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DNA Gyrase Mutations in Quinolone-Resistant Clinical Isolates of *Neisseria gonorrhoeae*

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Eight quinolone-resistant clinical isolates of *Neisseria gonorrhoeae* were shown to carry mutations in their GyrA proteins. Six isolates had a single amino acid change of serine to phenylalanine at the position corresponding to Ser-83 in *Escherichia coli*. In addition to the change of serine to phenylalanine, two isolates had another change of aspartic acid to asparagine at the position corresponding to Asp-87 in *E. coli*.

Fluoroquinolones have a broad spectrum of antimicrobial activity including activity against *Neisseria gonorrhoeae* (6). These drugs have been demonstrated to be effective in treating gonococcal urethritis (1). However, treatment failure has been reported (3), and an increase in the number of clinical isolates of *N. gonorrheae* resistant to quinolones has been demonstrated (8, 12). Although a mutation in the *gyrB* gene analogous to that found in *Escherichia coli* (22) was suggested to be responsible for low-level quinolone resistance in *N. gonorrhoeae* (16), the mechanisms of high-level quinolone resistance are poorly understood. In this study, we sought to determine a partial sequence of the *gyrA* gene of *N. gonorrhoeae*, including the region corresponding to the quinolone resistance-determining region (21), and to identify and characterize mutations in the *gyrA* genes of clinical isolates showing decreased susceptibility to quinolones.

The type strain of *N. gonorrhoeae*, ATCC 19424, was purchased from the American Type Culture Collection to determine the partial sequence of the *gyrA* gene. Ten clinical isolates were obtained from patients with gonococcal urethritis from four independent sources in Gifu and Tokyo, Japan to analyze mutations in the *gyrA* gene. None of the patients had received antibiotic treatment before attending the clinics. The isolates were not epidemiologically related.

To amplify a DNA fragment of the *gyrA* gene from chromosomal DNA of the type strain, two oligonucleotides (GYRA-A4, 5′-GATGGTCTAAAGCCTGTACA-3′; GYRA-B, 5′-ATGTTGGTTGCCATACCTAC-3′) were synthesized. These primers were located within consensus amino acids of the bacterial GyrA proteins (9, 10, 17–19). GYRA-A4 was derived from nucleotide positions 115 to 134 with three mismatched bases, and GYRA-B was identical to nucleotide positions 526 to 545 of the *E. coli gyrA* gene, respectively. Chromosomal DNA of the type strain was isolated by sodium dodecyl sulfate lysis, followed by phenol-chloroform extraction. PCR amplification was performed in 100-μl reaction mixtures containing 10 μl of 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl (pH 8.3), 15mM $MgCl_2$, 0.01% gelatin), 1 μl of deoxynucleotide triphosphate mixture (20 mM concentrations of dATP, dCTP, dGTP, and dTTP), 5 μl of each oligonucleotide primer (4 μM), 0.5 μl of AmpliTaq DNA polymerase (5 U/μl), and 100 ng of genomic DNA of the type strain. Thirty PCR incubation cycles were performed, with one cycle consisting of a 60-s denaturation step at 95°C, a 50-s annealing step at 52°C, and a 50 s extension step at 72°C. Ligation of the PCR product to the pCR II vector, transformation into the competent *E. coli* cells (INVαF′ strain) and selection of colorless recombinant colonies were performed with the TA Cloning Kit following the manufacturer's instructions. Double-stranded plasmid DNA including the PCR product was purified and sequenced with the Sequence High-Cycle Kit and the Non-Radio-Isotopic Detection Kit (Toyobo, Osaka, Japan).

Antimicrobial susceptibilities were determined by the agar dilution method (11). The following quinolones for agar dilution testing were kindly provided by the indicated manufacturers: nalidixic acid and ofloxacin, Daiichi Pharmaceutical, Tokyo, Japan; norfloxacin and fleroxacin, Kyorin, Tokyo, Japan; ciprofloxacin, Bayer, Osaka, Japan; tosufloxacin, Toyama Chemical Company, Tokyo, Japan; and sparfloxacin, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan. Susceptibilities were determined on GC II agar base medium (Becton Dickinson, Cockeysville, Md.) supplemented with 1% IsovitaleX (Becton Dickinson) and containing serial twofold dilutions of each agent. Media were inoculated with 10^4 CFU and incubated at 37°C overnight. The MIC was defined as the lowest concentration inhibiting growth to ≤1 CFU.

To analyze mutations of the *gyrA* gene in clinical isolates, two primers (NG-GYRA-A, 5′-CGCGATGCACGAGCTGAAAA-3′; NG-GYRA-B, 5′-ATTTCGGTATAGCGCATGGCTG-3′) were synthesized on the basis of the partial sequence of the *gyrA* gene of the type strain determined in this study. PCR with NG-GYRA-A and NG-GYRA-B was expected to amplify the DNA fragment corresponding to nucleotide positions 150 to 374 of the *E. coli gyrA* gene. Chromosomal DNAs were isolated from 10 clinical isolates and subjected to PCR. PCR amplification and subsequent determination of sequences of PCR products were performed in the manner identical to that described above.

The primer set of GYRA-A4 and GYRA-B derived from two conserved regions in the N-terminal sequence of bacterial GyrA proteins amplified a DNA fragment having the expected size of 432 bp from the chromosomal DNA of the type strain. The PCR product was cloned, and its sequence was determined (Fig. 1). As expected, the nucleotide sequence of the amplified 391-bp DNA between the primers showed 72% identity with corresponding region of nucleotide positions 135 to 525 of the *E. coli gyrA* gene (17) and exhibited about 50%

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FIG. 1. Nucleotide sequence of the DNA fragment amplified from chromosomal DNA of *N. gonorrhoeae* type strain ATCC 19242 by PCR with primers GRYA-A4 and GYRA-B. The deduced amino acid sequence is also presented. The sequences of two primers, NG-GYRA-A and NG-GYRA-B, used for PCR amplification of the *gyrA* gene from the chromosomal DNAs of clinical isolates of *N. gonorrhoeae* are underlined. The sites in which nucleotide changes are observed in quinolone-resistant isolates of *N. gonorrhoeae* are denoted by asterisks.

identity with all other known *gyrA* genes (9, 10, 18, 19). The deduced 130-amino-acid sequence exhibited similarities ranging from 58 to 75% with the N-terminal positions of the bacterial GyrA proteins (Fig. 2). The amino acids equivalent to Ser-83 in the *E. coli* GyrA protein, whose mutations have been most frequently found and demonstrated to confer quinolone resistance in *E. coli* (2, 4, 5, 13, 20, 21), and corresponding to Tyr-122, which is the binding site of transiently cleaved DNA (7), are also serine and tyrosine, respectively.

TABLE 1. MICs of quinolones for the *N. gonorrhoeae* ATCC 19424 type strain and 10 clinical isolates of *N. gonorrhoeae*

Strain or isolate	MIC (μg/ml)[a]						
	NA	NFLX	OFLX	CPFX	TFLX	FLRX	SPFX
Type strain	0.25	0.008	0.015	0.002	0.008	0.015	0.002
GU1	256	4	1	0.5	0.5	2	0.25
GU2	256	1	1	0.5	0.25	2	0.25
GU3	256	4	0.5	0.25	0.5	2	0.25
GU4	256	8	4	2	0.25	8	1
GU5	256	8	4	2	0.25	8	0.5
GU6	256	4	1	0.25	0.25	2	0.25
GU7	1	0.06	0.06	0.015	0.015	0.06	0.008
GU8	256	4	1	0.5	0.5	4	0.25
GU9	1	0.06	0.03	0.015	0.015	0.03	0.008
GU10	128	4	0.5	0.5	0.5	2	0.25

[a] NA, nalidixic acid; NFLX, norfloxacin; OFLX, ofloxacin; CPFX, ciprofloxacin; TFLX, tosufloxacin; FLRX, fleroxacin; SPFX, sparfloxacin.

Table 1 presents the summary of MIC testing of the type strain and 10 clinical isolates. The type strain and isolates GU7 and GU9 were susceptible to all drugs tested. GU1, GU2, GU3, GU4, GU5, GU6, GU8, and GU10 were resistant to high levels of nalidixic acid and exhibited a 16-fold or greater increase in the MICs of fluoroquinolones compared with those of susceptible isolates. Among these isolates, GU4 and GU5 were more resistant to most fluoroquinolones than the remaining six isolates. From the chromosomal DNAs of these clinical isolates, single DNA fragments of 225 bp were amplified by PCR with NG-GYRA-A and NG-GYRA-B primers, and DNA sequence analyses of the PCR products were performed. In two quinolone-susceptible isolates of GU7 and GU9, the nucleotide sequences were in agreement with that of the type strain (Fig. 3). Six quinolone-resistant isolates of GU1, GU2, GU3, GU6, GU8, and GU10 exhibited a single C-to-T transi-

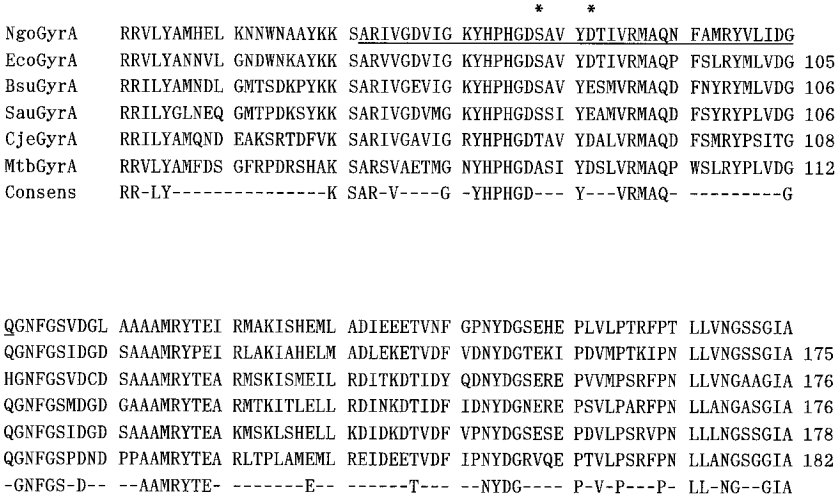

FIG. 2. Comparison of the amino acid sequence deduced from the partial sequence of the *N. gonorrhoeae gyrA* gene (NgoGyrA) with the corresponding sequences from *E. coli* (EcoGyrA) (18), *Bacillus subtilis* (BsuGyrA) (11), *S. aureus* (SauGyrA) (10), *C. jejuni* (CjeGyrA) (20), and *M. tuberculosis* (MtbGyrA) (19). Identical amino acids are listed on the consensus line (Consens). The region corresponding to the quinolone resistance-determining region of the *E. coli* GyrA protein are underlined. The sites in which amino acid changes are observed in quinolone-resistant clinical isolates of *N. gonorrhoeae* are denoted by asterisks. Dashes on the consensus line indicate amino acids which are not identical in the GyrA proteins.

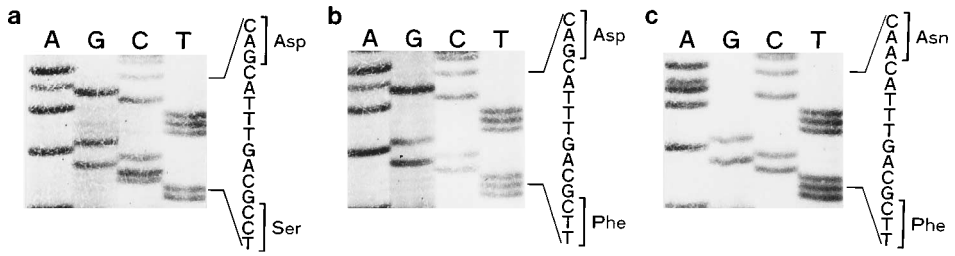

FIG. 3. Nucleotide changes in the DNA fragment of the *gyrA* gene amplified from clinical isolates of *N. gonorrhoeae* by PCR. (a) The nucleotide sequence of the DNA fragment from GU7 is in agreement with that determined from the type strain of *N. gonorrhoeae*, ATCC 19424. The amino acids equivalent to Ser-83 and 87-Asp in the *E. coli* GyrA protein are also serine and aspartic acid, respectively. The sequence of the DNA fragment from GU9 is also in agreement with that from the type strain. (b) The sequence analysis of the DNA fragment from GU1 indicates a C-to-T transition, resulting in an amino acid change of serine to phenylalanine at the position corresponding to Ser-83 in the *E. coli* GyrA protein. The identical amino acid change is found in the DNA fragments from GU2, GU3, GU6, GU8, and GU10. (c) The sequence analysis of the DNA fragment from GU4 indicates an additional G-to-A transition, resulting in an amino acid change of aspartic acid to asparagine at the position corresponding to Asp-87 in the *E. coli* GyrA protein. The identical double mutation is also found in the DNA fragment from GU5.

tion at the nucleotide position corresponding to position 248 (Ser-83) in the *E. coli gyrA* gene, resulting in an amino acid change of serine to phenylalanine (Fig. 3). Mutations of Ser-83 were most frequently found among clinical isolates of *E. coli* and alterations of the amino acid at the equivalent position were also found in other quinolone-resistant bacteria (14, 15, 18, 19). In addition to a C-to-T transition, GU4 and GU5 that were more resistant to most fluoroquinolones carried another G-to-A transition at the nucleotide position equivalent to position 259 (Asp-87) in the *E. coli gyrA* gene, producing Asp→Asn substitution (Fig. 3). The identical amino acid change of Asp-87 to Asn in *E. coli* (21) and of the equivalent Asp to Asn in *Campylobacter jejuni* (19) and *Mycobacterium tuberculosis* (18) were found. Other alterations at this amino acid position were also frequently found in quinolone-resistant bacteria (5, 13, 18).

GU4 and GU5 carrying a double mutation were more resistant to most fluoroquinolones. Double mutations in the *gyrA* genes were also demonstrated in the strains of *E. coli* (5) and *Staphylococcus aureus* (14), which exhibited an increase in resistance to fluoroquinolones, which contrasts with the strains carrying a single mutation (5, 13, 21). Accumulation of additional mutations in the *gyrA* gene seems to contribute to the increase in resistance to fluoroquinolones.

In this study, we examined mutations in the region of the *N. gonorrhoeae gyrA* gene equivalent to the quinolone resistance-determining region of the *gyrA* gene. The mutations demonstrated here would confer high-level quinolone resistance in *N. gonorrhoeae*. This is the first report to identify the mutations in the *gyrA* gene associated with quinolone resistance in clinical isolates of *N. gonorrhoeae*. This study should provide useful information for understanding the molecular mechanisms of high-level quinolone resistance in *N. gonorrhoeae.*

Nucleotide sequence accession number. The GenBank/EMBL accession number for the partial sequence of the *N. gonorrhoeae gyrA* gene reported here is D32252.

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