Shedding of *Salmonella* in single age caged commercial layer flock at an early stage of lay

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**Abstract**

The shedding of *Salmonella* in a single age commercial egg layer flock was investigated at the onset of lay (18 weeks) followed by two longitudinal samplings at 24 and 30 weeks. At the age of 18 weeks, when the first sampling was performed, the prevalence of *Salmonella* in faeces was 82.14% whereas all egg belt and dust samples were *Salmonella* positive by culture method. In later samplings, at the age of 24 and 30 weeks, the prevalence of *Salmonella* in faeces was significantly reduced (p = 0.001) to 38.88% and 12.95% respectively, however all egg belt and dust samples remained positive by culture method. The prevalence of *Salmonella* in faeces collected from the low tier cages was significantly higher (p = 0.009) as compared with samples from the high tier cages. In all types of samples processed by culture method, *S. Mbandaka* was the most frequently (54.40%) isolated serovar followed by *S. Worthington* (37.60%), *S. Anatum* (0.8%), and *S. Infantis* (0.8%). All samples were also tested by real-time PCR method. The observed agreement between culture method and real-time PCR in detecting *Salmonella*-positive dust and egg belt samples was 100%. There was almost perfect agreement (observed agreement = 99.21%) for the detection of *Salmonella*-positive eggshells. Observed agreement between culture method and real-time PCR for detecting *Salmonella*-positive shoe cover and faecal samples was, however, moderate (80%) and low (54.27%) respectively. Real-time PCR results showed that there was a significant increase in the load of *Salmonella* on egg belt, dust and shoe cover samples at the 24 and 30 weeks of lay as compared to the 18 weeks of lay. Real-time PCR provided a more rapid and reliable method of detection of *Salmonella* on all dry sample types whereas the traditional culture method proved much more reliable when trying to detect *Salmonella* in wet faecal samples.

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1. **Introduction**

The genus *Salmonella* is a member of *Enterobacteriaceae* family. *Salmonella* is a rod shaped Gram negative, non-spore forming bacteria. In Australia, the rate of *Salmonella* infection has been reported to be 53.7 cases per 100,000 people in 2010 and eggs are often implicated as a major source of *Salmonella* infection (OzFood Net, 2012).

At oviposition, 90% of eggs are free of bacteria (Board, 1966). The Eggshell surface can, however, become contaminated during contact with any surface. Furthermore, the contamination of eggs with *Salmonella* on layer farms could be variable. Even though, the incidence of *Salmonella* egg contamination is very low, the numbers of eggs produced/consumed per year contribute to the large number of *Salmonella* contaminated eggs entering the consumer market. This sporadic rate of egg contamination could affect the trace back of egg related *Salmonella* food poisoning outbreaks. The presence of *Salmonella* in layer flock can be identified with the help of environmental sampling (Holt et al., 2011). The present infection status of the flock can be better indicated by faecal samples whereas dust samples may indicate prior *Salmonella* infection (Carrique-Mas and Davies, 2008).

During the laying production cycle, birds could experience various stressful events. It was observed that stress can impair humoral and cell mediated immune response of the birds (El-Lethey et al., 2003). Thus, due to the impaired immune response, birds might become more susceptible to *Salmonella* infection which in turn may lead to increased *Salmonella* shedding in faeces. The results of our previous study indicated that increase in the shedding of *Salmonella* in faeces could increase the chances of eggshell contamination (Gole et al., 2014). One of the most important stressful events in laying hens is the onset of sexual maturity and or lay which generally also coincides with the transfer of birds from one production system (rearing shed) to another (layer shed) (Humphrey, 2006). It could, therefore, be hypothesised that when birds reach sexual maturity (with addition of transport stress), they may be more susceptible to *Salmonella* infection. However, there is little information available in literature regarding the shedding of *Salmonella* at the initial stages of the laying period.

Laying birds may also be stressed with the level of traffic (of workers) or noisy cleaning methods in layer shed (Edwards, 2011).
In a large egg layer farm (having capacity ~30,000 birds/shed), outer lanes (high traffic area) could be more frequently used by workers as compared to inner lanes (low traffic area) to access shed controls. In the presence of biosecurity measures, Davies et al. (1997) reported that the footware of workers working on processing plants was identified as a risk factor for Salmonella infection of the premises. Similarly, the birds in low tiers may experience greater disturbance with the movement of the workers as compared with birds in higher tiers. It could be hypothesised that all these factors may contribute to an increased susceptibility of birds to Salmonella infection. However, there is little known about the relationship between these stress factors and Salmonella shedding.

The culture method protocol, for identification of Salmonella-positive samples, involves multiple steps and generally takes four to six days (Uyttendaele et al., 2003). The use of a real-time polymerase chain reaction (RT-PCR) method could be helpful to reduce the time involved in detecting Salmonella-positive samples and enable the quantification of bacteria in samples. The data generated would be beneficial for the development of Salmonella monitoring and control programmes.

The objectives of present study were: 1) to investigate the Salmonella shedding (in terms of faecal positivity) and also environmental prevalence (measured by sampling egg belt, dust, feed, and shoe cover samples) at an early stage of lay 2) to study the effect of traffic (low and high) and the various level of tiers on the shedding of Salmonella (in terms of faecal positivity) and also environmental prevalence (measured by sampling egg belt, dust, feed, and shoe cover samples) were collected from 54 cages (6 cages × 3 tiers × 3 rows) and processed for Salmonella isolation. Similarly, 9 egg belts (3 tiers × 3 rows), 3 dusts, 3 shoe covers and 4 feed samples were collected. During t + 6 and t + 12 samplings, all the laid eggs at the front of the sampled cages were collected from the low and high tiers of two rows (rows A and F). Eggs were placed in a sterile Whirl-Pak plastic bag (150 × 230 mm, Thermo Fisher Scientific, Australia). A pool of six eggs was considered as one sample.

2. Materials and methods

The study farm was an egg layer farm that has three different sheds connected with a common egg conveyor belt. Each shed housed bird cohorts of the same age (early lay < 40 weeks, mid lay 40 to 65 weeks and late lay > 65 weeks). The early lay shed was selected for conducting a prospective cohort study. The birds were transferred to the shed two weeks prior to the commencement of the study. The shed included six rows containing five tiers of 49 cages each and in each cage twenty birds were housed. The total size of the flock in the study shed was approximately 36,750 birds. The study shed was first sampled at 18 weeks of bird age (t0) followed by two longitudinal samplings at 24 and 30 weeks (t + 6 and t + 12, respectively). In each time point, faecal, egg belt, dust, feed, and shoe cover samples were collected. Eggs were collected in only last two samplings from the cages of low and high tiers as the eggs from 18 weeks of age were directly sent for egg pulping (whole egg pulp processing).

2.1. Specimen collection

During the first sampling, a larger cross-sectional sampling was conducted to map the initial infection distribution within the flock. In total, 56 composite cage faecal samples, 12 egg belt swabs, 6 dust samples, 4 shoe cover samples and 4 feed samples were collected. The cages were systematically sampled at an approximate interval of 16 cages (cage order: 1, 16, 33, 49) from tiers 1, 2 and 5. Additional faecal samples from tier 5 were collected from cages near to fan end (cage number 49). During the sample collection of each row, shoe covers were removed and placed in a 250 ml sterile plastic container (Pacific Laboratory Products, Australia). Following the first sampling (t0), sampling at t + 6 and t + 12 were restricted to 3 rows, two in high (rows A and F) and one in low traffic area (row D) (Fig. 1). Side rows, which have been most frequently used by farm workers, were considered high traffic areas, and middle rows, which were less frequently used, were considered low traffic areas. From each selected row, six cages were systematically sampled (cage numbers: 1, 8, 16, 33, 40, 49) from the tiers 1, 2 and 5. Altogether, composite faecal samples were collected from 54 cages (6 cages × 3 tiers × 3 rows) and processed for Salmonella isolation. Similarly, 9 egg belts (3 tiers × 3 rows), 3 dusts, 3 shoe covers and 4 feed samples were collected. During t + 6 and t + 12 samplings, all the laid eggs at the front of the sampled cages were collected from the low and high tiers of two rows (rows A and F). Eggs were placed in a sterile Whirl-Pak plastic bag (150 × 230 mm, Thermo Fisher Scientific, Australia). A pool of six eggs was considered as one sample.

2.2. Isolation of Salmonella from different samples

For isolation of Salmonella spp. the 2 g of faecal, feed and dust samples were inoculated in 4 ml of BPW (1:4) (Cox et al., 2002). The inoculated samples were incubated at 37 °C overnight and 100 μl of this sample was transferred into 10 ml of Rappaport Vassiladi Soya pepton broth (RVS, Oxoid, Australia) 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 Australia) and xylose lysine deoxycholate (XLD, Oxoid, Australia) agar plates. Two to three presumptive Salmonella colonies from BSA and XLD agar were selected and used to stab inoculate triple sugar iron (TSI; Oxoid, Australia) agar slopes. 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 (Oxo, Australia), lysine decarboxylase (LDC) and urease (Oxoid, Australia) activity. Depending upon the results of biochemical reactions, the presumptive Salmonella isolates were sent for serotyping to Salmonella Reference Laboratory, Adelaide, Australia.

In laboratory, egg belt samples were again (to avoid drying of swabs) moistened with BPW and processed for Salmonella isolation as mentioned above. Eggshell and egg internal content samples were individually processed. Individual sample (a pool of six eggs) was placed in 60 ml of sterile BPW in Whirl-Pak bags and rinsed by massaging for 4 min. Before rinsing, BPW was pre-warmed to 37 °C to facilitate bacterial recovery. After a rinse sample was obtained, each pool was removed and transferred to a new sterile bag. The egg’s internal contents, collected in sterile containers, were thoroughly mixed and 2 ml of egg internal content per pool was inoculated into 8 ml of BPW. The inoculated BPW was further processed for Salmonella isolation as mentioned above.

2.3. Real-time polymerase chain reaction detection of Salmonella

2.3.1. The DNA extraction from pre-enriched samples

The Wizard genomic DNA purification kit (Promega, Australia) was used to extract DNA from the pre-enriched BPW samples (faecal, egg belt, dust, feed, shoe cover and eggshell) as per manufacturer’s instructions with slight modifications. Briefly, 5 ml of pre-enriched samples was centrifuged at 14,000 g for 2 min to pellet bacterial cells. Cells were resuspended in 600 μl nuclei lysis solution followed by incubation at 80 °C for 5 min. The samples were allowed to cool down at room temperature and 3 μl of RNase solution was added to the cell lysate. The samples were vortexed and incubated at 37 °C for 30 min. To the RNase treated cell lysate, a 200 μl protein precipitation solution was added, followed by centrifugation at 14,000 g for 3 min. The containing supernatant was transferred to a clean 1.5 ml microcentrifuge tube with 600 μl of isopropanol and centrifuged at 14,000 g for 2 min. The DNA
pellet was further washed with 600 μl of 70% ethanol. A 100 μl of DNA rehydration solution was finally added to microcentrifuge tube to rehydrate DNA by incubating tubes at 4 °C overnight. The concentration of DNA in sample was determined using a spectrophotometer (Nano drop ND 1000, Biolab, Australia). Further dilution was performed using nuclease free water to achieve final 5 ng/μl DNA concentration. Finally, these diluted DNA samples were used in real-time PCR.

2.3.2. The DNA extraction from raw faecal samples

In order to compare the efficiency of raw faecal samples versus pre-enriched BPW to detect Salmonella, DNA was also extracted from raw faecal samples using QIAamp DNA Stool Mini Kit (Qiagen, Australia) as per manufacturer instructions. A 0.2 g faecal sample, was weighed and dispensed into microcentrifuge tubes containing 2 ml ASL buffer. The samples were vortexed and heated in a 70 °C water bath for 5 min. The samples were centrifuged at 4800 × g for 10 min and 120 μl of the supernannuant was transferred to another clean microcentrifuge tube containing an Inhibitex tablet (Qiagen, Australia). The samples were vortexed and stored at room temperature for 1 min. The samples were then centrifuged at 4800 × g for 10 min and 200 μl of resulting supernatant was treated with 15 μl of proteinase K and 200 μl of AL buffer. The mixture was reheated at 70 °C and transferred to a spin column. Washing and elution was performed according to the manufacturer’s instructions. The elution volume was 100 μl. Extracted DNA was quantified using Nanodrop and stored at −20 °C until used for real time PCR. Further dilution was performed using nuclease free water to achieve the final 5 ng/μl DNA concentration. Finally, these diluted DNA samples were used in real-time PCR.

2.3.3. The real-time PCR reaction

The PCR detection of Salmonella was done using the TaqMan Salmonella enterica detection kit system (Applied Biosystems, Australia) in a total reaction volume of 15 μl containing 6 μl sample (5 ng/μl), 7.5 μl of 2× Environmental Master Mix and 1.5 μl of 10× Target Assay Mix. All reactions were run on a Corbett Research (Adelab Scientific, Australia) with the following PCR 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 analysed using the software version Rotor-gene 1.7.75. The TaqMan Salmonella enterica detection kit does not provide quantification of positive samples. To determine the limit of detection and quantification of positive samples, a standard curve was prepared by generating a serial 10-fold dilution of faecal samples spiked with various concentrations of Salmonella. Briefly, 1 g of faecal samples was spiked with different 10 fold dilutions (3 × 10⁴ CFU to 3 CFU/g) of Salmonella. As described above, DNA extraction from spiked faecal samples was performed using QIAamp DNA Stool Mini Kit (Qiagen, Australia). The real-time PCR was performed on the diluted DNA samples (5 ng/μl). In each PCR reaction cycle, positive and negative controls were used to confirm the success of reaction. A cut-off Ct of 32 was used to exclude detection of false positives. A Ct of 32 corresponded to 30 CFU of Salmonella.

2.4. Statistical analysis

The prevalence of Salmonella-positive cages was estimated at each sampling point with 95% binomial exact confidence intervals. Multilevel logistic regression was used to compare prevalence estimates to account for the fact that the cages were sampled repeatedly and the fact that the cages were clustered within tier and tiers within row.
The default mixed model included random effects for ‘row’, ‘tier within row’ and for ‘cage within tier’. This model was then used to investigate the fixed effect of the following factors on Salmonella-positive isolation: sampling points (t0, t + 6, t + 12), high and low traffic areas, tier level, and cage location within a tier.

Agreement between detection methods (culture and real-time PCR) was estimated by simply using the proportion of samples for which the test result (either positive or negative) agreed (i.e. observe agreement). Kappa statistics was intentionally not used because of the recognised limitations of this index including its instability with samples with extreme prevalence (~20% or >80%).

Kruskal–Wallis test was used to determine the variation in the load of Salmonella (log-transformed CFU) in different types of samples over a period of three samplings. p-Values were interpreted at 5% significance level. Model assumptions were assessed using standard diagnostic plots. Statistical analyses were performed using the statistical package STATA v12.1 (STAT v12.1, 2011).

3. Results

3.1. Prevalence of Salmonella in faecal samples

During the first sampling t0 (flock was 18 weeks of age), 82.14% (95% CI: 69.6%–91.1%) of the cage faecal samples was Salmonella-positive. When compared to t0, the prevalence of Salmonella-positive samples significantly decreased to 38.88% at t + 6 (95% CI: 25.9%–54.1%) and to 12.96% (95% CI: 5.4%–24.9%) at t + 12 (p < 0.001). Overall (combining all three sampling points t0, t + 6 and t + 12), the prevalence of Salmonella in low tier cages (prevalence = 64.38%, CI: 0.53–0.74) was significantly higher (p = 0.009) as compared with high tier cages (prevalence = 24.39%, CI: 0.15–0.35). There was no significant difference (p > 0.05) in the prevalence of Salmonella-positive cage across cage location, between specific rows, and between high and low traffic areas in the shed.

3.2. Prevalence of Salmonella in other type of samples

Table 1 summarizes the numbers of samples and the test outcomes for each type of specimen collected at the 3 sampling points. All egg belt and dust samples collected during three sampling points were tested positive for Salmonella. Out of the 10 dust samples collected with shoe covers, nine (90%) were Salmonella-positive. At t + 6, out of the 55 eggshells tested, all were Salmonella-negative, and, at t + 12, two of the 72 eggshells were Salmonella-positive (2.7%). All feed samples and egg internal contents were Salmonella negative.

3.3. Serotyping of Salmonella isolates

Serotyping results confirmed that, in all types of samples, S. Mbandaka was the most frequently (54.40%) isolated serovar followed by S. Worthington (37.60%), S. Anatum (0.8%), and S. Infantis (0.8%). In faecal samples, S. Worthington was the most prevalent serovar whereas S. Mbandaka was predominantly isolated from the egg belt, dust, shoe cover and eggshell samples (Table 2).

3.4. Comparison between real-time PCR and culture

The limit of detection for real time PCR was 30 CFU/g of sample (Ct value = 32) with reaction efficiency above 100%. The real-time PCR identified 69 positive samples out of 343 tested samples. Out of 69 positive samples, 12 were negative by the culture method. On the other hand, RT-PCR failed to detect 70 samples which were positive by culture method (Table 3).

Table 3 provides the details of agreement between culture method and real-time PCR to detect Salmonella overall and in the different types of specimens. Overall, the two methods agreed on the detection outcome of 76.1% of the tested samples. This observed agreement was perfect (100%) for the dust (n = 12) and egg belt (n = 30) samples, and almost perfect for eggshell samples (99.2%, n = 127). On other hand, this agreement was moderate for shoe cover samples (80%, n = 10) and low for faecal samples (54.3%, n = 164). For better detection of Salmonella in faecal samples with real-time PCR, the samples were also pre-enriched in BPW. Agreement between raw and BPW enriched faecal samples tested with real-time PCR was moderate (86.6%) with more samples testing positive with the raw vs pre-enriched BPW faecal samples (19 and 11 positives, respectively).

3.5. Salmonella quantification

Table 4 shows the load of Salmonella (average log colony forming unit (CFU) per PCR reaction) in faeces, egg belt, dust, shoe cover and eggshells across the three different sampling points. At t0, the average load of Salmonella on the egg belt was 3.02 log CFU ± 0.26 and increased significantly at t + 6 (4.59 log CFU ± 0.13) and t + 12 (5.26 log CFU ± 0.24). In shoe cover samples, the load of detected Salmonella also built-up significantly between t0 (1.48 log CFU ± 0.59), t + 6 (3.98 log CFU ± 0.46), and t + 12 (4.21 log CFU ± 0.46) (p = 0.03). Salmonella loads in shoe covers were not significantly different between t + 6 and t + 12 (p = 0.51). Similar results were observed for dust samples where Salmonella loads increased between t0 (0.83 log CFU ± 0.58), t + 6 (3.32 log CFU ± 0.38), and t + 12 (4.13 log CFU ± 0.44) (p < 0.05). In faecal samples, the Salmonella load was significantly lower at t + 12 as compared to t0 and t + 6 (p < 0.05).

4. Discussion

In the present study, the shedding of Salmonella in single age layer flock was investigated at the onset of lay. Results of culture method indicated that at t0 (the age of 18 weeks), the prevalence of Salmonella in faeces was highest (82.14%). However, in latter samplings, at t + 6

**Table 1**

Prevalence of Salmonella during three longitudinal sampling.

<table>
<thead>
<tr>
<th>Sampling</th>
<th>Week of lay</th>
<th>Faeces</th>
<th>Egg belt</th>
<th>Dust</th>
<th>Shoe cover</th>
<th>Egg shells</th>
<th>Salmonella serovars*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>24</td>
<td>38.88% (21/54)</td>
<td>100% (9/9)</td>
<td>100% (3/3)</td>
<td>100% (3/3)</td>
<td>0% (0/55)</td>
<td>Faeces: S. Agona (1), S. Worthington (4), S. Mbandaka (16), Egg belt: S. Worthington (2), S. Mbandaka (7), Dust: S. Mbandaka (3), Shoe cover: S. Mbandaka (2), S. Worthington (1).</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>12.96% (7/54)</td>
<td>100% (9/9)</td>
<td>100% (3/3)</td>
<td>66.33% (2/3)</td>
<td>2.77% (0/72)</td>
<td>Faeces: S. Worthington (5), S. Mbandaka (2), Egg belt: S. Mbandaka (9), Dust: S. Mbandaka (3), Shoe cover: S. Mbandaka (2), Eggshell: S. Mbandaka (2).</td>
</tr>
</tbody>
</table>

Count of positive isolation/total number of samples for each sample type (faeces, egg belt, dust, shoe cover and eggshells). S.: Salmonella.

* Salmonell serovar isolated (number of positive samples).
and t + 12, the prevalence of *Salmonella* in faeces was reduced significantly (p < 0.001) to 38.88% and 12.95% respectively. There is a dearth in the literature to compare these findings as layer flocks are rarely sampled at the very early stage of lay. Residual *Salmonella* contamination on the layer farm is responsible for re-introduction of *Salmonella* in a flock (Van de Giessen et al., 1994; Gradel et al., 2004). It is possible that, in the present study, newly arrived pullets on the farm were suffering from transport, handling and relocation stress along with the stress related to onset of lay. In laying hens, stress can negatively influence immune response (El-Lethey et al., 2003; Humphrey, 2006) which may have increased the susceptibility of young pullets to acquire *Salmonella* infection from the shed environment. This in turn may have resulted in the higher shedding of *Salmonella* at the age of 18 weeks. However, further controlled experiments are essential to establish association between stress and *Salmonella* shedding. Once the birds settled in cages (weeks 24 and 30), the shedding of *Salmonella* was reduced. Another possibility is that the birds already had *Salmonella* in the GIT which had been acquired in the rearing house and that they started to shed this as a result of the transport stress.

The prevalence of *Salmonella* in low tier cages was significantly higher (p = 0.009) as compared to high tier cages. The higher prevalence of *Salmonella* in low tier cages could be explained by several factors. First, the birds in lower tier cages were more exposed to the dust on the floor. McDerrmid and Lever ( McDerrmid and Lever, 1996) demonstrated that *Salmonella* can survive in aerosols, maintained using a rotating drum, for a considerable period of time. In the present study, dust samples, in all three samplings, were consistently positive for *Salmonella* which may have resulted in the higher lateral spread of infection in lower tiers as compared to higher tiers. Secondly, birds housed in lower tiers are more exposed to the movement of workers and cleaning equipment as compared to the birds in higher tiers which may have resulted in stress and ultimately higher *Salmonella* shedding in cages belonging to lower tiers. However, there was no significant difference observed in the shedding of *Salmonella* in the cages belonging to low and high traffic areas. There is little or no information in literature to compare these finding. Further experiments are necessary involving the estimation of stress inducing parameters in high and low traffic areas which may provide better information regarding *Salmonella* shedding.

The real-time PCR was able to detect 25.67% (19/74) of faecal samples which were also culture positive. With the objective to improve the detection of *Salmonella*-positive faecal samples, real-time PCR was also performed using pre-enriched BPW from faecal samples. However, with this protocol, real-time PCR was able to detect only 14.86% (11/74) of culture positive samples. This clearly suggested that BPW pre-enrichment or single enrichment did not improve the detection by PCR of *Salmonella* from faecal samples. When samples were processed for *Salmonella* detection by real-time PCR method, the limit of detection was 30 CFU. The comparative results between culture method and real-time PCR assay indicated that, culture method was able to detect less than 30 CFU of *Salmonella* spp. These findings are in agreement with Jensen et al. (2013) who also reported a lower relative sensitivity of real-time PCR (20%) as compared to a culture method. Low sensitivity of real time PCR compared to culture method could be attributed to the PCR inhibitors in environmental samples.

In the present study, most of the egg belt, dust and shoe cover samples tested positive for *Salmonella* throughout sampling period. *Salmonella* prevalence in a layer farm can be affected by various factors such as farm and flock size (Van Hoorebeke et al., 2011). The bird holding capacity of the flock sampled in the present study was 36,750. Larger flock size increases the risk of introduction of *Salmonella* infection (Van Hoorebeke et al., 2011). However, it has been observed that the persistence of *Salmonella* was not significantly related to flock size (Carrique-Mas et al., 2009a,b). The presence of multiple flocks on the same farm enhances the risk of cross contamination between sheds especially when they are connected by common egg conveyor belt (Carrique-Mas et al., 2008). In the present study, sheds on farm were connected with common egg conveyor belt. Another important factor for the continuous presence of *Salmonella* in battery cage layer farms is difficulty in cleaning and disinfection of interior of cages, egg belt and feeders (Carrique-Mas et al., 2009a; Davies and Breslin, 2003).

At age of 30 weeks (t + 12), in faecal samples, the level of *Salmonella* dropped significantly. This could have been attributed to the recovery of the birds from the stress and acclimatization to the shed environment. However, there was significant increase in the load of *Salmonella* on egg belt, dust and shoe cover samples at t + 6 and t + 12 as compared to t0. Cleaning of shed and removal of dust at regular interval may help to reduce the level of environmental contamination in layer shed. In the present study, real-time PCR results indicated that, at t + 12 (week 30), three eggshell samples were *Salmonella*-positive. Serovar isolated from all eggshell samples was S. Mbandaka. The same serovar was most frequently reported on egg belt, dust and shoe cover samples indicating the source of eggshell contamination. In the present study, even though

### Table 2

![Table 2](https://example.com/table2.png)

<table>
<thead>
<tr>
<th>Serovellla serovar</th>
<th>Faeces</th>
<th>Egg belt</th>
<th>Dust</th>
<th>Shoe cover</th>
<th>Eggshells</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. Worthington</td>
<td>60.27%</td>
<td>6.60%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>11.11%</td>
</tr>
<tr>
<td>S. Mbandaka</td>
<td>38.36%</td>
<td>73.33%</td>
<td>81.81%</td>
<td>77.78%</td>
<td>100%</td>
<td>54.40%</td>
</tr>
<tr>
<td>S. Infantis</td>
<td>1.37%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0.8%</td>
</tr>
<tr>
<td>S. Anatum</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>S. Worthington + S. Mbandaka</td>
<td>0%</td>
<td>10%</td>
<td>0.09%</td>
<td>11.11%</td>
<td>0%</td>
<td>10.8%</td>
</tr>
<tr>
<td>S. Worthington + S. Mbandaka + S. Infantis</td>
<td>0%</td>
<td>3.33%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0.8%</td>
</tr>
<tr>
<td>S. Mbandaka + S. Anatum</td>
<td>0%</td>
<td>6.66%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>1.6%</td>
</tr>
</tbody>
</table>

Table 3

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Samples identified by culture method</th>
<th>Samples identified by real time PCR</th>
<th>Real time PCR positive</th>
<th>Real time PCR negative</th>
<th>Culture total</th>
<th>Observed agreement [%]</th>
</tr>
</thead>
</table>

**Eggs**
- Culture positive: 2
- Culture negative: 1
- Real time PCR total: 3

**Faeces**
- Culture positive: 9
- Culture negative: 10
- Real time PCR total: 19

**Egg belt**
- Culture positive: 30
- Culture negative: 0
- Real time PCR total: 30

**Dust**
- Culture positive: 12
- Culture negative: 0
- Real time PCR total: 12

**Shoe cover**
- Culture positive: 8
- Culture negative: 1
- Real time PCR total: 9

**All sample types**
- Culture positive: 57
- Culture negative: 12
- Real time PCR total: 69

The table demonstrates the agreement between culture and real-time PCR methods for detecting *Salmonella*.
the birds were infected with *Salmonella*, egg internal contents were *Salmonella* negative. The vertical transmission ability of most prevalent *Salmonella* serovars isolated from this study needs further investigation. Previously, it has been reported that *Salmonella* Infantis was not isolated from egg internal contents of known positive birds (Cox et al., 2002). *Salmonella* Mbandaka has been isolated from egg shell surface (Little et al., 2007), however the controlled studies are essential to study the vertical transmission ability of predominant *Salmonella* serovars isolated during the present investigation. The serovars isolated in the present study may lack the ability to transmit vertically (vertical transmission) or may have little capacity to survive in egg internal contents (horizontal transmission). However, to confirm this, further studies are essential. A quarterly report released from Institute of Medical and Veterinary Science (IMVS), Adelaide, Australia, indicated that, *Salmonella* Mbandaka has been isolated from human population (Australian *Salmonella* Reference Centre, 2014). However this was not related to egg or egg product related food poisoning. *Salmonella* Worthington isolated from egg farms has not been reported in human population. Further studies are essential to investigate the virulence/pathogenicity of egg farm related *Salmonella* serovars.

In conclusion, during this experiment, at the start of lay (18 weeks), within first week of housing, the shedding of *Salmonella* in faecal samples was at a peak compared with later sampling times. However, over the time, *Salmonella* infection subsided in subsequent samplings. The prevalence of *Salmonella* in birds housed in the lower tiers was higher as compared to birds in higher tiers. The sensitivity of real-time PCR was lower as compared to culture method in detecting *Salmonella*–positive faecal samples. The sensitivity of real-time PCR was also not improved with use of a pre-enrichment step. This might be due to the presence PCR inhibitory factors in faeces, a low number of target microorganism as well as large number of competing bacteria in faeces. As per real-time PCR results, load of *Salmonella* on egg belt, shoe cover and dust increased with the age of the flock. Hence, regular monitoring and intervention strategies are required to reduce the environmental load of *Salmonella* in layer shed which could be helpful to reduce the chances of eggshell contamination.

### Acknowledgements

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### Table 4

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Week of lay (sampling number)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18 (1)</td>
<td>24 (2)</td>
</tr>
<tr>
<td><strong>Faeces</strong></td>
<td>0.25 ± 0.09*</td>
<td>0.49 ± 0.14*</td>
</tr>
<tr>
<td><strong>Egg belt</strong></td>
<td>3.02 ± 0.26*</td>
<td>4.59 ± 0.13*</td>
</tr>
<tr>
<td><strong>Shoe cover</strong></td>
<td>1.48 ± 0.59*</td>
<td>3.98 ± 0.46*</td>
</tr>
<tr>
<td><strong>Dust</strong></td>
<td>0.83 ± 0.58*</td>
<td>3.32 ± 0.38*</td>
</tr>
<tr>
<td><strong>Eggshells</strong></td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

The different superscripts in the same sample type are statistically and significantly different (p < 0.05) from each other. Log CFU ± standard error.

ND: not detected.
NA: not applicable.

### References


