Research Note: Evaluation of several inoculation procedures for colonization of day-old broiler chicks with *Salmonella* Heidelberg

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ABSTRACT Before starting a study with many birds, it helps to know the method of chick inoculation. The objective was to compare 3 methods of *Salmonella* challenge (oral gavage [**OR**], intracloacal inoculation [IC], and seeder bird [**SB**]). Day-old broiler chicks (n = 100) were inoculated with 10^6 colony forming units (**CFU**) per chick of a marker strain of *Salmonella* Heidelberg (**SH**) with each route of inoculation. Chicks (n = 25) inoculated by each route were placed in floor pens on fresh pine shavings litter. For the seeder batch, 5 colonized chicks, each orally gavaged with 10^6 CFUs, were placed with 20 pen mates. Two weeks after inoculation, 10 birds from each pen and the 5 inoculated seeder birds were euthanized, the ceca were aseptically removed and macerated with a rubber mallet and weighed, and 3 times (w/v) buffered peptone was added and stomached for 60 s. Serial dilutions were made and plated onto Brilliant Green Sulfa plates containing 200 ppm nalidixic acid. Plates were incubated along with the stomached ceca for 24 h at 37°C. If no colonies appeared on the plates, an additional plate was streaked from the preenriched bag and incubated for 24 h at 37°C. In addition to all seeder birds being positive, the number of SH-positive birds out of 20 sampled in each group was 13, 17, and 7 for OR, IC, and SB, respectively. The level of SH per g of ceca and cecal contents was log (SE) 3.0 (0.7), 2.0 (0.4), and 2.6 (0.4) for OR, IC, and SB, respectively. After enrichment, the number of colonized birds out of 20 was 18, 20, and 10 for OR, IC, and SB, respectively. In conclusion, this study suggests that IC is the method to use to ensure most of the challenged birds are colonized. However, if you prefer to have a smaller percentage of the birds colonized with higher levels, then OR might be better.

Key words: *Salmonella Heidelberg*, inoculation method, day-old broiler, chick

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INTRODUCTION

Nontyphoidal *Salmonella* is among the leading causes of bacterial foodborne illness in the United States (Scallan et al., 2011). *Salmonella enterica* subspecies *enterica* serovar Heidelberg (SH) is an important serotype in human infections and the third most isolated serotype from retail chicken samples (Gieraltowski et al., 2016; Edirmanasinghe et al., 2017). Chickens are exposed orally by feeding on the droppings of pen mates and by feeding on contaminated litter, water, or feed. Also, uptake can occur when a chick's cloaca comes in contact with contaminated litter, most likely while resting or sitting (Cox et al., 1990a,b).

The route of exposure or inoculation plays a critical role in the colonization of young chicks with *Salmonella*. Cox et al. have shown that a lower dose of *Salmonella* was required to colonize the ceca of chicks via the intracloacal route than via oral gavage (Cox et al., 1990a,b; Bailey et al., 2005). They concluded that the acidic pH of the proventriculus and gizzard may be too harsh for *Salmonella* survival (Cox et al., 1972; Bailey et al., 2005). Therefore, before beginning a research project that involves a large number of birds, it may be helpful to know what inoculation procedure would be best for the experiment in question. The objective of this preliminary study was to compare several methods of SH challenge (oral gavage, intracloacal inoculation, and the seeder bird approach) to determine which method produced the highest incidence and level of colonization in the ceca of day-old broiler chicks.

MATERIALS AND METHODS

All animal experiments were approved by the University of Georgia Office of Animal Care and Use under Animal Use Protocol: A2017 04-028-A2.

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Salmonella Heidelberg Strain Characteristics and Inoculum Preparation

The SH-2813 strain was previously recovered from a broiler chicken carcass rinsate and has been characterized by whole genome sequencing (Oladeinde et al., 2018). Briefly, this strain belongs to the multilocus sequence type 15, carries a 37-kb conjugative plasmid, and is resistant to erythromycin, tylosin, and fosfomycin. For selective enumeration, the SH strain was made resistant to 200 ppm of nalidixic acid (nal). The strain was preconditioned in poultry litter extract (PLE) prepared as described by Oladeinde et al., 2018 before inoculation of day-old broilers. Five single colonies from Brilliant Green Sulfa agar supplemented with 200-ppm nal were transferred to 10 mL of PLE and incubated in a water bath shaker (85 rpm) at 37°C for 24 h. After overnight growth, 20 µL of culture was transferred to 20 mL of fresh PLE (1000-fold dilution) and incubated overnight in a water bath shaker at 37°C. The SH inoculum was prepared from the 24-h culture after centrifuging at 4,600 × g for 5 min and resuspending the pellets in 1X phosphate buffered saline.

Experimental Design

Day-old broiler chicks (n = 100) were obtained from a commercial hatchery and were inoculated orally (OR) with 0.1-mL volume, intracloacally (IC) with 0.1-mL volume, or using seeder birds (SB). Each route (OR or IC) received approximately 10^6 CFU of SH-2813. Chicks (n = 25) inoculated by each route were placed in floor pens at a stocking density of 0.65 m^2/chick on fresh pine shavings litter. For the seeder batch, 5 orally gavaged colonized chicks were mingled with 20 uninoculated pen mates. After a few days, they were sacrificed to determine the extent of colonization. All birds were given water and feed *ad libitum* and were fed a standard starter diet and managed according to commercial broiler guidelines. Two weeks after inoculation, 10 birds from each pen were euthanized, the abdominal cavity was sprayed with 70% alcohol, and the ceca were aseptically removed, placed in a stomacher bag, put on ice, and brought to the laboratory for analysis. Ceca were weighed, and buffered peptone water was added 3× volume to the weight and stomached for 60 s. Serial dilutions were made and plated onto BG Sulfa plates containing 200-ppm nal. Plates were incubated along with the macerated ceca and broth for 24 h at 37°C. If no colonies appeared on the direct streaked plate, then an additional plate was streaked from the enriched ceca + buffered peptone water broth bag and was incubated for an additional 24 h at 37°C. After incubation, plates were examined for the presence of the nalidixic acid–resistant inoculum. Incidence was compared by chi-square test for independence. Number of *Salmonella* detected per g was compared by *t* test. Significance was assigned at $P \leq 0.05$.

RESULTS

Table 1 presents the results of challenging day-old broiler chicks with a 10^6 SH inoculum by 3 different methods, in 2 separate trials and the resulting incidence and level of colonization. In trial 1, the number of SH-positive birds out of 10 sampled was 5, 8, and 5 for OR, IC, and SB, respectively; in trial 2, this was 8, 9, and 2. Also, the average CFU per g of ceca and cecal contents for both trials was 3.0, 2.0, and 2.6 for OR, IC, and SB, respectively. When both trials are combined, the total number of birds colonized out of the 20 challenged was 13, 17, and 7 for OR, IC, and SB, respectively. After enrichment, the number of colonized birds out of 20 were 18, 20, and 10 for OR, IC, and SB, respectively.

DISCUSSION

Data from this study suggest that IC would be the best inoculation method to be sure that most challenged birds are colonized. However, for a smaller percentage of birds colonized with a higher level of cecal colonization, OR or SB challenge may be better. One can envision all 3 of these routes of entry into the young commercial broiler. The bird can ingest the droppings of another bird or a mouse or insect, so the OR route is very feasible. For the IC route, the cloaca of a young bird may contact contaminated surfaces in a commercial hatchery or fresh droppings in a grow house. The wet percloacum of a newly hatched chick provides a route of entry for salmonellae contamination (Cox et al., 1990a,b). Fluids can be rapidly drawn into the cloaca by antiperistaltic reflex action (Shaffner et al., 1974), taking with them any bacteria (including salmonellae) present in the fluid.

For the SB route, horizontal transfer of *Salmonella* by using seeder birds has been shown to be an important route for colonization of newly hatched chicks (Bailey et al., 1998). It is also easy to see how this readily occurs in the broiler house because a small percentage of chicks

Table 1. Incidence and level of colonization of broiler chicks after oral, intracloacal, and seeder bird inoculations with 10^6 *S.* Heidelberg.

Method of inoculation	Trial #	No. of birds colonized/ no. of birds inoculated	Log CFU/g ceca and cecal contents	After 24 h enrichment
OR	1	5/10	2.7 ± 1.0	8/10
	2	8/10	3.2 ± 1.1	10/10
Total/mean		13/20[a,b]	3.0 ± 0.7[a]	18/20[a]
IC	1	8/10	1.7 ± 0.4	10/10
	2	9/10	2.3 ± 0.7	10/10
Total/mean		17/20[a]	2.0 ± 0.4[b]	20/20[a]
SB	1	5/10	2.7 ± 0.6	7/10
	2	2/10	2.4 ± 0.1	3/10
Total/mean		7/20[b]	2.6 ± 0.4[a,b]	10/20[b]

Abbreviations: CFU, colony forming unit; IC, intracloacal inoculation; OR, oral gavage; SB, seeder bird.

[a,b]Total/mean values within columns with no like superscripts are significantly different ($P < 0.05$). Chi square test for independence was used for prevalence; Student's *t* test was used for Log CFU/g.

come from the hatchery already colonized and they can be shedding a significant number of *Salmonella* in their droppings. Then, grow house mates may be exposed to this contaminated material and also become colonized. Seeder bird inoculation is a realistic approach that happens in the real world; however, it has some limitations. To begin with, some young birds may not get challenged until they are 3 D old and their gut microflora has begun to mature. This may explain why fewer birds were colonized in this study by this route (SB) than via OR or IC. In a 1990 study by Cox et al., the number of birds colonized was greater at day 1 than at day 3 for both OR and IC. The organism used in that study was *S. typhimurium*, and 100% of the birds were colonized on day 1 by a 10^6 challenge, and by day 3, a 10^8 challenge only resulted in 91% colonized. In that study, the IC results were similar to those of OR with 100% colonization on day 1 with a 10^5 challenge and only 70% colonized with the same challenge on day 3. In another study, SH was demonstrated to colonize 34, 3.8, and 49% of the ceca of birds inoculated via OR, IC, and intratracheal routes, respectively (Chadwick, 2017).

It is difficult to compare current results to past studies because the serotype may be different, the ability of the marker strain to colonize may be different, the birds being challenged are different, and so forth. For instance, some strains may carry extra genetic elements such as plasmids that may confer multidrug resistance or virulence phenotypes associated with successful gut colonization (Nair et al., 2018). Also, when using animal models, there is much variation that cannot be controlled. For example, with SB, the actual challenge to the pen mates is unknown because it is difficult to determine how much contaminated material is ingested by each bird. There can be multiple challenges in each hour. With OR, it is unknown what portion of the challenge is damaged or killed by the acidity in the proventriculus and gizzard. For IC, the number of cells cloacally introduced is known, but it is not known how much of the inoculum gets into each cecum.

The nature of live bird research results in inconsistency and variability; the conclusions from this study should be somewhat tempered. It would appear that the IC method should be the one to use if the intention is to colonize most of the challenged birds. However, it is preferred to have a smaller percentage of the birds colonized with higher levels, then OR would be the better way to challenge.

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