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Shiga-Toxigenic *Escherichia coli* Detection in Stool Samples Screened for Viral Gastroenteritis in Alberta, Canada▽

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Shiga-toxigenic *Escherichia coli* (STEC) is an important cause of diarrheal disease. The most notorious STEC serotype is O157:H7, which is associated with hemorrhagic colitis and hemolytic-uremic syndrome (HUS). As a result, this serotype is routinely screened for in clinical microbiology laboratories. With the bias toward the identification of the O157 serogroup in routine diagnostic processes, non-O157 STEC has been largely underrepresented in the epidemiology of STEC infections. This diagnostic bias is further complicated by the fact that many non-O157 STEC infections cause nonspecific gastroenteritis symptoms reminiscent of enteric viral infections. In this study, real-time PCR was used to amplify Shiga toxin genetic determinants (*stx*$_1$ and *stx*$_2$) from enriched stool samples that were initially submitted for the testing of enteric viruses in patients with suspected viral gastroenteritis between May and September of 2006, 2007, and 2008 (n = 2,702). Samples were submitted from the province of Alberta, Yukon, the Northwest Territories, and Nunavut, Canada. A total of 38 samples (1.4%) tested positive for Shiga toxin genes, and 15 isolates were cultured for further characterization. Several of the serotypes identified (O157:H7, O26:HNM, O26:H11, O103:H25, O121:H19, and O145:HNM) have been previously associated with outbreaks and HUS. This study outlines the importance of combining molecular methods with classical culture techniques to enhance the detection of emerging non-O157 as well as O157 serotypes in diarrheal stool samples. Furthermore, atypical diarrhea disease caused by non-O157 STEC can be routinely missed due to screening only for viral agents.

Serogroup O157 *Escherichia coli* represents one of the most notorious human pathogens and can be linked to ingestion of contaminated food and/or water. O157 Shiga toxin (ST)-producing *E. coli* (STEC) is routinely screened for in many clinical laboratories using selective sorbitol-containing MacConkey medium (SMAC), which exploits the non-sorbitol-fermenting phenotype of O157. However, sorbitol-fermenting O157 strains (in particular O157:H-nonmotile) have been identified recently, and these strains have also been associated with the development of hemolytic-uremic syndrome (HUS) in Europe (18). This discovery has reinforced the need for additional screening methods to identify STEC. While most of the medical attention has been focused on O157:H7, several North American studies have found that up to 50% of STEC strains in diarrheal illnesses belong to the "non-O157" serogroups (10, 16, 19, 20, 28). These numbers are consistent with the observations that non-O157 serogroups are common causes of outbreaks in Europe and Australia (17). To date, over 100 non-O157 serogroups have been identified (17). Non-O157 serogroups of STEC have been quickly recognized as underappreciated and important emerging causes of human diarrheal disease because of their propensity to cause severe complications such as HUS. STEC infections are of particular concern for elderly and pediatric patients because of the higher risk of development of HUS in these populations (8). Though screening methods to detect non-O157 STEC are available, it has been reported that many laboratories still limit testing to culture-based identification (13, 15). However, many STEC infections, especially those caused by the non-O157 serogroups, present similarly to infections by viruses such as norovirus, rotavirus, enteric adenovirus, astrovirus, and enterovirus (31). The symptoms include nonbloody watery diarrhea, vomiting, headache, low-grade fever, and/or abdominal cramps (27, 31). In the setting of the Provincial Laboratory for Public Health in Alberta (ProvLab), many stool samples from patients submitted for virus testing have not been tested for enteric bacteria.

The major determinants of STEC infection are the Stx1 and Stx2 Shiga toxins (encoded by *stx*$_1$ and *stx*$_2$). These toxins are directly implicated in both hemorrhagic and systemic infections (23). Patients infected with STEC strains that produce the Stx2 toxin are statistically more likely to develop HUS, as reflected by epidemiological data from the United States (14, 17). Multiple methods exist for the detection of STEC, including screening samples for the presence of these toxins or their genetic determinants. Screening for the toxins or the genetic determinants is advantageous because it is not limited to the detection of specific serogroups such as O157. In fact, the Centers for Disease Control and Prevention released updated guidelines in October 2009 for the detection of STEC in relation to acute community-acquired diarrhea, which included specific testing for Shiga toxins or their genetic determinants in addition to traditional culture (http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5812a1.htm). However, some traditional methods, such as the verocytotoxicity assay, are laborious, and PCR-based assays have been designed to detect *stx*$_1$ and *stx*$_2$ genes in *E. coli* with higher sensitivity and fewer technical demands (2, 4, 5, 11, 24, 25, 29, 32). We recently compared

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four real-time PCR assays as well as a conventional PCR method for the detection of STEC stx_1 and stx_2 genes (6) and found that our in-house TaqMan real-time PCR assay was the most sensitive and specific and had the lowest limit of detection.

In this study, we determined the prevalence of STEC and characterized the serotypes of these *E. coli* strains in stool samples that were originally submitted for testing for enteric viruses. Stool samples from Alberta and the Canadian territories (Yukon, Northwest Territories, and Nunavut) were analyzed using the above-mentioned real-time PCR assay for stx_1 and stx_2 in the periods of May to September of three consecutive years (2006 to 2008).

MATERIALS AND METHODS

Stool samples, culture enrichment, and DNA preparation. Stool samples submitted to the ProvLab from patients accessing care in the province of Alberta (population of 3.4 million in 2007 and divided into nine health regions during the study period) and the Northern Territories (Yukon, Northwest Territories and Nunavut, population of ~110,000) for testing for viruses, including electron microscopy (EM), viral culture, and/or nucleic acid amplification test (NAT) for norovirus, between the months of May and September of 2006, 2007, and 2008 were included in the study. At ProvLab, EM is the routine diagnostic test performed on stool samples for the detection of enteric viruses. Viral culture is set up if the submitter has requested testing for enterovirus, and NAT for norovirus is performed only in outbreak investigations. Enteric bacterial culture is set up if requested by the submitter on the requisition. The period of May to September was chosen for 2006 to 2008 because these months had a higher incidence of enteric bacterial infections based on historical data (data not shown). Stool samples were stored at −20°C since the time of receipt at ProvLab, and storage times were approximately 2 years, 1 year, and less than 1 year for 2006, 2007, and 2008, respectively. An aliquot of each sample was enriched in Trypticase soy broth and lysed for DNA isolation as described previously (12). Each stool sample was visually analyzed for color and consistency. The consistency was grouped as either liquid, semisolid, or solid. Patient age, gender, and geographic location were retrieved from the ProvLab laboratory information system.

TaqMan real-time PCR of *stx* genes. Oligonucleotide primers and fluorescent probes for stx_1 and stx_2 were described previously (6). The probe for stx_2 was modified from the original (STX2-TM-P) in that the VIC dye was replaced by the 6-carboxyfluorescein (FAM) dye. Real-time NAT was performed as separate reactions for stx_1 and stx_2 on an ABI Prism 7000 sequence detection system using the following amplification conditions: 95°C for 10 min, 55 cycles of 95°C for 15 s and 60°C for 1 min, and a final hold at 25°C for 1 min.

STEC isolation. Enriched stool samples that tested positive by the TaqMan real-time PCR assay for stx_1 and/or stx_2 were plated on CHROMagar O157 solid medium (Dalynn Biologicals, Calgary, Alberta, Canada), MacConkey agar plates with crystal violet (Dalynn Biologicals), and sheep blood agar plates (Dalynn Biologicals). Mauve and/or blue colonies from CHROMagar O157 (representing *E. coli* O157 and non-O157, respectively) and pink colonies from MacConkey agar (representing lactose-fermenting coliforms, presumptive *E. coli*) were streaked to MacConkey solid medium and subsequently plated onto sheep blood agar plates (Dalynn Biologicals). DNA was extracted by suspending individual colonies in 200 μl of rapid lysis buffer (100 mM NaCl, 10 mM Tris-HCl [pH 8.3], 1 mM EDTA [pH 9.0], 1% Triton X-100), boiling for 15 min, and centrifuging at 14,000 × *g*. The supernatant from each isolate was analyzed by TaqMan real-time PCR to confirm the presence of stx_1 and/or stx_2. A total of 16 colonies were tested for each positive stool sample, and a maximum of 36 colonies were tested when an initial screen did not reveal *stx*-positive colonies. Isolates were also tested for O157 serogroup by O157 direct antibody agglutination (BD Difco, Burlington, Ontario, Canada).

Data analysis. Differences in the ages of patients among the three study periods were analyzed using the Kruskal-Wallis test. Differences in gender ratio and annual rates of specimens testing positive for enteric virus, stx_1, and/or stx_2 and specimens where *E. coli* were successfully isolated were analyzed using the chi-square or Fisher exact test as appropriate. All analyses were performed using PASW Statistics 17.0 or Stata v.10 with the level of significance set at a P value of <0.05.

RESULTS

A total of 2,725 stool samples were included in the study, with 97.2% of the samples submitted from Alberta (Table 1); only 4.7% ($n = 45$), 2.9% ($n = 21$), and 0.8% ($n = 8$) of the submitted samples were from the Northern Territories in 2006, 2007, and 2008, respectively. The female-to-male ratio was 1.0 for all three study periods. There was a significant difference in the median age of patients among the three years, i.e., 11 years in 2006, 7 years in 2007, and 19 years in 2008 ($P < 0.05$ by the Kruskal-Wallis test). Overall, 2,539 samples were tested by EM, and enteric viruses were identified in 124 samples (4.9%). There was a significant difference in the percentage of samples testing positive for enteric virus among the three years, with a higher detection rate when the median age was lower: 5.4% in 2006, 6.5% in 2007, and 3.4% in 2008 ($P < 0.05$ by the chi-square test) (Table 1). The median age of patients whose samples tested positive for enteric virus by EM was 1.4 (interquartile range, 0.8 to 2). Norovirus was identified in 80 out of 183 samples tested by NAT, and 7 of the 130 samples set up for viral culture grew adenovirus ($n = 1$) and enterovirus ($n = 6$). A total of 437 samples included in the study were set up for bacterial culture, and 14 were positive: 3 for *Salmonella enterica* serovar Typhimurium, 3 for *Campylobacter jejuni*, 2 for *Clostridium perfringens*, 3 for *Aeromonas caviae*, 1 for *Salmonella enterica* serovar Poona, 1 for *Salmonella enterica* serovar Hadar, and 1 for *Plesiomonas shigelloides*. There was no significant difference in the percentage of samples testing positive using NAT for norovirus, viral culture, and enteric bacterial culture among the three years.

A total of 38 of 2,725 stool samples (1.4%) tested positive for STEC using the real-time PCR assay; 23 tested positive for stx_1 only and 4 for stx_2 only (Table 1). The percentages of stool samples testing positive for stx_1 and/or stx_2 were 1.0% in 2006, 2.3% in 2007, and 1.1% in 2008 ($P = 0.08$ by the chi-square test). Only one of the 38 samples that tested positive for STEC had been previously set up for enteric bacterial culture (which was reported as negative), and two samples that were tested for virus by viral culture were negative. Thirty-seven of the STEC-positive samples were also tested by EM, and all were negative for enteric virus (none of these samples were tested for norovirus). The median age of patients who tested positive for STEC was 10 (interquartile range, 1.8 to 50). There was no significant difference in median age (Table 1) or female-to-male ratio (4.0, 1.0, and 0.7 for 2006, 2007, and 2008, respectively) among the three years.

STEC strains from 15 samples were isolated by culture and further characterized. The samples from the most recent year (2008) had higher recovery than those from 2007 and 2006 ($P < 0.05$ by the Fisher exact test) (Table 1). None of the 10 *stx*-positive stool samples in 2006 yielded bacterial growth on selective or nonselective media. For 2007, six *stx*-positive stool samples yielded growth on selective media; however, *stx*-positive isolates were obtained for only five of the samples (Table 2). One of these five stool samples was found to be coinfected with STEC belonging to the serogroups O6:H16 and O103:H25. For 2008, nine samples were culturable, and STEC isolates were obtained for each and were serotyped. Overall, isolates from three samples were serogrouped as O157 (two

TABLE 1. Electronic microscopy, viral culture, real-time PCR, and *E. coli* culture results for 2006-2008 "viral" stool samples in Alberta and northern territories of Canada

Yr (no. of samples)	Median patient age (yr) (interquartile range) for all included samples	No. of samples positive/total no. tested (% positive) for:				Median patient age (yr) (interquartile range) for Stx-positive samples)	Types of *stx* genes (*n*)	No. culture positive on nonselective and/or selective solid medium/ total no. Stx positive (%)
		Enteric virus, tested by EM[a]	Norovirus	Virus, tested by viral culture[b]	Enteric bacteria, tested by enteric culture[c]	Stx, tested by real-time PCR		
2006 (960)	11 (1.2–60)[d]	46/855 (5.4)[e]	45/86 (52.3)[f]	6/61 (9.8)[g]	6/189 (3.2)[g]	10/960 (1.0)[f]	6.5 (4.8–58)[h]	*stx*$_1$ (5), *stx*$_2$ (1), *stx*$_1$ and *stx*$_2$ (4)	0/10 (0)[i]
2007 (712)	7 (1.1–49)[d]	43/666 (6.5)[e]	18/52 (34.6)[f]	1/47 (2.1)[g]	6/140 (4.3)[g]	16/712 (2.3)[f]	2.5 (1.4–52)[h]	*stx*$_1$ (11), *stx*$_2$ (2), *stx*$_1$ and *stx*$_2$ (3)	6/16 (38)[i]
2008 (1,053)	19 (1.4–54)[d]	35/1018 (3.4)[e]	17/45 (37.8)[f]	0/22 (0)[g]	2/108 (1/9)[g]	12/1053 (1.1)[f]	10 (1.4–22)[h]	*stx*$_1$ (7), *stx*$_2$ (1), *stx*$_1$ and *stx*$_2$ (4)	9/12 (75)[i]
Total (2,725)	12.5 (1.2–55)	124/2539 (4.9)	80/183 (37.8)	7/130 (5.4)	14/437 (3.2)	38/2725 (1.4)	10 (1.8–50)	*stx*$_1$ (23), *stx*$_2$ (4), *stx*$_1$ and *stx*$_2$ (11)	15/38 (39.5)

[a] Enteric viruses detected by electron microscopy (EM) in the different years: 2006, 5 adenovirus and 41 rotavirus; 2007, 3 adenovirus, 39 rotavirus, and 1 small round structured virus; 2008, 8 adenovirus, 25 rotavirus, and 2 small round structured virus.
[b] Viruses isolated by culture in the different years: 2006, 1 adenovirus and 5 enterovirus; 2007, 1 enterovirus.
[c] Bacteria isolated by enteric culture in the different years: 2006, 2 *Campylobacter jejuni*, 2 *Clostridium perfringens*, 1 *Aeromonas caviae*, and 1 *Salmonella* Poona; 2007, 3 *Salmonella* Typhimurium, 1 *Salmonella* Hadar, 1 *Aeromonas caviae*, and 1 *Campylobacter jejuni*; 2008, 1 *Aeromonas caviae* and 1 *Plesiomonas shigelloides*.
[d] $P < 0.05$ by Kruskal-Wallis test.
[e] $P < 0.05$ by chi-square test.
[f] Not significant by chi-square test.
[g] Not significant by Fisher exact test.
[h] Not significant by Kruskal-Wallis test.
[i] $P < 0.05$ by Fisher exact test.

TABLE 2. Shiga-toxigenic *E. coli* isolated from patient stool from Alberta and Northern Territories

Yr	Serotype	Patient age	*stx* type(s)	Stool appearance
2007	O26:H11	83 yr	*stx*$_1$	Liquid, brown
	Orough:H11	3 yr	*stx*$_1$	Liquid, brown
	O11:H30	3 yr	*stx*$_1$	Liquid, brown
	O157:H16	2 yr	*stx*$_1$/*stx*$_2$	Solid, brown
	O6:H16/O103:H25	31 yr	*stx*$_1$	Liquid, brown
2008	O145:HNM	8 mo	*stx*$_1$	Liquid, tan
	O26:HNM	13 yr	*stx*$_1$	Liquid, brown
	O69:H11	52 yr	*stx*$_1$	Solid, brown
	O26:H11	6 mo	*stx*$_1$	Liquid, yellow
	Orough:HNM	16 mo	*stx*$_1$	Semisolid, brown
	O121:H19	10 yr	*stx*$_1$/*stx*$_2$	Liquid, brown
	O157:H7	48 yr	*stx*$_1$/*stx*$_2$	Liquid, brown
	O157:H7	3 yr	*stx*$_1$/*stx*$_2$	Liquid, brown
	O103:H25	16 yr	*stx*$_1$	Semisolid, brown

O157:H7 and one O157:H16), while the majority of the isolates belonged to non-O157 serogroups (Table 2).

The majority of stool samples containing *stx*-positive *E. coli* represented typical diarrhea (liquid and brown); however, 11 of the positive samples were identified solid or semisolid stools. Most of the samples were submitted without history on the requisition, but the submitting physician for one of the *stx*$_1$- and *stx*$_2$-positive samples in 2006 has indicated that the patient had bloody diarrhea; however, no bacteria was isolated from the sample for further characterization. A limited review of this patient case history revealed that there was a stool sample submitted for enteric bacteria culture at a regional laboratory during the illness that had tested negative for *E. coli* O157:H7 and other common enteric bacteria.

DISCUSSION

Viral and bacterial infections can present remarkably similar clinical symptoms in gastroenteritis. Most enteric viral infections cause mild to moderate diarrhea (with the exception of rotavirus) (27). Though many severe cases of O157 STEC gastrointestinal infections, especially those of the O157:H7 serotype, yield bloody diarrhea (9, 14), most infections do not progress to this stage and therefore can sometimes be thought to have a viral etiology. This is further complicated by non-O157 STEC infections, which are not commonly associated with bloody stool and can often resemble viral gastroenteritis. Based on our provincial historical data, the season in which an enteric infection is identified can often predict whether the causative agent is viral or bacterial, as enteric viral infections are more frequently found during the winter months, whereas enteric bacterial infections are more prevalent from May to September. The overall ambiguity of non-O157 STEC infections and viral gastroenteritis led us to screen stool samples that were referred for viral diagnostics between May and September for the presence of STEC to better understand the extent of these potentially undiagnosed infections. The median age of patients for which enteric viruses were isolated was lower than the median age of those who had STEC infection in our study. However, a large proportion of STEC-positive stools were from children younger than 6 years (19/38), which is highly concerning as young children are at a higher risk for developing HUS (3).

For the stool samples investigated in this study, non-O157 STEC strains represent the majority of causative agents of disease in culture-positive samples. Of the 15 isolates obtained in this study, only three were O157, and only two of these were the prototypical O157:H7 (Table 2). This is particularly concerning because the remaining 12 isolates would not have been detected using the current O157-biased detection methods even if they had been referred for enteric bacterial culture. Furthermore, three samples containing O157 isolates were not screened for enteric bacteria; otherwise the bacteria would have been identified using conventional culture. It should also be noted that none of the STEC-positive samples appeared to be coinfected with enteric viruses, suggesting that the STEC was likely the cause of symptoms. Several of these STEC-positive stool samples were solid or semisolid, which further complicates the diagnostic process as the organisms do not appear to cause typical diarrhea-like symptoms in all hosts. It was possible that the stool samples were submitted late in the illness or that the organisms were not causing a typical diarrheal illness. Of additional concern is the identification of five non-O157 serogroups previously associated with severe illness (O26:HNM, O26:H11, O103:H25, O121:H19, and O145:HNM), including HUS (1, 7, 9, 21, 22, 26, 30). O103:H25 was isolated from patients in both 2007 and 2008; however, the isolate from 2007 was stx_1 positive only, whereas the 2008 isolate was positive for both stx_1 and stx_2. Of note, the recent O103:H25 outbreak associated with HUS in Norway was stx_2 positive only (26). Given the revised CDC recommendations for STEC testing and the serious clinical implications of non-O157 STEC, our data support an algorithm in which Shiga toxin testing is conducted on all stool samples that are negative for viral etiologies.

One important observation in this study is the percentage of *stx*-positive samples that were culture positive for STEC. We observed a lower culturability for *stx*-positive stools from the 2006 and 2007 samples. These samples had been frozen and thawed several times during previous diagnostic processes, while the 2008 samples were frozen after the initial diagnostic test and had not been thawed prior to this study. It is also possible that the prolonged freezer storage of the 2006 and 2007 samples led to decreased culturability, since no cryoprotective medium was added to the stool samples, killing the bacteria, and only residual DNA was detected by the real-time assay. Alternatively, the bacteria might have survived the handling process but resided in a viable-but-nonculturable state. Regardless of the explanation, the lack of culturability and subsequent lack of serotyping hindered the characterization of the organisms and could contribute to an underrepresentation of STEC disease and incomplete outbreak reporting. These observations reinforce the need for stool samples to be processed for STEC by enrichment and real-time PCR soon after collection, with particular care given to the storage conditions used for specimen transport and storage.

Non-O157 STEC strains are rapidly becoming recognized as emerging pathogens with great clinical importance. The advent of molecular methods targeting *stx* genes in concert with classical culture techniques allows clinical laboratories to detect most STEC serogroups as opposed to only O157. This report shows that the symptoms caused by non-O157 STEC infection and occasionally O157 STEC infection can lead to misdiagnosis as viral gastroenteritis. Additionally, by using real-time PCR, these STEC strains can be identified in stool samples quickly, to allow accurate diagnosis of the causative agent of disease without relying exclusively on classical culturing techniques.

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