Characterization of *gyrA* mutations associated with fluoroquinolone resistance in *Campylobacter coli* by DNA sequence analysis and MAMA PCR

Authors

Abstract

Increasing numbers of fluoroquinolone-resistant *Campylobacter coli* isolates received at the Minnesota State Public Health Laboratory and at the Centers for Disease Control and Prevention have been a cause for concern. The *gyrA* quinolone resistance-determining regions of several fluoroquinolone-resistant isolates were sequenced to examine the mechanism of resistance. Ciprofloxacin-resistant *C. coli* isolates examined by DNA sequencing had a Thr-86 to Ile (ACT→ATT) *gyrA* mutation, leading to resistance to fluoroquinolone antibiotics. A mismatch amplification mutation assay polymerase chain reaction protocol was developed to detect this *gyrA* mutation.

1Introduction

Diarrhea caused by *Campylobacter coli* and *Campylobacter jejuni* is generally a self-limiting disease. However, early antibiotic treatment is recommended to reduce severity and duration. Fluoroquinolones are the treatment of choice for adults and are also prescribed for prophylactic treatment against traveller's diarrhea [1–6]. The existence of fluoroquinolone-resistant *Campylobacter* isolates has been reported in various countries for several years, including the Netherlands [6], Sweden [7], Thailand [8], UK [9] and USA [10,11]. The use of fluoroquinolones in agriculture to heal diseases of poultry [11,12] is thought to be a contributing factor for the emergence of fluoroquinolone-resistant enteric bacteria. Reports of the emergence of quinolone-resistant *Campylobacter* isolates in vivo after quinolone therapy [13,14] lend additional support to the contention that resistant isolates may develop after therapeutic treatment with quinolone antibiotics.

For these reasons it is of interest to us to investigate the mechanisms by which *C. coli* isolates are becoming resistant to quinolone antibiotics. DNA gyrase, a type II topoisomerase which introduces negative supercoils into DNA and which is essential for bacterial viability, is a major target for quinolone antibiotics in bacteria. The binding of quinolones to the GyrA subunits to prevent gyrase function is the primary mechanism [15,16]. Several studies have shown that mutations in the *gyrA* gene of several *Campylobacter* species are primarily responsible for the resistance of most *Campylobacter* clinical isolates to quinolone antibiotics [9,10,17,18].

In *Escherichia coli*, most mutations resulting in fluoroquinolone resistance map between codons 67 and 106, near the Tyr-122 catalytic site of DNA gyrase. Thus, this region has been termed the quinolone resistance-determining region (QRDR) [19]. An analogous QRDR

region exists in the *C. jejuni gyrA* gene, but the *gyrA* Tyr-125 residue appears to be the catalytic site [18,20]. A single mutation of Ser-83 to Ala in the GyrA protein of *E. coli* allows DNA gyrase activity to become resistant to the action of ciprofloxacin and nalidixic acid in vitro [21]. Mutations in the QRDR region of the *C. coli gyrA* gene analogous to known quinolone resistance mutations in *C. jejuni* and *E. coli* could reasonably be expected to be responsible for quinolone antibiotic resistance in *C. coli* isolates.

Several fluoroquinolone-resistant *C. coli* isolates were recently received by the Minnesota State Public Health Laboratory (SPHL) and the Centers for Disease Control and Prevention (CDC). This study was initiated to investigate the type and frequency of *gyrA* mutations in *C. coli* isolates with resistance to nalidixic acid and ciprofloxacin, and to develop a rapid polymerase chain reaction (PCR) protocol for the detection of *gyrA* mutations relevant to these resistance phenotypes that could serve as an alternative to previously reported rapid methods [22]. In addition, we wished to determine a greater amount of the DNA sequence surrounding the *gyrA* QRDR region of *C. coli* isolates than had been previously submitted to GenBank (Everett and Piddock, accession number U63413) in order to allow for greater flexibility in the choice of primers for PCR amplification, direct sequencing of the PCR amplification products, and rapid analysis of the actual DNA point mutations themselves. To our knowledge, the DNA sequence of *C. coli gyrA* quinolone-resistance point mutations has never been published or submitted to GenBank. Fluoroquinolone antibiotics are currently one of the most effective antimicrobial agents for the elimination of *C. coli* infections and a rapid, specific, inexpensive, molecular method for the detection of quinolone-resistance mutations should be able to serve as an aid to determine if fluoroquinolone antibiotics are appropriate treatment for specific clinical cases of the infection or for epidemiological studies.

2Materials and methods

2.1Strains, culture conditions and antibiotic susceptibility testing

Quinolone-susceptible *C. coli* isolates used in this study included nine isolates from the CDC and Minnesota SPHL collections. Fifteen quinolone-resistant isolates examined in this study were isolated in Minnesota and provided by the Minnesota SPHL; two were from CDC.

Culture media, growth environment and biochemical tests were performed as previously reported [10]. PCR amplification for the identification of the genus *Campylobacter* was performed according to the method of Linton et al. [23]. Further characterization to the species level was performed by using a PCR test based on the *ceuE* gene developed by Gonzalez et al. [24]. Screening for antimicrobial resistance was performed with E-test strips (AB Biodisk, Solna, Sweden) as previously published [10]. The nalidixic acid and ciprofloxacin antibiotypes were confirmed by agar dilution testing, as described by the National Committee for Clinical Laboratory Standards (NCCLS) [25], with modifications as previously published [10]. The interpretive breakpoints for the minimum inhibitory concentrations (MICs) to nalidixic acid were $\leq$8 μg ml^{-1} as susceptible and $\geq$32 μg ml^{-1} as resistant. The interpretive breakpoints for the MICs to ciprofloxacin were $\leq$1 μg ml^{-1} as susceptible and $\geq$4 μg ml^{-1} as resistant [26].

2.2DNA isolation, PCR, DNA sequencing and nucleotide sequence analysis

Chromosomal DNA was purified, as previously published [10]. Primers GZ*gyrA*5 and GZ*gyrA*6 (Table 1) were originally chosen for PCR amplification of a 673-bp product containing the QRDR region of the *gyrA* gene of quinolone-resistant and -sensitive *C. jejuni*[10] after analysis of the *C. jejuni gyrA* gene sequence published by Wang et al. [18]. In this study, primers GZ*gyrA*5 and GZ*gyrA*6 were used for *gyrA* PCR, which resulted in DNA bands of approximately 673 bp from the majority of *C. coli* isolates from this study. However, direct sequencing of these *C. coli gyrA* PCR products only yielded reliable DNA sequence data with primer GZ*gyrA*6 from three of the *C. coli* isolates. Good *gyrA* DNA sequence data from *C. coli* type strain ATCC 33559 using primer GZ*gyrA*6 was used to select new primers for further PCR and sequencing. Primers GZ*gyrA*Ccoli3F and GZ*gyrA*Ccoli4R (Table 1) were chosen for the final PCR and sequencing of the *gyrA* QRDR region of the *C. coli* isolates, producing a PCR product 505 bp in length.

Table 1. Primers used in this study

Name	Sequence (5′ to 3′)	Direction, purpose	Reference
GZ*gyrA*5	ATT TTT AGC AAA GAT TCT GAT	forward, *C. jejuni gyrA* primer	[10]
GZ*gyrA*6	CCA TAA ATT ATT CCA CCT GT	reverse, *C. jejuni gyrA* primer	[10]
GZ*gyrA*Ccoli3F	TAT GAG CGT TAT TAT CGG TC	forward, specific for *C. coli gyrA*	this study
GZ*gyrA*Ccoli4R	GTC CAT CTA CAA GCT CGT TA	reverse, specific for *C. coli gyrA*	this study
CampyMAMA*gyrA*8	TAA GGC ATC GTA AAC AGC CA	reverse, *C. coli* putative codon 86 *gyrA* mutation detection primer	this study

PCR reactions (100 µl each) contained 75 ng of purified chromosomal DNA and were performed under conditions previously described [10]. Primers were synthesized by the CDC Biotechnology Core Facility. After amplification, 5 µl of the product was loaded onto a 2.0%, 0.5×TBE, horizontal agarose gel and stained with ethidium bromide for analysis after electrophoresis at 150 V for 1 h. The remaining *gyrA* PCR products were purified and used in *Taq* dye terminator cycle sequencing, as previously described [10].

DNA sequence analysis and primer selection for the mismatch amplification mutation assay (MAMA) PCR [27] were performed in a manner similar to that previously described [10]. Briefly, conserved primers were chosen with OLIGO Primer Analysis Software version 5.0 (National Biosciences, Plymouth, MN, USA) and mutation primers were chosen after a manual analysis of the DNA sequence near putative codon 86 of the *C. coli gyrA* gene of fluoroquinolone-susceptible and -resistant isolates. DNA sequences were analyzed with DNASIS version 2.5 (Hitachi Software Engineering Co., San Francisco, CA, USA).

2.3MAMA PCR protocol

The mutation primer was chosen after a visual analysis of the DNA sequences near putative codon 86 of the *C. coli gyrA* genes. A conserved, forward primer, GZ*gyrA*Ccoli3F, and a reverse, mutation detection primer, CampyMAMA*gyrA*8 (Table 1), were used together in a PCR reaction to generate a 192-bp PCR product that was a positive indication of the presence of the Thr-86 to Ile (ACT→ATT) mutation in the *C. coli gyrA* gene. Primer GZ*gyrA*Ccoli4R,

a conserved reverse primer, was used in conjunction with primer GZ*gyrA*Ccoli3F to produce a positive PCR control product of 505 bp from the *gyrA* gene of all *C. coli* DNA preparations used in this study. PCR reactions (100 μl each) were performed as above, except only 10 pmol of each primer was used. PCR cycling conditions were as follows: initial denaturation was at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 20 s. Eight-μl aliquots of each PCR product were loaded onto horizontal, 2.0%, 0.5×TBE agarose gels and stained with ethidium bromide for analysis after electrophoresis.

3Results

3.1Detection of gyrA mutations associated with quinolone resistance

We used primers based on the *C. jejuni* sequence [10] to generate PCR fragments for preliminary sequencing of *C. coli gyrA*. Only primer GZ*gyrA*6 produced reliable *gyrA* sequence data with DNA from only a few *C. coli* isolates. Reliable *gyrA* DNA sequence data from *C. coli* type strain ATCC 33559, using primer GZ*gyrA*6, was employed to select new primers for PCR and sequencing (Table 1 and Fig. 1). The identity between the 633 bases of the *gyrA* gene of *C. coli* ATCC 33559 and the analogous region of the *gyrA* gene of *C. jejuni* UA580 was 82%, with some areas much more conserved than others (Fig. 1). An examination of the *gyrA* DNA sequence data, some of which are shown in Fig. 1, enabled us to determine the presence or absence of non-synonymous mutations in the QRDR region of the Minnesota isolates and compare this information to the nalidixic acid and ciprofloxacin MICs for the *C. coli* isolates (Table 2). The mutation at putative amino acid (aa) position 86 of the *gyrA* gene correlates with resistance to ciprofloxacin and nalidixic acid in the *C. coli* isolates examined. The relationship of the Thr-86 to Ile mutation to the acquisition of quinolone resistance in other *Campylobacter* isolates has been previously characterized [9,10,18].

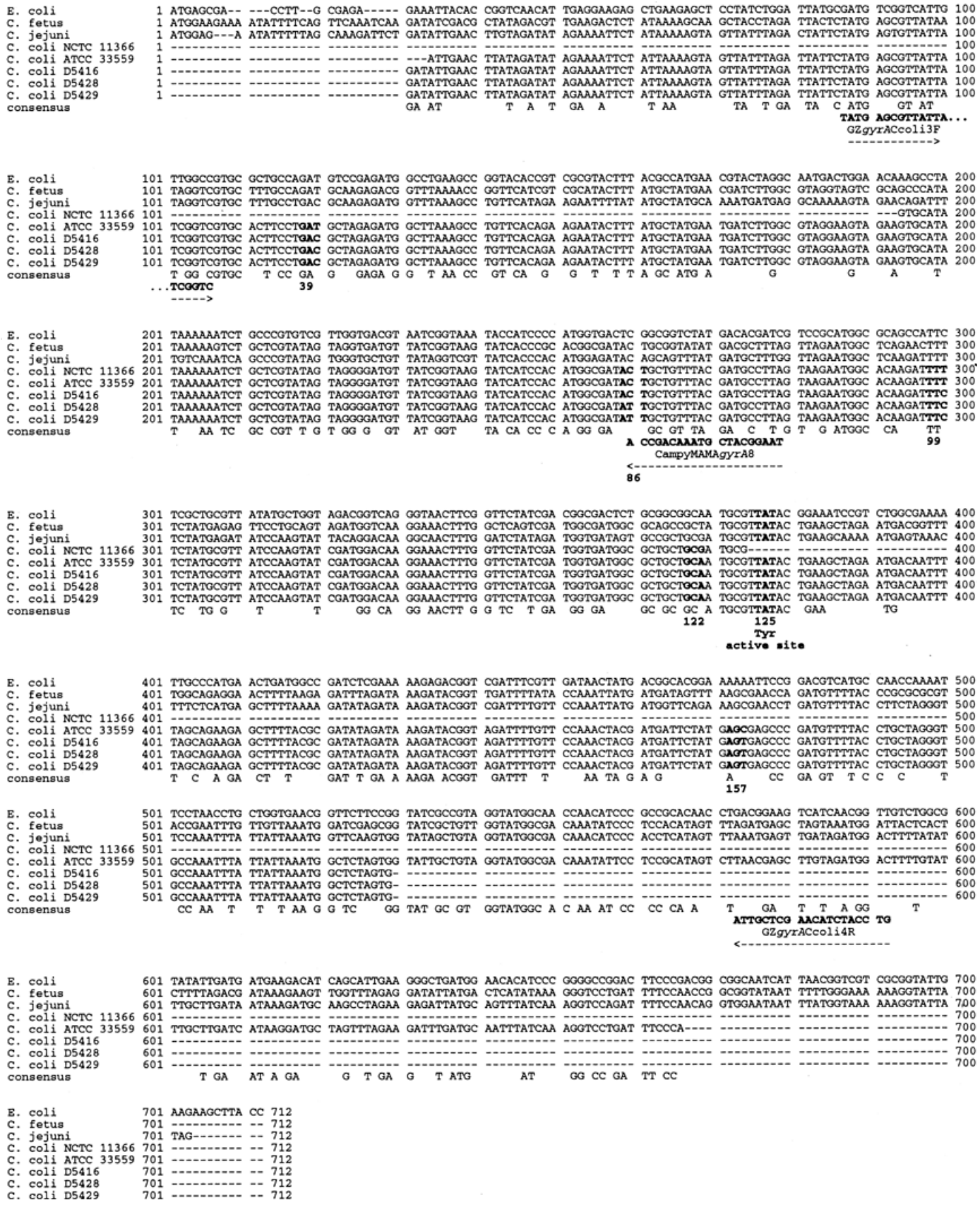

Figure 1.

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Nucleotide sequence alignment of partial *gyrA* gene sequences of *C. coli* isolates from this study compared to other *Campylobacter* and *E. coli gyrA* sequences from the GenBank database. The sequences of *E. coli* KL-16 (accession number x06744), *Campylobacter fetus* ATCC 27374 (accession number u25640) and *C. jejuni* UA580 (accession number l04566) were compared to the partial *gyrA* gene sequences of *C. coli* NCTC 11366 (accession number u63413), *C. coli* ATCC 33559 (accession number af092101, this study) and *C. coli* isolates D5416, D5428 and D5429 from Minnesota. Codon numbers are based on codon positions of the complete *C. jejuni* UA580 *gyrA* gene, and are only putative for *C. coli* sequences, since the first few bases of the *C. coli gyrA* genes were not sequenced.

Table 2. Mutations in the *gyrA* QRDR region of *C. coli* isolates and MICs to nalidixic acid and ciprofloxacin

1. [a]Mutants have a substitution of Thr-86 to Ile (ACT to ATT). NA indicates isolates were not sequenced.
2. [b]*C. coli* ATCC 33559 type strain.

CDC No.	Health laboratory/source	Putative *gyrA* aa 86 mutation[a]	Nalidixic acid MIC (μg ml^{-1})	Ciprofloxacin MIC (μg ml^{-1})	MAMA PCR result
D126	Canada, animal	NA	16	0.25	negative
D134	Canada	NA	8	0.06	negative
D145[b]	animal	no	16	0.25	negative
D730	WA	NA	8	0.03	negative
D2591	USA	NA	8	0.06	negative
D2592	Australia	NA	8	0.125	negative
D2595	Belgium	NA	16	0.125	negative
D2596	animal	NA	32	0.125	negative
D5416	MN	no	16	0.25	negative
D5403	MN	yes	256	8	positive
D5404	MN	yes	128	4	positive
D5413	MN	yes	128	8	positive
D5414	MN	yes	128	8	positive
D5415	MN	yes	256	16	positive
D5417	MN	yes	256	16	positive
D5418	MN	yes	128	8	positive
D5419	MN	yes	256	16	positive
D5420	MN	yes	256	16	positive
D5423	MN	yes	128	4	positive
D5427	MN	yes	256	8	positive
D5428	MN	yes	256	16	positive
D5429	MN	yes	256	16	positive
D5430	MN	yes	256	16	positive
D5431	MN	yes	128	16	positive

D5472	MA	NA	128	16	positive
D5496	MA	NA	128	32	positive

With the exception of isolates D5404 and D5413, all ciprofloxacin-resistant Minnesota isolates possessed identical *gyrA* DNA sequences with isolates D5428 and D5429 (Fig. 1). Isolate D5404 shared identical 39 and 99 *gyrA* DNA codons as the *C. coli* ATCC 33559 type strain, and isolate D5413 shared the same 39 and 157 *gyrA* DNA codons as the *C. coli* ATCC 33559 type strain, while the remainder of the D5404 and D5413 *gyrA* DNA sequences were identical to D5428 and D5429 (refer to Fig. 1).

3.2Detection of the Thr to Ile mutation (ACT→ATT) by use of MAMA PCR

A rapid PCR method was developed for the detection of the Thr to Ile (ACT to ATT) mutation at the putative aa position 86 in the *gyrA* DNA sequence of *C. coli* isolates. Isolates with the wild-type aa 86 codon (ACT, quinolone-susceptible) were not amplified with the reverse mutation primer, CampyMAMA*gyrA*8 (Fig. 1), whereas the isolates with the mutated aa 86 codon (ATT, quinolone-resistant) generated a 192-bp PCR product with the CampyMAMA*gyrA*8 reverse mutation primer and the GZ*gyrA*Ccoli3F forward conserved primer (Table 2, Fig. 1). Conserved primers GZ*gyrA*Ccoli3F and GZ*gyrA*Ccoli4R generated a 505-bp *gyrA* PCR product with DNA isolated from all of the isolates listed in this study.

Seventeen of the strains in Table 2 were examined by direct DNA sequencing of *gyrA* gene PCR products. CDC isolate D145 (the ATCC 33559 type strain) and isolate D5416 (from Minnesota) were both ciprofloxacin-susceptible, had wild-type *gyrA* DNA sequences and had negative results with the MAMA PCR mutation detection primer. Isolates D5403, D5404, D5413, D5414, D5415, D5417, D5418, D5419, D5420, D5423, D5427, D5428, D5429, D5430 and D5431 were all from Minnesota, were all ciprofloxacin-resistant, all had the Thr to Ile (ACT to ATT) mutation at the putative aa 86 position of the *gyrA* gene, and all had positive results with the MAMA PCR mutation detection primer.

To determine if the MAMA PCR assay was valid with other CDC *C. coli* isolates from varied sources and locations, spanning a number of years, nine more CDC isolates, D126, D134, D730, D2591, D2592, D2595, D2596, D5472 and D5496, were examined by MAMA PCR alone to verify whether or not the MAMA PCR assay could serve as a replacement for sequencing data when the antibiotype of the isolate is taken into account (Table 2). Results indicate the putative aa position 86 ciprofloxacin-resistance mutation was correctly identified in all ciprofloxacin-resistant isolates and that the MAMA PCR never gave a positive PCR response with any ciprofloxacin-sensitive isolates from this group. We were satisfied that the MAMA PCR assay worked well for all isolates tested.

4Discussion

The effectiveness of fluoroquinolone antibiotics for the treatment of traveller's diarrhea [3], the use of fluoroquinolones in agriculture to heal diseases of poultry [12], and the common practice of prescribing fluoroquinolone antibiotics for gastroenteritis for the purpose of shortening absences from work [2] have led to exposure to this class of antibiotics. The incidence of quinolone-resistant *Campylobacter* infection has increased recently, and evidence suggests that a significant number of human isolates are acquired from poultry products [11]. Reports of the emergence of quinolone-resistant *Campylobacter* isolates in

vivo after quinolone therapy [13,14] can also be found in the literature. Several fluoroquinolone-resistant *C. coli* isolates received by the SPHL and the CDC prompted us to perform this study to investigate the type and frequency of *gyrA* mutations in *C. coli* isolates with resistance to nalidixic acid and ciprofloxacin, and to develop a rapid PCR protocol for the detection of *gyrA* mutations relevant to these resistance phenotypes that could serve as an alternative to previously reported rapid methods [22]. Another goal of this study was to obtain a greater amount of the DNA sequence surrounding the *gyrA* QRDR region of *C. coli* isolates than had been previously submitted to GenBank (Everett and Piddock, accession number U63413) in order to allow for greater flexibility in the choice of primers for PCR amplification, direct sequencing of the PCR amplification products and rapid analysis of the actual DNA point mutations themselves.

A single mutation of Ser-83 to Ala in the GyrA protein of *E. coli* allows DNA gyrase activity to become resistant to the action of ciprofloxacin and nalidixic acid in vitro [21]. A *C. jejuni* Thr-86 to Ile *gyrA* mutation, in a position equivalent to the *E. coli* Ser-83 to Ala mutation, has been reported as responsible for high resistance to fluoroquinolones in *C. jejuni* in vivo [9,18]. We reported that other US isolates of *C. jejuni* that were resistant to nalidixic acid (MIC $\geq$32 μg ml^{-1}) were also resistant to ciprofloxacin (MIC $\geq$4 μg ml^{-1}), and all had the Thr-86 to Ile (ACA to ATA) mutation [10]. The same mechanism, a Thr-86 to Ile mutation (ACT to ATT) in the QRDR region of the *gyrA* gene, appears to be responsible for the increased resistance to fluoroquinolone antibiotics noted for the *C. coli* isolates, since isolates without this mutation were sensitive to ciprofloxacin (Table 1). *C. coli* isolates tested that were highly resistant to nalidixic acid (MIC $\geq$128 μg ml^{-1}) were also resistant to ciprofloxacin (MIC $\geq$4 μg ml^{-1}). Only isolate D2596 had a nalidixic acid MIC of 32 μg ml^{-1}, which is at the breakpoint for resistance, while being susceptible to ciprofloxacin (Table 1). Factors other than *gyrA* QRDR mutations, such as mutations in *gyrB, parC* and *parE,* efflux pumps, permeability factors or detoxification [20], may be responsible for some of the variability in nalidixic acid and ciprofloxacin MICs among resistant *C. coli* isolates in Table 1.

Since the Thr to Ile (ACT to ATT) mutation in putative codon 86 of the *C. coli gyrA* gene appears to be a commonly encountered mutation leading to fluoroquinolone resistance and one for which we have reported DNA sequence data (this study), we developed a MAMA protocol for the specific detection of this mutation. The design of this type of mismatch primer for mutation detection has been explained in detail previously [10,27]. The scientific basis for the ability of the mutation detection primer, CampyMAMA*gyrA*8, to generate a PCR product with mutant DNAs and not wild-type DNAs is based on the fact that the 3′ end of the mutation detection primer has only one mismatch with the mutant, fluoroquinolone-resistant, *gyrA* sequences, and is able to generate a PCR product, while two mismatches occur between the extreme 3′ end of the mutation detection primer and the wild-type, fluoroquinolone-susceptible, *gyrA* sequences, and no PCR product is generated (see Fig. 1) [27]. The primary variable used to eliminate false positive PCR signals between the mutation detection primer and wild-type DNAs was to increase the annealing temperature of the PCR reaction. With a PCR annealing temperature of 50°C, no PCR DNA bands were observed when wild-type DNA was used with the mutation detection primer, but a very strong PCR DNA band was always observed on the gel when the mutant DNA was included with the mutation detection primer in the PCR reaction. The likelihood of false negative reactions can be greatly reduced by careful addition of measured amounts of DNA template and by including PCR controls with each experiment which include conserved primers (GZ*gyrA*Ccoli3F and GZ*gyrA*Ccoli4R) to help determine if the DNA template is degraded.

It appears that most of the *C. coli* ciprofloxacin-resistant isolates are more highly related to one another than to the *C. coli* ATCC 33559 type strain or isolates D5404 and D5413 and may be from a related source. However, the number of isolates studied is very limited, and they come from a geographically restricted area, which makes it difficult to generalize the findings to other parts of the USA or the world. The analysis of a larger number of isolates from different areas and different periods in time would enable a better evaluation of the frequencies of silent mutations within *gyrA* and would more thoroughly explain the differences between the MN isolates and the reference strain. Ciprofloxacin-susceptible isolate D5416 appears to be similar to the parent strain from which the majority of the ciprofloxacin-resistant isolates apparently arose, since the only difference is the lack of a *gyrA* mutation at codon 86. However, since there is little data on the stability of the mutations in the *gyrA* gene of *C. coli*, then it is also theoretically possible that isolate D5416 may have reverted from Cip^R to Cip^S, particularly if antibiotics were no longer present in the growth environment.

The MAMA PCR method developed for the *C. coli gyrA* Thr-86 to Ile mutation (ACT to ATT) should serve as a simple, specific, rapid, inexpensive and portable alternative to methods such as the nonradioisotopic, single-strand conformation polymorphism method previously published [22] or DNA sequencing for detection of this important fluoroquinolone resistance mutation and may prove useful for clinical diagnosis or epidemiological studies.