

## ***Campylobacter* in housed broiler chickens: a longitudinal study of risk factors**

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### **SUMMARY**

Infections by *Campylobacter* spp. are a major cause of gastrointestinal disease in the United Kingdom. Most cases are associated with the consumption of chicken that has become contaminated during production. We investigated the epidemiology of *Campylobacter* spp. in chickens in a 3-year longitudinal study of flocks reared on 30 farms in the United Kingdom. We used Generalized Linear Mixed Effect Models (GLMM) to investigate putative risk factors associated with incidence and prevalence of flock infection arising from farm and flock management and local environmental conditions during rearing. We used survival analysis to investigate infection events and associated risk factors over the course of the study using two marginal models – the independent increment approach, which assumed that individual infection events were independent; and a conditional approach, which assumed that events were conditional on those preceding. Models of flock prevalence were highly overdispersed suggesting that infection within flocks was aggregated. The key predictors of flock infection identified from the GLMM analyses were mean temperature and mean rainfall in the month of slaughter and also the presence of natural ventilation. Mean temperature in the month of slaughter was also a significant predictor of flock infection, although the analyses suggested that the risk in flocks increased in a unimodal way in relation to temperature, peaking at 12 °C. The extent of pad burn was also identified as a predictor in these analyses. We conclude that predicting prevalence within flocks with linear modelling approaches is likely to be difficult, but that it may be possible to predict when flocks are at risk of *Campylobacter* infection. This is a key first step in managing disease and reducing the risks posed to the human food chain.

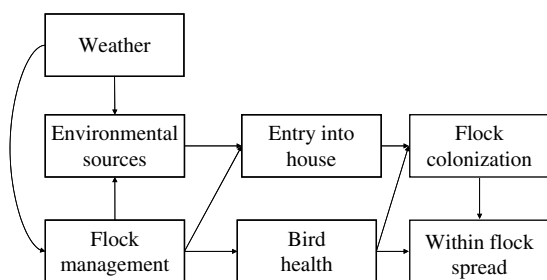
**Key words:** *Campylobacter*, mixed effect models, survival analysis.

### **INTRODUCTION**

Infections by *Campylobacter* spp. are the single biggest cause of bacterial gastroenteritis in the developed world. Reported cases exceeded 46 000 in the United

Kingdom in 2004 [1] and there is evidence that disease is under-reported [2, 3]. Cases of *Campylobacter* infections in The Netherlands alone have been estimated at costing €21–36 million annually [4]. Identification of *Campylobacter* spp. as pathogens was noted in the 1970s [5], and the subsequent search for cause of infection in humans identified poultry as a risk factor over 20 years ago [1]. Production of broilers for the

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**Fig. 1.** Causal path map showing likely pathways to infection of broiler chickens by *Campylobacter*.

United Kingdom in 2002 was about 1.2 million tonnes of carcasses [6]. A survey of meat on retail sale in the United Kingdom in 2002 [7] found that between 46% and 52% of raw poultry was contaminated with *Campylobacter*. On this basis, potential sources of entry of the bacteria into the human food chain are great. Although negative flocks can be cross contaminated with *Campylobacter* during poultry processing, the number of organisms on these carcasses is far less than on carcasses originating from *Campylobacter*-positive flocks [8]. Thus the farm on which they are reared is the most important place for control to reduce carcass contamination. Identifying risk factors for infection of chickens on the farm is the first point for addressing this problem.

There have been many studies of risk factors for *Campylobacter* in broilers and a key feature of many of these is the collation of data describing the prevalence of infection in flocks and measures of a series of putative covariates deemed to play a role in this process. Many factors have been identified as contributing to risk of infection and these factors are associated with processes that operate over several scales. Many of these factors interact, with processes at one scale impacting on factors more proximal to the infection of the individual broiler chicken, indicating that pathways to infection are multifactorial and complex (Fig. 1). *Campylobacter* forms long-term associations with its host [9] and the organism is found in a wide variety of wild mammals and birds and the faeces they produce. The outside environment therefore acts as the ultimate source of infection for housed broiler flocks and unsurprisingly flock infection has been related to these external sources, such as the presence of livestock outside [10–14] and wild rodents [15]. However, the incidence of infection in humans [16, 17] and broilers [18] also shows seasonality. In humans cases peak in spring, earlier following mild winters [16]. This suggests that larger scale processes involving weather and climate impact on

the sources and survival of the pathogen in the outside environment. Weather may also have indirect effects on the processes involved in introduction of the pathogen to the broiler house, since the activity of fly vectors is dependent on temperature. Management of the external environment and the broiler house itself also impact on the incidence of infection. External manure storage has been recorded as having a protective [19] as well as promoting effect [11], although the effects in the former case may have been related to local storage practice rather than a beneficial effect of storage *sensu lato*. Risk of infection has also been positively associated with the size of the enterprise, specifically the number of sheds and the size of the flocks [19]. Internal management within the shed may also mitigate the impacts of environmental sources through such activities as disinfection [15, 20] and removal of internal sources of infection such as dead birds [20] and ventilation [21]. Broiler houses are likely to be continuously surrounded by many potential sources of contamination and, as such, there are many risk factors that could contribute to individual infection events. Disentangling cause and effect is made complex because of the interaction of the processes over different scales. One approach that might provide more insight into the relative significance of different risk factors would be to focus on longitudinal studies of incidence of infection on a restricted number of farms, where many of the covariates associated with environmental risk can be measured, held constant or accounted for in the design of the monitoring. A longitudinal study allows for investigation of fixed farm and house effects, and analyses of seasonality all of which have been previously associated with *Campylobacter* infection in poultry [17].

We recently completed a large study on the incidence of *Campylobacter* in 789 housed broiler flocks in Great Britain. Aspects of that study will be published elsewhere and will include an assessment of the impact of bird health and season. Flocks from 214 farms were examined at least once. However, 30 farms were tested up to 13 times. In the present study we analyse data on the incidence of *Campylobacter* in chickens from this longitudinal subset of farms from one integrated poultry company using Generalized Linear Mixed Effect Models (GLMMs) and survival analysis. We used GLMMs to investigate the extent to which the occurrence of *Campylobacter* infection in broiler flocks was related to environmental and management risk factors. We then used survival analysis to analyse the time to infection using two underlying

conceptual models. In the first of these we tested the extent to which infection events on farms were opportunistic and independent, whilst in the second we tested the extent to which events were predicated by previous events.

## METHODS

### Study population and data collection

The sample design for this study follows that described in Bull *et al.* [22] and differs from it insofar as only those farms subjected to a sequence of repeated sampling are considered here. Briefly, samples of chickens were collected from indoor flocks reared on 30 farms supplying one integrated poultry company over the period 2004–2006. The majority of flocks sampled were Ross or Cobb standard broilers. Where possible, farms were visited in successive crop cycles. In practice this was not possible and the dataset was unbalanced with the maximum number of flocks per farm sampled ranging from six to 13 times.

Thirty birds were sampled at random at slaughter from each flock at first full or partial harvest and intact pairs of caeca were collected from each immediately after evisceration. Each bird was tested separately for the presence of *Campylobacter*. This sampling protocol allowed us to detect infection where the prevalence was >10% in the flock with 95% confidence [23]. In our study, caecal contents from each pair collected were sampled by immersing a swab (Medical Wire and Equipment, Wiltshire, UK) into a caecum, which was opened by an incision at the blind end. The swab was streaked onto modified charcoal cefoperazone deoxycholate agar (mCCDA) (Oxoid CM739 with SR155 supplement), which was incubated at 37 °C for 48 h in a microaerobic atmosphere. The atmosphere was achieved by evacuating the air from gas jars (Don Whitley Scientific Ltd, West Yorkshire, UK; Launch Diagnostics Ltd, Kent, UK) and replacing it with a gas mixture that resulted in an atmosphere comprising: 5–6% O<sub>2</sub>, 3–7% CO<sub>2</sub> and 7% H<sub>2</sub>, in a balance of nitrogen. After incubation, mCCDA plates were examined for the presence of typical *Campylobacter* colonies (greyish, flat and moist colonies, with a tendency to spread). One to three typical colonies per sample were subcultured onto duplicate plates of Columbia blood agar with 5% (v/v) defibrinated horse blood (COLBA; Oxoid Ltd, Hants, UK) and confirmed as *Campylobacter* using standard tests.

Table 1. *List of covariates collected from farms*

Locality
Outward postcode (first part of postcode)
Environment
Mean temperature in the month of bird placement
Mean temperature in the month of bird slaughter
Total rainfall in the month of bird placement
Total rainfall in the month of bird slaughter
Month from start of study (March 2003 = 1)
Management
Number of broiler houses on farm
Number of people entering individual houses
Food and rearing regime – freedom foods, standard
Method of removal of dead birds from houses
Type of ventilation (natural, fan-assisted, side, roof vents, inlets)
Type of drinker (drinking cups, nipples, bells)
Number of chickens on farm per crop cycle
Livestock presence on farm
Manure presence on farm

### Selection of covariates

We selected covariates for inclusion in the modelling on the basis of their reported role in colonization and subsequent within-flock spread and their likely theoretical involvement in both processes. The key variables were potential sources of *Campylobacter* in the outside environment (land use patterns, dung heaps, etc.); weather which might impact on survival of *Campylobacter* in the environment or influence ingress to broiler houses (specifically temperature and rainfall); and management factors that might allow ingress of the bacteria (people entering the house) and those that might accentuate spread amongst birds in the house (the form of water provision). These farm- and flock-specific covariates were collected at the time of flock harvest and they included indicators of bird health collected post mortem which has been considered a risk factor for colonization and spread (see Table 1 and ref. [22]).

### Data analysis

We used GLMMs and survival analysis to investigate the longitudinal nature of infection in chicken flocks. In the mixed-effect modelling we undertook two types of analysis. First we used the number of positive cases out of 30 birds tested in each flock as an indicator of flock prevalence of infection. We did not use a Poisson error structure in these models because the number of chickens sampled was finite and all chickens in the sample on some farms were colonized

with *Campylobacter* (offending the underlying assumptions of the Poisson model). Second, we used the presence or absence of infection in a flock as a categorical variable, in effect a simple descriptor of whether or not the flock was infected. Therefore, the flock prevalence analyses were undertaken with binomial error structures, the former with numbers of cases and a denominator of total number of birds tested, the latter simply a binomial trial of yes/no the flock being infected. We adopted a progressive model-building strategy following Pinheiro & Bates [24]. We first used a step-down modelling strategy with simple binomial regression to identify covariates associated with the presence of *Campylobacter* in chickens and flocks (with binomial error structure and relevant weights), removing those that were non-significant in simple models. Models and parameter estimates derived with this analysis might be biased because of serial dependency in the response variable and the potential impact of random effects arising from unmeasured farm-specific factors. We went on to investigate farm effects as a factor using GLMMs with a binomial error structure and the inclusion of different random effects for the intercepts at the level of the farm. We then tested whether the inclusion of random effects for individual covariates on farms improved model fit under the assumption that the response to the covariates varied with farm. We assessed the extent to which there was serial correlation in the data by assessing autocorrelation in the models and then re-fitted the models with a correlation structure allowing for serial dependence where appropriate. We assessed the distributional assumptions of the models by analysis of the residuals, and abandoned models if distributional assumptions were not met. Models were fitted in the statistical package R [25] using `glm` and the `glmmML` library of Broström [26].

In order to investigate the hazard of farms becoming infected with *Campylobacter* over time, we used Cox proportional hazard models to assess the extent to which the timing of infection events on farms could be explained in terms of the measured covariates. In these analyses we assumed that the detection of *Campylobacter* in a flock at slaughter constituted an ‘event’. The Cox model assumes that there is an underlying unspecified baseline hazard of events occurring, which stays constant through time (in this case of farms becoming infected) that is influenced by covariates that enhance or mitigate the risk of the event occurring. We used three models that made different assumptions about the infection process on

the farms. We used time to the first event as a baseline model. We investigated the effects of event number on individual farms as a covariate. We then compared the baseline model against two models which treated incidents of infection at the flock level as ordered events. In the first of these we assumed that each infection event on a farm was independent of the others; the independent increment or Andersen–Gill model. In the second we used a conditional model in which it was assumed that infection events were predicated by preceding events (the conditional or Prentice–Williams–Petersen model). The two models effectively assess the dependency of infection events on individual farms. The independent increment and conditional models treat the data as time-ordered outcomes and differ in their use of stratification [27]. In the latter model the data are stratified by the event number, i.e. the sequential numbering of infected status since the start of the study. We used step-wise reduction to identify the parsimonious model from a full model with all covariates. We tested assumptions of proportionality in the parsimonious models in two ways. First, we plotted time-dependent coefficients for each covariate against time and assessed the change in coefficients with time visually. Second, we undertook a formal test correlating the scaled Schoenfeld residuals for each model with time for each covariate and assessed significance with a two-sided  $\chi^2$  test (significance denoting evidence for deviation from a constant hazard of infection in relation to that covariate through time). All models were fitted with the Survival library of Therneau in R [25].

## RESULTS

Of the flocks from 30 farms tested, four farms never produced a *Campylobacter*-positive flock. A total of 289 flocks were sampled, of which 94 (32.5%) were positive for *Campylobacter*. The number of *Campylobacter*-positive flocks recorded over the study period at each farm ranged from two to 10 (median 3). The distribution of the within-flock prevalence data had a tendency to bimodality with numbers of positive birds in individual flocks being low (mean of 3.94 out of 30) or being very high with all animals testing positive.

### Incidence of *Campylobacter* at farm level

Results of a simple GLM analysing the incidence of *Campylobacter* at the level of the farm, with a

Table 2. Regression diagnostics for a GLM relating presence or absence of *Campylobacter* infection in individual flocks to different covariates for 289 flocks sampled from 30 farms, 2003–2006

	Estimate	S.E.	z value	Pr(> z )
(Intercept)	<b>-8.771 × 10</b>	<b>3.712 × 10</b>	<b>-2.363</b>	<b>0.01815</b>
Month from start	<b>-8.438 × 10<sup>-2</sup></b>	<b>2.865 × 10<sup>-2</sup></b>	<b>-2.945</b>	<b>0.00323</b>
Number of sheds	-2.552 × 10 <sup>-1</sup>	1.330 × 10 <sup>-1</sup>	-1.923	0.05448
Food regime (freedom foods)	-6.232 × 10 <sup>-1</sup>	7.308 × 10 <sup>-1</sup>	-0.856	0.39185
Food regime (standard)	-9.242 × 10 <sup>-1</sup>	8.416 × 10 <sup>-1</sup>	-1.098	0.30211
Age at slaughter	1.047 × 10 <sup>-1</sup>	6.838 × 10 <sup>-2</sup>	1.531	0.12568
Chickens on farm	<b>1.761 × 10<sup>-5</sup></b>	<b>7.914 × 10<sup>-6</sup></b>	<b>2.225</b>	<b>0.02610</b>
Mean temperature at month of slaughter	<b>1.939 × 10<sup>-1</sup></b>	<b>7.631 × 10<sup>-2</sup></b>	<b>2.541</b>	<b>0.01106</b>
Rain at slaughter	<b>9.647 × 10<sup>-3</sup></b>	<b>4.062 × 10<sup>-3</sup></b>	<b>2.375</b>	<b>0.01757</b>
Mean temperature at month of placement	1.045 × 10 <sup>-1</sup>	7.175 × 10 <sup>-2</sup>	1.456	0.14541
Rain at placement	6.631 × 10 <sup>-4</sup>	4.654 × 10 <sup>-3</sup>	0.142	0.88669
Livestock present	<b>1.540 × 10</b>	<b>7.129 × 10<sup>-1</sup></b>	<b>2.160</b>	<b>0.03077</b>
People entering house	7.934 × 10 <sup>-2</sup>	1.408 × 10 <sup>-1</sup>	0.564	0.57301
Surrounding manure	4.177 × 10 <sup>-1</sup>	3.666 × 10 <sup>-1</sup>	1.139	0.25450
Ventilation type: natural	<b>9.512 × 10<sup>-1</sup></b>	<b>4.763 × 10<sup>-1</sup></b>	<b>1.997</b>	<b>0.04583</b>
Water supply: drinking cup	5.022 × 10 <sup>-1</sup>	4.075 × 10 <sup>-1</sup>	1.232	0.21787

Null deviance: 364.59 on 288 degrees of freedom; residual deviance: 255.77 on 303 degrees of freedom; Akaike's Information Criterion: 287.77.

Significant covariates are shown in bold.

Table 3. Regression diagnostics for a parsimonious GLM relating presence or absence of *Campylobacter* infection in individual in flocks to different covariates for 289 flocks sampled from 30 farms, 2003–2006

	Estimate	S.E.	z value	Pr(> z )
Intercept	-2.83126	0.72903	-3.884	0.000103
Month from start	-0.06258	0.02178	-2.873	0.004066
Number of houses	-0.13688	0.07245	-1.889	0.058869
Mean temperature at month of slaughter	0.22208	0.03918	5.668	1.44 × 10 <sup>-8</sup>
Rain at slaughter	0.01144	0.00307	3.728	0.000193
Ventilation type: natural	1.30389	0.41429	3.147	0.001648

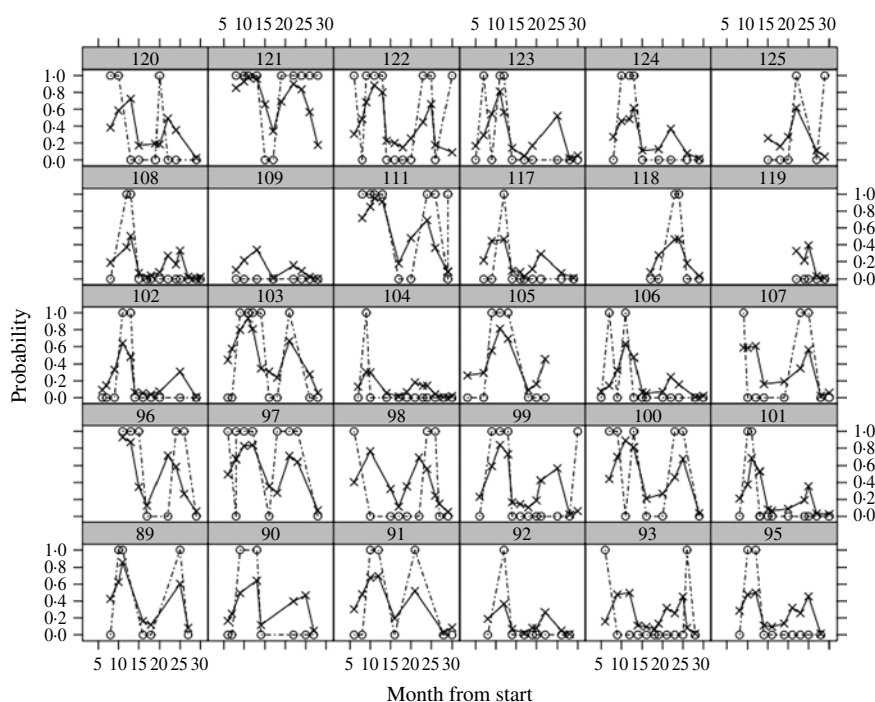
Null deviance: 364.59 on 288 degrees of freedom; residual deviance: 283.64 on 283 degrees of freedom; Akaike's Information Criterion: 295.64.

binomial error structure (Table 2) suggested that incidence of infection declined with time and that weather conditions during the month of slaughter were important predictors. The residual deviance for the model was similar to the number of degrees of freedom suggesting that the binomial error model was a reasonable error structure for the data. The residuals from the parsimonious model (Table 3) containing the mean monthly temperature at slaughter and the total rainfall during the month of slaughter and the use of natural ventilation, were not serially correlated when farm was included as a factor, suggesting that the incidence of *Campylobacter* in a

flock was not dependent on previous incidence of the pathogen. The results of the parsimonious GLMM, where farm was included as a random effect, are shown in Table 4. The mean monthly temperature and total rainfall during the month of slaughter, the use of natural ventilation, the use of nipples with drinking cups and the month from the start of the study were significant predictors. None of these variables were significant when modelled as random effects at the farm level; the best model included random effects for intercept only. This means that there were no differences in the farm-level response to the driving variables, but only differences in the

Table 4. Parsimonious binomial GLMMs relating incidence of *Campylobacter* spp. (presence or absence in flock) in 289 flocks sampled from 30 farms, 2003–2006

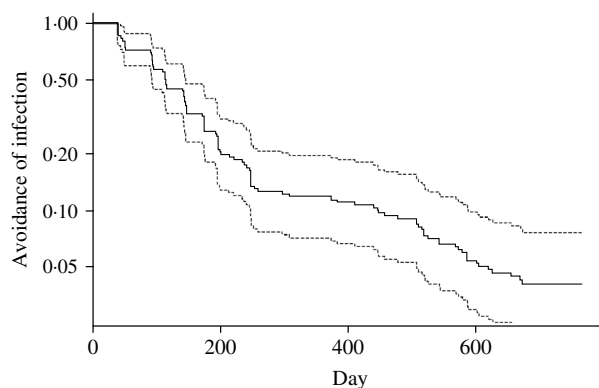
Random effects (intercept for farm) Standard deviation	Intercept	Residual			
	Value	S.E.	D.F.	<i>t</i> value	Pr(>   <i>z</i>  )
Intercept	−4.285054	0.7760265	256	−5.521788	0.0000
Month from start	−0.071552	0.0223273	256	−3.204687	0.0015
Temperature at slaughter	0.240769	0.0388643	256	6.195119	0.0000
Rainfall at slaughter	0.012847	0.0031411	256	4.089858	0.0001
Ventilation type: natural	1.275664	0.6102330	27	2.090453	0.0461
Water supply: drinking cup	0.958984	0.4704503	27	2.038438	0.0514



**Fig. 2.** Observed incidence (presence or absence in a flock) of *Campylobacter* over the period 2003–2006 in flocks on 30 farms compared with predicted probability of occurrence derived from a Generalized Linear Mixed Model with binomial error structure relating incidence to temperature and rainfall at slaughter, month, use of natural ventilation and drinking cups. ○---○, Observed data; ×—×, predictions from best model.

underlying risk of infection on each farm. In other words the increase in risk of infection per unit increment in mean monthly temperature and rainfall was the same for all farms. Analysis of the residuals from the models showed that there was no serial dependency in that none of the residuals were significantly correlated up to a lag of 12 months. Inclusion of an off-diagonal error structure to account for serial dependency, whilst not necessary on this evidence, did not markedly alter the magnitude of the regression coefficients. This suggests that flock infections were

independent of each other in time. The observed incidence of *Campylobacter* over time along with the predicted probability of incidence from the mixed-effect model is shown in Figure 2. Whilst it is difficult to make a simple comparison between categorical (0, 1) and binomial data (0 to 1), there is a close match between predicted and observed for many of the farms with peak and low incidences and the predicted probability roughly overlapping over time. The correlation between observed and predicted *Campylobacter* status of flocks was 0.646 ( $t=14.35$ ,



**Fig. 3.** Survival function for *Campylobacter* infections on the study farms, with associated 95% confidence limits. Curve represents probability of flock on a farm not becoming infected over time.

$P < 2.2 \times 10^{-16}$ ) and this provides a crude estimate of the utility of the model. Clearly, weather (temperature and rainfall) is a key predictor in this model. Of the 289 flocks sampled the residuals were  $> 1.97$  standard deviations from zero for only nine flocks. Five of these occurred at the last sampling date in 2006, when *Campylobacter* was observed but not predicted. Analysis of the random-effect intercepts for the farms from the GLMM showed that these were negatively correlated with the easting geographical position of the farm ( $r = -0.406$ ,  $t = -2.314$ ,  $P < 0.0282$ ). This suggests that the incidence of infections was generally higher in flocks on western farms, even though the farms were all drawn from a small geographical region.

#### Prevalence of *Campylobacter* within chicken flocks

Analyses of the prevalence of *Campylobacter* in the 30 chickens sampled from each flock indicated that whilst most covariates were apparent significant predictors of the prevalence in flocks, the residual deviance (the unexplained variation) in the model was approximately an order of magnitude greater than the degrees of freedom. The data were markedly overdispersed suggesting that either the binomial error structure postulated was inappropriate and/or there were key variables missing from the model. Inclusion of a factor for farm did decrease the residual deviance by about 20% but still left the simple GLM overdispersed and effectively invalid. Further extension of the modelling to include farm as a random effect did not decrease the residual deviance. Extension to include a negative binomial error structure (not shown) markedly decreased the residual deviance in a

parsimonious GLM, rendering it underdispersed, suggesting that the incidence of positive status in birds in flocks was aggregated. This error distribution was not strictly appropriate for the data given the upper constraint on the number of birds sampled. Given the large amount of overdispersion in the flock data and the intractability of modelling mixed-effect models where the response shows aggregation, no further analyses of prevalence of infection in flock were considered.

#### Survival analysis

A survival curve showing the flock infection over time across farms is shown in Figure 3. The ordinate represents the inverse of the hazard of infection (i.e. avoidance of infection) in flocks and is plotted on a log scale. In effect this shows that the probability of there *not* being an infection event on a farm declined exponentially with time. The most obvious feature is that the curve is steeper in the period up to 250 days, suggesting that hazard varied with time – with infections greater than expected (or those after 250 lower than expected) from an exponential model. However, there was a significant negative correlation between mean temperature at month of slaughter and the time of slaughter over the course of the study ( $r = -0.5459$ ,  $P < 1.8 \times 10^{-11}$ ) indicating that the temperature at slaughter was generally higher in the first year of the study. The extent to which hazard of infection varied with time and temperature is analysed more fully below.

For the time to first-event model, with each farm included once only, mean temperature and rainfall at month of slaughter were significant positive predictors (not shown) whilst the presence of stock was also a significant predictor having a protective effect (odds ratio 0.494, 95% confidence interval 0.346–0.703). Inclusion of event number as a factor in a simple Cox model showed that the magnitude of coefficients for the event number (not shown) did not increase monotonically with the event number as might be expected if there was a serial dependency. Incidence of the third and fourth infections had negative coefficients relative to the first suggesting that there was a reduced risk of infection of a flock becoming infected after the first. However, given the small sample size ( $n = 30$ ) relative to the total number of infection events and census points recorded over the study (130) the first-event model is not considered further.

Table 5. Regression diagnostics for parsimonious Cox proportional hazards models for infection events from the 30 study farms

Covariate	Model: Independent increments				Model: Conditional			
	Coefficient	Robust S.E.	Z	P	Coefficient	Robust S.E.	Z	P
Mean temperature at month of slaughter	0.99260	0.2404	5.26	0.00000	1.1169	0.1772	6.2900	0.0000
Mean temperature at month of slaughter squared	-0.03909	0.0093	-4.64	0.00000	-0.0468	0.0076	-6.1400	0.0000
Extent of pad burn (%)	0.08742	0.0357	3.44	0.00050	0.0501	0.0174	2.8700	0.0042
Pad burn squared	-0.00125	0.0009	-2.25	0.02400	—	—	—	—
$R^2$	0.3590				0.2970			
Likelihood ratio test	57.4000	(5 D.F.)		0.0000	45.4000	(4 D.F.)		0.0000
Wald	90.50	(4 D.F.)		0.0000	51.00	(3 D.F.)		0.0000
$\chi^2$ tests for non-proportionality	Model: Independent increments			Model: Conditional				
	rho	$\chi^2$	P	rho	$\chi^2$	P		
Mean temperature at month of slaughter	0.0184	0.0257	0.8726	0.0529	0.1590	0.6903		
Mean temperature at month of slaughter squared	-0.0001	0.0000	0.9992	-0.0473	0.1230	0.7261		
Extent of pad burn (%)	-0.2358	5.5700	0.0183	-0.1907	4.6570	0.0309		
Pad burn squared	0.1713	1.7800	0.1821	—	—	—		
Global	n.a.	6.9700	0.1374	n.a.	5.4400	0.1423		

n.a., Not available.

For the independent increment and conditional models there were many more events and in common with the first-event model mean monthly temperature at the month of slaughter (for both models) and the extent of pad burn for the independent increment model, were positively associated with the risk of infection. There was evidence that the effects of temperature and pad burn were not proportional in either model when analysed as monotonic covariates. Penalized models fitted with splines for mean temperature in the month of slaughter and pad burn showed that there was a significant nonlinear component to the effects of both covariates. This suggests that either a key variable was missing from the models or that the underlying assumptions of proportionality were violated. Inclusion of a quadratic term for mean monthly temperature at slaughter and pad in the independent event model showed that hazard increased to a peak at about 12.7 °C and 34.9% pad burn at slaughter. This suggests that hazard of infection peaked at 12–13 °C and declined above that peak. The best conditional model whilst also including a quadratic term for mean monthly temperature at slaughter did not include a quadratic term for

pad burn. Regression diagnostics for the best models are shown in Table 5. Plots of time-dependent coefficients against time for each covariate and their quadratic terms (when included) in the best independent and conditional models were flat in all cases, suggesting that there was no time dependence in the covariates. In effect this meant that the hazard of a flock becoming infected as a result of increased temperature was the same over the study period. Tests for non-proportionality for all covariates (including quadratic terms where included) in both models were not significant (Table 5) indicating that the assumption of proportional hazards for both models was valid.

The likelihood ratio and Wald tests for the independent increment model had higher levels of significance than for the conditional model suggesting that this was a better model for the data. This meant that the model which assumed that infection events on farms were independent of each other was a better model for the data than the one which assumed that there was serial dependency in events. In other words, infection events on farms were opportunistic rather than serially dependent.



## DISCUSSION

Identifying risk factors for infection in animal production systems is a major issue in veterinary research as it provides the basis to control infection, to maximize productivity and minimize problems of animal welfare. Where the pathogen is widely distributed in the local environment and is carried by other organisms, disentangling causal relationships that link processes to infection for one host species is particularly difficult. The study presented here aimed at identifying risk factors for *Campylobacter* in chicken flocks using linear and mixed-effect models in combination with survival analysis to investigate prevalence patterns on farms over time. Our rationale was to undertake a longitudinal analysis on a comparatively small number of farms, where many of the risk factors would be either consistent or consistently measurable over the study period.

The mixed-effect models were undertaken at two scales, one considering prevalence of infection in a sample of 30 birds from each flock and the other considering flock infection as a simple binary yes/no response. The results of the within-flock prevalence analyses were poor, in that all models were overdispersed by at least one order of magnitude. The binomial error structure was clearly not appropriate for the data. In this context, overdispersion is likely to have arisen because the individual chicken responses – infected/not infected – were not independent samples [28]. It is likely that the incidence of infection in chickens is dependent on dynamic processes like contact with chickens that are themselves infected; indeed, feed, drinkers, and air become contaminated [29]. The overall pattern of flock prevalence across all farms was bimodal. Similar patterns of prevalence have been observed in theoretical analyses of infection dynamics in flocks of broilers [30].

Analysing longitudinal data with random effects with non-normal error structures is problematic because the mathematical solution is complex [31] and relies on numerical approximation. This notwithstanding, the results of the farm-level models when analysed through a mixed-effect modelling approach were more clear-cut than those that included information on flock prevalence. These probably reflect the fact that by moving ‘up scale’ flock-dependent infection dynamics were factored out of the modelled response. There was a suggestion that infection events declined over the course of the study. This was manifested in terms of a significant trend in relation to time

in the study from the GLMM analyses and a flattened tail for the survivorship curve, where the hazard of infection appeared to decline with time from the start. This was explained in part by differences in weather conditions (it was warmer at the beginning of the study), but it may also reflect other unmeasured farm-level risk factors, because there was a suggestion from the survival analyses that third and fourth events were less likely to occur following a first. More importantly, both the GLMM and the survival analyses showed that weather conditions during the month of slaughter were major features determining whether or not a farm had an infected flock through the sample period. Seasonality in *Campylobacter* infections is well known in human cases [16, 32] and has also been reported in broiler flocks in many studies [17, 33–38]. The organism survives best in wet conditions and at low temperatures [39] and survival is poor in dry conditions [40]. Within-flock transmission is also higher in conditions of wet litter [41], which is also a risk factor for pad burn ([42], see below). The fact that weather conditions in the month of slaughter were significant predictors of whether or not a flock was infected whilst conditions in the month of placement were not probably reflects the aetiology of infection in flocks. One study noted that flocks become increasingly infected as the rearing period progressed reaching a peak around 10 days prior to slaughter [20]. It is at this time when the space available for individual chickens declines, growth rate (and physiological stress) are greatest [43]. Thus, one might expect that *Campylobacter* spread within a flock would be greatest at this time, a factor emphasized by the impact of drinkers, which would provide a rapid means of spreading infection. Ventilation varied on the farms in terms of where the air entered (sides, vent and inlets) and whether or not it was forced by fan or natural. The apparent significance of natural ventilation in enhancing disease might reflect either a magnified effect of external weather conditions – since the temperature in a fan-ventilated environment is likely to be lower than that provided by natural ventilation – or an indirect effect arising from a *Campylobacter* vector such as flies. In addition forced ventilation might lead to fly mortality as flies hit fan blades, giving rise to the contrast with natural ventilation, but this could only be verified by further research. Ventilation has been cited as a risk factor in a study of Canadian broilers [19], while Ekdahl *et al.* [44] argued that flies not only carry *Campylobacter* but can pass the bacterium onto chickens. Natural ventilation in warm

temperatures, which encourage fly movement, would clearly increase the risk of the pathogen entering a broiler house. Excluding flies from broiler houses has been shown to reduce infection rates from 54% to 15% [45].

The lack of overdispersion in the GLMM models suggests that there was no aggregation, i.e. no serial dependence in infection within farm. This coupled with the fact that the incremental independent model provided a better explanation of infection events in the survival analyses are both suggestive that infection at the flock level is to a certain extent opportunistic given the right environmental conditions. This is perhaps of little help in managing disease. One feature that differed between the results of the survival analyses and those of the GLMMs was the apparent significance of pad burn, which was identified as a significant predictor in the survival analyses but not the GLMMs. Bull *et al.* [22] noted that the closely related condition of hock burn was an important predictor of flock infection in a larger study of 760 farms (of which these data form a small subset). Indeed, the extent of pad and hock burn in flocks was significantly correlated ( $r=0.358$ ,  $P<0.002$ ); the relationship between survival and pad burn detected here could reflect this correlation. Whilst the relationship between hazard of infection and extent of pad burn clearly exists, it is likely that incidence of both reflects another unmeasured underlying factor associated with flock health, rather than a true causal relationship. It is possible that the underlying process is related to enhanced survival of the pathogen in the litter under the wet conditions that give rise to pad burn. Alternatively, it is possible that pad burn simply reflects a general low health status in the individual chicken, which also influences the extent to which they can become colonized by *Campylobacter*. Nonetheless, the existence of the relationship perhaps has an agronomic value in that it might be of use in predicting the likely risk of flocks having become infected at the point of slaughter. Of equal interest is the apparent lack of significance in our models of other previously identified risk factors, specifically, the number of people entering chicken houses, the size and number of houses present [19, 21] and the presence of livestock, which can carry *Campylobacter* [19, 46, 47]. It is possible that our measures of these risk factors for farms were rather crude, but one key difference between our analysis and those of the above authors is the scale and longitudinal nature of our sampling. Many previous studies have involved

large-scale surveys of large numbers of farms where the range of covariates that might be potential risk factors was also large. Identifying risk factors under these circumstances is problematic whilst correlations between predictors and outcomes are easy – particularly with large samples sizes – establishing causal links less so. Studying repeated infections, on a small number of farms over a longer time period, as we did here, means that many putative covariates were effectively factored out of the infection process. In addition we might also expect that focusing study on farms belonging to one company might reduce modifiable variation in some covariates because the farms are likely to have similar management procedures. The focus on mixed-effect modelling approach allowed us to focus on between-farm variations through time. Farms clearly differed in their underlying risk of flocks becoming infected – as demonstrated by the random-effect models. The fact that these effects were significantly related to the geographical position of the farm – with risk of infection greater in the wetter west suggests that this is a contributor to risk. Large-scale geographical variation in risk of *Campylobacter* infection has been recorded in New Zealand and it has been concluded that the infection process varies spatially, although analyses of the pattern of incidence on farms has been shown to be consistent with within-farm rather than between-farm transmission [48].

There are two major conclusions from our work. First, that we need considerably more research on the pattern of spread within broiler flocks. The evidence indicates that within-flock spread is highly variable and not easy to predict. The dynamics of disease spread mean that the use of simple linear models to relate flock prevalence to putative risk factors is likely to be difficult. Processes which encapsulate the dynamic and allow for the fact that disease dynamics will vary through time and across farms are needed to investigate the disease at the appropriate scale. More interestingly, the results of our farm-level mixed-effect models gave considerable insight into the factors determining incidence of disease at this scale. In fact the goodness-of-fit of model predictions to observed behaviour suggests that it may be possible to predict when flocks on farms are at risk of *Campylobacter* infection, both in terms of weather and possible intervention through manipulation of ventilation regimes. This is a first step towards managing disease and reducing the risks posed to the human population.

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## DECLARATION OF INTEREST

None.

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