# Persistent *Salmonella* Enteritidis environmental contamination on layer farms in the context of an implemented national control program with obligatory vaccination

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**ABSTRACT** The aim of this study was to closely examine the *Salmonella enterica* serovar Enteritidis environmental contamination on persistently positive layer farms in Belgium during successive laying cycles. All of the farms were required to vaccinate their layers under the national control program for *Salmonella*. Seven farms with previous or current *Salmonella* Enteritidis contamination were monitored during different stages of the laying period and after cleaning and disinfection (CD). Environmental samples, including from the equipment and vermin, were taken in the henhouse and egg-collecting area. Dilutions were performed to define the degree of *Salmonella* Enteritidis contamination. Eggshells, egg contents, and ceca were also tested for *Salmonella*. At the end of the first sampled laying period, 41.6% of the environmental samples were contaminated with *Salmonella* Enteritidis. After CD, the prevalence dropped to 11.4%. On average, the prevalence in the second laying period increased again: 17.8, 18.4, and 22.3% at the onset, middle, and end of the lay period, respectively. After CD before the third laying period, the prevalence decreased to 6.6% and stabilized at the onset of lay (6.3%). During lay, as well as after CD, a wide variety of contaminated environmental samples were found; for example, in the henhouse, in the egg-collecting area, on mobile equipment and in or on vermin. In the henhouse during laying, the most recurrent and highly contaminated sites were the overshoes, floor, manure belt, and hen feces. The egg-collecting area had a significantly higher number of contaminated samples compared with that of the henhouse. For both sites, the floor appeared to be the most suitable sampling site to estimate the *Salmonella* Enteritidis status of the farms. Eggshell and egg content contamination varied between 0.18 and 1.8% and between 0.04 and 0.4%, respectively. In total, 2.2% of the analyzed ceca contained *Salmonella* Enteritidis. This study revealed that *Salmonella* Enteritidis is present in the environment of persistently *Salmonella* Enteritidis-contaminated layer farms, demonstrated that in many cases *Salmonella* Enteritidis contamination was not eliminated after CD, and identified the egg-collecting area as a critical point on most farms.

**Key words:** *Salmonella* Enteritidis, environment, layer farm

2012 Poultry Science 91:282–291
doi:10.3382/ps.2011-01673

## INTRODUCTION

*Salmonella* is the second most commonly reported zoonotic infection in humans in the European Union. The most frequently reported *Salmonella* serovar in 2009 was *Salmonella enterica* Enteritidis (European Food Safety Authority, 2011). Eggs are the main source of human *Salmonella* Enteritidis infections (Davies and Breslin, 2004; European Food Safety Authority, 2011). In recent years in the European Union, the annual number of confirmed human cases of *Salmonella* infection has gradually decreased, which is primarily due to the lower incidence of human *Salmonella* Enteritidis infection. Parallel to the reduction of human cases, a decrease in the number of *Salmonella*-infected layer flocks has been observed (European Food Safety Authority, 2011). It is assumed that the implementation of European Regulations (Anonymous, 2003; Anonymous, 2006) and the vaccination of commercial laying hens (Collard et al., 2008; European Food Safety Author-


Received June 11, 2011.
Accepted September 28, 2011.
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ity, 2007a) have caused a sharp reduction of reported *Salmonella* Enteritidis in layers. The EU regulation no. 2160/2003 (Anonymous, 2003) requires member states to take effective measures to detect and control *Salmonella* serovars of public health significance at all relevant stages of the poultry production chain through a national control program. The implementation of this regulation (no. 1168/2006; Anonymous, 2006) makes strict sampling schemes mandatory in the member states to provide information about *Salmonella* flock contamination. To reduce the fecal shedding and colonization of the reproductive tract of laying hens with *Salmonella* (vertical transmission route; Gantois et al., 2009), vaccination against *Salmonella* Enteritidis is mandatory in many member states, including Belgium. Although the vaccination of laying hens against *Salmonella* Enteritidis only became mandatory in June of 2007, the Belgian Federal Agency for the safety of the food chain has recommended vaccination since 2004 (Collard et al., 2008). This recommendation did have an effect: the prevalence of *Salmonella* in Belgian laying hen flocks has decreased remarkably from 27.2% in 2004 (rearing and production) to 11.2 and 7.3% (production) in 2008 and 2009, respectively (European Food Safety Authority, 2007b, 2011).

Despite these efforts, some layer farms have persistent *Salmonella* Enteritidis contamination. Understanding the reasons for these persistent infections is becoming crucial to the future success of the *Salmonella* control program.

The main goal of the present study was to investigate in detail *Salmonella* Enteritidis environmental contamination on persistently positive layer farms during successive laying cycles in the new epidemiological context of obligatory vaccination against *Salmonella* as imposed by the national control program. Our specific aims were to 1) follow the prevalence of *Salmonella* Enteritidis-contaminated environmental samples on persistently *Salmonella* Enteritidis-positive farms during the laying period and after cleaning and disinfection (**CD**), 2) define the degree of *Salmonella* Enteritidis contamination in the various sampling sites, 3) identify the recurrently contaminated sites associated with *Salmonella* Enteritidis infection during subsequent laying rounds, and 4) identify the sites that were still contaminated after CD. These data can help the *Salmonella* Enteritidis-contaminated layer farms to control their persistent environmental contamination. In addition, this information will help *Salmonella* Enteritidis-negative layer farms to maintain their status, as vaccination is only effective in a well-managed farm environment.

## MATERIALS AND METHODS

### Sampled Layer Farms and Frequency of Sampling

Seven Belgian layer farms (farms A–G), chosen for their recent or current *Salmonella* Enteritidis-positive status (based on fecal samples and overshoes taken in cage and noncage systems, respectively) in the official monitoring and control program, were intensively sampled once permission was granted by the farmer. All flocks were vaccinated against *Salmonella* during rearing. Most flocks were vaccinated with the commercial live vaccine Avipro *Salmonella* Vac E (Lohmann A. H., Cuxhaven, Germany). The hens of farm B received the live vaccine Nobilis SG9R (Intervet, Milton Keynes, UK), and during the second laying round on farm C, hens received the inactivated Nobilis Salenvac vaccine (Intervet). Layer farms had one (farms C and G) or 2 (farm F) conventional cages (**CC**); others had a furnished cage system (**FC**; farms A and E) or an aviary (AV; farms B and D) in addition to the CC. Various breeds of hens were kept, including Lohmann Brown, Lohmann LSL, Dekalb White, and Isa Brown. Some layer farms kept flocks with hens of different ages (farms B, D, and E). The farms were monitored during successive laying cycles at the onset, middle, and end of lay and after CD. Additional sampling occasions were introduced when the laying cycle was prolonged or when molting was induced. The cleaning procedure included both dry and wet cleaning. Most farms used a specialized company to do the disinfection. After each sampling occasion, the farmer was notified of which samples were contaminated.

### Sampling

During each sampling event, 20 to 26 sites in each henhouse and 8 to 11 sites in the egg-collecting area were sampled (Table 1), depending on the presence and accessibility of the sample type. One sample was taken per sample type. Surfaces (when possible, were approximately 0.5 $m^2$) in the henhouse were swabbed using pieces of sterile cotton or several cotton swabs (used for less-accessible surfaces) soaked in buffered peptone water (**BPW**; CM0509, Oxoid, Basingstoke, UK). Air samples (400 L of air) were taken in the henhouse using an Air Sampler RCS (Biotest AG, Dreieich, Germany) with a Brain Heart Infusion (CM0375, Oxoid) airstrip. Flies and red mites were collected and crushed for culturing. Mouse and rat corpses were collected as available. From the henhouse, 200 freshly laid eggs were collected and examined for *Salmonella* presence (100 on the eggshell and 100 in the egg content). In addition, with the permission of the farmer, at the end of the laying period, 50 hens (Van Hoorebeke et al., 2010) were randomly selected to test for *Salmonella* in the ceca. Immediately after sampling, the samples were transported to the laboratory at ambient temperatures and analyses were started the same day.

### Isolation and Identification of Salmonella Enteritidis

*Salmonella* was isolated according to the ISO6579:2002 Annex D protocol (Anonymous, 2002). Briefly, each

**Table 1.** Environmental samples taken from various locations

| Location | Specific area |
|---|---|
| Henhouse | Ceiling |
| | Air inlet |
| | Overshoes |
| | Floor |
| | Crack/gap floor |
| | Wall |
| | Crack/gap wall |
| | Ventilator |
| | Gate |
| | Manure belt |
| | Hen feces |
| | Feed hopper |
| | Feed trough |
| | Feed |
| | Drinking nipple/cup |
| | Water reservoir (inside) |
| | Cage |
| | Drain |
| | Dust |
| | Air |
| | Hygiene mat |
| | Boots |
| | Egg belt at cage/laying nest |
| | Egg cross conveyor |
| Egg-collecting area | Floor |
| | Wall |
| | Wash basin |
| | Toilet |
| | Container egg tray |
| | Pallet truck |
| | Pallet |
| | Egg collector/sorter |
| | Egg sorter |
| | Egg-packer head |
| | Conveyor egg tray |
| | Control-panel conveyor |
| Equipment | Cleaning machine |
| | Scraper |
| | Ladder |
| | Wheelbarrow |
| | Shovel |
| | Wiper |
| | Dust pan |
| | Bucket |
| | Brush |
| Vermin | Mouse/rat feces |
| | Mouse/rat intestine |
| | Flies |
| | Red mite |
| Other | Feces cat |
| | Feces dog |
| | Cat litter box |
| | Mousetrap |

cotton piece, group of swabs, air strip, or other sample type was added to 225 mL of BPW and homogenized. Further decimal dilutions of this initial suspension were prepared up to $10^{-3}$ by sequentially adding 25 mL of the previous dilution to 225 mL of BPW. Samples that were positive in the initial suspension and the $10^{-1}$ dilution were considered to have a low contamination level, whereas samples that were positive in the $10^{-2}$ and $10^{-3}$ dilutions were considered to be highly contaminated.

The eggshell was analyzed by washing each egg in 10 mL of BPW as described previously (De Reu et al., 2006a,b). Next, the BPW volume of 10 washed eggs was pooled for further analysis. After aseptically removing the egg content, as described previously (De Reu et al., 2006a,b) of the remaining 100 eggs per henhouse, the egg contents were pooled by 10 eggs in 1 L of BPW supplemented with 20 μg/mL of ammonium [$Fe^{3+}$] citrate for further analysis. From the mice and rats, the liver, spleen, and intestines were removed and homogenized in 225 mL of BPW. Fifty hens were killed by cervical dislocation according to Close et al. (1996) and necropsied; both ceca were aseptically removed and homogenized in 225 mL of BPW.

The BPW was incubated for approximately 18 h at 37°C. Subsequently, 3 droplets (total volume of 100 μL) of the pre-enrichment culture were inoculated onto modified semi-solid Rappaport-Vassiliadis (355–6139, Bio-Rad, Marnes La Coquette, France) agar plates containing 0.001% novobiocine and incubated for approximately 24 h at 41.5°C. If an incubated plate was negative (absence of a halo of growth originating from the inoculation spots) after incubation for approximately 24 h, it was incubated for another 24 h. One microliter loop from the edge of a suspect halo growth zone was inoculated on xylose lysine deoxycholate agar (221192, Becton Dickinson, Franklin Lakes, NJ) and BBL CHROMagar *Salmonella* (214983, Becton Dickinson), followed by incubation for approximately 24 h at 37°C. Suspected colonies were biochemically confirmed using ureum agar (TV5007N, Oxoid), triple sugar iron agar (TV5074D, Oxoid), and lysine-decarboxylase broth (TV5028N, Oxoid). The serogroup was determined by the Poly A-I-Vi test (222641, Becton Dickinson). A specific PCR targeting the *Sdf*I region was applied to confirm the isolates belonging to the D-serogroup as the serotype *Salmonella* Enteritidis (Botteldoorn et al., 2010). Isolates not belonging to the D-serogroup or showing a negative PCR result were serotyped according to the Kauffmann-White scheme, performed at the Veterinary and Agrochemical Research Centre (Brussels, Belgium).

### Statistical Analysis

All statistical analyses were performed using Statistica (version 9.0; StatSoft, Tulsa, OK). A main effects model was chosen, because the interaction term sampling time × sampling site was not significant. For the sampling site, a distinction was made between samples of the henhouse and the egg-collecting area. The significance level α was set at 0.05. Individual differences were compared by Tukey's honestly significant difference test.

## RESULTS

### General Prevalence of Salmonella Enteritidis

At the end of the first sampled laying period, the overall prevalence of *Salmonella* Enteritidis-contami-

nated samples for the sampled farms varied between 7.0 and 80.1% (average 36.7%) in the henhouse and between 20.0 and 80.0% (average 51.3%) in the egg-collecting area. After the first CD, the prevalence declined (on the sampled farms) and varied between 0 and 15.0% (average 5.46%) in the henhouse and 0 and 45.5% (average 23.3%) in the egg-collecting area. At the onset of lay for the second sampled laying cycle, the prevalence increased again to a level of between 0 and 57.7% (average 12.5%) in the henhouse and between 0 and 63.0% in the egg-collecting area (average 27.6%). During this second sampled laying cycle, the prevalence in the henhouse remained constant and ranged between 0 and 67.8% (average 15.6%) and between 0 and 62.5% (average 21.7%) at the middle and end of lay, respectively. At those times, in the egg-collecting area, the percentage ranged between 0 and 62.5% (average 24.5%) and between 0 and 43.0% (average 23.7%) at the middle and end of lay, respectively. After the second CD (before the third laying cycle) the prevalence declined again and varied between 0 and 29.6% (average 6.12%) in the henhouse and between 0 and 30.8% (average 7.70%) in the egg-collecting area. Finally, at the onset of the third sampled laying period, the prevalence ranged between 0 and 37.5% (average 5.47%) in the henhouse and between 0 and 18.2% in the egg-collecting area (average 8.40%).

For all of the sampled farms during the laying period, the proportion of *Salmonella* Enteritidis-contaminated environmental samples is given over time in Figure 1. The main effect model that was fitted to the data demonstrated a significant effect on the proportion of *Salmonella* Enteritidis-contaminated samples for the sampling time (end lay,after CD,begin lay, and mid lay; $P = 0.00001$) and sampling area (henhouse and egg-collecting area; $P = 0.007$). In general, the proportion of *Salmonella* Enteritidis-contaminated samples was found to be significantly higher at the end of the first sampled laying period compared with the following sampled laying periods, more specifically, after the first followed CD ($P < 0.001$), onset ($P < 0.01$), mid ($P < 0.001$), and end lay ($P < 0.05$) of the second laying cycle, after the second CD ($P < 0.001$) and onset lay of the third laying cycle ($P < 0.001$). Between the other sampling times, no significant differences were found for the proportion of *Salmonella* Enteritidis-contaminated samples ($P > 0.05$). Averaged over all sampling occasions, a significantly higher proportion of contaminated samples was detected in the egg-collecting area compared with the henhouse ($P < 0.01$).

## Prevalence of Salmonella Enteritidis on 2 Individual Farms

The prevalence of *Salmonella* Enteritidis-contaminated samples on farms A and B is given in detail during the sampled laying periods for each henhouse as well as for the egg-collecting area (Figure 2). On both farms, a

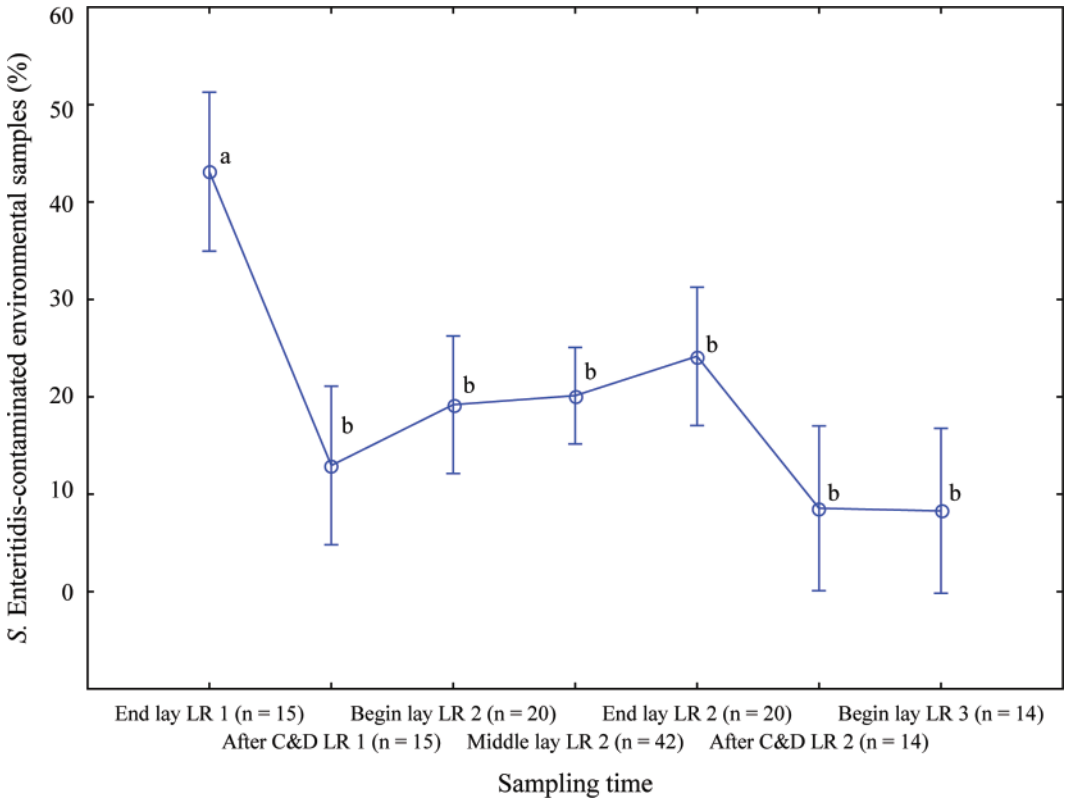


**Figure 1.** Percentage of *Salmonella* serovar Enteritidis-contaminated environmental samples across all of the farms during the different laying periods (sampling time). Common letters on each curve indicate no significant differences ($P > 0.05$). LR = laying round; CD = cleaning and disinfection; and n = number of sampled henhouses/egg-collecting areas. Vertical bars denote 0.95 CI.

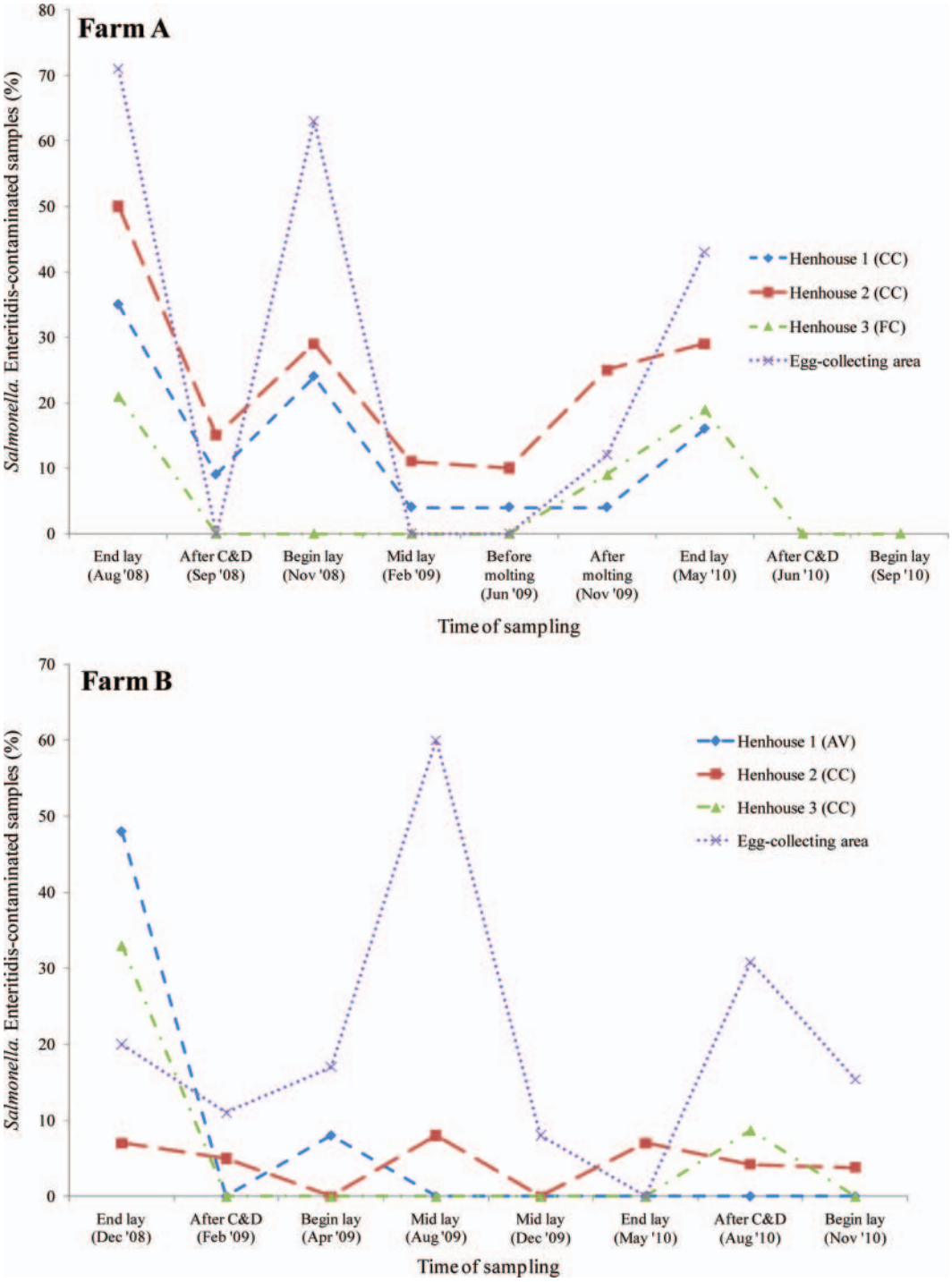


**Figure 2.** Detailed prevalence of *Salmonella* serovar Enteritidis-contaminated samples on 2 farms (A and B) during successive laying periods for each henhouse as well as for the egg-collecting area with the corresponding month and year of sampling. CD = cleaning and disinfection; CC = conventional cage; FC = furnished cage; and AV = aviary.

high percentage of contaminated samples was detected in the henhouses at the end of the first sampled laying period, ranging between 21.3 and 50.4% and between 7.1 and 48.0% on farms A and B, respectively. The egg-collecting area was also found to be highly contaminated, with 71.5 and 20.0% contaminated samples for farms A and B, respectively. After CD, a reduction in the number of contaminated samples but no complete elimination was observed. During the following sampled laying period, the percentage of henhouse contamination fluctuated between 0 and 29.1% on farm A and between 0 and 8.7% on farm B. The egg-collecting area remained contaminated, with the percentage of contaminated samples varying between 0 and 63.4% and between 0 and 60.1% on farms A and B, respectively. After CD on farm B, before the third sampled laying cycle, no improvement was noticed in the contamination of the henhouses and the egg-collecting area.

**Table 2.** Summary of contaminated *Salmonella* serovar Enteritidis samples during the laying period averaged over 7 farms[1]

| Location | | Contaminated sample[2] (%) | Highly contaminated sample[3] (%) | No. of contaminated farms[4] |
|---|---|---|---|---|
| Henhouse | Overshoes | 42.3 (n = 71) | 33.3 (n = 30) | 7/7 |
| | Floor | 34.2 (n = 73) | 30.0 (n = 25) | 7/7 |
| | Manure belt | 31.3 (n = 64) | 35.0 (n = 20) | 7/7 |
| | Hen feces | 29.4 (n = 68) | 55.0 (n = 20) | 7/7 |
| | Feed trough | 22.1 (n = 68) | 20.0 (n = 15) | 7/7 |
| | Hygiene mat | 21.7 (n = 23) | 40.0 (n = 5) | 3/4 |
| | Egg belt at cage/laying nest | 20.0 (n = 60) | 8.33 (n = 12) | 5/7 |
| | Dust | 20.0 (n = 20) | 0.00 (n = 4) | 3/6 |
| | Ventilator | 19.1 (n = 68) | 7.69 (n = 13) | 7/7 |
| | Wall | 18.8 (n = 69) | 0.00 (n = 13) | 5/7 |
| | Egg cross conveyor | 18.7 (n = 75) | 7.14 (n = 14) | 5/7 |
| | Drain | 18.4 (n = 38) | 14.3 (n = 7) | 4/5 |
| | Crack/gap floor | 17.2 (n = 64) | 36.4 (n = 11) | 6/7 |
| | Feed | 15.8 (n = 57) | 33.3 (n = 9) | 6/7 |
| | Feed hopper | 13.0 (n = 69) | 0.00 (n = 9) | 6/7 |
| | Crack/gap wall | 12.9 (n = 70) | 11.1 (n = 9) | 4/7 |
| | Boots | 11.1 (n = 9) | 100 (n = 1) | 1/3 |
| | Air | 10.5 (n = 57) | 0.00 (n = 6) | 5/7 |
| | Gate | 8.60 (n = 58) | 0.00 (n = 5) | 4/7 |
| | Air inlet | 8.30 (n = 48) | 0.00 (n = 4) | 3/6 |
| | Cage | 8.00 (n = 88) | 14.3 (n = 7) | 5/7 |
| | Ceiling | 7.00 (n = 43) | 33.3 (n = 3) | 3/6 |
| | Drinking nipple/cup | 5.90 (n = 68) | 25.0 (n = 4) | 4/7 |
| | Water reservoir (inside) | 0.00 (n = 45) | 0.00 (n = 0) | 0/7 |
| Egg-collecting area | Floor | 47.2 (n = 36) | 17.6 (n = 17) | 7/7 |
| | Pallet truck | 45.5 (n = 22) | 0.00 (n = 10) | 4/6 |
| | Conveyor egg tray | 40.0 (n = 35) | 7.14 (n = 14) | 7/7 |
| | Egg sorter | 28.6 (n = 28) | 12.5 (n = 8) | 4/7 |
| | Control-panel conveyor | 23.1 (n = 26) | 0.00 (n = 6) | 4/6 |
| | Egg-packer head | 15.0 (n = 20) | 0.00 (n = 3) | 2/6 |
| | Container egg tray | 13.3 (n = 15) | 0.00 (n = 2) | 2/5 |
| | Wash basin | 8.00 (n = 25) | 0.00 (n = 2) | 2/7 |
| | Wall | 6.30 (n = 32) | 0.00 (n = 2) | 2/7 |
| | Pallet | 0.00 (n = 8) | 0.00 (n = 0) | 0/4 |
| | Toilet | 0.00 (n = 3) | 0.00 (n = 0) | 0/2 |
| Equipment | Cleaning machine | 45.2 (n = 13) | 33.3 (n = 6) | 3/3 |
| | Scraper | 44.4 (n = 9) | 0.00 (n = 4) | 3/4 |
| | Ladder | 35.5 (n = 31) | 18.2 (n = 11) | 4/7 |
| | Wheelbarrow | 34.5 (n = 29) | 10.0 (n = 10) | 4/5 |
| | Shovel | 30.8 (n = 39) | 25.0 (n = 12) | 3/5 |
| | Wiper | 25.0 (n = 28) | 28.6 (n = 7) | 3/4 |
| | Dust pan | 25.0 (n = 16) | 0.00 (n = 4) | 2/4 |
| | Bucket | 23.1 (n = 13) | 0.00 (n = 3) | 2/4 |
| | Brush | 16.7 (n = 60) | 10.0 (n = 10) | 5/7 |
| Vermin | Mouse/rat fece | 72.7 (n = 11) | 12.5 (n = 8) | 5/5 |
| | Mouse/rat intestine | 60.0 (n = 5) | 33.3 (n = 3) | 3/5 |
| | Flies | 41.2 (n = 17) | 42.9 (n = 9) | 4/4 |
| | Red mite | 40.0 (n = 15) | 50.0 (n = 6) | 5/6 |

[1]Sample types were listed in decreasing order based on the proportion of contaminated samples.

[2]Total number of samples analyzed is given in parentheses.

[3]Percentage of contaminated samples that were highly contaminated (tested positive in the $10^{-2}$ and $10^{-3}$ dilutions). Total number of contaminated samples in parentheses.

[4]Number of farms on which the sample type was found contaminated (x/y; x = number of farms on which the sample type was found contaminated and y = number of farms on which the sample type was sampled).

## *Salmonella* Enteritidis Environmental Contamination

The percentage and the degree of contaminated environmental samples during lay (onset, middle, and end) are summarized for the henhouse, the egg-collecting area, on the equipment, and in and on vermin (Table 2).

In the henhouse, the most frequently *Salmonella* Enteritidis-contaminated sampled sites were the overshoes, floor, manure belt, and hen feces. These sites also had the largest proportion of highly contaminated samples and were found to be contaminated on all 7 farms. In the egg-collecting area, the most frequently *Salmonella* Enteritidis-contaminated sampled sites were the floor, pallet truck, and conveyor egg trays. Again, the floor had the largest proportion of highly contaminated samples and was found to be contaminated on all 7 farms.

The percentage of highly *Salmonella* Enteritidis-contaminated samples was not found to be significantly different in the different stages of the laying period (all $P$-values $> 0.05$). In total, at the onset, middle, and

end of lay, 17.6% (n = 74), 18.5% (n = 157), and 21.4% (n = 173) of the contaminated samples, respectively, were found to be highly contaminated.

## *Salmonella* Enteritidis Contamination of Eggshells, Egg Contents, and Ceca

*Salmonella* Enteritidis was detected on eggshells from 5 of the 7 farms. Positive eggshells were found at the onset (1 time), middle (7 times), and end of lay (1 time); at the same time, the henhouse environmental contamination ranged from 12.5 to 67.8%. In total, 9 of the 490 pooled eggs were contaminated on the eggshell, indicating possible eggshell contamination ranging from 0.18 to 1.8% of the sampled eggs. The egg content was found to be *Salmonella* Enteritidis-positive form 2 of the 7 farms, once at mid lay and once at end of lay, with the environmental contamination of the henhouse being 53.2 and 61.5%, respectively. In total, 2 of 490 egg pools or 0.04 to 0.4% of the egg content of the sampled eggs were found to be contaminated. At the end of the laying period, ceca sampled from 6 of the farms were found to be *Salmonella* Enteritidis-contaminated in 2 of 10 sampled henhouses (on 2 farms). In total, 11 of 500 sampled ceca (2.2%) contained *Salmonella* Enteritidis.

## *Salmonella* Enteritidis Contamination After CD

After CD, all 6 sampled farms still yielded *Salmonella* Enteritidis-contaminated samples (one farm could not be sampled after CD). More specifically, in 60% of the sampled henhouses and 50% of the sampled egg-collecting areas, contaminated samples were still found. A summary of samples that were still contaminated after the CD procedure for each separate farm is shown (Table 3). Again, the floor or overshoes were found to be contaminated on all sampled farms after CD. The

**Table 3.** Summary of environmental samples in the henhouse and egg-collecting area still contaminated with *Salmonella* Enteritidis after cleaning and disinfection

| Farm | Sample location | Area[1] | Degree of contamination[2] |
|---|---|---|---|
| A | Feed trough | HH 1,2 | L |
| | Drain | HH 2 | L |
| | Crack/gap floor | HH 2 | L |
| | Overshoes | HH 1 | L |
| B | Manure belt | HH 2 | L |
| | Wheelbarrow | HH 2 | L |
| | Egg belt at cage | HH 3 | L |
| | Mouse intestine | HH 3 | L |
| | Conveyor egg tray | ECA | L |
| | Dustpan | ECA | L |
| | Cardboard flat egg tray | ECA | L |
| | Pallet truck | ECA | H |
| | Floor | ECA | H |
| | Container egg tray | ECA | H |
| C | Mouse intestine | HH | L |
| | Wheelbarrow | HH | L |
| | Floor | HH | L |
| | Crack/gap floor | HH | L |
| | Flies | ECA | L |
| | Floor | ECA | H |
| | Conveyor egg tray | ECA | H |
| | Pallet truck | ECA | H |
| | Cat litter box | ECA | H |
| D | Rat intestine | HH 2 | L |
| | Floor | HH 2 | L |
| | Manure belt | HH 3 | L |
| E | Wall | HH 2 | L |
| | Gate | HH 2 | L |
| | Cage | HH 2 | L |
| | Overshoes | HH 2 | L |
| | Mouse intestine | HH 2 | L |
| | Mouse feces | HH 2 | L |
| | Corpse laying hen | HH 2 | L |
| | Mouse trap | HH 2 | L |
| G | Mouse intestine | HH | L |
| | Cage | HH | L |
| | Ladder | ECA | L |
| | Brush | ECA | L |
| | Floor | ECA | L |

[1]Area where the sample was found contaminated: HH = henhouse; 1, 2, and 3 = identification of the henhouse; and ECA = egg-collecting area.

[2]Degree of contamination: L = low, initial suspension or $10^{-1}$ dilution of initial suspension; H = high, $10^{-2}$ or $10^{-3}$ dilution of initial suspension.

remaining mice or rats were found to be *Salmonella* Enteritidis-contaminated on 5 farms. Highly contaminated samples were only found in the egg-collecting area.

Among the sampled farms, Enteritidis was the persistent serotype. On 3 farms, a few other serotypes were found only once. On farm A, one isolate of *Salmonella* Livingstone and one isolate of *Salmonella* Brandenburg were found. On farms C and D, one isolate of *Salmonella* Oranienburg and one isolate of *Salmonella* Typhimurium were found, respectively.

## DISCUSSION

Several studies have investigated the *Salmonella* environmental contamination on layer farms (Poppe et al., 1992; Davies and Breslin, 2001; Davies and Breslin, 2003b; Wales et al., 2007; Carrique-Mas et al., 2009; Snow et al., 2010). To our knowledge, however, this is the first study that provides a detailed, semiquantitative evaluation of the sites of *Salmonella* Enteritidis environmental contamination on persistently positive layer farms in the new epidemiological context of flocks vaccinated with mainly live *Salmonella* Enteritidis vaccines.

*Salmonella* Enteritidis detection in the henhouse environment may not reflect actual *Salmonella* Enteritidis colonization or excretion by the birds (Kinde et al., 1996; Davies and Breslin, 2001). Nevertheless, environmental sampling is considered to be a representative indicator for the presence of *Salmonella* in layer flocks and for the probability that hens would lay contaminated eggs (Davies and Breslin, 2001; Namata et al., 2008). In addition, environmental sampling using a semiquantitative *Salmonella* analysis can indicate problems in the infrastructure of the henhouse, in farm management, and CD practices that may contribute to the persistence and spread of *Salmonella.*

Since the implementation of the national control program based on intensive monitoring, hygiene measures, and obligatory vaccination, the prevalence of *Salmonella*-contaminated flocks and human cases have gradually decreased in Belgium (European Food Safety Authority, 2007b, 2011). However, in view of the results obtained in the present study, it is clear that the remaining persistently *Salmonella* Enteritidis-positive layer farms had a high prevalence of *Salmonella* Enteritidis in their environment and that CD on these farms did not eliminate the contamination. This study clearly showed that vaccination alone cannot solve the *Salmonella* Enteritidis problem in the laying hen industry. The present study found contaminated ceca at the end of the laying period on 2 of the 6 farms, which shows that vaccinated hens can become colonized with *Salmonella* Enteritidis. Vaccination reduces the risk for inter- and intraflock *Salmonella* Enteritidis contamination (Woodward et al., 2002; Davies and Breslin, 2003a), but it must be combined with several other measures, including biosecurity. The majority of the sampled layer farms were found to have inadequate bio-security. All of the farms were lacking a strict and well-applied hygiene barrier in the henhouses and egg-collecting areas.

The prevalence of *Salmonella* Enteritidis on the contaminated layer farms was found to be relatively high in the henhouse as well as in the egg-collecting area, especially at the end of the first sampled laying round. After CD, a reduction in *Salmonella* Enteritidis-positive samples was noticed, but *Salmonella* Enteritidis contamination was not eliminated. The overall percentage of contaminated samples increased again during the next sampled laying period. In individual layer farms and henhouses, the percentage of positive samples fluctuated between the onset and end of lay, showing substantial variation from one visit to the next, an observation that is in accordance with Wales et al. (2007).

In the present study, several critical points were identified in the environment that may contribute to the persistence of contamination. A wide variety of sample types were found to be *Salmonella* Enteritidis-positive during laying, clearly illustrating the persistence of the contamination. The overshoes, floor, manure belt, and hen feces were the most recurrent and frequent highly contaminated samples in the henhouse. The air, together with the ventilators and air inlets, was found to be *Salmonella* Enteritidis-contaminated in several henhouses. This indicates that contaminated dust could spread through the henhouse, to other henhouses, to the egg-collecting area, and possibly even to the external farm environment. This highlights the importance of dust removal. Feed, feed troughs, feed hoppers, and drinking cups and nipples in the henhouse were found to be *Salmonella* Enteritidis-contaminated. The hens may therefore be contaminated with *Salmonella* Enteritidis from their feed or drinking water. Of the feed samples in the henhouse, one-third were highly contaminated on 3 of the 6 contaminated farms. In cases where the hens ingest high numbers of *Salmonella*, vaccination may be insufficient to provide protection (Woodward et al., 2002; De Buck et al., 2005; Atterbury et al., 2009). Freshly laid eggs were found to be *Salmonella* Enteritidis-positive on the eggshell and in the egg content on persisting farms, which shows the risk of egg contamination in an *Salmonella* Enteritidis-contaminated layer flock environment. In addition, our results show the high risk of cross contamination of eggshells in the egg-collecting area.

The aim of performing CD in layer houses is to eliminate organic matter and contamination of the construction and equipment. However, on all 6 sampled farms, *Salmonella* Enteritidis was still detected after CD in at least one henhouse, which was also true of the egg-collecting area on 3 of these farms. Some of those samples were even highly contaminated. Information provided by the farmer revealed that the CD of the egg-collecting area was often inadequate (e.g., incomplete removal of organic material) and was not even performed in some cases. The present study showed, however, that the egg-collecting area can be a reservoir for cross contamination. On multi-age farms, all henhouses were not

cleaned and disinfected at the same time, which poses a risk for cross contamination of cleaned and disinfected henhouses. Adjacent henhouses were connected by a common egg belt and passageways, making it difficult to maintain henhouse-specific bio-security.

This study revealed frequent *Salmonella* Enteritidis contamination of mobile equipment on all of the farms. Equipment, such as shovels, ladders, and wheelbarrows that are often moved between henhouses, pose a risk for *Salmonella* Enteritidis transmission between henhouses. Almost all sampled henhouses had problems with rodents, red mites, and flies, which were shown to be *Salmonella* Enteritidis carriers even after CD. They pose a risk for transmission of *Salmonella* Enteritidis within and between henhouses and the persistence of *Salmonella* Enteritidis after disinfection. A correlation between *Salmonella* Enteritidis persistence and a high number of rodents has already been illustrated (Carrique-Mas et al., 2009). Moreover, it has been shown experimentally that the poultry red mite could act as a vector and reservoir of *Salmonella* Enteritidis and that hens can be infected by ingesting contaminated mites (Moro et al., 2007). The present study showed that mites on 5 of the 6 farms were naturally infected with *Salmonella* Enteritidis. *Salmonella*-infected red mites could contaminate the newly housed birds after CD of the henhouse, given that *Salmonella* can survive in the mite for several months (Zeman et al., 1982). As demonstrated by Holt et al. (2007), flies residing in an *Salmonella* Enteritidis-contaminated environment can become contaminated themselves. Ingesting *Salmonella* Enteritidis-contaminated flies results in gut colonization of the birds. On farm A, feces of a cat and dog in the henhouse were found to be *Salmonella* Enteritidis-contaminated, which illustrates the importance of keeping pets out of the henhouse and egg-collecting area. Although Snow et al. (2010) suggest that the presence of cats and dogs can reduce the risk of *Salmonella* presence, given that they play a role in deterring rodents, it has been shown in other studies that they can excrete *Salmonella* (Van Immerseel et al., 2004; Leonard et al., 2011)

In conclusion, despite the implementation of a strict monitoring and control program, including obligatory vaccination in layers in Belgium, some layer farms still have persistent *Salmonella* Enteritidis contamination. Environmental contamination on persistently infected layer farms is largely associated with the same critical points as identified previously. This study, however, pointed out some deficiencies in the hygiene programs and identified several contamination hot spots. This information should help to focus the approach for *Salmonella* control on these farms in the future.

## ACKNOWLEDGMENTS

We gratefully thank the farmers, Galluvet (Lummen, Belgium), Degudap Veterinary Practices (Izegem, Belgium), and Luc Ver Eecke (De Biest, Kruishoutem, Belgium) for participating in this study. Also, many thanks to B. Vandevelde and M. Geerinckx [Animal Health Care Flanders (DGZ), Torhout, Belgium] who helped with the sampling. Thanks to T. Fevery (DGZ), T. Loncke (DGZ), and A. Van de Walle [Itstitue for Agricultural and Fisheries Research (ILVO), Melle, Belgium] for their excellent technical assistance and M. Levenson (ILVO) for English-language editing. This research is funded by the Belgian Federal Public Service for Health, Food Chain Safety, and Environment (RF 6195; Brussels, Belgium).

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