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Comparison of a Direct Fecal Shiga-Like Toxin Assay and Sorbitol-MacConkey Agar Culture for Laboratory Diagnosis of Enterohemorrhagic *Escherichia coli* Infection

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A direct fecal Shiga-like toxin assay (DSLTA) was used to prospectively screen 9,449 unselected stool samples, received at the British Columbia Provincial Health Laboratories and the Metropolitan Laboratories of Vancouver, for Shiga-like toxin I and Shiga-like toxin II. The results were compared with results of routine stool culture on sorbitol-MacConkey agar (SMAC) for *Escherichia coli* O157:H7. Of 80 specimens positive by either method, 59 (74%) and 74 (93%) were positive by SMAC and DSLTA, respectively; 53 (66%) were positive by both methods, 21 (26%) were positive by DSLTA only, and 6 (7%) were positive by SMAC only. On further screening, Shiga-like toxin-producing *E. coli* were detected in 8 (38%) of the 21 stools positive by DSLTA only, including serotypes O157:H7 (1 stool), O26:K60 (5 stools), O128:K67 (1 stool), and O103:H2 (1 stool). For the remaining 13 stools in which no SLTEC was found but DSLTA was positive, clinical information revealed that 11 of 12 patients had diarrheal illnesses, and 4 of these 11 had bloody diarrhea or hemolytic-uremic syndrome. Stools positive only by SMAC were collected earlier in the illness than stools positive by DSLTA, suggesting that free fecal toxin levels may be too low to detect at this time. Overall we found that DSLTA detected 19% more positive specimens than SMAC and that Shiga-like toxin-producing *E. coli* serotypes other than *E. coli* O157:H7 are causing disease in the province of British Columbia, Canada.

Since the first recognition of Shiga-like toxin-producing *Escherichia coli* (SLTEC) as a cause of bloody diarrhea in the early 1980s, a variety of clinical syndromes ranging from asymptomatic carriage to severe abdominal cramps, watery diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS) have been associated with infection by these organisms. It has also been shown that human isolates of SLTEC may produce one or both of at least two antigenically distinct toxins, called Shiga-like toxin I (SLT-I) and Shiga-like toxin II (SLT-II), otherwise known as verotoxins VT1 and VT2 (6, 14–17). Diagnosis of infections caused by SLTEC has been primarily by the detection of one specific serotype of SLTEC, *E. coli* O157:H7. As this serotype does not ferment sorbitol at 24 h, the diagnostic laboratory can easily screen for its presence in stool by using a sorbitol-MacConkey agar (SMA) plate. However, many other serotypes of *E. coli* have been shown to produce SLT and have been associated with clinical disease (5, 6, 8). These will not be detected with SMA. Karmali has suggested that the best method to diagnose SLTEC infections is to show the presence of free fecal verotoxin (3, 6). However, no clinical studies comparing the performance of the direct stool assay for SLT with the use of SMA culture (SMAC) have been reported.

Our objectives in this study were (i) to compare stool culture for *E. coli* O157:H7 on SMA with a direct fecal SLT assay (DSLTA) for the laboratory diagnosis of SLTEC infections, (ii) in those specimens positive by DSLTA but negative by SMAC, to test the individual *E. coli* colonies for SLT production and to serotype positive strains to see what serotypes other than O157:H7 produce SLT(s), and (iii) to collect clinical information on patients with stools positive by either or both methods to aid in determining the diagnostic significance of results.

Our results indicated that the DSLTA was more sensitive for the laboratory diagnosis of SLTEC than SMAC, that serotypes other than O157:H7 are important for production of verotoxin-associated disease, and that DSLTA and SMAC results are of equivalent clinical significance.

(Preliminary findings of this research were presented at the Annual Meeting of the American Society for Microbiology [12].)

MATERIALS AND METHODS

Stool specimens. Between April 1989 and August 1990, 9,449 stool specimens sent for culture to either the British Columbia Provincial Health Laboratories or Metropolitan Laboratories in Vancouver, Canada, were entered into the study. These laboratories receive specimens from physicians' offices, hospitals, and Provincial Health units throughout the province of British Columbia, which has a population of approximately 3.5 million people. Specimens at the British Columbia Public Health Laboratories were collected and transported in Cary-Blair transport medium and processed within 24 h of receipt in the laboratory. At Metropolitan Laboratories specimens were collected in sterile containers and cultured within 4 h of receipt in the laboratory.

Stool culture. Specimens were inoculated to media routinely used for the isolation of enteric pathogens, including SMA for *E. coli* O157:H7; bismuth-sulfite, desoxycholate-citrate-lactose-sucrose, and Tergitol-7 for isolation of *Salmonella* and *Shigella* spp.; selective agar for *Campylobacter* spp.; cefsulodin-irgasan-novobiocin agar for *Yersinia* species; sheep blood agar for *Aeromonas*, *Plesiomonas*, and *Vibrio* species; tetrathionate enrichment broth subcultured

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to bismuth-sulfite after 48 h; and phosphate-buffered saline subcultured to cefsulodin-irgasan-novobiocin agar after 12 days of incubation. Sorbitol-nonfermenting colonies growing on SMA were identified by classical biochemical testing. Isolates identified as *E. coli* were serotyped by a latex agglutination technique (Difco Laboratories) to determine whether they were O157:H7. Suspicious colonies growing on other media were selected for biochemical and serological identification according to standard techniques (2).

DSLTA control organisms and monoclonal antibodies (MAbs). Control organisms included *E. coli* C600-933J (SLT-I producer), *E. coli* C600-933W (SLT-II producer), *E. coli* CL8 (SLT-I and -II producer), and *E. coli* O157:H16 (non-SLT-producer). Each control organism was grown overnight in 20 ml of heart infusion broth at 37°C. The culture was centrifuged at 12,000 × *g* for 30 min at 4°C, and the supernatant was frozen in 500-μl aliquots at −70°C and used at a 1:200 dilution in Hybricare medium (American Type Culture Collection) with 10% fetal calf serum for the assays.

The MAbs used in the neutralization of SLTs were (i) anti-SLT-I, MAb 13C4 (ATCC 1794) in the form of mouse ascitic fluid, with a 1:1,000 neutralizing titer against 25 50% cytotoxic doses of SLT-I from *E. coli* C600-933J and used at a final dilution in the test well of 1:800 (working dilution, 1:50), and (ii) anti-SLT-II, MAb BC5-BB12 in the form of mouse ascitic fluid (provided by Nancy Strockbine, Centers for Disease Control, Atlanta, Ga.) with a 1:50,000 neutralizing titer against 25 50% cytotoxic doses of SLT-II from *E. coli* C600-933W and used at a final dilution of 1:20,000 in the test well (working dilution, 1:1,250). These dilutions were chosen so that antibody would be in excess in the DSLTA and neutralize any SLT that may be in the stool filtrates.

Assay controls. Assay controls included 50 μl of each control organism culture supernatant; 50 μl of a mix of equal quantities of each control organism culture supernatant and the MAb mixture, incubated at 37°C for 1.5 h; 50 μl of tissue culture medium; and 50 μl of MAb control (a 1:1 mixture of the working dilutions of each MAb).

Stool SLT assay. Approximately 1.0 ml of each stool specimen was pipetted into a 1.5-ml microcentrifuge tube and centrifuged at 12,000 × *g* for 30 min at 4°C in an Eppendorf centrifuge (model no. 5414). The supernatant was recovered and, if necessary, stored at −70°C before screening in the DSLTA.

Stool supernatants in both 1:1 and 1:10 dilutions in Hybricare medium were tested for free SLT activity as described by Karmali (3), with some modifications as follows. Vero cells were maintained in tissue culture medium (Hybricare; American Type Culture Collection) with 10% fetal calf serum and antimicrobial agents (penicillin-streptomycin-gentamicin-amphotericin B [Fungizone]). The cells were grown in monolayers in 500-ml flasks, seeded to 96-well flat-bottomed plates at 1.6×10^4 cells per well in 200 μl of tissue culture medium, and used within 24 h of seeding. Each supernatant dilution was tested simultaneously for fecal SLT and for neutralization as follows. Fifty microliters of the supernatant was preincubated with an equal volume of a 1:1 mixture of the SLT-I and SLT-II MAb working dilutions at 37°C for 1.5 h. Fifty microliters of each of the supernatants, the supernatant-MAb mixtures, and the controls was then added to separate wells of the seeded microtiter plate, and the plate was incubated at 37°C in 5% CO_2. After 48 h of incubation the plate was read under an inverted microscope and each well was scored on a scale of 0 to 4 (0 being equivalent to 0% of cells showing cytotoxicity typical of SLT). Supernatants producing typical cytotoxicity and any degree of neutralization were considered positive. Stools that were 100% toxic to cells in both the direct and neutralization assays were diluted further for resolution, and stools with questionable cytotoxicity were retested. Stools positive by DSLTA were tested against the individual MAbs to determine the type(s) of SLT present.

TABLE 1. Results of stools screened by DSLTA and SMAC and summary of SLTEC serotypes detected

No. of stools (no. of patients)	Result of:		No. of stools of SLTEC serotype:		
	DSLTA	SMAC	O157:H7	Non-O157	No SLTEC
53 (48)	+	+	53	0	0
6 (6)	−	+	4	2	0
21 (20)	+	−	1	7	13
9,369	−	−	0	0	9,369

Screening of DSLTA-positive stools for SLT$^+$ isolates. Stools positive by DSLTA but with negative SMAC were screened for *E. coli* colonies, and 20 of these colonies were tested for SLT production as follows. Isolated colonies from either the sheep blood agar or SMA were grown overnight in 1 ml of heart infusion broth at 37°C. The culture was centrifuged at 12,000 × *g* for 15 min (4°C), and the supernatant was frozen at −70°C before screening for verocytotoxicity. Serial 10-fold dilutions of each supernatant were mixed with anti-SLT-I alone, anti-SLT-II alone, and a mixture of both anti-SLT-I and anti-SLT-II, such that the final dilution of each anti-SLT in the well was similar to that used in the DSLTA. The incubation steps and test reading were carried out as for the DSLTA. All organisms with neutralizable cytotoxicity in their supernatants were identified and serotyped according to standard procedures (2).

Clinical evaluation. When a stool was positive by either DSLTA or SMAC, the referring physician was contacted and asked to provide the information listed below. If permission was granted by the physician, the patient was contacted directly to confirm or obtain further information. For specimens referred in by health units, the patient was contacted directly. The following information was recorded: patient age, sex, history of inflammatory bowel disease, other enteric pathogens isolated, diarrhea (≥3 stools in 24 h), nature of stools (color, consistency, and presence of blood or mucus), nausea, vomiting, abdominal cramps and severity, fever or chills, duration of symptoms, antibiotic use and response (if for treatment), hospitalization in association with present illness, and clinical outcome. Other information recorded included history of recent travel, water source (treated or untreated), food history (especially consumption of undercooked meats or unpasteurized milk), and any other family members with similar illness.

Patients were considered to have disease consistent with SLTEC infection if they had SLTEC isolated from their stools in association with (i) bloody diarrhea ± HUS or (ii) HUS after a recent (≤14 days) gastrointestinal illness.

RESULTS

Eighty of the 9,449 stools screened were positive by one or both methods: 59 (74%) and 74 (93%) were positive by SMAC and DSLTA, respectively, and 53 (66%) stools were positive by both methods (Table 1). None of the stools in which SLTEC was isolated had any other bacterial pathogen found. The isolation rate for any SLTEC from stools was 0.7% (67 of 9,449), and 90% of these SLTEC samples were

E. coli O157 strains. The 53 stools positive by both tests came from 48 patients, 35 (66%) of whom had bloody diarrhea with or without HUS, 11 (20%) of whom had nonspecific diarrheal illnesses, and 2 (4%) of whom were asymptomatic. The four stools positive only by SMAC and with *E. coli* O157:H7 came from four patients, three of whom had bloody diarrhea and one of whom had a nonspecific diarrheal illness. The two non-O157:H7 serotypes detected in the stools which were positive by SMAC but negative by DSLTA were O157:H? and O157:NM, and the patients had bloody diarrhea and nonspecific diarrhea, respectively.

The seven non-O157:H7 SLTEC serotypes detected in the DSLTA$^+$-SMAC$^-$ stools were O26:K60 (five stools), O128:K67 (one stool), and O103:H2 (one stool). These stools came from six patients, two of whom had bloody diarrhea (both with O26:K60 isolates). The first patient was a 50-year-old woman who had prepared a hamburger meal for her family and was the only one to become ill, and the second patient was a 7-month-old female whose parents had concomitant but nonbloody diarrhea. Three patients with serotypes O26:K60 (two patients) and O103:H2 (one patient) had nonspecific diarrheal illnesses. The sixth patient had severe abdominal cramps in association with gas and bloating but no diarrhea (serotype O128:H67). The one DSLTA$^+$-SMAC$^-$ stool from which *E. coli* O157:H7 was isolated came from an asymptomatic individual who had been screened during an investigation of an outbreak with the same organism.

The 13 stools in the DSLTA$^+$-SMAC$^-$-SLTEC$^-$ category came from 13 patients, and clinical information was available for 12. Of these 12 patients, 3 had bloody diarrhea and a fourth, a 4.5-year-old male, had HUS approximately 1 week after a nonspecific diarrheal illness. His 2-year-old sister was also ill with diarrhea, and *E. coli* O157:H7 was isolated from her stool (not included in this study). Six patients had nonspecific diarrheal illnesses. Of these, two had *Shigella sonnei* isolated from their stool cultures (both isolates negative for SLT production in vitro) and one (patient 70) had profuse watery diarrhea 4 days after eating a convenience store hamburger that was briefly microwaved. He submitted one stool on each of two successive days. The first stool, 5 days after the onset of illness, was DSLTA$^+$-SMAC$^-$-SLTEC$^-$, and the second stool was DSLTA$^+$-SMAC$^+$. One patient had a 3-day illness characterized by severe lower abdominal cramping but no diarrhea, and the final patient was an asymptomatic individual who was tested in a routine screening procedure prior to working in a watershed area.

It is unknown how many of the patients had stools submitted for ovum and parasite studies. One patient with a DSLTA$^+$-SMAC$^+$ stool had concomitant *Giardia lamblia*, but his illness was consistent with SLTEC infection: he was hospitalized for a bloody diarrhea after eating at a social function. Several of his friends who attended also became ill.

The average interval between the onset of illness and stool collection (O-C interval) in patients with DSLTA$^+$-SMAC$^\pm$-SLTEC$^+$ stools (i.e., DSLTA$^+$ where SLTEC was detected) and in those patients with DSLTA$^+$-SMAC$^-$-SLTEC$^-$ stools (i.e., DSLTA$^+$ where no SLTEC was detected) was 5.3 and 7.7 days, respectively. The O-C interval in patients with DSLTA$^-$-SMAC$^+$ stools was 3.5 days.

Toxin typing was performed on 70 stool supernatants and 59 SLTEC isolates. Overall, there was agreement between supernatant and isolate toxin typing in 43 of 52 (83%) stools in which SLTEC was found and the typing of both stool supernatant and isolate was possible. Of the 52 *E. coli* O157:H7 samples typed, 47 (90%) produced both SLT-I and SLT-II, 2 (4%) produced SLT-I only, and 3 (6%) produced SLT-II only. All five *E. coli* O26:K60 strains and the *E. coli* O103:H2 strain produced SLT-I only, and the serotypes O128:K67 and O157:H? produced both SLT-I and SLT-II. *E. coli* O157:NM was unavailable for toxin testing. For the stools in which no SLTEC isolates were found but which were DSLTA positive, five were typed as having SLT-II only, four had SLT-I only, and two had SLT-I and II. Seven stools had supernatants with only SLT-II detectable but had SLTEC isolates which were all SLT-I and SLT-II producers.

DISCUSSION

Despite the growing evidence that direct fecal SLT detection is the preferred method for diagnosis of SLTEC infections (3, 4, 7, 8, 10, 11, 13, 19), there have been no studies to date directly comparing the performance of fecal SLT detection with that of SMAC in unselected stool specimens from a general population. Our results show that screening stool supernatants for free fecal SLT is preferable to SMAC for both the detection of non-O157-serotype SLTEC (10% of all SLTEC detected in this study) and the establishment of an etiology of bloody diarrhea or HUS when no SLTEC can be isolated from stools. This confirms the findings of previous studies which employed a variety of methods to screen for SLTEC, including picking between 3 and 20 colonies from a MacConkey or sorbitol-MacConkey plate (5, 8), use of a polymyxin extraction-colony sweep technique (4, 11), and use of specific DNA probes for SLT-I or -II genes (13, 18). In each of these studies there were stool specimens from patients with HUS or hemorrhagic colitis in which the free fecal SLT assay was the only positive test.

We have also shown that both tests identify patients from across the spectrum of clinical syndromes produced by SLTEC organisms, from the asymptomatic individual to those with bloody diarrhea or HUS. Neither test detects all positives. In particular, some specimens can be positive by SMAC yet negative by DSLTA. These specimens tended to be collected earlier in the course of the illness, as shown by the smaller O-C interval, and we speculate that free toxin levels were not yet high enough to be detected in the stools.

From the clinical perspective, a positive DSLTA was associated with SLTEC isolation and/or a typical syndrome of bloody diarrhea or HUS in 90% (66 of 73) of cases, despite our use of a fairly insensitive technique to screen for SLTEC colonies. This screening method (20 colonies picked from either the SMA or blood agar plates) is labor intensive and certainly not as sensitive as the DNA probe technique used by Scotland et al. (13) which can detect as few as 1 in 1,200 SLTEC colonies, but it is a method easily done in any diagnostic laboratory. Until methods such as the polymerase chain reaction or DNA probing become more widely accepted or an assay such as the enzyme-linked immunosorbent assay is made commercially available (1), diagnosis of SLTEC other than O157:H7 will remain cumbersome for the general diagnostic laboratory.

Five of 12 (42%) DSLTA$^+$-SLTEC$^-$ stools came from patients with typical SLTEC disease (bloody diarrhea or HUS) or a patient from whom SLTEC was subsequently isolated (patient 70). We believe that these are true positives for the following reasons. First, it has been shown that cultures usually become negative within 4 days of the onset of illness and that toxin can still be detected in the stool long after the organism has disappeared (10). Four of these five patients had stools collected after the fourth day of illness. And second, on questioning the patients and by the labora-

tory tests, there was no clinical, epidemiological, or bacteriologic evidence to suggest any other cause for their symptoms.

Further studies are needed to clarify the significance of the 7 of 12 DSLTA$^+$-SLTEC$^-$ stools from patients with nonspecific diarrhea or no illness. However, with the knowledge that 28% of SMAC-positive specimens in this study came from patients with no symptoms or nonspecific diarrheal illnesses, it is clearly possible that these are true positives. Use of a more sensitive culture-screening technique may have resolved some of these.

The typical syndrome of hemorrhagic colitis was seen in only 39 of 54 (72%) of our patients with O157-serogroup SLTEC. This differs somewhat from the study by Pai et al. (9) in which they state that "almost all" index cases with *E. coli* O157:H7 had hemorrhagic colitis. Our rate of hemorrhagic colitis in patients with non-O157 SLTEC (33%) was similar to theirs. Interestingly, 90% of our *E. coli* O157:H7 isolates produced both SLT-I and -II, compared with 70% of those isolates from Washington state (18), which is geographically adjacent to British Columbia.

In summary, we have shown that direct fecal SLT detection picks up 19% more positive specimens than SMAC and that neither test alone will diagnose all cases of disease. By using the DSLTA we have shown that SLTEC serotypes other than serotype O157:H7 are present in the province of British Columbia, Canada, and are causing disease which would otherwise have gone undiagnosed. However, we also found that 7% of positives were detected only by the SMAC and that DSLTA may be falsely negative early in the course of the disease.

As a result of this study, we continue to use the DSLTA in parallel with SMAC at the Provincial Health Laboratory in British Columbia. The procedure has been modified to screen stool supernatants for verocytotoxicity and to use the neutralization step for confirmation of the positive results. The technologists find the assay easy to set up and have quickly become proficient at reading the plates. Other types of toxicity, e.g., that of *Clostridium difficile*, have been observed but are readily discernible by comparison with the SLT and neutralization controls. For practicality's sake, it is impossible for us to recommend that all clinical laboratories use the DSLTA—not all laboratories have the facility to do cell culture techniques or technologists with time to devote to it. We do recommend, however, that studies such as this be carried out in other regions to obtain prevalence data for non-O157:H7 illness and that these data then be used to decide whether DSLTA should be a routine test. We also recommend that reference laboratories make the test available, especially for outbreak situations and cases of HUS in which stools may be collected some time after the enteric illness.

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