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Impact of Free Verotoxin Testing on Epidemiology of Diarrhea Caused by Verotoxin-Producing *Escherichia coli*

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During a 10-week period in the summer of 1990, an epidemiologic investigation of the prevalence of verotoxin (VT)-producing *Escherichia coli* infection was conducted in Calgary, Alberta, Canada. Consecutive stool specimens (n = 3,577) were cultured for *E. coli* O157:H7, and fecal filtrates were tested for free VTs (FVTs). *E. coli* O157:H7 was recovered from 22 specimens (0.6%), but VT was detected in 74 specimens (2.1%). Sixty-nine stool specimens positive for FVTs or *E. coli* O157:H7 were probed for VT genes by colony blot hybridization; 22 of 38 VT gene probe-positive isolates were non-O157:H7 *E. coli* organisms. Fourteen of 22 strains could not be induced to produce VT in vitro, despite the presence of FVTs in the stool sample, positivity on colony blot hybridization, positive PCR probes with the primers described by Pollard et al. (D. R. Pollard, W. M. Johnson, H. Lior, S. D. Tyler, and K. R. Rozee, J. Clin. Microbiol. 28:540–545, 1990) or Gannon et al. (V. P. Gannon, R. K. King, J. Y. Kim, and E. J. Golsteyn-Thomas, Appl. Environ. Microbiol. 58:3809–3815, 1992) (but not those described by Karch and Meyer [H. Karch and T. Meyer, J. Clin. Microbiol. 27:2751–2757, 1989]), and positive Southern blot analysis of isolates in 10 of 14 strains. The patient survey questionnaire showed that *E. coli* O157:H7 infection was associated with bloody diarrhea of short duration, whereas infection with other serotypes or persistence of FVT only was associated with longer-duration nonbloody diarrheal illness. We conclude that (i) detection of FVT in stools enhances the diagnosis of VT infection threefold over cultures for *E. coli* O157:H7, (ii) cultures for *E. coli* O157:H7 detect the majority of organisms of that serotype, (iii) the spectrum of disease produced by organisms of non-O157:H7 serotypes may include less severe but more protracted illness, and (iv) differences in the in vivo and in vitro expression of toxin and results of genetic probe studies highlight the need to examine control mechanisms of toxin production.

The verotoxin (VT) or Shiga-like toxin family is a group of closely related subunit toxins produced by certain strains of *Escherichia coli*. Several variants of these toxins have been described, most notably, VT-1, VT-2, VT-2v, and VT-e. Despite variations in these toxins, they have identical biological activities (8, 9). *E. coli* strains producing VT-1 and VT-2 have been closely associated with hemorrhagic colitis, hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura in humans. VT-producing *E. coli* (VTEC) consists of a number of serotypes, of which O157:H7 is the most commonly recognized and the one most often recovered from the feces of humans with diarrhea or HUS (21).

The epidemiology of VTEC infection is incompletely defined at present. Infections occur as sporadic illnesses or outbreaks, are distributed throughout North and South America, Europe, and Asia, and are suspected of being common (but underrecognized) in many regions. In temperate climates, the peak incidence of infection is observed during the summer months. Investigations of the prevalence of VTEC infections in patients with diarrheal illness have more commonly been confined to the recovery of serotype O157:H7 from stools because of technical limitations in the detection of other serotypes. The recovery rate of *E. coli* O157:H7 from stools has ranged between 0.08 and 0.7% (6, 17, 26, 32, 43). However, the characterization of the genes dictating VT-1 and VT-2 production and the construction of specific DNA probes has facilitated more sophisticated epidemiologic studies. These probes have been used to detect VT-producing *E. coli* in stool samples from children and adults in Thailand with sporadic cases of diarrhea, outbreaks of O157:H7-associated HUS, and studies of food epidemiology (3–5, 16, 29, 39, 41, 42).

Studies done previously in the Calgary, Alberta, Canada, area were confined to patients presenting at hospital emergency rooms with watery or bloody diarrhea and demonstrated that VTEC was the most commonly recovered enteric pathogen, with organisms of serotype O157:H7 predominating. However, the prevalence of organisms of the non-O157:H7 serotypes may have been underestimated because of the low sensitivity of stool cultures (30, 31). Alberta is estimated to have one of the highest rates of sporadic *E. coli* O157:H7 enteric infections (13/100,000 per person-year) (48) and is the region in Canada with the highest rate of HUS (38).

The objectives of the study described here were (i) to determine the prevalence of VTEC infection in the Calgary area by contrasting hospital emergency room patients to patients presenting to family physicians and walk-in clinics, (ii) to compare three methods of detection of VTEC organisms (routine sorbitol-MacConkey agar plates, detection of free verotoxin [FVT]) with monoclonal antibody neutralization of direct fecal filtrates, and colony blot hybridization assays), (iii) to correlate in vivo toxin production as represented by FVT results in stools to in vitro toxin production, colony blot hybridization, and PCR characterization of isolates to determine if genetic information is routinely expressed in vivo, and (iv) to conduct a patient survey to characterize illness in the community.

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MATERIALS AND METHODS

Study population. During a 10-week period from July 1990 to September 1990, consecutive stool specimens submitted for routine culture for diarrheal pathogens were collected from the Calgary Medical Laboratories and the Calgary General Hospital Clinical Microbiology Laboratory. Diagnosis of diarrheal disease was the primary indication for test requisition for the vast majority of samples. Calgary Medical Laboratories is the largest commercial laboratory in Calgary and consists of 71 outlets or stations serving an estimated population of 300,000 people (approximately 40% of the population). The Calgary General Hospital is an 810-bed university-affiliated teaching hospital close to the downtown core of the city.

Enteric cultures. All stool specimens were cultured for bacterial enteric pathogens including *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Aeromonas* spp., *Yersinia* spp., and *Plesiomonas* spp. by standard methods (1) and for *E. coli* O157:H7 on sorbitol-MacConkey medium and then for O and H serogroups (23). They were also examined for intestinal parasites by standard techniques (14). Residual stool samples were collected from the clinical laboratory within 24 h of submission.

Fecal cytotoxin assay. Testing of stool samples for FVT was done as described by Karmali et al. (22). Stool filtrates were made by mixing 1 g of stool with 1 ml of phosphate-buffered saline (PBS) and were tested undiluted for cytotoxic activity on Vero cell monolayers in microtiter plates. Cytotoxic filtrates were retested, titrated, and confirmed for VT-1 and VT-2 by using specific neutralizing monoclonal antibodies against the respective toxins. Briefly, stool filtrates were serially diluted twofold and were mixed with either sterile PBS or 10 U of monoclonal antibody 13C4 (specific for VT-1) (44), monoclonal antibody BC5 BB12 (specific for VT-2) (11), or a combination of both monoclonal antibodies. One unit of antibody is the amount of antibody necessary to neutralize 1 50% cytotoxic dose of homologous toxin. After incubation for 1 h at 37°C the preparations were layered onto Vero cells and the cells were incubated for 72 h at 37°C in 5% CO_2. *E. coli* C600 (H19B) and *E. coli* C600 (933W) were used as positive controls for VT-1 and VT-2, respectively. *E. coli* ATCC 25922 was used as a negative control. Complete neutralization was defined as the absence of any cytotoxic activity in all wells, whereas significant neutralization was considered to be at least a fourfold reduction in titer. Residual stool samples were stored at −70°C in 10% glycerol-nutrient broth for colony blot assays.

Epidemiologic survey. Patients whose stools demonstrated Vero cell cytotoxic activity were contacted, after receiving permission from their physicians, and were asked to complete a mailed questionnaire requesting details of their clinical symptoms and general demographic information.

DNA probes. The DNA probes used in the study for the detection of VT-1 and VT-2 were those developed by Newland and Neill (29). These probes consist of an 1,142-bp fragment that encodes 98% of the A subunit and all of the B subunit of VT-1 and an 842-bp fragment that encodes 95% of the A subunit of VT-2. Probing for VT-positive *E. coli* in stool samples was done by a nonradioactive labelling method and chemiluminescence detection with a commercially available kit (DIG DNA labeling and detection kit; Boehringer Mannheim Canada Ltd., Laval, Quebec, Canada). Probes were labelled with digoxigenin-UTP by random priming according to the manufacturer's instructions.

Colony blot hybridization assays. One gram of previously frozen stool was serially diluted 10-fold in sterile PBS. Samples (0.1 ml) of each dilution were uniformly spread onto MacConkey agar, and the plates were incubated overnight at 37°C. A minimum of 50 colonies were picked and subcultured onto fresh plates, and the plates were incubated for 8 h at 37°C. Colonies were lifted onto nylon membranes (0.45-μm pore size; Magnagraph Nylon; MSI Separations Inc., Westboro, Mass.) and were processed for hybridization as described elsewhere (18). Hybridization was carried out overnight at 68°C with 50 ng of digoxigenin-labelled probe per ml in hybridization solution (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1% blocking reagent, 0.1% sarcosine, and 1% sodium dodecyl sulfate [SDS]). The membranes were washed twice with 2× SSC–0.1% SDS for 5 min each time at room temperature; this was followed by two washes with 0.1× SSC–0.1% SDS for 15 min each time at 68°C. The membranes were developed according to the instructions accompanying the DIG kit and were exposed to Kodak X-Omat AR film for 15 min. *E. coli* C600 (H19B) and *E. coli* C600 (933W) were used as positive controls for VT-1 and VT-2, respectively. *E. coli* ATCC 25922 was used as the negative control. A single probe-positive colony from each stool was picked, subcultured, and confirmed as *E. coli* by the API 20E system (API Analytab Products, Plainview, N.Y.).

In vitro toxin production. Media used for toxin production included brain heart infusion broth (Difco, Detroit, Mich.), syncase broth (Difco) without and with iron depletion with Chelex 100 (Bio-Rad Laboratories, Richmond, Calif.) (25), and Luria-Bertani (LB) medium (Difco) supplemented with 0.2 mM 2,2′-dipyridyl (Sigma Chemical Co.) (7). A single colony was inoculated into 5 ml of broth and was incubated overnight at 37°C in room air in a shaking incubator at 300 rpm. The sample was spun at 10,000 rpm (Eppendorf 5415C microcentrifuge); the supernatant was filtered through a 0.45-μm-pore-size filter and was tested for verocytoxin activity. The bacterial pellet was treated with polymyxin B (34), and the filtrate was tested for toxin activity. Tests were repeated at least three times on each non-O157:H7 *E. coli* serotype.

PCR and Southern hybridization. All VTEC isolates recovered from stools by colony blot hybridization were confirmed to be of the VT-1 or the VT-2 genotype by PCR. For PCR, whole-cell DNA was extracted (40) and was amplified with the primers described by Pollard et al. (33). DNA samples (1 μg of nucleic acids) were amplified in a 50-μl reaction volume in a DNA thermal cycler (Perkin-Elmer Corp., Norwalk, Conn.) for 40 cycles. The VT-1- and VT-2-amplified fragments were visualized on 2.5% agarose gels stained with ethidium bromide. The positive and negative control strains outlined above in the section on colony blot hybridization assays were used in each PCR test procedure, and positive bands had to correspond to the control strains and published sizes by using a 1-kb-molecular-mass-ladder (Gibco BRL, Gaithersburg, Md.). VTEC isolates which failed to produce toxin in vitro but which were colony blot positive and which were recovered from stools containing FVT were further tested by PCR with additional primers described by Gannon et al. (13) and Karch and Meyer (19) in triplicate. Furthermore, these isolates were further analyzed by Southern hybridization of an *Eco*RI digest of chromosomal DNA (18) by using the probes described by Newland and Neill (29). Therefore, in the present study the term VTEC refers both to *E. coli* strains that produce VT in vitro and to strains that are VT gene positive but that are unable to produce toxin in vitro.

Determination of serotypes. Serotyping of O157 was done by using a commercial typing kit (*E. coli* O157 Latex Test; Oxoid, Hampshire, England) according to the manufacturer's instructions. O157-positive isolates were then serotyped for the H antigen by using *E. coli* H7 antiserum (Difco) (12). Non-O157:H7 serotypes which produced toxin in vitro were kindly serotyped by H. Lior and the National Laboratory for Enteric Pathogens, Laboratory Centre for Disease Control, Ottawa, Ontario, Canada. Strains which did not produce toxin in vitro were serotyped by Richard Wilson of the Pennsylvania State University *E. coli* Reference Center.

Statistics. Relative risks with 95% confidence intervals, Fisher's exact test for categorical data, and Student's *t* test for continuous data were calculated by using Epi Info, version 5.01B, to compare the FVT and non-FVT groups.

TABLE 1. Recovery of enteric pathogens and results of FVT testing of stool specimens

Enteric pathogen	No. (%) of stool specimens		
	VT negative (n = 3,503)	VT positive (n = 74)	Total (n = 3,577)
Campylobacter jejuni	125	0	125 (3.5)
Salmonella spp.	80	2	82 (2.3)
Aeromonas spp.	30	1	31 (0.9)
E. coli O157:H7	2	20	22 (0.6)[a]
Shigella spp.	12	0	12 (0.3)
Yersinia spp.	10	0	10 (0.3)
Plesiomonas spp.	5	0	5 (0.1)
Total	264 (7.4)	23 (0.6)	287 (8.0)

[a] The 22 specimens were obtained from 15 patients; 13 of these patients had FVT-positive stools, 1 patient was FVT negative, and 1 patient submitted two stool specimens, one of which was FVT positive and one of which was FVT negative.

RESULTS

Over a 10-week period from July to September 1990, 3,577 consecutive stool specimens were collected. Ninety-two percent of these (3,296 samples) were obtained from the Calgary Medical Laboratories, and the other 8% (281 samples) were obtained from the Calgary General Hospital. The patients' ages ranged from 1 month to 90 years, with 33% of the patients being less than 15 years old and 40% being in the 16- to 40-year-old age range. Fifty-five percent of the patients were male.

Overall, 287 (8.0%) of the stool samples collected were culture positive for bacterial enteric pathogens (Table 1). *E. coli* O157:H7 was recovered from 22 samples (0.6%). The initial screen of stool filtrates yielded Vero cell cytotoxicity for 197 of 3,577 samples (5.5%). Seventy-four (2.1%) of these were neutralizable by monoclonal antibodies to VT-1 or VT-2, or both. The age distributions of patients who submitted FVT-positive stool samples (n = 66 patients) were as follows: ages ≤5 years, 42%; ages 6 to 15 years, 9%; ages 16 to 40 years, 27%; ages >40 years, 21%. Titers for the filtrates ranged from 1:4 to ≥1:256, with a median titer of 1:16. Titers of FVT were

TABLE 2. Results of colony blot hybridization for verotoxigenic *E. coli*

FVT and O157:H7 category[a]	No. of stool specimens/no. of patients				
	Total	Tested	Probe positive	O157:H7 recovered	Other serotypes recovered
FVT neg, O157:H7 pos	2/2	1/1	1/1	0/0	1/1
FVT pos, O157:H7 pos	20/14	16/12	15/11	14/10	1/1
FVT pos, O157:H7 neg	54/51	52/49	22/20	2/1	20/19
Total	76/67[b]	69/62	38/32	16/11	22/21[c]

[a] Initial results of FVT assay and sorbitol-MacConkey agar cultures; neg, negative; pos, positive.
[b] One patient submitted two stool samples: one was FVT negative and O157:H7 positive and the second was FVT positive and O157:H7 positive.
[c] VT type obtained on colony blot hybridization, PCR VT gene, and serotype results are given in Table 3.

similar whether patients had *E. coli* O157:H7 infection, had infection with an organism of a non-O157:H7 serotype which produced toxin in vitro, infected or colonized with a VT gene-positive strain which did not produce toxin in vitro, or only had stools in which FVT was detected. Testing for FVT increased overall pathogen identification to 9.5% (341 of 3,577) of all samples. On the basis of culture and toxin results, VTEC organisms were identified as potential pathogens in 76 (2.1%) samples (66 patients), rivaling *Salmonella* spp. in the frequency of identification. With the exception of three stool samples (two infected with *Salmonella* spp. and one infected with an *Aeromonas* sp.), FVT-positive stools were associated with *E. coli* O157:H7 or no identified pathogen. Infectious diarrhea caused by other enteric pathogens was not associated with FVT activity. There were two stool samples from which *E. coli* O157:H7 was recovered but that were negative for FVT. Nine of the 22 *E. coli* O157:H7 isolates and 14 of the 74 VT-positive samples were submitted through the emergency room and outpatient clinics of the Calgary General Hospital.

Results of colony blot hybridization, PCR, and in vitro toxin production studies. A total of 69 samples recovered from 62 patients were available for colony blot probe studies (Table 2); 7 stool samples were not tested because of no growth on colony blot cultures ($n = 3$) or failure to retrieve the sample ($n = 4$) from the 3,577 stored stool samples. Of these seven missing samples, five were initially culture positive for O157:H7 and no pathogen was recovered from two stool samples. Quantitative cultures during colony blot hybridization testing showed that the majority of probe-positive samples contained 10^5 to 10^7 CFU of VTEC organisms per g (wet weight) of stool. Few samples had lower or higher numbers of colony blot-positive colonies.

Of the 17 stool samples from which *E. coli* O157:H7 was recovered by using Sorbitol-MacConkey agar plates, 16 were colony blot positive, with recovery of 14 *E. coli* O157:H7 strains and 1 strain each of *E. coli* serotypes O75:NM (FVT-2 and VT-2 by PCR) and O16:H48 (FVT-1 and VT-1 by PCR). Selection of only a single colony to represent the probe-positive stool sample may account for the discrepancy between the initial culture result and the colony blot identification of the pathogen. With the latter two samples, both *E. coli* O157:H7 and a second *E. coli* VTEC strain were present in the sample. The relative contribution of different VTEC strains to diarrheal disease is unclear in cases of coinfection or colonization.

Colony blot hybridization of 52 FVT-positive, O157:H7-negative stool samples identified 22 probe-positive stool samples from 20 patients; 19 patients were infected with a non-O157:H7 serotype strain. *E. coli* O157:H7 was recovered by colony blot hybridization when the initial culture results were negative on the sorbitol-MacConkey agar plates for two specimens from one patient. All non-O157:H7 *E. coli* isolates ($n = 22$) recovered from stools by colony blot hybridization were positive for the VT-1 or the VT-2 genotype, or both, by PCR with one or more primers. Organisms recovered by colony blot hybridization but which could not be induced to produce toxin in vitro were tested with all three PCR primer sets. Although the primers of Pollard et al. (33) and Gannon et al. (13) showed positive results for VT-1 gene homology in 13 of 14 strains, the primers of Karch and Meyer (19) showed negative results. The latter result was also found by H. Lior of the Laboratory Centre for Disease Control (Table 3). Eight of the 20 strains produced VT-1 or VT-2, or both, in vitro, whereas the remaining 14 strains could not be induced to produce verocytotoxins in vitro; this was also the case when the bacterial pellet was treated with polymyxin B. Two of the 52 VT-positive stool samples were culture positive for a *Salmonella* sp. (FVT-2 titer = 8) and an *Aeromonas* sp. (FVT-1 and FVT-2, titer = 4), but colony blot studies were negative.

Table 4 shows a correlation of the presence of FVT in stool filtrates, in vitro toxin production by the recovered isolates, colony blot probe results for fecal cultures, and PCR gene probe results for toxin genes. The concordance of all four parameters was 29%. The greatest concordance was between colony blot probes and PCR (88%); this was followed by production of FVT in vivo and production of toxin in vitro (71%). Seven of the 16 *E. coli* O157:H7 strains possessed both VT-1 and VT-2 genes, but produced VT-2 in vivo and in vitro. There appeared to be a 46 to 67% correlation between tests indicating the presence of the toxin gene and the in vivo and in vitro expression of toxin.

Results of epidemiologic survey. Fifty-eight of 65 patients whose stools were positive for FVT were sent questionnaires, and replies were obtained from 46 of them (79.3%). The majority of patients (86% of 22 patients with bloody diarrhea and 82% of 22 patients with watery diarrhea) resided within the city of Calgary. The clinical and demographic characteristics of these patients are given in Table 5. In both groups (those with bloody diarrhea and those with watery diarrhea), close to 50% of the patients were under 15 years of age. There were no differences between the two groups in the duration of diarrhea or clinical symptoms such as abdominal cramping, nausea, vomiting, and fever ($P > 0.05$; Fisher's exact test). Patients with bloody diarrhea were twice as likely to be hospitalized (relative risk = 2.20; confidence interval = 1.31 to 3.53).

The mean time that elapsed between the onset of symptoms and the collection of the first stool sample was 12.9 ± 11.5 days for all patients. However, this interval for the subset of 11 patients with diarrhea and positive for *E. coli* O157:H7 was 4.8 ± 2.7 days, whereas it was 16.6 ± 11.1 days for those infected with other VTEC serotypes (9 patients) or 15.5 ± 12.7 days for those not infected with a defined pathogen (22 patients). Patients with bloody diarrhea and not infected with a defined pathogen did not have inflammatory bowel disease or some other underlying disease to explain bloody stools. Similar to previous studies, *E. coli* O157:H7 infection was associated with a short duration of diarrheal illness. However, infection with other *E. coli* serotypes and verocytotoxin-positive diarrheal illness without a demonstrable pathogen was of relatively long duration. The similarity between the time of onset of diarrhea to the time that cultures were obtained and the duration of diarrhea indicates that many patients were at the end of the natural history of their illnesses before presenting to their physicians' offices.

TABLE 3. Laboratory characteristics of 22 non-O157:H7 *E. coli* serotype isolates[a]

Strain designation	FVT type	FVT titer	Colony blot type	Serotype	Type by in vitro toxin production	Type by PCR[b]			Southern hybridization
						P	G	K	
KR32	1, 2	2	1, 2	O2:H29	1, 2	1, 2	ND[c]	+	ND
KR29	1, 2	32	1, 2	OR:H?	2	1, 2	ND	+	ND
KR41	1	4	1, 2	O103:H2	1	1	ND	+	ND
KR48A	1	8	1	O103:H25	1	1	ND	+	ND
KR47	1	16	1	O103:H25	1	1	ND	+	ND
KR39	1	16	1	O153:H11	1	1	ND	+	ND
KR40	1	8	1	O26:H11	1	1, 2	ND	+	ND
KR18	1	64	1, 2	O26:H11	1, 2	1, 2	ND	+	ND
KR49	1, 2	4	1	O142,X13:U	Neg	1	1	Neg	1
KR42	1	64	1	O17:U	Neg	1	1	Neg	1
KR13	1	4	1	O19,O133:NM	Neg	Neg	1	Neg	1
KR15	1, 2	32	1	O26:NM	Neg	1	1	Neg	1
KR45	1	4	1	O2:1,4,32	Neg	1	1	Neg	1
KR43	1	8	1	O2:U	Neg	1	1	Neg	1
KR20	1, 2	4	1	OR:U	Neg	1	1	Neg	1
KR50[d]	1	8	1	O16:H48	Neg	1	1	Neg	1
KR14[d]	Neg	Neg	2	O75:NM	Neg	2	Neg	Neg	1
KR19	1	4	1	O2:1,4,32,38	Neg	Neg	1	Neg	1
KR46	1, 2	16	1	OX13:NM	Neg	1	1	Neg	Neg
KR38	1, 2	4	1	—:48	Neg	Neg	1	Neg	Neg
KR36	1, 2	32	1, 2	—:M	Neg	1	1	Neg	Neg
KR70	1	8	1	Mult:U	Neg	Neg	1	Neg	1

[a] The designations 1 and 2 indicate VT-1 and VT-2, respectively. Neg, negative.
[b] PCR was done with the primers of Pollard et al. (P) (33), Gannon et al. (G) (13), and Karch and Meyer (K) (19).
[c] ND, not done.
[d] E. coli O157:H7 also was cultured initially.

Patients with bloody diarrhea were significantly more likely to be culture or colony blot probe positive for *E. coli* O157:H7 than were those with watery diarrhea (10 of 22 versus 1 of 22, respectively; relative risk = 2.50 [95% confidence interval = 1.53 to 4.07]). One of the O157:H7 strains was recovered from a patient previously negative for this organism by culture. Conversely, six of the seven VTEC isolates recovered from patients with watery diarrhea were serotypes other than O157:H7 (serotypes O26:H11, O103:H25, OR:H?, OX13:NM, O142, X13:U, and —:48). The four serotypes associated with bloody diarrhea included O2:H29, OR:U, O2:1,4,32, and −:M; strains of the last three serotypes did not produce toxin in vitro. Overall, 14 of 22 patients (64%) with bloody diarrhea were positive for VTEC organisms in their stools, whereas 7 of 22 patients (32%) with watery diarrhea were positive for VTEC organisms in their stools.

Two patients did not have acute diarrheal disease. One patient had upper gastrointestinal cramps, and the other patient was being investigated for eosinophilia and *Strongyloides stercoralis* infection. Stools from both patients were VT positive and also contained VTEC organisms on probing.

DISCUSSION

The 2-year prospective study by Pai et al. (30) demonstrated that VTEC was the most frequent cause of bloody diarrhea in patients presenting at hospital emergency departments in Calgary and was most prevalent in the summer months. However,

TABLE 4. Concordance of presence of FVT, colony blot type, in vitro toxin production, and PCR toxin gene probe studies[a]

Serotype (no. of stool samples)	FVT type in stool samples	Type by in vitro toxin production by stool isolates	Type from results of colony blot of stools	Type by PCR of stool isolates[b]
E. coli O157:H7				
2	1, 2	1, 2	1, 2	1, 2
1	2	2	2	2
1	1, 2	1, 2	1, 2	1
5	1, 2	2	1, 2	1, 2
7	2	2	1, 2	1, 2
OR:H? (1)	1, 2	2	1, 2	1, 2
O103:H2 (3) O103:H25 (2)	1	1	1	1
O153:H11, O26:H11 (2)	1	1	1	1, 2
O26:H11 (1)	1	1, 2	1, 2	1, 2
O2:H29 (1)	1, 2	1, 2	1, 2	1, 2

[a] Concordance: all four parameters, 7 of 24 (29%) samples; colony blot, in vitro toxin production, 11 of 24 (46%) samples; FVT, PCR gene probe, 13 of 24 (54%) samples; FVT, colony blot, 16 of 24 (67%) samples; FVT, in vitro toxin production, 17 of 24 (71%) samples; colony blot, PCR gene probes, 21 of 24 (88%) samples. The designations 1 and 2 indicate VT-1 and VT-2, respectively.
[b] With the primers of Pollard et al. (33).

TABLE 5. Characteristics of 44 patients with diarrhea whose stools were FVT positive

Characteristic	Bloody diarrhea (n = 22)	Watery diarrhea (n = 22)	Total (n = 44)
Sex (no. of males/no. of females)	10/12	6/16	16/28
Age (yr) (no. of patients)			
0–2	5	10	15
3–9	1	4	5
10–15	2	0	2
16–40	5	6	11
41–65	7	1	8
>65	2	1	3
No. of days (mean ± SD) between onset of illness and sample submission for:			
All patients	12.9 ± 12.9 (20)[a]	13.1 ± 10.4 (22)	12.9 ± 11.5 (42)
Patients with O157:H7 infection	5.0 ± 2.8 (10)	3 (1)	4.8 ± 2.7 (11)
Patients infected with other VTEC serotypes	28.3 ± 3.8 (3)	10.7 ± 8.1 (6)	16.6 ± 11.1 (9)
Patients with FVT only	19.2 ± 16.9 (7)	14.7 ± 11.3 (15)	15.5 ± 12.7 (22)
Duration of diarrhea (days [mean ± SD])			
All patients	14.7 ± 13.4 (15)	16.0 ± 12.2 (21)	15.5 ± 12.6 (36)
Patients with O157:H7 infection	4.7 ± 2.0 (7)	4.0 (1)	4.6 ± 1.9 (8)
Patients infected with other VTEC serotypes	30.0 (1)	10.5 ± 8.5 (6)	13.3 ± 10.7 (7)
Patients with FVT only	22.6 ± 14.0 (7)	19.2 ± 12.8 (14)	20.3 ± 13.0 (21)
No. of patients with:			
Abdominal cramps	17 (19)	13 (18)	30 (37)
Nausea	9 (15)	9 (16)	18 (31)
Vomiting	7 (16)	6 (17)	13 (33)
Fever	10 (17)	14 (19)	24 (36)
Hospitalization	7 (20)	1 (20)[b]	8 (40)
Antibiotic treatment	7 (19)	3 (21)	9 (39)
Antidiarrheal agent treatment	5 (16)	11 (21)	17 (37)

[a] Numbers in parentheses are numbers of patients who responded to the questionnaire.
[b] Relative risk, 2.20 (95% confidence interval = 1.31, 3.53).

it is unlikely that patients presenting at hospital emergency departments are similar to patients seen in physicians' offices and walk-in clinics. This latter population may more accurately reflect the incidence of VTEC infection in the general population since patients or parents would see their own physicians in preference to making an emergency room visit. For this reason, a combination of community-based and hospital-based laboratory facilities serving approximately a third of the population was used in the present study.

The results from our 10-week summer survey demonstrated that *E. coli* O157:H7 serotypes were recovered from only 0.6% of stool samples from patients with diarrhea. This result is similar to those obtained in two other Canadian studies (6, 42) and in studies in the United States (17, 26, 27) and Europe (32). The rate of isolation of *E. coli* O157:H7 from the Calgary General Hospital outpatient samples (9 of 281; 3.2%) was severalfold higher than that from the community-based samples (13 of 3,296; 0.4%) and was similar to that from samples in the previous hospital-based survey in Calgary (30). This suggests that there has been no change in the frequency of infection owing to *E. coli* O157:H7 in this geographical area over the past 5 years.

Toxin testing indicated that the prevalence of VTEC was more than threefold greater. This suggests that a significant proportion of VTEC infections are not being diagnosed in this area of high endemicity. On the basis of culture results and the presence of FVT in stools, VTEC was identified as a potential pathogen in 76 (2.1%) samples (66 patients) and rivaled *Campylobacter* and *Salmonella* infections in frequency. The Vero cell assay for FVT with monoclonal antibody neutralization appeared to be specific for VTEC-associated infection since the presence of FVT in the stool was associated with *E. coli* O157:H7, other VT-positive serotypes, and no other enteric pathogen. The stools of three patients infected with *Salmonella* spp. and *Aeromonas* sp. were negative by the colony blot hybridization assay, suggesting fecal clearance of a VTEC organism and coinfection with another potential pathogen. This study shows that, on the basis of toxin testing, 1 in 50 patients with diarrhea presenting for medical care in Calgary in the summer months had VTEC infection.

Several hybridization probes have been developed for detecting VTEC organisms. These include the VT-1- and VT-2-specific intragenic probes developed by Newland and Neill (29) which were highly specific for VTEC (VT-1 and VT-2) and *Shigella dysenteriae* type 1 (VT-1). These two probes have been used in investigations to determine VTEC infection in a series of studies of diarrhea in Thailand (3–5, 42). Using these probes, investigators isolated VTEC from 16 of 17 stool samples (94.1%) initially positive for *E. coli* O157:H7 by culture. Fourteen of the isolates recovered were serotype O157:H7. It is likely that the other two specimens contained both *E. coli* O157:H7 and organisms of another VTEC serotype, and the O157:H7 isolate was not recovered because only one probe-positive colony (which was subsequently shown to be unable to produce toxin in vitro) was selected for serotyping. It is therefore important to select more than one probe-positive colony for typing in future studies.

We were concerned that the efficiency of sorbitol-MacCon-

key agar for the identification of *E. coli* O157:H7 may be suboptimal since data from comparative studies performed by several methods and in non-outbreak-related situations are not available. From stool samples that were FVT positive only, *E. coli* O157:H7 was missed in only two specimens from one patient, indicating that cultures for *E. coli* O157:H7 with sorbitol-MacConkey agar will identify more than 90% of infected patients.

Probing for VTEC in 52 FVT-positive, culture-negative stools identified 22 samples infected with VTEC organisms not of the O157 serotype. Although colony blot testing may have been insufficiently sensitive for the detection of low numbers of VTEC organisms in the remaining FVT-positive stools, others have observed the short duration of fecal shedding of the pathogen, with persistence of toxin activity (37, 45). Furthermore, direct PCR of these colony blot-negative stool samples in another study (35) showed that only 1 of 20 samples was PCR positive. Whereas the pathogenic association between recovery of *E. coli* O157:H7 and diarrheal disease is very strong, the association between recovery of organisms of non-O157:H7 serotypes and pathogenicity is less certain. The eight strains which produced toxin in vitro and in vivo likely are true pathogens. The remaining 14 strains should be viewed as potential pathogens because of their inability to produce toxin in vitro. PCR studies with the primers of Karch and Meyer (19), which were designed to amplify both VT-1 and VT-2 sequences together but which also contained degenerate sequences to overcome differences in VT genes, were negative for these strains. However, other primers which specifically probed for VT-1 or VT-2 sequences were positive. Southern blot analyses were strongly positive in 10 of 14 strains. Further proof of the pathogenic potentials of these strains would require the sequencing of the specific gene segments. Coinfection with another VTEC strain also may account for the observations in some of these cases. It is possible that loss of Shiga-like toxin genes from clinical isolates (20) or alterations in genetic control mechanisms could account for our observations. Against the former possibility is the finding that the strains in the present study have been reprobed by colony blotting and PCR on at least three other occasions, with identical results obtained each time.

The proportion of patients with disease caused by *E. coli* O157:H7 versus those with disease caused by other serotypes was unanswered in the present study since 43% (30 of 69) of positive stool samples had the presence of FVT only as the indicator of VTEC infection. It is unclear why toxin persists without identification of the source pathogen. Possible explanations include delayed clearance of toxin from tissues, low numbers of organisms in the lumen, or mucosal adherence. A prospective natural history study with multiple sequential samples would be required to address this issue.

In the present study, the interval between the onset of diarrhea and the submission of *E. coli* O157:H7-positive stools was a mean of 4.8 days, whereas the interval was considerably longer for other patients. It appears that patients infected with *E. coli* O157:H7 presented to the health care system more promptly because of symptom severity not defined in the patient survey. Patients infected with organisms of other VTEC serotypes or who had only FVT persistence in their stools presented because of chronic persistent diarrhea of unknown cause. While the public health focus is on *E. coli* O157:H7 infections, the morbidity from non-O157:H7 VTEC infection may be underappreciated.

Hemorrhagic colitis is characterized by watery diarrhea followed by grossly bloody diarrhea, severe abdominal cramping, nausea, and vomiting, with little or no fever. This disease occurs as both outbreaks and sporadically with geographical and seasonal variations. *E. coli* O157:H7 has been identified as the infecting pathogen in 15 to 45% of cases of hemorrhagic colitis (30, 36, 49). In the present study, since not all the infected patients responded to the survey, it is not possible to determine the actual incidence of bloody diarrhea. However, half of those who responded and who had bloody diarrhea were infected with *E. coli* O157:H7 and 65% were infected with VTEC. Patients with bloody diarrhea were significantly more likely than those with watery diarrhea to be infected with *E. coli* O157:H7. This suggests that *E. coli* O157:H7 causes more severe acute diarrheal disease. This is consistent with similar findings of Pai et al. (31) and Lopez et al. (24).

In the present study we did not observe patients progressing to HUS. Several cases of HUS were observed immediately before and after the study period in this community. With the 5 to 15% estimated risk of developing HUS in those infected with *E. coli* O157:H7, the size of our population sample consisting of those under 15 years of age was not sufficient to make that observation.

The relatively long mean duration of diarrhea in the group of patients described here is unusual, since most diarrheal illnesses last a mean of 5 to 6 days. Possible explanations include lactase deficiency or persistence of mucosal abnormalities produced by attaching-and-effacing lesions associated with adherent and verocytotoxic *E. coli* (2, 28, 46, 47). Other studies have shown that non-VT-producing enteropathogenic *E. coli* strains and newly identified enteroadherent strains are associated with persistent diarrhea and small bowel mucosal lesions (10, 15). It is unclear whether the strains recovered in the present study have such properties and whether they possess *eae* adherence genes. It is possible that earlier diagnosis would have increased pathogen recovery and reduce the proportion of FVT-positive patients from whom no pathogen was recovered.

The nature of the epidemiologic survey conducted days to weeks after the episode did not allow for intervention, investigation of household carriage, or a detailed analysis of potential food sources. Because children are more prone to develop HUS after VTEC infection, diarrheal disease caused by VTEC is being investigated as a pediatric-focused disease by the Canadian Pediatric Kidney Disease Reference Centre (38). The results of the present study, however, indicate that adults may play a role in the epidemiology of VTEC disease since half of the study population with this infection was older than 15 years of age.

We conclude that direct free VT testing of stools amplifies the detection of VTEC diarrheal disease severalfold over routine cultures for *E. coli* O157:H7, that sorbitol-MacConkey agar cultures for *E. coli* O157:H7 identify the majority of patients infected with this pathogen, and that the spectrum and epidemiology of VTEC disease include all age groups and encompass both acute and subacute diarrheal illnesses. Future studies of this disease need to address the relationship between the presence of toxin genes, their control mechanisms, and the interplay of other pathogenic cofactors.

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