JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1997, p. 892–899 Vol. 35, No. 4
0095-1137/97/$04.00+0

Characterization of *Escherichia coli* O157:H7 and Other Shiga Toxin-Producing *E. coli* Serotypes Isolated from Sheep

INDIRA T. KUDVA,[1] PATRICK G. HATFIELD,[2] AND CAROLYN J. HOVDE[1]*

Department of Microbiology, Molecular Biology, and Biochemistry University of Idaho, Moscow, Idaho 83843,[1] and United States Sheep Experiment Station, Agriculture Research Service, U.S. Department of Agriculture, Dubois, Idaho 83423[2]

Received 16 September 1996/Returned for modification 16 October 1996/Accepted 6 January 1997

The isolation and characterization of *Escherichia coli* O157:H7 and non-O157 Shiga toxin-producing *E. coli* (STEC) strains from sheep are described. One flock was investigated for *E. coli* O157:H7 over a 16-month period that spanned two summer and two autumn seasons. Variation in the occurrence of *E. coli* O157:H7-positive sheep was observed, with animals being culture positive only in the summer months but not in the spring, autumn, or winter. *E. coli* O157:H7 isolates were distinguished by pulsed-field gel electrophoresis (PFGE) of chromosomal DNA and toxin gene restriction fragment length polymorphism (RFLP) analysis. Ten PFGE patterns and five RFLP patterns, identified among the isolates, showed that multiple *E. coli* O157:H7 strains were isolated from one flock, that a single animal simultaneously shed multiple *E. coli* O157:H7 strains, and that the strains shed by individuals changed over time. *E. coli* O157:H7 was isolated only by selective enrichment culture of 10 g of ovine feces. In contrast, strains of eight STEC serotypes other than O157:H7 were cultured from feces of sheep from a separate flock without enrichment. The predominant non-O157 STEC serotype found was O91:NM (NM indicates nonmotile), and others included O128:NM, O88:NM, O6:H49, and O5:NM. Irrespective of serotype, 98% of the ovine STEC isolates possessed various combinations of the virulence-associated genes for Shiga toxin(s) and the attaching-and-effacing lesion (*stx*$_1$, *stx*$_2$, and *eae*), suggesting their potential for human pathogenicity. The most common toxin-*eae* genotype was positive for *stx*$_1$, *stx*$_2$, and *eae*. A Vero cell cytotoxicity assay demonstrated that 90% of the representative STEC isolates tested expressed the toxin gene. The report demonstrates that sheep transiently shed a variety of STEC strains, including *E. coli* O157:H7, that have potential as human pathogens.

The enterohemorrhagic *Escherichia coli* (EHEC) and some other Shiga toxin-producing *E. coli* (STEC) strains are associated with disease in humans and animals. In humans, clinical manifestation of EHEC disease initiates as hemorrhagic colitis (HC), which can progress to the hemolytic-uremic syndrome or thrombotic thrombocytopenic purpura (15). The predominant EHEC serotype associated with human infection and death in the United States is O157:H7 (15). In addition, outbreaks of disease have been traced to non-O157 STEC serotypes, especially in Europe, Australia, and Asia (1, 13, 35). These include O5:NM (NM indicates nonmotile), O6:H31, O26:H11, O26:NM, O48:H21, O91:NM, O111:NM, O113:H21, O128:H2, and O128:NM. Two of these serotypes (O111:NM and O26:H11) can cause postweaning diarrhea in calves.

Multiple virulence factors contribute to the pathogenicity of EHEC and include the production of a Shiga toxin (Stx) or Stxs, the ability to cause attaching-and-effacing lesions that disrupt microvilli on the intestinal wall of the host, and the possession of large plasmids encoding adhesins and hemolysins (14, 40). *E. coli* Stxs, together with the Stx produced by *Shigella dysenteriae* type 1, comprise the Shiga cytotoxin family (39). The *E. coli* Stxs have been classified into two types on the basis of the ability of anti-Stx sera to neutralize their activity. *E. coli* Stx type 1 (Stx1) is neutralized by anti-Stx, while *E. coli* Stx type 2 (Stx2) is not neutralized by the anti-Stx sera (31). The ability to cause attaching-and-effacing lesions in the host intestine is associated with the presence of the *eae* (*E. coli* attaching and effacing) or the highly related *eae*$_{O157}$ gene (45, 46). Originally described for enteropathogenic *E. coli* (EPEC), *eae* encodes a 94-kDa outer membrane protein called intimin (22, 23). The presence of the F107 and K99 fimbrial adhesins is associated with virulence in STEC strains isolated from animals. The F107 fimbria was first identified in the edema disease strain 107/86 and is encoded by a cluster of genes (20). The K99 fimbria was first reported for enterotoxigenic strains of *E. coli*, which utilize it to adhere to the small intestines of calves, lambs, and piglets (10).

Human infections with *E. coli* O157:H7 are usually linked to the consumption of contaminated and improperly cooked beef, unpasteurized milk, or feces-contaminated vegetables, water, or apple cider (6, 15, 18). While several STEC serotypes can cause disease in pigs and calves, most are nonpathogenic and are harbored by asymptomatic, healthy animals. Non-O157 STEC isolates have been isolated from a variety of ruminant and nonruminant animals such as cattle, sheep, goat, pigs, poultry, dogs, and cats (8, 9, 44). However, thus far, *E. coli* O157:H7 has been reported to occur naturally only in cattle, sheep, and deer (17, 26, 34).

This report describes the isolation and characterization of *E. coli* O157:H7 and other STEC isolates acquired from 70 sheep. To determine the isolates' potential as human pathogens, they were characterized for the presence of five virulence genes (*stx*$_1$, *stx*$_2$, *eae*, and the K99 and F107 gene loci) and Stx expression. In addition, chromosomal DNAs from the *E. coli* O157:H7 isolates were analyzed by pulsed-field gel electrophoresis (PFGE), and Stx genes were compared by restriction fragment length polymorphism (RFLP) analysis. This information demonstrated strain variation in *E. coli* O157:H7-positive

* Corresponding author. Mailing address: Department of Microbiology, Molecular Biology, and Biochemistry, University of Idaho, Moscow, ID 83843. Phone: (208) 885-5906. Fax: (208) 885-6518. E-mail: cbohach@uidaho.edu.

individuals and among members of the same flock at single culture times and over the 16 months of this study.

MATERIALS AND METHODS

Animals. Samples from 35 Targhee lambs, designated flock I, were available, one time, for culture for STEC. Samples from a separate, previously described (26) group of 35 ewes, designated flock II, of either Rambouillet or Rambouillet-Finn-Targhee mixed breed were cultured for *E. coli* O157:H7 over a 16-month period at approximately 60-day intervals. A single 10-g fecal sample was obtained from each animal for a sampling month. During the summer and early fall the sheep grazed a native sagebrush-bunchgrass range at the U.S. Department of Agriculture's Agricultural Research Station Sheep Experiment Station near Dubois, Idaho. During the remainder of the year, from October through May, the animals grazed alfalfa and barley aftermath or barley straw near Aberdeen, Idaho. No supplements were included in their diets except in the month of March, when 25% crude protein was added. The differences in their diets correlated with seasonal availability. The weight and diet of each animal were recorded monthly, and climatic conditions during the study were recorded by the weather services near Dubois, Idaho, and Aberdeen, Idaho.

Bacterial strains. American Type Culture Collection (ATCC) *E. coli* O157:H7 strains ATCC 43890 stx_1 positive [stx_1^+] and stx_2 negative [stx_2^-]), ATCC 43889 (stx_1^- and stx_2^+), and ATCC 43894 (stx_1^+ and stx_2^+), all of which have the *eae* gene and gene loci for the K99 and F107 fimbriae, were used as positive controls in biochemical, serological, and colony blot hybridization assays. In the assays used to detect toxin genes or toxin production, *E. coli* O157:H7 ATCC 43888 (stx_1^- and stx_2^-) was used as the negative control. In all other assays, *E. coli* HB101, which lacks the O157 and H7 antigens, the *stx* and *eae* genes, and loci for the K99 and F107 fimbria genes, was used as a negative control.

Isolation of *E. coli* O157:H7. The ewes in flock II were monitored for 16 months, and periodic enrichment culture of fecal samples was done to detect *E. coli* O157:H7 but not other STEC isolates. Fecal samples were cultured by a previously described selective enrichment protocol (25). Briefly, 10 g of feces was incubated at 37°C for 18 to 20 h in Trypticase soy broth (BBL/Becton Dickinson, Detroit, Mich.), supplemented with cefixime (50 μg/liter; Lederle Laboratories, Pearle River, N.Y.; provided by D. Hancock, Washington State University), potassium tellurite (2.5 mg/liter; Sigma Chemical Co., St. Louis, Mo.), and vancomycin (40 mg/liter; Sigma). Serial dilutions of the cultures were plated onto sorbitol MacConkey medium (SMAC; Difco Laboratories, Detroit, Mich.) supplemented with cefixime, tellurite, and 4-methylumbeliferyl-β-D-glucuronide (MUG; 100 mg/liter; Biosynth Ag Biochemica and Synthetica, Skokie, Ill.). Samples collected from flock II in August 1994 were also cultured by a previously described nonenrichment protocol in which fecal samples were directly plated onto SMAC supplemented with MUG (100 mg/liter) by the streak plate technique (3). Colonies were screened for sorbitol fermentation and MUG utilization (fluorescence at 363 nm) after overnight incubation at 37°C. Ten to 30 sorbitol-negative, MUG-negative colonies were selected from cultures of each sample and were tested biochemically and serologically to identify *E. coli* O157:H7.

Isolation of non-O157 STEC strains. Fecal samples from the lambs in flock I were analyzed for STEC strains once in January 1994. Lamb fecal samples collected aseptically by rectal palpation were transported to the laboratory on ice. Upon receipt, the feces were cultured on SMAC agar (Difco) by the streak plate technique. After overnight incubation of the plates at 37°C, 10 isolated colonies per sample (total of 350 colonies) were tested for STEC biochemically and by colony blot hybridization.

Biochemical tests and serotyping. All isolates were confirmed to be *E. coli* by the indole, methyl red, Voges-Proskauer, and citrate tests (IMViC) (11). When required, isolates were differentiated from *Escherichia hermannii* by testing for cellobiose utilization and growth in potassium cyanide (KCN) broth (32). *E. coli* O157:H7 isolates were serotyped for the O157 and H7 antigens as described previously (26). Non-O157 STEC isolates were serotyped by standard technique at the *E. coli* Reference Center (Pennsylvania State University, College Station). Stock cultures of each STEC isolate were maintained at −80°C in 30% glycerol.

DNA probes. Toxin gene probes for stx_1 and stx_2, used in the colony blot DNA hybridization studies, were derived from the 656-bp *Pst*I-*Hin*dIII fragment of pSC25 (19) and the 1,750-bp *Sph*I-*Pst*I fragment of pMJ331 (42), respectively. For RFLP hybridizations, an 842-bp *Sma*I-*Pst*I fragment of pMJ331 was used as the stx_2 probe. To detect the *eae* and the K99 and F107 genes, the following probes were used: a 1,000-bp *Sal*I-*Kpn*I derivative of pCVD434 (23), a 468-bp *Pst*I-*Xba*I derivative of pTL3 (38), and a 500-bp *Eco*RI derivative of pCRII (20), respectively.

Colony blot DNA hybridization. Colony blots of STEC isolates were prepared with Nytran membranes (pore size, 0.45 μm; Schleicher & Schuell, Keene, N.H.) and were hybridized with psoralen-biotin-labelled probes (stx_1, stx_2, and *eae* and the K99 and F107 genes) according to the protocol in the Radfree Probe Labelling and Detection System (Schleicher & Schuell). To enhance stringency, the duration of all posthybridization washes was increased to 10 min. The toxin gene hybridization results were confirmed with fresh stx_1 and the stx_2 probe DNA labelled with [α-^{32}P]dCTP by using the Radprime DNA Labelling System (Gibco BRL, Grand Island, N.Y.).

PFGE. Agarose plugs, embedded with genomic DNA from the ovine *E. coli* O157:H7 isolates, were prepared as described by Barrett et al. (5). The agarose-embedded DNA was digested with 10 U of *Xba*I/plug (Gibco BRL) at 37°C overnight. PFGE was performed in a CHEF-DR II unit (Bio-Rad Laboratories, Hercules, Calif.) by using 1% PFGE-grade (Boehringer Mannheim, Indianapolis, Ind.) agarose-Tris borate buffer gels (5). The DNA was electrophoresed for 20 h at a constant voltage of 200 V (6 V/cm) with a pulse time of 5 to 50 s, an electric field angle of 120°, and a temperature of 15°C before being stained with ethidium bromide. The resulting patterns were analyzed on a DNA ProScan ProRFLP program (DNA Proscan, Inc., Nashville, Tenn.), and the number and size of the DNA fragments were used as criteria for categorizing distinct patterns (see Fig. 2).

RFLP analysis. *E. coli* O157:H7 chromosomal DNA was prepared either by a miniprep protocol for bacterial genomic DNA (2) or by using the Purgene DNA Isolation kit (Gentra Systems Inc., Minneapolis, Minn.) according to the instructions of the manufacturer. Ten micrograms of DNA was digested with either *Pvu*II or *Eco*RI (Gibco BRL) restriction enzymes. Following agarose gel electrophoresis of digested DNA, Southern blots were performed. Briefly, DNA was transferred to Nytran membranes (Schleicher & Schuell) and hybridized with [α-^{32}P]dCTP-labelled stx_1 and stx_2 probes at 65°C in Rapid-hyb buffer (Amersham Life Science).

Vero cell cytotoxicity assay. Eleven representative STEC isolates and 18 representative *E. coli* O157:H7 isolates were selected and tested for toxin expression by a previously described technique (21). Briefly, bacteria were grown in Penassay broth, and culture supernatants, potentially containing toxin, were applied to confluent Vero cell monolayers. The cell-toxin mixtures were incubated at 37°C for 48 to 72 h in a 7.5% CO_2 atmosphere and were examined microscopically for cytopathic effects. Toxicity in >50% of the Vero cells was considered toxin positive. *E. coli* O157:H7 ATCC 43894 (stx_1^+ and stx_2^+) and ATCC 43888 (stx_1^- and stx_2^-) were used as cytotoxicity-positive and -negative controls, respectively.

RESULTS

Animals. All animals in both flocks were free of disease and remained healthy, even when they were shedding STEC. Physical, dietary, and environmental parameters for flock II during the 6 months from June through November 1994 were reported previously (26). Ewes were 13 to 15 months old and weighed an average of 59 kg in June 1994 (26). By September 1995 their average weight was 69 ± 7 kg, a gain within normal expectations. The minimum and maximum temperatures for June and August 1994 and 1995 were similar, and the temperatures ranged from 5.7 ± 3 to 31 ± 2°C. The coldest months were January and February, with minimum temperatures of −12 and −7.2°C, respectively. Monthly precipitation ranged from 0.4 cm/month in February 1995 to 12.3 cm/month in June 1995. Interestingly, the precipitation in June 1995 was a record high and did not correlate with a near record low precipitation of 5.7 cm/month in June 1994.

***E. coli* O157:H7 strains isolated from ewes in flock II.** A total of 140 isolates had IMViC reactions characteristic of *E. coli* (++−−), were negative for the cellobiose and KCN tests, and, following agglutination tests with O157 and H7 antisera, were confirmed to be of the O157:H7 serotype. The number of *E. coli* O157:H7 isolates cultured from individual ewes ranged from 1 to 26.

Seasonal variation in the occurrence of *E. coli* O157:H7-positive ewes was observed. The percentage of *E. coli* O157:H7-positive animals in flock II during the 16 months of the study is presented in Fig. 1. *E. coli* O157:H7 isolates were recovered from ovine fecal samples only by the selective enrichment culture protocol and not by the nonenrichment protocol. No correlation between animal breed and shedding of *E. coli* O157:H7 was observed. Table 1 indicates the ewes (designated A through J) that were culture positive for *E. coli* O157:H7. Sheep A, G, and I were culture positive for *E. coli* O157:H7 at two sampling times.

Characterization of ovine *E. coli* O157:H7 isolates. Because *E. coli* O157:H7 was isolated from different sheep within the same flock, from animals at sampling times separated by 60 to 365 days, and from sheep that were culture negative in the interim between *E. coli* O157:H7 isolations, we wanted to determine if isolates could be distinguished from one another.

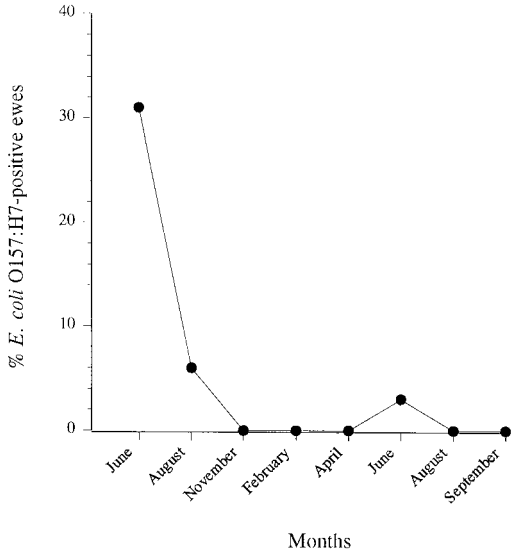

FIG. 1. Seasonal variation in *E. coli* O157:H7-positive sheep. Fecal samples from the ewes in flock II were cultured for *E. coli* O157:H7 on eight occasions (months indicated on the *x* axis) over a 16-month period. The numbers of *E. coli* O157:H7-positive animals are indicated as a percentage of the total numbers of animals tested.

We characterized the *E. coli* O157:H7 isolates on the basis of the presence of virulence genes, chromosomal DNA PFGE patterns, toxin gene RFLP patterns, and cytotoxicity.

(i) Detection of virulence genes. The predominant toxin and *eae* genotype among the *E. coli* O157:H7 isolates tested was stx_1^+, stx_2^+, and eae^+, accounting for 105 of the 140 (75%) colonies tested (Table 2). Of the remaining isolates, 32 (22.9%) were stx_1^+, stx_2^-, and eae^+ and 3 (2.1%) were stx_1^-, stx_2^-, and eae^- (Table 2). The three isolates that tested negative for the toxin and *eae* genes were positive for the K99 and F107 gene loci (data not shown). Colonies were tested for toxin genes by using either psoralen-biotin- or [α-^{32}P]dCTP-labelled probes; however, colony hybridization results were comparable to the toxin gene RFLP results (see the section "Toxin gene RFLP patterns of ovine *E. coli* O157:H7 isolates" below) only when ^{32}P-labelled stx_1 and stx_2 probes were used. In contrast, hybridization results for 90 of the 140 colonies tested by using psoralen-biotin-labelled probes did not correlate with RFLP results. For this reason, only the colony hybridization results obtained with the ^{32}P-labelled stx_1 and stx_2 probes are presented in Table 2. The majority of *E. coli* O157:H7-positive ewes shed organisms with virulence genes with one pattern. In two instances, *E. coli* O157:H7 isolates with different toxin and *eae* genotypes were isolated from single animals. Among the 11 *E. coli* O157:H7 isolates cultured from sheep C in June 1994, six were stx_1^+ stx_2^- eae^+ and five were stx_1^+ stx_2^+ eae^+. Similarly, among the isolates cultured from sheep G in August 1994, three colonies had stx_1^-, stx_2^-, and eae^- and one colony was stx_1^+, stx_2^+, and eae^+.

(ii) PFGE profiles of the ovine *E. coli* O157:H7 isolates. Eleven different PFGE patterns, numbered I through XI, were found among the 140 *E. coli* O157:H7 isolates (Fig. 2). As indicated in Table 1, the predominant PFGE pattern seen from the ovine *E. coli* O157:H7 isolates was pattern I (32%), followed by pattern XI (21.4%) and pattern X (18.5%). *E. coli* O157:H7 isolates with different PFGE patterns were isolated from 10 of 14 culture-positive ewes. For example, two isolates were recovered from sheep A in June 1994; one was PFGE pattern III and the other was PFGE pattern XI. This is in contrast to the 23 isolates recovered from animal G in June 1994, all of which were PFGE pattern I. There was a strong correlation between the PFGE pattern and the presence of virulence genes. All PFGE patterns except pattern I were associated with a single toxin gene profile. For instance, isolates with PFGE patterns III, IV, V, VI, VII, VIII, and XI all had the stx_1^+ and stx_2^+ genotype. However, *E. coli* O157:H7 isolates with the PFGE pattern I possessed a variety of toxin genotypes, such as stx_1^+ and stx_2^+ (sheep D, E, F, and G in June 1994), stx_1^+ and stx_2^- (sheep C in June 1994), or stx_1^- and stx_2^+ (sheep G in August 1994).

(iii) Toxin gene RFLP patterns of ovine *E. coli* O157:H7 isolates. We used the RFLP procedure developed by Samadpour (36) to distinguish human *E. coli* O157:H7 strains to analyze the ovine isolates. This technique uses two restriction enzymes, *Eco*RI, which lacks a restriction site within the toxin genes, and *Pvu*II, which has a unique site in both stx_1 and stx_2

TABLE 1. PFGE patterns of ovine *E. coli* O157:H7 strains isolated from individual ewes at various times

Mo and yr[a]	*E. coli* O157:H7-positive animal	No. of *E. coli* O157:H7 isolates	No. of isolates with the following PFGE patterns:										
			I	II	III	IV	V	VI	VII	VIII	IX	X	XI
June 1994	A	2			1								1
	B	1											1
	C	11	8	1									2
	D	5	2			3							
	E	11	6			1							4
	F	7	4			3							
	G	23	23										
	H	8				1							7
	I	9				1							8
	J	2											2
	K	5											5
August 1994	A	26					4	5	9	8			
	G	4	2								2		
June 1995	I	26										26	
Total		140	45	1	1	9	4	5	9	8	2	26	30

[a] Month and year in which *E. coli* O157:H7-positive sheep were detected in flock II.

TABLE 2. Serotypes and toxin genotypes of ovine STEC isolates

Animal flock	Mo and yr[a]	Serotype	No. of positive animals[b]	No. of STEC isolates	No. of isolates with the following hybridization pattern[c]:			
					1^+	2^+	1^+ and 2^+	1^- and 2^-
I	January 1994	O91:NM	8	23	2		21	
	January 1994	U:NM	6	16	1		15	
	January 1994	O5:NM	6	8	1	2	5	
	January 1994	U:U	1	1		1		
	January 1994	O5:U	1	1	1			
	January 1994	O6:H49	1	2	1	1		
	January 1994	O88:NM	1	1			1	
	January 1994	O128:NM	1	1			1	
II	June 1994	O157:H7	11	84	6		78	
	August 1994	O157:H7	2	30			27	3
	June 1995	O157:H7	1	26	26			

[a] Month and year in which STEC strains were isolated from ovine feces.
[b] A total of 35 animals were tested.
[c] 1, probe for stx_1; 2, probe for stx_2; superscript plus sign, hybridization positive; superscript minus sign, hybridization negative.

and which enables the detection of restriction site polymorphism in and near the toxin genes. Southern blots of *Eco*RI- or *Pvu*II-digested *E. coli* O157:H7 genomic DNA in which the DNA was hybridized separately with the stx_1 or stx_2 probes resulted in six unique DNA band size patterns (Table 3). Among the 140 *E. coli* O157:H7 isolates, the combined results gave five distinct RFLP patterns, designated patterns 1 through 5 (Fig. 3). The presence or absence of Stx genes in the RFLP analysis correlated with the colony blot hybridization results (Tables 2 and 3). Comparisons of the toxin gene RFLP and chromosomal PFGE patterns showed that each PFGE pattern corresponded to a single RFLP pattern, with the exception of isolates with PFGE pattern I, among which RFLP patterns 2, 3, or 5 were found (Table 3).

(iv) Expression of toxin genes. Eighteen isolates were selected to represent the various toxin genotypes, PFGE patterns, and RFLP patterns found among the 140 ovine *E. coli* O157:H7 isolates recovered in this study. These select strains were tested for expression of their toxin genes, and 17 were cytotoxic (data not shown). The one *E. coli* O157:H7 colony (from animal G in August 1994) that was negative for toxin activity lacked both toxin genes. Toxin-positive and -negative control strains were appropriately cytotoxic and noncytotoxic, respectively (data not shown).

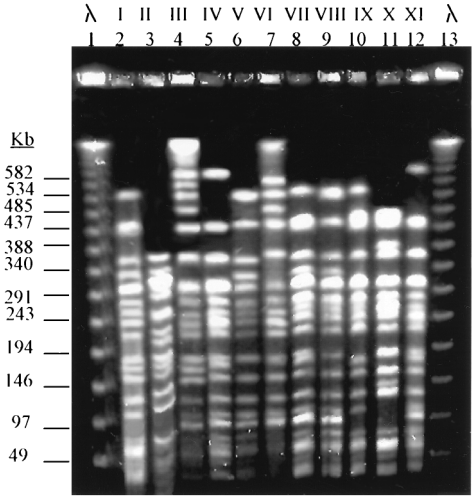

FIG. 2. PFGE patterns of ovine *E. coli* O157:H7 isolates. Eleven representative patterns, designated I to XI (indicated at the top), are shown: I (lane 2), II (lane 3), III (lane 4), IV (lane 5), V (lane 6), VI (lane 7), VII (lane 8), VIII (lane 9), IX (lane 10), X (lane 11) and XI (lane 12). Lanes 1 and 13, bacteriophage lambda DNA ladder standard for PFGE applications (Bio-Rad). The numbers at the left indicate molecular sizes (in kilobases).

Comparisons of *E. coli* O157:H7 strains shed by individual ewes over time. Three ewes in flock II were culture positive for *E. coli* O157:H7 at two sampling times. The distinguishing features of the isolates from these animals are presented in Table 4. Much variability in the ovine *E. coli* O157:H7 strains recovered from individuals was found. Although all isolates from animal A maintained the same toxin-*eae* genotype in both June and August 1994, both the PFGE patterns and the RFLP patterns among the isolates from these two months were distinct. All isolates obtained from this animal in June 1994 were RFLP pattern 1, but all isolates obtained from this same animal in August 1994 were RFLP pattern 2. The 2 isolates recovered from animal A in June 1994 had different PFGE patterns (pattern III or XI), and the 26 isolates recovered from animal A in August 1994 had four PFGE patterns that were different from those seen in June 1994 (patterns V, VI, VII, and VIII) (Table 4). Multiple PFGE and RFLP patterns among the isolates from an individual were most commonly found; however, this was not true in two instances. All 26 of the *E. coli* O157:H7 isolates from animal I cultured in June 1995 had the same profile: stx_1^+, stx_2^+, and eae^+, PFGE pattern X, and RFLP pattern 4. All 23 isolates from animal G, cultured in June 1994, had the same profile: stx_1^+, stx_2^+, and eae^+, PFGE pattern I, and RFLP pattern 2. All isolates from an individual ewe at a given time had the same toxin and *eae* genotype except among the isolates from sheep G obtained in August 1994. At that time, four *E. coli* O157:H7 colonies were recovered from the fecal sample; three colonies were negative for toxin and *eae* genes and one was stx_1^+, stx_2^+, and eae^+. The one stx_1^+, stx_2^+, and eae^+ *E. coli* O157:H7 isolate had PFGE pattern I and RFLP pattern 2. This profile was identical to those of all *E. coli* O157:H7 organisms isolated from this animal 2 months earlier, in June 1994. Of the other three stx_1^-, stx_2^-, and eae^- *E. coli* O157:H7 isolates obtained from the same animal at the same time, two were PFGE pattern IX and RFLP pattern 5 and one was PFGE pattern I and RFLP pattern 5.

Non-O157 STEC isolates from lambs in flock I. The age of the animals in flock I was 8.5 months, and the average weight was 27.8 ± 5 kg when the fecal samples were collected. Forty-three percent (15 of 35 animals) of the animals were positive

TABLE 3. Toxin gene RFLP patterns and PFGE patterns for the ovine *E. coli* O157:H7 isolates

RFLP pattern[a]	DNA band size (kb)				No. (%) of isolates[b]	PFGE pattern[a]	Sheep (mo and yr)[c]
	stx$_1$		*stx*$_2$				
	*Eco*RI	*Pvu*II	*Eco*RI	*Pvu*II			
1	8	3	5	5–6	40 (28.6)	III, IV, XI	A, B, C, D, E, F, H, I, J, and K (June 1994)
2	8	3	6–7	>12	65 (46.4)	I, V, VI, VII, VIII	C, D, E, F, and G (June 1994) A and G (August 1994)
3	8	3			6 (4.3)	I, II	C (June 1994)
4	4	8			26 (18.6)	X	I (June 1995)
5					3 (2.1)	I, IX	G (August 1994)

[a] RFLP and PFGE patterns were generated as described in Materials and Methods.
[b] Number of *E. coli* O157:H7 isolates (percentage of all isolates).
[c] Month and year of fecal sampling.

for STEC. Fifty-three non-O157 STEC colonies (53 of 350, or 15% of the colonies tested) were isolated. Each non-O157 STEC isolate had IMViC reactions characteristic of *E. coli* (++−−), and unlike *E. coli* O157:H7, the isolates were MUG utilization positive.

Characterization of non-O157 ovine STEC. (i) Serotyping. Eight STEC serotypes were recovered from flock I (Table 2). One to 26 STEC isolates were recovered from each of the 15 culture-positive lambs, and among these lambs, 53% shed STEC isolates of only one serotype, 27% shed STEC isolates of two serotypes, and 20% shed STEC isolates of three serotypes. The most common serotype, O91:NM, was cultured from 23% of the lambs, and 62.5% of the O91:NM-positive lambs simultaneously shed STEC isolates of at least one other serotype. The next most common serotypes isolated were O5:NM and U:NM (U indicates untypeable); both were found in 17% of the culture-positive lambs. Each of the remaining serotypes recovered, O128:NM, O88:NM, O6:H49, O5:U, and U:U, were cultured only once, each from a different lamb. Among the 53 isolates, 43% were O91:NM, 30% were O5:NM, and 15% were U:NM.

(ii) Detection of virulence genes. Among the 53 non-O157 STEC isolates, 43 (81%) were *stx*$_1^+$ and *stx*$_2^+$, 6 (11%) were *stx*$_1^+$, and 4 (8%) were *stx*$_2^+$ (Table 2). In addition, colony blot hybridizations demonstrated that all non-O157 STEC isolates were positive for *eae* and both the K99 and F107 gene loci (data not shown).

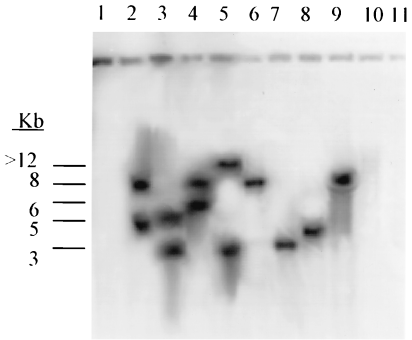

FIG. 3. RFLP patterns of ovine *E. coli* O157:H7 isolates. Chromosomal DNA was digested with *Eco*RI (even-numbered lanes) and *Pvu*II (odd-numbered lanes) and was simultaneously hybridized with radiolabelled oligonucleotide probes specific for *stx*$_1$ or *stx*$_2$. Representative patterns, designated 1 through 5, are shown: pattern 1 (lanes 2 and 3), pattern 2 (lanes 4 and 5), pattern 3 (lanes 6 and 7), pattern 4 (lanes 8 and 9), and pattern 5 (lanes 10 and 11). Lane 1, 1-kb ladder (Gibco BRL) and *Hin*dIII-digested bacteriophage lambda DNA ladder (International Biotechnologies, Inc., New Haven, Conn.) (not shown) as size standards. The numbers at the left indicate molecular sizes (in kilobases).

(iii) Expression of toxin genes. Eleven non-O157 STEC isolates were selected as representative strains on the basis of their serotypes and toxin genotypes. These 11 isolates were tested for toxin expression, and 9 were cytotoxic and 2 were not cytotoxic (data not shown). Interestingly, both nontoxic strains were positive for the toxin gene(s) by DNA hybridization. One isolate, an O88:NM isolate, was positive for both *stx*$_1$ and *stx*$_2$, while the other isolate, an O5:U isolate, was positive for *stx*$_1$. Toxin-positive and toxin-negative control strains were appropriately cytotoxic and noncytotoxic, respectively (data not shown).

DISCUSSION

The most significant findings of this study were that sheep shed *E. coli* O157:H7 in a seasonal pattern, that different strains of *E. coli* O157:H7 were shed simultaneously from individuals and by members of the same flock, and that these strains changed over time. In addition, we showed that sheep shed non-O157 STEC serotypes, several of which have been implicated in human disease.

We reported earlier that, like cattle, sheep are a natural reservoir for *E. coli* O157:H7 (26). This study expanded on that observation and showed that, like cattle, the most frequent occurrence of culture-positive sheep was in the summer (17). Variation in the occurrence of *E. coli* O157:H7-positive sheep was observed, and animals were culture positive only in the summer, not in the spring, autumn, or winter (Fig. 1). This seasonal variation coincides with the seasonal variation in reported cases of infection with *E. coli* O157:H7 in humans (18). Because we were able to distinguish individual ewes by use of ear tags, we were able to show that ewes shedding *E. coli* O157:H7 in the summer months became culture negative through the autumn, winter, and spring seasons, but could shed the bacterium again the next summer. Thus, we had animals that shed *E. coli* O157:H7 on widely spaced sampling days interspersed by periods in which the animals were culture negative for *E. coli* O157:H7, a phenomenon not yet reported for individual cattle.

Since all the sheep remained healthy throughout the study, we hypothesized that changes in diet and/or environment influenced the seasonal shedding of *E. coli* O157:H7. Culture-positive animals were found only when the animals grazed sagebrush and bunchgrass (June through August). A change in the grazing range at this time may have allowed the *E. coli* O157:H7 isolates to flourish in the intestinal tracts of the sheep, and over time on this same diet, animals became negative for the bacteria (by September, the only other month in which animals were on this diet, the animals were culture negative). We demonstrated previously that dietary influences,

such as changing the feed from alfalfa pellets to native sagebrush and bunchgrass, induced increased shedding of *E. coli* O157:H7 by experimentally inoculated sheep (25). We hypothesized that dietary nutrients or fiber content influences the colonization and clearance of *E. coli* O157:H7 in a ruminant gut (25). Rassmussen et al. (33) and others have suggested that the mechanism of dietary effects that influence *E. coli* colonization patterns and survival in the ruminant gut are volatile fatty acid concentrations. Although we did not measure the fiber, nutrient, or volatile fatty acid contents in the feeds or digesta of animals in flock II, changes in these parameters may have occurred with dietary change. In contrast to grazing on sagebrush and bunchgrass, all animals were culture negative while grazing alfalfa and barley aftermath (November through March) or while eating barley straw with or without supplements (April and May).

Another factor that may have influenced the seasonal nature of *E. coli* O157:H7 shedding by sheep was environmental conditions. *E. coli* O157:H7-positive sheep were detected in flock II only when the average daily temperatures and total precipitation were relatively high. Although we did not test for the bacterium in the pastures, feeds, or water used in this study, it has been shown that environmental conditions (temperature and moisture) can affect the survival of *E. coli* O157:H7 (41). The number of positive animals decreased in the second summer of the study. This difference may have been caused by the increased animal age, which may influence their susceptibility to *E. coli* O157:H7 carriage, subtle differences in their food supply because more precipitation occurred in the second summer compared with the amount in the first summer of the study, or unknown differences in copasturage with wild animals that may serve as *E. coli* O157:H7 reservoirs, or it may have been an artifact of the small number of animals in the flock.

Similar to the results of our previous studies, the use of the sensitive enrichment protocol was essential for the detection of *E. coli* O157:H7 in sheep fecal samples (25, 26). The selective enrichment protocol that we used has a detection limit for *E. coli* O157:H7 of 1 CFU/10 g of feces. *E. coli* O157:H7 was not detected even when as much as 1 g of feces from each animal was tested by a less sensitive nonenrichment protocol. The selective enrichment culture technique has also significantly increased the number of *E. coli* O157:H7-positive animals detected in several recent studies with bovines. Zhao et al. (47) almost doubled the number of *E. coli* O157:H7-positive calves (31 versus 16) detectable when 10-g fecal samples were tested by the enrichment culture technique. Sanderson et al. (37) also determined that selective enrichment culture was most sensitive in detecting *E. coli* O157:H7-positive cattle, but they found no significant difference when 10- or 0.1-g samples were tested.

With one exception, the ovine *E. coli* O157:H7 isolates appeared to be potential human pathogens since they were positive for one or more toxin genes and expressed Stx. We previously reported the isolation of 12 eae^- and stx^+ *E. coli* O157:H7 isolates from animals G, H, and I in June 1994 (26). In contrast, when this unusual lack of *eae* genes in the presence of a toxin gene(s) was analyzed by Southern blot hybridizations with the A and C probes specific for the locus of enterocyte effacement (*lee*) (23a, 27) or by colony blot hybridizations with ^{32}P-labelled probes, the presence of homologous *eae* regions in all 12 isolates was revealed. In addition, we had conflicting results for several toxin and *eae* genotypes reported previously and determined with psoralen-biotin-labelled probes (26). These false-negative results may have been due to inconsistencies in membrane or reagent quality or differences in the sensitivities of the techniques.

An advantage of being able to distinguish individual animals

TABLE 4. Comparison of virulence genotypes and PFGE and RFLP profiles among the *E. coli* O157:H7 strains isolated from culture-positive ewes at different sampling times

Sampling time	Animal A				Animal G				Animal I			
	Culture result	Virulence gene profile	PFGE pattern	RFLP pattern	Culture result	Virulence gene profile	PFGE pattern	RFLP pattern	Culture result	Virulence gene profile	PFGE pattern	RFLP pattern
June 1994	+	stx_1^+, stx_2^+, eae^+ (2/2)[a]	III (1/2), XI (1/2)	1 (2/2)	+	stx_1^+, stx_2^+, eae^+ (23/23)	I (23/23)	2 (23/23)	+	stx_1^+, stx_2^+, *eae* (9/9)	IV (1/9), XI (8/9)	1 (9/9)
August 1994	+	stx_1^+, stx_2^+, eae^+ (26/26)	V (4/26), VI (5/26), VII (9/26), VIII (8/26)	2 (26/26)	+	stx_1^+, stx_2^+, eae^+ (1/4), stx_1^-, stx_2^-, eae^- (3/4)	I (2/4), IX (2/4)	2 (1/4), 5 (3/4)	–	NA[b]	NA	NA
June 1995	–	NA	NA	NA	–	NA	NA		+	stx_1^+, eae^+ (26/26)	X (26/26)	4 (26/26)

[a] Values in parentheses indicate number of *E. coli* O157:H7 isolates with that profile/number of *E. coli* O157:H7 organisms isolated.
[b] NA, not applicable.

during the monitoring for the natural occurrence of *E. coli* O157:H7 was that we were able to compare variations in strains from single animals over time. Similar to reports by others who tested bovine *E. coli* O157:H7 strains (16, 28, 36), the combined use of PFGE and RFLP analyses provided the criteria to distinguish the ovine *E. coli* O157:H7 strains isolated in this study. The PFGE and RFLP patterns showed that multiple *E. coli* O157:H7 strains were isolated from one flock, that a single animal simultaneously shed multiple *E. coli* O157:H7 strains, and that the strains shed by individual animals changed over time. This is similar to a recently reported analysis of bovine *E. coli* O157:H7 isolates. Faith et al. (12) observed 20 distinct PFGE patterns among 160 *E. coli* O157:H7 isolates from 29 cattle and three cattle trough water samples over an 8-month period. They identified more than one strain of *E. coli* O157:H7 in a given herd and demonstrated that individual cattle could harbor more than one *E. coli* O157:H7 strain simultaneously.

On the basis of combined genotype, PFGE profile, and RFLP profile, we were able to distinguish ovine *E. coli* O157:H7 isolates obtained in cultures of fecal samples from individual animals over relatively short and long time periods. For example, from June to August of the same year, the *E. coli* O157:H7 strains shed by individual animals and among animals in the same flock changed. Also, the second most prevalent PFGE pattern among the isolates recovered in June 1994 (pattern XI) was not represented among any isolates recovered from animals in this flock at any other time (Table 1). The strong prevalence of PFGE patterns I and XI in June 1994 may suggest that all animals in the flock had access to a common environmental source (water, feed, pasture) that allowed them to become infected with these strains or that they were passed from one infected sheep to others in the flock. Animal-to-animal transmission has been shown to occur between experimentally infected sheep and noninfected pen mates (25). The wide variation in strains over time could mean that animals acquired new strains of *E. coli* O157:H7 from the environment or that the strain differences arose from bacterial mutations acquired during bacterial growth in the ruminant gastrointestinal tract. Because the strain differences among animals in the same flock over time are unique, we think that it is unlikely that the variation is due to exchange between animals. Only one ewe (animal G) shed *E. coli* O157:H7 isolates with identical genotypes, PFGE profiles, and RFLP profiles in samples separated by 60 days. However, this one incidence of strain stability is consistent with the finding that some sheep experimentally inoculated with *E. coli* O157:H7 shed the inoculum strain for 50 days (25).

In contrast to the requirement for selective enrichment culture for the detection of the O157:H7 serotype, other STEC serotypes were detected in the feces of sheep in flock I without enrichment culture. We isolated eight different non-O157 STEC serotypes from healthy sheep on a single sampling day. Of these, O5:NM, O91:NM, and O128:NM have previously been associated with human cases of HC and hemolytic-uremic syndrome, and O5:NM can cause HC-like disease in calves (1, 4, 35). We simultaneously cultured multiple non-O157 STEC serotypes from individual ewes, and this multiplicity of non-O157 STEC infection has been reported by others in both sheep and cattle (7). The predominant toxin genotype of the ovine non-O157 STEC isolates in this study was stx_1^+ and stx_2^+, and isolates were positive for *eae* and the K99 and F107 gene loci. The consistent presence of the *eae* gene is not surprising since most of the serotypes recovered have previously been categorized as EHEC or EPEC (22, 23). The presence of the K99 and F107 gene loci may reflect a requirement for colonization of the sheep gastrointestinal mucosa (10, 20, 30). However, since these fimbriae are frequently associated with enterotoxigenic *E. coli*, further investigation of their presence in ovine STEC isolates is planned. The majority of non-O157 STEC isolates tested (82%) were cytotoxic to Vero cells in culture, indicating that they expressed the toxin gene(s). The two isolates that were hybridization positive for *stx*, but that did not express the toxin (O88:NM and O5:U), may have resulted from pseudo lysogeny or toxin gene(s) loss or mutation (24).

Of the eight different serotypes isolated in this study, only the most common serotypes (O91:NM, U:NM, and O5:NM) have been isolated from healthy sheep by other investigators (7). Interestingly, two of the most common ovine STEC serotypes found in our study were also among the most commonly cultured ovine STEC serotypes in a study of sheep in Germany (7). Among the isolates from that European study, 24% were O5:NM and 23% were O91:NM. The U:NM strains, which accounted for 30% of the non-O157 isolates in this study, are fairly rare among the European ovine STEC isolates, in which they account for only 4% of the isolates. Although many STEC serotypes have been reported among bovine STEC isolates isolated from sheep, the predominant serotypes vary. Among cattle STEC strains isolated in Europe, the most common serotypes include O116:H21, U:U, and O113:H21 (29). In contrast, STEC isolates from cattle in North America have different predominant serotypes, the most prevalent being O171:H2, O121:H7, and a serotype related to O25 and O84:NM (43). These variations in the STEC serotypes of strains from cattle and sheep may be an artifact of the small numbers of animals sampled or a real difference due to animals, breeds, or location.

Irrespective of serotype, 98% of the ovine STEC isolates possessed various combinations of the virulence-associated genes, suggesting their potential for human pathogenicity. The most common toxin gene profile was stx_1^+ and stx_2^+. This is similar to the most common toxin genotype among human outbreak-related *E. coli* O157:H7 strains, in which 76% of the isolates are stx_1^+ and stx_2^+, 20% are stx_1^- and stx_2^+, and 3% are stx_1^+ and stx_2^- (14). In addition, a Vero cell cytotoxicity assay demonstrated that 90% of the representative STEC isolates tested expressed the toxin gene. These observations support the concept that sheep are a natural reservoir for potentially virulent *E. coli* O157:H7 and non-O157 STEC strains. In addition, the results of this study indicate that the occurrence of multiple strains of *E. coli* O157:H7 are common in a culture-positive flock or animal and that these multiple strains change over time.

ACKNOWLEDGMENTS

This work was supported, in part, by the Idaho Agriculture Experiment Station, U.S. Department of Agriculture, NRICGP grants 92-04350 and 95-37201-1979, Public Health Service grant AI33981 from the National Institutes of Health, and a grant from the United Dairymen of Idaho.

We thank T. E. Besser for editorial suggestions regarding the manuscript and for cytotoxicity work. We acknowledge D. S. Bradway for cytotoxic analysis of both O157 and non-O157 STEC strains. We thank R. C. Heimsch for expert negotiations. We thank D. D. Hancock for the Trypticase soy broth-cefixime tellurite vancomycin recipe and for providing the cefixime. We thank R. A. Wilson for serotyping the non-O157 STEC strains and for providing plasmid CRII. We gratefully acknowledge J. B. Kaper and T. K. McDaniel for the analysis of *E. coli* O157:H7 strains with the *lee* A and C probes and for supplying plasmid CVD434. We also thank M. P. Jackson for plasmid MJ331 and Y. F. Chang for plasmid TL3. Finally, we acknowledge the technical assistance provided by J. A. Hopkins and E. Vadnais, K. Blanch, D. H. Rice, and P. R. Austin.

REFERENCES

1. **Acheson, D. W. K., and G. T. Keusch.** 1996. Which Shiga toxin-producing types of *E. coli* are important? ASM News **62:**302–306.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1994. Preparation of genomic DNA from bacteria—miniprep of bacterial genomic DNA, p. 241–242. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley & Sons, Inc., Boston, Mass.
3. **Baron, E., and S. M. Finegold.** 1990. Diagnostic microbiology. The C. V. Mosby Company, St. Louis, Mo.
4. **Barrett, T. J., J. B. Kaper, A. E. Jerse, and I. K. Wachsmuth.** 1992. Virulence factors in Shiga-like toxin producing *Escherichia coli* isolated from humans and cattle. J. Infect. Dis. **165:**979–980.
5. **Barrett, T. J., H. Lior, J. H. Green, R. Khakhria, J. G. Wells, B. P. Bell, K. D. Greene, J. Lewis, and P. M. Griffin.** 1994. Laboratory investigation of a multistate food-borne outbreak of *Escherichia coli* O157:H7 by using pulsed-field gel electrophoresis and phage typing. J. Clin. Microbiol. **32:**3013–3017.
6. **Besser, R. E., S. M. Lett, J. T. Weber, M. P. Doyle, T. J. Barrett, J. G. Wells, and P. M. Griffin.** 1993. An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. JAMA **269:**2217–2220.
7. **Beutin, L., D. Geier, H. Steinrück, S. Zimmermann, and F. Scheutz.** 1993. Prevalence and some properties of verotoxin (Shiga-like toxin)-producing *Escherichia coli* in seven different species of healthy domestic animals. J. Clin. Microbiol. **31:**2483–2488.
8. **Beutin, L., D. Geier, S. Zimmermann, and H. Karch.** 1995. Virulence markers of Shiga-like toxin-producing *Escherichia coli* strains originating from healthy domestic animals of different species. J. Clin. Microbiol. **33:**631–635.
9. **Blanco, M., J. Blanco, J. E. Blanco, E. A. Gonzalez, T. A. T. Gomes, L. F. Zerbini, T. Yano, and A. F. P. De Castro.** 1994. Genes coding for Shiga-like toxins in bovine verotoxin-producing *Escherichia coli* (VTEC) strains belonging to different O:K:H serotypes. Vet. Microbiol. **42:**105–110.
10. **DeGraaf, F. K., B. E. Krenn, and P. Klaasen.** 1984. Organization and expression of genes involved in the biosynthesis of K99 fimbriae. Infect. Immun. **43:**508–514.
11. **Ewing, W. H., and W. J. Martin.** 1974. *Enterobacteriaceae*, p. 189–221. *In* E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), Manual of clinical microbiology, 2nd ed. American Society for Microbiology, Washington, D.C.
12. **Faith, N. G., J. A. Shere, R. Brosch, K. W. Arnold, S. E. Ansay, M. S. Lee, J. B. Luchansky, and C. W. Kaspar.** 1996. Prevalence and clonal nature of *Escherichia coli* O157:H7 on dairy farms in Wisconsin. Appl. Environ. Microbiol. **62:**1519–1525.
13. **Goldwater, P. N., and K. A. Bettelheim.** 1995. Hemolytic uremic syndrome due to Shiga-like toxin producing *Escherichia coli* O48:H21 in South Australia. Emerg. Infect. Dis. **1:**132–133.
14. **Griffin, P. M.** 1995. *Escherichia coli* O157:H7 and other enterohemorrhagic *Escherichia coli*, p. 739–761. *In* M. F. Blaser, P. D. Smith, J. I. Ravdin, H. B. Greenberg, and R. L. Guerrant (ed.), Infections of the gastrointestinal tract. Raven Press, Ltd., New York, N.Y.
15. **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.
16. **Grimm, L. M., M. Goldoft, J. Kobayashi, J. H. Lewis, D. Alfi, A. Perdichizzi, P. I. Tarr, J. E. Ongerth, S. L. Moseley, and M. Samadpour.** 1995. Molecular epidemiology of a fast-food restaurant-associated outbreak of *Escherichia coli* O157:H7 in Washington state. J. Clin. Microbiol. **33:**2155–2158.
17. **Hancock, D. D., T. E. Besser, 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. J. Epidemiol. Infect. **113:**199–207.
18. **Hofmann, S. L.** 1993. Southwestern internal medicine conference: Shiga-like toxins in hemolytic-uremic syndrome and thrombotic thrombocytopenic purpura. Am. J. Med. Sci. **306:**398–406.
19. **Hovde, C. J., S. B. Calderwood, J. J. Mekalanos, and R. J. Collier.** 1988. Evidence that glutamic acid 167 is an active-site residue of Shiga-like toxin I. Proc. Natl. Acad. Sci. USA **85:**2568–2572.
20. **Imberechts, H., H. de Greve, C. Schlicker, H. Bouchet, P. Pohl, G. Charlier, H. Bertschinger, P. Wild, J. Vandekerckhove, J. van Damme, M. van Montagu, and P. Lintermans.** 1992. Characterization of F107 fimbriae of *Escherichia coli* 107/86, which causes edema disease in pigs, and nucleotide sequence of the F107 major fimbrial subunit gene, *fedA*. Infect. Immun. **60:**1963–1971.
21. **Janke, B. H., D. H. Francis, J. E. Collins, M. C. Libal, D. H. Zeman, D. D. Johnson, and R. D. Neiger.** 1990. Attaching and effacing *Escherichia coli* infection as a cause of diarrhoea in young calves. J. Am. Vet. Med. Assoc. **196:**897–901.
22. **Jerse, A. E., K. G. Gicquelais, and J. B. Kaper.** 1991. Plasmid and chromosomal elements involved in the pathogenesis of attaching and effacing *Escherichia coli*. Infect. Immun. **59:**3869–3875.
23. **Jerse, A. E., J. Yu, B. D. Tall, and J. B. Kaper.** 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc. Natl. Acad. Sci. USA **87:**7839–7843.

23a. **Kaper, J. B. (University of Maryland).** Personal communication.

24. **Karch, H., T. Meyer, H. Russman, and J. Heesemann.** 1992. Frequent loss of Shiga-like toxin genes in clinical isolates of *Escherichia coli* upon subcultivation. Infect. Immun. **60:**3464–3467.
25. **Kudva, I. T., P. G. Hatfield, and C. J. Hovde.** 1995. Effect of diet on the shedding of *Escherichia coli* O157:H7 in a sheep model. Appl. Environ. Microbiol. **61:**1363–1370.
26. **Kudva, I. T., P. G. Hatfield, and C. J. Hovde.** 1996. *Escherichia coli* O157:H7 in microbial flora of sheep. J. Clin. Microbiol. **34:**431–433.
27. **McDaniel, T. K., K. G. Jarvis, M. S. Donnenberg, and J. B. Kaper.** 1995. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. Proc. Natl. Acad. Sci. USA **92:**1664–1668.
28. **Meng, J., S. Zhao, T. Zhao, and M. P. Doyle.** 1995. Molecular characterisation of *Escherichia coli* O157:H7 isolates by pulsed-field gel electrophoresis and plasmid DNA analysis. J. Med. Microbiol. **42:**258–263.
29. **Montenegro, M. A., M. Bülte, T. Trumpf, S. Aleksic, G. Reuter, E. Bulling, and R. Helmuth.** 1990. Detection and characterization of fecal verotoxin-producing *Escherichia coli* from healthy cattle. J. Clin. Microbiol. **28:**1417–1421.
30. **Morris, J. A., G. A. H. Wells, A. C. Scott, and W. J. Sojka.** 1983. Colonisation of the small intestine of lambs by an enterotoxigenic *Escherichia coli* producing F41 fimbriae. Vet. Rec. **113:**471.
31. **O'Brien, A. D., and R. K. Holmes.** 1987. Shiga and Shiga-like toxins. Microbiol. Rev. **51:**206–220.
32. **Perry, M. B., and D. R. Bundle.** 1990. Antigenic relationships of the lipopolysaccharides of *Escherichia hermannii* with those of *Escherichia coli* O157:H7, *Brucella melitensis*, and *Brucella abortus*. Infect. Immun. **58:**1391–1395.
33. **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.
34. **Rice, D. H., D. D. Hancock, and T. E. Besser.** 1995. Verotoxigenic *E. coli* O157 colonisation of wild deer and range cattle. Vet. Rec. **137:**524.
35. **Russmann, H., E. Kothe, H. Schmidt, S. Franke, D. Harmsen, A. Caprioli, and H. Karch.** 1995. Genotyping of Shiga-like toxin genes in non-O157 *Escherichia coli* strains associated with haemolytic uraemic syndrome. J. Med. Microbiol. **42:**404–410.
36. **Samadpour, M.** 1995. Molecular epidemiology of *Escherichia coli* O157:H7 by restriction fragment length polymorphism using Shiga-like toxin genes. J. Clin. Microbiol. **33:**2150–2154.
37. **Sanderson, M. W., J. M. Gay, T. E. Besser, D. D. Hancock, L. K. Fox, and C. C. Gay.** 1995. Sensitivity of bacteriologic culture for detection of *Escherichia coli* O157:H7 in bovine feces. J. Clin. Microbiol. **33:**2616–2619.
38. **Shin, S. J., Y. F. Chang, M. Timour, T. L. Lauderdale, and D. H. Lein.** 1994. Hybridization of clinical *Escherichia coli* isolates from calves and piglets in New York State with gene probes for enterotoxins (STaP, STb, LT), Shiga-like toxins (SLT-I, SLT-II) and adhesion factors (K88, K99, F41, 987P). Vet. Microbiol. **38:**217–225.
39. **Tesh, V. L., and A. D. O'Brien.** 1991. The pathogenic mechanisms of Shiga toxin and the Shiga-like toxins. Mol. Microbiol. **5:**1817–1822.
40. **Tesh, V. L., and A. D. O'Brien.** 1992. Adherence and colonization mechanisms of enteropathogenic and enterohemorrhagic *Escherichia coli*. Microb. Pathog. **12:**245–254.
41. **Wang, G., T. Zhao, and M. P. Doyle.** 1996. Fate of enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces. Appl. Environ. Microbiol. **62:**2567–2570.
42. **Weinstein, D. L., M. P. Jackson, L. P. Perera, R. K. Holmes, and A. D. O'Brien.** 1989. In vivo formation of hybrid toxins comprising Shiga toxin and the Shiga-like toxins and the role of the B subunit in localization and cytotoxic activity. Infect. Immun. **57:**3743–3750.
43. **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.
44. **Wieler, L., R. Bauerfeind, and G. Baljer.** 1992. Characterization of shiga-like toxin producing *Escherichia coli* (SLTEC) isolated from calves and without diarrhoea. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **276:**243–253.
45. **Willshaw, G. A., S. M. Scotland, H. R. Smith, T. Cheasty, A. Thomas, and B. Rowe.** 1994. Hybridization of strains of *Escherichia coli* O157 with probes derived from the *eaeA* gene of enteropathogenic *E. coli* and the *eaeA* homolog from a Vero cytotoxin-producing strain of *E. coli* O157. J. Clin. Microbiol. **32:**897–902.
46. **Yu, J., and J. B. Kaper.** 1992. Cloning and characterization of the *eae* gene of enterohaemorrhagic *Escherichia coli* O157:H7. Mol. Microbiol. **6:**411–417.
47. **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.