Journal of Antimicrobial Chemotherapy (2004) **53**, 872–874
DOI: 10.1093/jac/dkh150
Advance Access publication 17 March 2004

JAC

Emergence of fluoroquinolone resistance in the native *Campylobacter coli* population of pigs exposed to enrofloxacin

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Received 10 November 2003; returned 5 December 2003; revised 6 January 2004; accepted 15 January 2004

***Objective*: The effect of a single 5 day enrofloxacin treatment on the native *Campylobacter coli* population in conventionally weaned 5-week-old pigs was investigated.**

***Materials*: Twelve pigs were split into two groups of six: one group was treated with a therapeutic dose (15 mg/pig/day) of enrofloxacin and the other remained untreated to act as the control. *Campylobacter coli* were isolated from faecal samples and tested for ciprofloxacin resistance by measuring MIC values. Mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene of resistant isolates were identified by sequencing and denaturing HPLC. Levels of enrofloxacin and its primary metabolite ciprofloxacin in the pig faeces were also measured by HPLC.**

***Results*: No quinolone-resistant *C. coli* ($n = 867$) were detected in any of the pigs prior to treatment, indicating <0.1% resistance in the group. Resistant *C. coli* were isolated from pigs for up to 35 days after treatment with a therapeutic dose. These resistant *C. coli* had MIC values of 128 mg/L and 8–16 mg/L for nalidixic acid and ciprofloxacin, respectively, and the same single point mutation causing a Thr-86 to Ile substitution in the QRDR was identified in each. The concentration of enrofloxacin in the pig faeces was 2–4 μg/g faeces for the duration of the 5 day therapeutic treatment and was detected up to 10 days post-treatment. Ciprofloxacin was also measured and peaked at 0.6 μg/g faeces in the treated group.**

***Conclusion*: This study provides evidence that a single course of enrofloxacin treatment contributes directly to the emergence and persistence of fluoroquinolone resistance in *C. coli*.**

Keywords: *C. coli*, animal models, quinolones

Introduction

Campylobacter species are the most common cause of gastroenteritis in humans worldwide.[1] Most human *Campylobacter* infections are self-limiting; however, in cases of severe invasive disease, antimicrobial treatment may become essential for recovery, with the macrolide erythromycin and the fluoroquinolone ciprofloxacin the drugs of choice.[2] The occurrence of antimicrobial resistance in *Campylobacter* is on the increase. In 2001, The Public Health Laboratory Service (UK) Campylobacter Reference Unit (UK) identified one in four *Campylobacter coli* isolated from human intestinal infections as resistant to ciprofloxacin, compared with one in five for *Campylobacter jejuni*. The incidence of erythromycin resistance was also consistently higher in *C. coli* (19%) than *C. jejuni* (1–3%).[3]

The principal mechanisms of resistance to quinolones are mutation(s) in genes encoding topoisomerase enzymes and genes encoding the regulation of efflux functions that decrease intracellular antimicrobial concentrations.[4,5]

The introduction of fluoroquinolones for veterinary purposes in the 1990s could be a cause of this increase in resistance. Consequently, calls to restrict the use of these in livestock production are being broadcast. Pigs remain one of the most highly medicated sectors in livestock production (Veterinary Medicines Directorate, 2002, UK;

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www.vmd.gov.uk, last accessed December 2003) and therefore could be a major contributor, particularly as the UK national abattoir survey (VLA, UK, 2000) reports an incidence rate of one in 10 contaminated carcasses with resistant strains.

In light of this, we have investigated the effect of a standard 5 day treatment of enrofloxacin on the emergence of resistance in *C. coli* in the pig. In parallel, the concentrations of both enrofloxacin and its primary metabolite ciprofloxacin excreted in the pig faeces were determined by HPLC[6] reflecting the level to which the enteric bacteria were exposed.

Materials and methods

Animals

Twelve 3-week-old weaned piglets were housed as a single group for 2 weeks. They were then divided randomly into groups of six in two pens with individual HEPA filtration, and fed a standard organic feed *ad libitum* (Organic Feed Company, grower/finisher pellets).

All procedures complied with the Animals (Scientific Procedures) Act 1986 and were performed under Home Office Licence.

Antibiotic treatment

One group was given a standard therapeutic dose (15 mg/pig/day) of enrofloxacin (Baytril piglet oral doser, Bayer, UK), whereas the second group remained untreated. Baytril was administered for 5 days.

Collection of faecal samples and isolation of active C. coli

Faecal samples were collected by digital manipulation once before treatment, on day 3 of treatment and on days 7, 14, 21 and 35 post-treatment. One gram of faeces from each pig was emulsified in 9 mL of PBS, and 10-fold serial dilutions were made. Aliquots of each dilution were spread onto half plates of modified charcoal cefoperazone desoxycholate agar (CCDA) with selective supplement (CM739B, Oxoid) and incubated in microaerophilic conditions at 37°C (5% O_2, 10% CO_2, 85% N_2). Isolates (2 cfu/plate) were confirmed as *Campylobacter* by morphology, motility and lack of growth in air at 20°C, then speciated by the biochemical tests: hippurate, indoxyl acetate and urease. Pre-treatment, all pigs were screened for the presence of nalidixic acid- and ciprofloxacin-resistant *Campylobacter* by aliquoting out 100 μL of 10^{-1}, 10^{-3} and 10^{-5} dilutions onto whole plates. Plates with 20–80 cfu were replica plated onto Iso-Sensitest agar supplemented with 5% defibrinated horse blood, one with ciprofloxacin 1 mg/L the other with nalidixic 16 mg/L, and incubated in microaerophilic conditions at 37°C.

Statistical analysis

Campylobacter counts from the treated pigs were compared. Two samples of equal variance *t*-tests were run separately, using results from before and during treatment versus 3 days post-treatment.

Determination of susceptibility to antibiotics

The MIC of antimicrobials was determined by an agar doubling dilution method, essentially according to the NCCLS guidelines for determining the MIC for Enterobacteriaceae,[7] using Iso-Sensitest agar (CM471, Oxoid) with 5% defibrinated horse blood. The MIC was recorded as the lowest concentration that inhibited growth.

DNA isolation and analysis of the quinolone resistance-determining region (QRDR) of gyrA

DNA was isolated from the bacteria using the DNAce spin cell culture kit (Bioline). A 246 bp fragment covering the QRDR of *gyrA* was amplified by PCR using primers ccgyrA1 (TCCTGATGCTAGAGATGGCT; forward primer) and ccgyrA2 (CCATCACCATCGATAGAACC; reverse primer). PCR was carried out in a 50 μL aliquot containing 100 ng of genomic DNA, PCR Master mix (1.5 mM $MgCl_2$) (Abgene, Epsom, UK) and 250 nM of each primer. The reaction was performed in a Techne thermal cycler with an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, then a final step at 72°C for 10 min. Mutations in the product were detected using denaturing HPLC (DHPLC) on the Wave DNA fragment analysis system (Transgenomic Inc., Crewe, UK).[8]

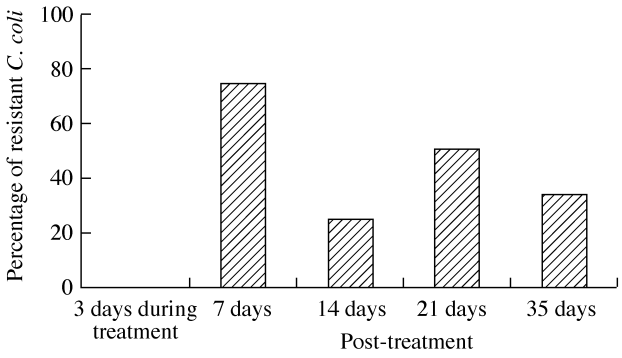

Figure 1. Proportion of resistant *C. coli* isolated from the treated pigs. Each time point is comprised of 12 isolates, except day 21, which is comprised of 10.

Determination of enrofloxacin and ciprofloxacin in pig faeces

Enrofloxacin and ciprofloxacin levels in pig faeces were measured by HPLC, exactly as described by Sunderland *et al.*[6]

Results

Effect of enrofloxacin on the native Campylobacter population

Campylobacter were isolated at 5×10^5 cfu/g faeces (mean: $5.76 \times 10^5 \pm 6.67 \times 10^4$) from the pigs prior to enrofloxacin treatment. Presumptive *Campylobacter* isolates (n = 144; 72 per group of pigs) were all confirmed as *C. coli*. Counts of *C. coli* in pigs treated with a standard dose of enrofloxacin decreased by 100-fold during treatment ($P = 0.099$), but returned to pre-treatment levels within 7 days post-treatment. No changes in counts of the *Campylobacter* population of untreated pigs were detected.

Prior to enrofloxacin treatment, all *Campylobacter* isolated ($n = 867$) were nalidixic-acid susceptible (MIC < 16 mg/L) and ciprofloxacin susceptible (MIC < 1 mg/L), indicating <0.1% resistance. Subsequently, isolates with high nalidixic (128 mg/L) and ciprofloxacin (8–16 mg/L) MICs were detected in the treated group up to 35 days post-treatment (Figure 1). These ($n = 21$) also showed high resistance to erythromycin (14 had MICs of 128 mg/L), tetracycline (all had MICs ≥ 4 mg/L) and ampicillin (all had MICs ≥ 8 mg/L).

Characterization of gyrA mutations

DHPLC and sequencing revealed that of the 21 resistant isolates tested, all had the same single point mutation in the *gyrA* QRDR that resulted in a Thr-86 to Ile substitution.

Measurements of enrofloxacin and ciprofloxacin in pig faeces

Enrofloxacin was measured during and 10 days post-treatment in the treated pigs, and the average concentrations in faeces during treat-

ment were in the range 2–4 μg/g. Ciprofloxacin was also measured and peaked at 0.6 μg/g faeces on day 3.

No enrofloxacin or ciprofloxacin was detected in the faeces from the control pigs.

Discussion

In this study, we have demonstrated that a standard 5 day treatment of enrofloxacin suffices to select for fluoroquinolone resistance in *C. coli* in the pig. The resistant *C. coli* were persistent colonizers as these could still be detected 5 weeks after treatment had ceased, although enrofloxacin could only be detected until 10 days post-treatment.

These findings correlate with results obtained previously by McDermott *et al.*[9] and Luo *et al.*[10] in poultry models. Although enrofloxacin treatment is different in poultry compared with pigs and the recommended dose is more than six times higher in poultry, the emergence of highly ciprofloxacin-resistant bacteria, which persisted at least 4 weeks post-treatment, occurred in both animal models, suggesting that resistant mutants are fit and can persist well beyond the completion of the treatment.

The distribution of fluoroquinolone MICs in *C. coli* isolated from this study was bimodal. Among the *C. coli* tested, ciprofloxacin MICs were either <0.5 mg/L or 8–16 mg/L. In addition, nalidixic-acid resistance (MIC = 64–128 mg/L) was only seen in ciprofloxacin-resistant isolates. The mechanism of resistance was identified as the common single point mutation substituting Thr-86 with Ile in GyrA.[4] If this point mutation is solely responsible for the high-level resistance identified, this could explain the bimodal nature of the phenotype and the rapid emergence of resistance in *Campylobacter* isolated from treated animals.

Two-thirds of the ciprofloxacin-resistant isolates were also resistant to erythromycin (MIC = 128 mg/L), indicating these were not the result of a single successful clone. The incidence of erythromycin resistance is very high in porcine *C. coli* and the UK National Surveillance Programme for Antimicrobial Resistance quoted 84.5% erythromycin resistance in 2001. Since the ban of five antimicrobials for growth promotion in livestock production, there has been a substantial increase in the use of therapeutic antimicrobials including macrolides[5] and this could explain the high prevalence of erythromycin resistance in *C. coli*.

Our results indicate that the use of fluoroquinolones in pigs selects for resistant *C. coli* that persist in the gastrointestinal tract well beyond the end of treatment, providing an opportunity to contaminate the carcass at slaughter. Surveys have reported that high levels of erythromycin resistance already exist in porcine *C. coli*, and consequently emergence of fluoroquinolone resistance poses a real threat to human patients needing treatment.

Acknowledgements

We thank Defra UK for funding this work (Defra project code OD 2003).

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