Journal of Food Protection, Vol. 65, No. 9, 2002, Pages 1371–1380

Detection and Quantitation of Enterohemorrhagic *Escherichia coli* O157, O111, and O26 in Beef and Bovine Feces by Real-Time Polymerase Chain Reaction†

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MS 01-490: Received 21 December 2001/Accepted 19 April 2002

ABSTRACT

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 and certain non-O157 EHEC serotypes (such as O26:H11, O26:NM, O111:H8, and O111:NM) have emerged as significant causes of human disease throughout the world. Important virulence attributes of EHEC are the intimin protein (encoded by the *eae* gene) and Shiga toxins 1 and 2 (encoded by the *stx1* and *stx2* genes, respectively). Two sets of real-time polymerase chain reaction (R-PCR) assays were developed for the simultaneous detection and quantitation of EHEC through the monitoring of the presence of the *eae* and *stx* genes, and these assays were evaluated. In the $eae_{\text{R-PCR}}$ assay, three sets of primers and TaqMan probes were designed for the amplification and real-time detection of a portion of the *eae* gene specific to the EHEC O26, O111, and O157 serotypes. In the $stx_{\text{R-PCR}}$ assay, two sets of primers and TaqMan probes were used to amplify and detect the *stx1* and *stx2* genes. DNA prepared from 67 bacterial strains carrying known virulence markers was tested to determine the specificities of the two assays. In the $eae_{\text{R-PCR}}$ assay, eae_{O157}- and eae_{O111}-specific primer-probe sets identified only EHEC O157 and O111 strains, respectively. The eae_{O26}-specific primer-probe set identified all EHEC O26 isolates and some Shiga toxin–negative serotypes of enteropathogenic *E. coli* and rabbit diarrheagenic *E. coli*. The $stx_{\text{R-PCR}}$ assay was able to identify only those strains carrying either or both of the Shiga toxin–encoding genes. The detection range of both R-PCR assays was linear over DNA concentrations corresponding to 10^3 to 10^8 CFU/ml of an EHEC strain. Both assays were able to detect and quantify very low levels (1 to 10 CFU/g of food or feces) of EHEC in feces and ground beef enriched for 16 h in a modified Trypticase soy broth. In conclusion, *eae*- and *stx*-based R-PCR assays are reliable and sensitive methods for the rapid screening and specific and quantitative detection of important serotypes of EHEC in cattle and in foods of bovine origin.

All enterohemorrhagic *Escherichia coli* (EHEC) *(13)* are Shiga toxin-producing *E. coli* (STEC) (Shiga toxins 1 and 2 are encoded by the *stx1* and *stx2* genes, respectively) and are capable of causing a broad spectrum of diseases, including uncomplicated diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome *(11)*. In addition to Shiga toxins that act on vascular endothelial cells to produce hemolytic-uremic syndrome *(19, 28),* EHEC strains harbor genes that mediate their adherence to intestinal epithelial cells by a characteristic attaching-and-effacing mechanism *(10)*. Production of attaching-and-effacing lesions requires the expression of intimin, an outer-membrane protein encoded by the *eae* gene that is part of the locus of enterocyte effacement *(16)*.

Although *E. coli* O157:H7, a prototype of EHEC, has been associated with the most important foodborne outbreaks in the United States *(30)* and Canada *(9),* other EHEC serotypes, particularly O26 and O111, have emerged as significant causes of human diseases over the past 20 years *(2, 8, 29)*. Cattle have been identified as a natural reservoir of EHEC, and some of these serotypes have been isolated from cattle and humans with disease. EHEC can survive in bovine feces for a long time and can retain the potential to produce Shiga toxins *(5)*. Thus, cattle feces represent a potential vehicle for the transmission of EHEC among cattle, food, and the environment. Foods of bovine origin, particularly contaminated meat, dairy products, or vegetables, are common sources of EHEC transmission to humans. The morbidity and mortality associated with EHEC infections have highlighted the need for the development of methods for automated and high-throughput screening of foods and feces for EHEC.

EHEC is genetically and phenotypically similar to other nonpathogenic *E. coli* commonly found in animal and human gastrointestinal tracts, making their detection and subsequent isolation from feces and foods difficult. Current methods for the identification of EHEC depend primarily on the presence of Shiga toxins, which are detected either by the Vero cell cytotoxicity assay *(12)* or by commercially available immunoassays *(20)*. However, these methods can neither distinguish multiple EHEC serotypes nor detect very low levels of EHEC in feces and foods containing high levels of competing microflora.

The polymerase chain reaction (PCR) is a very sensi-

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tive and relatively inexpensive tool, and it has been used in both single and multiplex formats to nonquantitatively detect EHEC and STEC in foods and feces *(6, 14, 22, 25, 33).* PCR tests that have been developed for the detection of EHEC have either used primers for the amplification and detection of *stx1*- and *stx2*-specific sequences *(22)* or targeted the *eae* gene to distinguish EHEC serotypes from each other and from enteropathogenic *E. coli* (EPEC) harboring *eae* but lacking *stx1* and *stx2 (14, 22, 31).* Quantitative detection of target gene–specific amplified products is not feasible in these PCR assays because amplified products are visualized in agarose gels after the completion of PCR. Recently, real-time PCR (R-PCR) assays have been developed for the detection and quantification of pathogen-specific gene products with the use of TaqMan probes or molecular beacons *(15, 17).* TaqMan probes and molecular beacons are oligonucleotides that are coupled with reporter and quencher dyes at the 5′ and 3′ ends, respectively. In an intact probe or 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 the annealing and extension phases of the PCR process results in an increase in the reporter dye's fluorescence intensity. The continuous measurement of the incremental fluorescence increase for each PCR cycle provides an accurate estimate of the number of cells of a bacterial pathogen present in contaminated food and fecal samples.

The objective of the present study was to design and evaluate the utility of multiplex R-PCR assays in the identification of EHEC O26, O111, and O157 on the basis of *eae* gene heterogeneity (the $eae_{\text{R-PCR}}$ assay) and on the basis of the presence of Shiga toxin–encoding genes (the $stx_{\text{R-PCR}}$ assay). We show that the $eae_{\text{R-PCR}}$ assay, when used in conjunction with the $stx_{\text{R-PCR}}$ assay, could distinguish the majority of EHEC serotypes from each other and from EPEC strains that carry identical *eae* alleles but lack Shiga toxin–encoding genes. We also demonstrate that these R-PCR assays can be used for the detection of very low levels of EHEC and their Shiga toxin types in foods and feces after overnight enrichment of these samples in a modified Trypticase soy broth (mTSB). The specificities and sensitivities of these assays are described and compared with those of other R-PCR assays designed for the detection of EHEC and STEC.

MATERIALS AND METHODS

Bacterial strains, culture media, and growth conditions. A total of 67 bacterial strains representing EHEC, STEC, EPEC, rabbit diarrheagenic *E. coli* (RDEC), enterotoxigenic *E. coli* (ETEC), and other bacterial species were used in this study (Table 1). The important genotypic characteristics of these strains have previously been described *(27).* These strains were obtained from the National Animal Disease Center (Ames, Iowa) and from Thomas Whittam (*E. coli* Reference Center, University Park, Pa.). The bacterial strains were propagated and maintained on Trypticase soy agar plates. Liquid cultures were obtained by growing bacteria in mTSB (TSB supplemented with 1 g of mannitol per liter, 2.5 g of sodium citrate per liter, and 0.25 g of sodium deoxycholate per liter) for 16 h at 37°C with continuous agitation (160 rpm) in a circulating air incubator (New Brunswick Scientific, Edison, N.J.). Trypticase soy agar and sorbitol-MacConkey agar were used to enumerate bacteria. Trypticase soy agar, TSB, and sorbitol-MacConkey agar were purchased from BBL (Becton Dickinson Microbiology Systems, Cockeysville, Md.).

Design of primers and fluorogenic probes. The published nucleotide sequences (Table 2) of *eae* genes harbored by EHEC O26, O111, and O157 were aligned using DNA sequence analysis software (DNAMAN, Lynnon BioSoft, Vaudreuil, Quebec, Canada), and primers and fluorogenic probes were selected from the nucleotide sequence spanning the highly divergent 3′ end of *eae* to amplify and detect 77-bp EHEC O26-specific, 88-bp O111-specific, and 106-bp O157-specific *eae* sequences. To detect Shiga toxin–encoding genes, the extensive nucleotide divergence between *stx1* and *stx2* genes was taken into account in selecting primer and probe sequences for the amplification and detection of 150-bp *stx1*-specific and 200-bp *stx2*-specific fragments *(1, 7).* The fluorogenic probes eae_{O26}, eae_{O111}, and eae_{O157} were labeled at the 5′ ends with FAM (6-carboxy-fluorescein), VIC (a proprietary fluorescent dye developed by PE Biosystems, Foster City, Calif.), and TET (6-carboxy-2′,4,7,7′-tetrachlorfluorescein) fluorescent reporter dyes, respectively. The 5′ ends of the *stx1*- and *stx2*-specific probes were conjugated with FAM and VIC reporter dyes, respectively. The quencher dye TAMRA (6-carboxytetramethyl-rhodamine) was attached at the 3′ ends of all five probes used in the R-PCR assays. The nucleotide sequences of primers and probes are listed in Table 2. Primers and probes (except for the eae_{O111} VIC-conjugated probe, synthesized by PE Biosystems) were synthesized by Integrated DNA Technologies (Coralville, Iowa).

Preparation of feces and beef for R-PCR. Bovine feces were collected from dairy cattle housed on a farm at Iowa State University (Ames, Iowa). Feces were collected directly from cattle that had been restrained in a chute. Ground beef was purchased from a local grocery store. These samples were determined to be free of EHEC and STEC by a PCR method. For this experiment, 1-g portions of feces or beef were inoculated into tubes containing 9 ml of mTSB and incubated at 37°C overnight. These cultures were centrifuged for 2 min at 1,000 × *g* to remove large particles. Culture supernatant (1 ml) was then centrifuged at 12,000 × *g* for 3 min, and the cell pellet was processed to isolate DNA (as described below). This DNA was used in the $eae_{\text{R-PCR}}$ and $stx_{\text{R-PCR}}$ assays. The amplification products were analyzed by electrophoresis through a 4% agarose gel (FMC BioProducts, Rockland, Maine) followed by ethidium bromide staining. The molecular sizes of visible bands were estimated from a 50-bp DNA-sizing ladder (Gibco-BRL). None of the fecal or beef samples tested positive for these genes. These samples were used for seeding experiments.

Detection of EHEC in bovine feces and ground beef. Feces and ground beef (1- or 25-g portions) testing negative for EHEC and STEC contamination were seeded with 0.1-ml aliquots of 10-fold serial dilutions of a culture containing equal proportions of overnight cultures of EHEC O157 (strain 2409), EHEC O26:H11 (strain DEC9A), and EHEC O111 (strain DEC8A). The seeded samples were mixed with mTSB (9 ml of mTSB per g of feces or beef) by vortexing and were incubated for 16 h at 37°C with shaking (150 rpm). One-milliliter aliquots from these cultures were then used in the preparation of DNA (described below). This DNA was used in R-PCR assays to assess the detection sensitivities of these assays and to generate standard curves that would facilitate the determination of levels of EHEC contamination in foods and feces that had also been subjected to an overnight enrichment.

TABLE 1. *Specificity of R-PCR assays*

Bacterial strain	Serotype[a]	Genotype[b]			Detection by $stx_{R\text{-}PCR}$ with probe		Detection by $EHEC_{R\text{-}PCR}$ with probe		
					stx1	*stx2*	eae_{O26}	eae_{O111}	eae_{O157}
Nontoxigenic *E. coli*	O111:H12 (2)	*stx1*	*stx2*	*eae*	−	−	−	−	−
	O111:H21 (3)	*stx1*	*stx2*	*eae*	−	−	−	−	−
	O149:HN	*stx1*	*stx2*	*eae*	−	−	−	−	−
	O149:H10	*stx1*	*stx2*	*eae*	−	−	−	−	−
	O157:H43	*stx1*	*stx2*	*eae*	−	−	−	−	−
	O128:H7 (2)	*stx1*	*stx2*	*eae*	−	−	−	−	−
	O128:H21	*stx1*	*stx2*	*eae*	−	−	−	−	−
	O128:H47	*stx1*	*stx2*	*eae*	−	−	−	−	−
ETEC	O101	*stx1*	*stx2*	*eae*	−	−	−	−	−
	O149:H10	*stx1*	*stx2*	*eae*	−	−	−	−	−
	O149:HN	*stx1*	*stx2*	*eae*	−	−	−	−	−
RDEC	O132	*stx1*	*stx2*	eae^+	−	−	−	−	−
	O15:HN	*stx1*	*stx2*	eae^+	−	−	−	−	−
EPEC	O55:NM (2)	*stx1*	*stx2*	eae^+	−	−	−	−	+
	O55:H6	*stx1*	*stx2*	eae^+	−	−	−	−	−
	O55:H7 (2)	*stx1*	*stx2*	eae^+	−	−	−	−	+
	O26:NM (2)	*stx1*	*stx2*	eae^+	−	−	+	−	−
	O26:H11	*stx1*	*stx2*	eae^+	−	−	+	−	−
	O111:H2 (3)	*stx1*	*stx2*	eae^+	−	−	+	−	−
	O128:H2 (4)	*stx1*	*stx2*	eae^+	−	−	+	−	−
STEC	O103:H2	$stx1^+$	*stx2*	*eae*	+	−	−	−	−
	O45:H2	*stx1*	$stx2^+$	*eae*	−	+	−	−	−
	O91:H21	*stx1*	$stx2^+$	*eae*	−	+	−	−	−
	O113	$stx1^+$	$stx2^+$	*eae*	+	+	−	−	−
	O119:H16	*stx1*	$stx2^+$	*eae*	−	+	−	−	−
	O121:H19	$stx1^+$	$stx2^+$	*eae*	+	+	−	−	−
	O126:H7	$stx1^+$	*stx2*	*eae*	+	−	−	−	−
	OX3:H2	$stx1^+$	$stx2^+$	*eae*	+	+	−	−	−
EHEC	O26:H11 (2)	$stx1^+$	$stx2^+$	eae^+	+	+	+	−	−
	O26:H11 (2)	$stx1^+$	*stx2*	eae^+	+	−	+	−	−
	O111:NM	$stx1^+$	$stx2^+$	eae^+	+	+	−	+	−
	O111:NM	$stx1^+$	*stx2*	eae^+	+	−	−	+	−
	O111:NM	$stx1^+$	*stx2*	eae^+	+	−	+	−	−
	O111:H8	$stx1^+$	$stx2^+$	eae^+	+	−	−	+	−
	O157:H7 (5)	$stx1^+$	$stx2^+$	eae^+	+	+	−	−	+
	O157:H7 (2)	*stx1*	$stx2^+$	eae^+	−	+	−	−	+
	O157:H7 (1)	$stx1^+$	*stx2*	eae^+	+	−	−	−	+
	O157:NM (3)	$stx1^+$	$stx2^+$	eae^+	+	+	−	−	+
	O157:NM (1)	*stx1*	$stx2^+$	eae^+	−	+	−	−	+
Salmonella Typhimurium		*stx1*	*stx2*	*eae*	−	−	−	−	−
Enterobacter cloacae		*stx1*	*stx2*	*eae*	−	−	−	−	−
Hafnia alveie		*stx1*	*stx2*	*eae*	−	−	−	−	−
Klebsiella pneumoniae		*stx1*	*stx2*	*eae*	−	−	−	−	−
Proteus vulgaris		*stx1*	*stx2*	*eae*	−	−	−	−	−
Pseudomonas aeruginosa		*stx1*	*stx2*	*eae*	−	−	−	−	−
Staphylococcus aureus		*stx1*	*stx2*	*eae*	−	−	−	−	−

[a] The number of isolates of the serotype is shown in parentheses.

[b] The presence of *eae* and *stx* genes in *E. coli* was determined by colony blot hybridization with probes specific for these genes *(2, 11)* (http://www.bio.psu.edu/people/faculty/whittam/lab/deca/).

Preparation of DNA. For DNA preparation, feces and beef cultured overnight in mTSB were centrifuged for 2 min at 1,000 × *g* to remove large fecal particles and tissue debris, respectively. Supernatants (1 ml) were centrifuged at 12,000 × *g*, and bacterial pellets were 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 at 100°C for 10 min, and centrifuged at 12,000 × *g* for 5 min. Supernatants (0.1 ml) were purified with a QIAquick PCR Purification Kit per the manufacturer's directions (Qiagen, Valencia, Calif.). This column purification procedure takes only 10 min and allows the removal of substances that inhibit the PCR process, such as pigments and lipids, from feces. The DNA was used immediately in R-PCR assays or stored at −70°C for later use. To determine the sensitivity of R-PCR assays for a pure culture, DNA

TABLE 2. *Nucleotide sequences of primers and fluorogenic probes*

Primer or probe	Sequence (5′→3′)	Location within target gene[a]	PCR product detected[b] (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	CTC TGC CAA AGA ACT GGT TAC AG	2312–2334	eae_{O26} (77)
eae-reverse	TTT CCA TGT GTA TTT TCC ATT GC	2388–2366	
eae-probe	ATG GTC AGG TTA AGC TAC AGG CAA CAG	2336–2362	
eae-forward	GCT CCG AAT TAT ATG ATA AGA GTG G	2516–2540	eae_{O111} (88)
eae-reverse	TCT GTG AGG ATG GTA ATA AAT TTC C	2603–2579	
eae-probe	CAA AAG GAC ATA GCA TTT GCA TAA CTG GCT T	2577–2547	
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 positions of the oligonucleotides are listed relative to the initiation codon (+1 adenine) of the gene.

[b] The nucleotide sequences used in the design of these primers and probes were retrieved from the GenBank (accession numbers M16625 [*stx1*], X07865 [*stx2*], AJ223063 [eae_{O26}], AF025311 [eae_{O111}], and AF081182 [$eae_{O157:H7}$]).

was extracted from 10-fold serial dilutions of a culture (prepared by mixing equal volumes of overnight cultures of EHEC O26, O111, and O157 strains) by centrifuging a 0.1 ml-aliquot of each dilution and processing cell pellets for DNA isolation as described above except that supernatants recovered after 100°C heat treatment were not column purified and used directly in R-PCR assays.

R-PCR amplification and quantitation of target DNA. R-PCR was carried out with a total volume of 50 μl, containing 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, 60 nM reference dye ROX (carboxy-X-rhodamine), and 2.5 U of AmpliTaq Gold DNA polymerase (PE Biosystems). The $eae_{R\text{-}PCR}$ assay contained primer (0.3 μM eae_{O111} and eae_{O157} and 0.6 μM eae_{26} primers) and probe (0.2 μM) sets to amplify and detect sequences specific to eae_{O26}, eae_{O111}, and eae_{O157}. The $stx_{R\text{-}PCR}$ assay contained primer (0.3 μM *stx1* and 0.75 μM *stx2*) and probe (0.2 μM each probe) sets for *stx1* and *stx2* amplification and detection. Amplification and detection were carried out in optical-grade 96-well plates in an ABI Prism 7700 Sequence Detection System (PE 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, and then samples were cooled to 25°C. The reaction conditions for amplification and the parameters for fluorescence data collection were programmed into a Power Macintosh 4400/20 (Apple Computer, Santa Clara, Calif.) linked directly to the ABI Prism 7700 Sequence Detection System with SDS 1.6 application software per the manufacturer's instructions (PE Biosystems).

After real-time data acquisition, the cycle threshold (C_T) value was calculated by determining the point at which fluorescence exceeded an arbitrary threshold signal. Any fluorescent signal that was 10-fold higher than the standard deviation of the mean baseline emission was indicative of positive detection. For the quantification of PCR products, the threshold signal was manually set across all samples so that it intersected the amplification curves in the exponential phase of the fluorescence signal increase. The C_T value is predictive of the quantity of target gene copies in a PCR sample. Standard curves were prepared by plotting C_T versus $\log_{10}$ of the CFU per milliliter (for serially diluted pure cultures) or per gram (for feces or beef artificially inoculated with 10-fold serial dilutions prepared from a culture containing equal proportions of overnight cultures of EHEC O26, O111, and O157) of a sample. The slopes of the standard curves were calculated either by a linear-regression analysis (for pure cultures) or by a regression analysis using a second-order polynomial equation (for feces and beef seeded with EHEC). Microsoft Excel 98 software was used to perform this analysis (Microsoft, Redmond, Wash.). The amplification efficiency (E) was estimated by the formula $E = [(10^{-1/\text{slope}}) - 1]$ *(24)*. A slope of −3.32 is considered to yield an amplification efficiency of 100%.

RESULTS

Optimization of PCR assays. Optimal PCR conditions were established by assessing the effects of various temperatures, Mg^{2+} levels, and primer concentrations on the amplification of predicted fragments of *eae* ($eae_{R\text{-}PCR}$) and *stx* ($stx_{R\text{-}PCR}$) genes. Genomic DNA prepared from STEC and EHEC strains harboring various combinations of *eae* and *stx* genes was used as a template in optimization assays. The optimized PCR conditions consistently yielded bands of the expected sizes in $eae_{R\text{-}PCR}$ and $stx_{R\text{-}PCR}$ assays with the template DNA of strains containing appropriate gene combinations (Fig. 1).

Specificity of the $eae_{R\text{-}PCR}$ and $stx_{R\text{-}PCR}$ assays. Genomic DNA prepared from a total of 67 bacterial strains representing EHEC, STEC, EPEC, RDEC, ETEC, and other bacterial species were tested to establish that the primers and probes used in these assays resulted in the amplification and detection of fragments specific to genes targeted in these assays. During R-PCR, positive amplification signals were produced only for those strains that harbored the genes targeted in these assays, and strains lacking these gene combinations were not detected (Table 1).

Detection sensitivity of R-PCR assays. Genomic DNAs extracted from 10-fold serial dilutions (prepared by

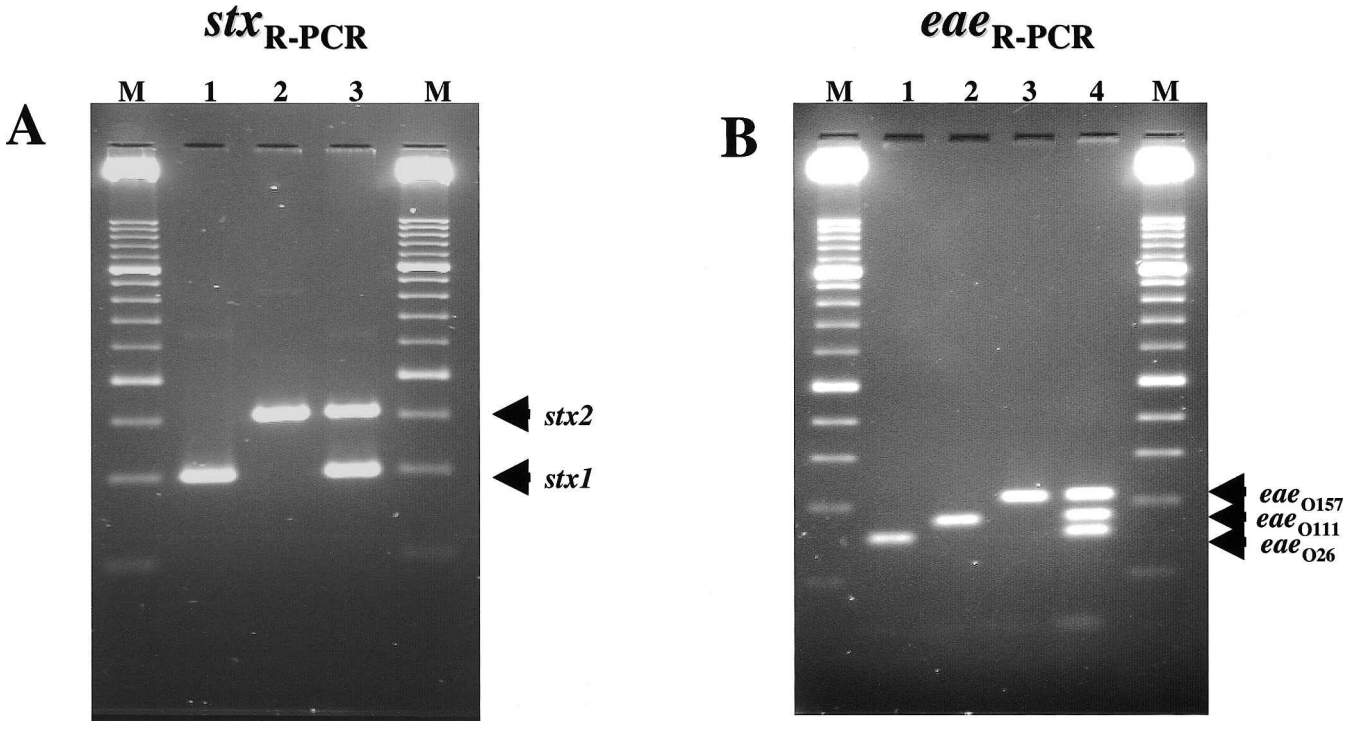

FIGURE 1. *Optimization of RT-PCR assays: agarose gel electrophoresis of PCR products from optimized PCR assays for EHEC* eae *and* stx *genes. (A) A gel with* $stx_{R\text{-}PCR}$ *products from template DNA of strains harboring* stx1 *(lane 1),* stx2 *(lane 2), and* stx1 *and* stx2 *(lane 3). (B) A gel for* $eae_{R\text{-}PCR}$ *products from template DNA of* eae*-harboring EHEC strains O26 (lane 1), O111 (lane 2), and O157: NM (lane 3) and a mixed culture of three strains (lane 4). The M lanes contain 50-bp molecular weight standards. Arrows indicate the relative positions of amplified products.*

mixing equal volumes of overnight cultures of EHEC O157 [$stx1^+$, $stx2^+$, eae^+], O26 [eae^+], and O111 [$stx2^+$, eae^+]) were used as templates to determine the detection sensitivity and dynamic range of R-PCR assays for *eae* and *stx* genes (Fig. 2). The fluorescence profile generated with the eae_{O26} primer-probe set used in the $eae_{R\text{-}PCR}$ assay is plotted in Figure 2A. The fluorescence profiles generated with other primer-probe sets were almost identical to that shown in Figure 2A (data not shown). Standard curves generated from fluorescence profiles showed a linear relationship between the $\log_{10}$ of input CFU and the threshold cycle (the PCR cycle at which the fluorescence intensity rises above the threshold) (Fig. 2B). The slopes of the curves for eae_{O26}, eae_{O111}, and eae_{O157} were -3.5981, -3.6894, and -3.604, respectively, and the squared regression coefficients for these genes after the linear regression ranged from 0.966 to 0.982. Similarly, the slopes of the standard curves for *stx1* and *stx2* were -3.6038 and -3.5415, respectively, and the squared regression coefficients for these genes after the linear regression were 0.972 to 0.986 (data not shown). The efficiency of the R-PCR assays (86 to 91%) was calculated from these standard curves by the formula given in "Materials and Methods." These results indicate that the primer-probe sets worked optimally in these assays and produced similar amplification yields and similar degrees of fluorogenic detection of the targeted genes. The lowest detection limits of $eae_{R\text{-}PCR}$ and $stx_{R\text{-}PCR}$ were approximately 18 CFU per PCR (equivalent to 10^3 CFU/ml). When the PCR products generated in the $eae_{R\text{-}PCR}$ and $stx_{R\text{-}PCR}$ assays were analyzed on an ethidium bromide–containing agarose gel, bands of predicted sizes were clearly detected within the linear range (10^3 to 10^8 CFU/ml) of the assays (data not shown), and these results were in agreement with the C_T values generated at these cell concentrations. However, the template DNA prepared from serial dilutions containing $\leq 10^2$ CFU/ml produced C_T values of ≥ 38, and the PCR products from these samples were not consistently detected in the agarose gels. Thus, to avoid the detection of false positives, C_T was adjusted to a value of ≤ 38.

Quantitation of very low levels of EHEC O157 and non-O157 serotypes in beef and feces. In combination with an overnight enrichment, the *eae* probes ($eae_{R\text{-}PCR}$) specific for EHEC O26, O111, and O157 could detect 15, 1.5, and 1.5 CFU/g of beef, respectively (Fig. 3A). The detection sensitivities for specific EHEC strains in feces (per gram) were 15 CFU with the eae_{O111} and eae_{O157} probes and 150 CFU with the eae_{O26} probe (Fig. 3B). The $stx_{R\text{-}PCR}$ assay resulted in the detection of 1.2 CFU of STEC per g of beef with probes specific for the *stx1* and *stx2* genes (Fig. 4A). The detection sensitivities of *stx1* and *stx2* probes in feces (per gram) were 1.2 CFU to 12 CFU, respectively (Fig. 4B). A regression analysis of the enrichment data allowed the generation of standard curves showing a linear correlation between C_T and $\log_{10}$ of 10^0 to 10^5 CFU seeded into 1 g of beef or feces. The C_T for samples seeded with 10^6 and 10^7 CFU was in the nonlinear range of standard curves.

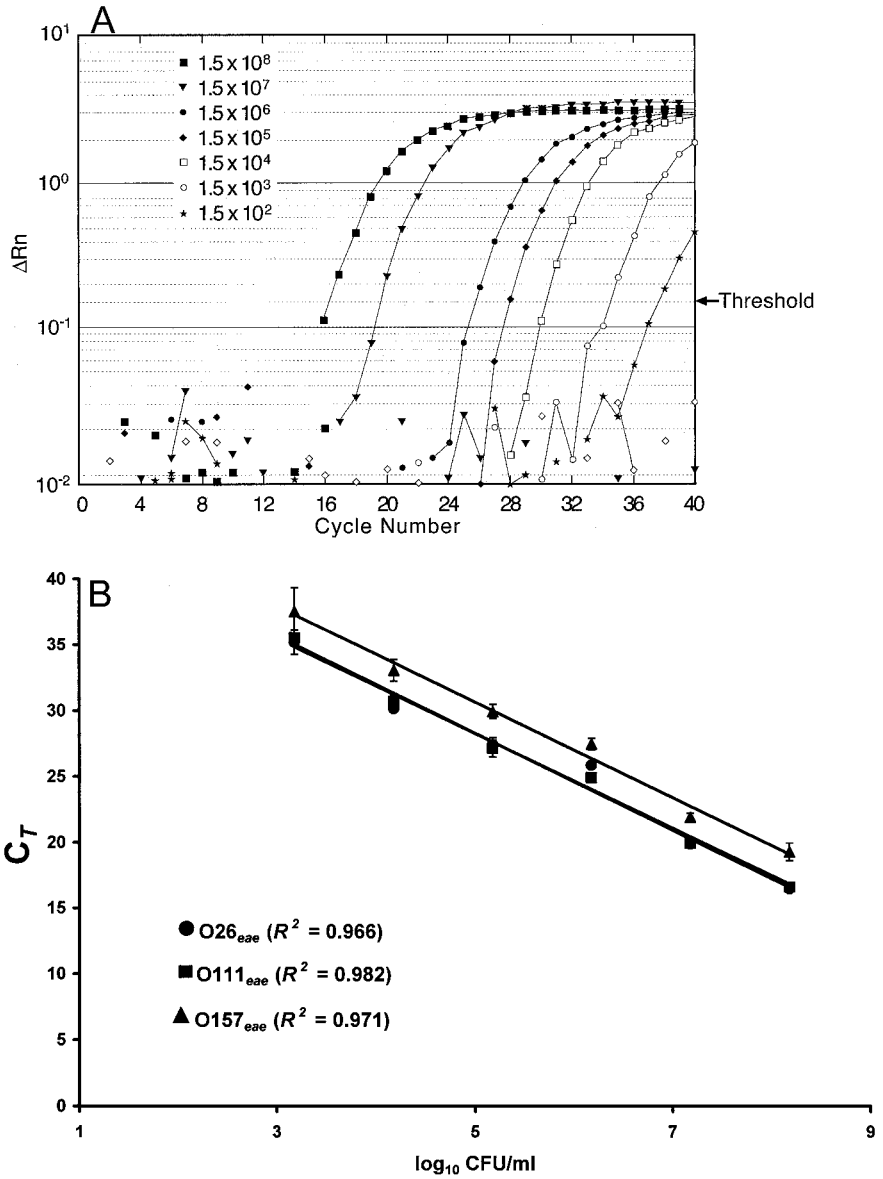

FIGURE 2. *Detection sensitivity of R-PCR assays. Genomic DNA extracted from serial 10-fold dilutions of a mixed culture of EHEC O26, O111, and O157 were tested with* $eae_{R\text{-}PCR}$ *and* $stx_{R\text{-}PCR}$ *assays. (A) The relative fluorescence intensity (ΔRn; measured as an increase in the reporter dye intensity relative to the intensity of the passive internal reference dye) produced by one of the primer-probe sets during PCR amplification is plotted (similar fluorescence profiles were produced by other primer-probe sets [data not shown]). Bacterial cell counts corresponding to each fluorescence profile are indicated in the graph. A horizontal bar representing the cycle threshold (*C_T*) is indicated by an arrow. (B) Standard curves generated from the fluorescence profile. The* C_T *value corresponding to each dilution is plotted. Linear-regression analysis allowed the calculation of straight lines and regression coefficients (*R^2*), which are indicated in the plot. Standard deviations based on three PCR reactions are indicated.*

DISCUSSION

Although more than 100 O serotypes of STEC have been identified, the majority of outbreaks and sporadic cases of hemorrhagic colitis and hemolytic-uremic syndrome have been attributed to members of only a few serogroups, such as O26, O111, and O157 *(8, 30).* Several multiplex PCR assays *(6, 22)* and a few fluorogenic PCR assays *(18, 27)* for the specific detection of EHEC O157 in foods and feces have been described. In contrast, only a few PCR-based assays have been reported for the simultaneous detection of EHEC O26, O111, and O157 serotypes *(14, 23).* Moreover, these PCR assays require laborious, less-sensitive, and specific gel-based analysis of PCR-amplified products and are not suitable for the quantitation of target organisms over a wide dynamic range. Thus, to improve sensitivity, specificity, and detection range and to afford the ability to screen large numbers of samples, two sets of multiplex RT-PCR assays were developed and validated for the rapid and semiautomated detection and quantification of EHEC O26, O111, and O157 in bovine feces and ground beef. Simultaneous real-time detection of EHEC O26, O111, and O157 was achieved by targeting the highly divergent 3′ end of the *eae* gene ($eae_{R\text{-}PCR}$) and by targeting the genes encoding Shiga toxins 1 and 2 ($stx_{R\text{-}PCR}$). The specificity of the $eae_{R\text{-}PCR}$ assay in detecting these three EHEC serotypes was comparable to that of conventional PCR assays *(14, 26)* with the exception that the eae_{O111} primer-probe set used in our assay allowed the specific detection of EHEC O111:H8 and some O111:NM isolates. These isolates harbor an *eae* allele that is different from the *eae* alleles associated with EPEC 1 (which carries the *eae* allele coding for α-intimin), EPEC 2 (which carries the *eae* allele coding for β-intimin), and EHEC 1 (which carries the *eae* allele encoding γ-intimin) groups of attaching and effacing *E. coli* (pathogenic *E. coli* serotypes that use an attaching-and-effacing mechanism to adhere to intestinal epithelial cells) *(26).* The EHEC 1 group includes serotype O157:H7 (and the closely related EPEC O55:H7), and the EPEC 2 group includes both non–Shiga toxin–producing and Shiga toxin–producing (EHEC 2) O26 and O111 se-

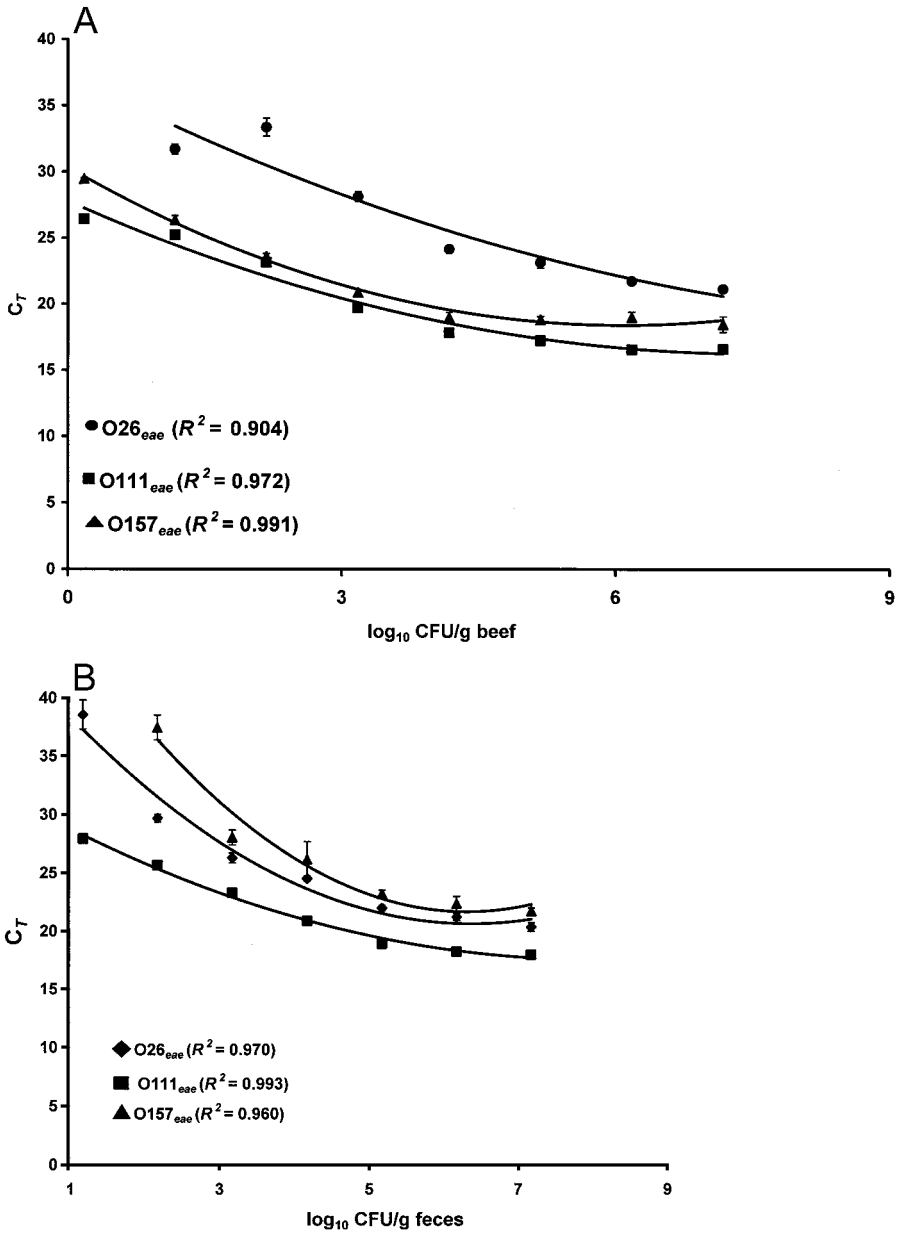

FIGURE 3. *Detection sensitivity of* $eae_{R\text{-}PCR}$. *Genomic DNAs extracted from ground beef or feces seeded with known numbers of three EHEC strains and enriched overnight were tested in R-PCR assays. The cycle threshold* (C_T) *values are plotted against CFU. The standard curves and corresponding regression coefficients* (R^2) *were calculated for (A) beef and (B) fecal samples on the basis of a second-order polynomial equation. Error bars indicate standard deviations based on three PCR reactions.*

rotypes *(26).* Thus, by designing a set of primers and a TaqMan probe from the nucleotide sequence at the 3′ end of the *eae* gene of an EHEC O111:NM strain *(21),* we were able to identify EHEC O111:H8 and some other EHEC O111:NM strains that did not cross-react with the eae_{O26} probe used for the detection of EHEC O26 in our PCR assay.

Of the 67 bacterial strains tested to validate the specificity of the $eae_{R\text{-}PCR}$ assay, only EHEC O157:NM and EPEC O55:H7 cross-reacted with the EHEC O157–specific *eae* primer-probe set. The detection of EHEC O157:NM and EPEC O55:H7 by the $eae_{O157:H7}$ probe is unavoidable, because most isolates of EHEC O157:NM are considered nonmotile variants of EHEC O157:H7 despite the presence of *fliC* (the gene encoding H7 flagellar antigen in EHEC O157:H7) *(6),* and EPEC O55:H7 harbors highly homologous *eae* genes and is closely related to EHEC O157:H7, as determined by multilocus enzyme electrophoresis *(31).* Nonetheless, the detection of EHEC O157:NM is advantageous because of the isolation of this serotype from patients with hemolytic-uremic syndrome *(3).*

The probe designed for the specific detection of EHEC O26 also cross-reacted with EPEC O111:H2, EPEC O128:H2, RDEC serotypes, and an EHEC O111:NM isolate. The cross-reactivity of the EHEC eae_{O26} probe with the *eae* gene of non-O26 serotypes of EPEC, EHEC, and RDEC has been observed in other *eae*-based multiplex PCR assays *(14, 26)* and is due to the presence of an identical *eae* allele (encoding β-intimin) *(26).* However, these serotypes, with the exception of EHEC O111:NM (which encodes β-intimin), were not detected in the $stx_{R\text{-}PCR}$ assay, confirming that these strains are not EHEC. Thus, when $stx_{R\text{-}PCR}$ is used in conjunction with $eae_{R\text{-}PCR}$, Shiga toxin–producing serotypes carrying an eae_{26} allele can be distinguished from non-Shiga-toxigenic EPEC and RDEC serotypes. The $stx_{R\text{-}PCR}$ assay was highly specific, as *stx1* and *stx2* primer and probe sets did not exhibit any cross-reactivity with strains lacking these genes.

We optimized the R-PCR assays to quantify EHEC

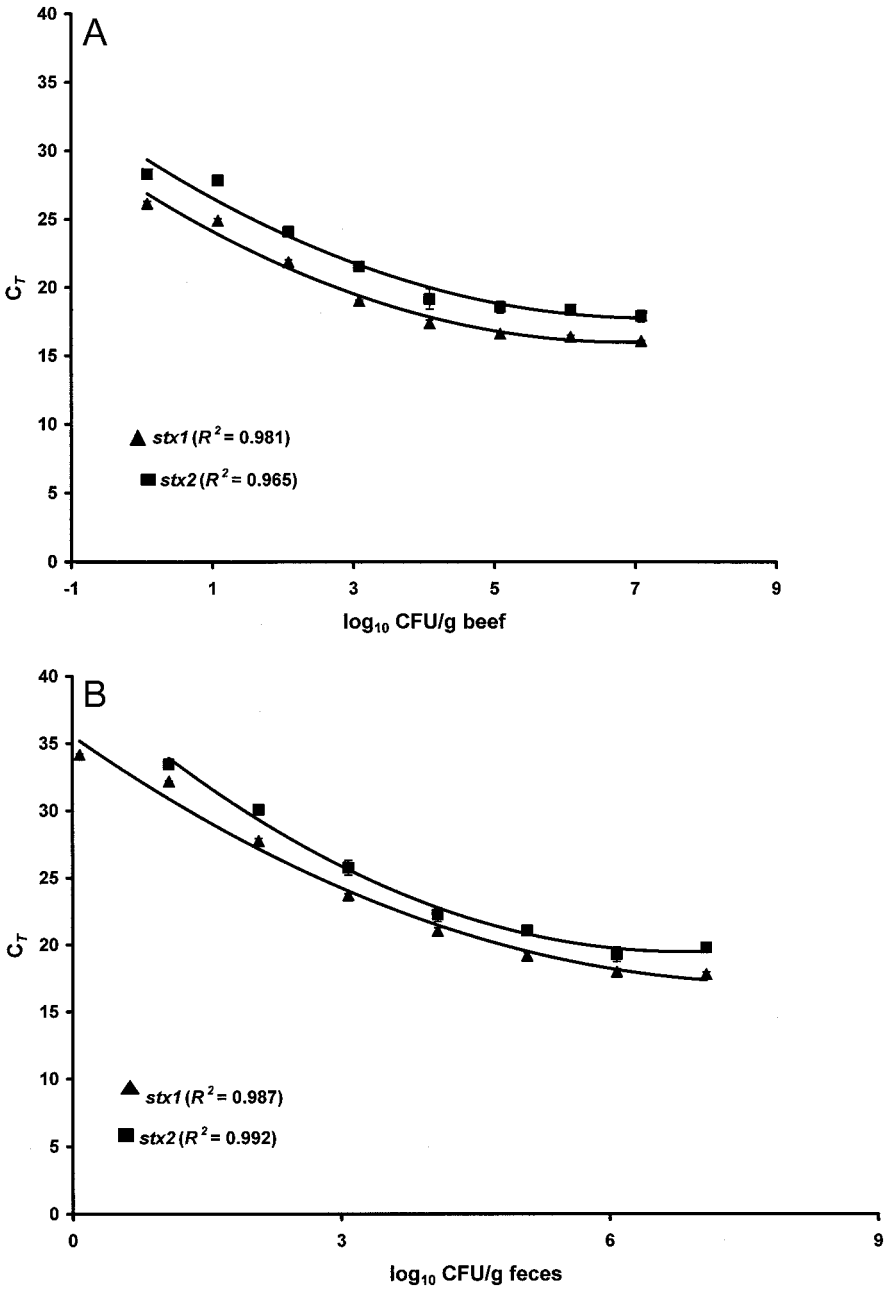

FIGURE 4. *Detection sensitivity of* $stx_{R\text{-}PCR}$. *Genomic DNAs extracted from ground beef or feces seeded with known numbers of three EHEC strains and enriched overnight were tested in R-PCR assays. The cycle threshold* (C_T) *values are plotted against CFU. Standard curves and corresponding regression coefficients* (R^2) *were calculated for (A) beef and (B) fecal samples on the basis of a second-order polynomial equation. Error bars indicate standard deviations based on three PCR reactions.*

over a wide dynamic range. The squared regression coefficients after the linear regression indicated good correlation between the $\log_{10}$ of input CFU and C_T in the standard curves for the *eae* and *stx* genes. The amplification efficiencies were only slightly lower than the theoretical limit of 100% *(21).* The linearity of the standard curves, as well as highly efficient and constant PCR performance, indicated that these assays were well suited for the quantification of EHEC serotypes and the associated Shiga toxin genes. The standard curves exhibited a wide dynamic range, with linear quantification of up to 10^8 CFU/ml, a value comparable to that reported for other R-PCR assays *(4, 15).* Thus, the dynamic range of R-PCR is 10^3 to 10^8 CFU/ml, which is 3 to 4 orders of magnitude higher than that for endpoint PCR (for which the detection range is approximately 10^3 to 10^4 CFU/ml) *(18, 27).* The lowest detection limit for both R-PCR assays was 10^3 CFU/ml, for the R-PCR assays in which genomic DNA isolated from mixed cultures containing as many as three different strains was used. At the lowest detection limit (10^3 CFU/ml), a C_T value of ≤ 37 was consistently observed, and PCR products of the expected sizes were easily detected by agarose gel electrophoresis (data not shown).

The infective dose of EHEC O157, and presumably of EHEC O26 and EHEC O111, capable of causing severe disease in humans is very low (10 to 100 CFU) *(32).* The detection sensitivity of most PCR assays for bacterial pathogens in unenriched feces and foods is generally very low (10^4 CFU/g of sample) *(22, 27)* because crude DNA preparations from feces and foods contain PCR inhibitors *(22).* Therefore, the detection of very low levels of bacterial contamination requires the culture of foods and feces in an appropriate enrichment broth for a certain length of time. This enrichment step dilutes out PCR-inhibitory substances

and provides conditions conducive to growth and the multiplication of bacterial pathogens to reach detectable levels. When an enrichment period of 16 h was incorporated, the two R-PCR assays allowed the detection of 1 to 150 CFU/g of a sample, the levels considered sufficient to cause disease in humans. Detection sensitivity was unaffected by endogenous bacteria at up to 10^7 CFU/g of food or fecal sample (data not shown). The detection range for enriched samples was linear as long as the level of inoculum seeded into a gram of beef or feces before overnight enrichment was within the range of 10^0 to 10^5 CFU. This detection range is at least 2 to 3 orders of magnitude higher than that for endpoint PCR assays (which have a detection range of 10^0 to 10^3 CFU/g) developed for the detection of EHEC O157:H7 and STEC *(18, 27).* Beef and feces seeded with $>10^5$ CFU/g contained 10^9 CFU after 16 h of growth in mTSB (determined by plating on sorbitol-MacConkey agar; data not shown). The amount of template DNA in these samples was so large that the R-PCR became saturated early in the PCR process, resulting in almost identical C_T values for these samples. Since these C_T values were in the nonlinear range of the assay, samples contaminated with very high levels ($>10^5$ CFU/g) of EHEC or STEC would not be distinguished from each other.

In conclusion, *eae*- and *stx*-based R-PCR assays provide a sensitive and accurate closed-tube method for the rapid screening of bovine feces and foods for contamination with EHEC. These assays are also suitable for the quantitation of important EHEC serotypes through the monitoring of the presence of serotype-specific *eae* alleles and Shiga toxin–encoding genes in feces and foods. Overnight enrichment of feces or beef in a nonselective medium is critical for the detection of very low levels (between 1 and 10 CFU/g of feces or beef) of EHEC contamination. Without the enrichment of feces or foods, these R-PCR assays could probably allow the detection of contamination levels of $\geq 10^3$ CFU/g, as the lowest detection limit of these assays was determined to be $\geq 10^3$ CFU for assays in which 10-fold serial dilutions of a pure culture were used. Because the detection range of the R-PCR assays described in this report spans several orders of magnitude, these assays are suitable for generating standard curves with the use of DNA prepared from unenriched or overnight-enriched feces or beef seeded with predetermined numbers of EHEC cells. These standard curves then can be used for determining levels of EHEC contamination in an unknown food or fecal sample. The instrumentation (ABI 7700 Sequence Detection System) used to perform these assays provides a platform for the automated amplification and detection of 96 samples simultaneously, facilitating the screening of large numbers of clinical, food, and water samples. These R-PCR assays should improve the detection and clinical diagnosis of EHEC and other STEC and have potential applications to the improvement of food safety assurance, to the study of the epidemiology of EHEC and STEC in animal reservoirs and the environment, and to the rapid detection of these pathogens in cattle and humans.

ACKNOWLEDGMENTS

The author thanks Robert Morgan for technical assistance, Sandy Johnson for assistance in the preparation of this manuscript, and John Bannantine and Cherie Ziemer for critical reading and constructive evaluation of this manuscript.

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