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Rapid Detection of Point Mutations of the *Neisseria gonorrhoeae gyrA* Gene Associated with Decreased Susceptibilities to Quinolones

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Mutations in the *gyrA* gene resulting in amino acid changes at Ser-91 and Asp-95 are significantly associated with decreased susceptibilities to quinolones in *Neisseria gonorrhoeae*. To detect these mutations, we developed a rapid and simple assay based on amplification of the region of the *gyrA* gene containing the mutation sites by PCR and digestion of the PCR product with a restriction enzyme. A naturally occurring *Hin*fI restriction site was present in the region containing the Ser-91 codon, and an artificial *Hin*fI restriction site was created in the region containing the Asp-95 codon by the method of primer-specified restriction site modification. The mutations generating alterations at Ser-91 and Asp-95 were detected as restriction fragment length polymorphisms of the PCR products digested with *Hin*fI. Fifty-five clinical strains of *N. gonorrhoeae* were examined for mutations in the *gyrA* gene by this method. Mutations at Ser-91 and/or Asp-95 were detected in all the 31 strains in which the mutations had been confirmed by DNA sequencing. Our method allows simultaneous testing of a large number of strains and provides results within 8 h. This rapid and simple assay could be a useful screening device for genetic alterations associated with decreased susceptibilities to quinolones in *N. gonorrhoeae* and could facilitate epidemiological studies on clinical isolates of *N. gonorrhoeae* with decreased susceptibilities to quinolones.

Fluoroquinolones having excellent in vitro activity against *Neisseria gonorrhoeae* (11) have been demonstrated to be highly effective in curing uncomplicated gonorrhea (7). However, treatment failure with fluoroquinolones and an increase in the number of clinical isolates showing decreased susceptibilities to fluoroquinolones have been reported (2, 3, 8, 12, 20). In laboratory mutants of *N. gonorrhoeae*, mutations in the *gyrA* and *gyrB* genes of DNA gyrase and in the *parC* gene of topoisomerase IV associated with decreased susceptibilities to quinolones have been identified (1, 16). An amino acid change in GyrB has been responsible for a small decrease in susceptibility to nalidixic acid (16). Alterations at Ser-91 and Asp-95 in GyrA have reduced susceptibilities to fluoroquinolones (1). Alterations in ParC have been identified only in strains having GyrA alterations and played a complementary role in further decreasing susceptibilities to fluoroquinolones (1). In these laboratory mutants, no alterations of outer membrane proteins have been found, though reduced fluoroquinolone uptake has been observed in clinical isolates of *N. gonorrhoeae* with decreased susceptibilities to fluoroquinolones (4, 19). In our previous studies (5, 6), we also have found that all clinical isolates with decreased susceptibilities to quinolones have amino acid changes at Ser-91 and/or Asp-95 in GyrA. These findings have indicated that DNA gyrase is a primary target of quinolones in *N. gonorrhoeae* and that alterations in the GyrA subunit of DNA gyrase, particularly at Ser-91 and Asp-95, are significantly associated with decreased susceptibilities to quinolones (1, 5, 6). Therefore, the analyses of alterations at these amino acid positions in GyrA are important and inevitable in understanding mechanisms of decreases in susceptibilities to quinolones and in carrying out epidemiological assessments of transmission and spread of strains with decreased susceptibilities to quinolones. However, detection of the alterations in GyrA has required labor-intensive and time-consuming procedures including (i) amplification of the DNA fragment including the region corresponding to the quinolone resistance-determining region of the *Escherichia coli gyrA* gene (25) from genomic DNA by PCR, (ii) ligation of the PCR product to a vector, (iii) transformation, (iv) selection of recombinant colonies, (v) purification of the plasmid containing the PCR product, and (vi) determination of the sequence of the PCR product (5).

Although present technology allows reliable direct sequence determination of PCR products without ligation, transformation, or other procedures, DNA sequencing still is not suitable for the analyses of a large number of clinical strains. Recently, a rapid and simple assay has been developed for the detection of point mutations in the region of the gene not containing naturally occurring restriction enzyme cleavage sites (9). This process involves the introduction of an artificial restriction enzyme cleavage site into the PCR product using a primer-specified restriction site modification method and restriction enzyme digestion of the PCR product. In this communication, we report the application of this assay system to the detection of mutations within the Ser-91 and Asp-95 codons of the *N. gonorrhoeae gyrA* gene associated with decreased susceptibilities to quinolones.

MATERIALS AND METHODS

Bacteria. Fifty-five clinical strains of *N. gonorrhoeae* were used in this study. They had been tested for their susceptibilities to selected quinolones including ciprofloxacin (5, 6). Thirty-one strains exhibited decreased susceptibilities to the quinolones. MICs of ciprofloxacin for these strains ranged from 0.06 to 8.0 μg/ml, in contrast with those for the remaining 24 strains (between 0.004 and 0.015 μg/ml). All the strains had also been examined for mutations in the region corresponding to the quinolone resistance-determining region of the *E. coli gyrA* gene by sequencing of amplified PCR products (5, 6). All the 31 strains with

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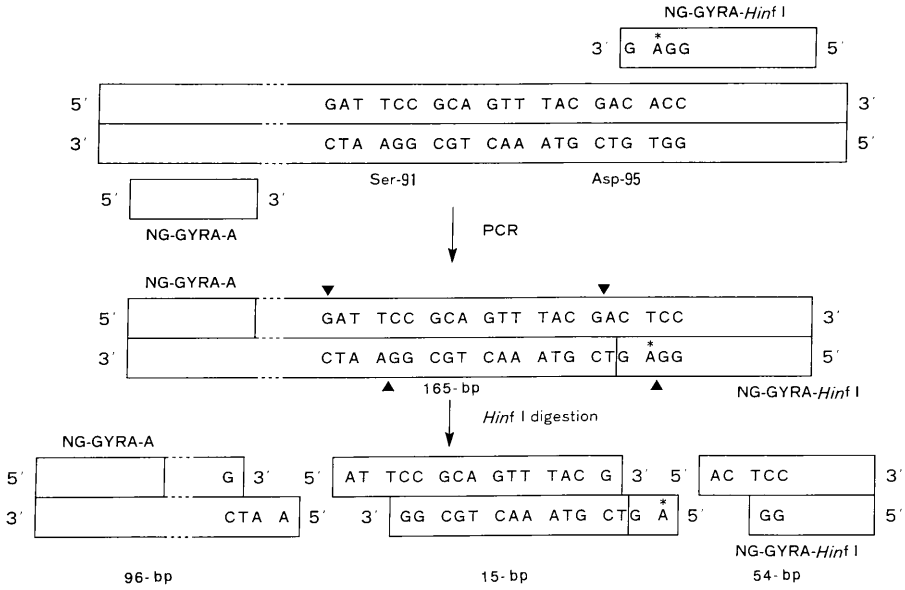

FIG. 1. A schematic diagram of the assay involving the introduction of an artificial restriction enzyme cleavage site into the PCR product using the primer-specified restriction site modification method and restriction enzyme digestion of the PCR product. The modified primer, NG-GYRA-*Hin*fI, is complementary apart from the incorporation of adenine in place of thymine at the position indicated by the asterisk. During PCR, the modified primer introduces a thymine into the PCR product. When a DNA fragment is amplified from a wild-type *gyrA* gene by PCR, introduction of thymine creates a *Hin*fI restriction site. Consequently, the PCR product (165 bp) has both naturally occurring and artificial *Hin*fI restriction sites (arrowheads) in the regions containing the codons of Ser-91 and Asp-95 and produces three fragments (96, 15, and 54 bp) upon *Hin*fI restriction enzyme digestion.

decreased susceptibilities to quinolones had mutations in the *gyrA* gene resulting in amino acid changes in GyrA, while the strains exhibiting lower MICs of quinolones had no mutations in the region sequenced. One strain had a double mutation consisting of a C-to-T transition at nucleotide position 272 (Ser-91→Phe) and an A-to-G transition at position 284 (Asp-95→Gly). Two strains had a double mutation of the same type of nucleotide transition at position 272 (Ser-91→Phe) and a G-to-A transition at position 283 (Asp-95→Asn). Twenty-two had a single C-to-T transition at nucleotide position 272 (Ser-91→Phe), and two had a single C-to-A transition at this position (Ser-91→Tyr). Four had a single G-to-A transition at position 283 (Asp-95→Asn). MICs of ciprofloxacin for the three strains with a double mutation ranged from 2.0 to 8.0 μg/ml, whereas MICs for the 28 strains with a single mutation were between 0.06 and 0.5 μg/ml.

PCR and restriction enzyme digestion. The primer NG-GYRA-A, described in the previous study, was used as a sense primer (5). The antisense primer NG-GYRA-*Hin*fI (5′-CCGTCTATCAGCACATAACGCATAGCGAAATTTTGC GCCATACGGACGATGGAG-3′ [nucleotides 285 to 338]) was made adjacent to the mutation sites within the Asp-95 codon with a nucleotide sequence that differed by the one underlined base from the gene sequence to create a new *Hin*fI cleavage site (Fig. 1). These primers were expected to produce a 165-bp DNA fragment. For preparation of template DNA, 10 colonies of the isolate cultured on GCII agar base medium (Becton Dickinson, Cockeysville, Md.) supplemented with 1% IsoVitaleX (Becton Dickinson) were collected in a tube containing 500 μl of TE (10 mM Tris, 1 mM EDTA [pH 8]). The tube was heated at 95°C for 10 min, then placed on ice, and then spun at 15,000 rpm at 4°C for 10 min. For PCR, a portion (5 μl) of the supernatant fluid was used in a 50-μl mixture consisting of 5 μl of 10× PCR (Takara Shuzo, Otsu, Japan), 200 μM deoxynucleoside triphosphates (Takara Shuzo), 0.2 μM each primer, and 1.25 U of thermostable polymerase (*TaKaRa Taq*; Takara Shuzo). PCR amplification consisted of 35 cycles of denaturation (94°C, 60 s), annealing (52°C, 50 s), and extension (72°C, 50 s). After the PCR amplification, ethanol precipitation of the PCR product was performed in the presence of glycogen and ammonium acetate. The DNA pellet was dried and then dissolved in 10 μl of distilled water. Restriction enzyme digestion with *Hin*fI (Takara Shuzo) was performed in a 10-μl mixture containing 8 μl of the PCR product, 1 μl (4 to 12 U) of *Hin*fI, and 1 μl of 10× digestion buffer supplied by the manufacturer. The digests were subjected to electrophoresis on 3% NuSieve 3:1 (FMC BioProduct, Rockland, Maine) agarose gel. The DNA fragments were stained with ethidium bromide and visualized with a UV transilluminator.

RESULTS

Theoretically, when a wild type of the *gyrA* gene is amplified with the primers NG-GYRA-A and NG-GYRA-*Hin*fI, the PCR product will contain both a naturally occurring *Hin*fI cleavage site in the region containing the Ser-91 codon and an artificially created *Hin*fI cleavage site in the region containing the Asp-95 codon (Fig. 1). Consequently, *Hin*fI will digest the amplified 165-bp DNA fragment to produce fragments of 96, 15, and 54 bp. When DNAs having mutations associated with decreased susceptibilities to quinolones are amplified, *Hin*fI cleavage sites at Ser-91 and/or Asp-95 will be destroyed. Digestion of the PCR products with the enzyme will give rise to restriction fragment length polymorphisms. Figure 2 presents representative agarose gel electrophoresis profiles of the assays. Two DNA fragments (96 and 54 bp) were produced from the strain with no mutations. A 15-bp fragment which was theoretically produced by *Hin*fI digestion was not observed on the gel, because it ran off the gel. In the strains with a C-to-T or C-to-A transition at nucleotide 272 generating an amino acid change of Ser-91→Phe or Ser-91→Tyr, *Hin*fI failed to digest the PCR products at the site containing the mutation but instead digested them at the site containing the codon of Asp-95. Thus, two DNA fragments (111 and 54 bp) were produced. In the strain with a G-to-A transition at position 283 resulting in an amino acid change of Asp-95→Asn, the cleavage site containing the Asp-95 codon was destroyed, but the other site containing Ser-91 was digested. From this strain, two DNA fragments (96 and 69 bp) were produced. In the strains with double mutations in the regions containing the Ser-91 and Asp-95 codons, the PCR products were not cut by *Hin*fI and produced 165-bp DNA fragments.

Fifty-five strains, including the six strains in Fig. 2, were analyzed by this method. Twenty-four strains with no mutations produced two DNA fragments of 96 and 54 bp. Twenty-four strains with a mutation at nucleotide position 272 resulting in a single amino acid change of Ser-91→Phe or Ser-91→Tyr produced two DNA fragments of 111 and 54 bp. Four strains with a mutation at nucleotide position 283 resulting in a single amino acid change of Asp-95→Asn produced two

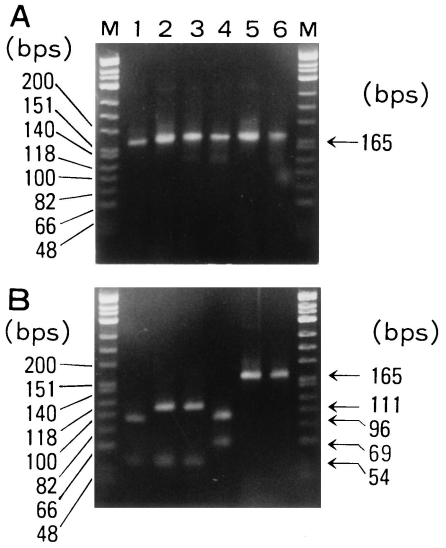

FIG. 2. *Hin*fI restriction fragment length polymorphisms. (A) The 165-bp products were amplified from the strains with no mutations (lane 1) in *gyrA*, with a C-to-T transition at nucleotide position 272 (Ser-91→Phe) (lane 2), with a C-to-A transition at nucleotide position 272 (Ser-91→Tyr) (lane 3), with a G-to-A transition at nucleotide position 283 (Asp-95→Asn) (lane 4), with C-to-T and G-to-A transitions at nucleotide positions 272 and 283 (Ser-91→Phe and Asp-95→Asn) (lane 5), and with C-to-T and A-to-G transitions at nucleotide positions 272 and 284 (Ser-91→Phe and Asp-95→Gly) by PCR. (B) The *Hin*fI restriction enzyme digestion gave rise to restriction fragment length polymorphisms of the PCR products. The PCR product having two *Hin*fI cleavage sites produced two fragments of 96 and 54 bp (lane 1). The products that lost a *Hin*fI cleavage site in the region containing the Ser-91 codon produced two fragments of 111 and 54 bp (lanes 2 and 3). The product that lost a *Hin*fI cleavage site in the region containing the Asp-95 codon produced two fragments of 96 and 69 bp (lane 4). The products that lost two *Hin*fI cleavage sites in the regions containing the Ser-91 and Asp-95 codons produced undigested fragments of 165 bp (lanes 5 and 6). Lane M, *Hin*fI-digested ϕX174 DNA molecular weight standards.

DNA fragments of 96 and 69 bp. Three strains with double mutations resulting in amino acid changes at Ser-91 and Asp-95 produced undigested DNA fragments of 165 bp. Overall, mutations at Ser-91 and/or at Asp-95 were detected by this method in all strains in which the mutations had been confirmed by sequencing of the PCR products (5, 6). In practice, 30 strains were simultaneously analyzed, and the results were obtained within 8 h.

DISCUSSION

Mutations in the *gyrA* gene resulting in amino acid changes at Ser-91 and/or Asp-95 in GyrA protein are significantly associated with decreased susceptibilities to quinolones in *N. gonorrhoeae* (1, 5, 6). In various bacterial species, analogous mutations in the *gyrA* gene responsible for decreased susceptibilities to quinolones have been identified (18, 23–25). The region containing the mutation site within the Ser-91 codon in *N. gonorrhoeae* has a naturally occurring *Hin*fI-specified restriction site (1, 5). In other bacterial species such as *E. coli* (17) and *Staphylococcus aureus* (14, 15), the *Hin*fI site is conserved in the region containing the codon equivalent to Ser-91 in *N. gonorrhoeae* GyrA. Therefore, this region is amplified by PCR, and the mutation there is detected when the *Hin*fI enzyme fails to digest the amplified product, as analyzed by electrophoresis on agarose gels. Direct PCR-*Hin*fI restriction fragment length polymorphism analysis has been used to screen *gyrA* mutations in *S. aureus* (14, 15). However, the region containing the mutation sites (nucleotide positions 283 and 284) within the Asp-95 codon is not involved in any restriction enzyme cleavage sites, so conventional PCR-restriction enzyme analysis could not be applied to detect mutations in this region. To detect the mutations at Asp-95, we introduced a base substitution near the mutation sites to create an artificial *Hin*fI cleavage site using the primer-specified restriction site modification method (9). The DNA fragment amplified from the wild-type *gyrA* gene had both naturally occurring and artificially created *Hin*fI cleavage sites within the Ser-91 and Asp-95 codons, respectively. By digesting the DNA fragment with *Hin*fI, therefore, this method could be used to simultaneously assess mutations occurring within the two codons, Ser-91 and Asp-95. In this study, mutations were detected in 31 of 55 clinical strains by this method. These results were concordant with those of DNA sequencing of the PCR-amplified DNAs (6). Thus, the validity of this assay in detection of point mutations in the *N. gonorrhoeae gyrA* gene associated with decreased susceptibilities to quinolones was confirmed.

Clinically the patients infected with the strains harboring the mutations had been treated successfully with broad-spectrum cephalosporins or spectinomycin. The association of the mutations in the *gyrA* gene with fluoroquinolone treatment failures had not been confirmed in our previous studies (5, 6). Treatment failures initially occurring with single doses of 250 mg of ciprofloxacin correlated with ciprofloxacin MICs ranging from 0.05 to 0.25 μg/ml (12). These MICs were equivalent to those (0.06 to 0.5 μg/ml) for the strains in which this screening method detected single mutations in the *gyrA* gene. Three more treatment failures at 500-mg doses correlated with ciprofloxacin MICs ranging from 1.0 to 16.0 μg/ml (21, 22). These MICs corresponded to those (2.0 to 8.0 μg/ml) for the strains in which double mutations were detected. Although the clinical significance of the results obtained with this screening method will depend on further study of the association between mutations in the *gyrA* gene and treatment failures, this assay may be able to detect strains which are clinically resistant to single 250- or 500-mg dose regimens of ciprofloxacin used for the treatment of uncomplicated gonorrhea.

Another type of technology, single-strand conformation polymorphism, analysis also has been used to detect point mutations in a variety of genes (10, 13). Compared with single-strand conformation polymorphism analysis, the method described here requires neither radioisotopes nor equipment other than that to perform PCR and agarose gel electrophoresis and also requires minimal training. In addition, this method allows simultaneous testing of a large number of strains and provides results within 8 h. Therefore, this method could be a relevant tool for assessing whether genetic alterations associated with decreased susceptibilities to quinolones are present in *N. gonorrhoeae*. However, this method has several limitations including the inability to predict what nucleotide transposition is present in the *gyrA* gene or to screen mutations occurring at other nucleotide positions of the gene. In *E. coli*, quinolone resistance-associated mutations have been identified at nucleotide positions in the *gyrA* gene differing from the codons equivalent to Ser-91 and Asp-95 of *N. gonorrhoeae* GyrA (25). In all the strains of *N. gonorrhoeae* with decreased susceptibilities to quinolones tested here, the mutations were found within only the Ser-91 and Asp-95 codons. However, our studies have not precluded clonality among the strains exhibiting decreased susceptibilities to quinolones and having alterations at Ser-91 and/or Asp-95 (5, 6). In *N. gonorrhoeae* as well as in *E. coli*, mutations at other nucleotide positions in the *gyrA* gene, which are not detectable with this assay, could also be associated with decreased susceptibilities to quinolones. Therefore, further studies will be needed to ascertain that alterations

at Ser-91 and/or Asp-95 in GyrA play a central role in decreasing susceptibilities to quinolones in *N. gonorrhoeae* by analyzing a large number of quinolone-resistant clinical isolates from other geographic regions. Nevertheless, our present study provides sufficient data suggesting that this rapid and simple assay will potentially develop into a relevant screen for determining quinolone resistance of *N. gonorrhoeae* and facilitate epidemiological studies on clinical isolates of *N. gonorrhoeae* with decreased susceptibilities to quinolones.

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