ORIGINAL ARTICLE

A longitudinal study of environmental salmonella contamination in caged and free-range layer flocks

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The environmental contamination by salmonella was examined over a 12-month period in 74 commercial layer flocks from eight farms in the UK, which previously had been identified as being contaminated with salmonella. Samples of faeces, dust, litter, egg belt spillage and wildlife vectors were taken, plus swabs of cages, feeders, drinkers, floors, egg belts and boots. Some sampling was performed in each month of the year. Numerous serovars were detected but *Salmonella enterica* serotype Enteritidis was the only persistent serotype found among single-age flocks. There was a significant correlation between qualitative environmental samples and semi-quantitative faeces samples. The level of environmental contamination increased significantly over time. There were significant temperature and seasonal effects upon contamination. Wildlife vectors proved to be sensitive samples for the detection of salmonella. The efficacy of cleaning and disinfection upon residual salmonella contamination, and upon subsequent flock contamination, was highly variable between and within premises. The variability between detected prevalences over time and between flocks indicates a need for regular, sensitive monitoring of flocks for salmonella to permit targeting of control measures aimed at eliminating contamination of the layer environment by salmonella. There is substantial scope for improvement of cleaning and disinfection procedures.

Introduction

Salmonella enterica serotype Enteritidis is the most common identified cause of human salmonellosis in the UK (Health Protection Agency, 2005) and is among the most significant *Salmonella* serotypes in public health elsewhere, including other parts of Europe (Fisher, 2004b) and North America (Centers for Disease Control and Prevention, 2004). Undercooked and raw eggs have been heavily implicated in human infection with *S.* Enteritidis (Coyle *et al.*, 1988; St Louis *et al.*, 1988; Hogue *et al.*, 1997; Palmer *et al.*, 2000; Centers for Disease Control and Prevention, 2004; De Buck *et al.*, 2004; Crespo *et al.*, 2005). This serotype is able to cause long-term colonization of the chicken reproductive tract and become deposited within egg contents (Berchieri *et al.*, 2001; Okamura *et al.*, 2001; Amy *et al.*, 2004; Guard-Bouldin *et al.*, 2004), and in addition will cause external contamination of the shell (Messens *et al.*, 2005).

Improved biosecurity and hygiene in the UK poultry industry plus vaccination of the majority of commercial laying birds and broiler breeders, introduced in the mid to late 1990s, has been followed by a large reduction in reported incidents of *S.* Enteritidis in poultry and in humans (Anonymous, 2005; Health Protection Agency, 2005). Similar improvements have also occurred in some other countries (Wegener *et al.*, 2003; Marcus *et al.*, 2004; Mumma *et al.*, 2004) but there is still a significant reservoir of infection in commercial laying flocks (Adak

& Gillespie, 2004; Crespo *et al.*, 2005). On commercial laying farms, persistent environmental contamination is currently considered to be the predominant problem (van de Giessen *et al.*, 1994; Davies & Breslin, 2003b; Gradel *et al.*, 2004). Vaccination and other interventions such as competitive exclusion products do not reliably eliminate infection (Davies & Breslin, 2003a; Davies *et al.*, 2003), and their effectiveness is reduced where there is a heavy environmental challenge (Nakamura *et al.*, 1994, 2004; Davies & Breslin, 2003a,b).

Sampling the hen house environment, when coupled with suitable cultural techniques, has proved a sensitive and cost-effective method of monitoring salmonella carriage and excretion by layer hens (Kradel & Miller, 1991; Henzler *et al.*, 1994), and there is good agreement between the level of environmental contamination and the level of internal egg contamination and associated human disease (Altekruse *et al.*, 1993; Henzler *et al.*, 1994, 1998; Schlosser *et al.*, 1995; Mallinson *et al.*, 2000). The sensitivity of environmental sampling varies between sample types (Davies & Wray, 1996), and in caged layers samples taken from egg belts, from dust close to birds or cage stacks and from naturally accumulated pooled chicken faeces on droppings belts or scrapers have proved especially useful (Davies & Breslin, 2001).

The prevalence of eggs contaminated internally or externally by salmonella from infected flocks appears to

vary substantially, between less than 0.03% and 1% overall, but with up to 35% of some batches positive (reviewed by Davies & Breslin, 2004; De Buck *et al.*, 2004). Although technical factors may account for some variation between studies, it is unclear to what extent factors such as the time since the introduction of salmonella into the flock, the stage of lay and stressors such as hot weather can affect the level of infection in the flock and the production of contaminated eggs. Longitudinal studies are an appropriate way to address these questions, but the requirements of resource and cooperation by producers over months or years tends to limit the number and scope of such investigations. Consequently, there are few reports that have examined the levels of salmonella in laying houses and hens in lay over an extended period of time (Davison *et al.*, 1999; Davies & Breslin, 2003b; Kinde *et al.*, 2005). The present report describes a longitudinal study conducted on several layer premises over a 12-month period.

Materials and Methods

Sample collection. A number of caged and free-range layer flocks were identified as having *S.* Enteritidis, by reference to the Zoonoses Order Database, or by personal contact or sampling. Approaches were made to the owners of these flocks and, when permission for intensive sampling had been obtained, they were visited; environmental samples were taken on successive occasions at differing stages of lay and also following depopulation, cleaning and disinfection. Sampling was systematic and targeted at sites and types of material that were likely to reveal salmonella contamination if present, based upon previous experience. The number and types of samples taken on each occasion was, in addition, determined by several factors. These included: the need for reasonable coverage of hen houses of differing sizes, the amount of certain sample types (e.g. dust, egg belt spillage and faeces) present according to design and usage, and constraints on access to certain sample types imposed by the hen house construction. On some visits an increased number of samples was taken to better define the distribution of salmonella within the house.

Samples were taken directly into 225 ml buffered peptone water (BPW: Merck) using gauze surgical swabs (Kleenex Readiwipes: Robinson Healthcare). For qualitative detection of salmonellas, samples consisted of approximately 25 g faecal material, spillage from egg belts and from floors under cages, litter from free-range houses, dust from within and around cages and nest boxes (10 to 15 g), rodent faeces (1 to 2 g) and flies from adhesive paper or contact insecticidal traps (1 to 2 g). In addition, sterile swabs soaked in BPW were used to sample the surfaces (0.5 m²) of egg belts, feeder troughs, cleaned droppings boards, floors beneath cages and the boots of free-range unit workers, The interiors of empty cages and drinker spillage cups or troughs were also swabbed, using composite samples of eight cages per swab. For semi-quantitative culture, bulked samples of chicken (approximately 30 g) and rodent (approximately 1 to 2 g) faeces were collected into dry pots.

All solid samples were returned to the laboratory at ambient temperature on the day of collection and processed immediately. Swab samples taken into BPW were, in addition, stored and transported in a cold box at below 10°C. Mouse and rat carcasses were collected as available and 3 g liver, spleen and intestines were removed aseptically for culture at the laboratory.

Culture technique. For standard (qualitative) isolation, samples in BPW were pre-enriched at 37°C for 18 h and then 0.1 ml pre-enriched mixture was inoculated onto modified semi-solid Rappaport–Vassiliadis agar with 0.01% novobiocin (MSRV; Difco 218681) and incubated at 41.5°C for 16 to 24 h. Where opaque growth was seen on MSRV, a 1 μl loop from the edge of the opaque growth zone was inoculated onto Rambach agar (Merck 107500). Rambach and associated MSRV plates were incubated at 37°C and 41.5°C, respectively, for 24 h. The plates were examined and any MSRV plates on which the growth had spread widely but that were negative for salmonella on the Rambach

plates were subcultured again onto Rambach agar. Representative *Salmonella* isolates were confirmed by complete serotyping at the Salmonella Reference Laboratory at VLA—Weybridge according to the Kaufmann–White Scheme (Popoff, 2001).

For semi-quantitative culture (Wales *et al.*, 2006a), 90 ml BPW was thoroughly mixed with each 10 g bulked chicken faeces sample, and similarly 80 ml BPW was mixed with 20 g samples. Ten or 20 ml BPW was added to pools of mouse and rat droppings, respectively. From each of these initial 10-fold or five-fold dilutions of chicken or rodent faeces, a 10% volume aliquot was dispensed into a universal container, to serve as the first in a decimal dilution series, which was continued by taking 1 ml from the initial dilution, mixing it with 9 ml BPW and successively repeating this step five times, adding 1 ml each consequent dilution to 9 ml BPW. A pre-enrichment incubation at 37°C for 18 h was performed on all preparations, comprising the primary 1:10 or 1:5 mixture of faeces in BPW ('0'), the 10% volume aliquot of the same ('1'), and the decimal dilutions ('2' to '7'). After incubation, 0.1 ml each of preparations '0' and '1' was cultured on selective (MSRV) and indicator (Rambach) media as described above. Preparations '2' to '7' were refrigerated, and were then similarly cultured if either the '0' or '1' preparations yielded growth. A semi-quantitative result was derived using the highest numbered preparation of each sample that yielded salmonellas to indicate the most probable range of salmonella colony-forming units in the original sample. A semi-quantitative score was given to each bulked chicken faeces sample, depending on the highest dilution that yielded salmonellas upon culture (Table 1).

Statistical analyses. *Prevalence versus semi-quantitative data*. For each sampling visit from which semi-quantitative data were obtained, the overall house prevalence of positive samples, derived from the standard (qualitative) isolation procedure, was assigned to one of four bands: <20%, 20% to 40%, 40% to 60% and >60%. These were tabulated against the semi-quantitative score (zero to five) from faeces (caged) or litter (free-range) samples. The exact probability for a linear by linear association test was calculated by StatXact software (Cytel, Cambridge, Massachusetts, USA).

Effects of season, temperature, stage of lay and vector activity. A non-linear mixed-effects method was implemented to model the binary outcome: positive or negative for salmonella for each qualitatively cultured sample. The data used for fitting the model were from caged single-aged flocks, excluding the A&L farm that was visited only once. The model was fitted using a logit-link and the binomial distribution within Proc NLMIXED, in SAS (SAS Institute, Cary, North Carolina, USA) (Gilmour *et al.*, 1985; Lindstrom & Bates, 1990; Pinheiro & Bates, 1995). The random effects matrix was estimated by a non-adaptive Gaussian quadrature method, with 201 points used. The linear predictor was:

$$\eta = \mu + \tau_i + \beta_0.MIH + \beta_1.Temp_i + Z$$

where μ is the intercept, τ_i is the farm ($i = 1, 2, \ldots 5$), *MIH* is the age of the birds prior to entering the active experiment, and $Temp_i$ is the average monthly temperature recorded at the weather station closest to each farm i, as supplied by the Meteorological Office (2006). The random effect matrix Z includes the flock effect, over time. The model fitting process tested terms for their strength of evidence in affecting the odds of infection, including fly and wildlife infestation from

Table 1. *Relationship between quantitative score and probable concentration of salmonella organisms in a sample*

	Range of salmonella colony-forming units	
Score	10 g sample (/g)	20 g sample (/g)
0	Not detected	Not detected
1	0.1 to 1	0.05 to 0.5
2	1 to 10	0.5 to 5
3	10 to 100	5 to 50
4	100 to 1000	50 to 500
5	1000 to 10 000	500 to 5000

semi-quantitative estimates of severity in each house, and the season of sample taken. Seasons were defined as: March to May (spring), June to August (summer), September to November (autumn) and December to February (winter). Since the temperature effect was completely nested within the season effect, nested models were compared and the likelihood ratio test was used to show whether there was any variation attributable to season in addition to that accounted for by temperature. For each effect found to be significant, confidence intervals were calculated for the odds ratio of a sample being salmonella-positive when one state was compared with another (e.g. a particular number of months in house compared with an additional increment of 1 month).

Results

Sampling was performed over a 12-month period from August 2004 to July 2005 and a total of 74 flocks (59 caged, 15 free range) were sampled from eight farms. All flocks had been vaccinated against salmonella, the majority using a live *S*. Enteritidis vaccine with or without a live *Salmonella* Typhimurium component. In the remaining flocks, an inactivated *S*. Enteritidis plus *S*. Typhimurium (Salenvac T) or a live *Salmonella* Gallinarum vaccine providing cross-protection against *S*. Enteritidis (Nobilis SG 9R) had been used. In addition, an oral competitive exclusion treatment had been given to approximately one-half of the flocks. All farms except two (Gra and Sut) operated an all-in-all-out policy for stocking hen houses, potentially allowing thorough cleaning and disinfection (C&D) of accommodation between flocks. Between one and four visits were made per flock at intervals of two to six months (Tables 2 and 3), with flock ages varying between less than one and 19 months in house. Overall, and excluding rodent and other vector samples, 19.4% (736/3793) of cage house and 10.2% (85/833) of free-range house samples yielded salmonellas. However, there was wide variation in the prevalence of positive samples between flocks and between farms (Tables 2 and 3).

Salmonella **serovars and phage types.** Where *S*. Enteritidis was present on a farm, often there were two or three (in one case four) phage types detected (Table 4), although sometimes these were closely related types, such as PT4 and PT7. The same *S*. Enteritidis phage types were usually present in more than one flock on any one farm, and were present in both caged and free-range flocks where both were kept on the premises. Samples taken after C&D, and samples from successive flocks in the same house (Table 4), showed that endemic *S*. Enteritidis phage types frequently persisted after C&D and were present in contamination sampled from a subsequent flock. Other serovars, by contrast, typically were restricted to an individual flock and appeared only once in a series of sampling visits (Table 4).

Semi-quantitative versus qualitative samples. Semi-quantitative culture using 20 g faeces was adopted for later samples following evidence of a superior sensitivity compared with a 10 g sample. Semi-quantitative data are included in Tables 1 and 2, and no distinction is made between scores from the two sample sizes since they were of the same order of magnitude. For values of prevalence and quantitative score from the same flock visit, the exact probability for a linear by linear association test is $P = 0.0001$, indicating a highly significant association between results from the two culture techniques. The same data are plotted against each other in

Figure 1, which shows an apparently higher sensitivity of the multiple qualitative samples compared with the single bulked faeces sample, manifested as many more non-zero prevalence values plotted against zero semi-quantitative scores than *vice versa*. Nonetheless, there were many samples that were negative for both techniques: there are 13 superimposed data points at the origin in Figure 1.

Stage of lay. Figure 2 shows the prevalence of salmonella-positive samples against time spent in house by all single-age layer flocks. There is a substantial range of prevalences observed, although the trend appears to be upwards over time for sequentially sampled flocks. Figure 3 shows the changes in salmonella prevalence over time in caged flocks where there had been two or three visits to occupied houses, for two sample sources: faeces/droppings boards and dust. Although the overall trends are upwards, this is more pronounced for the dust than for the faeces and droppings boards samples.

In the fitted non-linear mixed-effects model, the time in house was significant in respect of prevalence ($P < 0.0001$), with an average increase in odds ratio of 1.20 (95% confidence interval, 1.13–1.26) for each additional month in house.

Effect of temperature and season. The wide range and variation in the data is illustrated in Figure 4, showing, month by month, the prevalence of salmonella-positive samples from each flock visit as a deviation above or below the year-round average prevalence for that particular farm.

The fitted non-linear mixed-effects model revealed a significant ($P = 0.0014$) effect between the odds of a positive sample and average monthly temperature. The average increase in odds ratio for salmonella detection in a sample was 1.08 (95% confidence interval, 1.03–1.12) for each additional degree Celsius. Furthermore, after additionally fitting the season effect, it was found that the summer months significantly increased the odds of a sample being positive, when compared with the winter months ($P = 0.0486$), with an associated odds ratio of 3.41 (95% confidence interval, 1.01–11.55).

Wildlife vectors and free-range paddocks. Details of isolations from rodents (predominantly pooled mouse faeces, but also rat faeces and one set of mouse viscera), flies, litter beetles and free-range paddocks are presented in Table 4. In flocks where no salmonellas were detected in samples from the house, any vector and paddock soil samples were also negative. The overall prevalence of salmonella-positive samples from wildlife vectors was 34/88 (38.6%), more than double the prevalence of positive samples from houses, which was 821/4626 (17.7%). Eleven positive samples were cultured by the quantitative method, yielding a range and mean of quantitative scores of 2 to 7 and 4.36, respectively. Where isolates from vectors were phage typed, the results generally correlated with those for samples from the same flock. In the statistical model, there was not enough evidence from the semi-quantitative wildlife data (not shown) to conclude that the severity of fly infestation caused a change in the odds of a positive sample ($P = 0.22$).

Table 2. *Details of overall salmonella prevalence (positive samples/total samples) from caged layer flock houses, and of quantitative scores of salmonella concentration from bulked faeces samples*

Farm, visit	Flock number, positive samples/total samples taken; semi-quantitative score[a]													
A&L	Flock 1	Flock 2												
November	0/25	0/28												
C&K	Flock 3	Flock 4	Flock 5	Flock 6	Flock 7	Flock 51	Flock 51 PCD[c]							
September	0/40; 0	6/40; 2	1/40; 0			5/40; 1	(9/60)							
November	1/40; 0	12/40; 1	0/40; 0	0/40; 0										
April	5/40; 0	13/40; 2		0/40; 0	3/40; 0									
Coc	Flock 8	Flock 9	Flock 10	Flock 11	Flock 12	Flock 13	Flock 14	Flock 15	Flock 16	Flock 17	Flock 18	Flock 70	Flock 73	Flock 74
November	2/19; 0	3/19; 4	3/18; 0	12/20; 2	0/20; 0	12/19; 2	10/18; 2	8/20; 5	2/19; 2			(16/57)		
February	5/20; 2	10/20; 0	7/19; 5	15/20; 0	1/20; 0	(9/63)			4/19	1/20; 0	4/20			
April	9/20; 2	7/20; 0		4/20; 2	1/20; 0				2/20; 3	0/20; 3			1/20; 0	1/20; 0
Cots	Flock 19	Flock 20	Flock 21	Flock 22	Flock 23	Flock 24	Flock 25	Flock 26	Flock 27	Flock 28	Flock 52	Flock 53	Flock 54	Flock 55
August											22/40; 0	28/40; 5		
September											(4/60)	(19/60)		
October	1/40; 0	2/40; 0											21/40	
November			7/40; 0	0/40; 0	0/40; 0	7/40; 2							(9/60)	32/40; 4
January	0/20; 0	3/20; 0												1/60
March						1/40; 2	8/40; 2							
May			10/40	10/40	8/40									
June			(0/60)	(0/60)	(1/60)									
July	7/40; 4	25/40; 3				29/40; 4	4/40; 2	16/40; 0	21/40; 1	24/40; 2				
Fld	Flock 29	Flock 30	Flock 31	Flock 32	Flock 33	Flock 34	Flock 35	Flock 36	Flock 37	Flock 38	Flock 56			
December	0/13	0/13	0/14								12/40; 0			
January	0/20	0/20	0/40	0/20	0/20	0/20	0/40				(5/60)			
July	7/20				1/20	0/20	1/20	7/20	1/20	3/20				
August	(10/60)													
Gra	Flock 39[b]	Flock 40[b]	Flock 41[b]											
April	13/35; 3	35/40; 5	18/40; 0											
Hum	Flock 42	Flock 43	Flock 44	Flock 45	Flock 46	Flock 47								
October	0/40; 0	6/40; 1	0/40; 0	1/40; 0										
January	11/40; 0	(2/59)	0/40; 0	4/40; 0										
June	(7/58)		0/40; 0		6/40; 0	5/38; 0								
Sut	Flock 48[b]	Flock 49[b]	Flock 50[b]											
March	2/40	45/60; 3	29/60; 0											

Visits are listed in chronological order. Results in parentheses are from samples taken immediately after C&D following removal of the indicated flock. [a]For key to numbers of salmonella per gram, see Table 1. [b]Multi-age flock. [c]Post C&D.

Table 3. *Details of overall salmonella prevalence (positive samples/total samples) from free-range layer flock houses, and of quantitative scores of salmonella concentration from bulked faeces samples*

Farm, visit	Flock number, positive/total samples taken; semi-quantitative score[a]				
A&L	Flock 57	Flock 58	Flock 59		
November	0/40	0/37	0/37		
Fld	Flock 60	Flock 61	Flock 62	Flock 63	Flock 71
January	0/20	0/18	0/40		(5/50)
July		25/39	23/39	8/40	
August		(9/50)			
Gra	Flock 64				
April	3/8				
Hum	Flock 65	Flock 66	Flock 67		
October	0/44	0/50			
January	0/44		0/40		
June	0/40		0/40; 0		
Sut	Flock 68	Flock 69	Flock 72		
September	0/51	7/41; 0			
October		(0/25)			
March	1/40; 0		1/40; 0		

Visits are listed in chronological order. Results in parentheses are from samples taken immediately after C&D following removal of the indicated flock. [a]For key to numbers of salmonella per gram, see Table 1.

Cleaning and disinfection. On 17 occasions a hen house was sampled immediately after C&D, and in 10 of these cases the house had in addition been sampled shortly before depopulation. Figure 5 illustrates the degree of salmonella contamination before and after C&D in these 17 flocks, showing that the prevalence of positive samples fell after C&D in 9/10 cases, with no detectable salmonella following C&D in three of the 17 houses. The extent of contamination, as measured by the prevalence of positive samples following C&D, did not correlate with that detected before C&D. Two notable examples are flocks 53 and 55, both from the same farm, with similarly high pre-depopulation salmonella prevalences but widely differing post-C&D contamination. For all flocks except 13, 29 and 70, an aldehyde disinfectant was used, which should have been effective even in the presence of residual organic matter. The breakdown of the data on contaminated sites seen post-C&D (Figure 5) illustrates that all areas of the hen houses were prone to residual contamination, including those sites (cages, drinkers and feeders) likely to pose an early challenge to a new flock.

Examination of the data from new flocks in cleaned houses (Tables 2–4, plus data not shown) reveals that in three cases (flocks 26, 27 and 28, all on the same farm), high salmonella prevalences (40 to 60%) were found on first visits within 5 weeks of occupying houses that had had no detectable contamination after C&D. In the 14 other cases where the changeover of flocks was monitored, post-repopulation salmonella prevalences were 20% or lower, even in houses with detected residual contamination post-C&D.

Discussion

The present study examined the environmental contamination by salmonella in 74 flocks from eight farms over a 12-month period. Sampling was performed in every month of the year, although the data from any one farm tended to be clustered within a few months, and two farms (A&L and Gra) were visited only once. Sampling and detection was predominantly qualitative, yielding a positive or negative result for each sample and an overall percentage of positive samples (prevalence) figure for each site or flock. An abbreviated most-probable-number technique was used to estimate the number of viable salmonellas (colony-forming units) in a single bulked faeces sample from many flock visits and in wildlife vector samples from a few. The detected prevalence of contamination at any particular visit varied widely, with many flocks (e.g. flocks 30, 31, 32 and 65) having no detectable salmonella on any occasion and others (flocks 40 and 55) having a single-visit prevalence in excess of 80%. Those flocks that were sampled more than once often showed substantial variations in prevalence from one visit to the next, as illustrated in Figure 2. In most of the flocks examined, additional private monitoring was being carried out by cloacal swabs and environmental stick swabs at the end of lay, and in only one case was contamination identified by these additional tests.

Typing of *Salmonella* isolates showed that *S*. Enteritidis was the only persistent serotype on any farm with the exception, on evidence from the present and previous studies, of *Salmonella* Livingstone and *Salmonella* Infantis on farm Sut, where houses with individual cages were operated on a multi-age, continuously occupied, regimen. While the occurrence of hen house contamination by non-*S*. Enteritidis serovars appears typically to be transient, these organisms are nonetheless found frequently and there is evidence that certain serovars (e.g. *S*. Infantis) will contaminate both shells and contents of eggs. Attention to biosecurity, particularly in respect of feed and wildlife, should help reduce the chances of flock, and therefore egg, contamination by these non-Enteritidis serovars.

Several different phage types of *S*. Enteritidis (4, 6, 7, 21b, 35) were detected and each farm exhibited a particular, and persistent, combination of these. While PT4, which has been strongly associated with infection of layers in the UK and Europe (Cogan & Humphrey, 2003), was detected on six of the eight farms, it was present in combination with other persistent *S*. Enteritidis phage types on five of these. This is consistent with previous findings (Liebana *et al*., 2001), whereby a variety of often closely related phage types was seen in samples from poultry farms in geographically varied UK

Table 4. *Details of* Salmonella *types isolated from layer houses and vectors*

Farm/house	First flock in house			Second flock in house		
	Flock	Serovars/PTs[a] (visit 1: visit 2: visit 3: visit 4)	Rodent (insect/soil) samples[b]	Flock	Serovars/PTs[a] (visit 1: visit 2: visit 3: visit 4)	Rodent (insect/soil) samples[b]
C&K/2	3	0: **e**21B: **e**21B	No samples			
C&K/3	51	**e**21B: **e**21B[c]	No samples			
C&K/4	4	**e**21B: **e**21B: **e**21B	2/2			
C&K/5	5	**e**21B: 0	No samples	7	t, o	0/1
Coc/1	8	a, bi: **e**4/7, n: **e**4/7, l, k	1/1			
Coc/2	9	**e**4/7, br: **e**4/7: **e**4/7, br, l	0/2			
Coc/3	10	a: **e**4/7	0/2			
Coc/6	11	**e**4/7: **e**4/7, k: **e**4/7	No samples			
Coc/7	12	0: **e**4/7: **e**4/7	No samples			
Coc/8	13	**e**4/7: **e**4/7[c]	0/3	73	**e**4/7	
Coc/9	14	**e**4/7	0/1	17	**e**4/7: 0	No samples
Coc/10	70	**e**4/7[c]	1/1	18	**e**4/7	0/1
Coc/11	15	**e**4/7, a	No samples	74	**e**4/7	0/1
Coc/12	16	**e**4/7, a: **e**4/7: **e**4/7	1/1			
Cots/1	21	**e**4&6: **e**6: 0[c]	1/2	26	vx, ou	5/6 (**e**6&35, vx)
Cots/2	52	**e**: **e**[c]	0/2	19	**e**6: 0: **e**6/35	2/2
Cots/3	22	0: **e**6&7: 0[c]	1/2 (**e**6)	27	**e**6/35, vx	6/6 (**e**6&35, vx)
Cots/4	23	0: **e**−, z: **e**−[c]	0/2	28	**e**6/35, co, m, te, y	3/6 (**e**6&35, a)
Cots/5	53	**e**6&35: **e**[c]	2/2	20	**e**6: **e**4&6: **e**6/35	1/2
Cots/6	54	**e**: **e**6[c]	2/2 (**e**35)	24	**e**4&6: r: **e**6/35	0/1
Cots/8	55	**e**4/6: **e**4	1/2 (**e**7)	25	**e**4, co, y: **e**4, a, cu	0/3
Fld/1R	29	0: 0: **e**4/7: **e**4/7/35[c]	2/5 (1/3 insect)			
Fld/1L	36	**e**4/7				
Fld/2R	37	**e**4/7	(0/1 insect)			
Fld/2L	38	**e**4/7, s				
Fld/3R	33	0: **e**4/7	(0/1 insect)			
Fld/4R	56	**e**: **e**4[c]	0/2	35	0: **e**4/7	1/3
Fld/4L (FR)	63	**e**4/7	1/3			
Fld/5R (FR)	61	0: **e**4/7: **e**4/7/35[c]	(0/10 soil)			
Fld/5L (FR)	71	**e**4[c]	No samples	62	0: **e**4/7	no samples
Gra/1	39	**e**6	0/1			
Gra/2	40	**e**6	1/1			
Gra/3	41	**e**4	No samples			
Gra/P (FR)	64	**e**4	(3/10 soil)			
Hum/2	42	0: **e**4, a: **e**4[c]	1/2			
Hum/3	43	a, ty: **e**4[c]	0/1	46	**e**4	no samples
Hum/6	45	**e**4: **e**4	(0/10 insect)	47	**e**4	(1/2)
Sut/A	48	L	No samples			
Sut/9	49	L	0/1			
Sut/10	50	l, i	0/1			
Sut/frA (FR)	69	i: 0[c]	(4/10 soil: 2/5 soil [c])	72	w	(0/10)
Sut/frB (FR)	68	0: l	No samples			

[a]Lower-case letters indicate serovars as detailed below; numbers and upper-case letters indicate phage types (PTs) for *S*. Enteritidis. '0', no isolates at that visit; '/', 'and/or'. Where no *S*. Enteritidis PT is given, the isolate was untypable (−), was not typed (Fld) or a phage infection prevented typing (Cots). [b]Results presented as the number of positive samples/total samples taken at all visits, or at separate visits for flock 69. Not all presumptive *Salmonella* isolates were typed. [c]Visit after cleaning and disinfection. FR, free-range flock.

Abbreviations: **e,** *Salmonella* Enteritidis; a, *Salmonella* Agona; bi, *Salmonella* Binza; br, *Salmonella* Braenderup; co, *Salmonella* Corvallis; cu, *Salmonella* Cubana; i, *S*. *Infantis*; k, *Salmonella* Kedougou; l, *S*. *Livingstone*; m, *Salmonella* Mbandaka; n, *Salmonella* Newport; o, *Salmonella* Oranienburg; ou, *Salmonella* Ouakam; r, *Salmonella* Rissen; s, *Salmonella* Senfenburg; te, *Salmonella* Tennessee; ty, *S*. *Typhimurium*; vx, *S*. Enteritidis vaccine strain; w, *S*. 4,12 incomplete; y, *S*. 3,19:i incomplete; z, *S*. 6,4:D:
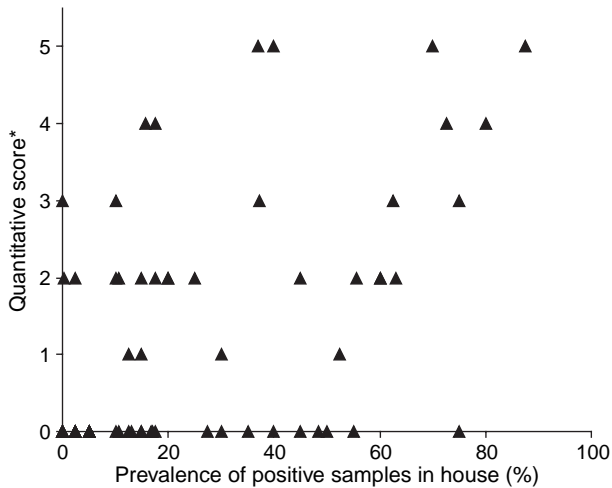
Figure 1. *House salmonella prevalence versus quantitative culture of bulked faeces. *Quantitative score is as detailed in Table 1.*

locations. In addition, our findings show that these phage type mixes can persist for an extended period of time on a farm. Where caged and free-range units existed on the same premises, a very similar mix of *S*. Enteritidis phage types and/or other serotypes was found in both production systems (Table 4). Egg surveys in the past 15 years have shown evidence of an increasing diversity of *S*. Enteritidis phage types isolated from eggs in the UK, with a waning dominance of PT4 (Food Standards Agency, 2004). A long-term decline in the incidence of PT4 has also been observed in isolates from humans in Europe (Fisher, 2004a; Health Protection Agency, 2005). The present findings indicate that a diversity of *S*. Enteritidis phage types is also present at the probable source of egg contamination.

Statistical investigations included a comparison of the qualitative and semi-quantitative culture techniques, which showed significant correlation. The latter technique shows promise as a research tool for the highlighting of areas and of vectors where a high level of challenge may be encountered (Wales *et al*., 2006a,b). In addition, a non-linear mixed-effects model was used to examine the effects of various factors on the prevalence of contamination, incorporating the random effect of flock. One limitation upon this statistical analysis is that, because many flocks were first sampled in the cooler months towards the end of the calendar year, the

environmental temperature and the number of months in-house exhibited some confounding; that is, the "time in house" and the "temperature" parameters both contributed to the same effect, and there is little that can be done to isolate the effects of each of the two variables on the response (Woodward, 1999). However, in most cases the overlap was not complete and it is considered that the confounding is not severe enough to negate the main statistical conclusions. The problem could have been ameliorated had a longer study period and random sampling start dates been possible. Nonetheless, confounding is difficult to avoid in epidemiological studies where (in contrast to prospective experimental studies) different variables frequently cannot be controlled separately. Temperature and season present another issue: as temperature is heavily dependent upon season in the UK, their effects cannot be considered independently. The effect of temperature was nested within season and the impacts of temperature and of season were assessed sequentially in the model. Therefore, the significance and odds ratio of temperature was unadjusted for season, whereas the effect of season was already adjusted for (i.e. in addition to) that of temperature.

When the stage of lay (or the duration of house occupation) is considered, the data show that the longest continuously occupied houses (multi-age flocks 39, 40, 41, 48, 49 and 50) typically were heavily contaminated with salmonella. The findings among the single-age flocks are more variable but there is a significant trend of an increase in the prevalence of contamination of the environment with time. The pattern of contamination appears to fluctuate more for faeces samples than for dust. As faeces are periodically removed in most systems, this difference may reflect fluctuating excretion by the hens (measured in faeces) against a background of a progressive build-up of *Salmonella* organisms in the henhouse environment (seen in dust).

When considering the effects of season, it might be expected that higher environmental temperatures in summer would increase bird stress and bacterial multiplication rates, resulting in higher levels of henhouse contamination. The statistical modelling does indeed show significant positive effects of temperature and, additionally, of season upon the detected prevalence of contamination. This is in the context of wide variation in detected contamination rates all year round. The season effect in addition to temperature may be mediated by
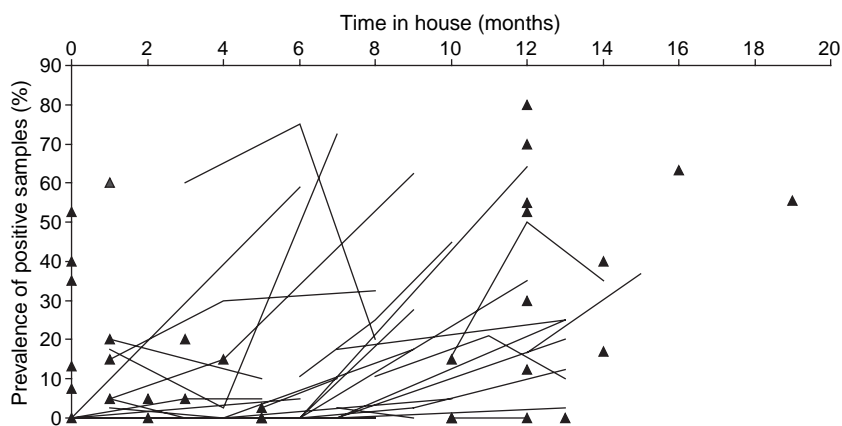

Figure 2. *Overall prevalence of salmonella-positive samples over time in 66 single-age layer flocks. Prevalence values from consecutive visits to the same flock are joined by straight lines. Values from flocks sampled only once are indicated by triangles.*
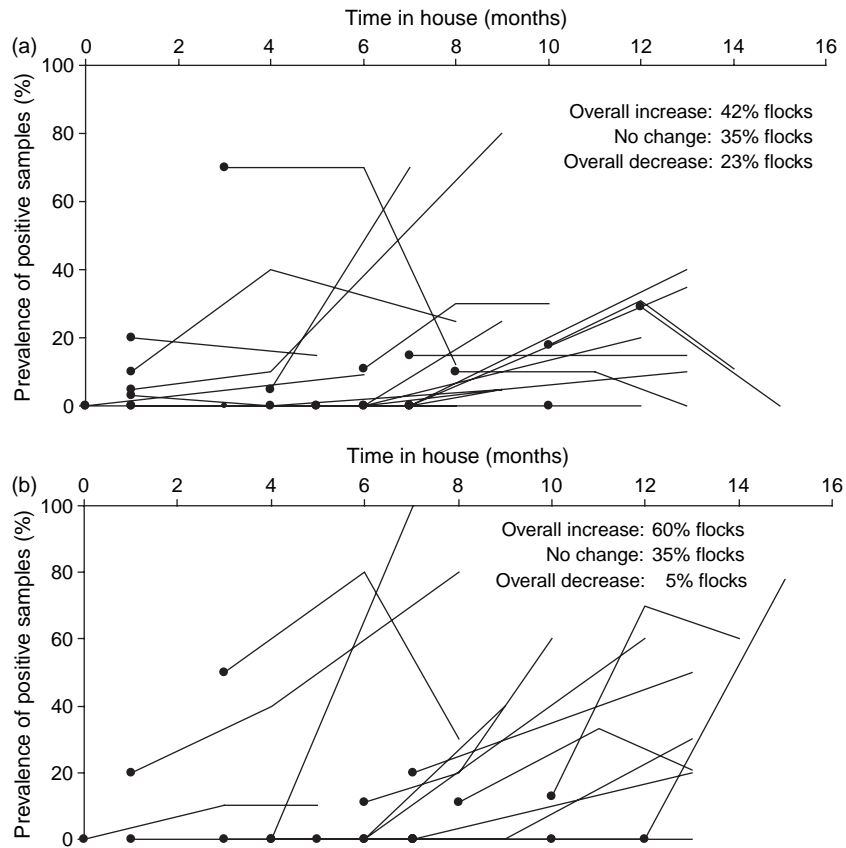
Figure 3. *Changes in the prevalence of salmonella-positive samples over time. 3a: Cage layers faeces/droppings board samples. 3b: Cage layers dust and floor samples. Prevalence values from initial visits are marked by solid circles and are connected to prevalence values from subsequent visits to the same flock by straight lines.*

factors such as an increase in vector numbers and activity. A more pronounced seasonal pattern is seen among the free-range flocks than the caged flocks in the present study (Figure 4), but there are too few of the former to draw any firm conclusions about seasonal differences between the two production systems. However, it may be that the control of bird stress and house temperatures differs significantly between the two production systems.

It is noteworthy in this context that in another UK study a seasonal effect was not seen for egg contamination (Davies & Breslin, 2004). In the British climate, well-designed and well-insulated hen houses should not be subject to excessive temperature fluctuations at any time of year, so a seasonal effect upon salmonella in eggs may be more marked in accommodation that has serious deficiencies in ventilation and insulation.

There is good evidence for the importance of wildlife vectors, especially rodents and flies, in the introduction to hen houses of salmonella and its maintenance thereafter (Davies & Breslin, 2001; Guard-Petter, 2001; Mian *et al.*, 2002; Garber *et al.*, 2003). Wildlife vectors may also spread infection between flocks, by virtue of their mobility. The observations that samples from vectors not only reflected the serotype and phage types of the corresponding flock (as noted previously by Davies & Breslin, 2003b), but were negative when the flock samples were negative and were positive at a high frequency in positive flocks, indicates the value of such samples for monitoring flock infection. Furthermore,
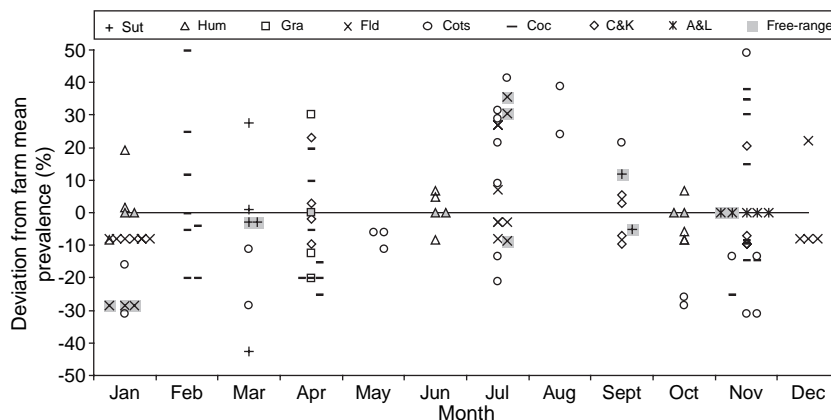

Figure 4. *Month-by-month deviation from farm/housing system averages of salmonella prevalence for all flocks.*
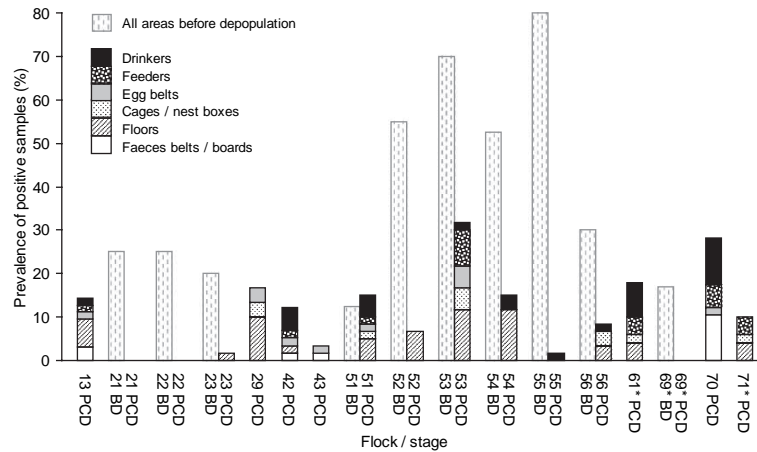
Figure 5. *Results of cleaning and disinfection in 17 hen houses. *Free-range flock. BD: Before duplication; PCD: Post cleaning and disinfection.*

they highlight the importance of the control of vectors, as the quantitative culture of rodent faeces showed that they frequently excrete high concentrations of salmonella, and they have the potential to amplify residual environmental contamination as well as to contaminate feeding and drinker systems directly. A strong association has been found between the number of mice and the detection of salmonella in layer houses (Garber *et al*., 2003), but in the limited data of the present study the association between wildlife score and prevalence was not found to be significant.

The variation in effectiveness of C&D between houses, even when in most cases similar disinfection agents were being used, suggests that other factors, such as the physical removal of organic matter and the mode of application of disinfectants, are highly significant in the eventual reductions in contamination that may be achieved. Among the farms in the current study, disinfection was poorly applied, in that products were usually used at insufficient concentration and application rates. Also, key areas such as drinkers, dropping belts and boards (which normally form the ceilings of cages) were often poorly cleaned, usually by dry cleaning only, and inconsistently sprayed with disinfectant. The use of multi-age houses that cannot effectively be cleaned and disinfected between flocks appears, on the present evidence (flocks 39, 40, 41, 48, 49 and 50; Table 2), to afford very poor control of salmonella contamination. It is interesting that, in some of the present cases, apparently good C&D was followed by high prevalences of contamination within a month or so of repopulation. It seems likely that there was significant but undetected residual contamination. The evident difficulties in eliminating salmonella from any part of empty hen houses, plus the tendency of wildlife vectors to re-introduce the organisms (Garber *et al*., 2003), implies that control measures such as vaccination and intestinal competitive exclusion will be important components of salmonella control for the foreseeable future, even with salmonella-free replacement stock. With free-range units there is an additional issue of the persistence of salmonella on paddocks even after the removal of detectable contamination in the house, as seen with flock 69 in the present study.

In conclusion, our investigation has shown a high degree of variation in the prevalence of salmonella contamination of hen houses, both between flocks on the same premises and within the same flocks over time. This has implications for monitoring programmes, when false negatives may occur. *S*. Enteritidis was predominant as the persistent serotype, and differing combinations of *S*. Enteritidis phage types proved stably persistent on various farms. The study reconfirmed the value of sampling wildlife vectors and their faeces, if present. There was a significant tendency to increased contamination with increasing flock age, but the temporal patterns of salmonella contamination in the first months of lay can be highly variable. There may be significant differences between contamination patterns over time in faeces and non-faeces samples. There were significant effects of temperature and season upon salmonella contamination. In all cases there was a clear need to improve both rodent control and C&D, and in order to make further progress the egg industry must acknowledge the additional cost in terms of baits, traps, house maintenance, disinfectants, additional down-time between flocks and the labour to achieve this when salmonella is present. It is also vitally important that sensitive monitoring is introduced for laying flocks so that additional controls can be introduced, since routine and repeated use of the measures required to eliminate salmonella from infected premises would be prohibitively costly.

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Non-English Abstracts

A longitudinal study of environmental salmonella contamination in caged and free-range layer flocks

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Etude longitudinale de la contamination environnementale par salmonella des troupeaux de pondeuses en cage et en plein air
La contamination environnementale par salmonella a été étudiée au cours d'une période de 12 mois à partir de 74 troupeaux de pondeuses, dans huit élevages au Royaume Uni, qui avaient été précédemment identifiés comme étant contaminés par salmonella. Des échantillons de fèces, poussière, litière, déchets sur les tapis à œufs, et de la faune sauvage potentiellement vectrice de salmonelles ont été prélevés, plus des chiffonnettes passées sur les cages, mangeoires, abreuvoirs, sols, tapis à œufs et bottes. Quelques échantillons ont été réalisés chaque mois de l'année. De nombreux sérovars ont été détectés mais *Salmonella enterica* sérotype Enteritidis a été le seul sérotype persistant trouvé dans les troupeaux à âge unique. Une corrélation significative a été mise en évidence entre les résultats des recherches qualitatives réalisées à partir des échantillons de l'environnement et ceux des analyses semi quantitatives à partir des échantillons de fèces. Le niveau de la contamination de l'environnement a augmenté significativement au cours du temps. Il a été noté des effets significatifs de la température et de la saison sur la contamination. La faune sauvage vecteur s'est révélée être un prélèvement sensible pour la détection des salmonelles. L'efficacité du nettoyage et de la désinfection sur la contamination salmonellique résiduelle et sur la contamination des troupeaux ultérieurs a été hautement variable entre et à l'intérieur des élevages. La variabilité entre les prévalences détectées au cours du temps et entre les troupeaux indique le besoin d'une surveillance régulière et sensible des troupeaux vis-à-vis de salmonella pour permettre le ciblage des mesures de contrôle destinées à l'élimination de la contamination salmonellique de l'environnement des pondeuses. Il y a des possibilités substantielles pour l'amélioration des procédures de nettoyage et désinfection.

Langzeitstudie zur Umgebungskontamination mit Salmonellen in Käfig- und Auslauf-Legehennenhaltungen
Über einen Zeitraum von 12 Monaten wurden in 74 kommerziellen Legehennenherden von acht Farmen in Großbritannien, in denen vorher Salmonellen nachgewiesen worden waren, die Belastung der Umgebung mit Salmonellen untersucht. Es wurden Proben von Fäzes, Staub, Einstreu, Rieselgut vom Eiertransportband und von wildlebenden Vektoren sowie Abstriche von Käfigen, Fütterungs- und Tränkeanlagen, Böden, Eiertransportbändern und Stiefeln entnommen. Einige Probeentnahmen wurden in jedem Monat des Jahres durchgeführt. Zahlreiche Salmonellenserovare wurden nachgewiesen, aber nur *Salmonella enterica* Serotyp Enteritidis war der einzige Serotyp, der in den Herden mit einer Altersgruppe persistierte. Es bestand eine signifikante Korrelation zwischen dem qualitativen Nachweis aus Umgebungsproben und dem halbqualitativen Nachweis aus Fäzesproben. Der Grad der Umgebungskontamination stieg mit der Zeit signifikant an. Außerdem ließen sich signifikante Effekte der Temperatur und der Jahreszeit auf die Kontamination feststellen. Wildlebende Vektoren erwiesen sich als sensitive Proben für den Salmonellennachweis. Die Wirksamkeit von Reinigung und Desinfektion auf die Rest-Salmonellenkontamination sowie auf die nachfolgende Herdenbelastung war hochgradig variabel zwischen und innerhalb der Bestände. Die Variabilität zwischen den ermittelten Prävalenzen über die Zeit und zwischen den Herden weist auf die Notwendigkeit von regelmäßiger, sensitiver Überprüfung von Herden auf Salmonellen hin, um eine Steuerung von Bekämpfungsmaßnahmen mit dem Ziel der Salmonelleneliminierung aus der Umgebung von Legehennen zu ermöglichen. Es gibt noch umfangreiche Möglichkeiten zur Verbesserung von Reinigungs- und Desinfektionsmaßnahmen.

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Estudio longitudinal de la contaminación ambiental por Salmonella en lotes de ponedoras alojadas en jaulas o en libertad

Se evaluó la contaminación ambiental por Salmonella durante un periodo de 12 meses en 74 lotes de ponedoras comerciales de ocho granjas de Reino Unido en las que previamente se había detectado contaminación con Salmonella. Se tomaron muestras de heces, polvo, cama, cinta de recogida de huevos y vectores salvajes, además de hisopos de jaulas, bebederos, comedereros, suelos, cinta de huevos y botas. Cada mes del año se realizó algún muestreo. Se detectaron numerosos serovares pero *Salmonella enterica* serotipo Enteritidis fue el único persistente en lotes de una sola edad. Se observó una correlación significativa entre muestras ambientales cualitativas y muestras fecales semi-cuantitativas. El nivel de contaminación ambiental aumentó significativamente en el tiempo. Se observó un efecto significativo de la temperatura y de la estación del año sobre la contaminación. Se mostró que los vectores salvajes eran muestras sensibles para la detección de salmonela. La eficacia de la limpieza y desinfección sobre la contaminación residual de salmonela y sobre la contaminación de los siguientes lotes mostró una elevada variabilidad entre naves y en las naves. La variabilidad de la prevalencia detectada en el tiempo y entre lotes indica la necesidad de una monitorización de salmonela en los lotes regular y con elevada sensibilidad que permita determinar las medidas de control para eliminar la contaminación del ambiente de las ponedoras. Existen posibilidades considerables de mejorar los procedimientos de limpieza y desinfección.