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A study of the molecular basis of quinolone and macrolide resistance in a selection of *Campylobacter* isolates from intensive poultry flocks

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ABSTRACT

The aim of this study was to investigate the molecular basis for observed high-level quinolone and macrolide resistance in poultry *Campylobacter* isolates. Seventeen *Campylobacter* isolates displaying high-level resistance to nalidixic acid, ciprofloxacin and/or erythromycin were investigated. Minimum inhibitory concentrations were initially determined using both the broth microdilution and E-test methods. The contribution of target gene mutations and active efflux to the observed resistances were then investigated using PCR and sequencing methods. High-level resistance to nalidixic acid was attributed to amino acid substitutions Thr-86-Ile and Asn-203-Ser in GyrA in some but not all isolates. Contrary to previous reports, the Thr-86-Ile substitution did not confer universal resistance to all quinolones. Strains displaying a high level of resistance to erythromycin carried the 23S rRNA transition mutation A2075G and/or carried mutations in the L4 and/or L22 ribosomal-encoding proteins. Interestingly and in contrast to previous studies, not all of the isolates carrying substitutions within the β-hairpin region of the L22 ribosomal protein displayed erythromycin resistance. With the exception of a single isolate, efflux did not contribute to either quinolone or macrolide resistance.

This study further expands our understanding of the molecular basis of quinolone and macrolide resistance in *Campylobacter* spp. and suggests that other factors, remaining to be elucidated, may also contribute to the resistant phenotypes observed.

1. Introduction

Campylobacteriosis is the most common cause of acute bacterial gastroenteritis in developed countries (Threfall, Ward, Frost, & Willshaw, 2000). While the majority of cases are self-limiting and do not require therapeutic intervention, severe cases are normally treated with erythromycin or ciprofloxacin (Engberg, Aarestrup, Taylor, Gerner-Smidt, & Nachamkin, 2001). However, over the last decade antibiotic resistance has been widely reported in *Campylobacter* giving rise to serious public health concerns (Soonthornchaikul et al., 2006). While antimicrobial resistance to quinolones and macrolides has been attributed, at least in part, to their use in poultry production (Humphrey et al., 2005; Luber, Bartelt, Genschow, Wagner, & Hahn, 2003), control of the emergence and dissemination of antimicrobial resistance in *Campylobacter* requires a fundamental understanding of the molecular basis of the observed resistant phenotypes.

Multiple mechanisms for antibiotic resistance have been reported for *Campylobacter* (Taylor & Tracz, 2005) including modification (mutation) of target genes and active efflux pump systems. In gram-negative bacteria DNA gyrase is the primary target of quinolones. Resistance to this class of antibiotics is usually associated with amino acid substitutions in the *gyrA*-encoding subunit of the DNA gyrase (Dionisi, Luzzi, & Carattoli, 2004; Griggs et al., 2005) within the DNA-binding domain in a region termed the *quinolone resistance determining region* (QRDR). In the absence of a secondary target for quinolones in *Campylobacter* (Payot et al., 2006), the Thr-86-Ile amino acid substitution in the QRDR is sufficient to confer a resistant phenotype in *Campylobacter jejuni* and *Campylobacter coli* (Cooper, Segal, Lastovica, & Elisha, 2002; Luo, Sahin, Lin, Michel, & Zhang, 2003; Payot, Cloeckaert, & Chaslus-Dancla, 2002; Piddock, Ricci, Pumbwe, Everett, & Griggs, 2003). Other modifications of the *gyrA*-encoding subunit have also been associated with quinolone resistance including Asp-203-Ser (Lucey et al., 2002; Luo et al., 2003; Piddock et al., 2003).

Macrolide drugs bind to bacterial ribosomes causing dissociation of the peptidyl-tRNA thereby interfering with protein synthesis and preventing bacterial growth. Two mechanisms of

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macrolide resistance have been described in *Campylobacter* including: [a] modification of the antibiotic target and [b] removal from the bacterial cell by efflux (Taylor & Tracz, 2005). The former is the most common mechanism and usually occurs by mutation. The large (50S) bacterial ribosomal subunit contains 23S rRNA that is the primary target of macrolides. Modification by mutation reduces macrolide binding thereby conferring a resistant phenotype. Point mutations at positions 2074 and/or 2075 have been associated with high levels of erythromycin resistance (Corcoran, Quinn, Cotter, & Fanning, 2006; Payot et al., 2004; Taylor & Tracz, 2005; Vacher, Menard, Bernard, & Megrau, 2003).

Macrolide binding may also be inhibited by mutations in the ribosomal proteins L4 and L22. However, not all mutations confer erythromycin resistance. The A103V substitution in the L22 protein has been identified in high-level erythromycin-resistant *C. jejuni* and *C. coli* but K15I, E111A, T114A in L22 and V121A and V196A in L4 are located outside the important target region and have been found in susceptible *Campylobacter* strains (Corcoran et al., 2006).

Other factors may also contribute to the resistance phenotype. Efflux of drug(s) was first proposed in 1995 as a mechanism that conferred a multi-drug resistance (MDR) phenotype to *Campylobacter*. In 2002, the chromosomally encoded multi-drug resistance-nodulation-cell division (RND) efflux system, CmeABC efflux pump, was described in *C. jejuni* (Lin, Michel, & Zhang, 2002; Luo et al., 2003) and in *C. coli* (Corcoran, Quinn, Cotter, & Fanning, 2005). This efflux pump is known to promote both intrinsic (Lin et al., 2002; Pumbwe & Piddock, 2000) and acquired resistance (Ge, McDermott, White, & Meng, 2005; Luo et al., 2003; Payot et al., 2002) to a range of antimicrobial agents including quinolones and macrolides in *Campylobacter* species. The structure of this pump includes an outer membrane protein (CmeC), an inner membrane efflux transporter of the RND superfamily (CmeB) and a periplasmic fusion protein (CmeA) (Payot et al., 2004). This pump, which is normally present in wild-type *Campylobacter* strains, is also capable of extruding a wide range of substances including antimicrobial agents, detergents and bile salts (Pumbwe & Piddock, 2000).

The aim of this study was therefore to investigate the role of both target gene mutations and efflux pump activity in both fluoroquinolone and macrolide resistance in *Campylobacter* isolates from intensively reared poultry thus adding to the body of knowledge that already exists in this area that is providing the scientific basis for the design of next generation antibiotics and the development of strategies to control antibiotic resistance in *Campylobacter*.

2. Materials and methods

2.1. Bacterial isolates and growth conditions

Fifteen poultry *Campylobacter* isolates, previously shown to have quinolone and/or macrolide resistance phenotypes, were obtained from the Teagasc *Campylobacter* collection at the Teagasc Food Research Centre. These had been isolated from several different flocks within 12 months of this study (Ashtown). Two reference strains NCTC 11168 (*C. jejuni*, human isolate) and NCTC 11366 (*C. coli*, porcine isolate) were also included.

2.2. Broth microdilution

Broth microdilution was performed as described by Luber et al. (2003) with minor modifications. Briefly, several *Campylobacter* colonies were transferred into a tube containing 5 ml Mueller-Hinton broth (CM 405; Oxoid) and incubated at 37 °C for 24 h under microaerophilic conditions in an atmosphere consisting of 5% O_2, 10% CO_2, and 85% N_2. The optical density of each culture was measured and the bacterial isolates diluted to obtain an OD_{600nm} of 0.4 (equivalent to approx. 1×10^6 cfu/ml). Volumes of 150 μl were seeded per well in a 96 well plate (Nunc microwell plates, catalogue no. 120013) and the plates were covered with a plastic plate seal film and incubated under microaerophilic conditions at 37 °C for 24 h. *Campylobacter* control strains were included in each batch of broth microdilution tests (as described above). Each test was repeated on 3 occasions per isolate.

2.3. Determination of MIC by E-test

E-tests were carried out as a confirmation check for the data obtained from broth microdilution. Minimum inhibitory concentrations (MIC) were determined for nalidixic acid, ciprofloxacin and erythromycin. Briefly, suspensions of *Campylobacter* strains were prepared with a turbidity equivalent to a 0.5 McFarland standard in sterile water. A cotton swab was saturated in the preparation and swabbed evenly over the entire surface of a Mueller-Hinton agar (Oxoid, Basingstoke, England) plate containing 5% lysed horse blood. Plate surfaces were allowed to dry thoroughly before E-test strips (AB Biodisk, Sweden) were applied. Plates were incubated under microaerophilic conditions at 37 °C for 24 h. MICs were recorded directly from the test strip according to the instructions of the manufacturer. Each test was repeated on 3 occasions per isolate.

2.4. Efflux pump inhibitors (EPI)

To examine the effect of phenylalanine arginine β-naphthylamide (PAβN), a well characterised EPI, on the active efflux of nalidixic acid, ciprofloxacin and erythromycin, MICs for each of these agents were determined in the presence of the efflux pump inhibitor. A stock solution of PAβN (concentrations) was prepared in deionised distilled water and sterilised by membrane filtration and stored at −20 °C. The inhibitor was incorporated into MH agar at a final concentration of 20 μg/ml. Concentrations of EPI up to 64 μg/ml had no visible effect on bacterial growth. The assessment of MIC for each strain was performed in triplicate in three independent experiments.

2.5. PCR amplification of the QRDR of the gyrA gene, the efflux pump gene cmeB and the 23S rRNA gene

The primers sets, target genes and referenced methods are listed in Table 1. A 673-bp fragment of the quinolone resistance determining region (QRDR) of the *gyrA* gene was amplified and sequenced according to the method previously described by Zirnstein, Li, Swaminathan, and Angulo (1999). The *cmeB* efflux pump component (1070 bp) was amplified using the method of Corcoran, Quinn, Cotter, O'Halloran, and Fanning (2005). The 23S rRNA gene (316 bp) was amplified as previously described by Corcoran et al. (2006). Sequence analysis was performed to detect mutations in the 23S rRNA gene. The ribosomal protein genes *rplD* and *rplV* encoding the L4 and L22 polypeptides from 6 high-level erythromycin-resistant isolates were analysed. The L4 and L22 ribosomal-encoding genes were amplified as previously described by Vacher et al. (2003). Amplified DNA products were resolved by electrophoresis in a 1.5% (wt/vol) agarose gel in Tris–Borate–EDTA buffer and imaged using a Gel Doc 2000 (BioRad, Hercules, CA).

2.6. DNA sequence analysis

Amplification products generated were purified using a QIAquick PCR Purification kit (Qiagen, GmbH, Germany). Purified amplicons were sequenced commercially (MWG Biotech, Germany). Sequences were manually edited and then compared to