JOURNAL OF CLINICAL MICROBIOLOGY, July 2005, p. 3213–3220 Vol. 43, No. 7
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.7.3213–3220.2005

Characterization of a Shiga Toxin-, Intimin-, and Enterotoxin Hemolysin-Producing *Escherichia coli* ONT:H25 Strain Commonly Isolated from Healthy Cattle

Haiqing Sheng,[1] Margaret A. Davis,[1] Hannah J. Knecht,[1] Dale D. Hancock,[2] Joyce Van Donkersgoed,[3] and Carolyn J. Hovde[1]*

University of Idaho, Department of Microbiology, Molecular Biology, and Biochemistry, Moscow, Idaho 83844-3052[1]; Washington State University, Department of Veterinary Medicine, Pullman, Washington[2]; and Veterinary Services Inc., Lacombe, Alberta, Canada[3]

Received 13 January 2005/Returned for modification 27 February 2005/Accepted 27 March 2005

Among bovine fecal and recto-anal mucosal swab samples cultured in our laboratory for *Escherichia coli* O157:H7, we frequently isolated *E. coli* organisms that were phenotypically similar to the O157:H7 serotype as non-sorbitol fermenting and negative for β-glucuronidase activity but serotyped O nontypeable:H25 (ONT:H25). This study determined the prevalence and virulence properties of the *E. coli* ONT:H25 isolates. Among dairy and feedlot cattle ($n = 170$) sampled in Washington, Idaho, and Alberta, Canada, the percentage of animals culture positive for *E. coli* ONT:H25 ranged from 7.5% to 22.5%, compared to the prevalence of *E. coli* O157:H7 that ranged from 0% to 15%. A longitudinal 8-month study of dairy heifers ($n = 40$) showed that 0 to 15% of the heifers were culture positive for *E. coli* O157:H7, while 15 to 22.5% of the animals were culture positive for *E. coli* ONT:H25. As determined by a multiplex PCR, the *E. coli* ONT:H25 isolates carried a combination of virulence genes characteristic of the enterohemorrhagic *E. coli*, including intimin, translocated intimin receptor, Stx2, and hemolysin (*eae*-β, *tir*, $stx_{2vh\text{-}a}$, and *hly*). *E. coli* ONT:H25 isolates from diverse geographic locations and over time were fingerprinted by separating XbaI-restricted chromosomal DNA by pulsed-field gel electrophoresis (PFGE) separation. Two strains of *E. coli* ONT:H25 were highly similar by PFGE pattern. Experimental inoculation of cattle showed that *E. coli* ONT:H25, like *E. coli* O157:H7, colonized the bovine recto-anal junction mucosa for more than 4 weeks following a single rectal application of bacteria.

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) organisms that cause hemorrhagic colitis and the potentially fatal hemolytic uremic syndrome (HUS) are classed as enterohemorrhagic *E. coli* (EHEC) (16, 28, 45). Although the majority of HUS cases in humans have been associated with the EHEC serotype O157:H7 (27, 32), non-O157 serotypes also cause disease and are more common than *E. coli* O157:H7 in some geographical areas (18, 22, 36, 46). There are over 200 different serotypes of STEC (32), and the number associated with human illness exceeds 100 (6). Domestic ruminants are important reservoirs of STEC and are the most common source for foodborne and animal-contact infections (13). Investigations throughout the world reveal that 10 to 80% of cattle are infected with STEC (5, 6, 49; http://www.who.int/emc-documents/zoonoses/whocsraph988c.html). Non-O157 STEC strains are more prevalent in animals and as contaminants in foods and water than *E. coli* O157 and, therefore, presumably humans are more frequently exposed to them (9, 10, 27). In the United States, it is estimated that 20% to 50% of STEC human infections are caused by non-O157:H7 serotypes (1, 2).

The recto-anal junction (RAJ) mucosa has recently been identified as a site of *E. coli* O157:H7 colonization in the bovine gastrointestinal tract (33). This finding was supported by earlier observations in mature cattle that *E. coli* O157:H7 persists in the lower gastrointestinal tract and feces but is not detected in the upper gastrointestinal tract after a few days post-oral inoculation (12, 23) and by the detection of the bacterium only in the distal rectal tissue in experimentally inoculated sheep (23). Our laboratory recently demonstrated that recto-anal mucosal swab (RAMS) culture is a more sensitive detection method for *E. coli* O157:H7 in cattle than traditional fecal culture (40), and a method to reliably colonize animals is application of the bacteria to the recto-anal junction mucosa (44).

Routine identification of *E. coli* O157:H7 from cattle samples includes culture on sorbitol MacConkey (SMAC) agar containing cefixime, potassium tellurite, vancomycin, and 4-methylumbelliferyl-beta-D-glucuronic acid dihydrate (MUG), a molecule that fluoresces when cleaved and therefore is used to detect β-glucuronidase activity. Differentiation of the O157:H7 serotype on this medium is easy because colonies not fermenting sorbitol (colorless) and not hydrolyzing MUG (no fluorescence at 363 nm) are presumptive *E. coli* O157:H7. The serotype is confirmed by a commercially available latex agglutination assay. While culturing bovine feces and RAMS samples for *E. coli* O157:H7, we frequently isolated an *E. coli* phenotypically similar to *E. coli* O157:H7 in that it was non-sorbitol fermenting and did not exhibit β-glucuronidase activity and it contained the gene for Stx type 2 (stx_2) but serotyped as O untypeable and H25 (ONT:H25). Because STEC ONT:H25 isolation from both human patients and cattle had been previously reported in Europe (7, 9, 50, 51), we further characterized the *E. coli* ONT:H25 isolates from representative

* Corresponding author. Mailing address: Department of Microbiology, Molecular Biology and Biochemistry, University of Idaho, Moscow, ID 83844-3052. Phone: (208) 885-5906. Fax: (208) 885-6518. E-mail: cbohach@uidaho.edu.

TABLE 1. Detection of virulence genes in *E. coli* ONT:H25 and O157:H25 strains by PCR

Gene[a]	Primers (5′ to 3′) and fragment size (reference)[b]	Gene detected in:				
		E. coli ONT:H25[c]			*E. coli* O157[d]	
		SH1	SH2	SH3	W1	W2
eaeA	F, GTGGCGAATACTGGCGAGACT R, CCCCATTCTTTTTCACCGTCG 890 bp (20)	+	+	+	+	+
stx_1	F, ACACTGGATGATCTCAGTGG R, CTGAATCCCCCTCCATTATG 614 bp (21)	−	−	−	+	+
stx_2	F, CCATGACAACGGACAGCAGTT R, CCTGTCAACTGAGCA(GCA)CTTTG 779 bp (21)	+	+	+	+	+
tir	F, CATTACCTTCACAAACCGAC R, CCCCGTTAATCCTCCCAT 1550 bp (29)	+	+	+	+	+
hylA	F, ACGATGTGGTTTATTCTGGA R, CTTCACGTGACCATACATAT 165 bp (19)	+	+	+	+	+
$fliC_{h7}$	F, GCGCTGTCGAGTTCTATCGAGC R, CAACGGTGACTTTATCGCCATTCC 625 bp (25)	−	−	−	+	+

[a] *eaeA*, stx_1, stx_2, *tir*, *hlyA*, and $fliC_{h7}$ refer to the genes which encode intimin, Shiga toxin type 1, Shiga toxin type 2, translocated intimin receptor, EHEC-hemolysin, and H7 flagellar antigen, respectively.
[b] F, forward primer; R, reverse primer. Predicted size of amplified product is indicated in bases; the corresponding reference is indicated in parentheses.
[c] Three strains of *E. coli* ONT:H25 isolated in this study, designated as SH1, SH2, and SH3. One hundred percent of 258 individual isolates representing these strains gave identical results.
[d] Two different *E. coli* strains isolated in this study, designated as W1 and W2.

dairy, feedlot, and research settings in our region. In this study we analyzed *E. coli* ONT:H25 for (i) prevalence among dairy and feedlot cattle, (ii) virulence factors and DNA fingerprint, and (iii) the ability to colonize the bovine recto-anal junction mucosa.

MATERIALS AND METHODS

Animals. All animals in this study were apparently healthy and had fully developed ruminant gastrointestinal tracts. Animals from three distinct settings provided representative populations of each group.

Dairy heifers. Forty Holstein heifers (2 months to 4 months old) in eastern Washington were sampled once per month, except for the month of September. Forty Holstein heifers (6 months to 14 months old) in southern Washington were sampled once.

Steers. Twenty-three Holstein steers at a University of Idaho research barn in northern Idaho were sampled twice, and 27 Holstein steers on a farm in central Washington were sampled once. The steers were 4 to 8 months old.

Feedlot cattle. A total of 40 cattle from four different feedlots in Alberta, Canada, were sampled; 30 animals were sampled twice, 1 month apart, and 10 animals were sampled once.

Research animals. Eight 7-month-old Holstein steers were used in the inoculation study. Animals were housed in quarantined facilities in which feed, water, bedding, manure, and other waste were not in contact with any nonexperimental animals. All personnel followed strict biosafety procedures, and all procedures were approved by the Institutional Animal Care and Use and Biosafety Committees. Steers were fed alfalfa hay twice and pellet grain feeds once daily, with free access to drinking water.

RAMS and fecal sample collection. The RAMS samples were collected from dairy heifers and Holstein steers as previously described (44). Briefly, a sterile foam-tipped applicator (catalog number 10812-022; VWR International, Buffalo Grove, IL) was inserted into the anus approximately 2 to 5 cm and the entire mucosal circumference of the recto-anal junction was swabbed using a rapid in-and-out motion. Each RAMS was placed into a culture tube containing 3 ml of Trypticase soy broth (TSB; Difco Laboratories, Detroit, Mich.). All RAMS samples in TSB tubes were kept on ice until they were processed in the laboratory within 6 h of collection. RAMS samples from the feedlot cattle in Alberta, Canada, could not be obtained. Instead, feces (0.2 to 0.5 g) from each animal were put into tubes containing 3 ml TSB and shipped on ice to the laboratory and processed within 24 h.

Bacterial culture. RAMS samples and fecal samples were processed by direct and enrichment (if direct culture was negative) culture methods as previously described (40). SMAC agar (Difco Laboratories) containing cefixime (50 ng/ml; Wyeth-Ayerst, Pearl River, N.Y.), potassium tellurite (2.5 mg/liter; Sigma Chemical Co., St. Louis, MO), vancomycin (40 mg/liter; Sigma), and MUG (100 μg/ml; Biosynth Ag, Staad, Switzerland) (SMAC-CTVM) was used for isolation of *E. coli* O157:H7 and ONT-H25 strains from RAMS and fecal samples. Sorbitol-nonfermenting and MUG-negative (SMN) colonies were assayed for the O157 antigen by a latex agglutination test (Pro-Lab Diagnostics, Toronto, Canada). Bacterial counts were estimated from plates inoculated with serial dilutions of samples by multiplying the colony counts by the appropriate dilution factor. Non-O157 SMN colonies were stabbed into Luria-Bertani agar until further analysis. All or five isolates (if ≥5 colonies were found on the plate) from each animal positive by direct culture and two isolates from each animal positive by enrichment culture were preserved at each sampling time.

Api test. The Api 20E identification system (BioMerieux, France) was used according to the manufacturer's instructions for bacterial genus species designation based on biochemical reactions.

Serotyping. Non-O157 SMN isolates were serotyped by the Gastroenteric Disease Center (Pennsylvania State University, State College) by standard technique. A total of 180 O-antigen-specific and 53 H-antigen-specific antisera were used for O and H typing, respectively. The O157 antigen was detected using a latex slide agglutination test (Pro Lab Diagnostics, Canada).

Chromosomal DNA fingerprinting. To analyze the genetic relationship between isolates of *E. coli* ONT:H25 and *E. coli* O157:H7, pulsed-field gel electrophoresis (PFGE) separation of digested chromosomal DNA was performed as previously described (15). DNA was restricted with XbaI, and fragments were resolved in 1% agarose gels with 0.5× Tris-borate-EDTA electrophoresis buffer using a contour-clamped homogeneous electric field DR-III electrophoresis chamber (Bio-Rad, Richmond, Calif.). Pulse times were ramped from 2.2 to 54.2 seconds over a run time of 20 h at 6 V/cm.

Detection of virulence genes. A multiplex PCR was designed to detect stx_1, stx_2, *eaeA*, *tir*, EHEC *hlyA*, and $fliC_{h7}$ gene sequences and performed with a GeneAmp PCR System 2400 (Applied Biosystems/Perkin-Elmer, Norwalk, Conn.). Oligonucleotide primers were commercially manufactured (Invitrogen, Carlsbad, CA). Primers and the predicted amplicon lengths are listed in Table 1. A single colony was transferred into a 50-μl reaction mixture containing 10× PCR

buffer (5 μl), deoxynucleoside triphosphates (0.2 mM), $MgCl_2$ (2.0 mM), primer sets (0.2 mM of each), and *Taq* polymerase (0.5 U; Invitrogen). Initial denaturation was done at 95°C for 3 min, followed by 35 cycles composed of incubation at 94°C for 1 min, 58°C for 45 s, and 72°C for 90 s. Amplified DNA fragments were resolved by gel electrophoresis in 1.5% (wt/vol) agarose gels. DNA was stained with ethidium bromide, visualized with UV illumination, and imaged with an AlphaDigiDoc gel documentation and analysis system (Alpha Innotech Corporation, San Leandro, CA). *E. coli* O157:H7 (ATCC 43894) and *E. coli* K-12 (MG1655) were used as positive and negative controls, respectively.

Shiga toxin expression. To compare production of Stx by *E. coli* isolates, cells were grown in Luria-Bertani broth and an enzyme immunoassay (Premier EHEC; Meridian Diagnostics Inc., Cincinnati, Ohio) was performed according to the manufacturer's instructions. Results were analyzed spectrophotometrically at two wavelengths, 450 and 630 nm, in a PowerWave XS reader (Bio-Tek, Winooski, Vermont). *E. coli* K-12 (MG1655) and *E. coli* O157:H7 (ATCC 43894) were used as negative and positive controls, respectively.

Enterohemolysin expression. Hemolysin expressed by the *E. coli* isolates was detected on tryptose agar plates (Difco Laboratories, Detroit, Mich.) supplemented with 10 mM $CaCl_2$ and 5% defibrinated sheep blood cells, as described previously (8). The inoculated plates were observed for hemolysis after 4 h of incubation (for detection of alpha-hemolysis) and after overnight incubation in ambient air at 37°C (for detection of hemolysis or nonhemolysis). *E. coli* K-12 (MG1655) and O157:H7 (ATCC 43894) were used as negative and positive controls, respectively.

Intimin subtyping. To subtype the *eae* genes, primers and conditions for amplification of 13 *eae* variants (α, β, γ1, δ/κ, ε, ζ, η, γ2/θ, ι, λ, μ, *ν*, and ξ) were used according to those described by Blanco et al. (9). Amplified DNAs were separated by electrophoresis through 2% agarose gels and visualized by UV fluorescence after staining with ethidium bromide. A DNA size standard 100-bp ladder (Invitrogen) was used in each gel.

***stx*$_2$ subtyping.** To identify the *stx*$_2$ types of the *E. coli* O157:H7 and ONT:H25 isolates, restriction fragment length polymorphism-PCR (RFLP-PCR) was used, as previously reported (3, 4, 11, 39, 48). The primers, restriction endonucleases, and expected sizes of DNA products are listed in Table 1. PCRs were performed with a GeneAmp PCR System 2400 (Applied Biosystems/Perkin-Elmer). Initial denaturation at 95°C for 3 min was followed by 35 cycles of incubation at 94°C for 2 min, 55°C for 2 min, and 72°C for 1 min. All PCRs were performed on isolated single colonies. PCR products were digested with the corresponding restriction enzymes: HincII, HaeII, PvuII, and RsaI (Invitrogen). DNA fragments were electrophoresed through 2% agarose gels and visualized as described above. *E. coli* O157:H7 (ATCC 43894) and *E. coli* K-12 (MG1655) were used as positive and negative controls, respectively.

Experimental inoculation. Two groups containing four animals each were penned separately and inoculated with *E. coli* O157:H7 ATCC 43894 or *E. coli* ONT:H25 strain SH2 by using a rectal application method, as previously described (44). Briefly, feces were manually removed from the terminal rectum. A 10-cm by 3.5-cm-diameter cylindrical sponge (Rubbermaid, St. Francis, WI) with a wooden handle was saturated with 10 ml of the *E. coli* O157:H7 or *E. coli* ONT:H25 overnight culture (1.9×10^9 CFU/ml or 1.7×10^9 CFU/ml, respectively) and then inserted into the anus and gently and rapidly rubbed against the recto-anal junction mucosa. After swabbing, defecation was prevented for 10 min by holding the steer tail firmly against the anus. This presumably ensured that the inocula were retained in the rectal lumen for a sufficient period of time for bacteria to attach to the mucosa.

Data analysis. To test the difference between the prevalence of *E. coli* ONT:H25 and that of *E. coli* O157:H7 in dairy heifers, the McNemar's chi-square *P* values for each sampling month were calculated using GraphPad Quickcalcs (http://www.graphpad.com/quickcalcs/McNemar1.cfm). The error bars presented below in Fig. 2 were also calculated using GraphPad based on the exact 95% confidence intervals around a binomial proportion. Bacterial counts were log transformed, and averages were taken of the log-transformed data for purposes of comparison between the two groups in the inoculation study. Error bars in Fig. 4, below, were calculated based on the standard errors of the means.

RESULTS

This study was spurred by our observation that in both bovine fecal and RAMS samples cultured for *E. coli* O157:H7, we frequently found non-O157 SMN colonies on SMAC-CTVM agar. Although very similar in morphology to *E. coli* O157:H7 colonies, the non-O157 SMN colonies were visually distinguishable on the SMAC-CTVM plates because of a lighter center and an overall slightly lighter color. From a collection of 433 RAMS and 70 fecal samples from healthy cattle in four locations of Washington and Idaho and from four feedlots in Alberta, Canada, we preserved 258 representative non-O157 SMN isolates. Also, among the bovine *E. coli* O157:H7 isolates recovered from these same animals during this study, two were designated as *E. coli* O157:H7 W1 and W2 and used for comparisons with the ONT:H25 isolates.

The non-O157 SMN isolates serotyped as ONT-H25 and were STEC. All non-O157 SMN isolates had the same API profile (0044112), indicating they were *E. coli*. Serotyping by the Gastroenteric Disease Center at the University of Pennsylvania and PFGE profiles of chromosomal DNA indicated that all SMN *E. coli* isolates belonged to a single serotype: ONT:H25. All isolates were designated as having no reaction with any O antisera. However, a weak and unusual reaction was noticed with O15 antisera, unlike the standard O15 control strain. Following heat treatment, strains showed a weak agglutination after 6 to 8 h that became flaky after 24 h. This unusual reaction was deemed nontypeable. All isolates were positive for *stx*$_2$ by PCR and expressed Stx2 by enzyme-linked immunosorbent assay, indicating they were STEC (Table 1 and data not shown).

The *E. coli* ONT:H25 isolates had three different PFGE DNA fingerprints. DNA from 87 of the 258 *E. coli* ONT:H25 isolates, which represented different animals, sampling times, and locations, were digested and analyzed by PFGE. Among the 87 *E. coli* ONT:H25 isolates there were three distinct PFGE profiles that differed by two or more bands and were designated as SH1, SH2, and SH3 (Fig. 1). SH1 was isolated from eastern, central, and southern Washington. SH2 was isolated from Idaho and Alberta, Canada. SH3 was isolated from both southern Washington and Alberta, Canada, locations separated by more than 800 miles. Each PFGE type (SH1, -2, and -3) was represented in the subset of isolates that were serotyped (Table 2). Two natural bovine *E. coli* O157:H7 isolates (W1 and W2) from this study and *E. coli* O157:H7 ATCC 43894 had PFGE profiles distinct from each other and from *E. coli* ONT:H25 (Fig. 1).

***E. coli* ONT:H25 was prevalent among cattle.** Among 170 cattle from the northwestern United States and Alberta, Canada, the percentage of animals positive for STEC ONT:H25 ranged from 7.5% to 22.5% (Table 2). In a longitudinal study the percentage of heifers positive for *E. coli* O157:H7 ranged from 0 to 15% ($n = 40$), whereas the percentage of animals positive for *E. coli* ONT:H25 ranged from 15 to 22.5% during the 8-month sampling (Fig. 2). *E. coli* ONT:H25 prevalence was significantly higher than *E. coli* O157:H7 prevalence in the months of October (*E. coli* ONT:H25 prevalence was 22.5% while *E. coli* O157:H7 prevalence was 5%; McNemar's chi-square *P* value = 0.05) and March (*E. coli* ONT:H25 prevalence was 20%, while *E. coli* O157:H7 prevalence was 0; McNemar's chi-square *P* value = 0.01). Most of the culture-positive heifers carried *E. coli* ONT:H25 transiently during the 8-month period, but three animals were consistently culture positive for the bacteria. In only one dairy heifer at one sampling time were both *E. coli* O157:H7 and *E. coli* ONT:H25 simultaneously isolated. Among the 40 feedlot animals, three

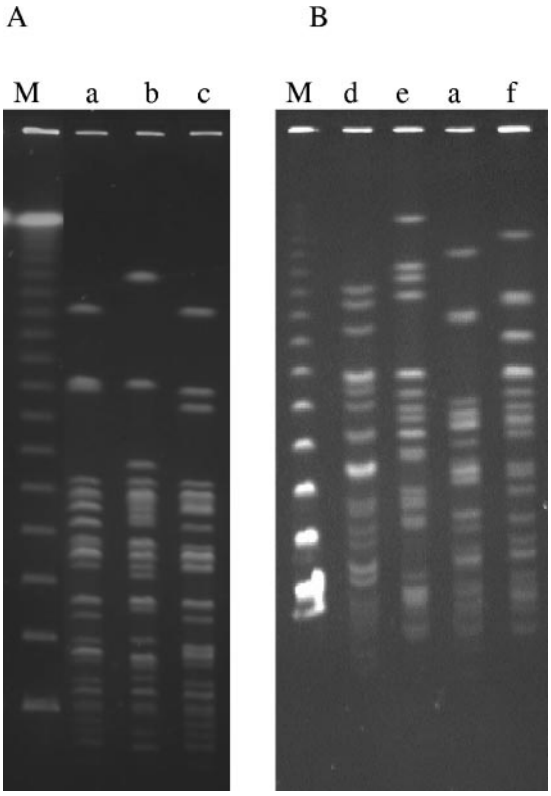

FIG. 1. PFGE profiles of *E. coli* ONT:H25 strains (A) and *E. coli* O157:H7 strains (B). Lanes: M, λ DNA ladder (DNA size standards); a, *E. coli* ONT:H25 strain SH1; b, *E. coli* ONT:H25 strain SH2; c, *E. coli* ONT:H25 strain SH3; d, *E. coli* O157:H7 strain W1; e, *E. coli* O157:H7 strain W2; f, *E. coli* O157:H7 ATCC 43894.

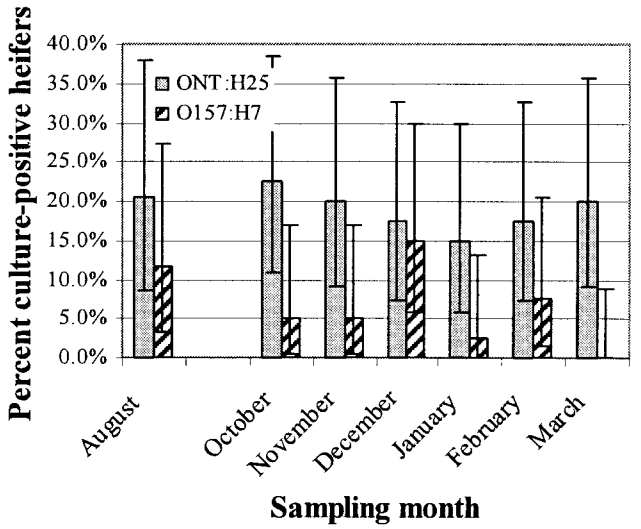

FIG. 2. Longitudinal study of the prevalence of *E. coli* ONT:H25 and *E. coli* O157:H7 in dairy heifers. RAMS samples from 40 heifers were cultured for *E. coli* ONT:H25 and *E. coli* O157:H7. Prevalence of ONT:H25 was significantly higher than for O157:H7 in the months of October and March ($P < 0.05$, McNemar's chi-square). No samples were obtained in September. Bars represent 95% confidence intervals.

(7.5%) were positive for *E. coli* ONT:H25 (Table 2) and four (10%) were positive for *E. coli* O157:H7 (data not shown).

***E. coli* ONT:H25 carried *eae*-β, *stx*$_2$, *tir*, and EHEC *hlyA* genes.** Multiplex PCR results from testing 258 individual SMN isolates (representing SH1, SH2, and SH3) and two *E. coli* O157:H7 strains showed that STEC in this study (*E. coli* O157:H7 W1 and W2 and *E. coli* ONT:H25 strains SH1, SH2, and SH3) carried *eae*, *stx*$_2$, *tir*, and EHEC *hlyA* gene sequences (Table 1). All three *E. coli* ONT:H25 strains carried only *stx*$_2$ and not *stx*$_1$, whereas the *E. coli* O157:H7 W1 and W2 isolates possessed both *stx*$_1$ and *stx*$_2$.

Among the five STEC strains characterized, *eae* gene subtyping results showed that *E. coli* O157:H7 W1 and W2 isolates carried *eae*-γ1, which were detected by the primer pair 5′-AAAACCGCGGAGATGACTTC-3′ and 5′-AGAACGCTGCTCACTAGATGTC-3′. The size of that PCR product matched the expected 804 bp (data not shown). Subtyping of *eae* variants from all three *E. coli* ONT:H25 isolates showed that they possessed *eae*-β, which was detected by the primer pair 5′-AAAACCGCGGAGATGACTTC-3′ and 5′-CTTGATACACTTGATGACTGT-3′. The sizes of the PCR-amplified products matched the expected 830 bp (data not shown).

TABLE 2. Prevalence[a] of *E. coli* ONT:H25 in cattle from North America

Location	No. of cattle sampled	Total fecal samples	Culture-positive cattle (%)	No. of SMN PFGE isolates/total banked isolates[b]	PFGE pattern
Northern Idaho (research barn)	23	46[c]	17.4–21.7	10/28	SH2
Eastern Washington (dairy)	40	320[d]	15.0–22.5	52/172	SH1
Central Washington (dairy)	27	27[e]	11.1	6/12	SH1
Southern Washington (dairy heifer raiser)	40	40[e]	15.0	12/37	SH1 (2)[f] SH3 (10)
Alberta, Canada (feedlot)	40	70[g]	7.5	7/9	SH2 (2)[h] SH3 (5)
Total	170	433	7.5–22.5	87/258	

[a] Values represent RAMS or fecal samples containing at least one ONT:H25 isolate.
[b] The number of SMN isolates tested by PFGE divided by the total number of SMN isolates banked.
[c] RAMS samples were obtained from the same steers in a research herd on two occasions, 3 months apart.
[d] RAMS samples were obtained from the same 40 heifers eight times, at 1-month intervals.
[e] RAMS samples were obtained from 27 Holstein steers and 40 heifers once.
[f] Two isolates from one of six positive cattle showed the SH1 PFGE type.
[g] A total of 40 feedlot cattle were sampled; 30 animals were sampled twice, 1 month apart, and 10 animals were sampled once.
[h] Two isolates from one of three positive cattle showed the SH2 PFGE type.

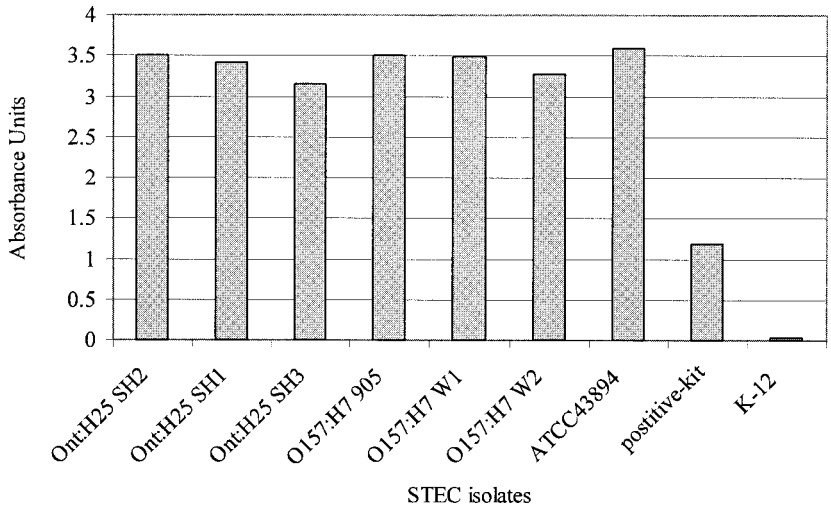

FIG. 3. Toxin production by STEC isolates. Two clinical isolates of *E. coli* O157:H7 902 (stx_2) and ATCC 43894 ($stx_1$and stx_2) were used as positive controls. The negative control was strain MG 1655 (K-12). "Positive-kit" was the positive control provided with the enzyme immunoassay kit. Values on the *y* axis correspond to absorbance units at two wavelengths (450 and 630 nm).

***E. coli* ONT:H25 isolates expressed enterohemolysin and Stx2vh-a.** All *E. coli* ONT:H25 strains displayed a typical EHEC-enterohemolytic phenotype with small, clear hemolytic zones along the sides of bacterial growth on blood agar plates after overnight incubation at 37°C and no visible hemolysis after 46 h of incubation at 37°C (8).

To test Stx production, the *E. coli* ONT:H25 strains SH1, SH2, and SH3 and the *E. coli* O157:H7 bovine strains W1 and W2 were compared by enzyme-linked immunosorbent assay to positive control strains: *E. coli* O157:H7 strain 905, a clinical isolate from a patient with HUS (a gift from M. K. Waldor, Tufts-New England Medical Center and Howard Hughes Medical Institute, Boston, MA) which possesses only stx_2 (41), and *E. coli* O157:H7 ATCC 43894, which possesses both stx_1 and stx_2. Toxin production by all ONT:H25 isolates and the bovine O157:H7 isolates was similar to that of the positive control strains (Fig. 3). All three strains of *E. coli* ONT:H25 (SH1, SH2, and SH3) produced Stx at levels similar to the clinical *E. coli* O157:H7 isolates 905 and ATCC 43894. The toxin-negative control *E. coli* K-12 did not produce toxin. On the basis of RFLP-PCR analysis, all five STEC strains, three *E. coli* ONT:H25 strains and two *E. coli* O157:H7 strains found in this study, carried the Stx2vh-a subtype (Table 3).

STEC ONT:H25 colonized the RAJ mucosa in a manner similar to *E. coli* O157:H7. Because STEC ONT:H25 strains were found in RAMS and fecal samples and carried a set of virulence genes similar to the O157:H7 serotype, we speculated that STEC ONT:H25 might colonize the same niche as *E. coli* O157:H7 in the bovine gastrointestinal tract. To test whether *E. coli* ONT:H25 was able to colonize the RAJ mucosa, one of the primary sites of colonization for *E. coli* O157:H7 (33), two groups of four steers were inoculated with a rectal application of the ONT:H25 or the O157:H7 *E. coli* serotypes. All animals remained healthy and had normal weight gain throughout the study. Figure 4, shows the average of the $\log_{10}$ bacteria counts detected by RAMS culture in each

TABLE 3. Subtyping of stx_2 genes by RFLP

Primer (size in bp)[b]	Enzyme	Fragment lengths (bp) in STEC strain (subtype designation):					
		43894 (VT2)	W1 (VT2vh-a)	W2 (VT2vh-a)	SH1 (VT2vh-a)	SH2 (VT2vh-a)	SH3 (VT2vh-a)
Lin FR[a] (900)	HincII	750 150	550 350	550 350	550 350	550 350	550 350
VT2ef[c] (348)	HeaII	348	216 132	216 132	216 132	216 132	216 132
	PvuII	323 25[d]	323 25[d]	323 25[d]	323 25[d]	323 25[d]	323 25[d]
Tyler[e] (285)	RsaI	216 69[d]	136 80 69[d]	136 80 69[d]	136 80 69[d]	136 80 69[d]	136 80 69[d]

[a] This primer pair was used according to the method of Lin et al. (30).
[b] Expected size of amplified product.
[c] This primer pair was used according to the method of Pierard et al. (39).
[d] Fragments did not resolve or were too small to be clearly visible under the electrophoresis conditions used.
[e] This primer pair was used according to the method of Tyler et al. (48).

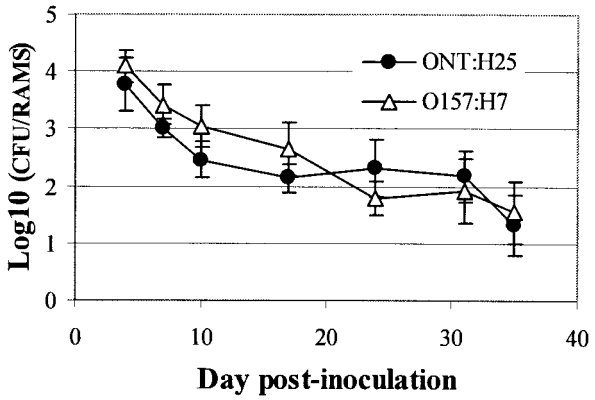

FIG. 4. Rectal application of *E. coli* ONT:H25 or *E. coli* O157:H7 in calves. The average number of bacteria detected by RAMS culture over time is shown. Two groups of four calves each were rectally inoculated with 1.7×10^{10} CFU of *E. coli* ONT:H25 strain SH2 (●) or with 1.9×10^{10} CFU of *E. coli* O157:H7 ATCC 43894 (△). There was no significant difference in the average $\log_{10}$ bacterial count between the two groups ($P > 0.05$, Student's *t* test).

group. Both *E. coli* O157:H7 and *E. coli* ONT:H25 continued to be detected throughout the 35-day experimental period, though the numbers of bacteria declined from 10^3 to 10^4 CFU/swab at day 4 to 10^1 CFU at day 35. The pattern of *E. coli* O157:H7 detected by RAMS was consistent with our previous study of steers following rectal application of *E. coli* O157:H7 (44), and *E. coli* ONT:H25 showed the same pattern of colonization. There was no significant difference in the average $\log_{10}$ bacteria counts/RAMS between the two groups (Fig. 4). These results indicated that *E. coli* ONT:H25 can colonize at the RAJ as well as *E. coli* O157:H7.

DISCUSSION

This is the first detailed report of a non-O157 bovine STEC, *E. coli* ONT:H25, which is characterized by genotypic and phenotypic traits usually associated with disease-causing EHEC. Like *E. coli* O157:H7, *E. coli* ONT:H25 does not ferment sorbitol and lacks β-glucuronidase activity. These characteristics allowed easy screening for both of these *E. coli* serotypes and were the initiating factor in our observations of *E. coli* ONT:H25. In addition, culture from RAMS samples and the persistence of *E. coli* ONT:H25 in cattle for more than 35 days after a single rectal application of the bacteria identify it as an *E. coli* serotype (in addition to the O157:H7 serotype) that can attach to the bovine RAJ mucosa.

An unexpected and striking finding in this study was the high prevalence of *E. coli* ONT:H25 in cattle from several locations in the Pacific Northwest. Our findings suggest that *E. coli* ONT:H25 may be more prevalent among cattle than *E. coli* O157:H7. We compared the prevalence of ONT:H25 to O157:H7 in dairy heifers sampled longitudinally over 8 months, using the same sampling method, and consistently found *E. coli* ONT:H25 more frequently over time in both RAMS samples and fecal samples. The lower prevalence (7.5%) found among feedlot animals may have been due to the fecal rather than RAMS sample, the 24-h delay in culture due to shipping distance, and the smaller number of animals tested only twice. Studies of total STEC in cattle populations have reported variable prevalence, although sampling and detection methods have been different and individual serotypes are not always reported (14). The ease with which we were able to visually distinguish *E. coli* ONT:H25 from other *E. coli* serotypes made the study of a STEC serotype other than O157 possible without elaborate culture and detection techniques. Development of new techniques and more careful study of other STEC may find a serotype(s) even more prevalent in the bovine RAJ mucosa microbiota.

In this study we identified three *E. coli* ONT:H25 strains with different chromosomal DNA patterns following restriction and PFGE separation. Although the three strains differed by at least two bands, very slight differences were observed between strains SH1 and SH3. These high similarities between banding patterns within a serotype may be interpreted as indicating a close genetic relatedness. Further, these PFGE data suggest a broad geographic distribution of the SH2 and SH3 strains over >800 miles among both dairy and feedlot animals.

In addition to its relatively high prevalence, *E. coli* ONT:H25 shares many genotypic and phenotypic traits with *E. coli* O157:H7. It is a slow sorbitol fermenter and lacks β-glucuronidase activity. Some investigators have reported an association between the sorbitol-negative phenotype and human disease (34). It has genes for traits that are considered important for the disease-causing ability of *E. coli* O157:H7, including Shiga toxin 2, the *eae* gene encoding intimin, the *tir* gene encoding the translocated intimin receptor, and the *hly* gene encoding hemolysin. Among the four well-characterized STEC virulence factors, Stx1, Stx2, intimin, and hemolysin, Stx2 is the most frequently associated with HUS (10, 24, 35) Eleven distinct variants of Stx2 have been identified (4, 26, 37–39, 42, 43), the most frequently reported being Stx2c (subdivided into Stx2vha or Stx2vhb), Stx2d, and Stx2e (39, 42). The *E. coli* ONT:H25 strains tested in this study carried the Stx2vha type. This toxin has also been called Shiga-like toxin (SLT) II-vha (31, 47), verotoxin (VT) 2vha (26), or stx2d1 (17) and is the mucus-activatable toxin found in strain B2F1 (31, 47). Readers are referred to Schultz et al. for a complete compilation of Stx nomenclature (41). Finally, although the small numbers of animals limited our ability to detect a difference, the results of the animal inoculation study indicate that *E. coli* ONT:H25 shares the ability to colonize the bovine RAJ mucosa with *E. coli* O157:H7 and that temporal patterns of shedding by animals rectally inoculated with *E. coli* ONT:H25 closely parallel those with *E. coli* O157:H7.

Although *E. coli* ONT:H25 carries all of the known genetic factors associated with disease-causing EHEC, and although it appears to be a common finding among cattle in our region, there is little evidence of an association with human disease. We have not ruled out the possibility that the presence of this strain has been missed from clinical stool specimens associated with bloody diarrhea or HUS. This is plausible, because clinical and hospital laboratories often do not screen for STEC other than *E. coli* O157:H7. However, a strong association with hemorrhagic colitis and HUS or involvement with outbreaks of human disease is unlikely to have gone unnoticed. One recent prospective study of post-diarrhea HUS in the United States found that STEC was isolated from 30 of 70 specimens and that of these 30, 25 were O157:H7. None of the non-O157

STEC was ONT:H25 (2). This raises the possibility that *E. coli* ONT:H25 may be a strain lacking in specific genes required to cause disease in humans. *E. coli* ONT:H25 may be an important tool in studies using close genetic comparisons to identify unique elements of the *E. coli* O157:H7 genome that are critical for causing human disease.

ACKNOWLEDGMENTS

We thank Lonie Austin and Dan Rice for assistance with sampling and handling the cattle.

This work was supported, in part, by the Idaho Agriculture Experiment Station, U.S. Department of Agriculture NRICGP grants 99-35201-8539 and 04-04562, Public Health Service grants NO1-HD-0-3309, U54-AI-57141, P20-RR16454, and P20-RR15587 from the National Institutes of Health, and by grants from the United Dairymen of Idaho and the Idaho Beef Council.

REFERENCES

1. **Andreoli, S. P., H. Trachtman, D. W. K. Acheson, R. L. Siegler, and T. G. Obrig.** 2002. Hemolytic uremic syndrome: epidemiology, pathophysiology, and therapy. Pediatr. Nephrol. **17:**293–298.
2. **Banatvala, N., P. M. Griffin, K. D. Greene, T. J. Barrett, W. F. Bibb, J. H. Green, and J. G. Wells.** 2001. The United States National Prospective Hemolytic Uremic Syndrome Study: microbiologic, serologic, clinical, and epidemiologic findings. J. Infect. Dis. **183:**1063–1070.
3. **Bastian, S. N., I. Carle, and F. Grimont.** 1998. Comparison of 14 PCR systems for the detection and subtyping of stx genes in Shiga-toxin-producing *Escherichia coli*. Res. Microbiol. **149:**457–472.
4. **Bertin, Y., K. Boukhors, N. Pradel, V. Livrelli, and C. Martin.** 2001. Stx2 subtyping of Shiga toxin-producing *Escherichia coli* isolated from cattle in France: detection of a new Stx2 subtype and correlation with additional virulence factors. J. Clin. Microbiol. **39:**3060–3065.
5. **Beutin, L., D. Geier, H. Steinruck, 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.
6. **Beutin, L., D. Geier, S. Zimmermann, S. Aleksic, H. A. Gillespie, and T. S. Whittam.** 1997. Epidemiological relatedness and clonal types of natural populations of *Escherichia coli* strains producing Shiga toxins in separate populations of cattle and sheep. Appl. Environ. Microbiol. **63:**2175–2180.
7. **Beutin, L., G. Krause, S. Zimmermann, S. Kaulfuss, and K. Gleier.** 2004. Characterization of Shiga toxin-producing *Escherichia coli* strains isolated from human patients in Germany over a 3-year period. J. Clin. Microbiol. **42:**1099–1108.
8. **Beutin, L., M. A. Montenegro, I. Orskov, F. Orskov, J. Prada, S. Zimmermann, and R. Stephan.** 1989. Close association of verotoxin (Shiga-like toxin) production with enterohemolysin production in strains of *Escherichia coli*. J. Clin. Microbiol. **27:**2559–2564.
9. **Blanco, M., J. E. Blanco, A. Mora, G. Dahbi, M. P. Alonso, E. A. Gonzalez, M. I. Bernardez, and J. Blanco.** 2004. Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing *Escherichia coli* isolates from cattle in Spain and identification of a new intimin variant gene (*eae-xi*). J. Clin. Microbiol. **42:**645–651.
10. **Boerlin, P., S. A. McEwen, F. Boerlin-Petzold, J. B. Wilson, R. P. Johnson, and C. L. Gyles.** 1999. Associations between virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans. J. Clin. Microbiol. **37:**497–503.
11. **Brett, K. N., M. A. Hornitzky, K. A. Bettelheim, M. J. Walker, and S. P. Djordjevic.** 2003. Bovine non-O157 Shiga toxin 2-containing *Escherichia coli* isolates commonly possess Stx2-EDL933 and/or Stx2vhb subtypes. J. Clin. Microbiol. **41:**2716–2722.
12. **Buchko, S. J., R. A. Holley, W. O. Olson, V. P. Gannon, and D. M. Veira.** 2000. The effect of different grain diets on fecal shedding of *Escherichia coli* O157:H7 by steers. J. Food Prot. **63:**1467–1474.
13. **Centers for Disease Control and Prevention.** 2000. Summary of outbreaks of *Escherichia coli* O157 and other Shiga toxin-producing *E. coli* reported to the CDC in 1999 (updated June, 2000). [Online.]. http://www.cdc.gov/ncidod/dbmd/diseaseinfo/files/ecoli_99summary.pdf.
14. **Cobbold, R. N., D. H. Rice, M. Szymanski, D. R. Call, and D. D. Hancock.** 2004. Comparison of Shiga-toxigenic *Escherichia coli* prevalences among dairy, feedlot, and cow-calf herds in Washington State. Appl. Environ. Microbiol. **70:**4375–4378.
15. **Davis, M. A., D. D. Hancock, D. H. Rice, D. R. Call, R. DiGiacomo, M. Samadpour, and T. E. Besser.** 2003. Feedstuffs as a vehicle of cattle exposure to *Escherichia coli* O157:H7 and *Salmonella enterica*. Vet. Microbiol. **95:**199–210.
16. **Edelman, R., M. A. Karmali, and P. A. Fleming.** 1988. From the National Institutes of Health. Summary of the International Symposium and Workshop on Infections Due to Verocytotoxin (Shiga-like toxin)-producing *Escherichia coli*. J. Infect. Dis. **157:**1102–1104.
17. **Eklund, M., K. Leino, and A. Siitonen.** 2002. Clinical *Escherichia coli* strains carrying *stx* genes: *stx* variants and *stx*-positive virulence profiles. J. Clin. Microbiol. **40:**4585–4593.
18. **Elliott, E. J., R. M. Robins-Browne, E. V. O'Loughlin, V. Bennett-Wood, J. Bourke, P. Henning, G. G. Hogg, J. Knight, H. Powell, and D. Redmond.** 2001. Nationwide study of haemolytic uraemic syndrome: clinical, microbiological, and epidemiological features. Arch. Dis. Child. **85:**125–131.
19. **Fratamico, P. M., S. K. Sackitey, M. Wiedmann, and M. Y. Deng.** 1995. Detection of *Escherichia coli* O157:H7 by multiplex PCR. J. Clin. Microbiol. **33:**2188–2191.
20. **Gannon, V. P., S. D'Souza, T. Graham, R. K. King, K. Rahn, and S. Read.** 1997. Use of the flagellar H7 gene as a target in multiplex PCR assays and improved specificity in identification of enterohemorrhagic *Escherichia coli* strains. J. Clin. Microbiol. **35:**656–662.
21. **Gannon, V. P., R. K. King, J. Y. Kim, and E. J. Thomas.** 1992. Rapid and sensitive method for detection of Shiga-like toxin-producing *Escherichia coli* in ground beef using the polymerase chain reaction. Appl. Environ. Microbiol. **58:**3809–3815.
22. **Goldwate, P., and K. Bettelheim.** 2002. *Escherichia coli* 'O' group serology of a haemolytic uraemic syndrome (HUS) epidemic. Scand. J. Infect. Dis. **32:**385–394.
23. **Grauke, L. J., I. T. Kudva, J. W. Yoon, C. W. Hunt, C. J. Williams, and C. J. Hovde.** 2002. Gastrointestinal tract location of *Escherichia coli* O157:H7 in ruminants. Appl. Environ. Microbiol. **68:**2269–2277.
24. **Gyles, C., R. Johnson, A. Gao, K. Ziebell, D. Pierard, S. Aleksic, and P. Boerlin.** 1998. Association of enterohemorrhagic *Escherichia coli* hemolysin with serotypes of Shiga-like-toxin-producing *Escherichia coli* of human and bovine origins. Appl. Environ. Microbiol. **64:**4134–4141.
25. **Hu, Y., Q. Zhang, and J. C. Meitzler.** 1999. Rapid and sensitive detection of *Escherichia coli* O157:H7 in bovine faeces by a multiplex PCR. J Appl. Microbiol. **87:**867–876.
26. **Ito, H., A. Terai, H. Kurazono, Y. Takeda, and M. Nishibuchi.** 1990. Cloning and nucleotide sequencing of Vero toxin 2 variant genes from *Escherichia coli* O91:H21 isolated from a patient with the hemolytic uremic syndrome. Microb. Pathog. **8:**47–60.
27. **Karmali, M.** 1989. Infection by verocytotoxin-producing *Escherichia coli*. Clin. Microbiol. Rev. **2:**15–38.
28. **Karmali, M. A., M. Petric, C. Lim, P. C. Fleming, G. S. Arbus, and H. Lior.** 1985. The association between idiopathic hemolytic uremic syndrome and infection by verotoxin-producing *Escherichia coli*. J. Infect. Dis. **151:**775–782.
29. **Kobayashi, H., J. Shimada, M. Nakazawa, T. Morozumi, T. Pohjanvirta, S. Pelkonen, and K. Yamamoto.** 2001. Prevalence and characteristics of Shiga toxin-producing *Escherichia coli* from healthy cattle in Japan. Appl. Environ. Microbiol. **67:**484–489.
30. **Lin, Z., H. Kurazono, S. Yamasaki, and Y. Takeda.** 1993. Detection of various variant verotoxin genes in *Escherichia coli* by polymerase chain reaction. Microbiol. Immunol. **37:**543–548.
31. **Melton-Celsa, A. R., S. C. Darnell, and A. D. O'Brien.** 1996. Activation of Shiga-like toxins by mouse and human intestinal mucus correlates with virulence of enterohemorrhagic *Escherichia coli* O91:H21 isolates in orally infected, streptomycin-treated mice. Infect. Immun **64:**1569–1576.
32. **Nataro, J. P., and J. B. Kaper.** 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. **11:**142–201.
33. **Naylor, S. W., J. C. Low, T. E. Besser, A. Mahajan, G. J. Gunn, M. C. Pearce, I. J. McKendrick, D. G. Smith, and D. L. Gally.** 2003. Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic *Escherichia coli* O157:H7 in the bovine host. Infect. Immun. **71:**1505–1512.
34. **Ojeda, A., V. Prado, J. Martinez, C. Arellano, A. Borczyk, W. Johnson, H. Lior, and M. M. Levine.** 1995. Sorbitol-negative phenotype among enterohemorrhagic *Escherichia coli* strains of different serotypes and from different sources. J. Clin. Microbiol. **33:**2199–2201.
35. **Ostroff, S. M., P. I. Tarr, M. A. Neill, J. H. Lewis, N. Hargrett-Bean, and J. M. Kobayashi.** 1989. Toxin genotypes and plasmid profiles as determinants of systemic sequelae in *Escherichia coli* O157:H7 infections. J. Infect. Dis. **160:**994–998.
36. **Parma, A. E., M. E. Sanz, J. E. Blanco, J. Blanco, M. R. Vinas, M. Blanco, N. L. Padola, and A. I. Etcheverria.** 2000. Virulence genotypes and serotypes of verotoxigenic *Escherichia coli* isolated from cattle and foods in Argentina. Importance in public health. Eur. J. Epidemiol. **16:**757–762.
37. **Paton, A. W., J. C. Paton, P. N. Goldwater, and P. A. Manning.** 1993. Direct detection of *Escherichia coli* Shiga-like toxin genes in primary fecal cultures by polymerase chain reaction. J. Clin. Microbiol. **31:**3063–3067.
38. **Paton, A. W., J. C. Paton, M. W. Heuzenroeder, P. N. Goldwater, and P. A. Manning.** 1992. Cloning and nucleotide sequence of a variant Shiga-like toxin II gene from *Escherichia coli* OX3:H21 isolated from a case of sudden infant death syndrome. Microb. Pathog. **13:**225–236.
39. **Pierard, D., G. Muyldermans, L. Moriau, D. Stevens, and S. Lauwers.** 1998.

Identification of new verocytotoxin type 2 variant B-subunit genes in human and animal *Escherichia coli* isolates. J. Clin. Microbiol. **36:**3317–3322.

40. **Rice, D. H., H. Q. Sheng, S. A. Wynia, and C. J. Hovde.** 2003. Rectoanal mucosal swab culture is more sensitive than fecal culture and distinguishes *Escherichia coli* O157:H7-colonized cattle and those transiently shedding the same organism. J. Clin. Microbiol. **41:**4924–4929.
41. **Scheutz, F., L. Beutin, D. Piérard, and H. R. Smith.** 2001. Appendix: nomenclature of verocytotoxins, p. 447–452. *In* G. Duffy, P. Garvey, and D. A. McDowell (ed.), Verocytotoxigenic *E. coli*. Food & Nutrition Press, Inc., Trumbull, Conn.
42. **Schmidt, H., J. Scheef, S. Morabito, A. Caprioli, L. H. Wieler, and H. Karch.** 2000. A new Shiga toxin 2 variant (Stx2f) from *Escherichia coli* isolated from pigeons. Appl. Environ. Microbiol. **66:**1205–1208.
43. **Schmitt, C. K., M. L. McKee, and A. D. O'Brien.** 1991. Two copies of Shiga-like toxin II-related genes common in enterohemorrhagic *Escherichia coli* strains are responsible for the antigenic heterogeneity of the O157:H- strain E32511. Infect. Immun **59:**1065–1073.
44. **Sheng, H., M. A. Davis, H. J. Knecht, and C. J. Hovde.** 2004. Rectal administration of *Escherichia coli* O157:H7: novel model for colonization of ruminants. Appl. Environ. Microbiol. **70:**4588–4595.
45. **Smith, H. R., and S. M. Scotland.** 1988. Vero cytotoxin-producing strains of *Escherichia coli*. J. Med. Microbiol. **26:**77–85.
46. **Strockbine, N. A., J. G. Wells, C. A. Bopp, and T. J. Barrett.** 1998. Overview of detection and subtyping methods, p. 331–356. *In* J. B. Kaper and A. D. O'Brien (ed.), *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains. American Society for Microbiology, Washington, D.C.
47. **Teel, L. D., A. R. Melton-Celsa, C. K. Schmitt, and A. D. O'Brien.** 2002. One of two copies of the gene for the activatable Shiga toxin type 2d in *Escherichia coli* O91:H21 strain B2F1 is associated with an inducible bacteriophage. Infect. Immun. **70:**4282–4291.
48. **Tyler, S. D., W. M. Johnson, H. Lior, G. Wang, and K. R. Rozee.** 1991. Identification of verotoxin type 2 variant B subunit genes in *Escherichia coli* by the polymerase chain reaction and restriction fragment length polymorphism analysis. J. Clin. Microbiol. **29:**1339–1343.
49. **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, et al.** 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.
50. **Wilson, J. B., R. C. Clarke, S. A. Renwick, K. Rahn, R. P. Johnson, M. A. Karmali, H. Lior, D. Alves, C. L. Gyles, K. S. Sandhu, S. A. McEwen, and J. S. Spika.** 1996. Vero cytotoxigenic *Escherichia coli* infection in dairy farm families. J. Infect. Dis. **174:**1021–1027.
51. **Wilson, J. B., S. A. McEwen, R. C. Clarke, K. E. Leslie, R. A. Wilson, D. Waltner-Toews, and C. L. Gyles.** 1992. Distribution and characteristics of verocytotoxigenic *Escherichia coli* isolated from Ontario dairy cattle. Epidemiol. Infect. **108:**423–439.