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PCR-Restriction Fragment Length Polymorphism Assay for Detection of *gyrA* Mutations Associated with Fluoroquinolone Resistance in *Campylobacter coli*

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A fragment of the *gyrA* gene was sequenced from 34 isolates of *Campylobacter coli*, including 23 isolates resistant to ciprofloxacin. All ciprofloxacin-resistant isolates examined by DNA sequencing carried a point mutation at position Thr-86 on the *gyrA* gene product, involving the replacement of Thr-86 by Ile. A combined PCR-restriction fragment length polymorphism technique using RsaI was developed to detect this mutation.

The thermotolerant species of *Campylobacter* are frequent causes of acute bacterial gastroenteritis in humans worldwide. More than 90% of these infections are caused by *C. jejuni*, with *C. coli* causing most of the remainder (5, 8). Gastroenteritis caused by *C. jejuni* and *C. coli* is normally self limiting. However, early antibiotic therapy is recommended to reduce severity and duration, mainly in severe cases with prolonged disease, in immunocompromised patients or if the infection is extraintestinal (2). In such cases, macrolides and fluoroquinolones, particularly ciprofloxacin, are the drugs of choice. Fluoroquinolones are also frequently used as prophylaxes for traveler's diarrhea. However, coinciding with the introduction of fluoroquinolones in veterinary medicine was the increased emergence of quinolone-resistant enteric bacteria strains (3, 9, 13). Such strains can be transmitted to humans. This is particularly important in *Campylobacter* because it is thought to be a food-borne pathogen. In fact, fluoroquinolone resistance in *Campylobacter* from food animals is now recognized as an emerging public health problem (4, 14).

The resistance of *C. jejuni* and *C. coli* to quinolones mainly depends on mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene and occasionally on mutations of the *parC* gene. Mutations in *gyrA* contribute to confer decreased sensitivity of DNA gyrase to quinolone antibiotics and a corresponding increase in their MICs (7, 15). Point mutations in the *gyrA* gene product at position Thr-86 have been associated with high-level resistance to ciprofloxacin (17).

Although direct sequencing is the more accurate technique for the detection of nucleotide mutations, DNA sequencing cannot be used as a routine assay for diagnosis because sequencing protocols usually are expensive and time-consuming. Therefore, alternative protocols for the detection of single-nucleotide changes of genes have been developed that can be applied to diagnosis in a routine clinical laboratory or epidemiological studies (11, 18, 19). In *Campylobacter*, most of these alternative protocols are focused on *C. jejuni*, a major cause of *Campylobacter* infection in humans. However, *C. coli* is often isolated together with *C. jejuni*, and it is the second cause of campylobacteriosis in humans. Furthermore, *C. coli* appears to display higher rates of resistance to ciprofloxacin than *C. jejuni* at present (6, 16).

For these reasons it is of interest to us to develop a PCR-based restriction fragment length polymorphism (PCR-RFLP) assay allowing rapid and reproducible identification of major mutations mediating fluoroquinolone resistance in *C. coli*. 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 nonculture method to the detection of mutants within the Thr-86 codon of the *C. coli* gene associated with decreased susceptibility to ciprofloxacin, *gyrA*.

In this study, 34 *C. coli* strains, 10 of human origin and 24 of food or animal origin, were first examined for ciprofloxacin susceptibility. MICs were determined by the agar dilution method on Mueller-Hinton agar (Oxoid) supplemented with 5% lysed horse blood (Oxoid), following the NCCLS recommendations (10), as there are no internationally accepted criteria for susceptibility testing for *Campylobacter*. The plates were incubated at 42°C under a microaerobic atmosphere (5% O_2, 10% CO_2, and 85% N_2) for 24 h. The breakpoint for ciprofloxacin was considered to be a MIC of $\geq$4 μg/ml. The results of ciprofloxacin susceptibility testing for the 34 strains are shown in Table 1. Twenty-three of the 34 isolates were resistant to ciprofloxacin, all of them showing high-level resistance (MICs from 16 to 64 μg/ml). Among the 23 ciprofloxacin-resistant strains, one strain was resistant to amoxicillin, nine strains were resistant to tetracycline, six strains were coresistant to tetracycline and erythromycin, and four strains were coresistant to tetracycline and amoxicillin.

On the basis of the DNA sequence of the *gyrA* gene of *C. coli* (GenBank accession number AF092101), we have designed an oligonucleotide adjacent to the mutation sites within the

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TABLE 1. *C. coli* strains tested in this study, PCR-RFLP results, and ciprofloxacin MICs for the corresponding strains

Isolate no.[a]	Source/country of origin	*gyrA* mutation at codon 86[b]	Ciprofloxacin MIC (μg/ml)
CNET 051	Human/France	Yes	32
CH 008	Human/Spain	Yes	32
CH 024	Human/Spain	No	0.5
CH 051	Human/Spain	Yes	32
CH 056	Human/Spain	No	0.125
CG001	Human/Spain	Yes	64
CG002	Human/Spain	Yes	64
CG003	Human/Spain	No	0.125
CG004	Human/Spain	Yes	64
CG005	Human/Spain	Yes	64
CNET 019	Flock outbreak/Netherlands	Yes	16
CNET 021	Flock outbreak/Netherlands	Yes	16
CNET 066	Pig/Netherlands	No	0.25
CNET 068	Pig/North Ireland	No	0.25
CNET 069	Pig/North Ireland	No	0.25
CNET 072	Pig/Denmark	No	0.25
CNET 064	Poultry/Denmark	No	0.125
CNET 082	Poultry/North Ireland	No	0.125
CPM 011	Poultry/Spain	Yes	32
CPM 021	Poultry/Spain	Yes	32
CPM 023-1	Poultry/Spain	Yes	16
CPM 042-7	Poultry/Spain	Yes	32
CPM 043-3	Poultry/Spain	Yes	32
CPM 043-4	Poultry/Spain	Yes	32
CPM 045	Poultry/Spain	Yes	32
CPM 053-3	Poultry/Spain	Yes	32
CPM 057-1	Poultry/Spain	Yes	64
CPM 058-2	Poultry/Spain	Yes	64
CPM 063-1	Poultry/Spain	Yes	32
CPM 063-4	Poultry/Spain	Yes	64
CPM 064-2	Poultry/Spain	No	0.5
CPM 069-2	Poultry/Spain	Yes	64
CPM 072-1	Poultry/Spain	Yes	32

[a] CNET, isolates from the CAMPYNET collection; CH and CG, isolates from humans with gastroenteritis; CPM, isolates from poultry samples at the retail level.

[b] Mutants had a Thr-86-to-Ile (ACT-to-ATT) substitution.

Thr-86 codon nucleotide sequence that differ from the gene sequence by one base (underlined) to create an artificial RsaI leavage site. This nucleotide was used in forward primer colgyrA (5′-AAATCTGCTCGTATAGTAGGGGATGT TAT CGGTAAGTATCATCCACATGGCGGT-3′). The reverse primer was cjgyrA2 as previously described (12). They were used to amplify a 179-bp fragment containing the QRDR of the *gyrA* gene of quinolone-resistant and -susceptible *C. coli* strains. Analysis of *gyrA* was started with the amplification of the 179-bp fragment by PCR using primers colgyrA and cjgyrA2, as mentioned above. The PCR was carried out in 25 μl of reaction mixture, containing 1× PCR buffer (10 mM Tris-HCl, 1.5 mM MgCl2, 50 mM KCl [pH 8.3]), 80 ng of genomic DNA, 10 pmol of each primer, 0.2 mM deoxynucleotide, and 1 U of *Taq* polymerase (Invitrogen). An initial denaturation step at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 45 s and a final extension at 72°C for 7 min were performed in a Robocycler 96 thermal cycler (Stratagene). Amplification products of the expected size (179 bp) were obtained for all strains, whether they had been resistant or susceptible to the ciprofloxacin. Finally, the PCR products were digested with RsaI (Roche) to screen for mutations at position Thr-86. Enzyme digestion was performed in a 10-μl mixture containing 7 μl of the PCR product and 10 U of enzyme. After digestion with RsaI and separation on a 3% MS-6 agarose gel (Pronadisa), two different profiles should be expected. As DNAs carrying mutations at the position corresponding to Thr-86 are amplified, the RsaI cleavage site is destroyed, and a 179-bp fragment identical in size to that of the nondigested PCR product after the digestion should be observed. As a wild-type *gyrA* gene is amplified, the PCR product should contain the artificial RsaI cleavage site. Consequently, RsaI digests the amplified 179-bp PCR product to produce two fragments of 54 and 125 bp, respectively. PCR-RFLP results from the 34 strains are recorded in Table 1 and shown in Fig. 1.

A complete correlation was observed between the PCR-RFLP results and the MICs of ciprofloxacin for the corresponding strains. Analysis of restriction patterns after digestion with RsaI showed that all resistant strains had the same RFLP, the 179-bp fragment. These strains were assumed to have mutations at Thr-86. The susceptible strains had two fragments of 54 and 125 bp, respectively, produced by RsaI digestion. These strains were assumed to have no mutation at Thr-86.

In order to confirm the mutation associated with resistance, an extended 235-bp fragment of the QRDR of *gyrA* from all tested strains was amplified using primers clgyrA1 and cjgyrA2, as previously described (12). The PCR products were purified with a QIAquick PCR purification kit (QIAGEN) and sequenced by Sistemas Genómicos SA (Valencia, Spain). The nucleotide sequence of the 235-bp fragment was compared to that of the wild-type *C. coli* isolate in the GenBank (accession number AF092101). On the basis of this comparison, a point mutation at codon 86 (ACT→ATT), leading to the replacement of Thr by Ile, was identified in all ciprofloxacin-resistant strains but not in ciprofloxacin-sensitive strains. Despite all isolates having identical amino acid substitutions in the QRDR of the *gyrA* product, the ciprofloxacin MICs varied from 16 to 64 μg/ml, suggesting that other factors could contribute to the resistance phenotype. It is possible that mutations outside the QRDR or in other genes were responsible for the increased ciprofloxacin MICs (12). Additional silent mutations were found among isolates that were resistant or susceptible strains. Three isolates each had a silent mutation at the position cor-

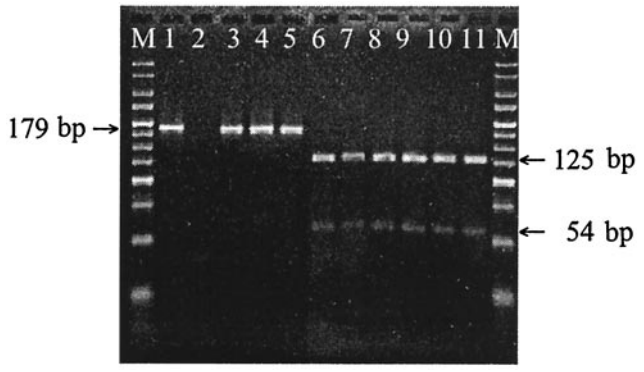

FIG. 1. PCR-restriction fragment length polymorphism patterns obtained after digestion with RsaI in *C. coli* strains. Lanes: M, 25-bp DNA Ladder (Bioline); 1, undigested PCR product of *gyrA* gene; 2, negative control; 3 to 5, ciprofloxacin-resistant strains; 6 to 11, ciprofloxacin-sensitive strains.

responding to Gly-84 (GGC→GGT), and all but three isolates had one at the position corresponding to Phe-99 (TTT→TTC).

As in *C. jejuni*, fluoroquinolone resistance in *C. coli* is primarily associated with a single Thr-86-to-Ile mutation in GyrA in isolates from both humans and animals (1, 12, 17). Our results agree with this criterion, since the Thr-86-to-Ile mutation correlated with high-level resistance to ciprofloxacin in the *C. coli* isolates examined, whether isolated from humans, animals, or food. Thus, the validity of this assay for detection of point mutations in the *C. coli gyrA* gene associated with decreased susceptibilities to fluoroquinolones was confirmed. This molecular approach was therefore able to overcome technical problems of MIC determination and to detect high-level fluoroquinolone-resistant *C. coli* strains regardless of the MICs for them.

The PCR-RFLP methodology described here is a simple and rapid method (it can be performed within 6 h) for the detection of ciprofloxacin-resistant *C. coli* strains. It should serve as a portable alternative to methods such as sequence-specific oligonucleotide hybridization and nonradioisotopic single-strand conformation polymorphism, which have been previously described (11, 18, 19). The present study provides sufficient data suggesting that this rapid and simple assay proves useful for clinical diagnosis or epidemiological studies of *C. coli* isolates of different origins with decreased susceptibilities to ciprofloxacin.

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