Spatial Distribution of *Salmonella enterica* in Poultry Shed Environments Observed by Intensive Longitudinal Environmental Sampling

Helen K. Crabb,^{a,b} Joanne Lee Allen,^b Joanne Maree Devlin,^b Colin Reginald Wilks,^b James Rudkin Gilkerson^{a,b}

^aNational Centre for Antimicrobial Stewardship, The Peter Doherty Institute for Infection and Immunity, University of Melbourne, Parkville, Victoria, Australia

^bAsia Pacific Centre of Animal Health, Veterinary Biosciences, Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Parkville, Victoria, Australia

ABSTRACT Detection of salmonellae within poultry environments is an important component of many food safety programs, but sampling approaches vary greatly and may not enable the detection of salmonellae when bacteria are present at a low prevalence or concentration. Intensive longitudinal sampling within caged sheds enabled us to undertake a longitudinal analysis of the spatial distribution of salmonellae in caged shed environments. Both the number of samples collected and location of sample collection within a poultry shed were important to ensure the best chance of detecting *Salmonella* spp. Differences in the within-shed spatial distribution of *Salmonella enterica* subspecies *enterica* serovar Typhimurium [$\chi^2(27, 1,538) = 54.4$; $P < 0.001$] and *Salmonella enterica* subspecies *enterica* serovar Infantis [$\chi^2(27, 1,538) = 79.8$; $P < 0.0001$] were identified. More than one *Salmonella enterica* serovar was detected in each shed on the same sampling occasion; 5% of all samples contained more than one serovar. Samples collected on the north side of the shed (odds ratio [OR], 1.77; 95% confidence interval [CI], 1.17–2.68), on the sheltered side of the shed (OR, 1.90; 95% CI, 1.26–2.89), and during winter (OR, 48.41; 95% CI, 23.56–104.19) were more likely to be positive for salmonellae. The within-shed differences observed in the both the sample prevalence and spatial location of the serovar detected indicate that there are important shed microenvironmental factors that influence the survival and/or distribution of salmonellae. These factors should be taken into consideration when environmental surveillance is undertaken for salmonellae in flocks housed in cage sheds.

IMPORTANCE Routine epidemiological surveillance for salmonellae in poultry relies initially on environmental sampling. Intensive, spatially homogenous sampling, as conducted within this study, confirmed that the sampling methodology conducted within a poultry environment is a nontrivial part of sampling design. The frequency of sampling is especially important when the prevalence of *Salmonella* spp. is low. These factors must be taken into consideration in the design of studies for the detection of salmonellae in poultry sheds.

KEYWORDS *S. enterica*, *Salmonella*, cage sheds, environment, poultry, sampling, spatial

The routine surveillance for salmonellae in poultry typically involves environmental sampling because it has long been established as a more cost-effective and sensitive method for detection (1) than individual bird sampling (2–4). Environmental sampling is indicative of flock infection with salmonellae, while the level of environmental contamination (both semiquantitative and sample prevalence) and egg prevalence may be correlated with prevalence of infection in the flock (here, termed flock prevalence) (5, 6). As flock prevalence declines, the number of environmental samples

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Address correspondence to Helen K. Crabb, helen.crabb@unimelb.edu.au.

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required to detect infection must increase to maintain adequate test sensitivity (7, 8). Importantly, animal welfare and production effects associated with live-bird handling are minimized, allowing environmental sampling to be conducted at frequent intervals with little to no flock interference.

A variety of environmental sampling methods and strategies have been developed for the detection of salmonellae in poultry environments (1, 9, 10), and the principal features of these methods have been incorporated into national surveillance and control programs (11–15). While extensive knowledge has been gained regarding environmental sampling for the identification of infected flocks for control purposes, few studies have described intensive longitudinal sampling for salmonellae within infected flocks or environments. Where longitudinal studies have been conducted, the interval between sampling is long (months), and samples of the same type are typically pooled for testing (16–19). Samples were collected intensively in this study and were tested by both sample type and location, with no pooling.

It is known that salmonellae will survive for extended periods of time in different environments and sample types and under many different environmental conditions (20–24). In poultry house environments, an increase in the number of positive samples has been observed in winter periods (9, 18) although it is not clear whether this is due to increased shedding from infected birds, prolonged survival during cooler weather, or multiplication of salmonellae within the environment. In addition, multiple *Salmonella enterica* subspecies and/or serovars may be detected in flocks at a single environmental sampling event (19).

Despite extensive discussion in the literature regarding sample type and sample pooling (1, 3, 4, 8, 10, 25–27), the method of sample site selection within a cage shed environment is infrequently described or discussed (9, 28). The method of choosing the environmental sites for sampling within a poultry cage shed environment may be a nontrivial part of surveillance design. Information about the spatial distribution of salmonellae within poultry environments and how this may affect the design of surveillance strategies are largely unknown or undescribed in the literature. No studies have reported repeatedly sampling the same locations within the same environment for the entire life span of the flocks contained therein. Thus, it is not known what, if any, environmental (temperature, humidity, moisture, airflow, and spatial distribution) or flock (age, breed, stocking density, and spatial placement) factors may influence the repeated detection of salmonellae during the life of the flock or what influence postcleaning decontamination may have on subsequent flock infection or the detection of environmental *Salmonella* spp.

In veterinary science, prevalence calculations typically use herd or flock size to calculate the appropriate sample size to detect infection at a given prevalence (29) so that the sampling strategy chosen is sufficiently powerful to provide sufficient confidence of detecting infection at this determined prevalence (30). When this information is extrapolated to environmental sampling for the detection of a specific pathogen, thus indirectly sampling the flock, several unknowns are encountered. Specifically, these unknowns include, but are not limited to, the following. Does repeated detection reflect flock shedding of salmonellae or, rather, the repeated detection of the same salmonellae persisting or multiplying in the contaminated environment? Is the failure to detect salmonellae due to inadequate sample site selection, insufficient sample size, or differences in distribution within a cage shed environment? How does one ensure that sufficient samples are collected that adequately represent both the environment and the distribution of infection within a flock, assuming infection may be heterogeneously distributed?

The study reported here was part of a larger study whose overall aim was to detect the novel introduction of salmonellae into poultry shed environments and trace the subsequent dissemination of salmonellae between locations. Our shed sampling design was intended to optimize the detection of salmonellae introduction while taking into account the fixed location of birds housed in caged environments. Our investigations and sampling approach as detailed within this paper led to the serendipitous but

TABLE 1 Summary of environmental testing results by housing type

Housing type	No. of units ^a	No. of samples	No. of positive samples (%)
Conventional cage	9	895	565 (63)
Colony cage	10	1,984	509 (26)
Total	19	2,879	1,074 (37)

^aThe number of flocks sampled per housing type.

potentially important finding that the spatial distribution of salmonellae within poultry sheds may be a critical parameter that needs to be taken into consideration in the design of comprehensive sampling strategies. It is expected that these results will be useful to other investigators in designing sampling approaches for *Salmonella* spp. detection in all poultry flocks.

RESULTS

Sampling results. A total of 19 caged flocks were sampled on 105 occasions, with 2,879 samples collected. Overall, 37% of all samples collected were positive for *S. enterica*, with more samples obtained from conventional cages testing positive than those collected from colony cages. *S. enterica* serovar Typhimurium and *S. enterica* serovar Infantis were the most frequently detected serovars, comprising 9.8% and 14.9% of the isolates, respectively. In the colony cages, *S. Typhimurium* and *S. Infantis* were the only serovars detected. In the conventional cages, other *S. enterica* serovars, including *S. Singapore*, *S. Agona*, and *S. Virchow*, were detected in addition to *S. Infantis* and *S. Typhimurium* (Table 1).

Predictive value of sampling method. The positive and negative predictive values of the combined sampling methodology for *S. enterica* were high (Table 2). Importantly, the negative predictive value of the combined sampling method was high regardless of the serovar. However, the positive predictive values of the sampling method differed between *S. enterica* serovars, with the probability of detecting *S. Typhimurium* lower than that of *S. Infantis*, reflecting the environmental prevalence of the respective serovars.

Sampling events. All flocks were sampled at least twice; the median number of sampling events for all flocks was 3 (range, 2 to 15). Twelve of the 19 flocks were longitudinally sampled; 8 flocks were sampled at least monthly until 40 weeks of age, and 4 flocks (A, B, C, and D) housed in colony cage sheds were sampled at 3-week intervals until flocks were ~65 weeks of age. All flocks were determined to be positive for *S. enterica* on at least one sampling occasion (Table 3), and multiple *S. enterica* serovars were detected in all sheds.

The odds of a sample being positive for *S. enterica* varied substantially by shed (odds ratio [OR] range, 3.04 to 144.53), indicating a substantial shed effect with regard to *S. enterica* persistence or survival within an individual shed, either within the birds or within the shed environment (see Table S3 in the supplemental material).

Negative sampling events were more likely to occur in colony cage houses than in conventional cage houses (OR, 60.12; 95% confidence interval [CI], 7.39–48.11; $\chi^2 = 34.24$; $P < 0.001$), but *S. Typhimurium* was slightly more likely to be detected in colony cage samples (OR, 2.19; 95% CI, 0.89–5.39; $\chi^2 = 2.99$; $P = 0.08$). *S. Infantis* was more

TABLE 2 Positive predictive values for the sampling methodology and the odds of detection for each sample type for *S. enterica*, *S. Typhimurium*, and *S. Infantis*

Organism	OR (range) by sample type				PPV for all samples ^a	NPV for all samples ^b
	Boot	Dust	Manure	Egg		
<i>S. enterica</i>	7.69 (5.60–11.35)	3.84 (2.94–5.31)	5.19 (3.94–7.28)	1.00	0.984 (0.979–0.989)	0.994 (0.979–1.00)
<i>S. Typhimurium</i>	2.12 (1.36–3.65)	2.76 (1.91–4.32)	2.94 (2.03–4.62)	1.00	0.907 (0.855–0.959)	0.999 (0.994–1.00)
<i>S. Infantis</i>	5.14 (3.66–7.78)	2.98 (2.20–4.31)	3.53 (2.59–5.17)	1.00	0.943 (0.902–0.985)	0.998 (0.991–1.00)

^aPPV, $P(B+ | A+)$, i.e., the probability that the sample type is positive if all the samples are positive.^bNPV, $P(B- | A-)$, i.e., the probability that the sample type is negative if all the samples are negative.

TABLE 3 Mean true sample *S. enterica* prevalence summarized by housing type and sampling event for each serovar

Housing type	No. of sampling events	No. of negative sampling events	Mean true prevalence (SE) at a sampling event of:		
			All <i>S. enterica</i> serovars	<i>S. Typhimurium</i>	<i>S. Infantis</i>
Conventional cage	31	1	0.64 (0.09)	0.03 (0.03)	0.17 (0.03)
Colony cage	74	14	0.26 (0.05)	0.13 (0.04)	0.14 (0.04)
All	105	15	0.37 (0.05)	0.10 (0.03)	0.15 (0.04)

likely to be isolated from positive samples, regardless of shed type (OR, 1.76; 95% CI, 1.49–2.08; $\chi^2 = 45.40$; $P < 0.001$).

Postcleaning sampling. All sheds were either wet or dry cleaned prior to repopulation. Twelve flocks were sampled postcleaning, and salmonellae were detected in 9 of 12 sheds. *S. Typhimurium* and *S. Infantis* were detected in 3 and 7 of the cleaned sheds, respectively. In sheds that were wet washed, the sample prevalence (number of samples positive) was lower in the first sampling event postcleaning [3.54, $P < 0.001$; Student's *t* test] than in the sampling event prior to cleaning and all subsequent sampling events [6.11, $P < 0.001$; Student's *t* test]. A wet-washed shed was more likely to have *S. enterica* negative sampling events during the subsequent flock production period than sheds that were dry cleaned only (OR, 1.44; 95% CI, 0.43–4.82; $\chi^2 = 0.35$; $P = 0.55$); however, this difference was not statistically significant.

Sample type. Results for all sheds aggregated by sample type and *S. enterica* serovar are presented in Table 4. The estimated true sample prevalence for all *S. enterica* serovars was 39%, with *S. Infantis* (14%) more frequently detected than *S. Typhimurium* (9%). When both the shed type and sampling event are taken into account, all sample types were more likely to be positive for *S. enterica* than egg belt samples [$F(3, 2,589) = 42.84$; $P < 0.001$], regardless of the serovar. For the detection of *S. Typhimurium* there was no statistically significant difference between sample types (dust = manure belt = boot swab); however, boot swabs were better than all other sample types for detecting *S. Infantis* (boot swab > dust = manure belt) [$F(3, 2,589) = 21.7$; $P < 0.001$].

Other factors. Samples collected on the north side of a shed ($P < 0.001$) or on the aspect of a shed sheltered by another shed (between sheds) ($P < 0.001$) were more likely to be positive for *S. enterica* (Table 5). There was a significant difference between flocks and sheds in the numbers of positive samples collected at each sampling event ($P < 0.001$), indicating that it is important to take into account the hierarchical effects of the sampling design.

When the month of sampling was considered, samples collected in June and August ($P < 0.001$) were more likely to be positive for *S. enterica*. There was a seasonal effect, with samples collected in winter ($P < 0.001$) more likely to be positive than those collected in the other seasons (winter > spring > summer > autumn). Full results of the weather variables considered in the analysis are detailed in the supplemental material (for weather results, see Tables S6 and S7).

Colony cage sheds. To further investigate the environmental factors affecting *S. enterica* prevalence and distribution in detail, the four most intensively sampled flocks were considered in depth. These flocks (A, B, C, and D) were sampled on 13 to 15

TABLE 4 True sample *S. enterica* prevalence and 95% confidence intervals for all flocks and sample events by sample type for each serovar

Sample type	No. of samples	No. of <i>Salmonella</i> -positive samples	True prevalence (95% CI) of:		
			<i>S. enterica</i>	<i>S. Typhimurium</i>	<i>S. Infantis</i>
Boot swab	400	184	0.50 (0.43–0.58)	0.09 (0.05–0.13)	0.23 (0.18–0.27)
Dust	1,002	376	0.40 (0.35–0.45)	0.10 (0.07–0.12)	0.15 (0.13–0.18)
Manure belt	715	276	0.31 (0.26–0.37)	0.09 (0.06–0.12)	0.13 (0.11–0.15)
Egg belt	762	228	0.29 (0.35–0.22)	0.05 (0.03–0.07)	0.07 (0.05–0.08)
All	2,879	1,074	0.39 (0.35–0.42)	0.09 (0.08–0.11)	0.14 (0.13–0.16)

TABLE 5 Summary of univariate analysis results of environmental variables associated with true sample *S. enterica* prevalence, aggregated for all sheds and flocks

Variable	Code ^a	OR	95% CI	χ^2	df	P value
Flock	1–19			117.1	18	<0.001
Shed	1–13			445.4	12	<0.001
Aspect	North South	1.33 Reference	1.12–1.57	10.7	1	<0.001
Betweenness	Yes No	1.96 Reference	1.64–2.33	56.9	1	<0.001
Month	1–12			196.7	11	<0.001
Sampling event	1–15			200.4	14	<0.001
Sample type	Boot swab Dust Manure belt Egg belt	2.42 1.63 1.71 Reference	1.85–3.17 1.31–2.03 1.35–2.15	45.4	3	<0.001
Season	Autumn Spring Summer Winter	Reference 0.46 0.74 2.02	0.36–0.59 0.59–0.94 1.57–2.59	133.5	3	<0.001

^aValues for flock, shed, month, and sampling event indicate the number of each in the aggregate (e.g., 19 flocks and 13 sheds). For shed and sampling event, see Tables S3 and S4, respectively, in the supplemental material.

occasions during the flock production period. A total of 1,538 environmental samples were collected; of these 24% were positive for *S. enterica*. Five percent of the samples contained more than one *S. enterica* serovar, but only *S. Typhimurium* and *S. Infantis* were identified in these sheds. In 3 of the 4 sheds, *S. Typhimurium* was isolated more frequently than *S. Infantis* (Table 6). The percentages of *S. enterica*-positive samples (8 to 56%) varied by shed and serovar, and the difference in sample prevalences between sheds was statistically significant [$\chi^2(3, 1538) = 246.8$; $P < 0.001$].

All sheds were negative for *Salmonella* spp. prior to the onset of flock placement. *S. enterica* was detected in all sheds on most sampling events (69 to 100%). However, the number of sampling events that were positive for each sample type varied for any one shed (Table S4). The odds of a sample being positive for *S. enterica* varied by time (sampling event), with samples collected on the first sampling event significantly more likely to be positive than those collected later in the sampling period (Table S5).

The sheds with fewer positive sampling events had fewer *S. enterica*-positive samples. In sheds A and C (high environmental sample prevalence sheds) any combination of boot or dust sample would have detected that these were sheds positive for *S. enterica* on any sampling event, whereas in sheds B or D (low environmental sample

TABLE 6 Summary of environmental sampling in four intensively sampled colony sheds

Parameter	Value for the parameter by shed			
	A	B	C	D
Total sampling period (wk)	36	36	38	41
Mean sampling interval (wk [range])	2.8 (2.0–3.0)	3.0 (0)	2.8 (2.0–3.0)	2.8 (2.0–3.0)
Total no. of sampling events	13	13	14	15
No. of positive events (%) ^a	13 (100)	9 (69)	14 (100)	1 (73)
No. of samples	364	362	392	420
No. of positive samples	203	35	103	33
True prevalence (95% CI)				
<i>S. enterica</i>	0.63 (0.57–0.69)	0.10 (0.07–0.14)	0.29 (0.25–0.35)	0.08 (0.06–0.12)
<i>S. Typhimurium</i>	0.39 (0.34–0.46)	0.09 (0.06–0.12)	0.006 (0.00–0.02)	0.07 (0.05–0.11)
<i>S. Infantis</i>	0.19 (0.15–0.24)	0.01 (0.003–0.04)	0.28 (0.23–0.34)	0.002 (0.001–0.02)

^aThe number of sampling events where at least one of 28 samples was positive.

FIG 1 Heterogeneity of *S. Typhimurium* and *S. Infantis* distribution within a colony cage environment. The environmental prevalence for each serovar is calculated from all samples collected from that sampling location. (A) *S. enterica* prevalence at the sampling location within the shed environment for *S. Typhimurium* and *S. Infantis* separately. The length of the bar indicates serovar prevalence at that sampling location. Sampling location is ordered by the site with the highest *S. Typhimurium* prevalence. (B) Heat map indicating *S. Typhimurium* prevalence in a two-dimensional space at each sampling location. The warmer the color, the greater is the prevalence at that location. A corresponding perspective plot of *S. Typhimurium* prevalence is shown in which height is proportional to the prevalence at the sampling location. (C) Heat map indicating *S. Infantis* prevalence in a two-dimensional space at each sampling location. The warmer the color, the greater is the prevalence at that location. A corresponding perspective plot of *S. Infantis* prevalence is shown in which height is proportional to the prevalence at the sampling location.

prevalence sheds), fewer than half the samples were positive on any sample event, regardless of the sample type. Only by combining the results of each sample type on each sampling event did the probability of detecting *S. enterica* increase, with a 9- to 12- fold increase in sheds B and D, respectively.

There was a significant difference between locations within a shed for the detection of *S. enterica* [$\chi^2(27, 1,538) = 96.6$; $P < 0.001$]. Samples taken on the north side of the shed (OR, 1.95; 95% CI, 1.54–2.49) and those collected from locations between sheds were more likely (OR, 1.92; 95% CI, 1.52–2.45) to be positive.

Thirteen of the 28 sampling locations were likely to be positive for *S. enterica*, regardless of the serovar (Table S6). *S. Typhimurium* was more likely to be found in 7 of the 28 locations [$\chi^2(27, 1,538) = 54.4$; $P < 0.001$], and *S. Infantis* was more likely to be identified in 7 different locations [$\chi^2(27, 1,538) = 79.8$; $P < 0.001$]. The detection of an *S. enterica*-positive sample at one location was not influenced by the detection of another positive sample in the same or similar spatial location (spatial autocorrelation, Moran I = -0.026 ; $P = 0.343$). The frequency at which each serovar was identified at a location is illustrated in Fig. 1A, and the sample prevalence for each serovar, illustrated in a two-dimensional (2D) space, is presented in Fig. 1B and C.

TABLE 7 Estimated regression coefficients and standard errors for the final mixed-effects logistic regression model of environmental risk factors to predict the number of *S. enterica*-positive environmental samples from a longitudinal investigation of caged chicken flocks (2013 to 2018)

Variable	No. of positive samples	Total no. of samples	Coefficient (SE)	Z	P value	OR (95% CI)
Intercept	367	1,458	−4.14 (0.32)	−12.96	<0.001	
Aspect						
North	229	728	0.58 (0.21)	2.71	0.007	1.78 (1.16–2.68)
South	138	726	Reference			1.00
Betweenness						
Yes	230	728	0.65 (0.21)	3.06	0.002	1.90 (1.25–2.89)
No	137	726	Reference			1.00
Sample type						
Egg belt	39	415	Reference			1.00
Dust	126	415	2.14 (0.25)	8.47	<0.001	8.53 (5.26–14.22)
Manure belt	123	416	2.11 (0.25)	8.31	<0.001	8.24 (5.08–13.75)
Boot swab	79	208	2.68 (0.28)	9.39	<0.001	14.39 (8.41–25.73)
Season						
Autumn	63	448	Reference			1.00
Winter	74	112	3.92 (0.38)	10.25	<0.001	50.22 (24.29–108.92)
Spring	113	446	0.92 (0.21)	4.47	<0.001	2.52 (1.81–3.79)
Summer	117	448	0.99 (0.20)	4.82	<0.001	2.70 (1.81–4.07)

Hierarchical mixed-effects multivariable modeling. The univariate results for all statistically significant explanatory variables considered for multivariable modeling are summarized in Table S9; a full description of all variables is available in Table S10. The final multivariable model considered the detection of an *S. enterica*-positive sample, irrespective of serovar. Estimated regression coefficients for the final model and the variability of the random effects term are provided in Table 7.

Two-level hierarchical models with random effects considered to account for both sample event- and shed-level effects were built. The final most parsimonious model accounted for the difference in the shed-level effects as a fixed term as there was no significant difference between the two models when both levels were included as random effects [$\log$ -likelihood (10) = −98.2; P = 1.0]. The Hosmer-Lemeshow goodness of fit test was significant at small values of g = 5 to 10 [statistical test values for the test at each of the two sample sizes were $\chi^2(3)$ = 2.18, P = 0.54, and $\chi^2(8)$ = 13.85, P = 0.086], indicating a relatively good fit to the data with this model. The residual variation due to unknown shed effects (variation partition coefficient) was estimated as $(1.858/1.858 + 3.29) = 0.39$.

The sensitivity and specificity of the model were estimated as 0.87 and 0.77, respectively. The diagnostic accuracy (Fig. S2) was estimated as 0.88 (95% CI, 0.86–0.90), indicating a good prediction of the outcome by the model. Samples that were collected from a location on the north side of a shed (P < 0.007) or between sheds (P < 0.002) were more likely to be positive for *S. enterica* serovars.

Both the location of sample collection and sample type were important, but only sample type was included in the final model. The odds of a sample being positive was greater for boot swabs (P < 0.001) than for any other sample type. As each sample type was collected by location, it could be interpreted as a proxy variable for location. Individual weather variables were not significant in the final model (Table S9), but the effect of season was very important as samples collected in the winter months (P < 0.001) were more likely to be positive than those collected in other seasons.

DISCUSSION

This study investigated the performance of environmental sampling for the detection of salmonellae in caged environments under Australian environmental conditions. Sample size estimations demonstrated that at least 28 samples were required to detect

salmonellae at a design prevalence of 1% with an imperfect test. As individual bird sampling for salmonellae is known to be less sensitive than environmental sampling (4), the number of birds to sample was estimated using the strictest criterion, proof of freedom of disease. This comparison (bird versus environment) was made to demonstrate the relative efficiency of environmental sampling versus individual bird sampling as a screening tool.

Both the logistics and practicality of selecting and testing hundreds of birds from each flock at regular intervals throughout production preclude this from being a real option for routine surveillance purposes. It also highlights that the number of samples (birds) required at very low flock prevalence is greater than the design criteria when an imperfect test is used. Under these environmental conditions, it is impossible to sample a sufficient number of birds to determine the lowest flock prevalence of 1 positive unit per 100 units. This has important implications for confirmatory testing of the *S. enterica* status in flocks after a positive environmental sampling result, particularly when the flock prevalence is low.

Analysis of the field results demonstrated that not only is the number of samples taken critical for detecting salmonellae within a caged environment but where the samples are collected is also important. Samples were more likely to be positive in the winter, and univariate analysis indicated that decreasing temperature and increasing moisture increased the odds of detection. Critically in the seasons studied, the winter was dry and cool (temperature, 7.9 to 21.9°C; rainfall, 2.00 mm) but not wet. It is not known what direct effect season has on the recovery of salmonellae as the shed conditions were not directly measured. A seasonal effect was observed in this study, which is consistent with findings from other studies (9). It is important to note that to maintain flock health and welfare, sheds in this study were cooled or warmed to maintain even flock housing conditions that may not directly replicate the outside environmental conditions.

Despite the small number of sheds intensively sampled, many samples were collected frequently from the same locations within the sheds. The analyses demonstrated that salmonellae were heterogeneously distributed within the shed and that the distributions differed by serovar. The absence of spatial autocorrelation by location supports the conclusion that specific microenvironmental conditions are likely to be important in the survival or persistence of *S. enterica* within the shed. The presence of different serovars in specific locations and sample types supports the hypothesis that there are differences between bird contact surfaces and other environmental surfaces for the detection of *S. enterica*.

Consequently, it is critical where sampling is conducted because the presence or survival of salmonellae within the shed does not appear to be a random event. The effectiveness of detecting salmonellae varied by shed, and this variation was related to the overall sample prevalence in the flock and, therefore, the level of contamination in a particular shed. As the shed environment accounted for 60% of the variation in the sample prevalence within the shed, it is critical to consider the shed environment and its structure (bird contact versus noncontact surfaces) in designing the sampling methodology.

The use of a multisample-type sampling strategy is not novel and is considered to be a standard sampling approach. This approach was used to allow comparison with international studies using similar methods (1, 3, 5, 19). However, the strategy for collecting samples was novel in that we designed the sampling strategy to ensure that samples were collected homogeneously within the shed, taking into account the vertical and horizontal placement of birds within this space. A critical gap identified in the literature was where to collect samples from (other than randomly), and there is robust discussion about whether it is better to take a small sample more frequently from multiple locations or use a large pooled sample of, for example, dust (1, 5). An underlying assumption with such discussions seems to be that salmonellae are homogeneously distributed within the shed and that the main variable to consider when sampling is the quantity of sample to collect of each sample type, assuming therefore

that it is representative of a certain number of birds housed within an environment. However, the findings of this study clearly demonstrate that the location within a shed as well as the serovar is an important variable to consider when sampling as well as variables already identified.

Additionally, sampling was conducted so that it was repeatable, and identical locations were repeatedly sampled at every sampling event to ensure that any novel salmonella introduction was detected as early as possible. Samples were deliberately collected systematically from within the shed at person height (tier two of each frame) to ensure that all frames of birds were sampled within easy reach, assuming dust samples are heavy and that any dust present at one level is from both the tier sampled and possibly the tiers above. Birds at this height are exposed to more interaction with people as they are in easy view, and it may be hypothesized that they experience more stress, resulting in more shedding of salmonellae.

Multiple sites, and thus different sample types, were selected from each frame to maximize the opportunity to detect salmonellae. Manure belt samples were obtained at the end of each frame. Samples were obtained from egg belts from one tier of each frame, and dust was collected from the same tier. Floor samples were collected from each half of the shed. It was hypothesized that samples from these sites, except boot swabs, were less likely to be cross-contaminated by movements of people in and out of the shed.

We included in this surveillance design the use of boot swabs on concrete floors. Boot swabs are typically used on litter or slatted floors in free-range layer sheds or broiler production sheds (14), but this study demonstrated that they are also a very sensitive method of sampling in the cage environments. Boot swabs were more likely to test positive for *S. Infantis* than for *S. Typhimurium*, which may reflect better survival of *S. Infantis* in the floor environment. Finally, manure samples were collected by directly sampling the end of the manure belts, where they are more easily accessed, rather than by collecting a pooled sample of feces. Depending on the shed design, access to fresh manure under cages can be very difficult; additionally, the timing of sampling is critical as manure belts may have been cleaned just prior to sampling, which will limit access to an adequately representative manure sample. Manure belt samples were as effective as dust samples for salmonella detection, and there was no difference in the detection of the two *S. enterica* serovars. Dust or manure belt swabs were easier to collect and preferable to handling large quantities of manure for collection, laboratory testing, and disposal.

These specific sampling sites were chosen to ensure that the location and type of sampling were repeatable on all sampling occasions by all samplers, and a 3-week interval between sampling events was chosen because it is known that shedding of salmonellae in newly infected flocks may resolve quickly, particularly if the infectious dose is low (31–33). It is important to note that, while obvious, birds housed in caged environments cannot roam freely within the whole shed space. Additionally, birds are housed both vertically and horizontally. This means that if infection within the flock is not homogeneously distributed, then failing to sample the entire shed space may bias the sampling results, particularly if the prevalence is low. Other studies have reported that the number of samples positive (sample prevalence) may be lower in birds housed in higher tiers of the frames (34).

The positive predictive value of the overall sampling strategy was high (98%), even when an imperfect test is accounted for. Each of the sample types performed well, with the key exception of egg belt swabs in this study, where the likelihood of detection was lower. Egg belts in these sheds were constructed of a polyethylene plastic material, and this may have hindered detection because the surface is prone to rapid drying and may not be a suitable environment for long-term survival. Also, it is likely that insufficient egg belt surfaces were swabbed. The egg belt is the only surface in these sheds that was not as readily cross-contaminated with dust or environmental material as the other exposed surfaces because it is protected from above.

A number of other potential factors were deliberately not included in this study but

may affect the presence of *S. enterica* within the shed as a consequence, or reflection of, bird stress. The higher prevalence of positive environmental samples on northern or sheltered aspects (hotter, in the Southern Hemisphere) of the shed may be an indicator of increased stress in the flock, with consequential increased shedding in that part of the flock, or it may indicate preferential long-term survival of salmonellae under those environmental conditions. Unfortunately, internal shed environmental records were available only for the whole shed rather than for specific areas of the shed, so these effects could not be investigated further.

These findings are particularly relevant to other housing designs, such as aviary housing where birds may be housed freely but have both vertical and horizontal access within a shed environment, in a similar organizational arrangement to birds in cage sheds, which makes sampling individual birds significantly more challenging. The sampling principles described here are easily applicable to these environments with the identical sampling considerations applied to the space.

Conclusion. This study confirmed that the number of samples chosen is important for *S. enterica* detection, but of greater importance is how and where samples are collected from within the environment within a caged-flock shed. *S. enterica* distribution within a shed is not homogenous, and the location of *S. enterica* serovars within a shed does not appear to be random. Factors influencing detection include the season, the weather (low rainfall and moderate temperatures), and the shed aspect (north or south side) or sheltering by another building. A multisample-type approach has high positive predictive value even when the diagnostic test sensitivity is low and the environmental prevalence is low.

S. enterica serovars were detected in different spatial locations within the shed, indicating that specific microenvironments may enhance survival and possibly multiplication and subsequent detection. All of these factors should be taken into account in designing a surveillance strategy for the detection of salmonellae in caged flocks. In sheds of this type, multiple samples should be collected from different areas, preferably in close contact with the birds, focusing on manure belts, dust, and boot swabs. If resources limit the number and type of sample that can be collected and processed, then boot swabs are a good first choice of sampling material. Regardless of the sample type chosen, sampling must be conducted to ensure that the whole shed space is sampled as homogeneously as possible.

MATERIALS AND METHODS

Study population. Poultry producers representing 19 cage layer production flocks from farms in Victoria, Australia, with a history of infection with salmonellae volunteered to participate in this study (35, 36). Each flock was housed as a single age group, with all-in all-out management. Each flock was placed in the shed after cleaning and disinfection of the shed prior to the onset of laying between 16 and 19 weeks of age.

Samples were collected over a 4-year period, from 2014 to 2018, with the following months aggregated for each Southern Hemisphere season: summer, December to February; autumn, March to May; winter, June to August; and spring, September to November. This study was conducted under normal farming operating conditions to evaluate routinely conducted sampling procedures for surveillance purposes. All environmental sampling was conducted as part of routine production management as recommended by standard industry procedures and guidelines (15, 37–39). Participation in the *Salmonella enterica* serovar Enteritidis surveillance program entails a multisample-type approach similar to that of the European (40) and U.S. poultry improvement programs (12), with sampling of flocks conducted toward the end of flock life. As all samples were collected as part of routine veterinary care and agricultural practice, this study did not require ethics approval (41).

Bird housing and shed design. Birds were housed in compliance with the Australian model code of practice for poultry (38) and state legislation (42). Birds were housed in multitiered frames in either enriched colony cages, with one cage width per frame, or conventional cages, with two cages back to back per frame width. Colony cage sheds housed ~24,000 birds in eight frames, 3- to 4 tiers high, in 48 colonies with each containing ~500 birds. Conventional cage sheds housed ~65,000 birds in 5 frames 6 tiers high, with each cage containing ~5 to 6 birds. Cages were of European design and construction from two major suppliers of cage equipment.

Sheds of the same type were identical in design and construction, only varying in their positioning next to another shed and whether the long length of the shed was exposed or sheltered by another shed (Fig. 2). Sheds were ~130 m by 30 m in dimension with concrete floors. Walls had short concrete walls from the floor (~600 mm) topped by an aluminum-bonded insulated panel to the ceiling with no

FIG 2 Shed arrangement and orientation. (A) Side-by-side shed arrangement. (B) Linear shed arrangement. For both panels, orientations are indicated on the figure as follows: A, northern aspect; B, southern aspect between sheds; C, northern aspect between sheds; D, southern aspect. The cool pad locations (E) tunnel ventilation direction of airflow (F) are also indicated.

exposed joists or framing. Ceilings were constructed of the same material as the walls and were greater than 6 m in height. Sheds were climate controlled with evaporative cooling (cool pads and fans) and heat exchange for warming. Eggs were automatically collected from nest boxes or cages to an egg belt delivered to a vertical elevator at the end of each frame where they were transported (via egg belts) to a central egg-packing room. Manure was collected on belts under the cages and removed at least weekly from one end of the shed. All sheds were placed with the same orientation to the sun, with the long length of the shed positioned west-east (Southern Hemisphere).

Sample size and prevalence calculations. The overall study was designed to detect the novel introduction of *Salmonella* spp. within a shed environment. Sample size was estimated for three low-to-moderate design prevalence estimates of 1, 5, or 10 *S. enterica*-positive units per 100 units at risk (1, birds; 2, cages; 3, environmental sites) with 95% confidence that the estimated prevalence was within 5% of the true prevalence in the unit tested, assuming an imperfect test.

To identify the minimum and maximum numbers of samples to collect for the given prevalence estimates, based on the unit at risk, two sample size calculation methodologies were used. To estimate the number of birds to sample, calculation of the number of samples needed to demonstrate freedom from infection using an imperfect test was used (43, 44). For the number of cage or environmental sites to be sampled, the true prevalence assuming an imperfect test was used (29, 45). For sample size calculations, diagnostic test sensitivity and specificity for *S. enterica* by culture were assumed to vary between 0.88 and 0.98 and between 0.99 and 1.00, respectively (46–48).

Prevalence estimates based on the results of testing (apparent prevalence) were distinguished from prevalence estimates corrected on the basis of imperfect diagnostic test performance (true prevalence) (49, 50). For the calculation of true prevalence, test sensitivity was assumed to be 0.88, and specificity was assumed to be 0.995.

Sample size estimation. The number of samples required to achieve 95% confidence that the estimate of prevalence was within 5% of the true population prevalence for each unit of interest (1, birds; 2, cages; 3, environment) is summarized in Table S1 in the supplemental material. At least 28 cages or environmental samples are required to estimate the *Salmonella* sp. sample prevalence with sufficient confidence at the lowest design prevalence. At the lowest design prevalence, at either diagnostic test sensitivity, the number of birds to be sampled was greater than the precision of the sample calculation and unable to be calculated.

Environmental sample sites. A total of 168 potential sampling sites were identified within each shed. Sampling sites considered were those with either direct bird contact or immediately within the bird surroundings. Sites were excluded from consideration where repeated sampling of the exact sites within the shed could not be guaranteed. These sampling sites included manure belts, frame surfaces, feed lines or feeders, nest box surfaces, fan covers, floor and wall surfaces, and egg belts. Sites were identified as either bird contact areas (hypothesized to reflect flock infection status), which are those immediately within the bird surroundings such as manure belts, egg belts, nest box surfaces, or the framing, or non-bird contact areas (hypothesized to reflect shed contamination status) indirectly contaminated by dust, dander, or feed residue such as walls, floors, fan covers, feed lines. Potential bird contact sampling locations are indicated in Fig. 3.

Environmental sampling methodology. Sheds were sampled immediately postcleaning (dry shed), within 2 weeks of placement of a new flock, and subsequently every 3 weeks until the end of the flock life. On each sampling occasion, 28 or 29 samples were collected, consisting of 8 to 10 egg belt samples, 8 to 10 dust samples, 5 to 8 manure belt samples, and 4 boot swabs. As caged birds are housed horizontally and vertically within a three-dimensional space, to ensure spatial homogeneity in sampling, all horizontal frames of the shed were sampled. The total number of samples collected per shed was dictated by the shed design to ensure that each vertical frame housing birds was sampled as long as the number was no less than the sampling design.

Four 10-by-10-cm cotton gauze swabs, premoistened with buffered peptone water, were used to collect each surface sample. Manure belt samples were collected by wiping the exposed edge of all belts

FIG 3 Bird contact sampling locations within cage house for each cage tier and cage frame (colony cage style illustrated). Green, boot swab; light blue, manure belt; orange, egg belt; dark blue, dust.

at one end of the shed, avoiding excessive shed dust and debris and concentrating on exposed fecal material on the leading surface and immediately under the belt rather than on the top exposed surface. Egg belts were sampled by wiping the length of the bottom surface of the egg belt from a single tier. Dust samples were collected by wiping the surface of the nest box the length of the frame. Clean dry boots, with new plastic boot covers, were worn on entry to the shed. Two pairs of boot swabs were worn by persons walking within the shed during sample collection. The second pair was exchanged during the midpoint of sampling the shed. Each sample type was pooled separately by frame and cage row into a Whirlpak bag and identified by shed, flock, sample type, and sample location. Samples were refrigerated immediately after collection.

Surface area sampled. On each sampling occasion, between 208 and 224 m² of surface area per shed was sampled. The total surface area of each shed sampled by sample type is described in Table S2.

Sample processing and microbiology. All samples were collected and then transported at 4°C to the laboratory for processing the same day. Each sample was processed separately; no samples were pooled. All samples were cultured in accordance with the Australian Standard 5013.10-2009 (ISO 6579:2002 MOD) for the detection of *Salmonella* spp. from environmental samples (51). Microbiological results were reported as positive or negative for *Salmonella* spp. for each sample collected (positive/sampling unit). All samples were processed in accordance with the Australian Standard 5013.10-2009 horizontal method for the detection of *Salmonella* spp. (ISO 6579:2002, MOD) (51). At least three isolates (single-colony pick) from each sample were confirmed as *S. enterica*, *S. enterica* subspecies *enterica* serovar Typhimurium or *S. enterica* subspecies *enterica* serovar Infantis by real-time PCR (Table S12) using previously published methods with no modifications to the primer design or method (52–54). Isolates of *S. Typhimurium*, *S. Agona*, and *S. Infantis* were used as positive controls, and *Escherichia coli* (NTCC 10418) (55) was used as a negative control. Representative isolates unable to be typed using PCR were submitted to the Victorian *Salmonella* Reference Laboratory (Microbiological Diagnostic Unit [MDU], University of Melbourne) for serotype confirmation. The full sample processing methodology is available online (56), and details are provided in the supplemental material (“Microbiological methods”).

Statistical analysis. All analyses were conducted in the R statistical package unless otherwise stated (57). Spatial analysis was conducted using the following packages: *spsurvey* (58), *rgeos* (59), *sparr* (60), and *spatstat* (61, 62). Measures of association, odds ratios, and positive predictive values were estimated using *epiR* (63). The R packages *nlme*, *lme4*, and *aod* were used for hierarchical mixed-effects model building (64–66).

Assessment of sample site homogeneity. The spatial distribution of *S. enterica* serovars within each shed was evaluated by aggregating all sampling events for each shed. Spatial heterogeneity was evaluated by spatial point pattern analysis and calculation of the spatial density (kernel) of each of the sampled sites using *spatstat* (61, 62). The distribution of *S. enterica* within the shed was visualized using heat maps to plot the calculated density at each sampling location within a two-dimensional representation of the shed environment. Spatial autocorrelation was evaluated using Moran’s I from the *ape* package (67), and the resulting variogram was visualized using *geoR* (68).

Univariate analysis. There were two outcomes of interest: (i) binary, i.e., the presence (1) or absence (0) of salmonellae in a sample, and (ii) the true sample prevalence at a given sampling event. Only explanatory variables associated with the sampling methodology and the housing environment were considered in this analysis. A full description of each variable considered for analysis is available in Table S10.

Measures of association between the binary outcome of interest and the explanatory variables were computed using the odds ratio. For categorical or continuous variables, simple linear or logistic regression was used where appropriate. If an explanatory variable was statistically associated with the binary outcome of interest in the univariate analysis ($P < 0.10$), it was considered for inclusion in the multivariable analysis.

Multivariable hierarchical mixed-effects logistic regression. Due to the hierarchical and longitudinal nature of the data (Table S11), with samples clustered within sampling events and sampling events clustered within sheds and sheds within farms, the model was extended to include sample event-, shed-, and farm-level random effect terms, where appropriate. This approach was utilized to ensure that the variance present under these analysis conditions is accounted for (69). Factors influencing the detection of *S. enterica* were categorized into three classes: those operating at the sample level, those at the sample event level, and those at the shed level. Sample-level effects considered included the sample type,

sampling location, whether the sample was collected from the northern aspect of a shed, or whether the sampling location in a shed was between two sheds. Sampling event-level effects included the month of sampling, the season of sampling event, and the weather (including rainfall, temperature, and solar radiation at the time of sampling or the 3 weeks prior to sampling). For this analysis, the shed-level effects considered were the *Salmonella* status of the previous flock and the presence of salmonellae in the shed prior to the onset of the study and the flock. Two fixed-effects multivariable logistic regression models were built according to which the probability of a sample being positive for *S. enterica* at a sampling event was parameterized as a function of the significant explanatory variables ($P < 0.10$) identified in the univariate analysis. Model 1 was built using all available data for all 19 flocks. Model 2 was built for the subset of 4 flocks that had intensive sampling for the life of the flock; the first 13 sampling events of these four flocks were included in this analysis.

The models were built in a stepwise manner with all variables included initially and nonsignificant explanatory variables removed sequentially from the model until all variables retained in the model were significant at an α of <0.05 . Variables removed from the model were retested in the final model and retained if their inclusion changed the regression coefficients significantly, using the Wald test. Where no significant difference was observed between two models, then the most parsimonious model was selected. Biologically plausible interactions were considered. Autocorrelated measures were identified and tested individually in the model; only one was kept in the model if it remained significant.

The assumptions of normality and homogeneity of variance of the final model were checked using frequency histograms of the residuals and plots of the residuals versus predicted values. The Hosmer-Lemeshow goodness of fit test was used to evaluate the fit of the final model, and the diagnostic accuracy was computed by evaluating the receiver operator curve (ROC) from the model estimates.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00333-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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REFERENCES

- Carrique-Mas JJ, Breslin M, Sayers AR, McLaren I, Arnold M, Davies R. 2008. Comparison of environmental sampling methods for detecting *Salmonella* in commercial laying flocks in the UK. *Lett Appl Microbiol* 47:514–519. <https://doi.org/10.1111/j.1472-765X.2008.02450.x>.
- Arnold ME, Carrique-Mas JJ, McLaren I, Davies RH. 2011. A comparison of pooled and individual bird sampling for detection of *Salmonella* in commercial egg laying flocks. *Prev Vet Med* 99:176–184. <https://doi.org/10.1016/j.prevetmed.2010.12.007>.
- Carrique-Mas JJ, Davies RH. 2008. Sampling and bacteriological detection of *Salmonella* in poultry and poultry premises: a review. *Rev Sci Tech* 27:665–677. <https://doi.org/10.20506/rst.27.3.1829>.
- Van Hoorebeke S, Van Immerseel F, De Vylder J, Ducatelle R, Haesebrouck F, Pasmans F, Dewulf J. 2009. Faecal sampling underestimates the actual prevalence of *Salmonella* in laying hen flocks. *Zoonoses Public Health* 56:471–476. <https://doi.org/10.1111/j.1863-2378.2008.01211.x>.
- Arnold ME, Carrique-Mas JJ, Davies RH. 2010. Sensitivity of environmental sampling methods for detecting *Salmonella* Enteritidis in commercial laying flocks relative to the within-flock prevalence. *Epidemiol Infect* 138:330–370. <https://doi.org/10.1017/S0950268809990598>.
- Arnold ME, Martelli F, McLaren I, Davies RH. 2014. Estimation of the rate of egg contamination from *Salmonella* infected chickens. *Zoonoses Public Health* 61:18–27. <https://doi.org/10.1111/zph.12038>.
- Carrique-Mas JJ, Breslin M, Snow L, Arnold ME, Wales A, McLaren I, Davies RH. 2008. Observations related to the *Salmonella* EU layer baseline survey in the United Kingdom: follow-up of positive flocks and sensitivity issues. *Epidemiol Infect* 136:1537–1546. <https://doi.org/10.1017/S095026880700012X>.
- Arnold ME, Martelli F, McLaren I, Davies RH. 2014. Estimation of the sensitivity of environmental sampling for detection of *Salmonella* in commercial layer flocks post-introduction of national control programs. *Epidemiol Infect* 142:1061–1069. <https://doi.org/10.1017/S0950268813002173>.
- Davies R, Breslin M. 2001. Environmental contamination and detection of *Salmonella enterica* serovar Enteritidis in laying flocks. *Vet Rec* 149:699–704.
- Davies RH, Wray C. 1996. Determination of an effective sampling regime to detect *Salmonella* Enteritidis in the environment of poultry units. *Vet Microbiol* 50:117–127. [https://doi.org/10.1016/0378-1135\(96\)00031-4](https://doi.org/10.1016/0378-1135(96)00031-4).
- Arnold ME, Papadopoulou C, Davies RH, Carrique-Mas JJ, Evans SJ, Hoinville LJ. 2010. Estimation of *Salmonella* prevalence in UK egg-laying holdings. *Prev Vet Med* 94:306–309. <https://doi.org/10.1016/j.prevetmed.2010.01.004>.
- USDA Animal and Plant Health Inspection Service. 2017. National poultry improvement plan (NPIP). <https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/nvap/Reference-Guide/Poultry/National-Poultry-Improvement-Plan>.
- European Commission. 2003. Directive 2003/99/EC of the European Parliament and of the Council of 17 November 2003 of zoonoses and zoonotic agents, amending Council Directive 90/42/EEC and repealing Council Directive 92/117/EEC. <http://data.europa.eu/eli/dir/2003/99/oj>.
- European Commission. 2003. Regulation (EC) no 2160/2003 of the European parliament and of the council of 17 November 2003 on the control of salmonella and other specified food-borne zoonotic agents. <http://data.europa.eu/eli/reg/2003/2160/oj>.
- Arzey G. 2005. Joint NSW/VIC *Salmonella* Enteritidis monitoring and accreditation program (JNSW/VIC SEMAP). Department of Primary Industries, Orange, NSW, Australia.
- Wales A, Breslin M, Davies R. 2006. Semiquantitative assessment of the distribution of *Salmonella* in the environment of caged layer flocks. *J Appl Microbiol* 101:309–318. <https://doi.org/10.1111/j.1365-2672.2006.02916.x>.
- Wales A, Breslin M, Carter B, Sayers R, Davies R. 2007. A longitudinal study of environmental *Salmonella* contamination in caged and free-range layer flocks. *Avian Pathol* 36:187–197. <https://doi.org/10.1080/03079450701338755>.
- Davies RH, Wray C. 1996. Seasonal variations in the isolation of *Salmo-*

- nella Typhimurium, Salmonella Enteritidis, Bacillus cereus and Clostridium perfringens from environmental samples. Zentralbl Veterinärmed B 43:119–127. <https://doi.org/10.1111/j.1439-0450.1996.tb00295.x>.
19. Kinde H, Castellán DM, Kerr D, Campbell J, Breitmeyer R, Ardans A. 2005. Longitudinal monitoring of two commercial layer flocks and their environments for Salmonella enterica serovar Enteritidis and other Salmonellae. Avian Dis 49:189–194. <https://doi.org/10.1637/7228-062704R>.
 20. Davies RH, Breslin M. 2003. Persistence of Salmonella Enteritidis phage type 4 in the environment and arthropod vectors on an empty free-range chicken farm. Environ Microbiol 5:79–84. <https://doi.org/10.1046/j.1462-2920.2003.00387.x>.
 21. Davies RH, Wray C. 1996. Persistence of Salmonella Enteritidis in poultry units and poultry food. Br Poult Sci 37:589–596. <https://doi.org/10.1080/00071669608417889>.
 22. Payne JB, Li X, Santos FBO, Sheldon BW. 2006. Characterization of Salmonella from three commercial North Carolina broiler farms. Int J Poult Sci 5:1102–1109. <https://doi.org/10.3923/ijps.2006.1102.1109>.
 23. Ellis JR, McCalla TM. 1978. Fate of pathogens in soils receiving animal wastes—a review. Trans ASAE 21:309–313. <https://doi.org/10.13031/2013.35294>.
 24. Williams JE, Benson ST. 1978. Survival of Salmonella Typhimurium in poultry feed and litter at three temperatures. Avian Dis 22:742–747. <https://doi.org/10.2307/1589652>.
 25. Kingston DJ. 1981. A comparison of culturing drag swabs and litter for identification of infections with Salmonella spp in commercial chicken flocks. Avian Dis 25:513–516. <https://doi.org/10.2307/1589943>.
 26. Skov MN, Carstensen B, Tornøe N, Madsen M. 1999. Evaluation of sampling methods for the detection of Salmonella in broiler flocks. J Appl Microbiol 86:695–700. <https://doi.org/10.1046/j.1365-2672.1999.00715.x>.
 27. Muñoz-Zanzi C, Thurmond M, Hietala S, Johnson W. 2006. Factors affecting the sensitivity and specificity of pooled-sample testing for diagnosis of low prevalence infections. Prev Vet Med 74:309–322. <https://doi.org/10.1016/j.prevetmed.2005.12.006>.
 28. Davies R, Breslin M. 2003. Observations on Salmonella contamination on commercial laying farms before and after cleaning and disinfection. Vet Rec 152:283–287. <https://doi.org/10.1136/vr.152.10.283>.
 29. Thrusfield M. 2007. Veterinary epidemiology, 3rd ed. Blackwell Science, Ltd., Oxford, United Kingdom.
 30. Aho M. 1992. Problems of Salmonella sampling. Int J Food Microbiol 15:225–235. [https://doi.org/10.1016/0168-1605\(92\)90053-6](https://doi.org/10.1016/0168-1605(92)90053-6).
 31. Sadler WW, Brownell JR, Fanelli MJ. 1969. Influence of age and inoculum level on shed pattern of Salmonella Typhimurium in chickens. Avian Dis 13:793–803. <https://doi.org/10.2307/1588587>.
 32. Hassan JO, Mockett APA, Catty D, Barrow PA. 1991. Infection and reinfection of chickens with Salmonella Typhimurium: bacteriology and immune responses. Avian Dis 35:809–819. <https://doi.org/10.2307/1591614>.
 33. Barrow PA, Simpson JM, Lovell MA. 1988. Intestinal colonisation in the chicken by food-poisoning Salmonella serotypes; Microbial characteristics associated with faecal excretion. Avian Pathol 17:571–588. <https://doi.org/10.1080/03079458808436478>.
 34. Gole VC, Caraguel CG, Sexton M, Fowler C, Chousalkar KK. 2014. Shedding of Salmonella in single age caged commercial layer flock at an early stage of lay. Int J Food Microbiol 189:61–66. <https://doi.org/10.1016/j.jfoodmicro.2014.07.030>.
 35. Crabb HK, Gilkerson JR, Browning GF. 2019. Does only the age of the hen matter in Salmonella enterica contamination of eggs? Food Microbiol 77:1–9. <https://doi.org/10.1016/j.fm.2018.08.006>.
 36. Crabb HK, Allen JL, Devlin JM, Firestone SM, Wilks CR, Gilkerson JR. 2018. Salmonella spp. transmission in a vertically integrated poultry operation: clustering and diversity analysis using phenotyping (serotyping, phage typing) and genotyping (MLVA). PLoS One 13:e0201031. <https://doi.org/10.1371/journal.pone.0201031>.
 37. Food Regulation Standing Committee. 2006. Australian standard for construction of premises and hygienic production of poultry meat for human consumption. AS 4465:2005. CSIRO Publishing, Collingwood, Victoria, Australia.
 38. Primary Industries Standing Committee. 2002. Model code of practice for the welfare of animals: domestic poultry, 4th ed. CSIRO Publishing, Collingwood, Victoria, Australia.
 39. Food Standards Australia New Zealand. 2012. Primary production and processing standard for poultry meat. <https://www.legislation.gov.au/Details/F2012L00292>.
 40. European Commission. 2005. Commission regulation (EC) No 1003/2005 of 30 June 2005 implementing Regulation (EC) No 2160/2003 as regards a Community target for the reduction of the prevalence of certain salmonella serotypes in breeding flocks of Gallus and amending regulation (EC) No 2160/2003. <https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32005R1003>.
 41. University of Melbourne Office of Research Ethics and Integrity. 2016. Animal care and use standards. <https://staff.unimelb.edu.au/research/ethics-integrity/animal-ethics/animal-care-and-use-standards>.
 42. State of Victoria Department of Agriculture. 2016. Prevention of cruelty to animals (domestic fowl) regulations 2016. S.R. no. 122/2016. <http://agriculture.vic.gov.au/agriculture/animal-health-and-welfare/animal-welfare/animal-welfare-legislation/victorian-codes-of-practice-for-animal-welfare/prevention-of-cruelty-to-animals-domestic-fowl-regulations-2006>.
 43. Cameron AR, Baldock FC. 1998. A new probability formula for surveys to substantiate freedom from disease. Prev Vet Med 34:1–17. [https://doi.org/10.1016/S0167-5877\(97\)00081-0](https://doi.org/10.1016/S0167-5877(97)00081-0).
 44. Cameron AR. 1999. Survey toolbox for livestock diseases—a practical manual and software package for active surveillance of livestock diseases in developing countries. Australian Centre for International Agricultural Research, Canberra, Australia.
 45. Humphry RW, Cameron A, Gunn GJ. 2004. A practical approach to calculate sample size for herd prevalence surveys. Prev Vet Med 65:173–188. <https://doi.org/10.1016/j.prevetmed.2004.07.003>.
 46. Kuijpers AFA, van de Kastelee J, Mooijman KA. 2012. EU Interlaboratory comparison study animal feed II (2012): detection of Salmonella in chicken feed. National Institute for Public Health and the Environment, Bilthoven, Netherlands.
 47. Kuijpers AFA, Mooijman KA. 2011. EU Interlaboratory comparison study veterinary XIV (2011): detection of Salmonella in chicken faeces. National Institute for Public Health and the Environment, Bilthoven, Netherlands.
 48. Kuijpers AFA, Mooijman KA. 2014. EU Interlaboratory comparison study primary production XVI (2013): Detection of Salmonella in chicken faeces adhering to boot socks. National Institute for Public Health and the Environment, Bilthoven, Netherlands.
 49. Rogan WJ, Gladen B. 1978. Estimating prevalence from the results of a screening test. Am J Epidemiol 107:71–76. <https://doi.org/10.1093/oxfordjournals.aje.a112510>.
 50. AusVet. 2015. Estimated true prevalence and predictive values from survey testing. <http://epitools.ausvet.com.au/content.php?page=TruePrevalence>.
 51. Australian Department of Agriculture and Water Resource. 2009. Microbiology of food and animal feeding stuffs: horizontal method for the detection of Salmonella spp. AS 5013.10-2009. SAI Global, Sydney, Australia.
 52. Shanmugasundaram M, Radhika M, Murali HS, Batra HV. 2009. Detection of Salmonella enterica serovar Typhimurium by selective amplification of fliC, fliB, iroB, rfbJ, STM2755, STM4497 genes by polymerase chain reaction in a monoplex and multiplex format. World J Microbiol Biotechnol 25:1385–1394. <https://doi.org/10.1007/s11274-009-0025-3>.
 53. Kardos G, Farkas T, Antal M, Nógrády N, Kiss I. 2007. Novel PCR assay for identification of Salmonella enterica serovar Infantis. Lett Appl Microbiol 45:421–425. <https://doi.org/10.1111/j.1472-765X.2007.02220.x>.
 54. Malorny B, Hoorfar J, Bunge C, Helmuth R. 2003. Multicenter validation of the analytical accuracy of Salmonella PCR: towards an International Standard. Appl Environ Microbiol 69:290–296. <https://doi.org/10.1128/AEM.69.1.290-296.2003>.
 55. Bell SM, Pham JN, Newton P, Nguyen TT. 2013. A manual for medical and veterinary laboratories 2013, 6th ed. Prince of Wales Hospital, Randwick, NSW, Australia.
 56. Crabb HK. 2018. Poultry enterprise environmental sample handling and processing for Salmonella spp. detection. <https://www.protocols.io/view/poultry-enterprise-environmental-sample-microbiolo-n6sdheh>.
 57. R Core Team. 2016. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org/>.
 58. Kincaid TM, Olsen AR. 2016. spsurvey: spatial survey design and analysis. <https://cran.r-project.org/web/packages/spsurvey/index.html>.
 59. Bivand R, Rundel C, Pebesma E, Stuetz R, Hufhamer KO. 2017. rgeos: interface to geometry engine—open source (GEOS), v0.3-22. <https://cran.r-project.org/web/packages/rgeos/index.html>.
 60. Tilman MD, Hazelton ML, Marshall JC. 2011. sparr: analyzing spatial

- relative risk using fixed and adaptive kernel density estimation in R. *J Stat Softw* 39:1–14.
61. Baddeley A, Rubak E, Turner R. 2015. Spatial point patterns: methodology and applications with R. Chapman and Hall/CRC Press, London, United Kingdom.
 62. Baddeley A, Turner R. 2005. spatstat: an R package for analysing spatial point patterns. *J Stat Softw* 12:1–42.
 63. Stevenson M, Nunes T, Heuer C, Marshall J, Sanchez J, Thornton R, Reiczgel J, Robison-Cox J, Sebastiani P, Solymos P, Yoshida K, Jones G, Pirikahu S, Firestone S. 2015. epiR: tools for the analysis of epidemiological data, v0.9-69. <https://rdrr.io/cran/epiR/>.
 64. Bates D, Maechler M, Bolker B, Walker S, Christensen BBB, Singmann H, Grothendieck G. 2015. Package 'lme4': linear mixed-effects models using 'Eigen' and S4, v1.1-10. <https://cran.r-project.org/web/packages/lme4/index.html>.
 65. Lesnoff M, Lancelot R. 2012. Package 'aod': analysis of overdispersed data, vR package version 1.3. <http://cran.r-project.org/package=aod>.
 66. Pinheiro J, Bates D, DebRoy S, Sarkar D, Team RC. 2015. Package 'nlme': linear and non-linear mixed effects models, vR package version 3.1-122. <https://cran.r-project.org/package=nlme>.
 67. Paradis E, Claude J, Strimmer K. 2004. APE: analyses of phylogenetics and evolution in R language. *Bioinformatics* 20:289–290.
 68. Ribeiro PJ, Jr, Diggle PJ. 2016. geoR: analysis of geostatistical data, v1.7-5.2. <https://cran.r-project.org/web/packages/geoR/index.html>.
 69. Dohoo I, Martin W, Stryhn H. 2009. Veterinary epidemiologic research, 2nd ed. VER, Inc., Charlottetown, Prince Edward Island, Canada.