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

N. M. Taylor[1], A. D. Wales[2], A. M. Ridley[2] and R. H. Davies[2]

[1]Veterinary Epidemiology and Economics Research Unit, School of Agriculture, Policy and Development, University of Reading, Earley Gate, P.O. Box 237, Reading, Berkshire RG6 6AR, UK
[2]Department of Bacteriology and Food Safety, Animal and Plant Health Agency – Weybridge, New Haw, Addlestone, Surrey KT15 3NB

Correspondence & reprints:
Nick M. Taylor, Veterinary Epidemiology and Economics Research Unit, School of Agriculture, Policy and Development, University of Reading, Earley Gate, P.O. Box 237, Reading, Berkshire RG6 6AR, UK
Tel: +44 (0) 118 931 6633
Fax: +44 (0) 118 926 2431
Email: n.m.taylor@reading.ac.uk

Running head: Fluoroquinolone resistance in poultry

Summary

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

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

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

Introduction

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

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

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

Aggregated European Community data for *E. coli* isolates from broilers showed, similarly to *Campylobacter* spp., that over 50% of isolates were resistant to ciprofloxacin (EFSA/ECDC, 2014). A sampling study provided evidence for the dissemination of individual and multiple antimicrobial resistances in *E. coli* from turkeys and broilers to their human handlers (van den Bogaard et al., 2001). Furthermore, FQ-resistant isolates from human bacteraemias and faeces were found to be more closely related to chicken isolates than to FQ-susceptible human isolates in another study (Johnson et al., 2006).

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

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

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

Materials and Methods

Data collection

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

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

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

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

Bacteriology

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

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

Statistical analyses

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

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

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

Results

Bacteriological findings

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

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

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

On both post-C&D broiler farms, FQr *E. coli* was found in cracks and crevices, pooled wash water, ante-rooms which had been less well disinfected and fresh droppings from wild birds collected from the house exterior.

Seventy two *E. coli* strains from the second sampling programme were examined for MIC and serotype. Isolates were from breeder units in mid-lay, broiler units and the hatchery. Ciprofloxacin MIC values were ≥ 8 mg/l, with a modal value of 16 mg/l. Eight serovars were identified, and 12 isolates proved untypable. There was no overlap between identified serovars isolated from breeder versus broiler flocks. Thus, there was no evident relationship between breeder and broiler isolates. One of the three serovars isolated from the hatchery was associated with the breeder flocks, and another with the broiler flocks.

From one company, *E. coli* O101:K+ (verocytotoxin-negative, MIC 32 mg/l) was isolated in five broiler flocks in mid-late rear on one farm.. The same serovar was also found on two other farms from the same company, in two sequential flocks on each farm. FQr *E. coli* O9:K+ was isolated from two of the breeding flocks (MIC 8 mg/l) and from a waste skip at the hatchery (MIC 16 mg/l). However, this serovar was not amongst the isolates tested from broiler units within the company.

The 72 serotyped *E. coli* isolates were also tested for resistance to antibiotics. Several patterns were found, with resistance to ampicillin (86% isolates), sulphamethoxazole/trimethoprim (65% isolates), tetracycline (67% isolates) and streptomycin (43% isolates) being the most frequently encountered, in addition to quinolone/FQ resistance.

Use of antibiotics and risk of fluoroquinolone resistance on the surveyed farms

The questionnaire response options in relation to use of FQ on farms were: 'within 12 months', 'between one and two years ago', 'over two years ago' and 'never'. The responses are summarised in Table 1. Use of FQ was reported on 22 of 88 (25%) poultry farms in the survey, with one no-response. FQ use was significantly (Chi^2 $p < 0.0001$) more common on turkey farms (14/21) than on broiler farms (8/67). Among the broiler farms, FQ use was significantly (Chi^2 $p < 0.0001$) more common by independent producers (7/18) than by large poultry company farms (1/49). Amongst turkey farms the most recent use had been within a year on nine of the 14 farms that reported use. On broiler farms, only two of the eight reporting use of FQ had done so within the last year (Table 1). On all except one farm, FQ were administered through water medication. In turkeys, the most common problem treated with FQ was reported as being '*E. coli* septicaemia'. Amongst broilers, the most common problems reportedly associated with FQ use were 'yolk sac infections' or 'stunted chicks'. Use, in the previous 12 months, of non-FQ antibiotics other than amoxicillin (41% of farms), lincospectin (22% of farms) and tetracycline (10% of farms) was uncommon. Just under one fifth of farms reported routine use of in-feed antibiotic.

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

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

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

Modelling of risk factors for the occurrence of FQ-resistance

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

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

- Independent farms were strongly *positively* correlated with the use of FQ, the presence of dogs or cats, slaughtering birds at an older age and cleaning and disinfecting ante rooms.
- Independent farms were strongly *negatively* correlated with the provision of masks and wheel dips, seeing more than five rats at depopulation, cleaning and disinfecting header tanks, and the use of digestive enzymes and growth promoters.

In addition, the correlation analysis indicated the following:

- Single-handed farms tended not to have wheel dips.
- Farms enclosed by a perimeter fence tended to provide wheel dips and have dogs or cats.
- Farms enclosed by a perimeter fence tended not to have big houses, tended not to be turkey farms and, therefore, tended not to use growth promoters and tetracycline.
- Larger farms tended to provide masks to staff.
- Dusting of all detailed areas was positively correlated with wet cleaning of all detailed areas and removal of all wash water from the site.
- C&D of ante rooms was strongly positively correlated with C&D of feed hoppers.
- In this particular sample of poultry farms, the variable ‘provision of a mask’ was also positively correlated with provision of hat and gloves and provision of hand sanitiser and provision of a toilet.

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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Conflicts of interest

None

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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	
				E. coli	*Campylobacter*
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	
	E. coli	*Campylobacter*
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 95%	Lower Limit C.I.s 90%	Odds ratio point estimate	Upper Limit C.I.s 90%	Upper Limit C.I.s 95%
Constant	- 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 95%	Lower limit C.I.s 90%	Odds ratio point estimate	Upper limit C.I.s 90%	Upper limit C.I.s 95%
constant	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.