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Topoisomerase Mutations in Trovafloxacin-Resistant *Staphylococcus aureus*

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A total of 201 *Staphylococcus aureus* isolates were surveyed for susceptibility to ciprofloxacin and trovafloxacin. Of 66 methicillin-resistant isolates, 89% were ciprofloxacin resistant and 6% were also trovafloxacin resistant. Trovafloxacin-resistant strains had unusual patterns of quinoline resistance mutations in DNA topoisomerase genes, including two mutations in the A subunit (encoded by *grlA*) of topoisomerase IV.

Acquired fluoroquinolone resistance is common among staphylococci, especially methicillin-resistant *Staphylococcus aureus* (MRSA). In some hospitals, the majority of MRSA isolates are highly resistant to ciprofloxacin (1), and such isolates tend to be cross-resistant to the newer agents levofloxacin and sparfloxacin (3, 16). The resistance is usually due to mutations in DNA topoisomerases which cluster in short stretches, the quinolone resistance-determining regions (QRDRs), of the genes for the A subunits of gyrase (*gyrA*) and of topoisomerase IV (*grlA*) (5, 8, 15–18). The most recently approved fluoroquinolone, trovafloxacin, has good activity against gram-positive bacteria, including many MRSA (2, 6, 14). In the present work, we surveyed recent *S. aureus* isolates for susceptibility to ciprofloxacin and trovafloxacin. Most MRSA were found to have high-level ciprofloxacin resistance but to be susceptible to trovafloxacin. A few were highly resistant to both agents, and we have sought the molecular basis of this resistance.

Independent clinical isolates ($n = 201$) were collected in 1996 from four New Jersey hospitals. We screened for susceptibility to oxacillin, ciprofloxacin, and trovafloxacin by agar dilution (12) over the range 0.125 to 8 μg/ml in twofold increments. Selected isolates were subjected to microdilution assays (12) for susceptibility to ciprofloxacin, trovafloxacin, sparfloxacin, and levofloxacin over the range 0.015 to 256 μg/ml. Oxacillin was purchased from Sigma Chemical Co., St. Louis, Mo., and ciprofloxacin was purchased from Bayer Corp., West Haven, Conn. Levofloxacin was provided by the RW Johnson Research Institute, Raritan, N.J., sparfloxacin was provided by Rhône-Poulenc Rorer, Collegeville, Pa., and trovafloxacin was provided by Pfizer, Inc., New York, N.Y.

For genotype analyses, DNA was prepared with Instagene Matrix (Bio-Rad Laboratories, Hercules, Calif.) or QIAamp Tissue Kit (Qiagen Inc., Santa Clarita, Calif.). PCRs were performed by adding 100 ng of DNA to the mixture of 50 pmol of each primer, 200 μM deoxynucleoside triphosphates, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 3 mM $MgCl_2$, and 2.5 U of *Taq* DNA polymerase and then cycling 30 times, with 1 cycle consisting of 1 min at 95°C, 2 min at 37°C, and 2 min at 72°C. PCR primers were based on conserved subsequences flanking QRDRs: for *gyrA*, nucleotides 1 to 20 and 472 to 455 (11); for *grlA*, nucleotides 1 to 22 and 772 to 751 (8). T3 or T7 promoters were appended to 5′ ends to permit sequencing with a Li-Cor 4000L automated sequencer (Li-Cor, Lincoln, Nebr.) using labelled T3 or T7 primers. Amplicons were purified on QIAquick spin columns (Qiagen), and then both strands were sequenced directly. Sequences were obtained for *gyrA* codons 8 through 133 and for *grlA* codons 41 through 205 (GenBank accession nos. AF044066 to -75 and AF044897 to -906), and analyzed with the Wisconsin Package (Genetics Computer Group, Madison, Wis.).

Sixty-six isolates were methicillin resistant (oxacillin MIC ≥ 4 μg/m); of these isolates, 59 (89%) were highly ciprofloxacin resistant (MIC ≥ 8 μg/ml) and only 4 (6%) were also highly trovafloxacin resistant (defined likewise). Of 135 methicillin-susceptible isolates, 7 were ciprofloxacin resistant and 2 were also trovafloxacin resistant. We subjected to further analysis seven MRSA: one fluoroquinolone-susceptible isolate, two ciprofloxacin-resistant, trovafloxacin-susceptible isolates, and the four ciprofloxacin-resistant, trovafloxacin-resistant isolates. Table 1 summarizes sequence results for known fluoroquinolone resistance mutational hot spots *gyrA* codons 84 and 88 and *grlA* codons 80 and 84, together with detailed MICs. Aside from changes involving the resistance hot spots, inferred protein sequences were as published for wild-type *S. aureus* (8, 11). Ciprofloxacin-resistant isolates, as expected (8, 15, 17, 18), had serine-to-leucine mutations at *gyrA* codon 84 (S84L) and serine-to-tyrosine or -phenylalanine mutations at *grlA* codon 80 (S80Y or S80F) and were cross-resistant to levofloxacin and sparfloxacin. The isolates that were also trovafloxacin resistant had a second mutation involving *grlA*, glutamic acid-to-lysine or -glycine at codon 84 (E84K or E84G). Although three of these isolates (SA22, SA32, and SA92) had identical sequences and may belong to the same clonal lineage, each was obtained from a different hospital and none was involved in an outbreak of infections. The clonal relationships among our isolates have yet to be investigated.

Our survey results are in accord with earlier work (2, 6) showing that high-level ciprofloxacin resistance is common among MRSA and that most ciprofloxacin-resistant isolates remain trovafloxacin susceptible. Our detailed analyses of particular strains indicate that the ciprofloxacin resistance was due to mutations in *gyrA* and *grlA* which have been previously described and which confer cross-resistance to sparfloxacin and levofloxacin. We did uncover several strains with high-level trovafloxacin resistance, but our results underscore the rarity

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TABLE 1. Sequence changes of and MICs for selected *S. aureus* isolates

Strain	Amino acid/nucleotides[a]				MIC (μg/ml) of antibiotic[b]			
	gyrA codon		*grlA* codon		CPFX	TRFX	SPFX	LVFX
	84	88	80	84				
Ciprofloxacin susceptible, trovafloxacin susceptible								
Wild type[c]	S/TCA	E/GAA	S/TCC	E/GAA	0.25	0.03	0.06	0.19
SA74	S/TCA	E/GAA	S/TCC	E/GAA	0.25	0.023	0.06	0.125
Ciprofloxacin resistant, trovafloxacin susceptible								
SA75	**L**/T**T**A	E/GAA	**F**/T**T**C	E/GAA	8	0.75	8	12
SA85	**L**/T**T**A	E/GAA	**Y**/T**A**C	E/GAA	64	0.75	8	8
Ciprofloxacin resistant, trovafloxacin resistant								
SA198	**L**/T**T**A	E/GAA	**F**/T**T**C	**K**/**A**AA	128	8	16	32
SA22	**L**/T**T**A	E/GAA	**Y**/T**A**C	**G**/G**G**A	128	8	8	32
SA32	**L**/T**T**A	E/GAA	**Y**/T**A**C	**G**/G**G**A	128	16	8	32
SA92	**L**/T**T**A	E/GAA	**Y**/T**A**C	**G**/G**G**A	128	8	16	32

[a] *S. aureus* numbering used throughout; mutations are in boldface type.
[b] MICs are the average of duplicate determinations; the duplicates did not differ by more than one twofold dilution. Antibiotic abbreviations: CPFX, ciprofloxacin; TRFX, trovafloxacin; SPFX, sparfloxacin; LVFX, levofloxacin.
[c] The wild-type sequence is from references 8 and 11; wild-type MICs were determined on a derivative of antibiotic-sensitive standard strain 8325 (13).

of such resistance. The sequencing results suggest a molecular explanation for this rarity, namely, that high-level resistance to trovafloxacin requires mutations in both amino acids 80 and 84 of GrlA, as well as amino acid 84 of GyrA; while, as found by others (8, 15, 17, 18), a single mutation each in the QRDRs of *gyrA* and *grlA* suffice for high-level resistance to ciprofloxacin, sparfloxacin, and levofloxacin. Testing this hypothesis will require introduction of appropriate mutations into otherwise isogenic strains.

Earlier work has shown that GrlA is the primary site of action of ciprofloxacin in *S. aureus* (8). *grlA* mutations S80F, S80Y, and E84K are most frequent (5, 8), and each can confer low-level ciprofloxacin resistance in the absence of other QRDR mutations (7, 8, 18). The *grlA* E84G mutation, which occurred in three of our high-level multiresistant isolates, has not been previously reported in *S. aureus* but has been implicated in fluoroquinolone resistance in members of the family *Enterobacteriaceae* (4, 9).

Our trovafloxacin-resistant isolates are the first American *S. aureus* isolates shown to contain three resistance mutations in topoisomerase QRDRs. However, fluoroquinolone-resistant Japanese isolates have recently been described with one mutation in *gyrA* (S84L) and two mutations in *grlA* (S80F plus E84K) (18) or with two mutations in *gyrA* (S84L plus E88K) and one mutation in *grlA* (S80F) (15). Further, Tanaka et al. (17) described an isolate with *gyrA* E88K and *grlA* S80F plus E84K; topoisomerase IV from this strain was more resistant to fluoroquinolones than topoisomerase IV with only S80F. The above Japanese strains showed high-level resistance to ciprofloxacin; trovafloxacin susceptibility was not determined.

Presumably the prevalent fluoroquinolone-resistant MRSA in our geographic area, those with single mutations in *gyrA* and *grlA*, have been selected by ciprofloxacin and, more recently, by levofloxacin and sparfloxacin. These isolates registered as susceptible to trovafloxacin by the recommended criterion of MIC of $\leq$2 μg/ml (10). However, they were overall less susceptible (trovafloxacin MIC at which 50% of the isolates are inhibited [MIC_{50}], 1 μg/ml) than ciprofloxacin-susceptible isolates (methicillin-sensitive and -resistant isolates, trovafloxacin MIC_{50}, 0.125 μg/ml), a factor which must be considered in designing trovafloxacin therapeutic regimens for *S. aureus* infections. The nature of selection pressure for the strains with high-level trovafloxacin resistance is problematic, as these strains predate the approval of trovafloxacin. They are marginally more resistant to ciprofloxacin and levofloxacin than some doubly mutated strains (Table 1), suggesting the possibility of selection by intensive use of high-dose ciprofloxacin or levofloxacin. Widespread use of trovafloxacin would likely exert greater pressure. Trovafloxacin at the same time is a candidate for treatment of infections due to multiresistant staphylococci; however, it must be used judiciously and at sufficient dosage to suppress the commonly occuring ciprofloxacin-resistant MRSA in order to minimize opportunities for generation of triple QRDR mutants that express high-level trovafloxacin resistance.

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