

Direct PCR detection of *Escherichia coli* O157:H7

K.A. Fode-Vaughan*, J.S. Maki[†], J.A. Benson and M.L.P. Collins

Department of Biological Sciences, University of Wisconsin-Milwaukee, Milwaukee, WI, USA

2003/0217: received 14 January 2003 and accepted 1 June 2003

ABSTRACT

K.A. FODE-VAUGHAN, J.S. MAKI, J.A. BENSON AND M.L.P. COLLINS. 2003.

Aims: This paper reports a simple, rapid approach for the detection of Shiga toxin (Stx)-producing *Escherichia coli* (STEC).

Methods and Results: Direct PCR (DPCR) obviates the need for the recovery of cells from the sample or DNA extraction prior to PCR. Primers specific for Stx-encoding genes *stx1* and *stx2* were used in DPCR for the detection of *E. coli* O157:H7 added to environmental water samples and milk.

Conclusions: PCR reactions containing one cell yielded a DPCR product.

Significance and Impact of the Study: This should provide an improved method to assess contamination of environmental and other samples by STEC and other pathogens.

Keywords: detection, *Escherichia coli* O157:H7, PCR, Shiga toxin, STEC, *stx*.

INTRODUCTION

Direct PCR (DPCR) provides an improved method for the detection and quantification of bacteria in environmental samples (Fode-Vaughan *et al.* 2001). This is a more rapid and simple approach because the untreated environmental sample is used directly as a template in PCR, eliminating the steps of cell recovery or DNA extraction. We have previously used primers specific for functional genes to detect methanotrophic and phototrophic bacteria by DPCR (Fode-Vaughan *et al.* 2001). This approach should also be valuable for the detection of pathogens in environmental and other samples. In this work, DPCR for the detection of Shiga toxin (Stx)-producing *Escherichia coli* (STEC) is demonstrated.

Disease caused by STEC has become a major public health problem. While other strains may cause outbreaks, including ones that are waterborne (World Health Organization 1998; McCarthy *et al.* 2001), the most common

STEC in the USA, Europe, and Japan is *E. coli* O157:H7. The majority of strains of this organism produce Stx2, some produce both Stx1 and Stx2, and a few produce Stx1 only (Law 2000). *Escherichia coli* O157:H7 may be transmitted by food or water, the latter including both recreational and drinking water (Chalmers *et al.* 2000; Lee *et al.* 2002). Outbreaks of *E. coli* O157:H7 infection have been attributed to the presence of this bacterium in groundwater and surface water (Chalmers *et al.* 2000; Lee *et al.* 2002). A likely source of contamination of aquatic systems is cattle manure and agricultural run-off. Dairy and beef cattle may be carriers of this organism because these animals lack receptors for specific toxins and do not exhibit disease (Pruimboom-Brees *et al.* 2000). *Escherichia coli* O157:H7 persists in cattle manure (Wang *et al.* 1996; Bolton *et al.* 1999; Fukushima *et al.* 1999; Osek 2002) and manure-amended soil (Jiang *et al.* 2002) and experiments with models have suggested that it may leach through soil (Gagliardi and Karns 2000).

Rapid methods to detect *E. coli* O157:H7 are important to identify the source of outbreaks and to assure public safety. Both molecular and culture-based methods have been used for the detection of *E. coli* O157:H7. Culture-based methods developed for clinical samples have been applied to environmental samples. These methods rely on enrichment cultures followed by confirmation based on metabolic and antigenic properties. A disadvantage of this approach is the

*Present address: K.A. Fode-Vaughan, Department of Biochemistry, Medical College of Wisconsin, 8701 W. Watertown Plank Rd, Milwaukee, WI 53226, USA.

[†]Present address: J.S. Maki, Department of Biology, Marquette University, PO Box 1881, Milwaukee, WI 53201, USA.

Correspondence to: Mary Lynne Perille Collins, Department of Biological Sciences, University of Wisconsin-Milwaukee, PO Box 413, Milwaukee, WI 53201, USA (e-mail: mlpcoll@uwm.edu).

lack of complete correlation of these antigenic and metabolic properties with Stx production (Karch and Bielaszewska 2001). As culture-based methods are slow and labour intensive, they are not ideal for the analysis of the large numbers of samples that would be tested when possible environmental sources of an outbreak are being investigated.

As the infectious dose is very small and the number of cells contaminating environmental samples or food may be low, immunomagnetic separation capture with anti-O157 antibody has been suggested as a means to concentrate and detect the target cells (Pyle *et al.* 1999). However, this approach is limited to cells displaying a specific antigen making it unsuitable for other STEC.

Molecular approaches for bacterial detection avoid the need for culture and can be designed to be specific. Primers specific for *stx1* and *stx2*, as well as *E. coli* O157:H7-specific targets, have been used in PCR and real-time PCR (Olsvik and Strockbine 1993; Fratamico *et al.* 2000; Fortin *et al.* 2001; Li and Drake 2001; Ibekwe *et al.* 2002; Ibekwe and Grieve 2003). In this work we apply DPCR to the detection of *E. coli* O157:H7 in water samples and milk.

MATERIALS AND METHODS

Bacterial growth and treatment conditions

An overnight culture of *E. coli* O157:H7 strain G5244 (provided by S. McLellan, University of Wisconsin-Milwaukee) was grown at 37°C. To prepare control cells that would not be infectious, conditions were empirically identified that would render *E. coli* non-culturable yet still suitable for use as a PCR template. Cells were harvested and resuspended to the original volume in fixative (0.5% w/v paraformaldehyde in 0.01 M phosphate buffered saline, pH 7.6) and incubated for 3 h. Cells treated in this manner for 15 min or more did not form colonies on plates. After fixation, a portion of the cells in fixative was retained for a direct cell count, which was performed as described previously (Maki and Remsen 1981). The remainder of the fixed cells was harvested and washed in phosphate-buffered saline. Cell pellets were frozen at -20°C until DPCR analysis.

Molecular techniques

Primer design (Table 1) and PCR conditions were optimized for DPCR using recommendations reported

previously (Fode-Vaughan *et al.* 2001). The PCR conditions for amplification of *stx1* and *stx2* were those used for *pmoA* (Fode-Vaughan *et al.* 2001). The *stx1F/stx1R* primer pair has ≤ 2 mismatches to all *stx1* sequences deposited in GenBank by February 2003. The *stx2F/stx2R* primer pair is a perfect match for all the *E. coli* O157:H7 sequences deposited with one exception that has an insertion within the *stx2* sequence. The *stx2* primer pair would also be expected to amplify *stx2* sequences from other STEC, and from *Citrobacter freundii* and *Enterobacter cloacae*.

Fixed bacterial cells were used in DPCR analysis. Immediately before DPCR analysis, the cell pellets were thawed, resuspended in 50 μ l and decimally diluted in double distilled water. Each dilution was used as a template for PCR. For quantitative analysis, 50 μ l of a cell suspension containing 10^7 cells was serially diluted to extinction in five replicate series. A negative control with no template was included in each dilution series. The final volume of each PCR was 100 μ l. We have previously described the calculation of most probable number-DPCR (MPN-DPCR) (Fode-Vaughan *et al.* 2001). The sensitivity of DPCR with each primer pair was evaluated by comparing the number of cells added to the DPCR reactions determined by direct count and the cell number estimated by MPN-DPCR.

Environmental and other samples

The groundwater sample designated GLRF has been previously described (Cheng *et al.* 1999). The river water was obtained from Mull Creek in Ozaukee County, Wisconsin, USA. Five microlitres containing 10^3 *E. coli* O157:H7 cells were added to 45 μ l of either groundwater, river water, or pasteurized milk. Serial 10-fold dilutions were made in double distilled water to a theoretical concentration of 10^{-2} cells and each dilution was used in DPCR analysis.

Analysis

PCR products were analysed on gels of 2% agarose with the MBI Fermentas (Amherst, New York, USA) 100 bp DNA Ladder Plus used as a size marker. PCR products were sequenced with AmpliTaq DNA polymerase FS with a model 373 DNA Sequencer Applied Biosystems (Foster City, CA, USA). Figures were prepared with Adobe Photoshop 5.5.

Table 1 Primers used for DPCR

Primer	Target Sequence	Design
<i>stx2F</i>	<i>stx2</i>	TTCTTCGGTATCCTATTCCC Modified from Olsvik and Strockbine (1993)
<i>stx2R</i>	<i>stx2</i>	ATGCATCTCTGGTCATTGTA Modified from Olsvik and Strockbine (1993)
<i>stx1F</i>	<i>stx1</i>	CAGTTAATGTGGTGGCGAAG Olsvik and Strockbine (1993)
<i>stx1R</i>	<i>stx1</i>	CTGTACAGTAACAAACCGT This work

RESULTS

DPCR of *E. coli* O157:H7

Escherichia coli O157:H7 was serially diluted in double distilled water and each dilution was used as a template for DPCR with the *stx2* primer pair. A 482-bp PCR product was formed in every tube with sufficient template (Fig. 1). One to 10 cells were sufficient to obtain a product.

As the infectious dose of STEC is very low, detection rather than quantification is the goal. In this work, a quantitative approach was taken in order to evaluate the sensitivity of the method. For this purpose, the MPN-DPCR estimation of the number of cells was compared with the direct count. For quantitative analysis, 10^7 cells in $50 \mu\text{l}$ (2×10^8 cells ml^{-1}), as determined by direct cell counts, were added to the initial tubes of five replicate dilution series. The cell concentration in the original suspension as estimated by MPN-DPCR was 1.58×10^8 cells ml^{-1} (95% confidence limits = 4.80×10^7 to 5.23×10^8).

The *stx1* primer pair amplified a 513 bp product and one to 10 cells were required to obtain a DPCR product (not shown). A sample determined to have 2×10^8 cells ml^{-1} by direct count was determined to have 6.95×10^7 (95% confidence limits = 2.11×10^7 to 2.30×10^8) cells by MPN-DPCR. The identity of all PCR products was confirmed by sequencing.

DPCR of STEC in environmental samples

DPCR of STEC suggests that this method should provide a means to detect *E. coli* O157:H7 and other STEC in environmental samples. To test this, groundwater was spiked with *E. coli* O157:H7. The samples were serially diluted and each of these dilutions was used as a template for PCR (Fig. 1). No PCR product was detected in the undiluted sample (10^3

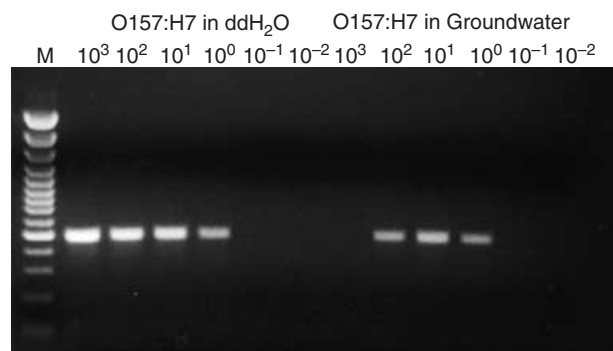


Fig. 1 DPCR of STEC. *Escherichia coli* O157:H7 (10^3 cells) was added to $50 \mu\text{l}$ of either double distilled water or groundwater. Serial dilutions were carried out in double distilled water to a theoretical concentration of 10^{-2} cells and each tube was used in PCR with the *stx2* primer pair. The control without template did not yield a PCR product (not shown). M, molecular weight markers

E. coli O157:H7 cells in groundwater). The absence of a PCR product in the undiluted groundwater is attributable to substances in the sample inhibitory to PCR (Fode-Vaughan *et al.* 2001). This inhibition was relieved by dilution. A product was amplified in tubes containing 10^2 , 10^1 and 10^0 cells in groundwater diluted 1 : 10, 1 : 100 and 1 : 1000, respectively. These results indicate that were *E. coli* O157:H7 present in such a sample at a level of 200 cells ml^{-1} (10 cells in $50 \mu\text{l}$ diluted 1 : 10 to relieve inhibition), it would test positive in DPCR. Similarly, river water samples spiked with *E. coli* O157:H7 cells were tested by DPCR and samples that should contain one cell on the basis of the direct count produced the expected DPCR product (not shown).

DPCR of STEC in milk

As raw or improperly pasteurized milk may also be a vector of *E. coli* O157:H7 (Keene *et al.* 1997), the use of DPCR with spiked milk was evaluated. One-thousand *E. coli* O157:H7 cells were added to $50 \mu\text{l}$ milk. Inhibition of PCR was relieved by dilution and the *stx2* PCR product was detected in the sample containing 10^2 cells in 1 : 10 milk (Fig. 2). The lower abundance of the PCR product in the 1 : 10 dilution containing 10^2 cells as compared with the 1 : 100 dilution containing 10 cells is probably attributable to substances in milk that partially inhibit the PCR.

DISCUSSION

DPCR provides a useful approach for the detection of pathogens in environmental and other samples. This simple

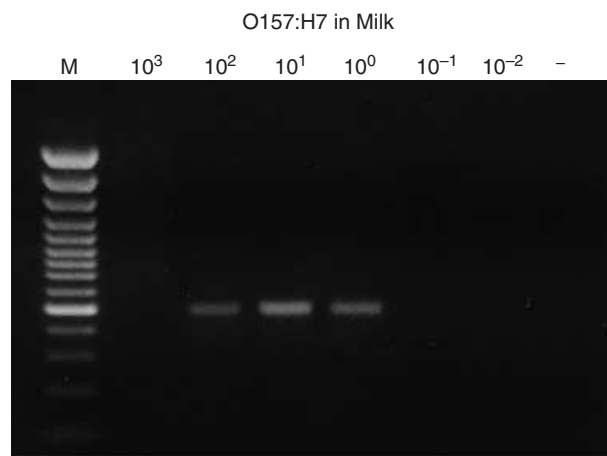


Fig. 2 DPCR detection of *E. coli* O157:H7 in milk. *Escherichia coli* O157:H7 (10^3 cells) was added to $50 \mu\text{l}$ skim milk. Serial dilutions were carried out in double distilled water to a theoretical concentration of 10^{-2} cells and each tube was used in PCR. (-) No DNA control. M, molecular weight markers. Similar results were obtained with whole milk (not shown)

method is an alternative to existing techniques or may be used in conjunction with these methods. The use of DPCR for the detection of STEC in environmental samples would require a minimum of 20 cells ml⁻¹. For samples requiring a 1 : 10 dilution, 200 cells ml⁻¹ would be required. This detection limit is equivalent to or more sensitive than that reported for conventional PCR or real-time PCR (Maurer *et al.* 1999; Fortin *et al.* 2001; Ibekwe *et al.* 2002; Ibekwe and Grieve 2003).

As STEC present below the detection limit could constitute a risk (Loge *et al.* 2002), the sensitivity may be increased by concentrating the water samples prior to analysis. Alternatively, DPCR could be performed on an enrichment culture provided that the cells are culturable. In this case, DPCR would provide a means to detect *E. coli* O157:H7 in an enrichment culture that would be faster than subculture on selective and differential media. The demonstration that a 10-fold dilution of a culture grown in EC broth (Difco, Detroit, MI, USA) is sufficient to relieve PCR inhibition (not shown) suggests that DPCR would provide a rapid means to confirm the presence of STEC in enrichment cultures. With optimized primer design and PCR conditions, DPCR could be used to detect other virulence genes of *E. coli* O157:H7 and distinguish *E. coli* O157:H7 from other STEC.

Investigation of a possible environmental source of an outbreak of STEC infection would require screening a large number of samples. DPCR could be adapted to a 96-well format for this purpose. Presumptive identification based on DPCR detection of *stx1* and/or *stx2* would be confirmed by further analyses which could include conventional culture-based methods or DPCR with other specific primers. Samples that yield negative results in DPCR should be tested with added STEC cells or DNA as an amplification control to determine that PCR was not inhibited in the particular sample.

In contrast to culture-based methods, DPCR and other PCR methods may detect cells that are non-culturable. Detection of STEC by DPCR does not necessarily indicate that a sample has live, infectious bacteria because dead cells or free DNA would also be detected. Such false positives would be expected in any PCR assay. In contrast, culture-based methods may result in false-negatives as *E. coli* O157:H7 has been reported to enter a viable, non-culturable state under environmental conditions (Rigsbee *et al.* 1997; Wang and Doyle 1998). Viable, non-culturable *E. coli* O157:H7, which may be potentially infectious, would not be detected by culture methods but would be detected by DPCR. The ability of DPCR to detect dead cells or free DNA could be advantageous for some purposes. For example, DPCR could be useful in tracing the source of an outbreak to identify STEC in a sample regardless of whether it still contains culturable or viable cells.

In summary, DPCR is applicable to the detection of STEC, including *E. coli* O157:H7, in environmental and other samples. DPCR should also be useful for the detection of other pathogens.

ACKNOWLEDGEMENTS

This work was supported with funds from the Wisconsin Department of Natural Resources (NMB00000187) and by the Center for Water Security at the Great Lakes WATER Institute through a grant from DARPA (NBCH1020016). This is contribution no. 443 from the Great Lakes WATER Institute.

REFERENCES

- Bolton, D.J., Byrne, C.M., Sheridan, J.J., McDowell, D.A. and Blair, I.S. (1999) The survival characteristics of a non-toxicogenic strain of *Escherichia coli* O157:H7. *Journal of Applied Microbiology* **86**, 407–411.
- Chalmers, R.M., Aird, H. and Bolton, F.J. (2000) Waterborne *Escherichia coli* O157. *Journal of Applied Microbiology* **88** (Suppl.), 124S–132S.
- Cheng, Y.S., Halsey, J.L., Fode, K.A., Remsen, C.C. and Collins, M.L.P. (1999) Detection of methanotrophs in groundwater by PCR. *Applied and Environmental Microbiology* **65**, 648–651.
- Fode-Vaughan, K.A., Wimpee, C.F., Remsen, C.C. and Collins, M.L.P. (2001) Detection of bacteria in environmental samples by direct PCR without DNA extraction. *Biotechniques* **31**, 598–607.
- Fortin, N.Y., Mulchandani, A. and Chen, W. (2001) Use of real-time polymerase chain reaction and molecular beacons for the detection of *Escherichia coli* O157:H7. *Analytical Biochemistry* **289**, 281–288.
- Fratamico, P.M., Bagi, L.K. and Pepe, T. (2000) A multiplex polymerase chain reaction assay for rapid detection and identification of *Escherichia coli* O157:H7 in foods and bovine feces. *Journal of Food Protection* **63**, 1032–1037.
- Fukushima, H., Hoshina, K. and Gomyoda, M. (1999) Long-term survival of Shiga toxin-producing *Escherichia coli* O26, O111, and O157 in bovine feces. *Applied and Environmental Microbiology* **65**, 5177–5181.
- Gagliardi, J.V. and Karns, J.S. (2000) Leaching of *Escherichia coli* O157:H7 in diverse soils under various agricultural management practices. *Applied and Environmental Microbiology* **66**, 877–883.
- Ibekwe, A.M. and Grieve, C.M. (2003) Detection and quantification of *Escherichia coli* O157:H7 in environmental samples by real-time PCR. *Journal of Applied Microbiology* **94**, 421–431.
- Ibekwe, A.M., Watt, P.M., Grieve, C.M., Sharma, V.K. and Lyons, S.R. (2002). Multiplex fluorogenic real-time PCR for detection and quantification of *Escherichia coli* O157:H7 in dairy wastewater wetlands. *Applied and Environmental Microbiology* **68**, 4853–4862.
- Jiang, X., Morgan, J. and Doyle, M.P. (2002) Fate of *Escherichia coli* O157:H7 in manure-amended soil. *Applied and Environmental Microbiology* **68**, 2605–2609.
- Karch, H. and Bielaszewska, M. (2001) Sorbitol-fermenting Shiga toxin-producing *Escherichia coli* O157:H(-) strains: epidemiology, phenotypic and molecular characteristics, and microbiological diagnosis. *Journal of Clinical Microbiology* **39**, 2043–2049.

- Keene, W.E., Hedberg, K., Herriott, D.E., Hancock, D.D., McKay, R.W., Barrett, T.J. and Fleming, D.W. (1997) A prolonged outbreak of *Escherichia coli* O157:H7 infections caused by commercially distributed raw milk. *Journal of Infectious Diseases* **176**, 815–818.
- Law, D. (2000) Virulence factors of *Escherichia coli* O157 and other Shiga toxin-producing *E. coli*. *Journal of Applied Microbiology* **88**, 729–745.
- Lee, S.H., Levy, D.A., Craun, G.F., Beach, M.J. and Calderon, R.L. (2002) Surveillance for Waterborne-disease outbreaks – United States, 1999–2000. *Morbidity and Mortality Weekly Report Surveill. Summ.* **51**, 1–47.
- Li, W. and Drake, M.A. (2001) Development of a quantitative competitive PCR assay for detection and quantification of *Escherichia coli* O157:H7 cells. *Applied and Environmental Microbiology* **67**, 3291–3294.
- Loge, F.J., Thompson, D.E. and Call, D.R. (2002) PCR detection of specific pathogens in water: a risk-based analysis. *Environmental Science Technology* **36**, 2754–2759.
- McCarthy, T.A., Barrett, N.L., Hadler, J.L., Salsbury, B., Howard, R.T., Dingman, D.W., Brinkman, C.D., Bibb, W.F. *et al.* (2001) Hemolytic-uremic syndrome and *Escherichia coli* O121 at a lake in Connecticut, 1999. *Pediatrics* **108**, E59.
- Maki, J.S. and Remsen, C.C. (1981) Comparison of two direct-count methods for determining metabolizing bacteria in freshwater. *Applied and Environmental Microbiology* **41**, 1132–1138.
- Maurer, J.J., Schmidt, D., Petrosko, P., Sanchez, S., Bolton, L. and Lee, M.D. (1999) Development of primers to O-antigen biosynthesis genes for specific detection of *Escherichia coli* O157 by PCR. *Applied and Environmental Microbiology* **65**, 2954–2960.
- Olsvik, O. and Strockbine, N.A. (1993) PCR Detection of heat-stable, heat-labile, and shiga-like toxin genes in *Escherichia coli*. In *Diagnostic Molecular Microbiology: Principles and Applications* ed. Persing, D.H., Smith, T.F., Tenover, F.C. and White, T.J. pp. 271–276. Washington, DC: American Society for Microbiology.
- Osek, J. (2002) Rapid and specific identification of Shiga-toxin-producing *Escherichia coli* in faeces by multiplex PCR. *Letters in Applied Microbiology* **34**, 304–310.
- Pruimboom-Brees, I.M., Morgan, T.W., Ackermann, M.R., Nystrom, E.D., Samuel, J.E., Cornick, N.A. and Moon, H.W. (2000) Cattle lack vascular receptors for *Escherichia coli* O157:H7 Shiga toxins. *Proceedings of the National Academy of Science of the United States of America* **97**, 10325–10329.
- Pyle, B.H., Broadaway, S.C. and McFeters, G.A. (1999) Sensitive detection of *Escherichia coli* O157:H7 in food and water by immunomagnetic separation and solid-phase laser cytometry. *Applied and Environmental Microbiology* **65**, 1966–1972.
- Rigsbee, W., Simpson, L.M. and Oliver, J.D. (1997) Detection of the viable but nonculturable state in *Escherichia coli* O157:H7. *Journal of Food Safety* **16**, 255–262.
- Wang, G. and Doyle, M.P. (1998) Survival of enterohemorrhagic *Escherichia coli* O157:H7 in water. *Journal of Food Protection* **61**, 662–667.
- Wang, G., Zhao, T. and Doyle, M.P. (1996) Fate of enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces. *Applied and Environmental Microbiology* **62**, 2567–2570.
- World Health Organization (1998) Zoonotic non-O157 Shiga toxin-producing *Escherichia coli* (STEC). Report of a WHO Scientific Working Group Meeting [online: <http://www.who.int/emc-documents/zoonoses/whocsraph988c.html>].