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Fate of Enterohemorrhagic *Escherichia coli* O157:H7 in Bovine Feces

GUODONG WANG, TONG ZHAO, AND MICHAEL P. DOYLE*

Center for Food Safety and Quality Enhancement, Department of Food Science and Technology, University of Georgia, Griffin, Georgia 30223-1797

Received 18 December 1995/Accepted 16 April 1996

Dairy cattle have been identified as a principal reservoir of *Escherichia coli* O157:H7. The fate of this pathogen in bovine feces at 5, 22, and 37°C was determined. Two levels of inocula (10^3 and 10^5 CFU/g) of a mixture of five nalidixic acid-resistant *E. coli* O157:H7 strains were used. *E. coli* O157:H7 survived at 37°C for 42 and 49 days with low and high inocula, respectively, and at 22°C for 49 and 56 days with low and high inocula, respectively. Fecal samples at both temperatures had low moisture contents (about 10%) and water activities (<0.5) near the end of the study. *E. coli* O157:H7 at 5°C survived for 63 to 70 days, with the moisture content (74%) of feces remaining high through the study. Chromosomal DNA fingerprinting of *E. coli* O157:H7 isolates surviving near the completion of the study revealed that the human isolate strain 932 was the only surviving strain at 22 or 37°C. All five strains were isolated near the end of incubation from feces held at 5°C. Isolates at each temperature were still capable of producing both verotoxin 1 and verotoxin 2. Results indicate that *E. coli* O157:H7 can survive in feces for a long period of time and retain its ability to produce verotoxins. Hence, bovine feces are a potential vehicle for transmitting *E. coli* O157:H7 to cattle, food, and the environment. Appropriate handling of bovine feces is important to control the spread of this pathogen.

Escherichia coli O157:H7 has emerged as an important food-borne pathogen, causing hemorrhagic colitis and hemolytic uremic syndrome with increasing frequency since 1982 (7, 11). Epidemiologic investigations have demonstrated that dairy cattle, especially young animals, are a principal reservoir of *E. coli* O157:H7. Farm surveys have frequently isolated verotoxin-producing *E. coli*, including serotype O157:H7, from dairy cattle (3, 5, 15, 18, 23, 25). The pathogen is typically carried by healthy cattle, and isolation of sick cattle is not likely to reduce the risk of transmission; hence, control of infection among cattle is difficult (9, 17). A recent survey of feces of dairy calves in 14 states of the United States revealed that 22% of control herds and 50% of case herds were *E. coli* O157:H7 positive (26). Populations of *E. coli* O157:H7 ranging from $<10^2$ to 10^5 CFU/g of feces were detected in the positive calves, and the animals were determined to intermittently shed *E. coli* O157:H7. Depending on survival of the pathogen, bovine feces containing *E. coli* O157:H7 could be an important source of reinfection of dairy herds and a possible source of contamination of the environment.

Bovine products such as undercooked ground beef and raw milk have most often been implicated in food-borne infections with *E. coli* O157:H7 (7, 8, 11). There also have been reports of *E. coli* O157:H7 outbreaks associated with both drinking and recreational water (13, 14, 22). Investigations indicated that this pathogen could remain viable in water for a period of long time or that the water might be repeatedly recontaminated from unknown sources (22). An outbreak of *E. coli* O157:H7 with 23 confirmed cases was associated with the consumption of freshly pressed apple cider that was made from fallen apples at a farm (2). It was suspected that the apples that had fallen onto soil in the orchard which had been contaminated by cow manure. Since the gastrointestinal tract of cattle is an identified reservoir of this pathogen (21), contamination of food by bovine feces is a likely route of transmitting *E. coli* O157:H7 to humans.

Changes in farm management practices introduced in the 1980s such as irrigating pastures with cow manure slurry have been suggested to have created an environment in which *E. coli* O157:H7 can persist (12). Recent studies suggested that *E. coli* O157:H7 has unusual tolerance against some environmental stresses, such as acidic and dry conditions, compared with most other serotypes of *E. coli* (1, 10). The fate of this pathogen in bovine feces after being shed by cattle into the environment is poorly understood. The purpose of this study was to determine the survival and growth characteristics of *E. coli* O157:H7 in bovine feces at different temperatures.

MATERIALS AND METHODS

Bacteria. A five-strain mixture of nalidixic acid-resistant (50 μg/ml) *E. coli* O157:H7, including strains 932 (human isolate), E0122 (calf fecal isolate), C7927 (human isolate), E09 (meat isolate), and E0018 (calf fecal isolate), was used in this study. Each strain was grown separately in 100 ml of Trypticase soy broth (TSB; Difco Laboratories, Detroit, Mich.) containing 50 μg of nalidixic acid (Sigma Chemical Co., St. Louis, Mo.) per ml for 18 h at 37°C with agitation (150 rpm). The bacteria were sedimented by centrifugation (4,000 × *g*, 12 min), washed three times in 0.1 M phosphate-buffered saline, pH 7.2 (PBS), and resuspended in PBS. Cells were adjusted with PBS to an optical density at 640 nm of 0.5 (approximately 10^8 CFU/ml). Five strains were combined at equal concentrations. The populations of each individual strain and the five-strain mixture were verified by enumeration on tryptic soy agar (Difco). Two levels of inocula (10^3 and 10^5 CFU of *E. coli* O157:H7 per g of feces for low and high inocula, respectively) were used on the basis of epidemiologic data (26).

Feces. Ten healthy dairy cattle from 4 months to 3 years of age from a local dairy farm were used as sources of feces. These cattle were on a diet containing approximately 25% grain, 10% soybean meal, 5% supplement, 40% pasture, and 20% hay. Feces were collected between May and August from each animal through rectum retrieval using sterile gloves. The samples were put in plastic bags and kept cold at 4°C with ice bags during transportation. Feces were used within 4 h after recovery from animals. All feces were mixed well in sterile stomacher bags (35 by 51 cm) at low speed for 8 min in a stomacher (model 3500; Tekmar Co., Cincinnati, Ohio). Before inoculation, feces from each animal were tested for the presence of *E. coli* O157:H7 according to the procedure described by Zhao et al. (26). Aerobic plate counts of fecal samples were determined by plating serial dilutions (1:10) of feces on plate count agar (Difco) and incubating them at 30°C for 48 h. The inoculum (2 ml) of the *E. coli* O157:H7 five-strain

* Corresponding author. Mailing address: Center for Food Safety and Quality Enhancement, University of Georgia, Griffin, GA 30223-1797. Phone: (770) 228-7284. Fax: (770) 229-3216. Electronic mail address: MDOYLE@GAES.GRIFFIN.PEACHNET.EDU.

mixture at the appropriate dilution was added to 198 g of feces and mixed thoroughly in sterile stomacher bags (18 by 29 cm) at high speed for 4 min in a stomacher (model 400; Tekmar) to obtain the desired bacterial concentrations.

Incubation and sampling. Bovine feces in opened stomacher bags were held at 5, 22, and 37°C. *E. coli* O157:H7 counts were determined at 0, 1, 2, 3, 4, and 7 days postinoculation and thereafter once every week until the pathogen could no longer be isolated after enrichment of samples in TSB. Fecal samples (1 g per sampling) were serially diluted (1:10) in saline (0.85% NaCl) solution and assayed for *E. coli* O157:H7 counts by direct plating (0.1 ml of each dilution) in duplicate on Sorbitol-MacConkey agar (Unipath, Oxoid Division, Ogdensburg, N.Y.) with 0.1% 4-methylumbelliferyl-β-D-glucuronide (Sigma Chemical Co.) and nalidixic acid (50 μg/ml). The nalidixic acid was added after the autoclaved Sorbitol-MacConkey agar media cooled to 50°C. Plates were incubated at 37°C for 48 h. When *E. coli* O157:H7 was not detectable by direct plating, surviving cells were detected by enrichment (18 h, 37°C, 150 rpm) in TSB with 50 μg of nalidixic acid per ml and subsequent plating on Sorbitol-MacConkey agar–4-methylumbelliferyl-β-D-glucuronide plates with the same concentration of nalidixic acid. The experiment was terminated when *E. coli* O157:H7 was not detected in three consecutive daily enrichment cultures. Sorbitol-negative, 4-methylumbelliferyl-β-D-glucuronide-negative colonies were counted as presumptive *E. coli* O157:H7. Colonies typical of *E. coli* O157:H7 were randomly selected from plates (average of five per plate) of the highest dilution and were confirmed by agglutination with an *E. coli* O157 antiserum-coated latex test kit (Unipath) and H7 antiserum (Difco) and by biochemical tests using the API-20E miniaturized diagnostic kit (Analytab Products, Plainview, N.Y.). Water activity (a_w), moisture content, and pH were measured at each sampling time. The a_w was determined in a AquaLab CX2 a_w meter (Decagon Devices, Inc., Pullman, Wash.), and moisture content was calculated after drying the fecal samples in a 105°C oven overnight. pH values were determined for fecal samples mixed with 9 ml of saline (0.85% NaCl) solution. The saline solution was made from Millipore E-pure water. All tests were performed in duplicate, and the entire study was duplicated. Averages of the data were reported. Confirmed *E. coli* O157:H7 colonies obtained from the longest survival period at each incubation temperature were assayed for verotoxin production and pulsed-field gel electrophoresis fingerprinting.

Determination of verotoxin production. A sandwich enzyme-linked immunosorbent assay was performed in a 96-well polystyrene enzyme immunoassay-radioimmunoassay plate (GIBCO, Grand Island, N.Y.). Each well was coated with 3 μg of monoclonal antibody 9C9, specific for verotoxin 1 (VT-1) (19, 24), or monoclonal antibody BC5BB12, specific for VT-2 (6) in 100 μl of 50 mM carbonate buffer (pH 9.6). The monoclonal antibodies were previously purified through a protein A affinity column (Bio-Rad Laboratories, Hercules, Calif.). The coated plate was held overnight at room temperature with agitation (150 rpm). Bovine serum albumin (5%; Sigma Chemical Co.) in 50 mM Tris, pH 7.4, containing 150 mM NaCl (TBS) was added to block nonbinding sites, and 100 μl of supernatant fluid of overnight culture in TSB of the longest-surviving *E. coli* O157:H7 isolates at each temperature was added to each well. The five strains used as the inoculum were used individually as positive controls, and TSB was used as a negative control. After four washes with TBS containing 0.05% Tween 20 (TBS-T), 1.0 μg of rabbit polyclonal antibodies that were raised against each toxin individually was added to each well as the secondary detection antibody. Then 100 μl of alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added and incubated at 37°C for 1 h. After four washes with TBS-T, 100 μl of *p*-nitrophenyl phosphate in 1 M 2-amino-2-methyl-1-propanol (Sigma Chemical Co.), pH 9.9 (1 mg/ml), was added to each well. The plate was incubated at 37°C for 1 h, and the optical density at 405 nm was determined with an EL 312e microplate reader (Bio-Tek Instruments, Winooski, Vt.). A reading of 0.15 above the negative control value was considered positive in production of verotoxin(s).

Pulsed-field gel electrophoresis DNA fingerprinting. Genomic DNA analysis by pulsed-field gel electrophoresis of the longest-surviving *E. coli* O157:H7 isolates was performed according to the method described by Meng et al. (16). A total of 12 surviving isolates were selected, including 1 isolate at day 42, 2 at day 49, and 3 at day 56 for 22°C and 1 at day 35, 2 at day 42, and 3 at day 49 for 37°C, for determination of the dominant surviving strain(s). A total of 12 surviving isolates, including 6 at day 63 and 6 at day 70, for 5°C were tested. An overnight culture of *E. coli* O157:H7 in Lenox broth (0.5 ml) at 37°C was transferred to 10 ml of Lenox broth and grown at 37°C to an optical density at 640 nm of 1.0. Cells were sedimented by centrifugation (14,000 × *g*, 5 min), washed three times with 75 mM NaCl–25 mM EDTA, pH 7.4 (SE), and resuspended in 1.0 ml of SE. This suspension (1.0 ml) was mixed with an equal volume of 1.6% (wt/vol) InCert agarose (FMC Bioproducts, Rockland, Maine) in buffer consisting of 10 mM Tris, 10 mM $MgCl_2$, and 0.1 mM EDTA, pH 7.5. The mixture was carefully transferred into sample molds (20 by 9 by 1.2 mm). After 10 min at 4°C, each solidified sample was cut into four plugs. Cells in the plugs were lysed overnight at 56°C with agitation (70 rpm) in 10 ml of a mixture of 50 mM Tris, 50 mM EDTA, *N*-laurylsarcosine (1%), and 2 mg of proteinase K per ml, pH 8.0. On the second day, the plugs were washed five times with 10 mM Tris–5 mM EDTA, pH 7.5 (TE) and then one time with 0.1× TE and stored in 50 mM EDTA. The agarose-embedded DNA was digested with 50 U of *Xba*I (Bethesda Research Laboratories, Gaithersburg, Md.) at 37°C overnight. The reaction was stopped by the addition of 20 μl of 0.5 M EDTA. Electrophoresis was performed in a contour-clamped homogeneous electric field device (CHEF DRII; Bio-Rad Laboratories) and 1.2% agarose gels in 0.5× TBE buffer (10× TBE is 0.89 M Tris, 0.025 M EDTA, and 0.89 M boric acid). A lambda concatamer ladder (Promega, Madison, Wis.) was used as the size marker. After electrophoresis for 46 h at a constant voltage of 150 V, pulse times of 15 to 70 s with linear ramping, and a constant temperature of 12°C, the gels were stained with ethidium bromide and bands were visualized with a UV transilluminator after destaining in water overnight.

RESULTS

The average initial aerobic plate count of the fecal samples was 2.7×10^7 CFU/g (range, 6.2×10^6 to 3.4×10^8 CFU/g), and the average initial pH was 7.1 (range, pH 6.8 to 7.4). No *E. coli* O157:H7 was detected in the feces before inoculation. At 37°C, the *E. coli* O157:H7 population increased about 2 $\log_{10}$ CFU/g 2 days postinoculation (Fig. 1). The population decreased gradually to a level detectable only by enrichment ($<10^1$ *E. coli* O157:H7 CFU/g) at day 21. The moisture content decreased from 81.2% at day 0 to about 7% at day 42. Surprisingly, even at a low a_w of 0.387 to 0.462, *E. coli* O157:H7 was still isolated by an enrichment procedure at day 42 for the low inoculum and day 49 for the high inoculum. The pH increased during the incubation period (Table 1).

At 22°C, *E. coli* O157:H7 survived 49 and 56 days for the low and high inocula, respectively (Fig. 1). During the first 3 days, the *E. coli* O157:H7 population increased 1.5 to 2.0 $\log_{10}$ CFU/g. The pathogen was undetectable by direct plating but detectable by enrichment culture for about 5 weeks (Fig. 1). The loss of moisture occurred at a lower rate than at 37°C (Table 1), which may be a factor in the pathogen surviving 1 week longer at 22°C than at 37°C.

At 5°C, there was no growth of *E. coli* O157:H7. The pathogen survived for 63 and 70 days for the low and high inocula, respectively (Fig. 1). There was no substantive decrease of a_w (Table 1).

Strain 932 was the only surviving strain detected in fecal samples held at either 22 or 37°C among the 12 surviving isolates fingerprinted. Perhaps strain 932 has a greater ability to survive in a dry environment than the others. All five strains (932, E0122, C7927, E09, and E0018) survived in feces at 5°C for up to 10 to 11 weeks. All of the longest-surviving isolates (a total of 24) from each incubation temperature retained the ability to produce both VT-1 and VT-2. Hence, holding the pathogen in a stressful environment for long periods of time did not eliminate the toxin-producing ability of the strains assayed.

DISCUSSION

Although the intestinal tract of cattle has been identified as an important reservoir of *E. coli* O157:H7, this is the first report on the survival and growth characteristics of this pathogen in bovine feces. Epidemiologic data from a study of dairy herds revealed that populations of *E. coli* O157:H7 in calf feces range from $<10^2$ to $\geq 10^5$ (but less than 10^6) CFU/g (26). Therefore, two inoculation levels (1.3×10^3 and 1.7×10^5 CFU/g) were chosen for this study. There were a 1.7 to 2.7 $\log_{10}$-CFU/g increase of *E. coli* O157:H7 within 2 days at 37°C and a 1.6 to 1.9 $\log_{10}$-CFU/$_g$ increase within 3 days at 22°C, indicating growth of the pathogen in feces at these temperatures. Thereafter, *E. coli* O157:H7 populations decreased more rapidly at 37°C than at 22°C. Death of *E. coli* O157:H7 may in part be due to the reduction of a_w associated with dehydration of the feces. Feces dehydrated more rapidly at 37°C than at 22°C (Table 1). However, it was unexpected that the pathogen could survive 7 to 8 weeks at 37°C and 8 to 9 weeks at 22°C, considering the low moisture content (<10%) and a_w (<0.5) of

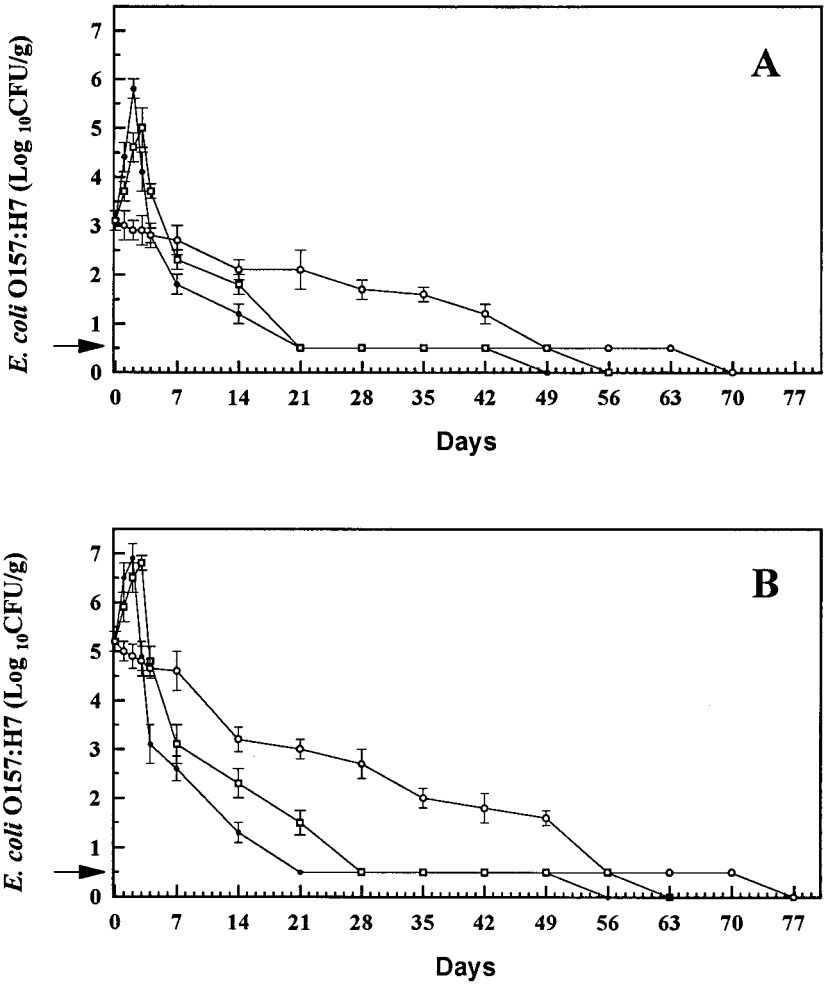

FIG. 1. Fate of *E. coli* O157:H7 in bovine feces at 5°C (○), 22°C (□), and 37°C (●). (A) Low inoculum (10^3 CFU/g); (B) high inoculum (10^5 CFU/g). Arrows indicate detection of *E. coli* O157:H7 by an enrichment procedure only. Error bars represent standard deviations ($n = 4$).

fecal samples in the later part of the study (Fig. 1). Although *E. coli* O157:H7 has unusual tolerance against some environmental stresses (1, 10), there is no documented evidence that this pathogen can survive and persist at low a_w. When feces started drying during incubation at 22 or 37°C, fecal samples in the open bags became powdery in areas near the edges and formed into grain-like pieces. Dehydration of the feces did not appear to occur uniformly, with higher moisture content near the center of the specimen.

DNA fingerprinting by pulsed-field gel electrophoresis was used to differentiate *E. coli* O157:H7 strains to determine if there was strain variation in survival and growth characteristics in feces. In the study, strain 932 survived longer at 22 and 37°C than did the four other strains. This is similar to the observations of Arnold and Kaspar (1), who determined that certain *E. coli* O157:H7 strains survived better than others in highly acidic conditions. All five *E. coli* O157:H7 strains survived for up to 10 to 11 weeks at 5°C, but moisture content or a_w did not appear to be a factor at this temperature, as there was relatively little change during 11 weeks of incubation. Perhaps at 5°C, *E. coli* O157:H7 cells died off because of their inability to continue protein synthesis at below 8°C (4). It is possible that *E. coli* O157:H7 loses its toxin-producing ability because of an instability of phage-carried VT-1 and VT-2 genes (7). We determined that all of the isolates surviving extended incubation in feces retained their ability to produce VT-1 and VT-2.

Our studies revealed that *E. coli* O157:H7 can survive well in feces, depending on temperature and perhaps a_w. Dairy herds have been identified as a reservoir of this pathogen (3, 5, 17, 18, 25). Irrigating pastures with manure slurry has increased in recent years (12). A presumptive association between manure slurry application to pastures and herds carrying *E. coli* O157:H7 was made, indicating that this may be a risk factor for transmitting the pathogen (9, 12). *E. coli* O157:H7-contaminated manure could increase the opportunity for transmitting the pathogen among cattle on farms as well as to produce grown on manure-fertilized soil. Recently, an outbreak of *E. coli* O157:H7 infection among more than 70 people in Montana was associated with consuming contaminated leafy red, green, and romaine lettuce (20). The lettuce may have been contaminated by *E. coli* O157:H7-contaminated water used to irrigate the field on which the lettuce was grown. *E. coli* O157:H7 contamination of dirt, soil, or water is difficult to avoid if there is contact with bovine feces.

Bovine feces is a potential source for spreading *E. coli* O157:H7 to the human food chain as well as to the environment. Effective control of *E. coli* O157:H7 carriage in dairy cattle and appropriate handling or usage of bovine feces are

TABLE 1. Changes in pH, a_w, and moisture content during the incubation of bovine feces

Temp (°C)	Inoculum (CFU/g)	Parameter	Result[a] on day:								
			0	3	7	14	42	49	56	63	70
37	10^5	pH	7.1	7.3	8.4	8.1	8.1	8.0	7.8	ND	ND
		a_w	1.00	0.94	0.88	0.78	0.49	0.42	0.39	ND	ND
		MC[b]	81.2	45.5	37.4	11.7	7.3	6.9	6.8	ND	ND
	10^3	pH	7.1	7.3	8.6	7.8	7.8	7.8	7.7	ND	ND
		a_w	1.00	0.94	0.87	0.76	0.49	0.44	0.36	ND	ND
		MC	81.2	46.5	36.9	12.1	7.6	7.43	6.9	ND	ND
22	10^5	pH	7.1	7.9	8.1	7.9	7.8	7.8	7.8	7.6	ND
		a_w	1.00	0.96	0.91	0.84	0.66	0.56	0.46	0.40	ND
		MC	81.2	55.3	43.9	28.7	18.2	11.2	8.7	7.4	ND
	10^3	pH	7.10	7.3	8.0	7.6	7.8	7.8	7.7	ND	ND
		a_w	1.00	0.98	0.91	0.84	0.60	0.54	0.49	ND	ND
		MC	81.2	53.6	40.5	24.1	15.0	8.7	7.4	ND	ND
5	10^5	pH	7.1	7.0	6.8	6.8	6.8	6.7	6.8	6.6	6.5
		a_w	1.00	1.00	0.99	0.99	0.99	0.99	0.99	0.99	0.99
		MC	81.2	81.0	80.9	80.5	76.9	77.0	75.1	74.5	74.2
	10^3	pH	7.1	6.9	6.9	6.5	6.4	6.5	6.5	6.4	6.3
		a_w	1.00	1.00	0.99	0.99	0.99	0.99	0.99	0.98	0.98
		MC	81.2	80.7	80.3	79.5	77.6	77.1	75.8	75.7	74.3

[a] Data represent the averages of four determinations. ND, not determined.
[b] MC, moisture content, reported as percent (weight/weight).

necessary so that contamination of the environment and food by this pathogen can be prevented.

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