Virulence and Antimicrobial Resistance in Campylobacter spp. from a Peruvian Pediatric Cohort

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The presence of virulence factors (VFs) and mechanisms of quinolones and macrolide resistance was analyzed in Campylobacter spp. from a pediatric cohort study in Lima. In 149 isolates (39 Campylobacter jejuni and 24 Campylobacter coli from diarrheic cases; 57 C. jejuni and 29 C. coli from controls), the presence of the cdtABC and cadF genes and iam marker was established. Nalidixic acid, ciprofloxacin, erythromycin, and azithromycin susceptibilities were established in 115 isolates and tetracycline-susceptibility was established in 100 isolates. The presence of mutations in the gyrA, parC, and 23S rRNA genes was determined. The cadF gene and all genes from the cdtABC operon were significantly more frequent among C. jejuni (P < 0.0001); the iam marker was more frequent in C. coli (P < 0.0001). No differences were observed in VFs between cases and controls. Almost all isolates were tetracycline-resistant; nalidixic acid and ciprofloxacin resistance reached levels of 90.4% and 88.7%, respectively. Resistance to macrolides was 13% (C. jejuni 4.3%; C. coli 26.1%). Resistance to ciprofloxacin was related to GyrA Thr86 substitutions, while 13 of 15 macrolide-resistant isolates possessed a 23S rRNA mutation (A2075G). Differences in the presence of VFs and alarming levels of resistance to tested antimicrobial agents were observed among C. jejuni and C. coli.

1. Introduction

Campylobacter spp. ranks among the most relevant causes of diarrheal illness worldwide, with recent estimations of around 166,000 cases/year, including 31,700 Guillain-Barré Syndromes, which lead to 37,604 deaths and 3,733,822 Disability Adjusted Life Years (DALYs) [1]. In addition, other severe sequelae, such as Miller-Fisher syndrome (a subtype of Guillain-Barré Syndrome), have been described [2, 3]. Although other Campylobacter species have clinical relevance, Campylobacter jejuni and Campylobacter coli have classically been considered the most relevant human pathogens belonging to this genus [2].

Although relatively little is known about the virulence of Campylobacter spp., these microorganisms possess different virulence factors (VFs) related to motility, adhesion, invasion, toxin-activity, immune evasion, and iron-uptake, among others [2]. Thus, while factors, like the cadF gene or the iam locus, are involved in different invasion steps [4, 5] others such as the cytolethal distending toxin, a tripartite toxin encoded in the cdtA, cdtB, and cdtC genes which is also present in other microorganisms [6], block the CDC2 kinase, leading to progressive cellular distension which results in cell death [2].

Diarrhea by Campylobacter spp. is usually a self-limited disease which only requires oral rehydration. However, in some cases (immunocompromised patients, long duration of symptoms, and patients with severe complications) the use of antimicrobial agents may be required [7]. Currently, macrolides are the drugs of choice, with fluoroquinolones as second-line drugs quinolones [7]. However, the presence of quinolone-resistant Campylobacter spp. isolates is not a novel event [8–10]. Moreover, the development of quinolone resistance during antibiotic treatment has also been reported [7, 11]. In general, the amino acid substitutions in the A subunits (GyrA and ParC) of the DNA-Gyrase and Topoisomerase IV are the most relevant mechanisms of quinolone resistance [12]. In addition, alterations in cytoplasmic quinolone
uptake and a series of transferable mechanisms of quinolone resistance (TMQR) also play a role in the increasing levels of quinolone resistance [12, 13]. Interestingly, Campylobacter spp. does not possess a Topoisomerase IV, and thus a single amino acid substitution at GyrA may result in high levels of quinolone resistance [12]. The most frequently described amino acid substitution in Campylobacter spp. affects positions 86 and 90 of GyrA, with the amino acid change Thr86-Ile being the most widely described [8, 14]. In addition, the relevant role of CmeABC, a resistance-nodulation-cell division (RND) efflux pump, has also been described [15]. Finally, to the best of our knowledge, up to now TMQR has not been described in Campylobacter spp.

Regarding macrolides, the isolation of resistant Campylobacter spp. is increasingly reported [16, 17], being especially of note in isolates of an animal origin [10, 18]. In both animal and human isolates, macrolide resistance is more frequent in C. coli [9, 10, 16, 18]. Macrolides interact with the 50S subunit of the ribosome, inhibiting protein elongation and thus protein synthesis [19]. Alterations at the interaction points of the 23S rRNA, L4, or L22 proteins result in the development of macrolide resistance in a wide range of microorganisms [19]. However, the clinical relevance of mutations in the 23S rRNA gene is closely related to the copy number of the gene that each microorganism possesses [19]. Thus, in Campylobacter spp., which has 3 copies of the 23S rRNA gene, mutations in more than one gene copy result in the development of macrolide resistance [20]. Mutations such as A2074G/T, A2075G, and A2076G (equivalent to A2057G/T, A2058G, and A2059G following E. coli numeration) have been described in Campylobacter spp., with those affecting A2075 being the most frequently detected [14, 16, 20]. Although L4 and L22 amino acid substitutions, such as the amino acid changes Gly74-Asp in L4 or Ala86-Glu in L22 or the insertions 86::Ala-Arg-Ala-Arg::87 or 98::Thr-Ser-His::99 in L22, have been related to the acquisition of macrolide resistance in Campylobacter spp. [14, 21], the role of alterations at L4 and L22 seems to be of less relevance in Campylobacter clinical isolates [16, 20]. In fact, it has been described that these alterations may lead to a negative effect on bacterial fitness levels [19]. Additionally, extrusion of macrolides from the bacterial cytoplasm by CmeABC has also been reported [21]. To the best of our knowledge, the erm(B) gene, which may be encoded within a transferable multidrug-resistant genomic island, is currently the only transferable mechanism of macrolide resistance (TMMR) described in Campylobacter spp. [22].

The aim of this study was to determine the presence of several VFs and the levels and molecular mechanisms of resistance to quinolones and macrolides in a series of Campylobacter spp. isolates recovered from children less than 18 months of age, in a periurban area of Lima, Peru.

2. Material and Methods

2.1. Microorganisms. One hundred forty-nine Campylobacter spp. (Supplemental material, available online at https://doi.org/10.1155/2017/7848926) recovered from feces of children less than 18 months old with (63 isolates) and without (86 isolates) diarrhea, during a double-blind controlled trial of bovine lactoferrin for the prevention of diarrhea in children in Lima between January 2008 and May 2011, were included in the study [25]. After initial culture at 42ºC in chocolate agar and microaerophilic conditions, followed by Campylobacter phenotypic identification (evaluation of colony morphology, Gram staining, and oxidase and catalase determinations), DNA was extracted by direct boiling of 1 colony of each isolate and both DNA and microorganisms were frozen until analysis. A C. coli clinical isolate kindly provided by the Instituto Nacional de Salud from Lima (Peru) and C. jejuni ATCC 33560, Escherichia coli ATCC 25922, and Staphylococcus aureus ATCC 25923 were used as control.

2.2. Species Determination. C. coli and C. jejuni were identified by PCR using the primers and conditions previously described (Table 1). The amplified products were analyzed in a 1.5% electrophoresis gel and stained with SYBR Safe (Invitrogen, Eugene, USA). Amplified products were selected at random and sequenced (Macrogen, Seoul, Korea) as quality control.

2.3. Virulence Factors. The presence of the cadF, cdTA, cdTB, and cdTC genes plus that of the full cdT cluster and the iam marker was determined by PCR [23] (Table 1).

2.4. Antimicrobial Susceptibility. The antimicrobial susceptibility to azithromycin (Azm, 15 μg), erythromycin (Ery, 15 μg), nalidixic acid (Nal, 30 μg), ciprofloxacin (Cip, 5 μg), and tetracycline (Tc, 30 μg) was established by disk diffusion following the EUCAST guidelines in the microorganisms recovered from frozen stock. The EUCAST (Ery, Cip, and Tc) (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v5.0_Breakpoint_table.pdf) and BSAC (Nal) (http://bsac.org.uk/wp-content/uploads/2012/02/Table-20.pdf) guidelines were used to interpret the obtained diameter. In the absence of established breakpoints, Azm was interpreted according to the following scheme: susceptible ≥ 18 mm and resistant ≤ 17 mm.

2.5. Analysis of Mutations in the gyrA and 23S rRNA Genes. In strains with susceptibility data, the presence of mutations in the gyrA and 23S rRNA genes was determined by PCR using the primers and conditions previously described (Table 1). In the case of the gyrA gene, the DNAs initially obtained for the nongrowing isolates were also included in the study. The amplified products were recovered and purified (PCR Clean-Up System (Promega, Madison, WI)) following the manufacturer's instructions. Both strands of purified products were sequenced (Macrogen, Seoul, Korea).

2.6. Statistical Analysis. Fisher’s exact test was used to analyze the data.

3. Results

3.1. Identification. Of the total strains analyzed, 96 (64.4%) were C. jejuni and 53 (35.6%) C. coli; of these, 39 C. jejuni and
### Table 1: Primers and PCR conditions used in the present study.

| Target | Description | Primer $(5' \rightarrow 3')$ | Size (bp) | Ann | Cycles | Ref |
|--------|-------------|-------------------------------|-----------|-----|--------|-----|
| **C. coli** | Identification | AGGCAAGGGAGCCCTTTAATC TATCCCTATCTACAAATTGCG | 364 | 61 | 30 | [23] |
| | | CATCTGCCCTAGTCAACCCCTT | 773 | 61 | 30 | [23] |
| **C. jejuni** | | AAG ATATGGGACTAGCAAGAC | | | | |
| **Resistance** | gyrA | DNA-Gyrase subunit A | ATGATGAGGCAAAAAGAGA TAATACCTAAAGTTGGAATGT | 410 | 55 | 30 | [8] |
| | 23SrRNA | GACCGAACTGTCTCACGACG | 699 | 52 | 35 | [24] |
| **Virulence** | cadF | Campylobacter adhesin to fibronectin | TTGAAGTGTAATTTAGATATG CTATTACCTAAGTGTGAAC | 400 | 45 | 30 | [23] |
| | cdtABC | Cytolethal distending Toxin subunits ABC | GGAATTTGTGATGCTGATATCTT TGCACTAACAACGGAGGATG | 1215 | 55 | 30 | [23] |
| | cdtA | Cytolethal distending Toxin subunit A | CCTTTGATGAGGCAACTAC ACATTCTCTTGGTTCCTG | 370 | 42 | 30 | [23] |
| | cdtB | Cytolethal distending Toxin subunit B | GTTAAATCTCCCTGCTATCAAACGA GTTGCGACTTTGGAATTTGCGAAGG | 495 | 42 | 30 | [23] |
| | cdtC | Cytolethal distending Toxin subunit C | CGATGAGTTAAAAACAAAAAGATA TGCCATTATAGAAAAACTAGTTT | 182 | 42 | 30 | [23] |
| | iam1 | Invasión-associated marker 1 | GGCACAAATATTATCCACCC TTCACGACTACATGGGG | 518 | 52 | 30 | [23] |
| | iam2 | Invasión-associated marker 2 | GGCCTTTAGGGAAGCTG CTATTTAATGAAATCACGGG | 1360 | 52 | 30 | [23] |
| | iam3 | Invasión-associated marker 3 | TGAGGAAGCTAAGGGGAATAATGACAACTATTCTTCACCAT | 270 | 52 | 30 | [23] |

bp: base pair; Ann: annealing; Ref: reference. *Primers used in a Multiplex PCR.

### Table 2: Samples type.

|          | Diarrhea $(n = 63)$ | Asymptomatic control $(n = 86)$ | Total $(n = 149)$ |
|----------|---------------------|---------------------------------|------------------|
| C. jejuni | 39 (61.9)           | 57 (66.3)                       | 96 (64.4)        |
| C. coli  | 24 (38.1)           | 29 (33.7)                       | 53 (35.6)        |
| Total    | 63 (100)            | 76 (100)                        | 149 (100)        |

24 C. coli were from diarrheic cases, while 57 C. jejuni and 29 C. coli were from healthy controls (Table 2). No differences were found in relation to sex in the prevalence of C. jejuni and C. coli.

### 3.2. Virulence Factor Analysis.

The cadF gene was present in all the isolates except 2 C. jejuni isolates from the control group. The complete cdtABC operon was amplified in 87 (58.4%) isolates (85 C. jejuni and 2 C. coli) being significantly more frequent among C. jejuni (88.7% versus 3.7%) $(P < 0.001)$. Regarding the cdt genes, cdtB was present in 121 isolates (81.2%), while cdtA and cdtC were present in 102 (67.1%) and 103 (68.7%) isolates, respectively. Independently, all 3 genes were significantly more present in C. jejuni than in C. coli $(P < 0.0001)$ (Table 3). In 1 C. jejuni full cdtABC amplification was achieved; however cdtA, cdtB, or cdtC genes could not be amplified. Similarly 11 C. jejuni and 4 C. coli amplify all genes in independent manner, but no PCR product was obtained when the primers for cdtABC were used. Regarding the iam marker the 3 sequences sought were more frequently detected in C. coli than in C. jejuni (93.1%, 89.7%, and 96.6% versus 4.0%, 4.0%, and 5.1% for iam1, iam2, and iam3, resp.) $(P < 0.0001)$. All 3 sequences were detected concomitantly in the 89.7% of C. coli and 4.0% of C. jejuni $(P = 0.0001)$ (Table 3). No differences in the prevalence of sought VFs were found among isolates from cases and control or sex groups.

### 3.3. Antimicrobial Resistance Levels.

The resistance levels to quinolones and macrolides were determined in 115 isolates (69 C. jejuni, 46 C. coli) able to grow from frozen stock, while the resistance levels to Tc were also established in 100 out of these isolates.
Table 3: *Campylobacter* virulence factors.

| VF         | C. jejuni | C. coli | P | All *Campylobacter* |
|------------|-----------|---------|---|---------------------|
|            | D (39)    | C (57)  | T (97) | D (24) | C (29) | T (53) | D (63) | C (86) | T (149) |
| cadF       | 39%       | 55%     | 96.4% | 95%     | 97.9% | 24%    | 100%   | 29%    | 100%    | 53%     | 100%   | 63%    | 100%   | 84%    | 97.7% | 147%   | 98.7% |
| cdtABC†    | 35%       | 50%     | 87.7% | 86%     | 88.7%* | 2%     | 8.3%   | 0%     | 0.0%    | 2%      | 3.8%*   | <0.0001 | 37%    | 58.7% | 50%    | 59.5% | 87%    | 58.4% |
| cdtA       | 39%       | 56%     | 98.2% | 96%     | 99.0%* | 3%     | 12.5%  | 4%     | 13.8%   | 7%      | 13.2%*  | <0.0001 | 42%    | 66.7% | 60%    | 71.4% | 102%   | 68.5% |
| cdtB       | 39%       | 56%     | 98.2% | 96%     | 99.0%* | 11%    | 45.8%  | 15%    | 51.7%   | 26%     | 49.1%*  | <0.0001 | 50%    | 79.4% | 71%    | 84.5% | 121%   | 81.2% |
| cdtC       | 39%       | 56%     | 98.2% | 96%     | 99.0%* | 3%     | 12.5%  | 5%     | 17.2%   | 8%      | 15.1%*  | <0.0001 | 42%    | 66.7% | 61%    | 72.6% | 103%   | 69.1% |
| iam        | 3%        | 7.7%    | 1%    | 1.7%    | 4%     | 4.0%*  | 21%    | 87.5%  | 26%     | 89.7%   | 47%     | 88.7%*  | <0.0001 | 24%    | 38.1% | 27%    | 32.1% | 51%    | 34.2% |
| iam1       | 3%        | 7.7%    | 1%    | 1.7%    | 4%     | 4.0%*  | 23%    | 95.8%  | 27%     | 93.1%   | 50%     | 94.3%*  | <0.0001 | 26%    | 41.3% | 28%    | 33.3% | 54%    | 36.2% |
| iam2       | 3%        | 7.7%    | 1%    | 1.7%    | 4%     | 4.0%*  | 21%    | 87.5%  | 26%     | 89.7%   | 47%     | 88.7%*  | <0.0001 | 24%    | 38.1% | 27%    | 32.1% | 51%    | 34.2% |
| iam3       | 4%        | 10.3%   | 1%    | 1.7%    | 5%     | 5.1%   | 23%    | 95.8%  | 28%     | 96.6%   | 51%     | 96.2%*  | <0.0001 | 27%    | 27.0% | 29%    | 34.5% | 56%    | 37.6% |

D: diarrhea; C: control; T: total; VF: virulence factor; N: number; %: percentage. *The presence of significant differences between specific groups. †In 1 C. jejuni the *cdtABC* operon was amplified but no individual gene amplification was obtained; similarly in 11 C. jejuni and 4 C. coli cases the 3 individual genes were amplified, but no amplification for the full *cdtABC* operon was obtained.
Regarding quinolones, the results showed almost full concordance (only 2 NalR C. jejuni isolates from the diarrhea group were not resistant to Cip) and also extremely high levels of resistance (104 isolates, 90.4% to Nal; 102 isolates, 88.7% to Cip). Likewise, extremely high levels of resistance to Tc were observed (96 isolates, 96.0%). Meanwhile, only 15 (13.0%) isolates showed resistance to both Ery and Azm. All macrolide-resistant microorganisms also showed resistance to the quinolones tested (Table 4).

Analysis by species only showed statistically significant differences in those regarding macrolide resistance. Thus C. coli showed higher levels of resistance than C. jejuni (12 isolates, 26.1% versus 3 isolates, 4.3%; P: 0.0012). The significance was also maintained between C. coli and C. jejuni from the control group (6 isolates, 24% versus 1 isolate, 2.6%; P = 0.0119), with borderline significance between C. coli and C. jejuni from the diarrhea group (P = 0.0521) (Table 4).

No association was observed between sex and macrolide or quinolone resistance. No association was found between susceptibility/resistance and a higher or lower presence of the VFs sought.

3.4. Analysis of the Mechanisms of Resistance. The analysis of the gyrA gene showed the presence of Thr86-Ile amino acid substitutions in the 102 NalR CipR and in 1 NalR CipS isolates, while in another C. jejuni, NalR CipS, the Thr86-Ala substitution was observed. Additionally, 3 C. jejuni isolates exhibiting susceptibility to both quinolones also possessed the Thr86-Ile substitution. Meanwhile, for the 34 nongrowing isolates the presence of Thr86-Ile was observed in 28 cases.

Resistance to macrolides was related to the presence of the base change A2075G in 13 out of 15 (86.7%) macrolide-resistant isolates. Interestingly in 2 out of these 13 isolates (both C. coli) double peaks were observed, highlighting the presence of mutations in only 1 or 2 of the 3 Campylobacter spp. 23S rRNA gene copies. Finally, 1 of the 2 macrolide-resistant isolates without a mutation in the 23S rRNA gene had an Ery halo of 19 mm and an azithromycin halo of 16 mm, while the remaining isolate had no halo to both of the macrolides tested.

4. Discussion

4.1. Microorganisms. Although a reduction in the burden of diarrhea has been observed in Peru, it has been estimated that in 2015 diarrhea led to 514 deaths in children less than 5 years of age (0.8 deaths/1,000 live births), accounting for 4.9% of deaths in this population (http://apps.who.int/gho/data/node.main.COCD?lang=en). In Peruvian rural zones and in periurban areas of Lima and other cities the lack of adequate sanitation conditions supports the high prevalence of diarrheic diseases. In these areas, Campylobacter spp. ranks after enteric viruses and enteropathogenic E. coli as etiologic cause of diarrhea [25].

The proportions of C. jejuni and C. coli in our study are quite different from previous studies performed in this area. Thus, analyzing 4652 Campylobacter spp. collected between January 2001 and December 2010 the presence of 3856 C. jejuni (82.9%) and 554 C. coli (11.9%) was detected together with other Campylobacter spp. [17]. Although the spread of a C. coli clone in the area may be suggested, there is no clear reason for these differences.

4.2. Virulence Factors. Previous studies have shown that almost all C. jejuni and C. coli possess the cadF gene [26, 27]. In this line, our results are as expected. Regarding the presence of 2 cadF negative C. jejuni isolates, although possible insertion inactivation or deletion can not be ruled out, the presence of a polymorphism which might affect PCR-positivity has been previously described [27]. Meanwhile, both in the case of cdt and iam, the use of different primer sets increased the reliability of PCR results, confirming the presence of significant differences in the carriage of these VFs among C. coli and C. jejuni.

Although presence of polymorphisms in the primers annealing regions may not be ruled out, while all C. jejuni presenting the cdt operon possessed the 3 components, a series of C. coli were positives for cdtB but not for cdtA and/or cdtC. This is a relevant finding because the lack of either cdtA or cdtC leads to an impaired production of CDT [28].

Some studies have shown that the IAM region was more frequent in C. coli independently of whether it was from children (83.3%) or chicken (100%), being also frequent (54.7%) in C. jejuni from chicken but almost absent (1.3%) in those isolated from children [23]. In accordance with this, our results showed that C. coli carried the IAM region significantly more frequently than C. jejuni.

4.3. Antimicrobial Resistance. Symptomatic and asymptomatic Campylobacter spp. infections have been involved in reduced weight gain over three-month periods in children [29]. Although symptomatic infections were marginally associated with reduced linear growth over nine-month periods, the severity of the episodes was correlated with greater deficits in both weight gain and linear growth, demonstrating the need for early control of Campylobacter infections [29].

A survey performed in Peru between 2001 and 2010 showed an increase in Cip resistance levels of both C. jejuni and C. coli. In Lima, the levels of Cip resistance were 73.1% and 48.1% for C. jejuni and C. coli, respectively, in the period 2001–2005, with those values rising to 91.1% and 87.4% in the period 2006–2010, respectively [17]. The most recent values are in accordance with the levels of Cip resistance detected in our isolates.

Similar to that described in other geographical areas [30], our results showed extremely high resistance levels to Tc of 100% among C. coli and 90% among C. jejuni. Though not used in the treatment of Campylobacter infections in young children, this scenario shows that Tc has lost all its utility in the treatment of Campylobacter spp. in Peru.

The macrolide resistance was higher in C. coli than in C. jejuni, similar to what has been observed in other studies [16, 17]. Overall, our macrolide resistance levels were higher than those previously reported in the area of Lima (C. jejuni 4.3% versus 1.9%; C. coli 26.1% versus 5.3% and 5.8%, Ery and Azm, resp.) [17]. In a previous study a significant increase in the C. coli Azm resistance over time in Lima was of
Table 4: *Campylobacter* antimicrobial resistance levels.

| Ab   | C. jejuni   | C. coli     | All *Campylobacter* |
|------|-------------|-------------|---------------------|
|      | Diarrhea    | Control     | Total               |
|      | n/N  | %  | n/N  | %  | n/N  | %  | n/N  | %  | n/N  | %  | n/N  | %  | n/N  | %  |
| Nal  | 28/30 | 93.3 | 34/39 | 87.2 | 62/69 | 89.8 | 20/21 | 95.2 | 22/25 | 88.0 | 42/46 | 91.3 | 48/51 | 94.1 | 56/64 | 87.5 | 104/115 | 90.4 |
| Cip  | 26/30 | 86.7 | 34/39 | 87.2 | 60/69 | 87.0 | 20/21 | 95.2 | 22/25 | 88.0 | 42/46 | 91.3 | 46/51 | 90.2 | 56/64 | 87.5 | 102/115 | 88.7 |
| Ery  | 2/30  | 6.7  | 1/39  | 2.6* | 3/69  | 4.3‡ | 6/21  | 28.6 | 6/25  | 24.0‡ | 12/46  | 26.1‡ | 8/51  | 15.7 | 7/64  | 10.9 | 15/115 | 13.0 |
| Azm  | 2/30  | 6.7  | 1/39  | 2.6* | 3/69  | 4.3‡ | 6/21  | 28.6 | 6/25  | 24.0‡ | 12/46  | 26.1‡ | 8/51  | 15.7 | 7/64  | 10.9 | 15/115 | 13.0 |
| Tc   | 24/27 | 88.9 | 32/33 | 97.0 | 56/60 | 93.3 | 19/19 | 100.0 | 21/21 | 100.0 | 40/40  | 100.0 | 43/46 | 93.5 | 53/54 | 98.1 | 96/100 | 96.0 |

Ab: antibiotic, Nal: nalidixic acid, Cip: ciprofloxacin; Ery: erythromycin; Azm: azithromycin; Tc: tetracycline; \( P < 0.05 \). Comparison between erythromycin resistance * and azithromycin resistance † of C. jejuni and C. coli from control groups; \( P < 0.005 \). Comparison between erythromycin resistance ‡ and azithromycin resistance # of total recovered C. jejuni and C. coli.
4.4. Mechanisms of Quinolone and Macrolide Resistance.

While most microorganisms possess 2 quinolone-targets (DNA-Gyrase and Topoisomerase IV), Campylobacter spp. only possess one of the DNA-Gyrase; thus a single target-mutation may lead to both high Nal and Cip resistance levels [8, 12, 31]. The GyrA amino acid change Thr86-Ile has been extensively described in Campylobacter spp. [8, 31]. The phenotype Nal^{R}Cip^{R} was observed in two C. jejuni, in one case related to the Thr86-Ala substitution. It has been observed that the Thr86-Ala substitution leads to increases in the Nal MIC, in some cases just low-bordering the resistance breakpoint, with a lesser effect on the Cip resistance levels [31]. In addition, microorganisms either having the wild type presence of Ala [32] or presenting a mutation leading to the presence of Ala in the equivalent position of GyrA [33] present Nal resistance patterns, albeit usually lower than those produced by other amino acid substitutions, and decreased susceptibility to fluoroquinolones. This may be related to lower alterations in the hydrophobic patterns of the DNA-Gyrase interaction point [12, 32]. The remaining Nal^{R}Cip^{S} as well as the 3 Nal^{R}Cip^{S} isolates carrying the Thr86-Ile substitution might be explained by an enhanced quinolone uptake that may be due to a malfunction of efflux pumps or to enhanced outer membrane permeability.

The presence of mutations at position A2075 was found in all but 2 macrolide-resistant isolates. In two cases the data suggested the presence of heterozygote isolates, with only one or two mutated 23S rRNA. In these cases, as 33–66% of the ribosomes were resistant to the action of the macrolides, the isolates remained resistant to both Azm and Ery. The presence of 2 macrolide-resistant isolates without alterations in the 23S rRNA gene may be due to an overexpression of the CmeABC [21, 34]. This option is highly probable in the isolate having a borderline macrolide halo [34], while another explanation, such as the presence of amino acid substitutions in L4 or L22, might be considered in the other case [19]. In addition, the presence of TMRR, such as Erm(B) recently described in Campylobacter genus [22] cannot be ruled out.

In summary, the present data demonstrates high levels of Tc and quinolone resistance in both C. jejuni and C. coli and increasing macrolide resistance among C. coli. Moreover, the concomitant resistance to quinolones and macrolides is serious and may lead to the expansion of difficult-to-treat Campylobacter spp. isolates. The implementation of control measures which result in a more rational antimicrobial use in human infections, but especially in veterinary settings, is a priority.

Conflicts of Interest

The authors declare that they have no conflicts of interest.
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