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A seventeen-year observation of the antimicrobial susceptibility of clinical *Campylobacter jejuni* and the molecular mechanisms of erythromycin-resistant isolates in Beijing, China

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SUMMARY

Objectives: To investigate the dynamic development of the antimicrobial resistance of *Campylobacter jejuni* isolated from human diarrhea in Beijing, China, between 1994 and 2010, and to further analyze the molecular mechanisms of erythromycin-resistant strains.
Methods: Susceptibility tests were performed on 203 non-duplicate clinical *C. jejuni* strains against eight common antibiotics using the standard agar dilution method. The molecular determinants were further studied in the erythromycin (ERY) non-susceptible strains. The analysis focused on the 23S rRNA gene, the *rplD* and *rplV* ribosomal genes, the *ermB* gene, and the regulatory region of the *CmeABC* efflux pump.
Results: The rates of resistance of *C. jejuni* to ciprofloxacin (CIP), nalidixic acid (NAL), doxycycline (DOX), tetracycline (TET), florfenicol (FFC), and chloramphenicol (CHL) increased significantly over the period studied (all $p < 0.05$). Similarly, the proportions of resistant patterns (CIP–NAL–DOX–TET, CIP–NAL–DOX–TET–FFC, and CIP–NAL–DOX–TET–CHL) increased remarkably. In this study, 4.4% (9/203) of *C. jejuni* strains were ERY non-susceptible. The A2075G mutation in the 23S rRNA was found in all of the resistant strains except cj8091, which harbored the *ermB* gene. Interestingly, the *ermB* gene was also detected in intermediately resistant isolates, and the earliest *ermB*-positive strain cj94473 was derived in 1994. Moreover, none of the ribosomal *rplD* or *rplV* genes harbored mutations that have been described to confer resistance to macrolides. Different mutations affecting the regulatory region of the *CmeABC* efflux pump were also found.
Conclusions: This is the first comprehensive study on the recent trend in antimicrobial resistance and the molecular mechanisms of macrolide resistance in clinical *C. jejuni* strains isolated in China. More stringent monitoring and regulation of human and animal antimicrobial use are warranted.

1. Introduction

Campylobacter jejuni, a food-borne zoonotic pathogen, is considered one of the most common causes of bacterial gastroenteritis worldwide, especially in developed countries.[1] Few cases of *C. jejuni* infection have been reported in developing countries, and these cases have been characterized by marked seasonality and localized outbreaks.[2] The transmission of *C. jejuni* to humans occurs largely through the consumption of contaminated food and animal products, especially poultry.[3] The common symptoms of campylobacteriosis are watery or bloody diarrhea, abdominal pain, fever, and nausea.[4,5] Additionally, Guillain–Barré syndrome (GBS) is a severe sequela that mainly occurs after *C. jejuni* infection and is mediated by an autoimmune demyelinating polyneuropathy of the peripheral nervous system.[4]

Although *C. jejuni* infections are typically self-limiting and usually resolve within a few days without antibiotic therapy,

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antibiotics are urgently required for routine prophylaxis in cases of acute diarrhea and immunocompromised or pregnant patients, particularly in developing countries. Macrolides and fluoroquinolones, such as erythromycin and ciprofloxacin, are recommended as the first and alternative choices, respectively, in the clinical treatment of campylobacteriosis. However, rapidly increasing frequencies of *C. jejuni* strains that are resistant to these antibiotics and isolated from various sources (e.g., humans, poultry, or food production) have been reported in numerous studies,[6–8] which could compromise future treatment.

Hence, the aim of this study was to describe the dynamic development of the antimicrobial resistance of *C. jejuni* isolated from human diarrhea between 1994 and 2010 in Beijing, China. The molecular mechanisms of resistance to macrolides were further analyzed.

2. Materials and methods

2.1. Bacterial isolates and culture conditions

A total of 203 non-duplicate strains were isolated from stool samples of diarrhea patients ($n = 203$) between January 1994 and December 2010 in the Department of Infectious Diseases, Peking University First Hospital, Beijing, China. All patients included in the study were over 14 years of age. The isolates were grown on Skirrow's medium (Columbia agar base supplemented with 5% sheep blood at 37 °C for 48 h in a microaerobic environment containing 5% O_2, 10% CO_2, and 85% N_2). The identification of *C. jejuni* was performed using multiple PCR, as reported previously.[9] All strains were preserved in Mueller–Hinton (MH) broth with 20% glycerol at −80 °C.

2.2. Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of eight antibiotics in the *C. jejuni* isolates were measured using the standard agar dilution method, according to the Clinical and Laboratory Standards Institute guidelines.[10] Mueller–Hinton agar plates were supplemented with 5% sheep blood and incubated for 48 h at 37 °C. *C. jejuni* ATCC 33560 was used as a quality control strain for susceptibility testing in the antimicrobial susceptibility determinations. The *C. jejuni* isolates were considered resistant to chloramphenicol (CHL), ciprofloxacin (CIP), nalidixic acid (NAL), and tetracycline (TET) at MICs of ≥32, ≥4, ≥64, and ≥16 μg/ml, respectively.[10,11] For gentamicin (GEN), florfenicol (FFC), and doxycycline (DOX), the isolates with MICs ≥8 μg/ml were considered resistant.[11] Additionally, the MIC breakpoints for erythromycin (ERY) were those defined by the CLSI, as follows: susceptible, ≤8 μg/ml; intermediate, 8–32 μg/ml; and resistant, ≥32 μg/ml.[12] Strains with MICs ≥256 μg/ml were considered to have high-level resistance to ERY.

2.3. PCR amplification of genes and sequence analysis

Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The 23S rRNA, the ribosomal protein coding genes *rplD* and *rplV*, the regulatory region of *CmeABC* (between *CmeR* and *CmeA*), and the *ermB* gene were detected in erythromycin non-susceptible (i.e., intermediate and resistant) *C. jejuni* strains (MIC ≥16 μg/ml) and susceptible strains. The genomic targets and primer sets used are listed in Table 1.[13–17]

The PCR products were purified and then sequenced by the Biomed Corporation (China, Beijing), and the sequences were analyzed to identify mutations using the BLAST program of the GenBank sequence database.

2.4. Statistical analysis

The statistical analysis was performed using GraphPad Prism 5.0 software. The Chi-square test and Fisher's exact two-tailed test were used to compare differences in the ratios of resistance between the different periods of time. The rates of resistance of the isolates and their 95% confidence intervals (95% CIs) were calculated overall. Differences were considered significant at p-values of <0.05.

3. Results

3.1. Changes in the antimicrobial susceptibility of C. jejuni

A total of 203 non-duplicate *C. jejuni* isolates obtained at Peking University First Hospital from 1994 to 2010 were analyzed in this study. By time period, the numbers of isolates obtained were 18 in 1994–1996, 25 in 1997–1999, 63 in 2000–2002, 27 in 2003–2005, 41 in 2006–2008, and 29 in 2009–2010. The frequencies of resistance of *C. jejuni* to eight common antimicrobial agents are summarized in Table 2. The data revealed a continuous increase in antimicrobial resistance to CIP, NAL, DOX, TET, FFC, and CHL during the period 1994–2010, and all of the changes were statistically significant (50–100%, $p < 0.001$; 50–100%, $p < 0.0001$; 66.7–100%, $p < 0.0094$; 72–100%, $p < 0.0006$; 12–62%, $p < 0.005$; 5.6–34.5%, $p < 0.05$, respectively). In contrast, the total proportions of isolates that were resistant to ERY and GEN were relatively low, and the changes in the resistance to these antibiotics remained non-significant ($p = 0.5797$ and 0.2621, respectively).

The proportions of the *C. jejuni* isolates that were resistant to at least four of the antimicrobial agents used in the study were also

Table 1
Primers used in this study

Gene	Primer	Sequence (5′→3′)	Annealing temperature (°C)	Reference
23S rRNA	F1-campy-23S	AAGAGGATGTATAGGGTGTGACG	55	14
	R1-campy-23S	AACGATTTCCAACCGTTCTG		
rplD	L4 Fwd	GTAGTTAAAGGTGCAGTACCA	44	15
	L4 Rev	GCGAAGTTTGAATAACTACG		
rplV	CJ20	TCCGGTTTATATTACTGAA	50	16
	CJ21	CTTTAGTTGGAAACCATCTTG		
ermB	Erm-F	CAGGTAAAGGGCATTTAACGACG	58	This study[b]
	Erm-R	CATCTGTGGTATGGCGGGTAAG		
IR region[a]	CmejejuniF	TTGCCAATTGGATAGAAAATAATC	58	17
	CmejejuniR	TCGTATTCCTTTTGAGAGATTGC		
	CmeR-F	TAGAAAAGTATATTTGTATACCCT	55	18
	CmeR-R	CGCCACTAACTTGAGGCTTTA		

[a] *IR* region, a *CmeR–CmeA* intergenic region upstream of the *CmeA* gene.
[b] The primers for the *ermB* gene were designed on the basis of GenBank accession number **KC575115.1**.