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Sources of Variation in the Ampicillin-Resistant *Escherichia coli* Concentration in the Feces of Organic Broiler Chickens▽

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Currently, there are limited published data for the population dynamics of antimicrobial-resistant commensal bacteria. This study was designed to evaluate both the proportions of the *Escherichia coli* populations that are resistant to ampicillin at the level of the individual chicken on commercial broiler farms and the feasibility of obtaining repeated measures of fecal *E. coli* concentrations. Short-term temporal variation in the concentration of fecal *E. coli* was investigated, and a preliminary assessment was made of potential factors involved in the shedding of high numbers of ampicillin-resistant *E. coli* by growing birds in the absence of the use of antimicrobial drugs. Multilevel linear regression modeling revealed that the largest component of random variation in log-transformed fecal *E. coli* concentrations was seen between sampling occasions for individual birds. The incorporation of fixed effects into the model demonstrated that the older, heavier birds in the study were significantly more likely ($P = 0.0003$) to shed higher numbers of ampicillin-resistant *E. coli*. This association between increasing weight and high shedding was not seen for the total fecal *E. coli* population ($P = 0.71$). This implies that, in the absence of the administration of antimicrobial drugs, the proportion of fecal *E. coli* that was resistant to ampicillin increased as the birds grew. This study has shown that it is possible to collect quantitative microbiological data on broiler farms and that such data could make valuable contributions to risk assessments concerning the transfer of resistant bacteria between animal and human populations.

Within a given environment, the commensal microfloras of birds and mammals act as useful markers of antimicrobial resistance (2, 21, 31). In the nutrient-rich environment of the intestinal tract, commensal microorganisms are present in high numbers. Many of these species of bacteria are adept at both carrying resistance genes (7, 22) and exchanging genetic material with members of their own and other species (4, 26). For these reasons, intestinal bacteria may constitute important reservoirs of antimicrobial resistance (30). Furthermore, investigations of commensal organisms in healthy host populations and antimicrobial drug-free environments have found that resistance genes can persist in commensal bacteria in the absence of antimicrobial drug-selective pressures (16, 25). Thus, research into antimicrobial resistance in commensal populations can provide valuable insights into the panoptic dynamics of antimicrobial resistance (5, 29).

When studying antimicrobial resistance, the microbiological methods utilized will strongly influence the interpretation of the results gained. This has been aptly demonstrated by two Danish studies of vancomycin-resistant *Enterococcus faecium* (VREF) occurring on broiler chicken farms following the 1995 Danish ban of the use of avoparcin as an in-feed growth-enhancing agent. One study used Danish surveillance data whereby the proportion of VREF among *E. faecium* isolates cultured from broiler samples had been ascertained. Analysis of these data sets showed a highly significant decline in the proportion of VREF among isolates that had been cultured between 1995 and 1998 (P, <0.00001) (3). However, the second study utilized isolation methods that directly selected for VREF from the broiler samples. This alternative technique showed that there was no significant decrease in the proportion of VREF-positive flocks in Denmark between 1998 and 2001 (P, >0.1) and that 5 years after the withdrawal of avoparcin, VREF was still detected within 74.3% of conventional broiler flocks (8). Furthermore, if one is aiming to elucidate the dynamics of populations of antimicrobial drug-resistant bacteria, then quantitative microbiological techniques are required. Currently, quantitative data of this nature are limited for commensal bacterial populations. Such data gaps may be impeding the development of comprehensive epidemiological models and precise quantitative microbial risk assessments (14), and models of this nature could make valuable contributions to the debate regarding the significance of the use of antimicrobials in the livestock industries with respect to the transmission of resistant bacteria to human populations (27).

The aims of this field study were to test the suitability of sampling and laboratory protocols that were designed to generate quantitative microbiological data for investigating antimicrobial resistance on broiler chicken farms; to use the data produced to examine the within-bird-over-time dynamics of ampicillin-resistant *Escherichia coli* (AREC) for broiler chickens that are not exposed to antimicrobial drugs; and to evaluate the relative contribution of a variety of potential components of variation to the concentration of fecal *E. coli* that is

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ampicillin resistant in an environment where exogenous antimicrobials are not administered.

MATERIALS AND METHODS

Farm details. The study took place over 5 consecutive days in October 2003, on a long-term organic, mixed-species livestock farm in southern England. The broiler unit consisted of 20,000 birds between 21 and 77 days old that were partitioned into single-age groups of approximately 1,500 birds each. These groups of 1,500 birds were housed in adjacent mobile barns and had unrestricted access to an area of range surrounding each barn during daylight hours. In the 4 years preceding this study, antibiotic therapy had been administered to poultry on just one occasion. This had occurred in 2001 when 3,000 birds less than 7 days of age had been dosed with enrofloxacin in the face of an outbreak of yolk sac infections in chicks arriving from a commercial hatchery.

Sampling protocol. Two male and two female birds were randomly chosen in each of six mobile barns. Each barn housed identically aged birds from a single flock, and those flocks encompassed three different ages of birds: 30 days, 57 days, and 70 days old. Dark-colored stock markers were used to enable individual identification of the study birds within each house. Sampling took place between 10:00 a.m and 12:30 p.m. on each morning of the study. The marked birds were caught, weighed, and then placed inside portable pet carriers lined with fresh paper for a period of up to 10 minutes. After this time, fecal droppings voided within the pet carrier were collected from the lining paper. If the birds did not defecate within the pet carrier, they were released and then observed until defecation occurred, at which point the droppings were immediately collected from the house floor or field surface. The fecal samples were scored for consistency, color, and volume by the first author and were then held at 4°C during transportation to the laboratory. All samples were processed within 12 h of collection.

Laboratory methods. Each fecal sample was weighed, and an equal amount (volume to weight) of buffered peptone water (BPW) was added prior to mixing using a vortex mixer. A 1:10 dilution was obtained by homogenizing 4 g of the fecal suspension with 16 ml BPW by using a Stomacher 400 circulator (Seward, Norfolk, United Kingdom). A 10-fold dilution series down to 1:10,000 was produced using maximum recovery diluent (MRD). Presumptive *E. coli* counts were obtained by plating the fecal dilutions onto CHROMagar ECC agar plates. A Whitley Automatic Spiral Plater (WASP1; Don Whitley Scientific Limited, Whitley, United Kingdom) was used in the logarithmic mode to dispense 50 or 100 μl of the 1:10,000 or 1:100 dilution onto the CHROMagar ECC plates. In order to assess variations in *E. coli* counts due to laboratory techniques, two dilution series were prepared from one sample per house per day, and all dilutions from all samples were plated in duplicate. The total presumptive *E. coli* counts were obtained by plating onto plain CHROMagar ECC medium, while presumptive ampicillin-resistant *E. coli* counts were obtained by plating onto CHROMagar ECC medium incorporating 8 mg/liter ampicillin. This level of ampicillin was chosen to correspond to the British Society of Antimicrobial Chemotherapy breakpoint MIC. After inoculation, the plates were incubated at 37°C for 18 to 24 h. The identities of inoculated plates were blinded by a separate member of the laboratory staff, and the blinded plates were counted manually by the first author. Presumptive *E. coli* isolates were selected using colony morphology.

The CHROMagar ECC agar plates and the BPW and MRD were prepared by the biological products unit at the Veterinary Laboratories Agency (VLA), following VLA standard operating procedures in line with ISO9001/2000 accreditation systems. Control organism strains NCTC 10418 and ATCC 25922 and two internal VLA controls of known ampicillin MICs, S/28/99 and LR22, were plated onto each batch of medium in order to check the performance of the selective medium. Plates containing ampicillin that had not been used within 48 h of preparation were discarded.

For the purpose of this study, we defined ampicillin-resistant *E. coli* as those colonies of typical morphology growing on CHROMagar ECC medium containing 8 mg/liter ampicillin. The accuracy of this definition was examined using real-time PCR techniques with a subset of 141 presumptive AREC isolates from the study. To confirm whether these isolates were *E. coli*, the glutamate decarboxylase *gadA* gene was detected using TaqMan real-time PCR (Applied Biosystems, Foster City, CA) based on the methods of Meiland et al. (20). The same panel of isolates was also screened for the presence of bla_{TEM} beta-lactamase genes using a LightCycler 2.0 system (Roche Diagnostics, Basel, Switzerland). The primers TEMf (5′TCG TGT CGC CCT TAT TCC CTT TTT) and TEMr (5′GCG GTT AGC TCC TTC GGT CCT C) were designed using DNASTAR software (DNASTAR, Madison, WI) and were based upon a published sequence of a $bla_{\mathrm{TEM\text{-}1}}$ gene located on a plasmid carried by a strain of *Klebsiella pneumoniae* (GenBank accession number AF309824).

house *i* = 1-6
→ **bird *j* = 1-24**
→ **bird-day *k* = 1-5**
→ **dilution series *l* = 1-2**
→ **dilution *m* = 1-2**
→ **aliquot (residual) = 1-2**

FIG. 1. The structure and notation used for the hierarchy of random effects incorporated within the preliminary multilevel mixed-effects linear regression models of log-transformed *E. coli* concentrations.

Data analysis. Trellis scatterplot matrices were produced using S-PLUS 4.6 software (MathSoft Incorporated, Cambridge, MA). Box-and-whisker plots were produced using the boxplot function in R 2.1.1 (R Development Core Team, Vienna, Austria. 2005. [http://www.R-project.org]).

Due to the longitudinal nature and hierarchical structure of the study, multilevel mixed-effect linear regression models were used to assess the sources of variation in log-transformed *E. coli* concentrations. Separate models were run for total *E. coli* concentrations and ampicillin-resistant *E. coli* concentrations. Repeated samples obtained from the same bird across time were designated as bird-days, and these were nested within birds, which in turn were clustered within houses. To allow an assessment of variance due to laboratory procedures, the hierarchy was extended so that the replica aliquots were nested within the double dilutions that were plated, which in turn were nested within the replica 10-fold dilution series that were constructed and nested within bird-days. The structure and notation used to describe the hierarchy are shown in Fig. 1.

Logarithmic transformations of *E. coli* concentrations in CFU per gram were approximately normally distributed; therefore, the following mixed-effect model was fitted by restricted maximum likelihood (REML) using the linear mixed-effect function (lme) in R (23). Writing Y_{ijklm} to refer to the log of the observed concentration of *E. coli* in plate *m* of dilution series *l* from bird *j* in house *i* on day *k*, we have the following:

$$Y_{ijklm} \sim N(\mu + X_{ijklm}\beta + A_i + B_{ij} + C_{ijk} + D_{ijkl}, \tau^2)$$

Here, μ is the mean intercept, and X_{ijklm} is a vector of fixed effects. The random effects A_i, B_{ij}, C_{ijk}, and D_{ijkl} are independent normally distributed random variables, each with a mean of zero and variances σ^2_A, σ^2_B, σ^2_C, and σ^2_D, respectively. The residual random error incorporating variation at the plate level is denoted by τ^2.

Initially, the contributions of the different levels of variation were explored using intercept-only models (i.e., without the incorporation of any fixed effects X_{ijklm}). After refining the random effects portion of the model, separate fixed-effect models were fitted by adding each covariate individually in order to assess their significance and thus determine which of the fixed-effect variables were suitable for inclusion within the multivariable models. The final model incorporated the significant covariates and random effects. To obtain 95% credible intervals for the random effects, this final model was then fitted using Markov chain Monte Carlo (MCMC) sampling within a Bayesian framework as implemented by WinBUGS software (WinBUGS 1.4.1; Imperial College and Medical Research Council, United Kingdom [http://www.mrc-bsu.cam.ac.uk/bugs/]). Noninformative priors were used for both fixed and random effects. Convergence was assessed by running multiple chains and examining sample paths. After a burn-in period of 10,000 iterations, the posterior distributions were sampled between iterations 10,001 and 30,000 using a thinning interval of 50. The results of the MCMC fit were compared with those of the REML fit.

In order to assess the relationship between the proportion of *E. coli* within a single fecal sample that was resistant to 8 mg/liter ampicillin and the weight of a bird, logit-transformed proportions were fitted using an additional REML mixed-effect model. The proportions fitted within this model were derived by dividing the mean concentration of ampicillin-resistant *E. coli* by the mean concentration of total *E. coli* for each dilution series plated per sample. Thus, the random effects hierarchy for the proportion models was only four levels: house, bird, and bird-day, with fecal dilution series incorporated within the residual random error.

RESULTS

Data summary. A total of 115 fecal samples were analyzed. The median concentration of total fecal *E. coli* for all the samples was 4×10^6 CFU/g (6.6 $\log_{10}$ units/g) within a range

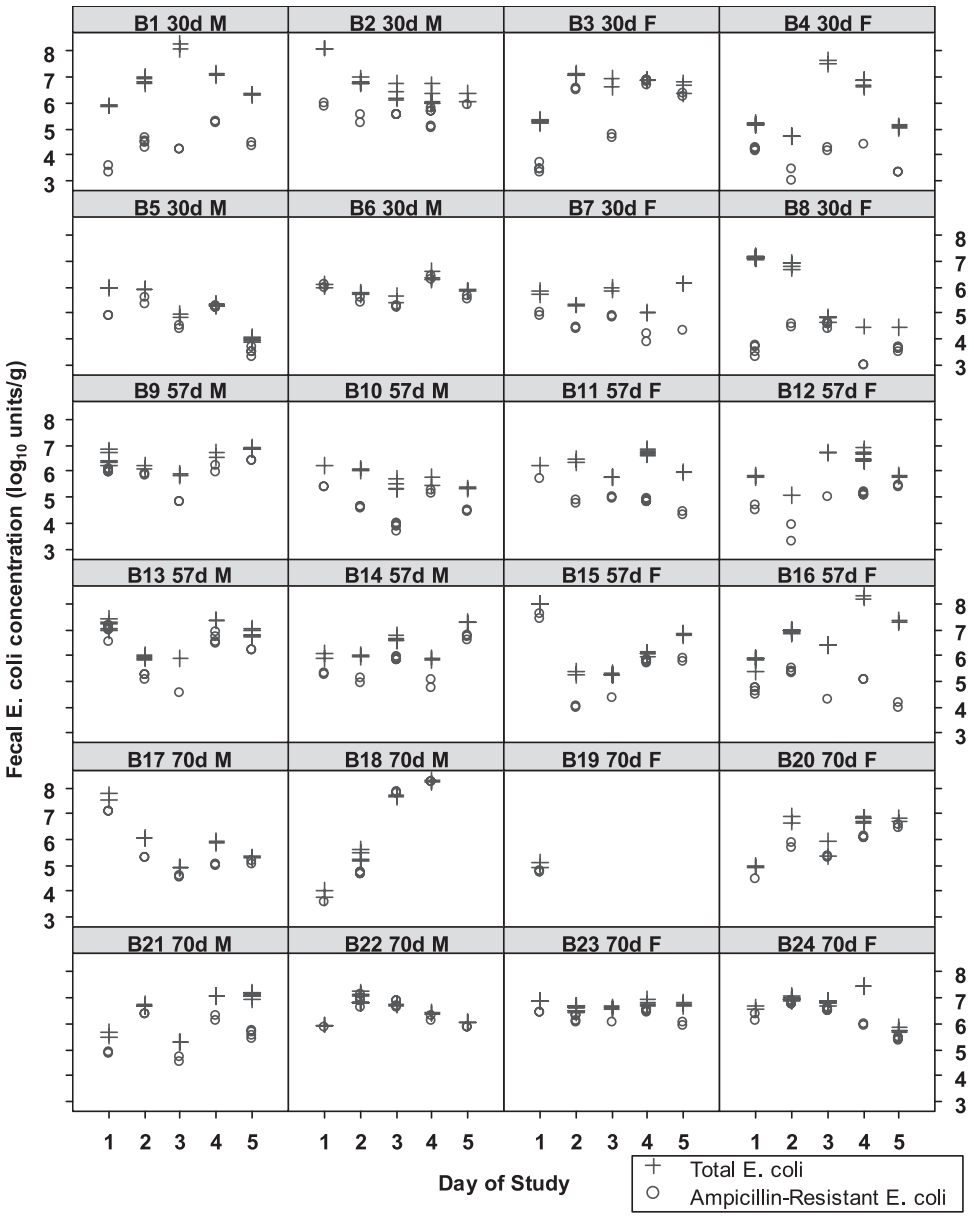

FIG. 2. A Trellis scatterplot matrix showing the daily fecal concentrations of total *E. coli* (+) and *E. coli* resistant to ≥8 mg/liter ampicillin (○) for each of the 24 birds over the 5 consecutive days of the study. B*n* identifies the individual birds, *n*d is the age of the bird on the first day of the study, and M/F is male or female. Each row of the plot represents birds from the same house.

extending from 6.0×10^3 to 2.1×10^8 CFU/g (3.8 to 8.3 $\log_{10}$ units/g). The median concentration of ampicillin-resistant *E. coli* was 10-fold lower at 2.1×10^5 CFU/g (5.3 $\log_{10}$ units/g); however, the range of ampicillin-resistant *E. coli* counts was of dimensions similar to that for the total counts, extending from 1.0×10^3 to 1.7×10^8 CFU/g (3.0 to 8.2 $\log_{10}$ units/g).

Figure 2 shows plots of the daily concentrations of both the total and the ampicillin-resistant fecal *E. coli* for each of the 24 marked birds. The ages of the birds increase from the top to the bottom of the figure. The plots show that there are considerable variations in the fecal *E. coli* concentrations both between birds and between sampling days for an individual bird. Likewise, the proportions of the total *E. coli* population that are ampicillin resistant also varies markedly between and within birds.

Figure 3a shows box-and-whiskers plots of total fecal *E. coli* concentration against potential conditioning variables. Some variation can be seen between the different poultry houses; however, there are no unidirectional trends in total *E. coli* counts associated with the sampling day, age, or weight of birds. Figure 3b also shows evidence of variation in counts of ampicillin-resistant *E. coli* between the different poultry houses; however, in contrast with Fig. 3a, Fig. 3b also shows the concentration of AREC increasing with the increasing age and weight of the birds. It is worth noting that the house-level variation in AREC seen here

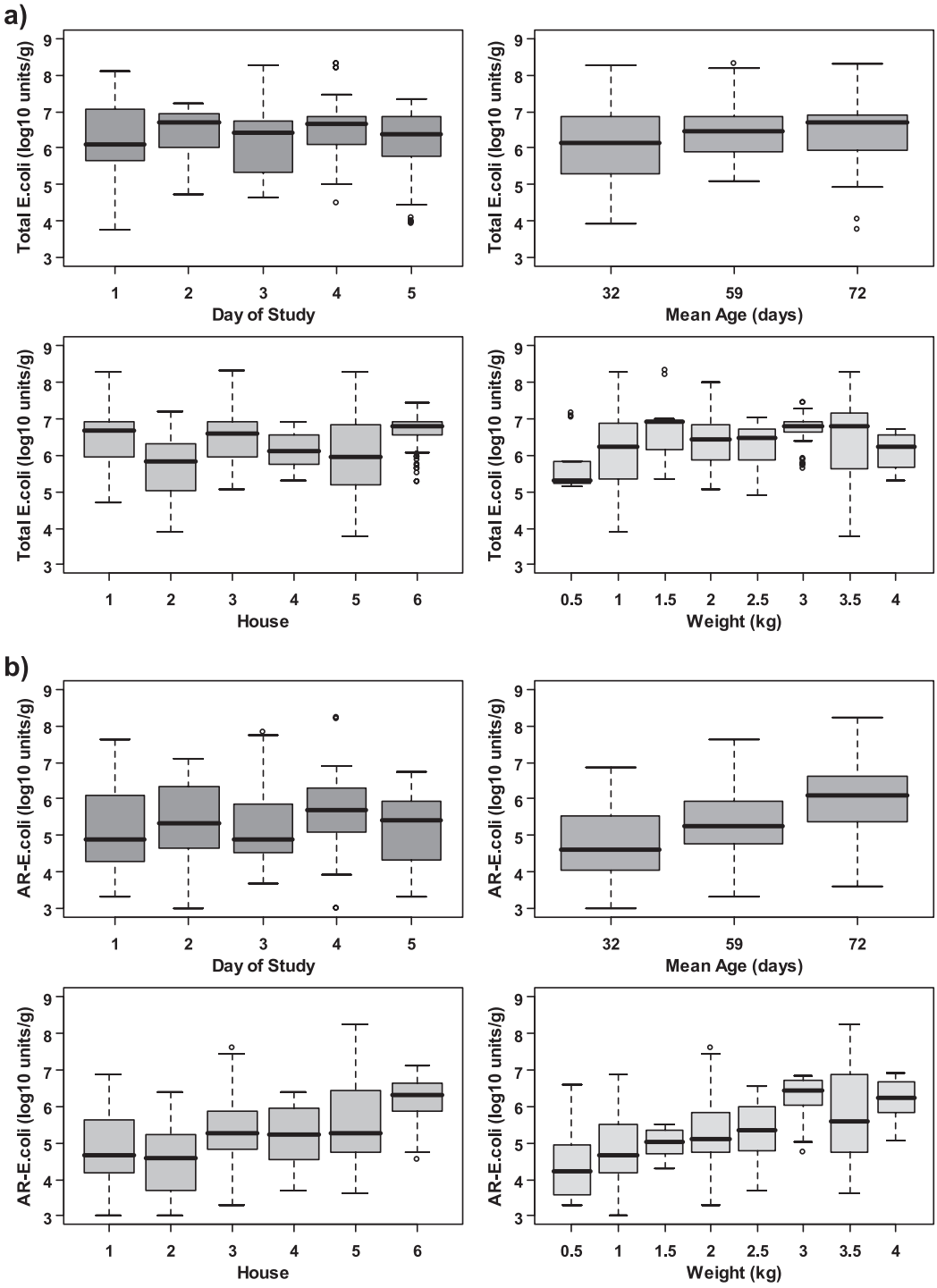

FIG. 3. Box-and-whiskers plots illustrating fecal *E. coli* concentrations against potential explanatory variables. (a) Total *E. coli* population. (b) Subset of *E. coli* that was resistant to ≥8 mg/liter ampicillin (AREC).

could also be associated with the weight and age of birds, because houses 1 and 2 contained the youngest birds in the study (30 to 35 days old), and houses 5 and 6 contained the eldest (70 to 75 days old).

The relationship between an increasing concentration of ampicillin-resistant *E. coli* within a fecal sample and the increasing weight of a bird was seen to be associated with an increase in the proportion of the total fecal *E. coli* population that was resistant to 8 mg/liter ampicillin (Fig. 4).

Statistical analysis. The upper section of Table 1 shows the results for the total and the AREC REML random effect models without the incorporation of fixed-effect covariates: the intercept-only models. Within the random effects hierarchy, in the absence of fixed effects, the greatest source of variation for both the total

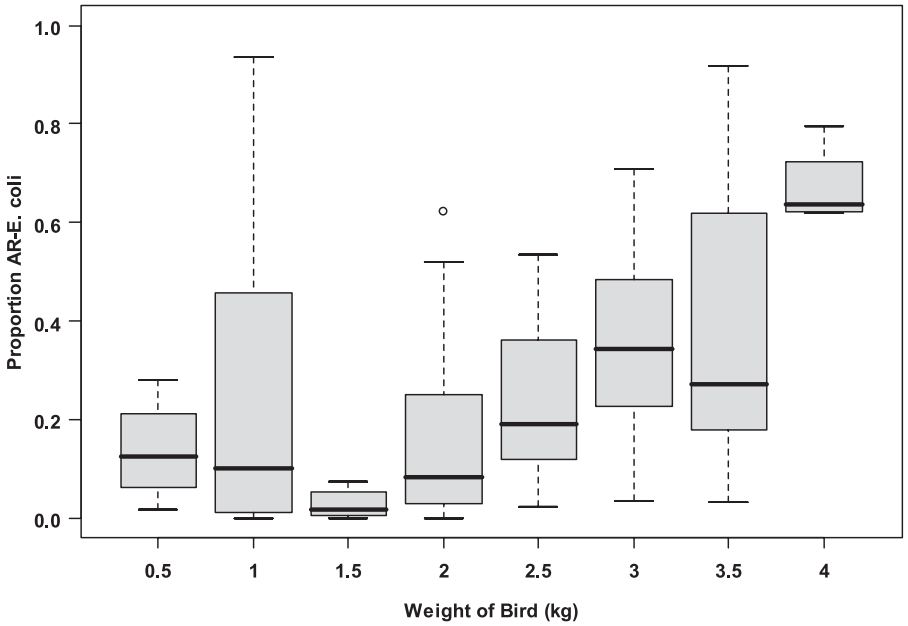

FIG. 4. Box-and-whiskers plot illustrating the proportions of the total fecal *E. coli* population that were resistant to ≥8 mg/liter ampicillin against the weights of the birds.

E. coli and the AREC was seen within an individual bird over time (σ^2 = 3.55 and 3.68, respectively). In these models, house effects also contributed to the variation within both the total population and the ampicillin-resistant subset (σ^2 = 0.59 and 0.81, respectively). However, while between-bird variation was negligible for the total *E. coli* concentration (σ^2 = 0.0003), the between-bird variation in ampicillin-resistant *E. coli* concentration was sizeable (σ^2 = 1.23). For both the total and ampicillin-

TABLE 1. Intercept-only random effects and single-fixed-variable mixed-effect REML models of $\log_e$ concentration of total and ampicillin-resistant *E. coli* (to ≥8 mg/liter) in fecal samples collected from organic table chickens

Variable	Total *E. coli* concn				Ampicillin-resistant *E. coli* concn			
	Variance (σ^2)	Coefficient (β)	95% CI	*P* value	Variance (σ^2)	Coefficient (β)	95% CI	*P* value
Random effects (intercept-only models)								
House	0.59				0.81			
Bird	0.0003				**1.23**			
Within bird over time	**3.55**[a]				**3.68**			
Fecal dilution series	0.0003				0.00002			
Dilution plated	0.05				0.07			
Sample aliquot (residual)	0.06				0.06			
Fixed effects (single-fixed-variable models)								
Age of bird (days)		0.02	−0.03–0.06	0.44		**0.06**	**0.03–0.09**	**0.0008**
Weight of bird (kg)		0.12	−0.53–0.78	0.71		**1.05**	**0.50–1.59**	**0.0003**
Sex of bird								
Male		Ref[b]				Ref		
Female		−0.20	−0.92–0.52	0.59		−0.97	−2.06–0.12	0.10
Source of sample								
Pet carrier		Ref				Ref		
House floor/field		−0.41	−1.31–0.48	0.36		0.13	−0.89–1.14	0.81
Fecal color								
Dark brown		Ref				Ref		
Light brown		0.43	−0.55–1.42	0.39		−0.22	−1.28–0.85	0.69
Red/brown		0.38	−0.60–1.37	0.45		−0.06	−1.11–0.10	0.92
Fecal consistency								
Well formed		Ref				Ref		
Loose		−0.17	−0.91–0.57	0.65		−0.22	−1.04–0.60	0.59
Liquid		0.52	−0.72–1.76	0.41		0.36	−0.97–1.70	0.60
Fecal volume								
Average		Ref				Ref		
Scanty		−0.78	−1.64–0.08	0.08		**−1.33**	**−2.23–−0.42**	**0.005**
Profuse		−0.13	−0.98–0.72	0.76		−0.15	−1.05–0.76	0.75

[a] The numbers reported in bold indicate marked effects (variance > 1 and *P* = 0.05).
[b] Ref indicates the reference category for that variable.

TABLE 2. Comparison of classical and Bayesian fits of a mixed-effect linear regression model in which the $\log_e$ concentration of ampicillin-resistant *E. coli* (to ≥8 mg/liter) in poultry feces depends on the covariates listed and a hierarchy of nested random effects

Variable	REML				MCMC[b]	
	Estimates		95% CI	*P* value	Coefficient	95% Bayesian credible intervals
	Variance (σ^2)	Coefficient (β)				
Random effects						
Bird	0.90				0.74	0.007–2.29
Within bird over time	3.32				3.52	2.60–4.91
Laboratory effects (residual)	0.22				0.22	0.18–0.27
Fixed effects						
Fecal volume						
Average to profuse		Ref[a]			Ref	
Scanty		−1.27	−2.13–−0.42	0.003	−1.19	−2.05–−0.33
Weight of bird		0.98	0.45–1.50	0.0004	0.99	0.47–1.52

[a] Ref indicates the reference category for that variable.
[b] Gibbs sampling.

resistant *E. coli*, the levels of variation in the measured concentrations due to laboratory effects were extremely low.

The lower section of Table 1 shows the results for each of 14 mixed-effect models. Each of these models incorporated the random effects hierarchy and a single fixed effect. Of those fixed effects studied, none was significantly associated (P, <0.05) with the concentration of total fecal *E. coli*. There was a suggestion of a negative association between the small volume of fecal samples and the concentration of total *E. coli*; however, this was not significant at the 5% level. Therefore, the null model, incorporating the random effects hierarchy alone, was the model of best fit for the total *E. coli* concentrations. In contrast, both the age ($P = 0.0008$) and the weight ($P = 0.0003$) of the bird were significantly positively associated with the concentration of AREC. Furthermore, a highly significant ($P = 0.005$) negative relationship was also seen between the small volume of fecal samples and the concentration of AREC.

Model optimization using comparative strategies found that the multivariable model of best fit for log-transformed ampicillin-resistant *E. coli* concentration incorporated two fixed-effect variables: the weight of the bird as a centered variable and the volume of the fecal sample recoded as a binary variable by combining normal and profuse samples into a single reference category. After the incorporation of these fixed effects, the variance at the level of the house was seen to be negligible ($\sigma^2 = 0.002$). Thus, the final random effects hierarchy incorporated bird effects, within-bird-over-time effects, and laboratory effects collapsed into a single level. The optimized model was then fitted using both REML and MCMC techniques. Table 2 shows the estimates from the two fits; there was good general agreement between the two fits for both the random and fixed effects.

Table 3 shows the results of the models of logit-transformed proportions of fecal *E. coli* that were resistant to ampicillin, confirming that there was a significant relationship between the increasing proportion of ampicillin-resistant *E. coli* and the increasing weight of the bird ($P = 0.007$). In contrast to the log-linear models of ampicillin-resistant *E. coli* concentration, a significant association was not found between the logit-transformed proportion of *E. coli* that was ampicillin resistant and the volume of the fecal sample tested ($P = 0.07$). Furthermore, the incorporation of fecal volume within the mixed-effect model did not enhance the model fit, and therefore the final mixed-effect model for the logit-transformed proportion data incorporated centered-weight-of-bird as a single fixed effect.

Isolate characterization. All 141 presumptive ampicillin-resistant *E. coli* isolates that were screened by PCR were found to be *gadA* positive, and 138/141 were found to be carrying a bla_{TEM} beta-lactamase gene. Thus, the working definition of ampicillin-resistant *E. coli* using morphological colony characteristics was validated.

DISCUSSION

This study has suggested that in the absence of exogenous antimicrobial administration, a positive relationship exists between the weight of growing broiler chickens and the proportion of the fecal *E. coli* population that is resistant to ampicillin. Single-variable fixed-effect linear regression models found that both the weight and the age of the bird were positively and significantly associated with AREC concentration. However, when weight and age were included as fixed effects within a single model, the standard errors of the regression coefficients increased such that neither variable was declared to be significantly associated with the response variable. Obviously, the weight of a bird is heavily influenced by both its age and its sex; therefore, age and weight are highly correlated variables. A comparison of models determined that the multivariable mod-

TABLE 3. Mixed-effects REML model of the logit-transformed proportion of total fecal *E. coli* bacteria that are resistant to ≥8 mg/liter ampicillin

Variable	Residual maximum likelihood fit			
	Variance (σ^2)	Coefficient (β)	95% CI	*P* value
Random effects				
House	0.40			
Bird	1.41			
Within bird over time	2.14			
Fecal dilution series (residual)	0.38			
Fixed effect				
Weight of bird		1.04	0.31–1.76	0.007

els giving the best fit were those incorporating weight as a fixed effect; therefore, age and sex were not incorporated within the final model. This makes biological sense as all the birds within a single house are of a single age, whereas each of the individual birds will have a unique weight. Therefore, measuring the weight of the bird also provides within-house distributions for a bird-level variable that is not available if age is used instead. Furthermore, because the birds were reared in single-age groups, age and weight will also be correlated within a house. This relationship would account for the large decrease in random variation at the house level between the null intercept-only model ($\sigma^2 = 0.81$) and the five-level hierarchy mixed-effect model ($\sigma^2 = 0.002$) for ampicillin-resistant *E. coli*.

The largest proportions of the random variation in *E. coli* concentration, for both total and ampicillin-resistant populations, were found to occur between sampling occasions for an individual bird. Furthermore, the variation at this level remained high even after the addition of the fixed effects into the model. This suggests that the intestinal microflora of these growing birds is in a highly dynamic state. In contrast, while the between-bird random variation had a negligible influence on total *E. coli* concentration, it exerted a marked influence on the concentration of AREC. The random variation in AREC concentration decreased with the addition of the fixed effects but still remained at a notable level ($\sigma^2 = 0.90$). This indicates that at the individual bird level, there are other factors that are playing a role in the proportion of the *E. coli* population that is ampicillin resistant that have not been explained by the final model presented here.

This work has shown that it is possible to obtain quantitative data at the individual chicken level for farm-based studies of antimicrobial resistance among the aerobic commensal floras of commercial poultry. However, as it is difficult to obtain a useable sample of completely liquid feces, such as may be produced by birds with severe enteritis, a degree of selection bias could be imposed by these methods. This did occur on one sampling occasion during this investigation. Furthermore, a significant negative association was found between the fecal samples of smallest volumes and the concentration of AREC. Generating a serviceable and accurate fecal dilution series can be difficult with very small samples, and it is likely that there is a higher level of laboratory-based errors contained within the bacterial counts obtained from small-volume samples. Therefore, it is uncertain as to whether this is a true effect, due possibly to differences in the proportion of AREC that may occur in different areas of the gastrointestinal tract, or whether this is simply a reflection of laboratory errors. Nonetheless, although these methods were labor intensive and time consuming, the results of the regression modeling showed that actually only a minor proportion of random variation was attributable to the laboratory methods. This result has allowed for an increase in efficiency in larger, subsequent investigations due to a reduction in the number of replica dilution series constructed for each sampling visit.

There are a number of explanations that could account for a potential relationship between the weight of the bird and the proportion of fecal *E. coli* that is resistant to ampicillin. For instance, many mobile genetic elements transfer more than a single resistance gene between bacteria, and therefore, it is possible that genes encoding beta-lactamases are linked to genes encoding factors involved with, for example, colonization and adhesion to the gut wall (1, 17, 19). It is also possible that this trend illustrates that there is a degree of active selection for ampicillin-resistant *E. coli* within the growing host. Such active selection of resistant strains could be due to either interbacterial competition causing alterations in the population structure of the enteric flora (6, 24) or the direct result of bacterial-host communication (28). Alternatively, the resistance genes themselves may be conferring other properties, besides drug resistance, on those bacteria that carry them. For instance, the carriage of bla_{TEM} genes could act to enhance the assembly of peptidoglycans during the production of the bacterial cell wall (18). There is little published work in this area; however, one study did compare the fitness of streptomycin-sulfadiazine-tetracycline-resistant *E. coli* that had been derived from young calves with the fitness of mutant strains that had been generated within the laboratory by knocking out the resistance genes. In this instance, no differences in fitness were seen between the wild-type resistant and the mutant sensitive strains, suggesting that the carriage of genes encoding those three resistances was not conferring a fitness advantage (15).

Associations between the age of calves and antimicrobial-resistant *E. coli* have previously been reported. In these studies, rapid colonization of neonatal calves with antimicrobial-resistant *E. coli* has been observed in the absence of antimicrobial administration (9, 11). One study found that the peak prevalence of shedding of ampicillin-resistant *E. coli* by beef calves was seen when the animals were 4 months of age; after this, the shedding of resistant *E. coli* declined with increasing age (12). Similarly, in another study, in vivo competition experiments demonstrated that strains of streptomycin-sulfadiazine-tetracycline-resistant *E. coli* inoculated into neonatal calves out-competed *E. coli* strains that were sensitive to those three drugs even in the absence of the administration of antimicrobial drugs. However, this trend was not seen when the same fitness studies were carried out using older animals; therefore, the authors concluded that calf-adapted *E. coli* bacteria were responsible for the maintenance of antimicrobial resistance in dairy calves (16). The oldest birds in the study presented here were just 10 weeks of age, and these birds were processed in the week following the study. Therefore, while this work may have highlighted true differences in the dynamics of resistant gut flora between chickens and calves, it is likely that the substantial differences in management practices (such as the age of slaughter or number of in-contact animals) are also important drivers in these apparent species differences. Nonetheless, the results of this study could also support a hypothesis that chicken-adapted strains of *E. coli* that are concurrently bearing antimicrobial resistance genes are acting to maintain resistance on broiler farms even in the absence of antimicrobial use. It would be interesting to ascertain whether the broiler strains of ampicillin-resistant *E. coli* that have been collected from this work are also capable of predominating within the gut flora of fully mature chickens.

This observational study focused on a single-drug resistance of a single bacterial species. In order to determine whether these results are likely to be applicable to other resistant bacteria, such as tetracycline-resistant *E. coli* for instance, then a greater degree of phenotypic and genotypic characterization of the ampicillin-resistant *E. coli* isolates is required. It currently remains unclear as to whether we are observing the clonal

expansion of resistant strains of *E. coli* or an increase in numbers of genes or gene vehicles within a more stable resident population of *E. coli* strains. Published molecular studies of ampicillin-resistant *E. coli* isolated from beef farms have demonstrated that distinct genetic strains of antimicrobial-resistant *E. coli* spread through cohorts of calves over time in a successive manner (10, 13). Assuming that the same phenomena could occur within poultry farms, it is of interest to determine whether this is principally due to strains freshly acquiring resistance determinants within the farm environment or whether successions of resistant strains of *E. coli* are either persisting in the farm environment or being repeatedly introduced onto the farm.

In terms of ascertaining the wider ecological implications of these results, it must be remembered that *E. coli* is actually a minority species within the flora of the intestinal tract, which predominantly consists of obligate anaerobic species. As many of these intestinal anaerobes are also capable of carrying and transferring resistance genes (26), *E. coli* offers only a narrow window onto the overall dynamics of the enteric flora. Nonetheless, this study has found that quantitative microbiological techniques can reveal trends within populations of resistant *E. coli*. Using these techniques has revealed that it may be possible for the ampicillin-resistant proportion of the fecal *E. coli* population to increase even in the absence of the use of exogenous antimicrobial agents. This observation is worthy of further investigation.

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