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Risk factors for occurrence of cephalosporin-resistant *Escherichia coli* in Norwegian broiler flocks

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ABSTRACT

A longitudinal study of 27 broiler farms including 182 broiler flocks was performed to determine risk factors for occurrence of cephalosporin-resistant *Escherichia coli* in Norwegian broiler flocks. Information regarding possible risk factors was collected by an online questionnaire and by samples obtained from broiler and parent flocks during the study period. Additional information was provided by the broiler production company. The prevalence of cephalosporin-resistant *E. coli* in parent flocks and broiler flocks sampled in the study was estimated.

Cephalosporin-resistant *E. coli* was detected in 13.8% of the parent flocks and 22.5% of the broiler flocks included in the study.

A multivariable generalized linear model was used to estimate risk factors. The risk for occurrence of cephalosporin-resistant *E. coli* was associated with the status of the previous flock in the broiler house (odds ratio = 12.7), number of parent flocks supplying the broiler flock with day-old chickens (odds ratio = 6.3), routines for disinfection of floor between production cycles (odds ratio = 0.1), and transport personnel entering the room where the broilers are raised (odds ratio = 9.3).

Our findings highlights that implementation of a high level of biosecurity with a minimal number of people entering the broiler house during production cycles, as well as rigorous cleaning and disinfection routines between production cycles will contribute to a decrease in the occurrence of cephalosporin-resistant *E. coli* in broiler flocks provided that there is no selection pressure from antimicrobial use in the broiler production.

1. Introduction

Escherichia coli (*E. coli*) with acquired resistance towards extended-spectrum cephalosporins is an increasing problem worldwide (Carattoli, 2008; Coque et al., 2008; Overdevest et al., 2011; Ewers et al., 2012). This is worrying, as cephalosporins are regarded as critically important antimicrobials for treatment of human infections (WHO-AGISAR, 2011). Broilers, including retail chicken meat, are associated with especially high occurrences of cephalosporin-resistant *E. coli* (Ewers et al., 2012). Presence of resistant bacteria in food is unwanted, as this can constitute a reservoir for human acquisition (Winokur et al., 2001; EFSA, 2011; Overdevest et al., 2011). In order to minimize the occurrence of cephalosporin-resistant *E. coli* in broiler flocks and thereby possibly also in retail chicken meat, it is necessary to identify and quantify risk factors for occurrence of cephalosporin-resistant *E. coli* in broiler flocks.

The usage of antimicrobial agents in the Norwegian broiler production is minimal (Mo et al., 2014), with only a single broiler flock treated in 2013 and 2014 (0.02%) (Animalia, 2015; Refsum, 2015) and four flocks treated in 2015 (0.08%) (Animalia, 2016). Furthermore, only two parent flocks (1.1%), have been treated yearly in the period 2013–2015 (Personal communication, Thorbjørn Refsum, Animalia, (Refsum, 2015)). Findings indicating vertical transmission of both cephalosporin-resistant *E. coli* and quinolone-resistant *E. coli* throughout the broiler production pyramid has been reported from Sweden (Nilsson et al., 2014; Börjesson et al., 2015), and import of breeding animals have been suggested as the

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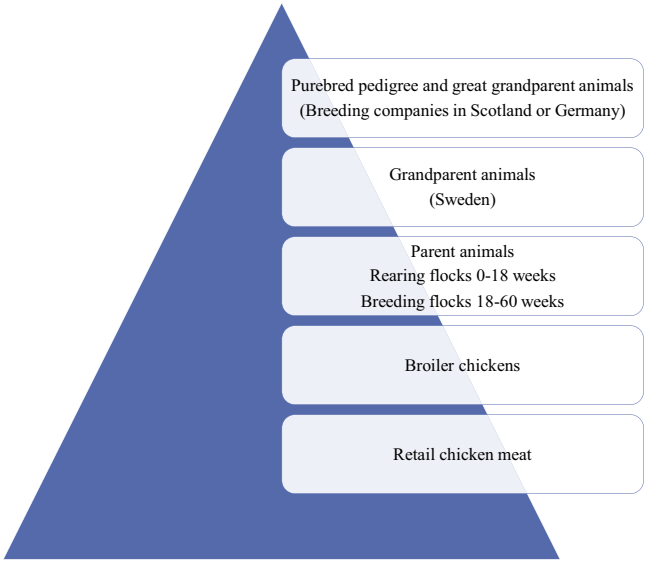

Fig. 1. Structure of the broiler production pyramid. Norway is dependent on import of hatching eggs produced by grandparent animals in Sweden. In Norway, only parent animals and broiler are produced.

probable source of resistant *E. coli* in both Sweden and Norway (NORM/NORM-VET, 2007; Sunde et al., 2009; Börjesson et al., 2013; Nilsson et al., 2014).

Norway is dependent on import of parent animals (imported as hatching eggs) from Sweden to supply the broiler production. Thus, only parent animals and broilers are produced in Norway (Fig. 1). One parent flock can supply several broiler flocks, and one broiler flock can receive chickens from several parent flocks per production cycle. Therefore, the potential for spread of newly introduced resistant bacteria is considerable (Dierikx et al., 2013b). In 2014, the Norwegian poultry industry started an action plan against cephalosporin-resistant *E. coli* in the Norwegian broiler production. As part of this project, samples were collected from imported parent animals after hatching. None of the samples collected at the parent hatchery of the largest chicken breeding company in Norway were positive for cephalosporin-resistant *E. coli* in 2014 (Animalia, 2015). However, cephalosporin-resistant *E. coli* were still detected in samples from retail chicken meat and faecal material from broilers collected in 2015 (NORM/NORM-VET, 2015). Therefore it is hypothesized that other factors than antimicrobial use and import of breeding animals contaminated with cephalosporin-resistant *E. coli* must play a role in the maintenance of cephalosporin-resistant *E. coli* in the Norwegian broiler production.

The aim of this study was to identify possible risk factors for occurrence of cephalosporin-resistant *E. coli* in broiler flocks affiliated to the largest broiler production company in Norway. Furthermore, the occurrence of cephalosporin-resistant *E. coli* in sampled parent- and broiler flocks during the study period was estimated.

2. Materials and methods

2.1. Study design and study population

The study was designed as a longitudinal study with each broiler flock as the study unit. The study population consisted of 62 randomly selected commercial broiler producers located in Hedmark county and affiliated to the largest broiler production company in Norway, Nortura Samvirkekylling. The random selection was generated using the command surveyselect in SAS Enterprise Guide 6.1 for Windows (SAS Institute Inc., Cary, NC, USA) from a dataset provided by Nortura SA. Criteria for inclusion in the study were as follows; active commercial broiler producer, farm located in Hedmark county, affiliation to Nortura Samvirkekylling, respond to questionnaire, and providing samples from a minimum of two broiler flocks housed at the farm during the study period.

To calculate the sample size (n), we used the following formula (Thrusfield, 2005) considering a large population size;

$$n = (Z_{\alpha}^2 \times p \times q)/L^2 \quad (1)$$

We expected a flock prevalence of 30% ($p = 0.30$, $q = 1–0.30 = 0.70$) based on previous knowledge on occurrence of cephalosporin-resistant *E. coli* in Norwegian broilers (NORM/NORM-VET, 2012). When applying a 95% confidence level ($Z\alpha = 1.96$) and an acceptable error of 5% ($L = 0.05$), this resulted in a sample size of 323 flocks. We further assumed that each producer would supply samples from an average of 6.5 flocks, proposing a required inclusion of at least 62 producers, as the expected response rate was 80%.

2.2. Data collection

An online questionnaire was designed in order to collect information about potential risk factors for the presence of cephalosporin-resistant *E. coli* in broiler flocks (Supplementary material). The questionnaire was based on a questionnaire previously used for collection of data regarding risk factors for campylobacteriosis in Norwegian broilers (Lyngstad et al., 2008). Furthermore, it was pilot tested on consultants working with broiler producers affiliated to Nortura SA. Before distribution of the questionnaire, a report was sent to the Norwegian Data Protection Authority.

A link to the online questionnaire and a request for participation in the study were sent per e-mail to the 62 selected broiler producers in November 2013, with three weeks' response time. The day after the deadline, reminders were sent to the producers that did not respond (n = 35). The same procedure was repeated one week later (n = 22). Three weeks after the first reminder, producers that still had not responded to the questionnaire (n = 17) were called and reminded again. Only broiler producers completing the questionnaire (n = 45) received equipment for sampling. This included boot swabs, prepaid envelopes for sending samples to the Norwegian Veterinary Institute in Oslo for analysis, and a thorough description of how to sample their broiler flocks. Broiler producers were instructed to sample broiler flocks housed at the farm from February 2014 through January 2015. Sampling was performed once during the production period using boot swabs. A flock was defined as broilers housed in a single house at the same farm during the same time period. All sampled flocks consisted exclusively of the hybrid Ross 308.

To obtain results regarding the occurrence of cephalosporin-resistant *E. coli* in the parent flocks providing hatching eggs for the broiler flocks, sampling was performed from November 2013 through October 2014. Samples were collected by the respective producer using boot swabs every four weeks during the production period. Nine parent flocks were not sampled in this study, but information regarding the presence of cephalosporin-resistant *E. coli* in these flocks was available from the poultry industry's action plan (Animalia, 2015). Sampling of these flocks was done once during the production period using boot swabs, and the samples were analysed using the same detection method in the laboratory. Furthermore, some grandparent flocks provided hatching eggs for a limited number of broiler flocks. Information regarding the occurrence of cephalosporin-resistant *E. coli* in grandparent flocks was provided by Nortura SA.

Broiler producers were reminded per e-mail if the sample was not received within the week the flock was going to be slaughtered,

while producers with parent flocks were reminded if the sample was not received five weeks after the previous sample.

Information about ancestry, flock size, slaughter date and slaughter age for all sampled broiler flocks was provided by Nortura SA.

2.3. Detection of cephalosporin-resistant E. coli

Boot swabs were dissolved in MacConkey broth (Beckton, Dickinson and Company, Le Pont de Claire, France) with 1 mg/L cefotaxime (Duchefa, Haarlem, The Netherlands) and incubated at 37 °C overnight. Subsequently, 10 μL of the broth was plated out on MacConkey agar (BD) supplemented with 1 mg/L cefotaxime, and MacConkey agar supplemented with 2 mg/L ceftazidime (Sigma-Aldrich, St. Louis, MO, USA). Agar plates were incubated at 37 °C for 24–48 h. Colonies with typical morphology were plated on blood agar, incubated at 37 °C overnight, and confirmed as *E. coli* using Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF, Bruker Daltonics). The cephalosporin resistance phenotype was investigated using the disk-diffusion method as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, www.eucast.org). Real-time PCR with previously published primers and probe (Schmidt et al., 2015) was applied to determine the genetic background of acquired cephalosporin resistance. The specificity of the detection method was assumed to be 100%, while a conservative estimate for the sensitivity was assumed to be 95% (Wasyl et al., 2010). The method corresponds to the method recommended by the European Food Safety Authority (EFSA, 2011).

2.4. Case definition and dependent variable

The occurrence of cephalosporin-resistant *E. coli* in a broiler flock was defined as the dependent variable. The status of a flock was defined as positive if *E. coli* from the sample were able to grow on MacConkey agar supplemented with cephalosporins, and an acquired cephalosporin resistance gene was detected by PCR. Otherwise, the flock was defined as negative.

2.5. Description of variables

The questionnaire included 58 multiple choice questions on potential risk factors for occurrence of cephalosporin-resistant *E. coli* in the broiler flock. The main subjects covered were information regarding the size of the production, management factors at the farm and in the broiler house(s), and areas surrounding the broiler house(s). Information regarding washing and disinfection routines was divided into single binary variables describing whether or not different parts of the house and equipment were washed or disinfected between each production cycle. Also, a binary variable indicating whether or not the disinfection routine was performed between each production cycle was included. For 13 of the variables derived from the questionnaire, two or more categories were merged to accomplish reasonable representation of each category.

The variable "Day sampled" represented the day a certain flock was sampled, and was defined as number of days after 31st January 2014, in accordance with the starting date for sampling of the included broiler flocks. Thus, "Day sampled" represented a variable explaining a possible seasonal variation in occurrence of cephalosporin-resistant *E. coli*.

For ten parent flocks, the status regarding occurrence of cephalosporin-resistant *E. coli* was unknown. To avoid missing data in the analysis, parent status was imputated once based on the distribution of the status of grandparent flocks and the status of the receiving parent flocks. Imputation was performed using the ranbin function in SAS Enterprise Guide 6.1 for Windows. If the status of the grandparent flock was negative, the probability of a receiving parent flock being positive was 0.12. Furthermore, if the status of the grandparent flock was positive, the probability of the receiving parent flock being positive was 0.14. In total, 25 broiler flocks were affected by the imputated parent status. In order to ensure that the imputation did not have a significant effect in the model, sensitivity analysis was performed on three datasets. The status of all parent flocks with an imputated status was changed to negative in the first dataset, positive in the second dataset, while all the 25 broiler flocks affected by the imputation were excluded in the third dataset. Univariable analyses with the status of the broiler flock as outcome and parent status as explanatory variable were performed on each dataset.

A variable describing the "overall status" of the parent flocks supplying hatching eggs for a single broiler flock was determined as follows; if at least one of the parent flocks were positive, the overall status was positive. If all parent flocks were negative, the overall status was negative.

Variables explaining the "total score cleaning" and "total score disinfection" were determined on the basis of how many parts of the broiler house and equipment were washed and disinfected between production cycles. One point was awarded for each part cleaned or disinfected with a maximum score of 11.

In order to distinguish between different houses at the same farm, a variable explaining the House ID was included in the dataset. If the farm only had one house, the House ID was the same as the Farm ID. A variable describing the number of parent flocks (i.e. 1, 2 or ≥3 flocks) supplying hatching eggs to each broiler flock and a variable explaining the size of the broiler flock (>10,000; 10,000–20,000; >20,000 chickens) was derived from the data provided by Nortura SA.

The start of the production period for a broiler flock was calculated as follows;

$$t_{ij} = s_{ij} - a_{ij} \quad (2)$$

where s was the slaughter date of flock i at farm j, and a is the slaughter age of flock i at farm j.

To calculate the number of empty days between two consecutive production cycles, the following formula was used;

$$t_{empty} = t_{ij} - s_{(i-1)j} \quad (3)$$

where t was the start of production of flock i at farm j, and s is the slaughter date of flock i at farm j. The number of empty days between production cycles was divided into three categories; <11 days, 11–20 days and >20 days.

A variable explaining the status of the previous broiler flock in the same house was included on the basis of the samples collected in this study. Consequently, the first flock sampled in each included house (n = 37) was excluded from the dataset to avoid missing values for the variable "Status of the previous broiler flock in the same house". In addition, farms or houses where samples from only a single flock were available were excluded in order to enable inclusion of a random nested effect of House ID within Farm ID in the model. The final dataset included 182 observations from 34 different broiler houses.

2.6. Data management and statistical analyses

Data management was performed in SAS Enterprise Guide 6.1 for Windows (SAS Institute Inc.), and statistical analyses were performed in R version 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria).

2.7. Univariable and multivariable analysis

All explanatory variables were fit into separate univariable logistic regression models using the function glm in R with status as the binary outcome variable. In parallel, the variables were also fit into separate univariable logistic regression models using the function glmer in the lme4 library in order to include a nested random effect of House ID within Farm ID. A p-value ≤ 0.20 was set as criterion for considering inclusion of the variable in the multivariable analysis.

"Day sampled" was modelled as a b-spline using the function bs in the R library splines.

The multivariable analysis was divided into two steps. First, variables were sorted into sub-groups, and a multivariable model was built for each sub-group. For each sub-group, associations between pairs of included variables were tested using Pearson Chi-squared tests (two categorical variables), ANOVA (one categorical and one numerical variable) or Spearman correlation (two numerical variables). If two variables were significantly associated, the variable with the highest biological plausibility or with the strongest association to the outcome was withheld for inclusion in the multivariable analysis. Each sub-model was built by backward selection. Only variables with a p-value ≤ 0.05 were retained in the sub-models. Subsequently, the significant variables from each of the sub-models were included in building of the final predictive model. This model was built as described for the sub-models. Due to the hierarchical structure of the data (i.e. multiple flocks and houses within farms), we performed the modelling with and without a nested random effect of House ID within Farm ID. Finally, all remaining variables, regardless of exclusion in univariable- and sub-models, were tested against the model one by one to investigate if any confounding effects were present. Furthermore, biologically plausible interactions between variables included in the final model were investigated. The models were compared using the likelihood ratio test and Akaike information criterion (AIC).

A receiver-operating characteristics (ROC)-curve was used to assess the overall predictive ability of the model, and the area under the curve was calculated. Residuals were plotted against predicted values and variables included in the final model.

Odds ratios and 95% confidence intervals were calculated from the estimated coefficients in the final model, and used as a prediction of the strength of the association between the variable and the outcome.

3. Results

3.1. Response rate

Two broiler producers randomly selected for participation in the project were excluded due to discontinuation of broiler production. The response-rate for the questionnaire was 72.6% (45/62). However, only 31 of the 45 responders contributed with samples from their broiler flocks, and two of these were excluded due to affiliation to another hatchery. Thus, the final response rate was 46.8% (29/62). An overview of included and excluded broiler producers is illustrated in Fig. 2.

3.2. Descriptive results

Cephalosporin-resistant *E. coli* were detected in samples from eight (13.8%) parent flocks (n = 58). Furthermore, 50 (22.5%) of all sampled broiler flocks (n = 222) and 41 (22.5%) of the broiler flocks included in the final dataset (n = 182) were positive. All isolates displayed an AmpC-phenotype and were found to carry the *bla*CMY-2 gene encoding cephalosporin resistance.

Five of the included broiler producers (n = 27) had more than one house at the farm, of which four producers had two houses and one producer had four houses, leading to a total of 34 houses included in the study. Overall, 28 (82.4%) of the houses housed only or mainly negative flocks (60–100% of flocks negative), while three houses (8.8%) housed only positive flocks. In three houses (8.8%), an equal distribution between positive and negative flocks was seen. The number of flocks sampled per house varied from two-eight, and the time span between sampling of consecutive flocks varied from 27 to 151 days.

The average flock size was approximately 14,000 broilers (range 3600–30,450), and the mean age of broilers at sampling was 17 days (range 6–32). The mean number of empty days between two production cycles was 22 (range 5–118 days).

The majority of the broiler flocks (n = 85, 46.7%) received day-old chickens from two parent flocks, while 58 (31.9%) broiler flocks received chickens from a single parent flock, and 39 (21.4%) received chickens from three or more parent flocks. None of the sampled broiler flocks were subjected to treatment with antimicrobial agents.

3.3. Multivariable analysis

Results from the univariable analyses are presented in Supplementary material (Tables S1a and b).

The variance for the nested random effect of House ID within Farm ID did not change when the variable describing floor disinfection was excluded, while the random effect of Farm ID increased from <0.001 to 0.4 (Tables S3a and b, Supplementary material). For the nested random effect of House ID within Farm ID, the variance increased from <0.001 to 0.12 when excluding the variable describing transport personnel entering the house. The variance for the random effect of Farm ID did not change (Tables S3a and c, Supplementary material). When comparing the different models including the nested random effect of House ID within Farm ID, the full model provided the best fit for the data ($p = 0.02$ and $p = 0.01$, respectively) (Table S3a, Supplementary material). The estimates for the included variables did not change significantly using the different models, but the AIC values were higher for the models including the nested random effect compared to the model excluding the nested random effect (Tables 1 and S3a–c, Supplementary material). Also, when the final multivariable models including and excluding the nested random effect of House ID within Farm ID were compared by the likelihood ratio test, the random effect was not significant ($p = 1.0$). Therefore, the simplest model possible with a high degree of explanation of the data without the nested random effect was chosen. The final multivariable model included the four variables "Status of previous broiler flock in the same house", "Transport personnel enter the room where the broilers are raised", "Always disinfect floor between production cycles" and "Number of parent flocks supplying the broiler flock" (Table 1). Disinfection of floor between production cycles reduced the odds of a positive status (OR = 0.1, $p = 0.01$, 95% confidence interval [CI] 0.03–0.6). On the other hand, a positive status of the previous flock (OR = 12.7, $p < 0.001$, 95% CI 4.8–33.5), having transport personnel entering the room where the broilers are raised (OR = 9.3, $p = 0.01$, 95% CI 1.6–55.1) and having three or more parent flocks supplying the broiler flock with day old chickens (OR = 6.3, $p = 0.01$, 95% CI 1.6–25.0) all increased the odds of a positive status (Table 1).

The area under the ROC curve was 0.86, indicating a good overall fit of the model to the observed data. The plot of residuals against the predicted values revealed no major shortcomings of the model.

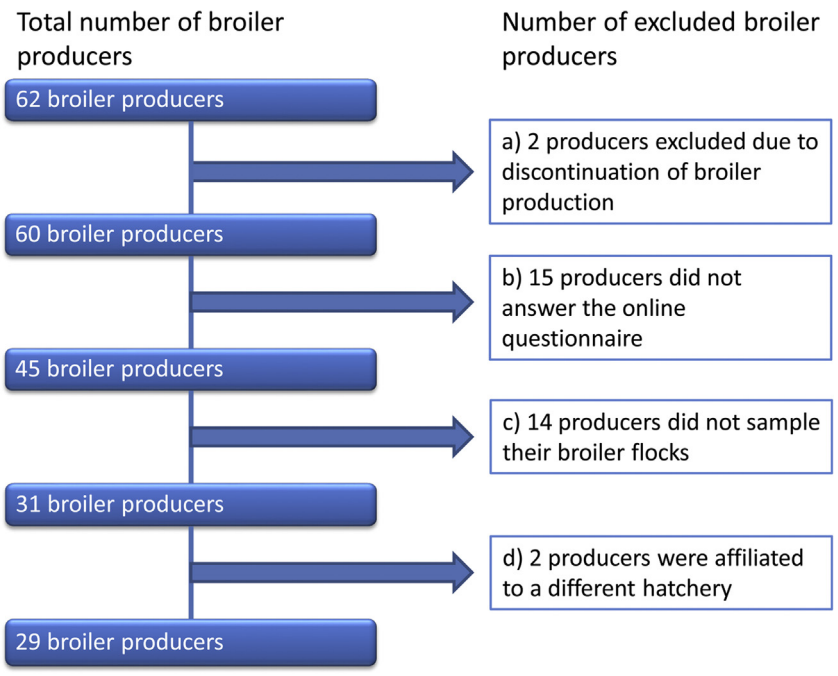

Fig. 2. Flow-chart showing included and excluded broiler producers in a study of risk factors for occurrence of cephalosporin-resistant *E. coli* in Norwegian broiler flocks from February 2014 through January 2015. The inclusion criteria were as follows; (1) Active commercial broiler producer, (2) respond to online questionnaire, (3) provide samples from broiler flocks housed at the farm during the study period, (4) affiliation to the hatchery of Nortura Samvirkekylling.

Table 1
Results from the multivariable generalized model used to identify risk factors for occurrence of cephalosporin-resistant *Escherichia coli* in 182 Norwegian broiler flocks originating from 34 houses on 27 farms during February 2014-January 2015. Nested random effect of House ID within Farm ID was not found to be significant (Likelihood ratio test, $p = 1.0$), and therefore not included in the final model.

Variable	Estimate	SE	OR (95% CI)	*p*-value
Status of previous broiler flock in the same house				
Negative	0		1	
Positive	2.5	0.5	12.7 (4.8–33.5)	<0.001
Transport personnel enter the room where the broilers are raised				
Never	0		1	
Occasionally	2.2	0.9	9.3 (1.6–55.1)	0.01
Always disinfect floor between production cycles				
No	0		1	
Yes	−2.0	0.8	0.1 (0.03–0.6)	0.01
Number of parent flocks supplying the broiler flock				0.02
1	0		1	
2	1.2	0.6	3.2 (0.9–11.1)	0.06
>2	1.8	0.7	6.3 (1.6–25.0)	0.01

Null deviance 194 on 181 ◦ of freedom, residual deviance 126 on 176 ◦ of freedom, and AIC = 138.0. SE: standard error, OR: odds ratio, CI: confidence interval.

4. Discussion

Our study indicated that the variables "status of previous broiler flock in the same house", "transport personnel entering the room where the broilers are raised", "always disinfect floor between production cycles" and "number of parent flocks supplying the broiler flock" were associated with a broiler flock being positive for cephalosporin-resistant *E. coli*.

The models including a nested random effect of House ID within Farm ID did not improve the models, as random effect provided the same explanation as the variables describing floor disinfection and transport personnel entering the house. These results led us to the conclusion that the multivariable model excluding the nested random effect provided the simplest and best fit for the data.

We identified a positive status of the previous broiler flock in the same broiler house as the most significant risk factor for occurrence of cephalosporin-resistant *E. coli* in a broiler flock. If the previous flock was positive, the odds of the next flock being positive was approximately thirteen times higher compared to if the previous flock was negative. Also, not always disinfecting the floor between production cycles was identified as a significant risk factor. This suggests that good cleaning and disinfection routines are essential in lowering the risk of transmission of cephalosporin-resistant *E. coli* from one flock to the next. Improvement of hygiene has also previously been suggested as a control measure to prevent spread of cephalosporin-resistant *E. coli*, and to prevent local recirculation of resistant strains in broiler houses (Liebana et al., 2013). Furthermore, we found that some broiler houses included in the study housed mainly positive flocks, while others housed none or only a few positive flocks. These findings enhance the theory that recirculation of cephalosporin-resistant *E. coli* in broiler houses may occur. A previous study from the Netherlands identified cephalosporin-resistant *E. coli* in empty broiler houses even after intensive cleaning and disinfection routines had been performed (Dierikx et al., 2013b). Recirculation of cephalosporin-resistant *E. coli* to consecutive flocks has also been suggested by others (Hiroi et al., 2012; Dierikx et al., 2013a; Laube et al., 2013; Agerso et al., 2014; Nilsson et al., 2014). These findings also underline the impor-

tance of sufficient cleaning and disinfection routines to minimize the risk of cephalosporin-resistant *E. coli* surviving in the broiler house and infecting the subsequent flock. However, our findings are in contrast to previous findings in a Belgian study, where it was suggested that a dirty environment may lead to a decrease in occurrence of resistant bacteria due to a more diverse microbiota and a dilution effect by susceptible bacteria (Persoons et al., 2011). Contamination of the environment surrounding the broiler farms with cephalosporin-resistant *E. coli* has been reported (Laube et al., 2014; Blaak et al., 2015). Thus, there is a possibility of cross-contamination from surrounding areas into the broiler house if hygienic measures are insufficient, or possibly via vectors such as flies (Blaak et al., 2014). The contamination of the surroundings, and thus the risk of cross-contamination will probably be higher on farms with many positive flocks, and might offer an explanation for why we observed some farms where the majority of flocks were positive, while other farms had no or only a few positive flocks.

The variable "Transport personnel enter the room where the broilers are raised" was identified as having a strong positive association with broiler flock status. Furthermore, this variable was not surprisingly, strongly associated with the variable "Average frequency of visitors in the broiler house per production cycle", and in the final model only one could be included. However, both variables represent an increased number of people entering the broiler house, which again might result in an increased risk of cross-contamination of resistant strains between hatchery and broiler house, between broiler houses at different farms, or between surrounding environment and the broiler house.

The odds of a positive status increased when three or more parent flocks supplied day-old chickens for a specific broiler flock. This may be explained by an increased probability of at least one of the supplying parent flocks being positive when the number of parent flocks increase, as only a limited occurrence of cephalosporin-resistant *E. coli* in parent flocks was found in this study. However, as no apparent association was found between status of supplying parent flocks and status of broiler flocks this theory does not represent a full explanation. Another possibility may be that several parent flocks can have a low level of cephalosporin-resistant *E. coli* circulating, and that this low level cannot be detected by the laboratory tests used in our study. However, when several parent flocks with low levels of cephalosporin-resistant *E. coli* supply one broiler flock with day old chickens, the "burden" of cephalosporin-resistant *E. coli* might accumulate, and the broiler flock could be positive. The lack of an association between the status of parent flocks and the status of broiler flocks receiving day-old chickens from these parent flocks was somewhat surprising. Vertical transmission of clonally related cephalosporin-resistant *E. coli* through the broiler production has been substantiated by findings in a study from Sweden (Nilsson et al., 2014), which has a broiler production highly similar to that in Norway. However, a decrease in the occurrence of cephalosporin-resistant *E. coli* in parent flocks has been reported in the Norwegian broiler production (Mo et al., 2014). Also, cephalosporin-resistant *E. coli* was not detected in any of the samples collected in conjunction with hatching of imported parent animals by Nortura Samvirkekylling in 2014 (Animalia, 2015). Thus, it is possible that the initial introduction of cephalosporin-resistant *E. coli* to the Norwegian broiler production was by vertical transmission from breeding animals as previously hypothesized (NORM/NORM-VET, 2007; Sunde et al., 2009), but that recirculation on farms already contaminated with resistant bacteria is more important when the burden of resistant bacteria from supplying parent flocks is low.

Previous studies have pointed at treatment with antimicrobials as an important risk factor for occurrence of cephalosporin-resistant *E. coli* in broiler flocks (Persoons et al., 2011; Agerso et al., 2014). In this study, none of the sampled broiler flocks were treated with antimicrobial agents. Furthermore, the consumption of antimicrobial agents in the Norwegian broiler production is minimal (Mo et al., 2014; Animalia, 2015, 2016; Refsum, 2015), and therefore this important risk factor cannot be identified in Norway.

The occurrence of cephalosporin-resistant *E. coli* in broiler flocks and parent flocks in this study was low to moderate compared to other countries (Persoons et al., 2011; Dierikx et al., 2013a; Reich et al., 2013; Agerso et al., 2014; MARAN, 2015). The plasmid-mediated AmpC-gene *bla*CMY-2, was identified in all cephalosporin-resistant isolates, which is in accordance with previous findings (Mo et al., 2014; NORM/NORM-VET, 2015).

A strength of the study was that the broiler flocks of included producers were sampled throughout a year. Thus, we would be able to identify possible seasonal variations in the occurrence of cephalosporin-resistant *E. coli*. However, no such variation was present in our data. Furthermore, we were able to get an overview of several flocks at each farm, meaning that we could identify whether included producers had houses where the majority of flocks were positive or negative. Also, parent flocks supplying the broiler flocks included in the study were sampled prior to the broiler flocks, allowing us to identify a possible association between status of the parent flocks and status of the broiler flocks. Data on the ancestry of all broiler flocks sampled in the study and the status of grandparent flocks supplying the included flocks was available from Nortura SA. However, this study also had some limitations. Only farms affiliated to a single hatchery were included, and the final response rate was low. In addition, samples were not provided for all flocks on all included farms, as we only received samples from 222 of 275 flocks (80.7%). This led to a smaller sample size than expected. Participation in the study was voluntary, possibly resulting in an over-representation of farmers with interest in issues regarding antimicrobial resistance and bacterial contamination. It is possible that these farmers also were extra careful with their cleaning and disinfection routines, and had better biosecurity routines than an average farmer. However, we still found that infrequent disinfection of floors, and transport personnel entering the broiler house increased the odds of occurrence of cephalosporin-resistant *E. coli* in broiler flocks. Thus, it is possible that the effect of these two variables have been underestimated in the current study. The online questionnaire was answered before the initiation of sampling. Therefore, it cannot be excluded that some of the answers provided might not be applicable for all flocks from the same farm, as routines could have changed during the study period. The direction of any possible bias resulting from this is difficult to estimate. The farms were located in a restricted geographical area, and only one hybrid, namely Ross 308, was represented. Thus, the results might not be directly extrapolated to the entire Norwegian broiler production. However, Nortura SA has the main share in the retail chicken meat market, and Ross 308 is by far the most common hybrid used in the Norwegian broiler production. Although the Norwegian broiler production is quite uniform, both hybrid and hatchery of origin has previously been identified as risk factors for cephalosporin-resistant *E. coli* (Persoons et al., 2011), and a possible effect of these two factors cannot be excluded on the basis of this study. Only one broiler producer reported that floors were not always disinfected between production cycles, and two broiler producers reported that transport personnel occasionally entered the room where the broilers are raised when delivering day-old chickens. Thus, it cannot be excluded that other factors than disinfection of floor and transport personnel entering the broiler house, not identified by the questionnaire, can be the reason that these producers differ from the others. However, it is highly biologically plausible that cephalosporin-resistant *E. coli* can survive in the broiler house if disinfection is not done, and that cross-contamination from hatcheries, other broiler farms or surrounding

areas can occur if transport personnel enter the room where the broilers are raised.

5. Conclusion

This is the first study identifying risk factors for occurrence of cephalosporin-resistant *E. coli* in Norwegian broilers. Our results indicate that implementation of a high level of biosecurity with a minimal number of persons entering the broiler house during each production cycle, and intensive washing and disinfection routines will contribute to a decrease in the odds of cephalosporin-resistant *E. coli* being detected in a broiler flock.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.prevetmed.2016.06.011.

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