2003

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Jelacic, Jill K.; Damrow, Todd; Chen, Gilbert S.; Jelacic, Srdjan; Bielaszewska, Martina; Ciol, Marcia; Carvalho, Humberto M.; Melton-Celsa, Angela R.; O'Brien, Alison D.; and Tarr, Phillip I., "Shiga Toxin–Producing *Escherichia coli* in Montana: Bacterial Genotypes and Clinical Profiles" (2003). *Uniformed Services University of the Health Sciences*. 96.
https://digitalcommons.unl.edu/usuhs/96

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Shiga Toxin–Producing *Escherichia coli* in Montana: Bacterial Genotypes and Clinical Profiles

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The diseases and virulence genes associated with Shiga toxin–producing *Escherichia coli* (STEC) are characterized incompletely. We analyzed, by polymerase chain reaction, 82 STEC isolates collected prospectively in Montana and profiled associated illnesses by patient chart review. All *E. coli* O157:H7 contained stx_2-group genes, as well as *eae, iha, espA,* and *ehxA;* 84% contained stx_1. Non-O157:H7 STEC less frequently contained stx_1 ($P = .046$), stx_2 ($P < .001$), *iha* ($P < .001$), *eae,* and *espA* ($P = .039$ for both), were isolated less often from patients treated in emergency departments ($P = .022$), and tended to be associated less frequently with bloody diarrhea ($P = .061$). There were no significant associations between *stx* genotype and bloody diarrhea, but isolates containing stx_{2c} or $stx_{2d-activatable}$ were recovered more often from patients who underwent diagnostic or therapeutic procedures ($P = .033$). Non-O157:H7 STEC are more heterogeneous and cause bloody diarrhea less frequently than do *E. coli* O157:H7. Bloody diarrhea cannot be attributed simply to the *stx* genotype of the infecting organism.

Many different *Escherichia coli* produce Shiga toxin (Stx). Stxs belong to 1 of 2 groups. Stx1 [1] is nearly identical to Stx, the principal extracellular cytotoxin produced by *Shigella dysenteriae* serotype 1. Members of the Stx_2 group (Stx_2, Stx_{2c}, Stx_{2d}, $Stx_{2d-activatable}$, Stx_{2e}, and Stx_{2f}) [2–5] have sequences that are less similar to Stx. *E. coli* O157:H7 is the best known and the most frequently isolated of the Stx-producing *E. coli* (STEC); the comparatively high rate of isolation and prominence of this serotype is attributable, at least in part,

to its inability to ferment sorbitol [6]. When plated on MacConkey agar that contains sorbitol in lieu of lactose, *E. coli* O157:H7 appear as colorless colonies. This non–sorbitol-fermenting phenotype is, therefore, an easily distinguishable characteristic that can be sought in economical and efficient screening protocols, if stool samples are plated on sorbitol MacConkey (SMAC) agar when they arrive in the laboratory [7]. However, it will not detect most non-O157:H7 STEC, which generally ferment sorbitol, so such organisms are easily overlooked.

The fact that *E. coli* O157:H7 causes diarrhea, bloody diarrhea, and hemolytic-uremic syndrome (HUS) has been well established since 1983, when *E. coli* O157:H7 was first associated with human disease [8, 9]. In contrast, the spectrum of illnesses associated with non-O157:H7 STEC, the characteristics of patients from whom they are isolated, the genotypes of these organisms, and the extent to which these organisms are pathogenic to humans, are less well established [10]. However, several studies suggest that illnesses associated with non-O157:H7 STEC differ from those caused by *E. coli*

O157:H7, particularly in the lesser ability of the former group of organisms to cause bloody diarrhea and HUS [4, 11–14].

The characteristics of STEC-induced illnesses in humans relate, presumably, to the genomic contents of the infecting strains. Ostroff et al. [15] reported that *E. coli* O157:H7 that contained stx_2-group genes but that did not contain stx_1 were more likely to be isolated from patients with HUS than were *E. coli* O157:H7 isolates containing both stx_1 and stx_2-group genes. This trend was observed again in a recent prospective study in the Pacific Northwest [16]. Indeed, Donohue-Rolfe et al. [17] have reported that the removal of stx_1 from an stx_1^+/stx_2^+ *E. coli* O157:H7 isolate augments its virulence in gnotobiotic piglets. Boerlin et al. [12] reported a strong association between the presence of stx_2-group genes in human STEC from 8 major serotypes and the severity of associated disease. Friedrich et al. [4] refined *stx* genotype analysis by examining allelic variants within the stx_2 group and suggested that STEC containing stx_{2d} or stx_{2e} were associated with less-severe disease or were not pathogenic to humans.

STEC contain a repertoire of putative virulence loci, in addition to their *stx* genes. *eae*, which encodes intimin, is in the locus of enterocyte effacement (LEE) [18] and is the best characterized of the non-*stx* virulence loci. *eae* is necessary for pathogenicity in several animal models of enteric infection [19–21]. Additional candidate virulence loci in STEC include other LEE genes, such as *espA*, which encodes a filamentous organelle on the surface of *E. coli* O157:H7 [22], as does its homologue in enteropathogenic *E. coli* [23]; *iha*, which is found on the tellurite-resistance, adherence-conferring island and encodes a novel adhesin [18] in *E. coli* O157:H7 (a homologue of Iha is found in the outer membranes of other STEC) [24]; and genes encoded on the large plasmid found in most presumptively pathogenic STEC, such as *ehxA*, which encodes the enterohemorrhagic *E. coli*–hemolysin [25–29].

Studies that have attempted to associate STEC genotypes and the characteristics of the illnesses in the patients from whom these organisms were isolated have often analyzed existing strain collections for the sake of convenience. Such selection could lead to unintentional biases in analyses and conclusions. In addition, there have been no detailed attempts to relate bacterial genotypes to clinical manifestations of non-O157:H7 STEC in the United States, and the analyses of non-O157:H7 STEC infections from North America have consisted of small numbers of patients or limited descriptions of their illnesses [13, 30–34]. Here, we analyze STEC belonging to a variety of serotypes that were collected from patients in Montana during a defined interval in a statewide surveillance project. We also profile the illnesses in the patients from whom these organisms were recovered and attempt to determine whether there are discernible associations between STEC genotype and illness observed.

MATERIALS AND METHODS

Patient isolates. Between June 1998 and May 2000, all stool samples submitted to each of 16 Montana microbiology laboratories (Billings Deaconess Clinic Health System and St. Vincent's Hospital, Billings; Bozeman Deaconess, Bozeman; Browning Indian Health Service, Browning; St. James Hospital, Butte; Crow Agency Indian Health Service, Crow Agency; Barrett Memorial Hospital, Dillon; Benefis Healthcare and Great Falls Clinic, Great Falls; Northern Montana Hospital, Havre; St. Peter's Hospital, Helena; Kalispell Regional Hospital, Kalispell; Central Montana Medical Center, Lewiston; Holy Rosary Health Center, Miles City; and Missoula Community Medical Center and St. Patrick's Hospital, Missoula) were evaluated for the presence of *Salmonella* species, *Shigella* species, and *Campylobacter jejuni* at point of receipt and then were sent as swab specimens in Cary-Blair transport media to the Montana State Public Health Laboratory (Helena) for the isolation and identification of STEC.

After arrival at the reference laboratory, the swabs were removed from the transport media and inoculated into 10 mL of MacConkey broth. Broths then were incubated overnight at 35°C. One hundred microliters of this culture was tested for the presence of Stx by use of the Premier EHEC EIA kit (Meridian Biosciences), according to the manufacturer's instructions. Broths that produced a signal indicating the presence of Stx were further cultured to standard MacConkey and to SMAC agar plates. After overnight incubation at 35°C, non–sorbitol-fermenting colonies were tested for the presence of the O157 antigen by use of the Wellcolex O157:H7 STEC latex agglutination test (Abbott Laboratories). If non–sorbitol-fermenting colonies were not observed or if the non–sorbitol-fermenting colonies failed to react with the O157-specific antibody, 2 or 3 lactose-fermenting colonies were grown overnight in MacConkey broth at 35°C and were tested the next day for the production of Stx by use of the Premier EHEC EIA kit on broth, as described above.

Isolates that produced Stx in broth were confirmed to be *E. coli* by use of a Vitek Junior Model 32 System J1733 analyzer (BioMerieux Vitek). Somatic antigens 26, 104, 111, 121, and 157 were sought by agglutination for all STEC. An isolate was considered to be motile if a line of diffuse emanation was observed in motility media after inoculation and overnight incubation. If an isolate did not grow diffusely from the inoculation line, it was considered to be nonmotile (NM). The presence or absence of the H7 antigen was determined by use of the Wellcolex *E. coli* latex agglutination test (Abbott Laboratories) for all isolates that expressed the O157 lipopolysaccharide. Isolates with undetermined O or H antigens were typed at the Centers for Disease Control and Prevention (Atlanta) for each of the 181 known O antigens and 52 of the 56 known H antigens. Isolates that agglutinated completely with all O-

specific antisera were classified as "Orough," because of their self-agglutination phenotype. Isolates that were nonreactive in *E. coli* O antigen antiserum pools were classified as "Onontypeable" (ONT).

Bacterial genotypes. Table 1 lists the primers for the alleles sought, the polymerase chain reaction (PCR) conditions, and the positive control strains for each primer pair used in this study. The negative control for each reaction was *E. coli* HB101 [35]. Bacteria were grown overnight in Luria-Bertani broth [35]. Template DNA was prepared by adding 45 μL of bacterial broth culture to 5 μL of 0.1% Triton-X in sterile Eppendorf tubes and by boiling the mixture for 20 min. dNTPs were purchased from Promega, and *Taq* DNA polymerase and restriction endonucleases were purchased from New England Biolabs. After amplification in a thermal cycler (iCycler; Bio-Rad), PCR products were analyzed by electrophoresis in 0.5× Tris-borate EDTA (TBE) [35] 1.5% agarose, followed by ethidium-bromide staining. stx_2 and stx_{2c} genes were preliminarily differentiated by restriction analysis of the GK3-GK4 amplification products, using *Hae*III and *Fok*I [36]. However, stx_{2c} alleles are indistinguishable from $stx_{2d\text{-}activatable}$ genes (A.R.M.-C., unpublished observation) by the initial PCR technique used in this study [36]; therefore, isolates with apparent stx_{2c} alleles were tested using $stx_{2d\text{-}activatable}$ primer pairs, as noted in table 1, to generate an 890-bp amplicon. *Pst*I cleaves this amplicon into 504- and 386-bp fragments if the target gene is stx_{2c} but does not cleave amplicons derived from $stx_{2d\text{-}activatable}$.

Because the stx_2-group amplicon of an *E. coli* O28ab:H28 failed to yield predicted fragments when digested with *Fok*I or *Hae*III, we produced an amplicon that spans the genes encoding the holotoxin, using primers 5′-CAAAGCAGCAATGGCGCT-AGG-3′ and 5′-TTTCACCAGTCGCCCCTCCAC-3′, digested the amplicon with *Eco*RV, modified the resulting fragments using the A-tailing procedure (Promega), inserted them into the pGEM-T Easy Vector (Promega), and sequenced them using an ABI PRISM 377 DNA Sequencer (Applied Biosystems). Then, we amplified a 382-bp amplicon spanning the presumed *Eco*RV site by use of primers 5′-TACTGGGTTTTTCTTCG-GTA-3′ and 5′-GTGGTATAACTGCTGTCCGT-3′, which also was inserted into the pGEM-T Easy Vector and sequenced.

Activation assay. Two isolates that contained $stx_{2d\text{-}activatable}$ as their sole *stx* genes were tested further, to determine whether the Stx that they produced exhibited the activatable phenotype. The activation assay was performed as described elsewhere [37]. In brief, culture supernatants from strains containing Stx_2-type toxins were incubated with mouse small-intestinal mucus or a buffer control and incubated at 37°C for 2 h. The cytotoxicity of the toxin-mucus or toxin-buffer mixture then was measured on Vero cells. The toxin is considered to be activatable if the resulting cytotoxicity increases at least 8-fold after incubation with the mouse mucus.

Clinical data. Clinical data were obtained by reviewing the medical records of Montana residents from whom STEC were isolated during the study period, after receiving permission from the Institutional Review Board of the University of Washington Medical Center. Data recorded included age, sex, date of first stool culture, site of first medical treatment, symptoms described in the chart (presence of blood in the stool, abdominal pain, nausea, vomiting, fever, headache, and muscle aches), laboratory results (white blood cell count and fecal leukocytes), procedures performed, and antimicrobial agents prescribed. A symptom was characterized as being present if it was specifically mentioned as having been experienced by the patient during the illness or as being absent if it was specifically denied. If a symptom was neither specifically mentioned nor specifically denied as being present, patients were not entered into analysis for that variable. A procedure was defined as any entry of a therapeutic or diagnostic device, including surgery, into the body, excluding phlebotomies and bladder catheterizations. If a procedure was not mentioned, it was characterized as not having been performed. If an antimicrobial was not specifically recorded in the chart, it was characterized as not having been prescribed. All data obtained were entered into a relational database for statistical analysis.

Statistics. We used the Wilcoxon rank sum test to assess the significance of the difference between median ages of patients infected with *E. coli* O157:H7 and with non-O157:H7 STEC. The association between pairs of categorical variables was assessed by Fisher's exact test. Logistic regression was performed to assess the association between the characteristics of the infecting organism and the probability of bloody diarrhea.

RESULTS

STEC recovered. Between June 1998 and May 2000, ~6300 stool samples were analyzed for STEC, as described above. STEC were recovered from 85 of these stool samples, and 1 stool sample with a positive EIA result failed to yield an STEC after further subculturing. Three patients whose stool samples yielded STEC belonging to serotypes O121:H19, O124:H19, and ONT:NM also were infected with *C. jejuni*. Because we were unable to attribute these patients' symptoms to the *C. jejuni* or the non-O157:H7 STEC, these strains and the illnesses of the patients from whom they were isolated were not entered into analysis. Of the 82 remaining STEC, 31 (38%) were *E. coli* O157:H7, and 1 (1%) was a non–sorbitol-fermenting *E. coli* O157:NM. The 32 isolates expressing the O157 lipopolysaccharide antigen were analyzed together as *E. coli* O157:H7. Forty-one of the 50 non-O157:H7 STEC expressed 8 different identifiable O antigens, 4 were Orough, and 5 were "Oundetermined" (table 2). Seventy-two percent and 63% of the re-

Table 1. Polymerase chain reaction (PCR) primers and conditions used in the present study.

Primer pair [reference]	Sequence	Target	Primer amounts, pmol of each	MgCl$_2$, mmol/L	Taq DNA polymerase, U	PCR condition[a]	Length of PCR product, bp	Positive E. coli control [reference]
STX1L STX1R [this study]	5'-CGCTTTGCTGATTTTTCACA-3' 5'-GTAACATCGCTCTTGCCACA-3'	stx_1A	50	3.0	1.25	A	208	O157:H7 87-01 [65]
LP43 LP44 [66]	5'-ATCCTATTCCCGGGAGTTTACG-3' 5'-GCGTCATCGTATACACAGGAGC-3'	stx_2A and variants[b]	50	2.5	2.5	B	587	O157:H7 EDL 933 [67]
GK3 GK4 [68]	5'-ATGAAGAAGATGTTTATG-3' 5'-TCAGTCATTATTAAAACTG-3'	stx_2B, and $stx_{2c}B$	100	1.5	2.5	C	270	O157:NM E32511 [69]
VT2-cm VT2-f [44]	5'-AAGAAGATATTTGTAGCGG-3' 5'-TAAACTGCACTTCAGCAAAT-3'	$stx_{2d}B$	50	2.0	2.0	D	256	ONT:H12 EH250 [44]
SLT-II-vc CKS2 [this study]	5'-ACCACTCTGCAACGTGTCGC-3' 5'-ACTGAATTGTGACACAGATTA-3'	$stx_{2d\text{-}activatable}$	1012	1.5	1.25	E	890	O91:H21 B2F1 [70, 71]
FK1 FK2 [72]	5'-CCCGGATCCAAGAAAGATGTTTATAG-3' 5'-CCCGAATTCTCAGTTAAACTTCACC-3'	$stx_{2e}B$	10	1.5	2.5	F	280	O101:NM ED-53 [73]
128-1 128-2 [74]	5'-AGATTGGGCGTCATTCACTGGTTG-3' 5'-TACTTTAATGGCCGCCCCTGTCTCC-3'	$stx_{2f}A$	30	1.5	2.0	G	428	O128:H2 T4/97 [75]
eae R eae L [this study]	5'-GCACTGGCATTAAGTGCTGA-3' 5'-CGGTGAAAAAGAATGGGGTA-3'	eae	50	3.0	1.25	A	199	O157:H7 86-24 [65]
espA1 espA2 [this study]	5'-GTGCGAACGCGAGTACTTCG-3' 5'-GGCCGAAATAGCGGCCTTCACCG-3'	espA	50	3.0	1.25	A	492	O157:H7 86-24 [65]
hlyA1 hlyA4 [25]	5'-GGTGCAGCAGAAAAAGTTGTAG-3' 5'-TCTCGCCTGATAGTGTTTGTTA-3'	ehxA	1.5	1.5	2.0	H	1551	O157:H7 86-24 [65]
IHA F IHA R [76]	5'-CTGGCGGAGGCTCTGAGATCA-3' 5'-TCCTTAAGCTCCCGCGGCTGA-3'	iha	50	3.0	1.25	A	827	O157:H7 86-24 [65]

NOTE. eae, enterohemorrhagic E. coli attaching and effacing gene; ehxA, enterohemolysin A gene; espA, E. coli secreted protein A gene; iha, IrgA homologue adhesin gene; stx, Shiga toxin gene.

[a] PCR conditions were as follows: A, denaturing at 94°C for 60 s, annealing at 55°C for 60 s, extension at 74°C for 120 s, and final extension step of 7 min at 72°C; B, denaturing at 94°C for 90 s, annealing at 64°C for 90 s, and extension at 72°C for 90 s; C, denaturing at 94°C for 30 s, annealing at 52°C for 60 s, and extension at 72°C for 40 s; D, initial denaturation step of 5 min at 94°C, denaturing at 94°C for 30 s, annealing at 60°C for 60 s, and extension at 72°C for 60 s; E, initial denaturation step of 5 min at 95°C, denaturing at 95°C for 60 s, annealing at 56°C for 60 s, and final extension step of 10 min at 72°C; F, denaturing at 94°C for 40 s, annealing at 53°C for 60 s, and extension at 72°C for 60 s; G, denaturing at 94°C for 30 s, annealing at 55°C for 60 s, extension at 72°C for 60 s, and final extension step of 5 min at 72°C; and H, initial denaturation step of 5 min at 94°C, denaturing at 94°C for 30 s, annealing at 57°C for 90 s, and extension at 72°C for 90 s. All PCRs were run for 30 cycles, except for $stx_{2c}A$ and variants, which used 35 cycles. All PCRs were performed in 50-μL final volume.

[b] $stx_{2c}A$, $stx_{2d}A$, and $stx_{2e}A$ [75].

Table 2. **stx genotype of Shiga toxin–producing *Escherichia coli* (STEC) isolates in this study, by serotype, and proportion with bloody diarrhea.**

Serotype (no.)	Genotype, no. of isolates						Bloody diarrhea[a]	Procedures[b]	Emergency department[c]
	stx_1	stx_2 group	stx_2	stx_{2c}	stx_{2c2}	$stx_{2d\text{-}activatable}$			
O157:H7 (31)	26	31	29	4	0	0	21/26 (81)	6	17
O157:NM (1)	1	1	0	1	0	0	1/1 (100)	1	1
O26:H11 (16)	16	0	0	0	0	0	5/11 (45)	1	7
O26:NM (2)	2	0	0	0	0	0	1/1 (100)	1	1
O28ab:H28 (1)	0	1	0	0	1	0	0/1 (0)	0	1
O28ac:H25 (1)	0	1	0	0	0	1	1/1 (100)	1	0
O73:H18 (1)	0	1	0	0	0	1	1/1 (100)	0	0
O103:H2 (1)	1	0	0	0	0	0	0/1 (0)	0	0
O103:H25 (1)	1	0	0	0	0	0	0/1 (0)	0	0
O119:H4 (15)	1	0	0	0	0	0	NA	0	0
O121:H19 (15)	1	15	15	0	0	0	7/13 (54)	2	3
O145:NM (1)	1	0	0	0	0	0	1/1 (100)	1	1
O165:H25 (1)	0	1	0	1	0	1	1/1 (100)	1	1
O177:NM (1)	1	1	0	1	0	0	1/1 (100)	0	0
O181:H49 (1)	0	1	1	0	0	0	1/1 (100)	0	0
Orough:H2 (1)	1	0	0	0	0	0	NA	0	0
Orough:H11 (2)	2	0	0	0	0	0	1/1 (100)	0	0
Orough:H18 (1)	1	1	1	0	0	0	NA	0	0
ONT:H25 (2)	2	1	1	0	0	0	NA	1	0
ONT:NM (1)	1	0	0	0	0	0	1/1 (100)	0	0
Total	58	55	47	7	1	3	43/63 (68)	15	32

NOTE. NA, symptoms not addressed for patients whose stool samples yielded these STEC.

[a] Data are no. reporting diarrhea/no. whose history addressed this symptom (%).

[b] Procedures performed on 7 patients in the *E. coli* O157:H7 group consisted of 1 appendectomy, 2 sigmoidoscopies, and 4 colonoscopies. Procedures performed on 8 patients in the non-O157:H7 STEC group consisted of 2 appendectomies (O26:H11 and ONT:H25), 2 sigmoidoscopies (O28ac:H25 and O121:H19), 3 colonoscopies (O121:H19, O145:NM, and O165:H25), and 1 esophagogastro-duodenoscopy and colonoscopy (O26:NM).

[c] Data are no. of patients whose cultures were submitted from an emergency department.

coveries of *E. coli* O157:H7 and non-O157:H7 STEC, respectively, occurred between June and October, inclusive.

Patients whose stool cultures yielded O157:H7 STEC had a tendency to report bloody diarrhea more frequently at presentation ($P = .061$) and to have had their cultures obtained in an emergency department ($P = .022$). Patients infected with *E. coli* O157:H7 were older than those infected with non-O157:H7 STEC (median age, 16.7 years [range 2.6–68 years] vs. 10.0 years [range, 0.6–87 years]; $P = .19$, Wilcoxon rank sum test). Otherwise, the characteristics of patients whose stool sample contained *E. coli* O157:H7 were similar to those whose stool samples contained non-O157:H7 STEC (table 3).

stx *alleles,* E. coli O157:H7 vs. non-O157:H7 STEC. All *E. coli* O157:H7 contained stx_2-group genes. Twenty-seven (84%) contained, in addition, stx_1. Two *E. coli* O157:H7 contained stx_{2c} as their only stx_2-group allele, and 2 contained stx_{2c} in addition to stx_2. None of the stx_{2c} genes in *E. coli* O157:H7 were determined after subsequent analysis to be an $stx_{2d\text{-}activatable}$ variant.

Of the 50 non-O157:H7 STEC, 27 contained stx_1 but not stx_2-group genes, 19 contained stx_2-group genes but not stx_1, and 4 contained both stx_1 and stx_2-group genes. Four contained stx_{2c} on preliminary analysis, but after subsequent analysis, 3 of these isolates contained $stx_{2d\text{-}activatable}$ genes, 1 in combination with an stx_{2c} gene.

***Non-*stx *alleles,* E. coli O157:H7 vs. non-O157:H7 STEC.** Non-O157:H7 STEC contained *iha, eae,* and *espA* significantly less frequently than the *E. coli* O157:H7 in this study, and none of the 3 non-O157:H7 STEC with $stx_{2d\text{-}activatable}$ genes contained *eae* or *espA*. Nonetheless, half or more of the non-O157:H7 STEC isolates in the present study did possess each non-*stx* locus sought.

Genotypes and bloody versus nonbloody diarrhea. Of the 50 patients infected with non-O157:H7 STEC, 15 denied and 21 reported having had bloody diarrhea. Of these 36 patients for whom the presence or absence of bloody diarrhea could be assessed, non-O157:H7 STEC containing stx_2-group genes were recovered from 7 of the 15 patients without bloody diarrhea and from 12 of the 21 patients with bloody diarrhea ($P = .736$).

Table 3. Comparison of clinical characteristics of patients infected with *Escherichia coli* O157:H7 and non-O157:H7 Shiga toxin–producing *E. coli* (STEC).

Variable	*E. coli* O157:H7 group	Non-O157:H7 STEC group	P^a
Male	12/32 (38)	30/50 (58)	.070
Headache	0/2 (0)	2/4 (50)	.467
Fecal leukocytes	20/26 (77)	20/29 (69)	.558
Procedure	7/32 (22)[b]	8/50 (16)[b]	.565
Antibiotics	11/32 (34)[c]	20/50 (38)[b]	.648
First site			
Emergency department	18/32 (56)	15/50 (30)	**.022**
Other ambulatory facility	14/32 (44)	35/50 (70)	
Bloody diarrhea	22/27 (81)	21/36 (58)	.061
Abdominal pain	24/27 (89)	33/40 (83)	.728
Nausea	8/13 (62)	8/12 (67)	1.0
Vomiting	8/20 (40)	15/29 (52)	.562
Fever (% of "yes")	7/23 (30)	8/35 (26)	.553
sx_1	27/32 (84)	31/50 (62)	**.046**
stx_2-group alleles	32/32 (100)	23/50 (46)	**<.001**
stx_2 allele	29/32 (91)	18/50 (78)	**<.001**
stx_{2c} allele	5/32 (16)	2/50 (8)	.104
stx_{2c2} allele	0/32 (0)	1/50 (2)	1.0
$stx_{2d\text{-}activatable}$ allele	0/32 (0)	3/50 (6)	.277
stx_1 or stx_2 group			
stx_1 only	0/32 (0)	27/50 (54)	**<.001**
stx_2- group only	5/32 (16)	19/50 (38)	**.046**
stx_1 and stx_2 group	27/32 (84)	4/50 (8)	**<.001**
eae	32/32 (100)	43/50 (86)	**.039**
espA	32/32 (100)	43/50 (86)	**.039**
ehxA	32/32 (100)	44/50 (88)	.077
iha	32/32 (100)	25/50 (50)	**<.001**

NOTE. For symptoms, data are no. of patients who mentioned that symptom was present/no. who mentioned or denied symptom (%). For genotypes, data are no. of isolates with specific genotypes/all isolates tested (%).

[a] Two-tailed *P* (Fisher's exact test). Significant values are in bold type.

[b] Procedures performed on 7 patients in the *E. coli* O157:H7 group consisted of 1 appendectomy, 2 sigmoidoscopies, and 4 colonoscopies. Procedures performed on 8 patients in the non-O157:H7 STEC group consisted of 2 appendectomies (O26:H11 and ONT:H25), 2 sigmoidoscopies (O28ac:H25 and O121:H19), 3 colonoscopies (O121:H19, O145:NM, and O165:H25), and 1 esophagogastroduodenoscopy and colonoscopy (O26:NM).

[c] Antibiotics administered to patients in the *E. coli* O157:H7 group included amoxicillin, cephalexin, ciprofloxacin, metronidazole, and trimethoprim-sulfamethoxazole. Antibiotics administered to patients in the non-O157:H7 STEC group included ampicillin, ceftriaxone, ciprofloxacin, erythromycin-sulfisoxazole, furazolidone, metronidazole, and trimethoprim-sulfamethoxazole.

Non-O157:H7 STEC containing stx_1 were recovered from 10 and 9 of these patient groups, respectively ($P = .192$). Of interest, of the 12 patients whose stool samples yielded *E. coli* O26 (none of which contained stx_2-group genes) and whose history addressed whether or not they had bloody diarrhea, 6 reported that this symptom was present. One isolate from these 36 patients (ONT:NM) contained stx_1 and stx_2, and that patient had bloody diarrhea. Of the 22 patients infected with *E. coli* O157:H7 who reported bloody diarrhea, 4 were infected with stx_1^-/stx_2-group$^+$ *E. coli* O157:H7, and 18 were infected with stx_1^+/ stx_2-group$^+$ *E. coli* O157:H7. Each of the 5 patients infected with *E. coli* O157:H7 who specifically denied bloody diarrhea was infected with stx_1^-/stx_2-group$^+$ *E. coli* O157:H7 ($P = .56$). Thus, there is no statistically significant association between *stx* group and the presence or absence of bloody diarrhea.

Each of the 2 patients with stx_{2c}^+ non-O157:H7 STEC reported having bloody diarrhea, as did each of the 4 patients whose stool samples contained stx_{2c}^+ *E. coli* O157:H7. Of interest, 4 of the 8 patients in this study whose infecting STEC contained variant stx_2 alleles (stx_{2c}, $stx_{2d\text{-}activatable}$, or both) underwent invasive procedures, compared with only 11 of 74 patients infected with STEC without these alleles ($P = .033$).

We next assessed the relationship between each of the genes sought and the presence or absence of bloody diarrhea, using logistic regression analysis and restricting analysis only to the patients whose stool samples yielded non-O157:H7 STEC. Bloody diarrhea ("yes" vs. "no") was the response variable, and the explanatory variable was whether the test was positive or negative for a certain characteristic. The last column of table 4 shows the *P* value of the test for the addition of the respective characteristic to the logistic model.

Novel Stx sequence. Primers GK3 and GK4 produce from *E. coli* O28ab:H28 a 270-bp amplicon that was cleaved by neither *Fok*I nor *Hae*III [36]. The gene encoding this B subunit is the same length as the genes encoding the B subunits in stx_2 and stx_{2c} and is 6 bp longer than the genes encoding the B subunits in stx_{2d}, stx_{2e}, and stx_{2f} (figure 1). Within the overlap region, this gene's sequence differs from stx_2, stx_{2c}, stx_{2d}, stx_{2e}, and stx_{2f} at 16, 10, 21, 51, and 64 sites, respectively. Each of the 10 polymorphic sites, compared with stx_{2c}, the most similar allele in the database, is in the 3′ two-thirds of the gene, which suggests intragenic recombination in its evolution. However, only 1 of these sites resulted in a change in an amino acid, compared with Stx$_{2c}$. We have designated this *E. coli* O28ab:H28 *stx* allele stx_{2c2} (GenBank accession no. AY095209).

DISCUSSION

This population-based, prospective study demonstrates that non-O157:H7 STEC are less likely to be isolated from patients with bloody diarrhea than are *E. coli* O157:H7. We speculated previously that the higher frequency of bloody diarrhea among patients infected with *E. coli* O157:H7 could be attributed to the higher frequency of stx_2 in this group of organisms [13, 30], especially in consideration of animal and in vitro studies that demonstrate that Stx$_2$ is the more toxic of the 2 Stxs [38, 39]. However, we did not confirm this association among the non-O157:H7 STEC in this study, largely because one-third of

Table 4. Logistic regression analysis using bloody diarrhea ("yes" or "no") as the response variable among the 36 patients whose stool samples contained non-O157:H7 Shiga toxin–producing *Escherichia coli* (STEC), whose histories specifically addressed this symptom, and the genotype of the recovered STEC.

Characteristic	Group without bloody diarrhea ($n = 15$)	Group with bloody diarrhea ($n = 21$)	P for logistic regression
eae	14 (93)	19 (91)	.461
espA	14 (93)	19 (91)	.461
ehxA	13 (87)	19 (91)	.722
iha	6 (40)	11 (52)	.462
stx_1	9 (60)	10 (48)	.462
stx_2 group (all alleles)	7 (47)	12 (57)	.535
stx_2 allele	6 (40)	8 (38)	.908
stx_{2c} allele	0 (0)	2 (10)	.135
stx_{2c2} allele	1 (7)	0 (0)	.181
$stx_{2d\text{-}activatable}$ allele	0 (0)	3 (21)	.064

NOTE. Data are no. (%) of patients whose isolate had each genotype.

the patients whose stool cultures yielded non-O157:H7 STEC containing stx_1, but not stx_2-group genes, reported observing bloody diarrhea. Therefore, factors other than the *stx* genotype are likely to be responsible for the bloody diarrhea observed during STEC infections, a restatement of the conclusion reached by Welinder-Olsson et al. [11]. This relationship is obviously complex, and further elucidation awaits a more complete enumeration of virulence loci and their allelic variants in non-O157:H7 STEC.

Our findings are noteworthy for several additional reasons. First, *E. coli* O121:H19 was isolated from an unexpectedly large number of cases and was recovered nearly as frequently as *E. coli* O26:H11. *E. coli* O121:H19 was associated with an epidemic in Connecticut [40], but its causative role in that report was based mainly on serologic testing and not on isolate recovery. None of the cases we report here were clustered in time or space, and their multicentric occurrence confounded attempts at source linkage. The high frequency of this serotype in this study contrasts with its rarity of isolation from patients in 2 recent large European series [4, 41].

Second, none of the serogroup O26 STEC isolated during the present study harbored an stx_2 gene, which is typically present in an STEC O26 clonal subgroup that recently emerged as a pathogen in Germany [42]. This result suggests that this new O26 STEC subgroup has not yet spread to the North American region covered by our study.

Third, we demonstrated that some alleles designated stx_{2c} in the detection protocol actually encoded a mucus-activatable toxin [43] on the basis of restriction fragment analysis. Thus, the $stx_{2d\text{-}activatable}$ allele may be more prevalent in human STEC than previously realized. It should also be emphasized that stx_{2d} variants, as defined by the analytical PCR conditions of Pierard et al. [44] and which were not found in this study, are distinct

from the genes encoding the protein designated as the $Stx_{2d\text{-}activatable}$ toxin.

Fourth, we provide data suggesting that *E. coli* containing stx_{2c} and $stx_{2d\text{-}activatable}$ alleles are associated with more-severe gastrointestinal disease in the human host (as manifested by higher frequencies of associated procedures), compared with patients whose infected STEC harbors other *stx* alleles. stx_{2c} was originally identified as a variant toxin in *E. coli* O157:NM [45], but in vitro and mouse lethality testing failed to demonstrate enhanced virulence of Stx_{2c}, compared with other Stx_2 alleles [46]. Compared with Stx, Stx_{2c} binds to a distinct, but overlapping, epitope on globotriaosylceramide, the glycosphingolipid receptor for Stxs on eukaryotic cells [47]. Furthermore, parenterally administered VT2c, which is identical to Stx_{2c}, preferentially binds to gut tissue, especially cecal tissue, in rabbits and causes severe hemorrhagic diarrhea [48], but it is probable that human gut has a different distribution of Stx receptors. Although the inference that *E. coli* containing stx_{2c} are more virulent for humans than those with other stx_2 alleles requires confirmation, it is worth noting that stx_{2c} was the only stx_2 variant identified in STEC from patients with HUS in a recent German study [4].

Fifth, surveillance efforts for non-O157:H7 STEC probably should include points of care other than emergency departments. In a recent study based largely on data gathered in an emergency department, *E. coli* O157:H7 were recovered more frequently than were non-O157:H7 STEC [13]. This discrepancy probably is attributable to the more common occurrence of bloody diarrhea in patients infected with *E. coli* O157:H7, a symptom that conceivably precipitates urgent medical evaluation, and to the overall less-severe illnesses associated with non-O157:H7 STEC. However, the possibility exists that non-microbiologic factors, such as access to care and geographic
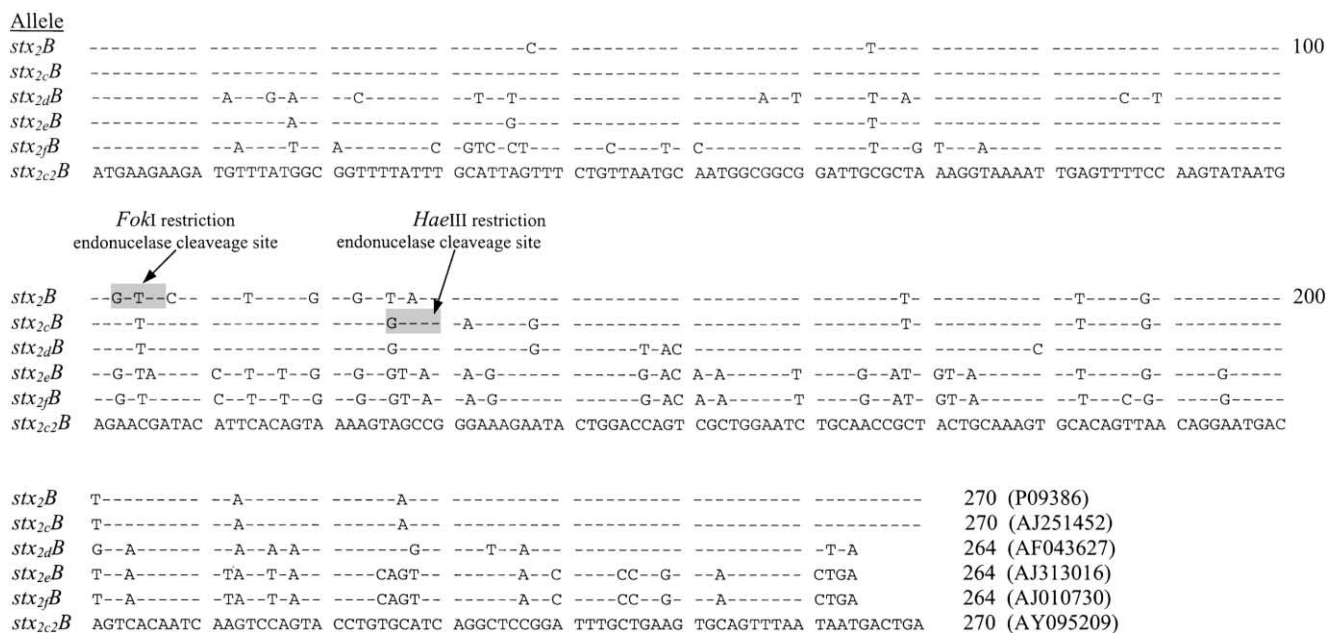
Figure 1. Comparison of *stx₂* B subunit allele nucleotide sequences currently available in the National Center for Biotechnology Information Database (available at: http://www.ncbi.nlm.nih.gov) with new *stx₂c₂* B subunit allele from *Escherichia coli* O28ab:H28. GenBank accession nos. of respective B subunits are provided in parentheses. Shaded nucleotides represent designated endonuclease sites in reference strains.

patient catchment patterns, influence the sites of presentation to care of these Montana residents.

Finally, we identified a new allele, *stx₂c₂*, which most closely resembles *stx₂c*. Designation of Stx₂c₂ as a bona fide and novel Stx₂ variant awaits biologic studies that differentiate it from Stx₂c [49]. However, although the DNA sequence suggests the novelty of this allele, Stx₂c₂ differs from Stx₂c by only a single amino acid.

Despite the geographically and temporally well-defined population investigated, our study has several limitations. First, we relied on a retrospective analysis of charts to ascertain symptoms; such a data set is less complete than one collected prospectively. We do not know whether nonmention of symptoms reflects nonseverity, or the extent to which the conclusions would have been different had data collection been prospective and more complete. Second, the study structure did not permit an assessment of the rate of development of HUS, because of the lack of standardized follow-up. We were also unable to examine the effect of antibiotics, which were administered to large subsets of patients in both groups, on outcome. Third, the possibility exists that STEC that were present were not recovered. The shipment of stool samples to a central laboratory might have diminished STEC viability while in transport. In addition, our protocol of evaluating only sorbitol-fermenting colonies for the ability to produce Stx if a serologic test for colonies that did not express the O157 antigen was negative would have overlooked unusual non-O157:H7 STEC that fail to ferment sorbitol, such as *E. coli* O104:H21 [50]. Furthermore,

the study is biased toward the detection of STEC that produce Stx that are detected in the EIA identification employed. However, this methodology might not have detected all Stx₁ [41] and Stx₂ [4] variants, which are produced more frequently by non-O157:H7 STEC than by *E. coli* O157:H7. Reduced Stx expression in vitro and antigenic variation in Stx structure could conceivably compromise the sensitivity of EIA. Vero cell assays, or nucleic acid detection methodologies, might more sensitively identify STEC that produce variant Stxs. Fourth, because stool samples were not uniformly plated initially on SMAC agar for *E. coli* O157:H7, some patients infected with this serotype might have gone undetected, because EIA is less sensitive than SMAC agar screening for the detection of this pathogenic serotype [13]. Fifth, it is possible that some of these cases represented undetected clustering of illnesses and that the strain and trait distributions are skewed by duplicate analyses of what is, in reality, the same organism. Sixth, because of the heterogeneity of virulence factors among non-O157:H7 STEC, it is important to note that the profiles of the illnesses associated with these organisms might not apply to all populations, where the distribution of causative serotypes might be different from the one we observed in Montana during the study period. Seventh, this heterogeneity also needs to be taken into account when considering non-O157:H7 infections as a group, in comparison to *E. coli* O157:H7 infections. Although multiple studies have demonstrated that, in aggregate, non-O157:H7 STEC are associated with diminished frequency of bloody diarrhea and

of HUS, compared with *E. coli* O157:H7, a subset of non-O157:H7 STEC are probably as virulent as *E. coli* O157:H7.

A final limitation of this study is that we cannot state with certainty that the non-O157:H7 STEC identified were the etiologic agents of the diarrhea, although we eliminated from analysis patients whose stool samples yielded another bacterial enteric pathogen. The patients whose stool samples contained non-O157:H7 STEC were plausibly made ill by them, but we cannot assign a categorically pathogenic role to these organisms, without control subjects. In this regard, it is noteworthy that, in several studies that attempted to address the pathogenicity of non-O157:H7 STEC, control subjects without diarrhea had the same frequency of fecal excretion of non-O157:H7 STEC as did patients with diarrhea [51–55]. Non-O157:H7 STEC are quite common in food [56, 57], in contrast to the comparative rarity of O157:H7 STEC [58]. Thus, human contact with non-O157:H7 STEC is probably frequent, and it is possible that, in some patients, the recovery of non-O157:H7 STEC in the stool reflects innocuous gastrointestinal pass-though and not infection. Nonetheless, some of the many non-O157:H7 STEC with which humans come in contact can cause epidemics [50, 59–61], and selected serotypes have well-substantiated associations with HUS [60, 62–64]. Furthermore, most of the non-O157:H7 STEC in this study contained auxiliary, and probably critical, virulence genes, such as *eae,* belong to serotypes that have been associated with HUS and epidemics, and were in comparatively high abundance among the patients' aerobic coliform flora. It is, therefore, likely that most of the non-O157:H7 identified in this study did, indeed, cause the enteric illnesses in the patients from whom they were isolated. Clearly, a definitive assessment and enumeration of the traits that are needed to cause disease will facilitate the identification of which of the many non-O157:H7 STEC are truly pathogens.

Acknowledgments

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