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# Increased Resistance to Quinolones in *Campylobacter jejuni*: A Genetic Analysis of *gyrA* Gene Mutations in Quinolone-Resistant Clinical Isolates

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***Abstract*: *Campylobacter jejuni* is a frequent cause of enteritis and sometimes it requires antimicrobial therapy. We have studied the evolution of resistance to nine antibiotics from 1990 to 1994 and investigated how frequently *gyrA* mutations are involved in the acquisition of quinolone resistance. The percentage of chloramphenicol-, clindamycin-, tertracycline- and amoxicillin plus clavulanic acid-resistant strains has remained practically unchanged and erythromycin and gentamicin resistance has decreased, whereas the percentage of ampicillin-, nalidixic acid- or ciprofloxacin-resistant strains has almost doubled in the follow-up period, from 56 to 76% for ampicillin- and from 47.5 to 88% for quinolone-resistant strains. This study clearly shows that a mutation in Thr-86 to Ile or Lys is a frequent mechanism associated with the acquisition of a high level of resistance to quinolones in clinical isolates of *C. jejuni*.**

***Key words*: *Campylobacter jejuni*, GyrA, Antibiotics, Quinolone resistance**

*Campylobacter jejuni* ranks second after *Salmonella* spp. as the cause of enteritis in adults and first as the cause of enteritis in children in most industrialized countries. Infectious diarrhea caused by *C. jejuni* is usually a self-limiting disease lasting 3 to 5 days but can persist for up to 2 weeks or longer (2, 24, 25); therefore, in some cases, it may by necessary to initiate antibiotic treatment. This microorganism is susceptible to several antimicrobial agents (7, 16, 27, 30) and the new fluoroquinolones present excellent *in vitro* activity (3, 7, 9, 23, 26). However, quinolone resistance developed during ciprofloxacin therapy has been reported (8). Gootz and Martin (10) have shown that a single-step mutation leading to high-level resistance can occur in *C. jejuni*. Several mutations have been shown in the *gyrA* gene from quinolone-resistant *C. jejuni*. (31). Besides the mutations in *gyrA*, *gyrB* or *parC* genes, reduced quinolone accumulation in the cells has been shown to be a mechanism of resistance to quinolones in *Escherichia coli* (4, 5, 11, 15, 20, 21, 28, 29).

The aim of this study was to analyze the prevalence of quinolone-resistant clinical isolates of *C. jejuni* from 1990 to 1994 and investigate how frequent *gyrA* mutations are involved in the acquisition of quinolone resistance.

A total of 313 *C. jejuni* clinical isolates were obtained over a period of four years from stool samples of outpatients with diarrhea submitted to the Clinical Microbiology Laboratory at the Hospital Clinic of Barcelona, Spain. The samples were cultured in a *Campylobacter* blood-free medium that was incubated for 48 hr at 42 C under microaerophilic conditions. After incubation, the plates were examined for characteristic morphology. Suspect colonies were Gram-stained to observe the typical *Campylobacter* morphology and tested for catalase and oxidase production. To identify *Campylobacter* to the species level, hippurate hydrolysis test and indoxyl acetate hydrolysis were used. Eighteen *C. jejuni* clinical isolates (six quinolone-susceptible strains and twelve quinolone-resistant strains) were randomly chosen to perform a genetic analysis of the *gyrA* gene.

Antimicrobial susceptibility tests were performed by

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an agar diffusion disk method as advocated by the National Committee for Clinical Laboratory Standards (18). Mueller-Hinton agar with 5% horse blood was obtained from Becton Dickinson (Cockeysville, Md., U.S.A.) and antimicrobial disks (erythromycin, 15 μg; chloramphenicol, 30 μg; gentamicin, 10 μg; clindamycin, 2 μg; ampicillin, 10 μg; amoxicillin plus clavulanic acid, 20/10 μg; tetracycline, 30 μg; nalidixic acid, 30 μg; and ciprofloxacin, 5 μg) were obtained from BBL Microbiology Systems, Becton Dickinson. The MICs of ciprofloxacin and nalidixic acid were performed using an agar dilution method in accordance with the guidelines of the National Committee for Clinical Laboratory Standards (19). Approximately $10^4$ CFUs of each isolate were inoculated in a freshly prepared medium containing serial dilutions of ciprofloxacin (Bayer, Leverkusen, Germany) or nalidixic acid (Prodesfarma, Barcelona, Spain) with a multipoint replicator.

The PCR to amplify a fragment from nucleotides 169 to 570 of the *gyrA* gene was performed using the conditions described in a previous report (29), with the following 20-mer oligonucleotide primers; 5'ATGATGAGGCAAAAAGTAGA3' and 5TAAACTATGAGGTGGGATGT3'.

The antimicrobial resistance among *C. jejuni* strains isolated from 1991 to 1994 is shown in Table 1. The percentages of chloramphenicol- and clindamycin-resistant strains remained practically unchanged in the studied period, with an overall percentage of resistance of 1% and 8%, respectively, as did the percentage of tetracycline-resistant strains which remained around 42%. The percentage of erythromycin-, gentamicin- and amoxicillin plus clavulanic acid-resistant strains has decreased in the last four years from 11, 5.45 and 4% to 5.3, 0 and 2.6%, respectively, whereas ampicillin, nalidixic acid and ciprofloxacin increased in the follow-up period from 56 to 76% for the ampicillin-resistant strains and from 47.5 to 88% for the quinolone-resistant strains. All the nalidixic acid-resistant strains showed resistance to ciprofloxacin.

A DNA fragment of 410 bp containing the quinolone resistance-determining region of the *gyrA* gene was obtained by PCR and DNA sequenced. All 12 isolates with a MIC of ciprofloxacin ≥ 16 μg/ml (16–64 μg/ml) and nalidixic acid ≥ 128 μg/ml (128–> 256 μg/ml) showed a change in Thr-86 to Ile, with one exception where a novel mutation generated a change of Thr-86 to Lys; whereas all the isolates with a MIC of ciprofloxacin ≤ 0.25 μg/ml and nalidixic acid ≤ 8 μg/ml did not show any change at Thr-86.

Erythromycin is considered the first-choice antibiotic. In this study, the percentage of erythromycin-resistant *C. jejuni* clinical isolates in 1994 was 5.3%, similar to that reported by Sanchez et al (22). Actually, fluoroquinolones are also considered antibiotics of choice for the treatment of infections caused by *Campylobacter* (1). Fliegelman et al (7) found ciprofloxacin to be the most active against *Campylobacter*; however, as it has been shown in this study, in the few last years, a dramatic increase of resistance to quinolones in *C. jejuni* clinical isolates has occurred in Catalonia, Spain. A mutation at Thr-86, homologous to Ser-83 in *E. coli*, has been described in *C. jejuni* which leads to the high level of resistance to nalidixic acid and ciprofloxacin. A mutation at Ala-70 or Asp-90, observed in mutants obtained in the laboratory, results in a high level of nalidixic acid resistance but a low level of ciprofloxacin resistance (31). In our study, a single change in the Thr-86 of GyrA to Ile or Lys seems associated with a high level of resistance to nalidixic acid and ciprofloxacin. These results agree with those obtained by Wang et al (31). In *E. coli*, a double mutation at Ser-83 and Asp-87 determines a greater level of resistance to ciprofloxacin (29); however, the mutation at amino acid codon 90 in *C. jejuni*, equivalent to Asp-87 in *E. coli*, was not found in any of the analyzed strains. A quinolone-resistant strain showed a novel and previously undescribed mutation at position 86 (Thr → Lys). That is in contrast with that obtained by

Table 1. Antimicrobial resistance among *C. jejuni* strains isolated from 1991 to 1994

| Antibiotic | % of resistant isolates | | | |
|---|---|---|---|---|
| | 1991 (*n*=55)[a] | 1992 (*n*=74) | 1993 (*n*=108) | 1994 (*n*=76) |
| Erythromycin | 11 | 7 | 7.4 | 5.3 |
| Chloramphenicol | 0 | 1.4 | 0 | 2.6 |
| Gentamicin | 5.45 | 4 | 1 | 0 |
| Clindamycin | 9 | 7 | 11 | 5 |
| Ampicillin | 56 | 63 | 57 | 76 |
| Ampicillin +clavulanic acid | 4 | 1.3 | 5.5 | 2.6 |
| Tetracycline | 45 | 35 | 49 | 45 |
| Nalidixic acid | 47.5 | 63.5 | 73 | 88 |
| Ciprofloxacin | 47.5 | 63.5 | 73 | 88 |

[a] Total of *Campylobacter jejuni* clinical isolates.

Yonezawa et al (32) with a laboratory constructed mutant of *E. coli*, in which the mutation Ser-83 to Lys did not confer quinolone resistance.

All the quinolone-resistant clinical isolates analyzed had a MIC of nalidixic acid ≥ 128 μg/ml and a MIC of ciprofloxacin ≥ 16 μg/ml. No clinical isolates with a MIC of ciprofloxacin between ≥ 1 μg/ml and ≤ 16 μg/ml were found. Gootz and Martin (10) showed that a single-step mutation could occur in the *gyrA* gene of "*in vitro*" nalidixic acid-selected mutants of *C. jejuni* resistant to quinolones, which produced clinically relevant levels of resistance to the newer quinolones. Our study supports these results and highly suggests that a single-step mutation also takes place in clinical isolates.

It has been suggested that the increase of quinolone resistance in *Campylobacter* strains of human origin, which was paralleled by a similar rise in quinolone resistance in *Campylobacter* strains isolated from poultry, probably reflects the veterinary use of quinolones (6) and also the increase of antibiotic consumption in humans (17). In our study, the high frequency of quinolone-resistant *C. jejuni* clinical isolates may be due to the abusive veterinary use of enrofloxacin (a derivative of ciprofloxacin) in Spain. Although no other mechanism of resistance to quinolone, such as *gyrB* or *parC* gene mutations or changes in permeability has been studied, our results indicate that mutation in the Thr-86 of the GyrA protein is a frequent mechanism associated with the acquisition of a high level of resistance to quinolones in clinical isolates of *C. jejuni*.

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