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Research paper

Genetic diversity and clonal characteristics of ciprofloxacin-resistant *Campylobacter jejuni* isolated from Chilean patients with gastroenteritis

Luis Collado[a,*], Nataly Muñoz[a], Lorena Porte[b], Sofía Ochoa[a], Carmen Varela[b], Ivo Muñoz[a]

[a] Instituto de Bioquímica y Microbiología, Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile
[b] Laboratorio Clínico, Clínica Alemana de Santiago, Facultad de Medicina Clínica Alemana, Universidad del Desarrollo, Santiago, Chile

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ABSTRACT

Campylobacter jejuni is a major cause of acute gastroenteritis worldwide. However, it has also been associated with other diseases such as bacteremia and with several post-infection sequelae. Although campylobacteriosis is usually a self-limited infection, antibiotics are indicated for severe and chronic conditions. Unfortunately, several industrialised nations have reported a substantial increase in antibiotic resistance of *C. jejuni*. However, there is still a lack of knowledge about the epidemiology of resistance developed by this pathogen in the developing world. For this reason, our objective was to determine the resistance of clinical *C. jejuni* strains to ciprofloxacin and erythromycin in Chile and their associated genotypes.

Fifty *C. jejuni* isolates recovered from fecal samples of people with acute gastroenteritis, in central and southern Chile between 2006 and 2015, were analysed. Resistance to erythromycin and ciprofloxacin was assessed by disk diffusion and agar dilution methods. Furthermore, these strains were genotyped by Multilocus Sequence Typing (MLST).

Only one of the isolates was resistant to erythromycin. However, 48% of them were resistant to ciprofloxacin. The minimal inhibitory concentration of these ciprofloxacin-resistant isolates was in the range between 4 and 32 μg/ml. Moreover, MLST analyses showed that most ciprofloxacin-resistant strains were grouped into three dominant clonal complexes (ST-21, ST-48 and ST-353), while the unique strain resistant to both antibiotics belonged to the ST-45 complex. Our results evidence a high ciprofloxacin resistance and suggest that there is a dissemination of resistant clonal lineages responsible for cases of campylobacteriosis in Chile. Further studies should elucidate the origin of these resistant genotypes.

1. Introduction

Campylobacter jejuni is the predominant etiologic agent of campylobacteriosis, one of the most frequent causes of acute bacterial gastroenteritis worldwide (Kaakoush et al., 2015; Tang et al., 2017). Besides the production of diarrhea and in a lesser extent bacteremia, this species is associated with a series of post-infection sequelae such as the Guillain Barre Syndrome (GBS), reactive arthritis (ReA) and irritable bowel syndrome (IBS) (Keithlin et al., 2014).

Although most cases of *Campylobacter* infection are acute and self-limited and do not require antibiotic therapy, this may be necessary in some patients with severe, prolonged and relapsing illness (Aaerestrup et al., 2008). Very young and elderly persons or patients with other conditions (immunocompromised patients, pregnant women, patients with extra intestinal infection, among others) also require antimicrobial treatment (Lapierre et al., 2016).

The most commonly used antibiotics for the treatment of campylobacteriosis are erythromycin (treatment of choice) and ciprofloxacin (second-line treatment) (Humphries and Schuetz, 2015; Lapierre et al., 2016). However, over the past two decades *Campylobacter* resistance to these and other types of antibiotics has been reported in several countries (Aaerestrup et al., 2008). This is why drug-resistant *Campylobacter* has been recently classified as a serious antimicrobial threat by the Centers for Disease Control and Prevention (CDC, 2013) and the fluoroquinolone-resistant *Campylobacter* spp. are recognised as a World Health Organization-high priority pathogen (WHO, 2017).

It has been observed that the increased antibiotic resistance (mainly for ciprofloxacin) is due to the use of fluoroquinolones (FQN) in poultry production (Skarp et al., 2016). However, ruminants also play a significant role in campylobacteriosis and a rising trend in FQN resistance in cattle has been reported (Cha et al., 2017, Tang et al., 2017).

The epidemiology of antibiotic resistance in *Campylobacter* has been widely studied in industrialised countries. However, there is a lack of

* Corresponding author at: Instituto de Bioquímica y Microbiología, Facultad de Ciencias, Universidad Austral de Chile, Campus Isla Teja, Valdivia, Chile.
E-mail address: luiscollado@uach.cl (L. Collado).

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Table 1
Distribution of antibiotic resistance of 50 *Campylobacter jejuni* strains isolated from patients in two Chilean cities.

City (N)/antibiotics	Disk diffusion method				Agar dilution method[a]										
	Number of strains				Percentage of strains susceptible at following MIC (μg/ml)										
	S	I	R	%R	0.25	0.5	1	2	4	8	16	32	64	128	256
Santiago (17 strains)															
Erythromycin	16	0	1	5.9										100	
Ciprofloxacin	8	0	9	52.9					22.2	11.1	44.4	22.2			
Valdivia (33strains)															
Erythromycin	32	1[b]	0	0	100										
Ciprofloxacin	18	0	15	45.5						40	33.3	26.7			
Total (50 strains)															
Erythromycin	48	1[b]	1	2.0[c]	50									50	
Ciprofloxacin	26	0	24	48.0[c]					8.3	29.2	37.5	25			

N, number of strains; S, susceptible; I, intermediate; R, resistant; %R, percentage of resistance.
[a] Only the strains that were intermediate or resistant by disk diffusion method were analysed by agar dilution method. The breakpoints for erythromycin and ciprofloxacin were ≥32 μg/ml and ≥4 μg/ml, respectively.
[b] This strain showed a MIC of 0.25 μg/ml therefore was considered susceptible for data analysis.
[c] No significant difference was found between geographic areas and resistance to erythromycin (Fisher's exact $P = 0.34$) and ciprofloxacin (X^2 $P = 0.61$).

knowledge of the situation in the developing world (Fernández and Pérez-Pérez, 2016). Therefore, our main interest was to assess the antibiotic resistance of *C. jejuni* strains isolated from patients with gastroenteritis in Chile and to determine the resistant genetic lineages circulating in the country.

2. Materials and methods

2.1. Bacterial isolates and culture condition

We examined 50 isolates of *Campylobacter jejuni* recovered from stool samples of patients with gastrointestinal illness in central (city of Santiago) and south (city of Valdivia) Chile between 2006 and 2015 as part of independent studies published elsewhere (Collado et al., 2013; Porte et al., 2016). The isolates were cultured on Columbia blood agar in a microaerophilic atmosphere (CampyGen; Thermo Scientific) at 37 °C for 48 to 72 h.

2.2. Antimicrobial susceptibility testing

The screening of antibiotic resistance in *Campylobacter* isolates was performed using the disk diffusion (DD) method as this technique is widely accessible to most laboratories that perform *Campylobacter* cultures. Briefly, bacterial culture of 0.5 McFarland OD was spread onto Muller-Hinton Agar plate containing 5% defibrinated sheep blood (BMHA) and erythromycin (15 μg) and ciprofloxacin (5 μg) disks (Oxoid) were placed onto the plates, which were incubated at 37 °C in microaerobic atmosphere for 48 h and reading was taken by measuring zone diameter. The Laboratory Standards Institute (CLSI) guidelines, were used as the interpretation criteria (CLSI, 2008, 2010). Subsequently, the minimal inhibitory concentration (MIC) was determined by agar dilution method in the resistant isolates or in those with intermediate resistance. The CLSI breakpoints for erythromycin and ciprofloxacin were ≥32 μg/ml and ≥4 μg/ml, respectively. The reagent powder of each drug (Sigma Aldrich, St. Louis, USA) was dissolved in appropriate solvents (ethanol and NaOH, respectively, and further diluted in water) and tested at the following dilutions 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128 and 256 μg/ml in BMHA. A plate without antibiotics (solvent-only) was used as a control. A suspension of each isolate in saline solution was adjusted to match the 0.5 McFarland standard. Plates were seeded with a multipoint inoculum replicator and incubated at 37 °C for 48 h. The MIC determinations were done twice for each strain. *Campylobacter jejuni* DSM 4688^T (= ATCC 33560^T) was used as a control in each run during the test.

2.3. Multilocus sequence typing

Campylobacter jejuni genotypes were characterised by Multilocus sequence typing (MLST) according to protocols described by Dingle et al. (2001). Bacterial DNA was purified from overnight cultures grown on blood agar using Wizard® Genomic DNA Purification Kit (Promega). Seven housekeeping (*aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt* and *uncA*) gene PCR's were carried out and then purified using the Wizard® SV Gel kit and PCR Clean-Up System (Promega) and quantified with the Nanoquant (TECAN). Both strands of the purified PCR products were sequenced by Macrogen (Seoul, Korea). Amplification and sequencing primers were obtained from PubMLST database http://pubmlst.org/campylobacter. Consensus sequences for each allele were assigned to an allele number, a 7-locus sequence type (STs), and a clonal complex (CCs) through interrogation of the database. Unassigned sequences were deposited to the database to obtain new allele or ST numbers according to the guidelines (Jolley and Maiden, 2010).

2.4. Statistical analysis

Statistical analyses were performed using Epi Info™ version 7.2 (CDC, Atlanta, GA, USA). The chi-square test was used to examine differences in the prevalence of antimicrobial resistance between cities and identity association between ciprofloxacin resistance profiles and genotypes. The Fisher's exact test was used for variables with ≤5 in at least one cell. A P value of < 0.05 was considered as statistically significant.

3. Results

Strains from two Chilean geographic areas showed similar antibiotic resistance patterns with no significant differences ($P > 0.05$) (Table 1). Only one *C. jejuni* strain from Santiago was resistant to erythromycin, while another strain from Valdivia showed intermediate susceptibility by DD method. However, this latter strain showed a MIC of 0.25 μg/ml therefore was considered susceptible for data analysis. The 48% of the strains were resistant to ciprofloxacin by both agar disk diffusion and agar dilution methods (52.9% resistance in Santiago and 45.5% in Valdivia). Ciprofloxacin-resistant isolates had a MIC range between 4 and 32 μg/ml (Table 1).

Forty-seven out of the 50 strains were successfully genotyped by MLST. As shown in Table 2, the 47 genotyped strains yielded 26 different STs, seven of which had not been previously reported (4 by novel allele sequences and 3 by novel combinations of preexisting alleles). The isolates were assigned to 12 clonal complexes (CCs), but 55.3% of

Table 2
Distribution of clonal complexes (CCs), sequence types (STs) and antibiotic resistance of 47 Chilean *Campylobacter jejuni* strains.

CC	ST	N° of strains (n° strains from Santiago/Valdivia)	N° of resistant strains (n° of resistant strains from Santiago/Valdivia)	
			Erythromycin	Ciprofloxacin[a]
21	21	1 (0/1)		1 (0/1)
21	50	6 (3/3)		4 (1/3)
21	**8938**[b]	3 (1/2)		3 (1/2)
22	22	1 (0/1)		1 (0/1)
41	41	1 (0/1)		
41	**8937**	1 (0/1)		
42	469	3 (2/1)		
45	2109	1 (1/0)	1 (1/0)	1 (1/0)
48	38	1 (0/1)		
48	429	1 (0/1)		1 (0/1)
48	475	5 (1/4)	1[c] (0/1)	4 (1/3)
49	3720	1 (1/0)		1 (1/0)
257	257	5 (2/3)		1 (1/0)
353	353	2 (0/2)		1 (0/1)
353	1953	2 (2/0)		2 (2/0)
353	2517	1 (0/1)		1 (0/1)
353	4053	2 (0/2)		
353	**8944**	1 (1/0)		1 (1/0)
362	587	2 (0/2)		
446	446	1 (1/0)		
1332	696	1 (0/1)		
	585	1 (0/1)		
	8940	1 (0/1)		
	8941	1 (0/1)		
	8942	1 (1/0)		
	8943	1 (1/0)		
Total		47 (17/30)	1 (1/0)	22 (9/13)

[a] Most ciprofloxacin-resistant strains were significantly associated with CCs ST-21, ST-48 and ST-353 (X^2 $P = 0.0002$).
[b] Novel STs found in the present study are in bold.
[c] One strain from Valdivia showed intermediate resistance to erythromycin by DD method. However, its MIC was 0.25 μg/ml therefore was considered susceptible for data analysis.

strains belonged to only four (ST-21, ST-48, ST-257 and ST-353) of them. The most prevalent STs were ST-50 (12.8%), followed by ST-475 (10.6%), and ST-257 (10.6%). Most ciprofloxacin-resistant strains were significantly associated with three dominant CCs (ST-21, ST-48 and ST-353) ($P < 0.05$), while the unique strain resistant to both antibiotics belonged to ST-2109 and ST-45 complex. Interestingly, distribution of the genotypes varied between some strains of both geographic regions. In fact, just five STs were common to strains from both cities while seven STs were detected exclusively in strains of Santiago and 14 STs were exclusive of Valdivia (Table 2).

4. Discussion

This study demonstrates a high prevalence of ciprofloxacin resistance as well as an incipient resistance to erythromycin in clinical *C. jejuni* strains in Chile. Indeed, an increase in resistance for both antibiotics is observed when comparing our results with previous nationwide studies. In fact, in a former study carried out in the city of Valdivia, no resistance to ciprofloxacin and erythromycin was found in 108*C. jejuni* clinical strains isolated between 1996 and 1997 (Fernández et al., 2000). However, more recent studies conducted in Santiago, Metropolitan Region of Chile, have demonstrated 30.3 to 60% resistance for ciprofloxacin and 0 to 1.5% for erythromycin (García et al., 2009; González-Hein et al., 2013; Lapierre et al., 2016). The reason for the high prevalence of ciprofloxacin resistance in Chile is unknown, but it might be driven by the use of FQN in animal production. In fact, Fernández and Pérez-Pérez (2016) claimed food-producing animals and the food chain to be the main source of the transmission of FQN resistant strains in most Latin-American countries.

In Chile, slaughterhouse chickens have elevated contamination rates with thermotolerant *Campylobacter* (on average 54%) (Figueroa et al., 2009) and have been reported resistance rates of 58.2% to ciprofloxacin 1.8% to erythromycin in *C. jejuni* isolates from chicken meat (González-Hein et al., 2013). However, in a recent study, carried out in Santiago, *C. jejuni* strains recovered from chicken (n strains = 25), turkey ($n = 29$) and bovine ($n = 17$) retail meat showed intermediate to low resistance to ciprofloxacin (12, 24 and 0% respectively) and complete susceptibility to erythromycin (Lapierre et al., 2016). These data suggest that, in addition to the acquired resistance of *C. jejuni* isolated from food, there must be other sources of FQN and macrolides resistance not yet identified in Chile.

Genotype distribution of *C. jejuni* strains from central and southern Chile, suggest that although there are common sources of campylobacteriosis along the country, each of the studied geographic areas might have additional environmental sources for this pathogen. It has already been proven that although the majority of human *Campylobacter* cases can be linked to food consumption, between 30% and 50% of cases may be a result of infection from the wider environment (Sanderson et al., 2017). Therefore, considering that both cities (Santiago and Valdivia) are approximately 840 Km away from each other, so differences could be attributed to local characteristics. Some potential factors, such as the greater production and contact with ruminant (cattle and sheep) in southern Chile, different climatic conditions, among other potential variables, should be analysed further. However, these differences should be carefully interpreted as they may reflect only a trend, influenced by the low number of strains under study. Therefore, further analyses are needed to confirm this hypothesis.

Four predominant CCs (ST-21, ST-48, ST-257 and ST-353) were found among our strains. This is in agreement with different studies demonstrating those genotypes as dominant among the human *C. jejuni* population in various geographic regions (Cody et al., 2012; Kovac et al., 2018; Ramonaite et al., 2017; Yang et al., 2017). According to PubMLST database and individual studies, ST-21 complex is considered a generalist lineage (having a wide host range) and one of the most prevalent and widely distributed genotypes among clinical strains (Woodcock et al., 2017). On the other hand, we only isolated one strain belonging to ST-45 complex, which is also a major CC and a generalist lineage frequently isolated from clinical samples in European studies (Cody et al., 2012; Woodcock et al., 2017).

Most of the MLST data present in the *Campylobacter* database correspond to strains isolated in Europe, Oceania and North America, while South America is the least represented area. A query in the database (last accessed: 08/06/2017) showed that, in addition to our strains, only Brazil and Uruguay have reported *C. jejuni* MLST profiles from human campylobacteriosis in Latin America. Therefore, there is a considerable lack of information in this area.

Despite the existence of a national *Campylobacter* surveillance program in Chile, the lack of implementation of diagnostic techniques in most clinical laboratories has precluded its effectiveness (Collado et al., 2013; Fernández et al., 2000; Porte et al., 2016). As far as we are aware, the alleles, STs and CCs described here correspond to the first Chilean genotypes deposited to the *Campylobacter* MLST database and therefore, these data will contribute to the understanding of the local epidemiology of campylobacteriosis and its antibiotic resistance.

5. Conclusions

Our results evidence a high ciprofloxacin resistance in *C. jejuni* and suggest the dissemination and expansion of resistant clonal lineages responsible for cases of human campylobacteriosis in Chile. Further studies should elucidate the origin or source of these resistant genotypes.

Conflict of interest

The authors declare no conflict of interest.

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