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Evaluation of Four Loop-Mediated Isothermal Amplification (LAMP) Assays for Identification of Shiga Toxin Producing *E.Coli* O157 (STEC) and Non-O157 Strains

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Abstract

Shiga toxin-producing strains of *E. coli* are a significant cause of food-borne outbreaks of gastroenteric disease. The objective of this study was to evaluate loop-mediated isothermal amplification (LAMP) assays for the identification of Shiga toxin producing *E. coli* O157 (STEC) and non-O157 strains. Four LAMP assays were developed for the detection of the *rfbE* gene of *E. coli* O157, the Shiga toxin genes *stx1/stx2* and *eae* intimin gene. The assays were run on a real time fluorometer (Genie II, Optigene, Horsham, UK) that displays real time amplification, the time to positivity and amplicon annealing temperature (*Tm*). The specificity of the LAMP assays was confirmed by testing a panel of 35 enteric bacteria, viruses and parasites, all of which tested negative in all four assays. The lower limit of detection for each of the gene targets was 10-100 genome equivalents and 1 *cfu* of *E. coli* O157. The LAMP assays were evaluated by testing a total of 135 stool specimens by LAMP, PCR or the xTAG® Gastrointestinal Pathogen Panel (GPP) assay (Luminex Molecular Diagnostics, Toronto, ON, Canada). Following resolution of the discordants and using positivity in two or more assays as the reference standard, the sensitivity of the LAMP assays was 100% (27/27), 98.4% (63/64) and 98.0% (47/48) for *rfbE*, *stx1/stx2* and *eae* genes respectively, while the specificity for the assays was 100% (107/107), 100% (71/71) and 98.5% (66/67), respectively. The LAMP assays had excellent sensitivity and specificity for detecting Shiga toxin-producing *E. coli* O157 (STEC) and non-O157 in stool specimens and they were faster and more accurate than PCR. We suggest that these assays could be incorporated into *E. coli* O157 (STEC) testing algorithms.

Keywords: *E. coli*; Isothermal amplifi ation; Shiga toxin

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) are food-borne pathogens that can cause serious illness, especially in young children and the elderly, and pose a serious global health concern [1,2]. *E. coli* O157 has been responsible for numerous food-borne outbreaks, and can result in hemorrhagic colitis or hemolytic uremic syndrome [3,4]. These bacteria are capable of producing large quantities of toxins (Shiga toxins) that can damage the intestinal lining and cause bloody diarrhea. More than 200 different serotypes of *E. coli* can produce Shiga toxin and at least 150 of these are human pathogens [5]. Shiga-toxin producing *E. coli* (STEC) strains cause approximately 176,000 illnesses, 2,400 hospitalizations, and 20 deaths annually in the USA [6]. Since surveillance for non-O157 STEC (O26, O45, O103, O111, O121, and O145) began in 2000, the incidence of non-O157 STEC infections surpassed that of O157 infections in the USA for the fi st time in 2010 [7,8]. Thus rapid and sensitive methods to detect both *E. coli* O157, as well as non-O157 serotypes, is required.

E. coli O157 and non-O157 STEC strains are usually detected using selective culture media, enzyme immunoassays or by commercial or in house PCR assays or by immunomagnetic separation (IMS) assays for food testing [9,10]. Immunomagnetic separation in combination with plating is the most common method used to detect *E. coli* O157 for food testing, but this approach is time-consuming and technically challenging. The recent outbreak of HUS in Germany attributed to a rare STEC serotype O104:H4 in sprouts adds additional demands on IMS testing [11]. In addition, the effective detection and isolation of non-O157 *E. coli* using traditional culture methods remains difficult. Currently many laboratories use enzyme immunoassays (EIAs) to test for O157 since only a few commercial assays or molecular tests have been approved by the FDA for the diagnosis of STEC infections. Nucleic acid amplifi ation techniques such as PCR have been applied to detect *E. coli* O157 and non-O157 strains. Many PCR assays have been described for the detection of *rfbE*, *stx1*, *stx2*, *eae* and other genes present in STEC [12-14]. More recently, loop-mediated isothermal amplifi ation (LAMP) has been applied to detect *E. coli* O157 in food, environmental water and human stools [15-20]. Our laboratory has recently shown that LAMP coupled with rapid specimen processing can detect respiratory viruses in nasal swabs in under 20 minutes [21,22]. More recently, other molecular epidemiological approaches including the detection of specific genetic loci as markers for STEC [23], and pulsed fi ld gel electrophoresis, multilocus variable-number tandem repeat analysis (MLVA) and whole genome sequencing have been used to characterize *E. coli* isolates and to monitor outbreaks [24].

In this report, we evaluated four LAMP assays for the identifi ation of STEC including O157 and non-O157 *E. coli* strains. The three assays detected four different *E. coli* genes, including the *rfbE* gene of *E. coli* O157, the Shiga toxin *stx1/stx2* genes using a multiplex assay, and the *eae* virulence factor gene. The assays all had excellent sensitivity and specific ty for detecting STEC and provide faster and more accurate results than PCR.

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Materials and Methods

Clinical specimens and pre-analytical procedures

A total of 135 stool specimens submitted to the Regional Virology Laboratory at St. Josephs Healthcare Hamilton or to the Alberta Provincial Laboratory in Edmonton were used in the study. Th s study was approved by St. Joseph's Healthcare Hamilton Research Ethics Review Board and the University of Alberta Ethics Review Board. In Hamilton, 20 stool specimens collected in March 2013 were processed as follows: 100-150 mg of bulk stool was added to SK38 bead tubes (Bertin Technologies, Montigny, France) containing Lysis Buffer (bioMérieux, St Laurent, Canada). Th s 10% stool suspension was vortexed for 5 minutes, allowed to stand at room temperature for 10 to 15 minutes, then centrifuged at 14,000 rpm for 2 minutes to pellet stool material, and 200 µL of the supernatant was used to extract total nucleic acid using the automated easyMag® (bioMerieux, St. Laurent, QC) as per the manufacturer's Specific A protocol. An additional 115 stool specimens were collected at the Provincial Laboratory for Public Health (ProvLab) in Edmonton. Ninety of the 115 were fi st enriched by culture overnight in MacConkey broth and 200 µL was removed, centrifuged at 13,000 × *g* for 3 min, and the pellet was washed with 1 ml wash buffer consisting of 12 mM Tris buffer, pH 7.4. After re-centrifugation, the pellet was suspended in 200 µL rapid lysis buffer (100 mM NaCl, 10 mM Tris-HCL, pH 8.3, 1 mM EDTA, pH 9.0, 1% Triton X-100), boiled for 15 min and clarifi d by centrifugation at 13,000 × *g* for 15 min. A 1:10 dilution was performed and an aliquot (5 µL) of the diluted sample was subsequently used for molecular testing. An additional 25 isolates of known serotypes of STEC containing either *stx1*, *stx2* or both *stx1/stx2* genes from the ProvLab collection were also evaluated. These isolates were grown on BAP and a single colony was touched with a pipette tip, dispensed into 200 µL of rapid lysis buffer (above), boiled for 15 min and following centrifugation an aliquot (5 µL)of the supernatant was tested by PCR and LAMP.

E. coli O157 serotyping

E. coli serotyping was performed using O157 direct antibody agglutination (BD Difco Burlington, ON, Canada) and H7 antiserum (BD Difco) by tube fl culation as previously described [7,8].

PCR testing

Two conventional PCR assays were used in the study. One end point assay that amplifies 614 bp and 779 bp regions of the *stx1* and *stx2* genes, respectively was performed as described [12] A second endpoint PCR assay was used to resolve discordant results. Th s assay which amplifies a 259 bp region of the *E. coli* O157 *rfb* gene, 180 bp and 255 bp regions of the *stx1* and *stx2* genes, respectively, and a 384 bp region of the *eae* gene and was performed as described by Paton and Paton [13]. For all PCR assays 5 µL of extracted nucleic acid was used for each reaction.

xTAG® GPP Assay

Nucleic acid extracted from 20 stools collected at St. Joseph's Healthcare Hamilton was tested in the xTAG® GPP Assay (Luminex Molecular Diagnostics, Toronto, ON, Canada) which detects 15 Gastrointestinal Pathogen targets including Campylobacter (*C. jejuni*, *C. coli* and *C. lari* only), *Clostridium difficile* toxin A/B, Cryptosporidium (*C. parvum* and *C. hominis* only), *E. coli* O157, Enterotoxigenic *E. coli* (ETEC) LT/ST, Giardia (*G. lamblia* only, also known as *G. intestinalis* and *G. duodenalis*), Norovirus GI/GII, Rotavirus A, Salmonella, Shiga Toxin-producing *E. coli* (STEC) stx1/stx2, and Shigella (*S. boydii*, *S. sonnei*, *S. flexneri* and *S. dysenteriae*). The xTAG® GPP assay was performed according to the manufacturer's instructions using 10 µL of extracted nucleic acid from bulk stool prepared as described above.

Preparation of transcripts

PCR amplicons containing full length gene targets for LAMP *viz. rfbE* gene of *E. coli* O157, the *stx1*/*stx2* genes and the *eae* gene were cloned into pGEM-T vector using standard methods. Transcripts were prepared using an *in vitro* transcription Kit (Ambion, Life Technologies, Burlington, ON) and RNA copy number was determined by reading absorbance at A260 nm (1 Absorbance unit equals 40 ug RNA).

LAMP assays

Four LAMP assays that detect either the *E. coli* O157 *rfbE* gene, the Shiga toxin genes *stx1* and *stx2*, or the *eae* virulence factor gene were used to test for *E. coli* O157 (STEC) and non-O157 *E. coli*. The primers consisted of a set of 5 or 6 primers for each gene target and were purchased as RUO reagents from Canadian Molecular Developments (Division of Pro-Lab Diagnostics, Richmond Hill, ON, Canada). The fi al reaction volume for LAMP was 25 µL and consisted of 15 µL of ISO-0001 MasterMix (Optigene, Horsham, UK), 5 µL primer mix and 5 µL of either easyMag® extracted nucleic acid or boiled lysate as described above. The 5 µL primer mix for each gene target consisted of the following: F3 and B3 primers at 0.2 µM, FIP and BIP primers at 0.8 µM, and LF and LB primers at 0.4 µM as described previously. The specific ty of each assay were determined by testing extracted nucleic acid from a range of other pathogens including both bacteria and viruses. The reactions were run on a real time fluorometer (Genie II from Optigene) at 65°C for 30-40 min, followed by heating and cooling steps of 98°C to 80°C (0.05°C/s) to allow re-annealing of any amplifi d DNA product. The Genie II instrument displays the amplifi ation curve, the amplifi ation time in min/sec and the annealing temperature of the amplifi d product. A positive result is indicated by either an amplifi ation time (min and seconds), an amplifi ation curve, or a melting temperature (Tm) within 2°C of the predicted Tm. A positive control consisting of *E. coli* O157 (EDL933) containing *rfbE*, *stx1*/*stx2*, and *eae* genes was included in each run. All specimens were tested blindly by LAMP, PCR and the Luminex xTAG® GPP® assay. The sensitivity and specific ty of the LAMP assays were calculated using a combined reference standard of positivity by two or more assays.

Results

We evaluated four LAMP assays for the detection of Shiga toxin-producing *E. coli* O157 and non-O157 strains. Four sets of primers were used for the detection of four genes, including the *rfbE* gene of serotype O157 and three virulence factor genes *viz. stx1,stx2* and *eae*. The LAMP assays for *rfbE* and *eae* were run as separate assays while the assays for *stx1*and *stx2* were combined into a single multiplex LAMP assay. The assays were run on a real time fluorometer that displayed amplifi ation signals in real time and at the end of the run displayed both the time to positivity and the annealing temperature (Tm) for each specimen that provided confi mation of a positive result. Figure 1 shows a typical amplifi ation curve and Tm value for the *stx2* gene. The Tm values for the four gene targets were between 83 and 88°C and were consistently within two degrees of the expected values for each target for all positive specimens. For the *stx1*/*stx2* multiplex assay the Tm values for *stx1* and *stx2* were overlapping so that a positive result could not distinguish which of the two Shiga toxin genes was present. LAMP was successfully run on extracted nucleic acid and also on aliquots of cell lysates obtained using a rapid lysis method where a single colony was picked from an

agar plate, boiled for 15 min, and 5 µL tested directly in the assay. Using this rapid method, we were able to identify the presence of all virulence genes in a single colony within 30-40 minutes.

To assess the specific ty of the LAMP assays, we fi st tested purifi d nucleic acid from 35 different enteric specimens which represented 29 enteric bacteria, four enteric viruses *viz.* Adenovirus, Rotavirus and Norovirus GI and GII, and two parasites, Giardia and Cryptococcus. Nucleic acid was purifi d from *S. aureus, S. epidermidis, E. faecalis, M. luteus, S. saprophyticus, P. mirabilis, Y. entercolitica, S.* Typhimurium,. *S. marcescnes, S. sonnei, S. flexneri, K. pneumonia, P. vulgaris, P. aeruginosa, E. cloacae, S. boydii*, ETEC LT/ST, *E. coli* O12826, *Campylobacter, C. difficile, S. dysenteriae*, Adenovirus, Norovirus, Rotavirus, *Giardia* and *Crytosporidium*. All extracted nucleic acid samples tested negative for all four LAMP targets (Table 1). *E. coli* O26:B6, *E. coli* O55:B5, *E. coli* O86: B7, *E. coli* O111:B4 and *E. coli* O121:B8 were negative for the O157 *rfbE* and *stx1/stx2* genes but tested positive for the *eae* gene by LAMP. These results were confi med by PCR using the primers described by Patton et al. [13]. Of note is the fact that specimens which typed as O rough:H7 and O22:H2 were positive by both LAMP and PCR for the *rfbE* gene using the Paton et al. *E. coli* O157 primers.

To determine the analytical sensitivity of the LAMP assays, we tested serial dilutions of *in vitro* transcripts prepared from cloned PCR products. All four LAMP assays had a lower limit of detection of between 10 and 100 genome equivalents (*ge*). The *rfbE* assay detected

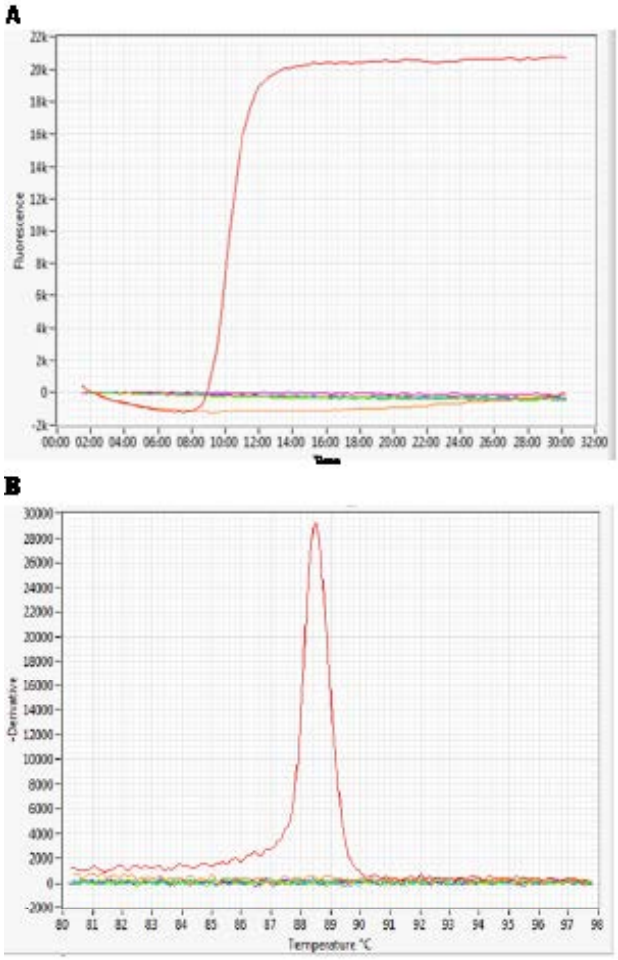

Figure 1: Typical amplification curve for the *E. coli stx2* gene.

Specimen ID	Amplification Time (mm:ss), Annealing Temperature (°C)		
	stx1/stx2	***eae***	***rfbE***
S. aureus (ATCC 25923)	-	-	-
S. epidermidis (ATCC 1228)	-	-	-
E. faecalis (ATCC 29212)	-	-	-
M. luteus (ATCC 49732)	-	-	-
S. saprophyticus (ATCC 15305)	-	-	-
P. mirabilis (ATCC 43071)	-	-	-
Y. entercolitica (ATCC 9610)	-	-	-
S. Typhimurium (ATCC 14028)	-	-	-
S. marcescens (ATCC 8100)	-	-	-
S. sonnei	-	-	-
S. flexneri (ATCC 12022)	-	-	-
K. pneumoniae (ATCC 13883)	-	-	-
P. vulgaris (ATCC 13315)	-	-	-
P. aeruginosa (ATCC 27853)	-	-	-
E. cloacae (ATCC 13047)	-	-	-
Shigella boydii	-	-	-
ETEC LT/ST (clinical stool)	-	ND	-
Salmonella (clinical stool)	-	ND	-
E. coli (ATCC 25922)	-	ND	-
E. coli O26:B6*	-	22:30, 83.40	-
E. coli O55:B5*	-	5.30, 83.67	-
E. coli O86:B7*	-	9.30, 83.80	-
E. coli O128:B12	-	-	-
E. coli O111:B4*	-	18:30, 83.58	-
E. coli O121:B8*	-	16:15, 83.48	-
Campylobacter (clinical stool)	-	ND	-
Shigella (clinical stool)	-	ND	-
C. difficile (clinical stool)	-	ND	-
Shigella dysenteriae	16:15, 86.09	-	-
Giardia (clinical stool)	-	ND	ND
Crytosporidium (clinical stool)	-	ND	-
Adenovirus (clinical stool)	-	ND	-
Norovirus GI (clinical stool)	-	ND	-
Norovirus GII (clinical stool)	-	ND	-
Rotavirus A (clinical stool)	-	ND	-

Note: **E. coli* O26:B6, *E. coli* O55:B5, *E. coli* O86:B7, *E. coli* O111:B4, *E. coli* O121:B8 also tested positive in the *eae* PCR from Paton et al. (1998). The *E coli* O157 LAMP assay detected samples typed to be Orough:H7 and O22:H2. But these samples also tested positive with the *E. coli* O157 primers from Paton et al. (1998).

Table 1: Specificity of the LAMP assays for *E. coli stx1/stx2*, *eae* and *rfbE* genes.

10 *ge* in 2 out of 3 replicates, the *stx1/stx*2 assay detected 10 *ge* in 1/3 replicates while the *stx*2 and e*ae* assays detected 100 *ge* in 2/3 and 3/3 replicates respectively (Table 2). All four LAMP assays were able to detect 10 CFU/mL (Table 3).

We next evaluated the LAMP assays using stool specimens that tested positive for one or more enteric pathogens in the xTAG® GPP Assay (Table 4). Elevan of the 20 stool specimens were positive by either the *rfbE* or the *stx1/stx2* LAMP assays. One specimen, GPP03-48B, tested positive for *stx1/stx2* by the xTAG® GPP, but was negative by the LAMP *stx1/stx2* assay. Sequencing of the xTAG® GPP amplfi d product showed that this sample was negative for the *stx1/stx 2* genes. A second specimen, GPP03-174B, was *E. coli* O157 positive in the xTAG®GPP test, but tested negative in the *rfbE* LAMP assay. Sequencing of the PCR amplicon showed the specimen to be *E. coli* O157 negative. After discordant resolution of these 20 specimens, the *stx1/stx2* LAMP assay had a sensitivity of 100% (8/8) and specific ty of 100% (12/12). Similarly, the *rfbE* LAMP assay had a sensitivity of 7/7 (100%) and specific ty of

Number of copies of rfbE target	Amplification Time (mm:ss), Annealing Temperature (°C)
10^6 copies	12:30, 85.47
10^5 copies	13:45, 85.54
10^4 copies	15:30, 85.82
10^3 copies	17:00, 85.81
10^3 copies	17:00, 85.34
10^3 copies	17:00, 85.37
10^2 copies	17.30, 85.60
10^2 copies	22:00, 85.60
10^2 copies	18:00, 85.59
10 copies	-
10 copies	17:45, 85.73
10 copies	18:00, 85.84
1 copy	-
Number of copies of stx1 or stx2 target	**Amplification Time (mm:ss), Annealing Temperature (°C)**
Stx1 10^6 copies	13:15, 85.79
Stx1 10^5 copies	16:00, 85.74
Stx1 10^4 copies	19:30, 85.83
Stx1 10^3 copies	21:30, 85.79
Stx1 10^3 copies	*, 85.68
Stx1 10^3 copies	22:45, 85.44
Stx1 10^2 copies	- , 85.99
Stx1 10^2 copies	-
Stx1 10^2 copies	-
Stx1 10 copies	29:00, 85.62
Stx1 10 copies	-
Stx1 10 copies	-
Stx2 10^6 copies	17:30, 86.99
Stx2 10^5 copies	18:45, 87.45
Stx2 10^4 copies	23:15, 87.40
Stx2 10^3 copies	- ,88.57
Stx2 10^3 copies	- ,88.69
Stx2 10^3 copies	-
Stx2 10^2 copies	- ,87.07
Stx2 10^2 copies	*,87.27
Stx2 10^2 copies	-
Stx2 10 copies	-
Stx2 10 copies	-
Stx2 10 copies	-
Number of copies of eae target	**Amplification Time (mm:ss), Annealing Temperature (°C)**
10^6 copies	11:00, 83.02
10^5 copies	12:30, 83.01
10^4 copies	13:30, 82.92
10^3 copies	16:00, 82.53
10^3 copies	14:45, 83.07
10^3 copies	15:45, 83.17
10^2 copies	15:15, 84.29
10^2 copies	17:15, 82.92
10^2 copies	17:15, 82.95
10 copies	-
10 copies	-
10 copies	-
1 copy	-
1 copy	-
Note: The result is considered to be positive if either an amplification time and/or a Tm value within 2 degrees of the predicted Tm is recorded or if an amplification curve is displayed but the time to positivity is absent and the Tm is within range.	

Table 2: Lower limit of detection of LAMP assays for *stx1/stx2*, *eae* and *rfbE* genes.

Concentration of *E. coli* O157 (EDL933)	Amplification Time (mm:ss), Annealing Temperature (°C)
10^6 CFU/ml	12:30, 84.19
10^5 CFU/ml	14:15, 84.18
10^4 CFU/ml	16:15, 84.24
10^3 CFU/ml	18:45, 84.20
10^2 CFU/ml	20:15, 84.29
10^1 CFU/ml	20:45, 84.18
Note: A stock culture of *E. coli* O157 (EDL 933) was diluted and aliquots containing 101-106 CFU were tested by LAMP using the *rfbE* primers. Similar results were obtained using the *stx1, stx2,* and *eae* LAMP assays.	

Table 3: Lower limit of detection of *rfbE* LAMP assay for detecting *E. coli* O157.

Specimen ID	xTAG®GPP test result	LAMPb *stx1/stx2* result (mm:ss,T*m*)	LAMPb *rfbE* result (mm:ss,T*m*)
S002	*E. coli* O157, STEC *stx*1/*stx*2	15:15, 87.18	14:45, 84.09
S010	*E. coli* O157, STEC *stx*1/*stx*2	11:15, 87.27	11:00, 84.19
S016	*E. coli* O157, STEC *stx*1/*stx*2	15:00, 87.22	15:30, 84.04
S028	*Campylobacter*, STEC *stx*1/*stx*2	23:30, 86.78	NA
S037	*E. coli* O157, STEC *stx*1/*stx*2	15:00, 87.40	15:00, 83.85
S092	*E. coli* O157, STEC *stx*1/*stx*2	13:00, 87.40	12:45, 84.25
GPP03-17B	Norovirus GI/GII, ETEC LT/ST, STEC *stx*1/*stx*2	-*, 85.62	-
GPP03-48B	Adenovirus 40/41, Rotavirus A, STEC *stx*1/*stx*2, Shigella	-	-
GPP03-90B	*E. coli* O157, Giardia, MS2 failure	-	20:15, 84.27
GPP03-25B	Adenovirus 40/41, Norovirus GI/GII, *E. coli* O157	-	-, 84.29
GPP03-174B	Rotavirus A, C. difficile toxin A/B, *E. coli* O157	-	-
GPP03-198B	Rotavirus A, STEC *stx*1/*stx*2	9:45, 87.16	-
GPP03-6B	*Campylobacter*, ETEC LT/ST	-	-
GPP03-10B	Norovirus GI/GII, *Campylobacter,* ETEC LT/ST, *Salmonella*	-	-
GPP03-11B	*Campylobacter*, Cryptosporidium	-	-
GPP03-14B	Adenovirus 40/41, Norovirus GI/GII	-	-
GPP03-156B	Rotavirus A, ETEC LT/ST, *Salmonella*	-	-
GPP03-160B	Rotavirus A, *Salmonella*	-	-
GPP03-170B	Rotavirus A, ETEC LT/ST, *Salmonella*	-	-
GPP03-15B	*Shigella*	-	-
E. coli O157		9:45, 87.16	9:30, 84.14
Note: aTwenty xTAG®GPP positive stool specimens were tested by LAMP for the presence of *stx1/stx2* and *rfbE* genes. LAMP results are expressed as time to positivity in mm:ss, Tm of the amplification curve. The result is considered to be positive if either an amplification time and/or a Tm value within 2 degrees of the predicted Tm is recorded or if an amplification curve is displayed but the time to positivity is absent and the Tm is within range.			

Table 4: LAMP results for 20 xTAG®GPP positive stool specimens.

12/12 (100%). The *stx1/stx2* and the *rfbE* LAMP results were negative for the stool specimens that were positive for *Giardia*, Adenovirus, Norovirus GI/GII, Rotavirus A, *Campylobacter, ETEC LT/ST, C. difficile A/B, Cryptosporidium, Shigella,* and *Salmonella*.

Next, we tested 25 samples from ProvLab's collection which included 22 *stx1/stx2* positive and where *stx1/stx2* subtyping were performed. Th *stx1/stx2* LAMP assay correctly detected 21/22 positive specimens including the following *stx* subtypes *stx1, 1a, 1c, 1d, 2a, 2b, 2c, 2d, 2e*, and *2g* for a sensitivity of 95.5% (21/22). One of the *stx2f* subtypes was missed (sample #24, Table 5). Th ee PCR negative specimens (#3, 4 and 6) were also negative by LAMP. Using PCR as the comparator, the LAMP *eae* and *rfbE* assays had a sensitivity of 100% (13/13) and 100% (7/7) and a specific ty of 100% (12/12) and 100% (18/18), respectively.

The LAMP assays were next evaluated by testing an additional 90 stools collected prospectively and submitted to the ProvLab testing for STEC testing in a research study. These were tested by routine enteric bacteria screening and by PCR for *stx1/stx2*. Eleven stools were positive for *E. coli* O157 by both conventional serotyping methods and the *rfbE* LAMP assay. Two additional stools were positive by *rfbE* LAMP assay but negative based on conventional serotyping method. These two discordants were also positive for the *rfbE* gene by PCR using the Paton primers. After discordant resolution, the sensitivity and specific ty of the *rfbE* assay was 100% (13/13) and 100% (77/77) respectively. Thi ty four of the stools were positive for either *stx1* or *stx2* using conventional PCR methods; 14 were positive for *stx1*, 5 were positive for *stx2* and 15 were positive for *stx1*and *stx2*. All 34 PCR positives were positive by the

ID #	Serotypes (Stx status)	PCR Results		LAMP Results (mm:ss), *Tm* (°C)		
		rfbE	***eae***	***stx1/stx2***	***rfbE***	***eae***
1	O111:H8 (*Stx1/2*)	-	+	11:45, 87.42	-	11:45, 83.41
2	O174:H8 (*Stx1*)	-	-	13:00, 85.83	-	-
3	Neg	-	-	-	-	-
4	Neg	-	-	-	-	-
5	O121:H11 (*Stx2*)	-	+	15:45, 87.48	-	17:45, 83.69
6	Neg	-	-	-	-	-
7	O25:H1 (*Stx2*)	-	+	12:45, 87.55	-	10:30, 83.49
8	O157:H7 (*Stx1/2*)	+	+	12:15, 87.55	13:15, 84.32	11:15, 83.39
9	O157:H7 (*Stx1/2*)	+	+	13:00, 87.77	14:00, 84.24	11:45, 84.64
10	O157:H7 (*Stx1/2*)	+	+	12:15, 87.64	12:45, 84.21	10:30, 84.71
11	O157:H8 (*Stx1/2*)	+	+	15:45, 87.82	18:15, 84.43	15:45, 84.91
12	O165:H25 (*Stx1/2*)	+	+	12:45, 87.76	12:45, 84.46	10:45, 84.90
13	ORough:H7 (*Stx1/2*)	+	+	11:30, 87.68	12:00, 84.46	10:15, 84.84
14	O26:H11 (*Stx1*)	-	+	19:00, 86.08	-	18:45, 84.92
15	Serotype ND (*Stx1a*)	+	+	13:15, 87.42	12:30, 84.40	11:15, 83.22
16	Serotype ND (*Stx1c*)	-	-	14:15, 85.82	-	-
17	Serotype ND (*Stx1d*)	-	-	15:30, 85.53	-	-
18	O103:H2 (*Stx1*)	-	+	13:45, 85.59	-	18:00, 83.11
19	Serotype ND (*Stx2a*)	-	-	13:30, 85.92	-	-
20	Serotype ND (*Stx2b*)	-	-	24:00, 87.15	-	-
21	Serotype ND (*Stx2c*)	-	-	16:00, 87.54	-	-
22	Serotype ND (*Stx2d*)	-	-	17:00, 87.34	-	-
23	Serotype ND (*Stx2e*)	-	-	20:15, 87.46	-	-
24	Serotype ND (*Stx2f*)	-	+	-	-	15:15, 83.87
25	Serotype ND (*Stx2g*)	-	-	20:15, 87.39	-	-

Table 5: Comparison of PCR and LAMP results for 25 enriched stool specimens.

LAMP assay*	% Sensitivity	% Specificity
rfbE	100 (27/27)	100 (107/107)
stx1/stx2	98.4% (63/64)	100 (71/71)
eae	98.0 (47/48)*	98.5 (66/67)

Note: *A total of 135 stool specimens from two different study sites were tested by three LAMP assays. Only 134 specimens were tested by the *rfbE* primers as one specimen had insufficient volume for *rfbE* testing.

Table 6: Overall performance of the LAMP assays for stool specimens.

stx1/stx2 LAMP assay. All 56 *stx1* or *stx2* negatives were also negative by the *stx1/stx2* LAMP assay. The LAMP *stx1/stx2* assay had a sensitivity and specific ty of 100% (34/34) and 100% (56/56), respectively. Of the 34 stools that were positive for *stx1* or *stx2*, there were 31 that tested positive by the *eae* LAMP assay. One of the *eae* LAMP negatives was negative by PCR using the Paton primers. There were four specimens that were negative by conventional methods that were positive by the LAMP *eae* assay, and three of these were also positive using the Paton PCR assay. Using a combined reference standard of positivity in two or more assays, the sensitivity and specific ty of the *eae* LAMP assay for these 90 specimens was 97.1% (34/35) and 98.2% (54/55), respectively.

When the data was combined for all 134 stool specimens, the overall sensitivity and specific ty for the *rfbE* LAMP assay was 100% (27/27) and 100% (107/107), respectively (Table 6). The overall sensitivity and specific ty for the *stx1/stx2* assay was 98.4% (63/64) and 100% (71/71) while the overall sensitivity and specific ty for the *eae* assay was 98.0% (47/48) and 98.5% (66/67), respectively.

Discussion

We evaluated four LAMP assays for detecting *rfbE*, *stx1/stx2* and *eae* genes for the identifi ation of Shiga toxin-producing *E. coli O157* (STEC) and non-O157 *E. coli*. The LAMP assays for *rfbE* and *eae* were uniplex assays while the assay for the *stx1/stx2* genes was a multiplex assay with primers for each gene. All assays were run on the Genie II real time fluorometer that displays the time to positivity and the annealing temperature of the product [21,22]. These assays could be used with either extracted nucleic acid from stool specimens or with overnight cultures (either broth or agar plate colonies) coupled with a rapid lysis method providing a rapid test result in under one hour. The time to positivity by LAMP was inversely correlated with the number of bacteria in the sample, increasing from 12.5 min for106 CFU /mL to 20.75 min for 1 CFU/mL (Table 3). All four LAMP assays had excellent analytical sensitivity with a lower limit of detection of 10 ge for *rfbE* and *stx1/stx2* targets and 100 ge for the *eae t*arget. The *rfbE* assay was capable of detecting 1°C FU/mL. All LAMP assays had excellent specific ty and gave negative results for stools that tested positive for other enteric pathogens (Table 1). We evaluated the performance of the three assays using a total of 135 stool specimens 45 of which were collected from two clinical studies and 90 were submitted for routine enteric bacteria screening. For processed stool specimens the sensitivity of the three assays was 100% (27/27) for *rfbE*, 98.4% (63/64) for *stx1/stx2* and 98.0% (47/48) for *eae* gene. The specific ty of the three assays was also excellent; 100% (107/107) for *rfbE*, 100% (71/71) for *stx1/stx2* and 98.5% (66/67) for *eae*. For cultured or processed stool specimens, including overnight culture enrichment followed by DNA extraction, the amplifi ation times for the three gene targets ranged from 7 to 29 minutes allowing the LAMP assays to provide results in under an hour which was considerably faster than the 2-4 hours required for conventional PCR assays.

The majority of clinical microbiology laboratories today rely on culture-based techniques to identify *E. coli* O157. These include the use

BBL CHROMagar (BD, Oakville, ON, Canada), which identifies O157 STEC based on a specific colony color or Sorbitol MacConkey agar plates which identify sorbitol non-fermenting *E. coli* O157. These culture based assays rely on phenotypic traits and typically require confi mation using molecular testing such as PCR. Testing stool specimens directly for Shiga toxin by either Vero cell culture requires cell culture expertise and can be time consuming. The ImmunoCardSTAT! Test from Meridian Bioscience (Cinncinati, OH, USA) test can be performed directly on stools and gives results in about 30 minutes but the sensitivity is poor. A new lateral fl w test called the QUIK CHEK assay has an improved sensitivity around 80% but both tests miss a signifi ant number of *E. coli* STEC positives [8,25]. Many different PCR assays have been developed for detecting *E. coli* O157. Although these assays show differences in performance, PCR generally has good sensitivity and high negative predictive values compared to culture and provide results much faster than the 22-30 hours required for culture. In one study, detecting *E. coli* O157 or non-O157 STEC by PCR had a lower specific ty and sensitivity compared with conventional methods especially when targeting the *stx2* gene which was due to genetic polymorphisms [9]. Recently, we proposed an algorithm for STEC screening and isolation using several well-characterized techniques including routine stool culturing, the QUIK CHEK assay and real-time PCR [10]. The QUIK CHEK assay alone was not suffici t, but could be combined with PCR to achieve acceptable sensitivity and specific ty. If either the QUIK CHEK assay or real-time PCR was positive, further isolation was recommended using BBLTM CHROMagar O157 and Colorex STEC plates, followed by confi mation with PCR. The LAMP assays described in this study had excellent sensitivity and specific ty for detecting *E. coli* O157 or non-O157 STEC and represent an improvement over PCR in both accuracy and provide a faster turn-around time. All the LAMP assays provided results in less than one hour compared with conventional PCR assays requiring 2-4 hours or real-time qPCR assays that can provide results in 45 minutes [25]. We show here the identifi ation of *E. coli* STEC in stool specimens using LAMP assays without the need for screening with immunoassays or enrichment by overnight culture. These LAMP assays could be included in a testing algorithm as either front line testing of immunoassay screen positive stool specimens or for confi matory testing of *E. coli* O157 and non-O157 following culture enrichment. We do not know the performance of these assays when used directly on stool specimens that have not been enriched by overnight culture. Th s is the subject of an ongoing study.

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Conflict of Interest

No conflict of interest

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