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Detection of *gyrA* Gene Mutations Associated with Ciprofloxacin Resistance in Methicillin-Resistant *Staphylococcus aureus*: Analysis by Polymerase Chain Reaction and Automated Direct DNA Sequencing

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A portion of the *gyrA* gene from amino acid codons 67 to 129 was sequenced in 34 methicillin-resistant *Staphylococcus aureus* strains (14 isolated in Minnesota, 10 isolated in Indiana, and 10 isolated in Tennessee). Twenty-eight of these strains were ciprofloxacin resistant. Sixteen of the strains contained a Ser→Leu mutation at codon 84; 3 contained strains a Ser→Ala mutation at codon 84; 3 strains contained two mutations, Ser→Leu at codon 84 and Ser→Pro at codon 85; and 6 strains contained a Glu→Lys mutation at codon 88. Six strains were wild type and ciprofloxacin susceptible. Several mutations from amino acid codons 84 through 88 can be associated with high-level quinolone resistance.

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections pose serious therapeutic difficulties in that few antimicrobial agents are effective against this pathogen (1). At one time, ciprofloxacin held promise for the treatment of these infections (1, 19). Presently, however, widespread resistance of MRSA to ciprofloxacin has developed in many centers and the mechanism of this resistance has provoked much study.

Ciprofloxacin is a 4-quinolone antibiotic that acts by inhibiting the A_2B_2 tetrameric bacterial enzyme DNA gyrase (19). DNA gyrase promotes the supercoiling of DNA by catalyzing transient double-stranded DNA breaks (9). Three possible mechanisms of staphylococcal resistance to ciprofloxacin have been described (10). One is a membrane-associated active efflux pump which excludes the agent from within the cell (21); a second is DNA gyrase (*gyrA*) gene mutations (3, 16); and a third is a recently described genetic locus distinct from the first two and which acts by an unknown mechanism (18). The described mutations in *gyrA* have been most closely associated with high-level (ciprofloxacin MIC, ≥16 μg/ml) quinolone resistance (4).

The *gyrA* and *gyrB* genes have been cloned in *S. aureus* and show close homology to the same genes in *Escherichia coli* (8). In *E. coli*, point mutations in the *gyrA* gene, specifically in the region of the Ser-83 codon, have been associated with ciprofloxacin resistance (3, 7, 20). Study of clinical MRSA isolates resistant to ciprofloxacin have revealed similar point mutations at Ser-84 (which is homologous to Ser-83 in *E. coli*), and Ser-85 (16).

Because mutations of the codons for Ser-84 and -85 in the *gyrA* gene are closely associated with ciprofloxacin resistance, we examined the occurrence of mutations by sequencing this region in 34 strains of MRSA from three geographically separate centers. Twenty-eight of these strains had a ciprofloxacin MIC of ≥16 μg/ml. The purpose of this investigation was to develop the largest bank of sequencing data thus far compiled for the MRSA *gyrA* gene in order to (i) evaluate possible *gyrA* mutations in quinolone-resistant MRSA isolated from distinct geographic locations and (ii) determine the frequency of these mutations in a large number of highly quinolone-resistant MRSA.

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Fourteen isolates of MRSA from patients residing in Minnesota and ten isolates from patients in both Indiana and Tennessee were included in this study. Each isolate was submitted to the authors as a distinct (nonduplicate) strain from individual patients. The strains were tested for ciprofloxacin susceptibility by using the microdilution broth method recommended by the National Committee for Clinical Laboratory Standards (12). Doubling dilutions of ciprofloxacin ranging from 0.125 to 128 μg/ml were tested.

Total genomic DNA from all 34 isolates was obtained by using a model 340A nucleic acid extractor (Applied Biosystems, Inc., Foster City, Calif.) (2b). This automated extractor performs proteinase K digestion, phenol-chloroform extraction, and sodium acetate-isopropanol precipitation, and then washes with ethanol. Four primers for a two-step asymmetric polymerase chain reaction (PCR) (11) were prepared by using an oligonucleotide synthesizer (model 3808; Applied Biosystems) after a phosphite triester synthesis method (2) with beta-cyanoethyl phosphoramidites as synthesis materials. The primer sequences and their positions relative to the *gyrA* gene are shown in Table 1. Primer 4 incorporated the −21 M13 universal priming sequence in order to facilitate binding of the sequencing dye primers to the final PCR product.

Amplification was carried out in a DNA thermocycler (Perkin Elmer-Cetus) by using the following final concentration cocktail: 1.5 mM magnesium chloride, 1× reaction buffer II (Perkin Elmer-Cetus part N808-0009), 2.5 U of *Taq* polymerase, and 200 μM each deoxynucleoside triphosphate. Thirty cycles were performed for each reaction as follows: 1 min at 94°C, 1 min at 55°C, and 30 s at 72°C with

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TABLE 1. Primers and sequences

Primer	Sequence	Position
1	5′-AGTACATCGTCGTATACTATATGG	Third base of codon 44 through second base of codon 52
2	5′-ATCACGTAACAGTTCAAGTGTG	Complementary to third base of codon 131 through codon 138
3	5′-AATCAGCACGTATCGTTGGTGACG	Second base of codon 66 through first base of codon 74
4	5′-tgtaaaacgacggccagtTAGTCATACGCGCTTC	−21 M13 universal primer[a] complementary to codon 125 through first base of codon 130

[a] −21 M13 universal primer is in lowercase letters.

5 min at 72°C after the final cycle. Rapid ramps were used between each temperature change. For each strain a symmetric PCR was carried out by using 20 pmol each of primers 1 and 2, with 2 μg of total genomic DNA in 100 μl of the reaction cocktail described above. Nine microliters of the product was electrophoresed on a 1.4% TAE agarose gel to ascertain the presence of amplified DNA. Symmetrically amplified products were precipitated by using ammonium acetate-ethanol, washed in 70% ethanol, dried, and rehydrated in 30 μl of H_2O before proceeding.

A second (asymmetric) stage of the amplification was carried out by using the same reaction cocktail, as described above. One microliter of the precipitated PCR product from the first reaction was used as the template. Primer 3 was used at 0.8 μM and primer 4 was used at 0.1 μM. Nine microliters of this reaction mixture was electrophoresed on a 1.4% agarose gel to check for presence of single-stranded DNA.

Asymmetric PCR products were used as templates in the sequencing reactions which followed Sanger's enzymatic dideoxy chain termination method (14). By the methods in Applied Biosystems User Bulletin 13 (2a), four separate reactions (A, T, C, and G) are set up for each template by using a distinct fluorescent dye-labeled primer and a specific chain-terminating base analog for each reaction. Unique fragment lengths are generated for each base of the template; the terminating base corresponds to the matching fluorescent dye. The sequencing reactions consisted of 10 cycles of 1 min at 90°C followed by 2 min at 70°C, with *Taq* DNA polymerase to catalyze the base additions. The completed reaction sets were combined, and the DNA was precipitated with sodium acetate-ethanol. Samples were electrophoresed on denaturing (urea) polyacrylamide gels at a gel concentration of either 6 or 8%. Sequence information was gathered in the automated instrument by detecting the color of fluorescence of each fragment length and then analyzing the results with ABI software version 1.3.

For each strain, at least two complete sets of PCR and sequencing reactions were carried out and electrophoresed on both 6 and 8% gels. Results of replicate sequencing were compared for consistency. Any strains in which the complete DNA sequence in the area of interest could not be clearly determined in both sets of sequencing runs had their cDNA strands evaluated by using a new set of PCR primers (results not shown).

Combining data from each of the sequencing reactions for each of the strains produced readable sequences for the region from amino acid (aa) codon 75 to aa codon 120 of the *gyrA* gene. The sequences were identical to the wild-type sequence obtained by Hopewell et al. (8) except for the mutations summarized in Table 2. The two Tennessee strains and the four Minnesota strains with the wild-type sequence were the only ciprofloxacin-susceptible strains in the study.

Each replicate set of sequences obtained for individual strains showed no discrepancies between each other. All quinolone-resistant isolates had at least one *gyrA* mutation between aa codon 84 and aa codon 88.

This is the largest series of MRSA strains to date for which partial sequences of the *gyrA* gene have been obtained. Mutations in the region of the gene that we sequenced have been implicated in fluoroquinolone resistance in MRSA as well as in *E. coli* (3, 7, 16). The TCA→TTA mutation at aa codon 84 as well as the double mutation at codon 85 (TCA TCT→TTA CCT) has been found in other MRSA strains in a previous study (16). The mutation at aa codon 88 found in six strains and the TCA→GCA mutation at codon 84 found in three strains have not been previously described for MRSA; however, the codon 88 mutation may be similar in effect to an Asp-87 mutation described for *E. coli* (13, 20), and the novel codon 84 mutation is concordant with a new Ser-83 mutation recently reported for *E. coli* (7).

The TCA→TTA codon 84 mutation was seen in strains from three separate geographic regions. The widespread nature of this mutation was suggested by previous work with oligonucleotide probes (5); however, that study missed the

TABLE 2. Mutations in various codons

Codon	Mutation	Amino acid change	No. of occurrences and origin of strains[a]	Ciprofloxacin MIC (range [μg/ml])
84	TCA→TTA	Ser→Leu	10, Indiana	16–>128
			3, Tennessee	32–>128
			3, Minnesota	32–128
84	TCA→GCA	Ser→Ala	3, Minnesota	16
84 and 85	TCA TCT→TTA CCT	Ser Ser→Leu Pro	3, Minnesota	64–128
88	GAA→AAA	Glu→Lys	5, Tennessee	16–32
			1, Minnesota	32
Wild type			2, Tennessee	0.5
			4, Minnesota	0.5–1

[a] Indiana, 10 strains total; Tennessee, 10 strains total; Minnesota, 14 strains total.

two new mutations detected by *gyrA* sequencing. Comparing the results of that previous study by using oligonucleotide probes with those of the current investigation by partial gene sequencing showed that there was complete concordance between the two techniques when there was a perfect match between the oligonucleotide probe and the target DNA sequence (6). However, if there was a single base mismatch in the middle of the probe sequence, or a two-base mismatch, probe binding rarely occurred (5 of 103 hybridizations with individual probes), and only the sequencing approach provided useful information with the new mutations. The codon 88 mutation was seen in two geographic regions while the double codon 84-85 mutation and the TCA → GCA mutation at codon 84 was seen in only one (Minnesota).

These results suggest that at least some *gyrA* mutations are similar from one region of the United States to another. From an epidemiologic point of view, it may be useful to know the specific mutations in strains of MRSA which acquire ciprofloxacin resistance. Such information can be obtained by using the methods described. Our own experience suggests that mutations in the region of aa codon 84 to 88 can occur frequently in a highly related clone of MRSA. Our MRSA epidemic is predominantly caused by a single bacterial clone (15), and we have recognized all currently reported staphylococcal *gyrA* mutations associated with ciprofloxacin resistance in our MRSA strains. This would appear to suggest that multiple, independent mutations associated with quinolone resistance can readily occur in MRSA and that the emergence of these mutations in *gyrA* does not need to be associated with the spread or transfer of genetic information from one strain to another.

It is postulated that mutation in this (aa codon 84 through 88) region of the *gyrA* gene confers ciprofloxacin resistance by altering the structure of the gyrase protein near its active site. It is interesting that the mutations we found result in a substitution of one type of amino acid for another. The Ser-84→Leu and the Ser-84→Ala mutations substitute nonpolar amino acids for a polar amino acid. The Glu-88→Lys mutation substitutes a positively for a negatively charged amino acid. These changes as well as the Ser-85→Pro mutation could alter the structure of the enzyme to interfere with quinolone activity. Also, our results are consistent with those of Hallett and Maxwell, who recently reported the Ser-83→Ala mutation in *E. coli* which corresponds to the same new amino acid change we found in Ser-84 of MRSA (7). Furthermore, it is of interest that only the Ser-84→Leu change led to the highest levels of ciprofloxacin resistance. This, too, is consistent with the report by Hallett and Maxwell in that the bulky, hydrophobic amino acid residues such as Leu and Trp result in the highest levels of quinolone resistance (7). All our isolates with a Ser-84→Leu change had a ciprofloxacin MIC of 16 to ≥128 μg/ml, whereas those with a different mutation showed an MIC range of 16 to 32 μg/ml (Table 2).

The use of the PCR combined with an automated sequencing system allowed for the relatively rapid sequencing of a specific segment of DNA from a large number of organisms. This work adds support to the theory that ciprofloxacin resistance in MRSA is highly associated with mutations in the Ser-84 region of the *gyrA* gene. All of the 28 highly quinolone-resistant (ciprofloxacin MIC, ≥16 μg/ml) isolates studied in this investigation had at least one mutation in this region. However, we recently reported the partial *gyrA* sequence of a laboratory-derived strain of MRSA that was highly resistant to ciprofloxacin yet had a wild-type sequence (17). This is the only such strain of quinolone-resistant MRSA we have encountered, but it illustrates the fact that there are additional resistance mechanisms associated with high-level quinolone resistance that are yet to be described. Trucksis et al. recently reported a new locus associated with quinolone resistance in *S. aureus*, but the ciprofloxacin MICs for these isolates were only 1 to 8 μg/ml (18). Because we sequenced only a portion of the gene, we cannot rule out the possibility of other mutations in *gyrA*, although to date it appears that the majority of significant mutations will be found in the region of aa 84 to 88. Further studies such as site-directed mutagenesis experiments may clarify this issue. *gyrA* mutations at or near aa codon 84 are highly associated with significant quinolone resistance in MRSA. The numerous mutations seen may explain the rapid, widespread development of quinolone resistance described in MRSA.

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