Detection and quantification of *Escherichia coli* O157:H7 in environmental samples by real-time PCR

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ABSTRACT

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Aims: To apply the real-time Polymerase chain reaction (PCR) method to detect and quantify *Escherichia coli* O157:H7 in soil, manure, faeces and dairy waste washwater.

Methods and Results: Soil samples were spiked with *E. coli* O157:H7 and subjected to a single enrichment step prior to multiplex PCR. Other environmental samples suspected of harbouring *E.coli* O157:H7 were also analysed. The sensitivity of the primers was confirmed with DNA from *E.coli* O157:H7 strain 3081 spiked into soil by multiplex PCR assay. A linear relationship was measured between the fluorescence threshold cycle (C_T) value and colony counts (CFU ml^{-1}) in spiked soil and other environmental samples. The detection limit for *E.coli* O157:H7 in the real-time PCR assay was $3{\cdot}5 \times 10^3$ CFU ml^{-1} in pure culture and $2{\cdot}6 \times 10^4$ CFU g^{-1} in the environmental samples. Use of a 16-h enrichment step for spiked samples enabled detection of <10 CFU g^{-1} soil. *E. coli* colony counts as determined by the real-time PCR assay, were in the range of $2{\cdot}0 \times 10^2$ to $6{\cdot}0 \times 10^5$ CFU PCR^{-1} in manure, faeces and waste washwater.

Conclusions: The real-time PCR-based assay enabled sensitive and rapid quantification of *E. coli* O157:H7 in soil and other environmental samples.

Significance and Impact of the Study: The ability to quantitatively determine cell counts of *E.coli* O157:H7 in large numbers of environmental samples, represents considerable advancement in the area of pathogen quantification for risk assessment and transport studies.

Keywords: *Escherichia coli* O157:H7, multiplex PCR, real-time PCR, fluorescence threshold, faeces, manure.

INTRODUCTION

Escherichia coli O157:H7 is one of the most important foodborne human pathogens of animal origin (Altekruse *et al.* 1997; Slutsker *et al.* 1998). Most human *E. coli* O157:H7 infections are caused by consumption of contaminated food or water. Cattle are generally considered the major reservoir for this organism (Dorn and Angrick 1991; Slutsker *et al.* 1998). The organism can be transported from feed lots to field plots where vegetables are grown, or through storm water after a heavy rainfall washing infected manure into the wells of the farming community and subsequently contaminating ground water and soil. Owing to the application of animal waste to agricultural land, large numbers of pathogenic bacteria may be released into the environment. Water contamination by *E. coli* is becoming common in rural areas of the US, with up to 40% of tested wells found to be contaminated (US-EPA 1996). In Walkerton, Ontario, more than 1000 people fell ill and five died of *E. coli* infections following a storm on the 12th of May, 2000 (O'Conner 2002). Intensification of regional livestock enterprises was named as the likely cause. A recent 1-year study of cattle herds suggested that 36·8% of herds carry the toxigenic strain of *E. coli* O157:H7 (Chapman *et al.* 1997). Another surveillance

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study showed a high prevalence of *E. coli* O157:H7 in beef cattle during processing (Elder *et al.* 2000).

Escherichia coli O157:H7 causes wide spectrum of disease symptoms in humans, ranging from mild to bloody diarrhoea, hemorrhagic colitis, and complications including haemolytic uraemic syndrome (HUS) and seizures that are particularly severe in children (Franke *et al.* 1995). *Escherichia coli* O157:H7 strains are generally lysogenized with one or more phages carrying genes for Shiga-like toxins 1 and 2 (O'Brien and Holmes 1987), and are encoded by genes *stx*1 and *stx*2. They play a major role in the pathogenesis of haemorrhagic colitis and HUS through cytotoxic effects on cells of the kidneys, intestines, central nervous system, and other organs (Karmali 1989; Obrig 1992). *Escherichia coli* O157:H7 also requires adherence to host intestinal cells to cause attaching and effacing (A/E) lesions. The ability to cause these lesions is localized to a *ca.* 43-kb region (the LEE pathogenicity island) of the *E. coli* O157:H7 chromosome (McDaniel *et al.* 1995). One gene in the locus, *eae*, encodes for the production of intimin, required for initial bacterial cell attachment to host cells and the formation of A/E lesions (Louie *et al.* 1993; McDaniel *et al.* 1995; McKee *et al.* 1995; Agin *et al.* 1996).

Numerous outbreaks of *E. coli* O157:H7 in humans have been traced to the consumption of contaminated plant materials (Ackers *et al.* 1998; Hillborn *et al.* 1999), but little research has been performed on the quantification of this pathogen in the environment. The recent availability of new technologies has greatly aided in the study of pathogens in the environment such as *E.coli* O157:H7 (Hu *et al.* 1999; Campbell *et al.* 2001). Real-time PCR using specific fluorogenic probes has facilitated the automated detection and quantification of amplified gene products (Heid *et al.* 1996). Real-time PCR has been used for the detection and quantification of *E. coli* O157:H7 in food and clinical samples (Oberst *et al.* 1998; Sharma *et al.* 1999; Sharma and Carlson 2000; Bellin *et al.* 2001), but this technology has not been tested vigorously with environmental samples. The objective of this study was to explore the potential of quantitative real-time PCR to estimate numbers of *E. coli* O157:H7 in environmental samples.

MATERIALS AND METHODS

Bacterial strains and culture media

Escherichia coli O157:H7 strain 3081 and non-O157:H7 strain 63 were used in this study as previously described (Sharma *et al.* 1999). These *E. coli* strains were cultured on Luria-Bertani (LB) broth and Sorbitol MacConkey (SMAC) agar plates at 37°C for 16 h.

Seeding experiments

Escherichia coli O157:H7-negative soil samples were used for the seeding experiments. An overnight culture of *E. coli* strain 3081 was serially diluted, and the number of colony forming units (CFU) of bacteria in each dilution was determined by plating on CT-SMAC (CT-SMAC-BCIG; CT, cefixime tellurite, BCIG, 5-bromo-4-chloro, 3-indoxyl-β-D-glucuronide) agar plates containing cefixime (0·05 mg l^{-1}) and tellurite (2·5 mg l^{-1}). The CT-SMAC-BCIG medium is a chromagenic medium for isolation and differentiation of *E. coli* O157:H7 (LAB M: IDG, Lancashire, UK). The titre of each dilution was determined by the average CFU ml^{-1} of the three replicate plates. For the spiked experiment, 10 g of soil was seeded with a 0–10-fold dilution series of *E. coli* strain 3081 in flasks containing 90 ml of modified LB broth (mLB) consisting of 8 mg l^{-1} vancomycin, 0·5 mg l^{-1} cefixime, and 10 mg l^{-1} cefsuludin. The inoculated flasks were incubated at 37°C with agitation at 200 rpm. Samples were vortexed for 30 s and 2-ml aliquots were taken after 0, 8, 16, and 24 h for DNA extraction. The DNA extracted from the enrichment broth was used as template in the multiplex PCR. To compare the multiplex PCR assay with the conventional culture method, the enrichment broth of the seeded soil samples were also plated onto CT-SMAC agar plates containing cefixime (0·05 mg l^{-1}) and tellurite (2·5 mg l^{-1}). Sorbitol-negative colonies were selected and analysed by multiplex PCR.

DNA isolation from pure culture and environmental samples

Genomic DNA was isolated from a pure culture of *E. coli* O157:H7 strain 3081, grown for 16 h at 37°C with the Qiagen tissue protocol (QIAamp DNA Mini Kit, Qiagen, Velencia, CA, USA), according to the manufacturer's protocol. Total bacterial community DNA was extracted from the environmental samples to determine the detection limits of *E. coli* O157:H7 strain 3081 by multiplex and real-time PCR. The DNA was extracted from 500 mg of soil or faecal samples, or 100 ml of water with UltraClean Soil, Faecal and Water DNA kits (MO BIO, Inc., Solana Beach, CA, USA). The 100 ml water was filtered and concentrated into 0·25 ml for DNA extraction with the Water DNA kit (MO BIO, Inc.,). All extractions were conducted according to the manufacturer's protocol.

In April and July 2000, triplicate fresh faecal samples were collected from calves and lactating cows from a commercial dairy farm in Chino, California. Manure that had been deposited for a few weeks was also collected, as well as dairy washwater prior to, during and after treatment in a constructed wetland. Untreated dairy washwater from milk cows was sent from a raw waste pond to an aeration pond,

and then through the constructed wetlands. The samples were collected from the raw pond, aeration pond, prewetland treatment and postwetland treatment and were analysed by real-time PCR for *E. coli* O157:H7. The top portions of fresh faecal samples were collected within minutes of defaecation with a stainless steel shovel for laboratory analysis. Total heterotrophic bacteria, *E. coli* and *E. coli* O157 were enumerated by culture methods on tryptic soya agar (TSA), SMAC, and CT-SMAC agar, respectively.

Primer and probe design

Primers and probes used for the detection and amplification of the *stx*1 and *eae* genes have been reported previously (Sharma *et al.* 1999). The reporter dyes FAM (6-carboxy-fluorescein) and Texas Red (Sulphorhodamine 101) were conjugated at the 5′ ends of the probes and quencher dyes, Black Hole Quencher (BHQ) I and II at the 3′ ends, respectively (Biosearch Technologies 2000). The FAM-labelled probe was used for the detection of the *stx*1 gene and the Texas Red-labelled probe was used for detection of the *eae* gene. The BHQ dyes were used as the quencher dyes because they are dark quenchers with a broad spectrum of light, and it has a larger signal-to-noise ratio when compared with other quenching dyes. Real-time PCR was conducted only with *stx*1 and the *eae* genes while *stx*2 was included in the conventional multiplex PCR.

Conventional multiplex PCR conditions

Multiplex PCR was performed in a 50-μl volume containing 200 mmol l^{-1} of dNTPs, 2 μl of DNA, 2·5 U of AmpliTaq Gold polymerase, 10X PCR buffer (PE Applied Biosystems, Foster City, CA, USA), 0·3 μM of each primer, and 5 mM of $MgCl_2$. Genomic DNA purified from *E. coli* O157:H7 strain 3081 was used as a positive control. PCR conditions consisted of 95°C for 10 min to denature the DNA, 35 cycles at 94°C for 20 s, 55°C for 30 s, 72°C for 40 s, followed by a 5-min extension at 72°C and a hold at 4°C.

Fluorogenic quantification of *E. coli* O157:H7 by PCR

For quantification of *E.coli* O157:H7, a master mix was made with the above reagents with the addition of 0·1 μM of each fluorescent probe. A 50-μl reaction volume including the DNA templates, was transferred to a 96-well thin-wall PCR plate. The plate set-up contained a positive DNA template from *E.coli* O157:H7 strain 3081, and two negative controls (one without *Taq* and the other without target DNA in triplicate). The plate was covered with optically clear sealing film and centrifuged briefly to bring all reagents to the bottom of the wells. PCR conditions were 10 min at 95°C, followed by 50 cycles of 20 s at 94°C, 30 s at 55°C, 40 s at 72°C, followed by a 5-min extension at 72°C and a hold at 4°C. PCR was performed with the iCycle iQ thermal cycler (BIO-RAD, Hercules, CA, USA) and the instrument's software was used for data analysis. This system used a thermal cycler, an optical module and detection-software to quantify PCR products in real-time, as revealed by the increase of fluorescence signal by 5′ nuclease activity during the amplification process. When the threshold cycle (C_T) for each standard was plotted against the log of starting quantity, a standard curve was obtained. This standard curve was used to quantify the unknown samples.

Standardization and amplification efficiency

Standard curves of the C_T vs CFU ml^{-1} of the starting DNA quantities (pg) were used to estimate viable cell numbers of unknown samples compared with samples with known cell number in the reaction. For a comparison of PCR amplification efficiencies and sensitivities among different experiments and the *E. coli* strain 3081, slopes of the standard curve lines constructed with *E. coli* DNA were calculated by performing a linear regression analysis. All data collection and analyses were performed with the iCycler software (BIO-RAD). The standard curve was constructed from genomic DNA extracted from *E. coli* O157: H7 strain 3081 carrying the *stx*1 and *eae* genes. DNA concentrations serially diluted from 7·9 pg to $7{\cdot}9 \times 10^{-9}$ pg ml^{-1} were used in a 50-μl reaction volume for PCR. The concentration of the extracted DNA was measured by an Ultrospec4000 spectrophotometer with SwiftII application software (PharmaciaBiotech, Cambridge, England, UK). From the slope of the standard curve, the amplification efficiency (E) was estimated by the formula $E = (10^{-1/\text{slope}}) - 1$. A reaction with 100% efficiency generated a slope of −3·32.

RESULTS AND DISCUSSION

Multiplex PCR detection of *E. coli* O157:H7 from spiked soil with and without enrichment

Culture-negative soil samples were spiked with various numbers of *E. coli* strain 3081, and incubated in LB broth for 0, 2, 8, 16 and 24 h. The enrichment cultures were analysed by multiplex PCR to determine the sensitivity of the PCR assay in analysing Shiga toxin-producing *E. coli* (STEC) in soil enrichment broths. Prior to soil enrichment, *E. coli* strain 3081 was grown for 16 h to a total cell count of $3{\cdot}5 \times 10^8$ CFU ml^{-1} and used for inoculation of the enrichment broth. This concentration was serially diluted and used as inoculum at various concentrations. At 0 h (no enrichment), $7{\cdot}8 \times 10^5$ CFU g^{-1} was recovered after spiking the soil with $3{\cdot}5 \times 10^8$ CFU ml^{-1} (Table 1). After 2, 8,

Table 1 Sensitivity of conventional multiplex PCR assay with enrichment for detection of the *stx*1 and *stx*2 genes of Shiga toxin-producing *Escherichia coli* (STEC) and the *eae* genes of *E. coli* O157:H7

Enrichment time (hours)	Genotypic amplification*			Inoculum density before enrichment CFU ml^{-1}
	stx1	*stx2*	*eae*	
0	–	–	–	0
0	+	+	ND	$3{\cdot}50 \times 10^{8}$
0	–	–	–	$3{\cdot}50 \times 10^{5}$
0	–	–	–	$3{\cdot}50 \times 10^{4}$
0	–	–	–	$3{\cdot}50 \times 10^{3}$
2	–	–	–	0
2	+	+	+	$3{\cdot}50 \times 10^{8}$
2	+	+	+	$3{\cdot}50 \times 10^{5}$
2	+	+	+	$3{\cdot}50 \times 10^{4}$
2	+	+	+	$3{\cdot}50 \times 10^{3}$
8	–	–	–	0
8	+	+	+	$3{\cdot}50 \times 10^{8}$
8	+	+	+	$3{\cdot}50 \times 10^{7}$
8	+	+	+	$3{\cdot}50 \times 10^{6}$
8	+	+	+	$3{\cdot}50 \times 10^{5}$
8	+	+	+	$3{\cdot}50 \times 10^{4}$
8	+	+	+	$3{\cdot}50 \times 10^{3}$
8	+	+	ND	$3{\cdot}50 \times 10^{2}$
8	+	+	ND	$3{\cdot}50 \times 10^{1}$
8	+	+	+	$3{\cdot}50 \times 10^{0}$
8	+	+	+	$3{\cdot}50 \times 10^{-1}$
16	–	–	–	0
16	+	+	+	$3{\cdot}50 \times 10^{8}$
16	+	+	+	$3{\cdot}50 \times 10^{7}$
16	+	+	+	$3{\cdot}50 \times 10^{6}$
16	+	+	+	$3{\cdot}50 \times 10^{5}$
16	+	+	+	$3{\cdot}50 \times 10^{4}$
16	+	+	+	$3{\cdot}50 \times 10^{3}$
16	+	+	+	$3{\cdot}50 \times 10^{2}$
16	+	+	+	$3{\cdot}50 \times 10^{1}$
16	+	+	+	$3{\cdot}50 \times 10^{0}$
16	+	+	+	$3{\cdot}50 \times 10^{-1}$
24	–	–	–	0
24	+	+	+	$3{\cdot}50 \times 10^{8}$
24	+	+	+	$3{\cdot}50 \times 10^{7}$
24	+	+	+	$3{\cdot}50 \times 10^{6}$
24	+	+	+	$3{\cdot}50 \times 10^{5}$
24	+	+	+	$3{\cdot}50 \times 10^{4}$
24	+	+	ND	$3{\cdot}50 \times 10^{3}$
24	+	+	ND	$3{\cdot}50 \times 10^{2}$
24	+	+	ND	$3{\cdot}50 \times 10^{1}$

+: detection; –: no detection; ND: not determined.

*The presence of these genes had already been determined by Sharma *et al.* (1999) with fluorogenic PCR using DNA probes specific for these genes and confirmed in this study by spiking different concentrations of *E. coli* O157:H7 strain 3081 into soil.

16 and 24 h of enrichment, cell numbers below 10^3 CFU ml^{-1}, increased to $5{\cdot}2 \times 10^6$, $2{\cdot}2 \times 10^8$, $1{\cdot}8 \times 10^8$ and $1{\cdot}9 \times 10^8$ CFU g^{-1}, respectively. Multiplex PCR clearly detected the three genes used in this study after 2 and 8 h of enrichment when the initial *E. coli* concentration used as inoculum was higher than 10^3 CFU ml^{-1}. After 16 h of enrichment, the three genes in the multiplex assay were detected in samples spiked with cell concentrations below 100 CFU ml^{-1}, and cell numbers on CT-SMAC had increased to $2{\cdot}2 \times 10^6$ CFU g^{-1}. These results indicate that the multiplex PCR is specific and sensitive enough to detect between 10 and 100 *E. coli* O157:H7 cells in soil samples when combined with an enrichment step. The results from this study with conventional multiplex PCR was very comparable with the results of Campbell *et al.* (2001), who showed a detection limit of 2 CFU g^{-1} of soil after a two-step enrichment. A study by Hu *et al.* (1999), reported pathogen detection down to initial inoculation values of 1 CFU g^{-1} of faecal matter. Sharma *et al.* (1999), using the same primers, reported detection limits for a semi-automated, real-time assay of 5·8–580 CFU and 1·2–1200 CFU for faeces and meat samples, respectively.

In combination with an enrichment step, the multiplex PCR was able to detect <10 CFU of *E. coli* O157:H7 PCR^{-1}, demonstrating the high sensitivity of the assay. Purification of the enrichment broth with the QIAamp Tissue Kit-removed PCR inhibitors present in the enrichment broth and was essential for increasing the sensitivity and reliability of the PCR assay. As shown in Table 1, the multiplex PCR assay provided a sensitive and reliable method for detecting *E. coli* O157:H7 in soil when combined with a single enrichment step.

Sensitivity and amplification efficiency of the real-time PCR assay

To explore the potential of quantitative real-time PCR to estimate cell numbers of *E. coli* O157:H7 in environmental samples, two standard dilution techniques were used. First, DNA concentrations from 7·9 to $7{\cdot}9 \times 10^{-9}$ pg ml^{-1} of genomic DNA from *E. coli* strain 3081 were quantified. Quantification was performed by a 10-fold serial dilution of 7·9 pg ml^{-1} DNA concentration extracted from $6{\cdot}4 \times 10^8$ CFU ml^{-1} of *E. coli* O157:H7 strain 3081. Secondly, $6{\cdot}4 \times 10^8$ CFU ml^{-1} of *E. coli* O157:H7 strain 3081was serially diluted 10-fold and plated onto CT-SMAC media. DNA was extracted from the plated dilution series, as well as the determination of CFU ml^{-1} from each dilution. All dilutions were tested in triplicate (Fig. 1a) and were reported as threshold cycle number vs CFU PCR^{-1} of *E. coli* O157:H7. Positive signals (those with C_T values above the C_T baseline) were found in all dilutions except those where the DNA concentrations were below

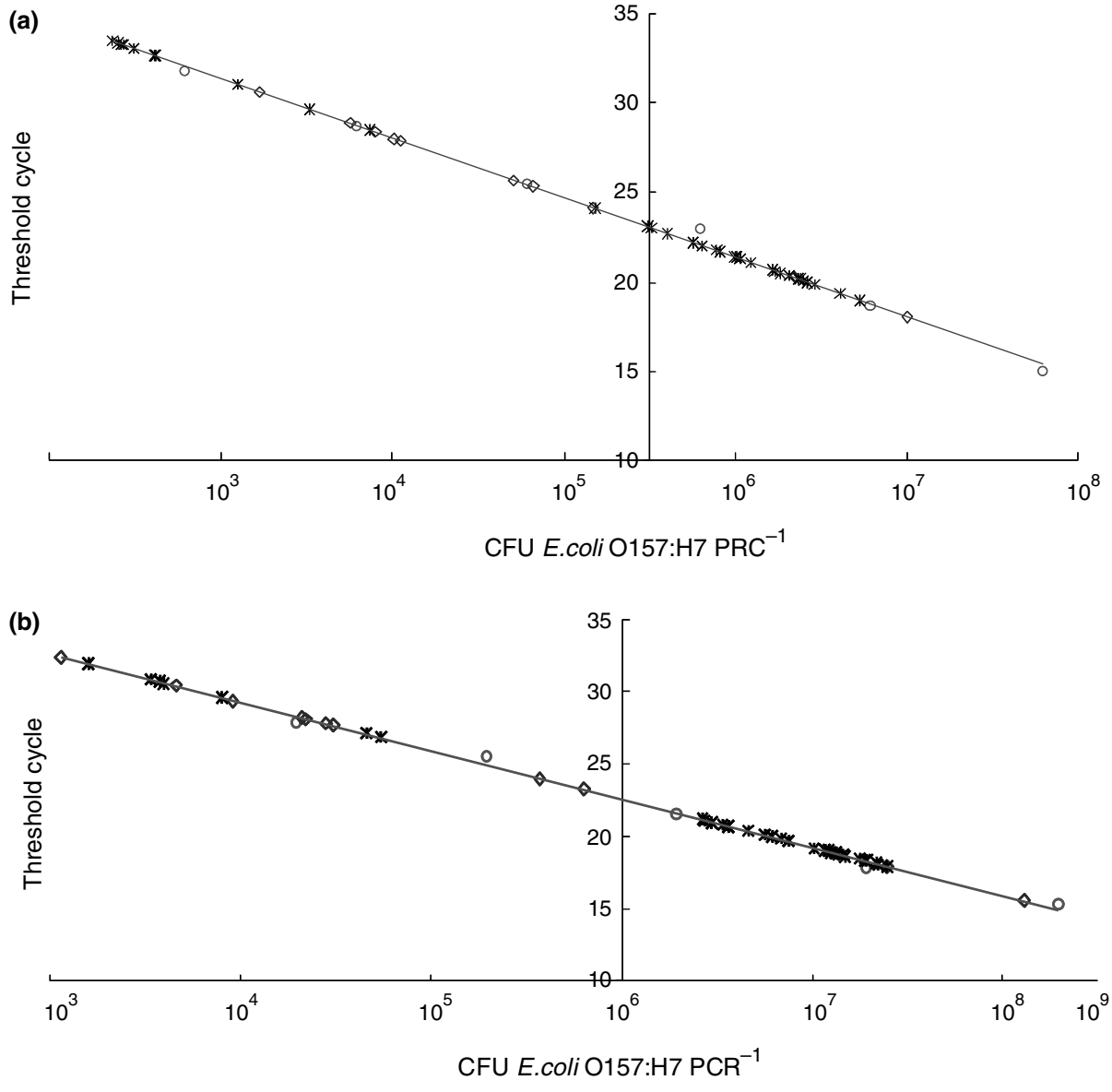


Fig. 1 Standard curves for *Escherichia coli* O157:H7 from spiked soil. Real-time analysis of 10-fold serial dilution, (○) *E. coli* O157:H7 DNA concentrations compared with the concentrations of (◇) *E. coli* O157:H7 CFU g^{-1} after plating (known) and, (×) *E. coli* O157:H7 concentrations in spiked soil samples detected after enrichment. (a) Detection with the FAM probe for *stx*1 gene ($Y = -3{\cdot}35x + 22{\cdot}57$, $R^2 = 0{\cdot}99$) (b) Texas Red probe for detection of *eae* gene ($Y = -3{\cdot}36x + 23{\cdot}03$, $R^2 = 0{\cdot}99$). The C_T values are plotted against *E. coli* O157:H7 CFU PCR^{-1}. Starting DNA concentrations calculated from instrument software are shown on the standard curve with numbers of *E. coli* O157:H7 CFU g^{-1} from plate count

$6{\cdot}4 \times 10^3$ CFU ml^{-1} ($7{\cdot}9 \times 10^{-5}$ g ml^{-1}) of genomic DNA from *E. coli* O157:H7. When both methods of serial dilutions were tested, a detection limit of $6{\cdot}4 \times 10^3$ CFU ml^{-1} was determined. This was equivalent to $7{\cdot}9 \times 10^{-5}$ pg ml^{-1} of DNA (Fig. 1a). The PCR products generated through the real-time assay were electrophoresed in a 2% low-melting agarose gel and stained with ethidium bromide. Bands of expected sizes were detected within the linear range and in agreement with the C_T values. The C_T values ranging from $15{\cdot}34 \pm 0{\cdot}46$ pg to $30{\cdot}66 \pm 0{\cdot}36$ pg of genomic DNA from *E. coli* O157:H7 showed a reproducible linearity over the range of detection (Fig. 1a). From the standard curve, the efficiency of the assay was calculated. Based on the formula in the Materials and Methods section, a reaction with 100% efficiency will generate a slope of −3·32 and an efficiency (E) value of 1. The amplification plot of this assay, using the *stx*1 primers and probe, generated a slope of −3·35 (98·8% efficiency) with a correlation coefficient of 0·994.

The evaluation of the assay sensitivity and amplification efficiency for the *eae* gene of *E. coli* strain 3081 followed the same procedure as was used for the *stx*1 gene. A detection limit was determined to be $6{\cdot}4 \times 10^3$ CFU ml^{-1} or a DNA concentration equivalent to $7{\cdot}9 \times 10^{-5}$ pg ml^{-1} (Fig. 1b). These results were almost identical to those for the *stx*1 gene. The C_T values ranged from $15{\cdot}10 \pm 0{\cdot}12$ pg to $32{\cdot}21 \pm 0{\cdot}60$ pg and showed a reproducible linearity over the range of detection (Fig. 1b) for triplicate samples. The amplification efficiency was 100% with a correlation coefficient of 0·996. The two approaches for the standard curve yielded results that were within the same log and were not significantly different from each other ($P = 0{\cdot}05$) by the Minitab Statistical Software (release 13; Minitab Inc., State College, PA, USA). Therefore, the two approaches provided data for absolute quantification to be expressed in terms of DNA quantity or CFU per PCR.

Table 2 Detection of *E.coli* O157:H7 DNA determined by real-time PCR in spiked soil

Inoculum density (CFU ml^{-1})	PCR g^{-1} *stx*1 gene†	PCR g^{-1} *eae* gene†	Enrichment time (hours)
$3{\cdot}50 \times 10^8$	$2{\cdot}67 \times 10^5 \pm 0{\cdot}4 \times 10^5$	$4{\cdot}69 \times 10^5 \pm 0{\cdot}3 \times 10^5$	0
$3{\cdot}50 \times 10^5$	$1{\cdot}84 \times 10^4 \pm 0{\cdot}6 \times 10^4$	$2{\cdot}09 \times 10^5 \pm 0{\cdot}1 \times 10^5$	0
$3{\cdot}50 \times 10^4$	$3{\cdot}91 \times 10^4 \pm 0{\cdot}5 \times 10^4$	$1{\cdot}97 \times 10^4 \pm 0{\cdot}5 \times 10^4$	0
$3{\cdot}50 \times 10^3$	$2{\cdot}23 \times 10^5 \pm 0{\cdot}3 \times 10^5$	$7{\cdot}88 \times 10^4 \pm 0{\cdot}6 \times 10^4$	0
$3{\cdot}50 \times 10^8$	$6{\cdot}54 \times 10^7 \pm 0{\cdot}6 \times 10^7$	$1{\cdot}68 \times 10^8 \pm 0{\cdot}5 \times 10^8$	2
$3{\cdot}50 \times 10^5$	$1{\cdot}11 \times 10^8 \pm 0{\cdot}4 \times 10^8$	$1{\cdot}49 \times 10^8 \pm 0{\cdot}6 \times 10^8$	2
$3{\cdot}50 \times 10^4$	$5{\cdot}75 \times 10^7 \pm 0{\cdot}2 \times 10^7$	$1{\cdot}68 \times 10^4 \pm 0{\cdot}6 \times 10^4$	2
$3{\cdot}50 \times 10^3$	$3{\cdot}04 \times 10^7 \pm 0{\cdot}1 \times 10^7$	$3{\cdot}37 \times 10^8 \pm 0{\cdot}4 \times 10^8$	2
$3{\cdot}50 \times 10^8$	$3{\cdot}75 \times 10^7 \pm 0{\cdot}5 \times 10^7$	$6{\cdot}32 \times 10^7 \pm 0{\cdot}3 \times 10^7$	8
$3{\cdot}50 \times 10^7$	$1{\cdot}80 \times 10^7 \pm 0{\cdot}2 \times 10^7$	$1{\cdot}05 \times 10^8 \pm 0{\cdot}1 \times 10^8$	8
$3{\cdot}50 \times 10^6$	$1{\cdot}48 \times 10^7 \pm 0{\cdot}1 \times 10^7$	$5{\cdot}14 \times 10^7 \pm 0{\cdot}1 \times 10^7$	8
$3{\cdot}50 \times 10^5$	$1{\cdot}80 \times 10^7 \pm 0{\cdot}7 \times 10^7$	$6{\cdot}74 \times 10^7 \pm 0{\cdot}4 \times 10^7$	8
$3{\cdot}50 \times 10^4$	$2{\cdot}80 \times 10^7 \pm 0{\cdot}8 \times 10^7$	$3{\cdot}61 \times 10^7 \pm 0{\cdot}5 \times 10^7$	8
$3{\cdot}50 \times 10^3$	$1{\cdot}30 \times 10^7 \pm 0{\cdot}2 \times 10^7$	$1{\cdot}96 \times 10^7 \pm 0{\cdot}8 \times 10^7$	8
$3{\cdot}50 \times 10^2$	$1{\cdot}65 \times 10^4 \pm 0{\cdot}5 \times 10^4$	$2{\cdot}55 \times 10^4 \pm 0{\cdot}4 \times 10^4$	8
$3{\cdot}50 \times 10^1$	$7{\cdot}87 \times 10^3 \pm 0{\cdot}7 \times 10^3$	$1{\cdot}57 \times 10^4 \pm 0{\cdot}5 \times 10^4$	8
$3{\cdot}50 \times 10^0$	$1{\cdot}93 \times 10^4 \pm 0{\cdot}6 \times 10^4$	$1{\cdot}70 \times 10^4 \pm 0{\cdot}6 \times 10^4$	8
$3{\cdot}50 \times 10^{-1}$	$7{\cdot}82 \times 10^3 \pm 0{\cdot}2 \times 10^3$	$1{\cdot}49 \times 10^4 \pm 0{\cdot}5 \times 10^4$	8
$3{\cdot}50 \times 10^8$	$5{\cdot}07 \times 10^7 \pm 0{\cdot}1 \times 10^7$	$9{\cdot}65 \times 10^6 \pm 0{\cdot}5 \times 10^6$	16
$3{\cdot}50 \times 10^7$	$4{\cdot}38 \times 10^7 \pm 0{\cdot}2 \times 10^7$	$1{\cdot}16 \times 10^8 \pm 0{\cdot}2 \times 10^8$	16
$3{\cdot}50 \times 10^6$	$8{\cdot}86 \times 10^7 \pm 0{\cdot}8 \times 10^7$	$2{\cdot}57 \times 10^8 \pm 0{\cdot}4 \times 10^8$	16
$3{\cdot}50 \times 10^5$	$6{\cdot}74 \times 10^7 \pm 0{\cdot}6 \times 10^7$	$1{\cdot}83 \times 10^8 \pm 0{\cdot}7 \times 10^8$	16
$3{\cdot}50 \times 10^4$	$7{\cdot}18 \times 10^7 \pm 0{\cdot}1 \times 10^7$	$1{\cdot}52 \times 10^8 \pm 0{\cdot}6 \times 10^8$	16
$3{\cdot}50 \times 10^3$	$1{\cdot}69 \times 10^7 \pm 0{\cdot}5 \times 10^7$	$1{\cdot}07 \times 10^8 \pm 0{\cdot}1 \times 10^8$	16
$3{\cdot}50 \times 10^2$	$3{\cdot}41 \times 10^7 \pm 0{\cdot}3 \times 10^7$	$4{\cdot}09 \times 10^7 \pm 0{\cdot}1 \times 10^7$	16
$3{\cdot}50 \times 10^1$	$2{\cdot}29 \times 10^7 \pm 0{\cdot}2 \times 10^7$	$3{\cdot}59 \times 10^7 \pm 0{\cdot}4 \times 10^7$	16
$3{\cdot}50 \times 10^0$	$1{\cdot}34 \times 10^7 \pm 0{\cdot}3 \times 10^7$	$4{\cdot}84 \times 10^7 \pm 0{\cdot}7 \times 10^7$	16
$3{\cdot}50 \times 10^{-1}$	$1{\cdot}47 \times 10^7 \pm 0{\cdot}5 \times 10^7$	$2{\cdot}53 \times 10^7 \pm 0{\cdot}6 \times 10^7$	16
$3{\cdot}50 \times 10^8$	$6{\cdot}10 \times 10^7 \pm 0{\cdot}1 \times 10^7$	$1{\cdot}29 \times 10^8 \pm 0{\cdot}3 \times 10^8$	24
$3{\cdot}50 \times 10^7$	$9{\cdot}54 \times 10^7 \pm 0{\cdot}5 \times 10^7$	$1{\cdot}29 \times 10^8 \pm 0{\cdot}3 \times 10^8$	24
$3{\cdot}50 \times 10^6$	$1{\cdot}23 \times 10^8 \pm 0{\cdot}2 \times 10^8$	$1{\cdot}89 \times 10^7 \pm 0{\cdot}7 \times 10^7$	24
$3{\cdot}50 \times 10^5$	$1{\cdot}06 \times 10^8 \pm 0{\cdot}1 \times 10^8$	$1{\cdot}66 \times 10^8 \pm 0{\cdot}6 \times 10^8$	24
$3{\cdot}50 \times 10^4$	$6{\cdot}10 \times 10^7 \pm 0{\cdot}1 \times 10^7$	$4{\cdot}15 \times 10^7 \pm 0{\cdot}1 \times 10^7$	24
$3{\cdot}50 \times 10^3$	$7{\cdot}23 \times 10^7 \pm 0{\cdot}2 \times 10^7$	$2{\cdot}09 \times 10^7 \pm 0{\cdot}1 \times 10^7$	24
$3{\cdot}50 \times 10^2$	$3{\cdot}74 \times 10^7 \pm 0{\cdot}6 \times 10^7$	$6{\cdot}24 \times 10^7 \pm 0{\cdot}2 \times 10^7$	24
$3{\cdot}50 \times 10^1$	$7{\cdot}38 \times 10^7 \pm 0{\cdot}3 \times 10^7$	$7{\cdot}89 \times 10^7 \pm 0{\cdot}8 \times 10^7$	24

*CFU ml^{-1} after serial dilution of *E. coli* strain 3081 in LB broth, used as inocula and enriched for 16 h.

†Calculated concentration of *E. coli* O157:H7 strain 3081 (PCR g^{-1} *stx*1 or *eae* genes) detected after enrichment and determined by real-time PCR with triplicate samples compared to known CFU g^{-1} spiked into the soil. All control values were below C_T.

Time (hours) used for the enrichment of strain 3081 in soil.

PCR quantification of *E. coli* O157:H7 from spiked soil with and without enrichment

Escherichia coli O157:H7 strain 3081was added to and recovered from soil samples to test the reliability of the real-time method under field conditions. The experiment allowed quantification (CFU per PCR) of *E. coli* strain 3081 from the spiked soils. The reliability of the assay was demonstrated when $2{\cdot}67 \times 10^5$ CFU per PCR of *E. coli* O157:H7 cells were recovered with the *stx*1 gene and $4{\cdot}96 \times 10^5$ CFU per PCR were recovered with the *eae* gene from soil spiked with $3{\cdot}5 \times 10^8$ CFU ml^{-1} and without an enrichment step. In soil samples without enrichment (0 h), the detection limit for the inoculation density was $3{\cdot}5 \times 10^3$ CFU ml^{-1} and was within the linear range of the curve. This was equivalent to $2{\cdot}23 \times 10^5$ CFU per PCR of DNA from soil as determined by quantitative PCR for the *stx*1 gene, and $7{\cdot}88 \times 10^4$ CFU per PCR of DNA from soil for the *eae* gene. Detection outside of this range was possible, but was not within the linear range of the standard curve for quantification. Data points outside the linear range were considered to be semi-quantitative and were determined by extrapolation. The information obtained outside the linear range can still be useful, as it confirmed the presence of this microorganism in the environment.

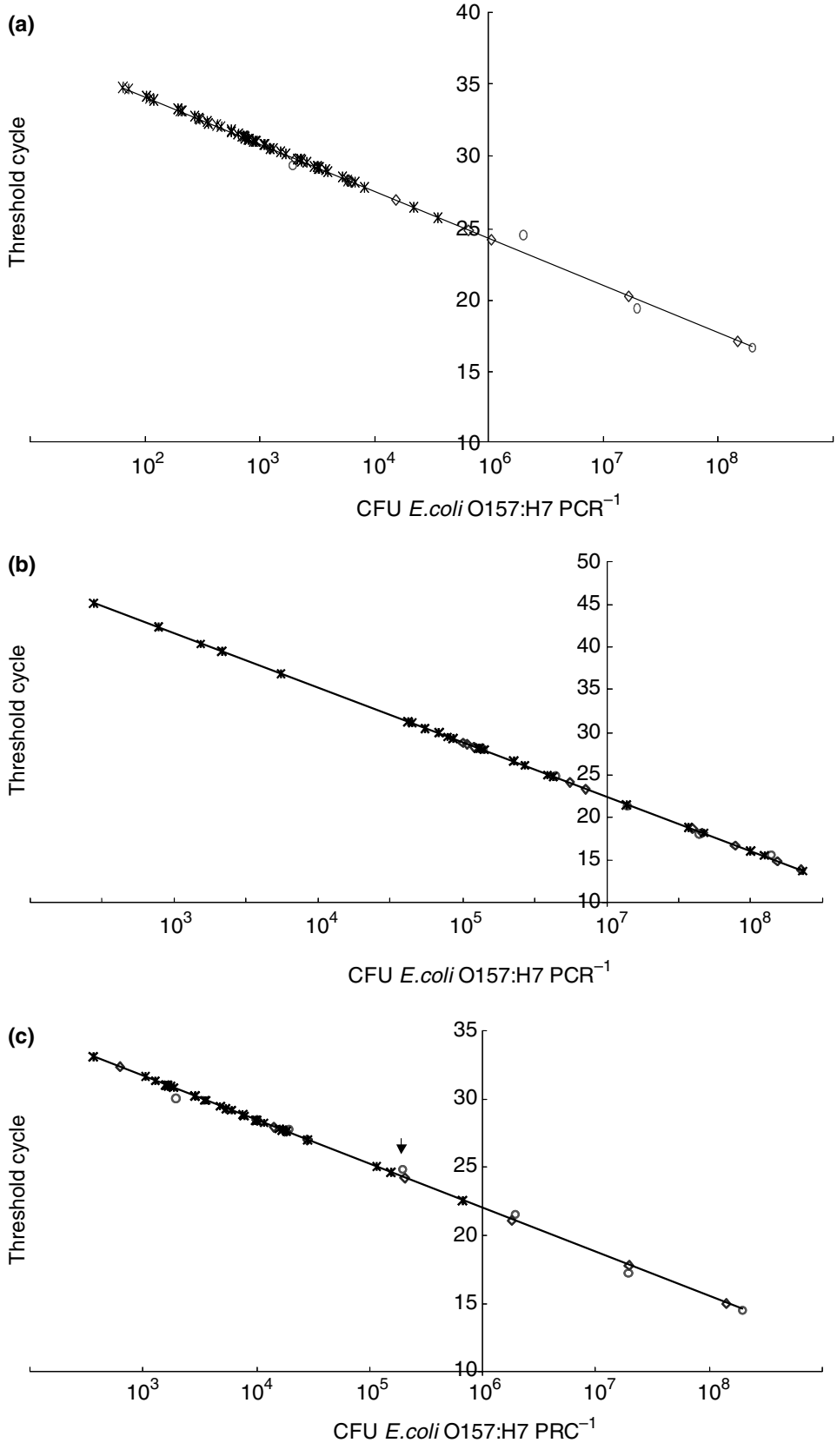
With a 2 h enrichment step, these numbers increased to $6{\cdot}54 \times 10^7$ CFU per PCR for the *stx*1 gene and $1{\cdot}68 \times 10^8$ CFU per PCR for the *eae* gene when $3{\cdot}50 \times 10^8$ CFU per ml was used as the inoculum density. Other dilutions showed an increase in numbers of organisms per PCR recovered after enrichment (Table 2). Most bacterial pathogens, including *E. coli* O157:H7, are present in very low numbers in soil, waste water and faeces. Soil and faeces also contain humic acid which is inhibitory to PCR amplification (Tebbe and Vahjen 1993; Taylor *et al.* 1997). Detection of very low levels of bacterial contamination in soil and faeces necessitates that these samples be cultured for a few hours in an appropriate enrichment broth to determine their presence. This enrichment serves a dual purpose of diluting out inhibitory substances and of providing conditions conducive to the growth and multiplication of bacterial pathogens to a detectable number.

For the enriched samples, aliquots were taken after 2, 8, 16 and 24 h of enrichment and the DNA was extracted for quantification. After 8 h of enrichment, the real-time PCR assay was able to detect $1{\cdot}65 \times 10^4$ and $2{\cdot}55 \times 10^4$ CFU per PCR of *stx*1 and *eae* genes, respectively, when $3{\cdot}5 \times 10^2$ CFU ml^{-1} of *E. coli* O157:H7 was spiked into the soil. There was a 2-log increase in cell density at the lowest point of the linear range for quantification after 8 h of enrichment. At the original cell density above $3{\cdot}5 \times 10^2$ CFU ml^{-1} in spiked soils, the enrichment process increased the concentration of cells to an average of 10^7 CFU g^{-1}. After 16 h, spiked cell

Table 3 Concentrations of different bacteria with plate count and real-time PCR from different matrices

Treatments	Total bacteria*	Total *E. coli*	Total *E. coli* O157	April *stx*1	July *stx*1	April *eae*	July *eae*
Dairy wastewater	$1{\cdot}8 \times 10^7$ Å $0{\cdot}52 \times 10^7$	$3{\cdot}8 \times 10^7$ Å $0{\cdot}58 \times 10^7$	$1{\cdot}1 \times 10^6$ Å $0{\cdot}12 \times 10^5$	$3{\cdot}6 \times 10^5$ Å $0{\cdot}21 \times 10^5$	$1{\cdot}8 \times 10^5$ Å $0{\cdot}12 \times 10^4$	$8{\cdot}7 \times 10^5$ Å $0{\cdot}50 \times 10^5$	$1{\cdot}8 \times 10^5$ Å $1{\cdot}0 \times 10^5$
Facultative Pond	$1{\cdot}5 \times 10^7$ Å $0{\cdot}41 \times 10^7$	$5{\cdot}6 \times 10^5$ Å $0{\cdot}22 \times 10^5$	$8{\cdot}2 \times 10^2$ Å $0{\cdot}45 \times 10^5$	$1{\cdot}8 \times 10^5$ Å $0{\cdot}31 \times 10^5$	$1{\cdot}4 \times 10^5$ Å $0{\cdot}11 \times 10^4$	$2{\cdot}8 \times 10^5$ Å $0{\cdot}75 \times 10^5$	$1{\cdot}3 \times 10^5$ Å $0{\cdot}75 \times 10^5$
Wetland 1 influent	$4{\cdot}6 \times 10^6$ Å $0{\cdot}15 \times 10^6$	$4{\cdot}7 \times 10^4$ Å $0{\cdot}38 \times 10^4$	$2{\cdot}1 \times 10^3$ Å $0{\cdot}25 \times 10^5$	$3{\cdot}5 \times 10^5$ Å $0{\cdot}18 \times 10^5$	$7{\cdot}9 \times 10^4$ Å $0{\cdot}18 \times 10^3$	$6{\cdot}2 \times 10^5$ Å $0{\cdot}18 \times 10^5$	$7{\cdot}3 \times 10^4$ Å $0{\cdot}18 \times 10^4$
Wetland 1 effluent	$2{\cdot}6 \times 10^5$ Å $0{\cdot}23 \times 10^5$	$7{\cdot}5 \times 10^2$ Å $0{\cdot}58 \times 10^2$	$4{\cdot}8 \times 10^1$ Å $0{\cdot}24 \times 10^1$	$4{\cdot}8 \times 10^4$ Å $0{\cdot}22 \times 10^4$	$8{\cdot}4 \times 10^4$ Å $0{\cdot}22 \times 10^3$	$3{\cdot}9 \times 10^4$ Å $0{\cdot}20 \times 10^4$	$9{\cdot}6 \times 10^4$ Å $0{\cdot}20 \times 10^4$
Wetland 2 influent	$4{\cdot}8 \times 10^7$ Å $0{\cdot}17 \times 10^7$	$5{\cdot}2 \times 10^3$ Å $0{\cdot}23 \times 10^5$	$1{\cdot}8 \times 10^3$ Å $0{\cdot}13 \times 10^3$	$2{\cdot}8 \times 10^4$ Å $0{\cdot}21 \times 10^4$	$9{\cdot}2 \times 10^4$ Å $0{\cdot}21 \times 10^3$	$3{\cdot}2 \times 10^5$ Å $0{\cdot}30 \times 10^5$	$1{\cdot}1 \times 10^5$ Å $0{\cdot}30 \times 10^4$
Wetland 2 effluent	$1{\cdot}9 \times 10^5$ Å $0{\cdot}28 \times 10^5$	$7{\cdot}5 \times 10^2$ Å $0{\cdot}18 \times 10^5$	$1{\cdot}2 \times 10^2$ Å $0{\cdot}21 \times 10^5$	$6{\cdot}3 \times 10^3$ Å $0{\cdot}21 \times 10^3$	$9{\cdot}3 \times 10^4$ Å $0{\cdot}20 \times 10^3$	$4{\cdot}8 \times 10^4$ Å $0{\cdot}60 \times 10^4$	$1{\cdot}0 \times 10^5$ Å $0{\cdot}66 \times 10^5$
Cow feces	$5{\cdot}8 \times 10^8$ Å $0{\cdot}82 \times 10^8$	$7{\cdot}8 \times 10^6$ Å $0{\cdot}35 \times 10^5$	$6{\cdot}7 \times 10^4$ Å $0{\cdot}78 \times 10^5$	$4{\cdot}1 \times 10^5$ Å $0{\cdot}81 \times 10^5$	$1{\cdot}1 \times 10^5$ Å $0{\cdot}81 \times 10^3$	$1{\cdot}3 \times 10^5$ Å $0{\cdot}36 \times 10^5$	$1{\cdot}6 \times 10^5$ Å $0{\cdot}36 \times 10^4$
Calf feces	$6{\cdot}4 \times 10^8$ Å $0{\cdot}21 \times 10^8$	$6{\cdot}1 \times 10^6$ Å $0{\cdot}12 \times 10^5$	$2{\cdot}5 \times 10^6$ Å $0{\cdot}46 \times 10^5$	$2{\cdot}4 \times 10^5$ Å $0{\cdot}5 \times 10^5$	$2{\cdot}6 \times 10^5$ Å $0{\cdot}25 \times 10^3$	$9{\cdot}1 \times 10^5$ Å $0{\cdot}23 \times 10^5$	$3{\cdot}2 \times 10^5$ Å $0{\cdot}23 \times 10^5$
Manure	$6{\cdot}9 \times 10^8$ Å $0{\cdot}56 \times 10^8$	$6{\cdot}5 \times 10^7$ Å $0{\cdot}45 \times 10^5$	$1{\cdot}1 \times 10^7$ Å $0{\cdot}28 \times 10^5$	$6{\cdot}4 \times 10^5$ Å $0{\cdot}23 \times 10^4$	$2{\cdot}1 \times 10^5$ Å $0{\cdot}23 \times 10^3$	$4{\cdot}1 \times 10^5$ Å $0{\cdot}67 \times 10^5$	$1{\cdot}7 \times 10^5$ Å $0{\cdot}66 \times 10^4$

*Total bacteria were plated on TSA, total *E coli* on SMAC, and *E. coli* O157 on CT-SMAC as described in Materials and Methods.


(a)
Threshold cycle
40
35
30
25
20
15
10
10^2
10^3
10^4
10^6
10^7
10^8
CFU *E.coli* O157:H7 PCR^{-1}
(b)
Threshold cycle
50
45
40
35
30
25
20
15
10
10^3
10^4
10^5
10^7
10^8
CFU *E.coli* O157:H7 PCR^{-1}
(c)
Threshold cycle
35
30
25
20
15
10
10^3
10^4
10^5
10^6
10^7
10^8
CFU *E.coli* O157:H7 PRC^{-1}

densities <1 CFU ml^{-1} were estimated by real-time PCR to increase to as high as $1{\cdot}47 \times 10^7$ CFU per PCR for the *stx*1 and $2{\cdot}53 \times 10^7$ CFU per PCR for the *eae* genes (Table 2). By using a single enrichment process, the fluorogenic, real-time PCR assay could detect between 1 and 10 CFU of *E. coli* O157:H7 after 16 h of enrichment in artificially inoculated soils. The presence of endogenous bacterial flora at levels up to 10^8 CFU g^{-1} in different matrices had no effect on the detection sensitivity of this assay (Table 3). The detection sensitivity obtained in this assay was comparable with the laborious and time-consuming methods of plate counts obtained by Hu *et al.* (1999) and from fluorogenic PCR (Sharma *et al.*1999; Sharma and Carlson 2000).

Enumeration of different bacteria and quantification of *E. coli* O157:H7 from different matrices

The number of heterotrophic bacteria from different matrices ranged from $1{\cdot}9 \times 10^5$ CFU ml^{-1} in the wetland 1 effluents to $6{\cdot}9 \times 10^8$ CFU g^{-1} in the manure samples (Table 3). Total *E. coli* concentrations in different matrices as determined by culture method on SMAC agar showed *E. coli* concentrations between $7{\cdot}5 \times 10^2$ CFU ml^{-1} (wetland effluent) and $6{\cdot}5 \times 10^7$ CFU g^{-1} (manure). There was a 3–4-log reduction in *E. coli* concentrations between the raw washwater and the final effluent water (Table 3). The numbers of *E. coli* O157 from CT-SMAC from the different matrices were between 48 CFU ml^{-1} in wetland 1 effluents and $1{\cdot}1 \times 10^7$ CFU g^{-1} in the manure samples. All samples were collected in April and July, 2000 in triplicate and combined after enumeration for average values.

Quantification of *E. coli* O157:H7 was determined by real-time PCR for samples collected during the 2 months. Linearity between the real-time C_T values and the target DNA in the environmental samples was observed over a five order-of-magnitude dilution series, demonstrating that quantification of *E. coli* O157:H7 in environmental samples was possible (Fig. 2a–c). *Escherichia coli* O157:H7 was detected in most of the samples, with higher numbers in April, than in July (Table 3) for both the *stx*1 and *eae* genes. The efficiencies of these assays were between 98 and 100%, as determined by the standard curve, suggesting a very reproducible assay. As high as $6{\cdot}0 \times 10^5$ *E.coli* O157:H7 PCR g^{-1} were quantified from cow and calf faeces, manure and raw wastewater, while about $6{\cdot}3 \times 10^3$ PCR g^{-1} was found in the effluent samples. This data is in agreement with the work of Zhao *et al.* (1995) and Shere *et al.* (1998) who reported that numbers of *E.coli* O157:H7 in cattle faeces range from 10^2 to 10^5 CFU g^{-1} faeces. Comparing real-time PCR data with the numbers of *E. coli* O157 from the plate counts showed very little differences from most of the samples. It is also known that PCR may sometimes detect dead cells and degraded DNA (Luna et al. 2002). This concept may be true in most instances, but based on data from this study, only a small proportion of this artifact may be of concern for absolute quantification of pathogens in the environment using rDNA as template for amplification.

When developing models of pathogen transport in the environment that are subsequently used for risk assessment, it is critical that reproducible quantitative data be used. Most models and transport studies demonstrating the risk of *E. coli* O157:H7 in the environment depend on culture techniques (Gagliardi and Karns 2000). Other studies have looked at the detection of *E.coli* O157:H7 in soil, water and faeces (Hu *et al.* 1999; Fratamico *et al.*, 2000; Campbell *et al.* 2001). In this study, fluorescent signal was converted into target cell densities and related directly to cell densities in soil, manure, faeces and washwater. This approach was made possible by relating the target DNA to the CFU ml^{-1} of a cultured *E. coli* strain. This is in contrast to other studies of *E. coli* O157:H7, where real-time PCR had been used to estimate the population size of *E.coli* O157:H7 (Oberst *et al.* 1998; Bellin *et al.* 2001). The quantification strategy used in this study was successful because of prior knowledge of *E.coli* O157:H7 rDNA copy numbers and genome size. This strategy has also recently been applied to the detection and quantification of methyl *tert*-butyl ether-degrading strain PMI (Hristova *et al.* 2001).

The use of 16 h enrichment, along with the multiplex PCR approach described here, offers the possibility for the sensitive detection of *E. coli* O157:H7 in soil within 1 day. *Escherichia coli* O157:H7 detection and quantification time in soil and other difficult matrices was further reduced by the use of fluorogenic probes in PCR reactions in this study, as well as from other studies (Bassler *et al.* 1995; Oberst *et al.* 1998; Sharma *et al.* 1999). This method avoids the need for agarose gel visualization of postamplification products because of the release of fluorogenic reporter dye during DNA polymerization (Lee *et al.* 1993). The application of rapid PCR thermal-cycling instrumentation coupled with the use of fluorogenic probes has resulted in reduced PCR assay times for detection of *Bacillus* spores (Belgrader *et al.* 2000) and the detection of *Listeria monocytogenes* (Bassler

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Fig. 2 Real-time PCR standard curve for *E. coli* O157:H7 DNA templates from faecal, manure and wetland samples collected in April and July, 2001. A 10-fold serial dilution, (○) *E. coli* O157:H7 DNA concentration per PCR reaction was used to compare with the concentrations, (◇) *E. coli* O157:H7 CFU g^{-1} obtained after plating (known) and the unknown, (×) *E. coli* O15:H7 concentrations per PCR in the samples. *stx*1 detection with (a) FAM – April ($Y = -3{\cdot}20x + 22{\cdot}40$, $R^2 = 0{\cdot}98$, (b) FAM – July ($Y = -3{\cdot}25x + 21{\cdot}96$, $R^2 = 0{\cdot}99$, and *eae* detection with (c) Texas Red – April and July ($Y = -3{\cdot}30x + 24{\cdot}30$,$R^2 = 0{\cdot}97$)

et al. 1995). The use of these technologies for the rapid detection and quantification of *E. coli* O157:H7 in environmental samples will greatly enhance the study of pathogens fate and transport in the environment.

The fluorogenic PCR assay described in this study provided a highly sensitive and specific technology for the detection and quantification of *E. coli* O157:H7 in environmental samples. In addition, the automated PCR amplification and detection of target gene amplicons was conducive for screening large numbers of environmental samples in a single assay. This method is a significant tool for monitoring large numbers of environmental samples contaminated with cattle faeces or manure, and which can subsequently be transported either by horizontal flow to larger bodies of water or by vertical movement to ground water.

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