Research Note: Horizontal transmission and internal organ colonization by *Salmonella Enteritidis* and *Salmonella* Kentucky in experimentally infected laying hens in indoor cage-free housing

Richard K. Gast ,[*,1] Deana R. Jones,[*] Rupa Guraya,[*] Kenneth E. Anderson,[†] and Darrin M. Karcher[‡]

*U. S. National Poultry Research Center, USDA Agricultural Research Service, Athens, GA 30605; [†]Department of Poultry Science, North Carolina State University, Raleigh, NC 29765; and [‡]Department of Animal Sciences, Purdue University, West Lafayette, IN 47907

ABSTRACT The transmission of *Salmonella* to humans via contaminated eggs is an international public health concern. *S. Enteritidis* is deposited inside eggs after colonizing reproductive tissues of infected hens. Diverse housing facility characteristics and flock management practices influence *Salmonella* persistence and transmission in poultry, but the food safety consequences of different housing systems for laying hens remain unresolved. The present study compared the horizontal transmission of infection and invasion of internal organs during the first 2 wk after experimental *S.* Enteritidis and *S.* Kentucky infection of laying hens in indoor cage-free housing. Groups of 72 hens were housed in isolation rooms simulating commercial cage-free barns, and 1/3 of the hens in each room were orally inoculated with either *S.* Enteritidis (2 rooms) or *S.* Kentucky (2 rooms). At 6 d and 12 d postinoculation, 12 inoculated and 24 contact-exposed hens in each room were euthanized, and samples of liver, spleen, ovary, oviduct, and intestinal tract were removed for bacteriologic culturing. All orally inoculated hens were positive for intestinal colonization by *S.* Enteritidis at 6 d postinfection, and 70.8% of contact-exposed hens had become colonized by 12 d. *S.* Enteritidis was isolated from 100% of livers and 50.0% of ovaries from inoculated birds at 6 d and from 41.7% of livers and 10.4% of ovaries from contact-exposed birds at 12 d. The majority of both orally inoculated and contact-exposed hens were positive for intestinal colonization by *S.* Kentucky at 6 d, but *S.* Kentucky was found in other internal organs of both inoculated and contact-exposed hens significantly ($P < 0.05$) less often than *S.* Enteritidis at both sampling intervals. These results indicate that *Salmonella* infection can spread rapidly and extensively among hens in cage-free indoor housing, including a high frequency of internal organ involvement for invasive *S.* Enteritidis.

Key words: *Salmonella* Enteritidis, *Salmonella* Kentucky, laying hens, cage-free housing, internal organs

2020 Poultry Science 99:6071–6074
https://doi.org/10.1016/j.psj.2020.08.006

INTRODUCTION

The transmission of *Salmonella* infections to humans via contaminated eggs is a continuing public health concern around the world (Chousalkar et al., 2018). Egg-laying flocks are the most commonly identified source for human salmonellosis in Europe, principally caused by *Salmonella enterica* serovar Enteritidis. Epidemiologic investigations have indicated that the incidence of human *S.* Enteritidis infections directly correlates with the prevalence of this pathogen in commercial layers, and *S.* Enteritidis isolates from human outbreaks are often genetically identical to those from poultry sources. Comprehensive programs for risk reduction and flock testing have been widely implemented in commercial egg production, but the reported incidence of human *S.* Enteritidis infections in the United States in 2018 had not declined for more than 10 yr. Other *Salmonella* serovars, such as *S.* Kentucky, are commonly found in laying flock environments but are not significantly associated with human illness.

During the first 12 h following oral infection of chickens with *S.* Enteritidis, the pathogen colonizes the gastrointestinal tract and invades to internal tissues such as the liver and spleen. In mature laying hens, infection may subsequently disseminate systemically to involve the ovaries and oviducts, leading to bacterial

Received January 25, 2020.
Accepted August 7, 2020.

[1]Corresponding author: Richard.Gast@usda.gov

deposition inside the yolk and albumen of forming eggs as they descend the reproductive tract and are encased within shells before oviposition (Gantois et al., 2009). Higher frequencies of internally contaminated eggs are typically observed after experimental infection with *S.* Enteritidis than with other serovars. *S.* Enteritidis is usually cleared from the internal organs of hens within a few weeks, but individual birds that shed the pathogen in their feces or remain persistently infected can spread *Salmonella* horizontally within flocks and lead to continuing egg contamination. Commercial lines of laying hens can differ in their susceptibility to *Salmonella* infection (Gast et al., 2019).

A wide range of housing facility characteristics and flock management practices affect the persistence and transmission of salmonellae in poultry. Primarily as a result of concerns about animal welfare, the commercial egg industry has been undergoing a transition in recent years from conventional cage-based hen housing to less space-intensive alternatives such as indoor cage-free aviaries or outdoor free-range systems. However, the food safety implications of different housing options remain incompletely understood. Prior research has produced highly variable results because accurate comparisons of housing types are often complicated by confounding differences in bird stocking densities, levels of exposure to contaminated dust and feces, and populations of biological vectors. Previous studies have suggested that noncage systems may pose some unique inherent pathogen control challenges (Jones et al., 2015). The objective of the present study was to compare the early phases of horizontal transmission of infection and invasion of internal organs during the first 2 wk after experimental *S.* Enteritidis and *S.* Kentucky infection of laying hens in indoor cage-free housing.

MATERIALS AND METHODS

Experimental Housing of Laying Hens

In each of 2 similar trials, 144 female Tetra Brown chicks (a strain used by the commercial egg industry) were obtained from a breeding company and reared in cage-free barns at Purdue University (West Lafayette, IN) without vaccination against *Salmonella* infection. At 21 wk of age, the young hens were transferred to the U.S. National Poultry Research Center (Athens, GA), distributed evenly between 2 separate rooms of a disease-containment facility and allowed to acclimate to experimental housing conditions for 3 wk before *Salmonella* infection. Each isolation room simulated a commercial cage-free barn with community kick-out nest boxes and perches. Hens were housed on wood shavings at a density of 1,710 cm^2 of floor space per bird. Water was supplied *ad libitum* via automatic nipple-type drinkers and feed (antibiotic-free, ground-mash layer ration) was provided according to standards for commercial egg production. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the U.S. National Poultry Research Laboratory.

Preinoculation Cloacal Swab Samples

Immediately before hens in each of the 4 rooms were inoculated, sterile cotton swabs were used to collect cloacal swab samples from 56 randomly selected hens in that room. Each sample was transferred into 10 mL of buffered peptone water (Acumedia, Neogen Corp., Lansing, MI) and incubated for 24 h at 37°C. A 0.1 mL portion of each culture was then transferred into 10 mL of Rappaport-Vassiliadis broth (Acumedia) and incubated for 24 h at 41.5°C. A 10-μL portion from each of these broth cultures was then streaked onto brilliant green (**BG**) agar supplemented with 0.02 mg/mL of novobiocin (Sigma Chemical Co., St. Louis, MO). These plates were incubated for 24 h at 37°C and then examined for the presence of typical *Salmonella* colonies.

Experimental Infection of Laying Hens With Salmonella

In each of 2 trials, 24 of the 72 hens in 1 isolation room were orally inoculated with a measured dose of a 2-strain mixture of *S.* Enteritidis and 24 of the 72 hens in the other room were similar infected with a 2-strain mixture of *S.* Kentucky. One room in each trial was infected at 24 wk of age and the other room at 27 to 28 wk, with the order of administration of the 2 serovars reversed between trials. All *Salmonella* strains were originally isolated from commercial poultry. Each inoculum strain was resuscitated by transfer into tryptic soy broth (Acumedia) for 2 successive cycles of 24-h incubation at 37°C. After cell numbers in each incubated culture were estimated by determining its optical density at 600 nm, equal numbers of the 2 inoculum component strains were combined, and further serial dilutions in 0.85% saline were performed to achieve the desired final cell concentration. Plate counts on BG agar (Acumedia) confirmed that the final cell concentration in each 1.0-mL oral inoculum dose was 6.5×10^7 cfu (equalized for both serovars). Orally infected hens were identified by colored leg bands.

Internal Organ Samples

At 6 d postinoculation in each trial, 36 hens were randomly selected (12 orally infected hens and 24 uninoculated hens) and humanely euthanized for bacteriologic culture of internal tissues. Portions (approximately 5–10 g) of the liver, spleen, ovary, oviduct (magnum-isthmus junction region), and intestinal tract (including the ileo-cecal junction and adjacent regions of both ceca) from each hen were aseptically removed, transferred to 20 mL of buffered peptone water (Acumedia), and mixed by stomaching for 30 s. After incubation for 24 h at 37°C, a 1-mL portion of each culture was transferred to 9 mL of tetrathionate broth and

incubated for 24 h at 37°C. A 10-μL aliquot of each culture was then streaked onto BG agar plus novobiocin. Following incubation of these plates for 24 h at 37°C, typical *S.* Enteritidis or *S.* Kentucky colonies were subjected to biochemical and serological confirmation. This sampling procedure was repeated for the remaining 36 hens in each isolation room at 12 d postinoculation.

Statistical Analysis

Significant differences ($P < 0.05$) between trials, sampling dates, inoculum strains, or sampled tissues in the mean frequencies of *Salmonella* isolation from internal organs were determined by Fisher's exact test. Because the 2 replicate trials did not differ significantly in *Salmonella* recovery from any of the 5 sampled tissues for either serovar, their results were combined for analysis and presentation. Data were analyzed with Instat biostatistics software (GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

No preinfection fecal samples were *Salmonella*-positive in either trial. In groups of orally infected hens sampled at 6 d postinoculation (Table 1), *S.* Enteritidis was isolated significantly more often than *S.* Kentucky from livers (100.0 vs. 33.3%; $P < 0.0001$), spleens (86.1 vs. 12.5%; $P < 0.0001$), ovaries (50.0 vs. 8.3%; $P = 0.0034$), and oviducts (41.7 vs. 12.5%; $P = 0.049$), but the 2 serovars were found at similar frequencies (100.0 vs. 86.1%) in ileocecal intestinal samples. Among orally infected hens sampled at 12 d (Table 1), *S.* Enteritidis was recovered significantly more often than *S.* Kentucky from livers (50.0 vs. 16.7%; $P = 0.0305$) and spleens (66.7 vs. 12.5%; $P < 0.0001$), but the serovars were isolated at similar frequencies from ovaries (12.5 vs. 4.2%), oviducts (4.2% for both), and intestines (100.0 vs. 91.7%). In groups of uninoculated hens exposed by horizontal contact with orally infected hens and sampled at 6 d postinoculation (Table 1), *S.* Enteritidis was recovered at a significantly higher frequency than *S.* Kentucky from samples of livers (35.4 vs. 8.3%; $P = 0.0025$) and spleens (14.6 vs. 0.0%; $P = 0.0124$), but the serovars were isolated similarly often from ovaries (8.3 vs. 2.1%), oviducts (6.3% for both), and intestines (64.6 vs. 54.2%). Among contact-exposed hens sampled at 12 d (Table 1), *S.* Enteritidis was found significantly more often than *S.* Kentucky in samples of livers (41.7 vs. 4.2%; $P < 0.0001$), spleens (22.9 vs. 4.2%; $P = 0.0144$), and intestines (70.8 vs. 45.8%; $P = 0.0222$), but the serovars were recovered at similar frequencies from ovaries (10.4 vs. 2.1%) and oviducts (6.3 vs. 2.1%).

A wide range of environmental and management influences affect the introduction, transmission, and persistence of *Salmonella* in poultry flocks. Dust, feces, rodents, and insects can all perpetuate *Salmonella* contamination and serve as vehicles for spreading infection horizontally. Salmonellae sometimes survive facility cleaning and disinfection regimens, allowing transmission between successive flocks. The risks of *Salmonella* infection are especially high in flocks that are older, larger, or housed in aged facilities.

When egg contamination by *S.* Enteritidis was first recognized as a major public health problem in the late 1980s, most commercial laying flocks were housed in conventional cages. Experimental infection models have characterized many aspects of *Salmonella* infections in caged poultry. *S.* Enteritidis was isolated more often from internal organs when hens were housed at higher stocking densities in cage-based systems (Gast et al. 2016), although no correlated effects on the frequency of egg contamination were observed. Much of what is known about *Salmonella* in noncage housing systems for laying flocks has been derived by comparison to caged housing. Higher frequencies of *Salmonella* environmental prevalence, hen infection, and egg contamination have sometimes been attributed to conventional cage systems, particularly when rodent populations are high (Denagamage et al., 2015). Other investigations reported more extensive horizontal dissemination of infection within cage-free flocks, as well as higher frequencies of *Salmonella* isolation from egg and environmental

Table 1. Recovery of *Salmonella* Enteritidis and *Salmonella* Kentucky from internal organs of orally infected and contact-exposed laying hens in cage-free housing.[1]

Salmonella-positive/total (%)	Liver	Spleen	Ovary	Oviduct	Ileum/ceca
6 d postinoculation					
S. Kentucky (inoculated)	8/24 (33.3)^{b,c}	3/24 (12.5)^{c,d,e}	2/24 (8.3)^{b}	3/24 (12.5)^{b}	23/24 (86.1)^{a}
S. Kentucky (contact-exposed)	4/48 (8.3)^{d}	0/48 (0.0)^{e}	1/48 (2.1)^{b}	3/48 (6.3)^{b}	26/48 (54.2)^{c,d}
S. Enteritidis (inoculated)	24/24 (100.0)^{a}	23/24 (86.1)^{a}	12/24 (50.0)^{a}	10/24 (41.7)^{a}	24/24 (100.0)^{a}
S. Enteritidis (contact-exposed)	17/48 (35.4)^{b,c}	7/48 (14.6)^{c,d}	4/48 (8.3)^{b}	3/48 (6.3)^{b}	31/48 (64.6)^{c,d}
12 d postinoculation					
S. Kentucky (inoculated)	4/24 (16.7)^{c,d}	1/24 (4.2)^{c,d,e}	1/24 (4.2)^{b}	1/24 (4.2)^{b}	22/24 (91.7)^{a,b}
S. Kentucky (contact-exposed)	2/48 (4.2)^{d}	2/48 (4.2)^{d,e}	1/48 (2.1)^{b}	1/48 (2.1)^{b}	22/48 (45.8)^{d}
S. Enteritidis (inoculated)	12/24 (50.0)^{b}	16/24 (66.7)^{b}	3/24 (12.5)^{b}	1/24 (4.2)^{b}	24/24 (100.0)^{a}
S. Enteritidis (contact-exposed)	20/48 (41.7)^{b}	11/48 (22.9)^{c}	5/48 (10.4)^{b}	3/48 (6.3)^{b}	34/48 (70.8)^{b,c}

^{a,b}Values in columns that share no common superscripts are significantly ($P < 0.05$) different.

[1]24 of 72 hens in each cage-free housing room were orally inoculated with approximately 6.5×10^7 cfu of 2-strain mixtures of either *S.* Enteritidis or *S.* Kentucky. The remaining hens were exposed to infection by horizontal contact.

samples (Snow et al., 2010). Under commercial management conditions, similar *Salmonella* prevalence was detected in egg shell and environmental samples from conventional cage, enriched colony, and aviary housing systems (Jones et al., 2015). Facility design characteristics and management practices intrinsic to each poultry housing system may create unique *Salmonella* reservoirs and require correspondingly targeted risk reduction strategies.

In the present study, *S.* Enteritidis infection of hens in cage-free indoor housing led to the establishment of intestinal colonization in all birds sampled at both 6 and 12 d after oral inoculation. A high degree of invasiveness was evident in the frequent isolation of the pathogen from internal tissues of these birds at 6 d postinoculation (100% from livers and 50% from ovaries). These values were similar to observations from a prior experiment in which similarly infected hens were housed in conventional cages (Gast et al., 2013). Extensive horizontal transmission of infection was observed, as the majority of contact-exposed hens exhibited intestinal colonization by 6 d postinoculation. Internal organ invasion was also common in birds infected by contact in this housing system, reaching peak values (approximately 42% of livers and 10% of ovaries) at 12 d after infection.

The deposition of *S.* Enteritidis in eggs has been ascribed to intracellular survival in chicken macrophages, adherence to reproductive tract mucosa, and invasion of ovarian granulosa cells (Babu et al., 2016). *Salmonella* serovars characterized by an affinity for reproductive tissues (such as Enteritidis and Typhimurium) possess substantial genetic similarities. *S.* Kentucky is known to be an effective and persistent intestinal colonizer in chickens and may be environmentally resistant, but it appears to lack key virulence determinants found in more invasive serovars (Cheng et al., 2015). As a result, this serovar is commonly found in laying flocks and environments but is not significantly associated with egg-transmitted human illness.

Intestinal colonization of hens infected with *S.* Kentucky in the present study was initially similar to the corresponding results for *S.* Enteritidis, as most orally inoculated birds were positive at 6 d postinfection as well as more than 50% of birds exposed by horizontal contact. However, at both 6 d and 12 d, *S.* Kentucky was found in internal organs of both inoculated and contact-exposed hens significantly less often than *S.* Enteritidis. Moreover, although continuing horizontal dissemination of *S.* Enteritidis infection led to an increase in the frequency of internal organ isolation from contact-exposed hens between 6 d and 12 d, no similar trend was observed for *S.* Kentucky. Bacteriological enumeration from sampled tissues, although beyond the logistical scope of the present investigation, might provide a fuller picture of the dynamics of infections with different serovars. The results of this study suggest that *Salmonella* infection can spread rapidly and extensively among hens in cage-free indoor housing, including a high frequency of internal organ involvement for invasive *S.* Enteritidis.

ACKNOWLEDGMENTS

The authors gratefully express appreciation for excellent technical assistance from Stephen Norris and Robin Woodroof (U. S. National Poultry Research Center, Athens, GA). This study was supported by appropriated funds of the USDA Agricultural Research Service and by U. S. Poultry and Egg Association grants program projects 075 and 701.

Conflict of Interest Statement: The authors did not provide any conflicts of interest.

REFERENCES

Babu, U. S., L. M. Harrison, I. R. Patel, G. A. Ramirez, K. M. Williams, M. Pereira, and K. V. Balan. 2016. Differential antibacterial response of chicken granulosa cells to invasion by *Salmonella* serovars. Poult. Sci. 95:1370–1379.

Cheng, Y., A. A. Pedroso, S. Porwollik, M. McClelland, M. D. Lee, T. Kwan, K. Zamperini, V. Soni, H. S. Sellers, S. M. Russell, and J. J. Maurer. 2015. *rpoS*-regulated genes involved in the competitive fitness of *Salmonella enterica* serovar Kentucky in the intestines of chickens. Appl. Environ. Microbiol. 81:502–514.

Chousalkar, K., R. Gast, F. Martelli, and V. Pande. 2018. Review of egg-related salmonellosis and reduction strategies in United States, Australia, United Kingdom and New Zealand. Crit. Rev. Microbiol. 44:290–303.

Denagamage, T., B. Jayarao, P. Patterson, E. Wallner-Pendleton, and S. Kariyawasam. 2015. Risk factors associated with *Salmonella* in laying hen farms: systematic review of observational studies. Avian Dis. 59:291–302.

Gantois, I., R. Ducatelle, F. Pasmans, F. Haesebrouck, R. Gast, T. J. Humphrey, and F. Van Immerseel. 2009. Mechanisms of egg contamination by *Salmonella* Enteritidis. FEMS. Microbiol. Rev. 33:718–738.

Gast, R. K., R. Guraya, D. R. Jones, and K. E. Anderson. 2013. Colonization of internal organs by *Salmonella* Enteritidis in experimentally infected laying hens housed in conventional or enriched cages. Poult. Sci. 92:468–473.

Gast, R. K., R. Guraya, D. R. Jones, K. E. Anderson, and D. M. Karcher. 2016. Colonization of internal organs by *Salmonella* Enteritidis in experimentally infected laying hens housed in enriched colony cages at different stocking densities. Poult. Sci. 95:1363–1369.

Gast, R. K., P. Regmi, R. Guraya, D. R. Jones, K. E. Anderson, and D. M. Karcher. 2019. Colonization of internal organs by *Salmonella* Enteritidis in experimentally infected laying hens of four commercial genetic lines in conventional cages and enriched colony housing. Poult. Sci. 98:1785–1790.

Jones, D. R., N. A. Cox, J. Guard, P. J. Fedorka-Cray, R. J. Buhr, R. K. Gast, Z. Abdo, L. L. Rigsby, J. R. Plumblee, D. M. Karcher, C. I. Robison, R. A. Blatchford, and M. M. Makagon. 2015. Microbiological impact of three commercial laying hen housing systems. Poult. Sci. 94:544–551.

Snow, L. C., R. H. Davies, K. H. Christiansen, J. J. Carrique-Mas, A. J. C. Cook, and S. J. Evans. 2010. Investigation of risk factors for *Salmonella* on commercial egg-laying farms in Great Britain, 2004-2005. Vet. Rec. 166:579–586.