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Experimental *Escherichia coli* O157:H7 Carriage in Calves

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Nine weaned calves (6 to 8 weeks of age) were given 10^{10} CFU of a five-strain mixture of enterohemorrhagic *Escherichia coli* O157:H7 by oral-gastric intubation. After an initial brief period of pyrexia in three calves and transient mild diarrhea in five calves, calves were clinically normal throughout the 13- to 27-day study. The population of *E. coli* O157:H7 in the feces decreased dramatically in all calves during the first 2 weeks after inoculation. Thereafter, small populations of *E. coli* O157:H7 persisted in all calves, where they were detected intermittently in the feces and rumen contents. While withholding food increased fecal shedding of *E. coli* O157:H7 by 1 to 2 $\log_{10}$/g in three of four calves previously shedding small populations of *E. coli* O157:H7, the effect of fasting on fecal shedding of *E. coli* O157:H7 was variable in calves shedding larger populations. At necropsy, *E. coli* O157:H7 was not isolated from sites outside the alimentary tract. *E. coli* O157:H7 was isolated from the forestomach or colon of all calves at necropsy. Greater numbers of *E. coli* O157:H7 were present in the gastrointestinal contents than in the corresponding mucosal sections, and there was no histologic or immunohistochemical evidence of *E. coli* O157:H7 adhering to the mucosa. In conclusion, under these experimental conditions, *E. coli* O157:H7 is not pathogenic in weaned calves, and while it does not appear to colonize mucosal surfaces for extended periods, *E. coli* O157:H7 persists in the contents of the rumen and colon as a source for fecal shedding.

During the past 10 years, an increasing number of disease outbreaks of hemorrhagic colitis and hemolytic-uremic syndrome in humans have been linked to the ingestion of beef and dairy products contaminated with *Escherichia coli* O157:H7 (1, 3–5, 11, 17). In the United States alone, *E. coli* O157:H7 causes an estimated 20,000 infections a year (5).

Microbiologic surveys indicate that up to 4.9% of clinically normal cattle shed *E. coli* O157:H7 in their feces (7, 8, 12, 18, 19). There is an apparent age susceptibility to infection, such that weaned calves and heifers are more likely than adult cattle to shed *E. coli* O157:H7 and, after experimental inoculation, shed *E. coli* O157:H7 for longer periods of time (18, 19). Clinical abnormalities have not been noted in calves naturally infected with *E. coli* O157:H7 and shedding the bacteria in their feces (18, 19). In addition, experimental studies suggest that *E. coli* O157:H7 does not cause clinical disease in cattle (6). Hence, *E. coli* O157:H7 appears to be nonpathogenic in cattle.

While cattle are considered a reservoir of *E. coli* O157:H7, very little is known about *E. coli* O157:H7 infection in cattle. The primary objective of this study was to determine the clinical response, the pattern and magnitude of fecal shedding, and the site of localization of *E. coli* O157:H7 in experimentally infected calves. The second objective was to develop a reproducible model of *E. coli* O157:H7 infection in calves which could be used in future studies designed to investigate the effects of management or treatment protocols on *E. coli* O157:H7 survival and shedding in the calf.

MATERIALS AND METHODS

Experimental animals. Twelve (two groups of six; trials I and II) single-source male Holstein calves were reared on milk replacer and weaned at 6 weeks of age prior to transfer to the University of Georgia. Calves were housed individually in climate-controlled BL-2 concrete rooms. Each room had an individual floor drain and was cleaned once daily with water. Calves were fed a mixture of alfalfa pellets and sweet feed (12% minimum crude protein, 2.5% minimum crude fat, 10% maximum crude fiber) twice daily and had free access to water. During a 2-week conditioning period, feces from all calves were sampled once and tested negative, via fluorescent-antibody staining, for bovine virus diarrhea, coronavirus, rotavirus, *E. coli* pilus antigens, and cryptosporidia. Fecal flotation for intestinal parasites and bacterial culture for salmonellae and *E. coli* O157:H7 were also performed. Three fecal samples from each calf, obtained on 3 consecutive days, were cultured for *E. coli* O157:H7. Trial II calves were treated with 9.6% amprolium (10 mg/kg of body weight) for 5 days since small numbers of *Eimeria* oocysts were detected on fecal flotation; all calves were subsequently negative for intestinal parasites. In an effort to minimize any potential effects of Amprolium on bacterial growth, treatment was stopped 7 days prior to the inoculation of calves with *E. coli* O157:H7. *Salmonella* cultures were negative in all calves, and a non-nalidixic-acid-resistant *E. coli* O157:H7 was cultured once by the enrichment procedure from the feces of one calf (trial I; necropsied at 27 days postinoculation [p.i.]) during the conditioning period. Two subsequent fecal cultures from this calf did not yield *E. coli* O157:H7. This calf was kept in the study since it was clinically normal and represented a calf that was naturally infected with a different strain of *E. coli* O157:H7. One trial I calf was eliminated from the study because of inappetence, weight loss, and diarrhea of an undetermined cause. All calves were 8 weeks of age and clinically normal at the time of inoculation.

Inoculation. Strains of *E. coli* O157:H7 may differ in their virulence attributes and in their ability to colonize an animal species. Therefore, calves were inoculated with a mixture of five different strains of toxigenic *E. coli* O157:H7. Strains 932 (ground-beef-associated outbreak) and C7927 (apple-cider-associated outbreak) were isolated from human feces, strain E009 (1) was isolated from ground beef, and strains E0018 and E0122 were isolated from cattle feces (19). To facilitate enumeration of *E. coli* O157:H7 bacteria, strains were selected for resistance to nalidixic acid by culturing on MacConkey sorbitol agar (MSA; Difco Laboratories, Detroit, Mich.) containing 50 μg of nalidixic acid per ml (MSA-NA). Nontoxigenic isolates of *E. coli* obtained from cattle feces were similarly selected for resistance to nalidixic acid. Nontoxigenic *E. coli* strains did not produce Shiga-like toxins and were negative via fluorescent-antibody examination for pilus antigens F41 and K99. Each strain of nalidixic acid-resistant *E. coli* was grown individually in 10 ml of tryptic soy broth (Difco) containing nalidixic acid (50 μg/ml) for 16 to 18 h at 37°C with agitation (150 rpm). Each isolate (2 ml) was transferred to 300 ml of tryptic soy broth containing nalidixic acid (50 μg/ml) and grown individually at 37°C for 16 to 18 h. The bacteria were sedimented by centrifugation (4,000 × *g* for 20 min) and washed three times in 0.01 M phosphate-buffered saline (PBS; pH 7.2). PBS was added to sedimented bacteria in an amount needed to obtain an optical density of 0.5 at 640 nm, representing approximately 10^9 CFU/ml. Two milliliters of each of the five isolates of *E. coli* O157:H7, representing a total bacterial inoculum of 10^{10} CFU,

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was added to 250 ml of 2% skim milk (Difco) just prior to oral inoculation into calves. Nontoxigenic strains of nalidixic acid-resistant *E. coli*, representing a total bacterial inoculum of 10^{10} CFU, were prepared in a similar manner for oral inoculation into control calves. The concentration of each isolate and the final concentration in the skim milk were confirmed by standard dilution plating on MSA in duplicate.

Experimental design. After withholding food for 36 h, calves were inoculated via oral-gastric intubation with skim milk containing *E. coli* O157:H7 (five calves per trial) or nontoxigenic *E. coli* (one calf per trial). Calves were examined daily for clinical signs, including lethargy, pyrexia, diarrhea, and anorexia. Rectal fecal samples were obtained daily for culture and enumeration of *E. coli* O157:H7 bacteria. Blood (12 ml) was collected twice weekly by jugular venipuncture for determination of total and differential leukocyte counts and fibrinogen concentration. In trial I, food was withheld from all calves on days 13 and 14 and 20 and 21 p.i. to determine the effect of fasting on fecal shedding of *E. coli*. One calf each was euthanatized and necropsied on days 15, 16, 22, and 27 p.i. The control calf, given nontoxigenic *E. coli*, was euthanatized and necropsied on day 28 p.i. A second group of six calves (trial II) was similarly inoculated. Because *E. coli* O157:H7 was consistently isolated from the forestomachs of calves in trial I at necropsy, rumen fluid was obtained from trial II calves by oral-gastric tube on days 7, 8, 11, 12, 18, and 19 p.i. to study the proliferation of *E. coli* O157:H7 in this site. Calves were fasted on days 6 and 7 and 11 and 12 p.i. A single calf was euthanatized and necropsied on each of days 13, 14, 19, 21, and 25 p.i. The control calf was euthanatized and necropsied on day 26 p.i.

Isolation of *E. coli* O157:H7 from feces and tissue. Feces (10 g) collected from the rectum of each calf were placed in 15 ml of Cary-Blair medium (Remel Co., Lenexa, Kans.), held at 5°C, and transported to the laboratory in 4 to 72 h (19). A volume of Cary-Blair medium containing 1 g of feces was serially diluted (10^{-1} to 10^{-5}) in 0.85% NaCl, and 0.1 ml from each dilution was plated in duplicate on MSA-NA plates for enumeration. In addition, 0.1 ml of undiluted feces in Cary Blair medium was plated in quadruplicate on MSA-NA plates. Tissue samples collected at necropsy were weighed and washed gently in PBS to remove the contents of the tubular organs. The rinsed tissue samples were resuspended in 9 ml of PBS and homogenized for 1 min at 9,500 rpm with a tissue homogenizer (Janke & Kunkel GmbH & Co., IKL Labortechnik, Staufen, Germany). Tissue sample suspensions (0.1 ml) and content samples (0.1 ml) were plated separately in quadruplicate on MSA-NA plates (18). All plates were incubated at 37°C for 24 h.

A selective enrichment technique was used to detect small populations of *E. coli* O157:H7 when the direct plating method was negative. Feces, tissue, or gastrointestinal contents (10 g) were added to 100 ml of modified Trypticase soy broth containing 3 g of Trypticase soy broth (BBL, Cockeysville, Md.), 0.15 g of bile salts no. 3 (Difco), 1 g of Casamino Acids (Difco), 0.135 g of KH_2PO_4, 0.6 g of Na_2HPO_4, and 5 mg of nalidixic acid (15). The samples were incubated at 37°C for 18 h with agitation (150 rpm). Enrichment broth (1 ml) was serially diluted (1:10) to 10^{-8} in 0.85% NaCl, and 0.1 ml of each dilution was plated onto MSA-NA in duplicate.

Isolates were confirmed as *E. coli* O157:H7 by picking five typical colonies from each MSA-NA plate and plating them again on MSA-NA plates. These isolates were then characterized by the *E. coli* O157:H7 latex agglutination assay (Oxoid Division, Unipath Co., Ogdensburg, N.Y.), an H7 antiserum motility inhibition assay, and the API-20E miniaturized diagnostic kit (Biomerieux) (19).

Genomic fingerprinting. Six isolates of nalidixic acid-resistant *E. coli* O157:H7, isolated from the feces and rumen of one calf in each trial (obtained 17 and 27 days p.i.; three different isolates per calf), were analyzed by genomic DNA fingerprinting to determine which strain(s) was present. Restriction enzyme digests of genomic DNA from the six isolates were compared with the genomic DNA of the five inoculated strains by pulsed-field gel electrophoresis (14). Briefly, bacteria were grown in 10 ml of tryptic soy broth at 37°C until reaching an optical density reading of 1.0 at 640 nm. The bacteria were then sedimented by centrifugation, washed three times in 75 mM NaCl containing 25 mM EDTA (pH 7.4), and resuspended in 0.5 ml of wash solution. This bacterial suspension was mixed with 0.5 ml of 2% (wt/vol) low-melting-point agarose (Bio-Rad Laboratories, Richmond, Calif.) in buffer consisting of 10 mM Tris, 10 mM $MgCl_2$, and 0.1 mM EDTA (pH 7.5). This mixture was dispensed into sample molds (Bio-Rad), and the agarose plugs were digested with proteinase K (2 mg of proteinase K per ml, 50 mM Tris, 50 mM EDTA, 1% *N*-lauroylsarcosine [pH 8.0]) at 56°C overnight. The samples were then washed in 10 mM Tris–5 mM EDTA (pH 7.5) and digested with 50 U of *Xba*I (Bethesda Research Laboratories, Gaithersburg, Md.). After incubating the samples at 37°C overnight, the reaction was stopped by the addition of 20 μl of 0.5 M EDTA. The DNA samples were electrophoresed on a 1.2% agarose gel in 0.5× Tris-borate-EDTA buffer by a contour-clamped homogeneous electric field (CHEF Mapper; Bio-Rad). After electrophoresis for 24 h at 200 V with pulse times of 5 to 50 s and linear ramping and an electrical field angle of 120° at 14°C, the gels were stained with ethidium bromide and bands were visualized and photographed with UV transillumination (14).

Necropsy. Animals were euthanatized with intravenous sodium pentobarbital. The gastrointestinal tract was clamped at the esophagus and rectum and removed in toto. Four- to six-centimeter lengths of duodenum, proximal, middle, and distal jejunum, proximal and distal ileum, proximal and distal cecum, proximal loop of the ascending colon, two centripetal turns and two centrifugal turns of the spiral colon, transverse colon, and descending colon were double tied to allow sampling of all sections for enumeration of *E. coli* O157:H7 bacteria in both the tissue and the intestinal contents without cross-contamination. Sections and contents of rumen, reticulum, omasum, and abomasum and sections of kidney, spleen, liver, gall bladder, jejunal lymph node, ileal lymph node, and cecal lymph node were also collected for culture and enumeration of *E. coli* O157:H7. Sections from all of these sites, as well as sections of prescapular lymph node, skeletal muscle, skin, tonsil, thyroid, thymus, esophagus, heart, pancreas, umbilicus, adrenal, urinary bladder, and testes were also placed in 10% buffered formalin for histologic examination.

Histopathology and immunohistochemistry. Fixed tissues were embedded in paraffin by standard procedures, sectioned at 5 μm, and stained with hematoxylin and eosin. Selected sections were Gram stained by the Lillie-Twort method (13).

Sections of tissue from gastrointestinal sites culture positive for *E. coli* O157:H7 and/or exhibiting large numbers of surface or luminal bacteria histologically were selected and treated by an alkaline phosphatase immunostaining procedure to identify *E. coli* O157:H7. Tissues were deparaffinized in xylene for 10 min, rehydrated through graded alcohols, and rinsed in PBS (Sigma Chemical Co., St. Louis, Mo.). The sections were covered with *E. coli* O157:H7-specific goat antiserum (0.1 μg/ml; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) and incubated in a humidity chamber for 30 min at room temperature. A positive control slide consisted of sections of muscle to which *E. coli* O157:H7 had been added prior to formalin fixation. Negative controls consisted of intestinal sections from non-*E. coli* O157:H7-infected control calves. After a 10-min rinse in PBS, slides were covered with rabbit biotinylated anti-goat antibody (Supersensitive link; BioGenex Laboratories, San Ramon, Calif.) for 20 min in a humidity chamber at room temperature. The slides were then rinsed for 10 min in PBS and returned to the humidity chamber, and tissue sections were covered with alkaline phosphatase-conjugated streptavidin (Supersensitive label; BioGenex Laboratories). After a 20-min incubation, the slides were rinsed in PBS for 10 min, placed in the humidity chamber, and overlaid with substrate solution (fast red substrate; BioGenex Laboratories) for 15 min. The slides were then rinsed in PBS for 10 min, counterstained for 3 min with Mayer's hematoxylin (Sigma), mounted with aqueous medium (Crystal/Mount; Biomeda Corp., Foster City, Calif.) followed by nonaqueous mounting medium (Permount; Fisher Scientific, Fair Lawn, N.J.), and examined microscopically.

RESULTS

Animal health. Two days following inoculation with 10^{10} CFU of the five-strain mixture of *E. coli* O157:H7, three of four calves in trial I developed pyrexia. Body temperatures were increased 0.9 to 1.6°C above the normal range (38.4 ± 0.8°C, mean preinoculation body temperature ± 2 standard deviations) and remained elevated for 1 day in one calf and for 5 to 6 days in the remaining two calves. One calf inoculated with *E. coli* O157:H7 and the control calf infected with nontoxigenic *E. coli* were not febrile. All calves inoculated with *E. coli* O157:H7 developed nonhemorrhagic watery diarrhea beginning 1 day p.i. Diarrhea was observed only on day 1 p.i. in two calves and on days 1 through 3 p.i. in the remaining two calves. The control calf had normal feces throughout the study. All calves maintained a normal appetite and remained alert and responsive.

Calves in trial II did not exhibit an increase in body temperature above the normal range (38.9 ± 0.9°C, mean preinoculation body temperature ± 2 standard deviations). One calf in trial II infected with *E. coli* O157:H7 developed mild diarrhea on day 1 p.i. This calf had normal feces on days 2 through 14 p.i. and then developed anorexia and diarrhea on day 15 p.i. which persisted until the calf was euthanatized on day 19 p.i. The remaining calves in trial II had normal feces, normal appetites, and remained bright and alert. While a number of these calves exhibited periodic coughing, tracheal or lung lesions were not observed at necropsy.

Individual calves periodically exhibited a mild neutrophilia (up to $8.5 \times 10^3/\mu l$; normal, 0.6×10^3 to $4.0 \times 10^3/\mu l$). However, these elevations were transient and occurred both before and after inoculation with *E. coli* O157:H7 (data not shown). Likewise, some calves demonstrated a mild lymphocytosis (up to $10.3 \times 10^3/\mu l$; normal, 2.5×10^3 to $7.5 \times 10^3/\mu l$). Fibrinogen was not elevated above the normal range in any calf (data not shown).

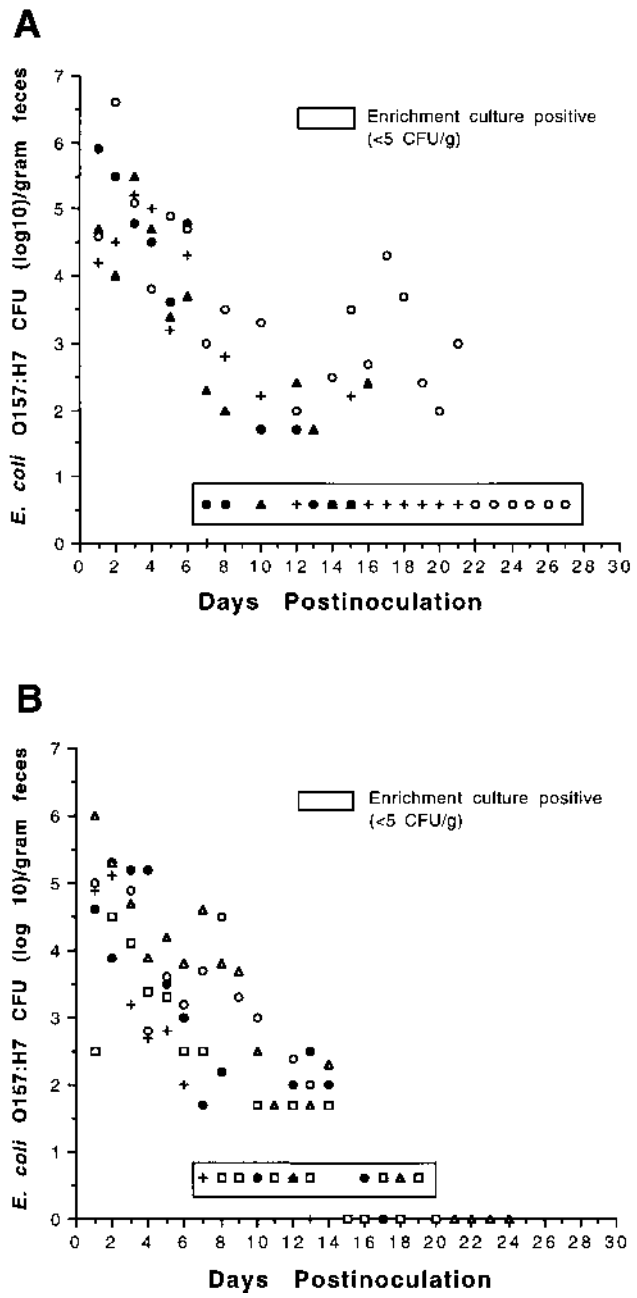

FIG. 1. Time course of fecal shedding of *E. coli* O157:H7 in calves in trials I (A) and II (B). Calves were inoculated with a five-strain mixture of *E. coli* O157:H7 on day 0. Fecal samples were collected daily for enumeration of *E. coli* O157:H7 bacteria. Each different symbol represents the CFU ($\log_{10}$) per gram of feces for an individual calf.

Duration and magnitude of fecal shedding of *E. coli* O157:H7. The numbers of *E. coli* O157:H7 bacteria shed in the feces decreased dramatically over the first 14 days p.i. (Fig. 1). In the first trial, fecal counts ranged from 1×10^4 to 4×10^6 CFU/g of feces at 48 h p.i., decreasing to $\leq 5 \times 10^2$ to 3.1×10^2 CFU/g of feces by day 14 p.i. (Fig. 1A). After 20 days p.i., *E. coli* O157:H7 was detected only by an enrichment procedure, reflecting *E. coli* O157:H7 populations of ≤ 5 CFU/g of feces.

In the second trial, fecal *E. coli* O157:H7 counts ranged from 7.9×10^3 to 2×10^5 CFU/g at 48 h p.i. and ranged from 5×10^1 to 2×10^2 at 14 days p.i. (Fig. 1B). After day 14, *E. coli* O157:H7 could be detected in the three remaining calves only by enrichment culture. Two of these calves shed intermittently; *E. coli* O157:H7 was not recovered from their feces at every sampling. The remaining calf was negative for fecal *E. coli* O157:H7 after 21 days p.i.

The levels of fecal shedding of *E. coli* O157:H7 before and after a 48-h fast were compared (Fig. 2). Overall, of 16 fasting events (seven calves fasted twice, two calves fasted once), fecal shedding of *E. coli* O157:H7 decreased in 7, increased in 7, and remained unchanged in 4. However, of the four calves that

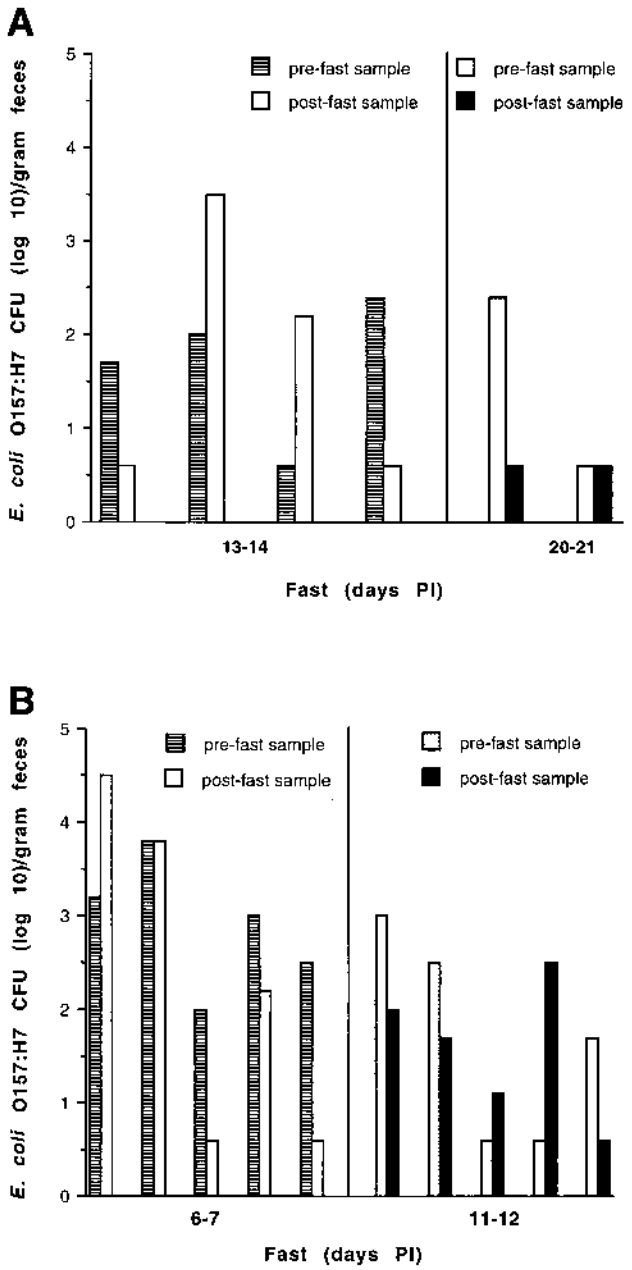

FIG. 2. Recovery of *E. coli* O157:H7 CFU ($\log_{10}$) per gram of feces from experimentally inoculated calves the day before (first bar of each pair) and the day after (second bar of each pair) a 48-h fast. (A) Trial I, food withheld on days 13 and 14 p.i. (left panel) and on days 20 and 21 p.i. (right panel); (B) trial II, calves fasted on days 6 and 7 p.i. (left panel) and on days 11 and 12 p.i. (right panel).

TABLE 1. Enumeration of *E. coli* O157:H7 bacteria in rumen contents (trial II)

Calf no.	No. of CFU (log_{10})/g at:						
	Days p.i.						Necropsy[a] (days p.i.)
	7	8	11	12	18	19	
1	1.2	1.4	<0.7[b]	2.2	—[c]	—	3.5 (14)
2	1.8	2.1	<0.7	0.9	<0.7	<0.7	<0.7 (25)
3	1.4	<0.7	<0.7	<0.7	—	—	<0.7 (13)
4	1.5	1.5	2.3	1.2	NR[d]	—	0.9 (19)
5	0.9	2.1	2.2	<0.7	NR	NR	0.9 (21)

[a] Rumen contents obtained at necropsy on indicated day p.i.
[b] Enrichment culture positive.
[c] —, calf previously euthanatized.
[d] NR, none recovered on enrichment culture.

were shedding low numbers (detectable by enrichment only) of *E. coli* O157:H7 bacteria prior to fasting, fecal shedding of *E. coli* O157:H7 increased after fasting in three calves and remained unchanged in one calf.

Enumeration of *E. coli* O157:H7 in rumen samples. *E. coli* O157:H7 was consistently isolated from fluid obtained via oral-gastric intubation of calves in trial II. The pH of the fluid varied from 6.0 to 8.0, eliminating the abomasum as the site of origin of the fluid. While passage of the tube into the reticulum could not be discounted, passage into the rumen was considered more likely based on the anatomy of the forestomachs. The population of *E. coli* O157:H7 in the rumen fluid decreased over time (Table 1). *E. coli* O157:H7 was isolated from the rumen of all five calves at necropsy.

Sites of localization of *E. coli* O157:H7. Calves were necropsied between 13 and 28 days p.i. *E. coli* O157:H7 was recovered from the rumen and omasum of all inoculated calves in both trials (Table 2). Large populations of *E. coli* O157:H7 were also isolated from the reticulum in seven of nine calves. *E. coli* O157:H7 was not detected in the abomasum of any calf. The populations of *E. coli* O157:H7 isolated and the frequency of positive isolations were low within the proximal small intestine and increased in sites distal to the ileum. Distal sites most commonly containing *E. coli* O157:H7 included the proximal cecum, spiral colon, and descending colon. *E. coli* O157:H7 was not isolated from tonsil, intestinal lymph nodes, spleen, lung, gall bladder, liver, or kidney.

TABLE 2. Recovery of *E. coli* O157:H7 at necropsy from experimentally infected calves (trials I and II)

Sample site	No. positive/ total no.	CFU/g of contents	
		Range[a]	Mean[b]
Rumen	9/9	$(<0.5 \times 10^1)$–(3.2×10^3)	3.8×10^2
Reticulum	7/9	$(<0.5 \times 10^1)$–(2.5×10^3)	4.1×10^2
Omasum	9/9	$(<0.5 \times 10^1)$–(2.5×10^3)	2.9×10^2
Abomasum	0/9	0	0
Duodenum	2/9	$<0.5 \times 10^1$	$<0.5 \times 10^1$
Jejunum	3/9	$(<0.5 \times 10^1)$–(0.8×10^1)	0.3×10^1
Ileum	4/9	$(<0.5 \times 10^1)$–(4.0×10^1)	1.4×10^1
Distal cecum	7/9	$(<0.5 \times 10^1)$–(2.5×10^1)	0.8×10^1
Proximal cecum	6/9	$(<0.5 \times 10^1)$–(4.0×10^2)	7.9×10^1
Ascending colon	5/9	$(<0.5 \times 10^1)$–(5.0×10^1)	2.5×10^1
Spiral colon	7/9	$(<0.5 \times 10^1)$–(6.3×10^2)	1.2×10^2
Transverse colon	5/9	$<0.5 \times 10^1$	$<0.5 \times 10^1$
Descending colon	5/9	$(<0.5 \times 10^1)$–(2.5×10^2)	6.8×10^1

[a] Range of recovery. Enrichment culture positive expressed as $<0.5 \times 10^1$.
[b] Geometric mean recovery.

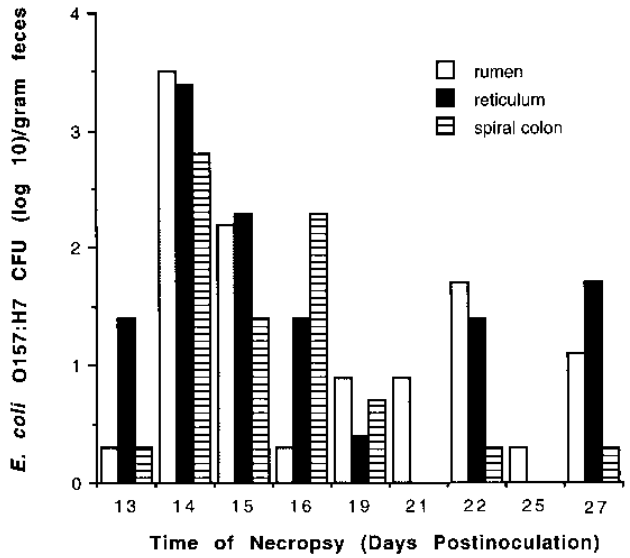

FIG. 3. Recovery of *E. coli* O157:H7 from selected gastrointestinal sites 13 to 27 days after experimental inoculation. *E. coli* O157:H7 CFU (log_{10}) per gram of contents were isolated from the rumen, reticulum, and spiral colon from each inoculated calf at necropsy.

The numbers of *E. coli* O157:H7 recovered from rumen, reticulum, and spiral colon contents during necropsy were compared (Fig. 3). Greater numbers of *E. coli* O157:H7 were recovered from the forestomachs than from the spiral colon in all but one calf. The numbers of bacteria isolated from the forestomachs and spiral colon generally decreased over time, with a greater decrease occurring in the spiral colon than in the rumen and/or reticulum.

To differentiate mucosal colonization from localization within luminal contents, *E. coli* O157:H7 bacteria were enumerated in gently washed sections of intestinal wall (including the mucosal surface) and luminal contents at all sites. In all sites, smaller populations of *E. coli* O157:H7 were isolated from tissue than from luminal contents (data not shown). *E. coli* O157:H7 was not isolated from tissue sites unless the corresponding contents were also positive. In general, enrichment procedures were required to isolate bacteria from tissue sites.

Gross and histopathologic observations. Two calves in trial II (necropsied on days 13 and 19 p.i.) had mild clubbing of the ruminal papilla. Other gross abnormalities of the mucosa of the forestomachs or intestines were not observed in any calf. One calf (calf 6) in trial I had fluid luminal contents within the jejunum, but no associated mucosal abnormalities were present. Histologic abnormalities were not observed in any calf. Large numbers of surface bacteria were observed in some sections of the gastrointestinal tract. Staining sections by an immunohistochemical technique did not identify any of these surface-associated bacteria as *E. coli* O157:H7. Attaching and effacing lesions were not observed.

Identification of *E. coli* O157:H7 strains. Three isolates of *E. coli* O157:H7 were obtained at necropsy from a calf in trial I (day 28 p.i.). The isolates, obtained from the distal ileum, proximal cecum, and rumen, were all identified as strain EO122 (Fig. 4). This strain was originally isolated from cattle feces. Three isolates of *E. coli* O157:H7 were also obtained from a calf in trial II (data not shown). Two fecal isolates, obtained 17 and 18 days p.i., were also identified as strain EO122. The third isolate, obtained from the rumen at necropsy

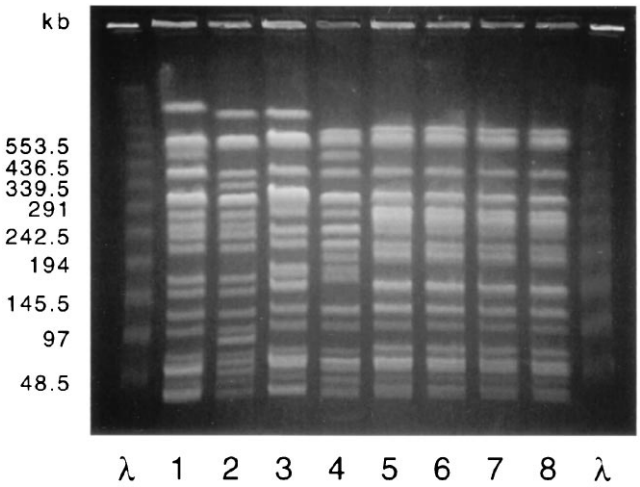

FIG. 4. Comparison of pulsed-field gel electrophoresis profiles of *E. coli* O157:H7 isolates in the inoculum (lanes 1 through 5) and from the distal ileum (lane 6), rumen (lane 7), and proximal cecum (lane 8) of a calf 28 days after inoculation. Lanes: 1, isolate E0018; 2, isolate C7927; 3, isolate 932; 4, isolate E009; 5, isolate E0122.

on day 18 p.i., was identified as strain 932. Strain 932 was originally isolated from human feces.

DISCUSSION

E. coli O157:H7 does not appear to be pathogenic in calves. Inoculation of calves with 10^{10} CFU of *E. coli* O157:H7 did not induce significant clinical disease. The initial fevers and transient nonbloody diarrhea noted in some calves may have been due to endotoxin absorption associated with the administration of massive numbers of gram-negative bacteria. Eight of nine calves remained alert and continued to eat well during the entire study. In addition, gross and microscopic lesions were not observed in calves necropsied between 13 and 28 days after inoculation with *E. coli* O157:H7. While no calves in this study were necropsied prior to 13 days after inoculation, lesions were not observed in calves necropsied 3 days after inoculation with *E. coli* O157:H7 in a previous study (6).

Fasting increases ruminal proliferation and fecal shedding of other serotypes of *E. coli* and salmonellae in sheep and cows (4, 9, 10). Likewise, an effect of fasting on the proliferation of *E. coli* O157:H7 has been demonstrated in vitro. While *E. coli* O157:H7 grows poorly in vitro in rumen fluid from well-fed animals, enhanced growth of *E. coli* O157:H7 is observed in rumen fluid collected from fasted animals (16). These findings suggest that ensuring adequate feed intake in cattle prior to slaughter may decrease the shedding of *E. coli* O157:H7. A clear association between fasting and increased fecal shedding of *E. coli* O157:H7 was not evident in this study. Animals were fasted at a time when the numbers of *E. coli* O157:H7 bacteria shed in the feces were decreasing. Therefore, the reduction of *E. coli* O157:H7 numbers after fasting seen in some calves may have been less than if the fast had not occurred. The most consistent increase in shedding of *E. coli* O157:H7 after fasting was observed in calves shedding low numbers of *E. coli* O157:H7 at the time the fast was instituted. Further studies using larger numbers of calves and including a nonfasted calf group for comparison are required to fully address the effects of fasting on the fecal shedding of *E. coli* O157:H7.

E. coli O157:H7 was confined to the gastrointestinal tract in inoculated calves, as has been previously reported (6). Within the gastrointestinal tract, the forestomachs were a primary site of *E. coli* O157:H7 localization and proliferation. *E. coli* O157:H7 was consistently isolated from rumen fluid throughout the second trial and from the rumen and omasum at necropsy in all calves. In two calves, necropsied on days 21 and 25 p.i., *E. coli* O157:H7 was found only in the rumen and omasum. Fecal cultures for *E. coli* O157:H7 were negative in both of these calves prior to necropsy. Therefore, the primary sites of localization of *E. coli* O157:H7 in calves shedding low numbers of bacteria or shedding intermittently appears to be the rumen and omasum. Based on the high level of acid tolerance exhibited by some strains of *E. coli* O157:H7 (2), a proportion of bacteria originating from the forestomachs likely survive passage through the acidic abomasum and eventually localize in the large intestine and/or are shed in the feces. Factors which influence the proliferation of *E. coli* O157:H7 in the forestomachs may then ultimately determine the magnitude of fecal shedding (16).

In gastrointestinal sites, *E. coli* O157:H7 appears to localize within the intestinal contents rather than colonize the mucosal surface. Attaching and effacing lesions, associated with mucosal colonization of *E. coli* O157:H7, were not observed in experimentally inoculated calves. Further, immunohistochemical staining of sections of intestine and rumen that were culture positive for *E. coli* O157:H7 failed to demonstrate *E. coli* O157:H7 associated with the mucosal surface. Lastly, culturing of gently washed mucosal samples, which would be expected to contain adherent bacteria, generally did not yield *E. coli* O157:H7. Intestinal contents, which would be expected to contain nonadherent bacteria, consistently contained greater numbers of *E. coli* O157:H7 than the corresponding tissue samples.

Five of six *E. coli* O157:H7 isolates obtained from two calves 17 to 28 days p.i. were identified by DNA fingerprinting as cattle fecal strain EO122. While only a limited number of isolates were DNA fingerprinted, this preliminary finding suggests that *E. coli* O157:H7 isolated from cattle feces may survive longer in calves than isolates from food or human sources do.

A predictable pattern of fecal shedding of *E. coli* O157:H7 following oral inoculation was observed. Large numbers of the bacteria were shed in the feces by 24 h p.i. and continued to be shed in large numbers for the subsequent 2 weeks. One calf, identified as shedding a non-nalidixic-acid-resistant strain of *E. coli* O157:H7 prior to experimental inoculation, shed large numbers of *E. coli* O157:H7 bacteria for a longer period of time and had relatively large populations of *E. coli* O157:H7 in the gastrointestinal tract at necropsy. While conclusions based on this observation in a single animal are speculative, it is possible that individual animals may be more likely to carry *E. coli* O157:H7 for longer periods of time and in larger numbers than others due to some unidentified factor(s). In addition, the findings in this calf agree with the observation that previous carriage of *E. coli* O157:H7 does not appear to decrease the magnitude or duration of fecal shedding of *E. coli* O157:H7 following reinoculation (6).

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REFERENCES

1. **Bell, B. P., M. Goldoft, P. M. Griffin, M. A. Davis, D. C. Gordon, P. I. Tarr, C. A. Bartleson, J. H. Lewis, T. J. Barrett, and J. G. Wells.** 1994. A multistate

outbreak of *Escherichia coli* O157:H7-associated bloody diarrhea and hemolytic uremic syndrome from hamburgers: the Washington experience. JAMA **272:**1349–1353.
2. **Benjamin, M. M., and A. R. Datta.** 1995. Acid tolerance of enterohemorrhagic *Escherichia coli*. Appl. Environ. Microbiol. **61:**1669–1672.
3. **Boyce, T. G., D. L. Swerdlow, and P. M. Griffin.** 1995. *Escherichia coli* O157:H7 and the hemolytic-uremic syndrome. N. Engl. J. Med. **333:**364–368.
4. **Brownlie, L. E., and F. H. Grau.** 1967. Effect of food intake on growth and survival of salmonellas and *Escherichia coli* in the bovine rumen. J. Gen. Microbiol. **46:**125–134.
5. **Centers for Disease Control and Prevention.** 1994. *Escherichia coli* O157:H7 outbreak linked to commercially distributed dry-cured salami—Washington and California. Morbid. Mortal. Weekly Rep. **44:**157–160.
6. **Cray, W. C., and H. W. Moon.** 1995. Experimental infection of calves and adult cattle with *Escherichia coli* O157:H7. Appl. Environ. Microbiol. **61:** 1586–1590.
7. **Dorn, C. R., and E. J. Angrick.** 1991. Serotype O157:H7 *Escherichia coli* from bovine and meat sources. J. Clin. Microbiol. **29:**1225–1231.
8. **Garber, L. P., S. J. Wells, D. D. Hancock, M. P. Doyle, J. Tuttle, J. A. Shere, and T. Zhao.** 1995. Risk factors for fecal shedding of *Escherichia coli* O157:H7 in dairy calves. J. Am. Vet. Med. Assoc. **207:**46–49.
9. **Grau, F. H., L. E. Brownlie, and E. A. Roberts.** 1968. Effect of some preslaughter treatments on the *Salmonella* population in the bovine rumen and faeces. J. Appl. Bacteriol. **31:**157–163.
10. **Grau, F. H., L. E. Brownlie, and M. G. Smith.** 1969. Effects of food intake on numbers of salmonellae and *Escherichia coli* in rumen and faeces of sheep. J. Appl. Bacteriol. **32:**112–117.
11. **Griffin, P. M., and R. V. Tauxe.** 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. Epidemiol. Rev. **13:**60–98.
12. **Hancock, D. D., T. E. Bessner, M. L. Kinsel, P. I. Tarr, D. H. Rice, and M. G. Paros.** 1994. The prevalence of *Escherichia coli* O157:H7 in dairy and beef cattle in Washington State. Epidemiol. Infect. **113:**199–207.
13. **Luna, L. G.** 1968. Manual of histologic staining methods of the Armed Forces Institute of Pathology, 3rd ed., p. 81–82. McGraw-Hill Book Co., New York, N.Y.
14. **Meng, J., S. Zhao, T. Zhao, and M. P. Doyle.** 1995. Molecular characterization of *Escherichia coli* O157:H7 isolates by pulsed-field gel electrophoresis and plasmid DNA analysis. J. Med. Microbiol. **42:**258–263.
15. **Padhye, N. V., and M. P. Doyle.** 1991. Rapid procedure for detecting enterohemorrhagic *Escherichia coli* O157:H7 in food. Appl. Environ. Microbiol. **57:**2693–2698.
16. **Rasmussen, M. A., W. C. Cray, T. A. Casey, and S. C. Whipp.** 1993. Rumen contents as a reservoir of enterohemorrhagic *Escherichia coli*. FEMS Microbiol. Lett. **114:**79–84.
17. **Riley, L. W., R. S. Remis, S. D. Helgerson, H. B. McGee, J. G. Wells, B. R. Davis, R. J. Hebert, E. S. Olcott, L. M. Johnson, N. T. Hargrett, P. A. Blake, and M. L. Cohen.** 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N. Engl. J. Med. **308:**681–685.
18. **Wells, J. G., L. D. Shipman, K. D. Greene, E. G. Sowers, J. H. Green, D. N. Cameron, F. P. Downes, M. L. Martin, P. M. Griffin, S. M. Ostroff, M. E. Potter, R. V. Tauxe, and I. K. Wachsmuth.** 1991. Isolation of *Escherichia coli* serotype O157:H7 and other shiga-like-toxin-producing *E. coli* from dairy cattle. J. Clin. Microbiol. **29:**985–989.
19. **Zhao, T., M. P. Doyle, J. Shere, and L. Garber.** 1995. Prevalence of enterohemorrhagic *Escherichia coli* O157:H7 in a survey of dairy herds. Appl. Environ. Microbiol. **61:**1290–1293.