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The dynamics of *Salmonella* occurrence in commercial laying hen flocks throughout a laying period

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### **Short title**

*Salmonella* occurrence in laying hens

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## Abstract

Contaminated eggs and egg products have been recognised since many years as an important source of *Salmonella* infections in humans in the European Union and in the United States. Longitudinal studies can help to increase our knowledge about the dynamics of the occurrence of *Salmonella* in the course of a laying period. The total of 41 laying hen flocks, 18 in Belgium, 6 in Denmark and 17 in Germany were followed during an entire laying period. Samples taken from the empty cleaned and disinfected poultry houses were all negative for *Salmonella*. After hens arrived on the farms five pooled faecal samples, one pooled dust sample and 40 cloacal swabs (Belgium and Germany) or 40 swabs from fresh droppings (Denmark) were taken four times from 18 flocks, three times from 21 flocks and two times from 2 flocks in the course of the laying period. Ten flocks (two Belgian and eight German flocks) were tested up to three times positive for *Salmonella*. Forty three out of 50 positive samples contained *Salmonella* Enteritidis phage type 4 (29 isolates) or phage type 8 (14 isolates). The probability of subsequent *Salmonella* positive findings increased significantly in *Salmonella* positive flocks ( $p < 0.05$ , odds ratio = 6.4). However, the probability of finding *Salmonella* did not depend on the time of sampling in the laying period or the season.

## Introduction

Salmonellosis belongs to the most frequently reported zoonotic food-borne diseases worldwide. It is known since many years that contaminated eggs and egg products are important vehicles for the transmission of these organisms to the consumer in the European Union and in the United States (Patrick *et al.*, 2004; European Food Safety Authority, 2009). *Salmonella* can be found either on the surface of the eggshell or in the egg content. The eggshell can become contaminated as a result of infection of the oviduct or by colonization of the intestine of a hen (De Reu *et al.*, 2008). Also the presence of *Salmonella* in faeces can lead to eggshell contamination (Gast & Beard, 1990). *Salmonella* in the egg content is resulting from an infection of the reproductive organ or is a consequence of the migration of these bacteria through the egg shell and membranes (Gantois *et al.*, 2009). The most common *Salmonella* serovar in eggs and egg products is *Salmonella* Enteritidis which is also the most frequently reported serovar in *Salmonella* infections in humans in the European Union and in the United states (Braden, 2006; European Food Safety Authority, 2009). Case-control studies and outbreak investigations have demonstrated the association between human cases and the presence of *Salmonella* Enteritidis in laying hen flocks (Altekruse *et al.*, 1993; Rampling, 1993; Trepka *et al.*, 1999).

Many studies have investigated the transmission routes and identified risk factors for *Salmonella* infections in laying hen flocks (Davies & Breslin, 2003; Gradel *et al.*, 2004; Mollenhorst, 2005; Humphrey, 2006; Carrique-Mas *et al.*, 2008; Namata *et al.*, 2008; Van Hoorebeke *et al.*, 2010). However, studies over longer time periods describing the dynamics of the within flock *Salmonella* prevalence during a production period are still rare. This may be caused by high costs of such complex field studies and simply by the difficulty to convince

farmers to take part in long lasting investigations (Wales *et al.*, 2007). Therefore, in the framework of the EU project SAFEHOUSE a longitudinal study was carried out in laying hen flocks on different farms in Belgium (BE), Denmark (DK) and Germany (DE). The flocks were sampled several times in the course of one laying period in order to describe the dynamics of *Salmonella* occurrence throughout a production period under field conditions.

## Materials and Methods

**Sampled flocks.** In total 41 flocks were included in the study: 18 flocks from BE (1 enriched cage system, 4 battery cage, 5 free range, 4 barn and 4 organic systems), 6 flocks from DK (1 free range system, 3 battery cage and 2 barn system) and 17 flocks from DE (4 battery cage, 5 free range, 5 barn and 3 small colony keeping systems). A flock was defined according to regulation (EC) 2160/2003 of the European Parliament and of the Council (Anon, 2003). That means all hens of the same health status kept on the same premises or in the same enclosure and constituting a single epidemiological unit. In case of housed hens this includes all birds sharing the same airspace. Only flocks larger than 1000 birds were included. On farms with several flocks (overall 24 farms and 21 of them with flocks of different ages) one flock was selected randomly for sampling. The housing systems were categorised according to the EU directive 2002/4/EC and council regulations (EC) No 1804/1999 (Anon., 1999; Anon., 2002). An exception was the German small colony keeping system which is defined by the German authorities (German Federal Ministry of Justice, 2006). This system resembles the EU furnished cage but offers the hens more space and extra perches at different heights.

**Periods of sampling.** Field studies were performed from February 2007 until July 2008 in BE and DE. In DK flocks were sampled from October 2007 until March 2009. First samples were taken from the empty and disinfected animal houses one week before the hens arrived at the farm. The age at placement varied between 15 and 22 weeks. In three flocks no samples were taken from empty houses because hens of different ages were housed in the same premise and the animal houses were never empty. In total 139 samplings were carried out at different times during one egg-laying period. Flocks were sampled three or four times at different intervals with the exception of two flocks which were sampled only twice. Between the different countries sampling intervals varied because of different logistics. The laying periods were divided in six 10-week intervals for data analysis. Table 1 shows the number of flocks sampled in each country during these intervals.

**Number and sample type taken on-farm.** One pooled dust sample, five nest-box samples or 5 cage samples were taken in the empty and disinfected houses. The dust was collected from 20 different locations in the animal house. The dusty material was transferred by a gloved hand or sterile brush into a sterile bag. The minimum amount was 25 g dust per pooled sample. If no suitable surfaces with dust were present in the cleaned houses, surfaces from 20 locations were swabbed with sterile swabs, which were placed in Ames medium (Oxoid, Deutschland GmbH). Swabs and Ames medium were also used to swab 5 randomly selected nest-boxes except in battery cages which do not provide nest-boxes. In the latter case the grids of 5 randomly selected cages were swabbed.

From each laying hen flock 5 nest-box samples or 5 cage samples and 1 pooled dust sample were taken per visit as described above. Furthermore 5 pooled faecal samples and individual cloacal swabs from 40 randomly selected laying hens (in BE and DE) were sampled at each farm visit. In Denmark, the 40 swabs were obtained from 40 randomly



selected fresh faecal droppings because farmers did not allow sampling their hens. Depending on the housing system, the pooled faecal samples were taken as follows:

- Cage systems and small colony keeping systems: 5 samples of mixed fresh faeces were either taken from dropping belts, scrapers or deep pits depending on the type of cage (conventional battery cage, furnished cage, small colony keeping system). Each pooled faecal sample consisted of approximately 250 g.
- Barn, free-range and organic systems: for each of the 5 pooled faecal samples 60 piles of fresh faeces were collected from the floor and the slats. Each pooled faecal sample consisted of approximately 250 g.

The pooled faecal samples were put in a sterile box. New gloves were used for collecting each pooled faecal sample.

Cloacal swabs were taken by inserting a cotton-tipped swab approximately 5 cm into the cloaca, taking care to avoid contact with the surrounding feathers and skin. Afterwards, the swabs were stored in tubes containing Ames medium. A sample size of 40 swabs allows detecting a within-flock prevalence of 7% at a level of confidence of 95% in a flock with more than 1000 hens (WinEpiscope 2.0). All samples were placed in leak-proof bags and transported in container boxes to the laboratory under ambient temperatures. The samples were analysed either immediately after arrival at the laboratory or stored at 4°C for analysis within the next 24 hours.

**Bacteriological analysis of samples.** All samples were analyzed according to ISO 6579:2002/Amd 1:2007 Annex D: Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage. From each of the pooled faecal and mixed dust sample, 25 g was added to 225 ml of buffered peptone water (BPW) (Oxoid, Basingstoke, Hampshire, UK). All cloacal swabs, swabs from nest-boxes and from dusty

surfaces were individually placed in 9 ml of BPW. Pooled faecal and mixed dust samples were mixed in a stomacher bag for 1 minute. All samples were incubated for  $18 \pm 2$  h at  $37 \pm 1$  °C. Three droplets of each pre-enrichment culture were dropped on the surface of a modified semi-solid Rappaport-Vassiliadis (MSRV) (Difco; Becton Dickinson) agar plate containing  $0.01 \text{ g l}^{-1}$  novobiocine. The plates were incubated for  $2 \times 24 \pm 3$  h at  $41.5 \pm 1$  °C. Material of suspected white cultures from the borderlines of the growth zones were transferred on Brilliant Green Agar (BGA; Oxoid) and Xylose Lysine Deoxycholate agar (XLD; Oxoid), followed by incubation for  $24 \pm 2$  h at  $37 \pm 1$  °C. Presumed *Salmonella* colonies on BGA and XLD were biochemically confirmed using urea agar, triple sugar iron agar and lysine-decarboxylase broth. Serotyping of *Salmonella*-isolates according to the White-Kauffmann-Le Minor scheme (Grimont & Weill, 2007) was performed in the national Reference Laboratories for *Salmonella* in each participating country. Phage typing of *S. Enteritidis* was carried out as described by Ward *et al.* (1987).

**Data processing and analysis.** In order to get reliable information on the sampled flocks (e.g. number and age of hens in a flock) and the farms (e.g. total number of flocks and hens, age of different flocks, cleaning measures and disinfection procedures) a questionnaire was developed and filled in by interviewing the farmer at one of the farm visits as described by Van Hoorebeke *et al.* (2010). Answers in the questionnaires were coded and transferred into an excel spread sheet (Microsoft Office Excel 2003, Microsoft Cooperation). The variable “period of sampling” was divided into six 10-weeks intervals. To define the category “season”, the meteorological seasons for Europe (spring begins on the first of March, summer on the first of June, autumn on the first of September and winter on the first of December) were chosen. The effect of the period of sampling and the season on the within flock prevalence was analysed using logistic regression as was the effect of a previous sample result

within the same production round on the next sample result. For statistical analysis the software SPSS 17.0 (SPSS Inc., Chicago, IL) was used.

## Results

**Salmonella detection.** In none of the animal houses members of *Salmonella* were found after cleaning and disinfecting prior to stocking. Ten out of the 41 sampled flocks were tested once, twice or three times positive for *Salmonella* in the course of one laying period. Two Belgian and eight German flocks were Salmonella-positive across five different housing systems. Table 2 gives an overview of the kind of positive samplings, the number of positive samples, sampling intervals and detected serovars (including *S. Enteritidis* phage types) in the positive tested flocks. Six of these flocks (no. 7, 8, 25, 26, 31 and 41) were housed in all-in and all-out systems, two flocks (no. 27 and 32) originate from farms with hens of different ages in different houses and two flocks (no. 33 and 40) were kept in multi-age systems. Two flocks (no. 25 and 41) hosted different serovars. The most prevalent isolate was *S. Enteritidis* found in 44 (88%) of the 50 positive tested samples (29 isolates of phage type 4, 14 isolates of phage type 8, phage type of 1 isolate could not be identified). The serovars *S. Infantis*, *S. London* and one unidentified serotype from serogroup B were isolated from 6 samples (12%). Table 2 shows that *Salmonella* were detected 18 times in 10 flocks by using three kinds of sampling techniques. Already 17 positive samplings in 10 flocks were obtained by environmental samplings (pooled faecal and pooled dust samples). The number of positive flocks (pooled faecal = 7, pooled dust = 6, cloacal swabs = 3) and samplings (pooled faecal = 12, pooled dust = 10, cloacal swabs = 5) decreases when only a single sampling technique is taken into account.

**Statistical analysis.** Observed *Salmonella* prevalence throughout the production period is given in Figure 1. Although differences are obvious between the different sampling intervals, these were not statistically significant. No significant effect of the season was found either. However, the risk to find *Salmonella* in a flock increased significantly ( $p < 0.01$ ) (odds ratio = 6.4, 95% confidence interval: 2.051 – 19.745) when *Salmonella* was found in the previous sampling of the same flock.

## Discussion

The occurrence of *Salmonella* was observed in Belgian and German laying hen flocks but not in Danish flocks what can be explained by the relatively small number of Danish flocks included into the study and the well-known low *Salmonella* prevalence in Danish laying hen flocks (Wegener *et al.*, 2003; European Food Safety Authority, 2006). Therefore swab samples taken from fresh droppings instead of cloacal swabs in Denmark had most likely no considerable impact on the overall results.

Ten out of the 41 sampled flocks were tested one, two or three times *Salmonella*-positive in the course of the investigated laying period by using cloacal swabs, dust samples and samples of faeces. Taking cloacal swabs was less useful to detect *Salmonella*. This was probably influenced by the relatively low within flock prevalence of shedding hens ( $< 7\%$ ) even in a *Salmonella* contaminated environment. Sampling the environment was rather successful and especially the combination of faeces and dust samplings seems to be more effective than using a single sampling technique. These findings support the recommendation to sample both faeces and dust to increase the sensitivity of *Salmonella* detection in laying

hen houses (Carrique-Mas & Davies, 2008). Although non-shedding hens with infected organs cannot be detected with the applied methods (Van Hoorebeke *et al.*, 2009), the use of more sensitive environmental samplings can help identify flocks at risk of producing *S. Enteritidis*-contaminated eggs (Henzler *et al.*, 1998).

A *Salmonella* positive finding in a flock significantly increased the probability of subsequent positive results in the same flock. In these cases generally the same serovars and phage types were detected. Especially the same phage types in environmental samples and in cloacal swabs of the same flock demonstrate the close relationship between the infected animals and the environment which seems to play an important role as a reservoir. Furthermore, in most of the positive tested flocks the infection was already observed in the early stage of the laying period. Although the samplings of the cleaned and disinfected laying hen houses were negative, undetected residual contamination may have contributed to an initial infection of hens. Especially in cage and multi-age systems, which represent seven (70%) of the positive tested flocks in this study, an effective cleaning and disinfection is difficult to perform in practice and may even fail (Davies & Breslin, 2004; Wales *et al.*, 2007). Another source for infections in the early laying period may be living vectors such as rodents and arthropods. This was not specifically investigated in this study. However, it is known that they can contribute to the horizontal transmission of infectious agents including *Salmonella* (Gast *et al.*, 1998; Carrique-Mas *et al.*, 2008). These vectors may also be responsible for infections even on farms with all-in all-out management because they can stay on the farm after depopulation and re-enter into the new flocks (Hald *et al.*, 1998). Beside horizontal transmission also vertical transmission may have contributed to the *Salmonella* presence in the investigated flocks. *Salmonella* infections in parent breeding flocks are described as an existing problem in Germany (European Food Safety Authority, 2009). Although vaccination is compulsory in German parent breeding flocks, this route of

transmission should not be neglected because even vaccinated hens can become infected with *Salmonella* Enteritidis and may transmit this organism to their descendants (Davies & Breslin, 2004).

The presented results show no significant differences concerning the *Salmonella* prevalence in the different sampling intervals. These findings contradict somehow other studies which described an increase of infected flocks with increasing age of the hens. A higher susceptibility of older hens to infections, molting and an accumulation of *Salmonella* in the environment were suggested as factors which lead to an increase of *Salmonella* positive flocks and an increase of *Salmonella* positive samples in ageing flocks respectively (Schlosser *et al.*, 1999; Garber *et al.*, 2003; Van de Giessen *et al.*, 2006; Wales *et al.*, 2007). In the present study induced molting was not practiced on the sampled farms and no higher numbers of *Salmonella* positive faecal, dust and cloacal swab samples were observed towards the end of the laying period. These findings may partly be influenced by cleaning procedures and the removal of faeces during the production cycle (Wales *et al.*, 2007). Hence, differences in the farm management and in the design of studies make it difficult to compare the results of this study and previous investigations. However, an early *Salmonella* contamination in the laying period did apparently not result in an increased number of shedding hens. In contrast to other opinions, it seems that an increasing number of shedding hens towards the end of a laying period does not hold true for infected flocks in general. This may be typical in particular for early infected flocks due to the decline of shedding hens within a few weeks after infection (De Vylder *et al.*, 2009) and should be considered in future longitudinal studies by taking samples in the very early stage of lay (in the first three weeks after housing).

Seasonal effects may also influence the detection of *Salmonella* in laying hen flocks in longitudinal studies (Wales *et al.*, 2007). Therefore the season was included in the logistic regression model but no significant seasonal effects were observed. The discussion about

seasonal effects is controversial and it seems that not the changing climatic conditions itself but rather their consequences for the management or the presence of living vectors are influencing *Salmonella* detection and transmission (Carrique-Mas *et al.*, 2008; Van Hoorebeke *et al.*, 2010).

The dynamics of *Salmonella* infection in laying hen flocks is very complex. It is strongly depending on the farm management (e.g. cleaning and disinfection procedures), the immune status of the birds, the presence of living vectors as carriers and an accumulation of *Salmonella* in the flock environment. This study indicates that early infections of laying hen flocks with the important human pathogen *S. Enteritidis* (PT4 and PT8) can lead to a continuous infection of hens and to a persistent contamination of their environment. This may increase also the risk of egg contaminations. Therefore more stringent action should be taken both to eliminate *Salmonella* from breeding and production flocks by applying comprehensive bio-security measures. Special regards should be paid to avoid *Salmonella* introduction in laying hen flocks at any stage. This will be a considerable contribution to decrease the risk for the consumer.

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## Figure Legends

**Figure 1.** *Salmonella* prevalence detected in six different sampling intervals throughout a laying period

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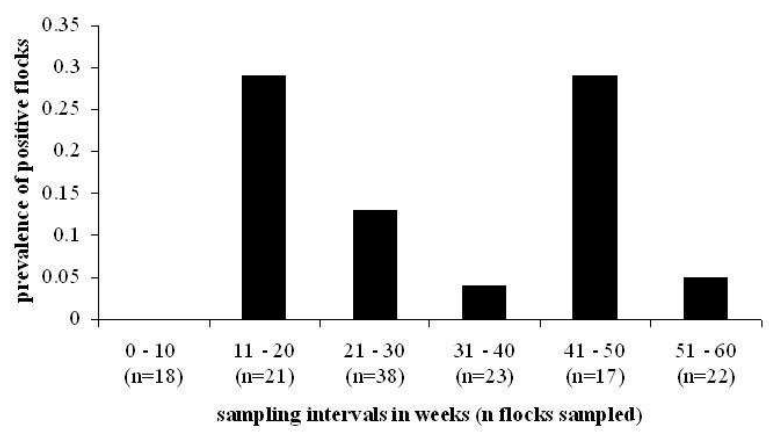
**Table 1.** Completed samplings during a laying period

No. of flocks, country	week of laying period						
	-1 (empty house)	0 - 10	11 - 20	21 - 30	31 - 40	41 - 50	51 - 60
2, DK	X		X		X		X
2, DK	X			X	X		X
1, DK	X		X	X		X	
1, DK	X		X		X		
18, BE	X	X		X	X		X
17, DE	X		X	X		X	

**Table 2.** Sampling intervals, negative samplings (0), number and kind of positive samples (p.f., p.d., c.s.) and detected serovars (phage types) of the positive tested flocks

Flock no., housing system and country	week of laying period						
	-1 (empty house)	0 - 10	11 - 20	21 - 30	31 - 40	41 - 50	51 - 60
7. barn, BE	0	0	–	0	0	–	1 p.f. (S.E. PT4)
8. enriched cage, BE	0	0	–	0	1 p.d. (S.E., nt)	–	0
25. battery cage, DE	0	–	4 p.f. (S.E. PT4; S.I.)	0	–	1 p.d. (S.E. PT4)	–
26. battery cage, DE	0	–	0	0	–	1 p.f. (S.E. PT4)	–
27. battery cage, DE	0	–	3 p.f. (S.E. PT4)	2 c.s. (S.E. PT4)	–	2 p.f. (S.E. PT4)	–
31. barn, DE	0	–	2 p.f., 1 p.d., 1 c.s. (S.E. PT8)	1 p.f., 2 c.s. (S.E. PT8)	–	3 p.f., 1 p.d., 1 c.s. (S.E. PT8)	–
32. barn, DE	0	–	0	1 p.d. (S.L.)	–	0	–
33. barn, DE	–	–	5 p.f., 1 p.d., 7 c.s. (S.E. PT4)	2 p.f., 1 p.d. (S.E. PT4)	–	–	–
40. small colony, DE	–	–	2 p.f. (S.E. PT4)	0	–	0	–
41. small colony, DE	0	–	1 p.d. (S. group B)	1 p.d. (S. group B)	–	1 p.f., 1 p.d. (S.E. PT8)	–

p.f. = pooled faecal sample; p.d. = pooled dust sample; c.s. = cloacal swab sample; – = no sample was taken in this interval; nt = not typable; S. E. = *Salmonella* Enteritidis; S.I. = *Salmonella* Infantis; S.L. = *Salmonella* London; PT = phage type



Salmonella prevalence detected in six different sampling intervals throughout a laying period

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