

Association between Indoor Environmental Contamination by *Salmonella enterica* and Contamination of Eggs on Layer Farms

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This study involves longitudinal and point-in-time surveys of *Salmonella* carriage and environmental contamination on two commercial cage layer farms positive for *Salmonella enterica* subsp. *enterica* serovar Typhimurium (flock A age, 32 weeks; flock B age, 34 weeks). *Salmonella*-positive fecal, egg belt, and dust samples were all unconditionally associated with eggshells testing positive for *Salmonella*. The odds of an eggshell testing positive for *Salmonella* were 91.8, 61.5, and 18.2 times higher when fecal, egg belt, and dust samples, respectively, tested positive for *Salmonella*. The agreement between the culture-based methods and real-time PCR on preenriched broths for detecting *Salmonella* was almost perfect for eggshell (observed agreement, 99.19%; kappa coefficient, 0.94) and egg belt samples (observed agreement, 95%; kappa coefficient, 0.88), and it was substantial for fecal (observed agreement, 87.14%; kappa coefficient, 0.47) and floor dust samples (observed agreement, 80.61%; kappa coefficient, 0.58). A 1-log increase in the load of *Salmonella* detected in the fecal, egg belt, and floor dust samples resulted in 35%, 43%, and 45% increases, respectively ($P < 0.001$), in the odds of an eggshell testing positive for *Salmonella*. The multilocus variable-number tandem-repeat analysis (MLVA) patterns of the *S. Typhimurium* strains isolated from flock A were distinct from those of flock B. *S. Typhimurium* strains detected from human food poisoning cases exhibited an MLVA pattern similar to those of the strains isolated from flocks A and B.

Eggs and their derived products are often linked to cases of *Salmonella* food poisoning. *Salmonella* outbreaks have been associated with uncooked products, like mayonnaise, ice cream, and cold desserts that contain raw egg (1). A very low dose of *Salmonella*, 10 to 20 CFU, is sufficient to cause human salmonellosis (2, 3). In Australia in 2010, the incidence risk of *Salmonella* infection was 53.7 cases per 100,000 people, almost 30% higher than the average risk of 41.8 cases per 100,000 people in the previous five years (1).

Salmonella enterica subsp. *enterica* serovar Enteritidis is a major concern for most egg industries around the world. Although *S. Enteritidis* is associated with the majority of egg-related outbreaks of human salmonellosis occurring in the European Union (77.2%) (4), it is not endemic to Australian layer flocks (5). Instead, *S. enterica* subsp. *enterica* serovar Typhimurium was the most frequently reported serovar in the 21 egg-related food poisoning outbreaks in Australia in 2010 (1). In Australia, a study showed that *S. enterica* subsp. *enterica* serovar Infantis was the most frequently reported serovar from the eggshell wash of eggs collected from 31 flocks (6). Furthermore, in Australia, *S. Infantis* has had the largest percentage increase in reported human infections, with 2.2 times more notifications reported nationally in 2010 than in the previous year (1).

Residual contamination of the environment with *Salmonella* is a major problem in commercial layer farms (7–9). Davies and Breslin (10) concluded that in cage systems, environmental samples, such as those from egg belts, dust near cages, and pooled accumulated feces should be tested while screening flocks for *Salmonella*. There is little information available in the literature about the risks of *Salmonella* contamination of eggs from infected birds and a contaminated shed environment. Chemaly et al. (11) investigated the prevalence of *Salmonella* on eggshells in infected layer flocks, whereas Wales et al. (12) correlated environmental contamination with fecal contamination by *Salmonella*. However,

the rate at which an infected flock can produce *Salmonella*-contaminated eggs is unclear. The possible transmission of *Salmonella* from the environment to the egg might be explained with the help of longitudinal studies (12). However, cooperation from egg producers over a period of months or years and the requirement of resources are limiting factors to such studies (12). There are a few reports in which the levels of *Salmonella* contamination in laying houses and hens were examined over time during lay (12–14). However, these studies did not investigate the degree of internal or external egg contamination. Furthermore, the focus of these studies was mainly on *S. Enteritidis*. Although *S. Typhimurium* has an established ability to be transmitted to humans via contaminated shell eggs, there is little published data on field studies, natural infections, and long-term experiments on this topic (15).

In the present study, longitudinal and point-in-time surveys were conducted on two known *S. Typhimurium*-contaminated commercial layer farms, both with multiaged flocks housed in the same shed. The primary objectives of this study were to (i) evaluate the association between *Salmonella* load in the shed environment and the probability of eggshells being contaminated with *Salmonella*, (ii) investigate the dynamics of *Salmonella* shedding of various serovars over a prolonged period of time during longi-

Received 21 March 2014 Returned for modification 27 April 2014

Accepted 17 June 2014

Published ahead of print 25 June 2014

Editor: E. Munson

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JCM.00816-14>.

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doi:10.1128/JCM.00816-14

tudinal samplings, (iii) detect *S. Typhimurium*- and *S. Infantis*-positive samples using multiplex PCR, and (iv) investigate the relatedness of various *S. Typhimurium* strains using multilocus variable-number tandem-repeat analysis (MLVA).

MATERIALS AND METHODS

This study was conducted in two stages. In stage 1, with the help of a cross-sectional study, cages infected with various serovars of *Salmonella* spp. were identified. Based on the results of the cross-sectional study, in stage 2, *Salmonella*-positive and -negative cases were selected for longitudinal study, and the association between eggs and environmental *Salmonella* contamination was investigated.

Stage 1, cross-sectional survey to select cages for longitudinal study.

Two commercial layer cage sheds were selected from two different farms with a history of *Salmonella* infection. The study sheds, shed A (from farm A) and shed B (from farm B), included multiaged flocks, with each age class housed in separate rows, and only a single age class flock was selected for sampling in each shed. In shed A, the selected flock included 1,320 cages of 32-week-old birds (5 birds per cage, for an approximate total of 6,600 birds), while the selected flock in shed B included 1,300 cages of 34-week-old birds (5 birds per cage, for an approximate total of 6,500 birds). To ensure that at least several cages positive for *Salmonella* would be identified, a representative sample size of 78 cages per flock was targeted. Accounting for field constraints, two adjacent cages were selected at equal intervals along the three lowest tiers (tiers 1, 2, and 3) out of the five tiers.

For the isolation of *Salmonella* spp., the fecal samples were inoculated in buffered peptone water (BPW) (Oxoid, Australia) (1:4). The inoculated samples were incubated at 37°C overnight, and 100 µl of this sample was transferred into Rappaport-Vassiliadis soya peptone (RVS) broth (Oxoid), which was then incubated at 42°C for 24 h. A loopful of the incubated RVS broth was streaked onto Brilliance *Salmonella* agar (BSA) (Oxoid) and xylose lysine deoxycholate agar (XLD) (Oxoid) plates. Two to three presumptive *Salmonella* colonies from BSA and XLD agars were selected and used to stab inoculate triple sugar iron (TSI) agar slopes (Oxoid). After incubation at 37°C, the inoculated TSI slopes were examined at intervals of 24 h up to 72 h for typical *Salmonella* reactions. The presumptive *Salmonella* colonies were also tested for *ortho*-nitrophenyl-β-D-galactopyranoside (Oxoid), lysine decarboxylase (LDC), and urease (Oxoid) activity. Depending upon the results of the biochemical reactions, the presumptive *Salmonella* isolates were sent for serotyping to the *Salmonella* Reference Laboratory, Adelaide, Australia.

Stage 2, longitudinal study to investigate the association between eggs and environmental *Salmonella* contamination. Based on the *Salmonella* typing results, five *Salmonella*-positive cages each from farm A (3 cages of *S. Typhimurium* phage type 9, 1 cage each of *S. Infantis* and *Salmonella enterica* subsp. *enterica* serovar Orion) and farm B (2 cages of *S. Typhimurium* phage type [PT] 9, 1 cage each of *S. Infantis*, *Salmonella enterica* subsp. *enterica* serovar Agona, and *Salmonella enterica* subsp. *enterica* serovar Oranienburg), as well as two *Salmonella*-negative cages per farm, were selected for the longitudinal study. The reason for selecting cages positive with different *Salmonella* serovars was to investigate the dynamics of *Salmonella* shedding of various serovars over a prolonged period of time during the longitudinal samplings. The selected cages were sampled at 4-week intervals. Both farms were sampled with a gap of 1 week. For each flock, 10 longitudinal samplings were performed over the period of 40 weeks (i.e., 4-week intervals).

Environmental and egg sampling. A composite fecal sample (one per study cage, $n = 7$) from each flock was collected in a sterile Whirl-Pak plastic bag (150 by 230 mm; Thermo Fisher Scientific, Australia) from underneath the individual selected cages. The full length of the manure belt under each cage was covered while collecting fecal samples. To avoid cross-contamination, disposable gloves were changed between each fecal sample collection. Egg belt samples (one per study cage, $n = 7$) were collected from the front of the cage. Whirl-Pak Speci-Sponge bags (115 by

239 mm; Thermo Fisher Scientific) were used for sample collection. The swabs were premoistened using 25 ml of BPW and dragged to cover the whole area in front of the individual cages. During each sampling period, five dust samples were collected from different parts of the poultry shed and from the floor near the selected cages for longitudinal study. Dust ($n = 5$) was collected in gamma-irradiated sterile containers (Pacific Laboratory Products, Australia). Figure 1a and b show the layout of the layer sheds, along with sample collection areas.

All the eggs at the front of the seven selected cages were collected. Each egg was collected in a separate sterile Whirl-Pak plastic bag to avoid cross-contamination. Flock A molted at the 67th week of lay; therefore, no eggs were obtained in the ninth week of sampling.

Sample processing for *Salmonella* isolation. For feces and dust, 2 g of sample was added to 8 ml of BPW. Fecal, egg belt swab, and dust samples were processed as mentioned above to isolate *Salmonella* spp. Eggshell and egg internal content samples were individually processed. Individual eggs were placed in 10 ml of sterile BPW in Whirl-Pak bags and rinsed by massaging for 2 min. Before rinsing, BPW was prewarmed to 37°C to facilitate bacterial recovery. After a rinse sample was obtained, each egg was removed and transferred to a new sterile bag. The BPW samples were incubated at 37°C overnight, and 100 µl of this sample was inoculated into RV broth (Oxoid, Australia), which was then incubated at 42°C for 24 h. The incubated RV broths were further processed for *Salmonella* isolation, as mentioned above. The egg internal contents, collected in sterile containers, were thoroughly mixed, and 2 ml of egg internal content was inoculated into 8 ml of BPW. The inoculated BPW samples were further processed for *Salmonella* isolation, as mentioned above.

Multilocus variable-number tandem-repeat analysis of *S. Typhimurium* isolates. After serotyping, all *Salmonella* strains that were identified as *S. Typhimurium* were further analyzed by MLVA, as described by Ross et al. (16), at the *Salmonella* Reference Laboratory, Adelaide, Australia.

Quantitative PCR detection of *S. enterica*. Total nucleic acid was extracted from samples using a modification (17) of a South Australian Research and Development Institute (SARDI) (Adelaide, Australia) proprietary method (18). All samples had been subjected to preenrichment in BPW, with 2 g of feces or dust samples in 8 ml BPW, egg belt swabs in 25 ml BPW, and six pooled individual eggshell washes in 10 ml BPW each. Following incubation at 37°C overnight, the incubated broths were frozen and freeze-dried. Ten milliliters of extraction buffer (17) was added to the freeze-dried samples and incubated at 70°C for an hour before proceeding with the SARDI proprietary extraction method. The quantitative PCR (qPCR) detection of *Salmonella* was done using the TaqMan *S. enterica* detection kit system (Applied Biosystems, Australia) in a total reaction volume of 20 µl containing an 8-µl sample, 10 µl of 2× environmental master mix, and 2 µl of 10× target assay mix. All real-time PCR assays were run in a 384-well format, with master mix and template being dispensed using a Biomek 3000 laboratory automation workstation (Beckman Coulter, USA). All reactions were run on a 7900HT sequence detection system (Applied Biosystems), with the following conditions: 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 60 s. All data were analyzed using the 7900HT v2.3 SDS software (Applied Biosystems). The raw data were analyzed for target-specific *S. enterica* and internal positive control (IPC) using a cycle threshold (C_T) of 0.8 and baseline of 3 to 10. *Salmonella* copies were calculated using a standard curve prepared by a serial 10-fold dilution of cultured *S. enterica* serovar Infantis. A cutoff C_T of 34 was used to exclude the detection of false positives. A C_T of 34 corresponded to 200 CFU of *Salmonella*.

Multiplex PCR to identify *S. Typhimurium*- and *S. Infantis*-positive samples. Two multiplex PCRs, one for the detection of *S. Typhimurium* and the other for *S. Infantis*, were performed using the primers published by Akiba et al. (19). The primer sequences and the expected amplification product sizes are shown in Table S1 in the supplemental material. The multiplex PCR assays were used on various *Salmonella* serovars isolated from the Australian layer industry to confirm the specificities of the assays (see Table S2 in the supplemental material). For the *S. Typhimurium*

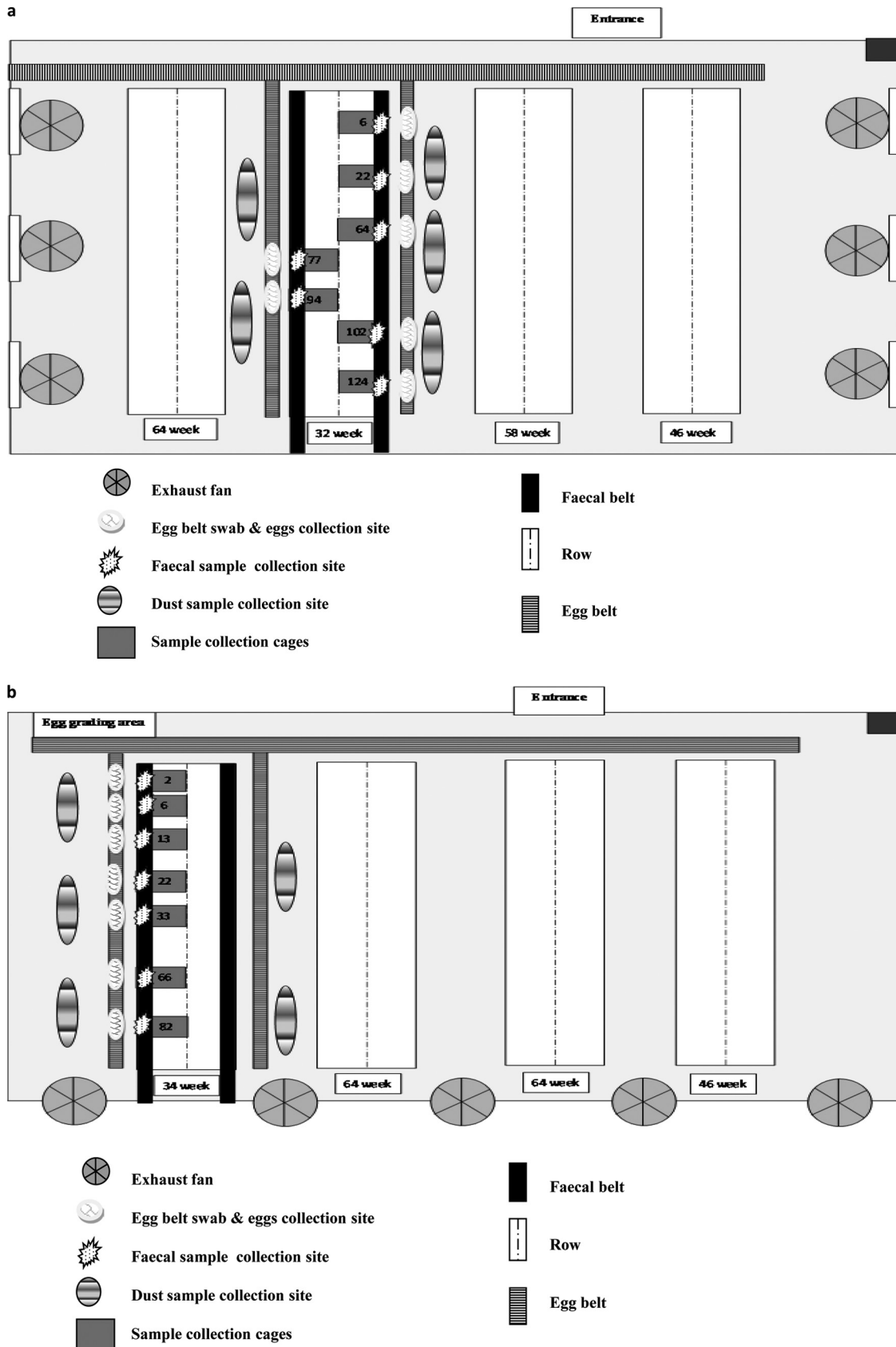


FIG 1 Layouts of the shed with flock A (A) and flock B (B) showing the areas of sample collection (with number of samples in the collection area).

TABLE 1 Prevalence of *Salmonella* in flock A during longitudinal sampling

Collection no.	Week of lay	No. of positive isolates/total no. of indicated sample type				Sample type(s) and <i>Salmonella</i> serovar(s) (no. of isolates) ^a
		Eggshells	Feces	Egg belt	Dust	
1	36	0/33	0/7	0/7	1/5	Dust: <i>S. Typhimurium</i> PT 9
2	40	0/33	0/7	1/7	1/5	Egg belt and dust: <i>S. Worthington</i>
3	44	0/30	1/7	0/7	3/5	Feces and dust: <i>S. Typhimurium</i> PT 9
4	48	0/30	0/7	0/7	0/5	
5	52	0/30	0/7	0/7	1/5	Dust: <i>S. Typhimurium</i> PT 9
6	56	0/30	0/7	0/7	1/5	Dust: <i>S. Typhimurium</i> PT 9
7	60	0/32	1/7	1/7	1/5	Feces: <i>S. Worthington</i> ; egg belt: <i>Salmonella</i> subsp. 1 serotype 4,5,12; dust: <i>S. Typhimurium</i> PT 9
8	64	0/28	1/7	1/7	0/5	Feces: <i>S. Worthington</i> ; egg belt: <i>Salmonella</i> subsp. 1 serotype 4,5,12:-:-
9	68		2/7	1/7	2/5	Feces and egg belt: <i>S. Worthington</i> ; dust: <i>S. Worthington</i> (1), <i>S. Typhimurium</i> PT 9 (1)
10	72	1/10	0/7	1/7	3/5	Egg: <i>S. Worthington</i> ; egg belt: <i>S. Typhimurium</i> PT 9; dust: <i>S. Typhimurium</i> PT 9 (2), <i>S. Worthington</i>

^a PT, phage type.

multiplex PCR, each reaction mixture contained 1× PCR buffer II (Applied Biosystems, Australia), 2.5 mM MgCl₂, 1.6 mM deoxynucleoside triphosphates (dNTPs) (Invitrogen, Australia), 0.5 μM (each) InvAF, InvAR, TMP2F, and TMP2R primers, 0.3 μM (each) TMP1F, TMP1R, TMP3F, and TMP3R primers, 1 U of AmpliTaq DNA polymerase LD (Applied Biosystems), and 5 μl DNA template in a reaction volume of 20 μl. For the *S. Infantis* multiplex PCR, each reaction mixture contained 1× PCR buffer II (Applied Biosystems), 2.5 mM MgCl₂, 1.6 mM dNTPs, 0.5 μM (each) InvAF, InvAR, IMP3F, and IMP3R primers, 0.3 μM (each) IMP1F, IMP1R, IMP2F, and IMP2R primers, 1 U of AmpliTaq DNA polymerase LD (Applied Biosystems), and 5 μl DNA template in a reaction volume of 20 μl. The samples were amplified in a MJ Research PTC-225 Peltier thermal cycler (GeneWorks, Adelaide, Australia), with an initial denaturation step at 95°C for 2 min, followed by 35 cycles of amplification (denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s), and a final extension step at 72°C for 10 min. The PCR products were separated by 2% agarose gel electrophoresis in Tris-acetate-EDTA (TAE) buffer, stained with GelRed (Jomar Diagnostics, Australia), and visualized under ultraviolet light.

In order to determine the limit of detection for each multiplex PCR assay, fecal samples were spiked with various concentrations (10⁸ CFU/ml to 10² CFU/ml) of the corresponding *Salmonella* serovar (*S. Typhimurium* or *S. Infantis*). DNA was extracted from *Salmonella*-spiked fecal samples, and the multiplex PCRs were performed as mentioned above. The limit of detection was determined by running the PCR products on a 2% agarose gel.

Statistical analysis. Binomial exact confidence intervals were computed for the prevalence of *Salmonella*-positive cages estimated in each flock. Multilevel logistic regression was used to estimate the association between an eggshell being *Salmonella* positive and *Salmonella*-positive feces from the cage that held the egg, a *Salmonella*-positive egg belt at the front of a cage, and *Salmonella*-positive floor dust at the front of a cage. Random effects for “flock” and for “cage within flock” were added to the model to account for the fact that eggs were clustered within the cage and within the flock. Multilevel logistic regression was also used to evaluate the association among the *Salmonella* test outcomes of the corresponding cage fecal, egg belts, and floor dust samples (i.e., only included “flock” as random effect). Kappa statistics were computed to assess the agreement between culture isolation and real-time PCR. The association between the *Salmonella* burden (using log-transformed C_T values from qPCR) in the fecal, egg belt, and floor dust samples and the odds of an eggshell testing positive for *Salmonella* was investigated using the same structure for multilevel logistic regressions. The parameters of all models (odds ratio) were interpreted at a 5% significance level. The models assumptions were as-

sessed using standard diagnostic plots. Statistical analyses were performed using the statistical package Stata v12.1 (20).

RESULTS

Selection of cages for longitudinal study. The culture isolation results indicated that in flock A, 21 cages (26.9%; confidence interval [CI], 17.5 to 38.2) were positive for *Salmonella* spp. at 32 weeks. Flock B had a higher prevalence of *Salmonella*-positive cages, with 31 cages (39.7%; CI, 28.8 to 51.5) reported to be positive for *Salmonella* at 34 weeks. Based on the *Salmonella* typing results, five *Salmonella*-positive cages each from farm A (3 cages for *S. Typhimurium* phage type 9 and 1 cage each for *S. Infantis* and *S. Orion*) and farm B (2 cages for *S. Typhimurium* PT 9, and 1 cage each for *S. Infantis*, *S. Agona*, and *S. Oranienburg*), as well as two *Salmonella*-negative cages per farm were selected for the longitudinal study.

***Salmonella* prevalence in flocks A and B in longitudinal study.** The details of the number of samples that were reported as *Salmonella* positive, along with types of serovars identified over the period of 40 weeks from flocks A and B, are described in Tables 1 and 2, respectively. Figure 2 shows the prevalence of *Salmonella* in different types of samples. In both flocks, the *Salmonella* prevalence was higher in dust samples than in egg belt, fecal, and eggshell samples. In flock B, from the 6th sampling (58 week onwards), there was an increase in the prevalence of *Salmonella* in all types of samples, with the highest prevalence in dust samples. It was observed that there was a higher fluctuation in *Salmonella* contamination in fecal samples than in the dust and egg belt samples. Out of all eggs tested, in flock B, 7.17% (19/265) of the eggshells were *Salmonella* positive; however, in flock A, only one eggshell out of 256 (0.39%) was reported as *Salmonella* positive. All of the egg internal contents from flocks A and B were *Salmonella* negative.

Serotyping results confirmed that *S. Oranienburg* was the most frequently (76.92%) reported *S. enterica* subsp. *enterica* serovar, followed by *S. Typhimurium* PT 9 (11.54%), *S. Worthington* (8.46%), *S. Agona* (3.08%), *Salmonella* subsp. 1 serotype 4,5,12:-:- (1.54%), and *Salmonella* subsp. 1 serotype rough g,s,t:- (0.77%). Table 3 provides the percentages of various *Salmonella* serovars isolated from different types of samples. The results of MLVA

TABLE 2 Prevalence of *Salmonella* in flock B during longitudinal sampling

Collection no.	Week of lay	No. of positive isolates/total no. of indicated sample type:				Sample type(s) and <i>Salmonella</i> serovar(s) (no. of isolates) ^a
		Eggshells	Feces	Egg belt	Dust	
1	38	0/31	1/7	0/7	1/5	Feces and dust: <i>S. Oranienburg</i>
2	42	0/36	0/7	0/7	1/5	Dust: <i>S. Oranienburg</i>
3	46	0/39	0/7	0/7	1/5	Dust: <i>S. Agona</i>
4	50	0/27	0/7	2/7	2/5	Egg belt: <i>S. Oranienburg</i> (1), <i>S. Agona</i> (1); dust: <i>S. Oranienburg</i> (1), <i>S. Agona</i> (1)
5	54	0/27	2/7	3/7	3/5	Feces, dust, and egg belt: <i>S. Oranienburg</i>
6	58	1/30	5/7	5/7	1/5	Feces, dust, egg belt, and eggshells: <i>S. Oranienburg</i>
7	62	10/34	5/7	6/7	5/5	Feces, dust, and egg belt: <i>S. Oranienburg</i> ; eggshells: <i>S. Oranienburg</i> (10), <i>S. Typhimurium</i> PT 9 (1)
8	66	8/33	6/7	7/7	5/5	Eggshells, feces, and egg belt: <i>S. Oranienburg</i> ; dust: <i>S. Oranienburg</i> (5), <i>S. Agona</i> (1)
9	70	0/1	2/7	6/7	5/5	Feces, dust, and egg belt: <i>S. Oranienburg</i>
10	74	0/4	2/7	6/7	5/5	Feces: <i>S. Oranienburg</i> (1), <i>S. Typhimurium</i> PT 9 (1); egg belt: <i>S. Oranienburg</i> (5), <i>S. Typhimurium</i> PT 9 (2); dust: <i>S. Oranienburg</i> (5), <i>Salmonella</i> subsp. 1 serovar rough: g,s,t:-

^a PT, phage type.

indicated that *S. Typhimurium* strains isolated from flocks A and B were genetically distinct. In flock B, all the *S. Typhimurium* isolates possessed the same MLVA pattern (03 15 07 11 550). On the other hand, the *S. Typhimurium* strains isolated from flock A exhibited three different MLVA patterns (03 24 11 10 523, 03 24 11 11 523, and 03 24 11 12 523).

Relationship between the environmental contamination of *Salmonella* with *Salmonella*-positive eggshells. *Salmonella*-positive fecal, egg belt, and dust samples were all unconditionally (analysis did not account for other factors) associated with eggshells testing positive for *Salmonella*. The odds of an eggshell testing positive for *Salmonella* were 91.8 times higher when the fecal sample from the cage tested positive for *Salmonella* (odds ratio, 91.8; $P < 0.001$; CI, 11.2 to 749.7). The odds of an eggshell testing positive for *Salmonella* were 61.5 times higher when the corresponding section of the egg belt tested positive for *Salmonella* (odds ratio, 61.5; $P < 0.001$; CI, 7.65 to 494.8). The odds of an eggshell testing positive for *Salmonella* were 18.2 (odds ratio, 18.2; $P < 0.001$; CI, 3.93 to 84.2) times higher when the corresponding floor dust sample tested positive for *Salmonella*. In the final multifactorial model (designed to study the possible environment/

bird/egg transmission of *Salmonella*), the fecal and dust sample results were conditionally (analysis accounted for other factors) associated with an eggshell testing positive for *Salmonella*. The odds of an eggshell testing positive for *Salmonella* were 58.9 times higher when the fecal sample from the cage tested positive for *Salmonella* (odds ratio, 58.9; $P < 0.001$; CI, 6.9 to 501.0) and 9.2 times higher when the corresponding floor dust tested positive for *Salmonella* (odds ratio, 9.2; $P = 0.007$; CI, 1.8 to 45.8).

Quantification of *Salmonella* load in environmental samples using qPCR and its relationship with *Salmonella* eggshell contamination. The TaqMan *S. enterica* detection system does not provide quantification of positive samples. Therefore, to determine the limit of detection of the assay, a standard curve prepared from a known concentration of *S. Infantis* (2×10^6 to 2×10^0 CFU *Salmonella* organisms per qPCR) was used. The standard curve produced a slope of -3.2 , a y intercept of 41, and R^2 of 0.99. Despite the good PCR assay efficiency (105%), confident detection was not possible at <200 CFU per qPCR or 25 CFU/ μ l extracted nucleic acid template. When a cutoff C_T of 34 was used (CFU, >200 per PCR), qPCR identified 87 *Salmonella*-positive samples, of which 7 were not detected by the culture-based

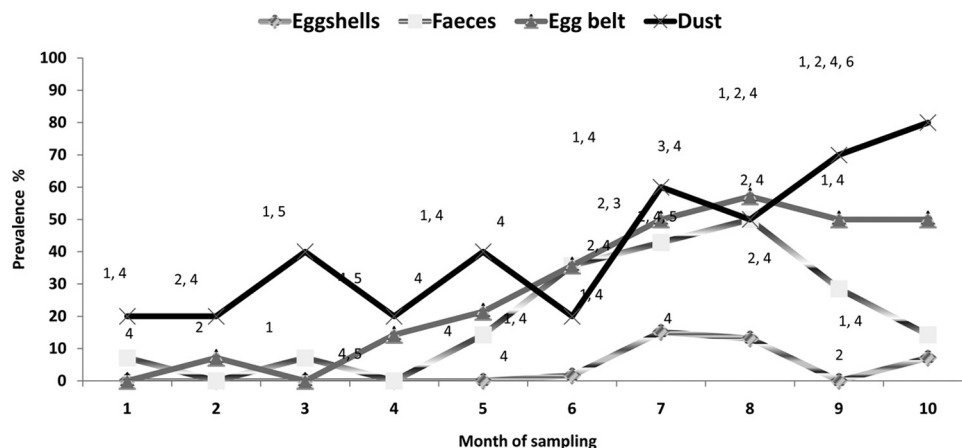


FIG 2 Percent prevalence of *Salmonella* in different types of samples over a period of 10 months. 1, *Salmonella* Typhimurium PT 9; 2, *Salmonella* Worthington; 3, *Salmonella* subsp. 1 serotype 4,5,12:-:-; 4, *Salmonella* Oranienburg; 5, *Salmonella* Agona; 6, *Salmonella* subsp. 1 serotype rough g,s,t:-.

TABLE 3 Serovars detected in various *Salmonella*-positive sample types from layer farms

<i>Salmonella</i> serovar ^a	Frequency (% [no. positive/total no.]) for indicated sample type				
	Feces	Egg belt	Dust	Eggshells	Total
<i>S. Typhimurium</i> PT 9	7.14 (2/28)	7.50 (3/40)	21.43 (9/42)	5 (1/20)	11.54 (15/130)
<i>S. Oranienburg</i>	78.57 (22/28)	82.5 (33/40)	64.28 (27/42)	90 (18/20)	76.92 (100/130)
<i>S. Worthington</i>	14.28 (4/28)	5 (2/40)	9.52 (4/42)	5 (1/20)	8.46 (11/130)
<i>S. Agona</i>	0 (0/28)	2.5 (1/40)	7.14 (3/42)	0 (0/20)	3.08 (4/130)
<i>Salmonella</i> subsp. 1 Serotype 4,5,12:-:-	0 (0/28)	5 (2/40)	0 (0/42)	0 (0/20)	1.54 (2/130)
<i>Salmonella</i> subsp. 1 serotype rough: g,s,t:-	0 (0/28)	0 (0/40)	2.38 (1/42)	0 (0/20)	0.77 (1/130)

^a PT, phage type.

method. qPCR failed to detect *Salmonella* in 38 samples from which *Salmonella* was cultured. The qPCR resulted in 68% (80/118) of the samples being identified as containing *Salmonella* by microbiological culturing also testing positive by qPCR (see Table S3 in the supplemental material).

Table 4 provides the details of the *Salmonella*-positive and -negative samples detected by culture-based analysis and qPCR. Agreement between culture-based methodology and qPCR in detecting *Salmonella* was almost perfect for eggshell (observed agreement, 99.19%; kappa coefficient, 0.94) and egg belt samples (observed agreement, 95%; kappa coefficient, 0.88), and it was substantial for fecal (observed agreement, 87.14%; kappa coefficient, 0.47) and floor dust samples (observed agreement, 80.61%; kappa coefficient, 0.58). The overall (in all samples) agreement between the culture-based and qPCR detections of *Salmonella* was good (observed agreement, 91.02%; kappa coefficient, 0.73).

Using the qPCR standard curve, the load of *Salmonella* in fecal, egg belt, eggshell, and dust samples was determined. Figure 3 shows the load of *Salmonella* (average log CFU per PCR) in feces, egg belt, dust, and eggshells. The results indicated that the levels of *Salmonella* detected in the fecal, egg belt, and floor dust samples were unconditionally associated with an eggshell testing positive

for *Salmonella*. A 1-log increase in the load of *Salmonella* detected in fecal samples resulted in a 35% increase (odds ratio, 1.35; $P < 0.001$) in the odds of an eggshell testing positive for *Salmonella*. Similarly, a 1-log increase in the load of *Salmonella* detected on the egg belt and floor dust samples resulted in 43% (odds ratio, 1.43; $P < 0.001$) and 45% (odds ratio, 1.45; $P < 0.001$) increases, respectively, in the odds of an eggshell testing positive for *Salmonella*. When averaging *Salmonella* environmental burden across the fecal, egg belt, and dust samples, a 1-log increase in environmental *Salmonella* burden resulted in a 51% (odds ratio, 1.51; $P < 0.001$) increase in the odds of an eggshell testing positive for *Salmonella*. In the final multifactorial model (not considering combined environment burden), only the *Salmonella* burden detected in the egg belt appeared to be conditionally associated with an eggshell testing positive for *Salmonella* (odds ratio, 1.43, $P < 0.001$).

Multiplex PCR to detect *S. Typhimurium*- and *S. Infantis*-positive samples. The multiplex PCR, specific for *S. Typhimurium* and *S. Infantis*, was used to test various *Salmonella* serovars isolated from Australian layer farms (see Table S2 in the supplemental material). All tested *Salmonella* serovars were amplified by the InvA primers, which identify *Salmonella* spp. All three primer pairs specific to either *S. Typhimurium* or *S. Infantis* were able to correctly detect the respective serovar. The primers specific for *S. Typhimurium* or *S. Infantis* did not produce specific PCR amplification with other *Salmonella* serovars. However, there was an exception for *Salmonella* subsp. 1 serotype 4,5,12:-:-, which produced PCR amplification patterns similar to those of *S. Typhimurium*. In addition to the *Salmonella*-specific InvA amplicon, the following *S. enterica* subsp. *enterica* serovars also produced an additional single amplicon with one of the TSR or ISR primer pairs: *S. Agona*, *S. Adelaide*, *S. Havana*, *S. Kiambu*, *S. Livingstone*, *S. Mbandaka*, and *S. Ohio* (see Table S2). To determine the limit of detection of multiplex PCR, the fecal samples were spiked with a known concentration of *Salmonella*. The results indicated that the limit of detection by multiplex PCR was either 2,000 CFU/PCR or 400 CFU/ μ l extracted nucleic acid template.

The samples that were *Salmonella* positive by qPCR ($n = 87$) were all analyzed by *S. Typhimurium* and *S. Infantis* multiplex PCR. Multiplex PCR identified six potential *S. Typhimurium*- and no *S. Infantis*-positive samples. The latter result is in agreement with the serotyping results. Of the six samples identified as potentially *S. Typhimurium* positive by multiplex PCR, only one sample had *S. Typhimurium* (in addition to *S. Oranienburg*) isolated by microbial culturing. The other five samples identified as *S. Typhimurium* by multiplex PCR had either *S. Worthington* ($n = 1$) or *S. Oranienburg* ($n = 3$) isolated by culturing. These multiplex-pos-

TABLE 4 Agreement between culture method and real-time PCR to detect *Salmonella*-positive and -negative samples

Sample type	Sample identification by culture	No. of samples identified by real-time PCR to be:		Total no. of samples	Observed agreement (%)	Kappa coefficient
		Positive	Negative			
Eggshells	Positive	8	1	9	99.14	0.94
	Negative	0	114	114		
	Total	8	115	123		
Feces	Positive	10	18	28	87.14	0.47
	Negative	0	112	112		
	Total	10	130	140		
Egg belt	Positive	37	3	40	95	0.87
	Negative	4	96	100		
	Total	41	99	140		
Dust	Positive	25	16	41	80.61	0.58
	Negative	3	54	57		
	Total	28	70	98		
All types	Positive	80	38	118	91.02	0.73
	Negative	7	376	383		
	Total	87	414	501		

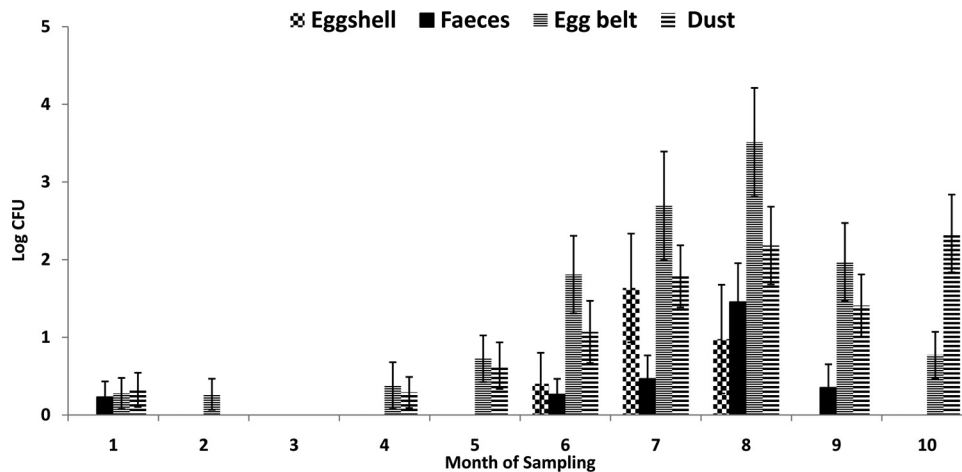


FIG 3 The load of *Salmonella* (average log CFU/qPCR) in feces, egg belt, dust, and eggshell samples over a period of 10 months. Error bars indicate standard errors.

itive samples all contained *Salmonella* at levels of >10,000 CFU/qPCR or 1,250 CFU/ μ l nucleic acid template. None of the four qPCR-positive samples that had *S. Typhimurium* isolated by culturing tested positive by the *S. Typhimurium* multiplex PCR assay.

DISCUSSION

The current study involved longitudinal and point-in-time surveys of *Salmonella* carriage and environmental contamination on two commercial cage layer farms. The initial prevalences of *Salmonella* (based on fecal sampling, $n = 78$) in flocks A and B were 26.9% and 39.7%, respectively.

In the longitudinal study of 40 weeks, the highest prevalence of *Salmonella* was detected in dust samples (42%), followed by in egg belt (28%), fecal (20%), and eggshell (4%) samples. The high prevalence of *Salmonella* in dust may result in an airborne spread of infection in the layer flock within the shed. It has been observed that *S. Typhimurium* is capable of surviving in aerosol form for long periods of time (21). A low dose of *S. Typhimurium* DT 104 infection (2×10^2 or 2×10^4 CFU per bird) resulted in increased *Salmonella* contamination of eggs (22). In the present study, the qPCR results indicated that the level of *Salmonella* in dust samples peaked up to 5 log CFU per qPCR, which may have resulted in the lateral spread of *Salmonella* in the flock. Hence, the presence of *Salmonella* in dust is a risk factor for the spread of infection in layer flocks.

Of the 140 fecal samples tested, 20% were reported as *Salmonella* positive. It was observed that there was higher variation in *Salmonella* contamination of feces than in that of the dust samples. This may be due to the increased frequency of the removal of feces from the systems compared to that of the dust (12). Fecal samples are believed to be better indicators of the infection status of flocks, whereas dust samples are more likely to indicate previous infection status (23).

The prevalence of *Salmonella* in both flocks A and B increased during the later stages of lay. There is no information available in the literature that clearly indicates the relationship between the stage of lay and degree of *Salmonella* shedding. In flock B, following 58 weeks of age, there was a substantial increase in the prevalence of *Salmonella* in all types of samples. During this period, a

new flock was introduced into the same shed. This new flock was housed adjacent to the flock that was sampled in the current study. There is a possibility that the introduction of a new batch of birds into the same shed may have stressed the birds under investigation, resulting in increased shedding of *Salmonella*. However, further studies are essential to confirm these observational findings.

Flock A molted at the 67th week of lay. In the following week (week 68), it was observed that the shedding of *Salmonella* in feces increased and, subsequently, dust contamination also increased. As a result of molting, no eggs were obtained for *Salmonella* isolation in the 68th week. However, in the 72nd week, one eggshell was reported as *Salmonella* positive. Molting along with immunosuppression can alter gut microbiota and physiology, and these changes may influence the host-pathogen relationship (24). Holt (25) reported that induced molting resulted in higher shedding of *Salmonella* Enteritidis in feces and increased colonization of internal organs. The higher numbers of *S. Enteritidis*-positive eggs were produced within the first 5 weeks after molting (25).

Out of all eggs tested, 4% (20/521) of the eggshells were reported to be *Salmonella* positive. The serovars that were detected on the eggshells were the same as those detected from the farm environmental samples. However, all the egg internal contents were *Salmonella* negative. These findings are in agreement with our previous survey (6), in which all egg internal contents were *Salmonella* negative. The egg penetration experiment indicated that *S. Worthington* has a capacity to penetrate the eggshell but lacks the ability to survive in the egg internal contents (V. C. Gole, unpublished data), whereas *S. Typhimurium* has a capacity to penetrate and survive in egg internal contents at 20°C (26). In the present experiment, even though chickens were positive for *S. Typhimurium*, the egg internal contents were *Salmonella* negative. There is a lack of reliable information regarding the ability of *S. Typhimurium* to transmit vertically.

In the current study, of 20 *Salmonella*-positive eggshell samples, 18 were positive for *S. Oranienburg*, whereas one sample was positive for *S. Worthington* and another positive for *S. Typhimurium*. In Australia, egg-associated *S. Oranienburg* outbreaks have not been reported so far. However, in Germany, a large chocolate-related outbreak of this serovar was reported in 2005 (27).

Salmonella-positive fecal, egg belt, and dust samples were all unconditionally associated with eggshells testing positive for *Salmonella*. The odds of an eggshell testing positive for *Salmonella* were 91.8, 61.5, and 18.2 times higher when fecal, egg belt, and dust samples tested *Salmonella* positive. This clearly suggests that fecal contamination with *Salmonella* is the most important factor for the production of *Salmonella*-positive eggshells. On other hand, the qPCR results suggest that not only the qualitative score (positive or negative) but also the quantitative level of *Salmonella* in environmental samples is very important in order to have *Salmonella*-positive eggshells. A 1-log increase in the load of *Salmonella* detected in fecal, egg belt, and dust samples resulted in 35%, 43%, and 45% increases, respectively, in the odds of an eggshell testing positive for *Salmonella*. These findings are very important for developing management strategies for reducing the incidences of *Salmonella*-positive eggshells by decreasing the level of *Salmonella* in the environment of layer shed.

The prevalence with which *Salmonella* was detected in samples using qPCR was lower than traditional microbiological culturing, with 68% of known *Salmonella* positives being identified. Furthermore, the sample type also influenced the variation in the agreement between the culture- and qPCR-based detection methods. An almost-perfect agreement was reported between the two methods for identifying *Salmonella*-positive eggshell and egg belt samples, with only a moderate agreement observed when fecal and dust samples were investigated. The microbiological culture-based method involved the preenrichment of samples in BPW, followed by selective enrichment in RVS, while qPCR analysis was done on the preenriched samples only. This might explain the lower probability of detection by qPCR, especially if samples were contaminated with only low levels of *Salmonella*, as has been observed by others (28). Despite the traditional microbiological culture-based methods being more sensitive due to selection, the qPCR results did indicate that the probability of eggshell contamination was significantly increased with as little as a 10-fold increase in *Salmonella* levels within the shed environment. Therefore, qPCR does have potential as an initial rapid and high-throughput screening tool for identifying *Salmonella* in the environment.

In the present study, MLVA was used to investigate the relatedness of the different *S. Typhimurium* strains isolated from the two study flocks. As per the Australian coding system, strains isolated from flock A were distinct from and unrelated to the strains isolated from flock B. All the *S. Typhimurium* strains from flock B exhibited no allelic variation. In contrast to this, there was allelic variation in the strains isolated from flock A. However, as per the Australian MLVA coding system (29), this variation was not significant to consider them unrelated or distinct *S. Typhimurium* isolates. A quarterly report released from Institute of Medical and Veterinary Science (IMVS), Adelaide, Australia, indicated that *S. Typhimurium* strains responsible for human food poisoning cases exhibited similar MLVA patterns to those of the strains isolated from flocks A and B (30). Serotyping confirmed that *S. Oranienburg* was the most frequently reported serovar, followed by *S. Typhimurium* PT 9, *S. Worthington*, *S. Agona*, *Salmonella* subsp. 1 serotype 4, 5, 12:-:-, and *Salmonella* subsp. 1 serotype rough g,s,t:-.

Multiplex PCR assays were used to identify *S. Typhimurium* and *S. Infantis* from samples that had been identified as *S. enterica* positive by qPCR. All the samples were negative for *S. Infantis*,

which is in agreement with the microbiological culture-based results. Of the qPCR-positive samples further analyzed by *S. Typhimurium* multiplex PCR, six potential *S. Typhimurium* positives were identified. Of these, only one sample had a positive *S. Typhimurium* strain isolated by culturing, while the others were positive for *S. Oranienburg* or *S. Worthington*. Four samples known to be positive for *S. Typhimurium* by culturing did not test positive by multiplex PCR. These results may be an indication of a mixed *Salmonella* infection within the sample and microbiological characterization based on a limited number of presumptive *Salmonella*-positive colonies. The limit of detection for multiplex PCR was 2,000 CFU/reaction, which may account for why no positive samples identified by culturing tested positive by multiplex PCR. This clearly suggests that culture-based methods are more sensitive and specific than multiplex PCR assays in characterizing *S. Typhimurium*. These findings are in agreement with previous experiment of Soumet et al. (31). They reported a poor sensitivity (10^7 CFU/ml) of multiplex PCR for the samples (obtained from poultry houses) preenriched in BPW and directly tested by multiplex PCR. Furthermore, when testing pure isolates of a range of *Salmonella* organisms, it was noted that *Salmonella* subsp. 1 serotype 4,5,12:-:- was amplified by all three *S. Typhimurium* primer pairs in multiplex PCR and hence was indistinguishable from *S. Typhimurium*. There is no previous information on the expected results for *Salmonella* subsp. 1 serotype 4,5,12:-:- using the multiplex PCR system described by Akiba et al. (19). In France, genomic analysis revealed that a nonmotile strain of *S. enterica* subsp. *enterica* with the antigenic formula 4,5,12:-:- is a nonmotile variant of *S. Typhimurium* and is responsible for egg-related food poisoning outbreaks (32). Within Australia, the present study is the first report of a nonmotile variant of *S. Typhimurium* in laying flocks. However, further genomic analysis is essential to reveal the similarity of this nonmotile variant with *S. Typhimurium*. In the future, such atypical *Salmonella* variants may emerge as a new challenge for the Australian layer industry.

In conclusion, the *Salmonella*-positive samples from feces, egg belt, and dust were significant predictors of eggshell contamination. A 1-log CFU increase in the level of *Salmonella* within the layer shed environment significantly increased the incidence of eggshell contamination. The flocks sampled during this study showed variation in *Salmonella* shedding over time. Stress induced by molting or the introduction of a new batch of birds within the shed may have resulted in higher shedding of *Salmonella* in the environment; however, further controlled studies are required to prove these observational findings. The results of this study might be helpful for determining the risks of having *Salmonella*-contaminated eggshells and also for developing strategies for risk management programs to control *Salmonella*.

ACKNOWLEDGMENTS

This research was conducted within the Poultry CRC, established and supported under the Australian Government's Cooperative Research Centres program.

Vaibhav Gole is an International Postgraduate Research Scholarship recipient.

We thank Amanda Kidsley, Geraldine Laven-Law, and Nigel Percy for their valuable technical help during this study.

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