Intra-cluster correlations for ceca *Salmonella* prevalence and enumeration from 40 experimental floor pen trials in broiler chickens using a seeder bird challenge model

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ABSTRACT Floor pen trials are an efficient way to evaluate the effectiveness of potential *Salmonella* control interventions in broiler chickens. When treatments are allocated at the pen level, and outcomes are measured at the individual bird level, floor pen studies are considered to be cluster randomized trials. Estimating the sample size required to achieve a desired level of statistical power for a cluster randomized trial requires an estimate of the intra-cluster correlation (**ICC**) as an input. In this study, ICCs were estimated for the untreated challenged control group from 40 broiler chicken *Salmonella* pen trials performed using a seeder bird challenge model. The ICCs for ceca *Salmonella* prevalences ranged from 0.00 to 0.64, with a median of 0.17. The ICCs for ceca *Salmonella* $\log_{10}(\text{MPN/g} + 1)$ ranged from 0.00 to 0.52, with a median of 0.14. These findings indicate that the effect of pen-level clustering is substantial in *Salmonella* floor pen trials, and it must be considered during both the study design and analysis. In a multivariable regression analysis, ICCs for ceca *Salmonella* prevalences were associated with the challenge status of sampled birds, age of birds at the time of challenge, and *Salmonella* serovar. ICCs were lower for studies in which a combination of direct (seeder) and indirect (horizontal) challenged birds were sampled, and for studies in which birds were challenged on the day of hatch or at one day of age. ICCs were higher for studies in which *Salmonella* Heidelberg was used as the challenge strain. These findings may be useful for investigators that are planning pen trials to evaluate *Salmonella* control interventions in broiler chickens. Choosing study design elements associated with a lower ICC may improve efficiency by leading to a larger effective sample size for the same number of experimental units.

Key words: broiler, *Salmonella*, pen trial, intra-cluster correlation

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INTRODUCTION

Nontyphoidal *Salmonella* has been estimated to cause in excess of 1 million foodborne illnesses, 19,000 hospitalizations, and 378 deaths in the United States each year (Scallan et al., 2011). Despite significant public health efforts to reduce *Salmonella* infections, the incidence in 2019 was estimated at 17.1 cases per 100,000 persons per year, which was a 5% increase compared to the period from 2016 to 2018 (Tack et al., 2020). While the relative frequency of detection for certain poultry-associated *Salmonella enterica* serotypes, including Typhimurium and Heidelberg, has decreased since 1996 to 1998, poultry is still considered to be an important source of human infections (Tack et al., 2020). One recent study estimated that chickens and eggs account for 10% and 12%, respectively, of all foodborne *Salmonella* illnesses in the United States (Batz et al., 2021).

Historically, *Salmonella* control efforts in broiler chickens have focused on processing (USDA-FSIS, 1996). In recent years, however, there has been increasing recognition that *Salmonella* prevalences and loads on the farm are positively associated with those at slaughter (Berghaus et al., 2013). Consequently, there is a desire to identify on-farm interventions such as vaccination that can effectively reduce *Salmonella* in the farm environment (Hofacre et al., 2021). Evaluating potential interventions in a commercial poultry farm environment is challenging because the scale of production is large, management is clustered at the level of the farm and house, and natural exposures to pathogens such as *Salmonella* are intermittent. Therefore, potential interventions are usually first evaluated in a small-scale controlled environment where birds can be administered a known disease challenge.

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Floor pen trials are commonly used to assess interventions in broiler chickens with a microenvironment that is similar to that encountered in large scale broiler production (Byrd et al., 2008; Cerisuelo et al., 2014; Fasina et al., 2021). Birds are placed on litter in small pens at a density that is consistent with that used in a production environment, and the pens are randomly allocated to different experimental treatments. Because the unit of randomization is the pen, floor pen studies that utilize measurements from individual birds such as *Salmonella* prevalences and loads are considered to be cluster randomized trials (Cornfield, 1978; Donner and Klar, 2000). This has implications for the study design and analysis because the responses of birds within the same pen are more similar to one another than they are to those of birds in different pens. That is, the responses of birds within the same pen are correlated. Intuitively, this makes sense because birds in the same pen share a common environment, and if one or more birds in the pen are shedding large numbers of *Salmonella*, then all of the other birds in that pen would share a high level of exposure. Practically, this means that many of the statistical methods investigators are familiar with from their early research training will not be appropriate for use with cluster randomized designs because such methods typically assume that all of the observations are statistically independent.

The magnitude of correlation between subjects (i.e., birds) within the same cluster (i.e., pen) is known as the intra-cluster correlation (**ICC**). The ICC is denoted using the Greek letter ρ, and it can be interpreted as the standard Pearson correlation between any 2 observations within the same cluster (Donner and Klar, 2000). The ICC can also be interpreted as the proportion of variance that is attributable to between-cluster variation, with possible values ranging between zero and one (Eldridge and Kerry, 2012). An ICC of zero implies that the responses of subjects within the same cluster are uncorrelated, and an ICC of one implies that the responses of all subjects within the same cluster are identical. In the context of floor pen trials, lower values of the ICC correspond to less variability between pens, and higher values of the ICC correspond to more variability between pens, in terms of the response.

From a study design standpoint, the magnitude of the ICC influences a study's effective sample size. If the ICC is zero, the effective sample size is equal to the number of individual birds because an ICC of zero implies that the responses of birds within the same pen are independent. If the ICC is one, the effective sample size is equal to the number of pens, because all of the birds in the same pen would have the same response. In most cases, the effective sample size for a cluster randomized trial lies somewhere between the number of clusters and the number of individual subjects, depending on the magnitude of the ICC.

It is for this reason that sample size calculations for cluster randomized trials require an estimate of the ICC as an input. One commonly used approach to account for clustering in sample size calculations is to inflate the sample size for each group that was calculated under the assumption of individual subject randomization by using the formula (Dohoo et al., 2009):

$$n' = n\,(1 + \rho\,(m - 1))$$

where n' is the cluster-adjusted sample size, n is the original sample size calculated under the assumption of independence, ρ is the ICC, and m is the number of subjects sampled per cluster.

Consequently, when one wishes to estimate the sample size required to achieve a certain level of statistical power for a *Salmonella* pen trial, it is necessary to provide an estimate of the ICC. To the authors' knowledge, however, estimates of ICCs for *Salmonella* pen trials in broiler chickens have not previously been published. Sometimes an investigator may have data from a previous trial that can be used to estimate the ICC, but it is difficult to know whether a single estimated value is representative of the population of ICCs that might be encountered in a larger number of similar trials. The primary objective of this study was to describe the distribution of ICCs for ceca *Salmonella* prevalences and log-transformed most probable number (**MPN**) per gram in the untreated challenged control group from 40 broiler chicken *Salmonella* pen trials that were conducted using a seeder bird challenge model. A secondary objective was to identify study characteristics that were associated with ICC.

MATERIALS AND METHODS

Selection of Studies

A consecutive sample of *Salmonella* pen trial studies performed at a private contract research facility (Southern Poultry Research Group, Inc., Watkinsville, GA) between March 2015 and February 2022 were included in the analysis. Studies were eligible for inclusion if: 1) the trial included 6 or more pens per treatment group with 10 or more birds sampled from each pen; 2) birds were challenged with *Salmonella* using a seeder bird challenge model by 7 d of age; and 3) ceca were collected when birds were between 35 and 46 d of age. Studies were excluded if the overall *Salmonella* prevalence in the untreated challenged control group was <5% or > 95%, since prevalences outside of this range limited the potential variability in responses between pens. Only data from the untreated challenged control group were analyzed for each study so that estimates of the ICCs would not be influenced by the inclusion of different interventions. Animal care practices for all studies conformed to the Guide for the Care and Use of Agricultural Animals in Research and Teaching (ADSA et al., 2020), and all studies were approved by the Southern Poultry Research Group Institutional Animal Care and Use Committee.

Housing

Broiler chickens were housed in modified conventional poultry houses with solid sides and concrete walkways.

Floor pens measured 1.5 m × 1.5 m (5 ft × 5 ft) or 1.5 m × 3.0 m (5 ft × 10 ft) with a stocking density of 0.09 m^2 (1.0 ft^2) per bird. Pens had dirt or concrete floors and were bedded with approximately 4 inches of fresh pine shavings at placement. Houses were fan cooled. Thermostatically controlled gas heaters were the primary heat source, and supplemental heat lamps were used when needed. Birds were raised under ambient humidity using a lighting program recommended by the primary breeder. Each pen contained one tube feeder and one bell drinker. Feed and water were supplied *ad libitum*.

Seeder Bird Challenge Model

In all studies, a seeder bird challenge model was used with one-half of the birds in each pen being tagged (color coded) and directly challenged by oral gavage of the individual birds with between 1×10^5 and 1×10^7 colony forming units of *Salmonella* per bird. The remaining birds in each pen were indirectly (horizontally) challenged by comingling them with the direct challenged birds.

Qualitative Ceca Salmonella *Cultures*

On the day of sampling, birds were euthanized by cervical dislocation and the ceca were aseptically removed. Ceca were placed in sterile plastic sample bags (Whirl-Pak; Nasco, Fort Atkinson, WI) and stored on wet ice until they were delivered to the onsite laboratory for *Salmonella* isolation. Upon arrival at the laboratory, ceca were weighed and 50 mL of tetrathionate broth (Difco, Division of Becton, Dickinson, and Co., Sparks, MD) was added to each sample prior to mixing by stomaching. A 3 mL sample of the stomachered mixture was removed for quantitative analysis, and the remaining volume was incubated overnight at 42°C. The next day, a 10 μL loopful of sample was struck onto xylose lysine tergitol-4 agar (**XLT-4**, Difco) plates with overnight incubation at 37°C. Up to 3 black colonies were selected and confirmed as *Salmonella* using Poly-O *Salmonella* specific antiserum (MiraVista, Indianapolis, IN). Samples with a negative primary enrichment culture were submitted to secondary enrichment by adding a 0.5 mL aliquot of the primary sample to 4.5 mL of tetrathionate broth with overnight incubation at 37°C. The next day, a loopful of sample was struck onto XLT-4 agar with overnight incubation at 37°C. One black colony from each plate was subsequently subcultured on blood agar and incubated overnight at 37°C before confirmation using *Salmonella*-specific antiserum.

Quantitative Ceca Salmonella *Cultures*

Quantitative cultures were performed using a micro-MPN procedure similar to that used in a previous study (Berghaus et al., 2013). Briefly, a 1 mL aliquot of the stomachered ceca samples was transferred to each of 3 adjacent wells in the first row of a 96-well 2 mL deep-well plate (VWR International, West Chester, PA). A 0.1 mL aliquot of the sample was then transferred to 0.9 mL of tetrathionate broth in the second row, and this process was repeated for the remaining rows to produce five 10-fold dilutions. Blocks were incubated for 24 hours at 42°C. One μL of each well was subsequently transferred to XLT-4 agar using a multi-channel pipet, and plates were incubated for 24 hours at 37°C. Suspect *Salmonella* isolates were confirmed by Poly-O *Salmonella*-specific antiserum. The number of positive replicates at each dilution was used to calculate the MPN of *Salmonella* per mL of media (Blodgett, 2020). The MPN per gram of ceca was calculated by multiplying the MPN/mL of media by the total volume of media added (e.g., 50 mL) and then dividing by the ceca weight in grams. Samples with a negative culture result by the MPN method but a positive culture result by primary or secondary enrichment were arbitrarily assigned an MPN value of 0.15 MPN/mL of media, which was approximately one-half the minimum detection limit of the MPN assay, for statistical analysis.

Statistical Methods

ICCs were calculated using the 1-way ANOVA estimator, which is appropriate for both binary and continuous outcomes (Donner, 1986; Ridout et al., 1999). Briefly, the ICC (ρ) was estimated using Stata's *loneway* command as:

$$\rho = \frac{\sigma_A^2}{\sigma_A^2 + \sigma_e^2} = (MSA - MSE)/(MSA + (n_0 - 1)MSE)$$
$$= (F - 1)/(F + n_0 - 1)$$

where σ_A^2 is the variance component due to the difference between groups (i.e., clusters); σ_e^2 is the variance component due to the difference between individuals within groups; MSA is the mean square among groups; MSE is the mean square error (within groups); $F = MSA/MSE$ is the variance ratio statistic from the 1-way ANOVA table; and

$$n_0 = \left(N - \sum_{i=1}^{k} n_i^2 / N \right) / (k - 1)$$

where n_i is the sample size of group i, and N is the sum of the n_i's over the k groups.

Descriptive statistics for ICCs were reported as the mean (SD) as well as a 5-number summary which included the minimum, 25th percentile, median, 75th percentile, and maximum. Wilcoxon's signed-rank test was used to compare the ICCs for *Salmonella* prevalences and *Salmonella* $\log_{10}$(MPN/g + 1) that were calculated from the same studies. Spearman's correlation was also used to evaluate the association between ICCs that were calculated for *Salmonella* prevalences and those that were calculated for log-transformed MPNs. Linear regression analysis was used to evaluate study characteristics as potential predictors of ICCs for the *Salmonella*

prevalences. Variables considered for inclusion in the regression analysis were: challenge status of the sampled birds (a mixture of direct and indirect challenged birds vs. indirect challenged only); *Salmonella* prevalence; challenge dose; age of birds at challenge; age of birds at sample collection; number of days between challenge and sample collection; pen size; pen floor type; number of pens per group; number of birds sampled per pen; and *Salmonella* serovar. All variables that had $P < 0.20$ in the univariable analysis were considered for inclusion in the multivariable analysis. Multivariable model selection was performed using a manual backward elimination procedure with variables being removed one-by-one from the maximum model in a stepwise approach until only those having $P < 0.05$ remained. Akaike's information criterion was used to choose between competing functional forms of continuous predictor variables (e.g., linear, quadratic, or categorical). The normality of residuals was assessed using the Shapiro–Wilk test. All tests assumed a 2-sided alternative hypothesis, and values of $P < 0.05$ were considered statistically significant. Analyses were performed using commercially available statistical software (Stata/SE 17.0, StataCorp LLC, College Station, TX).

RESULTS

Characteristics of the 40 *Salmonella* floor pen trials included in the analysis are summarized in Table 1. In total, qualitative *Salmonella* cultures were performed on 5,351 ceca samples and quantitative cultures were performed on 5,261 ceca samples across 405 different pens assigned to the untreated challenged control groups in the 40 studies. Ceca *Salmonella* prevalences in the studies ranged from 9 to 76% with a median of 47%. Values of *Salmonella* $\log_{10}$(MPN/g +1) for the culture-positive samples (n = 2,336) ranged from 0.08 to 5.77 with a median of 0.35.

The distribution of ICCs for the binary (prevalence) and continuous ($\log_{10}$[MPN/g + 1]) ceca *Salmonella* culture results is illustrated in Figure 1. For the analysis based on *Salmonella* prevalences, the mean (SD) ICC was 0.20 (0.17), and the 5-number summary statistics (minimum, 25th percentile, median, 75th percentile, maximum) were: 0.00, 0.08, 0.17, 0.30, and 0.64. For the analysis based on the log-transformed *Salmonella* MPNs, the mean (SD) ICC was 0.15 (0.15), and the 5-number summary statistics were: 0.00, 0.03, 0.14, 0.20, and 0.52. In a paired comparison, ICCs calculated for the *Salmonella* prevalences were significantly higher than those calculated for the log-transformed MPNs (Wilcoxon signed-rank test, $P = 0.006$). ICCs for the *Salmonella* prevalences were also strongly correlated with ICCs for the log-transformed MPNs from the same studies (Spearman's rho = 0.81; $P < 0.001$). Collectively, these results indicate that the effect of pen-level clustering is substantial in *Salmonella* floor pen trials, and it must be considered during both the study design and analysis.

Within the 32 studies in which a combination of both direct and indirect challenged birds was sampled, direct challenged birds had a higher median *Salmonella* prevalence than the indirect challenged birds (55% vs. 42%, respectively; Wilcoxon signed-rank test, $P < 0.001$). Within these same 32 studies, there was no significant difference between the median ICCs that were calculated for the *Salmonella* prevalences if the calculations were restricted to only the direct challenged birds or only the indirect challenged birds (0.14 vs. 0.15, respectively; Wilcoxon signed-rank test, $P = 0.54$).

Table 1. Characteristics of 40 experimental *Salmonella* floor pen trials conducted in broiler chickens between March 2015 and February 2022 using a seeder bird challenge model.

Variable	Median (Min, Max) or frequency (%)
Number of pens (clusters) per treatment group	10 (6, 16)
Number of birds sampled per pen	15 (10, 15)
Percent of sampled birds that were indirectly (i.e., horizontally) challenged	67 (33, 100)
Ceca *Salmonella* prevalence (%) in the untreated challenged control group	47 (9, 76)
[1]Day of age at *Salmonella* challenge	1 (0, 7)
[1]Day of age at sample collection	43 (35, 46)
Salmonella enterica serovar used for challenge	
Enteritidis	3 (7.5)
Heidelberg	33 (82.5)
Infantis	1 (2.5)
Kentucky	2 (5.0)
Typhimurium	1 (2.5)
Salmonella challenge dose (CFU/bird)	
1×10^5	4 (10.0)
1×10^6	7 (17.5)
1×10^7	29 (72.5)
Broiler strain	
Cobb × Cobb	3 (7.5)
Ross × Ross	37 (92.5)
[2]Pen size	
1.5 m × 1.5 m (5 ft × 5 ft)	10 (25.0)
1.5 m × 3.0 m (5 ft × 10 ft)	30 (75.0)
Pen floor type	
Dirt	26 (65.0)
Concrete	14 (35.0)

Abbreviation: CFU, colony forming units.
[1]Day 0 was the day of hatch.
[2]All studies had a placement density of 0.09 m^2 (1.0 ft^2) per bird.

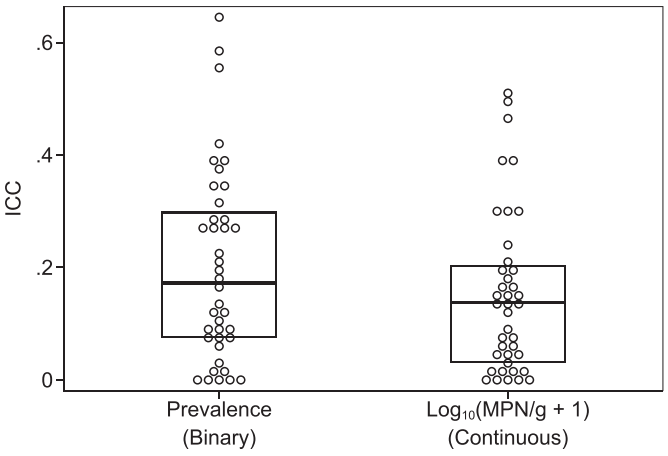

Figure 1. Dot plot showing the distribution of intra-cluster correlations (ICCs) for ceca *Salmonella* prevalences and ceca *Salmonella* $\log_{10}$(MPN/g + 1) from 40 experimental floor pen trials in broiler chickens using a seeder bird challenge model. Boxes illustrate the interquartile range (25th–75th percentiles), and the horizontal line in the interior of each box represents the median.

Table 2. Multivariable linear regression model to identify variables associated with the intra-cluster correlation (ICC) for ceca *Salmonella* prevalences in the untreated challenged control groups from 40 broiler chicken floor pen trials conducted between March 2015 and February 2022 using a seeder bird challenge model ($R^2 = 0.46$).

Variable	Coefficient (Std. error)	95% confidence interval	*P*
[1]Challenge status of sampled birds			
Direct and indirect (n = 32)	Reference		
Indirect only (n = 8)	0.13 (0.05)	0.02, 0.24	0.020
[2]Day of age at *Salmonella* challenge			
0–1 (n = 21)	Reference		
2–7 (n = 19)	0.16 (0.04)	0.07, 0.25	0.001
Salmonella enterica serovar			
Heidelberg (n = 33)	Reference		
Other (n = 7)	−0.14 (0.06)	−0.25, −0.03	0.018
Constant	0.12 (0.03)	0.06, 0.18	<0.001

[1]Direct challenged birds (seeder birds) were individually orally gavaged with *Salmonella*. Indirect (horizontal) challenged birds were only exposed to *Salmonella* via comingling with the direct challenged birds.
[2]Day 0 was the day of hatch.

Consequently, while the direct challenged birds were more likely to be colonized with *Salmonella*, they did not differ from the indirect challenged birds with respect to their ICCs.

In a multivariable linear regression analysis, ICCs based on the *Salmonella* prevalences were significantly associated with the challenge status of sampled birds (i.e., a mixture of direct and indirect challenged birds vs. only indirect challenged birds), the age of birds at challenge, and the *Salmonella* serovar (Table 2). The coefficient of determination for the final multivariable model was $R^2 = 0.46$, suggesting that the model accounted for approximately 46% of the variability in study ICCs. Compared to studies that sampled a combination of both direct and indirect challenged birds, studies that sampled only indirect (horizontal) challenged birds had a mean ICC that was 0.13 units higher, after adjusting for the age of birds at challenge and the *Salmonella* serovar. Compared to studies in which birds were challenged on day 0 (day of hatch) or day 1, studies in which birds were challenged on days 2 to 7 had a mean ICC that was 0.16 units higher. And compared to studies in which birds were challenged with *Salmonella* Heidelberg, studies in which birds were challenged with a different *Salmonella* serovar had a mean ICC that was 0.14 units lower. Figure 2 illustrates the bivariate ICC distributions for each of these three variables. In the multivariable analysis, ICCs were not significantly associated with: *Salmonella* prevalence; challenge dose; pen size; pen floor type; number of pens per group; number of birds sampled per pen; age of birds at sample collection; or the number of days between challenge and sample collection. Residuals from the final regression model were approximately normally distributed (Shapiro–Wilk test, $P = 0.29$). One pertinent finding of this analysis is

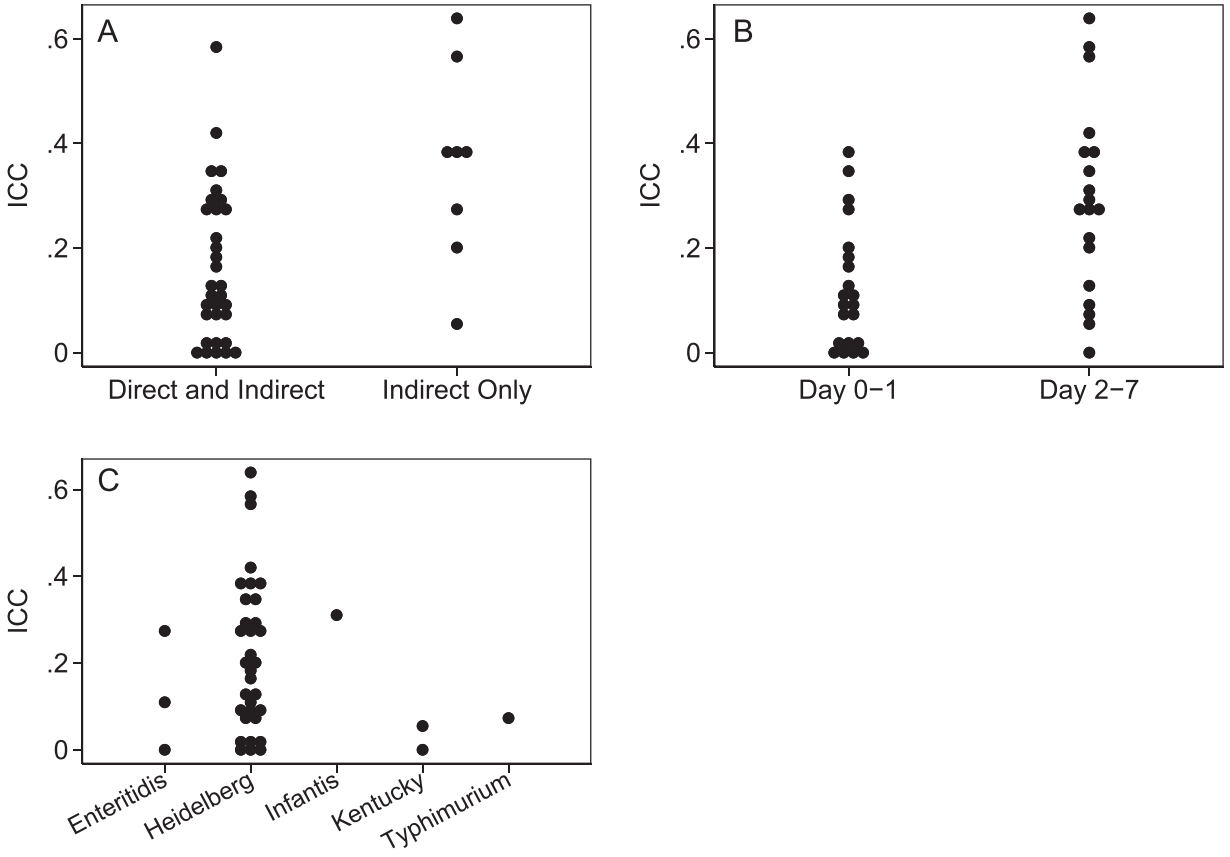

Figure 2. Dot plots showing the distribution of intra-cluster correlations (ICCs) for ceca *Salmonella* prevalences from 40 experimental floor pen trials in broiler chickens using a seeder bird challenge model. Panel A shows ICCs for studies in which a mixture of direct and indirect (horizontal) challenged birds were sampled compared to studies in which only indirect challenged birds were sampled. Panel B shows ICCs for studies in which birds were challenged with *Salmonella* on day 0 (day of hatch) or day 1 compared to studies in which birds were challenged on days 2 to 7. Panel C shows ICCs for studies by *Salmonella* challenge serovar.

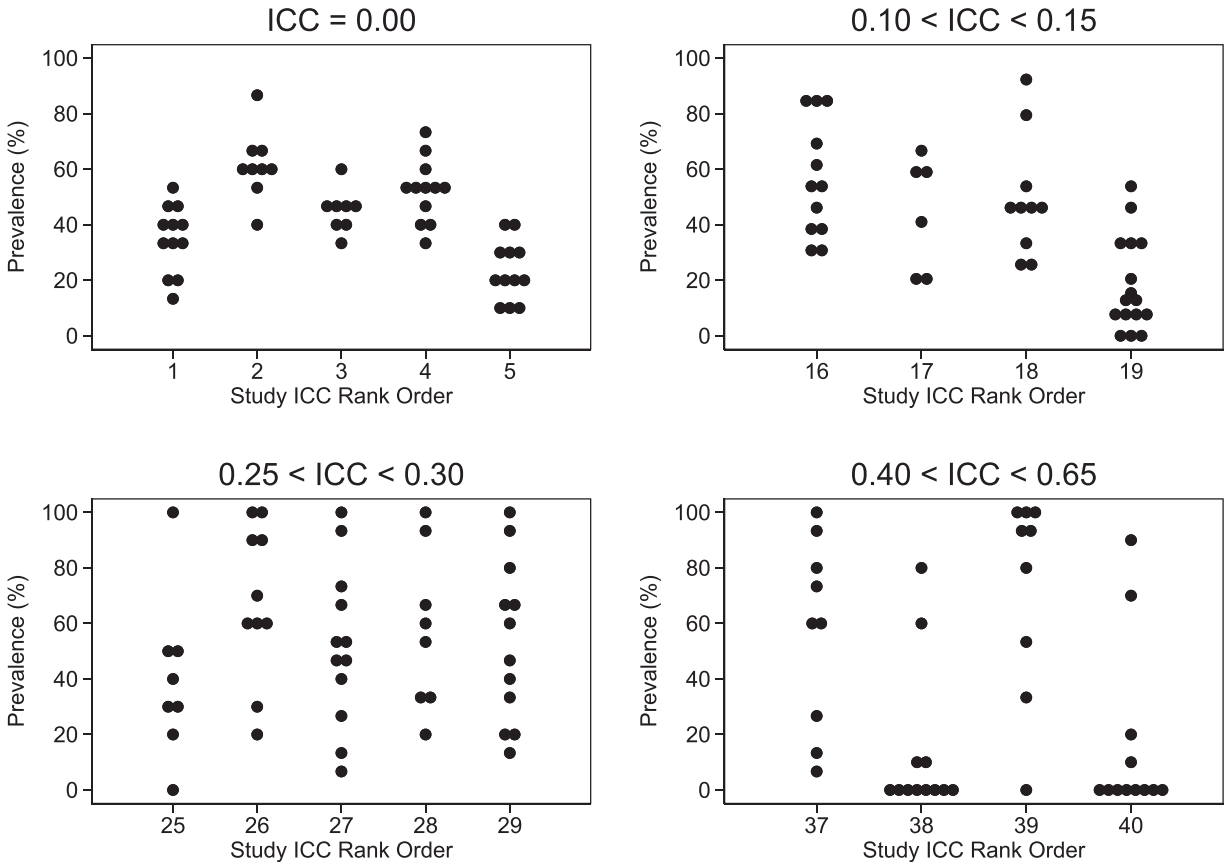

Figure 3. Dot plots of pen-level *Salmonella* prevalences for studies with different values of the intra-cluster correlation (ICC). Ceca were collected from 10 to 15 birds in each of 6 to 16 pens assigned to the untreated challenged control group in each study. Studies with higher ICCs exhibit greater variability in the distribution of pen-level prevalences.

that certain elements of the study design may influence the ICC. Choosing study design elements that are associated with a smaller ICC may improve efficiency by leading to a larger effective sample size for the same number of experimental units.

The distribution of pen-level *Salmonella* prevalences for a subset of studies is shown in Figure 3 to illustrate the between-pen variability for studies with different ranges of ICCs. For studies with an estimated ICC of 0.00, pen-level prevalences were minimally dispersed around a central value. Comparatively, studies with an ICC between 0.10 and 0.15 had somewhat broader pen-level prevalence distributions, and those with an ICC between 0.25 and 0.30 had markedly broader pen-level prevalence distributions. Studies with an ICC between 0.40 and 0.65 had either a bimodal distribution of pen-level prevalences, or a strongly skewed distribution. In summary, studies with lower ICCs exhibited less variability between pens, and studies with higher ICCs exhibited more variability between pens, in terms of their response.

DISCUSSION

ICCs for the *Salmonella* pen trials evaluated in this study were substantial, with a median (25th percentile, 75th percentile) ICC of 0.17 (0.08, 0.30) for the analysis based on *Salmonella* prevalences. The practical consequences of an ICC of this magnitude can be assessed by calculating the previously mentioned inflation factor that is used to determine the cluster-adjusted sample size. For example, with an ICC of 0.17 and a sample size of 15 birds per pen, the sample size for a cluster randomized trial would need to be inflated by a factor of $(1+\rho\,(m-1)) = (1+0.17\,(15-1)) = 3.38$ relative to a study where randomization occurs at the level of the individual bird in order to achieve the same level of statistical power. So, if the required sample size under the assumption of individual bird-level randomization was 60 birds per group, a cluster-randomized trial with an ICC of 0.17 and 15 birds sampled per pen would require a minimum total sample size of $3.38 \times 60 = 202.8 \approx 203$ birds per group, and at 15 birds per pen this would require $203/15 = 13.5 \approx 14$ pens per group. Note that the inflation factor depends on the number of birds sampled per pen as well as the ICC. Sampling a smaller number of birds per pen will reduce the inflation factor and reduce the total sample size required, but it will also require a larger number of pens. Generally, for a given number of birds, one can achieve a greater increase in power by increasing the number of pens assigned to each treatment rather than by increasing the number of birds sampled per pen. Logistically, of course, the number of pens available for a study may be limited.

In the multivariable regression analysis, ICCs calculated from the *Salmonella* prevalences were associated

with challenge status of the sampled birds, age of birds at the time of challenge, and *Salmonella* serovar. When only indirect (horizontal) challenged birds were sampled, the mean ICC was higher than when a combination of direct and indirect challenged birds was sampled. This may be because the colonization of indirectly challenged birds is dependent on secondary transmission from the direct challenged birds, and the rate of secondary transmission may vary across pens as *Salmonella* shedding varies across birds. Including some proportion of directly challenged birds among those sampled might decrease the dependence on secondary transmission.

The ICCs for studies where birds were challenged on the day of hatch or at one day of age were lower than those for studies where birds were challenged between 2 and 7 d of age, suggesting more consistent transmission and colonization in birds that were challenged at an earlier age. In one previous study, birds that were challenged using a seeder bird approach in day-old chicks were found to have lower *Salmonella* prevalences than birds that were challenged directly through oral or cloacal routes (Cox et al., 2020). The authors of that study speculated that in part this may be because the indirectly challenged birds are not exposed to *Salmonella* until day 3 when their gut microflora has already started to mature. Hence, it is possible that delaying the challenge for a longer period of time may lead to less consistent colonization of the direct challenged birds as well as greater variability in the transmission to horizontally challenged birds.

Studies in which birds were challenged with *Salmonella* Heidelberg had a higher mean ICC than studies in which birds were challenged with other *Salmonella* serotypes, but this finding should be interpreted with caution. Only 7 of the 40 studies evaluated in this analysis used a serotype other than *Salmonella* Heidelberg as the challenge strain (3 used Enteritidis, 2 used Kentucky, 1 used Infantis, and 1 used Typhimurium). In addition, the *Salmonella* Heidelberg isolate used in these studies is resistant to nalidixic acid, and it may not be representative of all strains of *Salmonella* Heidelberg. Nonetheless, the finding that ICCs varied with the challenge serotype does suggest that different *Salmonella* strains may have different transmissibility profiles.

There are several limitations of the current analysis. We focused on an assessment of only the untreated challenged control groups from the included studies to avoid the influence of different interventions across studies. To the extent that certain interventions may affect the transmissibility of *Salmonella*, they may also affect the ICCs. Regardless, we believe there is value in quantifying the range of ICC values that might be observed even in the absence of any treatments.

We also included only studies in which a seeder bird challenge model was used. While this is a commonly used approach in *Salmonella* challenge studies, it is not the only approach (Cox et al., 2020). Sampling only direct challenged birds might be expected to lead to lower ICCs since birds that are individually challenged do not need to rely on secondary transmission from other birds to be exposed. We did not find this to be the case, however, when the calculation of ICCs was restricted to only the direct challenged birds or only the indirect challenged birds in studies where both direct and indirect challenged birds were sampled. Perhaps the results would be different if all of the birds in each pen had been directly challenged rather than only half the birds, but we were unable to assess such an approach with the current data. One potential advantage of using the seeder bird approach is that it mimics natural routes of exposure for the indirectly challenged birds.

As noted previously, an assumption of many commonly used statistical methods is that the observations are independent. This assumption is violated in *Salmonella* pen trials, where the responses of birds in the same pen are correlated. Using a naïve approach to the statistical analysis that ignores pen-level clustering would be expected to lead to inappropriately small standard errors and P values. Likewise, ignoring the effect of clustering during sample size calculations would be expected to lead to an under-powered study. Thankfully, analytical methods that explicitly account for clustering, such as generalized linear mixed models and generalized estimating equation models are readily accessible using modern statistical software. And calculation of a cluster-adjusted sample size is straightforward given information on the ICC and the number of subjects sampled per cluster.

Considering the wide range of ICCs observed in the trials that were summarized in the current study, it may be reasonable to ask what value should be used for sample size calculations. We propose that for similarly designed studies, the median observed value of the ICC ($\rho = 0.17$ for the *Salmonella* prevalence) would be a sensible starting point, since half of the trials in this study had an ICC that was smaller and half had an ICC that was larger. Certainly, it would also be reasonable to perform sample size calculations using a range of ICC values, perhaps from the 25th percentile (e.g., $\rho = 0.08$) to the 75th percentile (e.g., $\rho = 0.30$), to gain an appreciation for how the required sample size may be impacted. If one wishes to be conservative, a larger value can always be used, although this may result in expending more resources than necessary. Inputs for sample size calculations are rarely known with certainty, which may lead some investigators to question their utility. Perhaps the greatest value in performing sample size and power calculations is on those occasions when one realizes that even under the most optimistic of circumstances, there would be an unacceptably low probability of achieving a trial's objectives.

CONCLUSIONS

ICCs should be considered when estimating sample sizes for *Salmonella* pen trials. Given the variability in ICCs between studies, it may be desirable to perform sample size calculations for a range of ICC values to gain an appreciation for how the required sample size would

be impacted. Challenging birds on the day of hatch or as day-old chicks, and sampling a combination of direct and indirect challenged birds may help reduce the ICC relative to studies that challenge birds at an older age and those that sample only indirect challenged birds within the context of a seeder bird challenge model. Choosing study design elements that are associated with smaller ICCs may improve efficiency by increasing the effective sample size for the same number of experimental units.

DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES

ADSA, ASAS, and PSA. 2020. Guide for the Care and Use of Agricultural Animals in Research and Teaching. 4th ed. American Dairy Science Association; American Society of Animal Science; and Poultry Science Association, Champaign, IL.

Batz, M. B., L. C. Richardson, M. C. Bazaco, C. C. Parker, S. J. Chirtel, D. Cole, N. J. Golden, P. M. Griffin, W. Gu, S. K. Schmitt, B. J. Wolpert, J. S. Z. Kufel, and R. M. Hoekstra. 2021. Recency-weighted statistical modeling approach to attribute illnesses caused by 4 pathogens to food sources using outbreak data, United States. Emerg. Infect. Dis. 27:214–222.

Berghaus, R. D., S. G. Thayer, B. F. Law, R. M. Mild, C. L. Hofacre, and R. S. Singer. 2013. Enumeration of *Salmonella* and *Campylobacter* spp. in environmental farm samples and processing plant carcass rinses from commercial broiler chicken flocks. Appl. Environ. Microbiol. 79:4106–4114.

Blodgett, R. J. 2020. Bacteriological analytical manual appendix 2: most probable number from serial dilutions. https://www.fda.gov/food/laboratory-methods-food/bam-appendix-2-most-probable-number-serial-dilutions. Accessed March 22, 2022.

Byrd, J. A., M. R. Burnham, J. L. McReynolds, R. C. Anderson, K. J. Genovese, T. R. Callaway, L. F. Kubena, and D. J. Nisbet. 2008. Evaluation of an experimental chlorate product as a preslaughter feed supplement to reduce *Salmonella* in meat-producing birds. Poult. Sci. 87:1883–1888.

Cerisuelo, A., C. Marin, F. Sanchez-Vizcaino, E. A. Gomez, J. M. de la Fuente, R. Duran, and C. Fernandez. 2014. The impact of a specific blend of essential oil components and sodium butyrate in feed on growth performance and *Salmonella* counts in experimentally challenged broilers. Poult. Sci. 93:599–606.

Cornfield, J. 1978. Randomization by group: a formal analysis. Am. J. Epidemiol. 108:100–102.

Cox, N. A., A. A. Oladeinde, K. L. Cook, G. S. Zock, M. E. Berrang, C. W. Ritz, and A. Hinton. 2020. Research note: evaluation of several inoculation procedures for colonization of day-old broiler chicks with *Salmonella* Heidelberg. Poult. Sci. 99:1615–1617.

Dohoo, I. R., S. W. Martin, and H. Stryhn. 2009. Sample-size determination. Pages 48−50 in Veterinary Epidemiologic Research. VER, Inc., Charlotte, P.E.I.

Donner, A. 1986. A review of inference procedures for the intraclass correlation-coefficient in the one-way random effects model. Int. Stat. Rev. 54:67–82.

Donner, A., and N. Klar. 2000. Design and Analysis of Cluster Randomization Trials in Health Research. Oxford University Press, New York, N.Y.

Eldridge, S., and S. Kerry. 2012. The intra-cluster correlation coefficient. Pages 172−195 in A Practical Guide to Cluster Randomised Trials in Health Services Research. John Wiley & Sons, Ltd, Chichester, UK.

Fasina, Y. O., T. O. Obanla, P. R. Ferket, and D. H. Shah. 2021. Comparative efficacy of spray-dried plasma and bacitracin methylene disalicylate in reducing cecal colonization by *Salmonella* Enteritidis in broiler chickens. Poult. Sci. 100:101134.

Hofacre, C. L., A. G. Rosales, M. D. Costa, K. Cookson, J. Schaeffer, and M. K. Jones. 2021. Immunity and protection provided by live modified vaccines against paratyphoid *Salmonella* in poultry-an applied perspective. Avian. Dis. 65:295–302.

Ridout, M. S., C. G. Demetrio, and D. Firth. 1999. Estimating intraclass correlation for binary data. Biometrics 55:137–148.

Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. 2011. Foodborne illness acquired in the United States−major pathogens. Emerg. Infect. Dis. 17:7–15.

Tack, D. M., L. Ray, P. M. Griffin, P. R. Cieslak, J. Dunn, T. Rissman, R. Jervis, S. Lathrop, A. Muse, M. Duwell, K. Smith, M. Tobin-D'Angelo, D. J. Vugia, J. Zablotsky Kufel, B. J. Wolpert, R. Tauxe, and D. C. Payne. 2020. Preliminary incidence and trends of infections with pathogens transmitted commonly through food - foodborne diseases active surveillance network, 10 U.S. sites, 2016-2019. MMWR Morb. Mortal. Wkly. Rep. 69:509–514.

USDA-FSIS. 1996. Pathogen reduction; Hazard Analysis and Critical Control Point (HACCP) systems.Fed. Regist. 61:38806–38989.