

C-178



Rapid Detection of Shiga Toxin-Producing *Escherichia coli* by Real-time PCR Using the Smart Cycler®

***S. D. Bélanger*, M. Boissinot, S. Paradis, C. Ménard, F. J. Picard,
M. Ouellette, P. H. Roy, and M. G. Bergeron***

**Infectious Disease Research Center, Laval University, Québec City,
Québec, Canada**

**Infectious Diseases Research Center
of Laval University
CHUL, RC 709
2705 boulevard Laurier
Sainte-Foy, Qc
G1V 4G2
Tel : (418) 654-2705
Fax : (418) 654-2715
E-mail : cri@crchul.ulaval.ca**

ABSTRACT

Escherichia coli O157:H7 and other Shiga toxin-producing *E. coli* (STEC) has emerged in recent years as important human pathogens causing bloody diarrhea and haemolytic uremic syndrome. *E. coli* O157:H7 is the serotype most frequently associated with human pathologies. However, infections involving various non-O157 serotypes (e.g. O111, O103, O26) are found with increasing frequency in many countries. Almost all of the currently available clinical methods for the detection of these enteric pathogens focus specifically on *E. coli* O157:H7 while neglecting the other serotypes. We have developed a 40-minute real-time fluorescence-based PCR assay using the Smart Cycler® (kindly provided by Cepheid, Sunnyvale, CA), a rapid thermalcycler composed of 16 individually-driven sites coupled with individual fluorescence detectors. Based on multiple sequence alignments, we have designed 2 pairs of PCR primers amplifying efficiently all variants of the two chromosomal Shiga toxin genes *stx*₁ and *stx*₂, respectively. These primer pairs were combined for use in a multiplex assay. Two molecular beacons bearing different fluorophores were used as internal probes specific for each amplicon. Assays performed using purified genomic DNA from a variety of STEC strains (n=18) from all around the world showed sensitivity levels around 10 genome copies per PCR reaction for all strains tested. Non-STEC strains (n=18) were also tested and no amplification was observed. The PCR results correlated perfectly with the phenotypic characterization of the toxin production in both STEC and non-STEC strains thereby confirming the specificity of the assay. In conclusion, this Smart Cycler® based PCR assay is rapid, sensitive, specific and allows detection of STEC irrespective of their serotypes. Future developments will include direct testing of fecal specimens.

INTRODUCTION

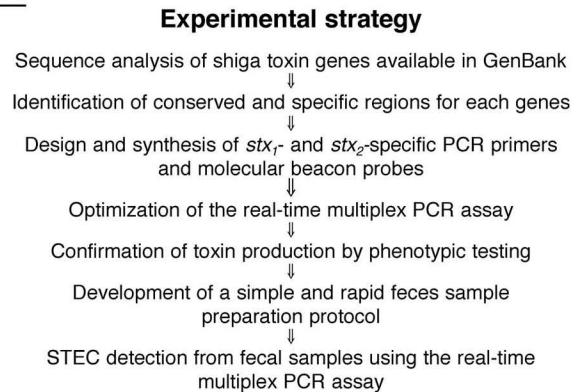
Although *Escherichia coli* is a common member of the normal flora of the gastrointestinal tract of human and animals, several pathogenic types of *E. coli* can lead to human diseases. *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* (STEC) have emerged in recent years as important human pathogens associated with a spectrum of diseases ranging from diarrhea to hemorrhagic colitis and hemolytic-uremic syndrome (HUS). Due to the morbidity and mortality associated with outbreaks and sporadic cases of STEC diseases, these pathogens are now considered a major public health problem of worldwide importance.

E. coli O157:H7 is the serotype most frequently isolated from patients, but STEC strains of other serotypes (e.g. O111, O103, O26) have also been associated with both outbreaks and sporadic cases of diseases. These pathogens share different virulence factors including a pathogenicity island (LEE) encoding proteins responsible for the intimate adherence of STEC to epithelial cells and the production of two Shiga toxins, Stx1 and Stx2. The production of Stx by STEC strains have a prominent role in pathogenesis, particularly in HUS. *E. coli* Stx1 is essentially identical to the Shiga toxin produced by *S. dysenteriae* whereas Stx2 has only 56% amino acid identity to Stx1.

Our ability to control diseases associated with STEC and to limit outbreaks depends upon the rapid detection of these pathogens. The methods currently used in clinical microbiology laboratories focus specifically on the identification of the O157 antigen while neglecting other STEC serotypes. We report here the development of a real-time PCR assay for the rapid detection of all STEC based on the amplification of the genes encoding Stx1 and Stx2, the major virulence factor of these organisms. This assay relies on PCR amplification using the Smart Cycler®, a rapid thermal cycler allowing real-time fluorescence detection.

MATERIALS & METHODS

FIGURE 1



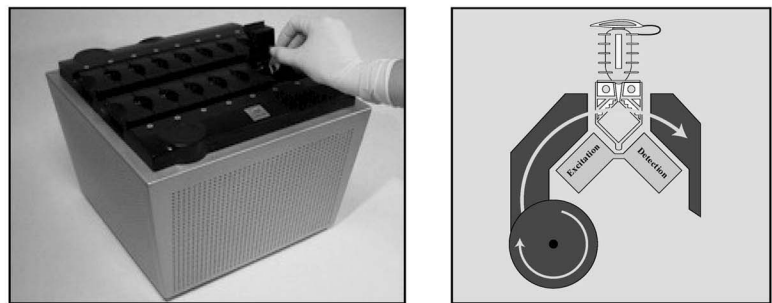
Bacterial Strains

The bacterial strains used in this study were obtained from the ATCC, the Centers for Disease Control and Prevention (Atlanta, GA) and the Laboratoire de Santé Publique du Québec (Sainte-Anne de Bellevue, Qc) (Table 1). The phenotypic characterization of toxin production for these strains was determined by using an immunoassay allowing the detection and identification of shiga toxins Stx1 and Stx2 by reverse passive latex agglutination (VTEC-RPLA kit from Denka Seiken Co., Ltd., Tokyo, Japan).

PCR assay

The optimization of the multiplex PCR assay was done using purified genomic DNA from the strains listed in table 1. The sensitivity of the assay was determined using serial dilutions of these genomic DNA. One microliter of these samples was added to a 24µl PCR reaction mixture. Amplification and detection were carried out on a Smart Cycler® (Cepheid, Sunnyvale, CA). This rapid thermal cycler consist of 16 independently programmable reaction sites (I-core modules), each allowing 4 color real-time optical detection at each site (Figure 2). The thermal cycling protocol used was 60 seconds at 95°C for initial denaturation, followed by 45 cycles of three steps consisting of 10 seconds at 95°C, 15 seconds at 56°C and 5 seconds at 72°C. Real-time detection of the PCR products was performed by measuring the fluorescent signal emitted by the molecular beacons hybridized to its target at the end of each annealing step.

FIGURE 2



Left: The Smart Cycler® consist of 16 independently programmable reaction sites. Right: Scheme of the I-core module and reaction tube.

Clinical samples

Feces samples obtained from the microbiology laboratory of the CHUL (Sainte-Foy, Qc) were spiked with 10-fold dilutions of STEC culture in the exponential phase of growth. Spiked samples were prepared for PCR by using the IDI DNA extraction kit (Infectio Diagnostic (I.D.I.) Inc., Sainte-Foy, Qc). An amplification control was used to monitor potential inhibition of the PCR.

RESULTS

Our real-time multiplex PCR assay demonstrated a detection limit of " 10 genome copies of purified genomic DNA for all 18 strains tested (Table 1).

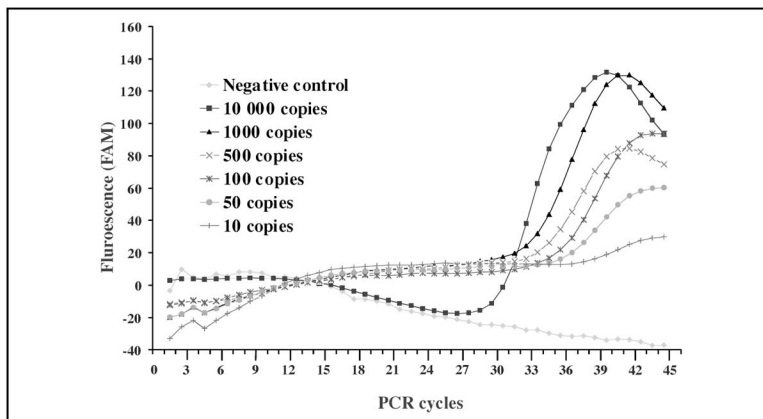
TABLE 1

Ubiquity, specificity and sensitivity tests performed with the *stx*-specific PCR assay using DNA from a variety of shiga toxin producing bacterial species.

Species* (strain number)	Serotype	Origin	Shiga toxin phenotype	Shiga toxin genotype (detection limit in genome copies)
<i>Escherichia coli</i> (ATCC 43890)	O157 :H7	USA (CA)	Stx1	<i>stx</i> ₁ (5)
<i>Escherichia coli</i> (ATCC 43894)	O157 :H7	USA (MI)	Stx1 and Stx2	<i>stx</i> ₁ (10), <i>stx</i> ₂ (5)
<i>Escherichia coli</i> (ATCC 43895)	O157 :H7	USA	Stx1 and Stx2	<i>stx</i> ₁ (10), <i>stx</i> ₂ (5)
<i>Escherichia coli</i> (CDC 97-3254)	O157 :H7	USA (MN)	Stx1	<i>stx</i> ₁ (5)
<i>Escherichia coli</i> (CDC 97-3330)	O157 :H7	USA (GA)	Stx2	<i>stx</i> ₂ (5)
<i>Escherichia coli</i> (CDC 98-3055)	O157 :H7	USA (TN)	Stx1 and Stx2	<i>stx</i> ₁ (5), <i>stx</i> ₂ (5)
<i>Escherichia coli</i> (LSPQ 2127)	O157 :H7	Canada (QC)	Stx1 and Stx2	<i>stx</i> ₁ (5), <i>stx</i> ₂ (5)
<i>Escherichia coli</i> (LSPQ 3760)	O157 :H7	Canada (QC)	Stx1 and Stx2	<i>stx</i> ₁ (5), <i>stx</i> ₂ (5)
<i>Escherichia coli</i> (LSPQ 3761)	O157 :H7	Canada (QC)	Stx2	<i>stx</i> ₂ (10)
<i>Escherichia coli</i> (LSPQ 3762)	O157 :H7	Canada (QC)	Stx1 and Stx2	<i>stx</i> ₁ (5), <i>stx</i> ₂ (10)
<i>Escherichia coli</i> (CDC 98-3169)	O157 :NM	USA (OR)	Stx2	<i>stx</i> ₂ (5)
<i>Escherichia coli</i> (CDC 98-3186)	O145 :NM	USA (MO)	Stx1 and Stx2	<i>stx</i> ₁ (5), <i>stx</i> ₂ (10)
<i>Escherichia coli</i> (CDC 99-3077)	O111 :NM	USA (VA)	Stx1 and Stx2	<i>stx</i> ₁ (10), <i>stx</i> ₂ (5)
<i>Escherichia coli</i> (CDC 97-3162)	O111 :NM	USA (MT)	Stx1 and Stx2	<i>stx</i> ₁ (5), <i>stx</i> ₂ (10)
<i>Escherichia coli</i> (CDC 89-3156)	OX3 :H21	USA (MI)	Stx2	<i>stx</i> ₂ (5)
<i>Shigella dysenteriae</i> (CDC C898)	type 1	Mexico	Stx1	<i>stx</i> ₁ (10)
<i>Shigella dysenteriae</i> (CDC BU2X1)	type 1	Burundi	Stx1	<i>stx</i> ₁ (10)
<i>Shigella dysenteriae</i> (CDCF4101)	type 1	Bangladesh	Stx1	<i>stx</i> ₁ (10)

* 18 non-STEC strains were also tested. These strains, which comprise 15 different serotypes of *E. coli* and 3 *Shigella* species (*S. boydii*, *S. sonnei* and *S. flexneri*), were all PCR-negative for both *stx*₁ and *stx*₂ genes.

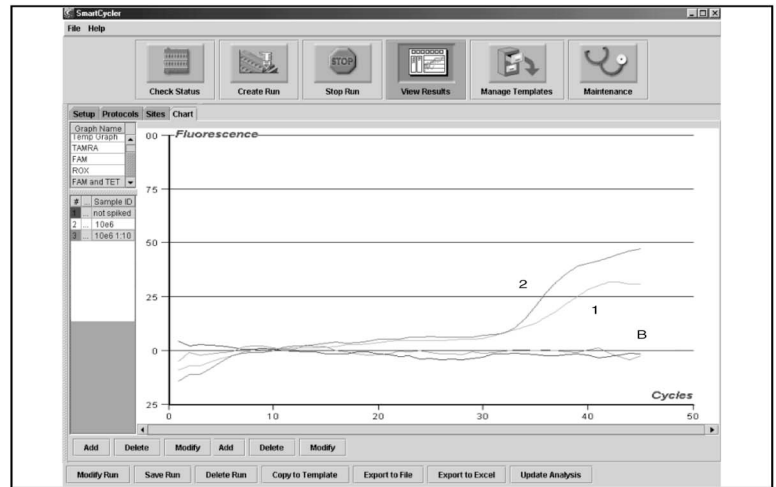
FIGURE 3



Example showing detection of *stx*₁ at different concentrations of purified STEC genomic DNA. *Stx*-specific amplification is measured by the fluorescence increase during the amplification process.

Tests with spiked fecal samples demonstrated the capacity of the 10 minutes IDI sample preparation protocol coupled with the multiplex PCR assay to detect efficiently STEC from these samples (Figure 4). The detection limit in fecal samples was found to be around 10⁵ bacteria per gram of feces. The amplification control showed that there was no inhibition of the PCR.

FIGURE 4



Smart Cycler[®] software user interface showing multiplex detection of Stx1 (1) and Stx2 (2) from a feces sample spiked with 10⁶ STEC cells (B: unspiked feces).

CONCLUSIONS

- The PCR assay developed in this study efficiently detected DNA from all STEC strains tested. There was no cross-amplification of DNA from any of the non-STEC strains tested.
- This PCR assay is very sensitive as it allows to detect 10 or less genome copies of STEC.
- The use of the novel Smart Cycler[®] technology coupled with real-time molecular beacon detection allowed the development of a very rapid PCR amplification protocol (40 minutes).
- Preliminary data using spiked feces samples prepared for PCR by using the 10 minutes sample preparation protocol developed by us demonstrated an excellent sensitivity of around 10⁵ bacteria per gram of feces.
- Detection of STEC from feces can be achieved within 50 minutes from sample reception.
- Future developments will include extended validation of the assay by testing feces from more patients.

Acknowledgements

This research project was supported by grant PA-15586 from the Medical Research Council of Canada and Infectio Diagnostic (I.D.I.) Inc. (Sainte-Foy, Qc., Canada). S.D.B. received a studentship from the Dr. Georges Phérix Foundation. We thank Cepheid (Sunnyvale, CA) for providing the Smart Cycler[®].



