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# Identification of Risk Factors for the Prevalence and Persistence of *Salmonella* in Belgian Broiler Chicken Flocks

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## Abstract

According to the European Food Safety Authority, salmonellosis is still one of the main causes of infectious foodborne gastroenteritis in humans. Broilers are an important source of salmonellosis after eggs and pork. Between 1987 and 1999 the trend of human salmonellosis incidence in Belgium increased constantly. However, from 2000 until 2005 a decrease in human cases was observed, probably following the sanitary measures implemented in the poultry breeder and laying sector. In order to decrease human infections it is essential to tackle the problem at the farm level to minimize cross contamination from farm to fork. This paper seeks to answer two questions (i) given the *Salmonella* status of the farm at a certain occasion (equal to the sampling time of the flock), what are the risk factors that the farm will be *Salmonella* positive at a following occasion? And (ii) what are the risk factors for a farm to be persistently positive for two consecutive flocks? We used surveillance data on 6,824 broiler flocks studied for *Salmonella* infectivity from 2005 to 2006 in Belgium. The farms were tested regularly (three weeks before slaughter of each broiler flock) for the presence of *Salmonella* based on multiple faecal samples per flock on a farm yielding clustered data. Generalized estimating equations, alternating logistic regression models, and random-intercept logistic regression models were employed to analyze these correlated binary data. Our results indicated that there are many factors that influence *Salmonella* risk in broiler flocks, and that they interact. Accounting for interactions between risk factors leads to an improved determination of those risk factors that increase infection with *Salmonella*. For the conditional analysis, the risk factors found to increase the risk of *Salmonella* infection on a farm at a current occasion given the previous *Salmonella* status included: *Salmonella* infection of day-old chicks (of the current flock); a previously infected flock even though the farm was equipped with a hygiene place to change clothes prior to entering the broiler house; having temporary workmen when there was a separation between birds of different species; and separating birds of different species in the Walloon region relative to the Flanders region. Sanitary measures such as a cleaning and disinfecting procedure conducted by an external cleaning firm, applying the all-in all-out procedure, and hand washing decreased the risk despite their interaction with other factors. From the joint analysis, the most important factors identified for increased risk for persistent *Salmonella* on a farm involved the interaction between having temporary workmen when there were poultry or farmers in contact with foreign poultry or persons, and the interaction between having temporary workmen when there were poultry or farmers in contact with external poultry or persons.

## Keywords:

Broiler chicken; Conditional probability; Joint probability; Intra-class correlation; Repeated data; Risk factors; *Salmonella*.

## Introduction

*Salmonella* is the second major cause of food borne diseases in Europe and in the World (Bouwknegt *et al.*, 2004; Collard *et al.*, 2007; EFSA (European Food Safety Authority), 2007). In addition to the health consequences, *Salmonella* infection also has a severe economical impact (Collard *et al.*, 2007; World Health Organisation (WHO), 2005). *Salmonella* spp with about 2600 existing serovars (Coburn *et al.*, 2007), are responsible for human illness and thus causes a real public health issue (Altekruse *et al.*, 2006; Collard *et al.*, 2007; Van Immerseel *et al.*, 2005). The symptoms in humans are most often characterised by the “non typhoid syndrome” which consists of an acute onset of fever, abdominal pain, nausea, and sometimes vomiting. These symptoms are self limiting in time. Humans become most often infected after consumption of contaminated eggs, poultry meat, pork, or, less frequently, bovine meat. In order to manage the risk to human health it is essential to tackle the problem at the farm level to reduce the cross contamination which can occur throughout the food chain process (Collard *et al.*, 2007; Van Immerseel *et al.*, 2005). Because animals most often are sub-clinically infected the disease tends to spread easily within a herd or flock, and because animals can become intermittent or persistent carriers, it is not easy to detect the prevalence of *Salmonella* other than by routine sampling for bacteriology testing (EFSA (European Food Safety Authority), 2007).

Belgium has implemented a *Salmonella* eradication programme in poultry in accordance with the European legislation 2160/2003 (E.U., 2003) in which a vaccination programme has been implemented in breeders and in layers but not in broilers because of the short life expectancy of broilers (42 days) (Anonymous, 2007; EFSA (European Food Safety Authority), 2004a). In broilers, a compulsory sampling, at least 3 weeks before slaughter, is requested from all farms with more than 5000 birds, as well as from farms who wish to trade their meat. A sanitary certificate is provided to the farm based on the results for *Salmonella* isolation (Anonymous, 1998; EFSA (European Food Safety Authority), 2004b). These results are requested by the slaughterhouses in order to programme their slaughter process, i.e., positive flocks must be slaughtered at the end of the day after slaughtering all negative flocks in order to avoid cross contamination within the slaughterhouse. Afterwards, the slaughterhouse is then thoroughly cleaned and disinfected. All positive farms are recorded in a notification system which exists since the 1st of January 2004 (Anonymous, 2007; EFSA (European Food Safety Authority), 2004b). One day-old chicks are sampled in the hatchery before being brought to the broiler farm. In Belgium, after a peak of infection in 1999, cases of salmonellosis in humans have been decreasing constantly, probably following vaccination and other sanitary measures implemented in poultry breeders and layers. In 2005 a total of 4872 human cases caused by *Salmonella* spp were registered (AFSCA (Agence Fédérale pour la sécurité de la chaîne alimentaire), 2007; Collard *et al.*, 2007; EFSA (European Food Safety Authority), 2004b; Van Pelt *et al.*, 2004).

Both vertical and horizontal transmissions play an important role in the contamination of flocks with *Salmonella*. Introducing only *Salmonella*-free chicks, e.g. by vaccinating the parental flocks against *Salmonella*, is an effective way to control the vertical transmission but will not prevent the contamination of the birds with the environment if in addition no hygienic measures are taken simultaneously (Van Immerseel *et al.*, 2005). Measures to reduce the horizontal transmission include ensuring *Salmonella*-free feed and water, effective cleaning and disinfection of the farm, the use of feed additives, applying all in all out procedures, appropriate biosecurity measures against animated or unanimated vectors, etc. (Anonymous, 2006; Davies and Breslin, 2001, 2004; Garber *et al.*, 2003; Gradel and Rattenborg, 2003;

Hald *et al.*, 1998; Renwick *et al.*, 1992; Skov *et al.*, 1999; Skov *et al.*, 2004; Van Immerseel *et al.*, 2005; Wales *et al.*, 2007; Wales *et al.*, 2006). A detailed description of the hygienic requirements for the farms in Belgium are described in the Belgian legislation (Anonymous, 1998). Even though these sanitary measures have been implemented as mentioned above, the burden of *Salmonella* infections on farms (mainly broilers) still exist, probably through contamination of the environment. For this purpose, further investigation of *Salmonella* on broiler farms with different flocks in time was essential. The first objective of the study was to examine the potential risk factors contributing to *Salmonella* infection of the current broiler flock on the farm given the *Salmonella* status of the previous flock. The other objective was to investigate the risk factors associated with the persistence (positive test result for the previous and current flocks) of *Salmonella* infection on the farm.

## **Materials and methods**

### **Data collection**

The database of the 2005-2006 Belgium *Salmonella* control programme carried out by the Federal Agency for the Safety of the Food Chain was used to investigate the *Salmonella* status at the entrance of one-day old broiler chicks and the status 3 weeks before slaughter (exit status). All the farms with more than 5000 birds and those willing to trade their meat must follow compulsory *Salmonella* sampling. Samples were taken by the owner. The epidemiological unit was a broiler flock. A flock is defined as a group of chickens from the same hatchery, belonging to the same herd, with the same sanitary and immune status, reared in the same room or barn, and having the following common characteristics: species, category (breeders, production), type (laying, broiler), stage of production (age), sanitary status (Anonymous, 1998, 2007; EFSA (European Food Safety Authority), 2004b). Each flock was sampled on entrance day and about 3 to 2 weeks before slaughter. To obtain the *Salmonella* status at entrance, day old chicks, arriving from the reproduction holding, were sampled by collecting specimens (20 pieces/flock, 5cm/5cm) of the inner lining of their transport boxes. The specimens were taken to the regional laboratory and tested for *Salmonella*. To obtain the *Salmonella* status at exit at about 3 to 2 weeks before slaughter faeces samples were sampled by one of the three following sampling methods: 1) a pooled sample (60 x 1g) taken with swabs, 2) 60 pooled faecal samples (300 to 600grams), 3) a pooled sample collected with 2 pairs of overshoes by walking in the barn. The samples were taken from different places of the barn where the flocks are kept and they were sent to an accredited laboratory within 48 hours according to standard norm ISO6579:2002 (Anonymous, 2007; ISO (Comité international de normalisation AW/9), 2002). A flock was considered positive when *Salmonella* was isolated from at least one sample and a farm was considered to be persistently positive if two consecutive flocks were positive on exit occasions. The information on the potential risk factors was obtained from a checklist questionnaire that was submitted to the different farmers during the 2003 Avian Influenza epidemic and answered on a voluntary basis. The risk factors which were investigated in our study are summarised in the data description part (Table 1). The information in the dataset of the 2005-2006 Belgian *Salmonella* national control program in broilers and that of the dataset identifying the risk factors were linked together using the farms identification number. The risk factors as well as the entrance *Salmonella* status for day-old chicks comprise the explanatory variables while the response variable refers to *Salmonella* status at exit. A more elaborate definition of the response variable follows in later sections.

## Data exploration

The design of the study was longitudinal with multiple observations collected on the same farms giving rise to correlated data. Table 1 shows the description of the variables that were recorded for the study. The response variables are binary outcomes of presence (outcome=1) or absence (outcome=0) of *Salmonella*. To get started, the data were re-structured to have the entrance outcome at a current occasion, the entrance outcome at the previous occasion, the exit outcome at a current occasion and the exit outcome at the single immediately previous occasion as separate variables. This implies that at least two flocks had to come on a farm thus eliminating farms that had one flock because they had no previous outcome. The interval in days between the consecutive flocks was calculated, thus creating a new variable (“duration”), split into three categories: less or equal to 6 weeks, between 6 and 12 weeks and over 12 weeks. The first objective of this paper used the exit outcome at a current occasion as the response variable. The previous exit outcome along with the current entrance outcome and other explanatory variables were used as predictor variables. The current entrance outcome was considered as baseline. For the second objective a new binary variable was created and denoted 1 if the current and previous exit outcomes were both positive and 0 otherwise. The explanatory variables included continuous and categorical variables. The frequencies of the data variables were explored as a check for sparseness. Moreover, the associations between each of the categorical predictor variables with the responses were examined using the Pearson chi square test of independence. A probability value of less than 0.05 leads to rejection of the null hypothesis of independence. Because of the availability of many independent variables per place of hygiene in a broiler house, a study of multicollinearity was imperative. Multicollinearity refers to the fact that independent variables are correlated with one another (Agresti, 2002; Neter *et al.*, 1996). To check this, Pearson chi square test was used to investigate the presence of association between any two classification variables while Pearson correlation coefficient was used to give an indication of the magnitude of this association. The variables were considered highly associated if their Pearson correlation coefficient was greater than 0.7. The relation of the continuous variables with the responses was investigated by estimating the difference in means between positive and negative *Salmonella* outcomes at exit.

Table 1: Variable descriptions. The binary variables take the value of 1 for a 'yes' reply to the question and 0 for a 'no' reply.

| Variable name                   | Description  | Variable type |
|---------------------------------|--|---------------|
| FarmID                          | Identifier for a farm.   | as given      |
| Broiler houseID                 | Identifier for broiler house.  | as given      |
| Samplingdate                    | Date the sample was taken.   | as given      |
| Analysedate                     | Date the sample was analysed.  | as given      |
| ReferenceID                     | Identifier for a sample.   | as given      |
| Sampletype                      | Type of sample.  | Categorical   |
| Entrance Result                 | positive <i>Salmonella</i> status for one-day old chicks?  | Binary        |
| Exit Result                     | positive <i>Salmonella</i> status for adult broilers before going for slaughter?                   | Binary        |
| Province                        | Province the data was obtained.  | Categorical   |
| Numberbroiler houses            | Number of broiler houses on a farm.  | Continuous    |
| NumberBroilers (Nbroilers)      | Number of broiler at the time of sampling.   | Continuous    |
| Distance (in km)                | The distance to the nearest poultry holding  | Continuous    |
| Production Type                 | Place where the broilers are reared.   | Categorical   |
| Shared materials                | Are there shared materials in broiler houses?  | Binary        |
| Species separation              | Is there separation between birds of different species on a holding?                               | Binary        |
| Protection Net                  | Is there a net protecting broilers from wild birds when there is an open air production type?      | Binary        |
| pre-broiler house Disinfection  | Is there one bucket to put in feet before entering the broiler house?                              | Binary        |
| pre-broiler house Hygiene place | Is there a place for changing clothes before entering the broiler house?                           | Binary        |
| broiler house hygiene place(HP) | Is there one place for hygiene per broiler house?  | Binary        |
| Hand-wash place                 | Is a place available to wash hands per hygiene place?  | Binary        |
| Undress place                   | Is a place available to undress per hygiene place?   | Binary        |
| HP Disinfection                 | Is a bucket for disinfection per hygiene place available?  | Binary        |
| Visitors dress                  | Are clean clothes for visitors available?  | Binary        |
| Ventilation                     | Is the ventilation mechanic?   | Binary        |
| Principle: All-in all-out       | Is the flock taken out and the place cleaned for about 3 days before the next flock is brought in? | Binary        |
| Cleaning Firm                   | Does an external firm clean the farm?  | Binary        |
| Feed producer                   | Is the feed from an accredited producer?   | Binary        |
| Town water                      | Is the water for drinking and cleaning from town?  | Binary        |
| Outside feeding                 | Do the broilers feed outside?  | Binary        |
| Temporary Workmen               | Are temporary workmen present on the farm?   | Binary        |
| International contacts          | Do poultry or farmers have contact with foreign poultry or persons?                                | Binary        |
| External contacts               | Do poultry or farmers have contact with external poultry or persons?                               | Binary        |

## Data Analysis

For comparative purposes we considered three statistical methods to incorporate and study the clustered type of data on *Salmonella* in Belgian broiler chicken flocks' farms. They are extensions of the well-known logistic regression that is a particular case of the generalized linear models with a logistic link function (McCullagh and Nelder, 1989). They are usually classified into marginal and random-effects models.

The generalized estimating equations (GEE) method, originally proposed by Liang and Zeger (1986) is a commonly used method for clustered data which models the correlation of a disease within clusters (for our case, *Salmonella* within farms). Let  $Y_{ij}$  denote the  $j$ th response at time point  $t_{ij}$  ( $j=1, \dots, n_i$ ) for farm  $i$  ( $i=1, \dots, N$ ) with expectation  $\pi_i$  and a working covariance matrix  $V_i$ . This covariance matrix  $V_i$  is an  $n_i$  by  $n_i$  matrix where the  $j$ th diagonal elements denote the variance for the  $j$ th observation (flock) in the  $i$ th farm and the off diagonal elements specify the correlation between two different flocks ( $j, k$ ) in the  $i$ th farm. Formally, this amounts to

$$V_i = \text{cov}(Y_{ij}, Y_{ik}) = \begin{cases} \pi_{ij}(1 - \pi_{ij}) & \text{if } j = k \\ \text{corr}(Y_{ij}, Y_{ik}) \times [\pi_{ij}(1 - \pi_{ij})\pi_{ik}(1 - \pi_{ik})]^{1/2} & \text{if } j \neq k \end{cases}$$

where  $\pi_{ij} = E(Y_{ij} = 1)$ . The term  $\text{corr}(Y_{ij}, Y_{ik})$  must be given a working correlation pattern in the analysis. Several choices are possible for the working form of the covariance matrix, ranging from the most simple assumption of independence ( $\text{corr}(Y_{ij}, Y_{ik}) = 0$  if  $j \neq k$ ) within clusters to the most complex unstructured form, where all parameters vary. It must be emphasized that estimation is consistent whatever the true correlation structure is, but efficiency is optimal when using an appropriate working covariance structure (Liang and Zeger, 1986).

Alternating logistic regression (ALR) is another method that explicitly models the clustering of *Salmonella* within farms. The ALR proposed by Carey *et al.*, (1993) also outlined by Bobashev and Anthony (1998) yields a readily interpretable statistical index of *Salmonella* clustering in the form of a “pairwise odds ratio” (PWOR). In the literal sense, the PWOR reflects how strongly *Salmonella* occurs in clusters. In more technical terms, the PWOR reflects odds of *Salmonella* for a flock in a cluster given that another randomly chosen flock from that cluster has *Salmonella*, relative to the odds if that randomly chosen flock does not have *Salmonella*. The logarithm of the PWOR can be expressed as a function of an indicator variable coded to show whether flocks  $j$  and  $k$  in a pair belong to the same or different farms:

$$\log(\text{PWOR}_{jk}) = \alpha F_{jk},$$

where  $F_{jk}$ , takes values 1 or 0, depending on whether the pair ( $j, k$ ) belongs to the same farm.

The third method incorporates clustering of *Salmonella* in farms through shared random effects. This involves the random components inside the linear predictor of ordinary logistic regression model, i.e random effects logistic regression model

$$\text{logit}(E(Y_{ij} | X_{ij}, Z_{ij}, u_i)) = X_{ij}^T \beta + Z_{ij}^T u_i$$

where the random effects  $u_i$  are assumed to vary independently from one farm to another according to a common distribution, usually the normal distribution with mean 0 and an unknown variance,  $\sigma^2$ .  $Z_{ij}$  is often a subvector of  $X_{ij}$ , which means that random effects apply only to a part of the covariates and/or the intercept. For simplicity, we refer to this model as the generalized linear mixed model (GLMM). The random effect variance is interpreted as the variation in  $\text{logit}(\pi_i)$  between farms after having accounted for fixed effects. With an approximate variance for the binary outcome the intra-class correlation (ICC) (correlation between two flocks in the same farm) can be computed as the sum of variance components of common random effects divided by the total variation (fixed effects variation plus random variation). More details about these models can be found in Agresti (2002) and Molenberghs and Verbeke (2005). Complete datasets, where the missing values are eliminated, were utilized for analyzes.

The analyzes were twofold: the first analysis uses the current exit outcome conditional on the previous outcome as the response variable while the second uses the joint outcome that the current and previous exit outcomes were both positive as response.

### Conditional Analysis

For each farm  $i$ , we distinguished the previous entrance response  $Y_{it}^e$  as the entrance response at time  $t$ , the current entrance response  $Y_{it+1}^e$  as the entrance response at time  $t+1$ , the previous exit response  $Y_{it}^o$  as the exit response at time  $t$ , and the current exit response  $Y_{it+1}^o$  as the exit response at time  $t+1$ . The statistical methods simultaneously account for clustering and the influence of covariates. For particular values of the explanatory variable,  $\mathbf{X}_i = (x_{i1}, \dots, x_{ip})$ , we modeled the current exit probability of *Salmonella* adjusting for the previous exit outcome and the current entrance response for one-day old chicks as baseline, using the logistic model

$$\text{logit}[P(Y_{it+1}^o = 1 | y_{it}^o, y_{it+1}^e)] = \beta_0 + \beta_1 y_{it}^o + \beta_2 y_{it+1}^e + \sum_p$$

where are effects of the corresponding explanatory variables. Here, the GEE method solves score equations of a marginal formulation of the likelihood function and uses a working correlation matrix (for our case, the exchangeable structure) to adjust for the correlation within clusters. The estimation using ALR is via iterative recalculation of PWORs (assuming an exchangeable log odds structure) and logistic regression on the outcomes (Agresti, 2002; Carey *et al.*, 1993).

In the GLMM we allowed the intercepts to vary for each farm and modeled the current exit probability of *Salmonella* adjusting for the previous exit outcome and the current entrance response  $y_{it+1}^e$  using the extended logistic model

$$\text{logit}[P(Y_{it+1}^o = 1 | u_i)] = \beta_0 + \beta_1 y_{it}^o + \beta_2 y_{it+1}^e + \sum_p + u_i \quad u_i \sim N(0, \sigma_u^2)$$

The model describes farm-specific intercepts instead of farm-averaged intercepts. Based on the underlying continuous variable coming from a logistic distribution, with a variance of  $\pi^2/3$ , which we substitute for the level 1 variance leads to a formulation of the intra-class correlation (ICC) (Browne *et al.*, 2005) across farms as

$$\frac{\sigma_u^2}{\sigma_u^2 + \pi^2/3}$$



More details about these model formulations as two-state discrete time Markov chains can be found in Agresti (2002) and Lindsey (1997).

### Joint Analysis

The same models as in the conditional analysis were adapted to model the persistence of *Salmonella* infection on a farm. We modelled the probability that at two consecutive occasions a farm was infected using the marginal models

$$\text{logit}[P(Y_{it}^o = 1, Y_{it+1}^o = 1)] = \beta_0 + \sum_p$$

and the farm-specific model

$$\text{logit}[P(Y_{it}^o = 1, Y_{it+1}^o = 1 | u_i)] = \beta_0 + \sum_p + u_i \quad u_i \sim N(0, \sigma_u^2)$$

### Model Selection

The data constituted more than 20 potential predictor variables (Table 1). Selecting a model from all main effects and their two-way or higher interactions often leads to a selection from a very large number of effects and produces a model that overfits the data. Moreover, when these effects include classification variables with several levels, the number of parameters available for selection is even larger. To determine what main effects and interactions to allow, we considered the dependence of each of the variables on the response and the presence of and magnitude of associations between predictor variables in order to avoid multicollinearity problems (see data description in Section 2.2). If multicollinearity existed, the choice of the variable to be included in the model was based on how strong it was related to the responses.

The model was constructed in a way that the response variable depends on the continuous variables and classification variables as well as on some two-way interactions of these effects. A parsimonious model was selected based on; the backward automatic selection procedure with the SAS LOGISTIC procedure, the Akaike Information Criterion (AIC) for the GLMM model and using the Quasi under Independence model Criterion (QIC) for the marginal models. The smaller the criteria value the better a particular model fits. The QIC criterion proposed by Pan (2001) and further discussed by Hardin and Hilbe (2003) is an analogue to the AIC extending its applicability to quasi-likelihood models. Like the AIC, the QIC adds a penalty term of twice the number of parameters in the model to the quasi-likelihood. The final GLMM model was fitted with the GLIMMIX SAS procedure. In the next Section we present the estimated effects of the fitted models.

## Results

This section presents the descriptive results and the results from model fitting. However, it is worth mentioning that when interpreting model fitting results, caution must be taken with those risk factors involved in higher order interactions since the interpretation of effects related to interaction terms involves the description of the effects of one variable depending on the value of the other variable.

### Data exploration

A description of all variables used in this paper is presented in Table 1. The frequencies, response rates and chi-square association probability values corresponding to the predictor variable categories in regard to both the conditional response (Table 2) and joint response (Table 3) are presented. Because of sparseness of data in some categories of province, we combined the provinces of Brabant Wallon, Hainaut, Liège, Luxembourg and Namur into the Walloon region (denoted 1) and the provinces of Antwerpen, Limburg, Oost-Vlaanderen, Vlaams Brabant and West-Vlaanderen to form the Flanders region (denoted 0). The upper part of the table includes binary predictor variables. During the period considered (2005-2006) 6824 broilers flocks on 723 farms were sampled. Of the 41 one-day old chicks which were positive for *Salmonella* at the current entry, 19.51% (Table 2) resulted positive at the current exit occasion. Given the 404 flocks that were infected at the previous exit occasion, 27.97% were also infected at the current exit occasion (Table 2). None of the one-day old chicks were infected at two consecutive entrance occasions. The proportion of broiler flocks that were infected at two consecutive exit occasions was 1.66%.

For the conditional response (Table 2), the following variables with chi-square p-values less than 0.05 were observed to be associated with the probability of *Salmonella* infectivity of a current exit flock: a previous positive *Salmonella* status at exit, a positive *Salmonella* status of one-day old chicks of the current flock during entrance, availability of shared materials in broiler houses, having a separation between the different bird species, presence of a hand wash place, use of an external cleaning firm, having temporary workmen, having poultry or farmers in contact with foreign poultry or persons and rearing birds in the Walloon versus Flanders region. For the joint response (Table 3), we observed the following variables to increase the probability that farms were infected at two consecutive exit occasions: existence of shared materials in broiler houses, separation between different bird species, applying the all-in all-out principle, using a external cleaning firm, having temporary workmen, having poultry or farmers in contact with foreign poultry or persons, having poultry or farmers in contact with external poultry or persons, rearing birds in Walloon versus Flanders region and the duration in between consecutive flocks. For the distributions of conditional and joint responses with the continuous variables (lower panels of Table 2 and Table 3), number of broilers and number of broilerhouses, we see that the mean predictor values were higher for the infected flocks relative to the non-infected ones suggesting these variables to be possible risk factors. Also the mean distance to the nearest poultry holding was smaller for the infected groups than for the non-infected indicating that reduced distance to the nearest poultry holding might be a potential risk factor.

The findings on multicollinearity using Pearson chi square test for independence showed highly significant (p-value <0.0001) associations between the pairs of the following variables: having a hand-wash place per hygiene place (HP), having an undressing place per HP, availability of a disinfection bucket per HP, presence of visitors special clothing and feed from accredited producers. Table 4 presents their Pearson correlation coefficients and they range from 0.72 to 0.84. Feed from accredited producers and use of town water for drinking

and cleaning were also highly associated with a correlation of 0.75. The presence of a hygiene place per broiler house was found to be associated with presence of the visitors' special clothing with a correlation of 0.70. Because the presence of a hand wash place per HP was more related to the responses (see  $\chi^2$  p-values, Table 2 and Table 3), it was used in substitute of the others to avoid multicollinearity. Some variables like production type and the number of broiler houses were not considered further for the analyses due to a large portion of missing values. Observations for feed from accredited producers and use of town water for drinking and cleaning and outside feeding existed for one category of the joint response and thus could not be considered for analysis as they would be inestimable.

It should be noted that all these results should be considered as indicative though not as formal inferential results, as they did not account for the clustered nature of the data. In the next section models and methods for clustered data as introduced in section 2, will be used to identify risk factors for *Salmonella*.

Table 2: Distribution of the conditional response with the study variables based on 6824 flocks from 723 farms. Chi-square ( $\chi^2$ ) association p-values < 0.05 show significant association between the response and each categorical variable.

| Binary Variable               | Variable Category 0 |                     | Variable Category 1    |                     | Chi-square             |
|-------------------------------|---------------------|---------------------|------------------------|---------------------|------------------------|
|                               | Flock Observations  | Positive Flocks (%) | Flock observations     | Positive Flocks (%) | P-value                |
| Previous exit response        | 6420                | 3.99                | 404                    | 27.97               | <.0001                 |
| Current entry response        | 6783                | 5.32                | 41                     | 19.51               | <.0001                 |
| Shared materials              | 3702                | 4.38                | 3122                   | 6.63                | <.0001                 |
| Species separation            | 6501                | 5.26                | 323                    | 8.36                | 0.0163                 |
| Protection Net                | 567                 | 5.64                | 6257                   | 5.39                | 0.7950                 |
| Pre-broilerhouse disinfection | 354                 | 4.80                | 6470                   | 5.44                | 0.6052                 |
| Pre-broilerhouse hygieneplace | 1096                | 4.84                | 5728                   | 5.52                | 0.3611                 |
| Broilerhouse HP               | 447                 | 7.16                | 6377                   | 5.28                | 0.0903                 |
| Handwash place/HP             | 354                 | 8.47                | 6470                   | 5.24                | 0.0088                 |
| Undressplace/HP               | 343                 | 7.58                | 6481                   | 5.29                | 0.0679                 |
| HP disinfection               | 324                 | 4.94                | 6500                   | 5.43                | 0.7021                 |
| Visitors dress                | 261                 | 6.13                | 6563                   | 5.38                | 0.5985                 |
| Mecanic ventilation           | 737                 | 5.56                | 6087                   | 5.39                | 0.8431                 |
| All-in All-out principle      | 1117                | 6.09                | 5707                   | 5.27                | 0.2716                 |
| Cleaning firm                 | 5137                | 5.88                | 1687                   | 3.97                | 0.0027                 |
| Feed producer                 | 232                 | 3.02                | 6592                   | 5.49                | 0.1015                 |
| Town water                    | 326                 | 3.99                | 6498                   | 5.48                | 0.2455                 |
| Outside feeding               | 6803                | 5.41                | 21                     | 4.76                | 0.8958                 |
| Temporary workmen             | 6287                | 5.22                | 537                    | 7.64                | 0.0174                 |
| International contacts        | 6652                | 5.31                | 172                    | 9.30                | 0.0222                 |
| External contacts             | 5926                | 5.30                | 898                    | 6.12                | 0.3078                 |
| Region                        |                     |                     |                        |                     |                        |
| Walloon(1) Flanders(0)        | 5878                | 5.78                | 910                    | 3.08                | 0.0008                 |
| Categorical Variable          | Flock observations  | Positive flocks(%)  | $\chi^2$ p-value       |                     |                        |
| Duration (in weeks)           |                     |                     |                        |                     |                        |
| up to 6                       | 1181                | 6.18                |                        |                     |                        |
| 6 to 12                       | 4537                | 5.11                |                        |                     |                        |
| more than 12                  | 1106                | 5.79                |                        |                     |                        |
| Production type               |                     |                     |                        |                     |                        |
| Bio                           | 1                   | 0.00                |                        |                     |                        |
| Cage                          | 50                  | 6.00                |                        |                     |                        |
| Free range                    | 45                  | 4.44                |                        |                     |                        |
| Barn                          | 1789                | 4.02                |                        |                     |                        |
| Continuous Variable           | Overall             | Salmonella negative |                        | Salmonella positive |                        |
|                               | Mean (SD)           | Flock observations  | Mean (SD)              | Flock observations  | Mean (SD)              |
| Number of broilers            | 35657.27<br>(23404) | 6388                | 35160.57<br>(22498.27) | 367                 | 44302.76<br>(34572.74) |
| Number broilerhouses          | 1.8671<br>(1.2630)  | 4941                | 1.8438<br>(1.2463)     | 297                 | 2.2559<br>(1.4618)     |
| Distance to poultryFarm       | 2.1289<br>(3.2293)  | 5764                | 2.1657<br>(3.2718)     | 317                 | 1.4606<br>(2.2238)     |

Note: 36, 4939, 69, 1586, 743 respectively, were missing data for region, production type, number of broilers, number of broiler houses and distance to nearest holding.

Table 3: Distribution of the joint response with the study variables based on 6824 flocks from 723 farms. Chi-square ( $\chi^2$ ) association p-values < 0.05 show significant association between the response and each categorical variable.

| Binary Variable                   | Variable Category 0 |                     | Variable Category 1    |                     | $\chi^2$ p-value       |
|-----------------------------------|---------------------|---------------------|------------------------|---------------------|------------------------|
|                                   | Flock observations  | Positive flocks(%)  | Flock observations     | Positive flocks(%)  |                        |
| Shared materials                  | 3702                | 1.19                | 3122                   | 2.21                | 0.001                  |
| Species separation                | 6501                | 1.46                | 323                    | 5.57                | <.0001                 |
| Protection Net                    | 567                 | 0.88                | 6257                   | 1.73                | 0.1314                 |
| pre-broilerhouse disinfection     | 354                 | 0.85                | 6470                   | 1.7                 | 0.2209                 |
| pre-broilerhouse hygieneplace     | 1096                | 1.19                | 5728                   | 1.75                | 0.1834                 |
| Broilerhouse HP                   | 447                 | 2.68                | 6377                   | 1.58                | 0.0779                 |
| Handwash place/HP                 | 354                 | 3.95                | 6470                   | 1.53                | 0.0005                 |
| Undressplace/HP                   | 343                 | 3.21                | 6481                   | 1.57                | 0.0209                 |
| HP disinfection                   | 324                 | 1.54                | 6500                   | 1.66                | 0.8706                 |
| Visitors dress                    | 261                 | 1.92                | 6563                   | 1.65                | 0.7374                 |
| Mecanic ventilation               | 737                 | 0.81                | 6087                   | 1.76                | 0.0579                 |
| All-in All-out principle          | 1117                | 2.78                | 5707                   | 1.44                | 0.0013                 |
| Cleaning firm                     | 5137                | 1.95                | 1687                   | 0.77                | 0.001                  |
| Feed producer                     | 232                 | 0.00                | 6592                   | 1.71                | 0.0443                 |
| Town water                        | 326                 | 0.00                | 6498                   | 1.74                | 0.0164                 |
| Outside feeding                   | 6803                | 1.66                | 21                     | 0.00                | 0.5515                 |
| Temporary workmen                 | 6287                | 1.48                | 537                    | 3.72                | <.0001                 |
| International contacts            | 6652                | 1.56                | 172                    | 5.23                | 0.0002                 |
| External contacts                 | 5926                | 1.50                | 898                    | 2.67                | 0.0104                 |
| Region: Walloon(1) vs Flanders(0) | 5878                | 1.80                | 910                    | 0.77                | 0.0233                 |
| Categorical Variable              | Flock observations  | Positive flocks(%)  | $\chi^2$ p-value       |                     |                        |
| <i>Duration (in weeks)</i>        |                     |                     | <.0001                 |                     |                        |
| up to 6                           | 1181                | 3.81                |                        |                     |                        |
| 6 to 12                           | 4537                | 1.28                |                        |                     |                        |
| more than 12                      | 1106                | 0.90                |                        |                     |                        |
| <i>Production type</i>            |                     |                     | 0.0609                 |                     |                        |
| Bio                               | 1                   | 0.00                |                        |                     |                        |
| Cage                              | 50                  | 6.00                |                        |                     |                        |
| Free range                        | 45                  | 0.00                |                        |                     |                        |
| Barn                              | 1789                | 1.45                |                        |                     |                        |
| Continuous Variable               | Overall             | Salmonella negative |                        | Salmonella positive |                        |
|                                   | Mean (SD)           | Flocks observations | Mean (SD)              | Flocks observations | Mean (SD)              |
| Number of broilers                | 35657.27<br>(23404) | 6642                | 35370.45<br>(22821.77) | 113                 | 52516.27<br>(43082.64) |
| Number broilerhouses              | 1.8671<br>(1.2630)  | 5137                | 1.8491<br>(1.2405)     | 101                 | 2.7822<br>(1.9058)     |
| Distance to poultryFarm           | 2.1289<br>(3.2293)  | 5985                | 2.1445<br>(3.2467)     | 96                  | 1.1563<br>(1.5783)     |

Note: 36, 4939, 69, 1586, 743 respectively, were missing data for region, production type, number of broilers, number of broilerhouses and distance to nearest holding.

Table 4: Pearson correlation coefficients for testing independence between any two of the designated covariates.

| Variable              | var1 | var2 | var3 | var4 | var5 | var6 | var7 |
|-----------------------|------|------|------|------|------|------|------|
| var1:Broilerhouse HP  | 1.00 | 0.65 | 0.68 | 0.62 | 0.70 | 0.63 | 0.52 |
| var2: Handwash place  |      | 1.00 | 0.78 | 0.78 | 0.80 | 0.80 | 0.59 |
| var3: Undress place   |      |      | 1.00 | 0.72 | 0.81 | 0.73 | 0.60 |
| var4: HP disinfection |      |      |      | 1.00 | 0.84 | 0.84 | 0.62 |
| var5:Visitors dress   |      |      |      |      | 1.00 | 0.84 | 0.70 |
| var6:Feed producer    |      |      |      |      |      | 1.00 | 0.75 |
| var7:Town water       |      |      |      |      |      |      | 1.00 |

Note: The p-value was <.0001 for all combinations rejecting the null hypothesis of independence.

### Conditional Analysis

The results from the conditional analysis, which investigated the risk factors associated with the probability of *Salmonella* infection of a current flock at exit from the farm given the *Salmonella* status of the previous flock using generalized estimating equations, alternating logistic regression models and logistic-normal random intercept model (GLMM) are presented in Table 5. From the three approaches, 15 predictors were shown to be associated with *Salmonella* infection of the current broiler flock. One-day old chicks at entrance infected with *Salmonella* was a highly significant risk factor for *Salmonella* to the current flock on the farm. The estimated farm-averaged odds ratios of *Salmonella* to one-day old chicks were  $e^{1.658} = 5.24$  and  $e^{1.503} = 4.50$ , respectively for GEE and ALR models while the estimated farm-specific odds ratio was  $e^{1.481} = 4.4$  using the GLMM model. Generally, the three approaches produced similar results in terms of statistical significance. Except for the one-day old chicks' predictor variable, the other predictors were found to interact with each other as they all influenced *Salmonella* infection of the current broiler flock on the farm.

The impact of the *Salmonella* status of the previous flock on the probability of *Salmonella* for the current flock was found to depend, pair wise, on five other factors. From GEE and ALR models, while having a hygiene place for changing clothes before entering the broiler house increased the odds for *Salmonella* for the current flock when the previous flock was infected with *Salmonella*, the existence of the hygiene place decreased the risk when a previous flock was uninfected. With the GLMM model, the presence of a hygiene place decreased the odds for *Salmonella* when the previous flock was infected, but decreased further when the previous flock was uninfected. Also from the GLMM model, the use of mechanic ventilation decreased the odds for *Salmonella* when the previous flock was infected, but the risk decreased further when the previous flock was uninfected. Still, applying the all-in all-out principle or using an external cleaning firm or introducing a new flock on a farm at least six weeks after the previous flock, decreased the odds for *Salmonella* when the previous flock was infected, with further decrease when the previous flock was not infected.

The effect of the number of broilers on the occurrence of *Salmonella* to the current flock, given the *Salmonella* status of the previous flock, interacted with five other predictors. Separation between birds of different species or having a hygiene place for changing clothes before entering the broiler house or region of location or employing an external cleaning firm to clean or using temporary workmen, decreased the odds for *Salmonella* when the number of broilers was less or equal to 2SDs from the mean number of broilers ( $N_{Broilers} \leq 82465$ ) (see Table 2). With this number of broilers, the odds for *Salmonella* decreased further in the Walloon region than in the Flanders region. Using the GLMM model, a larger number of broilers ( $N_{Broilers} = \text{mean} + 3SDs$ ) increased the odds for *Salmonella* when an external firm

cleaned, while from the ALR model this larger number of broilers increased the risk when there were temporary workmen. For illustration purposes, the interaction effect of the number of broilers and the cleaning external firm using the GEE model was derived as

$$\begin{aligned}\text{Log(odds)} &= -3.985 + 3.1\text{E-}5*\text{NBroilers} - 3.289*(0) + 3.4\text{E-}5*\text{NBroilers}*(0) && \text{for Firm} = 0 \\ \text{Log(odds)} &= -3.985 + 3.1\text{E-}5*\text{NBroilers} - 3.289*(1) + 3.4\text{E-}5*\text{NBroilers}*(1) && \text{for Firm} = 1\end{aligned}$$

The three models also revealed that separating between birds of different species or having a hand wash place in the hygiene place; decreased the odds for *Salmonella* with a one kilometer increase in the distance to the nearest poultry holding. The GEE and ALR models showed that using mechanic ventilation reduced the odds for *Salmonella* when the distance to the nearest poultry holding was increased. While using temporary workmen increased the risk for *Salmonella* when there was a separation between birds of different species, the odds decreased when birds of different species were separated and there were no temporary workmen. Similarly, farms located in the Walloon region had increased odds for *Salmonella* when there was a separation between birds of different species, but the odds decreased when there was a separation between birds of different species for farms located in the Flanders region.

Using an external cleaning firm decreased the odds for *Salmonella* when there was a protection net sheltering the broilers from wild birds, but the odds went down further when the external firm was employed and the protection net was not available. The presence of a hand wash place decreased the odds for *Salmonella* when there were poultry or farmers in contact with external poultry or persons, but absence of a hand wash place and presence of external contacts led to an increase in the odds for *Salmonella*. Finally, using an external cleaning firm decreased the odds for *Salmonella* regardless of the existence of temporary workmen, but the odds decreased further when there were temporary workmen than when they did not exist.

The GLMM model estimated the variance of the farm-specific intercepts as  $\hat{\sigma}_u^2 = 0.6526$  giving an estimated intra-class correlation of 0.165. In contrast, the estimated exchangeable correlation based on GEE was  $\hat{\rho} = 0.032$ . The pairwise exchangeable odds ratio using the ALR was 0.758 and it is highly significant.

Table 5: Parameter estimates and their standard errors and their significance p-values from the conditional analysis for GEE and ALR Marginal Models and for the Random intercepts model(GLMM)

|                            | Marginal model GEE |         | Marginal model ALR |         | GLMM               |         |
|----------------------------|--------------------|---------|--------------------|---------|--------------------|---------|
|                            | $\hat{\beta}$ (SE) | P-value | $\hat{\beta}$ (SE) | P-value | $\hat{\beta}$ (SE) | P-value |
| Intercept                  | -3.985(0.441)      | <.0001  | -3.932(0.473)      | <.0001  | -3.994(0.773)      | <.0001  |
| Previous exit $y_t^o$      | 1.802(0.781)       | 0.0210  | 1.523(0.849)       | 0.073   | 0.323(1.000)       | 0.7469  |
| Current entry $y_{t+1}^e$  | 1.658(0.523)       | 0.0015  | 1.503(0.568)       | 0.008   | 1.481(0.464)       | 0.0014  |
| Number of Broilers         | 3.1E-5 (3.7E-6)    | <.0001  | 3.1E-5(4.5E-6)     | <.0001  | 3.1E-5 (8.5E-6)    | 0.0003  |
| Distance to poultryFarm    | -2.918(0.869)      | 0.0008  | -2.919(0.777)      | 0.0002  | -2.578(1.084)      | 0.0175  |
| Species separation         | 2.447(0.684)       | 0.0003  | 2.561(0.645)       | 0.0001  | 2.676(0.967)       | 0.0057  |
| Protection Net             | -0.767 (0.330)     | 0.0203  | -0.651(0.409)      | 0.1111  | -                  | -       |
| Pre-broilerhouse           | 1.2417 (0.316)     | 0.0001  | 1.270(0.322)       | 0.0001  | 0.968(0.413)       | 0.0190  |
| Hygiene place              | -0.0225(0.307)     | 0.9415  | -0.036(0.371)      | 0.9219  | -0.699(0.617)      | 0.2566  |
| Handwash place             | -0.0307 (0.256)    | 0.9045  | -0.131(0.273)      | 0.6320  | -0.172(0.273)      | 0.5286  |
| Mecanic ventilation        | 0.0566(0.236)      | 0.8101  | 0.015(0.233)       | 0.9489  | 0.005(0.263)       | 0.98    |
| All-inAll-outPinciple      | -3.289 (0.914)     | 0.0003  | -2.883(0.969)      | 0.0029  | -0.903(0.369)      | 0.0143  |
| Cleaning Firm              | -0.8658(0.4826)    | 0.0728  | -0.832(0.521)      | 0.1101  | -0.480(0.336)      | 0.1537  |
| Temp workmen               | 5.298 (1.726)      | 0.0021  | 5.242(1.524)       | 0.0006  | 4.384(2.033)       | 0.0311  |
| External contacts          | -0.516 (0.295)     | 0.0797  | -0.490(0.294)      | 0.0949  | 0.481(0.503)       | 0.339   |
| Region: Wal vs Fla         |                    |         |                    |         |                    |         |
| <i>Duration (weeks)</i>    |                    |         |                    |         |                    |         |
| dur1: 6 to 12              | 0.491 (0.236)      | 0.0375  | 0.489(0.239)       | 0.0406  | 0.576(0.245)       | 0.0188  |
| dur2: > 12                 | 0.860 (0.276)      | 0.0019  | 0.848(0.278)       | 0.0022  | 0.972(0.277)       | 0.0005  |
| PrevY*hygieneplace         | 1.896 (0.637)      | 0.0029  | 1.466(0.680)       | 0.0311  | 1.757(0.685)       | 0.0103  |
| PrevY*principle            | -1.361 (0.505)     | 0.0070  | -1.210(0.531)      | 0.0228  | -1.227(0.493)      | 0.0128  |
| PrevY*Clean'gFirm          | -1.035 (0.518)     | 0.0457  | -                  | -       | -1.032(0.466)      | 0.0268  |
| <i>PrevY* Duration</i>     |                    |         |                    |         |                    |         |
| prevY*dur1                 | -0.740 (0.407)     | 0.0690  | -0.733(0.457)      | 0.1082  | -0.973(0.391)      | <.0129  |
| prevY*dur2                 | -1.728 (0.686)     | 0.0118  | -1.657(0.806)      | 0.0399  | -1.911(0.580)      | <.0010  |
| PrevY*MecanicV             | -                  | -       | -                  | -       | 1.541(0.696)       | 0.026   |
| NBroilers*Species          | -8.3E-5 (2.6E-5)   | 0.0012  | -8.3E-5(2.4E-5)    | 0.0005  | -8.0E-5(3.0E-5)    | 0.0051  |
| NBroilers*hygieneplace     | -3.1E-5 (5.5E-6)   | <.0001  | -3.1E-5(6.1E-6)    | <.0001  | -2.0E-5 (9.4E-6)   | 0.0100  |
| NBroilers*Clean'gFirm      | 3.4E-5 (8.0E-6)    | <.0001  | 2.8E-5(8.4E-6)     | 0.0008  | 2.1E-5(7.7E-6)     | 0.0066  |
| NBroilers*region           | -                  | -       | -                  | -       | -3.0E-5(1.2E-5)    | 0.0339  |
| NBroilers*Workmen          | 1.4E-5 (5.4E-6)    | 0.0114  | 1.5E-5 (5.9E-6)    | 0.0140  | -                  | -       |
| Distance*Species           | -1.425 (0.362)     | 0.0001  | -1.376(0.348)      | 0.0001  | -1.375(0.510)      | 0.0070  |
| Distance*Handwash          | 2.408 (0.850)      | 0.0046  | 2.428 (0.760)      | 0.0014  | 2.520(1.085)       | 0.0202  |
| Distance*MecanicV          | 0.474 (0.176)      | 0.0070  | 0.455 (0.168)      | 0.0067  | -                  | -       |
| Species*Workmen            | 7.410 (1.483)      | <.0001  | 7.123(1.444)       | <.0001  | 6.981(1.843)       | 0.0002  |
| Species*Region             | 5.051 (1.356)      | 0.0002  | 4.857 (1.310)      | 0.0002  | 4.212(1.873)       | 0.0245  |
| Nets*Clean'gFirm           | 2.019 (0.682)      | 0.0030  | 1.713 (0.788)      | 0.0296  | -                  | -       |
| Handwash*External          | -5.191 (1.733)     | 0.0027  | -5.190(1.534)      | 0.0007  | -4.313(2.048)      | 0.0352  |
| Clean'gFirm*Workmen        | -2.387 (0.676)     | 0.0004  | -2.357(0.700)      | 0.0008  | -                  | -       |
| $\hat{\rho}$               | 0.032              |         |                    |         |                    |         |
| logOR                      |                    |         | 0.758(0.171)       | <.0001  |                    |         |
| $\hat{\sigma}_u^2$ (flock) |                    |         |                    |         | 0.653(0.1492)      |         |



## Joint Analysis

In the joint analysis, we investigated risk factors impacting the probability that two consecutive flocks (previous and current) were positive for *Salmonella*. The results are shown in Table 6. The persistence of *Salmonella* on a farm by having two consecutive flocks with positive test results was associated with four variables in addition to five interaction terms.

Employing an external cleaning firm led to a decrease in the risk for persistent *Salmonella*. The estimated farm-averaged odds ratios of *Salmonella* were  $e^{-1.645} = 0.23$  and  $e^{-1.222} = 0.29$ , respectively for GEE and ALR models while the estimated farm-specific odds ratio was  $e^{-1.076} = 0.34$  using the GLMM model. Also, the duration between the consecutive flocks of at least six weeks led to a significant decrease of the risk for *Salmonella*. Furthermore, applying the all-in all-out principle decreased the risk for persistent *Salmonella* infection on a farm. In the GLMM model the effect of the number of broilers did not interact with other variables and it was found to increase the risk by a small magnitude but statistically significant (odds ratio=1.000014 and confidence interval [1.000001, 1.000027]).

The odds for *Salmonella* decreased with an increase in the number of broilers ( $\text{NBroilers} \leq \text{mean} + 2\text{SDs}$ ) when there were poultry or farmers in contact with external poultry or persons, but the odds even decreased more when there were no external contacts. Also, the odds for *Salmonella* decreased with an increase in the number of broilers for farms located in the Flanders region, but decreased more for farms in the Walloon region. Although the risk for *Salmonella* infection decreased with the presence of a hand washing place whether or not there were temporary workmen, the risk decreased further when there were temporary workmen. While having poultry or farmers in contact with foreign poultry or persons increased the risk for *Salmonella* when there were temporary workmen, the risk decreased when there were international contacts but no temporary workmen. Likewise while external contacts increased the odds for *Salmonella* when there were temporary workmen, external contacts decreased the odds for *Salmonella* when there were no temporary workmen.

The estimated variance of the farm-specific random effects was  $\hat{\sigma}_u^2 = 3.178$  for the GLMM model giving an estimated intra-class correlation of 0.491. In contrast, the estimated exchangeable correlation was  $\hat{\rho} = 0.009$  for the model based on GEE. The difference in the magnitudes of the parameter estimates from the GLMM models and those from GEE is based on the approximate relationship  $\beta_{\text{GLMM}} \approx \beta_{\text{GEE}} \sqrt{1 + 0.346\sigma_u^2}$  (Schukken *et al.*, 2003; Kim *et al.*, 2006).

Table 6: Parameter estimates and their standard errors and their significance p-values from the joint analysis for GEE and ALR Marginal Models and for the Random intercepts model(GLMM)

|                            | Marginal model GEE |         | Marginal model ALR |         | GLMM               |         |
|----------------------------|--------------------|---------|--------------------|---------|--------------------|---------|
|                            | $\hat{\beta}$ (SE) | P-value | $\hat{\beta}$ (SE) | P-value | $\hat{\beta}$ (SE) | P-value |
| Intercept                  | -4.837(0.869)      | <.0001  | -5.183(0.994)      | <.0001  | -5.415(1.078)      | <.0001  |
| Number of Broilers         | 1.4E-5 (5.4E-6)    | 0.0082  | 2.2E-5 (5.5E-6)    | <.0001  | 1.4E-5(6.8E-6)     | 0.0369  |
| Handwash place             | 2.510 (0.939)      | 0.0075  | 2.133(0.975)       | 0.0286  | 0.985(1.034)       | 0.3408  |
| All-inAll-out Principle    | -1.645 (0.611)     | 0.0071  | -1.309 (0.464)     | 0.0048  | -                  | -       |
| Cleaning Firm              | -1.459(0.432)      | 0.0007  | -1.222(0.473)      | 0.0098  | -1.076(0.510)      | 0.0350  |
| TempWorkmen                | 3.291(0.870)       | 0.0002  | 3.142(1.293)       | 0.0151  | 4.424(1.570)       | 0.0048  |
| Int'l contacts             | -0.226(0.669)      | 0.7353  | -0.023 (0.829)     | 0.9774  | 0.107(1.068)       | 0.9202  |
| External contacts          | -2.522 (1.366)     | 0.0650  | 0.490 (0.502)      | 0.3294  | -                  | -       |
| Region: Wal vs Fla         | 2.075 (1.442)      | 0.1502  | 1.674 (1.347)      | 0.2138  | -                  | -       |
| <i>Duration (weeks)</i>    |                    |         |                    |         |                    |         |
| 6 to 12                    | -1.008 (0.258)     | 0.0001  | -0.842(0.262)      | 0.0013  | -1.039(0.269)      | 0.0001  |
| > 12                       | -1.360(0.336)      | 0.0001  | -1.282(0.362)      | 0.0003  | -1.451(0.432)      | 0.0008  |
| NBroilers*External         | 6.4E-5 (1.9E-5)    | 0.0011  | -                  | -       | -                  | -       |
| NBroilers*Region           | -12.0E-5 (3.1E-5)  | 0.0001  | -12.0E-5(2.5E-5)   | <.0001  | -                  | -       |
| Handwash*Workmen           | -11.066 (2.872)    | 0.0001  | -6.809(1.934)      | 0.0004  | -6.306(1.976)      | 0.0014  |
| Workmen*Int'l              | 9.345(2.443)       | 0.0001  | 4.921(1.680)       | 0.0034  | 4.670(1.958)       | 0.0171  |
| Workmen*Ext                | 4.960 (1.811)      | 0.0062  | 3.368(1.320)       | 0.0107  | -                  | -       |
| $\hat{\rho}$               | 0.009              |         |                    |         |                    |         |
| logOR                      |                    |         | 3.185(0.413)       | <.0001  |                    |         |
| $\hat{\sigma}_u^2$ (flock) |                    |         |                    |         | 3.178(0.434)       |         |

## Discussion

The investigations from this study showed that *Salmonella* infection in broiler chicken flocks involves several risk factors and their interactions. *Salmonella* prevalence for current broiler flock conditional on the previous *Salmonella* status, according to the 2005-2006 data, was estimated as 27.9% which is rather close to the community observed prevalence of 23.7% in the year 2005 (EU, 2005). Using the three modelling approaches, the conditional analysis revealed one-day old chicks infected with *Salmonella* as an important risk factor to a farm, as also observed in other studies by Kim *et al.* (2007) and Van Immerseel *et al.* (2004, 2005). Positive chicks can spread the infection through their faeces and quickly contaminate the farm. The boxes in which they arrive may constitute a way for introducing the infection as well (Kim *et al.*, 2007; Renwick *et al.*, 1992; Van Immerseel *et al.*, 2004). Thus the first control measure is having *Salmonella* free breeding flocks (Bailey, 1993; Bouwknecht *et al.*, 2004; Breytenbach, 2004; Collard *et al.*, 2007; Garber *et al.*, 2003; Skov *et al.*, 1999; Van Immerseel *et al.*, 2005; Van Immerseel *et al.*, 2004). This can be easily achieved for instance through vaccination of parental lines. In Belgium since a few years, hatcheries have managed to obtain a good control of *Salmonella* infection even though at the time of this study, vaccination was only performed on a voluntary basis in breeders and in layers. Vaccination on broiler farms is never considered due to the short life expectancy of broilers and a diverse range of *Salmonella* serovars implicated. Thus vaccination of the broiler breeders is important, and has proven being effective in reducing the possibility of human infection through contaminated poultry products consumption (Cogan and Humphrey, 2003; Van Immerseel *et al.*, 2005). Vaccination is now since June 2007 a legal obligation in Belgium in breeders and in layers (Anonymous, 2007).

The above risk factor is associated with the vertical transmission of *Salmonella*, but other factors associated with the horizontal transfer of *Salmonella*, mainly through the environment (Breytenbach, 2004; Davies and Breslin, 2003b; Kim *et al.*, 2007; Renwick *et al.*, 1992; Van Immerseel *et al.*, 2004; Wales *et al.*, 2007), were found in this study. *Salmonella*'s capability of resisting desiccation, allows it to survive for long periods in the environment. It has been found to remain for several months in dust of ventilation filters (Davies and Wray, 1994; Kim *et al.*, 2007; Renwick *et al.*, 1992). A proper cleaning and disinfecting procedure conducted by external firms, especially trained for that purpose, seemed to be a major decreasing risk factor in our study and as proven before (Davies and Breslin, 2001, 2003a; Huneau-Salaun *et al.*, 2007). A sanitary break (i.e. the duration between the previous and current flock of at least 6 weeks), or applying the all-in all-out procedure, or using mechanical ventilation, all contributed as well to reducing the risk of *Salmonella* to the current flock when the previous flock was infected.

An increase in risk was observed, according to the conditional analysis using GEE and ALR models, with having a hygiene place to change clothes prior to entering the broiler house when the previous flock was infected. This suggests that having proper biosecurity measures such as a clean hygiene place before entering a unit is probably not sufficient enough if a proper maintenance of those rooms is not ensured. Equipping the barns with individual hygiene places would only be effective if in addition the barns are equipped with their individual ventilation systems, and biosecurity measures are extended to all entering objects such as vehicles, litter, feed, water in order to be fully effective (Anonymous, 2006; De Zutter *et al.*, 2001; Hald *et al.*, 2000; Heyndrickx *et al.*, 2002; Huneau-Salaun *et al.*, 2007; Renwick *et al.*, 1992; Wales *et al.*, 2007). Management of those places, such as the cleaning and disinfecting procedure applied to them must be taken in account as well. Not only is it

important to have an effective cleaning and disinfecting procedure, but also controlling its efficacy (Barker *et al.*, 2003; Wales *et al.*, 2007; Wales *et al.*, 2006). A crucial element is the choice of the right products. Bacteria can persist in biofilms, which is organic matter accumulating, for instance, in water pipes (Garber *et al.*, 2003; Morgan-Jones, 1980; Renwick *et al.*, 1992; Van Immerseel *et al.*, 2004). Chlorine which is often used to disinfect those systems does not remove organic matter. Therefore a possible cause of *Salmonella* presence could be due to these biofilms (Alchalabi, 2007; Davies and Breslin, 2003b; Renwick *et al.*, 1992; Ziggity Systems Inc, 2006). It would be interesting in future studies of risk factors on farms to integrate the presence or absence of hygiene places and their maintenance, i.e. the cleaning and disinfecting procedure products and frequency of application. The efficacy of such procedures should be assessed in a standardised manner for all the farms.

From the joint analysis, studying the persistence of *Salmonella* on a farm, the main factors influencing this outcome were as previously seen in the conditional analysis, i.e., a cleaning and disinfection procedure conducted by an external farm, as well as applying all-in all-out procedure and at least a period of weeks of sanitary break decreased the risk. A possible explanation for the increase in risk due to the interactions of external contacts and international contacts with temporary workmen could be an introduction of bacteria through contaminated tools or persons, as previously seen in other studies (Hald *et al.*, 2000; Huneau-Salaun *et al.*, 2007).

In conclusion, although a lot of risk factors have been investigated in this study, due to sparseness of data, some of them had to be omitted such as water, feed, and litter supply and the storing of these supplies. It would be interesting to also include in future studies the performance of the different ventilation systems, the temperature in the houses, as these factors have been recognised to greatly influence the poultry sector performance (Woelford, 2007). Measures against rodents, flies and manure disposing are key points in controlling the infection and avoiding persistence (Anonymous, 2006; Breytenbach, 2004; Davies *et al.*, 1997; Henken *et al.*, 1992; MacKenzie and Bains, 1976) but these are not currently available in the Belgian database. It is therefore necessary, in future, to collect information on these measures in order to enrich the Belgian database for studying potential risk factors contributing to *Salmonella* infection.

It is worth noting that this study was not explicitly designed for the study of risk factors associated with *Salmonella* infection but data on risk factors were obtained from the Avian Influenza check list, filled in on a voluntary basis by the farm owner. Nevertheless, risk factors recognised to play a critical role in avian influenza infection appear to be the same as those triggering *Salmonella* infection. Also, the fact that the farm owner was responsible for the filling in of the questionnaire and to collect samples might highlight a problem of bias in the data. To avoid such bias in the future it is important to have an independent person filling in the questionnaire in a standardised way as well as an independent standardised sampling method in order to have reliable good quality data.

Accounting for interactions leads to an improved determination of the risk factors that propagate the susceptibility to *Salmonella*. The epidemiological studies of *Salmonella* or other diseases should be designed with interactions in mind. The consistency in the results with the three modelling approaches is encouraging and strengthens their usefulness in identifying risk factors for *Salmonella* when faced with many variables and repeated data. These techniques can also handle higher order interactions than two-way interactions but these are seldom investigated due to small sample sizes.

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