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Detection of Ciprofloxacin Resistance Mutations in *Campylobacter jejuni gyrA* by Nonradioisotopic Single-Strand Conformation Polymorphism and Direct DNA Sequencing

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A total of 27 strains of *Campylobacter jejuni* (24 clinical strains and three laboratory strains) were examined for the presence of point mutations in the quinolone resistance determining region (QRDR) of *gyrA* gene by nonradioisotopic single-strand conformation polymorphism (non-RI SSCP) analysis with silver stain. Direct DNA sequencing of the polymerase chain reaction (PCR)-amplified DNA fragments confirmed the results obtained by non-RI SSCP analysis and revealed that in clinical strains high-level quinolone resistance [minimal inhibitory concentration (MIC) to ciprofloxacin ≥ 16 μg/ml] was closely associated with one type of single-point mutation at codon 86 (Thr-Ile). Two strains with MICs of 8 and 1 μg/ml showed point mutations at codons 86 and 70, respectively. Furthermore, transitions at codon 119 of the *gyrA* QRDR were identified in 17 strains. Six types of bands were separated in a single electrophoretic step with silver stain within 2 hours after PCR amplification of the *gyrA* QRDR as follows: type I associated to mutation at codon 70 (Ala-Thr), type II to mutation at codon 90 (Asp-Asn), type III to variant with transition at 119, type IV to wild-type, type V to mutation at codon 86 (Thr-Ile), and type VI to mutation at codon 86 (Thr-Ile) and transition at codon 119. Using four DNA extracts from *Cambylobacter coli* organisms as templates for amplification of the *gyrA* QRDR, no PCR products were obtained. Non-RI SSCP was proved to be a simple, rapid, and useful screening method for detecting *gyrA* mutations associated with ciprofloxacin resistance in *C. jejuni*. © 1996 Wiley-Liss, Inc.

Key words: *Campylobacter jejuni*, *gyrA* mutations, Non-RI SSCP

INTRODUCTION

New fluoroquinolones are broad-spectrum antimicrobial agents that inhibit DNA gyrase A subunit activity in DNA supercoiling in vitro and rapidly arrest DNA replication in vivo (1,2). In gram-negative bacteria, the mechanisms of resistance to quinolones essentially fall into two classes: 1) mutations in the genes encoding for the DNA gyrase A or B subunit (3–5); and/or 2) decreased accumulation of quinolones in the bacterial cell (6–8).

Campylobacter jejuni, a gram-negative enteric pathogen is recognized as a leading cause of diarrhea worldwide (9–10). Quinolones have powerful effects against this bacterium, but development of resistance during therapy can occur (11–13). Little is known about mechanisms of resistance to fluoroquinolones in *C. jejuni*. However, a high level of resistance to ciprofloxacin and its analogs has been associated with single nucleotide change in the highly conserved 5′ end of the *gyrA* gene, leading to amino acid change in the N-terminal region of the gyrase A protein (14). Various methods have been reported for the detection of single nucleotide change of genes, including direct sequencing (15), sequence-specific oligonucleotide probe hybridization (16), and restriction fragment length polymorphism (17). Although these methods have been proved to be useful they are unsatisfactory for a routine clinical laboratory, or for the performance of epidemiological studies. Single-strand conformation polymorphism analysis presents a simpler method than those mentioned above for the detection of point mutations (18). In single-strand conformation polymorphism (SSCP) analysis a mutated sequence is detected by change of mobility in polyacrylamide gel electrophoresis as a result of its altered folded structure (19,20). In nonradioisotopic (non-RI) SSCP using the sensitive silver stain the bands are sharp, so that even a slight difference of migration

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can be detected in a small slab gel (21). In addition, when compared with radioisotopic labeling of DNA, the method is more rapid and can be performed in routine laboratories.

We used the non-RI SSCP analysis in a pilot study for diagnosis of point mutations in the *gyrA* gene associated with high-level ciprofloxacin resistance in *C. jejuni*.

MATERIALS AND METHODS

Bacterial Strains and Cultivation

The 24 clinical *C. jejuni* strains used in this study were isolated in the University Hospital laboratory of Crete, Greece. All strains were isolated from stool specimens on antibiotic selective medium (Campylosel, Merieux), confirmed as *Campylobacter* by the culture confirmation system Accuprobe (Gen-probe), and then identified as *C. jejuni*, by the API Camp system.

One laboratory ciprofloxacin-resistant strain used in this study, the *C. jejuni* strain 34PEF^r [minimal inhibitory concentration (MIC) 32 μg/ml], was derived from a pefloxacin-susceptible clinical strain by serial passages on pefloxacin gradient-containing plates. All *C. jejuni* strains were cultured under microaerophilic conditions at 37°C for 48 hours. Mueller Hinton (MH) agar including 0.25% L-cysteine was used for selection of the strain 34PEF^r.

Antibiotics and Chemicals

Mueller Hinton (MH) agar was from Diagnostics Pasteur (France), pefloxacin was from Rhone Poulenc (Rorer, France), ciprofloxacin was from Bayer (France), L-cysteine was from Merck (Rahway, NJ), and the oligonucleotides were provided by the Department of Microchemistry, Institute of Molecular Biology, Crete, Greece.

Susceptibility Testing

MICs of ciprofloxacin were determined by the agar dilution method. The inoclulum, about 2×10^5 CFU/spot was deposited with a multiple-inoculum replicator on the surface of MH agar-containing plates with 5% sheep blood added and incubated under microaerobic conditions at 37°C for 48 hours. Resistance to ciprofloxacin was indicated by MICs ~ 16 μg/ml.

PCR Amplification of *C. jejuni gyrA* QRDR

To amplify the QRDR of *C. jejuni*, two 20-mer oligonucleotide primers were chosen, 5´ GCT ATG CAA AAT GAT GAG GC 3´ and 5´ CAG TAT AAC GCA TCG CAG CGG 3´, as described by Wang et al. (14). Chromosomal DNA was prepared by sodium dodecyl sulfate (SDS) lysis of bacteria, followed by phenol chloroform extraction. The DNA extract, approximately 300 ng, was amplified by PCR in 100 μl of reaction mixture containing 1× high salt concentration reaction buffer (Amersham), 100 μM dCTP, dGTP, 200 μM dATP, dTTP, 60 pM of each primer, and 1 U of Tab polymerase (Hot Tab polymerase, Amersham). Thirty cycles were performed for each reaction, with one cycle consisting of 50 sec at 90°C for denaturation, 50 sec at 52°C for annealing, and 30 sec at 72°C for extension. Reactions were carried out in a DNA Thermal Cycler 480 (Perkin-Elmer Cetus, Emeryville, CA). The PCR product of each strain was purified by the nucleotrap system (Nucleotrap CR, Macherey Nagel AG, Oesingen, Switzerland). The sample was then directly processed for DNA sequencing with the Taq DyedeoxyTerminator Cycle sequencing kit and analyzed in an automatic DNA Sequencer ABI (Applied Biosystems, 373A).

Non-RI SSCP

In non-RI SSCP analysis, 3 μl aliquots of PCR product were mixed with 7 μl of loading solution containing 95% deionized formamide, 20 mM EDTA, 0.05% xylene cyanol, and 0.10% bromophenol blue. After denaturation at 90°C for 5 min, 2.5 μl of sample were applied to a 12.5% homogenous polyacrylamide (30:1 acrylamide/bis-acrylamide ratio) gel with a 6% polyacrylamide stacking gel (PhastGel, Pharmacia). The gel was 0.45 mm thick, and the buffer system in the gel contained 0.112 M acetate, 0.112 M Tris, pH 6.5. The gel was 45 mm long, with a 13 mm stacking gel zone and a 32 mm separation zone. The gels were run with PhastGel native buffer strips, which were made with 2% agarose isoelectric focusing. The buffer system in these strips was 0.88 M L-alanine and 0.25 M Tris, pH 8.8. Electrophoresis was performed at 15°C for 40 min at 250 V, 10 mA, 3W. Each gel was then subjected to silver staining by the PhastGel development method and the PhastGel development kit purchased from Pharmacia using the procedure recommended by the manufacturer.

RESULTS

MICs

Figure 1 shows the susceptibility profile of 27 *C. jejuni* strains used in this study including the MICs of two reference strains, UA67 and UA580R3. The MICs of these strains were 1 and 4 μg/ml, respectively, as estimated previously (14). As can be observed, nine strains were resistant to ciprofloxacin.

Amplification and Nucleotide Sequence of the *gyrA* QRDR

A DNA fragment of ~250 bp was generated by PCR, enclosing the QRDR of *C. jejuni* strains. The direct sequence analysis of this fragment revealed a single-point mutation at codon 86 of the *gyrA* gene in all the quinolone-resistant strains with MIC ≥ 16 μg/ml. Transitions at codon 119 were seen in eight resistant strains and nine susceptible strains. The PCR

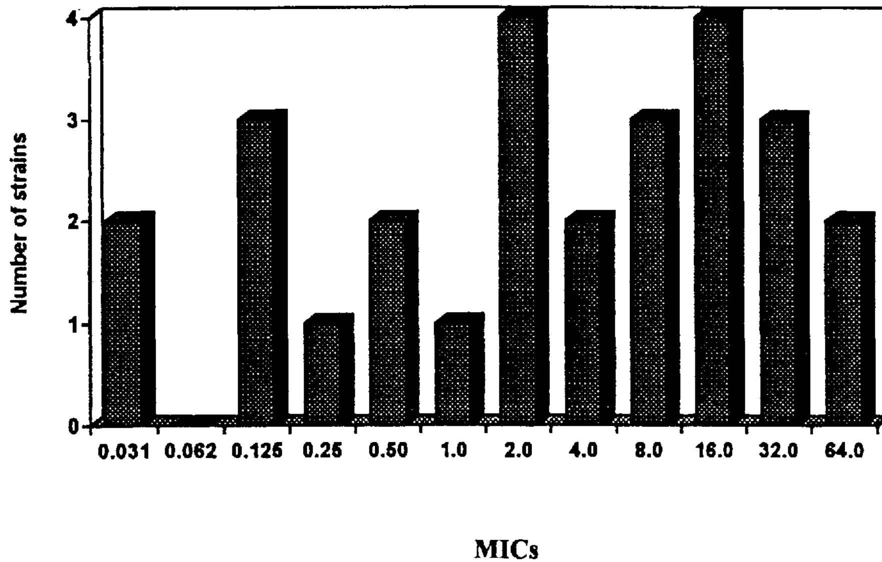

Fig. 1. Susceptibility to ciprofloxacin profile of 27 *C. jejuni* strains.

products from strains UA67 and UA580R3, kindly provided by professor Diane Taylor (Department of Medical Microbiology and Infectious Diseases, University of Alberta, Edmonton, Canada), contained nucleotide changes at codon 70 (Ala-Thr) and codon 90 (Asp-Asn), respectively, as in reference (14). One clinical strain with a MIC to ciprofloxacin of 1 µg/ml showed point mutation at codon 70, and one of the strains with a MIC to ciprofloxacin of 8 µg/ml showed point mutation at codon 86 (Thr-Ile). Using DNA templates from four *Campylobacter coli* organisms amplification as templates of *gyrA* QRDR by the procedure mentioned above was inefficient.

Non-RI SSCP

In non-RI SSCP analysis using the QRDR from *C. jejuni* strains, six types of bands with different mobilities were separated within 2 hours after PCR amplification (Fig. 2). Of the six types of bands, types V and VI [corresponding respectively to single nucleotide changes at codons 86 (Thr-Ile), and 86 (Thr-Ile) with a transition at codon 119] were associated with high-level ciprofloxacin resistance. One strain with low-level resistance to ciprofloxacin showed a type VI non-RI SSCP profile, as a consequence of nucleotide changes at codons 86 and 119. Types III and IV corresponded to a variant with transition at codon 119 and to a susceptible wild-

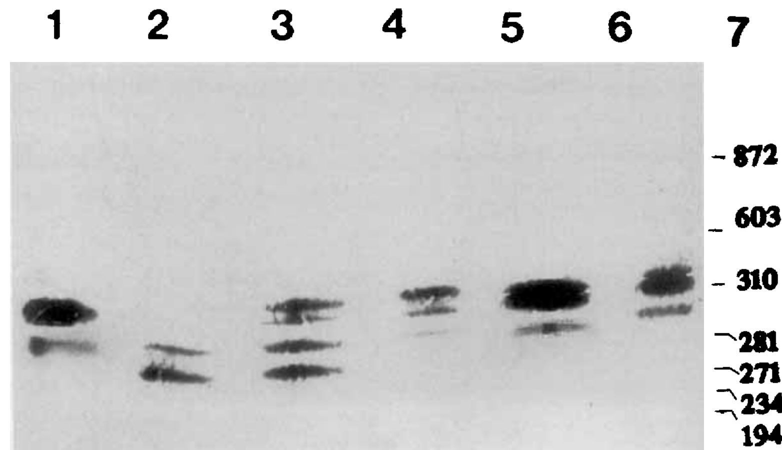

Fig. 2. Detection of *gyrA* mutations by non-RI SSCP analysis. Six types of bands with different mobilities were separated. Lane 1, type VI, point mutation at codon 86, transition at 119; lane 2, type V, point mutation at codon 86; lane 3, type IV, wild-type; lane 4, type III, variant with transition at codon 119; lane 5, type II, point mutation at codon 90; lane 6, type I, point mutation at codon 70; lane 7, molecular size markers in bp, φx/*Hae*III.

type, respectively. Types I and II corresponded to nucleotide changes at codons 70 and 90 and were associated with MICs to ciprofloxacin of 1 and 4 μg/ml, respectively.

DISCUSSION

The quinolone resistance-determining region in *gyrA* of *E. coli* consists of a chromosomal region where single nucleotide changes result in high-level quinolone resistance (4). From the knowledge gained from fluoroquinolone resistance in *E. coli,* other workers have cloned and sequenced the *gyrA* genes of several bacterial pathogens including *Staphylococcus aureus* (17), *Shegella dysenteriae* (22), *Klebsiella pneumoniae* (23), and *C. jejuni* (14). Using the fact that the 5′ ends of *gyrA* genes from different bacterial species contain corresponding areas that are highly conserved, PCR became a suitable approach for analysis of the QRDR.

Amplification by PCR of the previously cloned and sequenced *C. jejuni* QRDR and analysis by non-RI SSCP have been shown to be powerful in detecting point mutations in *gyrA* associated with high-level ciprofloxacin resistance in *C. jejuni.* Although the method seems to be accurate, it should be validated on hundreds of other isolates to confirm its accuracy. The method is simple and rapid and can be performed in 2 hours using PhastGel and the PhastGel development system after PCR. Under these conditions electrophoresis resulted in two to four bands, which indicated that ssDNA possessed multiple conformations. When the electrophoretic conditions and/or polyacrylamide gel concentrations were changed, the non-RI SSCP profiles were changed significantly.

Interestingly, in only one of the clinical strains with low-level ciprofloxacin resistance (MIC, 8 μg/ml) was a point mutation identified. This finding suggests that additional mechanisms could contribute to low-level *C. jejuni* quinolone resistance such as alterations in the B subunit of DNA gyrase and/or decreased drug accumulation (24).

Use of non-RI SSCP analysis allows for relatively rapid analysis of DNA from a large number of strains. Since the electrophoretic profile for each mutation is specific, identification of point mutations is possible by comparison of the mobilities of the sample DNAs with those of control DNAs carrying known mutations. Thus, non-RI SSCP analysis is not only simple and useful for the detection of point mutations associated with fluoroquinolone resistance but may also be a promising method for the investigation of epidemiologic markers.

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