Validation of the T86I mutation in the gyrA gene as a highly reliable real time PCR target to detect Fluoroquinolone-resistant Campylobacter jejuni

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Abstract

Background: Campylobacter jejuni is a leading cause of bacterial diarrhea worldwide, and increasing rates of fluoroquinolone (FQ) resistance in C. jejuni are a major public health concern. The rapid detection and tracking of FQ resistance are critical needs in developing countries, as these antimicrobials are widely used against C. jejuni infections. Detection of point mutations at T86I in the gyrA gene by real-time polymerase chain reaction (RT-PCR) is a rapid detection tool that may improve FQ resistance tracking.

Methods: C. jejuni isolates obtained from children with diarrhea in Peru were tested by RT-PCR to detect point mutations at T86I in gyrA. Further confirmation was performed by sequencing of the gyrA gene.

Results: We detected point mutations at T86I in the gyrA gene in 100% (141/141) of C. jejuni clinical isolates that were previously confirmed as ciprofloxacin-resistant by E-test. No mutations were detected at T86I in gyrA in any ciprofloxacin-sensitive isolates.

Conclusions: Detection of T86I mutations in C. jejuni is a rapid, sensitive, and specific method to identify fluoroquinolone resistance in Peru. This detection approach could be broadly employed in epidemiologic surveillance, therefore reducing time and cost in regions with limited resources.

Keywords: Campylobacter jejuni, T86I, gyrA, Ciprofloxacin, Antimicrobial resistance

Background

Campylobacter infection is the leading zoonotic cause of foodborne illness in the world, with 400 million acute cases of diarrheal disease reported worldwide annually [1]. C. jejuni is a leading bacterial cause of human diarrheal infections [2, 3] in high income countries, but the burden of campylobacteriosis is markedly elevated in low-to-middle income countries (LMIC) and contributes significantly to childhood mortality and poor linear growth in these regions [1, 4–6]. In addition to potentially severe dysentery, campylobacteriosis may also result in longer-term secondary complications including Guillain-Barre syndrome and reactive arthritis [1]. Antibiotics usually considered for treatment of campylobacteriosis include macrolides and fluoroquinolones (FQs) due to their relatively low cost, limited short-term side effects and ease of administration [7].

Globally, the emergence of FQ resistance in C. jejuni has become a major problem in treating undifferentiated dysentery and campylobacteriosis [7–10]. FQs, including
ciprofloxacin, are among the most widely used antibiotics for the treatment of travelers’ diarrhea. However, increasing rates of resistance to these drugs have been reported worldwide [11], with significant treatment implications for the management of Campylobacter infections in developing settings with limited access to resources and alternative therapeutic choices [12–14].

The antibacterial activity of ciprofloxacin and other FQs is due to their ability to bind and inhibit the DNA gyrase necessary for genomic DNA replication. Resistance to ciprofloxacin is mediated by mutations in the gyrA gene, with the single-step T86I amino acid change as one of the most common mutations associated with decreased susceptibility in Campylobacter [1, 7–10]. Alterations of nucleotide 257 (codon 86) from ACA to ATA of the gyrA gene result in a threonine to isoleucine substitution in the GyrA protein and confer high-level resistance to ciprofloxacin [15–20].

Numerous molecular techniques based on polymerase chain reaction (PCR) testing may detect mutations associated with resistance to FQ and include sequencing, detection of polymorphisms, and denaturing gradient gel electrophoresis [9, 16, 21, 22]. In recent years, several of these genotypic methods for the detection of FQ-resistant Campylobacter have been validated. To date, all have identified the T86I mutation through nucleic acid amplification [17, 23–25]. However, there are few validation studies among Campylobacter isolates from LMICs where rates of antimicrobial resistance (AMR) are particularly high [26, 27].

The primary goal of this study was to evaluate a RT-PCR assay for detection of the T86I gyrA gene mutation among C. jejuni isolates from Peru, a middle-income country with rapidly rising rates of FQ-resistant Campylobacter [27] and a substantial burden of campylobacteriosis [6, 26–28].

**Table 1** Primers and probes used

| Target gene | Oligonucleotide sequence (5’-3’) | Reference |
|-------------|---------------------------------|-----------|
| gyrA Wild type | HEX 5’-CCCACATGGAGATACAGCAGTTTATG-3’ | This study |
| gyrA point mutation T86I | 6-FAM 5’-CCCACATGGAGATATAGCAGTTTATG-3’ | This study |
| gyrA | GCCTGACCAGAAGAGATGGTT | Bakeli, et al. [17] |
| gyrA | TTTTCCCGATACCTACAGC | Espinoza et al. BMC Infectious Diseases (2020) 20:518 |

Methods

**Study sites**

A surveillance study for AMR in enteric pathogens in Peru occurred between January 2001 to December 2010 at nine hospital and community laboratories in the cities of Lima, Cusco, and Iquitos [27]. Stool isolates identified as Campylobacter spp. were placed in Cary-Blair transport media and stored at 4°C at the study site [27]. Every two weeks, isolates from each study site were transported to the U.S. Naval Medical Research Unit No. 6 (NAMRU-6) laboratory at 4°C for further testing. After microbiological confirmation using routine culture and biochemical methods, all isolates were stored at –80°C in Peptone Broth with 15% glycerol.

A random selection of 200 archived stool isolates from children between 1 month to 15 years old during period 2006–2010 were selected from three hospitals in Lima, Peru (Instituto Nacional de Salud del Niño, Hospital Nacional Docente Madre-Niño, and Emergencias Pediátricas) for antimicrobial susceptibility testing and detection of T86I gyrA mutation.

**Culture and isolation of C. jejuni**

All archived isolates that were previously identified as hippurate hydrolysis-positive were re-cultured and re-isolated according to methods described elsewhere [27] for further testing.

**Extraction of C. jejuni DNA**

Isolates were reactivated on Columbia agar (Becton, Dickinson and Company, Sparks, MD 21152 USA) that was supplemented with 5% sheep blood after incubation for 48 h at 42°C under micro-aerobic conditions. One loop of colony growth was then used for DNA extraction which was performed by QIAamp DNA Mini Kit (Qiagen GmbH, D40724 Hilden, Germany), following the manufacturer’s guidelines. The final concentration of DNA was adjusted to 10 ng/μl.

**Molecular confirmation of C. jejuni species**

C. jejuni isolates underwent further species confirmation by PCR. Primers designed by Vandamme et al. [29] were used to amplify internal glyA fragments and detect a 773-bp amplification product consistent with a C. jejuni species (Table 1). For PCR reaction, 11 μl of AmpliTaq...
Gold PCR Master Mix 2X, (AB Applied Biosystems, Foster City, CA 94404, USA), 2 μl of DNA [10 ng/μl] and primers at final concentration of 0.4 μM. DNA were mixed for a final volume of 25 μl. Amplification was performed by Veriti Thermal Cycler (AB Applied Biosystems, Singapore) using the parameters: initial denaturation (5 min 95 °C), twice (1 min 94 °C, 1 min 64 °C, 1 min 72 °C), twice (1 min 94 °C, 1 min 62 °C, 1 min 72 °C), twice (1 min 94 °C, 1 min 60 °C, 1 min 72 °C), twice (1 min 94 °C, 1 min 58 °C, 1 min 72 °C)), twice (1 min 94 °C, 1 min 56 °C, 1 min 72 °C) 30 cycles (1 min 94 °C, 1 min 54 °C, 1 min 72 °C) and final extension step 10 min at 72 °C as recommended by Vandamme et al. [29]. Post-amplification, PCR products were visualised by 2% UltraPure agarose gel (Invitrogen, Carlsbad, CA 92008, USA) with a SYBR Safe stain (1X) (Invitrogen, Eugene, Oregon, 541.4658300, USA).

Determination of phenotypic resistance

Positively-identified C. jejuni isolates were tested for antibiotic susceptibility using the E-test to determine the minimum inhibitory concentration (MIC) for ciprofloxacin at a range of 0.002-32 μg/mL (AB BioMérieux, bio-Mérieux SA RCS LYON 69280 Marcy-l’Etoile, France) [30]. Isolates also underwent disk-diffusion testing [27] using Clinical and Laboratory Standards Institute (CLSI) guidelines to determine susceptibility [31]. C. jejuni ATCC 33560 was used as the quality control organism (American Type Culture Collection, Manassas, Virginia, USA).

Determination of genotypic resistance

A blinded molecular detection of antimicrobial susceptibility was performed on all isolates to generate unbiased results. Isolates were tested for the presence of the T86I gyrA resistance mutation using real-time PCR. Specific TaqMan probes and primers (Table 1) were designed by AlleleID 7 software (PREMIER Biosoft International, Palo Alto, California, USA). For a final volume of 20 μl for the PCR reaction, the following were used: 10 μl of Rotor Gene Multiplex PCR (QIAGEN GmbH, Hilden, Germany), 1 μl of DNA [10 ng/μl], primers, and probes at final concentration of 0.5 μM and 0.25 μM respectively.

DNA amplification and the resultant PCR product was analysed using the Rotor Gene Q version 1.7.94 (QIAGEN GmbH, Hilden, Germany), using the following parameters: pre-denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 30 s, and extension at 72 °C for 20 s with acquisition in green and yellow channels.

Detection of ciprofloxacin resistance for allelic discrimination

Detection of T86I point mutation in the gyrA gene of C. jejuni was carried out by allelic discrimination. A fluorescent signal from only the HEX dye with acquisition in the yellow channel indicated detection of the wild type (mutation-negative) gene, while the presence of 6-FAM dye fluorescence with acquisition in green channel indicated detection of the T86I mutation (mutation-positive). Absence of both fluorescent signals indicated the gyrA gene was not amplified as observed with C. jejuni in no-template controls (NTCs) and C. coli isolates. For discrimination of positive and negative mutants, we used a wild-type control (ATCC 33560), a T86I mutant that was identified by sequencing the gyrA gene, and a non-template controls for each run, with a threshold of 0.1 for genotypic discrimination. Fluorescent signals were interpreted automatically using sequence detection software using the Rotor Gene Q version 1.7.94 (QIAGEN GmbH, Hilden, Germany).

Validation of T86I gyrA real-time PCR assay diagnostic performance

Sensitivity and specificity were determined for the T86I gyrA resistance mutation real-time PCR assay using disk-diffusion and E-test as gold standards. Confidence intervals (95%) for sensitivity and specificity were determined without continuity correction [32, 33].

Detection of amino acid mutations in gyrA to confirm PCR results in a subset of isolates

Sequencing of the amplified fragment of gyrA was identified by sequencing the gyrA gene, and a non-template controls for each run, with a threshold of 0.1 for genotypic discrimination. Fluorescent signals were interpreted automatically using sequence detection software using the Rotor Gene Q version 1.7.94 (QIAGEN GmbH, Hilden, Germany).

Table 2 Susceptibility of 189 C. jejuni isolates, and point mutation T86I of the gyrA gene

| Phenotype | No. of Isolates | CIP_Disk diffusion Range (mm) | NA_Disk diffusion Range (mm) | CIP_MIC Range (μg/mL) | Real-Time PCR gyrA gene (n) |
|-----------|----------------|------------------------------|------------------------------|-----------------------|---------------------------|
| CIP-S and NA-S | 44 | 29–50 | 19–37 | 0.008–1.0 | Wild type 44 |
| CIP-R and NA-R | 141 | 6–11 | 6–9 | 4–32 | T86I mutation 141 |
| CIP-S and NA-R | 4 | 24–38 | 6 | 0.047–0.19 | Wild type 4 |

CIP-S ciprofloxacin sensitive, CIP-R ciprofloxacin resistant, NA-S nalidixic acid sensitive, NA-R nalidixic acid resistant, MIC minimal inhibitory concentration
The sequencing results of 189 Campylobacter jejuni isolates were further selected for DNA sequencing of the gyrA gene as previously described [17]. The sequencing results of gyrA mutant and wild-type isolates are presented in Table 4. All 72 C. jejuni isolates demonstrated amino-acid substitutions at codon 86 (T → I). None of the 28 wild-type isolates demonstrated a point mutation at gyrA nucleotide position 257. The amino-acid substitution V149I was also observed in 28 ciprofloxacin resistant isolates as previously reported, with none of the ciprofloxacin-susceptible isolates showing this change. Table 5 shows the distribution of C. jejuni isolate by hospital and by year, as well as the percentage of those that carried the T86I gyrA mutation from 2006 to 2010. Overall, the prevalence of the gene mutation appeared in 65–75% of all isolates for each year until 2010, when it was identified in 100% of all isolates tested.

Discussion

Increasing AMR in C. jejuni is a major global public health threat. It is important to seek alternatives to conventional phenotypic testing of AMR in C. jejuni due to the limited availability of resources to isolate and characterize enteric pathogens in developing countries. This study is one of only a few studies that have validated this method in LMICs, including Latin America.

We have previously described increasing rates of AMR in Campylobacter isolates in Peru [27]. Our data in this study suggest that an increased prevalence of the T86I gyrA mutation in C. jejuni may be the cause of this rise in FQ resistance (Table 5). The very high sensitivity (100%) and specificity (100%) demonstrated by T86I gyrA mutation real-time PCR assay confirms it as a valid alternative resistance assay to conventional fluoroquinolone susceptibility testing in C. jejuni isolates, consistent with the high sensitivity and specificity described elsewhere [15, 16, 18]. This data set may shed light upon the feasibility of this technique to be broadly employed in other regions for detecting changes in ciprofloxacin resistance among C. jejuni. Although there are other

| Real Time PCR T86I Mutation | Total no. of isolates | Total no. of isolates |
|-----------------------------|-----------------------|-----------------------|
|                            | CIP_MIC_Sensitive     | CIP_MIC_Resistant     |
| Wild type (48)              | 48                    | 0                     |
| Mutation (141)              | 0                     | 141                   |

**Table 4** Correlation between Real-Time PCR T86I mutation MICs values of ciprofloxacin and mutations of the gyrA gene

| Strains | CIP_MIC (µg/mL) | T86I | A120V | D144N | V149I | H81 | G110 | S119 | S152 | S157 | V161 |
|---------|-----------------|------|-------|-------|-------|-----|------|------|------|------|------|
| Positive 28 | 12–32 | T    | T     | –     | T     | –   | C    | –    | T    | C    |
| Positive 28 | 12–32 | T    | T     | –     | A     | T   | –    | C    | –    | T    |
| Positive 7   | 32    | T    | T     | –     | –     | T   | T    | C    | –    | T    |
| Positive 3   | 12–32 | T    | T     | –     | T     | T   | –    | C    | C    | T    |
| Positive 6   | 6–32  | T    | –     | –     | –     | –   | –    | –    | –    | –    |
| Negative 21  | 0.016–0.190 | –    | T     | –     | –     | T   | –    | C    | –    | T    |
| Negative 1   | 0.064 | –    | T     | –     | T     | T   | C    | –    | T    |
| Negative 1   | 0.032 | –    | T     | A     | –     | T   | C    | –    | T    |
| Negative 5   | 0.023–0.047 | –    | –    | –     | –     | –   | –    | –    | –    |
mutations associated with ciprofloxacin resistance in gyrA gene of C. jejuni, the T86I mutation is the most frequent mutation encountered worldwide [17]. A different mutation at the same codon, T86A, has also been described in C. jejuni isolates with resistance to nalidixic acid [16–18], but its association with resistance to later-generation FQs such as ciprofloxacin is less clear. The present study found no T86A mutations in C. jejuni isolates throughout gyrA sequencing.

In these Peruvian isolates, the T86I substitution was the main amino-acid change associated with high-level resistance to ciprofloxacin, as described in other settings [9, 16, 17, 22, 28]. Nonetheless, these findings may be specific to given geographical regions with other common amino-acid substitutions (e.g., V149I) being associated with FQ resistance elsewhere [34]. However for some of these amino-acid substitutions their relevance as a mediator of AMR have been placed into question [16]. A limitation of our study was the lack of isolates with intermediate susceptibility to FQs, for which molecular methods that simultaneously detect the mutations associated with resistance to both quinolones and macrolides. Effective molecular diagnostics may be a feasible approach to guide the antimicrobial treatment of C. jejuni infections.

Conclusions
The present study developed a rapid, sensitive, and specific molecular technique for the detection of T86I mutations in the gyrA gene of C. jejuni that appears strongly associated with FQ resistance. Because of the high prevalence of C. jejuni in LMIC and the concern for increasing FQ resistance, this detection approach could be broadly employed in epidemiologic surveillance to reduce time and cost in regions with limited resources.

Table 5  Real time PCR detection of ciprofloxacin sensitive or wild type (WT) C. jejuni isolates compared to resistant or T86I positive C. jejuni isolates per year by hospital

| Hospital                  | 2006 T86I (%) | 2006 WT | 2007 T86I (%) | 2007 WT | 2008 T86I (%) | 2008 WT | 2009 T86I (%) | 2009 WT | 2010 T86I (%) | 2010 WT |
|---------------------------|---------------|---------|---------------|---------|---------------|---------|---------------|---------|---------------|---------|
| Hospital Materno Infantil| 11 (92)       | 1       | 9 (75)        | 3       | 1 (25)        | 3       | 4 (100)       | 0       | 9 (100)       | 0       |
| Emergencias Pediátricas   | 12 (63)       | 7       | 9 (53)        | 8       | 13 (68)       | 6       | 13 (65)       | 7       | 12 (100)      | 0       |
| Hospital del Niño         | 9 (75)        | 3       | 16 (76)       | 5       | 11 (92)       | 1       | 3 (43)        | 4       | 9 (100)       | 0       |
| Total                     | 32 (74)       | 11      | 34 (68)       | 16      | 25 (72)       | 10      | 20 (65)       | 11      | 30 (100)      | 0       |
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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
The Institutional Review Board of the U.S. Naval Medical Research Unit-6 in Peru (FWA 00010031) determined that the testing of these bacterial isolates (Protocol No PJT.NMRCD.HURS03) did not constitute human subjects research.

Consent for publication
Not applicable.

Competing interests
The authors declare that there are no financial, institutional or other relationships that might lead to bias or a conflict of interest.

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