Risk factors for *Salmonella* prevalence in laying-hen farms in Japan

Y. SASAKI[1]*, M. MURAKAMI[1], N. MARUYAMA[2], Y. TSUJIYAMA[1], M. KUSUKAWA[1], T. ASAI[3] AND Y. YAMADA[1]

[1] *Food Safety and Consumer Affairs Bureau, Ministry of Agriculture, Forestry and Fisheries, Kasumigaseki, Chiyoda-ku, Tokyo, Japan*
[2] *Research Institute for Animal Science in Biochemistry and Toxicology, Hashimotodai, Midori-ku, Sagamihara, Kanagawa, Japan*
[3] *National Veterinary Assay Laboratory, Tokura, Kokubunji, Tokyo, Japan*

SUMMARY

Human salmonellosis cases, particularly those caused by *Salmonella* Enteritidis, have been closely linked to egg consumption. This epidemiological survey was conducted to determine the baseline *Salmonella* prevalence and identify the risk factors for *Salmonella* prevalence in laying-hen farms in Japan. Caecal excrement samples and dust samples were obtained from 400 flocks in 338 laying-hen farms. *Salmonella* was identified in 20·7% of the farms and 19·5% of the flocks. The prevalence of *Salmonella* was significantly higher in flocks reared in windowless houses than in those reared in open houses. In addition, the risk of *Salmonella* presence was significantly higher when the windowless house farms implemented induced moulting or in-line egg processing. Efforts to reduce human salmonellosis in Japan should continue to focus on the establishment of control measures in laying-hen farms, especially those with windowless houses implementing induced moulting and equipped with in-line egg processing.

Key words: Antibiotic resistance, enteric bacteria, *Salmonella enterica*, veterinary epidemiology and bacteriology, zoonotic foodborne diseases.

INTRODUCTION

Human salmonellosis is one of the most common foodborne illnesses worldwide. In Japan, more than 10 000 cases of foodborne salmonellosis were reported annually from 1996 to 1999 [1]. During this period, *Salmonella enterica* subsp. *enterica* serovar Enteritidis

* Author for correspondence: Y. Sasaki, DVM, Ph.D., Food Safety and Consumer Affairs Bureau, Ministry of Agriculture, Forestry and Fisheries, 1-2-1 Kasumigaseki, Chiyoda-ku, Tokyo 100-8950, Japan.
(Email: yoshimasa_sasaki@nm.maff.go.jp)

(*S*. Enteritidis) was isolated most frequently in salmonellosis cases, accounting for 58% of cases in 1996, 55% in 1997, 62% in 1998, and 46% in 1999. Because food poisoning caused by *S*. Enteritidis was closely linked to egg consumption [1], the Enforcement Regulations of the Food Sanitation Law (Law No. 23 of 1948) were amended for safe distribution of raw shell eggs and liquid egg products in 1998. The number of foodborne salmonellosis cases decreased significantly between 2000 and 2004 [2]. However, *Salmonella* remains one of the top two causative agents of bacterial food poisoning.

S. Enteritidis remains the most predominant serovar, observed in more than 30% of foodborne salmonellosis cases [3].

Eggs and eggshells can be contaminated with *Salmonella* at the farm level. *Salmonella* in eggs results from infection of the oviduct. The presence of *Salmonella* on the shells is attributable to environmental contamination secondary to bacterial shedding from infected birds. The reduction in *Salmonella* prevalence in layer chickens is, therefore, one of the most effective ways to decrease *Salmonella* contamination of eggs. The Ministry of Agriculture, Forestry and Fisheries of Japan (MAFF) issued the 'Guidelines on Control Measures on *Salmonella* in Manufacturing Feed' [4] and 'Guidelines on Integrated Control Measures on *Salmonella* in Eggs' [5] in 1998 and 2005, respectively. Concurrently, vaccines against *S.* Enteritidis only and those against both *S.* Enteritidis and *S.* Typhimurium have been commercially available since 1998 and 2004, respectively. The total number of human salmonellosis incidents was 757 in 1998, and rapidly decreased to 67 in 2009, according to the statistics of food poisoning by the Ministry of Health, Labour and Welfare [6]. Although these figures imply that the control measures introduced in recent years have effectively reduced the prevalence of *Salmonella* in laying-hen farms, corroborative evidence is lacking, because *Salmonella* prevalence in laying-hen farms had never been investigated before the present study was conducted.

The objective of this study was to determine the baseline *Salmonella* prevalence in laying-hen farms in Japan and to examine the relationship between *Salmonella* presence and farm management factors. In addition, serovars and antimicrobial resistance profiles in *Salmonella* isolates were determined for comparison with those from human salmonellosis.

METHODS

Sampling

We asked owners of laying-hen farms to participate in the present study through the Japan Poultry Association (JPA). Overall, owners of 338 laying-hen farms voluntarily participated in this survey. Each farm kept ≥1000 laying birds. The farms tested were located in 45 prefectures in Japan. Samples were collected from 400 flocks as follows. Five fresh, pooled caecal excrement samples and two dust samples were obtained from each house. Each fresh, pooled caecal

excrement sample (∼10 g) consisted of caecal excrement from several birds. Each dust sample (∼25 g) was produced by mixing the primary samples obtained from the vicinity of several ventilators. The samples were collected from a laying-hen flock at the end of the laying period (within a maximum of 2 months before depopulation) (237 flocks) and/or at the beginning of egg production after induced moulting (77 flocks). Alternatively, if none of the flocks in a farm reached these production stages during the study period, samples were collected from the oldest laying-hen flock in the farm (86 flocks). Such a flexible arrangement allowed collection of as many samples as possible from the selected farms during the study period. A flock was defined as a group of birds raised in a house during the same period of time.

Each sample was placed in sterilized plastic vials and transported to the Research Institute for Animal Science in Biochemistry and Toxicology by express delivery under refrigeration. At the laboratory, the samples were kept refrigerated until examination, which was performed within a week after their arrival.

Bacteriological examination

Ten grams of the caecal sample or 25 g of the dust sample were mixed in 10 or 100 ml buffered peptone water (BPW) (Oxoid Ltd, UK), respectively. Next, 10 ml of the suspension was incubated for 24 h at 35 °C for pre-enrichment in 40 ml BPW. After incubation, 0·1 and 1 ml of the culture was added to 10 ml Rappaport–Vassiliadis broth (Oxoid) and 15 ml Hajna tetrathionate broth (Eiken Kagaku, Japan), respectively, and incubated for 18–20 h at 42 °C. After incubation, each culture was streaked onto two selective isolation agar plates: ES *Salmonella* agar II (Eiken Kagaku) and desoxycholate hydrogen sulphide lactose agar (Eiken Kagaku) containing 20 μg/ml of novobiocin (Wako Junyaku, Japan). The remaining Hajna tetrathionate broth was cultured for 5–7 days at room temperature as delayed secondary enrichment culture and then streaked onto the two selective isolation agar plates. Candidate colonies were biochemically identified. *Salmonella* isolates were tested by slide agglutination with O antisera (Denka Seiken Co., Japan) and tube agglutination with H antisera (Denka Seiken). Serovars were determined on the basis of reaction with O- and H-group antigens according to the Kauffmann–White scheme [7]. A flock was considered positive if

Salmonella was isolated from one or more of the seven samples (five caecal samples, two dust samples) obtained.

Antimicrobial susceptibility

The minimum inhibitory concentration of various antimicrobials was determined using the agar dilution method of the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) [8]. *Enterococcus faecalis* ATCC29212, *Escherichia coli* ATCC25922, and *Staphylococcus aureus* ATCC29213 were used as quality control strains. The following antimicrobials were tested: ampicillin (ABPC), cefazolin (CEZ), ceftiofur (CTF), dihydrostreptomycin (DSM), gentamicin (GM), kanamycin (KM), apramycin (APM), oxytetracycline (OTC), bicozamycin (BCM), chloramphenicol (CP), colistin (CL), nalidixic acid (NA), and enrofloxacin (ERFX). The resistant breakpoints were adopted from those defined by CLSI [9]. The breakpoints not defined by CLSI were obtained from a previous report [10].

Statistical analysis

A questionnaire was conducted to examine the relationship between *Salmonella* prevalence and farm management factors. The risk factor analysis was performed at the farm level. If a farm had both *Salmonella*-positive and *Salmonella*-negative flocks, the farm was considered positive. A windowless house (WLH) was defined as a layer house with negative or positive pressure ventilators. During negative ventilation, fans inside the house created a negative pressure that allowed a supply of fresh air into the house from the wall. During positive pressure ventilation, fresh air was introduced by the fans. An open house (OH) had open windows for ventilation. Water supplied to the tested farms was obtained from public drinking-water systems or groundwater, either chlorinated or without any disinfection process. Public drinking water and chlorinated groundwater were defined as disinfected water for the purpose of identifying risk factors in this study.

Initially, univariate analyses were performed. If the expected value of a variable was >5, the χ^2 test was used; otherwise, Fisher's exact test was used. Variables with a *P* value <0.25 in these tests were used for multivariate logistic regression analysis. However, if a strong association ($P<0.05$) in any combination of these variables was observed, one of the two variables

of interest was selected to avoid multi-collinearity. A logistic regression model was then constructed using a stepwise approach with a backward elimination procedure until all the remaining variables were statistically significant ($P<0.05$). For this final model, two-way interactions between the remaining factors were investigated. Finally, the overall fit of the model was assessed using the Hosmer–Lemeshow goodness-of-fit test. All data analyses were performed using SPSS 17.0J (SPSS Japan Inc., Japan).

RESULTS

Salmonella prevalence and serovars

Sampling was conducted between September 2007 and March 2008. In 81·4% (275/338) of farms, samples were collected during 2 months between October and November 2007. *Salmonella* was isolated from 78 [19·5%, 95% confidence interval (CI) 15·6–23·4) of 400 flocks (Table 1). In the case of 41 flocks, both caecal and dust samples were *Salmonella* positive, whereas in the case of 14 and 23 flocks, only caecal and only dust samples, respectively, were *Salmonella* positive. The prevalence (16·0%, 64/400) of *Salmonella* based on dust samples trended higher than that (13·5%, 54/400) based on caecal samples, although the difference was not statistically significant ($P=0.32$). The prevalence of *Salmonella* was significantly ($P<0.01$) higher in flocks reared in WLHs than in those reared in OHs, regardless of the production stages of the sampled flocks.

In all, 132 isolates were obtained from 70 (20·7%, 95% CI 16·4–25·0) of 338 laying-hen farms. They were serotyped into 32 serovars and five untypable *Salmonella*. In 35 farms, only one serovar was isolated, whereas in the remaining farms, multiple serovars were isolated (2 serovars in 21 farms, 3 serovars in 7 farms, and >3 serovars in 7 farms). Of 62 farms at which samples were obtained from a flock at the end of its laying period as well as from a flock at the beginning of egg production after induced moulting, both types of samples tested positive in eight farms. In five of these eight farms, isolated serovars (two or three per farm) were identical between the two flocks in each farm. Seventeen serovars were isolated from multiple farms (Table 2). *S.* Cerro was isolated from 15 farms and was the most prevalent serovar. *S.* Enteritidis was isolated from 10 (3·0%) farms (six WLH farms and four OH farms). *S.* Typhimurium was not isolated in this study.

Table 1. *Number of* Salmonella-*positive flocks in 400 layer flocks*

	N	No. of *Salmonella*-positive flocks (%)	Mean age of flocks (weeks)
Flock in WLH	101	50 (49·5)	81·3
Flock at the end of laying period	55	25 (45·5)	86·5
Flock after induced moulting	24	12 (50·0)	74·3
Oldest flock in a farm	22	13 (59·1)	78·2
Flock in OH	299	28 (9·4)	82·0
Flock at the end of laying period	182	17 (9·3)	84·0
Flock after induced moulting	53	5 (9·4)	77·1
Oldest flock in a farm	64	6 (9·4)	79·3
Total	400	78 (19·5)	81·8

WLH, Windowless house; OH, open house.

Table 2. *Frequency distribution of isolated* Salmonella *serovars in laying-hen farms*

Serovar	No. of *Salmonella*-positive farms
S. Cerro	15
S. Braenderup	14
S. Infantis	14
S. Corvallis	10
S. Enteritidis	10
S. Mbandaka	8
S. Livingstone	6
S. Thompson	6
S. Bareilly	4
S. Oranienberg	4
S. Derby	2
S. Javiana	2
S. Montevideo	2
S. Putten	2
S. Saintpaul	2
S. Singapore	2
S. Virchow	2
Other serovars	22

Antimicrobial susceptibility

Of 132 isolates, 106 (80·3%) were sensitive to all the antimicrobials tested. Resistance to 8/13 antimicrobials tested was observed (Table 3). Only five (3·8%) isolates were multi-antimicrobial resistant. Eleven S. Enteritidis isolates were obtained from 10 farms, eight (72·7%) of which were sensitive to all the antimicrobials tested and three of which were resistant to one (BCM or NA) or two (DSM and CL) antimicrobials. Fourteen S. Infantis isolates were obtained from 14 farms, 13 (92·9%) of which were sensitive to

all the antimicrobials tested and one of which was resistant to OTC.

Identification of risk factors for *Salmonella* presence in laying-hen farms

Of 338 farms tested in the present study, 85 (25·1%) had WLHs and constituted more than half (59·4%) of all laying hens reared in all the farms tested in this study. The average number of raised birds in a farm was 116 000 (1000–2 346 000). Of the 400 flocks, the age of 370 (92·5%) flocks was >51 weeks. The average number of chicken houses in a farm was seven (range 1–48). The mean age of the flocks sampled was 81·8 weeks (range 25·7–185·7 weeks). Complete answers to the questionnaire were returned by the owners of 313 farms. The prevalence of *Salmonella* in WLH farms (44/85, 51·8%) was significantly ($P < 0·01$) higher than that in OH farms (26/253, 10·3%) [odds ratio (OR) 9·4, 95% CI 5·2–16·9]. The data derived from the questionnaire indicated that rearing and biosecurity management practices differed significantly between WLH farms and OH farms (Tables 4 and 5), with more stringent rearing and biosecurity management practices followed at the former farms. These were considered to be two distinct subpopulations with potentially different risk factors. Therefore, a separate analysis was performed for each subpopulation.

For the WLH farm subpopulation, 13 variables were used for univariate analysis (Table 4). Four variables ('number of layer houses in a farm', 'implementation of induced moulting', 'in-line egg processing', and 'use of disinfected water for drinking') were associated ($P < 0·25$) with *Salmonella* prevalence.

Table 3. *Antimicroial resistance profiles of* Salmonella *isolates*

No. of antimicrobials	Antimicrobial resistance profile	No. of isolates	Serovars
1	ABPC	2	*S.* Potsdam (1), untypable (1)
	DSM	1	*S.* Albany (1)
	OTC	4	*S.* Javiana (1), *S.* Infantis (1), *S.* Mbandaka (1), *S.* Putten (1)
	BCM	13	*S.* Schwarzengrund (1), *S.* Derby (1), *S.* Montevideo (3), *S.* Oranienberg (3), *S.* Enteritidis (1), *S.* Singapore (1), *S.* Ljubljana (1), *S.* Mbandaka (1), *S.* Gaminara (1)
	NA	1	*S.* Enteritidis (1)
2	DSM + CL	1	*S.* Enteritidis (1)
	DSM + OTC	1	*S.* Virchow (1)
	BCM + CP	1	*S.* Braenderup (1)
4	ABPC + DSM + KM + OTC	1	*S.* Muenchen (1)
5	ABPC + DSM + OTC + BCM + CL	1	*S.* Lockleaze (1)
Total		26	

ABPC, Ampicillin; DSM, dihydrostreptomycin; OTC, oxytetracycline; BCM, bicozamycin; NA, nalidixic acid; CL, colistin; CP, chloramphenicol; KM, kanamycin.

Table 4. *Results of univariate analysis of risks for* Salmonella *prevalence in WLH farms*

Variables		N	No. of Salmonella-positive farms (%)	P value	OR	95% CI
Area of Japan	East	58	29 (50·0)	0·47	0·69	0·26–1·87
	West	22	13 (59·1)			
Number of layer houses in a farm	< 6 houses	40	18 (45·0)	0·18	0·55	0·22–1·33
	⩾ 6 houses	40	24 (60·0)			
Age of sampled flock	< 80 weeks	37	18 (48·6)	0·52	0·75	0·31–1·81
	⩾ 80 weeks	43	24 (55·8)			
Implementation of induced moulting	Yes	63	38 (60·3)	< 0·01	4·94	1·45–16·8
	No	17	4 (23·5)			
Regular examination of *Salmonella*	Yes	74	39 (52·7)	0·61	1·11	0·21–5·88
	No	6	3 (50·0)			
In-line egg processing	Yes	62	39 (62·9)	< 0·01	8·48	2·21–32·5
	No	18	3 (16·7)			
Vaccination for *Salmonella*	Yes	46	25 (54·3)	0·7	1·19	0·49–2·89
	No	34	17 (50·0)			
Disinfection of vehicles before entering the farm	Yes	76	41 (53·9)	0·27	3·51	0·35–35·3
	No	4	1 (25·0)			
Use of disinfected water for drinking	Yes	53	25 (47·2)	0·18	0·53	0·20–1·36
	No	27	17 (63·0)			
Change of working clothes every day	Yes	76	40 (52·6)	0·65	1·11	0·15–8·30
	No	4	2 (50·0)			
Regular extermination of rodents	Yes	71	36 (50·7)	0·29	0·51	0·12–2·22
	No	9	6 (66·7)			
Restricted access to the farm against outsiders	Yes	79	41 (51·9)	0·53	0	
	No	1	1 (100·0)			
Ventilation	Positive pressure	26	14 (53·8)	0·53	1·08	0·42–2·77
	Negative pressure	54	28 (51·9)			

WLH, Windowless house; OR, odds ratio; CI, confidence interval.

After the stepwise procedure, two variables, 'implementation of induced moulting' and 'in-line egg processing', remained in the final multiple logistic regression model (Table 6). The OR of *Salmonella* presence was significantly higher when WLH farms implemented induced moulting (OR 5·24, 95%

Table 5. *Results of univariate analysis of risks for* Salmonella *prevalence in OH farms*

Variables		N	No. of *Salmonella*-positive farms (%)	P value	OR	95% CI
Area of Japan	East	132	9 (6·8)	0·03	0·39	0·16–0·92
	West	101	16 (15·8)			
Number of layer houses in a farm	<6 houses	107	10 (9·3)	0·53	0·76	0·33–1·78
	≥6 houses	126	15 (11·9)			
Age of sampled flock	<80 weeks	107	8 (7·5)	0·14	0·52	0·21–1·25
	≥80 weeks	126	17 (13·5)			
Implementation of induced moulting	Yes	129	15 (11·6)	0·62	1·24	0·53–2·88
	No	104	10 (9·6)			
Regular examination of *Salmonella*	Yes	174	20 (11·5)	0·52	1·40	0·50–3·92
	No	59	5 (8·5)			
In-line egg processing	Yes	76	13 (17·1)	0·03	2·49	1·08–5·77
	No	157	12 (7·6)			
Vaccination for *Salmonella*	Yes	61	9 (14·8)	0·24	1·69	0·70–4·05
	No	172	16 (9·3)			
Disinfection of vehicles before entering the farm	Yes	128	22 (17·2)	<0·01	7·06	2·05–24·3
	No	105	3 (2·9)			
Use of disinfected water for drinking	Yes	144	18 (12·5)	0·27	1·67	0·69–4·18
	No	89	7 (7·9)			
Change of working clothes every day	Yes	185	22 (11·9)	0·26	2·02	0·58–7·07
	No	48	3 (6·3)			
Regular extermination of rodents	Yes	139	17 (12·2)	0·37	1·50	0·62–3·63
	No	94	8 (8·5)			
Use of screens for wild birds	Yes	206	22 (10·7)	0·95	0·96	0·27–3·44
	No	27	3 (11·1)			
Restricted access to the farm against outsiders	Yes	186	22 (11·8)	0·28	1·97	0·56–6·88
	No	47	3 (6·4)			
On floor or free range	Yes	27	2 (7·4)	0·55	0·64	0·14–2·87
	No	206	23 (11·1)			

OH, Open house; OR, odds ratio; CI, confidence interval.

Table 6. *Results of a multivariate analysis of variables in associated with* Salmonella *prevalence in WLH farms*

Variables	B	S.E.	OR	95%	CI	P value (Wald's)
Implementation of induced moulting	1·66	0·66	5·24	1·43	19·21	0·012
In-line egg processing	2·18	0·71	8·88	2·23	35·38	0·002

WLH, Windowless house; S.E., standard error; OR, odds ratio; CI, confidence interval.
Hosmer and Lemeshow $\chi^2 = 0.313$, $P = 0.855$.

CI 1·43–19·21, $P = 0.012$) or in-line egg processing (OR 8·89, 95% CI 2·23–35·38, $P = 0.002$).

For the OH farm subpopulation, 14 variables were used for univariate analysis (Table 5). Five variables were associated ($P < 0.25$) with *Salmonella* prevalence. Among the five variables, the variable 'in-line egg processing' was significantly ($P < 0.05$) related to the two variables 'area of Japan' and 'vaccination for *Salmonella*'; the variable 'age of sampled birds' was significantly ($P < 0.05$) related to the variable 'disinfection of vehicle before entering a farm'. Three variables, 'area of Japan', 'vaccination for *Salmonella*', and 'disinfection of vehicle before entering a farm', were excluded from the multivariate logistic regression analysis, because 'in-line egg processing' was associated with *Salmonella* presence in WLH farms and 'disinfection of vehicle before entering a farm' is a general biosecurity measure that could not promote *Salmonella* prevalence. After the stepwise procedure, only one variable, 'in-line egg

processing', remained in the final multiple logistic regression model. The OR of *Salmonella* presence was significantly higher when OH farms implemented in-line egg processing (OR 2·49, 95% CI 1·08–5·77, $P = 0.03$) than when they implemented off-line egg processing.

DISCUSSION

To determine the *Salmonella* status of laying-hen flocks, both caecal and dust samples were used in the present study. Although this study suggested the prevalence of *Salmonella* based on dust samples, both caecal and dust samples will be needed to determine *Salmonella* status, because *Salmonella* might be isolated only from caecal samples, as was the case with 14/78 positive flocks in this study. Iwabuchi *et al.* [11] recently reported that *Salmonella* was isolated from 48 (23·6%) of 203 laying-hen farms in Japan between December 2004 and March 2005. Their study used only airborne dust for *Salmonella* isolation. The corresponding value in our study was 16·9% (57/338 farms). A 6·7% decrease in *Salmonella* prevalence in laying-hen farms has been observed; this deceasing trend may reflect adherence by farmers to MAFF guidelines [4, 5] and the use of *Salmonella* vaccines, although the decrease is insignificant ($P = 0.057$).

We recently isolated *S.* Enteritidis, *S.* Derby, *S.* Livingstone, and *S.* Cerro from eggshells of commercial raw shell eggs between August 2007 and January 2008 [12], around the same time as this study. These four serovars were also isolated in multiple laying-hen farms, suggesting that *Salmonella* contamination of shell eggs occurs at laying-hen farms or egg grading and packaging centres. The present study showed that various *Salmonella* serovars are sporadically found in laying-hen farms around Japan. *S.* Enteritidis, the fourth most common serovar, was found in 3·0% of laying-hen farms, whereas *S.* Typhimurium was not isolated. This profile is completely different from that in the European Union, where *S.* Enteritidis is by far the most common serovar, isolated in half of *Salmonella*-positive flocks, and *S.* Typhimurium was the third most common serovar in laying-hen flocks [13, 14]. Although there are no official statistics on the vaccination rate of *Salmonella* in laying-hen farms, vaccination was conducted in one-third (107/313) of the farms in this study. The low prevalence rates of *S.* Enteritidis and *S.* Typhimurium in laying-hen farms in Japan

might indicate the presence of a vaccination-related effect in eliminating these serovars from laying-hen farms.

This study indicates that *S.* Infantis is the third most dominant serovar in laying-hen farms in Japan. Although *S.* Infantis has been reported to be an important cause of human salmonellosis in Japan [3], it is unlikely that the *S.* Infantis present in laying-hen farms is responsible for these cases. Most *S.* Infantis isolates from laying-hen farms were only resistant to OTC in the present study. *S.* Infantis isolates from broilers in Japan [15] and human isolates (T. Asai, personal communication) were frequently resistant to OTC, DSM, KM, and trimethoprim. As previously reported by Noda *et al.* [16], pulsed-field gel electrophoresis and amplified fragment length polymorphism revealed that genotypes of *S.* Infantis isolates from humans were similar to those from chicken meat, whereas there were no common profiles between humans and chicken egg isolates. This strongly suggests that human *S.* Infantis infection in Japan is caused by the consumption of broiler meat and not by the consumption of eggs.

In Japan, rates of multi-antimicrobial resistance were lower in *Salmonella* isolates from layer chickens than in those from cattle, pigs, and broilers [17]. The present study conducted between September 2007 and March 2008 showed that the resistance rate of strains to two or more of the antimicrobials was low (3·8%), and this is consistent with the findings of other studies in Japan. Asai *et al.* [17] reported that the resistance rate of *Salmonella* isolates from layer chickens to two or more antimicrobials was 10·7% between 2000 and 2003. We recently reported that none of *Salmonella* isolates from eggshells of commercial raw shell eggs were resistant to more than two antimicrobials [11]. These data suggest that the multi-antimicrobial resistance rate of *Salmonella* in laying-hen farms remains low.

The ages of 370 (92·5%) flocks tested in this study were >51 weeks. The difference in the timing of sampling in the production cycle of laying hens did not influence the prevalence of *Salmonella*. This may have been the result of flock-to-flock spread of *Salmonella*, causing indiscriminate infection, regardless of flock age. The isolation of identical sets of serovars from two flocks in a farm, observed in the case of five farms, strongly suggests the horizontal transfer of *Salmonella* between different flocks. In addition, considering the normal rearing period of laying hens (<2 years), the age of 51 weeks would

be sufficiently advanced to detect the presence of *Salmonella* in laying-hen farms.

Half of WLH farms were found to be *Salmonella* positive. The prevalence of *S*. Enteritidis was statistically ($P = 0.02$) higher in WLH farms (7.1%) than in OH farms (1.6%). Laying hens in WLH farms constituted more than half (59.4%) of all the laying hens reared in all the farms tested in this study. These results suggest that measures against *Salmonella* in WLH farms should be prioritized to decrease *Salmonella* contamination of eggs.

The present study identified 'induced moulting' as a risk factor associated with *Salmonella* presence in WLH farms. Induced moulting is generally used to stimulate multiple laying cycles and restore egg quality. Although induced moulting is an important economic tool for the egg industry, it places undue stress on birds, suppressing the immune system and creating an environment suitable for *S*. Enteritidis growth. Holt [18] reported that moulted hens excreted greater numbers of *S*. Enteritidis in faeces in a simulated situation. Murase *et al*. [19] showed that the levels of environmental *Salmonella* increased markedly in commercial flocks after induced moulting. Although discontinuation of the practice of induced moulting might effectively reduce the prevalence of *Salmonella* in WLH farms, it may not be a feasible risk management option, because induced moulting is widely practised in large-scale layer terms [e.g. 78.8% (63/80) of WLH farms in this study] for economic reasons. Establishment of control measures to protect layer hens from *Salmonella* infection during induced moulting in WLH farms is necessary.

In-line egg processing was identified as a risk factor associated with *Salmonella* presence in both WLH and OH farms, and this suggests that egg conveyor belts are vehicles for *Salmonella*, facilitating house-to-house spread once *Salmonella* is introduced to some houses in a farm. In Japan, all-in/all-out management at the farm level is not common; thus, it is common to find flocks of different ages in a farm. Therefore, *Salmonella* maintained in older flocks might invade new flocks immediately after their introduction through egg conveyors.

The results of the questionnaire survey show that WLH farms tended to apply stricter biosecurity measures than OH farms. For example, almost all of WLH farms in this study ($>88\%$) applied biosecurity measures such as disinfection of vehicles before entering the farm, change of working clothes every day, regular extermination of rodents, and restricted access of outsiders to the farm. It is necessary to evaluate the effectiveness of these biosecurity measures in protecting laying-hen farms from the introduction of *Salmonella*, and to confirm that laying-hen farms rigorously implement these biosecurity measures. A preliminary examination of the effectiveness of rodent control programmes and vaccination against *S*. Enteritidis is provided below.

The present study did not indicate any positive impacts of the regular extermination of rodents in preventing *Salmonella* contamination in WLHs. Matsumoto *et al*. [20] reported that 22 (17.9%) of 123 roof rats captured in WLHs were *Salmonella* positive, suggesting that roof rats are one of the most important factors in *Salmonella* control in WLHs. Lapuz *et al*. [21] also indicated that roof rats are carriers of *S*. Enteritidis and *S*. Infantis, and may have played a major role in the spread and maintenance of these pathogens inside WLHs. Furthermore, Lapuz *et al*. [21] reported that roof rats infected with *S*. Enteritidis were still observed after implementation of a rodent control programme. These findings suggest that the rodent control programme conducted in this study could not exterminate rodents. We interviewed several farmers who regularly implement rodent control programmes in WLHs regarding the effectiveness of these programmes; they indicated that (1) it is very likely that rodents play an important role in the spread and maintenance of *Salmonella* in poultry houses, and (2) rodent control programmes cannot decrease the numbers of rodents, not to mention exterminate them, once rodents reside in poultry houses, and are only effective in preventing their increase.

In this study, *S*. Enteritidis-positive flocks were found in 4/46 WLH farms conducting vaccination against only *S*. Enteritidis or both *S*. Enteritidis and *S*. Typhimurium. However, this result should not be construed as the ineffectiveness of vaccination against *S*. Enteritidis in eliminating the bacterium. It should be noted that all of these farms have implemented regular examinations for *Salmonella*. It is quite likely that vaccination is conducted after *Salmonella* is detected in these farms, giving a false impression of the impacts of *Salmonella* vaccination when viewed purely in terms of the correlation between vaccination records and *Salmonella* isolation.

Because *S*. Enteritidis remains the most predominant serovar in human salmonellosis in Japan, and because eggs are suspected to be the main source of human exposure, efforts to reduce human salmonellosis in Japan should focus on the establishment of

control measures against *S.* Enteritidis in laying-hen farms. The primary focus should be on control measures to prevent the spread of *Salmonella* during induced moulting and/or through conveyer belts for in-line processing in WLH and OH farms.

Although there might be selection biases owing to voluntary participation, this study design is considered optimal for estimation of the prevalence of *Salmonella* in laying-hen farms, because at present, participation in such surveys cannot be enforced on laying-hen farmers in Japan. This study should be regarded as the first step towards the accurate estimation of the prevalence of *Salmonella* in laying-hen farms. It is very important to conduct comparable studies on a regular basis to enhance farmer participation and more accurately estimate the prevalence of *Salmonella*.

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DECLARATION OF INTEREST

None.

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