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Incidence and Mechanism of Ciprofloxacin Resistance in *Campylobacter* spp. Isolated from Commercial Poultry Flocks in the United Kingdom before, during, and after Fluoroquinolone Treatment

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Five commercial broiler flocks were treated with a fluoroquinolone for a clinically relevant infection. Fresh feces from individual chickens and environmental samples were cultured for campylobacters before, during, and weekly posttreatment until slaughter. Both *Campylobacter jejuni* and *C. coli* were isolated during all treatment phases. An increased proportion of quinolone-resistant strains was seen during treatment, and these strains persisted posttreatment. One quinolone-resistant isolate of each species, each serotype, and each phage type from each sample at all treatment phases was examined for its phenotype and mechanism of resistance. Two resistant phenotypes were isolated: Nal^r Cip^r and Nal^r Cip^s. The majority (269 of 290) of fluoroquinolone-resistant isolates, whether they were *C. jejuni* or *C. coli*, had a mutation in *gyrA* that resulted in the substitution Thr-86→Ile. The other *gyrA* mutations detected were Thr-86→Ala ($n = 17$) and Asp-90→Asn ($n = 10$). The genotypic variation, based on the silent mutations in *gyrA* identified by the denaturing high-performance liquid chromatography pattern and DNA sequencing, was used to supplement typing data and provided evidence for both the spread of preexisting resistant strains and the selection of spontaneous resistant mutants in treated flocks. Multidrug resistance was significantly ($P < 0.01$) associated with resistance to ciprofloxacin. Twenty-five percent (73 of 290) of ciprofloxacin-resistant isolates but only 13% (24 of 179) of susceptible isolates were resistant to three or more unrelated antimicrobial agents. In conclusion, quinolone-resistant campylobacters were isolated from commercial chicken flocks in high numbers following therapy with a veterinary fluoroquinolone. Most ciprofloxacin-resistant isolates had the GyrA substitution Thr-86→Ile. Resistant isolates were isolated from the feces of some flocks up to the point of slaughter, which may have consequences for public health.

Campylobacter spp. are a common cause of gastroenteritis in humans, and while most cases of campylobacteriosis do not require antimicrobial therapy, treatment may be essential for vulnerable patients and for the management of invasive disease. Fluoroquinolones have been widely used for the treatment of *Campylobacter* infections, but the incidence of resistance among *Campylobacter jejuni* and *C. coli* strains isolated from humans increased significantly throughout the world during the 1990s (10, 29).

It has been widely postulated that the increase in the numbers of fluoroquinolone-resistant campylobacters isolated from human infections results from the emergence of resistant strains in poultry and their subsequent consumption (9, 15, 37). Recent studies have reported a high frequency of fluoroquinolone-resistant campylobacters among poultry flocks (7, 37). Fluoroquinolone-resistant campylobacters have not been detected in domestically acquired human infections in Australia, and this has been attributed to the fact that fluoroquinolones have not been licensed for use in food animals (36).

The primary target of fluoroquinolones in *Campylobacter* is DNA gyrase, and resistance arises as a result of mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene, which encodes the A subunit of the target enzyme (17, 38). The majority of highly fluoroquinolone-resistant clinical isolates of *C. jejuni* have the GyrA substitution Thr-86 to Ile (5, 15, 30, 33, 38, 41), which is sufficient to confer high-level resistance. Other less common substitutions, as well as silent polymorphisms, have been reported in the QRDR (20, 30, 33, 38, 41). Substitutions in the B-subunit gene, *gyrB*, have yet to be documented in fluoroquinolone-resistant campylobacters. One report (16) has described mutations in *parC* associated with fluoroquinolone resistance in *C. jejuni*, but that finding has not been supported by other studies (1, 22, 27, 30). Evidence for an efflux pump with broad specificity was found in two laboratory-derived multiply antibiotic-resistant *C. jejuni* strains (6). Recently, a gene encoding an efflux pump protein,

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CmeB, has been described, and inactivation of *cmeB* by insertional mutagenesis has been shown to increase the susceptibility of *C. jejuni* to several antibiotics, including ciprofloxacin (21, 31).

To explore the hypothesis that fluoroquinolone-resistant campylobacters arise in poultry during treatment, this study investigated the incidence and mechanism of fluoroquinolone resistance in *Campylobacter* strains isolated from commercial broiler flocks treated for a clinically relevant infection with a veterinary fluoroquinolone. The aims of the study were to determine (i) the baseline incidence of fluoroquinolone resistance in flocks prior to fluoroquinolone exposure; (ii) the level of resistance in treated flocks; (iii) whether fluoroquinolone-resistant campylobacters spread through a flock as a result of the selection of one or several resistant clones; (iv) whether the types of fluoroquinolone-resistant campylobacters and the mechanisms of resistance that emerge in poultry isolates are similar or identical to those in isolates from humans; and finally, (v) whether the mechanisms of resistance in poultry isolates confer sufficiently high-level resistance to be untreatable in human infections, i.e., whether they confer full clinical resistance.

This large study was performed by three groups in the United Kingdom: the Food Microbiology Collaborating Unit, Bristol; the Campylobacter Reference Unit (CRU), London; and the Antimicrobial Agents Research Group, Birmingham. The prevalence and subtypes of ciprofloxacin-resistant campylobacters isolated from five commercial chicken flocks treated with a therapeutic fluoroquinolone are described in detail in the accompanying article (20a). This report describes the incidence and mechanism of fluoroquinolone resistance in campylobacters isolated from fluoroquinolone-treated flocks.

MATERIALS AND METHODS

Treatment of flocks and sampling procedures. An alert system was put in place for chicken flocks reared in southwest England and elsewhere, whereby the Food Microbiology Collaborating Unit was informed when a commercial flock was about to be treated with a fluoroquinolone for a clinically relevant infection (20a). Six flocks were treated, although one flock (flock 2) remained campylobacter free throughout the sampling period. Birds from flock 1 (ca. 500 barn-reared birds), flock 3 (a free-range flock of ca. 300 birds), flock 4 (a large broiler flock of ca. 20,000 birds), and flock 5 (ca. 1,250 free-range birds) were all treated with difloxacin (Dicural; Fort Dodge Animal Health); and flock 6 (ca. 5,000 free-range birds) received enrofloxacin (Baytril; Bayer). All flocks were treated as instructed by a veterinarian, in accordance with the procedure recommended by the manufacturer (10 mg per kg per bird for 5 days administered in the drinking water).

Up to 14 samples of fresh feces were collected from individual chickens pretreatment (1 to 5 days prior to the start of treatment), during treatment (2 to 5 days after the start of treatment), and after treatment (weekly for up to 4 weeks posttreatment) until the flocks were slaughtered. Samples were also collected from the environment (pooled feed and litter, drinking or puddle water, and swabs of broiler house walls and floors). *Campylobacter* was isolated by direct or enrichment culture, as described in the accompanying report (20a). Up to six isolates from each fecal sample and three isolates from each environmental sample (a total of 1,630 isolates) were sent to CRU for species identification, serotyping, and phage typing and were screened for fluoroquinolone resistance by using breakpoint testing (20a, 35).

Referral of isolates from CRU. One quinolone-resistant isolate of each species, phage type, and serotype was investigated from each fecal or environmental sample from each flock during each treatment phase. At least three sensitive isolates from each treatment phase per flock were chosen for comparison with resistant isolates. Bacteria were grown on Mueller-Hinton agar plus 5% horse blood at 37°C in 7.5% CO_2 and were stored at −80°C on Protect beads (Technical Service Consultants Ltd., Heywood, United Kingdom).

Determination of antibiotic resistance. The agar doubling dilution procedure recommended by the NCCLS Campylobacter Working Group (24) was used throughout the study. Mueller-Hinton medium plus 5% defibrinated horse blood and incubation at 37°C in 7.5% CO_2 were used to determine the MICs of marker antibiotics of each chemical class (ciprofloxacin, nalidixic acid, erythromycin, tetracycline, chloramphenicol, trimethoprim, kanamycin, and ampicillin), marker dyes (acridine orange and ethidium bromide), marker detergents (sodium deoxycholate and sodium dodecyl sulfate), and marker disinfectants (triclosan and cetrimide). *C. jejuni* NCTC 11168 and *C. coli* NCTC 11366 were used as control strains. As no internationally recognized breakpoint concentrations are available for *Campylobacter* spp., designation of antibiotic susceptible or resistant was made with reference to the guidelines of the British Society for Antimicrobial Chemotherapy and NCCLS for human infections (23, 25) to determine the relevance of any resistance observed to public health. MICs of ≥8 μg/ml for ethidium bromide or ≥16 μg/ml for acridine orange, sodium deoxycholate, and sodium dodecyl sulfate were taken to indicate resistance. All susceptibility data were confirmed on at least two separate occasions.

Detection of mutations in *gyrA* and *gyrB*. Bacteria were grown on Mueller-Hinton agar containing 5% defibrinated horse blood at 37°C in 7.5% CO_2 for 48 h. Bacterial colonies were harvested from the agar plate, and a turbid suspension was prepared in sterile distilled water. Genomic DNA was extracted with a DNAace spin cell culture kit (Bioline, London, United Kingdom). The QRDR of *C. jejuni gyrA* was amplified by PCR with primers 293 (5′-GCCTGACGCAA GAGATGGTT-3′) and 343 (5′-CATCGCAGCGGCACTATCAC-3′) to generate an amplimer of 259 bp covering codons 39 to 123 of *gyrA*. The QRDR of *C. coli gyrA* was amplified with primers 344 (5′-TCCTGATGCTAGAGATGGCT-3′) and 345 (5′-CCATCACCATCGATAGAACC-3′) to generate an amplimer of 246 bp covering codons 39 to 118 of *gyrA*. PCR was performed with a reaction mixture volume of 50 μl by using PCR Master Mix (Abgene, Epsom, United Kingdom), 0.5 μg of DNA, and 250 nM each primer. An initial denaturation was carried out at 94°C for 5 min, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 10 min. The QRDR of *gyrB* was amplified as two fragments: amplimer A was amplified with primers 341 (5′-TAGAGGAAGAGAAGCAGCGA-3′) and 342 (5′-CTTCACCTATACCA CAGCCA-3′), and amplimer B was amplified with primers 348 (5′-AGCTATA CTGCCTTTGCGTG-3′) and 349 (5′-GATCCATCAACATCCGCATC-3′), which generated amplimers of 305 bp (codons 380 to 480) and 186 bp (codons 442 to 502), respectively. PCR of *gyrB* was essentially the same as that for *gyrA*, except that a lower annealing temperature of 48°C was used. All primers were synthesized commercially by MWG Biotech (Milton Keynes, United Kingdom).

Mutations in *gyrA* were detected by denaturing high-performance liquid chromatography (DHPLC) analysis (8). PCR amplimers from each isolate were mixed with an equal quantity of DNA (5 μg) amplified from a wild-type control strain (*C. jejuni* NCTC 11168 or *C. coli* NCTC 11366, as appropriate). The DNA mixture was denatured at 95°C for 4 min and then slowly reannealed by cooling to 35°C at 1°C per min to allow the formation of heteroduplexes, as described previously (8). Duplex products were screened for mutations in *gyrA* by using the WAVE nucleic acid fragment analysis system (Transgenomic, Crewe, United Kingdom), essentially as described previously (8). The column temperature used for analysis of *C. jejuni gyrA* was 58°C, and that used for analysis of *C. coli* was 57°C. Elution profiles were analyzed with Navigator software (version 1.4.1 and, later, version 1.5.1). DNA sequencing of novel DHPLC patterns was performed commercially by the Functional Genomics Laboratory, University of Birmingham. The sequences were compared to the published DNA sequences of *C. jejuni* (38; GenBank accession number L04566) and *C. coli* (40; GenBank accession number AF092101).

Mutations in *C. jejuni gyrB* were also detected by DHPLC analysis. Analysis of the *gyrB* gene with Navigator software showed that mutations could be detected by DHPLC only in the first 200 bp of the PCR amplimer (amplimer A) due to the melting profile of this section of the gene. Therefore, a set of primers was designed to amplify a further 186 bp (amplimer B). Both amplimers were analyzed by DHPLC as described above; amplimer A was analyzed with a column temperature of 57°C, and amplimer B was analyzed with two column temperatures: 55 and 56°C. The data from all analyses were compared to enable the QRDR of *gyrB* to be screened for mutations. DNA was sequenced as described above for any strains with novel DHPLC patterns.

RESULTS

Prevalence and subtypes of ciprofloxacin-resistant *Campylobacter* spp. in fluoroquinolone-treated flocks. Both *C. jejuni* and *C. coli* were isolated from samples collected during all

TABLE 1. Cross-resistance to antimicrobial agents and dyes of *Campylobacter* spp. isolated from commercial poultry flocks[a]

Agent	Concn (≥μg/ml) used to determine resistance	% Resistance among Cip^s isolates (no. of isolates) in flock:					% Resistance among Cip^r isolates (no. of isolates) in flock:				
		1	3	4	5	6	1	3	4	5	6
ERY	4	2 (1)	5 (2)	24 (6)	26 (10)	7 (2)	29 (23)	2 (1)	29 (12)	3 (2)	25 (15)
CHL	8	2 (1)	2 (1)	16 (4)	0 (0)	4 (1)	5 (4)	0 (0)	20 (8)	0 (0)	2 (1)
TET	8	9 (4)	0 (0)	36 (9)	0 (0)	78 (21)	51 (40)	0 (0)	68 (28)	19 (13)	61 (37)
KAN	16	13 (6)	10 (4)	56 (14)	10 (4)	7 (2)	10 (8)	19 (8)	49 (20)	13 (9)	3 (2)
AMP	16	28 (13)	71 (30)	20 (5)	13 (5)	59 (16)	83 (65)	74 (31)	80 (33)	25 (17)	36 (22)
EtBr	8	15 (7)	17 (7)	40 (10)	18 (7)	59 (16)	41 (32)	2 (1)	20 (8)	54 (37)	77 (47)
MDR		4 (2)	5 (2)	24 (6)	5 (2)	44 (12)	32 (25)	0 (0)	46 (19)	9 (6)	38 (23)
Total		46	42	25	39	27	78	42	41	68	61

[a] Cip^s, MICs ≤ 1 μg/ml; Cip^r, MICs ≥ 2 μg/ml. MDR indicates resistance to three or more of the following agents: erythromycin (ERY), chloramphenicol (CHL), tetracycline (TET), kanamycin (KAN), ampicillin (AMP), or ethidium bromide (EtBr).

treatment phases. Ciprofloxacin-resistant *C. jejuni* and/or *C. coli* strains were detected pretreatment in four flocks, but they constituted a very small proportion of the campylobacters present. A rapid increase in the proportion of ciprofloxacin-resistant campylobacters was observed during treatment, and this increase persisted posttreatment. During treatment nearly 100% of campylobacters were resistant, and in some flocks a high proportion of resistant strains persisted for up to 4 weeks after treatment.

Prior to treatment a variety of campylobacter subtypes were present, predominantly susceptible subtypes of *C. jejuni*. Considerable changes in both species and subtype prevalence were observed during and after treatment, but no single fluoroquinolone-resistant clone became dominant. Instead, resistant *C. coli* strains or a mixture of resistant *C. coli* and *C. jejuni* strains became dominant. The resistant subtypes which emerged and became dominant were not always the same as those detected pretreatment. A detailed analysis of the prevalence and the subtypes of *Campylobacter* spp. is reported in the accompanying article (20a).

Antimicrobial susceptibility. One resistant isolate of each species, serotype, and phage type was selected from each fecal or environmental sample for further study; and three or more susceptible isolates were selected at random from each flock during each treatment phase for comparison. The Antimicrobial Agents Research Group in Birmingham received a total of 469 isolates from CRU, and these represented 29% of all isolates typed by CRU (20a). Of these 469 isolates, 290 isolates were ciprofloxacin resistant (MICs ≥ 2 μg/ml). Fifty-three percent (174 of 326) of the *C. jejuni* isolates and 82% (115 of 141) of the *C. coli* isolates were ciprofloxacin resistant. Most ciprofloxacin-resistant isolates (282 of 290) were correctly identified as resistant by breakpoint testing by CRU.

Determination of the nalidixic acid and ciprofloxacin MICs revealed that there were two quinolone-resistant phenotypes: both nalidixic acid and ciprofloxacin resistant (Nal^r Cip^r; n = 290) and nalidixic acid resistant and ciprofloxacin susceptible (Nal^r Cip^s; n = 13). Eight *C. jejuni* isolates and five *C. coli* isolates had the Nal^r Cip^s phenotype and were typically inhibited by 0.25 or 0.5 μg of ciprofloxacin per ml (see Table 3).

MDR. As there is increasing evidence that exposure to a fluoroquinolone can select for bacteria that are resistant to multiple agents of unrelated chemical classes as well as fluoroquinolones, all isolates were examined for their susceptibilities to at least one agent representative of each chemical class used in human medicine. Isolates were divided into those that were ciprofloxacin susceptible (MIC ≤ 1 μg/ml) and those that were ciprofloxacin resistant (MIC ≥ 2 μg/ml) to determine whether there was an association between fluoroquinolone resistance and resistance to any other agents (Table 1). All isolates were resistant to trimethoprim (MICs ≥ 2 μg/ml); and most were resistant to acridine orange, sodium dodecyl sulfate, and sodium deoxycholate (MICs ≥ 16 μg/ml), regardless of their fluoroquinolone susceptibilities. Both trimethoprim and sodium deoxycholate were present in the charcoal cefoperazone deoxycholate agar (Oxoid) selective medium used for the isolation of campylobacter (20a). These agents were therefore excluded from the definition of a multiple-drug-resistant (MDR) phenotype. Chloramphenicol resistance was rare (Table 1).

TABLE 2. Substitutions in GyrA detected in flocks 1 and 3 to 6

GyrA substitution[a]	No. of isolates[b]								
	Flock 1, Nal^r Cip^r	Flock 3		Flock 4		Flock 5		Flock 6, Nal^r Cip^r	Total
		Nal^r Cip^r	Nal^r Cip^s	Nal^r Cip^r	Nal^r Cip^s	NAL^r Cip^r	Q^s		
Ile-86	72	40	0	40	1	65	0	51	269
Ala-86	1	1	12	0	0	1	0	2	17
Asn-90	1	0	0	0	0	1	0	8	10
Ile-60	0	0	0	0	0	0	1	0	1

[a] Ile-86, Thr-86→Ile; Ala-86, Thr-86→Ala; Asn-90, Asp-90→Asn; Ile-60, Val-60→Ile.
[b] Nal^r Cip^r, resistant to nalidixic acid (MICs ≥ 32 μg/ml) and ciprofloxacin (MICs ≥ 2 μg/ml); Nal^r Cip^s, nalidixic acid resistant (MICs ≥ 32 μg/ml) and ciprofloxacin sensitive (MICs ≤ 1 μg/ml); Q^s, quinolone sensitive.

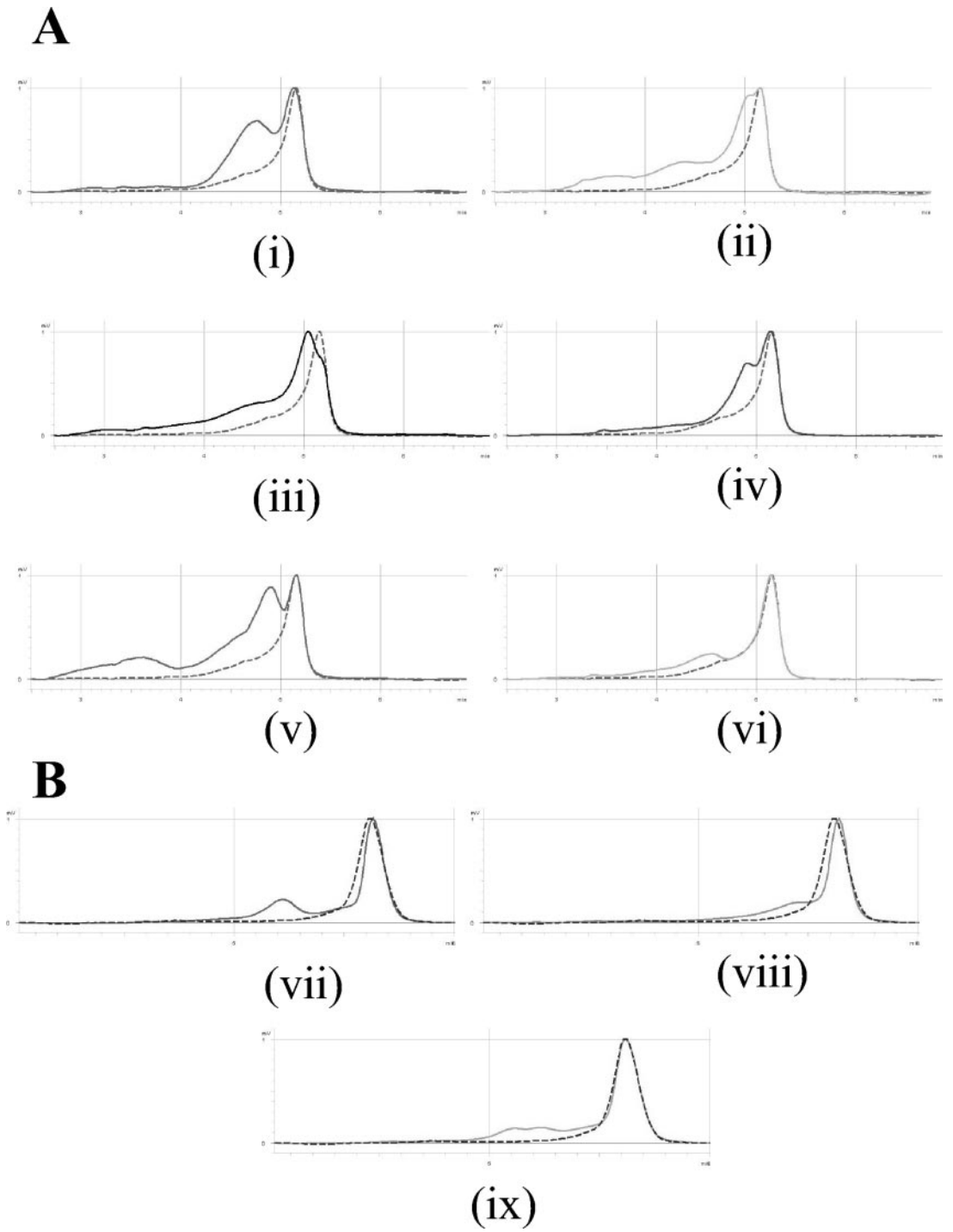

FIG. 1. DHPLC elution traces for *C. jejuni* and *C. coli* isolates with mutations in *gyrA*. (A) *C. jejuni* (i) type A (Thr-86→Ile, ACA→ATA), (ii) type B (His-81, CAC→CAT; Thr-86→Ile, ACA→ATA), (iii) type C (His-81, CAC→CAT; Thr-86→Ile, ACA→ATA; Ser-119, AGT→AGC; Ala-120, GCC→GCT), (iv) type G (Asp-90→Asn, GAT→AAT), (v) type E (His-81, CAC→CAT; Thr-86→Ile, ACA→ATA; Gly-110, GGC→GGT), and (vi) type F (His-81, CAC→CAT; Thr-86→Ala, ACA→GCA). (B) *C. coli* (vii) CC/A (Thr-86→Ile, ACT→ATT; Phe-99, TTT→TTC), (viii) CC/B (His-81, CAC→CAT; Thr-86→Ile, ACA→ATA; Gly-113, GGA→GGT; Ile-115, ATA→ATC), and (ix) CC/C (Val-60→Ile, GTA→ATA; Phe-99, TTT→TTC). The wild-type pattern (dotted line) is shown on each elution trace: *C. jejuni* NCTC 11168 (A) and *C. coli* NCTC 11366 (B). Isolates with *gyrA* code CC/B were *C. coli* but had a *gyrA* sequence with a closer identity to that of *C. jejuni* (the nucleotide changes shown are differences from the *C. jejuni gyrA* sequence). The polymorphisms seen at His-81 (CAT), Gly-113 (GGT), Ile-115 (ATC), and Ala-120 (GCT) in *C. jejuni* are present in wild-type *C. coli* (40).

Isolates in each flock were designated MDR and were resistant to three or more of the following agents: chloramphenicol, kanamycin, ampicillin, erythromycin, and ethidium bromide. Apart from flock 3, larger numbers of fluoroquinolone-resistant strains from each flock were MDR than sensitive. Only two isolates from flock 3 were MDR (Table 1). Twenty-five percent (73 of 290) of ciprofloxacin-resistant isolates and 13% (24 of 179) of ciprofloxacin-susceptible isolates were MDR. Statistical analysis by the χ^2 test showed that MDR was significantly associated with ciprofloxacin resistance ($P < 0.01$). Only 1 of the 13 Nal^r Cip^s isolates was MDR.

The MICs of the disinfectants triclosan and cetrimide were determined for all MDR isolates ($n = 97$). The MICs of the agents for *C. jejuni* NCTC 11168 were 4 and 8 μg/ml, respectively. For the MDR isolates, the triclosan MIC range was 2 to 128 μg/ml, with an MIC at which 50% of isolates are inhibited (MIC_{50}; median), MIC_{90}, and mode MIC of 64 μg/ml and a geometric mean MIC of 55.1 μg/ml. The MIC range for cetrimide was 1 to >128 μg/ml, with an MIC_{50} (median) of 32 μg/ml, an MIC_{90} and a mode MIC of 64 μg/ml, and a geometric mean MIC of 35.2 μg/ml. These values did not differ for ciprofloxacin-susceptible and -resistant MDR isolates.

Role of *gyrA* in fluoroquinolone resistance. DNA sequencing of each novel DHPLC pattern identified 15 *gyrA* genotypes among the *C. jejuni* isolates. Nine patterns were associated with quinolone-resistant (Nal^r Cip^r and Nal^r Cip^s) isolates, and six patterns were associated with sensitive strains, including wild-type *C. jejuni* NCTC 11168. Each pattern corresponded to one or more nucleotide changes in the DNA sequence compared with the sequence of the wild type. However, the majority of these changes did not confer an amino acid substitution. DNA sequencing of several representative isolates with each pattern revealed that the majority of resistant isolates possessed an amino acid substitution in *gyrA*, in which Thr-86 was replaced with Ile (Table 2). The different patterns were due to the presence of different silent mutations, in addition to the substitution mutation (types A to E and L; Fig. 1). For 17 strains, Thr-86 was replaced by Ala (types F and N), and 10 *C. jejuni* strains had the *gyrA* mutation Asp-90→Asn (type G; Fig. 1).

Fewer DHPLC patterns were found among the *C. coli* isolates. The majority of *C. coli* isolates produced pattern CC/A, which corresponded to two nucleotide changes, compared to the sequence of the wild type (*C. coli* NCTC 11366), that conferred the substitution Thr-86 to Ile and a nonsubstitution mutation at codon Phe-99 (Fig. 1). Isolates with the pattern CC/B also had an Ile-86 change, but the *gyrA* sequence was more similar to that of *C. jejuni* than to that of *C. coli* throughout the QRDR. One quinolone-sensitive *C. coli* isolate had a novel pattern (designated CC/C) that corresponded to the substitution Val-60→Ile (Fig. 1).

To determine whether there was any association between a specific mutation in *gyrA* and the MICs of nalidixic acid and ciprofloxacin for each species, a detailed analysis of the *gyrA* DHPLC patterns and MICs was performed (Table 3). Previous studies (30) found that it is difficult to assign an MIC for *Campylobacter* strains with a specific mutation, whereas previously published data for *Escherichia coli* (11) and *Salmonella* (18, 30) have suggested that defined mutations confer specific MICs (within the doubling dilution error of this technique). As had been found previously, no clear MIC was associated with a specific amino acid substitution, and for most patterns a range of ciprofloxacin MICs was obtained (Table 3). The MIC_{50}s (median), MIC_{90}s, and geometric mean MICs for strains with each *gyrA* substitution are shown in Table 3. For *C. jejuni* with the Ile-86 substitution, the mode MIC of ciprofloxacin was 32 μg/ml and the mode MIC of nalidixic acid was 64 μg/ml; for *C. coli* the mode MIC of ciprofloxacin was 8 μg/ml and the mode MIC of nalidixic acid was 128 μg/ml. For *C. jejuni* strains with the Asn-90 substitution, the mode MICs of ciprofloxacin and nalidixic acid were 128 μg/ml. However, the Ala-86 substitution was typically associated with a Nal^r Cip^s phenotype, with 12 of 17 strains inhibited by 0.25 to 0.5 μg of ciprofloxacin per ml and 64 to 128 μg of nalidixic acid per ml (Table 3).

It was noted that *C. coli* became the more prevalent species during treatment but that *C. jejuni* became reestablished posttherapy (20a). It was hypothesized that *C. coli* not only may be more prevalent but also may be more highly resistant than *C. jejuni*, enabling survival during fluoroquinolone exposure. However, these data indicate that the same amino acid is replaced in the QRDR of GyrA of resistant *C. coli* and *C. jejuni* strains. In fact the MICs for the *C. coli* strains containing Ile-86 were lower than those for many of the *C. jejuni* strains with the same substitution (Table 3).

Seventy-three isolates were cultured from the barn environments of four flocks (none were isolated from flock 4). Of these, 36 (49%) were ciprofloxacin resistant and were isolated from drinking water, feed, litter, and surface swabs up to 3 weeks posttreatment. The most common species, serotypes, and phage types of ciprofloxacin-resistant environmental strains were also seen among the fecal isolates (20a), and the same GyrA substitutions were detected among isolates from both sources.

Role of *gyrB* in fluoroquinolone resistance. Sixty of the 83 isolates from flock 3 were screened for mutations in *gyrB* by DHPLC, and representative isolates including all those with novel patterns ($n = 20$) were sequenced. Twenty-two of the 60 isolates screened were of the wild type. Four patterns in addition to the wild type were found, each of which had silent mutations at two loci in *gyrB*; these occurred at Leu-407 (TTA→TTG), Pro-415 (CCA→CCG), Phe-440 (TTC→TTT), or Leu-458 (CTA→TTA or CTG). All 38 of these isolates were either quinolone sensitive or resistant with an identified mutation in *gyrA*. No substitution mutations were found in *gyrB* in any isolate; therefore, it was decided to screen quinolone-resistant isolates from subsequent flocks for *gyrB* mutations by DHPLC only if no *gyrA* mutation was detected. However, no resistant isolates lacking a mutation in *gyrA* were found in flocks 4 to 6.

Role of *gyrA* genotype in isolate typing. It was thought that genotypic variation in *gyrA*, based on the patterns of silent mutations, could be used to supplement the typing data from CRU for detailed strain fingerprinting. These data were used to investigate whether fluoroquinolone exposure selected a preexisting antibiotic-resistant strain which became dominant or whether a spontaneous mutant was selected from the preexisting susceptible population. In practice, this was difficult to analyze because of the diversity of campylobacter serotypes and phage types within each flock, but there was evidence that

TABLE 3. Relationship between ciprofloxacin and nalidixic acid MICs and GyrA substitution in *C. jejuni* and *C. coli*

Resistance phenotype[a]	Species	GyrA substitution[b]	No. of isolates	Ciprofloxacin MIC (μg/ml)				Nalidixic acid MIC (μg/ml)			
				50%	90%	Range	Geometric mean	50%	90%	Range	Geometric mean
Nal^r Cip^r	*C. jejuni*	Ile-86	157	32	64	2–128	22.7	64	128	8–>128	73.2
		Ala-86	4	—[c]	—	4–32	—	—	—	16–>128	—
		Asn-90	10	32	128	8–128	39.4	128	>128	64–>128	97.0
	C. coli	Ile-86	111	8	16	4–64	12.0	128	128	32–>128	97.3
		Ala-86	1	—	—	8	—	—	—	64	—
Nal^r Cip^s	*C. jejuni*	Ile-86	1	—	—	1	—	—	—	128	—
		Ala-86	7	—	—	0.25–0.5	—	—	—	64–128	—
	C. coli	Ala-86	5	—	—	0.25–0.5	—	—	—	64	—

[a] Nal^r Cip^r, resistant to nalidixic acid (MICs ≥ 32 μg/ml) and ciprofloxacin (MICs ≥ 2 μg/ml); Nal^r Cip^s, nalidixic acid resistant (MICs ≥ 32 μg/ml) and ciprofloxacin sensitive (MICs ≤ 1 μg/ml).
[b] Ile-86, Thr-86→Ile; Ala-86, Thr-86→Ala; Asn-90, Asp-90→Asn.
[c] —, the MIC_{50}, MIC_{90}, and geometric mean were not calculated when there were <10 isolates.

both events occurred. In flock 1, both ciprofloxacin-sensitive isolates (*gyrA* type I; $n = 2$) and ciprofloxacin-resistant isolates (*gyrA* type C, $n = 1$) of *C. jejuni* serotype HS31 phage type 1 (PT1) were isolated pretherapy. During treatment, only *gyrA* type C resistant isolates ($n = 19$) were seen. A mutation at Thr-86 (ACA→ATA) in a *gyrA* type I strain would give rise to a C genotype, but as resistant isolates of type C were seen pretreatment and no further susceptible type I isolates of this serotype and phage type were isolated, it is suggested that the preexisting antibiotic-resistant strain became dominant. In flock 3, susceptible *C. jejuni* HS13 PT1 isolates (*gyrA* type H, *gyrB* type A) were isolated from feces and the barn environment pretherapy and during treatment. However, resistant isolates of *gyrA* type F ($n = 2$) and *gyrA* type B ($n = 2$) were also isolated during treatment, and all isolates had the same *gyrB* genotype and the MICs of all agents for these isolates (Table 4) were identical to those for the susceptible isolates. A single nucleotide change in *gyrA* type H would give rise to an F or a B genotype. This is evidence that preexisting susceptible strains may have acquired resistance mutations during treatment. Alternatively, type F and type B resistant strains may have been present at low densities in the flock prior to treatment but became dominant as a result of fluoroquinolone exposure. There was no association between the *gyrA* genotype and any specific serotype or phage type; the different *gyrA* genotypes were distributed at random throughout the different types identified by CRU.

DISCUSSION

The study described here and in the accompanying article (20a) investigated the incidence and mechanism of fluoroquinolone resistance among campylobacters isolated from five commercial chicken flocks treated with a fluoroquinolone for a

TABLE 4. Evidence for emergence of spontaneous quinolone-resistant mutants from preexisting susceptible *C. jejuni* HS13 PT1 strains from flock 3

Treatment phase	Source	Isolate	MIC (μg/ml)[a]		Genotype[b]		Resistance phenotype[c]
			CIP	NAL	*gyrA*	*gyrB*	
Pretreatment	Puddle	P751	0.12	8	H	A	Q^S
	Feces	P765	0.12	8	H	A	Q^S
Treatment	Litter	P762	0.12	8	H	A	Q^S
	Feces	P769	0.12	8	H	A	Q^S
	Feces	P757	0.25	64	F	ND	Nal^r Cip^s
	Feces	P753	0.5	64	F	A	Nal^r Cip^s
	Drinking water	P764	8	128	B	A	Nal^r Cip^r
	Feces	P756	16	64	B	A	Nal^r Cip^r

[a] CIP, ciprofloxacin; NAL, nalidixic acid. The MICs of sodium dodecyl sulfate, ethidium bromide, tetracycline, chloramphenicol, kanamycin, ampicillin, trimethyprim acridine orange, and sodium deoxycholate were identical (±1 dilution) for all eight isolates.
[b] *gyrA* genotypes were determined by DHPLC analysis and are designated as follows: H, His-81 (CAC→CAT); F and B, as shown in Fig. 1; a single nucleotide change in *gyrA* type H would give rise to an F or B genotype; *gyrB* genotype A, Phe-440 (TTC→TTT) and Leu-458 (CTA→TTA); ND, not done.
[c] Nal^r Cip^r, resistant to nalidixic acid (MICs ≥ 32 μg/ml) and ciprofloxacin (MICs ≥ 2 μg/ml); Nal^r Cip^s, nalidixic acid resistant (MICs ≥ 32 μg/ml) and ciprofloxacin sensitive (MICs ≤ 1 μg/ml); Q^S, quinolone sensitive.

clinically relevant infection. The hypothesis explored was that fluoroquinolone use in poultry gives rise to fluoroquinolone-resistant campylobacters that can enter the food chain. This is the first comprehensive, detailed study of commercial flocks rather than models. The proportion of ciprofloxacin-resistant campylobacters increased rapidly during treatment and then declined posttherapy (20a). Changes of the subtypes present in the campylobacter population were observed during and after treatment. Exposure to fluoroquinolones appeared to transiently select for *C. coli* over *C. jejuni* (20a). This report focuses on the mechanism of resistance in the quinolone-resistant campylobacters from the five farms.

Previous studies have used various techniques to screen campylobacters for mutations in the QRDR of *gyrA*. Some, such as single-strand conformation polymorphism analysis (5, 30), are able to distinguish mutations at several locations in the QRDR, while other techniques (restriction fragment length polymorphism analysis [30], real-time PCR with the LightCycler instrument [4], fluorogenic PCR [39], and mismatch amplification mutation assay PCR [40, 41]) screen only for changes at Thr-86. This is the first report to describe the application of DHPLC (8) to the detection of mutations in a campylobacter gene. Fifteen variants of the *gyrA* gene in *C. jejuni* were distinguished by use of this technology, and nine of these were associated with a substitution in GyrA. Most commonly, Thr-86 was replaced by Ile, which has previously been shown to be sufficient to render campylobacters resistant to fluoroquinolones, including enrofloxacin and ciprofloxacin. This has also been the predominant substitution in quinolone-resistant isolates from humans in the United Kingdom (15, 30).

Four *gyrA* patterns were detected among the *C. coli* isolates, including the wild-type strain. The most common pattern corresponded to a Thr-86→Ile substitution. DNA sequencing of one pattern (pattern CC/B) showed that it was quite different from the published sequence for *C. coli* (40; GenBank accession number AF092101) and was more similar to that of *C. jejuni* (38; GenBank accession number L04566). The species of all isolates were determined at CRU, and all isolates were typed at CRU by using established procedures (2, 3, 13, 14). The seven isolates with this pattern were confirmed to be *C. coli* by the PCR identification method of Fermer and Engvall (12) (data not shown). This phenomenon has previously been observed by the Antimicrobial Agents Research Group (30). The genome of *C. jejuni* is known to contain hypervariable sequences (26), and the high frequency of local repeats within the genome suggests that there is an increased likelihood of recombination events (28), which may account for this apparent anomaly.

No multiple GyrA substitutions were found in the campylobacters evaluated in this study, and they have been described only rarely in other studies (20, 30). Therefore, it seems unlikely that campylobacters with a Nal^r Cip^s phenotype would become Nal^r Cip^r by acquiring a second mutation in *gyrA*, whereas double mutations in *gyrA* are common in other species, such as *E. coli* and *Salmonella* (8, 11).

The presence of additional mechanisms of resistance could contribute to the wide range of fluoroquinolone MICs observed for isolates with the same mutation in *gyrA*. No mutations in *gyrB* have been described in quinolone-resistant strains, and campylobacters appear to lack the *parC* gene; mutations in both these genes have been shown to contribute to high-level resistance in other species (11). The strains screened for changes in *gyrB* in the present study contained no substitutions that resulted in mutations. There was a clear association between ciprofloxacin resistance and resistance to other agents, with a quarter of the resistant isolates being MDR. Hakanen et al. (19) recently reported a strong association between MDR and resistance to ciprofloxacin in *C. jejuni* strains isolated from clinical specimens. In a recent study, *C. jejuni* strains that had a mutation in *gyrA* and that overexpressed the efflux pump gene *cmeB* were less susceptible to ciprofloxacin than isolates in which either a *gyrA* mutation or overexpression of *cmeB* occurred alone (32). There is evidence that efflux systems other than *cmeB* and *cmeF* that may contribute to MDR are present in campylobacters (32). The additive effect of efflux and mutations in *gyrA* may account, in part, for the range of ciprofloxacin MICs observed for strains with identical mutations in the QRDR. The contributions of efflux and multiple mechanisms of resistance to MDR in these isolates are being investigated.

For all resistant *C. coli* isolates (except six isolates from flock 3) the mutation in *gyrA* resulted in the replacement of Thr-86 with Ile. While most resistant *C. jejuni* strains also had this substitution, other mutations were observed in a few strains from four of the five farms. These data suggest that there is more variation within *gyrA* of *C. jejuni* than in *gyrA* of *C. coli* and that *C. coli* may be more clonal than *C. jejuni*. The increased numbers of *C. coli* isolates recovered during fluoroquinolone treatment (20a) cannot be explained by the level of resistance (i.e., the MIC) or by the mechanism of resistance. The factor that caused the surge in the numbers of isolates of this species and the subsequent decrease posttreatment remains to be established.

Not all resistant isolates were of the same type, demonstrating that resistance was not a result of the spread of a single resistant clone but that numerous clones were selected by fluoroquinolone treatment (20a). The persistence of highly quinolone-resistant campylobacters up to 4 weeks posttreatment has possible consequences for human health. The *gyrA* mutations identified among poultry isolates are identical to those described in human clinical isolates, and the majority conferred high-level ciprofloxacin resistance. Although it has been presumed that a human infection caused by a food-borne ciprofloxacin-resistant campylobacter results in treatment failure, there is little evidence to confirm that this occurs. The proportion of campylobacter infections in humans that are treated with an antibiotic is also thought to be low, but no published data are yet available. However, it has been shown that the duration of diarrhea is prolonged in patients infected with quinolone-resistant *C. jejuni* (34).

In conclusion, quinolone-resistant campylobacters were isolated from commercial chicken flocks in high numbers following therapy with a veterinary fluoroquinolone. Most ciprofloxacin-resistant isolates had a *gyrA* mutation, with the replacement of Thr-86 by Ile. However, the high incidence was not due to the spread of a single resistant clone throughout each flock, as resistant isolates of different species, serotypes, and phage types were identified within each flock. Resistant isolates were isolated from

the feces of some flocks up to the point of slaughter, which may have consequences for public health.

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