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An investigation of the molecular mechanisms contributing to high-level erythromycin resistance in *Campylobacter*

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Abstract

The molecular mechanisms contributing to high-level erythromycin resistance in *Campylobacter jejuni* and *Campylobacter coli* isolates were investigated. The A2075G mutation in the 23S rRNA target genes was identified in all high-level erythromycin-resistant isolates. A number of amino acid substitutions together with insertions and deletions were identified in the corresponding genes encoding L4 and L22 ribosomal proteins both of resistant and susceptible isolates. Amino acid substitutions identified in the resistant strains were located outside regions known to be altered in these proteins. The efflux pump inhibitor L-phenylalanine-L-arginine-β-naphthylamide (PAβN) increased the susceptibility to erythromycin in one of four isolates displaying high-level erythromycin resistance, and reduced the minimal inhibitory concentration displayed by an erythromycin-susceptible *C. coli* isolate. The A2075G mutation in the 23S rRNA appeared to be the main contributor to high-level erythromycin resistance in *Campylobacter*. Other mutations/amino acid substitutions found in the 50S ribosomal subunit encoding proteins L4 and L22 do not appear to be linked to the high-level erythromycin-resistant phenotype. Active efflux contributes to the intrinsic resistance to erythromycin in *Campylobacter* and may contribute to high-level resistance in some isolates.

Keywords: *Campylobacter*; Erythromycin; Ribosomal proteins; Efflux

1. Introduction

Campylobacter is the leading cause of acute diarrhoeal disease in humans in the Western world [1,2]. Most human infections are caused by *Campylobacter jejuni* and to a lesser extent by *Campylobacter coli*. *Campylobacter* gastroenteritis is normally self-limiting, resolving in 3–5 days. However, in immunocompromised individuals and in cases of severe disease, antimicrobial therapy is warranted [1,2]. Currently, macrolides and fluoroquinolones are the antimicrobials of choice for the treatment of life-threatening *Campylobacter* gastroenteritis [3,4]. The prevalence of resistance to these antibiotics [5–8] heightens the risk of treatment failure, posing a serious concern to public health.

Macrolides act by inhibiting protein synthesis, resulting in the premature dissociation of the peptidyl-tRNA from the ribosome [9]. To date, macrolide resistance in *C. jejuni* and *C. coli* has mainly been attributed to target gene mutations in domain V of the 23S rRNA genes at positions 2074 and 2075 (corresponding to positions 2058 and 2059 in *Escherichia coli*) [10–15]. Recent studies have also indicated that the resistance nodulation cell division (RND) efflux pump family plays an active role in both intrinsic and acquired resistance to macrolides both in *C. jejuni* and *C. coli* [12,16]. In other bacterial species, macrolide resistance has been reported to result from mutations in the 50S ribosomal subunit encoding genes of the L4 and L22 ribosomal proteins [17–19].

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The possible involvement of these ribosomal structural proteins in macrolide resistance in *Campylobacter* has not been explored.

In this study, the molecular mechanisms contributing to high-level erythromycin resistance in human clinical *Campylobacter* isolates were investigated. This study analysed the 23S rRNA gene together with novel work that characterised the L4 and L22 ribosomal proteins at a molecular level. The contribution of efflux pump activity to high-level erythromycin resistance was also examined.

2. Materials and methods

2.1. Bacterial isolates

Fourteen human clinical erythromycin-resistant *Campylobacter* isolates were used throughout this study. Thirteen of these isolates were obtained from the French National Reference Centre of *Campylobacters* and *Helicobacters*, and one was from the James Connolly Memorial Hospital, Dublin, Ireland. In addition, four erythromycin-susceptible isolates and two reference strains, NCTC 11168 (*C. jejuni*, human isolate) and NCTC 11366 (*C. coli*, porcine isolate), were included. All the isolates used are listed in Table 1.

2.2. Bacterial susceptibility testing

Susceptibility to erythromycin was determined by the standard agar dilution method in accordance with recommended procedures and breakpoints of the Comité de l'Antibiogramme de la Société Francaise de Microbiologie [20] or by Etest (AB Biodisk, Solna, Sweden). Isolates with a minimal inhibitory concentration (MIC) > 8 μg/mL were considered resistant to erythromycin.

2.3. Polymerase chain reaction (PCR) amplification and DNA sequencing

The primers used for amplification and sequencing of a 316 bp fragment of domain V of the 23S rRNA gene and the *rplD* and *rplV* genes that encode the L4 and L22 ribosomal proteins, respectively, are listed in Table 2. PCR reactions were carried out in a final volume of 100 μL containing 200 ng of genomic DNA, 10 μL of 10× PCR buffer (100 mM Tris–HCl, pH 9.0), 500 mM KCl, 1% (v/v) Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 2.5 U *Taq* DNA polymerase (Promega, Madison, WI) and 25 pmol each of the forward and reverse primers (MWG-Biotech, Ebserberg, Germany). After an initial denaturation of 5 min at 95 °C, amplification was performed over 30 cycles each consisting of 95 °C for 1 min, annealing temperature for 1 min (Table 2) and 72 °C for 1 min with a final extension of 7 min at 72 °C. Amplification was carried out in a DNA Thermal Cycler (MJ Research Inc., Watertown, MA). Amplified DNA products were resolved by electrophoresis in a 1.5% (w/v) agarose gel in 1.0× Tris–borate–EDTA buffer containing 0.5 μg/mL ethidium bromide and imaged using a Gel Doc 2000 (BioRad, Hercules, CA). PCR products were purified using a QIAquick PCR Purification kit (Qiagen GmbH, Hilden, Germany) and

Table 1
Characteristics of *Campylobacter* isolates used in this study

Strains			Erythromycin MIC (μg/mL)[a]	Nucleotide/amino acid substitutions		
Number	Species	Source		23S rRNA (nucleotide)	L4 (amino acid)	L22 (amino acid)
CIT-428	*C. jejuni*	Human (I)	>256 [32]	A2075G	V196A	I65V, S109A
00072	*C. jejuni*	Human (F)	>128	A2075G	S2R, V121A	–
94279	*C. jejuni*	Human (F)	>128	A2075G	V196A	A103V, S109A
93034	*C. jejuni*	Human (F)	>256 [256]	A2075G	–	–
87072	*C. jejuni*	Human (F)	>256 [256]	A2075G	V196A	I65V, S109A
97095	*C. jejuni*	Human (F)	>128	A2075G	V121A, V196A, I200F	–
88375	*C. jejuni*	Human (F)	>128	A2075G	–	I65V, A74G, S109T, E111A, T114A
88283	*C. coli*	Human (F)	>256 [256]	A2075G	M192I, V196A	I65V, A74G, A103V, S109A
94177	*C. coli*	Human (F)	>128	A2075G	M192I, V196A	I65V, A74G, S109T, E111A, T114A
00222	*C. coli*	Human (F)	>128	A2075G	V196A	I65V, A74G, S109T, E111A, T114A
00135	*C. coli*	Human (F)	>128	A2075G	V196A	I65V, A74G, S109T, E111A, T114A
94051	*C. coli*	Human (F)	>128	A2075G	V196A	I65V, A74G, S109T, E111A, T114A
88436	*C. coli*	Human (F)	>128	A2075G	V196A	I65V, A74G, S109T, E111A, T114A
94142	*C. coli*	Human (F)	>128	A2075G	V196A	I65V, A74G, S109T, E111A, T114A
CIT-423	*C. jejuni*	Poultry (I)	0.094	–	V121A, V196A	–
CIT-424	*C. jejuni*	Poultry (I)	0.094	–	V196A	I65V, S109A
98054	*C. jejuni*	Human (F)	4	–	–	–
98178	*C. coli*	Human (F)	4 [0.38]	–	–	K15I, I65V, A74G, S109T, E111A, T114A
Reference strains						
NCTC 11168	*C. jejuni*	Human	0.25 [0.032]	–	–	–
NCTC 11366	*C. coli*	Porcine	0.5 [0.047]	–	–	–

I, Irish isolates; F, French isolates; MIC, minimal inhibitory concentration.
[a] Values in square brackets are in the presence of the efflux pump inhibitor Phe-Arg-β-naphthylamide at 20 μg/mL.