Applicability of a multiplex PCR to detect O26, O45, O103, O111, O121, O145, and O157 serogroups of Escherichia coli in cattle feces

Zachary Paddock, Xiaorong Shi, Jianfa Bai, and T. G. Nagaraja

Published Version Information

Citation: Paddock, Z., Shi, X., Bai, J., & Nagaraja, T. G. (2012). Applicability of a multiplex PCR to detect O26, O45, O103, O111, O121, O145, and O157 serogroups of Escherichia coli in cattle feces. Veterinary Microbiology, 156(3-4), 381-388.

Digital Object Identifier (DOI): doi: 10.1016/j.vetmic.2011.11.017

Publisher's Link: http://www.sciencedirect.com/science/journal/03781135/156/3-4

Applicability of a multiplex PCR to detect O26, O45, O103, O111, O121, O145, and O157 serogroups of *Escherichia coli* in cattle feces[1]

Zachary Paddock, Xiaorong Shi, Jianfa Bai, and T.G. Nagaraja*

Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University, 1800 Denison Avenue, Manhattan, KS 66506-5606, USA

Key Words: Shiga toxin-producing *E. coli*, Multiplex PCR, Cattle, Feces

[1] Contribution no. 11-344-J from the Kansas Agricultural Experimental Station, Manhattan.

Corresponding author. Tel.: +1 785 532 1214; fax: +1 785 532 4851.

E-mail address: tnagaraj@vet.k-state.edu (T.G. Nagaraja).

Abstract

Shiga toxin-producing *Escherichia coli* (STEC), particularly O157, are major food borne pathogens. Non-O157 STEC, particularly O26, O45, O103, O111, O121, and O145, have also been recognized as a major public health concern. Unlike O157, detection procedures for non-O157 have not been fully developed. Our objective was to develop a multiplex PCR to distinguish O157 and the 'top six' non-O157 serogroups (O26, O45, O103, O111, O121, and O145) and evaluate the applicability of the multiplex PCR to detect the seven serogroups of *E. coli* in cattle feces. Published sequences of O-specific antigen coding genes, *rfbE* (O157) and *wzx* and *wbqE-F* (non-O157), were analyzed to design serogroup-specific primers. The specificity of amplifications was confirmed with 138 known STEC strains and the reaction yielded the expected amplicons for each serogroup. In feces spiked with pooled 7 STEC strains, the sensitivity of the detection was 4.1×10^5 CFU/g before enrichment and 2.3×10^2 after 6 h enrichment in *Escherichia coli* broth. Additionally, 216 fecal samples from cattle were collected and tested by multiplex PCR and cultural methods. The multiplex PCR revealed a high prevalence of all seven serogroups (178 [O26], 108 [O45], 149 [O103], 30 [O111], 103 [O121], 5 [O145], and 160 [O157]) of 216 samples) in fecal samples. Cultural procedures identified 33.1% (53/160) and 35.5 % (11/31) of PCR-positive samples for *E. coli* O157 and non-O157 serogroups, respectively. Samples that were culture-positive were all positive by the multiplex PCR. The multiplex PCR can be used to identify serogroups of putative STEC isolates.

1. Introduction

Shiga toxin-producing *E. coli* (STEC), particularly O157:H7, are major food borne pathogens. Non-O157 serogroups, belonging to six O groups, O26, O111, O103, O121, O45, and O145, have also been recognized as a growing public health concern (Brooks et al., 2005). Cattle are believed to be the major reservoir of many of the non-O157 STEC (Karmali et al., 2010). Much of the data on prevalence of non-O157 STEC in cattle is based on detection of the serogroups in carcass samples (Arthur et al., 2002; Barkocy-Gallagher et al., 2003; Bosilevac et al., 2007, Bosilevac and Koohmaraie, 2011). A limited number of studies have reported on prevalence of a number of non-O157 STEC serogroups in cattle feces (Blanco et al., 1997; Jenkins et al., 2003; Renter et al., 2005). The limited information on fecal prevalence is because methods for isolation and detection of non-O157 STEC in feces have not been fully developed (Hussein and Bollinger, 2008). The IMS beads are commercially available only for the O26, O103, O111, O145, and O157 O-groups. A selective medium to phenotypically distinguish non-O157 STEC serogroups has not been developed. Detection of the putative non-O157 serogroups are performed by latex agglutination with O-group specific antiserum coupled with detection of Shiga toxins or *stx* genes (Bettelheim, 2007).

Multiplex PCR (mPCR) targeting different non-O157 serogroups has been developed. . Recently, DebRoy et al. (2011) have reported on a mPCR to detect 8 serogroups of *E. coli* in pure cultures (O26, O45, O103, O111, O113, O121, O145 and O157) by targeting the *wzx* (O-antigen flippase) genes of all O-antigen gene clusters. However, applicability of the mPCR for detecting the serogroups in fecal samples has not been evaluated; therefore, our objectives were to develop a mPCR that can distinguish the O157 and the 'top six' non-O157 serogroup (O26, O45, O103, O111, O121, and O145) recognized by the Centers for Disease Control and

Prevention, which can be used to identify and confirm presumptive isolates of *E. coli.* Also, we evaluated the applicability of the method to screen fecal samples for the presence of the seven O-serogroups before subjecting the samples to cultural procedures for detection and isolation.

2. Materials and Methods

2.1. Gene target selection and primer design.

The gene sequences that code for O-antigen regions of the six major non-O157 STEC serogroups were downloaded from the NCBI, and analyzed to design serotype-specific primers (Table 1). The *wzx,* which encodes for a flippase required for O-polysaccharide export (Liu et al., 1996), was used to design primers (Monday et al., 2007) for serogroups O26, O45, O103, O111, and O145 with resulting amplicons ranging from 200 to 900 bps. The *wbq*E gene, which encodes for a putative glycosyl transferase, and *wbq*F, which encodes for a putative acetyl transferase, were used to design primers for O121. The primer set for *rfbE*, described in Bai et al. (2010), was included for the O157 serotype identification.

2.2. PCR conditions

The mPCR conditions used were similar to that of Bai et al. (2010). Briefly, a reaction volume of 20 µl was used with 1 µl of DNA template and final concentrations of 0.36 µM of each primer (1 µl of the mixture of equal amount of the100 µM primer stocks), 10 µl iQ Multiplex Powermix (Bio-Rad, Hercules, CA). The PCR program was: 94°C denaturation for 5 min, 25 cycles for pure culture DNA or 35 cycles for fecal DNA, 94°C denaturation for 30 sec, and 67°C annealing and extension for 80 sec. The final step was a 68°C extension for 7

min. The amplified DNA was separated on 1.2% agarose gel and stained with 0.5 µg/ml of ethidium bromide. The DNA bands were visualized and documented with a Bio-Rad GelDoc 2000 Fluorescent Imaging System.

2.3. Validation with pure cultures

The specificities of primer sets were validated, initially, with one strain of each STEC serotype individually and subsequently with individual primer set with a pooled mixture of the seven STEC serotypes. The strains used were TW O1597 (O26), 2566:58 (O45) (The Thomas S. Whittam Microbial Evolution Laboratory, Michigan State University), 15612-1 (O103), 4190 (O121), 7726-1 (O111), 1234-1 (O145) (Renter et al., 2005), and ATCC 43894 (O157; American Type Culture Collection, Manassas, VA). The strains, stored on protect beads at -80°C, were streaked on blood agar plates (Remel, Lenexa, KS), and incubated overnight at 37°C. One or two colonies of each isolate were suspended in 1 ml of distilled water and boiled for 10 min. After centrifugation of the boiled suspension, 1 µl of the supernatant was used as DNA template in PCR reactions. PCR products from a single primer set reaction of each STEC were purified by GeneClean Turbo kit (MP Biomedicals, Irvine, CA) and eluted DNA samples were submitted to the Kansas State University DNA Sequencing and Genotyping Facility for nucleotide sequencing. The sequences of each STEC were then compared to the known DNA sequences from the NCBI database.

After validating the specificity of the primer set and standardizing the PCR conditions, an additional 138 strains of seven STEC serotypes (18 [O26]; 3 [O45]; 23 [O103]; 28 [O111]; 9 [O121]; 13 [O145]; 44 [O157]) were tested. These strains were from our culture collection (86 strains; Renter et al., 2005), Michigan State University (46 strains; The Thomas S. Whittam

Microbial Evolution Laboratory), Pennsylvania State University (2 strains; *Escherichia coli* Reference Center), and ATCC (4 strains). In each mPCR run, pooled culture of the seven strains listed above was used as a positive control.

2.4. Fecal samples spiked with STEC strains

Freshly defecated feedlot or dairy cattle fecal samples were inoculated with different concentrations of pooled cultures of seven STEC strains to determine the applicability of the mPCR for detecting the seven serogroups in feces, and the minimal concentration of cells needed in the feces for detection. The STEC strains were streaked on blood agar plates and incubated overnight at 37°C. A single colony was inoculated into 10 mL of Luria-Bertani (LB; Becton Dickinson Co., Sparks, MD) broth and incubated at 37°C overnight, then 100 µl was transferred into 10 ml of LB broth and incubated at 37°C to an absorbance of 0.6 (~5 h) at 600 nm. One milliliter of each strain was then pooled and subjected to serial 10-fold dilutions. Cell concentrations of the pooled cultures were determined by spread-plate count on MacConkey agar. One milliliter of each dilution (10^{-1} to 10^{-6}) was thoroughly mixed with 10 g fecal sample with sterile wooden sticks, and 0.2 g of each spiked fecal sample was placed in 2-ml microcentrifuge tubes for DNA extraction with a QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencia, CA). One gram of fecal sample spiked with different concentrations of pooled STEC was enriched in different enrichment broths (described in the next section). Then 1 ml of each spiked fecal sample was boiled for 10 min and centrifuged (9,390 x g for 5 min), then the supernatant was subjected to either QIAquick PCR Purification Kit (Qiagen Inc.) or GeneClean Turbo (MP Biomedicals, Irvine, CA). This sensitivity experiment was repeated three times, each with a different fecal sample from dairy or feedlot cattle.

135

136 *2.5. Evaluation of enrichment procedures of spiked fecal samples*

137 The following enrichment broths and incubation conditions were evaluated for fecal
138 samples spiked with different concentrations of pooled cultures of seven STEC: *Escherichia*
139 *coli* broth (EC; Oxoid Ltd., Hampshire, England) incubated at 40°C for 6 or 24 h (Lahti et al.,
140 2003) or Tryptic Soy broth (TSB; Becton Dickinson Co., Sparks, MD) with 1.5 g/L of bile
141 salts (Sigma-Aldrich, St. Louis, MO), 2.0 mg/L of rifampicin, 16.0 mg/L of vancomycin, and
142 either 0 (TSB_{BRV}) or 8.0 mg/L of novobiocin (TSB_{BRVN}) incubated at 42°C for 24 h (Possé et
143 al., 2008b; Vimont et al., 2007b). All enrichment procedures utilized 9 mL of the broth with 1
144 g of feces spiked with different concentrations (10^6 to 10^0 CFU/ml). One milliliter of each
145 enriched fecal sample was boiled for 10 min and centrifuged, and then the supernatant was
146 subjected to either QIAquick PCR Purification Kit (Qiagen In.) or GeneClean Turbo Kit (MP
147 Biochemicals). The lowest concentration of the enriched sample in which all seven
148 serogroup's amplicons were visualized on agarose gel was considered the detection limit of the
149 procedure.

150

151 *2.6. Evaluation of mPCR with fecal samples*

152 Cattle fecal samples (n=216; 108 samples from feedlot cattle and 108 from dairy cows)
153 were collected from pen floors using plastic spoons with care taken to avoid ground
154 contamination. The spoons with feces were placed in Whirl-pack bags (Nasco, Ft. Atkinson,
155 WI), and transported in a cooler with ice packs to the Pre-harvest Food Safety Laboratory for
156 processing. Samples were kneaded for 30 sec and approximately 1 g of feces was placed in
157 tubes containing 9 ml EC broth and incubated for 6 h at 40°C, which was determined to be the

optimal enrichment procedure during the sensitivity experiment. All enriched samples were subjected to mPCR for the seven STEC and cultured to detect O157. Initially, a subset of 24 samples (12 from feedlot and 12 from dairy cattle) were subjected to mPCR before and after enrichment and only enriched samples were cultured to detect the six serogroups in order to compare results of mPCR with that of culture-based methods. One milliliter of the fecal suspension before or after sample was boiled and DNA was extracted. In addition, immunomagnetic bead separations (except O45 and O121) of enriched samples were performed, followed by plating onto MacConkey agar (MAC) for non-O157 and sorbitol MacConkey agar (SMAC) with cefixime (0.5 mg/L) and potassium tellurite (2.5 mg/L; CT-SMAC) for O157 isolations and detections. Plates were incubated overnight at 37°C, and sorbitol negative colonies on CT-SMAC or random lactose positive colonies on MAC (at least 6 and up to 15 colonies) were streaked on to blood agar plates for identification of *E. coli* serogroup. Isolated colonies from blood agar were placed in microcentrifuge tubes with 1 mL of ddH_2O, boiled for 10 min, and centrifuged at 9,390 x g for 5 min. One microliter of the resulting DNA was then subjected to the mPCR. Additionally, the serogroup positive isolates were tested by mPCR to identify the major virulence genes, *eae* (intimin), *stx*1 (Shiga toxin 1), *stx*2 (Shiga toxin 2), and *hlyA* (hemolysin), and genes that code for O157 serogroup (*rfbE*) and H7 flagellar antigen (*fliC*) (Bai et al., 2010).

3. Results

3.1. Validation with pure cultures

The designed primers and optimized mPCR reaction conditions resulted in amplicons of 241, 296, 371, 451, 587, 716, and 890 bps for serogroups O26, O157, O145, O111, O121,

O103, and O45, respectively. The correct and specific amplifications, with no cross-amplification, were obtained with individual serogroups and the mixture of seven STEC strains (Fig. 1). Nucleotide sequences of the excised amplicons showed 99 % (O26, O45, O103, O111, O121, O145 and O157) homology to published sequences. The specificity of amplifications was tested with 138 STEC strains of known serogroups and with each serogroup the reaction yielded the expected amplicon size. When a pooled culture of seven serogroups was serially diluted (ten-fold dilutions) and subjected to mPCR amplification, the lowest concentration at which all seven bands were detectable was 10^4 CFU/ml or 10 CFU per PCR reaction (Fig. 2).

3.2. Fecal sample spiked with STEC strains

The lowest concentration of the pooled STEC culture in a spiked sample that yielded distinct bands was 4.1×10^5 ($\pm 1.8 \times 10^5$) CFU/g (Fig. 3). The two DNA purification kits, Qiagen and GeneClean Turbo, exhibited no difference in detection sensitivity (data not shown).

3.3. Evaluation of enrichment procedures of spiked fecal samples

The EC broth incubated at 40°C for 6 h showed a 1 to 2 log greater sensitivity than EC incubated at 40°C, and TSB_{BRV} or TSB_{BRVN} incubated at 42°C for 24 h (Table 2). Subsequently, all fecal samples in this study were enriched by EC broth at 40°C for 6 h at which the lowest concentration of pooled culture that yielded distinct bands of all seven STEC was 2.3×10^2 ($\pm 2.1 \times 10^2$) CFU/g of fecal sample (Table 2; Fig. 4).

3.4. Evaluation of the assay with fecal samples

Fresh bovine fecal samples from pen floors (216 total; 108 from feedlot cattle and 108 from dairy cows) were collected, enriched in EC broth, and subjected to the seven serogroup mPCR (Table 3). Fecal samples suspended in EC broth and subjected to mPCR before enrichment (n=24) showed no bands for any of the seven serogroups. After enrichment at 40° C for 6 h (n=216), prevalence levels ranged from 2.3 % (5 out of 216) of samples positive for O45 to 82 % (178 out of 216) of samples positive for O26. Interestingly, only a small number of samples (5 out of 216; 2.3%) were negative for any of the seven serogroups. The mPCR identified 58.3% (63/108) and 89.8% (97/108) of dairy and feedlot cattle fecal samples as positive for O157, respectively. However, based on culture-based detection method, only 15.7% (17/108) of dairy and 33.3% (36/108) feedlot fecal samples were positive for the O157 serogroup. Of the initial subset of samples (n=24) that were cultured for non-O157 serogroups, only O26 (5/24), O103 (4/24) and O121 (2/24) were isolated and 9 out of 24 were culture positive for at least one non-O157 serogroup. All samples that were culture-positive for the seven serogroup were also positive by mPCR; however, the mPCR identified many more positive samples. Majority of O157 isolates was positive for *stx*2, *eae*, *fli*C, and *hly*A genes. One isolate was positive for *stx*1 and two isolates were positive for *rfb*E and negative for the major virulence genes (Table 4). Of the 11 non-O157 serogroup isolates, one O26 strain was positive for *stx*1, one O103 was positive for *hly*A, one O121 was positive for *fli*C and none was positive for *stx*2 gene (Table 4).

4. Discussion

At least 100 serotypes of STEC are associated with human infections and cause illnesses that range from mild to severe diarrhea, with or without blood, to hemolytic uremic syndrome (Johnson et al., 2006). Although *E. coli* O157:H7 is the primary STEC serotype associated with human infections in the US, non-O157 STEC also have been recognized as major food borne pathogens (Scallan et al., 2011). According to the CDC, six O-groups were responsible for 71% of non-O157 STEC infections from 1983 to 2002 in the US (Brooks et al., 2005). Of the total non-O157 STEC, the six O-groups, in the order of prevalence, are O26 (22%), O111 (16%), O103 (12%), O121 (8%), O45 (7%), and O145 (5%). Similar to *E. coli* O157, cattle are also considered as asymptomatic carriers of non-O157 STEC (Karmali et al., 2010); however, very little information exists on fecal prevalence of non-O157 STEC in cattle. Several studies have reported the prevalence of a variety of non-O157 STEC serogroups, both 'top six' and others, in carcass samples of cattle (Arthur et al., 2002; Barkocy-Gallagher et al., 2003; Bosilevac et al., 2007, Bosilevac and Koohmaraie, 2011; Valadez et al., 2011). In a recent study (Bosilevac and Koohmaraie, 2011), the most prevalent non-O157 serogroups in commercial ground beef were O8, O20, O22, O113, O116, O117, O163, O171, and O174 and only a small number of the isolates (10 of 338 isolates) carried the major virulence genes. These O-groups are not often implicated in foodborne illnesses in the US, but a serotype of O113 with H21 was frequently associated with severe STEC infections, including hemolytic uremic syndrome in Australia (Paton and Paton, 1998). Only a limited number of studies have determined prevalence of one or more non-O157 serogroups in cattle feces in North America (Cobbold et al., 2004; Renter et al., 2005, 2007) and in other countries (Barlow and Mellor, 2010; Pradel et al., 2000; Oporto et al., 2008; Jenkins et al., 2003; Pearce et al., 2004). The paucity of fecal prevalence data is perhaps because culture-based methodologies to detect or

249 isolate and identify the non-O57 serogroups have not been standardized. Fecal detection and
250 isolation of O157 STEC are based on enrichment, IMS, plating on selective medium for
251 presumptive phenotypic identification (non-sorbitol fermentation) and final confirmation by
252 latex agglutination with O157 antiserum or by PCR for *stx*1, *stx*2, *rfb*E and *fli*C genes (Bai et
253 al., 2010). An optimal enrichment medium and a selective medium for presumptive
254 identification of the six non-O157 serogroups have not been developed. Commercial IMS
255 beads are not available for O45 and O121 STEC. The lack of phenotypic biochemical
256 characteristic that can distinguish non-O157 STEC from O157 or generic *E. coli* makes
257 detection of non-O157 STEC somewhat challenging. Novel differential media based on a
258 mixture of sugars, β-D-galactosidase activity and selective components, which allow color-
259 based separation of some non-O157 STEC (O26, O103, O111, and O145) have been proposed
260 (Possé et al., 2008a), but the methodology has not been fully validated. Regardless, culture-
261 based methodologies for detection of O157 and all six non-O157 serogroups in feces will be
262 time consuming, expensive, and will have logistical constrains in studies involving a large
263 number of samples. Several studies have used serogroup identification using PCR,
264 conventional (Auvray et al., 2007; DebRoy et al., 2011; Jenkins et al., 2003; Monday et al.,
265 2007; Paton and Paton, 1999; Valadez et al., 2011) or real-time (Madic et al., 2011; Perelle et
266 al., 2007), targeting specific regions of genes encoding the O antigen of serogroups. The genes
267 that have been targeted to serogroup putative isolates include *wzx* (O-antigen flippase) and *wzy*
268 (O-antigen polymerase, Feng et al., 2005). In this study, we targeted *wzx* for serogroups O26,
269 O45, O103, O111, and O145, and *wbq*E (putative glycosyl transferase) and *wbq*F (putative
270 acetyl transferase) for O121.

Our mPCR targets a specific region of the genetic loci encoding biosynthesis of the O antigen and the assay is unique because it is designed to detect the top seven serogroups (O157, O26, O111, O103, O121, O45, and O145) of *E. coli* in the US. However, identification of the O-group does not necessarily mean it carries the Shiga toxin genes. We validated the assay with 138 strains of STEC with 100% agreement and no cross amplification. The method will be useful in identifying or confirming the presumptive isolates of *E. coli* from any sample. We also evaluated the applicability of the method for detecting the seven serogroups in feces. In feces spiked with pooled cultures of seven STEC, a concentration of 10^4 CFU/g or 10 CFU/µl (sample volume) was needed, which is in agreement with the sensitivity reported by DebRoy et al. (2011) for the mPCR of 8 STEC. We improved the sensitivity to 10^1 to 10^2 CFU/g by subjecting the fecal sample to enrichment in EC broth for 6 h. A wide variety of enrichment media (brain heart infusion, buffered peptone water, EC, enterohemorrhagic EC, MacConkey, and tryptic soy broths), modifications (bile salts, tellurite, cefixime, novobiocin, rifampicin, vancomycin, cefsulodin, and acriflavin), incubation temperatures (35 to 42°C) and periods (6 to 24 h) have been compared (Hussein and Bollinger, 2008). Inclusion of antibiotics in media has been shown to inhibit certain serotypes (Uemura et al., 2003; Vimont et al., 2007a), and strains within serotype. Based on the minimum concentration needed to detect all seven serogroup in spiked feces, EC broth was considered to be the best, which agrees with the results of Vimont et al. (2007b). The improved sensitivity is because of the increased copy number of the target sequence and dilution of inhibitors present in a complex matrix like feces. The requirement for an enrichment step is not surprising because it is a necessary step in detecting O157 in feces by either a culture-based or molecular technique. The concentration of non-O157 serogroups of *E. coli* in feces of naturally-shedding cattle has not been determined.

294 Based on O157 data, a majority of cattle shed O157 at concentrations below the detection limit
295 by direct culture or PCR-based method (Jacob et al., 2010).
296 Many of the fecal prevalence studies (Barlow and Mellor, 2010; Cobbold et al., 2004; Fach
297 et al., 2001; Pradel et al., 2000; Renter et al., 2005) have utilized a PCR assay, generally
298 targeting the *stx* genes, to prescreen samples before subjecting the positive samples to cultural
299 procedures. In one such study (Pradel et al., 2000), 70% of fecal samples were positive for *stx*
300 gene and only 36% of the positive samples were culture-positive for STEC. To assess the
301 applicability of mPCR as a screening method, we tested 216 fecal samples for the prevalence
302 of the seven serogroups by mPCR and culture method. A large proportion of the fecal samples
303 (47 to 82%) were positive for five (O26, O45, O103, O121, and O157) of the seven
304 serogroups. Only 2.3% and 13.4% of the fecal samples were positive for O111 and O145 by
305 mPCR, respectively. In the case of O157 serogroup, 74% (160/216) of the fecal samples were
306 positive by mPCR (for *rfbE*) and only 29.2% (63/216) were positive by the culture method,
307 which means that we were able to isolate *E. coli* O157 from 39.4% (63/160) of the PCR-
308 positive samples. We only tested sorbitol-negative colonies from the CT-SMAC to detect
309 O157 serogroup in the samples. Cattle can also be a reservoir of sorbitol-fermenting *E. coli*
310 O157 (Bielaszewska et al. 2000), therefore, samples positive by mPCR would include both
311 sorbitol-negative and-positive O157. All isolates of *E. coli* O157 were positive for the major
312 virulence genes (*rfb*E, *stx*2, *eae*, and *fli*C). We tested only a limited number of samples (n=24)
313 by culture method to relate the prevalence of non-O157 to mPCR assay. We were able to
314 isolate 5 of 14 for O26, 0 of 2 for O45, 4 of 10 for O103, and 2 of 5 for O121, which means
315 that we were able to isolate non-O157 from 35.5% (11 of 31) of the samples positive by
316 mPCR. The proportions of the culture-positive samples were similar between O157 and non-

O157 although isolation methods for non-O157 serogroups did not have a selective medium with a phenotypic marker and only 4 of 6 non-O157 serogroups involved the IMS step. Interestingly, none of the non-O157 was positive for the major virulence genes (*stx*1, *stx*2, *eae*, and *hly*A), except one isolate of O26 positive for *stx*1 and a strain of O103 positive for *hly*A. We have shown that the same primer pairs used in the study were able to amplify the *stx*1 and *stx*2 of pure cultures non-O157 serogroup (Bai et al., 2010). Generally, PCR methods reveal a large number of positive samples, but only a portion of those samples yield cultures. It is interesting that none of the samples negative for mPCR was positive by culture-based method.

In conclusion, our mPCR that distinguishes the 'top seven' serogroups of *E. coli* will be a useful method to confirm putative isolates from any source. Data generated from a limited number of fecal samples suggest that approximately two-thirds of PCR-positive samples were negative by culture-based method for the seven serogroups. This suggests that either nonspecific amplifications or culture-based method is not sensitive enough to identify all the positive samples. Therefore applicability of multiplex PCR as a prescreening method before subjecting fecal samples positive for serogroups of *E. coli* to labor intensive culture-based methods requires further assessment.

Acknowledgments

This research was supported by a grant from the U.S. Department of Agriculture (2010-34359-20714).

References

Arthur T.M., Barkocy-Gallagher G.A., Rivera-Betancourt M., Koohmaraie M., 2002. Prevalence and characterization of non-O157 Shiga toxin-producing *Escherichia coli* on carcasses in commercial beef cattle processing plants. Appl. Environ. Microbiol. 68, 4847-4852.

Auvray, F., Lecureuil, C., Taché, J., Leclerc, V., Deperrois, V., Lombard, B., 2007, Detection, isolation and characterization of Shiga toxin-producing Escherichia coli in retail-minced beef using PCR-based techniques, immunoassays and colony hybridization. Let. Appl. Microbiol. 45, 646-651.

Bai J., Shi X., Nagaraja T.G., 2010. A multiplex PCR procedure for the detection of six major virulence genes in *Escherichia coli* 0157: H7. J. Microbiol. Meth. 82, 85-89.

Barkocy-Gallagher G.A., Arthur T.M., Rivera-Betancourt M., Nou X., Shackelford S.D., Wheeler T.L., Koohmaraie M., 2003. Seasonal prevalence of Shiga toxin-producing *Escherichia coli*, including O157:H7 and non-O157 serotypes, and *Salmonella* in commercial beef processing plants. J. Food Prot. 66, 1978-1986.

Barlow R.S., Mellor G.E., 2010. Prevalence of enterohemorrhagic *Escherichia coli* serotypes in Australian beef cattle. Foodborne Path. Dis. 7, 1239-1245.

Bettelheim K.A., 2007. The non-O157 Shiga-toxigenic (verocytotoxigenic) *Escherichia coli*; under-rated pathogens. Crit. Rev. Microbiol. 33, 67-87.

Bielaszewska M., Schmidt H., Liesegang A., Prager R., Rabsch W., Tschäpe H., Cízek A., Janda J., Bláhová K., Karch H., 2000. Cattle can be a reservoir of sorbitol-fermenting Shiga toxin-producing *Escherichia coli* O157:H7 strains and a source of human diseases. J. Clin. Microbiol. 38, 3470-3473.

Blanco M., Blanco J.E., Blanco J., Mora A., Prado M.P., Alonso M., Mouriño C., Madrid, Balsalobre C., Juarez A., 1997. Distribution and characterization of faecal verotoxin-producing *Escherichia coli* (VTEC) isolated from healthy cattle. Vet. Microbiol. 54, 309-319.

Bosilevac J.M., Guerini M.N., Brichta-Harhay D.M., Arthur T.M., Koohmaraie M., 2007. Microbiological characterization of imported and domestic boneless beef trim used for ground beef. J. Food Prot. 70, 440-449.

Bosilevac J.M., Koohmaraie M., 2011. Prevalence and characterization of non-O157 Shiga toxin producing *Escherichia coli* isolated from commercial ground beef in the United States. Appl. Environ. Microbiol. 77, 2103-2112.

Brooks J.T., Sowers E.G., Wells J.G., Greene K.D., Griffin P.M., Hoekstra R.M., Strockbine N.A., 2005. Non-O157 Shiga toxin–producing *Escherichia coli* infections in the United States, 1983-2002. J. Infect. Dis. 192, 1422-1429.

Cobbold R.N., Rice D.H., Szymanski M., Call D.R., Hancock D.D., 2004. Comparison of Shiga-toxigenic *Escherichia coli* prevalences among dairy, feedlot, and cow-calf herds in Washington State. Appl. Environ. Microbiol. 70, 4375-4378.

DebRoy C., Roberts E., Valadez A.M., Dudley E.G., Cutter C.N., 2011. Detection of Shiga toxin-producing *Escherichia coli* O26, O45, O103, O111, O113, O121, O145, and O157 serogroups by multiplex polymerase chain reaction of the *wzx* gene of the O-antigen gene cluster. Foodborne Path. Dis. 8, 651-652.

Fach P., Perelle S., Dilasser F., Grout J., 2001. Comparison between a PCR-ELISA test and the vero cell assay for detecting Shiga toxin-producing *Escherichia coli* in dairy products and characterization of virulence traits of the isolated strains. J. Appl. Microbiol. 90, 809-818.

Feng L., Senchenkova S.N., Tao J., Shashkov A.S., Liu B., Shevelev S.D., Reeves P.R., Xu J., Knirel Y.A., Wang L., 2005. Structural and genetic characterization of enterohemorrhagic *Escherichia coli* O145 O antigen and development of an O145 serogroup-specific PCR assay. J. Bacteriol. 187, 758-764.

Hussein H.S., Bollinger L.M., 2008. Influence of selective media on successful detection of Shiga toxin–producing *Escherichia coli* in food, fecal, and environmental samples. Foodborne Path. Dis. 5, 227-244.

Jacob M.E., Paddock Z.D., Renter D.G., Lechtenberg K.F., Nagaraja T.G., 2010. Inclusion of dried or wet distillers' grains at different levels in diets of feedlot cattle affects fecal shedding of *Escherichia coli* O157:H7. Appl. Environ. Microbiol. 76, 7238-7242.

Jenkins C., Pearce M.C., Smith A.W., Knight H.I., Shaw D.J., Cheasty T., Foster G., Gunn G.J., Dougan G., Smith H.R., Frankel G., 2003. Detection of *Escherichia coli* serogroups O26, O103, O111 and O145 from bovine faeces using immunomagnetic separation and PCR/DNA probe techniques. Lett. Appl. Microbiol. 37, 207-212.

Johnson K.A., Thorpe C.M., Sears C.L., 2006. The emerging clinical importance of non-O157 Shiga toxin-producing *Escherichia coli.* Clin. Infect. Dis. 43, 1587-1595.

Karmali M.A., Gannon V., Sargeant J.M., 2010. Verocytotoxin-producing *Escherichia coli* (VTEC). Vet. Microbiol. 140, 360-370.

Lahti E., Ruoho O., Rantala L., Hanninen M.L., Honkanen-Buzalski T., 2003. Longitudinal study of *Escherichia coli* O157 in a cattle finishing unit. Appl. Environ. Microbiol. 69, 554-561.

Liu D., Cole R., Reeves P., 1996. An O-antigen processing function for *wzx* (*rfbX*): a promising candidate for O-unit flippase. J. Bacteriol. 178, 2102-2107.

Madic J., Vingadassalon N., de Garam C.P., Marault M., Scheutz F., Brugere H., Jamet E., Auvray F., 2011. Detection of Shiga toxin-producing *Escherichia coli* serotypes O26:H11, O103:H2, O111:H8, O145:H28, and O157:H7 in raw-milk cheeses by using multiplex real-time PCR. Appl. Environ. Microbiol. 77, 2035-2041.

Monday S.R., Beisaw A., Feng P.C.H., 2007. Identification of Shiga toxigenic *Escherichia coli* seropathotypes A and B by multiplex PCR. Mol. Cell. Probes. 21, 308-311.

Oporto B., Esteban J.I., Aduriz G., Juste R.A., Hurtado A., 2008. *Escherichia coli* O157:H7 and non-O157 Shiga toxin-producing *E. coli* in healthy cattle, sheep and swine herds in northern Spain. Zoonoses Pub. Hlth. 55, 73-81.

Paton J.C., Paton A.W., 1998. Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. Clin. Microbiol. Rev. 11, 450-479.

Paton A.W., Paton J.C., 1999. Direct detection of Shiga toxigenic *Escherichia coli* strains belonging to serogroups O111, O157, and O113 by multiplex PCR. J. Clin. Microbiol. 37, 3362-3365.

Pearce M.C., Jenkins C., Vali L., Smith A.W., Knight H.I., Cheasty T., Smith H.R., Gunn G.J., Woolhouse M.E.J., Amyes S.G.B., Frankel G., 2004. Temporal shedding patterns and virulence factors of *Escherichia coli* serogroups O26, O103, O111, O145, and O157 in a cohort of beef calves and their dams. Appl. Environ. Microbiol. 70, 1708-1716.

425 Perelle S., Dilasser F., Grout J., Fach P., 2007. Screening food raw materials for the presence
426 of the world's most frequent clinical cases of Shiga toxin-encoding *Escherichia coli* O26,
427 O103, O111, O145 and O157. Int. J. Food. Microbiol. 113, 284-288.
428 Possé B., De Zutter L., Heyndrickx M., Herman L., 2008a. Novel differential and confirmation
429 plating media for Shiga toxin-producing *Escherichia coli* serotypes O26, O103, O111,
430 O145 and sorbitol-positive and -negative O157. FEMS Microbiol. Lett. 282, 124-131.
431 Possé B., De Zutter L., Heyndrickx M., Herman L., 2008b. Quantitative isolation efficiency of
432 O26, O103, O111, O145 and O157 STEC serotypes from artificially contaminated food
433 and cattle faeces samples using a new isolation protocol. J. Appl. Microbiol. 105, 227-235.
434 Pradel N., Livrelli V., De Champs C., Palcoux J.B., Reynaud A., Scheutz F., Sirot J., Joly B.,
435 Forestier C., 2000. Prevalence and characterization of Shiga toxin-producing *Escherichia*
436 *coli* isolated from cattle, food, and children during a one-year prospective study in France.
437 J. Clin. Microbiol. 38, 1023-1031.
438 Renter D.G., Bohaychuk V., Van Donkersgoed J., King R., 2007. Presence of non-O157 Shiga
439 toxin-producing *Escherichia coli* in feces from feedlot cattle in Alberta and absence on
440 corresponding beef carcasses. Can. J. Vet. Res. 71, 230.
441 Renter D.G., Morris J.G., Sargeant J.M., Hungerford L.L., Berezowski J., Ngo T., Williams K.,
442 Acheson D.W.K., 2005. Prevalence, risk factors, O serogroups, and virulence profiles of
443 Shiga toxin-producing bacteria from cattle production environments. J. Food Prot. 68,
444 1556-1565.
445 Scallan E., Hoekstra R.M., Angulo F.J., Tauxe R.V., Widdowson M.A., Roy S.L., Jones J.L.,
446 Griffin P.M., 2011. Foodborne illness acquired in the United States—major pathogens.
447 Emerg. Infect. Dis. 17, 7-15.

Uemura R., Sueyoshi R.M., Nagayoshi M., Nagatomo H., 2003. Antimicrobial susceptibilities of Shiga toxin-producing *Escherichia coli* isolates from pigs with edema disease in Japan. Microbiol. Immunol. 47, 57-61.

Valadez A.M., DebRoy C., Dudley E., Cutter C.N., 2011. Multiplex PCR detection of Shiga-toxin producing *Escherichia coli* strains belonging to serogroups O157, O103, O91, O113, O145, O111, and O26 experimentally inoculated in beef carcass swabs, beef trim, and ground beef. J. Food Prot. 74, 228-239.

Vimont A., Delignette-Muller M.L., Vernozy-Rozand C., 2007a. Supplementation of enrichment broths by novobiocin for detecting Shiga toxin-producing *Escherichia coli* from food: a controversial use. Lett. Appl. Microbiol. 44, 326-331.

Vimont A., Vernozy-Rozand C., Montet M.P., Bavai C., Fremaux B., Delignette-Muller M.L., Vernozy-Rozand C., 2007b. Growth of Shiga-toxin producing *Escherichia coli* (STEC) and bovine feces background microflora in various enrichment protocols. Vet. Microbiol. 123, 274-281.

Table 1

Primer sequences and strains used as positive control

Serogroup	Strain[1]	Primer sequences (5' to 3')	Amplicon size, bps	Reference
O26	TW O1597	Forward: GGGGGTGGGTACTATATTGG Reverse: AGCGCCTATTTCAGCAAAGA	241	This study
O45	KSU 2566:58	Forward: GGGCTGTCCAGACAGTTCAT Reverse: TGTACTGCACCAATGCACCT	890	This study
O103	KSU 15612-1	Forward: TAAGTACGGGGGTGCTTTTT Reverse: AAGCTCCCGAGCACGTATAA	716	This study
O111	KSU 7726-1	Forward: CAAGAGTGCTCTGGGCTTCT Reverse: AACGCAAGACAAGGCAAAAC	451	This study
O121	KSU 4190	Forward: TCATTAGCGGTAGCGAAAGG Reverse: TTCTGCATCACCAGTCCAGA	587	This study
O145	KSU 1234-1	Forward: TGCTCGACTTTTACCATCAAC Reverse: AACCAACACCATACACCTTGTCTT	374	This study
O157	ATCC 43894	Forward: CAGGTGAAGGTGGAATGGTTGTC Reverse: TTAGAATTGAGACCATCCAATAAG	296	Bai et al., 2010

[1] TW strain was from Thomas S. Whittam Microbial Evolution Laboratory at Michigan State University; KSU strains were from Renter et al., 2005; ATCC strain was from the American Type Culture Collection.

Table 2
Detection limit of multiplex PCR of fecal samples spiked with a mixture of O26, O45, O103, O111, O121, O145, and O157 serogroups of *Escherichia coli*

Enrichment broth			Replicates			
Additives	Incubation		1	2	3	Mean
	Temperature,°C	Hours				
None	-	-	5.3×10^5	6.5×10^5	6.5×10^4	4.2×10^5
E. coli broth	40	6	5.3×10^1	6.5×10^2	0.65	2.3×10^2
E. coli broth	40	24	5.3×10^3	6.5×10^2	0.65	2.0×10^3
Tryptic soy broth with: 2.0 mg/L of rifampicin, 16.0 mg/L of vancomycin, and 1.5 g/L of bile salts	42	24	5.3×10^3	6.5×10^3	6.5×10^1	4.0×10^3
Tryptic soy broth with: 2.0 mg/L of rifampicin, 16.0 mg/L of vancomycin, 1.5 g/L of bile salts, and 8.0 mg/L of novobiocin	42	24	5.3×10^3	6.5×10^3	6.5×10^3	6.1×10^3

Table 3

Fecal prevalence of O26, O45, O103, O111, O121, O145, and O157 serogroups *Escherichia coli* based on multiplex PCR or culture detection method

	Serogroups						
Sample	O26	O45	O103	O111	O121	O145	O157
Dairy cattle, n=12							
Multiplex PCR positive (%)	7 (58)	1 (8)	4 (33)	0 (0)	3 (25)	0 (0)	9 (75)
Culture positive (%)	3 (25)	0 (0)	1 (8)	0 (0)	2 (17)	0 (0)	2 (17)
Dairy cattle, n=96							
Multiplex PCR positive (%)	73 (85)	41 (43)	51 (53)	13 (14)	34 (35)	5 (5)	54 (56)
Culture positive (%)	-	-	-	-	-	-	15 (16)
Feedlot cattle, n=12							
Multiplex PCR positive (%)	7 (58)	1 (8)	6 (50)	0 (0)	2 (17)	0 (0)	5 (42)
Culture positive (%)	2 (17)	0 (0)	3 (25)	0 (0)	0 (0)	0 (0)	2 (17)
Feedlot cattle, n=96							
Multiplex PCR positive (%)	91 (95)	65 (68)	88 (92)	17 (18)	64 (67)	0 (0)	92 (96)
Culture positive (%)	-	-	-	-	-	-	34 (35)

Table 4

Virulence genes and genes that code for O157 and H7 antigens of O26, O45, O103, O111, O121, O145, and O157 serogroups of *Escherichia coli* strains isolated from fecal samples.

Serogroups	Number of strains	Genes					
		stx1	*stx2*	*eae*	*rfbE*	*fliC*	*hlyA*
O26	5	1/5	0/5	0/5	0/5	0/5	0/5
O45	0	-	-	-	-	-	-
O103	4	0/4	0/4	0/4	0/4	0/4	1/4
O111	0	-	-	-	-	-	-
O121	2	0/2	0/2	0/2	0/2	1/2	0/2
O145	0	-	-	-	-	-	-
O157	54	1/53	51/53	51/53	53/53	49/53	51/53

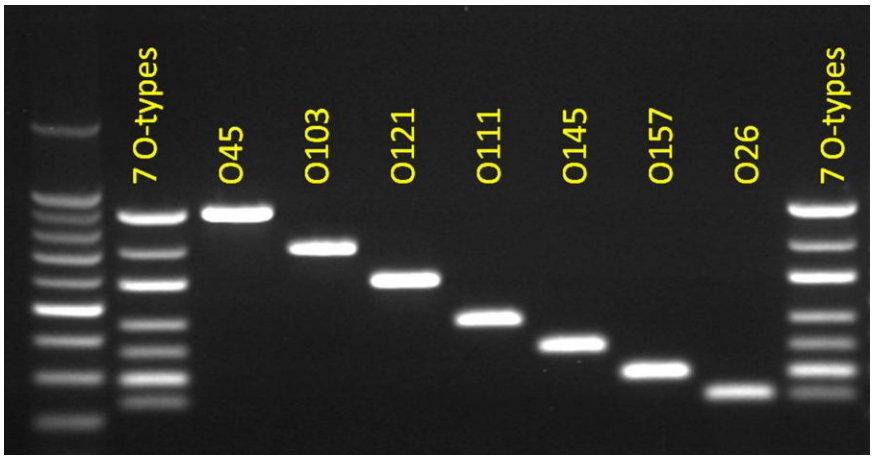

FIG. 1. Agarose gel image of amplicons obtained by multiplex PCR of individual or a pooled culture of Shiga-toxin producing *Escherichia coli* (STEC) serotypes. Lane 1: Molecular size markers; Lanes 2 and 10: Mixtures of seven STEC O-types; Lanes 3-9: Individual STEC O-types

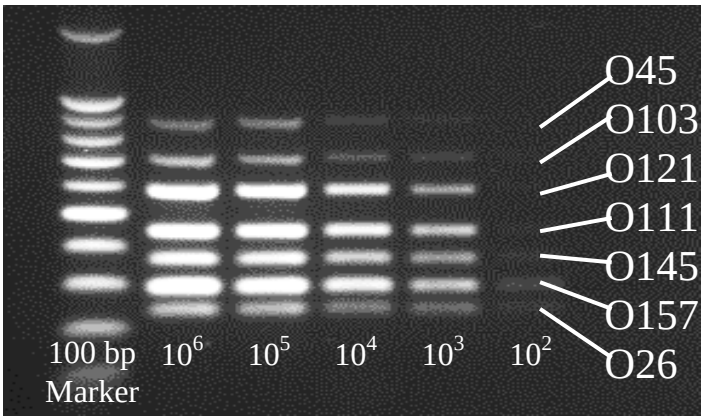

FIG. 2. Agarose gel image of amplicons obtained by multiplex PCR of serially diluted pooled culture of seven STEC strains (5.3×10^6 to 5.3×10^0 CFU/ml).

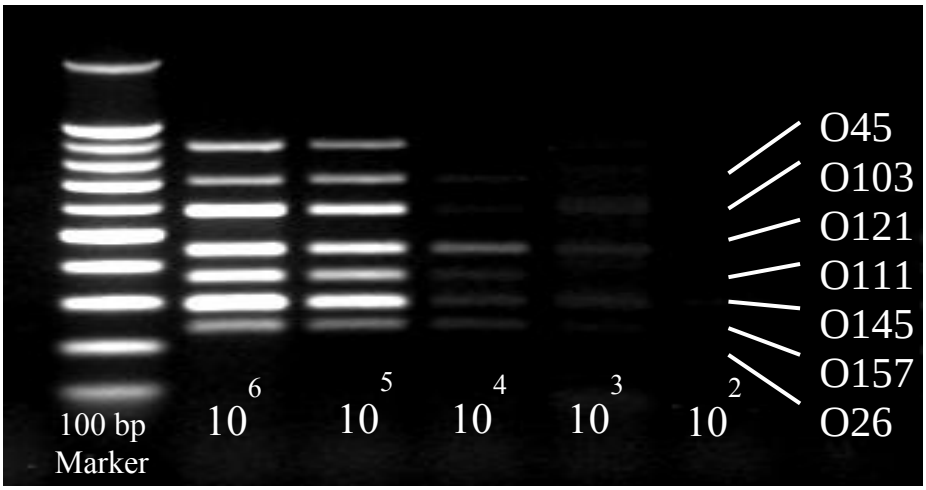

FIG. 3. Agarose gel image of amplicons obtained by multiplex PCR of serially diluted fecal samples inoculated with pooled cultures of seven STEC strains (5.3×10^6 to 5.3×10^0 CFU/ml).

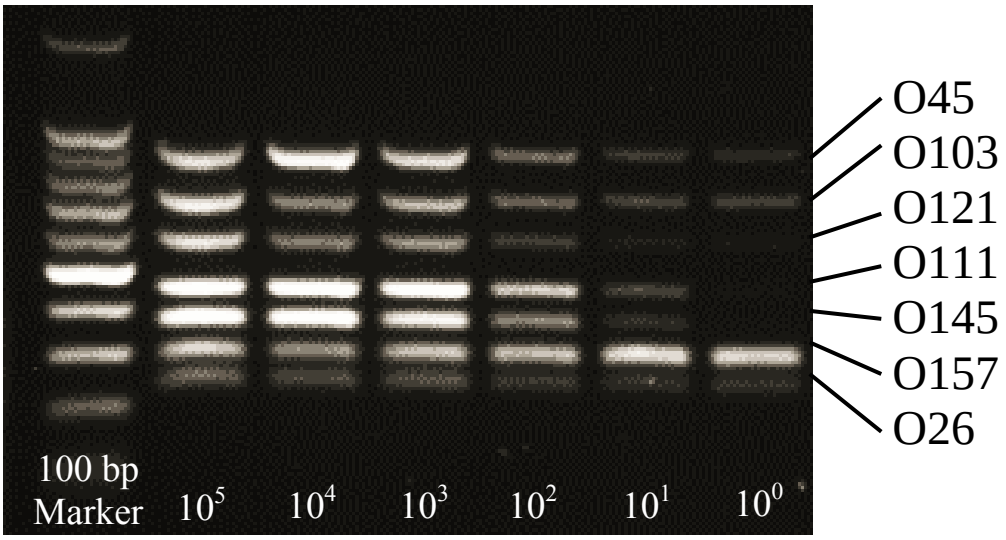

FIG. 4. Agarose gel image of amplicons obtained by multiplex PCR of serially diluted fecal samples inoculated with pooled cultures of STEC stains (5.3×10^6 to 5.3×10^0 CFU/ml) after 6 h enrichment in *Escherichia coli* broth at 40°C.