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Real-Time Isothermal Detection of Shiga Toxin–Producing *Escherichia coli* Using Recombinase Polymerase Amplification

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Abstract

Shiga toxin–producing *Escherichia coli* (STEC) are a major family of foodborne pathogens of public health, zoonotic, and economic significance in the United States and worldwide. To date, there are no published reports on use of recombinase polymerase amplification (RPA) for STEC detection. The primary goal of this study was to assess the potential application of RPA in detection of STEC. This study focused on designing and evaluating RPA primers and fluorescent probes for isothermal (39°C) detection of STEC. Compatible sets of candidate primers and probes were designed for detection of Shiga toxin 1 and 2 (Stx1 and 2), respectively. The sets were evaluated for specificity and sensitivity against STEC ($n=12$) of various *stx* genotypes (*stx1/stx2*, *stx1*, or *stx2*, respectively), including non-Stx-producing *E. coli* ($n=28$) and other genera ($n=7$). The primers and probes that were designed targeted amplification of the subunit A moiety of *stx1* and *stx2*. The assay detected STEC in real time (within 5–10 min at 39°C) with high sensitivity (93.5% vs. 90%; *stx1* vs. *stx2*), specificity (99.1% vs. 100%; *stx1* vs. *stx2*), and predictive value (97.9% for both *stx1* vs. *stx2*). Limits of detection of ~5–50 colony-forming units/mL were achieved in serially diluted cultures grown in brain heart infusion broth. This study successfully demonstrated for the first time that RPA can be used for isothermal real-time detection of STEC.

Introduction

Shiga toxin–producing *Escherichia coli* (STEC), in particular serotype O157:H7, are prominent foodborne pathogens of public health and clinical significance. STEC are estimated to cause more than 265,000 illnesses in the United States, with more than 3600 hospitalizations and 30 deaths each year. STEC infections often cause diarrhea, sometimes bloody diarrhea (hemorrhagic colitis), and some patients develop hemolytic uremic syndrome (HUS), a severe complication characterized by the triad of renal failure, hemolytic anemia, and thrombocytopenia that can be fatal. Most outbreaks of STEC infection and most cases of HUS in the United States have been caused by STEC serotype O157. Non-O157 STEC have also caused outbreaks in the United States (Scallan *et al*., 2011). STEC, particularly *E. coli* O157:H7, have low infectious doses ranging from 2 to 2000 cells (Buchanan and Doyle, 1997; Greig *et al*., 2010).

Since 2006, several reports have been published on use of recombinase polymerase amplification (RPA) for detection of a variety of pathogens including methicillin-resistant *Staphylococcus aureus* (MRSA) (Piepenburg *et al*., 2006), Rift Valley fever virus (Euler *et al*., 2012a), HIV DNA (Rohrman and Richards-Kortam, 2012; Boyle *et al*., 2013), bovine corona virus (Amer *et al*., 2013), the biothreat agent, *Francisella tularensis* (Euler *et al*., 2012b), and a panel of 10 biothreat agents (bacteria and viruses) that included, *Francisella tularensis*, *Yersinia pestis*, *Bacillus anthracis*, variola virus, Rift Valley fever virus, Ebola virus, Sudan virus, and Marburg virus (Euler *et al*., 2013). The ability of RPA to detect MRSA was previously demonstrated in the first publication on use of this RPA technology (Piepenburg *et al*., 2006) and was later elaborated by Lutz *et al*. (2010), who employed a foil-based centrifugal microfluidic cartridge device, and Shen *et al*. (2011), who used digital RPA in a SlipChip device to detect this pathogen. TwistaDx recently developed and launched a variety of RPA kits that are now commercially available for detection of prominent foodborne bacterial pathogens, namely, *Salmonella, Listeria monocytogenes*, and *Campylobacter* species, including *C. jejuni,*

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(www.twistdx.co.uk). RPA was also recently employed in detection of single-point mutations, suggesting that the RPA technique can be useful in future for the detection of DNA sequence alterations that are useful DNA biomarkers in human diseases (Shin *et al*., 2013).

To our knowledge, this study reports the first use of RPA for detection of STEC. We focused on designing and evaluating RPA primers and fluorescent probes that were used for isothermal detection of STEC in real time. RPA is a novel isothermal DNA amplification technology that enables the tests to produce results faster than other DNA detection tests available currently (reviewed by Gill and Ghaemi, 2008; Kim and Easly, 2011) while maintaining a very high level of specificity and sensitivity (Piepenburg *et al*., 2006). RPA uses the TwistAmp amplification process from TwistDx (www.twistdx.co.uk) and the Twista device (other fluorescence readers can be employed) (i.e., Twista™ Portable, Real-Time Fluorometer, an easy-to-use RPA reader [TwistaDx Ltd., Cambridge, UK] to detect disease-causing agents (Euler *et al*., 2012a; Euler *et al.*, 2012b, 2013; Amer *et al*., 2013) or DNA base mutations (Shin *et al*., 2013).

TwistDx's RPA process employs enzymes, known as recombinases, which are capable of pairing oligonucleotide primers with homologous sequence in duplex DNA. Through this method, DNA synthesis is directed to defined points in a sample DNA. If the target sequence is indeed present, DNA amplification reaction is initiated. The RPA process enables the Twista device to monitor the amplification of target DNA in real time (www.TwistaDx, Cambridge, UK). The detection system uses DNA probes that hybridize to the template or newly synthesized complementary DNA for detection. When the probe is broken down by an exonuclease enzyme present in the master mix, the fluorophore (e.g., 6-carboxyfluorescein, FAM) is separated from the quencher (commonly Black Hole Quencher-1, BHQ-1) and it subsequently fluoresces. The amount of released FAM fluorophore and detection signal increase with progress in amplification, enabling monitoring in real time.

The objectives of the current study were to develop RPA probe and primer sets that are suitable for detection of STEC. Our hypothesis was that some of these probes and primers will have high sensitivity and specificity to be used to detect STEC in real time, under isothermal DNA amplification conditions. The long-term goal is to develop RPA protocols that can be used directly in the field, at the point-of-care, for rapid real-time detection of pathogens, by, for example, produce growers, shippers, packers and processors, as well as livestock producers and food and feed processors.

Materials and Methods

RPA exo primer and probe design

STEC Stx gene sequences of interest were derived from National Center for Biotechnology Information databases (http://www.ncbi.nlm.nih.gov/). The STEC represented a broad diversity of serotypes (e.g., O8, O26, O104, O111, O113, O136, O157, O174, O178, O179 and O185). The *stx* GenBank Accession numbers were the following: FR850035, AJ543499, CP003301, AM230663, FR850032, GQ919289, JX161807, AF500188, FR850037, FR850031, and FR850038). Other Stx producers whose DNA sequences were included were Stx-converting bacteriophages (AP0000400 and FJ188381), the bacteria: *Shigella dysenteriae* type 1 (M19437), *Shigella sonnei* (AJ132761), *Acinetobacter hemolyticus* (DQ344636), and *Aeromonas caviae* (GU130282-GU130285 and GU130286), which share common *stx* sequences. The GenBank Accession numbers appear in parentheses. The retrieved FASTA format Stx gene sequences for subunit A_1, A_2, and B were aligned to find consensus base sequences. The probes and primers were designed using Geneious software (Version 5.6, Biomatters Ltd., Auckland, New Zealand) following guidelines for desirable characteristics for designing of TwistDx exo RPA primers and probes (www.twistdx.co.uk). Sequences for the B subunits of Stx were smaller than subunit A; consequently, the design focused on the longer subunit A moiety since the subunit B would have little probability of yielding long consensus sequences ideal for placement of both primers and probes (Fig. 1). Furthermore, there was lack of identity in the B subunit sequences across multiple sources of the *stx* gene sequences (data not shown). For RPA, ideally, 100–200 bases are required to fit in the RPA probes in areas of the template DNA that are flanked by the forward and reverse primers. Consensus sequences of the desirable length were not readily available as most identity (based on our alignment comparisons of more than 200 sequences from databases that had Stx 1 or Stx 2 encoding sequences from different STEC) was only about 15–17 tandem bases at most (data not shown). Therefore, the two toxins were considered independently while focusing the design of primers and probes on the longer A subunit encoding sequences. Once the forward and reverse primers were established for *stx1* and *stx2*, two separate probes flanked by the primers were designed following TwistDx guidelines. A series of primer and probe candidate sets were designed and optimized for use in sets to avoid unwanted cross-interactions (e.g., hybridization, hairpin-loop structures, and dimer formation). Table 1 shows the two sets that were used in this study. Basic Local Alignment Search Tool alignments of the primers and

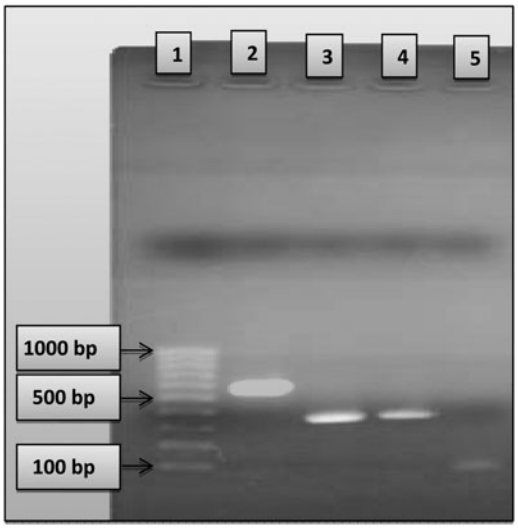

FIG. 1. Midori green–stained gel used for detection of Stx gene encoding sequences amplified by polymerase chain reaction. Results were used to verify recombinase polymerase amplification data. Lane 1 shows molecular weight markers (100-bp ladder). Lanes 2, 3, and 4, respectively, show *Escherichia coli* strains positive for only *stx2 (*ATCC 43889*)*, *stx1* (ATCC 43890), and *stx1* (*E. coli* 109), respectively, whereas *E. coli* ATCC 43888, in lane 5, was negative for both *stx1* and *stx2*.

Table 1. Exo Primer and Probe Sets Designed and Used in Detection of Shiga Toxin–Producing *Escherichia coli* Using Recombinase Polymerase Amplification

Set	Shiga toxin type	Prime/ probe	Direction	Sequence (5′-3′)	Number of bases
2	A1	Primer	Forward	TATTTCATCAGGAGGTACGTCTTTACTGATGATTGATAGTG	41
		Primer	Reverse	ATAAAAAACATTATTTGTCCTGTTAACAAATCCTGTCAC	39
		Probe	Forward	GTCAGAGGGATAGATCCAGAGGAAGGGCGGT-T(FAM)-T-dSpacer-A-T(BHQ-1)-AATCTACGGCTTATT (3’ blocker)	50
	A2	Primer	Forward	TATCAGGCGCGTTTTGACCATCTTCGTCTGATTATTGAGC	40
		Primer	Reverse	GGTGTGACAACGGTTTCCATGACAACGGACAGCAGTTATA	40
		Probe	Forward	TTCGTTAATACGGCAACAAATACTTTCTACCG-T(FAM)-T-dSpacer-T-T(BHQ-1)-CAGATTTTACACATAT (3’ blocker)	52
8	A1	Primer	Reverse	AACCGTAACATCGCTCTTGCCACAGACTGCGTCAGTGAGG	40
		Primer	Forward	TTTTTCACATGTTACCTTTCCAGGTACAACAGCGGTTACA	40
		Probe	Reverse	AGATAAGAAGTAGTCAACGAATGGCGA-T(FAM)-T-dSpacer-A-T(BHQ-1)-CTGCATCCCCGTACG (3′ blocker)	46
	A2	Primer	Reverse	AACTGCTCTGGATGCATCTCTGGTCATTGTATTACCACTG	40
		Primer	Forward	ATATATCAGTGCCCGGTGTGACAACGGTTTCCATGACAACGG	42
		Probe	Reverse	ATATGATGAAACCAGTGAGTGACGACTGATT-T(FAM)-G-dSpacer-A-T(BHQ-1)-TCCGGAACGTTCCAG (3′ blocker)	50

FAM, 6 carboxyfluorescein; BHQ-1, Black Hole 1Quencher-1; dSpacer, tetrahydrofuran; 3′ blocker prevents amplification of probe; A1, A subunit of *stx1*; A2, A subunit of *stx2*.

probes suggested 100% identity with most STEC and non–*E. coli* Stx-producing organisms, indicated above, emphasizing the robustness of the designs. For the two probes that were synthesized, FAM was used as a fluorophore, BHQ-1 as a quencher, and tetrahydrofuran (THF) as a spacer (Table 1). The THF was placed between the fluorophore and quencher. A block placed at the 3′-end prevented the oligonucleotide from acting as an amplification primer (www.twistdx.co.uk). The probes and primers were manufactured by Biosearch Technologies, Inc. (Novato, CA) and Integrated DNA Technologies (IDT, Coralville, IA), respectively.

Bacterial strains, toxin genotypes, and DNA extraction

The bacterial strains (Table 2) that were used in this study ranged from *E. coli* positive for Stx production (*stx1* or *stx2* alone, or both; $n=12$), *E. coli* negative for *stx* ($n=28$), and non–*E. coli* strains ($n=6$). Test bacteria were obtained from the American Type Culture Collection (ATCC, Manassas, VA), *E. coli* Reference Center (Pennsylvania State University, State College, PA), and the Agricultural Research Service (ARS, United States Department of Agriculture, Riverside, CA). The strains were revived from a frozen-stored culture (-80°C) that was inoculated into Brain Heart Infusion (BHI) broth (BD, Franklin Lakes, NJ) and grown at 37°C overnight. The subsequent culture was streaked on Tryptic Soy Agar (TSA) (BD) slants for refrigerated storage, and to TSA plates for isolation, Gram staining, or DNA extraction. Both media were incubated as previously stated.

The quality-control bacterial strains were verified using colony morphology, Gram staining techniques, and microscopy. The genotypes were confirmed using polymerase chain reaction (PCR) and agarose gel electrophoresis as described by Vidal *et al*. (2005). Gels were stained with (2 μL/100 g) Midori green nucleic acid staining solution (Cat. # MG04, Bulldog Bio Inc., Portsmouth, NH), before pouring. Gel band visualization was done on an ultraviolet transilluminator (model T10-M; UVP, Upland, CA), and documentation was conducted using a Samsung Galaxy S smart phone (Samsung Electronics America, Ridgefield Park, NJ) (Fig. 1). Bacterial DNA extraction was conducted using InstaGene Matrix following the manufacturer’s protocol (Bio-Rad Laboratories, Hercules, CA). A NanoDrop Spectrophotometer (model 2000, NanoDrop Products, Wilmington, DE) was used to assess the purity and concentration of the extracted DNA before it was used in RPA tests.

Recombinase polymerase amplification

RPA exo kits were procured from TwistaDx Ltd. (Cambridge, UK). RPA was conducted following the instructions outlined in the TwistAmp™ exo kit manual. For the laboratory tests, the probes and primers were pulled from a mother stock (100 μM) and diluted down to working concentrations (10 μM) in small quantities that were used within a week to avoid deterioration. A rehydration solution containing primer, probe, DNA template, distilled deionized water, and buffer was prepared. This was added to the lyophilized RPA exo master mix in a PCR tube, and lastly, magnesium acetate (TwistaDx Ltd.) was added to initiate amplification. The Twista device, an eight-channel spectrophotometer (model # Twista™, TwistaDx Ltd.), was used to monitor the amplification process isothermally, at 39°C. All tests were run under the same conditions and were repeated three to six times, as needed. A 16-PCR tube Mini Centrifuge (Iscope Corporation, Irvine, CA) was used to flash-spin samples after the initial mixing before incubating in the Twista device. As recommended, after 4 minutes’ incubation, the samples were flash-spun again and incubation was continued for an additional 15 min. Four organisms were tested in each RPA run in separate channels for *stx1* and *stx2*, respectively.

Sensitivity and specificity tests

For sensitivity and specificity tests, *E. coli* strains that are known to produce Stx1 or Stx2 only, or both toxins were used as positive controls, whereas non-Stx producing *E. coli* strains and non-*E coli* bacteria were used as negative controls

Table 2. Bacterial Strains Used in This Study for Evaluating Recombinase Polymerase Amplification Primers and Probes

Test strain	*Lab. #*	*Genotype stx1/stx2*	*RPA test stx1s:stx2*	*Test strain*	*Lab #*	*Genotype stx1/stx2*	*RPA test stx1:stx2*
E. coli				*E. coli*			
O157:H4	PSU1	−/−	2/2:2/2[a]	O157:H7	ATCC 43895	+/+	3/3:3/3[b]
O157:H7	PSU2	−/−	2/2:2/2	O157:H7	00362	+/+	3/3:3/3
O157:H7	PSU3	−/−	2/2:2/2	O157:H7	(111) ATCC 700927	+/+	3/3:2/3
O157:H7	PSU4	−/−	2/2:2/2	O157:H7	ATCC 43890	+/−	5/6:5/6[c]
O157:H7	PSU5	−/−	2/2:2/2	O157:H7	ATCC 43894	+/+	3/3:3/3
O157:H12	PSU6	−/−	2/2:2/2	O157:H7	ATCC 43889	−/+	5/6:4/6
O157:H12	PSU7	−/−	2/2:2/2	O157:H7	933	+/+	3/3:3/3
O157:H12	PSU8	−/−	2/2:2/2	O26:H21	O26	+/−	3/3:3/3
O157:H12	PSU9	−/−	2/2:2/2	O91:H21	O91	−/+	3/3:3/3
O157:H12	PSU10	−/−	2/2:2/2	O103:H2	103	+/+	3/3:3/3
O157:H19	PSU11	−/−	3/3:3/3	O157:H7	108	+/+	4/5:3/5
O157:H19	PSU12	−/−	3/3:3/3	O157:H7	109	+/−	2/2:2/2
O157:H19	PSU13	−/−	3/3:3/3				
O157:H29	PSU14	−/−	3/3:3/3	Non–*E. coli*			
O157:H29	PSU15	−/−	3/3:3/3	*Salmonella* Braenderup	ATCC BAA664	−/−	4/4
O157:H29	PSU16	−/−	3/3:3/3	*Salmonella* Choleraesuis	(65) ATCC 10708	−/−	4/4
O157:H29	PSU17	−/−	3/3:3/3	*Staphylococcus aureus*	(71) ATCC 29737	−/−	4/4
O157:H42	PSU18	−/−	3/3:3/3	*Staphylococcus aureus*	(9) ATCC 6538	−/−	4/4
O157:H42	PSU19	−/−	3/3:3/3	*Klebsiella pneumoniae*	ATCC 700603	−/−	4/4
O157:H42	PSU20	−/−	3/3:3/3	*Pseudomonas aeruginosa*	ATCC 27853	−/−	4/4
ON:H48	PSU21	−/−	3/3:3/3				
ON:H6/H4	PSU22	−/−	3/3:3/3				
O143:N/A	PSU23	−/−	3/3:3/3				
O101:NM	PSU24	−/−	3/3:3/3				
N/A	(HB101) ATCC 33694	−/−	4/4:4/4				
O157:H7	ATCC 43888	−/−	3/4:4/4				
N/A	4554	−/−	4/4:4/4				
N/A	(63) ATCC 8739	−/−	3/3:2/3				

[a]For this isolate, 2 of 2 tests for *stx1* were negative: 2 of 2 tests for *stx2* were negative.
[b]For this isolate, 3 of 3 tests for *stx1* were positive: 3 of 3 tests for *stx2* were positive.
[c]For this isolate, 5 of 6 tests for *stx1* were positive: 5 of 6 tests for *stx2* were negative.
stx; Shiga toxin–encoding gene sequence (presence of *stx1* and *stx2 was* verified using polymerase chain reaction [PCR]).
N/A, *E. coli* serotype is not available; -/-, negative for both s*tx1* and *stx2* using PCR;
−/+, Negative for *stx1* and positive for *stx2* using PCR; +/−, positive for *stx1* and negative for *stx2* using PCR; +/+, positive for both *stx1* and *stx2* using PCR.
RPA, recombinase polymerase amplification; *E. coli, Escherichia coli; Salmonella* Choleraesuis, *Salmonella choleraesuis subsp. choleraesuis* serovar Choleraesuis; *Salmonella* Braenderup, *Salmonella enterica subsp. enterica* serovar Braenderup.

(Table 2). The diversity of strains enabled determination of sensitivity and specificity of the RPA exo primer and probe sets. Sensitivity, specificity, and predictive values of the determinations were calculated following the procedure described by Gibson (1990), and were expressed as a percent.

Limit of detection tests

STEC test organisms (*E. coli* O157:H7 ATCC 700927 and ATCC 43895) were grown in BHI broth (BD) at 37°C for 6 h. The absorbance at 600 nm, OD 600, was measured using a spectrophotometer (SmartSpec™, Bio-Rad Laboratories) and was adjusted to 0.9 with BHI and the samples were serially diluted 10-fold and 2-fold as appropriate. Dilutions were plated on TSA in triplicate using sterile calibrated 10-μL loops (Cat. # 166-0471; Bio-Rad Laboratories). The plates were incubated at 37°C for 24 h and the resulting colonies (colony-forming units [CFU]/mL) were enumerated.

Results

Evaluation of RPA exo primer and probe sets

Control DNA supplied with the RPA exo kit was used to ascertain that the kit components and Twista device were working as anticipated. The control DNA amplified, whereas samples without DNA did not amplify. NanoDrop readings were used to determine concentration and purity of DNA

extracts. Absorbance 260/280 ratios ranged from 1.80 to 2.00, and ideally values around 1.60 were suitable for RPA amplification. A positive detection of either *stx1* or *stx2* is determined by the emission of fluorescence by the FAM fluorophore, which can only be released if the target DNA was successfully amplified. A negative result does not exceed the baseline relative fluorescence value of 200, which was determined empirically (appears as intensity millivolts; Int. mV in graphs), and yields a more or less horizontal line in the amplification graphs (Figs. 2 and 3). Figure 2A–C are representative graphs demonstrating use of RPA for detection of STEC using exo primer and probe set #8. Similarly, Figure 3A and B are representative graphs demonstrating the use of RPA for detection of STEC using exo primer and probe set #2. It was evident that primer/probe set #8 was more effective that primer/probe set #2 with regard to specificity, sensitivity, and limit of detection. Consequently, most tests were conducted with primer/probe set #8. Except for a few false negatives and false positives (Table 2), most *stx* gene sequences were amplified as expected, whereas *stx*-negative organisms did not amplify.

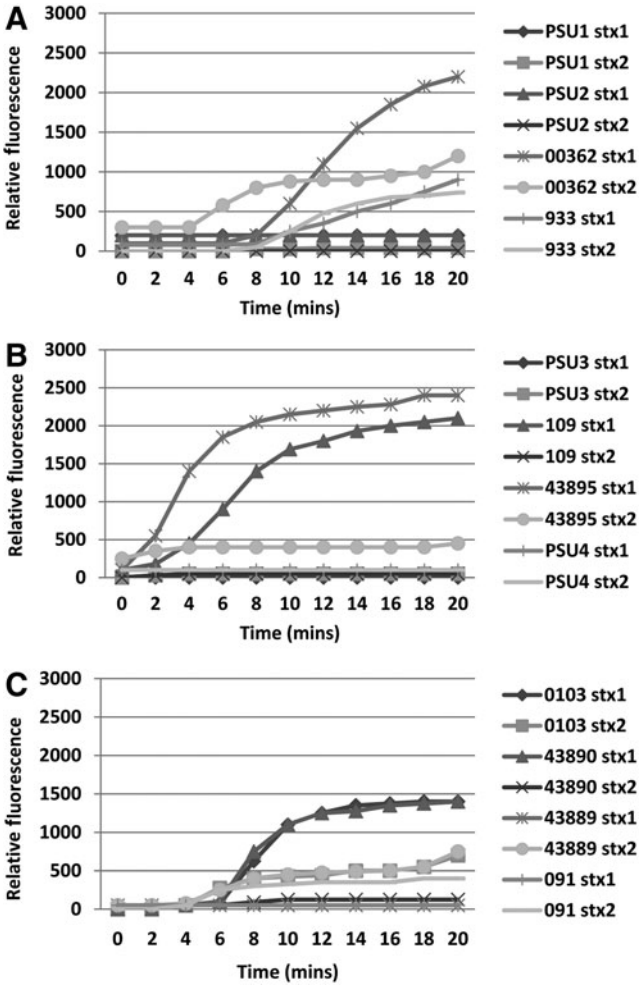

FIG. 2. Recombinase polymerase amplification for Shiga toxin–producing *Escherichia coli* detection using primer/probe set 8. The cut-off relative fluorescence value was set empirically at 200. All *stx* genotypes verified with polymerase chain reaction were correct. **(A)** *E. coli* strains PSU 1 and PSU 2 were negative for *stx1* and *stx2*, whereas *E. coli* 00362 and 933 were positive for both *stx1* and *stx2*. **(B)** *E. coli* strains PSU 3 and PSU 4 were negative for both *stx1* and *stx2*. *E. coli* strain ATCC 4389 was positive for both toxins. *E. coli* 109 was positive for *stx1* and negative for *stx2*. **(C)** *E. coli* O103:H2 was positive for *stx1* and *stx2*. *E. coli* 43890 was positive for *stx1* and negative for *stx2*. *E. coli* 43889 and O91:H21 were negative for *stx1* and positive for *stx2*.

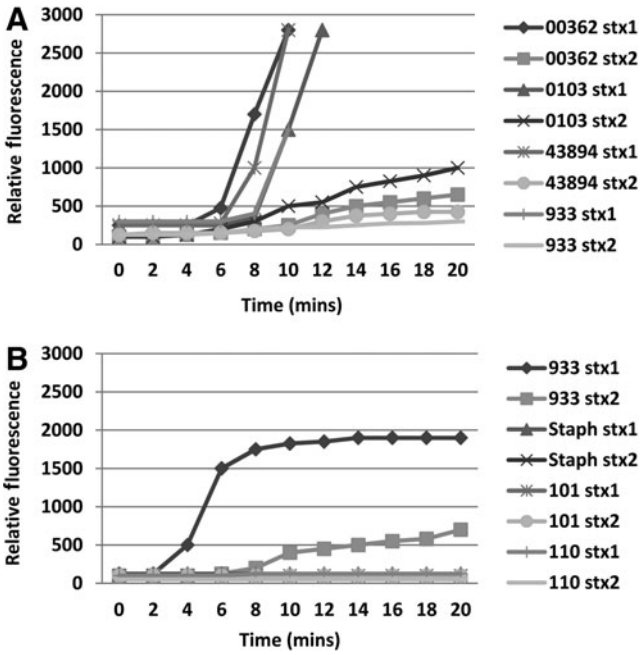

FIG. 3. Use of recombinase polymerase amplification for Shiga toxin *Escherichia coli* detection using primer/probe set 2. The cut-off relative fluorescence value was set empirically at 200. All *stx* genotypes verified with PCR were correct. **(A)** *E. coli* 00362, O103:H2, 43894, and 933 were positive for *stx1* and *stx2*. **(B)** *E. coli* 933 was positive for *stx1* and *stx2*, whereas SA 9 (*Staphylococcus aureus*), HB 101 (*E. coli*) and 110 (*E. coli* ATCC 43888), were *stx* negative, as anticipated.

Sensitivity and specificity tests

Evidently, it was sufficiently demonstrated that RPA can detect STEC with high sensitivity and specificity using exo primers and probes. Primer/probe set #8 yielded more accurate results than primer/probe set #2 (Table 3). Stx1 A subunit encoding sequences gave higher fluorescent signals than the Stx2 for both primer/probe sets (Figs. 2 and 3). Set 8 was also superior to set 2 in terms of overall sensitivity, specificity, and predictive value. It had a high predictive value of 97.9% for both toxin subunits. When overall results were considered for set 8, with regard to success of detection of both toxins per bacterial strain, the sensitivity, specificity, and predictive values were 93%, 98%, and 96%, respectively. For set 8 primers and probes, of the 93 tests on *stx*-negative bacteria that were conducted, 91/93 (97.8%) tests were correct in identifying *stx*-negative bacteria. For the 43 tests on *stx*-positive bacterial strains that were conducted, 36/43 (83.7%) gave correct results for the toxin genotypes (i.e., +/+, −/+, +/− for *stx1/stx2* outcomes). However, if detection of one of

Table 3. Sensitivity, Specificity, and Predictive Value of Primer/Probe Sets That Were Evaluated for Detection of Shiga Toxins (Stx) Using Recombinase Polymerase Amplification

	Primer/probe set #8 (%)		*Primer/probe set #2 (%)*	
Criteria[a]	*stx1*	*stx2*	*stx1*	*stx2*
Sensitivity	93.5	90.0	100.0	71.4
Specificity	99.1	100.0	71.4	57.1
Predictive value	97.9	97.9	85.7	64.3

A-subunits of Stx1 and 2 encoding sequences (*stx1 and stx2*) were targeted.
[a]Calculated as outlined by Gibson (1990).
Sensitivity: (True Positive) ÷ (True Positive + False Negative).
Specificity: (True Negative) ÷ (True Negative + False Positive).
Predictive Value: (True Positive + True Negative) ÷ (True Positive + False Positive + True Negative + False Negative).

the toxin genotypes was considered as a positive identification for STEC (less stringent), 40/43 (i.e., 93%) of the tests were positive for toxin detection.

Limit of detection

In two tests that were conducted in triplicate with serial 10-fold dilutions of *stx*-positive strains, the lowest detectable STEC numbers using RPA with primer and probe set #8 were ~5 and 7 CFU/mL, respectively (Fig. 4). Primer and probe set #2 was less sensitive and yielded a higher detection limit of 10^4 CFU/mL (data not shown), which was ~570–800 times less sensitive than set #8 in terms of CFU/mL. In both tests, only detection of *stx1* was monitored since *stx1* invariably yielded a stronger fluorescent signal than *stx2*.

Discussion

Since the discovery of RPA (Piepenburg *et al*., 2006) several studies have reported development of RPA tests to detect a variety of bacterial (Lutz *et al*., 2010; Shen *et al*., 2011; Euler *et al.*, 2012b; Euler *et al.*, 2013) and viral pathogens (Euler *et al.*, 2012a, 2013; Boyle *et al*., 2013; Rohrman and Richards-Kortam, 2012; Amer *et al.*, 2013), including

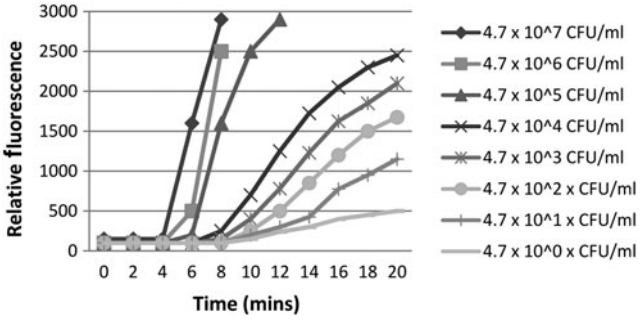

FIG. 4. Limit of detection verification for Shiga toxin–producing *Escherichia coli* using recombinase polymerase amplification (RPA). Serial 10-fold dilutions of *E. coli* strain ATCC 700927 were made. After DNA extraction, *stx1* was targeted for detection with RPA. The sample labeled 10^8 is the lowest dilution (4.7×10^7 colony-forming units [CFU]/mL), whereas 10^0 is the highest dilution (4.7 CFU/mL).

detection of single-point base mutations (Shin *et al*., 2013). A few RPA kits that are now commercially available have been developed for rapid detection of the foodborne pathogens, namely, *L. monocytogenes*, *Salmonella* species, and *Campylobacter* species, including *C. jejuni* (www.twistdx.co.uk). However, no one has yet reported use of RPA in detection of STEC (Forrest M, personal communication. TwistDx.co.uk. Senior Scientist, Technical Support, January 28, 2013), and our study is the first to achieve that goal. We demonstrated that RPA can be used for isothermal, real-time detection of STEC with high sensitivity, specificity, and predictive value.

Shiga toxins (Stx) are the major virulence factors of STEC (Nataro and Kaper, 1998; Paton and Paton, 1998). The number of encoding nucleotides of the A and B subunits of Stx1 and 2 vary in size from 878 (Stx1) and 888 (Stx2) for subunit A, and 207 (Stx1) and 210 (Stx2) for B subunits (Weinstein *et al*., 1988). The Stx1 operon, which encodes all subunits is about 1470 base pairs (Brett *et al*., 2003). Stx1 and Stx2 are compound toxins comprising one 32-kDa A subunit and 5 identical 7-kDa B subunits (Lee *et al*., 2007). The A subunit is noncovalently linked to multiple copies ($n = 5$) of the B subunit. The latter bind to globotriaosylceramide (Gb_3), their (most common) cognate receptor on target cells (Weinstein *et al*., 1988; Lee *et al*., 2007). The nucleotide and amino acid sequences of *Shigella dysenteriae* type 1 toxin and Stx1 are essentially identical, and differ only on three nucleotides (i.e., one amino acid) in the A subunit genes (Weinstein *et al*., 1988). Stx1 and Stx2 share 55–60% genetic and amino acid identity (Weinstein *et al*., 1988; Lee *et al*., 2007). These regions of nucleotide identity can serve as targets for coamplification and detection of Stx1 and Stx2 encoding genes, as has been demonstrated by some researchers (e.g., Karch and Meyer, 1989; Kido *et al*., 2000) who developed universal primers for detection of the two toxins using PCR.

We also demonstrated that STEC detection with RPA poses unique challenges. Unlike PCR or real-time PCR, which employ shorter primers (~15–25 bases), the design of RPA primers and probes is more stringent as it requires longer primers and probes (~45 bases), and necessarily, there are fewer opportunities for finding such sequences. We took an innovative approach to the probe and primer design, whereby we considered the *stx1* and *stx2* encoding genes independently, due to their poor alignment and lack of usable homologies if considered together, while also recognizing that A subunits are longer than B subunits. We therefore focused on the longer A subunit sequences to design probes that were flanked by the primers.

The *stx1* probe and primer subsets showed greater sensitivity to the targeted template than the *stx2* probe and primer subsets. This lower sensitivity resulted in reduced amplification and a weaker fluorescent signal for s*tx2*. Despite this, our results unequivocally demonstrated that STEC can be successfully detected using RPA, in real time (i.e., within 5–10 min), under isothermal incubation conditions.

It is anticipated that some of the primer/probe sets we designed (data not shown) could yield better or comparable results to those obtained using primer/probe sets #8 and #2. Future studies will include testing more probe and primer sets and refining the design (e.g., shortening or lengthening) of the functional sets for optimization. These studies will also focus on improving the detection limit to < 5 CFU/mL

since STEC have low minimal infectious doses, as well as spiking tests in different matrices (e.g., vegetables and meat), and ultimately, conducting field tests on, for example, produce or farm animals. Furthermore, it would be prudent to investigate the possibility of serotype-specific detection of STEC using RPA.

Additional challenges in use of RPA in STEC detection include multiplexing to enable simultaneous detection of *stx1* and *stx2* using compatible probes, and ability to detect the variant *stx*, particularly *stx2* (Lee *et al*., 2007). Although most nucleotide sequences of Stx1 genes, unlike Stx2, are conserved, several variants of Stx1 (Stx1c and Stx1d) and Stx2 (Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g) have been described (Lee *et al*., 2007; Scheutz *et al*., 2012). Achieving lower limits of detection is paramount for STEC detection, since these pathogens can cause disease in very low numbers (e.g., 2–2000 cells have been reported for STEC O157:H7 infections) (Buchanan and Doyle, 1997; Greig *et al*., 2010).

The approach we reported herein is a proof-of-concept for refinement and routine use of RPA in STEC detection. Although our study focused on *stx* sequences, alternative common gene sequences or virulence factor encoding genes found in STEC (reviewed by Nataro and Kaper, 1998; Paton and Paton, 1998; Gyles, 2007) could be used as targets for detection of these pathogens using RPA, in either singleplex or multiplex formats. There are possibilities for developing instrument-free, portable, miniaturized tools, or disposable devices/biosensors for application in research and clinics that use the RPA technology for STEC detection (e.g., microfluidic/lateral-flow strips that can be deployed at the point-of-care, such as produce field, cowside, or hospital bedside).

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Disclosure Statement

No competing financial interests exist.

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