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Salmonella enteritidis, Phage Type 4 Infection in a Commercial Layer Flock in Southern California: Bacteriologic and Epidemiologic Findings


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SUMMARY. Salmonella enteritidis, phage type 4 (SE PT4), was isolated from five of six 27-wk-old layer chickens submitted for necropsy from a flock of 43,000. Bacteriologic and epidemiologic investigations on the ranch revealed that five of the eight flocks (n = 176,000) were infected. The prevalence of SE PT4 in randomly selected healthy birds ranged from 1.7% (in caged birds) to 50% (in free-range birds) and prevalence in culled birds (kept on dirt floor houses) ranged from 14% to 42%. The estimated overall prevalence of group D Salmonella in eggs contaminated with group D Salmonella was 2.28 per 10,000. The estimated prevalence of group D Salmonella in eggs from caged birds in three infected houses ranged from 1.5 to 4.1 per 10,000, whereas in two houses of free-range birds, prevalence was 14.9 to 19.1 per 10,000. Three of the eight flocks on the ranch remained negative for Salmonella between May 1994 and December 1995 or until removed from the ranch. Salmonella enteritidis PT4 was also isolated from 12.5% (6 of 48) of mice; 57% (four of seven) of cats; and two of two skunks tested. Environmental drag swabs and well water samples yielded multiple serotypes of Salmonella (23/180 and 5/14, respectively) but not S. enteritidis.

RESUMEN. Infección por Salmonella enteritidis fago tipo 4 en un lote de ponedoras comerciales al sur de California: Hallazgos bacteriológicos y epidemiológicos.

Se aisló Salmonella enteritidis fago tipo 4 en 5 de 6 ponedoras comerciales de 27 semanas de edad recibidas para necropsia. Las aves provenían de un lote de 43000 aves. Las investigaciones bacteriológicas y serológicas en la granja revelaron que 5 de los 8 lotes (n = 176,000) estaban infectados. La prevalencia de Salmonella enteritidis fago tipo 4 en aves sanas seleccionadas al azar varió de 1.7% en las aves en jaula, al 50% en aves sueltas fuera del galpón. La prevalencia en aves de desecho, mantenidas en galpones con piso de tierra, varió de 14 a 42%. La prevalencia total calculada de Salmonella del grupo D en los huevos contaminados con Salmonella del grupo D fue del 2.28 por 10000. La prevalencia general calculada para Salmonella del grupo D en huevos de aves en jaulas en 3 galpones infectados varió de 1.5 a 4.1 por 10000, mientras que en los dos galpones de aves sueltas, la prevalencia fue del 14.9 al 19.1 por 10000. Tres de los 8 lotes en la granja permanecieron negativos a Salmonella entre Mayo de 1994 y Diciembre de 1995 o hasta cuando salieron de la granja.
También se aisló *Salmonella enteritidis*: fago tipo 4 de 12.5% (6 de 48) de los ratones, 57% (4 de 7) de los gatos y 100% (2 de 2) de moftas examinadas. Los hisopos de arrastre tomados del medio ambiente y las muestras de agua de pozo fueron positivas a varios serotipos de *Salmonella* (23 de 180 y 5 de 14, respectivamente) pero no a *Salmonella enteritidis*.

Key words: *Salmonella enteritidis*, phage type 4, layer flock.

Abbreviations: BGN = Brilliant Green with novobiocin; SE PT4 = *Salmonella enteritidis*, phage type 4; TBG = tetrathionate Brilliant Green; USDA = U.S. Department of Agriculture; XLD = xylose-lysine-deoxycholate.

*Salmonella enteritidis*, phage type 4 (SE PT4), infection in poultry is considered a foreign animal disease in the United States by the United States Department of Agriculture (USDA), and importation of animals known to be infected is prohibited. *Salmonella enteritidis* PT4 is a well-recognized pathogen of humans and animals in the United Kingdom (1,14). In the United States, human infections with S. *enteritidis* increased dramatically during 1976–86, particularly in the northeastern part of the country, and public health investigators linked many of these outbreaks to the consumption of grade A shell eggs or foods containing eggs (18).

*Salmonella enteritidis* PT4 has not previously been identified in food-producing animals in the United States, although it has been isolated from human patients in California since 1990 (S. Abbot, pers. comm.). During the last 6 yr (1989–94) the overall human salmonellosis in the five southern California counties (Los Angeles, Orange, Riverside, San Bernardino, and San Diego) has increased 57%, while infections due to S. *enteritidis* increased to 1056% (S. Mack, pers. comm.). Although these cases have been associated with the consumption of table eggs or egg products, no definitive evidence has been presented linking eggs to the human cases of S. *enteritidis* in California.

This paper is the first of two that describe the occurrence of PT4 infection in a commercial layer flock and report the bacteriologic and epidemiologic findings.

**MATERIALS AND METHODS**

**Flock history.** The ranch consisted of 176,000 leghorn layer chickens (representing strains A and B) housed in eight different houses and producing commercial shell eggs, as well as several categories of specialty eggs. Features of the houses included: typical four-deck cage systems; three houses with an average of 44,000 birds each (houses 1, 2, and 5); two houses of cage-free birds on wooden slats over a deep pit, 12,000 fertile (with males) and 12,000 nonfertile (without males) (houses 3 and 4); one house containing a combination of 12,000 cage-free fertile, and nonfertile birds (with and without males), on low plastic slats with a central litter area (house 6); and two adjacent houses with free-range birds kept on a dirt and litter floor with 4000 birds in each (houses 7 and 8).

Manure from two houses (houses 1 and 2) was removed daily by manure belts that run under cages and was transported to a collection receptacle that was then transferred to a 5-acre field about 500 ft away for composting. Manure from another house (house 5) was also removed by manure belts, but was allowed to dry under the cages before it left the house. The dried manure was transported and spread on a field about 700 ft away. Manure in houses 3 and 4 was collected in deep pits and cleaned out between flocks. The flock in house 6 was kept on a floor with a shallow pit and manure was scraped out between flocks. In the free-range birds (houses 7 and 8) the manure was scraped between flocks. The ranch was supplied with water from a 550-ft-deep well; the water was chlorinated (0.1 PPM) and pumped to a 60,000-ft³ holding tank. Feral cats, rodents, skunks, opossums, wild birds, and other wildlife was seen in the shrubs and bushes surrounding the houses. The north border of the ranch is formed by a creek originating from a city sewage treatment plant that flows toward the ranch (westbound). The creek was the only source of drinking water for the resident feral animals, especially in summer months. In some cases, houses were only 50 m away from the creek.

The owner operates three other ranches, including a pullet grower ranch, that are located about 40 miles away from the ranch under study. The owner of the ranch participated in a routine mortality surveillance program and submitted dead birds for gross necropsy examination on a weekly basis. Prior to the diagnostic case submission, for a 2–3-wk period, a pattern of fibrinous peritonitis, perihepatitis, and reproductive disorders was noted in one house (house 1). Subsequently, in May 1994, the owner submitted six dead birds from the same house with a complaint of in-
creased mortality (1.6%/month) and decreased egg production (8% over a period of 6 wk).

**Bacteriologic examination. Initial diagnostic case submission.** Samples of livers from six chickens with lesions were plated on 5% blood and MacConkey agar plates (Remel Laboratories, Lenexa, Kans.) and incubated aerobically at 37°C for 24 hr. Intestinal pools were collected in 10 times the volume of selenite broth (Remel) and incubated at 37°C for 18–24 hr. Following incubation, the selenite broth cultures were streaked onto Brilliant Green with novobiocin (BGN), xylose–lysine–tergitol 4 (XLT4), and MacConkey agar plates (Remel). The plates were incubated at 37°C for 24 hr. Following the incubation, plates were evaluated for bacterial pathogens. Identifications were performed according to standard procedures (3). At least three *Salmonella*-suspect colonies were picked from each plate to triple sugar iron and lysine iron agar slants (Remel). Following overnight incubation at 37°C, the cultures were serogrouped using commercial *Salmonella* grouping sera (Difco Laboratories, Detroit, Mich.). Isolates were confirmed to be *Salmonella* by biochemical tests (hydrogen sulfide production, methyl red positive, Voges–Proskauer negative, indole negative, citrate positive, urease negative, lysine positive, ornithine positive, o-nitrophenyl-β-galactopyranoside negative, motility, and gas production). *Salmonella* serotyping was performed according to procedures described previously (3). Phage typing of *S. enteritidis* was performed at the National Veterinary Services Laboratory, Ames, Iowa.

**Follow-up submissions.** A total of 469 randomly selected clinically healthy chickens (49 birds from the index flock and 60 birds from each of the other seven houses) were submitted for bacteriologic examination. Organ collections in these flocks were done in accordance with the U.S. Department of Agriculture and Plant Health Inspection Service *S. enteritidis* regulations (20). Chickens were aseptically opened and organ pools (pericardium, heart, liver, gallbladder, spleen, ovary, and proximal oviduct) were collected from each carcass in a sterile plastic bag. Laboratory culturing procedures were performed as described by the regulations (21). In a course of 16 wk, 180 culled birds were submitted for necropsy from the free-range houses (houses 7 and 8). Organ pools (selected as in the previous group) and intestinal pools (ileum, jejunum, and cecum) were collected separately from each carcass in sterile plastic bags. Specimens were homogenized by hand or with the use of a stomacher and 10 times the volume of Hajna tetrahionate broth (Difco) was added. The culture broth was incubated at 37°C for 24 hr. Following incubation, the broth was streaked onto BGN, XLT4, and MacConkey agar plates and processed as previously described.

**Evaluation of replacement pullets.** Fourteen 10-wk-old pullets from the grower ranch (located 40 miles away from the index ranch) were submitted for necropsy and livers and intestinal pools (small intestine and cecum) were collected in separate sterile bags and cultured as previously described.

**Feed samples (n = 7).** Feed samples were obtained from the storage tank on the index ranch and cultured for *Salmonella* as previously described (4).

**Environmental drag swabs (n = 180).** Drag swabs from egg belts, floor litter, nest boxes, and dust samples were collected from all the houses from the index ranch and the pullet ranch. These swabs were taken in accordance with the National Poultry Improvement Plan (19) guidelines and submitted for *Salmonella* culture. Drag swabs were aseptically weighed and placed in 10 times the volume of tetrathionate Brilliant Green (TBG, Difco) broth. Dust and litter samples (25 g) were placed in 225 ml of TBG broth. Broth cultures were incubated at 41.5°C for 24 hr. The broth cultures were then plated on to BGN and xylose–lysine–deoxycholate (XLD) (Remel) agar. Following overnight incubation at 37°C, plates were examined for colonies of typical of *Salmonella*. Further characterization by biochemical tests and serologic typing was performed as previously described.

**Feral animals.** Forty-eight mice were trapped from houses 2, 3, 4, 5, 6, and 8. There were no rats on the premises. Other animals trapped included two skunks, one opossum, one sparrow, and one pigeon. Samples of livers and intestinal pools collected in separate sterile plastic bags from individual animals were cultured for *Salmonella* as previously described. Suspect colonies for *Salmonella* were further characterized and serologic and phage typing were performed as described.

**Tank water.** The tank water was tested for *Salmonella* using a modified Moore swab technique as previously described (17). The swab was wrapped at one end of a 16-gauge wire and secured by staples, then appropriately folded in brown paper and sterilized. The swab was then placed just below the surface of the tank water and the free end was anchored to the opening (lid) of the tank. Following exposure for 2 wk in the tank, the swab was collected aseptically, placed in a sterile plastic bag, and immediately transported to the laboratory. The swab was incubated in 10 times its volume of tetrathionate broth at 41.5°C for 24 hr. Following incubation, the broth was streaked on to BGN and XLT4 agars. In addition, 1 ml of tetrathionate broth culture was transferred to a fresh tube containing the same medium daily for 5 days of continued incubation to enhance recovery on streaked plates. The broth cultures were incubated at 41.5°C for 24 hr and streaked to BGN and XLT4 agars. Following overnight incubation at 37°C the plates were examined for suspect *Salmonella* colonies. Further characterization and serologic typing of *Salmonella* were performed as previously described.
Eggs. Initially, 1000 eggs were submitted from each of the eight houses and examined for *Salmonella* as previously described (12). Eggs were disinfected with Lugol's iodine for 20 min. The contents of 20 eggs were aseptically pooled into a sterile bag and thoroughly mixed by hand until all the yolks were completely blended with the albumen. The blended egg mixture was left at room temperature (20–24°C) for 3 days. Following incubation, the culture was inoculated on XLD, BGN, and MacConkey agar plates and incubated overnight at 37°C. The plates were then examined for suspect *Salmonella* colonies and processed as previously described.

Further monitoring of the flocks for *Salmonella* consisted of testing 1000 eggs from the infected houses (houses 1, 2, 6, 7, and 8) every 2 wk until results were negative on a series of four consecutive lots. After a house was deemed negative, 480 eggs were tested every month for the production life of the flock. Houses that had never tested positive for *S. enteritidis* (houses 3, 4, and 5) continued to be monitored every 3 mo (480 eggs/house) for the production life of the flock. Biweekly testing (1000 eggs/house) would resume if, at any time, eggs tested positive for *S. enteritidis* during the surveillance period.

**Statistical analysis.** Using a pool size of 20, point and 95% confidence interval estimates for the proportions of eggs positive for group D *Salmonella* were derived as previously described (7). Briefly, this method estimates an asymmetric confidence interval for the proportion of positive pools and substitutes the confidence limits in an equation that relates the pooled estimate of prevalence to the individual estimate of prevalence. This method avoids the need to make assumptions about the number of infected eggs in each pool. The overall flock estimate of *Salmonella*-positive eggs was calculated by weighting the estimated prevalence in each house by the number of birds in each house. For the latter calculation, egg production per bird was assumed to be equal in all houses during sampling period.

**RESULTS**

Aerobic culture of five of six livers and intestinal pools from the diagnostic case submission (six dead birds) yielded *S. enteritidis*. Four liver isolates were selected for phage typing and were typed as SE PT4. In addition, two of six livers were positive for *E. coli* or *P. haemolytica*-like organisms. *Salmonella enteritidis* PT4 was isolated from organ pools of 7% (33 of 469) randomly selected healthy chickens from four of eight flocks. The prevalence of SE PT4 in chickens based on tissue pool culture was 1.7% (1/60) in house 7, 2% (1/49) in house 1, 50% (30/60) in house 8, and 1.7% (1/60) in house 6. *Salmonella enteritidis* was isolated from 22% (40/180) of organ pools of culled birds from houses 7 and 8 that were examined over a period of 16 wk. Of the 40 *S. enteritidis* isolates, 36 were SE PT4 and four were SE PT7. *Salmonella* was isolated from 23% (43/180) of the intestinal pools of culled birds. Of these 43 isolates, 49% (23/43) were SE PT4; 42.2% (19/43) were *S. heidelberg* and 2.3% each (1 each) were *S. cerro*, *S. braenderup*, and SE PT1. During the prospective monitoring of flocks through egg culturing an additional flock (house 2) was found to be infected. A total of six group D *Salmonella* isolates from the egg pools (one or more from each flock) were serotyped as *S. enteritidis* and later phage typed as SE PT4. *Salmonella heidelberg* was the only other serotype isolated (only once) from all the egg pools cultured from the flocks. Three flocks on the ranch (houses 3, 4, and 5) have continually been monitored and showed no evidence of infection throughout the observation period. Between May 1994 and January 1996, approximately 89,000 eggs from all eight houses were tested for *Salmonella*. The weighted estimate of the proportion of group D *Salmonella* positive eggs produced on the ranch was 2.28 per 10,000.

*Salmonella enteritidis* PT4 was isolated from the organ pools of four of seven cats, 6 of 48 mice, and two of two skunks tested. *Salmonella heidelberg* was isolated from organ pools of one cat and one mouse. An organ pool from an opossum yielded *Salmonella newport* and S. 40: g, z51 (arizonae). Organ pools from a sparrow and a pigeon were negative for *Salmonella*. Culture results of *S. enteritidis* from egg pools, chicken organs, and mice (organ or intestinal pools) are summarized in Table 1.

Twenty-three of 180 drag swabs (13%) were culture positive for *Salmonella*. *Salmonella heidelberg* was most frequently isolated, comprising 57% (13/23) of the total. The following serotypes were also isolated only once: *S. agona*, *S. kottbus*, *S. kentucky*, S. 48: g, z51 (arizonae), and S. 62:z4, z32 (arizonae). *Salmonella enteritidis* was not isolated from any of the drag swabs.

*Salmonella broughton* was isolated from one of seven feed samples. *Salmonella worthington* was isolated from an organ pool of 1 of 10 pullets obtained from the grower ranch.
Table 1. Culture results of *Salmonella enteritidis* from egg pools (n = 20).^a

<table>
<thead>
<tr>
<th>House number</th>
<th>Egg Pools (n = 20)</th>
<th>Birds (n = 469) (organs)</th>
<th>Mice (n = 48) (organ/ intestines)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive/no. of egg pools</td>
<td>Positive eggs Per 10,000 (95% C.I.)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2/656</td>
<td>1.53</td>
<td>(0.26 – 6.14)</td>
</tr>
<tr>
<td>2</td>
<td>6/718</td>
<td>4.19</td>
<td>(1.7 – 9.61)</td>
</tr>
<tr>
<td>3, 4, 5</td>
<td>0/756</td>
<td>0</td>
<td>(0 – 3.16)</td>
</tr>
<tr>
<td>6</td>
<td>3/742</td>
<td>2.03</td>
<td>(0.52 – 6.44)</td>
</tr>
<tr>
<td>7c</td>
<td>19/648</td>
<td>14.87</td>
<td>(9.20 – 23.66)</td>
</tr>
<tr>
<td>8c</td>
<td>28/748</td>
<td>19.06</td>
<td>(12.89 – 27.90)</td>
</tr>
</tbody>
</table>

^a*Salmonella enteritidis* was also isolated from four of seven cats and two of two skunks (outside house 2).

^bOne *S. enteritidis* isolate from each house.

^cFree range birds.

Five of the 14 tank water samples were positive for *Salmonella mbandaka* (twice), *S. tennesse, S. senftenberg* (twice), *S. montevideo*, *S. give, S. cerro*, and *S. eling*. *Salmonella enteritidis* was not isolated from the tank water.

Stool samples taken from the ranch employees were negative for *Salmonella*.

**DISCUSSION**

The finding of SE PT4 in the flock caused a serious concern to the egg industry in California because SE PT4 infection in chickens has been classified as a foreign animal disease by the USDA. The most probable source of the infection was effluent from a municipal sewage treatment plant located half a mile upstream on the creek that borders the infected ranch (unpubl. data). The creek, which is entirely composed of sewage effluent, is the only source of drinking water for resident feral animals and wild birds, especially in the summer months. Rodents, particularly mice, were considered to be the biological vectors and amplifiers of SE PT4 on this ranch. This was supported by the fact that the plasmid analyses and restriction endonuclease analysis of SE PT4 isolates from the effluent, rodents, skunks, and chickens (organ and egg pools) were identical (unpubl. data).

The prevalence rate of group D *Salmonella* in the index flock (house 1) was very low compared to other flocks on the ranch based on examination of eggs and randomly selected, healthy chickens. This was perhaps due to minimal exposure of birds to rodents, as chickens were kept in cages of multideck units and had better manure management. The overall estimate of positive eggs from the infected ranch was 2.28 per 10,000, which is similar to the 2.75 per 10,000 reported in the northeastern part of the United States where *S. enteritidis* is considered endemic in chickens (16). However, there was substantial variation between houses, with the highest prevalence being in the free-range birds kept on dirt floors (houses 7 and 8). The higher prevalence of group D *Salmonella* in these flocks was probably due to the ease of access of rodents to the houses, which contaminated the feed bins with feces. As many as 2.3 × 10^5 *S. enteritidis* organisms could be isolated from a single fecal pellet of a mouse (8). Infection of hens most likely occurred by the fecal–oral route through contamination of feed with feces. Mice droppings can be actively sought out by birds when mixed in the feed and or bedding because of their seedlike size and appearance (2).

Factors that contribute to shedding *Salmonella* include age, environmental stress, molting, and time of housing, among others (9,10,12,16). The birds were placed in houses at 18 (cage birds) and 16 (free range) weeks of age and infection was not recognized until several weeks after birds had moved into the houses (9 and 11 weeks, respectively). However, exact time of infection could not be established in the flocks because no active surveillance program existed on the ranch. Reduction of *Salmonella* shedding in feces has been reported following vaccination with an autogenous bacterin (6,13). In this study 2 ml of an autogenous bacterin of SE PT4 was injected subcutaneously twice at a 4-wk interval (at 31 and 35 weeks of age). Shedding of group D *Salmonella* in eggs continued more frequently for several weeks...
(up to 49 weeks of age) in house 7 and very sporadically in the other houses. The effect of vaccination on shedding of group D Salmonella in eggs among these flocks could not be evaluated as there was no control flock on the ranch for comparison. The results of egg cultures as an indication of infection status in a flock should be interpreted with caution. Experimental studies have shown that hens infected with S. enteritidis may lay infected eggs only for a short time even though organ culture results remain positive for much longer periods (1,5).

In the early part of the investigation environmental culturing for Salmonella was carried out for a brief time and later the focus was changed to culturing eggs for S. enteritidis. Salmonella enteritidis was not isolated from the environment (drag swabs), whereas other serotypes were recovered. This was perhaps due to the inadequacy of the conventional culturing method used for the environmental samples. The delayed secondary enrichment method has been used to enhance the total Salmonella isolation rates by over 40% compared to the conventional method (21). Egg cultures from houses 3, 4, and 5 remained negative throughout the observation period; however, 3 of 25 mice cultured from these houses yielded S. enteritidis PT4.

Environmental status of a house can serve as a potential indicator that the flock may produce S. enteritidis-contaminated eggs, and it has been used to determine egg diversion under the current S. enteritidis regulation (15). California is developing a partnership with the Food and Drug Administration for a trace-back system if and when a commercial egg production flock is implicated in an outbreak of human Salmonella enteritidis infection. The infection of the layer flock at this ranch was not implicated at any time before or after the outbreak of human S. enteritidis infection. Once the infection at the layer flock was confirmed the owner of the ranch voluntarily diverted all eggs from the infected flocks to pasteurization.

REFERENCES


