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Evaluation of real time PCR assays for the detection and enumeration of enterohemorrhagic Escherichia coli directly from cattle feces

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A B S T R A C T

Shiga toxin-producing Escherichia coli are a growing concern in the area of food safety, and the United States Department of Agriculture Food Safety and Inspection Service has identified the serotypes O26, O45, O103, O111, O121, O145, and O157 as adulterants in certain types of raw beef. The most relevant to human disease are the enterohemorrhagic E. coli (EHEC) strains that possess intimin (eae), Shiga toxin 1 and/or 2 (stx1–2), and in most cases the conserved pO157 or pO157 like virulence plasmid. Contamination of raw beef with EHEC is likely to occur via the transfer of cattle feces on hides to the carcass. To detect EHEC directly from cattle feces, we evaluated the utility of a multiplex real time PCR assay that targets the EHEC associated gene target eae and stx1–2. Our assay had an increased sensitivity and provided a reliable limit of detection (LOD) of 1.25 × 10^3 colony-forming units per mL (CFUs/mL) in an EHEC spiked fecal background. In addition, we evaluated the use of a duplex qPCR assay using ecf1 for the enumeration of total EHEC directly from cattle feces. The reliable limit of quantification (LQ) was determined to be 1.25 × 10^3 CFUs/mL. Our assay requires minimal sample processing and provides LOD and LOQ of EHEC directly from cattle feces that are the lowest reported. The application of this assay towards the identification of cattle shedding EHEC at a level above 1.25 × 10^3 CFUs/mL could be a first line of defense in identifying cattle shedding these pathogens.

1. Introduction

The contamination of food products by Shiga toxin-producing Escherichia coli (STEC) is a worldwide problem and can result in outbreaks of human disease (Mathusa et al., 2010). In most outbreaks, human illness is attributed to one of the top 7 STEC serotypes, O26, O45, O103, O111, O121, O145, and O157 (Gyles, 2007), while sporadic cases of other non-top 7 serotypes have been observed (Buchholz et al., 2011). The degree of illness can range from low grade fever, vomiting, and abdominal pain with nonbloody or bloody diarrhea. Children under 10 and the elderly are the most likely to develop hemorrhagic colitis and/or hemolytic uremic syndrome, which can be fatal (Goldwater and Bettelheim, 2012). Transmission of STEC occurs via the fecal oral route and can be spread zoonotically and from person to person (Erickson and Doyle, 2007).

In the environment, wild and domestic animals are the primary reservoir of STEC while domesticated ruminants have the highest association with contributing to human disease (Mathusa et al., 2010). A recent concern in the beef industry is the effect that super shedding cattle have in food safety. Super shedding cattle are defined as releasing >10^5 STEC CFUs/g of feces (Matthews et al., 2006; Menrath et al., 2010). Interestingly, it has been shown that 20% of super shedding cattle in a herd can be accountable for transmission of STEC O157:H7 to 80% of the herd (Matthews et al., 2006) while cattle contained in pens absent of a super shedder were found to be five times less likely to shed STEC O157:H7 (Cobbold et al., 2007). Moreover, super shedding cattle have a high propensity for the cross contamination of hides in the pre-harvest environment, and it was suggested to keep herd prevalence below 20% and the fecal shedding of STEC O157:H7 below the high shedding level of 200 CFU/g to minimize carcass contamination at harvest (Arthur et al., 2009). Although not as well studied, non-O157 STEC are likely to follow a similar trend (Menrath et al., 2010), and recently the United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) has deemed the top 6 non-O157 STEC as adulterants in non-intact beef (Almanza, 2011). Further control of spreading is thought to be attainable if colonization is decreased by 5% amongst super shedding cattle (Matthews et al., 2006). However, a cost efficient and rapid quantitative detection method to identify cattle shedding the top 7 and non-top 7 STEC serotypes directly from cattle feces is currently not available.

The detection of STEC from feces has classically been performed using enrichment cultures with or without serotype specific immunomagnetic bead separation prior to plating on selective media followed by PCR confirmation (Jacob et al., 2010; Walker et al., 2010) while the enumeration of STEC has been performed using most probable number determination
(Fox et al., 2007) and direct or spiral plating (Arthur et al., 2009). Specific molecular targets have been established for the detection and genetic characterization of STEC (Paton and Paton, 1999; Wasilenko et al., 2012), but the use of molecular methods to enumerate STEC directly from fecal samples has been limited. The current detection and enumeration methods for STEC from fecal samples use a combination of genetic targets stx1, stx2, eae, uidA, rfbE, and fliC alleles in real time PCR (qPCR) assays (Jacob et al., 2012; Jinneman et al., 2003; Sharma and Dean-Nystrom, 2003). These markers have been used primarily to detect and enumerate O157:H7 or a subset of non-O157:H7 serotypes. Moreover, some reports have shown an inability to discriminate between stx1, stx2, and eae of non-O157 STEC serotypes (Ibekwe et al., 2002; Jacob et al., 2012). This lack of discrimination could lead to the detection of false positives and inflate the estimation of O157:H7 CFUs/g in cattle co-colonized with O157 non-H7, and/or a non-O157 STEC, and/or background microflora. To detect and assess the total STEC load from cattle fecal samples, with relevance towards human pathogenesis, the subgroup of STEC classified as enterohemorrhagic E. coli (EHEC), which possesses eae, stx, and in 90% to 99% of isolates the virulence plasmid encoded enterohemolysin A (exha) (Lorenz et al., 2013) would be a valuable target.

Here we evaluated the use of the EHEC specific target E. coli attaching and effacing gene-positive conserved fragment 1 (ecf1), which is conserved on the virulence plasmid pO157 and pO157 like plasmids (Boerlin et al., 1998; Ogura et al., 2009; Groschel and Becker, 2013), in multiplex qPCR with eae, stx1, and stx2 targets and in duplex qPCR with eae for the detection and enumeration of EHEC directly from cattle feces, respectively. In addition, we evaluated the use of three commercial master mixes to identify a DNA polymerase that is insensitive to PCR inhibitors commonly found in feces and capable of multiplexing. We identified a master mix that had high sensitivity and a reliable limit of detection (LOD) of 1.25 × 10^3 CFUs/mL in a multiplex assay and had a reliable limit of quantification (LOQ) of 1.25 × 10^2 CFUs/mL in a duplex reaction. Moreover, the inclusion of ecf1 as a target in a multiplex detection would limit EHEC false positives due to samples containing separate organisms possessing either eae or stx and provide for the detection of atypical EPECs, which retain the pO157 or pO157 like plasmid but have lost stx during the culturing process (Bielaszewska et al., 2007). By using this qPCR protocol, we eliminated the need to perform time-consuming enrichment steps or extensive DNA purification procedures that can result in the loss of template. To our knowledge, this is the first study to describe the direct detection and enumeration of EHEC loads in cattle feces.

2. Materials and methods

2.1. Standard curve development and fecal samples

Standard curves were constructed using the E. coli O157:H7 reference strain EDL 932 (ATCC 43894), which was grown from a freezer stock in Luria–Bertani (LB) broth overnight at 37 °C overnight. A 1 mL aliquot was then harvested by centrifugation and washed once with phosphate buffered saline (PBS). The pellet was resuspended in 1 mL of PBS. Serial dilutions of the aliquot were made using PBS and a dilution providing a countable number of colony-forming units (CFUs) was plated in quadruplicate on aerobic plate count Petrifilm™ (3M Microbiology, St. Paul, MN) and grown at 37 °C overnight prior to enumeration using a Petrifilm® reader. Concurrently with the Petrifilm dilutions, a 10-fold dilution of the culture was made using BAX® system lysis buffer (DuPont, Wilmington, DE). To liberate the template DNA, cells were lysed and proteins were degraded at 37 °C for 20 min using the BAX® system protease. The BAX® system protease was inactivated by heating to 95 °C for 10 min. Further 10-fold dilutions were made in inactivated BAX® system lysis buffer. In addition, cattle feces were collected by rectal–anl mucosal swabs (RAMS) and suspended in 5 mL phosphate buffered Tryptic Soy Broth (pTBS) (Arthur et al., 2009) then diluted in BAX® system lysis buffer (Fig. 1). qPCR was used to identify fecal samples that were negative for stx, eae, and ecf1. Negative fecal samples were pooled and used as diluent for the construction of a six log standard curve. All standard curves were stored at −20 °C in single use aliquots.

Additional cattle fecal samples were collected by RAMS. After sampling, RAMS were placed in sterile 15 mL conical tubes containing 5 mL of pTBS and stored on ice until returning to the laboratory. A portion of the resuspended fecal sample was processed using the BAX® lysis n as described above and stored at −20 °C (Fig. 1). The RAMS tube was then incubated at 42 °C for 12 h to enrich for EHEC. After enrichment of the sample, a 1 mL portion was removed and prepared in a Roka G2 Sample Transfer Tube (Roka Biosciences, San Diego, CA) and then submitted to Roka Biosciences laboratory for analysis to identify samples that were positive for ecf1. A second 1 mL portion was used to generate a DNA boil lysis and screened for the presence of stx, eae, and any of the top 7 serogroups according to established protocols (Boislevac and Koohmaraie, 2012). Glycerol was added to the remainder of the bacterial enrichment and stored at −20 °C. Samples that were indicative of a top 7 EHEC were thawed and processed for immunomagnetic separation using magnetic beads conjugated with antibodies against a specific serogroup (Romler Labs, Newark, DE). Samples containing the respective magnetic beads were shaken at room temperature for 15 min prior to the automated processing using a King-Fisher 96 magnetic separator as previously described (Boislevac et al., 2009). The immuno-separated samples were diluted for plating on washed sheep blood agar containing 0.5 mg/L mitomycin C (Sugiyama et al., 2007).
et al., 2001) and STEC Differential Agar (Kalchayanand et al., 2013) using an Autoplate 4000 spiral plater (Advanced Instruments, Norwood MA). Plates were incubated overnight at 37 °C. Individual colonies were picked into 96 well blocks containing TSB and incubated at 37 °C overnight. All isolates were screened by PCR for serotype and genes associated with EHEC. Isolates possessing eae and stx, regardless of serotype, were suspended in 12.5% glycerol and stored at −20 °C. Select fecal samples with confirmed EHEC were used for additional qPCR analyses.

2.2. Evaluation of qPCR master mixes in multiplex reactions

The multiplex qPCR assays were performed on the EDL 932 standard curves developed using the BAX® system lysis buffer with an EHEC negative active cattle fecal background and five selected field samples of cattle feces that had characterized EHEC isolates. Samples were run in triplicate and no template controls run in duplicate 25 μl reactions that consisted of 12.5 μl of either TaqMan® Environmental Master Mix 2.0 (Applied Biosystems® by Life Technologies, Carlsbad, CA), GoTaq® Probe qPCR master mix with the addition of carboxy-X-rhodamine at 30 nM (Promega, Madison, WI), or PerfeCTa® qPCR ToughMix® master mix (Quanta Biosciences, Gaithersburg, MD), primers and probes targeting eae, ecf1, and stx1 and stx2 were used at the final concentrations indicated in Table 1, and 8 μl of template DNA (Fig. 1). For multiplex assays, the fluorescent dye on the ecf1 probe was Cy5. The maximum volume of template DNA in a fecal background was empirically determined, using the PerfeCTa® qPCR ToughMix® master mix. A 96-well plate format was used for all assays and run using an ABI 7500 Fast Real-Time PCR system with version 2.0.6 software (Applied Biosystems® by Life Technologies). Cycling conditions were 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 99 °C for 1 min as described in the USDA FSIS MLG 5B Appendix 1.01 protocol (USDA, 2012). A quantification cycle (Cq) threshold of 0.2 was manually set for all gene targets after an automatic adjustment of the baseline. The PCR efficiencies and correlation coefficients were evaluated using the trend line created from the standard curve, which was generated using MS Excel 2007 (Microsoft, Redmond, WA).

2.3. Evaluation of ecf1 for enumeration of EHEC

To address variations in the copy number of pO157 and pO157 like plasmids between serotypes, isolates recovered from cattle feces of the top 7 serotypes were used to create template DNA using the BAX system lysis buffer. The resulting template DNA from six O26, four O45, six O103, five O111, one O121, six O145, and six O157 serotypes was used in duplicate duplex qPCR reactions targeting the plasmid encoded gene ecf1 and the single copy number chromosomal gene eae. Duplex qPCR reactions were performed as described previously in this study. Plasmid copy number was determined using a relative quantification method as previously described (Skulj et al., 2008). In addition, direct sequencing data was analyzed to determine the pO157 and pO157 like copy number from 37 EHEC isolates, which included eight O26, two O45, five O111, five O103, five O121, three O145, three O157, one O118, one O123, and one O186. Plasmid copy numbers were determined by comparing the chromosome sequence coverage to the plasmid sequence coverage as previously described (Rasko et al., 2007).

The delta Cq (ΔCq) between eae and ecf1 in duplex and in simplex qPCR assays over a five log standard curve of the EDL 932 reference strain DNA was compared to determine the changes in the ΔCq due to differences in fluorescent intensity of FAM. Simplex qPCR assays were performed in triplicate 25 μl reactions containing 12.5 μl of the Power SYBR® Green (Applied Biosystems® by Life Technologies), 0.5 μM of each primer set, eae98 and ecf1, 8 μl of template DNA, and 3.5 μl of PCR grade H2O. Duplex qPCR assays were composed as indicated here and used similar amounts of the same DNA preparation. Real-time reactions were run and analyzed as previously described here.

Duplex qPCR reactions targeting ecf1 and eae were performed in triplicate 25 μl reactions containing 12.5 μl of the PerfeCTa® qPCR ToughMix® master mix, the indicated final concentration of primers and probes (Table 1), 8 μl of template DNA (Fig. 1), and 1.5 μl of PCR grade H2O. For duplex assays, the ecf1 probe was labeled with FAM. The EDL 932 reference strain was used for template DNA in the standard curves made with a BAX® system lysis buffer with or without a cattle feces background. Five selected field samples of cattle feces that had characterized EHEC isolates were used for enumeration. The plate format, cycling conditions using the ABI 7500 Fast Real-Time PCR system, determination of Cq threshold, and calculation of PCR efficiencies and correlation coefficients were as previously stated.

2.4. Statistics

All statistical tests were performed using the SigmaStat 3.1 software (Systat Software, San Jose CA). Statistical significance between the resulting Cq values at each dilution for the three master mixes was determined using a one-way ANOVA and the Holm–Šidák post hoc test. A paired T-test was used to detect a statistical significance between the Cq values at each dilution for the three master mixes.

Table 1

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Sequence</th>
<th>Final concentration (μM)</th>
<th>Location within sequence</th>
<th>GenBank accession</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>eae-Pr</td>
<td>5’-MAXN/ATAGTCTGGCAGATATTCGCCAAATACC/3BbFQ/-3’</td>
<td>0.2</td>
<td>4,394,309–4,394,338</td>
<td>CP003109</td>
<td>Wasilenko et al. (2012)</td>
</tr>
<tr>
<td>eae-F</td>
<td>5’-CATTGACAGAAGTTTTCTTGTGATA-3’</td>
<td>1.0</td>
<td>4,394,375–4,394,350</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eae-R</td>
<td>5’-CTATGCGAGAATACCTGTGTM-3’</td>
<td>1.0</td>
<td>4,394,274–4,394,294</td>
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<td></td>
</tr>
<tr>
<td>ecf1B-F</td>
<td>5’-CAATGATGCTGCTGGCACG-3’</td>
<td>0.25</td>
<td>4,666,080–4,666,099</td>
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<td></td>
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<tr>
<td>ecf1B-R</td>
<td>5’-AGCTGGTTAATACCTGACCC-3’</td>
<td>0.5</td>
<td>4,666,002–4,666,021</td>
<td></td>
<td></td>
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<tr>
<td>ecf1F</td>
<td>5’-/FAM/AAGGCGCTTTCGATGAAGCCGGAAA/3BbFQ/-3’</td>
<td>0.5</td>
<td>18,692–18,717</td>
<td>AE005174</td>
<td>This study</td>
</tr>
<tr>
<td>stx1-F</td>
<td>5’-TTCGAAGGAACGACGAACAG-3’</td>
<td>0.25</td>
<td>18,692–18,717</td>
<td></td>
<td></td>
</tr>
<tr>
<td>stx1-R</td>
<td>5’-CCTTCAAGACGCGCTGACCGA-3’</td>
<td>1.0</td>
<td>18,668–18,691</td>
<td></td>
<td></td>
</tr>
<tr>
<td>stx2-F</td>
<td>5’-CTGCGCTAGCTGAAGCGAAGGGAC-3’</td>
<td>0.25</td>
<td>18,742–18,762</td>
<td></td>
<td></td>
</tr>
<tr>
<td>stx2-R</td>
<td>5’-CCTTCAAGACGCGCTGACCGA-3’</td>
<td>1.0</td>
<td>18,766–18,782</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Pr = Probe, F = Forward, R = Reverse.

a Degenerate nucleotide codons as follows: Y (C, T), W (A, T), R (A, G), M (A, C), D (A, G, T), and S (C, G).

b Used for SYBR Green based qPCR.

† Location within sequence for stx1.

‡ Location within sequence for stx2.
values at each dilution for eae and ecf1 in reactions with a pooled fecal background and BAX® system lysis buffer alone.

3. Results

3.1. Performance of three qPCR master mixes in multiplex assays using a cattle fecal background

The performance of three commercial qPCR master mixes was evaluated in a multiplex qPCR reaction using the E. coli O157:H7 EDL 932 reference strain in feces. To increase the diversity of the complex fecal background for the reactions, 16 cattle fecal samples, suspended in BAX® system lysis buffer, were found by qPCR to be negative for eae, ecf1, stx1, and stx2. These negative samples were pooled and used for a six log dilution series with the initial spiking amount being equivalent to 1.25 × 10^7 EDL 932 CFUs/mL. The reliable LOD for the GoTaq® Probe qPCR master mix was 1.25 × 10^4 CFUs/mL for eae and 1.25 × 10^3 CFUs/mL for ecf1 and stx1–2 (Fig. 2B) while the TaqMan® Environmental Master Mix 2.0 (Fig. 2A) and PerfeCTa® qPCR ToughMix® (Fig. 2C) master mix had a reliable detection limit of 1.25 × 10^3 CFUs/mL for each of the targets. Template was detectable for all targets at 1.25 × 10^2 CFUs/mL using the TaqMan® Environmental Master Mix 2.0 and PerfeCTa® qPCR ToughMix® master mix, but not all replicates had a detectable level of template and were not considered as a reliable LOD (Table 2). In addition, the sensitivity based on the Cq when the fluorescence of each dye was above that of the background was significantly different between each of the master mixes (p < 0.05), with the PerfeCTa® qPCR ToughMix® master mix returning the lowest Cq values for each target at each dilution (Table 2). The efficiency and correlation coefficient of each target for each of the master mixes was calculated using the 1.25 × 10^7 to 1.25 × 10^3 dilution range. The PCR efficiencies for each of the targets using the PerfeCTa® qPCR ToughMix® master mix ranged from 103 to 108% and were in the acceptable efficiency range of 100 ± 10%. Using the TaqMan® Environmental Master Mix 2.0 provided amplification efficiencies that ranged from 112 to 120% and were above the acceptable efficiency range. The amplification efficiency for eae using the GoTaq® Probe qPCR master mix could not be calculated since eae was not detectable at the 1.25 × 10^3 dilution, however ecf1 was in the acceptable range at 110% while stx1–2 was below the acceptable range at 86%. Where able to calculate, the correlation coefficient was > 0.99 for each of the targets regardless of the master mix used. All no template controls for the targets were consistently negative for each master mix.

Individual field samples of cattle feces suspended in the BAX® system lysis buffer were used in multiplex qPCR reactions to evaluate the three master mixes. Five fecal samples (S1336, S1337, S1346, S1352, and S2089) were all found to be positive for eae, ecf1, and stx1–2. Each was confirmed to contain an EHEC: an O26 EHEC in S1336, O26 and O157 EHEC in S1337, an O103 EHEC in S1346, an O157 EHEC in S1352, and an O177 EHEC in S2089 (data not shown). For each of the gene targets in all of the field samples, the PerfeCTa® qPCR ToughMix® master mix returned the lowest Cq values followed by the GoTaq® Probe qPCR master mix and TaqMan® Environmental Master Mix 2.0, respectively (Table 3). In addition, sample S1337 was consistent for the detection of eae and ecf1 while stx1–2 was not detectable amongst the master mixes.

3.2. Evaluation of ecf1 for enumeration of EHEC in cattle feces

To increase the fluorescent intensity of the ecf1 probe, the dye label FAM was used in place of CY5 for duplex qPCR reactions. Previous qPCR reactions using the CY5 labeled probe returned Cq values that were approximately 1 to 2 Cq values higher than the FAM labeled probe (data not shown). Probes labeled with FAM and MAXN are commonly used in duplex reactions for compatible fluorescent signals that possess similar intensities. Using this duplex qPCR strategy, the average plasmid copy number was determined using isolates, obtained from cattle feces, of the top 7 serotypes. Amongst the top 7 serotype isolates, the average copy number of the plasmid ranged from approximately 5 to 3 copies per genome with an overall average copy number across the top 7 of approximately 4 (Supplementary Table 1). The respective PCR reaction efficiency for eae and ecf1 was 93% and 95% and the correlation coefficient for both genes was > 0.99. Using direct sequence analysis the plasmid copy number for the 37 isolates ranged from approximately 1 to 2.5 copies per genome with an average of 2 copies (Supplementary Table 2). Additional changes in the ΔCq due to different dye intensities, which would affect gene copy number determination, were tested for by using the same concentration of EDL 932 template DNA using Power SYBR® Green master mix in simplex reactions targeting eae and ecf1 and duplex reactions using PerfeCTa® qPCR ToughMix® master mix with FAM labeled ecf1 probe and MAXN labeled eae probe. The EDL 932 reference strain was determined to have approximately 2 copies of the plasmid per genome while the duplex reaction estimated approximately 4 copies. The PCR reaction efficiency over a 5 log curve (1.25 × 10^8 CFUs/mL to 1.25 × 10^3 CFUs/mL) using the Power SYBR® Green master mix was 94% for eae and 90% for ecf1 and the duplex reactions had an efficiency of 95% for eae and 94% for ecf1 while the correlation coefficient for Fig. 2. Comparison of three commercial qPCR master mixes for the multiplexed limit of detection of E. coli O157:H7 strain EDL 932 genomic DNA using the qPCR targets eae, ecf1, and stx1–2 over an identical 5 log standard curve with a pooled fecal background. (A) Standard curve for the use of the TaqMan® Environmental Master Mix 2.0 over 5 orders of magnitude for each gene target. (B) Standard curve for the use of the GoTaq® Probe qPCR master mix over 5 orders of magnitude for each gene target. (C) Standard curve for the use of the PerfeCTa® qPCR ToughMix® master mix over 5 orders of magnitude for each gene target.
Table 2

<table>
<thead>
<tr>
<th>log CFUs/mL</th>
<th>eae</th>
<th>ecf1</th>
<th>stx1</th>
<th>stx2f</th>
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<tbody>
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<td>7.10</td>
<td>27.82 ± 0.02 (3/3)</td>
<td>27.56 ± 0.01 (3/3)</td>
<td>27.33 ± 0.00 (3/3)</td>
<td>27.27 ± 0.01 (3/3)</td>
</tr>
<tr>
<td>6.10</td>
<td>31.12 ± 0.02 (3/3)</td>
<td>29.94 ± 0.01 (3/3)</td>
<td>30.48 ± 0.01 (3/3)</td>
<td>30.52 ± 0.01 (3/3)</td>
</tr>
<tr>
<td>5.10</td>
<td>34.04 ± 0.02 (3/3)</td>
<td>32.82 ± 0.01 (3/3)</td>
<td>33.38 ± 0.01 (3/3)</td>
<td>34.17 ± 0.01 (3/3)</td>
</tr>
<tr>
<td>4.10</td>
<td>37.17 ± 0.02 (3/3)</td>
<td>35.85 ± 0.01 (3/3)</td>
<td>36.61 ± 0.01 (3/3)</td>
<td>40.37 ± 0.01 (3/3)</td>
</tr>
<tr>
<td>2.10</td>
<td>43.18 ± 0.02 (2/3)</td>
<td>42.02 ± 0.01 (3/3)</td>
<td>43.02 ± 0.01 (3/3)</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND = No Detection.

1.25 × 10^3 CFUs/mL and the respective PCR efficiency for eae and ecf1 was 93% and 97% with a >0.99 correlation coefficient. In addition, at each dilution, there was no significant difference (p > 0.05) between the Cq values for eae and ecf1 using the BAX® system lysis buffer with or without a fecal background (Table 4).

Five additional fecal samples (S0028, S1476, S2003, S3218, and S6414) were found to be positive for eae and ecf1 and the EHEC serotypes Ount, O45, O145, O121, and O26 and O111, which were cultured and PCR confirmed from the respective samples (data not shown). These samples were used in the duplex qPCR assay to enumerate the total EHEC load. Table 5 displays the average Cq and CFUs/ml for eae and ecf1 for the five fecal samples. Each of the samples returned Cq values for eae and ecf1 in each of the 3 replicates except for S1476, in which eae could not be detected and ecf1 returned Cq values in 2 of the 3 replicates. In addition, the samples S0028 and S1476 and S2003 had an enumerable total EHEC load but were below the reliable LOQ for ecf1 while samples S0028 and S1476 were enumerable but below the reliable LOQ for eae. Using ecf1 and eae to enumerate the EHEC load provided a similar estimation of CFUs/ml in samples S0028, S3218, and S6414, while eae returned more than a log_{10} higher estimation than ecf1 in sample S2003. The respective PCR efficiency, over a six log standard curve (1.25 × 10^8 to 1.25 × 10^3 CFUs/ml), for eae and ecf1 was 90 and 96% while the correlation coefficient was >0.99 for both targets.

4. Discussion

Real time PCR is a rapid and sensitive diagnostic tool that can be used for the detection and quantification of pathogens. In the area of food safety, the transmission of pathogenic EHEC serotypes via fecal contamination is a growing concern. Current strategies for EHEC detection and quantification primarily involve targeting the O157:H7 serotype from various sources, yet non-O157 EHEC associated with food contamination and human disease is increasing. Here we investigated the utility of multiplex and duplex qPCR assays for the detection and enumeration of total EHEC directly from cattle feces, respectively.

For the multiplex qPCR assay, the gene targets eae, ecf1, and stx1–2 were selected to provide a non-discriminatory detection of STEC with a primary focus on EHEC in cattle feces. This wide detection range is due to the ability of the primers and probes used for eae and stx1–2 to amplify all known subtypes of eae and all subtypes of stx except for stx2f (Wasilenko et al., 2012). To focus the detection towards the EHEC subset of STEC, we chose to include the EHEC marker ecf1, which is encoded on the highly conserved pO157 and pO157 like plasmids (Lim et al., 2010). Amongst a culture collection and field samples composed of the top 7 and non-top 7 E. coli, K.W. Livezey (personal communication) and Groschel and Becker (2013) have indicated strong specificity and association of ecf1 with E. coli possessing eae, stx1–2, and exha, which further supports the initial observations by Boerlin et al. (1998) linking these virulence plasmids with EHEC. However, in 12 of the 231 top 7 culture collection isolates possessing eae and stx1–2, ecf1 was not detectable. Nine of the 12 non-O157 top 6 isolates lacking ecf1 apparently lost the pO157 like plasmid while the other 3 non-O157 isolates retained the plasmid but lost the coding region for ecf1 and other genes (K.W. Livezey, personal communication). A spontaneous loss of pO157 like plasmids and the 75 kb O104:H4 virulence plasmid,
pAA, has been observed and attributed to culturing conditions (Grad et al., 2013; Wieler et al., 1996). Culturing and environmental conditions have also been associated with the spontaneous loss of stx (Bielaszewska et al., 2007; Karch et al., 1992). Interestingly, it has been shown that isogenic EHECs that have lost stx are capable of regaining stx via phage transduction (Bielaszewska et al., 2007) while pO157 is suggested to be nonconjugative (Lim et al., 2010). The gene encoding eae has not been shown to be spontaneously lost during culturing nor is it known if the virulence plasmid and eae are linked (Bielaszewska et al., 2007; Karch et al., 1992). Interestingly, it has been shown that isogenic EHECs that have lost stx are capable of regaining stx via phage transduction (Bielaszewska et al., 2007) while pO157 is suggested to be nonconjugative (Lim et al., 2010).

A recent study found the genes Z2098 and Z2099 to be primarily associated with EHEC but both markers had a varied detection range of 67.6% to 94.9% for Z2098 and 78.6% to 96.8% for Z2099 of the top 7 associated with EHEC but both markers had a varied detection range. To overcome the limitations of an individual marker a combination of targets for multiplex qPCR is ideal for the detection of EHEC (Delannoy et al., 2013a; 2013b), which may lead to false positives in complex environmental samples. Since the presence of infectious stx-converting bacteriophages (EPEC) is associated with STEC with eae, which further decreases the chance for false positives. However, the potential for the loss of ecfI or stx1–2 during culturing indicates a need for a sensitive assay with minimal enrichment and subculturing.

Our assay is based on the FSIS MLG 5B Appendix 1.01 protocol (USDA, 2012), but FSIS recently supported the use of the BAX® System Real-Time PCR STEC Suite (USDA, 2013) and made the MLG 5B Appendix 1.01 and 1.03 protocols an alternative method for STEC detection. In both methods a 15 to 24 h enrichment step is required. With the alternative protocol an extensive DNA extraction process is required while the BAX® system utilizes a lysis buffer, which is described here. By using the BAX® system lysis buffer, a sample can be directly added so template DNA is not lost or mechanically damaged during the purification process, which can occur with different extraction procedures (Yuan et al., 2012). However, the direct lysis of an environmental field sample could introduce qPCR inhibitors into the reaction and would be indicated by an increased PCR efficiency (Kavanagh et al., 2011). A decrease in PCR efficiency due to the competition for shared reagents can be observed in multiplex qPCR reactions. Both PCR inhibition and reagent competition would lead to a decreased sensitivity and reliability of the reaction. In addition, the proprietary composition of commercial master mixes can result in significant differences in the resistance to PCR inhibitors and the sensitivity of the reaction (Morgan et al., 2012).

From our evaluation of three commercial master mixes, which were indicated by the manufacturers to be insensitive to PCR inhibitors, it was found that the PerfeCta® qPCR ToughMix® master mix provided the most sensitive detection and acceptable PCR efficiency of each target using spiked BAX® system lysis buffer with a complex fecal background. With the increased sensitivity, the reliable LOD of 1.25 × 102 CFU/mL was reached in less than 36 cycles. To save run time, it would be possible to decrease the number of cycles indicated by the FSIS method from 45 to 40. The PerfeCta® qPCR ToughMix® master mix also returned the lowest Cq values for each of the targets from five field samples. Interestingly, the field sample S1337 was consistently negative between the three master mixes for stx1–2 despite having detectable amounts of eae and ecfI and culture confirmed O26 and O157 EHEC isolates. Sample S1337 could have stx1–2 copies below the detectable level while the combined amount of eae and ecfI from the O26 and O157 could be maintained above the LOD. In addition, K.W. Livezey (personal communication) has found isolates that are positive for eae and ecfI but lacked stx1–2 and the typical enteropathogenic E. coli (EPEC) marker bundle-forming pili (bfpA), which suggests that these isolates are atypical EPECs. With the presence of infectious stx-converting bacteriophages in the environment, including ground beef, it cannot be overlooked that atypical EPECs could regain stx under favorable conditions and cause disease (Martinez-Castillo et al., 2013). This linkage of ecfI with EHEC and atypical EPECs further highlights the utility of our multiplex qPCR assay not to limit positive samples to those possessing a combination of eae, stx1–2, and a targeted serotype, which could cause false positives or misidentify a potential EHEC as an EPEC. However, fecal samples with detectable amounts of eae and stx1–2 should be further investigated for possible EHEC as the ecfI containing virulence plasmid could have been lost in the environment or the strain(s) did not possess the plasmid.

With the ability to directly detect ecfI in cattle feces, we investigated the use of qPCR to enumerate EHEC directly from cattle feces. Since stx1–2 has an increased propensity over eae to be associated with background microflora (Renter et al., 2005) and can be transiently lost in the environment (Bielaszewska et al., 2007), we chose to target eae and ecfI in a duplex qPCR reaction. Since ecfI resides on a plasmid the plasmid copy number could affect the EHEC enumeration. In addition, little is known about the plasmid copy number of pO157 and pO157 like plasmids between serogroups. Amongst the 34 top 7 serotypes, we found that the plasmid copy number ranged from 5 to 3 copies with a mean of 4 copies per genome by using our duplex qPCR assay. However, by using direct sequencing analysis of another set of EHEC isolates, we
found the plasmid copy number to range from approximately 2.5 to 1 copies per genome with an average of 2 copies. This difference in plasmid copy number estimation between qPCR and direct sequencing was further investigated using a SYBR Green based qPCR assay. The SYBR Green based assay suggested a difference in the fluorescent intensity between FAM and MAXN, which would explain why the 34 top 7 serotypes had double the estimated plasmid copy numbers compared to direct sequencing analysis and SYBR Green based qPCR. Our observation for plasmid copy number derived from direct sequencing analysis and SYBR Green based qPCR agrees with the recent determination of the \( E. coli \) O157:H7 strain Xu Zhou21 having approximately 2 copies of the \( p \)O157 (Zhao et al., 2013). Moreover, Straub et al. (2013) showed a significant under-estimation of plasmid copy numbers of the Bacillus anthracis \( pXO1 \) and \( pXO2 \) plasmids using TaqMan based qPCR compared to digital PCR and direct sequencing analysis, which were similar. Since the EDLS92 reference strain was found to possess approximately two \( p \)O157 plasmids per chromosome, which was found to be the average across the analyzed EHEC isolates, we deemed it acceptable for the generation of a standard curve to enumerate the EHEC load in cattle feces. In addition, since we used a direct lysis of the fecal sample as a template, a standard curve constructed from spiking a known amount of template into a target negative fecal background could be challenging to procure. To overcome this, we found that the use of BAX\(^\circ\) system lysis buffer without a fecal background was not significantly different (\( p > 0.05 \)), at any of the dilution points, than a standard curve prepared using a template spiked into a fecal background. Using FAM in place of Cy5 for \( ecf1 \) detection did decrease the returned \( Cq \) value, but the reliable LOQ was not lowered. The decrease in \( Cq \) value was likely due to differences in fluorescent intensity between Cy5 and FAM while a stochastic effect at the most dilute concentration of template did not lower the reliable LOQ. Our reliable LOQ of \( 1.25 \times 10^8 \) CFUs/mL is the lowest reported for EHEC quantification from cattle feces without using an enrichment and/or DNA purification protocols (Ilbeke et al., 2002; Jacob et al., 2012; Sharma and Dean-Nystrom, 2003). Current methods using only eae or stx could cause an over estimation of EHEC CFUs since eae is not specific to EHEC, as observed in sample S2003, and since stx may present in numerous background flora that can possess multiple alleles of the gene (Renter et al., 2005). However, since \( ecf1 \) is not specific to a single serotype the application of this assay to identify super shedding cattle should be caution as samples could be colonized by more than one EHEC serotype as it is not known if two or more EHEC serotypes individually shed below \( 10^6 \) CFUs/g but collectively shed at levels greater than \( 10^6 \) CFUs/g would constitute the animal as a super shedder. The population dynamics of multiple EHEC serotypes in individual cattle fecal samples is not known, and using \( ecf1 \) solely to quantify EHEC from fecal samples can only represent the total EHEC load. How the total EHEC load in cattle feces relates to downstream contamination and pathogenesis is not clear, but with the low infectious dose of 10 to 100 cells for O157:H7 and a speculated comparable amount for non-O157 signify the importance of monitoring cattle for shedding of high amounts of EHEC prior to harvesting (Pihlakala et al., 2012).

In conclusion, this combination of gene targets differentiates our assay from other qPCR detection protocols that target specific serotypes and/or relies on virulence associated genes, which may not be jointly possessed by the target organism (Jacob et al., 2012). Using our multiplex qPCR assay, which does not target a specific EHEC serotype, we were able to reliably detect eae, \( ecf1 \), and \( stx1-2 \) at a LOD comparable to \( 1.25 \times 10^3 \) CFUs/mL. Moreover, we were able to enumerate total EHEC with a similar LOQ to the LOD. To our knowledge, this is the lowest reported LOD and LOQ, using qPCR, for the detection and enumeration of EHEC from cattle feces without enrichment and/or DNA extraction. These attributes make this protocol applicable for high-throughput and rapid analysis of cattle feces for EHEC and EHEC levels.

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**Appendix A: Supplementary data**

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2014.07.015.

**References**


