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Farm level risk factors for fluoroquinolone resistance in *E. coli* and thermophilic *Campylobacter* spp. on poultry farms

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Running head: Fluoroquinolone resistance in poultry

1 **Summary**

2 Data on husbandry practices, performance, disease and drug use were collected using
3 detailed questionnaires during a cross-sectional survey of 89 poultry meat farms in
4 England and Wales to provide information on possible risk factors for the occurrence
5 of fluoroquinolone (FQ) resistant bacteria on poultry meat farms. Faeces samples
6 taken as part of the surveys were used to classify farms as 'affected' or 'not affected'
7 by FQ-resistant *E. coli* or *Campylobacter* spp., and statistical analyses were
8 performed to identify factors associated with the farms' FQ resistance status.

9 The use of FQ on the farms was by far the most important factor influencing the
10 occurrence of FQ-resistant bacteria. Resistant *E. coli* and/or *Campylobacter* spp. were
11 found on 86% of the farms with a reported history of FQ use. However, resistant
12 bacteria were also found on a substantial proportion of farms with no history of FQ
13 use, suggesting that resistant organisms may spread between farms. Further analysis
14 suggested that there are differences in the importance of various factors between the
15 two organisms. For *Campylobacter* spp., on-farm hygiene, cleaning and disinfection
16 between batches of birds and wildlife control were of most significance. For *E. coli*,
17 biosecurity factors protecting the premises from outside contamination were of
18 particular importance, although the statistical modelling indicated that other factors
19 are likely to be involved. Detailed studies on a small number of poultry sites showed
20 that FQ-resistant *E. coli* can survive routine cleaning and disinfection, so this must be
21 of a high standard to reduce the persistence of resistant organisms on the farm.

22 It appears difficult to avoid the occurrence of resistant bacteria when FQ are used on a
23 farm, but the present findings provide evidence to support recommendations to reduce
24 the substantial risk of the incidental acquisition of such resistance by farms where FQ
25 are not used.

26

27 **Introduction**

28 Antimicrobial resistance amongst farm strains of enteric zoonotic bacteria, such as
29 *E. coli* and thermotolerant *Campylobacter* spp., is of concern, particularly in view of
30 the risk it presents for human disease, persistent enteric colonisation and
31 (theoretically) transmission of resistance to other enteric bacteria
32 (ECDC/EFSA/EMA, 2015). *E. coli* is a ubiquitous enteric commensal in both human
33 and veterinary species, with a small subset of strains that present veterinary, human
34 and cross-species disease hazards due to particular colonisation factors and/or toxins
35 (Hartl and Dykhuizen, 1984). *Campylobacter* spp. are the most commonly identified
36 human gastrointestinal pathogens reported in the European Union, confirmed in over
37 220000 cases in 2011 (EFSA/ECDC, 2013).

38

39 In recent community-wide data from the European Union resistance to the
40 fluoroquinolone (FQ) antibiotic ciprofloxacin was found to be high (44% to 78% of
41 isolates overall, depending on source and subspecies) among *Campylobacter jejuni*
42 and *Campylobacter coli* isolates from human (mostly clinical) and broiler
43 (monitoring) sources (EFSA/ECDC, 2014). A survey of 145 *Campylobacter* spp.
44 isolates from human, milk, poultry and cattle sources in Italy similarly found 63%
45 exhibiting ciprofloxacin resistance but comparatively little resistance to other tested
46 antimicrobials, with the exception of tetracycline (Di Giannatale et al., 2014). A
47 survey in Chile revealed a similarly high frequency of ciprofloxacin resistance among
48 poultry and human *Campylobacter* spp. isolates (around 60%), whilst only 18% of
49 isolates from cattle were resistant (Gonzalez-Hein et al., 2013). For *Campylobacter*,
50 all these data are in the context of subtyping studies indicating that 50% to 80% of

51 human cases may be linked, directly or indirectly, to the chicken reservoir, and of FQ
52 being one of the principal drugs of choice for treating human campylobacteriosis
53 (Agunos et al., 2013; EFSA, 2010).

54

55 Aggregated European Community data for *E. coli* isolates from broilers showed,
56 similarly to *Campylobacter* spp., that over 50% of isolates were resistant to
57 ciprofloxacin (EFSA/ECDC, 2014). A sampling study provided evidence for the
58 dissemination of individual and multiple antimicrobial resistances in *E. coli* from
59 turkeys and broilers to their human handlers (van den Bogaard et al., 2001).

60 Furthermore, FQ-resistant isolates from human bacteraemias and faeces were found to
61 be more closely related to chicken isolates than to FQ-susceptible human isolates in
62 another study (Johnson et al., 2006).

63

64 Data from Australia, where FQ are restricted in the medical field and not used in food
65 animals, has shown that FQ resistance among human *Campylobacter* spp. isolates has
66 been slow to emerge, compared with other territories. Similarly, there is a low
67 frequency of FQ resistance among Australian human disease-causing *E. coli* isolates
68 (Cheng et al., 2012).

69

70 Attempts at restricting antimicrobial resistance on farms have included various
71 guidelines for the prudent use of veterinary antimicrobials (AAAP-AVMA, 2015;
72 OIE, 2014; RUMA, 2005). However, these have been based in large part upon expert
73 opinion, as published analyses of risk factors for the development of such resistances
74 are lacking.

75

76 The present report details a risk factor analysis performed following a survey for the
77 prevalence of FQ resistance among *E. coli* and *Campylobacter* spp. on poultry units in
78 the UK. Questionnaire data was used in conjunction with the prevalence results to
79 analyse FQ resistance with respect to a range of environmental and management
80 factors. The overall prevalence results for poultry and pigs and the analysis for risk
81 factors on pig farms have been reported elsewhere (Taylor et al., 2009, 2008)

82

83 **Materials and Methods**

84 **Data collection**

85 Two programmes of sampling were undertaken. For the first, 89 poultry meat farms
86 were included in a cross-sectional survey of FQ-resistant (FQr) *E. coli* and
87 *Campylobacter* spp., the details of which are described elsewhere (Taylor et al.,
88 2008). Briefly, 68 broiler and 21 turkey farms were each sampled once between June
89 2001 and June 2003, with 64 separate fresh floor droppings being collected from
90 random locations in up to four houses and combined into eight pools of eight samples
91 each. The sample size and sampling strategy were designed to give a 95% probability
92 of detecting resistant isolates if at least 5% of animals in the sampled houses were
93 shedding resistant bacteria and laboratory detection was 90% sensitive.

94

95 Sampling on poultry company premises was performed either by company-appointed
96 poultry veterinarians or by poultry company staff under the supervision of the
97 company veterinarian. Independent poultry producers (20 farms) carried out the
98 sampling themselves. To provide information on possible factors associated with
99 farms' FQ resistance status, data about husbandry practices, performance, disease and

100 drug use, including use of non-FQ antibiotics, were collected using detailed
101 questionnaires filled in by the farm manager with the veterinarian doing the sampling,
102 or by independent producers themselves. Data on antibiotic use was acquired, in the
103 large majority of cases and by all large units, by reference to detailed treatment
104 records in the farm diaries. These records are audited regularly for the purposes of
105 quality assurance and food chain protection.

106

107 The second (follow-up) programme investigated the potential for dissemination and
108 persistence of FQr organisms by carrying out farm-level sampling at representative
109 stages of breeding and production networks in two integrated companies. Faeces
110 sampling and data collection were carried out by the farm manager, according to the
111 protocols used for the first study, in five breeding flocks on repopulation, nine
112 breeding flocks in mid to late lay and 28 broiler flocks in mid to late rear. On a
113 selected proportion of sites where FQr organisms were found, intensive sampling was
114 performed by staff from the research team to investigate the distribution of resistant
115 *E. coli* on premises and to study their survival after cleaning and disinfection (C&D).
116 Samples taken on VLA sampling visits included faeces, water, dust and surface swabs
117 from building structures and equipment, as well as swabs from deep cracks in walls
118 and floors.

119

120 **Bacteriology**

121 Bacteriological analysis of faeces pools was performed using liquid media (buffered
122 peptone water [BPW] and Exeter's Enrichment Broth for *E. coli* and *Campylobacter*
123 spp., respectively) and selective solid media with added 1.0 mg/l ciprofloxacin
124 (Chromagar ECC for *E. coli*; sheep blood agar plus Skirrow's antibiotic supplement

125 and cefoperazone [BASAC] for *Campylobacter* spp.) as previously described (Taylor
126 et al., 2008). Farms were thus classified as ‘affected’ or ‘not affected’ with respect to
127 FQr *E. coli* or *Campylobacter* spp., using a selective concentration of ciprofloxacin
128 that is similar both to contemporaneous tentative breakpoints (Luber et al., 2003;
129 USDA, 2005), and the current European clinical breakpoint (EUCAST, 2014).
130 Putative *E. coli* colonies were confirmed using standard biochemical tests,
131 campylobacters were identified to species level by standard microbiological
132 procedures, and minimum inhibitory concentration (MIC) values of ciprofloxacin
133 were determined as described elsewhere (Taylor et al., 2008). Non-faeces samples
134 from intensive sampling visits **in the second sampling programme** were incubated in
135 approximately 10-fold volumes of BPW (225 ml for surface swabs) and incubated as
136 for faeces samples, before plating onto Chromagar ECC. Serotyping, toxin testing and
137 antibiograms (not including FQ) by the disc diffusion method were carried out using
138 standard protocols.

139

140 **Statistical analyses**

141 Statistical analyses were conducted using data from the first sampling programme
142 only. Associations between FQ use and farm types, and between FQ use and the
143 presence of FQr target organisms, were investigated using Chi-squared and Fisher’s
144 exact tests. Calculations of relative risks associated with reported FQ use, with 95%
145 confidence intervals, were carried out using EpiInfo version 6 (Centers for Disease
146 Control and Prevention U.S.A. & World Health Organisation, Geneva, Switzerland).

147

148 Correlation and cluster analyses and logistic regression modelling were carried out
149 using SAS version 8 (SAS, 1999). The approach taken was exactly the same as that
150 used in analysing data from pig farms (Taylor et al., 2009). Briefly, the questionnaire
151 data were first placed in blocks according to subject matter (e.g. farm characteristics,
152 farm hygiene, biosecurity, drug usage including other antibiotics) and then the
153 variables within each block were screened using Ward's minimum variance cluster
154 analysis to identify groups of related variables (Everitt, 1980; Ward, 1963). From each
155 group thus identified, a representative variable was selected (using epidemiological
156 significance plus data variability and completeness as criteria) as a candidate
157 explanatory variable in logistic regression modelling *within* each block of variables,
158 with the presence on a farm of FQr *E. coli* or FQr *Campylobacter* spp. as outcome
159 variables.

160 By this method a number of candidate explanatory variables were identified from
161 each block. These variables were re-analysed by Ward's minimum variance cluster
162 analysis regardless of their block of origin. Some variables closely correlated with
163 other, more epidemiologically pertinent, ones were removed from the analysis at this
164 stage. The retained candidate variables from all blocks were then tried together in
165 logistic regression modelling, with results given as a list of risk factors for occurrence
166 of FQ resistance in each bacterial species, quantified in terms of adjusted odds ratios.
167 An r^2 value, that estimates the proportion of variation in the data explained by the
168 model, was calculated for each model, according to the method of Nagelkerke (1991)
169 as recommended by Collett (2003).

170 **Results**

171 **Bacteriological findings**

172 *First sampling programme.* Findings have been reported in detail by Taylor *et al.*
173 (2008). FQr *E. coli* were isolated from 53 of the 89 farms. FQr *Campylobacter* spp.
174 were isolated from 20 of the 89 farms. Of tested isolates obtained from the 1.0 mg/ml
175 ciprofloxacin screening plates used, 79% of *E. coli* and 70% of *Campylobacter* spp.
176 isolates had MIC values for ciprofloxacin of 16 mg/l or greater.

177

178 *Second sampling programme.* Of the five breeding flocks tested on repopulation in
179 this follow-up investigation, none reported use of FQ during the previous six months
180 or yielded FQr *E. coli* or *Campylobacter* spp.. Among the nine breeding flocks tested
181 in mid- to late lay, FQr *E. coli* was isolated from two, but FQr *Campylobacter* spp.
182 was not isolated. One of these nine flocks reported FQ use (in one of two houses) in
183 the previous six months. Of the 28 broiler farms tested in mid-late rear, 25 yielded
184 FQr *E. coli*. No FQr *Campylobacter* spp. was isolated. FQ had been administered
185 during the previous six months on only one of these farms, in non-sampled parts of
186 the farm, and all samples from this farm yielded FQr *E. coli*.

187

188 Further intensive sampling visits, for FQr *E. coli*, were carried out at one of the mid-
189 lay breeding flocks, a linked company hatchery and after C&D on two of the
190 commercial broiler sites. From the breeding flock, FQr *E. coli* was isolated from 16 of
191 100 environmental samples. It was most frequently found in fresh faeces and litter
192 (rather than nest boxes), but also found in guttering and on the concrete apron outside
193 the house. At the hatchery, FQr *E. coli* was found in six of the 100 samples taken
194 from meconium and egg/chick waste, as well as on cleaned and disinfected surfaces.

195 On both post-C&D broiler farms, FQr *E. coli* was found in cracks and crevices,
196 pooled wash water, ante-rooms which had been less well disinfected and fresh
197 droppings from wild birds collected from the house exterior.
198
199 **Seventy two** *E. coli* strains from the second sampling programme were examined for
200 MIC and serotype. Isolates were from breeder units in mid-lay, broiler units and the
201 hatchery. Ciprofloxacin MIC values were ≥ 8 mg/l, with a modal value of 16 mg/l.
202 **Eight** serovars were identified, and **12** isolates proved untypable. There was no
203 overlap between identified serovars isolated from breeder versus broiler flocks. Thus,
204 there was no evident relationship between breeder and broiler isolates. One of the
205 three serovars isolated from the hatchery was associated with the breeder flocks, and
206 another with the broiler flocks.
207
208 From one company, *E. coli* O101:K+ (verocytotoxin-negative, MIC 32 mg/l) was
209 isolated in five broiler flocks in mid-late rear on one farm.. The same serovar was also
210 found on two other farms from the same company, in two sequential flocks on each
211 farm. FQr *E. coli* O9:K+ was isolated from two of the breeding flocks (MIC 8 mg/l)
212 and from a waste skip at the hatchery (MIC 16 mg/l). However, this serovar was not
213 amongst the isolates tested from broiler units within the company.
214
215 The **72 serotyped** *E. coli* isolates were also tested for resistance to antibiotics. Several
216 patterns were found, with resistance to ampicillin (86% isolates),
217 sulphamethoxazole/trimethoprim (65% isolates), tetracycline (67% isolates) and
218 streptomycin (43% isolates) being the most frequently encountered, in addition to
219 quinolone/FQ resistance.

220

221 **Use of antibiotics and risk of fluoroquinolone resistance on the**
222 **surveyed farms**

223 The questionnaire response options in relation to use of FQ on farms were: ‘within 12
224 months’, ‘between one and two years ago’, ‘over two years ago’ and ‘never’. The
225 responses are summarised in Table 1. Use of FQ was reported on 22 of 88 (25%)
226 poultry farms in the survey, with one no-response. FQ use was significantly (Chi²
227 $p < 0.0001$) more common on turkey farms (14/21) than on broiler farms (8/67).

228 Among the broiler farms, FQ use was significantly (Chi² $p < 0.0001$) more common
229 by independent producers (7/18) than by large poultry company farms (1/49).

230 Amongst turkey farms the most recent use had been within a year on nine of the 14
231 farms that reported use. On broiler farms, only two of the eight reporting use of FQ
232 had done so within the last year (Table 1). On all except one farm, FQ were
233 administered through water medication. In turkeys, the most common problem treated
234 with FQ was reported as being ‘*E. coli* septicaemia’. Amongst broilers, the most
235 common problems reportedly associated with FQ use were ‘yolk sac infections’ or
236 ‘stunted chicks’. Use, in the previous 12 months, of non-FQ antibiotics other than
237 amoxicillin (41% of farms), lincospectin (22% of farms) and tetracycline (10% of
238 farms) was uncommon. Just under one fifth of farms reported routine use of in-feed
239 antibiotic.

240

241 FQr *E. coli* or FQr *Campylobacter* spp. were detected on 19 (86%) of the 22 farms
242 that had used FQ and 40 (61%) of the 66 farms that reported never using FQ. The
243 prevalence of farms positive for FQr *E. coli* or FQr *Campylobacter* spp. was not
244 significantly different between farms with most recent use of FQ over one year ago,

245 compared with those using FQ within the last year. Therefore, farms where any FQ
246 use was reported were grouped together for comparison with those farms reporting
247 that they had never used FQ in further analyses. Table 2 shows the relative risks (with
248 95% confidence intervals) for the occurrence of FQ resistance on poultry farms,
249 associated with the use of FQ.

250

251 Overall within-farm prevalence values for FQr *Campylobacter* spp. and *E. coli* were
252 around 5% and 20% of faecal pools, respectively. On some premises, resistant
253 *Campylobacter* were shed by birds in only one or two houses, but there were others
254 where shedding birds were present across the farm. Birds shedding FQr *E. coli* tended
255 to be distributed throughout the houses on affected farms.

256

257 **Modelling of risk factors for the occurrence of FQ-resistance**

258 Correlation and clustering analysis revealed that farm type (turkey or broiler;
259 independent grower or large company) was strongly correlated with several of the
260 variables. Specifically:

- 261 • Turkey farms were strongly *positively* correlated with the use of FQ, cleaning
262 and disinfecting header tanks, seeing more than five rats at depopulation, the use
263 of plastic drinkers for chicks, and the use of growth promoters and tetracyclines.
- 264 • Turkey farms were strongly *negatively* correlated with single-handed operation,
265 enclosure by a perimeter fence, the provision of wheel dips, wild bird access to
266 poultry houses, the presence of dogs or cats, cleaning and disinfecting ante
267 rooms, feed hoppers and areas outside houses, and the use of nipple drinkers and
268 digestive enzymes.

269 • Independent farms were strongly *positively* correlated with the use of FQ, the
270 presence of dogs or cats, slaughtering birds at an older age and cleaning and
271 disinfecting ante rooms.

272 • Independent farms were strongly *negatively* correlated with the provision of
273 masks and wheel dips, seeing more than five rats at depopulation, cleaning and
274 disinfecting header tanks, and the use of digestive enzymes and growth
275 promoters.

276

277 In addition, the correlation analysis indicated the following:

278 • Single-handed farms tended not to have wheel dips.

279 • Farms enclosed by a perimeter fence tended to provide wheel dips and have
280 dogs or cats.

281 • Farms enclosed by a perimeter fence tended not to have big houses, tended not
282 to be turkey farms and, therefore, tended not to use growth promoters and
283 tetracycline.

284 • Larger farms tended to provide masks to staff.

285 • Dusting of all detailed areas was positively correlated with wet cleaning of all
286 detailed areas and removal of all wash water from the site.

287 • C&D of ante rooms was strongly positively correlated with C&D of feed
288 hoppers.

289 • In this particular sample of poultry farms, the variable ‘provision of a mask’ was
290 also positively correlated with provision of hat and gloves and provision of hand
291 sanitiser and provision of a toilet.

292

293 The turkey farm type was very strongly associated with the use of FQ. The turkey
294 farm variable itself was not significant in the final models. This implies that the
295 reason for the increased proportion of turkey farms with FQr *E. coli* or
296 *Campylobacter* spp., compared with broiler farms, as reported previously (Taylor et
297 al., 2008), is fully explained by other variables in the model, chiefly the use of FQ on
298 the farms.

299

300 The results of the final regression modelling are presented in tables 3 and 4 showing
301 the variables included as risk factors, estimates of coefficients with p-values, the
302 estimated adjusted odds ratios with 90% and 95% confidence intervals and the r^2
303 value.

304

305 Having fitted main effects, several interactions were identified as statistically
306 significant but inclusion of these in the regression models always resulted in estimates
307 for some odds ratios approaching infinity or zero. This was considered to be the result
308 of small sample sizes, such that inclusion of too many effects, notably the
309 interactions, produced models that were ‘over-fitted’, as described by Collett (2003).
310 To avoid the possibility of over-fitting and implausible interpretations, models were
311 finalised without interactions.

312

313 Table 3 provides a summary of the factors included in the final fitted logistic
314 regression model for the risk of occurrence of FQr *E. coli*. Significant factors
315 increasing risk are: use of FQ in past, single-handed operation of the site, and the
316 existence of a public footpath on the periphery of the site. The sole significant factor
317 decreasing risk is enclosure of the site by a perimeter fence. The r^2 value of the fitted

318 model is fairly low, which indicates that other, unidentified, explanatory risk factors
319 are likely to be involved.

320

321 Table 4 provides a summary of the factors included in the final fitted logistic
322 regression model for the risk of occurrence of FQr *Campylobacter* spp. Significant
323 risk factors increasing risk are: the use of FQ in the past and wild birds having access
324 to poultry houses. Significant factors decreasing risk are: more than the median (for
325 all broiler or turkey farms in the sample, as appropriate) number of birds on site, the
326 site operated by an independent grower, masks provided for staff, detailed areas
327 dusted before wet cleaning, and feed hoppers cleaned and disinfected.

328

329 The r^2 value is over 50%, indicating that the model provides a good explanation of
330 factors affecting the occurrence of FQ-resistant *Campylobacter* spp.. However, the
331 model is fitted with quite a large number of variables (seven) in relation to the dataset
332 size ($n = 84$) and is in danger of being ‘over-fitted’. The result of this is the relatively
333 wide confidence intervals for the adjusted odds ratios. Nevertheless, the fitted
334 variables are statistically significant. The conclusion is that the factors in the model
335 affect risk significantly, and perhaps greatly, but the data are not sufficient to allow
336 the risk effect to be quantified very precisely.

337

338 **Discussion**

339 The bacteriological findings of the initial survey (Taylor et al., 2008) and the follow-
340 up studies reported here have identified the frequent occurrence of *E. coli* and
341 *Campylobacter* spp. with FQ resistance on a substantial proportion of turkey and

342 broiler commercial production facilities. FQr *E. coli* were also isolated on breeding
343 flock premises. Moreover, the FQr *E. coli* and *Campylobacter* spp. typically exhibited
344 clinically-significant elevations in MIC values (Becnel Boyd et al., 2009; EUCAST,
345 2014) and the FQr *E. coli* often showed resistance to other classes of antimicrobial
346 agents. The present findings for *E. coli* are similar, in terms of frequency of isolation
347 on FQ resistance-selective media, MIC values observed, and common co-resistances
348 with other classes of antimicrobial drugs, with the findings of Gosling *et al.* (2012).
349 That study used UK-wide samples from turkey units taken for a European Union
350 baseline survey.

351

352 It was initially hypothesised that FQr organisms would be found on a small
353 percentage of farms, principally those where FQ were used. However, in the first
354 (structured) survey FQr organisms (mostly *E. coli*) were detected on a heavy majority
355 (86%) of farms that had used FQ in the past, and also on over half (61%) of the farms
356 that reported never using FQ. This finding is similar to that of a concurrent survey in
357 pig production (Taylor et al., 2009). A history of FQ use was associated with an
358 approximately doubled risk that FQr *E. coli* or *Campylobacter* spp. would be found on
359 a farm, and with the highest odds ratios among all the factors considered in the
360 logistic regression models for FQ resistance on farm.

361

362 The substantial prevalence of FQ resistance-affected farms that had never used FQ
363 suggests that FQr organisms may commonly be imported onto farms, either with
364 replacement birds in the case of *E.coli*, or from environmental sources in the case of
365 *Campylobacter* spp.. The persistence of such strains correlates with experimental data
366 suggesting little or no fitness cost associated with a moderate degree of FQ resistance

367 in *E. coli* (Schrag et al., 1997) and *Campylobacter* spp. (Q. Zhang et al., 2003). This is
368 consistent with the experience in countries where FQ are either prohibited or not
369 specifically licensed in poultry farming (USA, Canada and Denmark), where FQ
370 resistance among *Campylobacter* spp. isolates from poultry sources has not
371 consistently declined following cessation of FQ use in the sector (Agunos et al., 2013;
372 DANMAP, 2014).

373

374 There are, inevitably, some reasons to be careful in interpreting the present analysis.
375 The influence of co-resistance involving FQ resistance plus other antibiotics needs
376 some consideration, despite no significant associations being found between FQ
377 resistance on premises and recent use of a specific antibiotic class.

378

379 In *Campylobacter* spp., resistance to FQ typically is mediated by mutation of a
380 chromosomally-encoded topoisomerase, which is a mechanism specific to quinolone
381 antibiotics (Gyles, 2008; Qijing Zhang et al., 2003). This is augmented in some cases
382 by overexpression of the chromosomally-encoded multi-drug efflux pump CmeB
383 (Fàbrega et al., 2008). Therefore, clinical resistance to FQ is unlikely to occur
384 consequent upon use of a different antibiotic class or by introduction on mobile
385 genetic elements. However, as shown in the present study and elsewhere (Pérez-Boto
386 et al., 2013), FQ resistance in *Campylobacter* spp. from poultry farms is often
387 accompanied by other antibiotic resistances in the same isolates. *If FQ resistance is,*
388 *for whatever reason, more common amongst antibiotic-resistant strains than among*
389 *susceptible strains, then* co-selection by other antibiotics may maintain pre-existing
390 FQr strains for a prolonged period, especially if *, as appears to be the case,* the fitness
391 cost of FQ resistance *among *Campylobacter* spp.* is low (Luo et al., 2005). It is

392 therefore important to note that, whereas FQ resistance clearly has the potential to
393 persist in the absence of FQ use by co-selection, it seems unlikely to be present in the
394 first instance without either being introduced from elsewhere, or following selection
395 by FQ use.

396

397 For *E. coli*, the picture is perhaps more complicated. High-level FQ resistance is
398 firmly associated with topoisomerase mutation(s) (Fàbrega et al., 2008; Gyles, 2008;
399 Vanni et al., 2014), although intermediate resistance or enhancement of clinical
400 resistance is possible by chromosomal efflux pump upregulation and/or plasmid-borne
401 genes encoding target site protection (*qnr*), efflux (*qepA*) or FQ modification by an
402 aminoglycoside acetyltransferase (*aac(6')-Ib-cr*) (Fàbrega et al., 2008; Veldman et
403 al., 2011; Yue et al., 2008). Therefore, intermediate FQ susceptibility may be
404 introduced or maintained by horizontal transfer and/or co-selection by the use of other
405 antibiotic classes. However, no non-FQ antibiotics are likely to select the spontaneous
406 topoisomerase mutations fundamental to clinical resistance levels.

407

408 Although the prevalence of FQ resistance among contemporaneous diagnostic avian
409 samples of *E. coli* in the UK was low (around 2% to 6% depending on region and
410 source), resistances to commonly-used antimicrobials were more prevalent, in the
411 range 23% to 65% of isolates for ampicillin, amoxicillin, spectinomycin and
412 trimethoprim/ sulphonamide (Anon., 2007), consistent with the resistance findings in
413 the present study. This suggests that many FQ-resistant *E. coli* would also have had
414 resistance to other therapeutic antibiotics. Like *Campylobacter* spp., this might
415 facilitate co-selection of FQ resistance by other antibiotics but would not be expected
416 to generate *de novo* the clinical degree of resistance seen in the present study.

417 The second sampling programme and typing studies **reinforce the finding of the initial**
418 **survey** that the presence of FQr *E. coli* on a farm may not necessarily be related to
419 recent recorded use of FQ on the premises. The FQr *E. coli* isolated belonged to
420 numerous serogroups and had a range of different antibiograms, indicating that they
421 did not belong to a single clone. Furthermore, the FQr *E. coli* on the two farms tested
422 after C&D were able to persist in the environment and were a potential source of
423 infection for a new flock. A pertinent allied observation **from the initial survey** is that,
424 on farms where FQ had been used, there was no significant effect seen of the time
425 elapsed since last use upon the risk of FQ resistance. It is interesting to note in this
426 context that Ingram *et al.* (2013) isolated FQr *E. coli* harbouring multidrug-resistance
427 plasmids from chicken carcasses in Australia (a territory where FQ are not licensed
428 for poultry), thereby showing that topoisomerase-mutants may be present commonly
429 in products from apparently FQ-free systems.

430

431 The second sampling study also provided observational evidence that, for *E. coli* at
432 least, FQr strains potentially can transfer between broiler premises within integrated
433 operations, presumably via personnel and fomites. There was no evidence of vertical
434 transmission of FQr *E. coli* from breeder to broiler flocks, which may reflect the
435 biosecurity barrier that can be achieved between these levels of production by
436 hygienic hatchery management.

437

438 The differences in risk factors identified for the two bacterial genera examined may
439 reflect differences in the usual modes of transfer of these organisms between
440 locations. Interested readers are directed to Taylor *et al.* (2009) for discussion of the
441 merits and limitations of the statistical modelling approach of the present study. In

442 addition to FQ use and single-handed operation, the two variables identified as
443 significant risk factors for the occurrence of FQr *E. coli* were the existence of a
444 perimeter fence (protective) and of a public footpath (increasing risk). Thus, in
445 common with pig units, biosecurity appears to be of high importance for FQr *E. coli*.
446 For poultry the physical integrity of the farm limits seems to be of primary
447 significance, whereas for pigs the proximity of other pig units and visitor biosecurity
448 was found to be important (Taylor et al., 2009).

449

450 These differences in the most significant biosecurity barriers for pigs versus poultry
451 farms may to some extent reflect differences in the frequency of visitors and of feed
452 and stock transporters, differences in the housing systems, in the typical farm sizes,
453 and in the typical local environments. Whilst risk factor analysis may identify areas of
454 particular vulnerability or strength for particular enterprise types, examination of any
455 particular unit would sensibly include a comprehensive overview of biosecurity
456 issues, especially as the relatively low r^2 value for the *E. coli* model indicates other
457 significant unidentified risk factors that may not be common to all or most units.

458

459 For *Campylobacter* spp., the risk factor model for the occurrence of FQ resistance
460 indicates the importance of farm hygiene, perhaps reflecting the greater importance of
461 shorter-range transmission between animals for this more environmentally labile
462 pathogen when compared with *E. coli*. One protective factor of particular interest was
463 provision of a mask. This factor was positively correlated with, and effectively a
464 proxy variable for, other factors including the provision of hand sanitisers, a toilet,
465 hats and gloves. The inclusion of this factor in the model can be taken as indication of
466 the protective effect of better hygiene facilities in general.

467

468 The significantly protective variables regarding dusting (of several difficult or
469 inaccessible parts of poultry houses before wet cleaning) and C&D of feed hoppers
470 are interpreted as indicators of generally superior farm cleaning. *Campylobacter* are
471 frequently recovered from puddles and other wet locations on farms, but typically not
472 from dry materials. The findings indicate the importance of attention to detail when
473 cleaning between crops, presumably by preventing carry-over of infection,
474 particularly of *Campylobacter* spp., between batches of stock.

475

476 The introduction of *Campylobacter* spp. (including, potentially, FQR strains) to a
477 poultry flock or premises is considered to be a more important issue than carry-over,
478 and may occur following the repeated entrance of staff with contaminated clothing,
479 hands or equipment (Newell et al., 2011). The risks of acquisition of *Campylobacter*
480 spp. by flocks before slaughter are related to several factors including: season, on-
481 farm hygiene, other animal species on the farm, more than one poultry house per
482 stockperson, thinning of slaughter-age flocks by catching crews and features of the
483 farm environmental surroundings, as reviewed by Vidal *et al.*, (2014). However,
484 Refregier-Petton *et al.* (2001) reported a risk factor analysis for the presence of
485 *Campylobacter* spp. in broilers at slaughter using a similar methodology to the present
486 one and found, amongst other things, that no specific stockperson hygiene practices
487 were significant. Discrepancies noted in that report between claimed and observed
488 hygiene practices may explain this finding, and its apparent lack of concordance with
489 the present evidence.

490

491 The transmission of FQr *Campylobacter* spp., and probably of *Campylobacter* spp.
492 more generally, may also be associated with wildlife vectors. Remarkable suppression
493 of seasonal peaks in flock *Campylobacter* spp. colonisation has been demonstrated, in
494 the context of good general hygiene, following the use of mesh screens to exclude
495 wildlife down to the level of flying insects from broiler houses (Bahrndorff et al.,
496 2013). The factor, ‘saw more than five rats at last depopulation’ was associated with
497 an increased risk, but was not significant in the final model. Access to the poultry
498 houses by wild birds was a significant factor for increasing risk in the final model,
499 with a large odds ratio. It has been documented that wild birds carry *Campylobacter*
500 spp., including FQr strains (Broman et al., 2002; Waldenstrom et al., 2005), although
501 wild bird strains generally differ from poultry and human strains (Broman et al.,
502 2004). Access by wild birds may be indicative of poorer biosecurity with respect to
503 wildlife more generally.

504

505 In conclusion, the present investigations have illustrated the strong association
506 between any use of FQ on poultry farms and the presence of *E. coli* and/or
507 *Campylobacter* spp. with clinically-relevant levels of resistance to FQ on the same
508 premises. Furthermore, the introduction or maintenance of FQr organisms on farms
509 appears significantly influenced by farm hygiene (*Campylobacter* spp.) and boundary
510 biosecurity (*E. coli*), with evidence also being found of cross-transfer of FQr *E. coli*
511 between premises linked in the production system. As has been discussed elsewhere
512 (Taylor et al., 2008), both *E. coli* and *Campylobacter* spp. are zoonotic organisms for
513 which FQ are therapeutic agents in humans. It appears, on the present evidence, to be
514 difficult for farms that use FQ to avoid the development of FQ-resistant *E. coli* and
515 *Campylobacter* spp. on farm. However, for those farms that do not use FQ, an

516 emphasis on excellence in biosecurity and on-farm hygiene is likely to prove
517 protective. The benefits of such a strategy are likely to extend to control or exclusion
518 of some other infectious agents also. This is in line with guidelines produced by the
519 UK ‘Responsible Use of Medicines in Agriculture Alliance’ (RUMA;
520 <http://www.ruma.org.uk>), which stress that the use of antimicrobials should be seen as
521 complementing good management, vaccination and site hygiene.

522

523

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529

530 **Conflicts of interest**

531 None

532

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688

Table 1: Detection of fluoroquinolone (FQ)-resistant bacteria on poultry farms, compared with reported use of FQ

Last use of FQ antibiotics	Broiler farms	Turkey farms	All farms	Number with FQ resistance	
				<i>E. coli</i>	<i>Campylobacter</i>
In last year	2	9	11	10 (91%)	4 (36%)
Over 1 year ago	6 ^a	5 ^b	11	9 (82%)	4 (36%)
Never used	59	7	66	33 (50%)	11 (17%)

a: 2 of 6 reported most recent use over 2 years ago

b: 1 of 5 reported most recent use over 2 years ago

Table 2: Relative risks (with 95% confidence intervals) for the occurrence of fluoroquinolone (FQ) resistance on poultry farms, associated with the reported use of FQ

	Proportion of farms with FQ resistance	
	<i>E. coli</i>	<i>Campylobacter</i>
FQ used (n = 22)	0.86	0.36
FQ never used (n = 66)	0.50	0.17
Relative Risk (95% C.I.)	1.73 (1.29 – 2.32)	2.18 (1.01 – 4.72)

Table 3: Estimated adjusted odds ratios, with confidence intervals (C.I.s), of variables included as risk factors in the final logistic regression model for the occurrence of fluoroquinolone (FQ)-resistant *E. coli* on poultry farms

Risk Factor	co-efficient	p-value*	Lower Limit C.I.s		Odds ratio point estimate	Upper Limit C.I.s	
			95%	90%		90%	95%
<i>Constant</i>	- 0.204	0.6294					
Use of FQ in the past	2.049	0.0016	1.85	2.31	7.76	26.04	32.48
Site operated single-handedly	0.948	0.073	0.89	1.06	2.58	6.30	7.46
Site enclosed by a perimeter fence	- 1.302	0.014	0.09	0.11	0.27	0.67	0.79
Site has public footpath on the perimeter	1.407	0.019	1.17	1.43	4.09	11.67	14.20

n = 83; maximum re-scaled $r^2 = 29.9\%$

*p-value is based on likelihood ratio test.

Table 4: Estimated adjusted odds ratios of variables, with confidence intervals (C.I.s), of variables included as risk factors in the final logistic regression model for the occurrence of fluoroquinolone (FQ)-resistant *Campylobacter* spp. on poultry farms

Risk Factor	co-efficient	p-value*	Lower limit C.I.s		Odds ratio point estimate	Upper limit C.I.s	
			95%	90%		90%	95%
<i>constant</i>	1.476	0.2387					
Use of FQ at any time in past	2.685	0.0052	1.64	2.32	14.65	92.59	130.59
No. of birds on site higher than median	- 2.182	0.0097	0.02	0.024	0.11	0.54	0.73
Site owned by an independent grower	- 3.156	0.0031	0.00	0.005	0.04	0.36	0.54
Masks provided for staff	- 1.412	0.081	0.05	0.062	0.24	0.96	1.24
All detailed areas are dusted	- 2.147	0.0089	0.02	0.026	0.12	0.52	0.69
Feed hoppers cleaned and disinfected	- 1.684	0.061	0.03	0.041	0.19	0.85	1.13
Wild birds have access to poultry houses	2.332	0.017	1.40	1.91	10.30	55.46	76.05

n = 84; maximum re-scaled $r^2 = 56.3\%$

*p-value is based on likelihood ratio test.