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Detection of Novel Mutations in the *gyrA* Gene of *Staphylococcus aureus* by Nonradioisotopic Single-Strand Conformation Polymorphism Analysis and Direct DNA Sequencing

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A total of 36 clinical isolates of *Staphylococcus aureus* (29 fluoroquinolone-resistant strains and 7 fluoroquinolone-susceptible strains) were studied for the presence of point mutations in the *gyrA* gene by nonradioisotopic single-strand conformation polymorphism (Non-RI SSCP) analysis with silver stain. Direct DNA sequencing analysis of the PCR-amplified DNA fragments confirmed the results obtained by Non-RI SSCP analysis and revealed that fluoroquinolone resistance is closely associated with six types of mutations in the *gyrA* gene, of which three types of mutations were newly identified: (i) Ser-84→Leu and Glu-88→Gly, (ii) Ser-84→Leu and Glu-88→Lys, and (iii) Glu-88→Gly. Furthermore, the novel ATT→ATC mutation at codon 86 (silent mutation) was seen in only one fluoroquinolone-susceptible strain. All seven mutational types were separated from the wild type in a single electrophoretic step within 3 h after PCR amplification. Thus, we conclude that this new technique is a rapid, simple, and useful screening method for the genotyping of *gyrA* mutations associated with fluoroquinolone resistance.

Staphylococcal infection, particularly that caused by methicillin-resistant *Staphylococcus aureus*, is a serious medical problem, since only a few effective therapeutic agents are available (12). Antibacterial fluoroquinolones, such as ofloxacin and ciprofloxacin, have been effective in the treatment of infections caused by multidrug-resistant strains of *S. aureus* (2). However, the widespread use of these agents has led to the marked emergence of fluoroquinolone-resistant *S. aureus*, especially among methicillin-resistant strains (10, 15). So far, three possible mechanisms of staphylococcal resistance to fluoroquinolones have been described. The first proposed mechanism of resistance is a membrane-associated active efflux pump, encoded by the *norA* gene, which excludes the agent from the cell (22). The second is a recently described *cfx-ofx* locus which acts by some unknown mechanism (20). The third is a mutational alteration of DNA gyrase, an essential bacterial enzyme involved in DNA replication and repair (21). DNA gyrase is a tetrameric protein consisting of two A subunits and two B subunits, encoded by the *gyrA* and *gyrB* genes, respectively. Several point mutations in the *gyrA* gene have been reported to be associated with high-level fluoroquinolone resistance (4, 5, 16, 17). Mutations in *gyrA* leading to quinolone resistance which have been identified in ciprofloxacin-resistant isolates of *S. aureus* are as follows: (i) Ser-84→Leu, (ii) Ser-84→Ala, (iii) Ser-84→Leu and Ser-85→Pro, and (iv) Glu-88→Lys (5).

Various methods have been reported for detection of point mutations of genes, including sequence-specific oligonucleotide probe hybridization (4), direct sequencing (5), and restriction fragment length polymorphism analysis (16, 17). Although these methods have been shown to be useful, they are unsatisfactory for a routine clinical laboratory. Single-strand conformation polymorphism (SSCP) analysis might be a simpler method for the detection of point mutations. In SSCP analysis, a mutated sequence is detected by a change of mobility in polyacrylamide gel electrophoresis as a result of its altered folded structure (7, 14). Since the use of ^{32}P requires a relatively long exposure time for autoradiography, we have previously reported a new technique using nonradioisotopic (Non-RI) SSCP analysis for detecting a point mutation of c-Ki-*ras*2 gene (18). In Non-RI SSCP analysis with silver stain, the bands are sharp, so that even a slight difference of migration can be detected in a small slab gel (18). Because mutations from amino acid codons 84 through 88 are closely associated with fluoroquinolone resistance, we used Non-RI SSCP to examine the occurrence of mutations in PCR-amplified DNA including this region. In this study, we classified the mutations in the *gyrA* gene of *S. aureus* with Non-RI SSCP; each class obtained is identified by direct DNA sequencing of the PCR-amplified DNA.

MATERIALS AND METHODS

Bacterial strains. Thirty-six strains of *S. aureus* isolated between 1992 and 1993 in the Division of Clinical Laboratory, National Cancer Center Hospital and in the Department of Respiratory Medicine, Institute of Development, Aging and Cancer, Tohoku University, were used in this study. One isolate was collected from each patient.

Antimicrobial susceptibility. The strains were tested for ciprofloxacin and ofloxacin susceptibility by a semiautomated microdilution method in Sceptor gram-positive broth with the Sceptor system (Becton Dickinson and Company, Cockeysville, Md.) with a final inoculum of 5×10^5 CFU/ml. Resistance to ciprofloxacin and ofloxacin was indicated by MICs equal to or more than 4 and 8 μg/ml, respectively.

Extraction of DNA and synthesis of oligonucleotides. DNA

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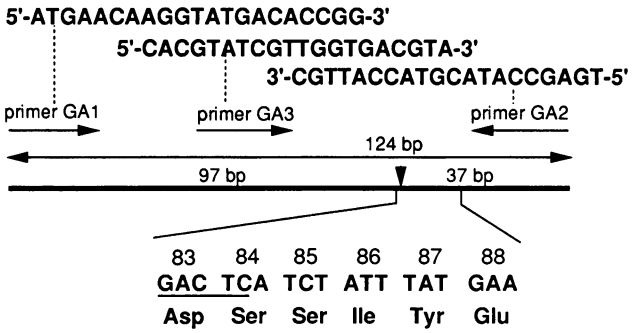

FIG. 1. Primers for PCR and direct DNA sequencing. A pair of primers (GA1 and GA2) yield a 124-bp *gyrA* fragment after PCR amplification. The presence of a *Hin*fI site (wild type) generates a pair of *Hin*fI DNA fragments 97 and 37 bp long, whereas its absence because of mutation yields a 124-bp fragment. The underlined sequence is a *Hin*fI site (GANTC), and the numbers identify the positions of the deduced amino acid residues. Primer GA3 was used as a sequencing primer.

was extracted by a method described previously (19). The oligonucleotides used as primers for PCR and direct sequencing were purchased from Intertech Co., Ltd. (Tokyo, Japan); their sequences are as shown in Fig. 1. A pair of primers (primers GA1 and GA2) yield a product of 124-bp DNA fragment after PCR amplification (9). Primer GA3 was used as a sequencing primer.

PCR. The DNA extract (1 μg) was amplified by PCR in 100 μl of a reaction mixture containing 25 pmol each of the two primers, 20 nmol each of the four deoxynucleoside triphosphates, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). Thirty cycles were performed for each reaction, with one cycle consisting of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C.

Restriction fragment length polymorphism analysis of PCR-amplified DNA. The PCR products were digested with *Hin*fI at 37°C for 1 h, and then cleavage of the PCR product was ascertained by electrophoresis through a 12% polyacrylamide gel and subsequent ethidium bromide staining.

Non-RI SSCP. In Non-RI SSCP analysis, 1-μl aliquots of PCR products were mixed with 10-μl portions of the loading solution containing 95% deionized formamide, 20 mM EDTA, 0.05% xylene cyanol, and bromophenol blue. After denaturation at 80°C for 5 min, 10 μl was applied to a 12% polyacrylamide gel (30:1 acrylamide/bisacrylamide ratio) containing 25 mM Tris and 192 mM glycine. The size of the gel was 85 by 86 mm. Electrophoresis was then performed for 2 h at 200 V with a 25 mM Tris–192 mM glycine buffer cooled to 17°C in a refrigerated circulating water bath. Each gel was then subjected to silver staining with a kit purchased from Daiichi Pure Chemical Co., Ltd. (Tokyo, Japan) by the procedure recommended by the manufacturer.

Direct DNA sequencing. PCR-amplified DNA was sequenced with synthetic oligonucleotide primer (primer GA3) by the cycle sequencing method (13), using the dsDNA Cycle Sequencing System (GIBCO BRL, Life Technologies Inc., Gaithersburg, Md.). The DNA sequencing primer was end labeled with [γ-^{33}P]ATP (Du Pont Company, NEN, Wilmington, Del.) by using T4 polynucleotide kinase (3). The samples were electrophoresed in a 6% polyacrylamide gel containing 7 M urea at 1,800 V for 2 h. The gel was dried and exposed to Kodak XAR film (Eastman Kodak Co., Rochester, N.Y.).

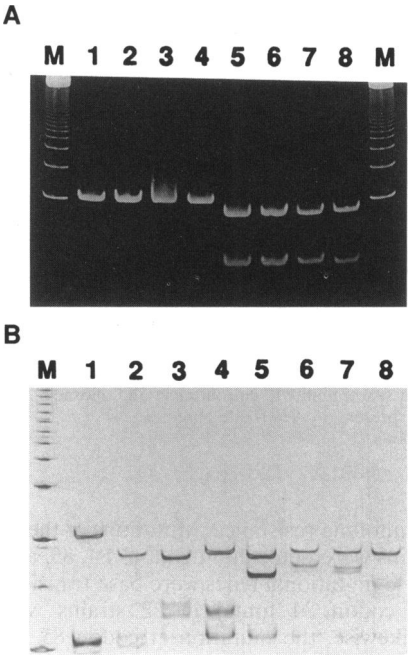

FIG. 2. (A) Polyacrylamide gel analysis of PCR-amplified DNA digested with *Hin*fI. PCR products were digested with *Hin*fI at 37°C for 1 h and subjected to electrophoresis through a 12% polyacrylamide gel at 20 mA for 40 min and subsequent ethidium bromide staining. (B) Detection of *gyrA* mutations by Non-RI SSCP analysis. Eight types of bands with different mobilities were separated. In all lanes, renatured double-stranded DNAs appeared at the positions seen for 124-bp fragments. Lanes: M, molecular size standards of 123-bp ladder plasmid DNA (GIBCO BRL); lane 1, mutant with Ser→Leu (TCA→TTA) at codon 84 and Ser→Pro (TCT→CCT) at codon 85; lane 2, mutant with Ser→Leu (TCA→TTA) at codon 84; lane 3, mutant with Ser→Leu (TCA→TTA) at codon 84 and Glu→Gly (GAA→GGA) at codon 88; lane 4, mutant with Ser→Leu (TCA→TTA) at codon 84 and Glu→Lys (GAA→AAA) at codon 88; lane 5, mutant with Glu→Gly (GAA→GGA) at codon 88; lane 6, mutant with Glu→Lys (GAA→AAA) at codon 88; lane 7, mutant with ATT→ATC at codon 84 (silent); lane 8, wild type. Lanes 1 through 6 were quinolone-resistant strains.

RESULTS

Of the 36 *S. aureus* strains used in this study, 29 strains were resistant to both ciprofloxacin and ofloxacin. Because mutations in amino acid codons 84 through 88 have been associated with ciprofloxacin resistance (4, 5, 16, 17), the primers used for PCR were designed to flank this region in the *gyrA* gene (Fig. 1). A 124-bp *gyrA* gene fragment was amplified from each isolate. The presence or absence of *gyrA* mutations at codon 84 was determined by digesting PCR products with *Hin*fI. The presence of the *Hin*fI site generates a pair of *Hin*fI DNA fragments, 97 and 37 bp long (9), whereas its absence caused by mutation yields a distinctive 124-bp product. In 36 strains analyzed, 14 generated two DNA fragments by *Hin*fI digestion of PCR products (Fig. 2A, lanes 5 to 8). However, seven strains were fluoroquinolone resistant, with the mutation at codon 88 but not at codon 84 (lanes 5 and 6 in Fig. 2A; Table 1).

In Non-RI SSCP analysis using DNAs derived from the isolates, eight types of bands with different mobilities were identified (Fig. 2B). Direct DNA sequencing analysis revealed that all abnormal bands were correlated with mutations in *gyrA* (Fig. 3). Of the eight types of mutations, six were associated

TABLE 1. Mutations in the *gyrA* gene in *S. aureus* detected by Non-RI SSCP

Strain[a] and codon(s)	Nucleotide mutation	Amino acid mutation	*Hin*fI[b]	No. of strains with mutation
Resistant				
84 and 85	TCA TCT→TTA CCT	Ser Ser→Leu Pro	−	2
84	TCA→TTA	Ser→Leu	−	16
84 and 88[c]	TCA GAA→TTA GGA	Ser Glu→Leu Gly	−	2
84 and 88[c]	TCA GAA→TTA AAA	Ser Glu→Leu Lys	−	2
88[c]	GAA→GGA	Glu→Gly	+	2
88	GAA→AAA	Glu→Lys	+	5
Susceptible				
86[c]	ATT→ATC	Ile→Ile	+	1
None			+	6

[a] Resistance or susceptibility to ciprofloxacin and ofloxacin.
[b] +, *Hin*fI site present; −, *Hin*fI site absent.
[c] Novel mutations.

with fluoroquinolone resistance. Mutations of the *gyrA* gene in resistant strains were detected at codons 84, 85, and 88 (Table 1). All of these mutational types were base transitions, i.e., the mutation at codon 84 found in 22 strains was a C-to-T transition. Likewise, the mutation at codon 85 found in two strains was a T-to-C transition, whereas the mutation at codon 88 was either a GAA→GGA or GAA→AAA transition. In 23 of 36 strains, mutation was found at a single codon, i.e., at codon 84 (16 strains) or 88 (7 strains), while 6 strains showed double mutations either at codons 84 and 85 (2 strains) or at codons 84 and 88 (4 strains). The GAA→GGA mutation at codon 88 and two types of double mutations, TCA GAA→TTA GGA or TTA AAA at codons 84 and 88, were not reported previously. Only one fluoroquinolone-susceptible strain contained an ATT→ATC transversion at codon 86, coding for the same amino acid (silent mutation). Six strains were wild type and fluoroquinolone susceptible.

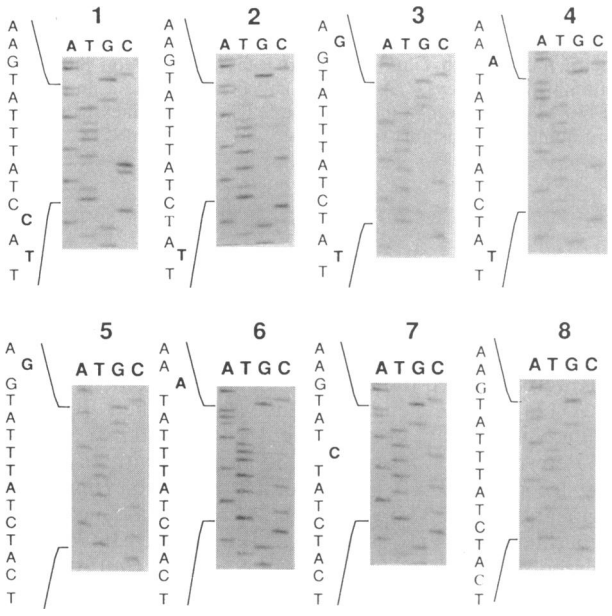

FIG. 3. Mutations in the *gyrA* gene from clinical isolates identified on DNA sequencing gels. PCR-amplified DNA was sequenced by the cycle sequencing method. The numbers at the top of the gels correspond to those of the lanes shown in Fig. 2. Gel 8 shows a sequence of wild type, whereas the others have either one or two base substitutions at codons 84 through 88. Mutant nucleotides are indicated by boldface type and are to the right of the sequence.

DISCUSSION

From the knowledge gained from fluoroquinolone resistance mechanisms in *Escherichia coli*, other workers have cloned and sequenced the *gyrA* gene of *S. aureus* (9, 16) and identified point mutations responsible for the resistance. In this study, we examined 36 strains of *S. aureus* by Non-RI SSCP analysis and demonstrated that six types of mutations were associated with quinolone resistance. The multiple mutation TCA→TTA at codon 84 and TCT→CCT at codon 85, the TCA→TTA mutation at codon 84, and the GAA→AAA mutation at codon 88 were reported in a previous study (5). Since SSCP analysis enables sensitive detection of single-base substitution, three novel mutations were detected in addition to the previously reported ones: two types of multiple mutations, TCA→TTA at codon 84 and GAA→GGA at codon 88, and TCA→TTA at codon 84 and GAA→AAA at codon 88, and one type of single mutation, GAA→AAA at codon 88. Furthermore, the novel ATT→ATC mutation at codon 86 was seen in one fluoroquinolone-susceptible strain, which could be distinguished as a silent mutation from the wild type. However, the previously reported mutation TCA→GCA at codon 84 (5) was not found in this study, so we produced a mutant with the TCA→GCA mutation at codon 84 by a site-directed mutagenesis technique (8) and performed electrophoresis under the conditions described above. This mutant was clearly separated from the above eight types (data not shown). In both Japan and the United States, the TCA→TTA mutation at codon 84 is found in about half of the quinolone-resistant strains. This mutation is the most important and the commonest change in quinolone-resistant *S. aureus* strains. All the 29 quinolone-resistant isolates studied in this investigation had at least one mutation in this region. Because we sequenced only a portion of the gene, we cannot rule out the possibility of other mutations present in the *gyrA* gene, although to date, it appears that the majority of significant mutations are found exclusively in this region.

From an epidemiologic point of view, it may be useful to know the specific mutations in strains of *S. aureus* which confer quinolone resistance. For identifying the sources and monitoring the spread of epidemic methicillin-resistant *S. aureus* strains, a number of epidemiologic markers have been re-

ported, including antibiogram, phage type, plasmid profile, ribotyping, and pulsed-field gel electrophoresis (1, 6, 11, 23). DNA analysis is useful for discrimination between individual species and between isolates of a given species. Since Non-RI SSCP analysis on one region could distinguish the eight types, combining these results with those from genotyping several mutational regions might provide more information for the discrimination of isolates. Classification of genotypic patterns by Non-RI SSCP may be a promising method for investigating the source, transmission, and spread of nosocomial methicillin-resistant *S. aureus* infections. The use of the Non-RI SSCP method allows for relatively rapid analysis of DNA from a large number of strains. Since the electrophoretic pattern is specific to each mutation, identification of a specific mutation is possible by comparison of the mobilities of the sample DNAs with that of the control DNA carrying known mutations. Thus, Non-RI SSCP analysis is a simple and useful method, not only for the detection of point mutations associated with fluoroquinolone resistance but also for the investigation of epidemiologic markers.

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