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 stx2-group genes, as well as eae, iha, espA, and ehxA; 84% contained stx2. Non-O157:H7 STEC less frequently contained stx1 (P < .046), stx2 (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 stx2e or stx2d-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 group (Stx1, Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, and Stx2f) [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 stx2-group genes but that did not contain stx1 were more likely to be isolated from patients with HUS than were E. coli O157:H7 isolates containing both stx1 and stx2-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 stx1 from an stx2+ stx2+ E. coli O157:H7 isolate augments its virulence in gnotobiotic piglets. Boerlin et al. [12] reported a strong association between the presence of stx2 variants within the stx1 major serotypes and the severity of associated disease. Friedrich et al. [4] refined stx genotype analysis by examining allelic variants within the stxi group and suggested that STEC containing stx2, or stx1, 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 “Ond-typeable” (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, and stx, genes were preliminarily differentiated by restriction analysis of the GK3-GK4 amplification products, using HaellII and FokI [36]. However, stx, alleles are indistinguishable from stx, (A.R.M.-C., unpublished observation) by the initial PCR technique used in this study [36]; therefore, isolates with apparent stx, alleles were tested using stx, primer pairs, as noted in table 1, to generate an 890-bp amplicon. PstI cleaves this amplicon into 504- and 386-bp fragments if the target gene is stx, but does not cleave amplicons derived from stx,.

Because the stx,-group amplicon of an E. coli O28ab:H28 failed to yield predicted fragments when digested with FokI or HaellII, we produced an amplicon that spans the genes encoding the holotoxin, using primers 5'-CAACAGCAATGGCCGT-AGG-3' and 5'-TTTCACCAGTCGCCCCTCCAC-3', digested the amplicon with EcoRV, 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 EcoRV site by use of primers 5'-TACTGGGTATTTCTGC-GTA-3' and 5'-GGGTATAACTGCTGTCGGG-3', which also was inserted into the pGEM-T Easy Vector and sequenced.

**Activation assay.** Two isolates that contained stx, as their sole stx genes were tested further, to determine whether the Stx they produced exhibited the activatable phenotype. The activation assay was performed as described elsewhere [37]. In brief, culture supernatants from strains containing Stx-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>
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<th>Primer pair [reference]</th>
<th>Sequence</th>
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<th>Primer amounts, pmol of each</th>
<th>MgCl₂, mmol/L</th>
<th>Taq DNA polymerase, U</th>
<th>PCR condition</th>
<th>Length of PCR product, bp</th>
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**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 120 s, and final extension step of 7 min at 72°C; B, denaturing at 94°C for 10 s, annealing at 52°C for 60 s, and extension at 72°C for 40 s; C, denaturing at 94°C for 60 s, annealing at 72°C for 60 s, and final extension step of 10 min at 72°C; 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, extension at 72°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 40 s; G, denaturing at 94°C for 30 s, annealing at 59°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 stxA and variants, which used 35 cycles. All PCRs were performed in 50-μL final volume.

b stx1A, stx1C, and stx1D [75].
<table>
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<td>Total</td>
<td>58</td>
<td>55</td>
<td>47</td>
<td>7</td>
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**NOTE.** NA, symptoms not addressed for patients whose stool samples yielded these STEC.

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

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 ONTH:H25), 2 sigmoidoscopies (O28ac:H25 and O121:H19), 3 colonoscopies (O121:H19, O145:NM, and O165:H25), and 1 esophagogastroduodenoscopy and colonoscopy (O26:NM).

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 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*-group genes. Twenty-seven (84%) contained, in addition, *stx*1. Two *E. coli* O157:H7 contained *stx*2 as their only *stx*-group allele, and 2 contained *stx*2p in addition to *stx*1. None of the *stx*-genes in *E. coli* O157:H7 were determined after subsequent analysis to be an *stx*2d-activatable variant.

Of the 50 non-O157:H7 STEC, 27 contained *stx*1, but not *stx*2-group genes, 19 contained *stx*1-group genes but not *stx*2p, and 4 contained both *stx*1 and *stx*2-group genes. Four contained *stx*2 on preliminary analysis, but after subsequent analysis, 3 of these isolates contained *stx*2d-activatable genes, 1 in combination with an *stx*1 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-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*1-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).**

<table>
<thead>
<tr>
<th>Variable</th>
<th>E. coli O157:H7 group</th>
<th>Non-O157:H7 STEC group</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>12/32 (38)</td>
<td>30/50 (58)</td>
<td>.70</td>
</tr>
<tr>
<td>Headache</td>
<td>0/2 (0)</td>
<td>2/4 (50)</td>
<td>.46</td>
</tr>
<tr>
<td>Fecal leukocytes</td>
<td>20/26 (77)</td>
<td>20/29 (69)</td>
<td>.55</td>
</tr>
<tr>
<td>Procedure</td>
<td>7/32 (22)</td>
<td>8/50 (16)</td>
<td>.56</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>11/32 (34)</td>
<td>20/50 (38)</td>
<td>.64</td>
</tr>
<tr>
<td>First site</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emergency department</td>
<td>18/32 (56)</td>
<td>15/50 (30)</td>
<td>.02</td>
</tr>
<tr>
<td>Other ambulatory facility</td>
<td>14/32 (44)</td>
<td>35/50 (70)</td>
<td></td>
</tr>
<tr>
<td>Bloody diarrhea</td>
<td>22/27 (81)</td>
<td>21/36 (58)</td>
<td>.06</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>24/27 (89)</td>
<td>33/40 (83)</td>
<td>.72</td>
</tr>
<tr>
<td>Nausea</td>
<td>8/13 (62)</td>
<td>8/12 (67)</td>
<td>1.0</td>
</tr>
<tr>
<td>Vomiting</td>
<td>8/20 (40)</td>
<td>15/29 (52)</td>
<td>.56</td>
</tr>
<tr>
<td>Fever (% of “yes”)</td>
<td>7/23 (30)</td>
<td>8/25 (26)</td>
<td>.53</td>
</tr>
<tr>
<td>sx</td>
<td>27/32 (84)</td>
<td>31/50 (62)</td>
<td>.04</td>
</tr>
<tr>
<td>stx -group alleles</td>
<td>32/32 (100)</td>
<td>23/50 (46)</td>
<td>&lt;.00</td>
</tr>
<tr>
<td>stx, allele</td>
<td>29/32 (91)</td>
<td>18/50 (78)</td>
<td>&lt;.00</td>
</tr>
<tr>
<td>stx, allele</td>
<td>5/32 (16)</td>
<td>2/50 (8)</td>
<td>.10</td>
</tr>
<tr>
<td>stx, allele</td>
<td>0/32 (0)</td>
<td>1/50 (2)</td>
<td>1.0</td>
</tr>
<tr>
<td>stx, or stx, allele</td>
<td>0/32 (0)</td>
<td>3/50 (6)</td>
<td>.27</td>
</tr>
<tr>
<td>stx, only</td>
<td>0/32 (0)</td>
<td>27/50 (54)</td>
<td>&lt;.00</td>
</tr>
<tr>
<td>stx, group only</td>
<td>5/32 (16)</td>
<td>19/50 (38)</td>
<td>.04</td>
</tr>
<tr>
<td>stx, and stx, group</td>
<td>27/32 (84)</td>
<td>4/50 (8)</td>
<td>&lt;.00</td>
</tr>
<tr>
<td>eae</td>
<td>32/32 (100)</td>
<td>43/50 (86)</td>
<td>.03</td>
</tr>
<tr>
<td>espA</td>
<td>32/32 (100)</td>
<td>43/50 (86)</td>
<td>.03</td>
</tr>
<tr>
<td>ehxA</td>
<td>32/32 (100)</td>
<td>44/50 (88)</td>
<td>.07</td>
</tr>
<tr>
<td>iha</td>
<td>32/32 (100)</td>
<td>25/50 (50)</td>
<td>&lt;.00</td>
</tr>
</tbody>
</table>

**NOTE.** For symptoms, data are no. of patients who mentioned that symptom was present—whether or not they had bloody diarrhea, 6 reported that this symptom was present. One isolate from these 36 patients (ONT:NM) contained stx, 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, /stx, -group+ E. coli O157:H7, and 18 were infected with stx, /stx, -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, /stx, -group+ E. coli O157:H7 (P = .56). Thus, there is no statistically significant association between stx, group and the present or absence of bloody diarrhea.

Each of the 2 patients with stx, -group non-O157:H7 STEC reported having bloody diarrhea, as did each of the 4 patients whose stool samples contained stx, -group+ E. coli O157:H7. Of interest, 4 of the 8 patients in this study whose infecting STEC contained variant stx, alleles (stx, stx,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 FokI nor HaeIII [36]. The gene encoding this B subunit is the same length as the genes encoding the B subunits in stx, and stx, and is 6 bp longer than the genes encoding the B subunits in stx, and stx, (figure 1). Within the overlap region, this gene’s sequence differs from stx, stx, and stx, at 16, 10, 21, 51, and 64 sites, respectively. Each of the 10 polymorphic sites, compared with stx, 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, We have designated this E. coli O28ab: H28 stx allele stx, (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, in this group of organisms [13, 30], especially in consideration of animal and in vitro studies that demonstrate that Stx, 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
the present study harbored an stx2, but not stx2-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 O157:H7, 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 American regions covered by our study.

Second, none of the serogroup O26 STEC isolated during the present study harbored an stx1 gene, which is typically present in an STEC O26 clonal subgroup that recently emerged 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 stx2c in the detection protocol actually encoded a mucus-activatable toxin [43] on the basis of restriction fragment analysis. Thus, the stx2c-activatable allele may be more prevalent in human STEC than previously realized. It should also be emphasized that stx2c alleles require confirmation, it is worth noting that stx2c was the only stx2 variant identified in STEC with HUS in a recent German study [4].

Fourth, we provide data suggesting that E. coli containing stx2 and stx2c-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. stx2 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 Stx2 compared with other Stx alleles [46]. Compared with Stx, Stx2c 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 Stx2c, 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 stx2c are more virulent for humans than those with other stx alleles requires confirmation, it is worth noting that stx2 was the only stx2 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 nonmicrobiologic factors, such as access to care and geographic

| 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            |
| stx1            | 9 (60)           | 10 (48)          | .462            |
| stx2 group (all alleles) | 7 (47)          | 12 (57)          | .535            |
| stx2 allele     | 6 (40)           | 8 (38)           | .908            |
| stx2c allele    | 0 (0)            | 2 (10)           | .135            |
| stx2c2 allele   | 1 (7)            | 0 (0)            | .181            |
| stx2d-activatable allele | 0 (0)       | 3 (21)           | .064            |

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

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Figure 1. Comparison of stx\textsubscript{2}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 stx\textsubscript{2c2}B subunit allele from Escherichia coli O\textsubscript{28ab}:H\textsubscript{28}. GenBank accession nos. of respective B subunits are provided in parentheses. Shaded nucleotides represent designated endonuclease sites in reference strains.

Finally, we identified a new allele, stx\textsubscript{2c2}, which most closely resembles stx\textsubscript{2c}. Designation of Stx\textsubscript{2c2} as a bona fide and novel Stx\textsubscript{2} variant awaits biologic studies that differentiate it from Stx\textsubscript{2c} [49]. However, although the DNA sequence suggests the novelty of this allele, Stx\textsubscript{2c2} differs from Stx\textsubscript{2c} 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:H\textsubscript{7} 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 Stx\textsubscript{1} [41] and Stx\textsubscript{2} [4] variants, which are produced more frequently by non-O157:H\textsubscript{7} STEC than by E. coli O157:H\textsubscript{7}. 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:H\textsubscript{7}, 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:H\textsubscript{7} 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:H\textsubscript{7} infections as a group, in comparison to E. coli O157:H\textsubscript{7} infections. Although multiple studies have demonstrated that, in aggregate, non-O157:H\textsubscript{7} STEC are associated with diminished frequency of bloody diarrhea and
of HUS, compared with \textit{E. coli} O157:H7, a subset of non-O157:
H7 STEC are probably as virulent as \textit{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 etio-
logic agents of the diarrhea, although we eliminated from analy-
sis patients whose stool samples yielded another bacterial en-
teric 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-through 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 associ-
ations 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 \textit{eae}, belong to serotypes that
have been associated with HUS and epidemics, and were in
comparatively high abundance among the patients’ aerobic co-
limon 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.

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