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Sensitive Detection of Shiga Toxin 2 and Some of Its Variants in Environmental Samples by a Novel Immuno-PCR Assay▽

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Shiga toxin-producing *Escherichia coli* (STEC) in the environment has been reported frequently. However, robust detection of STEC in environmental samples remains difficult because the numbers of bacteria in samples are often below the detection threshold of the method. We developed a novel and sensitive immuno-PCR (IPCR) assay for the detection of Shiga toxin 2 (Stx2) and Stx2 variants. The assay involves immunocapture of Stx2 at the B subunit and real-time PCR amplification of a DNA marker linked to a detection antibody recognizing the Stx2 A subunit. The qualitative detection limit of the assay is 0.1 pg/ml in phosphate-buffered saline (PBS), with a quantification range of 10 to 100,000 pg/ml. The IPCR method was 10,000-fold more sensitive than an analogue conventional enzyme-linked immunosorbent assay (ELISA) in PBS. Although the sensitivity of the IPCR for detection of Stx2 was affected by environmental sample matrices of feces, feral swine colons, soil, and water from watersheds, application of the IPCR assay to 23 enriched cultures of fecal, feral swine colon, soil, and watershed samples collected from the environment revealed that the IPCR detected Stx2 in all 15 samples that were shown to be STEC positive by real-time PCR and culture methods, demonstrating a 100% sensitivity and specificity. The modification of the sandwich IPCR we have described in this study will be a sensitive and specific screening method for evaluating the occurrence of STEC in the environment.

Shiga toxin-producing *Escherichia coli* (STEC) is a frequent cause of food-borne outbreaks of diarrhea and hemorrhagic colitis (26) and can produce the life-threatening complication of hemolytic-uremic syndrome (29). STEC strains comprise a group of >150 serovars (2), with STEC O157:H7 reported as the most common serotype associated with human diseases (36). However, serovars O26, O45, O103, O111, O121, and O145 have emerged as other important STEC serovars associated with human illness in the United States (5).

Shiga toxins (Stxs) are the major virulence factors contributing to STEC pathogenicity. Stxs are AB_5 holotoxins and are comprised of one A subunit (32 kDa) and five B subunits (7.7 kDa) (13, 14). The Stx A subunit is an enzymatically active *N*-glycosidase that inhibits the activity of rRNA by cleavage of an adenine base from the 28S rRNA component of the eukaryotic ribosomal 60S subunit, causing protein synthesis to cease and resulting in cell death (10). The Stx B subunit is responsible for binding to host cells through interaction with globotriaosylceramide (Gb3) present on the surfaces of cells (28), leading to subsequent internalization of the toxin. Stx2e is an exception, binding preferentially to globotetraosylceramide (Gb4) (8). Stx genes are carried by bacteriophages in *E. coli* and comprise two major groups, Stx1 and Stx2 (37). The expression of both Stx1 and Stx2 is linked directly to the phage lytic cycle (48) and is induced by DNA-damaging agents such as mitomycin C (31). Recent epidemiological and molecular typing studies have suggested that STEC strains expressing Stx2 may be more virulent than strains expressing either Stx1 or both Stx1 and Stx2 (4, 40).

The Stx2 group has several distinct variants (18, 33), and the Stx2, Stx2c, Stx2d, and $Stx2d_{activatable}$ variants are reported most frequently as causing human illness (34, 39). Stx2e is associated primarily with the edema disease of swine (49) and is rarely isolated from humans (24, 30). Stx2f has been isolated from feral pigeons (45), but STEC strains harboring Stx2f were recently reported to cause human illness (42). Sequence analysis revealed that Stx2e and Stx2f display the most divergence from Stx2 at the amino acid level. The expanding number of Stx2 variants discovered in diverse environmental reservoirs and subtle differences in DNA and encoded amino acid structures emphasize the need for improved methods for sensitive and specific detection of these toxins.

Ruminants are the major known reservoir of STEC strains (16, 19, 22), and food-borne transmission of pathogens is the most common means of infection (5). Although the occurrence of STEC strains in the environment has been reported in numerous studies (9, 32), the evaluation of STEC in environmental samples is still difficult because of the large numbers of nontarget bacteria in complex environmental samples such as feces, water, plants, and soil and the small number of pathogens needed to cause illness (7, 47). Culture methods have been the "gold standard" for detection of STEC strains in environmental samples (3, 43). However, they are time-consuming and require well-trained technologists to review culture plates. There are several commercial media (e.g., sorbitol MacConkey agar, Rainbow agar, and Chromagar O157) available

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TABLE 1. Characteristics of STEC strains used to obtain pure bacterial culture supernatants

Strain	Other name	Serotype	Stx type	Origin	Reference
RM4876		O157:H7	Stx negative[a]	Watershed, Monterey County, CA	44
RM1625	KSU-33	O157:H7	Stx2c	Cow feces	27
RM2084	EDL933	O157:H7	Stx2	Meat, outbreak	38
RM7005	EH250	O118:H12	Stx2d	Human, clinical	41
RM7110	S1191	O139:NM	Stx2e	Pig	49
RM7007	T4/97	O128:H2	Stx2f	Feral pigeon	45

[a] The strain is *rfbE*$^+$ *eae*$^+$ *hlyA*$^+$ as determined by previously described methods (7).

that permit screening for O157 STEC by culture within 24 h, based on the presence of biochemical markers. However, there are no comparable culture methods for detecting non-O157 STEC. In addition, culture methods may underestimate the number of bacteria due to the stress caused by some environmental factors, such as changes in osmolarity, lowered pH, nutrient starvation, and UV irradiation, or the need for specific nutritional requirements that make it difficult to grow bacteria on agar plates. Although cultural isolation remains important for more complete characterization of STEC strains in a sample (e.g., genotyping for microbial source tracking), a rapid and sensitive method is desirable for identifying positive samples and/or for situations requiring a rapid response.

A variety of methods have been developed for the identification of STEC strains in test samples that are less complex than many environmental samples or in artificially inoculated food samples (3, 15). These methods detect Stx produced by bacterial strains or screen for genes encoding Stx production. For example, colony immunoblot assays and enzyme-linked immunosorbent assay (ELISA) rely on the use of antibodies to capture bacterial cells or Stxs released after bacterial lysis (3, 46). These assays are easy to use but usually are not sensitive enough to detect the small numbers of pathogens in environmental samples (3). PCR methods have been developed to improve the speed of detection of STEC strains in stool and environmental samples through amplification of genes coding for virulence factors, and real-time PCR methods have also increased the speed and sensitivity of detection of potential STEC strains (17, 23). However, these approaches are limited by the small sample volumes designed for reactions, in light of the low concentrations of STEC in naturally contaminated environmental samples. Thus, concentration of STEC from large sample volumes is an additional step required for sensitive detection with current methods. Moreover, the presence of PCR inhibitors in complex matrices often leads to a high frequency of false-negative or unclear results (11), and false-positive results can also be obtained if cryptic target gene sequences (such as free toxin phages or defective Stx genes in bacteria) are present in the sample or if PCR amplification products from alternate DNA sequences are formed (3, 25). Cell-free (21) and cell-based (3, 44) cytotoxicity assays based on the activity of Stx produced by STEC have also been used, but the sensitivity of these assays to the presence of potential active components and inhibitors limits their application to complex samples. Highly sensitive, rapid, and specific assays are required to increase the effectiveness of detecting STEC strains and to prevent potential outbreaks, and immuno-PCR (IPCR) combines the advantages of specificity of an immunoassay and magnification by PCR, as reported previously for detection of trace amounts of antigens (1, 6, 12, 20, 35, 50). We developed a novel IPCR assay that detects Stx at lower concentrations than those for any other reported IPCR detection system. This assay can selectively capture and concentrate Stx, thus minimizing the effects of the surrounding matrix on the detection of Stx2 and Stx2 variants produced by STEC strains present in naturally contaminated fecal, feral swine colon, soil, and watershed samples. The sensitivity of the IPCR assay is significantly improved over that of a conventional ELISA for detecting Stx in environmental samples.

MATERIALS AND METHODS

Stx2 and antibodies used in IPCR. Purified Stx2 was purchased from List Biological Laboratories Inc. (Campbell, CA). Mouse monoclonal antibodies (MAbs) against Stx2, designated, Sifin 2A (clone VT135/6-B9) and Sifin 2B (clone VT136/8-H4), were purchased from Sifin Institute (Berlin, Germany). The Sifin 2A and Sifin 2B MAbs belong to the immunoglobulin G1 class and react with the A and B subunits of Stx2, respectively.

Sampling and preparation of environmental samples. Environmental samples were collected and processed as described previously (7). Briefly, 10-g soil, fecal, and feral swine colon samples were transferred into 250-ml sterile flasks containing 90 ml tryptic soy broth (TSB; Becton Dickinson, Sparks, MD) and incubated for 2 h at 25°C and then for 8 h at 42°C with shaking. For induction of Stx2, mitomycin C was added to cultures enriched in TSB at a final concentration of 50 ng/ml and further incubated overnight at 37°C with shaking at 180 rpm. Watershed samples were enriched by adding 11 ml of 10× TSB to 100 ml of sample and were incubated as described above. One milliliter of the enrichment broth was filter sterilized (0.45-μm filter) and analyzed for the presence of Stx2 by IPCR.

To test the matrix effect of environmental samples on IPCR performance, STEC- and Stx-negative samples were prepared as described above, and aliquots of the filtered enrichment broth were spiked with serial dilutions of Stx2 (ranging from 1 pg/ml to 100 ng/ml) and analyzed for Stx2 by IPCR.

Pure bacterial culture supernatants were prepared from the strains listed in Table 1. The cells were grown overnight in Luria-Bertani (LB) liquid medium at 37°C to an optical density at 600 nm (OD_{600}) of approximately 1.8. Following centrifugation at 13,000 × *g* for 10 min at 4°C, the supernatants were collected and filtered through a 0.2-μm filter to remove intact cells and other debris.

Preparation of streptavidin-antibody conjugate. To assemble the signal-generating immunocomplex used in IPCR, streptavidin-conjugated anti-Stx2A MAb was prepared using a Lightning-Link (LL) streptavidin conjugation kit (Innova Biosciences Ltd., Cambridge, United Kingdom) by following the manufacturer's instructions. Briefly, 100 μl of Sifin 2A MAb (1 μg/μl) was premixed with 10 μl of LL-modifier reagent (1 μg/μl) and added to a vial containing 100 μg of lyophilized LL-streptavidin. After incubating the mixture for 3 h at room temperature (RT), 10 μl of LL-quencher reagent was added. The conjugate was used after incubation for 30 min at RT or stored at 4°C until further use.

Preparation of biotinylated DNA marker. A monobiotinylated DNA marker was prepared by PCR, using the pUC19 plasmid as the template and the primer pair pUC-bio (50) (5′-biotin-CCCGGATCCCAGCAATAAACCAGCCAGCC-3′) and F1 (5′-TATGCAGTGCTGCCATAACCATGA-3′). A BamHI restriction site was included in the pUC-bio primer to allow removal of the DNA marker from the immunocomplex. The resulting PCR product was about 340 bp

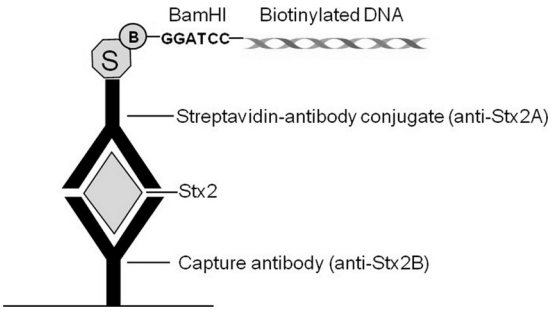

FIG. 1. Schematic diagram of the sandwich IPCR model, depicting the analytical complex on the surface of an assay well. B, biotin; S, streptavidin.

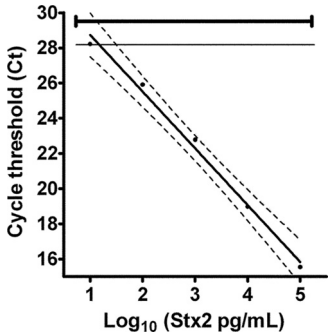

FIG. 2. Plots of average C_T values versus the log of the Stx2 concentration (pg/ml) for three replicate measurements of Stx2 in PBS. The linear regression of the calibration curve has a correlation coefficient (r^2) of 0.99. The dashed lines are the upper and lower 95% confidence limits. The thick horizontal line denotes the average blank C_T value, with the standard deviation drawn at each end of this line. The thin horizontal line intersecting the linear regression line indicates the detection threshold of the IPCR assay as defined in the text.

long and was purified by using a QIAquick PCR purification kit (Qiagen, Valencia, CA).

IPCR. IPCR was performed as described for sandwich IPCR for ricin (20), with minor modifications (Fig. 1). Briefly, 30 μl of purified Sifin 2B MAb (8 μg/ml) in borate buffer was used to coat microtiter wells overnight at 4°C. After blocking of nonadsorbed sites with 150 μl of blocking buffer for 1 h at RT, 30 μl of Stx2 standards or samples was added to each well, and the plates were incubated at RT for 1 h. The plates were washed with TBS-EDTA-Tween (Tris-buffered saline containing 5 mM EDTA and 0.05% Tween 20), and 30 μl of streptavidin-conjugated Sifin 2A MAb (500 ng/ml) was added to each well and incubated at 37°C for 30 min. After washing of the plates, 30 μl of biotinylated DNA marker (0.5 ng/μl) was added to each well and incubated at RT for 30 min. After thorough washing to remove unbound DNA, the bound DNA was detached from the immunocomplex by the restriction enzyme BamHI, and a 6-μl portion was used as a template in a real-time PCR (20 μl).

ELISA. ELISA was performed as described previously (20), with some modifications. Briefly, microtiter wells were coated with 100 μl of MAb specific for the Stx2 B subunit (VT136/8-H4) at 8 μg/ml in borate buffer and incubated overnight at 4°C. After a blocking step, 100 μl of toxin standards or samples was added to each well, and the plates were incubated at RT for 1 h. After washing of the plates with TBS-EDTA-Tween, 100 μl of streptavidin-conjugated Sifin 2A subunit MAb (500 ng/ml) was added to each well, and the plates were incubated at 37°C for 1 h. The wells were washed, and 100 μl of biotin-horseradish peroxidase (biotin-HRP) (Invitrogen Corp., Carlsbad, CA) at 1:5,000 in TBS-EDTA-Tween was added. The plates were incubated at RT for 1 h. After washing of the plates, Enhanced K-Blue substrate (Neogen Corp., Lexington, KY) was added, and the plates were incubated at RT for 30 min. The limit of detection (LOD) was defined as the lowest concentration used for the standard curve at which the average absorbance reading at 450 nm was higher than the negative-control absorbance plus 3 standard deviations (SD).

PCR data analysis. The cycle threshold (C_T) was calculated automatically by the instrument and represents the first PCR cycle at which the fluorescent reporter signal exceeded the signal of a given uniform threshold provided by the instrument software. Duplicate experiments were performed. Mean values and standard deviations of C_T for intra-assay triplicate measurements of IPCR were calculated using Microsoft Excel software. A linear regression fit of the data was determined, along with 95% confidence limits, using GraphPad Prism 5.03 software (La Jolla, CA). The LOD of the IPCR assay was defined as the lowest concentration used for the standard curve at which the C_T value fell below the average C_T value of the negative control minus 3 standard deviations (35). The C_T value is inversely proportional to the concentration of target: the lower the target concentration is, the higher the C_T value will be. Note that the LOD was not absolute but was calculated relative to the value of the negative control. Therefore, different ranges of C_T values were considered negative or positive between experiments depending upon the incubation conditions, purity of the Stx used, the Stx type, and sample matrix.

RESULTS

Development of immuno-PCR for detection of Stx2. In preliminary studies, we tested a direct IPCR by coating wells of a microplate directly with Stx2, probing samples with streptavidin-conjugated Sifin 2A MAb, and detecting bound MAb by PCR with a secondary reagent (biotin-DNA conjugate). The sensitivity of this method was ≥1 ng/ml of Stx2 (Table 2). As an alternative, a sandwich IPCR was tested, involving Stx2 capture by Sifin 2B MAb attached to the wells of a microplate followed by detection with a second antibody, streptavidin-conjugated Sifin 2A MAb (Fig. 1). This format greatly increased the sensitivity of the assay. Figure 2 shows the average C_T values obtained by sandwich IPCR versus the log of the Stx2 concentration for three replicate measurements of Stx2 in

TABLE 2. Detection of Stx2 in PBS by direct IPCR, sandwich IPCR, and sandwich ELISA[a]

Stx2 concn (pg/ml)	Direct IPCR		Sandwich IPCR		Sandwich ELISA	
	Avg C_T	SD	Avg C_T	SD	Avg A_{450}	SD
0	33.29	0.27	29.47	0.01	0.07	0.02
0.1	ND		28.69	0.10	ND	
1	ND		28.40	0.53	ND	
10	32.99	0.49	28.22	0.25	0.07	0.01
100	32.78	0.22	25.92	0.32	0.12	0.01
1,000	32.28	0.16	22.78	0.18	0.58	0.01
10,000	31.90	0.10	18.98	0.01	3.18	0.03
100,000	30.70	0.26	15.55	0.30	3.17	0.01
1,000,000	29.81	0.20	15.46	0.26	3.14	0.01

[a] The LOD for direct IPCR was 1,000 pg/ml, that for sandwich IPCR was 0.1 pg/ml, and that for sandwich ELISA was 1,000 pg/ml. ND, not determined.

TABLE 3. Detection of Stx2 variants in supernatants of STEC strains

Strain	Stx type	Dilution	Avg C_T	SD	Qualitative result[a]
Control (no strain)		TSB medium	29.66	0.02	−
RM4876	Negative[b]	Undiluted	29.40	0.25	− (control)
RM2084	Stx2	Undiluted	16.47	0.25	+
		1:10	17.47	0.03	+
		1:100	20.56	0.00	+
		1:1,000	24.97	0.60	+
		1:10,000	26.42	0.20	+
		1:100,000	27.46	0.11	+
		1:1,000,000	29.02	0.35	−
RM1625	Stx2c	Undiluted	23.43	0.07	+
		1:10	27.17	0.27	+
		1:100	28.69	0.04	−
RM7005	Stx2d	Undiluted	21.76	0.12	+
		1:10	24.74	0.06	+
		1:100	27.12	0.04	+
		1:1,000	28.70	0.32	−
RM7110	Stx2e	Undiluted	22.66	0.04	+
		1:10	26.53	0.46	+
		1:100	28.84	0.13	−
RM7007	Stx2f	Undiluted	30.33	0.54	−

[a] Qualitative results were determined based on average C_T values. If the C_T value of the sample was below the threshold (C_T value for the negative control minus 3 SD), the sample was considered Stx2 positive; otherwise, the sample was considered Stx2 negative. The average C_T values were obtained from three replicate measurements in the IPCR assays.
[b] Negative for Stx genes.

PBS. The quantitative LOD for this assay format was 10 pg/ml, based on the linear regression and 95% confidence limits. The log-linear quantification range of the assay was 10 to 100,000 pg/ml, but a qualitative LOD of ≥0.1 pg/ml was evident by the clear separation of the signals at 0.1 pg/ml (mean $C_T \pm$ SD = 28.69 ± 0.10) from those for the negative controls ($C_T \pm$ SD = 29.47 ± 0.01) (Table 2).

The sensitivity of the sandwich IPCR assay for Stx2 was compared with that of traditional ELISA. Table 2 shows that the detection threshold for Stx2 in PBS by sandwich ELISA was 1,000 pg/ml, which is 10,000-fold less sensitive than our results by sandwich IPCR. In an attempt to simplify the sandwich IPCR protocol, IPCR was performed using DNA markers in the same wells without transferring them to separate wells. The LOD for Stx2 in the simplified format was 100 pg/ml (data not shown), which is 1,000-fold less sensitive than the normal sandwich IPCR. Therefore, we used the sandwich IPCR for detection of Stx2 in different environmental sample matrices.

Validation of sandwich IPCR assay for detection of Stx2 variants. The success of the sandwich IPCR relies largely on the specificities and affinities of the antibodies used to capture and detect Stx2. In this assay, Sifin 2B and Sifin 2A MAbs were used as the capture and detecting antibodies, respectively. Pure overnight culture supernatants of reference STEC strains characterized as producing a single Stx2 or Stx2 variant were used as the toxin sources and tested by IPCR. As indicated in Table 3, this sandwich IPCR system could detect Stx2, Stx2c, Stx2d, and Stx2e in bacterial culture supernatants. The highest dilutions of supernatant resulting in positive signals by IPCR were 1:100,000 for the Stx2-producing strain RM2084, 1:100 for the Stx2d strain RM7005, and 1:10 for the Stx2c and Stx2e strains RM1625 and RM7110. The IPCR failed to detect Stx2f. Stx2 in the culture supernatant of RM2084 was detectable by ELISA, but the detection limit was at a dilution of only 1:100. None of the Stx2 variants present in the nondiluted bacterial culture supernatants were detected by ELISA. It is not clear whether the differences in detection of Stx2 variants observed for reference STEC strains by IPCR were due to differences in antibody affinity, toxin production, or a combination of the two, because there are no Stx2 standards or specific antibodies available for each variant to allow us to estimate the absolute amounts of toxins in the different cultures.

To confirm the presence of Stx2f and other toxin variants in culture supernatants of bacterial reference strains, we performed a cell-free translation assay developed previously for the detection of biologically active Stx (21). This assay measures Stx based on its ability to inhibit protein synthesis by using rabbit reticulocyte lysate containing luciferase mRNA. The relative biological activity was determined by comparing luminescence levels in treated samples to those of untreated controls. The results shown in Fig. 3 indicate that Stx2f from strain RM7007 was expressed and active, although it was not detected by the IPCR, and that the toxin activity was second only to that of Stx2 produced by strain RM2084.

Sensitivity of sandwich IPCR for detecting Stx2 in environmental sample matrices. To determine the sensitivity of the IPCR method for detection of Stx2 present in environmental sample matrices, soil, cow feces, feral swine colon, and water from watersheds (creek/stream or ditch) were enriched in TSB; the TSB was confirmed as Stx negative by PCR and culture methods (7) and then spiked with purified Stx2. Figure 4 shows representative curves for average IPCR C_T values versus the concentration of Stx2 spiked in one sample of each matrix. Three samples from each matrix (cow feces, feral swine colon,

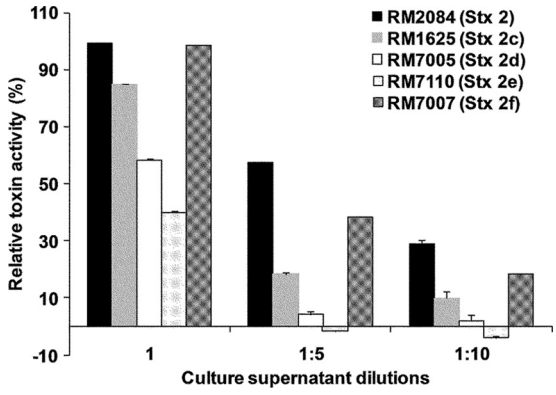

FIG. 3. *In vitro* inhibition of bacterial culture supernatants in a cell-free translation assay. The inhibitory activities of culture supernatants from bacterial strains RM2084 (Stx2), RM1625 (Stx2c), RM7005 (Stx2d), RM7110 (Stx2e), and RM7007 (Stx2f) were measured with the CFT assay. Relative activity was calculated by normalizing the measured activity to the activity of Stx2 in an undiluted culture supernatant of RM2084 (100%). Bacterial strain RM4876 (Stx negative) was used as a negative control (data not shown). Results represent means with standard deviations for three replicates from one representative experiment. Three individual experiments were performed.

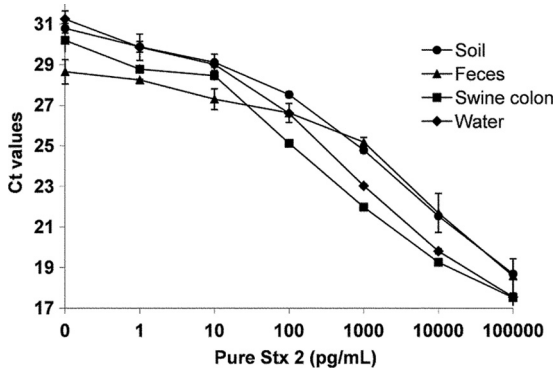

FIG. 4. Representative curves for IPCRs with one sample each of soil (solid circles), cow feces (solid triangles), feral swine colon (solid squares), and water (solid diamonds) spiked with 10-fold serial dilutions of Stx2, expressed as C_T values versus Stx2 concentration (pg/ml). Three replicate measurements were carried out for each serial dilution of Stx2 in each sample.

soil, and water) were tested. The qualitative LODs for Stx2 were shown to vary from 1 to 10 pg/ml for the broths from three different water samples and from 1 to 100 pg/ml for fecal, feral swine colon, and soil samples. These numbers were 10- to 1,000-fold higher than the LOD obtained with PBS, suggesting that complex environmental samples may contain components that are inhibitory to IPCR and that further optimization of the method to remove negative components may greatly improve the sensitivity of the IPCR for detecting Stx2 in these environmental samples.

Application of sandwich IPCR for detection of STEC in environmental samples based on detection of Stx2. We next evaluated the IPCR assay in a blinded study with environmental samples determined to be STEC and Stx2 positive by culture and PCR, respectively. Six soil, seven cow feces, four feral swine colon, and six water samples were tested. The samples were enriched in TSB medium containing 50 ng/ml mitomycin C to induce the phage lytic cycle, and supernatants of the enriched cultures were tested using the IPCR and ELISA. Table 4 shows that the IPCR detected Stx2 in all samples that were shown to be STEC positive by culture and real-time PCR methods (7), and no Stx2 was detected in any of the samples confirmed to be STEC and Stx negative, demonstrating that the IPCR assay had 100% sensitivity and specificity compared to the results of culture methods and real-time PCR with the initial enrichment broth. The average C_T values obtained by IPCR for STEC-positive samples were all less than the C_T values for the STEC-negative samples minus 3 standard deviations. The amounts of Stx2 in enrichment cultures of naturally contaminated soil, feces, and water were in the ranges of 0.1 to 10 ng/ml, 1 to 10 ng/ml, and 0.1 to 1 ng/ml, respectively, and the amounts in enriched cultures of feral swine colon samples were in the range of 10 to 100 pg/ml; these data were based on the calibration curves constructed with the corresponding Stx2-negative samples (data not shown). In contrast, the ELISA method did not detect Stx2 in all of the samples determined to be STEC positive by culture and real-time PCR methods (Table 4).

DISCUSSION

We developed a novel IPCR assay for the sensitive detection of Stx2 and validated the effectiveness of detection with complex samples. The results indicate that the sandwich IPCR assay can accurately detect levels of ≥0.1 pg/ml of purified Stx2, a 100-fold increase in sensitivity compared to results (10 pg/ml) reported previously (50). One of the major improvements of this IPCR assay is the use of a streptavidin-conjugated detection antibody facilitating attachment of a biotinylated DNA marker directly and specifically to the immobilized antigen, rather than using streptavidin as a bridge between the biotinylated detection antibody and the biotinylated DNA marker (50). The removal of this step improves speed and specificity, because each additional step in an amplification technology can result in significant nonspecific signals. In addition, the procedure we used for labeling the antibody with streptavidin is simpler and faster (setup takes about 1 min) than the procedure used for biotinylating an antibody. Comparison of the two IPCR protocols by use of the same streptavidin-antibody conjugate indicated that the LOD for the sandwich IPCR in PBS was 0.1 pg/ml qualitatively and 10 pg/ml quantitatively, whereas the LOD for the direct IPCR was 1 ng/ml (quantitatively and qualitatively), indicating that the sandwich IPCR is 100- to 10,000-fold more sensitive than the direct IPCR. Theoretically, the immobilization of an antigen in the sandwich IPCR should be more efficient than in the direct

TABLE 4. Results of PCR, IPCR, and ELISA for detection of Stx2 in samples collected from the environment

Sample type	Sample ID	Culture and PCR result[c]	Mean IPCR C_T ± SD	Mean ELISA A_{450} ± SD
Soil	S0997	+	24.00 ± 0.07[a]	0.08 ± 0.01
	S1079	−	26.52 ± 0.11	0.06 ± 0.01
	S1080	+	21.86 ± 0.32[a]	0.14 ± 0.01[b]
	S1081	−	26.88 ± 0.28	0.05 ± 0.01
	S1082	+	18.63 ± 0.33[a]	1.60 ± 0.13[b]
	S1086	+	21.69 ± 0.24[a]	0.16 ± 0.00[b]
Cow feces	F840	+	23.03 ± 0.16[a]	0.27 ± 0.04[b]
	F841	+	23.90 ± 0.57[a]	0.69 ± 0.01[b]
	F842	−	26.31 ± 0.17	0.09 ± 0.01
	F843	+	23.27 ± 0.18[a]	0.44 ± 0.01[b]
	F844	+	24.99 ± 0.10[a]	0.39 ± 0.01[b]
	F845	−	26.36 ± 0.06	0.06 ± 0.01
	F847	+	21.42 ± 0.16[a]	0.41 ± 0.05[b]
Feral swine colon	SP146	+	25.61 ± 0.02[a]	0.11 ± 0.01[b]
	SP147	+	24.56 ± 0.35[a]	0.05 ± 0.01
	SP148	−	27.47 ± 0.42	0.06 ± 0.01
	SP149	+	23.27 ± 0.27[a]	0.08 ± 0.01
Water	W208	+	24.92 ± 0.01[a]	0.17 ± 0.03[b]
	W263	+	19.41 ± 0.48[a]	0.62 ± 0.13[b]
	W264	−	26.02 ± 0.21	0.07 ± 0.01
	W265	+	22.62 ± 0.24[a]	0.09 ± 0.01
	W266	−	26.16 ± 0.19	0.10 ± 0.01
	W267	−	26.53 ± 0.39	0.07 ± 0.01

[a] IPCR results were considered Stx2 positive because the average C_T values for these samples for three replicate measurements were lower than the average C_T value for Stx2-negative samples minus 3 standard deviations.

[b] ELISA results were considered Stx2 positive because the average absorbances at 450 nm for these samples for three replicate measurements were higher than that for the Stx2-negative samples plus 3 standard deviations.

[c] Result of detection of STEC or Stx gene by culture or PCR.

IPCR. In the sandwich IPCR, the anti-Stx2 MAb binds to a specific epitope of the Stx2 B subunit, allowing the A subunit epitope to be presented optimally for binding of the anti-2A MAb, whereas in the direct IPCR, the attachment of the analyte to the surface of the microplate is a nonspecific adsorption process that may not present antigens/epitopes in the optimal configuration. This could be exacerbated in detecting analytes present in complex matrices, such as environmental samples, because other components competing for attachment to the well surface in the direct IPCR cause variability in the attachment of analytes and give increased background signals. The sandwich IPCR minimizes this problem, because analytes are captured by a highly specific MAb, facilitating removal of other components in the sample matrix by washing.

We determined that the sandwich IPCR is 10,000-fold more sensitive than ELISA; not only can Stx2 be detected in bacterial culture supernatants, but the Stx2c, Stx2d, and Stx2e variants can also be detected. Stx2f is not detected with this assay. This is probably due to the lack of specificity of the anti-Stx2 A and/or B MAb used in the IPCR for Stx2f. This conclusion is supported by the number of amino acid substitutions in Stx2f compared to that in other Stx2 variants. The A and B subunits of Stx2f are only 71% and 82% homologous to the A and B subunits of Stx2 by amino acid sequence.

In this study, we observed that the amounts of Stx2 and variant Stx2 produced by STEC reference strains and detected by IPCR (Table 3) did not correlate with the toxicity detected by the cell-free translation assay (Fig. 3). This may be due to different mechanisms of detection of Stx2 by these two methods. We reason that the specificity and affinity of the antibodies are critical for optimal IPCR assay performance. The application of the IPCR could be expanded and improved by identifying and/or producing new antibodies that recognize all Stx2 variants or antibodies that are highly specific for individual variants for identification or that have higher affinities for antigens. This project is under way in our laboratory.

One of the challenges to identifying pathogens in environmental samples is that the number of bacterial cells required to cause an illness is often below the threshold of sensitivity of the detection method, but the cells are present along with larger (by orders of magnitude) numbers of nonpathogenic bacteria and other microorganisms. A variety of methods, including culture methods (required for diagnostic purposes but very time-consuming), immunological methods, and nucleic acid-based methods, have been used and proven to be useful for identifying bacterial pathogens in simple matrices. However, improved detection methods are needed to determine the presence of pathogens in different complex matrices in the environment. The method described in this study for detection of STEC includes the preenrichment of STEC, induction of Stxs by mitomycin C, and detection of Stx2 by IPCR assay. Our data indicate that the sensitivity of the sandwich IPCR is decreased by complex matrices of cow feces, feral swine colon, soil, and water (the inhibition ranged from 10- to 1,000-fold reductions in assay sensitivity compared with that for PBS). Further optimization of the sample preparation step may assist in improving assay sensitivity. Despite the matrix effect measured in spiking experiments, application of the IPCR method to natural STEC-positive environmental samples revealed that it was able to detect the presence of Stx in all isolates determined to be STEC positive by real-time PCR and culture methods (Table 4), even though the numbers of STEC organisms in these samples were low. Therefore, we concluded that the IPCR we described provides a rapid and sensitive alternative method for screening environmental samples for the presence of STEC expressing Stx2 and the most common Stx2 variants. Detection of Stx2 by the IPCR method can be compared to the detection of stx_2 genes by other methods to analyze gene product versus gene content. This IPCR method can be adapted for detection of other Stx2 proteins and virulence factors as specific antibodies become available.

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