Journal of Microbiological Methods 159 (2019) 51–55

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

General detection of Shiga toxin 2 and subtyping of Shiga toxin 1 and 2 in *Escherichia coli* using qPCR

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ARTICLE INFO

Keywords:
Shiga toxin-producing *E. coli* (STEC)
qPCR
Subtyping
General detection

ABSTRACT

Shiga toxin-producing *E. coli* (STEC) is a gastrointestinal pathogen and has been recognized as one of the serious problems in public health. Shiga toxin genes (*stx*) can be grouped into different types according to their differences in sequence and biological activities. The two main groups of *stx* are stx_1 and stx_2 with each group containing several subtypes. It is essential to develop rapid stx_1/stx_2 subtyping assays and accurate stx_2 general detection assays to provide a quicker turn-around time to predict disease outcome and also to provide data for surveillance purposes. The stx_2 general detection qPCR assay developed in this study showed 100% sensitivity and 100% specificity with no cross reactivity with stx_2 negative STEC and non-STEC isolates. This stx_2 general detection assay was able to detect all seven different stx_2 subtypes at low level of detection and with good PCR efficiency. In addition, stx_1/stx_2 subtyping qPCR assays were succesfully developed to detect all stx_1 subtypes and stx_2 subtypes with the exception of one stx_{2b} subtype carried by one strain. The qPCR stx_1/stx_2 subtyping assays showed 100% specificity with no cross reactivity on subtypes not targeted by each assay. The rapidity with faster turn-around time along with high throughput volume of the stx_1/stx_2 subtyping and stx_2 general detection qPCR assays will have great value as tools for STEC associated risk assessment, outbreak monitoring, epidemiology studies, and clinical management.

1. Introduction

Shiga toxin-producing *E. coli* (STEC) is a gastrointestinal pathogen and has been recognized as one of the serious problems in public health as it can cause major outbreaks with great economic impact as well as disease burden in the health care system (Bartsch et al., 2018). STEC can cause infection in humans ranging from mild diarrhea to hemorrhagic colitis with complications such as hemolytic uremic syndrome (Smith et al., 2014; Thorpe, 2004) and even death. STEC has been found in large and small ruminants, dogs, and birds (Persad and LeJeune, 2014). Most of the animals are asymptomatic upon STEC colonization and can act as the reservoir (Bryan et al., 2015; Pruimboom-Brees et al., 2000; Smith et al., 2014). Transmission to humans can be through contaminated food, water, or direct contact with animals or person-to-person (Kintz et al., 2017).

The primary virulence factor of all STEC is the production of Shiga toxins (Stx) (Melton-Celsa, 2014). These toxins belong to a group of AB5 proteins consisting of two major subunits: an A subunit that joins noncovalently to a pentamer of five identical B subunits (Melton-Celsa, 2014); the B subunit targets the glycolipid globotriaosylceramide (Gb3) of the host cell. Once bound to the receptor, Stx can enter host cells and the A subunit can remove an adenine from 28S rRNA and therefore inhibit protein synthesis (Bryan et al., 2015; Melton-Celsa, 2014).

Shiga toxin genes (*stx*) can be grouped into different types according to differences in their sequence and biological activity (Melton-Celsa, 2014; Scheutz et al., 2012). The two main groups of *stx* are stx_1 and stx_2 with each group containing several subtypes. stx_1 has three subtypes stx_{1a}, stx_{1c}, and stx_{1d} while stx_2 has seven subtypes, stx_{2a} to stx_{2g}. Different stx_1/stx_2 subtypes vary in their pathogenicity, for example, stx_{1c} and stx_{1d} are rarely associated with human infection (Melton-Celsa, 2014); stx_{2a} has been reported to be more virulent in human infection as compared to isolates containing stx_{2e}, stx_{2f}, and stx_{2g} (Kruger and Lucchesi, 2015). Currently, the most commonly used stx_1/stx_2 subtyping assays were developed by Scheutz et al. (2012). However, these assays (Scheutz et al., 2012) use conventional PCR that are time consuming, labor intensive and with a lengthy turn-around time for reporting. Some studies have developed qPCR assays to detect different stx_1/stx_2 subtypes but only focused on certain subtypes, such as stx_{1c},

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https://doi.org/10.1016/j.mimet.2019.02.008
Received 21 January 2019; Received in revised form 13 February 2019; Accepted 13 February 2019
Available online 14 February 2019

stx_{1d}, and stx_{2f} (Harada et al., 2015; Kuczius et al., 2004). With real-time PCR (qPCR) being routinely used in frontline microbiology diagnostic laboratories and reference laboratories, it is essential to develop rapid stx_1/stx_2 subtyping assays to provide a quicker turn-around time for detecting all STEC subtypes to predict disease outcome and also to provide data for surveillance purposes.

In addition to stx_1/stx_2 subtyping assays, the general detection of all stx_1 and stx_2 are essential in STEC detection. At present, various assays have been developed for the general detection of stx_1 and stx_2 (Feng et al., 2011; Margot et al., 2013; Scheutz et al., 2012; Wasilenko et al., 2012). However, some detection methods miss certain subtypes, have low sensitivity, or were developed as a gel-based PCR assay (Feng et al., 2011; Margot et al., 2013; Scheutz et al., 2012; Wasilenko et al., 2012). In addition, the DNA sequence polymorphism of stx_1 and stx_2 can be a challenge in designing primers and probes for general detection of stx_1 or stx_2 by molecular assays. This is in particular challenging for stx_2 (Chui et al., 2010) as it has seven subtypes and more sequence polymorphisms. Therefore, the two objectives of this study were to develop rapid qPCR assays for 1) the general detection of stx_2 and 2) subtyping of all stx_1/stx_2 subtypes.

2. Materials and methods

A total of 113 STEC strains were used in this study. Among this STEC collection, 39 strains were previously subtyped by conventional PCR assays developed by Scheutz et al. (2012) and were used in the stx_1/stx_2 subtyping qPCR assays in this study for our validation. The remaining 74 clinical isolates confirmed as STEC by enzyme immune assay (SHIGA TOXIN QUIK CHECK, [TechLab, Blacksburg, VA, USA]) and our in-house qPCR assay (Chui et al., 2010) were used in the stx_2 general detection qPCR assay. In addition, a panel of non-STEC bacterial strains including 8 clinical and 13 ATCC strains were used to determine the specificity of the primers and probes for stx_2 general detection. The isolates consisted of the following: clinical isolates of *Aeromonas hydrophila*, *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Shigella boydii* (SEROTYPE 2), *Shigella dysenteriae* (SEROTYPE 2), *Shigella flexneri* (SEROTYPE 2), *Shigella sonnei*, *Yersinia enterocolitica*, *Proteus mirabilis* (ATCC12453), *Campylobacter coli* (ATCC33559), *Campylobacter jejuni* (ATCC33291), *Citrobacter ferundii* (ATCC8090), *Klebsiella pneumoniae* (ATCC31488), *Enterobacter aerogenes* (ATCC13048), *Pseudomonas aeruginosa* (ATCC27853), *Enterobacter cloacae* (ATCC13047), *Staphylococcus aureus* (ATCC25923), *Staphylococcus aureus* (ATCC25913), *Escherichia coli* (ATCC25922), *Bacillus cereus* (ATCC14579), *Staphylococcus epidermidis* (ATCC12228).

For DNA extraction, all isolates were grown on sheep blood agar plates overnight and a single colony was inoculated into 200 µL of rapid lysis buffer (100 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 8.3, 1 mmol/L EDTA, pH 9.0; 1% Triton X-100), boiled for 15 min followed by centrifugation at 13, 000 x g for 15 min. The supernatant was used as DNA template.

For stx_2 general detection qPCR assay, Taqman probe (Applied Biosystems, Foster City, CA, USA) was used (Table 1). Taqman probe and its corresponding primers were designed using Primer3 (Untergasser et al., 2012). The total reaction contained 10 µL TaqMan® Fast Advanced Master Mix (Applied Biosystems, Foster City, CA, USA), 0.9 µM of each primer, 0.25 µM probe, 5 µL DNA template, and molecular biology grade water added to a total volume of 20 µL. The qPCR amplification conditions for this assay were 95 °C for 20 s followed by 40 cycles of 95 °C for three seconds and 60 °C for 30 s. All qPCR assays in this study were performed on the Applied Biosystems® 7500 fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) and nuclease-free water (Invitrogen, Carlsbad, CA, USA) was used as no template control (NTC). To determine the sensitivity and specificity of stx_2 general-detection qPCR assay, 113 STEC strains (111 positive for stx_2 and two negative for stx_2 but positive for stx_1) and 21 non-STEC specificity panel strains were used. The sensitivity was calculated as the proportion of stx_2 positive samples that was determined as positive by the stx_2 general-detection qPCR assay, while the specificity was calculated as the proportion of stx_2 negative strains that are determined as negative by the assay. The limit of detection (LOD) for this stx_2 general detection assay was determined using DNA extracted from ten-fold cell dilutions of seven STEC strains (each is positive for one of the seven different stx_2 subtypes). In brief, 1 mL of cell suspension for each strain was adjusted to an optical density of 0.5 in 10 mmol/L Tris buffer using the Microscan® Turbidity Meter (DADE Behring, Inc., Deerfield, US) and dilutions were made from 10^{-1} to 10^{-8}. Colony forming units (CFU) were determined by inoculating 100 µL of each of the dilutions onto Luria Bertani (LB) plates in triplicates, and incubated at 37 °C overnight. In parallel, the original cell suspensions were also diluted in lysis buffer from 10^{-1} to 10^{-8} for DNA extraction as described in the previous paragraph. All PCR assays for LOD calculation were performed in triplicates in three separate runs. The limit of detection (with 95% confidence intervals [LOD95]) was calculated using a program based on Microsoft Office Excel (Wilrich and Wilrich, 2009).

For stx_1/stx_2 subtyping assays, either probe or SYBR green based qPCR approaches were used. The primers and probes sequences are listed in Table 1. In probe based qPCR assays, the probes are Locked Nucleic Acid (LNA) probes (Integrated DNA Technology, Skokie, Illinois) which has increased hybridization temperature through the modification to the ribose backbone. The probes and their corresponding primers were designed and checked by the Biophysics tool at http://biophysics.idtdna.com/ based on the multiple sequence alignment of the reference sequences listed in Table 1. All probe based qPCR stx_1/stx_2 subtyping are duplex assays. The total reaction contained 10 µL TaqMan® Fast Advanced Master Mix, 0.9 µM of each primer, 0.25 µM probe, 5 µL DNA template, and molecular biology grade water added to a total volume of 20 µL. The qPCR amplification conditions for probe based assays were 95 °C for 20 s followed by 40 cycles of 95 °C for three seconds and 60 °C for 30 s. For SYBR green based stx_1/stx_2 subtyping assays, the primers were adopted from a previous study (Scheutz et al., 2012). The reaction volume contained 5 µL of *E. coli* genomic DNA template and 10 µL of Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), 0.3 µM of each primer, and molecular biology grade water added to a total volume of 20 µL. The qPCR amplification conditions for SYBR green based qPCR were 95 °C for 20 s followed by 40 cycles of 95 °C for three seconds and 66 °C for 30 s. Melting curves for SYBR green based qPCR were generated by monitoring the fluorescence of SYBR green signal from 65 °C to 95 °C by a linear temperature increase rate at 0.3 °C/s. Both probe or SYBR green based qPCR stx_1/stx_2 subtyping assays were performed in duplicates.

One challenge of this study is the limited number of subtypes available in our STEC collection. To increase the robustness of our stx_1/stx_2 subtyping assays, the stx_1/stx_2 sequences with known subtype information from the public domain in the NCBI nucleotide database were used to supplement our data. These sequences were compared to the primers and probes sequences used in our stx_1/stx_2 subtyping assays. The sequences which have 100% concordance to the primers and probes used in our subtyping assays were listed in the results.

3. Results and discussion

For the stx_2 general detection by qPCR assay, 111 stx_2 positive STEC isolates, two stx_2 negative STEC isolates, and 21 non-STEC isolates were included. This assay showed 100% sensitivity and 100% specificity with no cross reactivity with stx_2 negative STEC and non-STEC isolates. The LOD and PCR efficiency of stx_2 general-detection qPCR assay were evaluated on seven stx_2 positive STEC strains with each of them possessing different stx_2 subtypes (stx_{2a} to stx_{2g}). The lowest LOD for stx_2 general-detection qPCR assay was achieved for subtype stx_{2c} at 1 CFU (95% confidence interval [CI_{95}] of 1 to 3 CFU) while the highest LOD were found in stx_{2e} at 20 CFU ($CI_{95:}$ 5 to 84). For subtypes stx_{2a}, stx_{2b}, stx_{2d}, and stx_{2g}, the LOD is 2 CFU (CI_{95}: 1 to 9). LOD for subtype stx_{2f} is

5 CFU (CI_{95}: 4 to 19). The amplification efficiencies of stx_2 general-detection qPCR assay for seven stx_2 subtypes range from 0.89 to 0.97 with a mean efficiency of 0.92 (CI_{95}: 0.90–0.95). In general, our stx_2 general detection qPCR assay was able to detect all seven different stx_2 subtypes at low LOD and with good PCR efficiency.

For stx_1/stx_2 subtyping assays, LNA probe based qPCR subtyping assays were developed for stx_{1a}, stx_{1c}, stx_{1d}, stx_{2a}, stx_{2b}, stx_{2e}, stx_{2f}, and stx_{2g}, while SYBR green qPCR subtyping assays were developed for stx_{2c} and stx_{2d} (see Table 1). In total, as shown in Tables 2, 50 stx_1 and stx_2 subtypes (six stx_{1a}, two stx_{1c}, one stx_{1d}, eight stx_{2a}, six stx_{2b}, five stx_{2c}, seven stx_{2d}, eight stx_{2e}, five stx_{2f}, and two stx_{2g}) were carried by the 39 STEC isolates used in this study. The result demonstrated that our qPCR stx_1/stx_2 subtyping assays succesfully detected all stx_1 subtypes and stx_2 subtypes of these isolates and only missed one stx_{2b} subtype carried by one strain. In addition, all qPCR stx_1/stx_2 subtyping assays showed 100% specificity with no cross reactivity on subtypes not targeted by each assay. Melting curve analysis was performed for the SYBR green qPCR subtyping assays (stx_{2c} and stx_{2d}). The results showed that the melting temperatures (Tm) for stx_{2c} are 73.39–74.42 °C. The SYBR green qPCR subtyping assay for stx_{2d} detection was a multiplex assay which incorporates three sets of primers targeting three variant stx_{2d} subtypes with amplicon sizes of 179, 235, and 280 bp. Before performing SYBR green multiplex qPCR for stx_{2d}, the amplicon size of stx_{2d} for each sample were determined by performing singleplex conventional PCR assays targeting the three stx_{2d} subtypes. The same set of primers that were used in the SYBR green multiplex qPCR for stx_{2d} was

Table 1
Primers and probes used in this study and sequence matches of stx_1/stx_2 subtypes in NCBI database.

Assays	Target	Forward primer, reverse primer, and probe sequene	Coordinates	Reference (NCBI Accession No.)	Amplicon size (bp)	Reference	NCBI Stx subtype sequence matches (Accession No.)
stx2 general detection	*stx2*	F: 5′-TTTGTYACWGTSAYAGCWGAAGC-3′ R: 5′-CCCCAGTTCARWGTRAGRTC-3′ 5′-[6-FAM]-ATACAGMGRGRATTYCGWCHGGCRCTGTC-TGA-[IABkFQ]-3′	722–744 830–849 761–792	Z33725.1	128	This study	NA
Subtyping multiplex one	*stx1a*	F: 5′-CTACGGCTTATTGTTGAACGAAAT-3′ R: 5′-GCTGTAACGTGGTATAGCTACTG-3′ 5′-[YakYel]-TT + TC + C + A + GG + T + AC-[IABkFQ]-3′	427–450 561–583 526–536	M19473.1	157	This study	AM230663.1, AM230662.1, Z36899.1, AB083044.1, Z36900.1, L04539.1, M19473.1
	stx1c	F: 5′-CTACGGCTTATTGTTGAACGAAAT-3′ R: 5′-GCTGTAACGTGGTATAGCTACTG-3′ 5′-[6-FAM]-ACAA + C + T + GCG + GT-[IABkFQ]-3′	420–443 554–576 528–538	Z36901.1	157	This study	AB071619.1, AB048237.1, DQ449666.1, Z36901.1, AB071623.1
Subtyping multiplex two	*stx1d*	F: 5′-CATCGGGAGGTACATCTTTACTG-3′ R: 5′-TGTTAAACCGCTCTTCCTCTG-3′ 5′-[Cy5]-ACA + G + G + C + GATAAT + T + TA-[IABkFQ]-3′	171–193 252–272 209–223	AY170851.1	102	This study	AY986980.1, AY986981.1, AY986982.1, AB050958.1, AB050959.1
	stx2b	F: 5′-TCATATCTGGCGTTAATGGAGTT-3′ R: 5′-TTGCCTGAACCGTAAGGC-3′ 5′-[Cy3]-TC + ATG + G + C + ATT + T + CC-[IABkFQ]-3′	654–676 744–761 681–693	AF043627.1	108	This study	AF043627.1, AJ313015.1, AJ567997.1, AB048229.1, AB048222.1, AB048226.1, AB048225.1
Subtyping multiplex three	*stx2a*	F: 5′-CTGTTAATGCAATGGCGGC-3′ R: 5′-GCAAATCCGGAGCCTGA-3′ 5′-[Cy3]-AG + G + AT + G + A + C + ACAT-[IABkFQ]-3′	1187-1205 1375-1391 1247-1258	Z33725.1	205	This study	AY443057.1, AY633472.1, EF441613.1, AF461173.1, DQ344636.3, AY633471.1, EF441599.1, EF441609.1, X07865.1, AY633459.1, EF441619.1, Z37725.1, EF441618.1, Z50754.1, AY443054.1, AF524944.1, AJ272135.1, FM998851.1, GQ429162.1
	stx2e	F: 5′-GAGCTACATCGGTATCCGTTATT-3′ R: 5′-CAATAATCAGACGAAGATGGTCAAA-3′ 5′-[Cy5]-TC + CG + T + A + G + GT + AT-[IABkFQ]-3′	408–430 497–521 458–468	M21534.1	114	This study	AM904726.1, AY332411.1, X81417.1, FM998844.1, M21534.1, FM998838.1, AJ313016.1, X81415.1, X81416.1, FM998846.1, FN182286.1, DQ449665.1, AJ249351.2, FN182284.1
Subtyping multiplex four	*stx2f*	F: 5′-TTCATATCTGGATTTAATGGAGTTCA-3′ R: 5′-TTTGTCTGAATCGCAGAGCT-3′ 5′-[6-FAM]-CA + TG + G + A + AC + G + TCC-[IABkFQ]-3′	544–569 634–653 572–583	AJ010730.1	110	This study	LC140877.1, AB472687.1, AB499802.1, AB232172.1, AJ271139.1, AJ010730.1
	stx2 g	F: 5′-TCATATCTGGCGTTAATGGAGTT-3′ R: 5′-TTGCCTGAACCGTAAGGC-3′ 5′-[Cy5]-TG + CTG + T + A + AC + A + GTG-[IABkFQ]-3′	668–690 758–775 742–754	AY286000.1	108	This study	AY286000.1, GU244510.1, AB048227.1, AB048236.1, AJ966783.1
Subtyping singleplex one	*stx2c*	F: 5′-GAAAGTCACAGTTTTTATATACAACGGGTA-3′ R: 5′-CCGGCCACYTTTACTGTGAATGTA-3′	NA	NA	177	Scheutz, F. *et al*[9]	NA
Subtyping singleplex two	*stx2d*	F: 5′-AAARTCACAGTCTTTATATACAACGGGTG-3′ R1: 5′-TTYCCGGCCACTTTTACTGTG-3′ R2: 5′-GCCTGATGCACAGGTACTGGAC-3′ O55-R: 5′-TCAACCGAGCACTTTGCAGTAG-3′	NA	NA	179 280 235	Scheutz, F. *et al*[9]	NA

Table 2
STEC strains used for stx_1/stx_2 subtyping in this study and their qPCR subtyping results.

STEC strain	Stx Subtypes	Positivity by qPCR	STEC strain	Stx Subtypes	Positivity by qPCR
STEC 1	*2c*	+	STEC 17	*2a*	+
STEC 2	*2a*	+	STEC 20	*2e*	+
	2c	+	STEC 21	*2e*	+
STEC 3	*2c*	+	STEC 22	*2b*	−
STEC 4	*2a*	+		*2d*	+
	2d	+	STEC 23	*2e*	+
STEC 5	*2d*	+	STEC 24	*2e*	+
STEC 6	*2a*	+	STEC 25	*2d*	+
STEC 7	*1c*	+	STEC 26	*2d*	+
	2b	+	STEC 27	*2e*	+
STEC 8	*2c*	+	STEC 28	*2e*	+
STEC 9	*1a*	+	STEC 29	*2e*	+
	2d	+	1335	*1a*	+
STEC 10	*2g*	+		*2a*	+
STEC 11	*2f*	+	1336	*2c*	+
STEC 12	*2f*	+	1337	*1a*	+
STEC 13	*2f*	+		*2a*	+
STEC 14	*2a*	+	1338	*2b*	+
STEC 15	*1a*	+	1339	*2d*	+
	2a	+	1340	*2g*	+
STEC 16	*2f*	+	1341	*1d*	+
STEC 18	*1a*	+	1342	*2f*	+
	2b	+	1343	*1c*	+
STEC 19	*1a*	+		*2b*	+
	2b	+	1344	*2e*	+

used in these singleplex assays. The same conventional PCR protocol was used as described previously (Scheutz et al., 2012). The melting curves results of the SYBR green multiplex qPCR for stx_{2d} showed that the Tm for stx_{2d} is 73.73–75.79, 75.93–76.11, and 77.85 °C for amplicon sizes of 179 bp, 235 bp, and 280 bp, respectively.

In addition to validating our stx_1/stx_2 subtyping qPCR assays against our own STEC collection, an *in silico* analysis was also performed to verify the robustness of our subtyping primers and probes by comparing the primers and probes sequences to stx_1/stx_2 sequences with known subtypes deposited in NCBI database. The number of stx_1/stx_2 subtype sequences (Table 1) that have 100% identity to the primers and probe for each subtype are as follows: stx_{1a} (7 matches), stx_{1c} (5 matches), stx_{1d} (5 matches), stx_{2a} (19 matches), stx_{2b} (7 matches), stx_{2e} (14 matches), stx_{2f} (6 matches), and stx_{2g} (5 matches). This result provides further evidence for the robustness of our qPCR stx_1/stx_2 subtyping assays.

In this study, stx_2 general detection and stx_1/stx_2 subtyping qPCR assays were successfully developed. The stx_2 general-detection qPCR assay developed in this study showed superior performance pertaining to assay specificity and sensitivity. Low LOD were also observed for this general detection assay across all stx_2 subtypes. This assay provided a rapid and accurate approach for detection of stx_2. With regard to stx_1/stx_2 subtyping, currently, the most commonly used assays were established by Scheutz et al. (2012), which is a conventional gel based PCR assay that is time consuming and labor intensive. The rapid stx_1/stx_2 subtyping qPCR assays developed in this study were able to detect all stx_1/stx_2 subtypes and only missed one stx_{2b} subtype carried by one strain. The failure to detect this stx_{2b} subtype is most likely caused by sequence mismatch between our stx_{2b} probe to this strain. Although one strain was missed, our stx_{2b} subtyping assay successfully detected the other five stx_{2b} subtype positive strains used in this study. The robustness of our stx_1/stx_2 subtyping assay was not only validated by testing against our own stx_1/stx_2 subtype collections but also verified against various stx_1/stx_2 subtype sequences deposited in the NCBI database. However, a larger collection of various subtypes of STEC collected from different parts of the world are required for further evaluation. Our qPCR assays for stx_1/stx_2 subtyping and stx_2 general detection can be completed within an hour in a 96 well plate format, while the conventional PCR may take up to 5–6 h and with limited sample throughput. In addition, as shown previously (Chui et al., 2010), the cost to perform qPCR (25 samples) is similar to the cost of conventional PCR. The cost for qPCR will be reduced when larger volumes of samples are processed as no extra labor is required for gel preparation, loading PCR products, electrophoresis and capturing images.

Both of the stx_1/stx_2 subtyping and stx_2 general detection qPCR assays developed in this study can be easily implemented by different types of laboratories such as for food safety, public health, frontline clinical, or research laboratories. Their rapidity with faster turn-around time along with high throughput volume will have great value as tools for STEC associated risk assessment, outbreaks monitoring, epidemiology studies, and clinical management.

Conflicts of interest

The authors declare that they have no competing interests.

Funding

Funding is provided by Alberta Health Services Residual Grant RES0023526.

Acknowledgements

We would like to thank Irene Young for providing technical help on confirming the *stx* subtypes of some strains. We would also like to thank Dr. Patrick Fach for providing some of the STEC strains.

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