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PCR Detection of Enterohemorrhagic *Escherichia coli* O145 in Food by Targeting Genes in the *E. coli* O145 O-Antigen Gene Cluster and the Shiga Toxin 1 and Shiga Toxin 2 Genes

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Abstract

Shiga toxin–producing *Escherichia coli* (STEC) strains belonging to serogroup O145 are an important cause of hemorrhagic colitis and hemolytic uremic syndrome worldwide. Cattle and other animals are potential reservoirs for this pathogen. To develop PCR assays for detection and identification of *E. coli* O145, the *wzx* (O-antigen flippase) and *wzy* (O-antigen polymerase) genes in the O145 O-antigen gene cluster that are specific for this serogroup were selected as targets. Oligonucleotide primers complementary to regions in the *E. coli* O145 *wzx* and *wzy* genes were designed to perform PCR assays with DNA from strains of *E. coli* O145, non–O145 *E. coli* serogroups, and other bacterial genera. The assays were highly specific for *E. coli* O145. A multiplex PCR assay targeting the *E. coli* O145 *wzx* and *wzy* genes and the Shiga toxin 1 (stx_1) and Shiga toxin 2 (stx_2) genes and a real-time multiplex PCR assay targeting the O145 *wzy*, stx_1, and stx_2 genes were developed for detection of STEC O145. The assays were used for detecting STEC O145 in seeded ground beef, lettuce, and raw milk initially inoculated with ca. 2, 20, or 200 CFU/25 g or 25 mL after 8 or 20 h of enrichment at 42°C in modified EC broth containing 20 mg/L of novobiocin. STEC O145 was detected in all samples inoculated with 2 CFU/25 g or 25 mL. The detection limit of the multiplex PCR assays was $\leq 7.9 \times 10^4$ CFU/mL, which corresponded to $\leq$400 CFU/PCR reaction. The PCR assays can be employed to identify enterohemorrhagic *E. coli* serogroup O145 and to detect low levels of the pathogen in food.

Introduction

It is estimated that enterohemorrhagic *Escherichia coli* (EHEC) O157:H7, which is also referred to as a Shiga toxin–producing *E. coli* (STEC) due to the production of Shiga toxins, causes greater than 73,000 cases of illness and 61 deaths each year in the United States. The term "EHEC" refers to STEC serotypes that share the same clinical, pathogenic, and epidemiologic features with *E. coli* O157:H7. Non–O157 EHEC, belonging to serogroups O26, O111, O145, and others, have become an important public health problem, and in the United States, they cause an estimated 37,000 cases of illness and 30 deaths each year (Mead *et al.*, 1999; Tozzi *et al.*, 2003; Sonntag *et al.*, 2004). Since 2000 in the United States, non–O157 STEC infections became notifiable to the National Notifiable Diseases Surveillance System. Increases in the incidence of disease caused by non–O157 EHEC may be due to an increased awareness of their role in human illness and because laboratories are making greater efforts to seek out these pathogens in clinical specimens and in food and environmental samples.

EHEC serogroup O145 strains, including Shiga toxin–producing strains of serotypes O145:NM, O145:H–, O145:H8, O145:H16, O145:H25, and O145:H28, have been associated

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with cases of bloody and nonbloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) worldwide (Piérard *et al.*, 1997; Beutin *et al.*, 1998; Eklund *et al.*, 2001; Friedrich *et al.*, 2002; Gerber *et al.*, 2002; Tozzi *et al.*, 2003; Blanco *et al.*, 2004; Sonntag *et al.*, 2004). Strains of STEC O145 isolated from patients with sporadic illness ranked among the top six non-O157 serogroups submitted to the CDC by 43 state public health laboratories between 1983 and 2002, isolated from patients with sporadic illness (Brooks *et al.*, 2005). A case of bloody diarrhea caused by *E. coli* O145 that led to HUS caused the death of a 22-month-old child in Slovenia (Kraigher *et al.*, 2005). Ground meat eaten a few days before the child became ill was thought to be the most likely cause of the infection. Ice cream contaminated with *E. coli* O145 was the source of an outbreak of severe diarrhea and HUS in Belgium (De Schrijver *et al.*, 2008). *E. coli* O145 isolates from patients, from the ice cream, and from samples collected from the farm where the ice cream was produced and sold were indistinguishable. STEC O145 strains have also been isolated from cattle and other food animals and from companion animals (Padola *et al.*, 2002; Schroeder *et al.*, 2002; Garcia and Fox, 2003; Pearce *et al.*, 2004; Krause *et al.*, 2005).

Unlike *E. coli* O157:H7 strains, which generally do not ferment sorbitol or have β-glucuronidase activity, the non–O157 STEC/EHEC do not have identifiable biochemical markers to facilitate screening for these pathogens. Detection of non–O157 STEC/EHEC requires testing for the presence of the Shiga toxins or for the Shiga toxin genes, which is followed by serotyping by agglutination using antisera raised in rabbits against ca. 180 different O surface polysaccharide antigens (Bettelheim and Beutin, 2003). However, serotyping is time consuming and labor intensive and is often nonspecific, as cross reactions of antisera with multiple O serogroups frequently occur. Further, the typing antisera can only be produced by specialized laboratories that have animal facilities. Genes involved in the synthesis of the O-specific polysaccharide are located in the O-antigen gene cluster between the *galF* and *gnd* genes on the *E. coli* chromosome (Samuel and Reeves, 2003). DNA sequence data of the cluster permit identification of unique genes or sequences that can be used to design serogroup-specific PCR assays. These assays can be employed for detection, as well as typing of *E. coli* as an alternative to serotyping.

Identification of the *E. coli* serogroup is not sufficient to determine if the *E. coli* strain is an EHEC/STEC because both Shiga toxin–positive and Shiga toxin–negative strains belonging to a particular serogroup exist. The production of Shiga toxin(s) or the presence of the Shiga toxin gene(s) and/or other virulence genes must also be determined. Although there have been reports on detection of *E. coli* O145 using PCR-based methods, the incidence of disease caused by EHEC O145 is likely underestimated due to the lack of simple, rapid, and reliable methods available for detection and typing of this pathogen (Jenkins *et al.*, 2003; Perelle *et al.*, 2003). Therefore, the objective of the current study was to design serogroup-specific PCR primers based on genes in the O-antigen gene cluster of *E. coli* O145 and to develop multiplex PCR and real-time PCR assays using primers targeting the O145 O-antigen gene cluster and the Shiga toxin 1 (stx_1) and Shiga toxin 2 (stx_2) genes to facilitate identification of STEC O145 and detection in artificially inoculated food, including ground beef, raw milk, and lettuce.

Materials and Methods

Bacterial strains and growth conditions

E. coli O145:NM 83-75 (Centers for Disease Control and Prevention [Atlanta, GA], isolated from a patient with bloody diarrhea), which harbors the stx_2 but not the stx_1 gene, was used for DNA sequencing and for the food inoculations. Bacteria used to test for specificity of the PCR included 71 strains belonging to *E. coli* serogroup O145, *E. coli* O145:NM 83-75, and *E. coli* O145:H– 94-0491 (Laboratory Centre for Disease Control, Ottawa, Canada) used for the multiplex PCR assays, one or more representative strains from each of the remaining O serogroups isolated from humans, animals, food, and water, including reference standard strains belonging to serogroups O1-O173, but excluding O14, O31, O47, O67, O72, O93, O94, and O122 strains, because these serogroup designations have been canceled, and OX3, OX6, OX7, OX9, OX10, OX13, OX18, OX19, OX21, OX23, OX25, OX28, OX38, and OX43 strains. In addition, strains representative of other bacterial genera, including 1 *Shigella boydii*, 29 *Salmonella* strains of various serotypes, 1 *Yersinia enterocolitica*, 13 *Vibrio* (5 different species), 2 *Serratia marcescens*, 2 *Klebsiella pneumoniae*, 2 *Citrobacter freundii*, 1 *Pseudomonas aeruginosa*, 2 *Proteus vulgaris*, 2 *Staphylococcus aureus*, 3 *Bacillus cereus*, 1 *Bacillus subtilus*, 1 *Hafnia alvei*, 2 *Enterococcus aerogenes*, 1 *Enterococcus faecalis*, 2 *Enterobacter cloacae*, 1 *Lactobacillus lactis*, and 1 *Listeria monocytogenes*, were tested. The bacteria were obtained from the strain collections at the *E. coli* Reference Center at The Pennsylvania State University (University Park, PA), the Microbial Food Safety Research Unit at the Eastern Regional Research Center (Wyndmoor, PA), and from the Laboratory of Food Hygienic Chemistry, Kyushu University (Fukuoka, Japan). The bacteria were routinely grown in tryptic soy broth or Luria-Bertani broth or the respective agars (Becton Dickinson, Sparks, MD).

DNA sequencing and analyses

E. coli O145:NM strain 83-75 was grown for 18 h in Luria-Bertani at 37°C, and sequencing of the O-antigen gene cluster was performed as described previously (Fratamico *et al.*, 2003). The assembled sequences were imported into Artemis, the open reading frames (ORFs) were located, and the putative coding regions were analyzed using the NCBI BlastX program against the nonredundant database (Altschul *et al.*, 1997).

Selection of PCR primers and specificity testing using singleplex and multiplex PCR assays

In a number of studies, PCR assays targeting the *E. coli wzx* and *wzy* genes were found to be serogroup specific; therefore, sequence similarity analyses were performed comparing the *E. coli* O145 *wzx* and *wzy* genes to similar genes in other *E. coli* serogroups. Results demonstrated that these genes were suitable targets for *E. coli* O145–specific PCR assays. Oligonucleotide primers, complementary to the *E. coli* O145:NM 83-75 *wzx* and *wzy* genes (accession no. AY863412), were designed and used in PCR assays to determine their specificity for this serogroup (Table 1). Template DNA from the bacteria was prepared by mixing a colony in sterile distilled water and heating at 100°C for 20 min. The PCR at the *E. coli* Reference Center was performed using a RapidCycler (Idaho Technol-

Table 1. Oligonucleotide Primers and Probes Used for Amplification of the *Escherichia coli* O145 *wzx* and *wzy* Genes, *stx*$_1$ and *stx*$_2$ Genes, and Internal Control

	Sequences 5′ to 3′	*Size of PCR product*
Primers		
O145wzx1	F - ACTGGGATTGGACGTGGATA	222
	R - AGGCAAGCTTTGGAAATGAA	
O145wzy1	F - CTGTTGCTTCAGCCCTTTTC	217
	R - GCAGCCCAATATGAAACCAT	
O145wzx2	F - TTTGGTTTGGTGGTACTGTGTCCG	555
	R - TGTAACGATCTTCTCTCATCGCGT	
O145wzy2	F - ATATTGGGCTGCCACTGATGGGAT	310
	R - TATGGCGTACAATGCACCGCAAAC	
Stx1A	F - CTCGACTGCAAAGACGTATG	199
	R - TCGTTCAACAATAAGCCGTA	
Stx2A	F - ACGATAGACTTTTCGACCCAACAA	140
	R - AAATAACTGCCCGGTGGGGT	
16S rRNA	F - CCTCTTGCCATCGGATGTG	99
	R - GGCTGGTCATCCTCTCAGACC	
Probes		
O145wzy-P	5′ 6FAM-AGCAGTGGTTCGCGCACAGCATGGT 3′BHQ-1	
Stx1A-P	5′-TexasRed-CGCTGAATGTCATTCGCTCTGCA-3′BHQ-2	
Stx2A-P	5′-TexasRed-AACAGACACCGATGTGGTCCCCTGAGAT-3′BHQ-2	
16S rRNA-P	5′-Cy5-GTGGGGTAACGGCTCACCTAGGCGAC-3′BHQ-2	

ogy, Salt Lake City, UT) using 11-μL total reaction volumes. The PCR mixture consisted of 3 μL of template DNA, 0.5 μM of primers (Integrated DNA Technologies, Coralville, IA), 0.18 mM of each of the four dNTPs, 3.0 mM $MgCl_2$, 0.4 U *Taq* DNA polymerase, 50 mM Tris (pH 8.3), BSA 250 μg/mL, 2% sucrose, and 0.1 mM Cresol Red. The PCR assays were performed using primer sets for *wzx* or for *wzy* separately in each of the reactions. The thermal cycling protocol was performed using the rapid cycle DNA amplification method (Wittwer *et al.*, 1994), which consisted of an initial denaturation step at 94°C for 30 sec, followed by 30 cycles of template denaturation at 94°C, primer annealing at 60°C, and extension at 72°C for 12 sec. For testing of the strains using the multiplex PCR, primer sets O145wzx1 and O145wzx2 were used; the annealing temperature was 55°C, and the extension time at 72°C was 20 sec. The PCR products were viewed with ethidium bromide after electrophoresis through 1% agarose gels.

Serogroup-specific PCR assays for detection of E. coli *O145*

Multiplex PCR assays of bacteria in broth cultures were performed targeting the *stx*$_1$, *stx*$_2$, (Shiga toxin A-subunit) *wzx*, and *wzy* genes in a single assay. Primers and probes shown in Table 1 were designed for the current study. The PCR was performed using a Smart Cycler II system (Cepheid, Sunnyvale, CA) with a cycling protocol consisting of an initial denaturation step at 94°C for 120 sec followed by 35 cycles of template denaturation at 94°C for 20 sec, primer annealing at 60°C for 60 sec, and primer extension at 72°C for 60 sec, and a final extension step at 72°C for 600 sec. The PCR mixture (25 μL total reaction volume) consisted of 2.5 μL of template DNA, 0.25 μM of primers, OmniMix HS beads (Cepheid), and a lyophilized predispensed blend of reagents containing 0.2 mM of each of the four dNTPs, 4.0 mM $MgCl_2$, 1.5 U TaKaRa hot start *Taq* DNA polymerase, 25 mM HEPES (pH 8.0), and rehydrated as instructed by the manufacturer. The PCR products were viewed with ethidium bromide after electrophoresis through 1.5% agarose gels.

Detection of E. coli *O145 in food using conventional and real-time multiplex PCR*

E. coli O145:NM 83-75 was grown for 18 h in tryptic soy broth (Becton Dickinson) at 37°C. Dilutions were made in 0.1% peptone, and 25-g or 25-mL samples of ground beef and lettuce (obtained from local supermarkets) and raw milk (obtained from a local agricultural high school) were added to 225 mL of modified *E. coli* broth (Becton Dickinson) containing novobiocin (20 mg/L) (mEC+n), and then inoculated with ca. 2, 20, and 200 CFU of *E. coli* O145:NM 83-75 per 25 g or 25 mL. Enumeration of CFU/mL was performed by plating dilutions onto tryptic soy agar and incubating at 37°C for 18 h. The inoculated food samples were incubated at 42°C at 150 rpm for 20 h. DNA was extracted from 1 mL of the enrichments using the PrepMan Ultra reagent (Applied Biosystems, Foster City, CA) as instructed by the manufacturer. The multiplex PCR assay targeting *wzx*, *wzy*, *stx*$_1$, and *stx*$_2$ genes was performed as described above.

A TaqMan-based real-time multiplex PCR assay targeting the O145 *wzy*, *stx*$_1$, and *stx*$_2$ genes was also performed using DNA extracted from ground beef samples inoculated with 2 and 20 CFU of *E. coli* O145:H– 94-0491 (carries *stx*$_1$ and *stx*$_2$) per 25 g and subjected to enrichment as described above. Each real-time multiplex PCR assay also included an internal control designed based on 16S rRNA gene sequences found in GenBank and yielding a 99-bp product (Fratamico *et al.*, 2008). The sequences of the primers and probe targeting the internal control and the other target genes are shown in Table 1. The PCR mixture (25-μL total reaction volume) consisted of 2.5 μL of template DNA, 0.25 μM and 0.625 μM of the primers and probes, respectively, and OmniMix HS beads. The PCR was performed using a Smart Cycler II system (Cepheid) with a cycling protocol consisting of an initial denaturation step at

94°C for 120 sec followed by 35 cycles of template denaturation at 94°C for 20 sec, primer annealing at 60°C for 60 sec, and primer extension at 72°C for 60 sec, and a final extension step at 72°C for 600 sec.

The detection limit of the multiplex PCR assay was determined by adding 100 μL of dilutions of an overnight culture of *E. coli* O145:H– 94-0941 to 900 μL aliquots of an uninoculated 8-hr ground beef enrichment for final concentrations ranging from 10^7 to 10^1 CFU/mL. The DNA was extracted using the PrepMan Ultra reagent as described above, and 2.5 or 1 μL of template DNA were used in the multiplex PCR assays. A standard curve was generated, and the limit of detection of the assay was determined as an average of the minimum number of CFU per PCR reaction required for a positive cycle threshold value over a specified background threshold (set at 15) for all three fluorescence signals.

Results and Discussion

Determination of E. coli O145–specific sequences

Feng *et al.* (2005) published the sequence of the O-antigen gene cluster of an *E. coli* O145 strain (type strain G1100; accession no. AY647260). The O145 O-antigen gene cluster had also been sequenced in our laboratory (O145:NM strain 83-75; accession no. AY863412) before the report by Feng and coworkers was published, and a comparison of the sequence data showed that there were several differences between the sequences of the O-antigen cluster of the two O145 strains. There are four point mutations located in the *nnaC* (ORF 3), *wzy* (ORF 6), and *wbeD* (ORF 9) genes. Although these point mutations do not create any stop codons, three of the point mutations did cause changes in the amino acids in the ORFs. A cytosine-to-thymine change in the *nnaC* gene resulted in an amino acid change from Leu (AY647260) to Ser (AY863412). A cytosine-to-adenine change in the *wzy* gene (nucleotide 5651) caused an amino acid change from Ile (AY647260) to Leu (AY863412), and a thymine-to-cytosine change within the *wbeD* gene (nucleotide 9317) resulted in a change in the amino acid from Pro (AY647260) to Leu (AY863412). An additional base difference within the *wbeD* gene (nucleotide 9277) was a silent mutation, and did not cause an amino acid change.

Several genes in the clusters, including the *wzx* (O-antigen flippase) and *wzy* (O-antigen polymerase) genes, show relatively low similarity among different *E. coli* serogroups, and PCR primers targeting these genes have been used to develop serogroup-specific PCR assays (D'Souza *et al.*, 2002; Wang *et al.*, 2002; Fratamico *et al.*, 2003; Beutin *et al.*, 2005). In the current study, the DNA sequence of the O-antigen gene cluster of *E. coli* O145:NM strain 83-75 was determined, and PCR-based methods for detection and identification of EHEC serogroup O145 were developed. Analysis of the ca. 15,631-bp region that was sequenced from the JUMPstart region to *gnd* of this strain showed that it contained 15 complete ORFs, with all having the same transcriptional direction (GenBank accession no. AY863412). The *wzx* (O-antigen flippase) and *wzy* (O-antigen polymerase) genes were predicted to encode integral membrane proteins with 12 and 11 transmembrane domains, respectively (Tusnády and Simon, 2001). PCR assays were developed targeting the *E. coli* O145 *wzx* and *wzy* genes, and the sequences of the primers are shown in Table 1. The structure of the *E. coli* O145 O–specific polysaccharide has not yet been reported.

Specificity testing

Primer sets O145wzx1 and O145wzy1 were used for the singleplex PCR assays, and O145wzx2 and O145wzy2 were combined with primer sets Stx1 and Stx2 in a multiplex PCR for detection of STEC O145. All of the *E. coli* O145 strains tested (71/71) were positive by the PCR showing amplicons of the expected sizes using primer sets O145wzx1 and O145wzy1, and 15/15 *E. coli* O145 strains were positive with primer sets O145wxz2 and O145wzy2 using a multiplex PCR assay. No PCR products of the expected sizes were observed using O145wzx1 and O145wzy1 in PCR assays using DNA from non–O145 *E. coli* strains and with non–*E. coli* bacteria (Table 2). Several weak nonspecific products were obtained with DNA from 5 *Salmonella* serotypes tested and from several strains of *Vibrio cholerae* and *Vibrio parahaemolyticus* using the multiplex PCR. However, the PCR did not produce amplicons of the expected sizes for the *E. coli* O145 *wzx* and *wzy* genes.

Detection of E. coli O145 in food by multiplex PCR

For over two decades, STEC strains belonging to serogroup O145 have been associated with sporadic cases and outbreaks of HC and HUS worldwide (Karmali *et al.*, 1985; Brooks *et al.*, 2005; Bettelheim, 2007; http://www.microbionet.com.au/vtectable.htm). Cattle are an important reservoir for STEC O145, and food of bovine origin has been linked to disease in humans (Hussein and Sakuma, 2004; Bettelheim, 2007; De Schrijver *et al.*, 2008). Therefore, rapid, sensitive, and reliable methods are needed to detect STEC O145 in different types of food samples.

In the current study, the multiplex PCR correctly identified strains of *E. coli* O145 that did not possess the stx_1 or stx_2 genes or that possessed only one or both toxin genes (Fig. 1).

TABLE 2. SPECIFICITY OF *ESCHERICHIA COLI* O145 SEROGROUP–SPECIFIC PCR ASSAYS

Bacteria tested (no. of strains)	*Primers*	*PCR results*
	O145wzx1 and O145wzy1	
E. coli O145 (71)		All strains positive
Non–O145 *E. coli* (64)		All strains negative
Non–*E. coli* (68)		All strains negative
	O145wzx2 and O145wzy2 multiplex PCR	
E. coli O145 (15)		All strains positive
Non–O145 *E. coli* (37)		All strains negative
Non–*E. coli* (68)		All strains negative

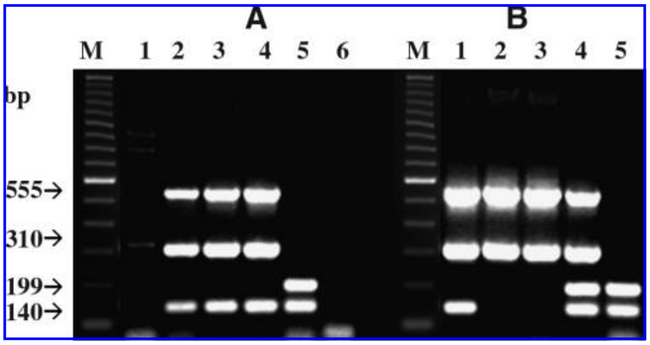

FIG. 1. Agarose gel showing results of a multiplex PCR assay using primer sets shown in Table 1 for amplification of the *Escherichia coli* O145 *wzx* (555 bp) and *wzy* (310 bp) genes and the stx_1 (199 bp) and stx_2 (140 bp) genes. (**A**) Agarose gel of multiplex PCR results using DNA extracted from uninoculated control (lane 1), from 20-h enrichments of ground beef seeded with ca. 2 (lane 2), 20 (lane 3), and 200 (lane 4) CFU of *E. coli* O145:NM strain 83–75 per 25 g, and from *E. coli* O157:H7 380-94 (possesses stx_1 and stx_2); no template control (lane 6). (**B**) Lane 1, *E. coli* O145:NM 83-75 (possesses stx_2); lane 2, *E. coli* O145:NM 88.0963 (*stx* negative); lane 3, *E. coli* O145:NM 87.1009 (*stx* negative); lane 4, *E. coli* O145:H– 94-0491 (possesses stx_1 and stx_2); lane 5, *E. coli* O157:H7 380-94; M, 100 bp DNA markers.

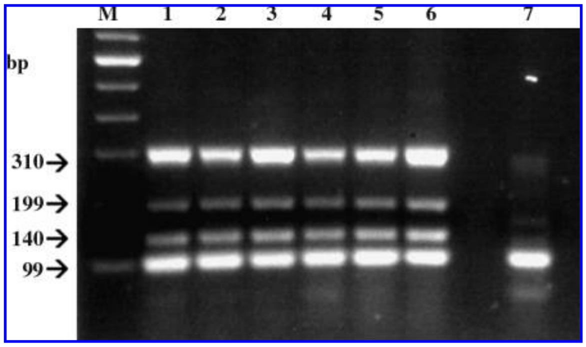

FIG. 2. Agarose gel showing results of a real-time multiplex PCR assay of DNA from ground beef samples inoculated with *Escherichia coli* O145:H– 94-0491 (carries stx_1 and stx_2) at 2 and 20 CFU and subjected to enrichment for 8 h (lanes 1–3) and 20 h (lanes 4–6). Lane 7 shows results of an uninoculated control ground beef sample; only the internal control band is visible. Lane M, 100-bp DNA markers. PCR products: 310 bp, O145 *wzy*; 199 bp, stx_1; 140 bp, stx_2; 99 bp, rRNA internal control.

Products of 555, 310, 199, and 140 bp were obtained for the O145 *wzx*, O145 *wzy*, stx_1, and stx_2 genes, respectively. *E. coli* O145:NM strain 83-75 and O145:H– 94-0941 were detected in ground beef, lettuce, and raw milk samples inoculated with 2, 20, and 200 CFU/25 g or 25 mL after 20 h of enrichment in mEC+n using the multiplex PCR assay (data not shown). The PCR results of ground beef samples that had been inoculated with *E. coli* O145:NM 83-75 (carries stx_2) are shown in Fig. 1.

Detection of E. coli *O145 in food by real-time multiplex PCR*

Methods have been described for detection of *E. coli* O145 and other non–O157 STEC based on immunomagnetic separation followed by PCR/DNA probe techniques or by real-time PCR (Jenkins *et al.*, 2003; Perelle *et al.*, 2003, 2007). However, these methods did not detect STEC O145 in food targeting both serogroup O145-specific sequences and the Shiga toxin genes in a single assay as was performed in the current study. *E. coli* O145:H– 94-0491 was detected by real-time PCR in ground beef samples inoculated with 2 and 20 CFU/25 g after 8 and 20 h of enrichment at 42°C in mEC+n. It is possible, however, that the PCR assay would be less sensitive if the bacteria had been exposed to stress, such as temperature or acid stress, and longer enrichments would be required. Analysis of the assay by gel electrophoresis showed that there were four PCR products of the expected sizes for stx_1, stx_2, O145wzy, and the rRNA internal control (Fig. 2). Testing of uninoculated ground beef enrichments to which dilutions of *E. coli* O145:H– were added showed a detection sensitivity of $\leq 7.9 \times 10^4$ CFU/mL using either 2.5 or 1 μL of template DNA per real-time PCR reaction. Therefore, because bacteria in 1 mL of enrichment were resuspended in 200 μL of PrepMan reagent and 1 μL was subsequently used in the PCR, the detection limit per PCR reaction was $\leq$400 CFU. Further optimization of the multiplex PCR assay may improve the sensitivity. These results are in agreement with those of Hsu *et al.* (2005), who reported a limit of detection of 10^5 CFU/g of ground beef for *E. coli* O157:H7 by real-time PCR. The detection limit for *E. coli* O157:H7 in ground beef using a multiplex PCR assay for simultaneous detection of *E. coli* O157:H7, *Salmonella*, and *Shigella* was 10^5 CFU/g, while it was 10^3 CFU/g for *Salmonella* Typhimurium and 10^4 CFU/g for *Shigella flexneri* (Wang *et al.*, 2007).

In summary, there is a need for rapid, sensitive, and specific methods to detect important non–O157 STEC serogroups in food. The conventional and real-time multiplex PCR assays described in the current study reliably and specifically identify STEC O145 and can be used to detect this pathogen in various types of foods and potentially also in human clinical specimens, animal fecal samples, and environmental samples. Methods for isolation of STEC O145 from food enrichments that are positive for the *E. coli* O145 O-antigen gene cluster and *stx* target genes are currently being developed in our laboratory. Isolated STEC O145 strains from food enrichments can then be further tested for additional virulence genes, including *eae* (intimin protein) and *hly* (hemolysin).

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Disclosure Statement

No competing financial interests exist.

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