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Salmonella enteritidis Colonization of the Reproductive Tract and Forming and Freshly Laid Eggs of Chickens

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Salmonella enteritidis **colonizes the tissues of the chicken ovary and oviduct, presumably contaminating eggs and thereby contributing to human outbreaks of salmonellosis. In this study, commercial adult laying hens were given an oral inoculation of 10^8** ***S. enteritidis*** **organisms. Tissues from various organs, the intestines, and the reproductive tract, including freshly laid eggs, were collected daily for up to 40 days postinoculation (p.i.). Within 2 days p.i.** ***S. enteritidis*** **was detected by culture in pools of the spleen, liver, heart, and gallbladder tissues, in intestinal tissues of all infected birds, and in various sections of the ovary and oviduct. Detection of organisms by immunohistochemical staining was rare for most tissues in spite of their culture-positive status, suggesting a low level of tissue colonization. However,** ***S. enteritidis*** **could be detected by immunohistochemical staining in oviduct tissues associated with four forming eggs, indicating the possibility of a heavier colonization in the egg during its development. In two subsequent experiments, forming eggs taken from the oviduct with their associated tissue, were found to be culture positive for** ***S. enteritidis*** **at a rate of 27.1 and 31.4%, while freshly laid eggs in these experiments were culture positive at the rate of 0 and 0.6%. These observations suggest that while forming eggs are significantly colonized in the reproductive tract, factors within the eggs may control the pathogen before the eggs are laid. The data show that prior to egg shell deposition, forming eggs are subject to descending infections from colonized ovarian tissue, ascending infections from colonized vaginal and cloacal tissues, and lateral infections from colonized upper oviduct tissues. The data are consistent with an ascending infection of freshly laid eggs from the cloaca, as the incidence of positive eggs in experiments 1 and 3 coincided with heavily contaminated cloacal tissues (50.7 and 80%, respectively), while no positive eggs were detected in experiment 2 when cloacal colonization was low (8.3%). The data do not support the possibility of egg invasion by bacterial translocation from the peritoneal cavity.**

The incidence of food-borne salmonellosis associated with *Salmonella enteritidis*-infected grade A table eggs has increased significantly since 1979 (24). The strains of *S. enteritidis* that currently are infecting flocks of table-egg-laying chickens do not generally cause clinical disease in these birds. Rather, it is the colonized chicken egg that causes clinical disease in human consumers. Routes of bacterial invasion of the egg have yet to be defined. Transovarial transmission has been proposed for *S. enteritidis* (22, 23), although there continues to be controversy over the resident site of the pathogen being in the yolk, in the follicular membrane surrounding the yolk, or associated with the albumen (6, 12, 23, 24). Translocation of organisms from the peritoneum to the yolk sac or to the oviduct via macrophages is a possibility suggested by previous studies (5, 9, 22, 24, 28, 29). Egg contamination by penetration of the shell by organisms present in chicken feces deposited on the outside of the egg as it passes through the cloaca is also feasible (24). Egg washing procedures and education of food handlers have improved this aspect of the problem (17), although food handlers in kitchens still contribute significantly to the outbreaks (10). An understanding of the mechanisms that are contributing to the colonization of eggs by *S. enteritidis* is essential to reducing the public health risk associated with consumption of foods containing infected eggs. The purpose of this study was to examine the course of invasion and colonization of forming and freshly laid eggs from *S. enteritidis*-infected laying hens by culturing their organs and especially their egg-associated oviductal tissues.

MATERIALS AND METHODS

Chickens. All chickens were housed in isolation facilities that are fumigated and tested for salmonellae after each use. The water supply and commercial bagged feed were regularly monitored for contaminants. Although the chickens were from different sources, the age of the laying hens in all experiments was between 23 and 25 weeks.

In experiment 1, 100 adult White Leghorn laying hens were obtained from a commercial Pennsylvania pullet flock whose environment was confirmed to be negative for *S. enteritidis* by surveillance in the Pennsylvania *S. enteritidis* Pilot Program. The hens were negative for *S. enteritidis* as monitored by culture of a cloacal swab after arrival at the New Bolton Center. Hens were housed in wire laying cages, three birds to a cage, with food and water available ad libitum.

In experiment 2, hens were obtained as in experiment 1 and were negative by cloacal swab for *S. enteritidis* after arrival at the New Bolton Center. However, screening of serum for *Salmonella* antibodies by a rapid serum plate test (30) revealed five positive reactors. These positive hens were not used in the study.

In experiment 3, 60 commercial brown-egg-laying hens were obtained from Robert Taylor, Jr. (Department of Poultry Science, University of New Hampshire). These hens were determined to be free of *S. enteritidis* by both antibody and antigen testing.

Bacterial inoculum. All hens were experimentally infected by the oral route with 10^8 organisms in 0.1 ml of sterile phosphate-buffered saline, pH 7.2 (PBS), using a small-bore, ball-tipped pipette. The inoculum was derived from an overnight, aerated brain heart infusion broth or Luria-Bertani broth culture grown at 37°C. The bacteria were collected by centrifugation, and the washed cells were resuspended in 10 ml of PBS. The concentration of the culture was quantitated utilizing a standard curve developed for *S. enteritidis*, which correlates A_{540} with CFU on brain heart infusion agar plates (14).

In experiments 1 and 3, hens were inoculated with *S. enteritidis* phage type 8 strain 575, which was derived from the first isolate from chicken egg yolks during the 1987 New York State trace-back investigation of a human salmonellosis outbreak. *S. enteritidis* 575 was used because it had been found to be twice as invasive as the original Y8P2 strain from which it was derived (2).

S. enteritidis SE6-E21 was used in experiment 2. As kindly provided by Jean

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Petter Guard (Southeast Poultry Research Laboratory, Athens, Ga.), it is a phage type 13A isolate that sheds into freshly laid eggs with high frequency after experimental inoculation of adult laying hens (18).

Experimental design. Experiment 1 was conducted as a preliminary study to determine if histochemical staining of *S. enteritidis* 575 in the reproductive tract tissues from infected hens could reveal the mechanisms of egg contamination. Sixty hens were inoculated with *S. enteritidis*, and two or three randomly selected hens were euthanized daily on days 1 to 21 and 32 to 40 postinoculation (p.i.). No control hens were included in this study. The following tissue samples were taken for detection of *S. enteritidis*: (i) ovary; (ii) oviduct, sections from the infundibulum, upper magnum, lower magnum, isthmus, uterus, vagina, and cloaca; (iii) an organ pool, which included liver, spleen, gallbladder, and heart; (iv) an intestinal pool, which included the small intestine and the cecum with their contents. Adjacent tissue samples were taken and either placed in selenite for culture and enzyme-linked immunosorbent assay (ELISA) detection or fixed in 10% phosphate-buffered formalin (pH 6.9 to 7.1 [Hydrol Chemical, Yeadon, Pa.]) for histochemical staining. All incomplete eggs were collected with their associated oviduct tissue and formalin fixed. Eggs laid by the hens were collected daily. Between days 22 and 31 p.i., only freshly laid eggs were collected.

In experiment 2, 48 hens received an oral inoculation of *S. enteritidis* SE6-E21 and 24 hens received an oral inoculation of only PBS to serve as controls. Tissues and eggs were collected from two infected and one control hen from days 1 to 20 p.i. for culture and histologic preparation, as described above. All incomplete eggs with their associated oviduct tissues were cultured for the presence of *S. enteritidis* rather than being formalin fixed for histochemical observation.

In experiment 3, 48 hens received *S. enteritidis* 575 and 24 hens received PBS, as in experiment 2. The tissues and eggs were collected for bacterial culture from days 1 to 20 p.i., as described above. No tissue samples were formalin fixed for histological examination.

Experiment 1 was terminated after 40 days, and experiments 2 and 3 were terminated after 20 days pi.

Sample preparation. (i) ELISA and bacterial culture detection of *S. enteritidis*. The protocols for ELISA and bacterial isolation have been previously described (14). Briefly, tissue samples taken for ELISA and bacterial isolation were placed in selenite broth for 24 h at 37°C. One milliliter of broth culture was then transferred to 10 ml of tetrathionate broth, incubated for 24 h at 37°C, and subsequently plated onto MacConkey (MAC) and XLD agars (BBL, Becton Dickinson Microbiological Systems, Cockeysville, Md.). For egg culture, freshly laid eggs were aseptically cracked after being dipped in iodine-alcohol solution, air dried, and incubated at room temperature for 48 h (experiment 1) or 72 h (experiments 2 and 3) prior to plating on MAC and XLD agars. Suspicious colonies were reisolated and serogrouped with Difco reagents (Difco Laboratories, Detroit, Michigan). In experiment 1, the contents of the eggs from nine hens were pooled (generally nine eggs per pool) and incubated at room temperature for 48 h. Samples for ELISA were obtained daily from the 48-h egg pool, and from either a brilliant green agar plate or a selenite broth culture of tissue samples, and tested for the presence of *S. enteritidis*. In addition to bacterial culture of samples in experiment 2, tissue samples from all hens were formalin fixed, but only those found to be culture positive were stained with hematoxylin and eosin (H&E) and observed for inflammatory lesions. In addition, in experiments 2 and 3 incomplete eggs taken from the oviduct with their associated tissue were carefully placed into 100 ml of selenite F broth and incubated for 24 h at 37°C. A sample of the selenite F broth was then removed, plated onto MAC and XLD agars, and incubated as before. The yolks of incomplete eggs were then broken, the mixed egg contents and associated tissue were incubated for an additional 24 h, and samples were plated onto agar for culture, as described above.

In experiments 2 and 3, freshly laid eggs were individually cultured by incubating them for 72 h at room temperature and then testing them for the presence of *S. enteritidis* by plate culture and ELISA.

In experiment 3, a swab of the peritoneal cavity, which was taken immediately after the hens were euthanized, was streaked onto MAC agar plates for overnight culture at 37°C.

(ii) Histochemical staining. (a) MAb. The development and preliminary characterization of monoclonal antibody (MAb) 1G10 has been described (14). Subsequent Western blot (immunoblot) analysis and ELISA screening have shown it to be a high-titered MAb with specificity for a lipopolysaccharide (LPS) epitope of *S. enteritidis*, *Salmonella dublin*, *Salmonella berta*, *Salmonella moscow*, and *Salmonella blegdam*. IG10 does not react with rough strains of salmonellae, which lack LPS (manuscript in preparation). It was used both for diagnostic ELISAs and histochemical staining in the present experiments. Dilutions of MAb 1G10 for use in tissue staining were optimized by using tissues from adult hens that were either *S. enteritidis* culture positive or culture negative, as well as air-dried smears of colonies of *S. enteritidis.*

(b) Sample preparation. Tissue samples taken for histochemical staining were placed in 10% formalin to await the results of ELISA and bacterial isolation. Only in experiment 1 were all incomplete eggs taken from the oviduct with their associated tissue and formalin fixed for histochemical staining.

When tissue samples were found to be culture positive, matched samples in formalin were embedded in paraffin. Five-micrometer sections were cut and mounted on chrom-alum-gelatin-coated slides, deparaffinized with xylene, and rehydrated through graded alcohols for staining. These slides were then washed in 0.1 M Tris-buffered saline (pH 7.5). The mounted tissues were first treated with 1 M citric acid, avidin, biotin, and normal serum from a Vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, Calif.) to block nonspecific background binding. Slides were submerged in an appropriate dilution of MAb in 0.1 M Tris buffer containing 0.2% (wt/vol) mannitol, 0.37% L-lysine, and 0.15% bovine serum albumin fraction V (pH 7.5) overnight at 4°C and then in Vectastain reporter reagents, according to the manufacturer's instruction. The presence of *S. enteritidis* in tissues was visualized with a HistoMark red stain kit (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.), according to the manufacturer's instructions and with a counterstain of hematoxylin (Gils formulation #2; Fisher Scientific, Pittsburgh, Pa.). Tissues were dehydrated in graded alcohols and xylene for mounting with xylene-based Permount (Fisher). Tissues from chickens that were either positive or negative for *S. enteritidis* served as controls for background staining. Air-dried smears of *S. enteritidis* were not rehydrated or dehydrated but otherwise were stained in the same way as tissue samples, beginning with the blocking steps. Coverslips were mounted on smears with water-based Biomeda Gel/Mount (Fisher).

TABLE 1. Detection of *S. enteritidis* 575 by bacterial culture and ELISA in the tissues of laying hens after oral inoculation

Tissue	No. of positive tissues/no. of samples at day(s) p.i.						
	1	2–4	5–8	9–12	13–16	17–21[a]	32–40
Ovary	0/2	6/6	2/6	0/8	0/8	0/17	1/24
Infundibulum	0/2	3/6	0/8	0/8	0/8	0/17	1/24
Upper magnum	0/2	1/6	0/8	0/8	0/8[b]	0/17	0/24
Lower magnum	0/2	2/6	0/8	0/8[b]	0/8	0/17[b]	0/24
Isthmus	0/2	3/6	1/8[b]	1/8	0/8	0/17	0/24
Uterus	0/2	4/6	0/8	0/8	0/8	0/17	0/24
Vagina	0/2	6/6	0/8	0/8	1/8	0/17	1/24
Cloaca	2/2	6/6	4/8	5/8	3/8	4/17	13/24
Organ pool[c]	0/2	6/6	8/8	6/8	1/8	3/17	1/24
Intestinal pool[d]	2/2	6/6	8/8	8/8	4/8	4/17	4/24
Egg pools	0/12	0/36	1/48	0/48	0/48	3/84	0/228

[a] No tissue samples were taken from days 22 to 31 p.i.

[b] *S. enteritidis* detection in egg-associated tissue only by immunohistochemistry.

[c] Organ pool = heart, liver, spleen, and gallbladder.

[d] Intestinal pool = small intestine and cecum, with contents.

(iii) H&E staining. H&E staining was carried out using Gill's #2 hematoxylin by traditional methods (21).

Statistics. The relative rates of tissue and egg colonization by *S. enteritidis* in the three experiments were assessed from two-by-two contingency tables using Pearson's chi-square from Statistix 4.0 (Analytical Software, St. Paul, Minn.).

RESULTS

Detection of *S. enteritidis* by bacterial isolation from, and immunohistochemical staining of, tissues. Table 1 summarizes the detection of *S. enteritidis* by bacterial isolation, ELISA, and histochemical staining in the tissues of infected hens from experiment 1. *S. enteritidis* was isolated from one egg pool at 5 days p.i. when the incidence of positive organ pools, intestinal pools, and cloacal samples was high. The egg pool from the same hens was culture negative on day 6 p.i. and for the remainder of the experiment. The first day after oral inoculation, only cloacal samples and intestinal pools were found to be positive for *S. enteritidis*. However, samples tested from days 2 to 4 p.i. revealed that 100% of the ovary, vagina, cloaca, organ pool, and intestinal pool samples and at least one in six of the remaining oviduct tissue sections contained *S. enteritidis*. Five days after infection, one egg pool was culture positive while the number of *S. enteritidis*-positive samples decreased considerably for most reproductive tissues. Organ and intestinal pool samples began to decrease from 100% positive after 12 to 14 days p.i. After the fifth day p.i., egg pools were negative until day 21 p.i. when one egg pool became positive for *S. enteritidis*. The egg pool from the same hens remained positive for three consecutive days. In contrast to the positive egg pool detected at 5 days p.i., the incidence of *S. enteritidis* in organ pools and

FIG. 1. MAb 1G10 staining of *S. enteritidis*. The Histomark red kit was used to visualize the MAb staining. Tissues were counterstained with hematoxylin (magnification, ×400). (a) Smear of *S. enteritidis* on microscope slide. (b) *S. enteritidis* associated with secretory cells (s) in the lumen (l) of the albumen-secreting magnum, which surrounded a forming egg. (c) Section of the albumen-secreting magnum surrounding a forming egg that was negative for *S. enteritidis*.

cloacal samples had dropped prior to detection of positive egg pools at 21 days p.i. When tissue sampling resumed at 32 days p.i., the incidence of *S. enteritidis* in all tissues remained low, with the exception of cloacal samples, and *S. enteritidis* was not detected in any subsequent egg pools.

With the exception of the oviductal tissues collected with four forming eggs, very few *S. enteritidis* organisms were detected in most tissues by histochemical staining, in spite of positive culture and ELISA results (Table 1). Staining was observed in a few interstitial cells in the medulla of the ovary or, in one case, as diffuse staining in the cortical stromal tissue of an atretic follicle. In two of the cases of tissues associated with forming eggs taken from the upper magnum or lower magnum, antigen staining was seen in and associated with distended secretory goblet cells of the mucosal epithelium extending into the albumen in the lumen (Fig. 1b). In both instances, the staining was seen in the magnum from the peritoneal epithelial layer to the lumen. Positive staining in the lower magnum section and in the isthmus section associated with the other two forming eggs was seen as a few bacterial aggregates associated with the peritoneal epithelium. A few areas of staining were also noted in association with ciliated epithelium in the lumen of the infundibulum and isthmus, with the peritoneal epithelium of the vagina, and with the stratified squamous epithelium of the vent portion of the cloaca.

TABLE 2. Detection of *S. enteritidis* SE6-E21 by bacterial culture and ELISA in tissues of laying hens after oral inoculation

Tissue	Treatment	No. of positive tissues/no. of tissues cultured at day(s) p.i.					
		1	2–4	5–8	9–12	13–16	17–20
Ovary	Control	0/1	0/3	0/4	0/4	2/4	0/4
	Infected	0/2	0/6	0/8	0/8	2/8	1/8
Infundibulum	Control	0/1	0/3	0/4	0/4	2/4	0/4
	Infected	0/2	1/6	0/8	0/8	2/8	0/8
Upper magnum	Control	0/1	0/3	0/4	0/4	2/4	0/4
	Infected	0/2	1/6	0/8	1/8	2/8	1/8
Lower magnum	Control	0/1	0/3	0/4	0/4	1/4	0/4
	Infected	0/2	1/6	0/8	1/8	4/8	0/8
Isthmus	Control	0/1	0/3	0/4	0/4	2/4	0/4
	Infected	0/2	2/6	0/8	0/8	2/8	1/8
Uterus	Control	0/1	0/3	0/4	0/4	2/4	0/4
	Infected	0/2	3/6	1/8	1/8	2/8	1/8
Vagina	Control	0/1	0/3	0/4	0/4	2/4	0/4
	Infected	0/2	2/6	3/8	6/8	2/8	1/8
Cloaca	Control	0/1	0/3	0/4	0/4	1/4	0/4
	Infected	1/2	1/6	0/8	0/8	2/8	0/8
Organ pool[a]	Control	0/1	0/3	0/4	1/4	1/4	2/4
	Infected	1/2	6/6	1/8	6/8	2/8	3/8
Intestinal pool[b]	Control	0/1	0/3	0/4	0/4	1/4	2/4
	Infected	2/2	2/6	3/8	5/8	4/8	3/8
Forming egg[c]	Control	0/1	0/3	0/4	0/4	1/4	2/4
	Infected	0/1	2/6	0/8	4/8	4/8	3/8
Egg[d]	Infected + control	0/72	0/198	0/222	0/174	0/126	0/78

[a] Organ pool = heart, liver, spleen, and gallbladder.
[b] Intestinal pool = small intestine and cecum, with contents.
[c] Forming egg with associated oviductal tissues.
[d] Freshly laid egg.

The design of experiment 2 was modified to take into consideration these histochemical findings, which suggested that the oviductal tissues surrounding forming eggs were highly colonized. Rather than formalin fixation, forming eggs and associated oviductal tissues were incubated for bacterial isolation by culturing the intact yolk with its surrounding tissue for 24 h in selenite, followed by a 24-h culture of the tissue with the yolk and membranes disrupted. Moreover, control hens, which were given an oral inoculation of PBS, were included in the trial to ensure that the chickens were *Salmonella*-free. Table 2 shows the results of this experiment. On day 12 p.i., tissues of control birds began to be culture positive for *S. enteritidis*, but by then the incidence of positive developing eggs from the infected hens had become significant, so the experiment was continued. Oviductal eggs taken from both control and experimentally infected hens were consistently found to be positive from days 2 to 19 p.i. Although in this experiment, the tissue type associated with each incomplete egg was not recorded, all of the positives except for the last sample came from the samples in which the yolks had been broken, and then the eggs and associated tissues had been incubated for an additional 24 h. From days 1 to 20 p.i., the incidence of tissue infection fluctuated but overall was not significantly different from the rate of infection in experiment 1 (Table 3). The lower magnum tissues in experiment 1 were significantly less colonized than those in experiment 2 (1.4 and 11.7%, respectively), while the cloacal tissues in experiment 1 were significantly more colonized than those of experiment 2 (54.7 and 8.3%, respectively). Also in contrast with experiment 1, no freshly laid eggs were found to be contaminated with *S. enteritidis* throughout the experimental period.

Experiment 3 was modified from experiment 2 in that no samples were examined histologically, because significant le-

TABLE 3. Percentage of *S. enteritidis* culture-positive tissues and eggs taken from infected hens in three experiments

Tissue	% Culture-positive tissues and eggs in expt:		
	1	2	3
Ovary	10.95[a]	8.3[a]	47.5[b]
Infundibulum	4.11[a]	8.3[a]	5.0[a]
Upper magnum	1.4[a]	11.7[b]	15.0[b]
Lower magnum	2.7[a]	10.0[a]	2.5[a]
Isthmus	6.9[a]	11.7[a]	5.0[a]
Uterus	5.5[a]	13.3[a]	5.0[a]
Vagina	9.6[a]	11.7[a]	37.5[b]
Cloaca	50.7[a]	8.3[b]	80.0[c]
Organ pool[d]	32.9[a]	38.3[a]	95.0[b]
Intestinal pool[e]	43.8[a]	36.7[a]	70.0[a]
Forming egg	NT[f]	27.1[a]	31.4[a]
Fresh egg	0.8[a]	0[b]	0.6[a]
Avg	15.07[a]	15.83[a]	36.25[b]

[a,b,c] Statistical significance is indicated by different letters within tissue type.

[d] Organ pool = heart, spleen, liver, and gallbladder.

[e] Intestinal pool = small intestine and cecum, with contents.

[f] NT, not tested by culture.

sion development was not evident in the previous experiments. However, the identity of the oviduct tissue that was associated with forming eggs was recorded in order to better assess the possible routes of bacterial invasion. The results of experiment 3 are presented in Table 4. All tissues from control birds were negative. High levels of colonization of the intestinal and organ pools and cloacal samples from infected hens were maintained throughout the experiment. Half the tissue samples from the ovary and vagina were positive as of day 2 p.i., and these levels were maintained as well. The incidence of infected tissue from the upper oviduct (infundibulum, upper and lower magnum, isthmus, and uterus) was low throughout. Eleven forming eggs from infected hens were found to be positive for *S. enteritidis* from days 2 to 20 p.i. The pathogen was cultured from the ovaries of eight of the hens from which these eggs were taken. Six of these eggs were isolated from the uterus, one from the infundibulum, and one from the upper magnum, with their associated tissues. Another positive egg was isolated with positive upper magnum tissue, but in this case the ovarian tissue was negative. The remaining two eggs were isolated with uterine tissue. Only the cloacal and vaginal tissues from these hens were positive. In these two cases, infection of the egg appeared to be the result of bacteria ascending to the uterus from the cloaca or vagina. Of the 24 remaining developing eggs that were cultured and found to be negative, 6 came from hens with positive ovaries.

Five freshly laid eggs from days 2, 3, 11, 12, and 13 p.i. were culture positive for *S. enteritidis*. Of the five hens, two had both a positive ovary and cloaca, one had a negative ovary but a positive cloaca, one had a positive ovary but a negative cloaca, and one had a negative ovary and cloaca but a positive organ pool, again denoting the possibilities for descending, ascending, and lateral infections of the eggs. All of the cultures from the peritoneal cavities of infected and control hens were negative for *S. enteritidis* throughout the experiment.

Nine of the forming-egg-associated tissue samples were found to be positive when cultured with intact egg yolk and albumen, while two were found to be positive only after the yolk and albumen had been disrupted and mixed with the tissue.

Table 3 provides a comparison among the experiments of the percentages of *S. enteritidis*-positive tissues and eggs taken from infected hens. Most notably, the ovarian, cloacal, and organ pool tissues from experiment 3 were significantly more colonized than those from experiments 1 and 2 and the overall percentage of tissue colonization in experiment 3 was twice that of experiments 1 and 2 ($P = 0.001$). Nevertheless, the incidence of positive freshly laid eggs in experiment 3 was not significantly different from that of experiment 1, nor was the incidence of positive forming eggs significantly different from that of experiment 2.

Clinical signs and gross necropsy. *S. enteritidis*-inoculated chickens from all three experiments appeared to be clinically normal throughout the experimental period. There were no signs of depression or diarrhea. Feeding, drinking behavior, and egg production remained normal. At necropsy, no gross lesions were observed in any of the birds.

Immunohistochemical staining for *S. enteritidis*. (i) MAb-based immunohistochemical staining. Dilutions of 1:500 and 1:1,000 of MAb 1G10 were found to stain *S. enteritidis* without background staining and were used for subsequent procedures. In air-dried smears, isolated bacteria could be differentiated easily, although the structural detail of organisms in aggregations tended to be lost (Fig. 1a). In tissue sections, in which individual organisms could rarely be found, antigen staining was most often seen as diffuse and amorphous. Figure 1b demonstrates the staining of *S. enteritidis* in albumen-secreting tissue surrounding an incomplete egg from an infected hen. Figure 1c is a control that shows staining of albumen-secreting tissue from an uninfected hen.

(ii) H&E staining for inflammatory lesions. In experiment 1, no significant inflammatory lesions were detected in any tissue, independent of the detection of antigen by histochemical staining. Very few significant lesions that were consistent with a bacterial infection were detected in tissues from experiment 2. In a culture-positive ovum taken from a hen 2 days p.i., a mild, multifocal, chronic oophoritis with granuloma formation was evident. In one positive vaginal section taken from a hen 7 days p.i., a cluster of basophilic bacteria was present but no significant lesions were observed. Seven culture-positive tissues from one hen, which was sacrificed on day 15 p.i., showed lesions consistent with a subacute inflammatory bacterial infection. In the ovary, the stroma was expanded by areas of fibrin and inflammatory cells consisting of heterophils, macrophages, and lymphocytes. Several follicles had heterophils transmigrating through the wall, and inflammatory cells were disseminated throughout the antrum. In sections of the upper oviduct, it was frequently observed that the serosa was folded and covered by plump mesothelial cells. There was multifocal expansion of the serosa by inflammatory infiltrates, and clumps of short and long bacilli were observed in the superficial exudate. The tissue of the uterus displayed moderate, multifocal, subacute, serositis with multifocal granuloma formation. A locally extensive region of serosa was expanded by deposits of fibrin and dense collections of inflammatory cells. Granuloma formation was characterized by collections of amorphous eosinophilic material surrounded by vacuolated multinucleate giant cells and macrophages.

DISCUSSION

This is the first report on the colonization of *S. enteritidis* in the sections of the oviduct as they relate to the formation of eggs. In this study, an average of 29.25% of the forming eggs taken from the oviduct of infected laying hens were positive for *S. enteritidis*, compared with a rate of 0 to 0.6% colonization of freshly laid eggs from the same hens.

Colonization of forming eggs was not measured in experi-

TABLE 4. Positive and negative culture status of tissues from infected[a] laying hens after oral inoculation with *S. enteritidis* 575

Day/hen	Culture status of tissue(s)							
	Ovary	Upper oviduct	Vagina	Cloaca	Organ pool[b]	Intestinal pool[c]	Forming egg	Related tissue[d]
1/A	−	−	+	+	+	+	None	None
1/B	−	−	+	+	+	+	None	None
2/A	−	−	+	+	+	+	+	+ (uterus)
2/B	−	−	+	+	+	+	−	− (uterus)
3/A	+	−	−	+	+	+	+	+ (uterus)
3/B	−	−	−	+	+	+	−	− (uterus)
4/A	−	+ (upper magnum)	−	−	−	+	None	None
4/B	+	−	+	−	−	+	None	None
5/A	+	+ (All)[e]	+	+	+	+	+	+ (upper magnum)
5/B	+	−	−	−	+	+	None	None
6/B	+	−	−	+	+	+	−	− (uterus)
6/B	−	−	+	−	+	+	−	− (uterus)
7/A	+	+ (uterus)	+	+	+	+	−	− (uterus)
7/B	+	−	−	−	+	+	+	+ (uterus)
8/A	−	−	+	+	+	+	−	− (uterus)
8/B	+	+ (lower magnum)	−	−	+	+	−	− (uterus)
9/A	−	−	−	+	+	+	−	− (uterus)
9/B	+	−	−	+	+	+	−	− (uterus)
10/A	−	+ (all)	+	+	+	+	+	+ (upper magnum)
10/B	+	−	−	−	+	+	−	− (uterus)
11/A	−	−	−	+	+	+	−	− (uterus)
11/B	−	−	−	−	+	+	−	− (uterus)
12/A	−	−	+	+	−	+	−	− (uterus)
12/B	−	−	−	−	−	+	−	− (uterus)
13/A	−	−	−	−	+	+	−	− (uterus)
13/B	−	−	−	−	−	+	−	− (lower magnum)
14/A	−	−	−	+	−	+	−	− (lower magnum)
14/B	+	+ (isthmus)	−	+	+	+	+	+ (uterus)
15/A	+	−	+	+	+	+	+	+ (infundibulum)
15/B	+	+ (infundibulum)	−	−	+	+	+	+ (uterus)
16/A	−	−	−	+	−	+	−	− (uterus)
16/B	−	−	−	+	−	+	−	− (uterus)
17/A	−	−	−	+	−	+	−	− (uterus)
17/B	−	−	−	−	+	−	−	− (uterus)
18/A	−	−	−	−	−	+	−	− (uterus)
18/B	+	−	−	+	−	+	+	− (uterus)
19/A	+	−	+	+	+	+	+	+ (uterus)
19/B	−	−	−	+	+	+	−	− (uterus)
20/A	−	−	+	+	−	+	+	− (uterus)
20/B	−	−	−	−	−	+	−	− (upper magnum)

[a] All tissues from control hens were negative for *S. enteritidis.*

[b] Organ pool = heart, spleen, liver, and gallbladder.

[c] Intestinal pool = small intestine and cecum, with contents.

[d] Culture status of the tissue associated with the forming egg.

[e] All upper oviduct tissues were positive.

ment 1. This trial was conducted to determine if immunohistochemical staining of tissues from *S. enteritidis*-infected laying hens could explain the invasion of eggs by the pathogen. In spite of positive culture results, most tissue sections showed very few *S. enteritidis* organisms. Lesion development and clinical signs were not evident in chickens infected with either strain 575 or strain E6-E21 of *S. enteritidis*, although both have been found to be highly invasive (2, 18). The present findings are in agreement with many other studies that have described limited infections with inconsistent colonization of organs and tissues, particularly in cases of oral inoculation of *S. enteritidis* (3, 9, 11, 12, 22, 23, 28, 29). The relatively high visibility of organisms in the oviduct associated with four forming eggs suggested the possibility of a higher degree of colonization in these tissues. It was also noted that two of the four forming eggs associated with positive tissues came from hens contributing to the egg pool that became positive at 21 days p.i. Therefore, experiments 2 and 3 were designed to more closely examine the culture status of forming eggs and their associated tissues taken from the oviducts of infected laying hens.

The hens in experiment 2 were inoculated with the highly invasive strain SE6-E21 to attain a higher level of *S. enteritidis* contamination of freshly laid eggs. It became apparent, however, that either these hens were preinfected with salmonellae or became contaminated after the initiation of the experiment, as tissues from control hens were found to be culture positive with *S. enteritidis* within 12 days p.i. with sterile PBS. Nevertheless, the significant incidence of positive forming eggs from infected hens prompted a continuation of the study. The hens in experiment 3 were, indeed, negative for *S. enteritidis* prior to infection with strain 575. No tissues from control hens were found to contain *S. enteritidis*, while positive tissues from infected hens, including both forming and freshly laid eggs, were detected from day 2 p.i. The overall rate of tissue infection in experiment 3 was significantly greater than that detected in experiments 1 and 2 (Table 3). This may relate to an inherent difference in susceptibility to *S. enteritidis* between the White Leghorn layers used in experiments 1 and 2 and the brown-egg layers used in experiment 3 (10a, 15).

The general course of infection of tissues and freshly laid eggs in all three experiments was consistent with previous reports (6, 7, 12, 23). The simple presence of *S. enteritidis* in upper oviduct tissues, regardless of the rate of colonization, was found to be related to the incidence of colonized forming eggs. The rate of colonization of forming eggs was not significantly different between experiments 2 and 3, in spite of the significantly higher percentage of colonized reproductive tissues from experiment 3 hens. However, no positive freshly laid eggs were found in experiment 2, while five freshly laid eggs were positive for *S. enteritidis* in experiment 3. This difference could be related to a primed immune response in the putatively preinfected hens of experiment 2, which would have been absent in the *Salmonella*-naive hens of experiment 3. Overall, these data favor the possibility of an ascending infection of the egg from the cloaca, as the production of positive freshly laid eggs in experiments 1 and 3 coincided with an overall high colonization rate of the cloaca, as compared with experiment 2, in which no positive fresh eggs were detected (Table 3). Moreover, in experiment 3, in which the culture status of tissues within a hen could be specifically related to forming and freshly laid eggs, the results further demonstrated the likelihood of descending infections from the ovary and the possibility of infections originating from the tissues of the albumen-secreting magnum and not associated with positive ovarian or cloacal colonization (Table 4). Negative culture results from experiment 3 argue against the possibility of egg infection originating from the peritoneal cavity.

In experiment 3, 9 of 11 egg-associated tissues were positive prior to mixing of the tissues with the yolk and albumen (Table 4). These data, and the high rate of positive forming eggs and associated tissues are consistent with the findings of several studies, which have reported that the albumen is more often positive for *S. enteritidis* than is the yolk (7, 8, 13, 20, 23). These data led investigators to postulate that the oviduct and the albumen could be the principal sites of egg contamination. Finding *S. enteritidis* associated with secretory cells of the upper and lower magnum by immunohistochemical staining is also compatible with the hypothesis that the pathogen may contaminate forming eggs through the albumen (Fig. 1). In order to make sense of these data, which show that one-third of the forming eggs but only 0 to 0.8% of freshly laid eggs were found to be positive for *S. enteritidis*, one must consider the journey of the forming egg through the oviduct. Of the average 26 h required for the formation of a completed egg, the ovum spends approximately 5 h in the magnum, where it is surrounded by thick albumen; this is followed by the addition of two shell membranes in the isthmus. The remaining 21 h are required for shell deposition in the uterus, after which the completed egg is moved through the vagina to pass through the cloaca as it is laid (1). The data presented herein show that 73% of *S. enteritidis*-positive forming eggs were associated with either colonized ovarian tissue or with the tissues of the upper oviduct. Of those positive forming eggs that did not have the above tissue associations, two of three were isolated with uterine tissue that was adjacent to highly colonized cloacal and vaginal tissue, all of which suggests that infection of the forming egg occurs in the upper oviduct, prior to egg shell deposition. The dramatic drop in the incidence of colonized freshly laid eggs compared with that of forming eggs strongly suggests that factors such as antibodies, antibacterial enzymes, and iron-sequestering and bacterial protease-inhibiting proteins within the egg yolk and albumen are able to control *S. enteritidis* infection of forming eggs before the shell is complete and the eggs are laid (1, 3, 8, 13, 15, 20, 26). Once the egg shell and its cuticle are in place, the likelihood of bacterial contamination from internal and external sources is greatly diminished. However, as the 41.9°C egg is laid into a room temperature environment, the pressure gradient created as the egg rapidly cools can be enough to draw bacteria into the egg (1, 14). Therefore, the possibility of contamination of freshly laid eggs as they pass through a heavily colonized vagina or cloaca cannot be ruled out.

Understanding the mechanisms of this host-pathogen interplay will permit the engineering of strategies for intervention and surveillance of *S. enteritidis* colonization of chicken eggs that will complement and invigorate those operative for the host. A focus on the mechanisms of colonization of the forming chicken egg, and its apparent natural ability to control that colonization, might be a more direct path toward the control of problems related to *S. enteritidis* food-borne illness.

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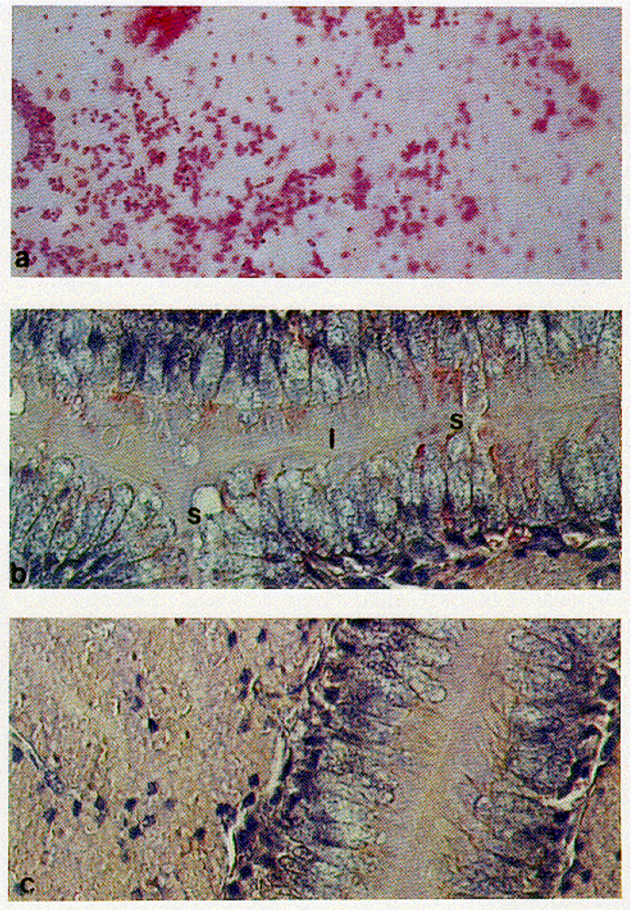
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