2003

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Available online at www.sciencedirect.com
SCIENCE DIRECT®
Veterinary Microbiology 93 (2003) 247–260
veterinary microbiology
www.elsevier.com/locate/vetmic

Detection of enterohemorrhagic *Escherichia coli* O157:H7 by using a multiplex real-time PCR assay for genes encoding intimin and Shiga toxins

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Received 9 August 2002; received in revised form 31 December 2002; accepted 20 January 2003

Abstract

A multiplex real-time PCR (R-PCR) assay was designed and evaluated on the ABI 7700 sequence detection system (TaqMan) to detect enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 in pure cultures, feces, and tissues. Three sets of primers and fluorogenic probes were used for amplification and real-time detection of a 106-bp region of the *eae* gene encoding EHEC O157:H7-specific intimin, and 150-bp and 200-bp segments of genes *stx1* and *stx2* encoding Shiga toxins 1 and 2, respectively. Analysis of 67 bacterial strains demonstrated that the R-PCR assay successfully distinguished EHEC O157:H7 serotype from non-O157 serotypes and provided accurate profiling of genes encoding intimin and Shiga toxins. Bacterial strains lacking these genes were not detected with this assay. The detection range of the R-PCR assay for the three genes was linear over DNA concentrations corresponding from 10^3 to 10^8 CFU/ml of EHEC O157:H7. The R-PCR allowed construction of standard curves that facilitated quantification of EHEC O157:H7 in feces and intestinal tissues. Detection sensitivity of the R-PCR assay ranged from 10^4 to 10^8 CFU/g of feces or tissues without enrichment. Enrichment of feces in a non-selective broth for 4 and 16 h resulted in the detection of levels (from 10^0 to 10^3 CFU/g of feces) considered sufficient for infection in humans. The R-PCR assay for $eae_{O157:H7}$, *stx1*, and *stx2* proved to be a rapid test for detection of EHEC O157:H7 in complex biological matrices and could also potentially be used for quantification of EHEC O157:H7 in foods or fecal samples.
Published by Elsevier Science B.V.

Keywords: *Escherichia coli*; Shiga toxins; Hemorrhagic colitis

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0378-1135/03/$ – see front matter. Published by Elsevier Science B.V.
doi:10.1016/S0378-1135(03)00039-7

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) and other Shiga toxin-producing *E. coli* (STEC) are important food-borne pathogens associated with severe gastrointestinal and systemic diseases, such as hemorrhagic colitis (HC) and hemorrhagic–uremic syndrome (HUS), in humans (Karmali, 1989). Although more than 200 serotypes of STEC have been identified, EHEC O157:H7 is implicated in the vast majority of outbreaks and sporadic cases of bloody diarrhea in North America (Wells et al., 1983). Most human EHEC O157:H7 infections are caused by consumption of contaminated food and water, and cattle are generally considered the major reservoir for EHEC O157:H7 and other STEC serotypes that have been isolated from human clinical cases (Griffin and Tauxe, 1991; Hancock et al., 1994).

PCR has become a useful technique for detection of pathogens because it is quick, specific, sensitive and relatively inexpensive. A number of PCR-based assays have recently been developed for detection of EHEC O157:H7 in foods and feces. Some of these assays have targeted only *stx* genes (Ramotar et al., 1995; Witham et al., 1996) while others have included *stx* and one or more EHEC O157:H7-specific genetic markers (Fratamico et al., 1995; Gannon et al., 1997a; Paton and Paton, 1998). PCR-based assays that used primers to amplify and detect *stx1* and *stx2* could not distinguish EHEC O157:H7 from non-O157 STEC. Although PCR assays targeting highly divergent 3′ one-third of the gene *eae* had facilitated detection of EHEC O157:H7 from other *eae*-harboring STEC, enteropathogenic *E. coli* (EPEC), *Citrobacter rodentium* and *Hafnia alvei*, these assays could not identify Shiga toxins associated with EHEC O157:H7. Moreover, quantitative detection of amplification products was not feasible in any of these PCR assays because agarose gel electrophoresis and ethidium bromide staining were used for visualizing PCR amplified products.

Recently, real-time PCR (R-PCR) assays have been developed for detection and quantification of pathogen-specific gene products by using TaqMan probes or molecular beacons (Lyons et al., 2000; Nogva et al., 2000). TaqMan probes and molecular beacons are oligonucleotides that are conjugated to reporter and quencher dyes at 5′ and 3′ ends, respectively. In an intact probe or a beacon, the quencher dye suppresses the fluorescence emission of the reporter dye. However, the modification of a TaqMan probe (hydrolysis by Taq polymerase to cleave reporter moiety from the probe) or a conformational change in a molecular beacon during annealing and extension phases of the PCR process results in an increase in the reporter dye's fluorescence intensity. The continuous measurement of incremental fluorescence increase at each PCR cycle provides an accurate estimate of the number of cells of a bacterial pathogen present in a contaminated food or fecal sample. Incorporation of fluorogenic probes in PCR assays provides an additional element of specificity that eliminates the need for downstream analysis of amplified products by agarose gel electrophoresis and use of additional tests for confirming identity of amplified products. A few R-PCR assays have been developed for the detection of EHEC O157:H7 (Bellin et al., 2001; Fortin et al., 2001; Oberst et al., 1998). In these assays, however, detection of EHEC O157:H7 was based on an amplification and detection of a single gene.

The objective of the present study was to develop a R-PCR assay for simultaneous amplification and detection of *eae*, *stx1*, and *stx2*, the three important virulence genes of

EHEC O157:H7 in a single PCR assay, and evaluate the utility of this PCR to detect EHEC O157:H7 in feces and tissues obtained from weaned calves and neonatal pigs inoculated with a streptomycin-resistant EHEC O157:H7. The detection sensitivity of this PCR assay was compared with the detection of a streptomycin-resistant EHEC O157:H7 by direct plating of feces and tissues on SMAC agar containing streptomycin. We also examined the effect of enrichment on detection sensitivity of the R-PCR assay by culturing feces for 4 to 16 h in a non-selective broth.

2. Materials and methods

2.1. Bacterial strains, culture media, and growth conditions

A total of 67 bacterial strains containing various combinations of *stx* and *eae* genes were used for evaluating the specificity of the real-time PCR assay for EHEC O157:H7 detection. These strains have previously been characterized genotypically (Sharma et al., 1999). Bacterial strains were propagated and maintained on trypticase soy agar (TSA) plates. Liquid cultures were obtained by growing bacteria in GNTSB [prepared by mixing equal volumes of gram-negative broth (GNB) and trypticase soy broth (TSB)] for 16 h at 37 °C with continuous agitation (160 rpm) in a circulating air incubator (New Brunswick Scientific, Edison, NJ, USA). TSA and sorbitol-MacConkey (SMAC; with or without streptomycin at 50 μg/ml) agar were used to enumerate bacteria. TSA, GNB, and TSB were purchased from BBL (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) and sorbitol-MacConkey agar (SMAC) was from DIFCO Laboratories (Becton Dickinson Laboratories, Sparks, MD, USA).

2.2. Design of primers and fluorogenic probes

The nucleotide sequences of primers and fluorogenic probes and expected sizes of amplified products are listed in Table 1. About 800 nucleotides at the 3′ end of a previously published sequence of an *eae* gene, encoding EHEC O157:H7-specific intimin (Beebakhee et al., 1992), were taken into consideration for selecting primers and a probe to amplify and detect 106-bp fragment specific for the *eae* gene of EHEC O157:H7 ($eae_{O157:H7}$). A comparison of the nucleotide sequences of $eae_{O157:H7}$-specific primers and probe set with the sequences deposited in the GenBank revealed that this primer and probe set could distinguish EHEC O157:H7 from non-O157 EHEC and EPEC serotypes (with the exception of EPEC O55:H7 which carry an *eae* allele homologous to that of EHEC O157:H7), and from bacterial species such as *Citrobacter freundii* and *Hafnia alvei* that harbor an *eae*-like gene (McGraw et al., 1999). Similarly, the *stx1* and *stx2* gene sequences available from GenBank (Calderwood et al., 1987; Jackson et al., 1987) were used for designing primers and probe sets to amplify and detect 150-bp and 200-bp fragments specific for *stx1* and *stx2*, respectively. Primers and probes were designed using the Primer Express® software (PE Applied Biosystems, Foster City, CA, USA). The FAM (6-carboxy-fluorescein), VIC (proprietary fluorescent dye developed at PE Applied Biosystems), and TET (6-carboxy-2′,4,7,7′-tetrachlorofluorescein) were used as fluorescent

Table 1
Nucleotide sequence of primers and fluorogenic probes

Primer[a] or probe	Sequence (5′ → 3′)	Location[b] within the target gene	PCR product detected (bp)
stx1-forward	GAC TGC AAA GAC GTA TGT AGA TTC G	90–114	*stx1* (150)
stx1-reverse	ATC TAT CCC TCT GAC ATC AAC TGC	240–217	
stx1-probe	TGA ATG TCA TTC GCT CTG CAA TAG GTA CTC	116–145	
stx2-forward	ATT AAC CAC ACC CCA CCG	184–201	*stx2* (200)
stx2-reverse	GTC ATG GAA ACC GTT GTC AC	392–373	
stx2-probe	CAG TTA TTT TGC TGT GGA TAT ACG AGG GCT TG	204–235	
eae-forward	GTA AGT TAC ACT ATA AAA GCA CCG TCG	2494–2524	$eae_{O157:H7}$ (106)
eae-reverse	TCT GTG TGG ATG GTA ATA AAT TTT TG	2599–2574	
eae-probe	AAA TGG ACA TAG CAT CAG CAT AAT AGG CTT GCT	2572–2540	

[a] The nucleotide sequences used in the design of these primers and probes were retrieved from the GenBank using accession numbers M16625 (*stx1*), X07865 (*stx2*), and AF081182 ($eae_{O157:H7}$).

[b] The positions of the oligonucleotides are listed relative to the initiation codon (+1 adenine) of the respective gene.

reporter dyes and conjugated to 5′ ends of probes to detect amplification products specific for *stx1*, *stx2*, and $eae_{O157:H7}$, respectively. The quencher dye TAMRA (6-carboxytetramethyl-rhodamine) was attached at the 3′ ends of these probes. Primers and probes were synthesized by Integrated DNA Technologies (Coralville, IA, USA) and PE Applied Biosystems.

2.3. PCR amplification and quantitation of stx1, stx2, and $eae_{O157:H7}$

PCR was performed in a total volume of 50 μl containing 2.5 μl of extracted DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 3.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 0.3 μM of *stx1* and $eae_{O157:H7}$ primers, 1.5 μM of *stx2* primers, 0.1 μM of each fluorogenic probe, 60 nM of reference dye ROX (carboxy-X-rhodamine), and 2.5 U of AmpliTaq Gold DNA polymerase (PE Applied Biosystems). Amplification and detection were carried out in optical-grade 96-well plates in an ABI Prism 7700 sequence detection system (PE Applied Biosystems) with an initial cycle of 95 °C for 10 min followed by 40 cycles of 94 °C for 20 s, 55 °C for 30 s, and 72 °C for 45 s. The final extension was carried out at 72 °C for 5 min followed by cooling of samples to 25 °C. Reaction conditions for amplification and parameters for fluorescence data collection were programmed into a Power Macintosh 4400/20 (Apple Computer, Santa Clara, CA, USA) linked directly to the ABI Prism 7700 sequence detection system using the SDS 1.6 application software as per manufacturer's instructions. After real-time data acquisition, the cycle threshold (C_T) value was calculated by determining the point at which fluorescence exceeds an arbitrary threshold signal (10-fold higher than the base line). The threshold signal was manually set so that it intersected the amplification curves in the linear region of semilog plot. The C_T value is predictive of the quantity of target gene copies in the PCR sample (Heid et al., 1996).

2.4. Preparation of feces and tissues for PCR

Feces and tissues used in this study were obtained from weaned calves and neonatal cesarean section-derived, colostrums-deprived (CDCD) pigs, respectively, that were the subjects of an ongoing study investigating the mechanisms of colonization of EHEC O157:H7 in cattle (Dean-Nystrom et al., 1997; Dean-Nystrom et al., 1999). These animals were inoculated via a stomach tube with 10^{10} CFU of a streptomycin-resistant EHEC O157:H7 strain 86-24 (*stx2*$^+$ and *eae*$^+$; Griffin et al., 1988; McKee et al., 1995). Bovine feces were collected 2 and 3 days post-inoculation, and porcine intestinal tissues (cecum and ileum) were taken at necropsy at 2 days after inoculation. Feces and tissues (1–4 g) were homogenized in peptone broth (30 ml final volume) at 30,000 rpm for 1 min using a microprocessor-controlled homogenizer (VIRTIS, Gardiener, NY, USA). Ten-fold dilutions of homogenates were plated on SMAC agar containing streptomycin to enumerate the inoculated strain. For DNA preparation, feces and tissue homogenates were centrifuged (2 min at $1000 \times g$) to remove large fecal particles and tissue debris, respectively. Supernatants (1 ml) were centrifuged ($12{,}000 \times g$), bacterial pellets suspended in 0.2 ml of a lysis solution (10 mM Tris–HCl, 1 mM Na_2-EDTA (pH 8.0) and 0.05% Triton X-100), heated (100 °C for 10 min) and then centrifuged ($12{,}000 \times g$ for 5 min). DNA was recovered from supernatants (0.1 ml) using a kit designed for the purification of PCR products as per manufacturer's directions (Qiagen, Valencia, CA, USA). DNA was used immediately in PCR assay or stored at −70 °C for later use. DNA was also prepared from 10-fold serial dilutions of an overnight culture of EHEC O157:H7 (*stx1*$^+$, *stx2*$^+$, and *eae*$^+$) strain 2409 (Sharma et al., 1999). Bacterial cells were collected from serial dilutions by centrifugation and processed for DNA isolation as described previously except that the supernatants recovered after 100 °C heat treatment were not column purified before use in the PCR assay.

2.5. Preparation of fecal enrichments

Feces used for determining the effect of enrichment on detection sensitivity of the R-PCR assay were collected from healthy cattle housed at the Animal Nutrition Study Farm, Iowa State University, Ames, IA, USA. These feces were tested for the presence of endogenous EHEC O157:H7 and STEC. For this, 1 g of feces were mixed with 9 ml of GNTSB, incubated for 16 h at 37 °C, and centrifuged (2 min at $1000 \times g$) to remove large particles. Culture supernatant (1 ml) was centrifuged ($12{,}000 \times g$ for 3 min) and the cell pellet was processed to isolate DNA (as described above). This DNA was used in PCR assays with primers listed in Table 1 to amplify and detect fragments specific for *stx1*, *stx2*, and $eae_{O157:H7}$. The amplification products were analyzed by electrophoresis through a 4% agarose gel (FMC BioProducts, Rockland, ME, USA) followed by ethidium bromide staining. The molecular size of bands was estimated from a 50-bp DNA ladder (Roche Diagnostic Laboratories, Indianapolis, IN, USA). Feces that produced no detectable *stx*- and $eae_{O157:H7}$-specific fragments on preliminary screening were used for preparing enrichment cultures. Enrichment cultures were prepared by seeding 1 g portions of feces with 10-fold serial dilutions of an overnight culture of EHEC O157 strain 2409. Inoculated feces were mixed with 9 ml of GNTSB and incubated at 37 °C with shaking. After 4 and

16 h incubations, 1-ml samples were removed and processed for isolation of genomic DNA by the procedure described previously.

3. Results

3.1. Specificity of real-time PCR assay

Genomic DNAs from an *E. coli* O26 (*eae*$^+$), O111 (*stx1*$^+$ and *eae*$^+$), O45 (*stx2*$^+$ and *eae*$^+$), O157:NM (*eae*$^+$), and O157:H7 (*stx1*$^+$, *stx2*$^+$, and *eae*$^+$) were tested initially to establish that primers and probes used in the PCR assay resulted in amplification and detection of amplicons specific to *stx1*, *stx2*, and $eae_{O157:H7}$ genes. Fig. 1 shows a correlation between a fluorescence signal and cycle numbers for each of the three probes. Fluorescent signal 10-fold higher than the standard deviation of the mean baseline emission was indicative of a positive detection. As shown in Fig. 1, *stx1*-, *stx2*-, and $eae_{O157:H7}$-specific probes produced exponential increase in fluorescence only when DNA from strains containing these genes was used as a template in the PCR assay. The amplified products generated in samples shown in Fig. 1 were also analyzed on a 4% agarose gel by standard horizontal gel electrophoresis. Samples resulting in an exponential increase in fluorescence with a particular probe also contained an amplicon of predicted size corresponding to the gene detected by the probe (data not shown). On additional testing of several other strains in the R-PCR assay, a positive amplification profile was produced only for those strains that harbored genes encoding for Shiga toxins and EHEC O157:H7-specific intimin (Table 2). Since EPEC O55:H7 harbors an *eae* homologue of $eae_{O157:H7}$, members of this serotype also produced a positive amplification signal with $eae_{O157:H7}$ primers and probe set.

3.2. Detection sensitivity of real-time PCR in pure cultures

Genomic DNAs prepared from 10-fold serial dilutions of an overnight culture of EHEC O157:H7 strain 2409 were used as templates to determine detection sensitivity of R-PCR and to construct standard curves by plotting colony-forming units (CFUs) versus threshold cycle (C_T) produced for each of the three target genes. The results are shown in Fig. 2. Standard curves showed a linear relationship between the $\log_{10}$ input CFUs and the C_T (PCR cycle at which the fluorescent intensity raises above the threshold). The slopes of the curves for *stx1*, *stx2*, and $eae_{O157:H7}$ were -1.41, -1.48, and -1.50 and the squared regression coefficients after the linear regression for these genes ranged from 0.96 to 0.98. The three primers and probe sets produced similar amplification yields and fluorogenic detection of target genes. The lowest detection limits of the PCR assay for $eae_{O157:H7}$ and *stx* genes were approximately 20 CFU/PCR reaction (equivalent to 10^3 CFUs/ml).

3.3. Detection of EHEC O157:H7 in feces and tissues

Feces and intestinal tissues from animals infected with EHEC O157:H7 (*stx2*$^+$ and *eae*$^+$) strain 86-24 were tested to evaluate the utility of the R-PCR assay for detecting

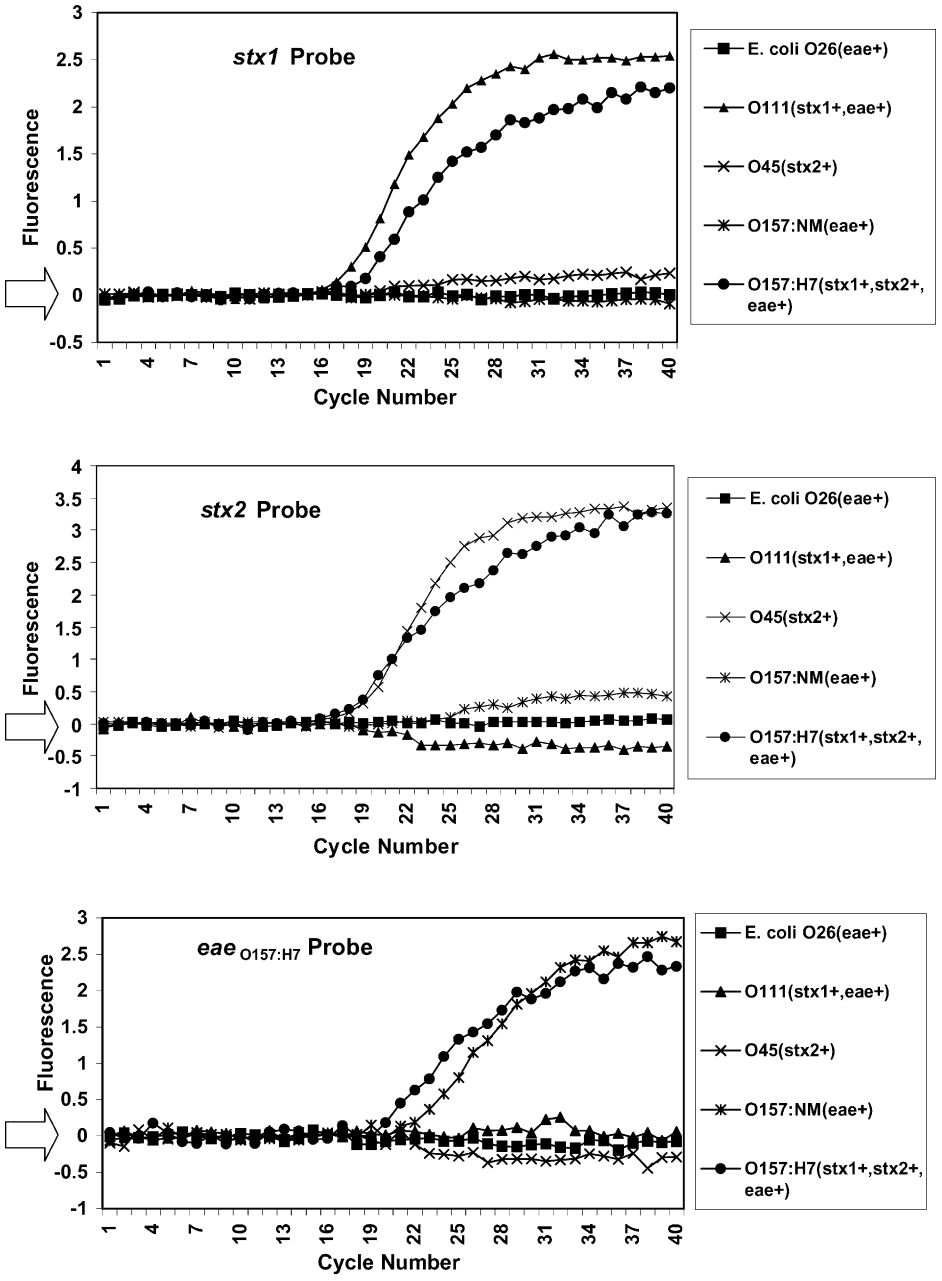

Fig. 1. Real-time PCR profiles for *stx1*, *stx2*, and $eae_{O157:H7}$ amplification. DNA extracted from five *E. coli* strains belonging to serotype O26, O111, O45, and O157 were tested in R-PCR using primer and probe sets designed to amplify and detect sequences specific to *stx1*, *stx2*, and $eae_{O157:H7}$. The relative fluorescence of each sample was plotted against PCR cycle number. The threshold fluorescence or the level at which the threshold cycle was determined is indicated by an arrow on the left.

Table 2
Specificity of real-time PCR for *stx1*, *stx2*, and $eae_{O157:H7}$ amplification

Bacterial strains	Genotype[a]	No. strains tested	No. strains detected with probes specific for		
			stx1	*stx2*	$eae_{O157:H7}$
STEC	$stx1^+$	37	37	0	0
	$stx2^+$	40	0	40	0
	$stx1^+$, $stx2^+$	14	14	14	0
EHEC O157:H7	$stx1^+$, eae^+	1	1	0	1
	$stx2^+$, eae^+	3	0	3	3
	$stx1^+$, $stx2^+$, eae^+	7	7	7	7
EHEC O26	$stx1^+$, eae^+	2	2	0	0
	$stx1^+$, $stx2^+$, eae^+	1	1	1	0
EHEC O111	$stx1^+$, eae^+	3	3	0	0
	$stx1^+$, $stx2^+$, eae^+	2	2	2	0
EPEC O55	$stx1^-$, $stx2^-$, eae^+	3	0	0	3
Other *E. coli* strains[b]	$stx1^-$, $stx2^-$, eae^+	16	0	0	0
Hafnia alvei	$stx1^-$, $stx2^-$, eae^+	1	0	0	0
Other bacterial species[c]	$stx1^-$, $stx2^-$, eae^-	8	0	0	0

[a] The virulence factor presence (+) or absence (−) previously established by a PCR assay (Sharma et al., 1999).

[b] Other *E. coli* tested were members of the enterotoxigenic *E. coli* (ETEC), enterpathogenic *E. coli* (EPEC), attaching and effacing *E. coli* (AEEC).

[c] Other bacterial species tested were *Enterobacter cloacae*, *Citrobacter freundii*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Yersinia enterocolitica*, *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*.

EHEC O157:H7 in cattle and swine. The DNA isolated from 10-fold serial dilutions of an overnight GNTSB culture of EHEC O157:H7 strain 2409 was used in this assay to construct standard curves for genes *stx2* and $eae_{O157:H7}$. The CFUs of EHEC O157:H7 present per gram of tissues or feces were interpolated from these standard curves and compared to bacterial counts determined by plating the same fecal and tissue samples on SMAC-streptomycin agar plates (Table 3). The CFUs per gram of feces or tissues estimated by the R-PCR assay using *stx2* and $eae_{O157:H7}$ probes were similar to those determined by plating the same samples on SMAC.

3.4. Detection of EHEC O157:H7 in feces following enrichment

Enrichment of feces, artificially contaminated with EHEC O157:H7 strain 86-24 and enriched for 4 or 16 h, allowed detection of as few as 25 CFUs/g of feces with the $eae_{O157:H7}$ probe. The detection range was linear over DNA concentrations corresponding to an initial inoculum of 10^0 to 10^5 CFU of EHEC O157:H7/g of feces (Fig. 3). The detection ranges for *stx1* and *stx2* were very similar to that of the *eae* gene except that the detection sensitivity was 1log higher (2.5 CFU/g of feces) for *stx* genes (data not shown).

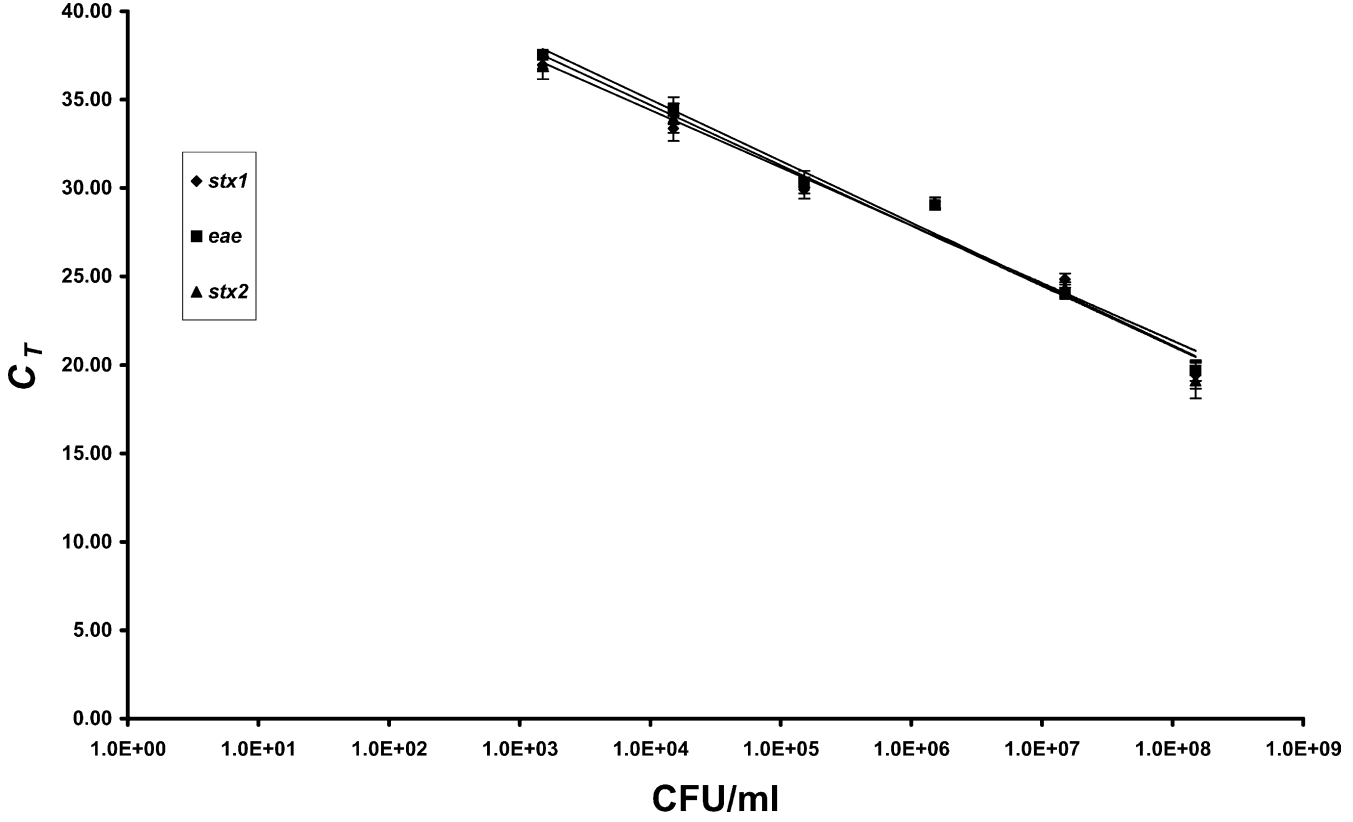

Fig. 2. Sensitivity of R-PCR. DNA extracted from 10-fold serial dilutions of an overnight culture of EHEC O157:H7 strain 2409 (*stx1*$^+$, *stx2*$^+$, and *eae*$^+$) was tested with *stx1*-, *stx2*-, and $eae_{O157:H7}$-specific primer and probe sets. The cycle threshold (C_T) values are plotted against bacterial growth (CFU/ml) of each dilution as determined by plating on SMAC-streptomycin agar. The straight lines calculated by linear regression yielded regression coefficients (R^2) of 0.966, 0.982, 0.971 for *stx1*, *stx2*, and $eae_{O157:H7}$, respectively. Error bars indicate the standard deviations based on three PCR reactions.

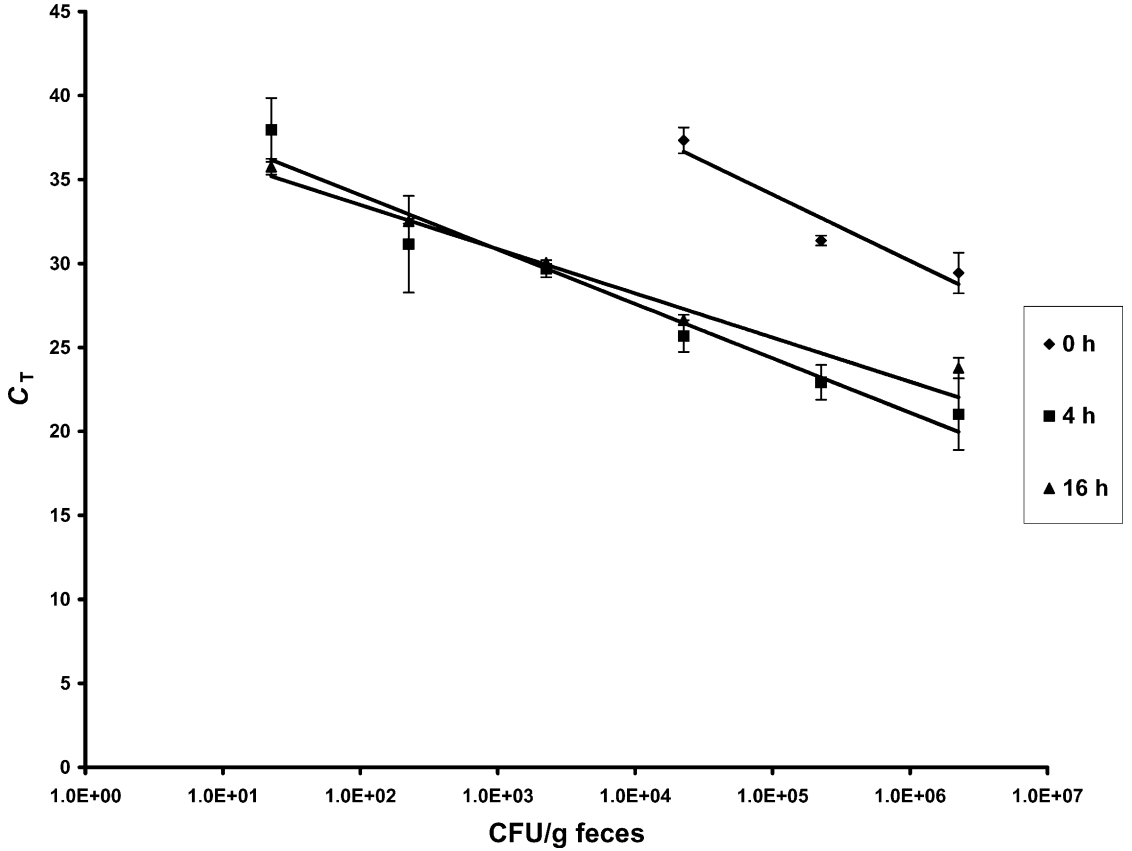

Fig. 3. Effect of enrichment on detection sensitivity of R-PCR. To detect very low levels of EHEC O157:H7, DNA prepared from fecal enrichments was tested in the R-PCR assay as described in Section 2. The cycle threshold (C_T) values were plotted against CFUs present in each serial dilution that was added to a fecal sample before enrichment (determined by plating on SMAC-streptomycin agar). The straight lines calculated by linear regression yielded regression coefficients (R^2) of 0.92, 0.96, 0.95 for 0, 4, and 16 h enrichments, respectively. Error bars indicate the standard deviations based on three PCR reactions.

Table 3
Detection of EHEC O157:H7 in feces and tissues

Animal number	Sample type[a]	Real-time PCR[b]		Plate counts[c]
		eae probe	*stx2* probe	
Calves				
325	Pre-feces	ND	ND	ND[d]
325	2-Day feces	6.6	7.1	6.5
325	3-Day feces	6.3	6.7	7.4
356	Pre-feces	ND	ND	ND
356	2-Day feces	5.4	6.2	5.3
356	3-Day feces	3	3.5	4.2
Pigs				
306	Cecum	8.1	8.2	7
306	Ileum	7.9	7.6	8
308	Cecum	9.1	9.1	8.6
308	Ileum	6.3	6.1	7.8
314	Cecum	7.2	7.5	7.9
314	Ileum	7.3	7.3	7
324	Cecum	9	9.3	9.2
324	Ileum	7.7	7.9	7.9

[a] Pre-feces were collected from weaned calves before inoculation with 10^{10} CFU of EHEC O157:H7 strain 86-24 ($eae_{O157:H7,stx2^+}{}^+$), and 2- and 3-day feces were collected at days 2 and 3 after inoculation. Cecum and ileum tissues were collected from neonatal CDCD pigs 2-days after inoculation.

[b] Log_{10} CFUs/g estimates were based on average of three PCR reactions.

[c] Log_{10} CFUs/g sample estimated.

[d] ND: Not detected. (Detection limits of real-time PCR and plating of samples on SMAC agar were $\geq 10^3$ CFU/g).

4. Discussion

Real-time PCR (R-PCR) assays that have recently been developed for the identification of EHEC O157:H7 are based on the detection of genes encoding Shiga toxins (Belanger et al., 2002; McKillip and Drake, 2000) or intimin (Oberst et al., 1998) or O antigen (Fortin et al., 2001). These assays, therefore, do not facilitate simultaneous detection of multiple virulence markers associated with EHEC O157:H7. The aim of this study was to develop a multiplex R-PCR assay for rapid and semi-automated detection and quantification of EHEC O157:H7 over a wider detection range by using three sets of primers and fluorogenic probes enabling amplification and immediate detection of gene *stx1*, *stx2*, and $eae_{O157:H7}$ in a single test tube. Testing DNA from several STEC serotypes and other *E. coli* strains and bacterial species verified the specific detection of these genes. The specificity of this PCR assay for detecting these genes was comparable to that reported for other R-PCR assays (Ibekwe et al., 2002; Oberst et al., 1998). Of the bacterial strains tested, only EHEC O157:NM and EPEC O55:H7 cross-reacted with the $eae_{EHEC\ O157:H7}$ primer-probe set. The reasons and significance of this cross-reactivity have been discussed in several previous

publications (Bilge et al., 1996; Feng et al., 1996; Gannon et al., 1997b; Louie et al., 1994; Whittam et al., 1993).

The detection range of the R-PCR assay for *stx* and $eae_{O157:H7}$ was linear when DNAs prepared from samples containing from 10^3 to 10^8 CFUs/ml of EHEC O157:H7 were used in this assay. This detection range is comparable to R-PCR assays that targeted *stx* (Belanger et al., 2002; McKillip and Drake, 2000) or *eae* (Oberst et al., 1998) or *rfbE* for detecting EHEC O157:H7 (Fortin et al., 2001). Overall, the detection range of R-PCR was three–four orders of magnitude higher than that of an end-point PCR (detection range from approximately 10^3 to 10^4 CFUs/ml (Sharma et al., 1999). All three primer and probe sets performed optimally in R-PCR as evidenced by the generation of almost identical slopes and squared regression coefficients.

The optimized R-PCR was evaluated for detecting three virulence markers in feces and tissues recovered from cattle and pigs inoculated with EHEC O157:H7. By using a simple and rapid bacterial DNA isolation procedure, we were able to detect as low as 10^4 CFUs of EHEC O157:H7/g of feces or tissues. The detection range, which was linear between 10^4 and 10^8 CFUs/g of feces or tissues, was very similar to that obtained by plating of fecal and tissue samples on a streptomycin-supplemented SMAC plates. However, the routine use of antibiotics in SMAC agar for specific selection and presumptive identification of EHEC O157:H7 in feces, tissues, or foods is precluded because the antibiotic resistance profile of the suspected EHEC O157:H7 isolate would not be known at the time of testing. Therefore, detection sensitivity of SMAC (without an antibiotic) plating will normally be much lower than that of a R-PCR.

By incorporating an enrichment period of 4 and 16 h, the real-time PCR allowed detection of 22 and 2 CFUs/g of feces using $eae_{O157:H7}$ and *stx* probes, respectively. The detection range of $eae_{O157:H7}$ and *stx* genes in enriched samples was linear as long as these samples contained from 10^0 to 10^5 CFUs/g of feces at the beginning of enrichment. However, the samples containing $\geq 10^6$ CFUs of EHEC O157:H7 before the start of an enrichment period could not be distinguished from each other in their cell counts by the R-PCR assay because these samples contained 10^9 CFUs/ml post-enrichment (data not shown), and the amount of DNA in these samples was so high that it saturated the amplification process within the first few cycles of PCR.

In summary, the R-PCR assay described in this study is sensitive and accurate method for specific detection and profiling of virulence markers of EHEC O157:H7 in complex samples such as feces and tissues. In addition, the real-time PCR format enables construction of standard curves that can be used for estimating concentration of EHEC O157:H7 or other STEC in feces and tissues. However, one potential limitation of this PCR would be the inability to distinguish a non-O157:H7 STEC from an EHEC O157:H7 strain if both were present in the same sample. Nonetheless, the capability for simultaneous detection of *stx1*, *stx2*, and $eae_{O157:H7}$ genes is important for rapid screening of feces and tissues to presumptively identify highly virulent EHEC O157:H7 from other less virulent serotypes, which are frequently isolated from asymptomatic carrier animals and healthy humans (Karmali, 1989). Moreover, this PCR assay represents an improvement over other real-time PCR assays that have either been developed for detecting only the Shiga toxin-encoding genes (Belanger et al., 2002; McKillip and Drake, 2000) or a single gene specific to EHEC O157:H7 (Fortin et al., 2001; Oberst et al., 1998).

Acknowledgements

The authors would like to thank Robert Morgan and Matt Inbody for expert technical support, and Sandy Johnson for secretarial assistance. We thank Irene Wesley and Richard Zuerner for critical review of this manuscript.

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