

## Simultaneous Detection of *Salmonella* Strains and *Escherichia coli* O157:H7 with Fluorogenic PCR and Single-Enrichment-Broth Culture

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**A multiplex fluorogenic PCR assay for simultaneous detection of pathogenic *Salmonella* strains and *Escherichia coli* O157:H7 was developed and evaluated for use in detecting very low levels of these pathogens in meat and feces. Two sets of primers were used to amplify a junctional segment of virulence genes *sipB* and *sipC* of *Salmonella* and an intragenic segment of gene *eae* of *E. coli* O157:H7. Fluorogenic reporter probes were included in the PCR assay for automated and specific detection of amplified products. The assay could detect <10 CFU of *Salmonella enterica* serovar Typhimurium or *E. coli* O157:H7 per g of meat or feces artificially inoculated with these pathogens and cultured for 6 to 18 h in a single enrichment broth. Detection of amplification products could be completed in ≤4 h after enrichment.**

*Salmonella* strains and enterohemorrhagic *Escherichia coli* O157:H7 are among the most important foodborne bacterial pathogens (9, 12). Cattle are important reservoirs of *E. coli* O157:H7 strains, and the majority of human infections due to this organism are associated with ingestion of undercooked, contaminated beef, water, or raw milk (6, 7). *Salmonella*, on the other hand, exhibits a broad host spectrum, which comprises most animal species, including mammals, birds, and cold-blooded animals (1). A variety of food products, especially contaminated poultry, beef, pork, and cheese, are the most important sources of human salmonellosis (3).

The objective of the present study was to assess the fidelity and utility of two sets of primers and two fluorogenic probes in a multiplex PCR format for simultaneous and semiautomated detection of pathogenic *Salmonella* strains and *E. coli* O157:H7. We also describe the use of a single nonselective broth for enrichment of meats and feces harboring very low numbers of these two pathogens. This PCR assay was optimized to obtain a strong and reproducible fluorescence signal from probes labeled with two reporter dyes that allowed immediate and specific detection of *Salmonella* and *E. coli* O157:H7.

**Bacterial strains, culture media, growth conditions, and sample preparation.** The bacterial strains that were used are listed in Table 1. The strains of *E. coli* and *Salmonella* were obtained from the National Animal Disease Center, Ames, Iowa. Thomas Whittam (The Pennsylvania State University, University Park) kindly provided some of the *E. coli* strains. 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 and Trypticase soy broth) for 18 h at 37°C with continuous agitation (160 rpm) in a circulating-air incubator (New Brunswick Scientific, Edison, N.J.). TSA and MacConkey agar were used to enumerate bacteria. TSA, gram-negative broth, Trypticase soy broth, and MacConkey agar were purchased from BBL (Becton Dickson Microbiology

Systems, Cockeysville, Md.). Meat and feces were tested, as described previously (11), for the presence of endogenous bacterial flora and for *Salmonella* and *E. coli* O157:H7 contamination. Meat and feces (1- or 25-g portions) found to be free of *Salmonella* and *E. coli* O157:H7 contamination by PCR (2, 11) were artificially inoculated with 0.1-ml aliquots of 10-fold serial dilutions (prepared from 1:1 mixture of an 18-h culture of *S. typhimurium* and *E. coli* O157:H7). Inoculated samples were cultured in GNTSB (9 ml of GNTSB was added to 1 g of meat or feces) for 6 to 18 h at 37°C. Cultures were centrifuged at 1,000 × g for 2 min to remove large particles. A washing step was performed by centrifuging (12,000 × g for 3 min) 0.05 ml of supernatant mixed with 0.95 ml of GNTSB. The bacterial pellet was processed for DNA isolation as described previously (11).

**Design of primers and fluorogenic probes.** The nucleotide sequences of primers and fluorogenic probes used in amplification and detection of genes *sipB-sipC* and *eae* have been reported previously (2, 11). The reporter dye FAM (6-carboxyfluorescein) or HEX (6-carboxyhexafluorescein) was conjugated at the 5' ends of these probes, and the quencher dye TAMRA (6-carboxytetramethylrhodamine) was conjugated at the 3' ends. The FAM-labeled probe was used for detecting the 250-bp *sipB-sipC* gene fragment of *Salmonella*, and the HEX-labeled probe facilitated the detection of the 150-bp *eae* gene fragment of *E. coli* O157:H7. Primers and probes were synthesized by Integrated DNA Technologies (Coralville, Iowa).

**PCR amplification.** Template DNA (5 μl) was added to 45 μl of a master mixture (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 10 mM Na<sub>2</sub> EDTA, 3 mM MgCl<sub>2</sub>, 0.2 mM deoxynucleoside triphosphates, 450 nM each *sipB-sipC* primer, 300 nM each *eae* primer, 100 nM each fluorogenic probe, and 2.5 Units of AmpliTaq Gold DNA polymerase [PE Applied Biosystems, Foster City, Calif.]) and amplified under conditions described previously (11).

**Fluorogenic detection of PCR products.** The specific detection of PCR-amplified products was achieved by reading a 96-well plate in a computer-controlled dual-scanning microplate spectrofluorometer (SPECTRAMax GEMINI; Molecular Devices, Sunnyvale, Calif.). A series of six no-DNA template controls (NTCs) were included in each reaction plate to

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TABLE 1. Specificity of fluorogenic PCR to detect *Salmonella* and *E. coli* O157:H7

Bacterial strains <sup>a</sup>	Serotype, species, or group <sup>b</sup>	<i>eae</i> gene present <sup>c</sup>	<i>sipB-sipC</i> genes present <sup>c</sup>	Fluorogenic PCR detection	
				<i>E. coli</i> O157:H7 specific	<i>Salmonella</i> specific
Nontoxigenic <i>E. coli</i>	O111:H12 (2)	-	-	-	-
	H21 (3)	-	-	-	-
	O149:HN	-	-	-	-
	O157:H43	-	-	-	-
	O128:H7 (2)	-	-	-	-
	H21	-	-	-	-
	H47	-	-	-	-
ETEC	O101	-	-	-	-
	O149:H10	-	-	-	-
RDEC	O132	+	-	-	-
	O15:HN	+	-	-	-
EPEC	O55:NM (2)	+	-	-	-
	H6 (2)	+	-	-	-
	O26:NM (3)	+	-	-	-
	O111:H2 (3)	+	-	-	-
	O128:H2 (4)	+	-	-	-
	O45:H2	+	-	-	-
	O55:H7 (2)	+	-	+	-
STEC	O111:NM (2)	+	-	-	-
	O111:H8	+	-	-	-
	O26:H11	+	-	-	-
	O45:H2	-	-	-	-
	O113	-	-	-	-
	OX3:H2	-	-	-	-
EHEC	O26:H11 (3)	+	-	-	-
	O111:NM	+	-	-	-
	O157:H7 (5)	+	-	+	-
	O157:NM (1)	+	-	+	-
<i>Salmonella</i>	Typhimurium	-	+	-	+
	Enteritidis	-	+	-	+
	Samftenberg	-	+	-	+
	Litchfield	-	+	-	+
	DT104 (2)	-	+	-	+
	BIRC8	-	+	-	+
	BIRC9	-	+	-	+
	EILC9	-	+	-	+
	EILC10	-	+	-	+
	C1CC117	-	+	-	+
<i>Enterobacter</i>	<i>E. cloacae</i>	-	-	-	-
<i>Hafnia</i>	<i>H. alvie</i>	+	-	-	-
<i>Klebsiella</i>	<i>K. pneumoniae</i>	-	-	-	-
<i>Proteus</i>	<i>P. vulgaris</i>	-	-	-	-
<i>Pseudomonas</i>	<i>P. aeruginosa</i>	-	-	-	-
<i>Staphylococcus</i>	<i>S. aureus</i>	-	-	-	-

<sup>a</sup> ETEC, enterotoxigenic *E. coli*; RDEC, rabbit diarrheagenic *E. coli*; EPEC, enteropathogenic *E. coli*; STEC, Shiga toxin-producing *E. coli*; EHEC, enterohemorrhagic *E. coli*.

<sup>b</sup> Parentheses indicate the number of isolates of a serotype tested.

<sup>c</sup> The presence of the *eae* gene in *E. coli* and that of *sipBC* in *Salmonella* was determined by colony blot hybridization using probes specific for these genes (2, 11; <http://www.bio.psu.edu/people/faculty/whittam/lab/deca/>).

establish the background fluorescence and to calculate the detection threshold (DT). The excitation (ex) and emission (em) wavelengths used for reporter (FAM and HEX) and quencher (TAMRA) dyes were as follows: FAM, ex at 490 nm and em at 515 nm; HEX, ex at 535 nm and em at 560 nm; and TAMRA, ex at 490 nm and em at 585. The fluorescence data

were collected and analyzed by using the fluorescence data management program SOFTmax PRO. Samples exhibiting reporter fluorescence, expressed as relative fluorescence units (RFU), higher than the DT were assigned a plus score, indicating the presence of the target gene(s). Samples exhibiting fluorescence equal to or less than the DT were assigned a minus score, indicating the absence of the target genes and thus no detectable amplification.

**Calculation of DT.** The DT was computed as [mean reporter fluorescence + confidence interval ( $\sigma/\sqrt{n}$ )]  $\times$  correction coefficient. The confidence interval was computed at a significance level of 99.9%, where  $\sigma$  represents the standard deviation of the mean and  $n$  is the number of NTCs. To calculate the correction coefficient, DNA from a 10-fold serial dilution (prepared from a 1:1 mixture of overnight-grown cultures of *S. enterica* serovar Typhimurium and *E. coli* O157:H7) was subjected to PCR amplification. The correction coefficient was calculated by dividing the mean fluorescence value of the highest 10-fold dilution producing detectable 250- and 150-bp amplicons on an agarose gel by the mean fluorescence value of the NTCs.

**Amplification of 250- and 150-bp fragments of target genes.** As shown in Fig. 1, the *sipB-sipC* and *eae* primer pair generated the predicted 250- and 150-bp DNA bands from *S. enterica* serovar Typhimurium and *E. coli* O157:H7 strain 2409, respectively. Amplified products were not detected from strains lacking these genes.

**Specificity of FAM- and HEX-labeled fluorogenic probes.** The ability of FAM- and HEX-labeled probes to specifically detect the *sipB-sipC* and *eae* genes was determined by measuring the RFU of each PCR sample. As shown in Fig. 2A, a FAM-specific fluorescence signal was generated by PCR samples containing DNA from *S. enterica* serovar Typhimurium harboring the *sipB* and *sipC* genes. Similarly, PCR samples containing DNA from *E. coli* O157:H7 produced a HEX-

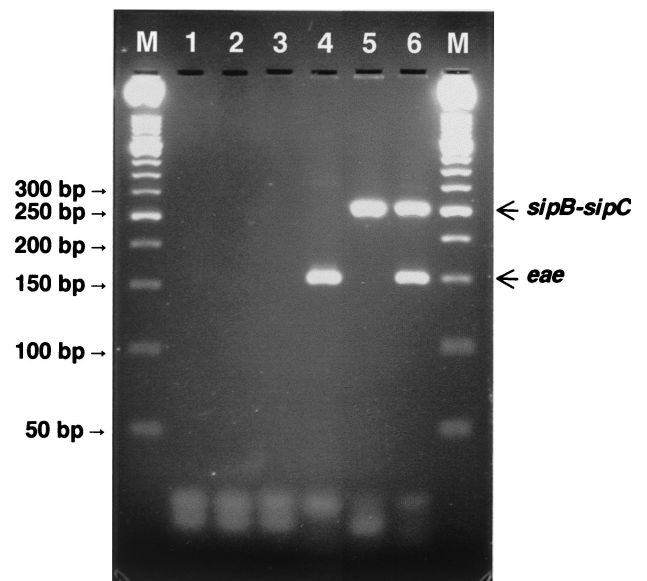


FIG. 1. Agarose gel electrophoresis of PCR products obtained from different strains of *Salmonella* and *E. coli* using primers designed to amplify the 250-bp junctional fragment of *sipB-sipC* and the 150-bp intragenic fragment of *eae*. Lanes: M, 50-bp molecular size standards; 1, NTC; 2, *E. coli* strain DEC A6 (*eae*) template DNA; 3, *Salmonella* strain A 1cc217 (*sipB-sipC*) template DNA; 4, *E. coli* O157:H7 (*eae*<sup>+</sup>) template DNA; 5, *S. enterica* serovar Typhimurium (*sipB*<sup>+</sup>-*sipC*<sup>+</sup>) template DNA; 6, *E. coli* O157:H7 and serovar Typhimurium template DNA. Arrows indicate the relative positions of amplified fragments.

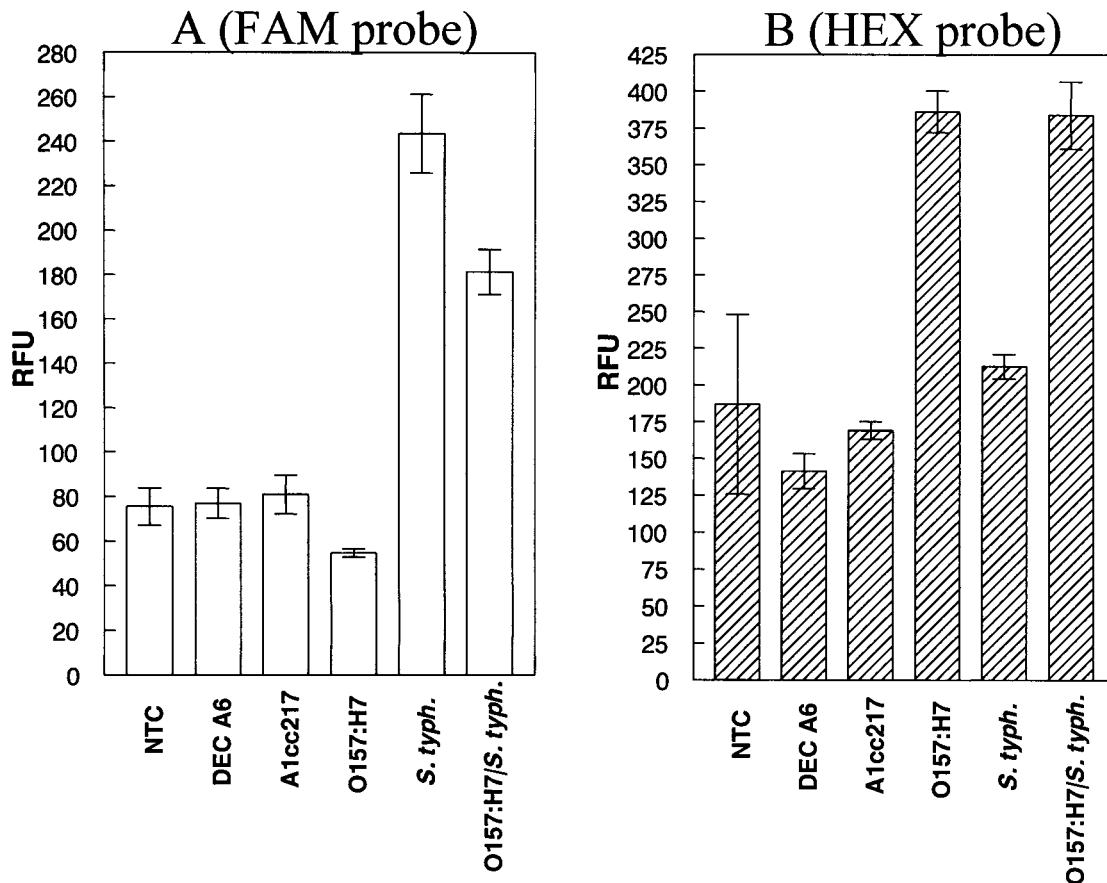


FIG. 2. The specificity of FAM- and HEX-conjugated probes for detection of the 250-bp *sipB-sipC* fragment of *S. enterica* serovar Typhimurium and the 150-bp *E. coli* O157:H7-specific *eae* fragment, respectively, was examined using DNA templates from different bacterial strains (indicated on the x axis). Following PCR, the RFU of each sample were determined (plotted on the y axis). A DT of 97.8 RFU was obtained for the *Salmonella*-specific FAM-labeled probe (A), and a DT of 314.0 RFU was obtained for the *E. coli* O157:H7-specific HEX-labeled probe (B). A PCR sample was scored positive if the RFU value was greater than the DT. Error bars indicated standard deviations of the mean ( $n = 3$ ).

specific signal (Fig. 2B). PCR samples that received the template DNA from serovar Typhimurium and *E. coli* O157:H7 produced a positive fluorescence signal for both reporter dyes.

**Specificity of fluorogenic PCR to detect pathogenic *Salmonella* and *E. coli* O157:H7.** As shown in Table 1, only those *E. coli* strains previously shown to harbor the *E. coli* O157:H7-specific *eae* gene (11) were scored positive for amplification by the fluorogenic detection system. Among the *E. coli* strains tested, all *E. coli* O157:H7 and O157:NM isolates were detected positive. These two serotypes cannot be distinguished from each other in *eae*-based PCR assays (4, 10). However, detection of *E. coli* O157:NM is an advantage considering the frequent isolation of this serotype from patients with hemolytic uremic syndrome (14). Recent studies have shown that many *E. coli* O157:NM isolates contain *fliC*, the gene encoding the H7 flagellar antigen (5). Based on this finding, *fliC*-harboring *E. coli* O157:NM isolates are considered the nonmotile variants of *E. coli* O157:H7. The *E. coli* O157:H7-specific *eae* probe also facilitated the detection of *E. coli* O55:H7, an enteropathogenic *E. coli* strain. All *Salmonella* isolates tested positive in this assay, and these isolates have been shown to harbor *sipB-sipC* (2). All 47 strains lacking *eae* of *E. coli* O157:H7 or the *sipB-sipC* genes of *Salmonella* resulted in RFU less than or equal to the DT and were scored negative for amplification by the detection system (Table 1).

**Detection limits of fluorogenic PCR in beef and feces.** The detection sensitivity of fluorogenic PCR using DNA isolated from a 10-fold serial dilution (prepared from a 1:1 mixture of *S. enterica* serovar Typhimurium and *E. coli* O157:H7 overnight cultures) was determined to be  $2.5 \times 10^3$  CFU per ml or 10 CFU per PCR (data not shown). However, most bacterial pathogens, including *E. coli* O157:H7 and *Salmonella*, are present in very low numbers in foods and feces. Foods and feces also contain substances that are inhibitory to the process of PCR amplification. Detection of very low levels of bacterial contamination in foods and feces necessitates that these samples be cultured for a few hours in an appropriate enrichment broth. This enrichment serves two purposes. First, it dilutes out substances inhibitory to the PCR process, and second, it provides conditions conducive for growth and multiplication of bacterial pathogens to a detectable number. The detection sensitivity of this PCR assay for *S. enterica* serovar Typhimurium ranged from 55 to 5.5 CFU per g of beef after 6 to 18 h of enrichment (Fig. 3A). For *E. coli* O157:H7, the detection sensitivity was 3.5 CFU per g of beef after 6 or 18 h of enrichment (Fig. 3B). In ground chicken, the detection sensitivity ranged from 47 to 0.47 CFU per g of chicken for *S. enterica* serovar Typhimurium (data not shown) and from 3.1 to 0.31 CFU per g of chicken for *E. coli* O157:H7 after 6 to 18 h of enrichment (data not shown). The detection sensitivity in feces

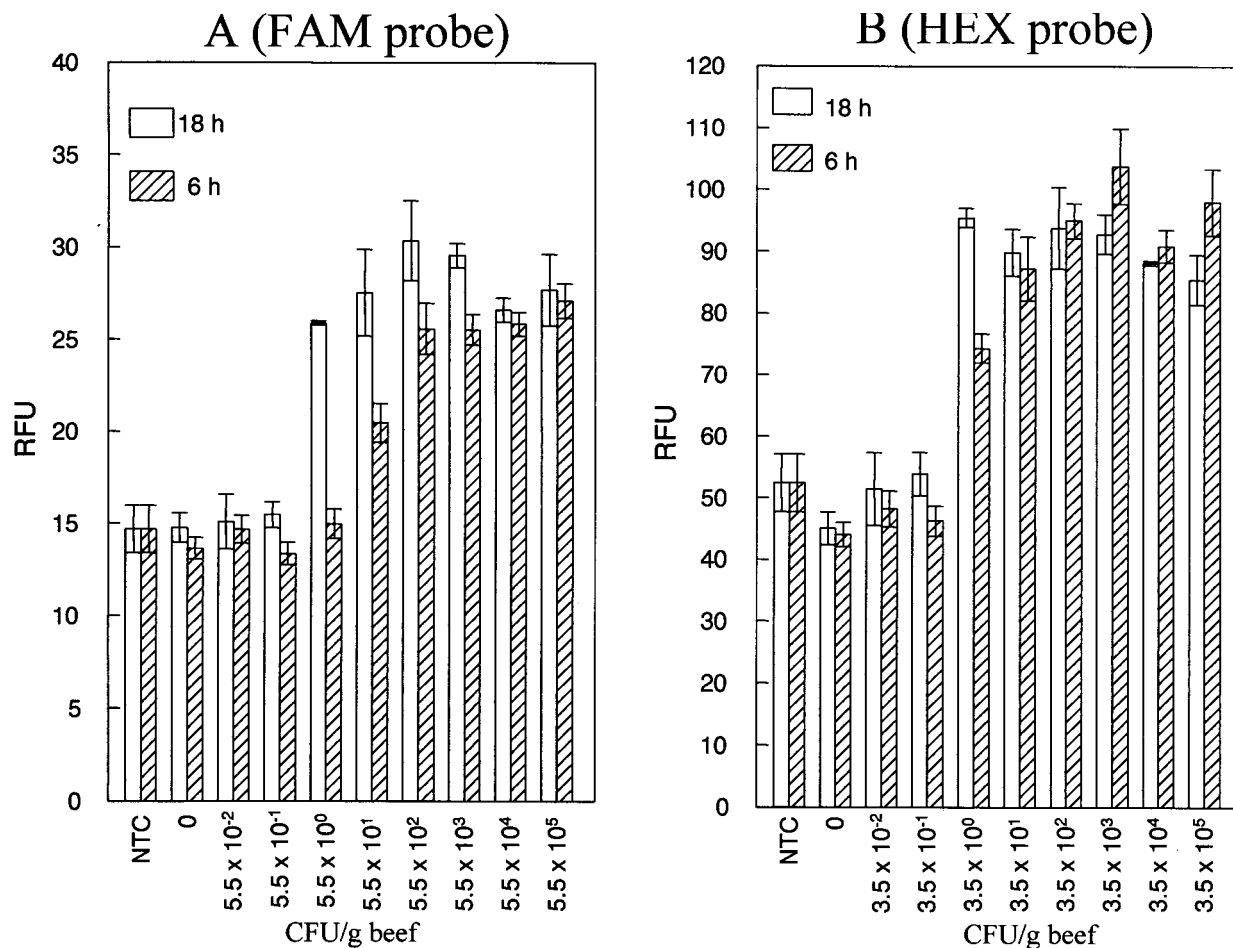


FIG. 3. Sensitivity of fluorogenic PCR in beef. DNA isolated from beef inoculated with various dilutions (plotted on the x axis) of a mixed culture of *Salmonella* and *E. coli* O157:H7 and enriched for 6 to 18 h was subjected to PCR amplification. The RFU of each sample were determined (plotted on the y axis). The DTs for the *Salmonella*-specific FAM-labeled probe and for the *E. coli* O157:H7-specific HEX-labeled probe were 18.4 and 66.0 RFU, respectively. A PCR sample was scored positive if the RFU value was greater than the DT. Error bars indicate the standard deviations of the mean ( $n = 3$ ).

ranged from  $5.8 \times 10^4$  to 5.8 CFU per g of feces for *Salmonella* (data not shown) and from 55 to 5.5 CFU per g of feces for *E. coli* O157:H7 (data not shown) after 6 to 18 h of enrichment. Thus, by using a single enrichment broth, the fluorogenic PCR assay could detect between 1 and 10 CFU of *Salmonella* and *E. coli* O157:H7 after 18 h enrichment of meat and feces artificially inoculated with these two pathogens. Neither strain interfered with the detection of the other strain in enriched meat or fecal samples. The presence of endogenous bacterial flora (determined as total aerobic plate counts) to the level of  $10^6$  CFU per g of beef and  $10^5$  CFU per g of feces has no effect on the detection sensitivity of this assay. This observation is in agreement with a previously reported study (11) showing that endogenous bacteria when present at or below  $10^8$  CFU per g of beef or feces do not interfere with detection of low numbers of target bacterial cells using our PCR assay. The lower detection sensitivity observed for *Salmonella* in feces enriched for only 6 h is probably due to the slow growth of *Salmonella* in these types of samples. However, once the samples (meat or feces) were enriched for 18 h, the detection sensitivity for *Salmonella* reached the same limits as for *E. coli* O157:H7. The detection sensitivity obtained in our assay is better than or comparable to those of protocols that rely on laborious and time-consuming methods to prepare DNA from meat and fecal samples (8, 10, 13). Immunomagnetic capture of target bacte-

rial cells, which is employed in some of these methods to increase detection sensitivity, is not required in our assay to detect very low levels of contamination.

In conclusion, the fluorogenic PCR assay described in the present study provides a highly sensitive and specific closed-tube system for simultaneous detection of pathogenic *Salmonella* and *E. coli* O157:H7 in meats and feces. In addition, the automated PCR amplification and detection of target gene amplicons are conducive for screening large number of samples in a single assay. This method, therefore, should be a significant tool in monitoring foods of animal origin and the environment where these animals are produced and processed into foods for the presence of potential human pathogens. The enrichment, sample preparation, DNA isolation, PCR amplification, and fluorogenic detection procedures described here can also be used for detection of other foodborne pathogens by combining appropriate primers and fluorogenic probes. The availability of additional reporter dyes and detection systems capable of detecting fluorescent signals from several reporters present in a single PCR tube will allow simultaneous detection of several pathogens in a single PCR assay.

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