; AMK, amikacin; VCM, vancomycin; RFP, rifampicin; CP, chloramphenicol. *, Strain isolated in China; †, strain isolated in Japan.

Materials and methods

Bacterial strains

Fluoroquinolone-susceptible and -resistant MRSA were from six hospitals in Japan (92 isolates) and from the Hua Shan Hospital, Shanghai, China (33 isolates) and were isolated during 1987–1990. A fluoroquinolone-resistant methicillin-susceptible *S. aureus* (MSSA) isolate from Japan and *S. aureus* FDA 209-P were also used as controls.

Antimicrobial agents

The 4-quinolones ofloxacin, levofloxacin, DU-6859a,¹² DV-7751a,¹³ ciprofloxacin, sparfloxacin, tosufloracin and norfloxacin as well as nalidixic acid were synthesised at Daiichi Pharmaceutical Co., Ltd. Methicillin, kanamycin, vancomycin, rifampicin and chloramphenicol were purchased from Sigma. Tetracycline and minocycline were obtained from Lederle Japan (Tokyo, Japan). Gentamicin and amikacin were obtained from Schering Plough (Osaka, Japan) and Banyu Pharmaceutical Co. (Tokyo, Japan), respectively.

MIC determination

MICs were determined by standard agar dilution methods¹⁴ with Mueller-Hinton Agar (Difco). One loopful (5 µl) of an inoculum from an overnight culture, corresponding to $c. 10^4$ cfu/spot, was inoculated on drug-containing agar plates and the plates were incubated for 18 h at 37°C. The MIC was defined as the lowest drug concentration that prevented visible growth of bacteria.

Inhibition of DNA gyrase

DNA gyrases were purified from *S. aureus* FDA 209-P and quinolone-resistant strains and assayed for quinolone inhibition of DNA supercoiling activity as described previously.¹⁵ The enzyme activity introduced into each reaction mixture was 1 unit¹⁵ and the purity of subunit A and B was almost 90% on SDS-PAGE.

Nucleotide sequence analysis of the *gyrA* gene

Polymerase chain reaction (PCR) amplification of part of the *gyrA* gene from quinolone-resistant strains was performed by the method of Hori *et al.*¹⁶ PCR-amplified DNA was sequenced by the cycle sequencing method¹⁷ with a commercially available kit (New England Biolabs Co.). Thirty-five cycles were performed for each reaction as follows: 1 min at 93°C, 1 min at 94°C, 1 min at 66°C, and 1.5 min at 74°C. The partial nucleotide sequences corresponding to the nucleotide positions 120–345 of the *gyrA* genes of *S. aureus* FDA 209-P and the resistant strains were determined.

Uptake of quinolones

The uptake of ofloxacin by *S. aureus* cells was

Table II. Comparison of MICs (mg/L) of 4-quinolones with their inhibitory effect against DNA gyrase

Strain no.	Ofloxacin		Levofloxacin		DU-6859a		DV-7751a		Ciprofloxacin		Sparfloxacin	
	MIC	IC50	MIC	IC50	MIC	IC50	MIC	IC50	MIC	IC50	MIC	IC50
FDA 209-P	0.39	26	0.2	19	0.006	0.65	0.025	0.86	0.1	36	0.05	25
87-53	0.39	110	0.2	65	0.025	7.3	0.05	—	0.39	110	0.05	45
89-33	12.5	> 1600	6.25	> 1600	0.78	85	1.56	—	25	> 800	6.25	1500
90-37	50	> 1600	12.5	1350	0.78	13	1.56	—	50	> 800	6.25	1100
891185	200	> 1600	100	1590	12.5	15	12.5	77	> 800	> 800	12.5	509
900165	100	> 1600	50	> 1600	3.13	61	6.25	270	400	> 800	12.5	600

IC50, dose required to give 50% inhibition of enzyme activity (mg/L).

monitored by a fluorometric method.¹⁸ Bacteria in the logarithmic phase were collected, washed and suspended in 50 mM sodium phosphate buffer (pH 7.2) corresponding to 40 g/L. Bacterial suspensions were incubated with ofloxacin for 10 min at 37°C at a final concentration of 10 mg/L in the presence or absence of carbonyl cyanide *m*-chlorophenylhydrazine (CCCP, Sigma) 5 mg/L (= 25 µM). The results indicate the amount of ofloxacin/mg of cells in the presence and absence of CCCP and the ratio of the amount of ofloxacin with CCCP to that without CCCP.

Restriction fragment length analysis of the *norA* gene

PCR amplification of part of the *norA* gene from 4-quinolone-resistant strains was performed by the method of Kaatz *et al.*¹⁹ PCR-amplified DNA (0.5 mg/L) was digested with the restriction endonuclease *SfcI* and subjected to agarose gel electrophoresis. *SfcI* digestion of the product which has cytosine at nucleotide position 1085 results in restriction fragments of 408 and 323 bp, whereas a substitution of adenine at this position eliminates the restriction site and leaves the 731-bp PCR product intact.

Results

Susceptibility testing

Table I shows the MICs of antibacterial agents against *S. aureus* FDA 209-P (standard strain), six MRSA (one 4-quinolone-susceptible and five -resist-

ant strains) isolates from China, and eight 4-quinolone-resistant strains (one MSSA and seven MRSA) isolated in Japan. Quinolone-resistant strains that showed higher MICs of ofloxacin among strains from each country were selected. Generally, the MICs of 4-quinolones against the Chinese *S. aureus* strains were lower than those of the Japanese strains. In contrast, 4-quinolone-resistant MRSA strains from China were resistant to aminoglycosides, tetracycline, minocycline, rifampicin and chloramphenicol. The MIC values in the resistant strains were increased for ofloxacin, levofloxacin and DV-7751a (32–512-fold) DU-6859a (64–2048-fold), sparfloxacin (32–256-fold), ciprofloxacin (128–> 8192-fold), tosufloxacin (128–> 4096-fold), norfloxacin (256–> 1024-fold) and nalidixic acid (4–> 16-fold) compared with the sensitive control strain *S. aureus* FDA 209-P.

Inhibitory effect on DNA gyrases

The inhibitory effects of 4-quinolones against the supercoiling activities of DNA gyrases are shown in table II. The 50% inhibitory concentrations (IC50s) of levofloxacin against purified DNA gyrases from quinolone-resistant MRSA were at least 20-fold higher than those against DNA gyrases from quinolone-susceptible MRSA 87-53 and *S. aureus* FDA 209-P. IC50s of sparfloxacin against *S. aureus* strains 89-33 and 90-37 were 2–3-fold higher than those against *S. aureus* strains 891185 and 900165 although the MIC values were in the reverse order. This tendency was also observed for levofloxacin. The difference in MICs against strains moderately and highly resistant to quinolones was not reflected in the inhibition of DNA gyrases. Although the MIC values of ofloxacin were the same in two quinolone-susceptible strains, the DNA gyrase from strain 87-53 had a four-fold increase in IC50 for ofloxacin than the enzyme from FDA 209-P. This might be caused by differences in the uptake of quinolones. Reconstitution assay of subunits A and B from quinolone-resistant and -susceptible *S. aureus* strains showed that quinolone-resistant MRSA had a mutation in subunit A of DNA gyrase (table III).

Table III. Inhibitory effects of 4-quinolones against reconstituted DNA gyrases

4-quinolone	IC50 (mg/L)			
	As-Bs	Ar-Bs	As-Br	Ar-Br
OFLX*	110	> 1600	32	> 1600
CPFX*	110	> 800	72	> 800
OFLX†	26	> 1600	38	> 1600
CPFX†	36	> 800	50	> 800

A, subunit A; B, subunit B; s, fluoroquinolone-susceptible MRSA (*S. aureus* 87-53)* or *S. aureus* FDA 209-P†; r, fluoroquinolone-resistant MRSA (*S. aureus* 89-33* or *S. aureus* 891185†). OFLX, ofloxacin; CPFX, ciprofloxacin.

Nucleotide sequence analysis of the *gyrA* gene

The partial nucleotide sequences corresponding to the nucleotide positions 120–345 of the *gyrA* genes of

Table IV. Mutations in *gyrA* in *S. aureus*

Strain no.	MIC of ofloxacin (mg/L)	Codon	Nucleotide mutation	Amino acid change
FDA 209-P	0.39	—	—	—
87-53	0.39	86	ATT → ATC	Ile → Ile
87-20	12.5	84, 86	TCA → TTA, ATT → ATC	Ser → Leu, Ile → Ile
87-21	25	88, 86	GAA → AAA, ATT → ATC	Glu → Lys, Ile → Ile
89-33	12.5	84, 86	TCA → TTA, ATT → ATC	Ser → Leu, Ile → Ile
89-36	12.5	84, 86	TCA → TTA, ATT → ATC	Ser → Leu, Ile → Ile
89-43	12.5	84, 86	TCA → TTA, ATT → ATC	Ser → Leu, Ile → Ile
90-37	50	84, 86	TCA → TTA, ATT → ATC	Ser → Leu, Ile → Ile
890405	25	88, 86	GAA → AAA, ATT → ATC	Glu → Lys, Ile → Ile
890664	100	84	TCA → TTA	Ser → Leu
890669	100	84	TCA → TTA	Ser → Leu
890670	100	84	TCA → TTA	Ser → Leu
890952	100	84	TCA → TTA	Ser → Leu
890958	50	88, 86	GAA → AAA, ATT → ATC	Glu → Lys, Ile → Ile
891185	200	84	TCA → TTA	Ser → Leu
900165	100	84	TCA → TTA	Ser → Leu

Table V. Uptake of ofloxacin and RFLP analysis by *SfcI* of *norA*

Strain no.	Amount of ofloxacin without CCCP*	Amount of ofloxacin with CCCP*	Ratio of uptake ofloxacin†	Retention of the <i>SfcI</i> restriction site‡
FDA 209-P	60.62	42.27	0.7	—
87-53	139.26	135.02	0.97	+
87-20	162.35	177.07	1.09	+
87-21	145.1	148.03	1.02	+
89-33	107.88	111.1	1.03	+
89-36	155.35	189.6	1.22	+
89-43	147.66	188.67	1.27	+
90-37	92.93	169.7	1.83	+
890405	77.23	107.28	1.39	—
890664	21.45	121.94	5.68	+
890669	34.86	168.16	4.82	+
890670	90.6	197.06	2.18	+
890952	59.6	193.73	3.25	+
890958	103.17	189.58	1.84	—
891185	94.88	158.5	1.67	+
900165	117.31	182.24	1.55	+

*ng/mg.

†The ratio of the amount of ofloxacin in cells with CCCP/that without CCCP after incubation for 10 min.

‡A '+' indicates that the DNA fragment has the mutation of cytosine at nucleotide position 1085, rather than adenine, and is thus susceptible to restriction digestion.

13 MRSA strains (five moderately resistant and eight highly resistant to 4-quinolones), one 4-quinolone-susceptible MRSA, one 4-quinolone-resistant MSSA and strain FDA 209-P were determined (table IV). Eleven of these 13 strains (four moderately resistant and seven highly resistant to quinolones) contained a Ser → Leu substitution caused by an alteration at codon 84, and two strains moderately resistant and one highly resistant to 4-quinolones contained a Glu → Lys substitution caused by an alteration at codon 88. Eight MRSA, including one 4-quinolone-susceptible strain and one MSSA, contained a silent mutation at codon 86.

Uptake of ofloxacin by MRSA

Uptake of ofloxacin by *S. aureus* cells is shown in table V. The amount of ofloxacin in 4-quinolone-susceptible strains remained unchanged when CCCP

was added. The ratio of the amount of ofloxacin in MRSA moderately resistant to 4-quinolones in the presence and absence of CCCP ranged from 1.02 to 1.39; these values are less than double the ratio in *S. aureus* FDA 209-P. In contrast, this ratio in strains highly resistant to 4-quinolones ranged from 1.55 to 5.68.

Restriction fragment length analysis of the *norA* gene

SfcI-digestion of the PCR product with cytosine instead of adenine at nucleotide position 1085 results in restriction fragments of 408 and 323 bp. Thirteen of 14 MRSA strains showed a mutation in *norA* at codon 362; strain FDA 209-P, one 4-quinolone-resistant MSSA (strain 890405) and one 4-quinolone-resistant MRSA (strain 890958) had no mutation in *norA* at codon 362 (table V). The latter two strains were less resistant to ofloxacin than the other six Japanese

strains (table I). This result showed no correlation with the uptake of ofloxacin.

Discussion

The pattern of susceptibility of MRSA strains to antimicrobial agents varied between the two countries. Japanese strains were less resistant to antibiotics than the Shanghai strains. In Japan, the use of rifampicin is generally limited only to the treatment of tuberculosis, and the use of tetracyclines and chloramphenicol is now insignificant. Vancomycin had a good antibacterial activity against all *S. aureus* strains and tetracyclines and gentamicin are expected to have clinical efficacy against MRSA infections in Japan. However, the level of resistance to quinolones was lower in China than Japan, where 4-quinolones have been used for 10 years. In China, although other antibiotics have long been widely used, 4-quinolones were only introduced in 1987 although their usage has recently increased (personal communication, Dr Wang Fu, Shanghai Medical University).

In the present study, the mechanism(s) of resistance to 4-quinolones among MRSA strains were investigated. DNA gyrase was purified and the MICs of 4-quinolones for various MRSA strains were determined. Although MIC values against MRSA were different, IC₅₀ values were similar, with the predominant mutation occurring in the A subunit of DNA gyrase. Nucleotide sequence analysis of the *gyrA* gene showed that 11 strains (four moderately resistant and seven highly resistant to 4-quinolones) contained a Ser → Leu substitution resulting from a mutation in codon 84, and that two strains moderately resistant

and one highly resistant to 4-quinolones contained a Glu → Lys substitution resulting from a mutation in codon 88. Mutations at codons 84, 85 and 88 have been reported to confer quinolone resistance in *S. aureus*;⁴⁻⁷ however, no mutation at codon 85 was found in this study. Moreover, a silent mutation at codon 86 was found in eight MRSA, including one 4-quinolone-susceptible strain, and in one MSSA. Tokue *et al.* reported this mutation in one fluoroquinolone-susceptible strain isolated in the Tohoku region of Japan.²⁰ All the Chinese, but only two of the Japanese strains contained this point mutation.

The *norA* gene, which is responsible for quinolone resistance related to the efflux system, has been cloned from the chromosomal DNA of *S. aureus*.⁸⁻¹⁰ To clarify the difference in susceptibility to quinolones, the uptake of ofloxacin into MRSA was also examined. In strains with ofloxacin MIC values of 12.5 and 25 mg/L, the ratio of the amount of uptake without CCCP compared to that with CCCP was within two-fold that of *S. aureus* FDA 209-P, whereas in those with MICs > 50 mg/L, this ratio ranged widely. From these results, we speculate that MRSA acquire resistance to 4-quinolones by the alteration of DNA gyrase and by the development of an active efflux system. Restriction fragment length analysis of the *norA* gene with the restriction endonuclease *Sfi*I showed a mutation in *norA* at codon 362. This result did not correspond to the uptake of ofloxacin, and showed no regional differences. Kaatz *et al.* reported that an increase in transcription of the *norA* gene was a cause of 4-quinolone-resistance.¹⁹

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