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Colonization of Chicken Cecae by *Escherichia coli* Associated with Hemorrhagic Colitis

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Bacterial enumeration, histologic examination, and immunoperoxidase staining demonstrated the ability of an *Escherichia coli* strain associated with hemorrhagic colitis (serotype O157:H7) to colonize chicken cecae for up to 90 days postinoculation after a peroral challenge at 1 day of age. The bacteria induced mild, transient, mucous membrane damage confined to the proximal cecae of healthy, normal-appearing chickens, principally at 14 to 28 days postinoculation. Attachment, effacement, and penetration of the cecal surface epithelium by *E. coli* O157:H7 were observed. With the exception of splenic, hepatic, and cecal tonsil immune-related changes and cecal damage and colonization, no other organ systems or portions of the gastrointestinal tract were affected by the bacteria. Bacterial counts indicated that *E. coli* O157:H7 was predominantly present in the cecae (often at levels greater than 10^6 CFU/g of tissue and contents) and to a lesser extent in the colon. Our results suggest that *E. coli* O157:H7 colonizes chicken cecae and is passed through the colon with fecal excrement. The ability of this organism to colonize chicken cecae indicates that chickens may serve as hosts and possibly as reservoirs for *E. coli* O157:H7.

Escherichia coli O157:H7 has been associated with three outbreaks of hemorrhagic colitis (4, 9, 10), an illness characterized by sudden onset of severe abdominal cramps, grossly bloody diarrhea, and no or low-grade fever. Two of the outbreaks were food associated, with ground-beef sandwiches epidemiologically implicated as the vehicle of transmission (9). To date, *E. coli* O157:H7 has been isolated from only two sources: stool specimens from infected humans and a raw ground-beef patty (12). Hence, little is known about the prevalence and potential sources of this organism in the environment and in foods. The purpose of this study was to determine whether chickens serve as hosts for *E. coli* O157:H7.

MATERIALS AND METHODS

Preparation of inoculum. *E. coli* O157:H7 (strain 932; obtained from G. K. Morris, Centers for Disease Control, Atlanta, Ga.), originally isolated from a patient with hemorrhagic colitis (9), was cultured at 37°C in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) containing 30 μg of nalidixic acid per ml, and a nalidixic acid-resistant isolate was selected for animal challenge studies. Like the parent strain, the nalidixic acid-resistant isolate produced Vero cell cytotoxin (8) and mouse lethality factor (7) in the growth medium. Cells used to challenge chicks were grown for 24 h at 37°C in agitated (100 gyrations per min) 250-ml Erlenmeyer flasks containing 50 ml of Trypticase soy broth. Cells were washed three times and resuspended in 0.01 M phosphate-buffered saline (PBS) (pH 7.5).

Inoculation of chicks. Two groups of 36 1-day-old White Leghorn chicks were used. Eight chicks in each group served as controls and received perorally 0.5 ml of PBS into the crop via a 20-gauge cannula, whereas the remaining chicks received 1.6×10^9 nalidixic acid-resistant *E. coli* O157:H7 by the same procedure. All chicks were held individually in wire cages that did not allow coprophagous activity. Chicks from each group (one control and three *E. coli* O157:H7-challenged chicks at each sampling time) were sacrificed by exposure to carbon dioxide at 2, 4, 7, 10, 14, 21, 28, and 90 days postinoculation. Group 1 chicks were used for histologic examination, whereas group 2 chicks were used for bacterial enumeration tests.

Examination of chicks and tissues. From inoculation until sacrifice, the chicks were monitored for abnormal behavioral signs, diarrhea, and weight changes. Immediately after sacrifice, the internal organs of group 1 chicks were surgically exposed and examined for gross pathological abnormalities of the heart, liver, gallbladder, spleen, kidneys, and gastrointestinal tract. These organs, including the gastrointestinal tract from the proventriculus to the cloaca, were removed, cut into small segments, and placed in 10% neutral buffered Formalin containing 0.5% cetyltrimethylammonium bromide for 48 h at room temperature. Representative segments were embedded in paraffin by standard procedures and in plastic by the cold glycol methacrylate (JB-4; Polysciences, Warrington, Pa.) method (1). Tissue blocks were cut into sections (paraffin, 4 to 7 μm; JB-4 plastic, 2 to 4 μm) and mounted on slides. The paraffin sections were stained with hematoxylin and eosin, and the JB-4 plastic sections were stained with hematoxylin and eosin (13) and azure A (5), and then both were examined microscopically.

The JB-4 plastic tissue sections exhibiting bacterial adherence or damage were selected and treated by an immunoperoxidase-staining procedure to confirm the presence of *E. coli* O157. This staining procedure was done as follows. The tissue sections were treated with 0.1% trypsin (1:250; GIBCO Laboratories, Grand Island, N.Y.) in 0.05 M Tris hydrochloride (pH 7.4) for 30 min at 37°C (2). They were then rinsed in PBS, and the trypsin activity was quenched by exposure to immunoglobulin G-free 5% fetal calf serum (GIBCO) for 15 min at 4°C. The sections were rinsed again briefly in PBS, and endogenous peroxidase was then inactivated by treatment with 1% H_2O_2 for 30 min to 1 h. After a 5-min rinse in PBS, the sections were covered with *E. coli* O157-specific rabbit antiserum (1:25 dilution; *E. coli* Reference Center, Pennsylvania State University, University Park, Pa.) for 1.5 h in a high-humidity chamber. After a 5-min rinse in PBS, the tissue sections were covered with

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goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (1:100 dilution; Sigma Chemical Co., St. Louis, Mo.) for 45 min, rinsed for 5 min in PBS, immersed for 15 min in Karnovsky complete mixture (3) containing 0.01 M imidazole (11), and rinsed for 5 min in 0.05 M Tris hydrochloride (pH 7.6). The tissue sections were processed through different concentrations of ethanol to xylene, and mounted in resin (Pro-Texx Scientific Products, McGaw Park, Ill.), and then examined microscopically.

Immediately after sacrifice, the internal organs of group 2 chicks were surgically exposed, and the heart, liver, kidneys, spleen, gizzard, small intestine (cut into three equal segments), colon, and cecae were aseptically removed and individually placed in preweighed conical plastic sterile tubes (50 ml; Becton Dickinson Labware, Oxnard, Calif.). Each tissue was weighed, diluted (1:10) in cold PBS, and aseptically homogenized for 1 min at 4°C with a Polytron tissue homogenizer (Brinkmann Instruments, Inc., Westbury, N.Y.). Samples were serially diluted (1:10) in PBS, and each dilution was pour plated in duplicate in MacConkey agar containing 30 μg of nalidixic acid per ml. The plates were incubated at 37°C for 48 h, and colonies typical of *E. coli* (purplish-red) were counted. At least one typical colony from a plate with an *E. coli* strain at the highest dilution of tissue was serologically confirmed as *E. coli* O157 by slide agglutination with 0157-specific antiserum (*E. coli* Reference Center).

RESULTS

The physical response of chickens challenged orally with *E. coli* O157:H7 was indistinguishable from that of control animals in that there was no apparent loss of appetite, no reduction in weight gain, no alteration in locomotion, and no diarrhea or respiratory distress. All animals, both inoculated and control, appeared healthy.

Gross pathology. Gross pathological examination of vital organs revealed changes in the cecae and spleens of all *E. coli* O157:H7-treated chickens beginning 10 to 14 days postinoculation. Changes in the cecae were principally observed in the proximal (closed-end) half. Cecae of *E. coli* 0157:H7-challenged chickens were generally distended due to the presence of gas and edema of the proximal mucous membrane, and the cecal tonsils of the inoculated animals were generally enlarged and remained so even at 90 days postinoculation. Cloacal bursae of inoculated and control chickens appeared normal. Splenic enlargement (ca. 25%) was observed in animals 14, 21, and 28 days postinoculation. At 90 days postinoculation, however, the spleens of inoculated and control chickens were similar in size and appearance.

Histopathology. Examination of tissue sections revealed changes in the spleen, liver, and cecae of *E. coli* O157:H7-inoculated animals but not in those of control animals. The heart, kidneys, and gallbladder were not affected and appeared indistinguishable from control tissues.

Splenic enlargement was observed in all *E. coli*-treated animals 14, 21, and 28 days postinoculation. This was due to (i) an increase in the number of lymphocytes in the splenic periarteriolar nodules and (ii) the development of germinal centers within periarteriolar lymphocytes. At 90 days postinoculation, spleens of treated animals closely resembled those of control animals in size and histology.

Liver tissues of inoculated animals were different from those of controls in that, at 10 and 14 days postinoculation, the hepatic reticuloendothelial cells were rounded and more readily discernable and, at 90 days postinoculation, accumulations of lymphocytes were observed in the portal triad connective tissues.

The cecae of inoculated chickens were the only portion of the gastrointestinal tract that evidenced an observable histologic difference from control animal cecae, and only those portions of the cecum that have a colonic, nonvillus epithelium (i.e., the proximal and middle sections of the cecum) were affected. The distal third of the cecum, which has a small intestine-like villus mucous membrane, was not affected.

Damage was localized to the surface epithelium and the underlying lamina propria of the mucous membrane of the proximal and middle sections of the cecum. The glandular (crypt) epithelium remained intact and unaffected. The observed damage was mild and transient, and three events were observed in the course of infection. These events were (i) attachment of *E. coli* O157:H7 to the surface epithelium, (ii) erosive effacement of the surface epithelium, and (iii) bacterial penetration of the surface epithelium to the subjacent connective tissue.

Bacterial attachment was observed as early as 4 days postinoculation, and by 7 to 10 days postinoculation about 80% of the inoculated animals showed both cecal attachment and beginning effacement. Effacement was characterized by the thinning and ultimate disappearance of the epithelial cell striated border and the appearance of epithelial cell cytoplasmic vacuoles. Maximal effacement involving 15 to 40% of the cecal surface epithelium (Fig. 1 and 2) and, occasionally, bacterial penetration to the surface epithelial lamina propria were observed in the cecae of animals 14 to 28 days postinoculation (Fig. 2).

To confirm that the attached and effacing bacteria were indeed *E. coli* O157:H7, we treated cecal tissue sections with an immunoperoxidase stain with *E. coli* O157-specific antiserum. The darkly stained areas on Fig. 3 indicate the presence of *E. coli* O157:H7. The organism is seen attached to the striated border of the surface epithelium and within the luminal contents (Fig. 3A), and, as a result of epithelial cell death and sloughing, is also observed penetrating to the subepithelial lamina propria (Fig. 3B).

Beginning in animals 7 to 10 days postinoculation and continuing in an advanced state 14 to 28 days postinoculation, the lamina propria subjacent to the cecal surface epithelium underwent several changes. These changes included (i) development of subepithelial edema; (ii) accumulation of macrophages, neutrophils, and lymphocytes; and (iii) death and lysis of connective tissue cells. However, the rest of the cecal lamina propria was indistinguishable from control tissues. All animals examined 90 days postinoculation showed evidence of bacterial attachment in the cecae. However, the cecal mucous membrane had recovered, giving no indication of epithelial effacement or bacterial penetration to the lamina propria.

Histologic examination of the enlarged cecal tonsils of inoculated animals revealed that the enlargement was due to a marked increase in the number of lymphocytes. No *E. coli* O157:H7 attachment to the colon of any of the inoculated chickens (1 through 90 days postinoculation) was observed by histologic examination.

Distribution of *E. coli* O157:H7 in organs. Bacterial enumeration of the organs of inoculated chickens indicated that the primary site of *E. coli* O157:H7 localization was the cecae, where greater than 10^6 *E. coli* O157:H7 cells per g were often detected (Table 1). Although present in smaller numbers (1 to 3 $\log_{10}$/g less than in cecae), the organism was also consistently found in the colon. Relatively few (gener-

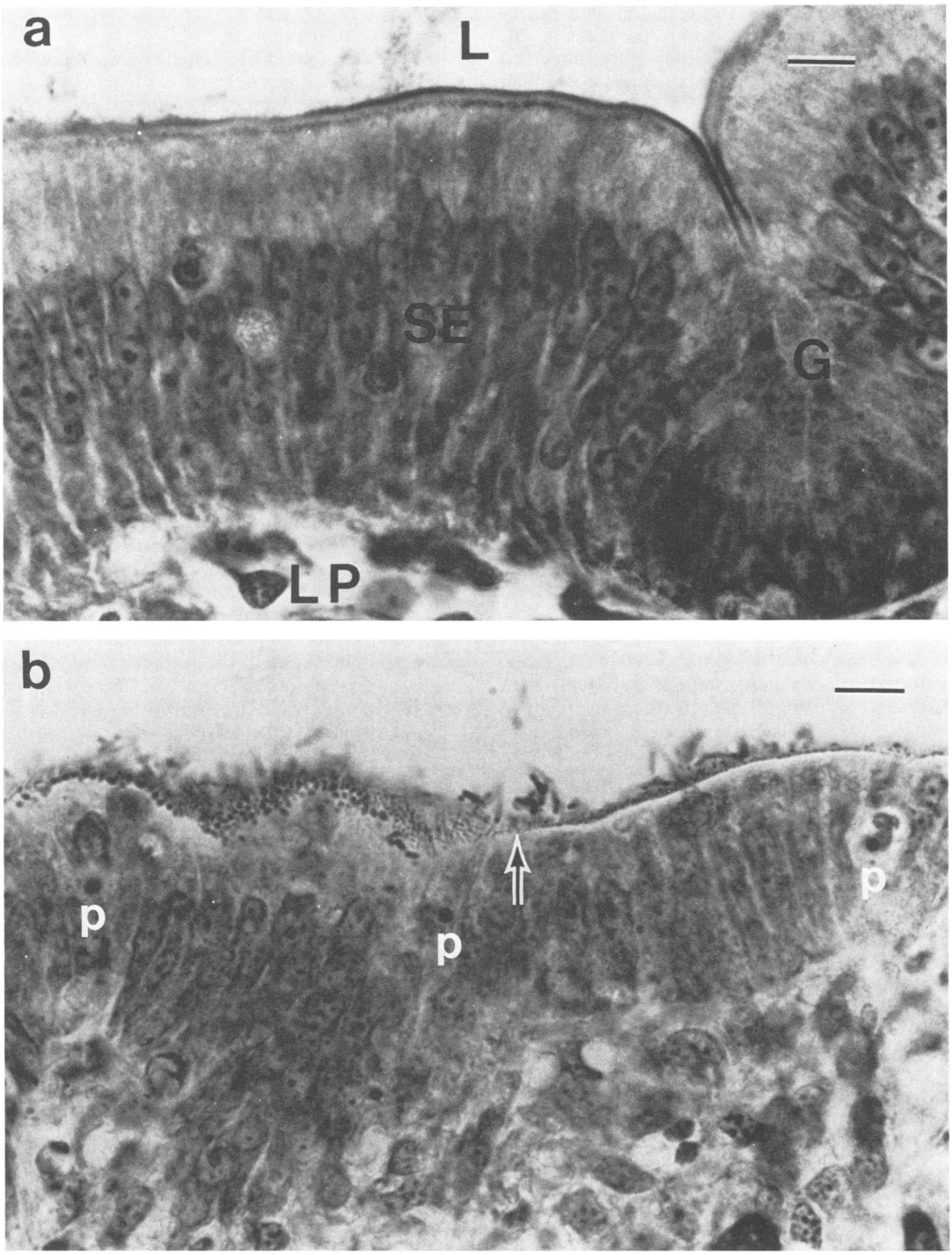

FIG. 1. (a) Histologic view of epithelium of proximal cecum of control chicken. Note the lumen (L), surface epithelium (SE), glandular epithelium (G), and connective tissue of the lamina propria (LP). The section shows the striated border (surface layer consisting of glycocalyx and microvilli) with no bacteria attached (azure A strain; glycol methacrylate section, 2 to 4 μm). (b) Histologic view of epithelium of proximal cecum of chicken 14 days after peroral administration of *E. coli* O157:H7. Bacterial attachment (right of arrow) and both attachment and effacement (left of arrow) are shown. The arrow denotes the transition point where effacement (left of arrow) has produced a loss of the epithelial striated border. The effaced surface epithelium is in a state of displacement, and as a result of vacuolization of the apical cytoplasm, an uneven, mottled area is present beneath the bacteria. Three pycnotic epithelial cell nuclei (p), indicating the death of surface epithelial cells, are also present (azure A stain; glycol methacrylate section, 2 to 4 μm). Bars, 10 μm.

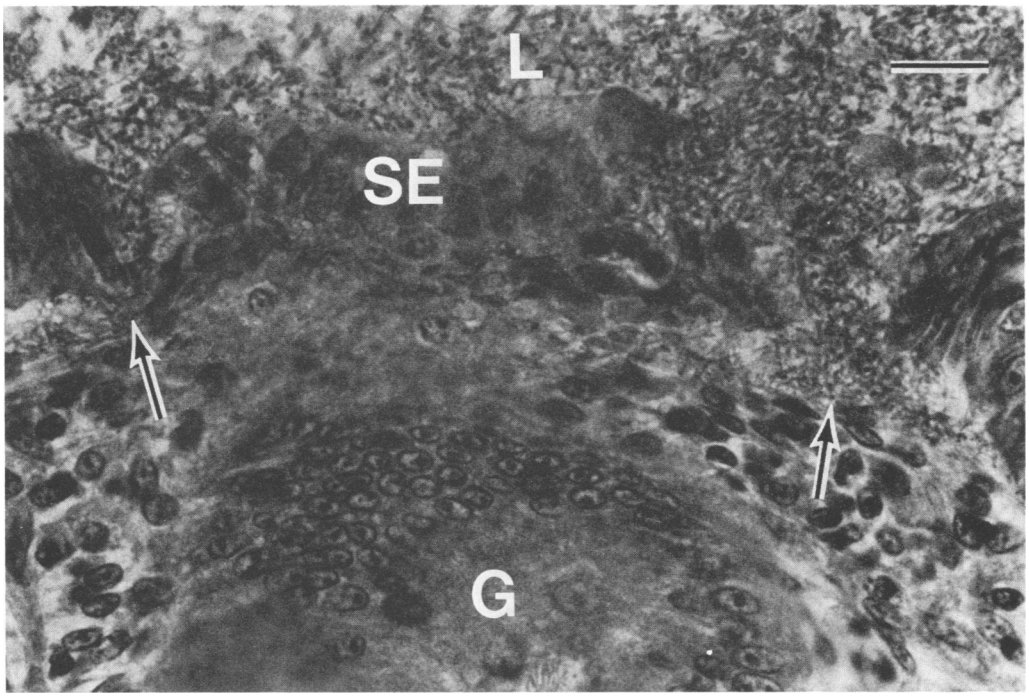

FIG. 2. Histologic view of epithelium of chicken proximal cecum 21 days after peroral administration of *E. coli* O157:H7. There is marked effacement and erosion of the surface epithelium (SE), and bacteria from the lumen (L) have penetrated the surface epithelium at two points (arrows) to the lamina propria. An obliquely cut crypt in the glandular epithelium (G) is shown in the lower center position (hematoxylin and eosin stain; glycol methacrylate section, 2 to 4 μm). Bar, 15 μm.

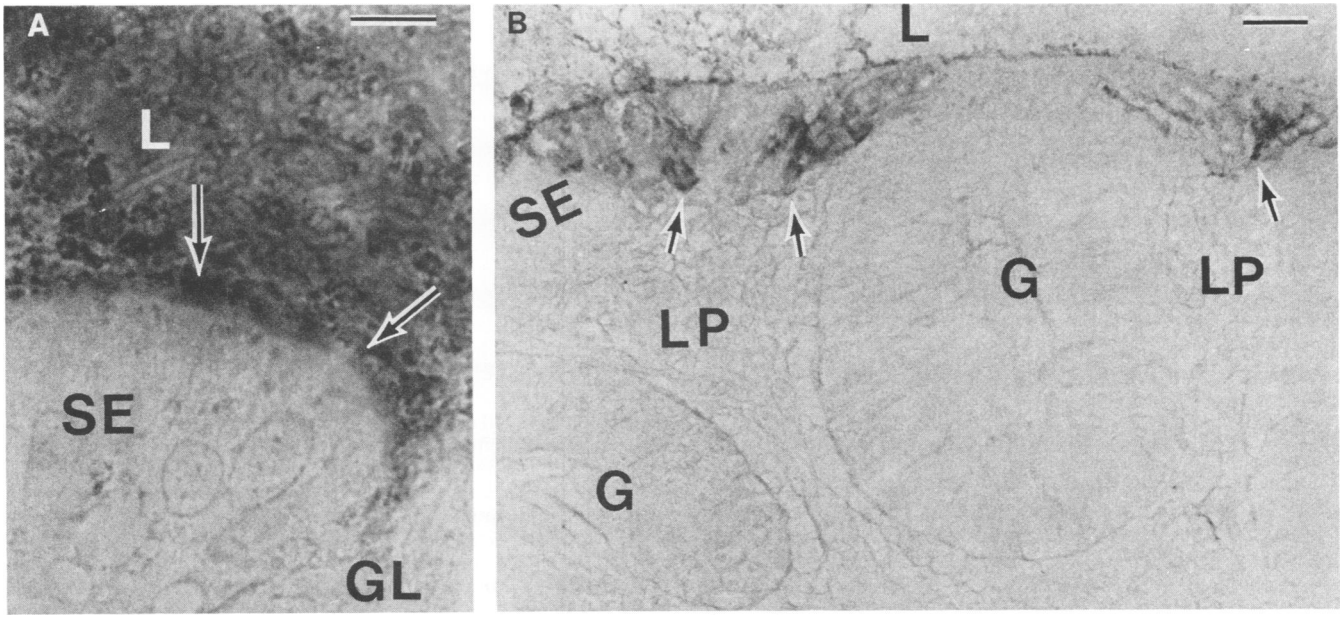

FIG. 3. (A) Histologic view of immunoperoxidase-treated epithelium of chicken proximal cecum 10 days after peroral administration of *E. coli* O157:H7. Dark-staining areas in the luminal contents (L) and on the striated border of the surface epithelium (arrows) are cells of *E. coli* O157:H7. The surface epithelium (SE) reflects downward and is continuous with the glandular epithelium (lower right) which surrounds the glandular lumen (GL) (peroxidase reaction [not counterstained]; glycol methacrylate section, 2 to 4 μm). Bar, 10 μm. (B) Histologic view of immunoperoxidase-treated epithelium of chicken proximal cecum 21 days after peroral administration of *E. coli* O157:H7. Dark-staining areas in the luminal contents (L), and on the surface of and among surface epithelial cells (SE) are cells of *E. coli* O157:H7. Bacterial penetration to the lamina propria (LP) beneath the epithelium has occurred (arrows). The glandular epithelium of crypts (G) is present at the lower left and center right positions (peroxidase reaction [not counterstained]; glycol methacrylate section, 2 to 4 μm). Bar, 15 μm.

TABLE 1. Distribution of *E. coli* O157:H7 in chicken organs after peroral inoculation[a]

Organ	Log_{10} *E. coli* O157:H7/g of tissue and contents at postinoculation times[b]:					
	4 days	7 days	14 days	21 days	28 days	90 days
Small intestine						
Proximal third	2.26 ± 0.60	1.76 ± 1.53	<1.0	<1.0	1.95 ± 1.84	<1.0
Middle third	3.59 ± 0.67	<1.0	<1.0	<1.0	<1.0	<1.0
Distal third	2.74 ± 0.34	1.96 ± 1.76	<1.0	1.78 ± 1.65	2.70 ± 2.64	<1.0
Colon	4.20 ± 1.52	5.17 ± 2.23	3.19 ± 1.33	2.80 ± 2.48	4.76 ± 1.76	2.27 ± 0.13
Cecum	6.53 ± 0.10	6.46 ± 1.55	4.05 ± 1.84	5.99 ± 0.32	6.09 ± 0.25	3.35 ± 0.24

[a] Less than 10 *E. coli* O157:H7 per g of tissue (minimum level of sensitivity) were found in the heart, liver, kidneys, spleen, or gizzard of inoculated chickens at each sampling time.

[b] Each value represents the average count ± standard deviation.

ally <500 CFU/g) *E. coli* O157:H7 cells were intermittently detected in different portions of the small intestine. Fewer bacteria were present in the cecae and colons of chickens 90 days postinoculation than in chickens assayed 4 to 28 days postinoculation; however, greater than 10^3 CFU/g of cecae were still present at 90 days postchallenge. *E. coli* O157:H7 was not detected in the heart, liver, kidneys, spleen, or gizzard of chickens 4 to 90 days postinoculation, nor in any organs of the control animals.

DISCUSSION

The ability of *E. coli* O157:H7 to colonize healthy young chickens was demonstrated by bacterial enumeration, histologic examination, and immunoperoxidase staining. The proximal end of the cecum was the primary site of colonization, where bacterial attachment, effacement, and penetration were observed. *E. coli* O157:H7 attachment to cecae was observed in chickens 4 to 90 days postinoculation, whereas effacement of the cecal epithelial cell striated border was only noted in chickens 7 to 28 days postinoculation. At 90 days postinoculation, proximal cecae appeared normal, with no indication of effacement or bacterial penetration.

The ability of *E. coli* O157:H7 to specifically colonize the proximal end of chicken cecae suggests that this tissue possesses specific receptor sites that allow attachment to occur. Although *E. coli* O157:H7 was consistently detected in the colon by bacterial enumeration, attachment to colonic tissue was not detected by histologic examination. These results suggest that *E. coli* O157:H7 does not colonize the colon but instead originates in the cecae and is transient through the colon via the luminal contents.

The attaching and effacing activities of *E. coli* O157:H7 are not unique to this organism. Moon et al. (6) have shown that certain strains of enteropathogenic *E. coli* can intimately attach to and efface the microvilli and cytoplasm of intestinal epithelial cells of pig and rabbit intestines. *E. coli* O157:H7 is known to produce a shiga-like toxin (7, 8), and filtrate prepared from an overnight culture of the organism has been shown to be cytotoxic to mouse colonic tissue (J. T. Beery, M. P. Doyle, and N. A. Nigley, Curr. Microbiol., in press), producing death and sloughing of colonic epithelium. Perhaps this toxic principal(s) is responsible for the effacement of the proximal cecal surface epithelium of chickens after bacterial attachment. Staining changes in the cecal surface epithelial cell cytoplasm (eosinophilia) before development of vacuolization and the death and lysis of connective tissue cells in the subepithelial lamina propria while the overlying epithelium remained intact and relatively free of cytoplasmic vacuoles provide additional evidence that a toxic principal(s) was elaborated by the attached *E. coli* O157:H7.

Our results indicate that chickens, if exposed to large numbers of *E. coli* O157:H7, may serve as reservoirs for these bacteria. Studies we are currently conducting indicate that large numbers (>10^5 CFU/g) of *E. coli* O157:H7 may be excreted in the feces of chickens more than 5 months after oral exposure to 10^8 cells of this organism. Studies are in progress to determine how long after exposure chickens will continue to fecally excrete *E. coli* O157:H7 and how many cells must be ingested to colonize chickens and result in the active excretion of this organism.

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