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1 **Longitudinal on-farm study of the development of antimicrobial resistance**  
2 **in *Campylobacter coli* from pigs before and after danofloxacin and tylosin**  
3 **treatments**

4

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22

23 Running title: Resistance in *C. coli* after danofloxacin and tylosin

24 **Abstract**

25 Effects of danofloxacin or consecutive fluoroquinolone and macrolide treatments on  
26 resistance development in *Campylobacter* have remained uncharacterised. Therefore we  
27 analysed the development of resistance in porcine *C. coli* before and after danofloxacin and  
28 tylosin treatments at a farrowing farm. Danofloxacin-treated ( $n = 12$ , group A) and control  
29 pigs ( $n = 15$ , group B) were subsequently treated with tylosin and sampled longitudinally. *C.*  
30 *coli* were isolated and susceptibilities to ciprofloxacin and erythromycin were assessed,  
31 isolates were genotyped with PFGE and resistance-related mutations were identified. Isolates  
32 from the danofloxacin-treated pigs had more frequently non-wild type MICs (above the  
33 epidemiological cut-off value (ECOFF)) for ciprofloxacin ( $P < 0.001$ ) and erythromycin ( $P <$   
34  $0.05$ ) than those isolated before danofloxacin or those from the controls. Subsequent tylosin  
35 treatment increased proportion of isolates with non-wild type MICs for erythromycin in both  
36 group A and B ( $P < 0.01$ ) and, interestingly, proportion of isolates with non-wild type MICs  
37 for ciprofloxacin in group B ( $P < 0.001$ ) with high MICs (128  $\mu\text{g/ml}$ ). PFGE analysis  
38 revealed treatments selecting predominant genotypes with variable resistance patterns and  
39 decreasing initial diversity of genotypes. The most common genotype had mainly high MICs  
40 for ciprofloxacin among danofloxacin-treated pigs but wild type MICs (below the ECOFF)  
41 among the controls housed in the same pens. This suggests that the non-wild type isolate was  
42 rarely transmitted or outcompeting wild type genotype in the control pigs without selection  
43 pressure. Isolates exhibiting non-wild type MICs for ciprofloxacin harboured the C257T  
44 (Thr-86-Ile) mutation in the *gyrA* gene. In conclusion, a high dose of danofloxacin used at the  
45 farm did not prevent emergence of isolates with high MICs for ciprofloxacin. After  
46 subsequent tylosin treatment isolates had even higher MICs for ciprofloxacin and  
47 erythromycin than before the treatment. Therefore, controlled use of antimicrobials in food  
48 animal production is essential.

## 49 1. Introduction

50 *Campylobacter jejuni* and *Campylobacter coli* are frequent causes of gastrointestinal disease  
51 worldwide. The drugs of choice for treating human campylobacteriosis are fluoroquinolones  
52 and macrolides (Allos, 2001; Blaser and Engberg, 2008). The use of antimicrobials in  
53 animals and their effects on resistance development in *Campylobacter* affecting human health  
54 has been a major concern for years (Aarestrup et al., 2008).

55 Fluoroquinolones are registered in Finland for the treatment of diarrhoea and respiratory tract  
56 infections in pigs (Fimea 2004; Fimea 2009). In addition to enrofloxacin, another  
57 fluoroquinolone, danofloxacin, was accepted for veterinary use in food producing animals in  
58 Finland in 1999 (Fimea 2004). Development of antimicrobial resistance after administration  
59 of enrofloxacin, but not danofloxacin, has been studied in chicken and porcine *C. jejuni* and  
60 *C. coli* isolates (McDermott et al., 2002; Luo et al., 2003; Delsol et al., 2004; Humphrey et  
61 al., 2005). Resistance mechanisms to fluoroquinolones in *Campylobacter* include point  
62 mutation(s) in the *gyrA* gene and the activity of the efflux pump CmeABC (Luo et al., 2003;  
63 Ge et al., 2005).

64 Tylosin, a macrolide, is used for the treatment of proliferative enteropathy in pigs (Lawson  
65 and Gebhart, 2000). The CmeABC efflux pump contributes to the macrolide resistance in  
66 campylobacters together with target modifications in 23S rRNA (Cagliero et al., 2005;  
67 Gibreel et al., 2005). Low level of resistance has been linked to mutations in *rplD* and *rplV*  
68 genes, encoding ribosomal proteins L4 and L22, respectively (Cagliero et al., 2006).

69 Our aim was to study the effects of consecutive danofloxacin and tylosin treatments on the  
70 development of ciprofloxacin and erythromycin resistance in porcine *C. coli* isolates at a  
71 large farrowing farm. We also characterized mutations induced by danofloxacin in the *gyrA*  
72 gene. Additionally, we investigated the effects of antimicrobial treatments on the dynamics of  
73 *C. coli* genotypes using pulsed-field gel electrophoresis.

74

## 75 **2. Materials and methods**

### 76 **2.1. Antimicrobial treatments of pigs at the farm and sampling**

77 A Finnish farrowing farm with 950 sows was selected for this study based on on-going  
78 danofloxacin and consecutive tylosin usage prescribed by a local veterinarian. Piglets were  
79 weaned at the age of three to four weeks, and the pigs with clinical symptoms of postweaning  
80 diarrhoea were treated with intramuscular danofloxacin (Advocin vet 25 mg/ml injection,  
81 Pfizer Oy, Animal Health, Helsinki, Finland) for three days (1 ml daily, approximately 3.3  
82 mg/kg which exceeds the drug label dose of 1.25 mg/kg). Danofloxacin treatment was based  
83 on susceptibility testing of the causative agent, *Escherichia coli*. All pigs were routinely  
84 administered tylosin (Tylan Premix 20 mg/g vet., Elanco Animal Health A/S, Lyngby,  
85 Denmark) twice daily in feed (100 mg/kg, equivalent to a body weight dose of 3 - 6  
86 mg/kg/day according to the drug label). A ten-day period of tylosin-supplemented feed was  
87 administered 11 days after weaning because of a persistent diarrhoea problem at the weaning  
88 unit suspected to be caused by *Lawsonia intracellularis*. A preliminary study on resistance  
89 development in *C. coli* from 19 pigs at the farm was performed in spring 2009. Based on  
90 these results (not shown), a main sampling schedule was designed: faecal samples were  
91 collected from 27 ear-tagged pigs at six time points (Figure 1) between October and  
92 December 2009 as described previously (Juntunen et al., 2010). Twelve of the sampled pigs  
93 received danofloxacin after approximately a five-day period at the weaning unit (group A),  
94 and 15 pigs remained as controls (group B). The pigs were reared in three pens each  
95 containing animals from both groups. In addition to other medications described, three pigs  
96 from both groups A and B received intramuscular amoxicillin (15 mg/kg) (Betamox vet 150  
97 mg/ml injection, Norbrook Laboratories Ltd, Newry, Co. Down, Northern Ireland) for three  
98 days for preweaning diarrhoea between sampling I and weaning.

## 99 2.2. Isolation of *Campylobacter* and species confirmation

100 *Campylobacter*s were isolated and confirmed as *C. coli* as described by Juntunen et al.  
101 (2010). The *C. coli* isolates were stored frozen at -70 °C in skim milk and glycerol.

## 102 2.3. MIC determination

103 The MIC determination of the *C. coli* isolates was performed with the agar dilution method  
104 (National Committee for Clinical Laboratory Standards, 2002) for ciprofloxacin (Sigma-  
105 Aldrich, Steinheim, Germany) and erythromycin (Sigma-Aldrich). The *C. jejuni* strain ATCC  
106 33560 was used as a control. The epidemiological cut-off values (ECOFFs) (MIC > 1 µg/ml  
107 for ciprofloxacin and MIC > 16 µg/ml for erythromycin) determined by the European  
108 Committee on Antimicrobial Susceptibility Testing (EUCAST, European committee on  
109 antimicrobial susceptibility testing) were applied to classify isolates as wild type (MIC below  
110 the ECOFF) or non-wild type (MIC above the ECOFF) (Schwarz et al., 2010).

## 111 2.4. Molecular analysis of *gyrA*, 23S rRNA, *rplD* and *rplV* genes

112 Chromosomal DNA was extracted using Pitcher's method (Pitcher et al., 1989). The *rplV*,  
113 *gyrA* and 23S rRNA genes were sequenced from 19 isolates and the *rplD* gene from 12  
114 isolates. The MIC values of these isolates varied from ≤ 0.125 to 128 µg/ml for ciprofloxacin  
115 and from 0.25 to > 512 µg/ml for erythromycin. The *gyrA* and 23S rRNA gene fragments  
116 were amplified with the primers described by Griggs et al. (2005) and Olkkola et al. (2010),  
117 respectively. The following primers were used, respectively, for *rplV* and *rplD*: CcRplV F 5'-  
118 AGGCCATAAAGGTTCTGTGC-3' and CcRplV R 5'-AACCATCTTGATTCCCAGTTTC-  
119 3' (based on the genome of *C. coli* RM2228) and CcRplD F 5'-  
120 AATGGTGCCATGGGTAAAAT-3' and CcRplD R 5'-CACCTTGCTCTTGCAAATTC-3'  
121 (based on the genome of *C. jejuni* NCTC 11168). Additionally, for the start of the *rplD* gene  
122 if it was not properly amplified with the previous primers, the primers CcrplD F5 5'-  
123 CCAGGTCGTGTTCAACCAG-3' and CcrplD R3 5'-AGCTCTTTCAAGCGCCAAT-3'

124 (based on the genome of *C. jejuni* NCTC 11168) were applied. The PCR products were  
125 sequenced at the Institute of Biotechnology, University of Helsinki  
126 ([http://www.biocenter.helsinki.fi/bi/dnagen/sequencing\\_service.htm](http://www.biocenter.helsinki.fi/bi/dnagen/sequencing_service.htm)). The sequence data was  
127 analyzed with the Staden Package (<http://staden.sourceforge.net/>) and BioNumerics version  
128 5.10 (Applied Maths NV, Sint-Martens-Latem, Belgium) and the consensus sequences were  
129 aligned in ClustalW version 2.0.12 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>).

### 130 **2.5. Pulsed-field gel electrophoresis (PFGE)**

131 Isolates ( $n = 133$ ) of 12 pigs from group A and 12 pigs from group B were genotyped with  
132 PFGE using *Sma*I and analysed with Bionumerics software (Applied Maths NV) as  
133 previously described (Juntunen et al., 2010). Similar genotypes by *Sma*I digestion but with  
134 different resistance patterns ( $n = 58$ ) were additionally digested with *Kpn*I restriction enzyme.  
135 Isolates with  $< 90\%$  similarity according to the dendrogram or at least one band difference  
136 were clustered as separate genotypes.

### 137 **2.6. Statistical analysis**

138 Statistical analyses of the data were performed with the SPSS for Windows, Rel. 15.0.1.  
139 (SPSS Inc, Chicago, IL, USA). Chi-squared and Fisher's exact tests, when appropriate, were  
140 applied to observe statistically significant differences ( $P < 0.05$ ) between the samplings and  
141 groups. The MIC values of the isolates from the samplings after weaning but before  
142 danofloxacin treatment (II and III) as well as the results from the samplings after  
143 danofloxacin but before tylosin treatment (IV and V) were combined to improve the power of  
144 statistical analyses.

145

## 146 **3. Results**

### 147 **3.1. Isolation of *Campylobacter***



148 In group A, 63/71 (88.7%) faecal samples were *C. coli*-positive, and in group B, 84/90  
149 (93.3%) samples were *C. coli*-positive. All isolates were confirmed as *C. coli* by PCR and *C.*  
150 *jejuni* was not detected. All pigs were *C. coli*-positive at least during three samplings.

### 151 **3.2. Resistance before and after weaning**

152 The percentages of resistance patterns of *C. coli* isolates, and the percentages of isolates with  
153 MICs higher than the ciprofloxacin and erythromycin ECOFFs in groups A and B at the six  
154 samplings are presented in Figures 2 and 3, respectively. Before weaning (sampling I), all  
155 isolates (9/9 in group A and 13/13 in group B) had wild type MICs for the studied  
156 antimicrobials. After weaning (samplings II and III), 17/20 (85.0%) isolates had wild type  
157 MICs in group A and 21/28 (75.0%) isolates had wild type MICs in group B (Figure 2). The  
158 most prevalent resistance pattern after weaning was ciprofloxacin resistance (6/48 isolates)  
159 (Figure 2).

### 160 **3.3. Resistance after the danofloxacin treatment**

161 After the danofloxacin treatment of group A (samplings IV and V), 6/22 (27.3%) isolates had  
162 wild type MICs for both antimicrobials in group A compared to 24/28 (85.7%) isolates in  
163 group B ( $P < 0.001$ ). The percentage of isolates with MICs above the ciprofloxacin ECOFF  
164 increased significantly in group A compared to group B ( $P < 0.001$ ). Within group A 3/20  
165 (15%) isolates had non-wild type MICs for ciprofloxacin before the danofloxacin treatment  
166 (samplings II and III) while 16/22 (72.7%) isolates had non-wild type MICs after the  
167 treatment (samplings IV and V) ( $P < 0.001$ ) (Figure 3). The proportion of isolates with non-  
168 wild type MICs for erythromycin also increased after the danofloxacin treatment in group A  
169 ( $P < 0.05$ , Fisher's exact test) and the rate of those isolates in group A was significantly  
170 higher compared to group B ( $P < 0.05$ , Fisher's exact test). However, the erythromycin MIC  
171 values of non-wild type isolates were at a low level (32 - 64  $\mu\text{g/ml}$ ) (Table 1). The most  
172 prevalent resistance patterns in group A were ciprofloxacin-erythromycin (8/22 isolates) and

173 ciprofloxacin resistance (8/22 isolates) and in group B erythromycin resistance (3/28 isolates)  
174 but with erythromycin MIC values of 32 µg/ml.

### 175 **3.4. Resistance after the tylosin treatment**

176 After the tylosin treatment of all pigs (sampling VI), 11/12 isolates (91.7%) in group A and  
177 14/15 (93.3%) isolates in group B had non-wild type MICs for at least one antimicrobial  
178 (Figure 2). Additionally, there were no differences in the percentages of non-wild type  
179 isolates to either antimicrobial agent studied between the groups ( $P > 0.05$ , Fisher's exact  
180 test) (Figure 3). The proportion of isolates exhibiting wild type MICs for the antimicrobials  
181 studied decreased significantly in group B in comparison to samplings IV and V prior to the  
182 tylosin treatment ( $P < 0.001$ ) (Figure 2). Isolates with non-wild type MICs for ciprofloxacin  
183 were predominant in both groups after the tylosin treatment and the increase in group B was  
184 significant in comparison to before tylosin ( $P < 0.001$ , Fisher's exact test). MIC values for  
185 ciprofloxacin also increased in both groups: all non-wild type isolates had ciprofloxacin MIC  
186 values between 2 and 64 µg/ml before the tylosin treatment while 10/21 non-wild type  
187 isolates had MIC = 128 µg/ml after tylosin (Table 1). The percentage of isolates with MICs  
188 higher than the erythromycin ECOFF increased both in group A ( $P < 0.01$ ) and B ( $P < 0.001$ )  
189 after the tylosin treatment and all non-wild type isolates had high MIC values ( $\geq 512$  µg/ml)  
190 (Table 1). The most prevalent resistance pattern in both groups after tylosin was  
191 ciprofloxacin-erythromycin resistance (8/12 and 11/15 isolates in group A and B,  
192 respectively). Of the 23 isolates with erythromycin MIC  $\geq 512$  µg/ml, 19 (82.6%) had  
193 simultaneously ciprofloxacin MICs higher than the ECOFF in the last sampling. Furthermore,  
194 31/38 (81.6%) isolates with non-wild type MICs for erythromycin collected over the entire  
195 study had non-wild type MICs for ciprofloxacin concurrently.

### 196 **3.5. PFGE analysis**

197 PFGE types were obtained from 133 *C. coli* isolates by SmaI and from 55 isolates by KpnI.  
198 The isolates with similar genotypes by SmaI but with differing resistance patterns had similar  
199 KpnI patterns as well. The PFGE types and resistance patterns are represented in Table 2. In  
200 total, the isolates were divided into 33 genotypes by SmaI. Twelve of 21 isolates from the  
201 preweaning stage (sampling I) belonged to genotype 8 or 33 but only three isolates of these  
202 genotypes were detected at weaning (sampling II) and none after that. The highest number of  
203 genotypes was observed after weaning (sampling II) when nine and eleven isolates were  
204 divided into eight and nine genotypes in group A and B, respectively. The number of  
205 genotypes decreased to four and six in the danofloxacin-treated and control pigs, respectively,  
206 after the danofloxacin treatment (sampling IV). However, 5/6 and 5/9 genotypes present after  
207 danofloxacin (samplings IV and V) in group A and B, respectively, were also observed  
208 before the danofloxacin treatment. Genotype 1 became predominant at sampling III and it  
209 was also the most prevalent after the danofloxacin treatment (samplings IV and V: 14/22 in  
210 group A and 7/23 in group B). The prevalence of isolates with non-wild type MICs for  
211 ciprofloxacin in genotype 1 was higher in the danofloxacin-treated (12/14) than control pigs  
212 (1/7) at samplings IV and V ( $P < 0.01$ , Fisher's exact test). Genotypes 6 ( $n = 5$ ) and 20 ( $n =$   
213 5) were predominant after the tylosin treatment (sampling VI). Neither genotype was  
214 observed at the previous samplings, and only three out of nine genotypes (1, 11 and 21)  
215 present at the sampling VI were detected previously. The isolates with MICs higher than the  
216 ECOFFs for ciprofloxacin ( $n = 43$ ) or erythromycin ( $n = 35$ ) were present in 12 and 7  
217 genotypes, respectively. Twenty isolates with high erythromycin MICs ( $\geq 512 \mu\text{g/ml}$ ) were  
218 divided into six genotypes, and genotype 11 included isolates with both wild type and non-  
219 wild type MICs for erythromycin. Five genotypes (1, 17, 18, 21 and 26) included isolates  
220 with both wild type and non-wild type MICs for ciprofloxacin.

### 221 3.6. Molecular analysis of the *gyrA*, 23S rRNA, *rplD* and *rplV* genes

222 All seven isolates with high ciprofloxacin MICs ( $\geq 32 \mu\text{g/ml}$ ) had the C257T mutation in the  
223 *gyrA* gene, and two isolates with high erythromycin MICs ( $> 512 \mu\text{g/ml}$ ) had the A2122G  
224 mutation in the 23S rRNA encoding gene. We detected no previously described erythromycin  
225 resistance-related mutations in the genes *rplV* and *rplD*, including isolates with erythromycin  
226 MIC values of 8 - 32  $\mu\text{g/ml}$  ( $n = 9/19$  for *rplV* and 6/12 for *rplD*). In *rplV*, several changes  
227 were detected in the C-terminal region (codons 103-130) of the predicted protein L22 as  
228 compared to *rplV* of *C. coli* RM2228 resulting in identical amino acid sequences as reported  
229 by Cagliero et al. (2006) in isolates Cc 12, C342, 207, C455 (data not shown). In *rplD*, the  
230 only changes found in the predicted protein L4 were V196A (GTG  $\rightarrow$  GCG) in all studied  
231 isolates and P28S (CCA  $\rightarrow$  TCA) in six isolates as compared to the *rplD* sequence of *C. jejuni*  
232 NCTC 11168. All P28S changes were in the isolates with erythromycin MIC of 16 - 32  $\mu\text{g/ml}$   
233 (data not shown). From seven *C. coli* isolates, we were unable to obtain whole gene sequence  
234 of *rplD*.

235

#### 236 4. Discussion

237 We assessed the development of antimicrobial resistance in porcine *C. coli* isolates. Pigs with  
238 symptoms of postweaning diarrhoea were treated with danofloxacin based on susceptibility  
239 testing of *E. coli*. Additionally, all pigs were administered tylosin after an 11-day period at  
240 the weaning unit because of chronic proliferative enteropathy problem at the unit. The study  
241 was performed in field conditions at a large farrowing farm, which best reflects the actual  
242 environment for antimicrobial resistance development in the food chain. We followed  
243 danofloxacin-medicated and control pigs that were reared in the same pens, which also  
244 allowed us to observe the likelihood of the transmission of non-wild type isolates from  
245 treated pigs to control pigs.

246 To our knowledge, the effects of the usage of danofloxacin, a fluoroquinolone accepted for  
247 veterinary use in Finland in 1999 (Fimea 2004), on resistance development in *Campylobacter*  
248 have not been studied before. Danofloxacin treatment selected isolates which had  
249 significantly more frequently MICs higher than the ECOFFs for ciprofloxacin as well as  
250 erythromycin than those collected before the treatment or from the control pigs. The  
251 danofloxacin dose used at the farm (approximately 3.3 mg/kg/day) was higher than the  
252 recommended therapeutic dose (1.25 mg/kg/day) (Fimea 2004) but it appeared not to be high  
253 enough to prevent the development and selection of *C. coli* isolates with fluoroquinolone  
254 MICs above the ECOFF. This is not concordant with the results of Stapleton et al. (2010)  
255 who concluded that a high dose of enrofloxacin prevents emergence of fluoroquinolone-  
256 resistant *C. jejuni* in chicken. The most common resistance pattern in the isolates from the  
257 danofloxacin-treated pigs was ciprofloxacin resistance with or without a low level of  
258 resistance to erythromycin. While the MIC values for ciprofloxacin were high (all isolates  
259 except one: MIC  $\geq 16 \mu\text{g/ml}$ ), those for erythromycin were just above or below the ECOFF  
260 set by EUCAST (MIC  $> 16 \mu\text{g/ml}$ ) for *C. coli*. After the tylosin treatment, the MIC values  
261 rose to  $\geq 512 \mu\text{g/ml}$  which more likely reflects real resistance. Amoxicillin therapy for three  
262 piglets in both groups for preweaning diarrhoea at the farrowing unit might have had  
263 selection pressure on *C. coli* isolates. Transfer of the piglets to the weaning unit exposed  
264 them to some pre-existing non-wild type *C. coli* isolates from the environment but no  
265 statistical increase was observed in resistance.

266 According to Grobbel et al. (2007), danofloxacin is significantly less active than enrofloxacin  
267 against *E. coli*, which impugns the use of danofloxacin for postweaning diarrhea caused by *E.*  
268 *coli*. In the U. S., danofloxacin is indicated only for bovine respiratory disease and extra-label  
269 use in food-producing animals is prohibited (FDA, Food and Drug Administration). In

270 Finland, danofloxacin is approved for the treatment of bovine and porcine respiratory disease  
271 and bovine enteritis (Fimea 2004).

272 Our results also indicated that danofloxacin usage decreases the susceptibility to  
273 erythromycin to a level which facilitates the development of high-level resistance if the  
274 bacteria are later challenged with a macrolide. Subsequent tylosin treatment selected isolates  
275 with high MICs for both erythromycin and ciprofloxacin. Interestingly, after the tylosin  
276 treatment of all pigs, the percentage of isolates with non-wild type MICs for ciprofloxacin  
277 also increased in the control group, and we observed up to fourfold increase in the  
278 ciprofloxacin MIC values among the isolates with ciprofloxacin MICs higher than the  
279 ECOFF. The most common resistance pattern after the tylosin treatment was ciprofloxacin-  
280 erythromycin resistance in both group A and B. In a previous study at another farm, we  
281 detected a similar increase in ciprofloxacin resistance after the tylosin treatment of pigs even  
282 though fluoroquinolones had not been administered at the farm for several years (Juntunen et  
283 al., 2010). It is of concern that isolates with high MICs for erythromycin had often high MICs  
284 for ciprofloxacin also in the control group that did not receive danofloxacin. This might be a  
285 consequence of the farm conditions as the danofloxacin-treated pigs were reared in the same  
286 pens with the controls.

287 Genotyping using PFGE was shown to be useful to study the spreading and persistence of  
288 isolates with non-wild type MICs. Even if there might have been present more genotypes  
289 than we were able to detect, PFGE analysis indicated a dynamic process of predominant  
290 genotypes. PFGE analysis revealed diversity in porcine *C. coli* populations, which were  
291 affected by transfer of the piglets from the farrowing unit to the weaning unit as well as the  
292 antimicrobial treatments. The genotypes with wild type MICs predominant at the preweaning  
293 stage disappeared after the transfer to the weaning unit. The genotype 26 present at the first  
294 sampling with wild type MICs was the only genotype detected to have non-wild type MICs

295 for ciprofloxacin at the subsequent samplings. None of the other non-wild type isolates  
296 sampled after weaning had PFGE genotypes observed before weaning. The genotype 1  
297 became predominant at the weaning unit and it was also the most prevalent genotype in the  
298 danofloxacin-treated pigs. Most of the genotype 1 isolates from danofloxacin-treated pigs had  
299 high MICs for ciprofloxacin. However, the same genotype from the control pigs had mostly  
300 wild type MICs for ciprofloxacin. This difference suggests that under farm conditions, *C. coli*  
301 isolates, which have non-wild type MICs for ciprofloxacin, are not frequently transmitted to  
302 untreated pigs and that they are not outcompeting wild type isolates without selection  
303 pressure even if the pigs are in close contact. However, in experimental conditions,  
304 fluoroquinolone-resistant *C. jejuni* isolates have been shown to outcompete susceptible ones  
305 when coinoculated into chickens (Luo et al., 2005).

306 A shift in the genotypes was observed after the tylosin treatment: the genotypes frequently  
307 detected before the treatment disappeared and new genotypes with high MICs emerged. A  
308 similar phenomenon was observed in our previous on-field study (Juntunen et al., 2010). The  
309 predominant genotypes 6 and 20 were not detected in the previous samplings and only three  
310 genotypes were observed in the earlier samplings. Genotype 11 had wild type MIC for  
311 erythromycin at samplings II and V but it had high erythromycin MIC after the tylosin  
312 treatment. Isolates with non-wild type MICs for ciprofloxacin and erythromycin were  
313 distributed among many genotypes. These findings support earlier results that resistance-  
314 conferring mutations occur independently in a set of isolates with variable genotypes (Keller  
315 and Perreten, 2006; Juntunen et al., 2010).

316 All isolates with high MICs for ciprofloxacin ( $\text{MIC} \geq 32 \mu\text{g/ml}$ ) had the C257T (Thr-86-Ile)  
317 change in the *gyrA* gene, as described with other fluoroquinolones (Ge et al., 2005).  
318 Therefore it seems that danofloxacin does not induce novel mutations in the quinolone-  
319 resistance-determining region of *gyrA* in *C. coli in vivo*. The two isolates with high

320 erythromycin MICs ( $> 512 \mu\text{g/ml}$ ) had the well characterised A2122G (*C. coli*<sub>rm2228</sub> -  
321 numbering) mutation the 23S rRNA gene. Although we studied several isolates with  
322 decreased susceptibility to erythromycin (MIC 8 - 32  $\mu\text{g/ml}$ ), no previously described,  
323 resistance-related mutations were detected in the genes *rplV* and *rplD*. In *rplV*, there were  
324 changes in the C-terminal region of the corresponding protein as compared to that of *C. coli*  
325 RM2228. In the 12 isolates with successful amplification of the whole *rplD* gene, we detected  
326 two changes in the predicted protein as compared to that of *C. jejuni* NCTC 11168. None of  
327 the changes have been connected to macrolide resistance in *Campylobacter* and they present  
328 more likely normal variation between isolates. Therefore our results did not support the  
329 hypothesis that mutations in the genes *rplD* or *rplV* would explain the decreased  
330 susceptibility to erythromycin in these isolates. The unsuccessful amplification of the whole  
331 *rplD* gene of seven *C. coli* isolates was possibly due to a different location of this gene as  
332 compared to *C. jejuni* NCTC 11168.

333

## 334 5. Conclusion

335 This was the first longitudinal field study conducted on the effects of danofloxacin as well as  
336 consecutive fluoroquinolone and macrolide treatments on resistance development in *C. coli*.  
337 Fluoroquinolone and macrolide-resistant *Campylobacter* are of particular concern because  
338 antimicrobial agents of those groups are important for the treatment of human  
339 campylobacteriosis. Treatment with a high dose of danofloxacin did not prevent emergence  
340 of isolates with non-wild type MICs for ciprofloxacin but it also increased the proportion of  
341 isolates with non-wild type MICs for erythromycin. Furthermore, subsequent tylosin  
342 treatment of all pigs increased considerably the MIC values for erythromycin. There was also  
343 a statistically significant increase in the percentage of isolates with non-wild type MICs for  
344 ciprofloxacin in the control group in which high MIC values for ciprofloxacin were detected.



345 These results emphasise the need for a prudent use of antimicrobials in food animal  
346 production.

347

### 348 **Conflict of interest statement**

349 None to declare.

350

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362

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Figure 1. Schedule of six samplings and antimicrobial treatments.

<sup>a</sup>a three-day danofloxacin treatment of group A after sampling III

<sup>b</sup>a ten-day tylosin treatment of group A and B after sampling V

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Figure 2. Percentage of resistance patterns of *C. coli* from 12 danofloxacin-treated (group A; left column) and 15 control pigs (group B; right column) at six samplings. Isolates with MICs above the epidemiological cut-off values for ciprofloxacin ( $> 1 \mu\text{g/ml}$ ) and erythromycin ( $> 16 \mu\text{g/ml}$ ) were considered as non-wild type isolates. CIP, ciprofloxacin; ERY, erythromycin.

<sup>a</sup>a three-day danofloxacin treatment of group A after sampling III

<sup>b</sup>a ten-day tylosin treatment of group A and B after sampling V

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Figure 3. Percentage of non-wild type *C. coli* isolates at six samplings in group A and B. Isolates with MICs above the epidemiological cut-off values for ciprofloxacin ( $> 1 \mu\text{g/ml}$ ) and erythromycin ( $> 16 \mu\text{g/ml}$ ) were considered as non-wild type isolates. CIP (A) or CIP (B); percentage of isolates with non-wild type MICs for ciprofloxacin in group A or B, ERY (A) or ERY (B); percentage of isolates with non-wild type MICs for erythromycin in group A or B.

<sup>a</sup>a three-day danofloxacin treatment of group A after sampling III

<sup>b</sup>a ten-day tylosin treatment of group A and B after sampling V



Table 1. Distribution of the MICs for ciprofloxacin and erythromycin against *C. coli* isolated from the danofloxacin-treated ( $n = 12$ ) and control pigs ( $n = 15$ ).

Antimicrobial agent	Range of dilutions tested ( $\mu\text{g/ml}$ )	Sampling	Pig group <sup>a</sup>	No. of isolates with MIC ( $\mu\text{g/ml}$ ) of:													No. (%) of isolates with MICs above the ECOFF		
				$\leq 0.125$	0.25	0.5	1	2	4	8	16	32	64	128	256	512		$\geq 1024$	
Ciprofloxacin	0.125-128	I	A	8	1													0 (0.0)	
			B	13														0 (0.0)	
		II	A	3	2	2	1					1							1 (11.1)
			B	4	1	4	1					3							3 (23.1)
		III <sup>b</sup>	A	4		4	1					2							2 (18.2)
			B	7	1	3						4							4 (26.7)
		IV	A	1		1	1					7	1						8 (72.7)
			B	7	1	5	1					1							1 (6.7)
		V <sup>c</sup>	A		1	2		1			2	4	1						8 (72.7)
			B	5	3	5													0 (0.0)
		VI	A		1	2						6		3					9 (75.0)
			B		1	1	1					4	1	7					12 (80.0)
Erythromycin	0.125-256	I	A			1	8											0 (0.0)	
			B		4	5	4											0 (0.0)	
		II	A				2	2	1	1	3								0 (0.0)
			B				4		3	1	4					1			1 (7.7)
		III <sup>b</sup>	A				1	2		3	4	1							1 (9.1)
			B				5	2	1	1	4	1				1			2 (13.3)
		IV	A				1			1	4	4	1						5 (45.5)
			B				7	1		2	4	1							1 (6.7)
		V <sup>c</sup>	A				3			1	4	3							3 (27.3)
			B				2	3	2	1	3	2							2 (15.4)
		VI	A				1				1						10		10 (83.3)
			B				1				1						13		13 (86.7)

Vertical lines indicate the epidemiological cut-off values (ECOFFs).

<sup>a</sup>A, danofloxacin-treated pigs; B, control pigs

<sup>b</sup>a three-day danofloxacin treatment for group A after sampling III

<sup>c</sup>a ten-day tylosin treatment for group A and B after sampling V

Table 2. PFGE types and resistance patterns of *C. coli* isolates from 12 danofloxacin-treated and 12 control pigs.

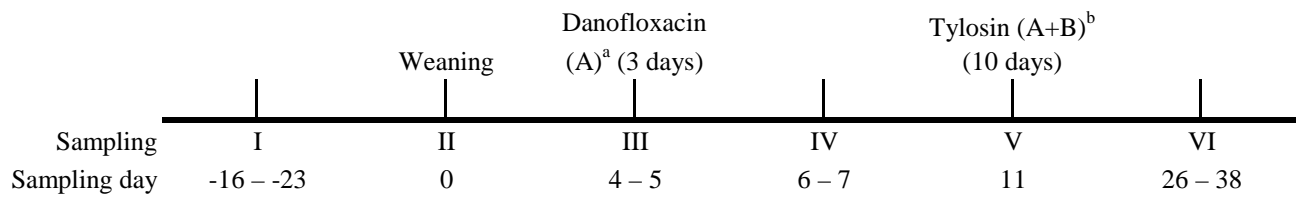
Pig	Pig group <sup>a</sup>	Sampling					
		I	II	III <sup>b</sup>	IV	V <sup>c</sup>	VI
1	A	ND	ND	1	1 (CIP-ERY)	17 (CIP)	22 (CIP-ERY)
2	A	ND	14	4	ND	26	11 (ERY)
3	A	25	26	23	1 (CIP-ERY)	26 (CIP)	20 (CIP-ERY)
4	A	8	26	ND	1 (CIP-ERY)	1 (CIP-ERY)	20 (CIP-ERY)
5	A	8	11	17 (CIP)	2 (CIP)	11	20 (CIP-ERY)
6	A	33	ND	1 (CIP-ERY)	23	26 (CIP)	21 (CIP)
7	A	27	17	31	1 (CIP)	1 (CIP-ERY)	7 (CIP-ERY)
8	A	16	24	23	1 (CIP-ERY)	1 (CIP)	20 (CIP-ERY)
9	A	9	3 (CIP)	1	1 (CIP)	1 (CIP)	11 (ERY)
10	A	26	18	1	1	1	1
11	A	33	33	12	1 (CIP-ERY)	1 (CIP-ERY)	6 (CIP-ERY)
12	A	ND	ND	17	17	ND	6 (CIP-ERY)
13	B	8	16	29	23	30	22 (CIP-ERY)
14	B	8	8	5	1	1 (ERY)	11 (ERY)
15	B	23	26	1	23	1	19 (CIP-ERY)
16	B	29	8	23	1 (ERY)	1 (ERY)	21 (CIP)
17	B	33	21	24	28	31	7 (CIP-ERY)
18	B	33	18 (CIP)	1 (CIP-ERY)	1 (CIP)	ND	6 (CIP-ERY)
19	B	8	ND	32	27	29	20 (CIP-ERY)
20	B	10	17	27	27	31	19 (CIP-ERY)
21	B	27	2 (CIP)	2 (CIP)	27	27	11 (ERY)
22	B	8	1	13	29	1	15
23	B	33	17	17	17	17	6 (CIP-ERY)
24	B	33	29	1 (CIP)	17	12	6 (CIP-ERY)

ND; not detected, CIP; ciprofloxacin, ERY; erythromycin

<sup>a</sup>A; danofloxacin-treated pigs, B; control pigs,

<sup>b</sup>a three-day danofloxacin treatment for group A after sampling III

<sup>c</sup>a ten-day tylosin treatment for group A and B after sampling V



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