Hindawi Scientifica Volume 2017, Article ID 7848926, 8 pages https://doi.org/10.1155/2017/7848926



Research Article

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

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Received 14 June 2017; Revised 10 August 2017; Accepted 29 August 2017; Published 9 October 2017

Academic Editor: Giuseppe Comi

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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

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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 results 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 42°C 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 μ g), erythromycin (Ery, 15 μ g), nalidixic acid (Nal, 30 μ g), ciprofloxacin (Cip, 5 μ g), and tetracycline (Tc, 30 μ g) 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/v_6.0_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'-3')	Size (bp)	Ann	Cycles	Ref
		Identification				
C. coli*		AGGCAAGGGAGCCTTTAATC	364	61	30	[22]
0. 0011		TATCCCTAT CTACAAATTCGC	301	01	30	[23]
C. jejuni*		CATCTTCCCTAGTCAAGCCT	773	61	30	[22]
. jejum		AAG ATATGGCACTAGCAAGAC		01		[23]
		Resistance				
gyrA	DNA-Gyrase subunit A	ATGATGAGGCAAAAAGAGA	410	55	30	[0]
8) 111	Divir Gyruse subunit 11	TAAACTATGAGGTGGGATGT	110	55	30	[8]
23S rRNA		GTAAACGGCGGCCGTAACTA	699	52	35	[24]
250 /10 /11		GACCGAACTGTCTCACGACG				[24]
		Virulence				
cadF	Campylobacter adhesin to fibronectin	TTGAAGGTAATTTAGATATG	400	45	30	[22]
caui	cumpytobacter addressin to horoneetin	CTAATACCTAAAGTTGAAAC	100	13	30	[23]
cdtABC	Cytolethal distending Toxin subunits ABC	GGAAATTGGATTTGGGGCTATACT	1215	55	30	[22]
Cullibe	Cytolethal distellating Toxin subulitis ADC	TTGCACATAACCAAAAGGAAG	1213	33	30	[23]
cdtA	Cytolethal distending Toxin subunit A	CCTTGTGATGCAAGCAATC	370	42	30	[22]
cutri	Cytolethal distellating Toxin subulife 11	ACACTCCATTTGCTTTCTG	370	72	30	[23]
cdtB	Cytolethal distending Toxin subunit B	GTTAAAATCCCCTGCTATCAACCA	495	42	30	[22]
cutb	Cytolethal distellang Tokin subulifit B	GTTGGCACTTGGAATTTGCAAGGC	173	12	30	[23]
cdtC	Cytolethal distending Toxin subunit C	CGATGAGTTAAAACAAAAAGATA	182	42	30	[22]
curo	Cytolethal distellating Tokin subulific C	TTGGCATTATAGAAAATACAGTT	102	12	30	[23]
iam1	Invasión-associated marker 1	GCGCAAAATATTATCACCC	518	52	30	[22]
IGIIII	invasion associated marker i	TTCACGACTACTATGCGG	310	32	30	[23]
iam2	Invasion-associated marker 2	GGCGCTTTAGGGAAGCTG	1360	52	30	[22]
Iuiii2	invasion associated marker 2	CTTTAAATTGAATCACGGG	1300	32	30	[23]
iam3	Invasion-associated marker 3	TGAGGAGCTAAGGGTGCAAA	270	52	30	[22]
101113	m, asion associated market 3	AATACTGATATTTTCCACAT	270	52	50	[23]

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

Table 2: Samples type.

		n (%)	
	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 cdtABCamplification 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.

	(46)	%	98.7	58.4	68.5	81.2	69.1	34.2	36.2	34.2	37.6
	T (1	Z	147	87	102	121	103	51	54	51	99
ylobacter	(98	%	2.76	59.5	71.4	84.5	72.6	32.1	33.3	32.1	34.5
All Camp	C	Z	84	50	09	71	61	27	28	27	53
	(63)	%	100	58.7	2.99	79.4	2.99	38.1	41.3	38.1	27.0
	D D	N	63	37	42	20	42	24	26	24	27 27.0
	Ь			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	(53)	%	53 100.0	3.8*	13.2^{*}	49.1^{*}	15.1^{*}	*88.7	94.3*	*88.7	96.2*
	Τ										
coli	(53)	%	29 100.0	0.0	13.8	51.7	17.2	89.7	93.1	89.7	9.96
C.	С	Z	56	0	4	15	5	76	27	56	28
	(24)	%	100.0	8.3	12.5	45.8	12.5	87.5	95.8	87.5	92.8
	Ω	N	24	7	3	11					23
	(26)	%	626	*288	99.0^{*}	99.0^{*}	99.0^{*}	4.0^*	4.0^*	4.0^{*}	5.1^*
	Τ	Z	95	98	96	96	96	4	4	4	5
iejuni	(57)	%	96.4	87.7	98.2	98.2	98.2	1.7	1.7	1.7	1.7
Ü	O	Z	55	20	99	99	99	1	1	1	1
	(38)	%	100.0	89.7	100.0	100.0	100.0	7.7	7.7	7.7	10.3
	Ω	Z	39	35	39	39		3		3	4
	VF		cadF	$cdtABC^{\dagger}$	cdtA	cdtB	cdtC	iam	iaml	iam2	iam3

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 genes were amplified, but no 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 Nal^R *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 $C.\ jejuni$ and $C.\ coli$ 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 Nal^RCip^R and in 1 Nal^RCip^S isolates, while in another *C. jejuni*, Nal^RCip^S, 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%) macrolideresistant 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 macrolideresistant 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.

									Antibioti	Antibiotic resistance	je,							
44			C. je	C. jejuni					C.	C. coli					All Camp	\text{\tint{\text{\tin}\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\tint{\text{\tint{\text{\tinit}\text{\text{\text{\text{\text{\text{\text{\text{\tex{\tex		
AD	Diar	Diarrhea	Control	trol	Total	al	Diar	Diarrhea	Cor	Control	Total	tal	Diarrhea	rhea	Control	trol	Total	al al
	N/u	%	N/u	%	N/u	%	N/u	%	N/u	%	N/u	%	N/u	%	N/n	%	N/u	%
Nal	28/30	93.3	34/39	87.2	65/69	8.68	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	69/09	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^{\ddagger}	6/21	28.6	6/25	24.0^{*}	12/46	26.1^{\ddagger}	8/51	15.7	7/64	10.9	15/115	13.0
Azm	2/30	6.7	1/39	2.6^{\dagger}	3/69	4.3#	6/21	28.6	6/25	24.0^{\dagger}	12/46	$26.1^{\#}$	8/51	15.7	7/64	10.9	15/115	13.0
Тc	24/27	88.9	32/33	97.0	26/60	93.3	19/19	100.0	21/21	100.0	40/40	100.0	43/46	93.5	53/54	98.1	96/100	0.96
Ab: anti	biotic, Nal:	nalidixic a	cid, Cip: cif	orofloxacir	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. oil; from control grouns: $D < 0.005$. Comparison between erythromycin resistance † and azithromycin resistance † and azithromycin resistance † and azithromycin resistance and a	omycin; A	zm: azithro	mycin; Tc:	tetracycline;	Azm: azithromycin; Tc: tetracycline; $P < 0.05$. Comparison between erythromycin resistance and existence and existence and existence and existence are selected processed C initial and C of C	Comparison	between er	ythromycin	resistance	* and azith	romycin re	sistance † of	C. jejuni
dia		incres Broad	7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7	o. compa	ison octuer	1 21) 4111 011	1) ciii i colota	mice + anice	teatin out year	ii i calarance	10 to tall 100 to	oreica c. J	yanı ana O.					

note [17]. Our data confirm this trend and also show an increase in macrolide resistance among *C. jejuni*. All the macrolide-resistant isolates detected also showed resistance to quinolones, highlighting the need of new antimicrobial agents to treat *Campylobacter* infections.

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-Gyrases; thus a single targetmutation 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^RCip^S 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^RCip^S as well as the 3 Nal^SCip^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 permeabil-

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 TMMR, 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.

Acknowledgments

This study was partially supported by the Agencia Española de Cooperación Internacional (AECID), Spain, and Programa de Cooperación Interuniversitaria e Investigación Científica con Iberoamérica (D/019499/08, D/024648/09). Joaquim Ruiz was supported by the I3 Program of the Ministerio de Economia y Competitividad, Spain (Grant no. CES11/012). "ISGlobal is a member of the CERCA Programme, Generalitat de Catalunya." The authors thank Donna Pringle for editorial assistance.

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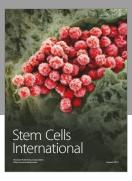
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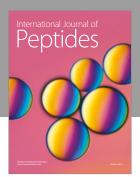
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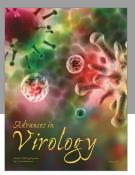
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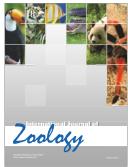


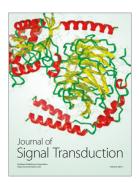






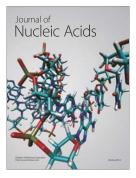






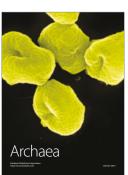


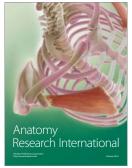
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