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Sensitive Receptor-Specified Enzyme-Linked Immunosorbent Assay for *Escherichia coli* Verocytotoxin

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The specific identification of verocytotoxin (VT)-producing *Escherichia coli* (VTEC) requires the detection of VTs in bacterial culture filtrates or the detection of genes encoding these toxins in bacterial cells by specific DNA probes. The standard method for detecting these toxins involves a time-consuming, labor-intensive, and expensive cytotoxicity assay. We have developed a specific, highly sensitive receptor-specified enzyme-linked immunosorbent assay (RELISA) to detect VT1, one of at least two VTs implicated in human disease. The assay is based on the affinity of VT1 for the glycolipid globotriosyl ceramide (Gb_3). Gb_3 was de-N-acylated to yield lyso-Gb_3, which is more polar but retains VT1 binding. Lyso-Gb_3 was used to sensitize microdilution plates to bind VT1 for subsequent immunodetection. This RELISA was used to detect VT1 in the culture supernatant of a variety of bacteria of known VT status. The assay was compared with the highly sensitive cell cytotoxicity assay for their abilities to detect VT. The RELISA was as sensitive as the cytotoxicity assay and, in a blind study, 100% specific. This assay will provide a quick, specific, efficient adjunct to the diagnosis and epidemiological study of VTEC infections and their relationship to human disease.

The significance of the apparent relationship between verocytotoxin (VT) (also referred to as Shiga-like toxin [SLT])-producing *Escherichia coli* (VTEC) and a wide spectrum of illnesses (8), including mild to moderate diarrhea, hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura, lies in reports of the isolation of VTEC or detection of free VT in stools of humans suffering from these illnesses in both sporadic cases (11, 20) and outbreaks (20). Indeed, it has been suggested that in some centers, VTEC are one of the major causes of diarrhea (20), rivaling *Campylobacter* and *Salmonella* spp. in the frequency with which they cause diarrhea.

Although VTEC were discovered in 1977 (13), epidemiological studies as well as systematic studies to define the prevalence of VTs among *E. coli* strains and to establish whether other serotypes or serovariants exist among the VTs of *E. coli* have been limited by the labor-intensive assay method for VT, which involves the determination of cytotoxicity to cells in culture. In view of the important clinical implication of this organism, a rapid, easily performed, specific, and sensitive test for detection of VT is vital for effective diagnosis and management of patients. In the present work, we describe a novel receptor-binding immunoassay for detection of VT which meets these criteria. In the assay we used the glycolipid globotriosyl ceramide [Gal(α1–4)Gal(β1–4)Glc-ceramide (Gb_3)] as a specific receptor for VT in an enzyme-linked immunosorbent assay (ELISA). We report here on the successful sensitization of the solid phase with deacylated Gb_3 (lyso-Gb_3) as a capture molecule for VT. A comparison of the receptor-specified ELISA (RELISA) sensitivity and specificity with that of the VT cell culture assay usually used for detecting VT is also presented.

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MATERIALS AND METHODS

Bacterial strains, medium, and cultures. For a list of the bacterial strains used in this study and their sources, see Table 2. The strains were maintained in cooked-meat medium and subcultured for purity onto 5% horse blood agar plates (Columbia blood agar base; GIBCO Diagnostics, Madison, Wis.). Cultures to be tested for VT were grown in Penassay broth (antibiotic medium no. 3; Difco Laboratories, Detroit, Mich.), which was incubated for 18 h at 37°C.

Cytotoxicity tests. Samples to be tested (culture supernatants) were filtered through membrane filters (pore size, 0.22 μm; Millipore Corp., Bedford, Mass.). Serial twofold dilutions of the filtrates (50 μl) were inoculated into a microdilution Vero cell assay system as described previously (10, 11). The microdilution plates were incubated at 37°C in a 5% CO_2 atmosphere and examined daily for the characteristic cytopathic effect of VT (13). The endpoint was taken as the highest dilution (expressed as a titer) of the sample that killed 50% of the Vero cell monolayer after 3 days of incubation. Each sample was tested for VT in triplicate, and the results were recorded as the geometric mean titer of the three assays.

Purification of VTs. VT1 was purified from *E. coli* H.30 (reference strain), as described previously (22), by ammonium sulfate precipitation, hydroxyapatite chromatography, chromatofocusing, Cibachron Blue chromatography, and gel filtration.

Antibodies. Rabbit anti-VT1 antibodies were generously supplied by S. Richardson, Department of Microbiology, The Hospital for Sick Children, Toronto, Ontario, Canada. These antisera were monitored for VT-neutralizing antibody as previously described (10) and had a VT1 neutralizing antibody titer of 1:2,500. The immunoglobulin G fraction was prepared by using protein A-Sepharose as described previously (5). Mouse monoclonal antibody 13C4 against the B subunit of Shiga-like toxin (SLTI) from *E. coli* was kindly provided by N. A. Strockbine, Centers for Disease Control,

Atlanta, Ga., as aliquots of cell culture supernatants and kept at −70°C until used.

Glycolipid receptor and deacylated glycolipid. Gb_3 was purified from human kidneys as described previously (1). The procedure for removal of the amide-linked fatty acid moiety to form lyso-Gb_3 was as follows. Aliquots (100 μg) of Gb_3 were each dried in a tube with a tight-fitting cap containing a Teflon liner and then dissolved in 1 ml of 1 N methanolic sodium hydroxide. The solution was maintained at 100°C for 3 h, acidified to pH 5 with 1 N HCl, and rotorevaporated to dryness. Chloroform-methanol (2:1, vol/vol) (2 ml) was added to the flask containing residue and gently sonicated. The mixture of lyso-Gb_3 and salt was filtered through glass wool, and the filtrate was dried. The filter was washed through with chloroform-methanol (2:1, vol/vol), and the combined filtrates were partitioned against 2 volumes of water. Approximately 50% deacylation was obtained. The upper phase was dried and used in the RELISA.

Glycolipid-binding VT. Gb_3 and lyso-Gb_3 (2.5 μg each) were separated by thin-layer chromatography (chloroform-methanol-water (60:40:9, vol/vol/vol) and tested for VT1 binding (17) as follows. The plate was blocked in 50 mM Tris-saline (pH 7.4) containing 3% gelatin at room temperature overnight. It was washed twice and incubated with VT (titer 5×10^3 [final concentration]) for 2 h at room temperature. It was then washed five times and incubated with rabbit anti-VT1 for a further 2 h at 4°C. After a further five washings, peroxidase-conjugated goat anti-rabbit IgG was added for 2 h at room temperature. Chloro-1-naphthol peroxidase substrate was added after washing as previously described (17).

RELISA. Flat-bottom wells in Costar microdilution plates (Bio-Rad Laboratories, Richmond, Calif.) were each coated by incubation with 100 μl of lyso-Gb_3 (10 μg/ml) diluted (from a stock solution of 1 mg per ml of methanol) in 0.01 M phosphate-buffered saline (pH 7.2) (PBS) overnight at room temperature. The solution was then removed, and the wells were washed three times with PBS. The wells were filled with 2% bovine serum albumin (BSA) dissolved in PBS, and the plates were incubated for 2 h at room temperature. They were then washed twice with PBS containing 0.05% Tween 20 (PBS-Tween). VT1 standard or culture supernatants of the isolated strain or known strains positive and negative for VT1 (100 μl) were diluted 1:10 in PBS-Tween and added in duplicate to each well. The plates were washed again three times with PBS-Tween after incubation for 1 h at room temperature. An optimal dilution of rabbit anti-VT1 or monoclonal anti-SLTI in PBS-Tween was added to each well (100 μl per well). The plates were allowed to incubate for a further 2 h at room temperature and were then washed three times with PBS-Tween. Peroxidase-conjugated anti-rabbit immunoglobulin G (or anti-mouse when appropriate) (Bio-Rad) diluted in PBS-Tween containing 2% BSA was added to all the wells, and the plates were incubated for 1 h at 37°C. Following three further washings, 100 μl of freshly made substrate solution containing 0.04% *o*-phenylenediamine and 0.045% H_2O_2 in phosphate-citrate buffer (pH 5) was added to each well and allowed to react for 20 min at room temperature. The enzyme reaction was stopped by the addition of 20 μl of 2 M sulfuric acid. Optical densities were measured at 492 nm by using a microplate ELISA reader.

RESULTS

Assay optimization. Attempts to develop a RELISA for detection of VT were initially disappointing. Results were erratic and unreliable when attempts were made to absorb Gb_3 glycolipid to a solid phase. This discrepancy was presumably due in part to the hydrophobic nature of the glycolipid and to nonuniform distribution of glycolipid when Gb_3 was used to sensitize the solid phase.

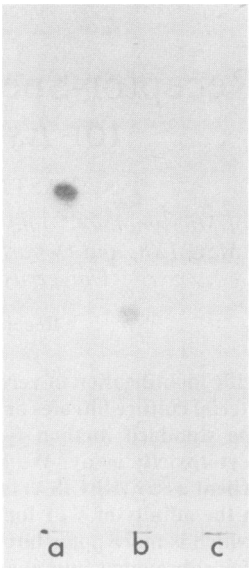

FIG. 1. VT1 binding to Gb_3 and lyso-Gb_3. Lyso-Gb_3 was prepared from Gb_3 by basic hydrolysis, as described in Materials and Methods. The deacylated glycolipid and native Gb_3 (2.5 μg of each) were separated by thin-layer chromatography and tested for binding by VT1 overlay (1). Lanes: a, Gb_3; b, lyso-Gb_3; c, digalactosyl diglyceride.

Deacylation of the VT receptor, Gb_3, results in the production of a less hydrophobic derivative (lyso-Gb_3) of the receptor, which remains an effective receptor for VT1 (Fig. 1). (Digalactosyl diglyceride, a glycerol base glycolipid that contains the same terminal carbohydrate sequence as Gb_3 but does not bind VTs [17, 26], was not recognized.) The release of the fatty acid makes lyso-Gb_3 more soluble in aqueous buffer solution. When lyso-Gb_3 at neutral pH was used to coat wells for RELISA, the assay results were highly reproducible in 10 replicate tests of toxin specimens in parallel (standard deviation, ±0.05) and in repeated tests performed on the same specimens 10 times on different days (standard deviation, ±0.065).

The optimal dilutions of the reagents used were determined by checkerboard titrations. Microdilution plates sensitized with increasing concentrations of lyso-Gb_3 were tested for the ability to bind VT1. The results indicate that the sensitivity of the assay is dependent on the immobilized receptor concentration (Fig. 2). We were unable to demonstrate any significant improvement in the sensitivity of the assay when more than 1,000 ng of lyso-Gb_3 was used to sensitize each well of the microdilution plate.

Sensitivity and specificity of RELISA. The sensitivity of the RELISA to purified VT1 was first determined. When 1 μg of lyso-Gb_3 was used to sensitize each well, the assay was sensitive enough to detect 5 pg of purified VT1 per well (Fig. 3). The lowest detectable concentration of VT1 was taken as the concentration which resulted in an optical density twice that of the medium control (unused culture medium substituted for VT1). The sensitivity could be increased by increasing the volume of sample, but this was deemed unnecessary in light of subsequent results. For culture

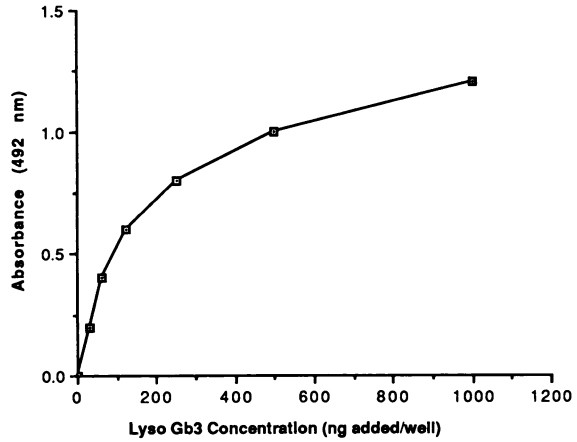

FIG. 2. Dose-response curve for lyso-Gb_3 in the RELISA. Increasing concentrations of lyso-Gb_3 were used to sensitize microdilution plates for VT1 detection. The assay was carried out as described in Materials and Methods, with 200 pg of VT1 per well.

supernatants, the RELISA was at least as sensitive as the cytotoxicity assay. A quantitative comparison for 10 different *E. coli* strains showed a remarkable correlation between VT titers measured by the two assays (Fig. 4).

The specificity of the RELISA was demonstrated by the negative results obtained with five different *E. coli* strains (heat-labile-enterotoxin-producing *E. coli*, heat-stable-enterotoxin-producing *E. coli*, enteroinvasive *E. coli*, reference *E. coli* ATCC 25922, and SLTII-producing *E. coli*). None of these strains produced cell cytotoxic activity that was neutralizable with antibody to VT1. Before any analysis was performed, it became evident that there were differences in cutoff points depending on the type of anti-VT antibodies used. A cutoff value above which RELISA results would be considered positive was established as twofold higher than the mean of the optical densities of the above five *E. coli* strains (Table 1). Values of 0.32 (2 × 0.16) and 0.18 (2 × 0.09) were chosen as cutoff points when rabbit anti-VT1 and monoclonal anti-SLTI were used, respectively.

Comparison of the RELISA with VT cytotoxicity assay. After standardization of the RELISA parameters, 39 coded culture supernatants of isolated or reference bacterial strains were tested blindly by the RELISA. These results were then compared with those of the cytotoxicity assay performed at

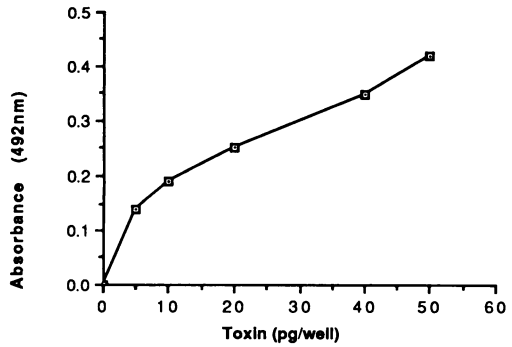

FIG. 3. Dose-response curve for purified VT1 in the RELISA. The RELISA (at 1 μg of lyso-Gb_3 per well) was used to detect increasing concentrations of purified VT1.

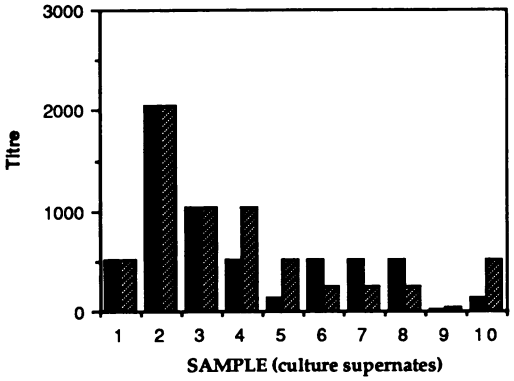

FIG. 4. Comparison of RELISA and cytotoxicity assay for VT1. Ten VTEC strains from different human isolates were grown in Penassay broth and, centrifuged and the titer of VT1 in the supernatant was measured simultaneously by the standard cytotoxicity assay (■) and the RELISA (▨).

the same time (Table 2). Of the 39 strains tested, 19 were positive by the cytotoxicity assay. Of the 19 strains, 15 were also positive by RELISA. Cytotoxin neutralization experiments with specific antisera showed that the four cytotoxin-positive, RELISA-negative cultures contained VT2 or SLTII only. One culture supernatant initially identified as producing SLTII only by cytotoxicity was rechecked on testing positive by RELISA and was found to produce both VT1 and VT2 (Table 2). One strain producing VT2 only, however, was detected in the RELISA. Thus, the overall agreement between the two assays was 98%.

DISCUSSION

VTEC have been recently recognized as significant human pathogens (8, 10, 11). These infections may result in mild diarrhea, hemorrhagic colitis, or renal endothelial lesions, leading to fibrotic occlusion of capillaries and hence to hemolytic uremic syndrome (10). The factors responsible for these different clinical scenarios are unknown but are likely to lie in a differential response to systemic verocytotoxemia. It is of interest that human kidneys contain high levels of Gb_3 but that kidney tissue of other mammals (which is generally unaffected during similar infections) contains no Gb_3 (1).

Epidemiological studies to establish the incidence of VTEC infections have been limited because of the lack of rapid and simple assays. The standard method for detecting these toxins is the expensive and labor-intensive cytotoxicity assay, which requires extensive cell culture facilities and expertise that is not generally available. The most sensitive approach for diagnosing VTEC infection in patients involves the detection of VT in fecal filtrates. VT is one of the most potent biological toxins. For this reason, classical ELISAs have been, for the most part, unsuccessful as monitors of VT (and therefore of VTEC infection) (12, 25). One ELISA method was able to detect Shiga toxin only at the level of 10 ng/ml (12), which is 200-fold less sensitive than the present RELISA. A more sensitive double-antibody ELISA was devised (4), but this was liable to interference by anti-immunoglobulin in stools (4). A colony ELISA has recently been developed for the detection of SLTI (25) and SLTII (21), but this was still less sensitive than the cytotoxicity assay. The present RELISA has overcome this shortfall in sensitivity by effectively concentrating the toxin from the sample for subsequent immunological detection.

TABLE 1. Results of RELISA with monoclonal and polyclonal anti-VT1 antibodies

Antibodies used	Optical density of supernatant of:						
	E. coli (VT1)	*E. coli* (heat-labile toxin)	*E. coli* (heat-stable toxin)	*E. coli* (EIEC)	*E. coli* ATCC 25922	*E. coli* (VTE)	*E. coli* (VT2)
Monoclonal antibodies	1.40	0.08	0.08	0.09	0.09	ND[a]	0.09
Immunoglobulin G fraction of polyclonal antibodies	1.26	0.16	0.15	0.19	0.15	0.16	0.17

[a] ND, Not determined.

We have previously demonstrated that both VT1 (17) and VT2 (26) specifically recognize the neutral glycolipid Gb_3 and that this glycolipid was likely to function as the receptor for these toxins in sensitive cells (3) and in human renal tissue (1). An ELISA in which this receptor is used might have the high sensitivity needed to match the sensitivity of the in vitro cell cytotoxicity assay. ELISAs for glycolipids have been reported (6, 7, 14, 15), but we were unable to achieve reasonable sensitivity or reliability by direct immobilization of Gb_3 in ELISA plates.

Other workers have coupled the carbohydrate sequence of Gb_3 to BSA (14) and used the conjugate in a RELISA (14, 15). The resulting assay has not been as sensitive as the cell culture procedure, and the degree of carbohydrate coupling to the BSA has been found to dramatically affect toxin binding (14). Indeed, it has been reported that Galα1–4Gal-BSA is 100-fold more sensitive than the native Gb_3-based RELISA in detecting Shiga toxin (15). Nevertheless, this assay was still much less sensitive than the cell cytotoxicity assay (15). We have demonstrated the importance of the lipid moiety in VT binding to Gb_3 (17, 26). We therefore decided to prepare lyso-Gb_3 to obtain a more water-soluble derivative of the toxin receptor for more efficient immobilization on the ELISA plate. The charge on the amino group would also facilitate immobilization.

Lyso-Gb_3 was prepared as described in Materials and Methods and found to bind VT1 in a manner similar to Gb_3 as monitored by the thin-layer chromatography overlay procedure (Fig. 1). Binding to Gb_3 and lyso-Gb_3 on the thin-layer chromatography plate cannot be directly compared, since lyso-Gb_3 is preferentially lost during the various washing steps. However, this experiment demonstrates that lyso-Gb_3 remains an effective receptor for VT1. The lyso-Gb_3 was purified and used to coat microdilution plates for the RELISA. By using a standard preparation of purified VT1 (22), a linear binding response was obtained between concentrations of 0 and 150 ng of lyso-Gb_3 added per well (Fig. 2). A linear response with increasing toxin concentration was obtained over the range of 5 to 50 pg of VT per well (Fig. 3). Under the standard conditions of the RELISA, we were able to detect VT levels down to 5 pg (Fig. 3). This is not quite as sensitive as the cell culture cytotoxicity procedure, which can detect purified toxin down to levels of 1 pg. However, when the RELISA was compared with the cell cytotoxicity assay for detecting VT in the culture supernatant of various VTEC strains, the RELISA (Fig. 4) was found to be at least as sensitive as the cell culture procedure. Since the RELISA will not be used to detect purified toxin, a comparison between the ability of the two assays to detect VT in a complex mixture of other proteins is more appropriate. In the measurement of toxin titer in the culture supernatants of various VTEC strains (Fig. 4), lower values of, at most, one well in double dilutions were obtained for the RELISA, whereas some RELISA values were two wells higher than the cytotoxicity assay. This suggests that the RELISA may be even slightly more sensitive than the cytotoxicity assay.

The Gb_3 RELISA showed no cross-reactivity with a variety of other *E. coli* products, including heat-labile and heat-stable toxins, VTE (the VT from the *E. coli* strain causing pig edema disease [16, 19]), and VT2 from *E. coli* that produces only this toxin (Tables 1 and 2). The results of experiments in which polyclonal as opposed to monoclonal anti-VT1 antibodies were used show slightly higher nonspecific background values; however, the overall specificity is equivalent for both antibodies (Table 1).

The specificity of the assay was convincingly demonstrated in a blind study of the culture supernatants of a variety of bacteria (Table 2). Only culture supernatants from *E. coli* producing VT1 and from *Shigella dysenteriae* type 1, which produces Shiga toxin (which is virtually identical to VT1 [24]), were positive in the RELISA. It is of interest that the strains of *Shigella sonnei* and *Shigella flexneri* used in this study were negative in both the RELISA and the cytotoxicity assay (Table 2), but other investigators have reported that strains of these species are positive for Shiga toxin by ELISA (4). Although VT2- and SLTII-producing *E. coli* strains were positive by the cytotoxicity assay, they were negative by the VT1-specific RELISA. A large variety of toxigenic and nontoxigenic bacterial strains were negative. CL15 was the sole exception, being neutralized in the cytotoxicity assay only by anti-VT2. Although anti-VT1 and anti-VT2 do not cross-neutralize, they do cross-react with the heterologous toxin, which must provide the basis for this reaction. Positive *E. coli* strains were of various serotypes, including O157:H7. One clone, *E. coli* KC6-1, was originally typed by antibody cytotoxicity neutralization tests as only an SLTII producer, but was rechecked for VT1 production following a positive reading in the RELISA and was subsequently found to be positive for VT1 by the cytotoxicity assay. Thus, in this instance, the RELISA was more sensitive than the cell cytotoxicity assay. A final correlation of 98% between the cell cytotoxicity assay and the RELISA was obtained.

It is evident that far more serotypes than O157:H7 are positive for VT, and hence the use of selective media specific for this serotype, such as sorbitol-MacConkey agar (18), will not result in the isolation of all VTEC serotypes.

The RELISA can be performed in 1 day, particularly if the receptor plates are prepared beforehand. In this regard, we have found that ELISA plates sensitized with lyso-Gb_3 are stable for at least 6 months at −20°C without any loss of VT1-binding capacity. The availability of such a quick, reliable, sensitive, and specific assay for the detection of VT1 in clinical specimens will facilitate rapid diagnosis and greatly aid the study of the epidemiology of these infections. The assay as described detects only VT1; however, use of anti-VT2 instead of anti-VT1 would render the assay capable of detecting VT2, and a mixture of both antibodies would detect both toxins. Further development may be required to

TABLE 2. Blind study of RELISA specificity

Culture	Serotype	Cytotoxicity assay result	Toxin	Gb_3 RELISA result
E. coli CL19(H30)[a]	O26:H11	+	VT1	+
E. coli CL13B[b]	Nontypable	+	VT1	+
E. coli CL16 (H19)[a]	O26:H11	+	VT1	+
E. coli E32511[c]	O157:H$^-$	+	VT2	−
E. coli B2FI[b]	O91:H21	+	VT2	−
E. coli CL3[b]	O113:H21	+	VT1, VT2	+
E. coli CL8[b]	O157:H7	+	VT1, VT2	+
E. coli CL12[b]	O111:H$^-$	+	VT1, VT2	+
E. coli CL40[b]	O157:H7	+	VT1, VT2	+
E. coli CL5[b]	O26:H11	+	VT1	+
E. coli CL15[b]	O113:H21	+	VT2	+
E. coli CL37[b]	O111:H8	+	VT1	+
E. coli CL55A[b]	Nontypable	+	VT1	+
E. coli CL56A[b]	O157:H7	+	VT1, VT2	+
E. coli R29[d]	O157:H7	+	SLTII	−
E. coli R30[d]	O157:H7	+	SLTII	−
E. coli CL112 (TD213C2)[e]		−	ST+	−
E. coli CL113 (TD427C2)[e]		−	LT+	−
E. coli CL114 (enteroinvasive)[e]	O164:H$^-$	−		−
S. flexneri S01983-83[f]		−		−
S. flexneri 1832-2[f]		−		−
S. sonnei 589[f]		−		−
S. sonnei S9228-71[f]		−		−
S. dysenteriae type 1 C3163-81[g]		+	Shiga	+
S. dysenteriae type 1 C6352-80[g]		+	Shiga	+
E. hermannii[g]		−		−
E. coli KC6-1[b]	O111:H8	+	VT1, SLTII[i]	+
E. coli S769522[f]	O111:H21	−		−
E. coli S761046[f]	O111:H21	−		−
E. coli S745267[f]	O111:H21	−		−
E. coli ATCC 25922		−		−
E. coli 82[h]		−		−
E. coli 84[h]		−		−
E. coli 85[h]		−		−
E. coli 90[h]		−		−
E. coli 97[h]		−		−
Salmonella agona S3020164[f]		−		−
Salmonella hadar S8013925[f]		−		−
Salmonella typhimurium S8016283[f]		−		−

[a] Reference strains provided by J. Konowalchuk, Ottawa, Ontario, Canada.

[b] Fecal isolates from patients with hemolytic uremic syndrome at the Hospital for Sick Children.

[c] Reference strains for VT2 provided by B. Rowe, Public Health Laboratory, Colindale, London, England.

[d] Reference strains for SLTII provided by I. K. Wachsmuth, Centers for Disease Control.

[e] Reference strains positive for heat-stable (ST) and heat-labile (LT) enterotoxins and enteroinvasion provided by H. Lior, Laboratory Centre for Disease Control, Ottawa, Ontario, Canada.

[f] Fecal isolates of *Shigella* spp., enteropathogenic *E. coli* serotype O111, and *Salmonella* spp. from patients with diarrhea at the Hospital for Sick Children.

[g] Reference strains provided by S. Toma, Ontario Ministry of Health.

[h] Isolates from nonenteric clinical sources at the Hospital for Sick Children.

[i] *E. coli* KC6-1 was originally typed by cytotoxicity as an SLTII producer but was rechecked for VT1 following the RELISA result and found to be positive.

enable the RELISA to detect VT in fecal filtrates and thus generate an assay that will have a wide clinical application. Such an assay would be a great benefit in management of patients, i.e., determination of people at risk and isolation of those infected, since it has been shown that these *E. coli* strains can be transmitted via contact (2, 9).

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