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Multiplex Quantitative PCR Assays for the Detection and Quantification of the Six Major Non-O157 *Escherichia coli* Serogroups in Cattle Feces†

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ABSTRACT

Shiga toxin–producing *Escherichia coli* (STEC) serogroups O26, O45, O103, O111, O121, and O145, called non-O157 STEC, are important foodborne pathogens. Cattle, a major reservoir, harbor the organisms in the hindgut and shed them in the feces. Although limited data exist on fecal shedding, concentrations of non-O157 STEC in feces have not been reported. The objectives of our study were (i) to develop and validate two multiplex quantitative PCR (mqPCR) assays, targeting O-antigen genes of O26, O103, and O111 (mqPCR-1) and O45, O121, and O145 (mqPCR-2); (ii) to utilize the two assays, together with a previously developed four-plex qPCR assay (mqPCR-3) targeting the O157 antigen and three virulence genes (stx_1, stx_2, and *eae*), to quantify seven serogroups and three virulence genes in cattle feces; and (iii) to compare the three mqPCR assays to a 10-plex conventional PCR (cPCR) targeting seven serogroups and three virulence genes and culture methods to detect seven *E. coli* serogroups in cattle feces. The two mqPCR assays (1 and 2) were shown to be specific to the target genes, and the detection limits were 4 and 2 log CFU/g of pure culture–spiked fecal samples, before and after enrichment, respectively. A total of 576 fecal samples collected from a feedlot were enriched in *E. coli* broth and were subjected to quantification (before enrichment) and detection (after enrichment). Of the 576 fecal samples subjected, before enrichment, to three mqPCR assays for quantification, 175 (30.4%) were quantifiable (≥4 log CFU/g) for at least one of the seven serogroups, with O157 being the most common serogroup. The three mqPCR assays detected higher proportions of postenriched fecal samples ($P < 0.01$) as positive for one or more serogroups compared with cPCR and culture methods. This is the first study to assess the applicability of qPCR assays to detect and quantify six non-O157 serogroups in cattle feces and to generate data on fecal concentration of the six serogroups.

Shiga toxin–producing *Escherichia coli* (STEC) is a major foodborne pathogen that causes human illnesses characterized by nonbloody and bloody diarrhea, with hemolytic uremic syndrome as a potential complication *(17)*. Serotype O157:H7 is the most common STEC responsible for the majority of foodborne STEC illnesses; however, other STEC serogroups, particularly O26, O45, O103, O111, O121, and O145, have gained more recognition in recent years because they account for more than 70% of non-O157 STEC infections in the United States *(33)*. In 2011, the U.S. Department of Agriculture, Food Safety and Inspection Service declared these six non-O157 STEC serogroups to be adulterants in ground beef and nonintact raw beef products *(35)*. Cattle, a major reservoir of STEC, harbor the organisms in the hindgut and shed them in their feces; this serves as a major source of contamination of food and water *(17)*.

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Not much is known about fecal shedding of the six non-O157 *E. coli* pathogens in cattle because detection methods, PCR- and culture-based, have been developed and validated only recently *(3, 5, 11, 21, 31)*. In addition to the presence of STEC pathogens in cattle feces, the concentration of these organisms plays a role in the spread between animals and subsequent hide and carcass contamination. A subset of cattle, known as "super shedders," shed the STEC O157:H7 organism at high concentrations ($\geq 10^4$ CFU/g of feces) *(7)*. Super-shedding cattle have been reported to be a major source of transmission of O157:H7 among cattle within the herd *(28)* and of subsequent contamination of hides and carcasses *(2, 12, 19)*. Because there are no data on fecal concentration of non-O157 STEC in cattle, it is not known whether a subset of cattle that are super shedders of non-O157 STEC (as with O157) exists in a population. Fecal concentration data of the six non-O157 serogroups (O26, O45, O103, O111, O121, and O145) in cattle, when factored in microbial risk assessment models, allow estimation of the potential contamination burden that fecal shedding represents in upstream production stages. Although real-time PCR assays have been developed to detect non-O157 STEC in food matrices *(14, 15, 24)*, the applicability of the real-time PCR assays to detect and quantify non-O157 *E. coli* in

cattle feces has not been evaluated. Anklam et al. *(1)* have developed four separate multiplex qPCR assays to target the seven serogroups (O26, O45, O103, O111, O121, O145, and O157) and four virulence genes (stx_1, stx_2, *eae,* and *ehxA*) in cattle feces. The assays were validated using pure cultures and culture-spiked cattle feces, but applicability of the assays for the detection and quantification of *E. coli* serogroups and associated virulence genes in feces of naturally shedding cattle was not evaluated. Luedtke et al. *(27)* developed a multiplex qPCR to target enterohemorrhagic *E. coli* (EHEC)–specific attaching and effacing gene-positive conserved fragment 1, *ecf1,* and *eae* for enumeration of EHEC directly from cattle feces; however, the assay does not quantify individual serogroups. The objectives of our study were (i) to develop and validate two sets of multiplex quantitative PCR (mqPCR) assays to target O-antigen genes of O26, O103, and O111 (mqPCR-1) and O45, O121, and O145 (mqPCR-2) and (ii) to evaluate the applicability of the two assays, together with a previously developed four-plex qPCR assay (mqPCR-3) targeting the O157 antigen gene and three virulence genes (stx_1, stx_2, and *eae (30)*), to quantify six non-O157 and O157 serogroups and three virulence genes in cattle feces ($n = 576$) from a commercial feed yard. Additionally, the detection of the seven STEC serogroups and three virulence genes in cattle feces by the three mqPCR assays was compared with detection by a 10-plex conventional PCR (cPCR) that targets the same genes (seven serogroup-specific and three virulence genes) and by culture methods.

MATERIALS AND METHODS

Design of the assays. Two sets of mqPCR assays (mqPCR-1 and mqPCR-2) were developed to detect and quantify O26, O45, O103, O111, O121, and O145 serogroups. The serogroups targeted by mqPCR-1 were O26, O103, and O111; those targeted by mqPCR-2 were O45, O121, and O145. The target gene for O26, O103, O111, O45, and O145 was *wzx (3),* which encodes for flippase involved in O-polysaccharide export *(25)*. The target genes for O121 were *wbqE* and *wbqF,* which encode for putative glycosyl and acetyl transferases, respectively *(3)*. The reporter dyes, FAM, VIC/MAX, and Texas Red, were conjugated at 5′ ends, and Black Hole Quencher dyes I and II were conjugated at 3′ ends of the probes to detect amplification products specific to each gene target. Primers and probes (Integrated DNA Technologies, Inc., Coralville, IA) designed to target O-antigen genes of the six non-O157 *E. coli* serogroups are shown in Table 1.

Optimization of the assays. The assays were optimized and validated with pure cultures of one strain each of six serogroups of non-O157 STEC (Table 2) individually and, subsequently, with pooled mixtures of two different combinations (O26, O103, and O111 for mqPCR-1 and O45, O121, and O145 for mqPCR-2). The strains of non-O157 STEC, stored at −80°C on cryobeads (CryoCare, Key Scientific Products, Round Rock, TX), were streaked onto blood agar plates (Remel, Lenexa, KS). Single colonies of each serogroup were inoculated and grown overnight in Luria-Bertani broth (Difco, BD, Sparks, MD) at 37°C, and then 100 μl of the culture was added to 10 ml of the broth and incubated at 37°C for 3 to 4 h until an absorbance of 0.4 at 600 nm (approximately 8 log CFU/ml) was reached. Equal volumes of the cultures of each serogroup were mixed into two combinations as described before. One milliliter of the culture suspension (individual serogroup and pooled mixtures) was boiled for 10 min and centrifuged at 9,300 × *g* for 5 min; the supernatant was used as DNA template for mqPCR assays. DNA was also subjected to 10-fold serial dilutions (10^{-1} to 10^{-7}), and standard curves were generated with the mqPCR assays.

Running conditions of the assays. The working concentrations of all primers in the primer mixture were 10 pmol/μl. The working concentrations of probes were 5 pmol/μl for O26 and O103, 3 pmol/μl for O121, 2.5 pmol/μl for O111 and O45, and 1.25 pmol/μl for O145. The PCR reaction mixture contained 10 μl of 2X Bio-Rad iQ Multiplex Powermix (Bio-Rad Laboratories, Life Science Group, Hercules, CA), 4 μl of nuclease-free water, 1 μl of primer mixture, 1 μl of each probe, and 2 μl of DNA template, making a total reaction volume of 20 μl. PCR was performed with the BioRad CFX96 mqPCR system, and data were analyzed using CFX Manager software version 3.1 (Bio-Rad Laboratories). The optimized PCR amplification protocol included a 10-min denaturation at 95°C, followed by 45 cycles of 95°C for 15 s, 56°C for 20 s, and 72°C for 40 s.

Specificity of the assays. Specificity of the assays was evaluated with a number of *E. coli* and non–*E. coli* strains from our culture collection. A total of 170 strains (human clinical and bovine origin) belonging to six non-O157 *E. coli* serogroups (35 strains of O26, 40 strains of O103, 40 strains of O111, 25 strains of O45, 12 strains of O121, and 18 strains of O145) were used as a positive control, and another 100 strains representing 42 *E. coli* serogroups other than the six non-O157 serogroups and other bacterial species (*Klebsiella pneumoniae, Proteus mirabilis,* and *Serratia marcescens*) were used as a negative control.

In addition, pure cultures of target serogroups for each assay were pooled by mixing each serogroup at equal and different concentrations. Each serogroup of the assay was added at equal concentration and at decreasing concentrations ($1{:}10^{0}$, $1{:}10^{-1}$, $1{:}10^{-2}$, $1{:}10^{-3}$, $1{:}10^{-4}$, $1{:}10^{-5}$, $1{:}10^{-6}$, $1{:}10^{-7}$) to a mixture containing the other two targets at fixed concentration and was inoculated into a cattle fecal sample that tested negative for the six target non-O157 serogroups. One gram of spiked fecal samples was added to 9 ml of *E. coli* (EC) broth (Difco, BD), vortexed, and incubated at 40°C for 6 h. Extracted DNA from pre- and postenrichment fecal samples was subjected to mqPCR assays.

Sensitivity of the assays using pure cultures of non-O157 STEC. Sensitivity of the assays was determined by using 10-fold serial dilutions of pure cultures of the six serogroups. Three different combinations of pooled pure cultures were prepared as described before: O26, O103, and O111 for mqPCR-1; O45, O121, and O145 for mqPCR-2; and O26, O45, O103, O111, O121, and O145 for both assays. Ten-fold serial dilutions (10^{-1} to 10^{-7}) of each pooled pure culture grown in Luria-Bertani broth were performed, and 100-μl aliquots of the last three dilutions (10^{-5}, 10^{-6}, and 10^{-7}) were spread plated onto blood agar plates (four plates per dilution) to determine initial cell concentrations (CFU per milliliter). One milliliter from each dilution (10^{0} to 10^{-7}) was removed, boiled for 10 min, and centrifuged at 9,300 × *g* for 5 min. Supernatants were used as DNA templates for mqPCR, and standard curves were generated to determine correlation coefficients, amplification efficiencies, and detection limits for each assay. Both mqPCR assays (targeting three serogroups each) were compared to the corresponding single (targeting a single serogroup) and duplex (targeting two serogroups) assays. The experiment was repeated on a different day.

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TABLE 1. *Primers and probes used in the multiplex quantitative PCR assays*[a]

Target genes	Primers/ probes	Sequence	Reference
wzx_{O26}	Forward	GTGCGAATGCCATATTTCCT	This study
	Reverse	TGCTAGTACTTCACCAACAGCAA	
	Probe	FAM-CCTTAGGGGAAAACATCTTTTCAA-BHQ1	
wzx_{O103}	Forward	AAAGGCGCATTAGTGTCTGC	This study
	Reverse	GAGGAGTATGTCCCACCCAGT	
	Probe	MAX-ATGCGGTTGCAGGTGTCTG-BHQ1	
wzx_{O111}	Forward	CCGGTTGTTTCATCAATCCT	This study
	Reverse	AAACTAAGTGAGACGCCACCA	
	Probe	TexasRed-TGAGTCAAAATGGATGTTTCTTCAA-BHQ2	
wzx_{O45}	Forward	GGTGTTGCCATATGGTGTTG	This study
	Reverse	TCCGGAAATTTTACCTTCCA	
	Probe	MAX-TGATCATGCAACAGGTCACTATGA-BHQ1	
$wbqE_{O121}$+$wbqF_{O121}$	Forward	TGGATATGCTTCGAATGAACC	This study
	Reverse	CAATGCGAGTTTTGTCTCCA	
	Probe	FAM-TTCCTACACCTACTCGATATACTCCCA-BHQ1	
wzx_{O145}	Forward	CATTGTTTTGCTTGCTCGAC	This study
	Reverse	CAACGAAAATACCATATCCTACAGC	
	Probe	TexasRed-CCATCAACAGATTTAGGAGTGTATGG-BHQ2	
$rfbE_{O157}$	Forward	CTGTCCACACGATGCCAATG	*20*
	Reverse	CGATAGGCTGGGGAAACTAGG	
	Probe	FAM-TTAATTCCACGCCAACCAAGATCCTCA-Iowa Black FQ	
stx_1	Forward	CAAGAGCGATGTTACGGTTTG	*20*
	Reverse	GTAAGATCAACATCTTCAGCAGTC	
	Probe	TexasRed-ACATAAGAACGCCCACTGAGATCATCCA-BHQ2	
stx_2	Forward	GCATCCAGAGCAGTTCTGC	*20*
	Reverse	GCGTCATCGTATACACAGGAG	
	Probe	Cy5-TGTCACTGTCACAGCAGAAGCCTTACG-BHQ2	
eae	Forward	AAAGCGGGAGTCAATGTAACG	*30*
	Reverse	GGCGATTACGCGAAAGATAC	
	Probe	MAX-CTCTGCAGATTAACCTCTGCCG-ZEN	

[a] BHQ, Black Hole Quencher dye.

Sensitivity of the assays using feces spiked with pure cultures of non-O157 STEC. Fifteen pen-floor fecal samples from cattle housed in the Kansas State University feedlot were collected and tested by both sets of mqPCR assays (1 and 2), and a sample that was negative for the six serogroups was selected to spike with pure cultures. Three different combinations of pooled pure cultures were prepared as described before: O26, O103, and O111 (mqPCR-1); O45, O121, and O145 (mqPCR-2); and O26, O45, O103, O111, O121, and O145 (both assays). Ten-fold serial dilutions (10^0 to 10^{-7}) of each mixture were performed in Luria-Bertani broth, and initial concentrations of each were determined. Aliquots of 10 g of fecal sample were inoculated with 1 ml of different dilutions (10^{-1} to 10^{-7}) of pooled pure cultures and were mixed as thoroughly as possible; 1 g of the spiked fecal sample was added to 9 ml of EC broth. The fecal suspension was vortexed and incubated at 40°C for 6 h. One-milliliter samples of pre- and postenrichment fecal suspensions were boiled for 10 min and centrifuged at 9,300 × *g* for 5 min. DNA cleanup of pre- and postenrichment fecal suspensions was performed using the GeneClean Turbo Kit (MP Biomedicals LLC, Solon, OH). Purified DNA from pre- and postenriched fecal suspensions and boiled DNA from pooled pure cultures used to spike the fecal sample were subjected to mqPCR, and standard curves were generated to determine the correlation coefficients, amplification efficiencies, and detection limits of each assay. The experiment was repeated with a different fecal sample.

TABLE 2. *Sources and virulence gene profiles of* Escherichia coli *strains used for optimization of multiplex quantitative PCR assays*

Serogroup	Strain	Host	stx_1	stx_2	*eae*
E. coli O26	TW 8569	Human	−	+	+
E. coli O103	TW 8103	Human	+	−	+
E. coli O111	7726-1	Bovine	+	+	+
E. coli O121	KDHE 48	Human	−	+	−
E. coli O45	KDHE 22	Human	+	−	+
E. coli O145	KDHE 53	Human	+	+	+

Application of mqPCR assays to quantify *E. coli* serogroups and virulence genes in fecal samples from feedlot cattle. A total of 576 pen-floor fecal samples from cattle housed in 24 pens (24 samples per pen) at a commercial feedlot in the central United States were collected the day before transportation of cattle to slaughter in the summer of 2013. Details regarding the design of the study and characteristics of the study population are available in Dewsbury et al. *(11)*. Fecal samples were suspended in EC broth, and DNA was extracted as described above. The DNA was subjected to the two mqPCR assays (mqPCR-1 and mqPCR-2) and to a previously developed four-plex qPCR assay (mqPCR-3) targeting O157 serogroup ($rfbE_{O157}$) and the three major virulence genes, *eae*, stx_1, and stx_2 *(30)*. Cycle threshold (C_T) values were determined to quantify seven major *E. coli* serogroups and three virulence genes. Concentration of each serogroup and virulence

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gene was determined based on standard curves obtained using cattle fecal samples spiked with known concentrations of pure cultures of *E. coli* O157 and non-O157 STEC serogroups.

Application of mqPCR assays and comparison with cPCR and culture methods for the detection of *E. coli* O157 and six non-O157 *E. coli* serogroups in fecal samples from feedlot cattle. Fecal samples ($n = 576$) suspended in EC broth were enriched at 40°C for 6 h and then were subjected to three mqPCR assays (mqPCR-1, mqPCR-2, and mqPCR-3), 10-plex cPCR assay targeting the same genes (seven serogroups and three virulence genes) *(3),* and culture-based methods *(11)* for the detection of the seven serogroups. For the culture-based detection method, postenriched fecal samples were subjected to immunomagnetic separation with seven serogroup-specific beads (Abraxis, Warminster, PA), and 50 μl of post–immunomagnetic separation bead suspensions were spread plated onto chromogenic Possé medium *(32)* that was modified to include novobiocin at 5 mg/liter and potassium tellurite at 0.5 mg/liter for non-O157 serogroups, and sorbitol MacConkey with cefixime (0.05 mg/liter) and potassium tellurite (2.5 mg/liter) for *E. coli* O157. The plates were then incubated at 37°C for 18 to 24 h, and six chromogenic colonies (shades of blue, purple, mauve, or green) from modified Possé medium and sorbitol-negative colonies from sorbitol MacConkey with cefixime and potassium tellurite were picked and streaked onto blood agar and incubated for 18 to 24 h. For non-O157 serogroups, colonies from each of the six streaks on the blood agar plate were pooled in 50 μl of distilled water, boiled for 10 min, and used as a DNA template for seven-plex cPCR targeting the seven serogroups *(31)*. For O157, colonies on blood agar were subjected to indole test, and indole-positive colonies were tested for O157 antigen using a latex agglutination assay (*E. coli* O157 latex test kit, Oxoid Ltd., Cheshire, England). Agglutination-positive colonies were then subjected to a six-plex cPCR assay ($rfbE_{O157}$, $fliC_{H7}$, stx_1, stx_2, *eae,* and *ehxA*) to confirm the O157 serogroup, H7 flagellar gene, and virulence genes *(4)*.

Statistical analyses. The proportion of positive samples for each serogroup and virulence gene based on the three detection methods (mqPCR, cPCR, and culture method) was calculated as the number of samples detected as positive for each gene by each detection method divided by the total number of samples tested by each detection method. The Cohen's kappa statistic was used to evaluate the agreement beyond that due to chance among mqPCR, cPCR, and culture methods for the detection of seven *E. coli* serogroups and three virulence genes. Interpretation of the kappa statistic was done based on the scale proposed by Landis and Koch *(22)*. The McNemar's chi-square test was used to compare the proportion of positive samples obtained by the three detection methods *(29)*. When the P value of McNemar's test is not significant ($P > 0.05$), there is little evidence to conclude that the tests are different; and, when the P value is significant ($P < 0.05$), there is a significant disagreement between tests, indicating that there is little value in further assessing agreement by Cohen's kappa statistic. In the latter case, Cohen's kappa statistics are provided for reference only. A receiver operating characteristic curve was generated by plotting the true positive against the false positive proportions across a range of reciprocal C_T values ($1/C_T$ value) to determine the C_T value that yields optimum sensitivity and specificity and the highest proportion of correctly classified samples by cPCR in relation to mqPCR. Statistical analyses were performed in Stata/MP version 12.0 (StataCorp LP, College Station, TX).

RESULTS

Specificity of the assays. All 170 strains belonging to O26, O103, O45, O111, O121, and O145 serogroups were detected by the corresponding mqPCR-1 and mqPCR-2 assays (data not shown). No cross-amplification occurred with nontargeted serogroups. None of the *E. coli* strains belonging to serogroups other than the six non-O157 serogroups were detected by the mqPCR assays, nor were other bacterial species. Both assays (mqPCR-1 and mqPCR-2) correctly detected the target serogroups when performed with pooled pure cultures and spiked fecal samples of two different combinations containing equal or different concentrations of target serogroups (data not shown).

Sensitivity of the assays with pure cultures and pure culture–spiked fecal samples. The detection limits of the two assays, mqPCR-1 and mqPCR-2, with pure cultures were 3 log CFU/ml, with mean C_T values of 37.1 and 37.4 and amplification efficiencies from 99 to 104% and 99 to 102%, respectively. The correlation coefficient was >0.99 for both assays (Table 3). Detection limits, correlation coefficients, and amplification efficiencies of both assays were similar to the corresponding single (targeting a single serogroup) or duplex (targeting two serogroups) assays (data not shown). In fecal samples spiked with serially diluted, pooled pure cultures, detection limits of both assays (mqPCR-1 and mqPCR-2) were 4 log CFU/g of feces, with mean C_T values of 37.2 and 37.4, respectively. After enrichment, detection limits of both assays improved to 2 log CFU/g of feces, with mean C_T values of 37.6 and 37.9 for mqPCR-1 and mqPCR-2, respectively. The correlation coefficients and amplification efficiencies are shown in Table 3.

Application of mqPCR assays to quantify *E. coli* serogroups and virulence genes in cattle feces. Preenriched fecal samples that yielded C_T values less than or equal to the mean cut-off C_T value were considered positive (37.2 for mqPCR 1, 37.4 for mqPCR-2, and 38.3 for mqPCR-3) for the serogroups. Of the 576 fecal samples (before enrichment), 175 (30.4%) were quantifiable for at least one of the seven serogroups. Serogroup O157 ($n = 66$; 11.5%) was the predominant serogroup quantified by mqPCR, followed by O45 ($n = 41$; 7.1%), O103 ($n = 41$; 7.1%), O121 ($n = 37$; 6.4%), O26 ($n = 29$; 5%), O111 ($n = 2$; 0.3%), and O145 ($n = 2$; 0.3%) (Table 4). The concentrations of *E. coli* serogroups ranged from 4 to 7 log CFU/g of feces. A greater proportion of fecal samples tested positive for *E. coli* serogroups at concentrations of 4 (19.3%) than 5 log CFU/g (17.4%), and none of the non-O157 *E. coli* serogroups had concentrations >6 log CFU/g. Seven fecal samples (1.2%) were positive for O157 at concentrations of 6 to <7 log CFU/g. Of the 175 fecal samples that tested positive for at least one of the seven *E. coli* serogroups at quantifiable concentrations (≥4 log CFU/g), 141 (80.6%) were positive for one serogroup, 28 (16%) for two serogroups, and three (1.7%) each for three and four serogroups. The concentrations of stx_1 and *eae* ranged from

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TABLE 3. *Detection limits, correlation coefficients, and amplification efficiencies of the multiplex quantitative PCR assays for pure cultures and cattle fecal samples spiked with pure cultures of non-O157 Shiga toxin–producing* Escherichia coli *strains*[a]

Template	Detection limit[b] (avg cycle threshold value)	Correlation coefficients	Amplification efficiencies (%)
Pure cultures	Log CFU/ml		
Assay 1: O26, O103, O111	3 (37.1)	>0.99	99–104
Assay 2: O45, O121, O145	3 (37.4)	>0.99	99–102
Cattle feces spiked with pure cultures	Log CFU/g		
Before enrichment			
Assay 1: O26, O103, O111	4 (37.2)	>0.96	90–95
Assay 1: O26, O103, O111, O45, O121, O145	4 (37.8)	>0.96	92–94
Assay 2: O45, O121, O145	4 (37.4)	>0.95	88–92
Assay 2: O26, O103, O111, O45, O121, O145	4 (37.5)	>0.95	89–91
After enrichment			
Assay 1: O26, O103, O111	2 (37.6)	>0.98	94–101
Assay 1: O26, O103, O111, O45, O121, O145	2 (37.7)	>0.98	92–101
Assay 2: O45, O121, O145	2 (37.9)	>0.98	91–98
Assay 2: O26, O103, O111, O45, O121, O145	2 (37.3)	>0.98	90–96

[a] Cattle fecal samples spiked with pure cultures of non-O157 STEC were suspended in *E. coli* broth (before enrichment) and incubated at 40°C for 6 h (after enrichment).
[b] Values represent means of two individual experiments.

4 to 8 log CFU/g, and that of stx_2 ranged from 4 to 7 log CFU/g (Table 4).

Application of mqPCR assays to detect *E. coli* serogroups and virulence genes in cattle feces. Post-enriched fecal samples that yielded C_T values less than or equal to the mean cut-off C_T value were considered positive (37.6 for mqPCR-1, 37.9 for mqPCR-2, and 37.9 for mqPCR-3) for serogroups. Of 576 samples, 566 (98.3%) were positive for at least one of the seven serogroups, and all except one sample were positive for at least one of the three virulence genes. Serogroup O157 (89.8%) was the predominant *E. coli* detected, followed by O103 (84.7%), O26 (59.0%), O121 (57.8%), O45 (55.9%), and O145 (5.9%). Only four samples (0.7%) tested positive for serogroup O111. Among the virulence genes, *eae* (99.7%) was predominant, followed by stx_1 (95.7%) and stx_2 (94.6%) (Table 5).

Based on cPCR assay, 484 (84.0%) of the 576 fecal samples tested positive for at least one of the seven serogroups, and 571 (99.1%) tested positive for at least one of the three virulence genes. Of the 10 samples that tested negative for any of the seven serogroups by three mqPCR assays, one sample was positive for O157 by cPCR. *E. coli* O103 (56.6%) was the most commonly detected serogroup by cPCR, followed by O157 (54.7%), O26 (44.4%), O121 (22.9%), O45 (17.9%), O145 (1.9%), and O111 (0.7%). Among the virulence genes, *eae* (97.4%) was predominant, followed by stx_2 (94.1%) and stx_1 (64.4%) (Table 5).

Based on the culture method, 481 (83.5%) fecal samples tested positive for at least one of the seven serogroups. *E. coli* O103 (60.2%) was the most commonly detected serogroup, followed by O157 (43.1%), O26 (22.7%), O45 (16.7%), O145 (3.0%), O121 (2.3%), and O111 (0.2%) (Table 5). Because the pooled colonies were screened by a seven-plex PCR targeting only the seven major serogroups, virulence gene detection was not part of the culture detection method.

TABLE 4. *Quantification of seven major* Escherichia coli *serogroups and three virulence genes in preenriched cattle feces using multiplex quantitative PCR assays*

Serogroup and virulence gene	Total no. (%) of negative samples	Total no. (%) of positive samples	Log CFU/g, no. (%) of samples positive			
			4–<5	5–<6	6–<7	7–<8
O26	547 (95.0)	29 (5.0)	19 (3.3)	10 (1.7)	0	0
O45	535 (92.9)	41 (7.1)	21 (3.6)	20 (3.5)	0	0
O103	535 (92.9)	41 (7.1)	27 (4.7)	14 (2.4)	0	0
O111	574 (99.7)	2 (0.3)	0	2 (0.3)	0	0
O121	539 (93.6)	37 (6.4)	21 (3.6)	16 (2.8)	0	0
O145	574 (99.7)	2 (0.3)	2 (0.3)	0	0	0
O157	516 (89.6)	66 (11.5)	21 (3.6)	38 (6.6)	7 (1.2)	0
Shiga toxin 1 (stx_1)	401 (69.6)	183 (31.8)	50 (8.7)	90 (15.6)	39 (6.8)	4 (0.7)
Shiga toxin 2 (stx_2)	435 (75.5)	134 (23.3)	31 (5.4)	88 (15.3)	15 (2.6)	0
Intimin (*eae*)	362 (62.8)	257 (44.6)	75 (13.0)	125 (21.7)	56 (9.7)	1 (0.2)

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TABLE 5. *Number of cattle fecal samples that tested positive for seven major* Escherichia coli *serogroups and three virulence genes by mqPCR, cPCR, and culture methods of detection, and agreement between tests*[a]

Serogroup and virulence gene	No. (%) of samples positive			mqPCR vs cPCR		mqPCR vs culture method	
	mqPCR	cPCR	Culture method	Kappa statistic (95% CI)	*P* value for kappa	Kappa statistic (95% CI)	*P* value for kappa
O26	340 (59.0)	256 (44.4)	131 (22.7)	0.71 (0.66–0.77)	<0.01	0.24 (0.18–0.29)	<0.01
O45	322 (55.9)	103 (17.9)	96 (16.7)	0.29 (0.24–0.35)	<0.01	0.21 (0.16–0.26)	<0.01
O103	488 (84.7)	326 (56.6)	347 (60.2)	0.38 (0.32–0.44)	<0.01	0.31 (0.24–0.38)	<0.01
O111	4 (0.7)	4 (0.7)	1 (0.2)	1.00 (1.00–1.00)	<0.01	−0.003 (−0.008–0.003)	0.53
O121	333 (57.8)	132 (22.9)	13 (2.3)	0.36 (0.30–0.41)	<0.01	0.03 (0.009–0.05)	<0.01
O145	34 (5.9)	11 (1.9)	17 (3.0)	0.47 (0.29–0.65)	<0.01	0.33 (0.16–0.50)	<0.01
O157	517 (89.8)	315 (54.7)	248 (43.1)	0.21 (0.16–0.27)	<0.01	0.12 (0.07–0.16)	<0.01
Shiga toxin 1 (stx_1)	551 (95.7)	371 (64.4)		0.12 (0.07–0.18)	<0.01		
Shiga toxin 2 (stx_2)	545 (94.6)	542 (94.1)		0.46 (0.31–0.62)	<0.01		
Intimin (*eae*)	574 (99.7)	561 (97.4)		0.11 (−0.10–0.32)	<0.01		

[a] mqPCR, multiplex quantitative PCR; cPCR, conventional PCR. $n = 576$.

The McNemar's test indicated a significant ($P < 0.01$) difference between the proportions of positive fecal samples detected by mqPCR and cPCR for all the target genes except for wzx_{O111} and stx_2. There was also a significant ($P < 0.01$) difference in the proportion of positive samples detected by mqPCR and the culture method for all target genes, except wzx_{O111}. In both cases, the kappa statistics were provided for reference only (Table 5). The receiver operating characteristic curve analysis showed that a mean C_T value of 32.3 yielded optimum sensitivity (83.3 to 100%), specificity (94.0 to 100%), and the highest number of correctly classified samples (93.0 to 100%) by cPCR in relation to mqPCR for all the seven serogroups.

Table 6 shows the number and proportion of fecal samples that tested positive or negative for each serogroup and virulence gene by three mqPCR assays, which tested positive or negative by cPCR or the culture method. Of the fecal samples that tested positive by three mqPCR assays for the seven serogroups, 0 to 68% tested negative by the cPCR, and 32.0 to 100% tested negative by the culture method, depending on the serogroup. Of the fecal samples that tested negative by the three mqPCR assays, the proportion of samples that were also negative by cPCR or culture method ranged from 83.0 to 100%. However, a few fecal samples that tested negative by mqPCR were positive by cPCR (4 for O157, 15 for stx_2, 3 for stx_1, and 1 for *eae*). Similarly, the culture method detected a few samples as positive that tested negative based on mqPCR (16 for O26, 15 for O103, 10 for O45, 8 for O145, 7 for O157, 1 for O111, and 1 for O121) (Table 6).

DISCUSSION

We have developed two multiplex qPCR assays that target serogroup-specific O-antigen genes to detect and quantify *E. coli* O26, O103, O111, O45, O121, and O145 serogroups in cattle feces. Both assays were specific to their corresponding target genes, and the detection limits of both assays in pure cultures were 3 log CFU/ml. The detection limit increased to 4 log CFU/g when fecal samples were spiked with known concentrations of pure cultures. The enrichment of spiked fecal samples in EC broth for 6 h at 40°C improved the detection limit of both assays to 2 log CFU/g feces. These two assays, in combination with the four-plex assay targeting O157 serogroup and the three major virulence genes *(30),* can be used to quantify (before enrichment) and detect (after enrichment) seven *E. coli* serogroups and three major virulence genes in cattle feces.

Real-time PCR assays have been developed for the rapid detection of non-O157 *E. coli* in food matrices *(14, 15, 24)*. Also, several commercially available real-time PCR-based detection systems have been evaluated for detection of non-O157 *E. coli* serogroups in beef and beef products *(13, 15, 37)*. Conventional PCR assays for the detection of non-O157 *E. coli* serogroups in cattle feces have been reported *(3, 6, 10, 31),* but, to date, there has been no published study on the utility of mqPCR assays to detect or quantify the six non-O157 *E. coli* serogroups in feces of naturally shedding feedlot cattle. Quantitative PCR assays targeting serogroup-specific virulence genes have been developed to detect non-O157 *E. coli* serogroups in cattle feces. Sharma *(34)* developed two multiplex qPCR assays that target a region of *eae* gene specific to O26, O111, and O157 serogroups (assay 1) and stx_1 and stx_2 (assay 2). Luedtke et al. *(27)* developed a four-plex qPCR to detect EHEC in cattle feces by targeting *ecf1,* an EHEC-specific gene, and the three major virulence genes, *eae,* stx_1, and stx_2. That assay was designed to detect EHEC but not individual serogroups of EHEC. Anklam et al. *(1)* developed four separate mqPCR assays to target O-antigen genes of seven (O26, O45, O103, O111, O121, O145, and O157) serogroups and four virulence genes (stx_1, stx_2, *eae,* and *ehxA*) in cattle feces. One assay targeted O26 (wzy_{O26}), O103 (wzx_{O103}), and O145 (wzx_{O145}); the second assay targeted O45 (wzy_{O45}), O111 ($manC_{O111}$), and O121 (wzx_{O121}); the third assay targeted O157 ($rfbE_{O157}$); and the fourth assay targeted stx_1, stx_2, *eae,* and *ehxA* (enterohemolysin). Our two mqPCR assays targeting the six non-O157 serogroups are, to some extent, similar to those of Anklam et al. *(1)*: the same genes were targeted for two serogroups (O103 and O145), but for the other four serogroups (O26, O45, O111, and O121), the targeted genes were different (wzx_{O26} instead of wzy_{O26};

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TABLE 6. *Comparison of mqPCR, cPCR, and culture method for the detection of seven* Escherichia coli *serogroups and three virulence genes in enriched fecal samples*[a]

Serogroup and virulence gene[b]	mqPCR, no. (%) positive	cPCR[c]		Culture method[c]		cPCR[d]		Culture method[d]	
		No. (%) positive	No. (%) negative	No. (%) positive	No. (%) negative	No. (%) positive	No. (%) negative	No. (%) positive	No. (%) negative
O26	340 (59.0)	256 (75.3)	84 (24.7)	115 (33.8)	225 (66.2)	236 (41.0)	0	236 (100)	16 (6.8)	220 (93.2)
O45	322 (55.9)	103 (32.0)	219 (68.0)	86 (26.7)	236 (73.3)	254 (44.1)	0	254 (100)	10 (3.9)	244 (96.1)
O103	488 (84.7)	326 (66.8)	162 (33.2)	332 (68.0)	156 (32.0)	88 (15.3)	0	88 (100)	15 (17.0)	73 (83.0)
O111	4 (0.7)	4 (100)	0	0	4 (100)	572 (99.3)	0	572 (100)	1 (0.2)	571 (99.8)
O121	333 (57.8)	132 (39.6)	201 (60.4)	12 (3.6)	321 (96.4)	243 (42.2)	0	243 (100)	1 (0.4)	242 (99.6)
O145	34 (5.9)	11 (32.4)	23 (67.6)	9 (26.5)	25 (73.5)	542 (94.1)	0	542 (100)	8 (1.5)	534 (98.5)
O157	517 (89.8)	311 (60.2)	206 (39.8)	241 (46.6)	276 (53.4)	59 (10.2)	4 (6.8)	55 (93.2)	7 (11.9)	52 (88.1)
Shiga toxin 1 (stx_1)	551 (95.7)	368 (66.8)	183 (33.2)			25 (4.3)	3 (12.0)	22 (88.0)		
Shiga toxin 2 (stx_2)	545 (94.6)	527 (96.7)	18 (3.3)			31 (5.4)	15 (48.4)	16 (51.6)		
Intimin (*eae*)	574 (99.7)	560 (97.6)	14 (2.4)			2 (0.3)	1 (50.0)	1 (50.0)		

[a] mqPCR, multiplex quantitative PCR; cPCR, conventional PCR. $n = 576$.
[b] McNemar's test indicated a significant ($P < 0.01$) difference between the proportion of positive fecal samples detected by mqPCR and cPCR for all the target genes except for wzx_{O111} and stx_2. McNemar's test indicated a significant ($P < 0.01$) difference in the proportion of positive samples detected by mqPCR and the culture method for all the target genes except for wzx_{O111}.
[c] Number of fecal samples that were positive or negative by cPCR or culture method from the samples that were positive by mqPCR for the respective serogroups and virulence genes.
[d] Number of fecal samples that were positive or negative by cPCR or culture method from the samples that were negative by mqPCR for the respective serogroups and virulence genes.

wzx_{O45} instead of wzy_{O45}; wzx_{O111} instead of $manC_{O111}$; and *wbqE* and *wbqF* instead of wzx_{O121}). We chose *wzx* genes for O26, O45, and O111 mainly because they worked better with the primers and probes of the other two O-antigen targets (O103 and O145) in the same reaction. As to O121, a *Shigella dysenteriae* strain (GenBank accession: AY380835.1) has an O-antigen gene cluster nearly identical to that of *E. coli* O121. We used minor differences in *wbqE* and *wbqF* genes to differentiate O121 from the *Shigella* strain. We used target regions of the *wzx* gene for O103 and O145 to make sure that there was no secondary structure or interference with each other; hence, the primers are different. Also, different combinations of the serogroups were included in the two assays, compared with Anklam et al. *(1)*. We did not include *ehxA* in our assay because the gene is present in many of the non–Shiga toxigenic *E. coli* pathogens *(9, 26)*. Anklam et al. *(1)* validated their assays in detecting *E. coli* serogroups and virulence genes by using pure cultures and cattle fecal samples spiked with pure cultures, but they did not evaluate the applicability of these assays to detect and quantify *E. coli* serogroups and virulence genes in feces of naturally shedding cattle. The detection limits of the assays reported by Anklam et al. *(1)* were 10^3 and 10^4 CFU/ml for pure cultures and spiked fecal samples, respectively, which improved to 10^0 after a 6-h enrichment of fecal samples. The detection limits of the pure cultures and spiked fecal samples are in agreement with the detection limits of our two assays. However, the detection limit for enriched samples was lower in their study than ours (10^0 versus 10^2), possibly because the fecal samples they used to inoculate with pure cultures were diluted 1:50 compared with the 1:10 dilution used in our assays.

To our knowledge, this is the first study to assess the applicability of mqPCR assays for detection and quantification of the six non-O157 *E. coli* serogroups in fecal samples from naturally shedding cattle. All three mqPCR assays detected more samples as positive for one or more of the serogroups compared with detection by cPCR and the culture method. McNemar's test indicated disagreement between the proportion of positive samples detected by mqPCR, cPCR, and the culture method. The disagreement between mqPCR and cPCR is explained by receiver operating characteristic curve analysis of C_T values, which indicated that cPCR is less sensitive than mqPCR. It is known that real-time PCR assay is more sensitive than conventional PCR- or culture-based testing methods *(23)*. As with any PCR assay, there is also a possibility of false positives because of amplification of DNA from nonviable cells in the feces. However, a few samples that were negative by mqPCR (16 for O26, 10 for O45, 15 for O103, 1 for O111, 1 for O121, 8 for O145, and 7 for O157) were positive by cPCR or the culture method. A likely reason for the difference could be the uneven distribution of target genes or serogroups in each aliquot that was subjected to the different methods of detection. Also, the misidentification of culture-positive samples by mqPCR is likely reflective of the difference in detection limits between the two methods. The mqPCR requires a concentration of 10^4 CFU/g for detection, whereas the culture method, which uses immunomagnetic separation beads, may randomly capture *E. coli* cells at

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lower concentrations. A similar disagreement between culture method and mqPCR assay has been reported for *E. coli* O157 in cattle feces *(18, 30)*. The number of fecal samples testing positive for intimin and Shiga toxin genes was generally higher than the number positive for the seven *E. coli* serogroups, which is likely due to the presence of STEC serogroups other than the seven targeted in this study as well as free bacteriophages carrying Shiga toxin genes in fecal samples *(8)*.

Apart from estimating the prevalence of *E. coli* serogroups and their virulence genes in cattle feces, determining their concentrations is essential for estimating the risk of foodborne illnesses associated with fecal shedding of *E. coli* serogroups and their associated virulence genes. Estimating the concentration of *E. coli* serogroups is beneficial for evaluating the efficacy of intervention strategies employed to reduce the pathogen load in feces so as to reduce hide and carcass contaminations *(2)*. Data on the concentration of non-O157 *E. coli* serogroups in cattle feces are nonexistent. Quantitative PCR has been used to quantify *E. coli* O157:H7 and virulence genes *(16, 18, 30, 36)* in cattle feces. In our study, 175 (30.4%) of 576 fecal samples were positive for at least one of the seven *E. coli* serogroups, with a concentration of $\geq$4 log CFU/g of feces. A majority of the samples (80.6%) that were quantifiable were positive for one of the seven serogroups, with *E. coli* O157 being the most common serogroup. However, an inherent limitation of the mqPCR assay, similar to cPCR, is that the presence of virulence genes cannot be directly associated to any particular serogroup in the sample.

In conclusion, the two sets of mqPCR assays are rapid diagnostic tools for the detection and quantification of six major non-O157 *E. coli* serogroups in cattle feces. These two assays, together with the four-plex assay targeting *E. coli* O157 and three virulence genes (stx_1, stx_2, and *eae*), can be used to detect and quantify seven major *E. coli* serogroups and three virulence genes in cattle feces. This is the first study to provide data on concentrations of non-O157 *E. coli* serogroups in cattle feces and to identify the existence of a subset of cattle, similar to super shedders of O157, that shed non-O157 at high concentrations.

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