ESCHERICHIA COLI O157: DETECTION AND QUANTIFICATION IN CATTLE FECES BY QUANTITATIVE PCR, CONVENTIONAL PCR, AND CULTURE METHODS

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Abstract

Shiga toxin-producing *E. coli* O157 is a major foodborne pathogen. The organism colonizes the hindgut of cattle and is shed in the feces, which serves as a source of contamination of food. Generally, cattle shed *E. coli* O157 at low concentrations ($\leq 10^2$ CFU/g), but a subset of cattle, known as "super-shedders", shed high concentrations ($>10^3$ CFU/g) and are responsible for increased transmission between animals and subsequent hide and carcass contamination. Therefore, concentration data are an important component of quantitative microbial risk assessment. A four-plex quantitative PCR (mqPCR) targeting *rfb*E_{O157}, *stx*1, *stx*2 and *eae* was developed and validated to detect and quantify *E. coli* O157 in cattle feces. Additionally, the applicability of the assay to detect *E. coli* O157 was compared to conventional PCR (cPCR) targeting the same four genes, and a culture method. Specificity of the assay to differentially detect the four genes was confirmed. In cattle feces spiked with pure cultures, detection limits were 2.8×10^4 and 2.8×10^0 CFU/g before and after enrichment, respectively. Detection of *E. coli* O157 in feedlot cattle fecal samples (n=278) was compared between mqPCR, cPCR, and a culture method. Of the 100 samples that were randomly picked from the 136 mqPCR-positive samples, 35 and 48 tested positive by cPCR and culture method, respectively. Of the 100 samples randomly chosen from the 142 mqPCR-negative samples, all were negative by cPCR, but 21 samples tested positive by the culture method. McNemar's chi-square tests indicated significant disagreement between the proportions of positive samples detected by the three methods. Applicability of the assay to quantify *E. coli* O157 was determined with feedlot cattle fecal samples (n=576) and compared to spiral plate method. Fecal samples that were quantifiable for O157 by mqPCR (62/576; 10.8%) were at concentrations of $\geq 10^4$ CFU/g of feces. Only 4.5% (26/576) of samples were positive by spiral plate method, with the majority

(17/26; 65.4%) at below 10^3 CFU/g. In conclusion, the mqPCR assay that targets four genes is a novel and more sensitive method than the cPCR or culture method to detect and quantify *E. coli* O157 in cattle feces.

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Chapter 1 - Concentration of *Escherichia coli* O157 in Cattle Feces: An Overview of Significance and Quantification Methods

Introduction

Shiga-toxin producing *E. coli* (STEC) account for 175,000 annual cases of illness in the United States, and the O157 serogroup represents the majority (36%) of those infections (Scallan *et al.*, 2011). Although over 380 STEC serotypes have been isolated from humans with gastrointestinal illnesses, *E. coli* O157:H7 remains the most frequently associated serotype with foodborne outbreaks and severe forms of the disease (Karmali *et al.*, 2010). *Escherichia coli* O157:H7 resides in the hindgut of cattle and is shed in the feces, which serves as a source of contamination of food products and water. During harvest procedures, feces from the hide can potentially contaminate the carcass. The risk of carcass contamination largely depends on the concentrations of *E. coli* O157:H7 in the feces and on the hide during transportation, lairage and at slaughter. However, cattle have been shown to shed *E. coli* O157 at variable concentrations that can fluctuate within the same animal over time (Munns *et al.*, 2014; Robinson *et al.*, 2009). Although it is estimated that 61-85% of adult cattle shed *E. coli* O157 at concentrations less than 10^2 CFU/g (Lahti *et al.*, 2003; Omisakin *et al.*, 2003; Pearce *et al.*, 2004), the organism has been reported at concentrations as high as 10^7 CFU/g in small populations of cattle (Chase-Topping *et al.*, 2008; Omisakin *et al.*, 2003; Robinson *et al.*, 2004b). In fact, a subset of the cattle population, known as super-shedders, shed *E. coli* O157 at much higher concentrations than what is typical. Most have categorized cattle that shed $\geq 10^4$ CFU/g of *E. coli* O157 in their feces as super-shedders (Arthur *et al.*, 2010; Chen *et al.*, 2013; Matthews *et al.*, 2006; Munns *et al.*, 2014; Stanford *et al.*, 2011), although some have defined a lower shedding threshold ($\geq 10^3$ CFU/g; Chase-Topping *et al.*, 2007; Low *et al.*, 2005). Super-shedders are responsible for increased

transmission within pens, during transportation and lairage, resulting in a significant increase in the risk for carcass contamination at slaughter (Fox *et al.*, 2008; Jacob *et al.*, 2010b; Omisakin *et al.*, 2003).

There are numerous pre-harvest intervention strategies aimed to reduce the transmission of *E. coli* O157 in cattle populations, including anti-colonization vaccines, probiotics, chlorate-treated water and antibiotics (Donnenberg, 2013). However, not all *E. coli* O157 are eliminated by these strategies and mere detection of the pathogen at the time of harvest is not sufficient to determine whether post-harvest intervention strategies can successfully eliminate the bacterial load that may still be present on the carcass (Stephens *et al.*, 2007). Therefore, quantification of *E. coli* O157 in cattle feces can provide an indication of relative risk of carcass contamination at harvest. Many studies have attempted to model risk assessment of foodborne outbreaks by examining shedding of *E. coli* O157:H7 in cattle feces. However, an overall lack of quantification data on *E. coli* O157:H7 shed in cattle feces still exists relative to prevalence data. Unlike other sample matrices (carcass, ground beef, hide, etc.), feces has a large number of background flora, making quantification of a target organism problematic (Stephens *et al.*, 2007). However, concentration data are an important component in developing an accurate quantifiable microbial risk assessment (Berry and Wells, 2008; Robinson *et al.*, 2004b). Data that are available are derived from various quantification methods, each of which can impact concentration data. Therefore, the objective of this review is to describe quantification methods for *E. coli* O157 in cattle feces, including both traditional and emerging techniques, and report on available enumeration data. Additionally, the role of super-shedders and their impact on transmission of *E. coli* O157 into the environment and subsequent hide contamination is discussed.

Super-shedders

Cattle are known to exhibit a high degree of variability in the frequency and concentration of *E. coli* O157 that is shed in the feces. Generally, cattle shed *E. coli* O157 at concentrations (10^2 or below per g of feces) below the limit of detection of most assays, therefore, an enrichment step of the sample is required for detection (Chapman, 1997). However, super-shedders excrete *E. coli* O157 at very high concentrations (>10^4 CFU/g) in their feces. Although super-shedders comprise only a small proportion of the total cattle population, it has been shown that these animals are responsible for the majority of *E. coli* O157 contamination in the environment. Matthews *et al.* (2006) have estimated that 80% of *E. coli* O157:H7 shed into the environment are from feces of super-shedders. This could explain why single pulsed-field *E. coli* O157 type is predominantly detected in slaughter pens (Elder *et al.*, 2000). Omisakin *et al.* (2003) detected *E. coli* O157 in 44/589 rectal grab samples from slaughtered cattle and after quantifying the organism, found that only 9% (4/44) of shedding cattle were super-shedders. Researchers further determined that these super-shedders represented more than 96% of the total *E. coli* produced by all cattle in the study population.

Super-shedders are also responsible for increased transmission of *E. coli* O157 within pens. Stephens *et al.* (2008) randomly introduced *E. coli* O157:H7 inoculated fecal pats (10^2 or 10^5 CFU/g) into pens of steers previously tested negative for the organism. Researchers collected fecal grabs, hide swabs, freshly voided fecal pats and rope samples from steers at multiple times during the 49-day trial. Fecal grabs and hide swabs from steers that shared pens with 10^5 CFU/g inoculated fecal pats had significantly higher ($P < 0.01$) concentration of *E. coli* O157:H7 compared to steers housed with 10^2 CFU/g or *E. coli*-negative fecal pats. Interestingly, freshly voided fecal pats and rope samples were not significantly different between cohorts. Cattle that had acquired *E. coli* O157:H7 shed the organism at low concentrations and for only a short

duration of time. Cobbold *et al.* (2007) have reported that cattle were five times less likely to shed *E. coli* O157 if they did not share a pen with a super-shedder. Arthur *et al.* (2009) found a significant association between contamination of hide and the presence of either one or more high density shedders (≥ 200 CFU/g) or super-shedders (≥ 10^4 CFU/g) in a pen or when pen-level fecal prevalence of *E. coli* O157 exceeded 20%. The authors suggested that pen-level fecal prevalence of *E. coli* O157 should be kept under 20% and shedding concentrations below 200 CFU/g to minimize risk of hide contamination. In the same study, when pen-level fecal prevalence exceeded 20%, hide prevalence was usually greater than 80%.

The importance of super-shedders in the transmission dynamics of *E. coli* O157, and whether these cattle should be targeted for interventions (Guy *et al.,* 2014) is still debatable. Munns *et al.* (2014) used a direct plating technique involving serially-diluted feces to enumerate *E. coli* O157:H7 from rectal grab samples of cattle at a commercial feedlot. Eleven super-shedding steers were identified in the population, which were then transported and penned individually. However, only five cattle were again identified as super-shedders when tested five days later. No super-shedders were identified six days after the first sampling, leading researchers to conclude that super shedding events in cattle are short-lived phenomena and that concentration of *E. coli* O157 from feces of a single animal can change from day to day.

Concentration data are further confounded by differences in frequency of sampling between studies. Many studies have sampled cattle feces weekly, biweekly or even monthly to examine *E. coli* O157:H7 shedding patterns (Cobbold *et al.,* 2007; Jacob *et al.,* 2010a; Menrath *et al.,* 2010; Omisakin *et al.,* 2003). Robinson *et al.* (2009) quantified *E. coli* O157:H7 in calf feces collected approximately every 3 hours for a five-day period and discovered more variation within an animal than between animals. Designation of an animal as "super-shedder" may

depend more upon the time and frequency of sample collection and less on characteristics of the host.

Methodology such as collection method (rectal grabs vs. freshly voided fecal pats) and time elapsed between collection and processing also vary between studies and may likely contribute to variability in data reported. In fact, a few studies have enumerated *E. coli* O157 in cattle feces only after prevalence had been determined, with samples stored in cold storage (2-4ºC) (Fegan *et al.*, 2004; Omisakin *et al.*, 2003). Such a delay in sample processing will likely impact true concentration data. Wang *et al.* (1996) reported an increase in *E. coli* O157 concentration, after the first two days of storage, for artificially inoculated feces kept at 37º C and 22º C, but not at 5ºC. Robinson *et al.* (2004a) collected feces from five naturally shedding calves that were shedding *E. coli* O157 at concentrations >200 CFU g^{-1}. Feces were covered then stored at ambient temperature and multiple subsamples per fecal pat were quantified by spiral plate technique at various time-points at and after defecation. Researchers reported that *E. coli* O157 increased 100-fold in the first few hours following initial defecation and was present in some samples up to two weeks later. There are factors that could possibly explain this spike. Fluctuations in temperature/moisture of the fecal pat after defecation may have affected growth of target bacteria. In addition, quantification may have occurred during the exponential growth phase of the target bacteria. However, this concentration fluctuation undoubtedly has implications for studies that aim to quantify *E. coli* O157 from cattle feces.

Although differences in methodology exist and will unavoidably impact quantification data, the concentration data are still an important component of microbial risk assessments and the further generation of such data will serve to enhance assessment of transmission risks associated with this organism (Berry and Wells 2008). It has been shown that animals shedding *E. coli*

O157 at much lower concentrations (< 10^2 CFU/g) are not contributing to environmental or food contamination nearly as much, relative to super-shedders (LeJeune *et al.,* 2006). Therefore, in order to minimize the public health risk, it may be important to concentrate pre-harvest intervention strategies aimed at super-shedders (Omisakin *et al.,* 2003). Consequently, prevalence of *E. coli* O157 in cattle may not be as important as the magnitude at which cattle are shedding the organism in their feces (Robinson *et al.,* 2004b), highlighting the importance of quantification methods used to enumerate the organism in cattle feces.

Super-shedder colonization and the potential impact on O157 quantification

Many host factors, including age, sex, breed, diet and stressors have been studied in relation to super-shedding events in cattle and have been described elsewhere (Gannon *et al.,* 2002; Jacob *et al.,* 2010a; Jeon *et al.,* 2013; Nielsen *et al.,* 2002; Shere *et al.,* 2002). Here, we describe the colonization of cattle by low- and super-shedder strains of *E. coli* O157 and unique genetic determinants of super-shedder O157 strains that have been shown to better facilitate recto-anal junction (RAJ) colonization and lead to increased shedding of the organism. Detection sensitivity of *E. coli* O157 from recto-anal mucosal swabs (RAMS) vs. voided fecal samples is also discussed, including the potential impact of these collection methods on quantification of *E. coli* O157.

There have been studies examining the role of host physiology as a contributing factor to super-shedding events in cattle (Gannon *et al.,* 2002; Jeon *et al.,* 2013; Nart *et al.,* 2008), however, these mechanisms are not well understood (Guy *et al.,* 2014; Lahti *et al.,* 2003). Naylor *et al.* (2003) have demonstrated that *E. coli* O157:H7 preferentially colonize the lymphoid follicles of the RAJ. Researchers examined principal sites of *E. coli* colonization in challenged and naturally colonized calves, and discovered that the majority of *E. coli* O157:H7

colonization occurred at the lymphoid follicle-dense mucosa at the terminal rectum. Furthermore, the organism was not detected or detected at significantly lower concentrations in lumen contents from the gastrointestinal tract. Interestingly, non-O157 *E. coli* serotypes were found at consistent levels throughout the large-intestine and researchers found some evidence for statistically lower concentrations nearer the RAJ ($P < 0.001$). Low *et al.* (2005) examined *E. coli* O157 populations in feces and from two locations (1 and 15 cm proximal to RAJ) from intact rectum samples collected at the time of slaughter, and found significantly higher prevalence and concentration of the organism at the location closer to the RAJ. Researchers also found significant association between "high-level carrier" animals, as determined by measurement of rectum tissue concentrations ($\geq 1 \times 10^3$ CFU/ml^{-1}), and high-level fecal shedding ($\geq 1 \times 10^3$ CFU/g^{-1}), lending support to an association between high fecal shedding of *E. coli* O157 and increased RAJ colonization. Davis *et al.* (2006) observed this phenomenon when comparing concentration of *E. coli* O157:H7 from RAMS and in feces of dairy heifers. The concentration of *E. coli* O157 in feces was positively associated ($r = 0.77$) with the duration of a culture-positive animal (as determined by RAMS method). In other words, cattle that were consecutively culture-positive by RAMS for a longer duration of the study (> 24 day period) shed higher concentrations of *E. coli* O157 in their feces compared to than those cattle that were culture-positive for less time (1-23 days). Researchers supported a link between RAMS positive animals and O157:H7 colonization, and reason that increased duration of RAJ colonization led to increased concentrations in fecal shedding.

The novel tropism that *E. coli* O157:H7 exhibits for the terminal rectum likely has implications on transmission of the organism between cattle. Cobbold *et al.* (2007) found a positive correlation between RAJ colonization of *E. coli* O157:H7 and fecal excretion of the

organism. In addition, there was less prevalence of *E. coli* O157:H7 in fecal samples from cattle without RAJ colonization of the organism. In the same study, it was determined that normal shedding cattle co-penned with super-shedders exhibited significantly greater mean pen RAJ colonization and fecal prevalence of *E. coli* O157:H7 during the 14-week sampling period compared to nonco-penned normal shedding cattle and that the isolates obtained from the co-penned cattle were highly related by pulsed-field gel electrophoresis (PFGE). This led researchers to support an association between RAJ colonization of cattle and exposure to high concentrations of *E. coli* O157:H7 in voided feces.

Since discovering that *E. coli* O157 preferentially colonizes the RAJ (Naylor *et al.*, 2003), many studies have demonstrated an increased sensitivity of detection of the organism from RAMS compared to voided fecal samples (Davis *et al.*, 2006; Greenquist *et al.*, 2005; Rice *et al.*, 2003), which likely has implications on quantification of the organism. Using experimentally infected cattle, Rice *et al.* (2003) have shown significantly increased ($P < 0.01$) sensitivity of detection of *E. coli* O157:H7 from enriched RAMS vs. enriched fecal cultures. However, there was no difference in detection ($P = 0.5$) of the organism between methods when examining enriched samples from naturally infected heifers. In a similar study using naturally infected dairy cattle, Davis *et al.* (2006) reported no difference in detection of *E. coli* O157 from enriched non-IMS RAMS culture vs enriched feces after IMS. In addition, direct plating of RAMs samples detected a significantly higher proportion of samples positive for *E. coli* O157 compared to direct plating of feces. This led researchers to conclude that the use of RAMS culture could prove to be a cost-effective and time-saving alternative to traditionally screening enriched feces with IMS, and in addition allow for more accurate quantification of the organism via direct-plating method.

Differences in bacterial genetics between low- and super-shedder strains of *E. coli* O157 have also been identified (Stanford *et al.*, 2012), and have been shown to contribute to increased RAJ colonization and super-shedding events in cattle (Cote *et al.*, 2015; Kudva *et al.*, 2012). Stanford *et al.* (2012) examined genetic diversity among *E. coli* O157 isolates (n = 658) from low- and super-shedding cattle and found that 77% (124/162) of all super-shedder isolates recovered during the study had distinct PFGE profiles compared to low-shedder isolates (n = 496) recovered from the same pen. Cote *et al.* (2015) have studied the whole genome sequence analysis of a super-shedder strain of *E. coli* O157 (SS17) and identified numerous single and multiple nucleotide polymorphisms in this strain compared to other laboratory O157 strains. The SS17 strain revealed polymorphisms in key adherence and virulence-related loci that allowed for a strong aggregative adherence on bovine RAJ stratified squamous epithelial (RSE) cells. Researchers observed significantly greater ($P < 0.01$) adherence patterns of the SS17 strain on RSE cells compared to adherence of non-super-shedder O157 strains (EDL933 and 86-24SM^{R}). Immunofluorescence staining revealed strong aggregative adherence properties associated with the SS17 cells, compared to a much more moderate to diffuse adherence pattern observed in the non-super-shedder O157 strains. Interestingly though, all strains exhibited diffuse adherence patterns on human HEp-2 cells. Using pooled antisera targeting locus of enterocyte and effacement (LEE)-encoded proteins (intimin, Tir, EspA and EspB), researchers then tested whether the LEE pathway was involved in the observed adherence patterns. Although the pooled antisera blocked SS17 adherence to HEp-2 cells, it decreased SS17 adherence to RSE cells by only 6%. Similar results were obtained when researchers used an SS17Δ*eae* strain to test adherence to RSE and HEp-2 cells, supporting the conclusion that non-LEE encoded proteins are likely responsible for increased RAJ colonization in super-shedder strains of *E. coli*

O157. Kudva *et al.* (2012) have observed similar patterns of O157 adherence on RSE cells when using antisera against LEE-encoded proteins or an O157Δ*eae* strain.

Although still not well understood, the super-shedding phenomenon is likely the result of a multilayered interaction of animal and bacterial factors. It is not the aim of this review to describe all of the nuances at play in super-shedding events, nor have we presented an exhaustive list of contributing factors to these events. We have discussed differences in bacterial genetics that likely contribute to increased RAJ colonization and how such colonization can impact O157 super-shedding events. We have additionally addressed how the use of RAMS vs. fecal culture for quantification of *E. coli* O157 may impact enumeration data and subsequent identification of super-shedders in the cattle population.

Culture-based enumeration techniques

Direct plating technique

Direct plating technique involves enumeration of the target organism directly from the sample matrix, prior to any enrichment or processing step. A fecal suspension is first prepared in a selective broth, at a typical volume-to-weight ratio of 9:1, and then an aliquot of the suspension is directly inoculated onto a selective medium for enumeration of *E. coli* O157. Alternatively, the prepared fecal suspension can be subjected to a series of dilutions and aliquots from each dilution can then be plated onto a selective medium. Using a direct plating technique involving serial dilution, Omisakin *et al.* (2003) reported that concentration of *E. coli* O157 in cattle feces (n=579) ranged from less than 10^2 to 10^5 CFU g^{-1} (Table 1.1) and that 61% (27/44) of infected cattle shed the organism below detectable levels (< 10^2 CFU g^{-1}) of the assay.

The direct plating method has been used to enumerate *E. coli* O157 in feces from cattle inoculated with nalidixic acid-resistant (*Nal*^{R}) *E. coli* O157 (Fox *et al.*, 2007). Researchers

reasoned that artificially inoculating cattle with the organism and then quantifying only the inoculated strain would allow for minimal variation in the data between methods. Fox *et al.* (2007) diluted feces from artificially inoculated cattle and then spread-plated aliquots to three sorbitol MacConkey agar with cefixime and potassium tellurite (CT-SMAC) plates. The resulting sorbitol-negative colonies were then confirmed by agglutination and PCR. Fox *et al.* (2007) reported 74.4% and 68.8% sensitivity and specificity, respectively, when enumerating samples with Nal^R *E. coli* O157 concentrations above 3.0 $\log_{10}$ CFU/g. Using a similar dilution technique, LeJeune *et al.* (2006) quantified Nal^R *E. coli* O157 from cattle feces and reported a minimum detection limit of >10

0 CFU/g (Table 1.1).

The direct plating method can yield expedient data without the extra cost associated with other enumeration techniques. However, the variable concentrations of background flora present in any given sample can complicate efforts to enumerate *E. coli* O157 from feces. Background flora can overcrowd target colonies on certain plates, while other plates can exhibit minimal overall growth. Serial dilutions of pre-enriched aliquots can be a solution, but consequently increase time and cost associated with the technique. Furthermore, additional time spent preparing the sample is often not amenable to processing a large number of samples with high throughput capacity (Brichta-Harhay *et al.*, 2007).

Most-probable-number technique

Most-probable number (MPN) is a direct plating technique that has been used for many years. Phelps (1908) used a tube-dilution assay to quantify *Bacillus coli* from sewage water, and researchers are still using the MPN technique to quantify *E. coli* from environmental samples. Serial dilutions of the sample are prepared, and the most diluted sample that yields a positive

result is determined. A quantification value is then reported in the reciprocal form of this dilution (Phelps, 1908). Enumerating bacteria with the MPN technique can be an effective alternative to direct plating without serial dilutions, especially when working with a fecal matrix with high background flora (Stephens *et al.,* 2007). MPN technique has been used to enumerate *E. coli* O157 in cattle feces, often in combination with immunomagnetic separation (IMS) and/or quantitative real-time PCR (qPCR) (Fegan *et al.,* 2004; Fox *et al.,* 2007; Guy *et al.,* 2014; Stephens *et al.,* 2007; Widiasih *et al.,* 2004). Guy *et al.* (2014) used qPCR to test pre-enriched feces for *E. coli* O157, then enriched feces and applied MPN technique to O157 bead suspensions after IMS of samples that were above the threshold of detection and quantification for direct qPCR. Researchers claimed that as few as one *E. coli* O157 organism per g of feces could be detected with this MPN assay (Table 1.1). Widiasih *et al.* (2004) described similar detection capabilities when using MPN in combination with IMS method, reporting a 3.5% (11/324) prevalence of *E. coli* O157 from naturally shedding cattle with concentration ranging from 4 CFU/10 g to > 110,000 CFU/10 g (Table 1.1). Fegan (2003) used MPN to quantify a small number of fecal samples from pasture-(n=10) and grain-fed cattle (n=12) that had tested qualitatively positive for *E. coli* O157. Data ranged from < 3 MPN g^{-1} of feces to 2.4 x 10^4 MPN g^{-1} of feces (Table 1.1), but no significant difference ($P = 0.06$) was found in concentrations of *E. coli* O157 between cohorts.

The MPN technique has also been used to quantify *E. coli* O157 in feces from artificially inoculated cattle. Fox *et al.* (2007) used MPN to quantify *E. coli* O157 from direct streaks of enriched cattle feces as well as from post-IMS bead suspensions. Mean MPN values for both techniques were similar, yielding 10^3 MPN/g (Table 1.1). Although previous studies have used 10 g of feces (Elder *et al.,* 2000; LeJeune *et al.,* 2006), researchers chose to use 2 g of feces for

quantification assays, citing the economical and logistical feasibility of processing such a large amount of feces as well as no sure possibility of always being able to acquire 10 g of feces from rectal grabs. However, Lahti *et al.* (2003) reports that *E. coli* O157 was isolated more often from 10 g of rectal grab feces compared to 1 g; ($P < 0.0001$)) from bulls. Lahti *et al.* (2003) reported concentration ranges from undetectable (< 0.2 MPN/g) up to and exceeding 1.6×10^5 MPN/g (Table 1.1). Interestingly, abattoir samples had lower to undetectable counts (< 0.4 MPN/g) more often than farm samples ($P < 0.05$). Stephens *et al.* (2007) artificially inoculated sterile and unsterile cattle feces with different concentrations of *E. coli* O157 (10^1 to 10^4), and then compared a MPN/IMS technique to direct plating for sensitivity and specificity of quantification. After plotting log MPN/g versus log CFU per milliliter (or gram) for all treatment interactions, the two procedures were correlated (r=0.93).

MPN technique is a relatively simple quantification method and allows for users to adjust accuracy by simply increasing the number of dilution tubes (Oblinger and Koburger, 1975). Although there are advantages to the MPN technique, this assay can be time-consuming and labor intensive, especially when coupled with additional IMS and qPCR steps. This procedure can also be expensive and low-throughput, as a single sample often requires preparation of multiple dilution tubes (Berry and Wells, 2008; Brichta-Harhay *et al.,* 2007; Stephens *et al.,* 2007). Furthermore, MPN calculations assume that the target organism is evenly distributed throughout the sample (Oblinger and Koburger, 1975), which may not always be true of *E. coli* O157 in feces. Another disadvantage of MPN method is that many times it relies on an enrichment period, during which time, competition between the target organism and background flora can have an impact on quantification data (Brichta-Harhay *et al.,* 2007).

Spiral plate count method

The spiral plate method was first described by Gilchrist *et al.* (1973) as a technique for quantification of bacteria in a suspension. A known volume of sample suspension is dispensed in an Archimedes spiral around the surface of a rotating agar plate. After incubation, a modified counting grid is used to enumerate colonies by relating the dispensed sample volume to area on the agar surface. Jarvis *et al.* (1977) was the first to use the spiral plate count method in food microbiology. This technique has also been used to enumerate *E. coli* O157 in cattle feces (Berry and Wells, 2008; Brichta-Harhay *et al.*, 2007; Fox *et al.*, 2007; Omisakin *et al.*, 2003; Robinson *et al.*, 2004a). Robinson *et al.* (2004a) used spiral plate count method to quantify serially diluted concentrations of *E. coli* O157 spiked in cattle feces and reported a good agreement between inoculated and recovered concentrations of *E. coli* O157. Researchers measured repeatability of quantification results by inoculating five replicate plates for each inoculum concentration and found that counts were most accurate between 1×10^3 to 1×10^8 CFU g^{-1}. Researchers rarely recovered *E. coli* O157 from feces spiked with less than 10^2 CFU g^{-1}, indicating the limit of detection (LOD) of this assay (Table 1.1). Brichta-Harhay *et al.* (2007) have reported a similar LOD (Table 1.1). They detected *E. coli* O157 in 16.7% (532/3,190) of enriched samples. Spiral plate technique was then used to quantify *E. coli* O157 from pre-enriched cattle feces (n=532). Researchers used 50 µl of 1 mL aliquots removed from a fecal slurry (10 g feces + 90 mL phosphate-buffered tryptone soy broth) for spiral plating, or approximately 0.0005 grams of feces per sample. A median observed value of 1.6×10^3 CFU g^{-1} was reported, where 71% of quantifiable samples (n=122) had observations between 10^2 and 10^3 CFU g^{-1}. Researchers reported 2.0×10^2 CFU g^{-1} as the LOD of the assay. Only one sample was reported with *E. coli* O157 > 10^5 CFU g^{-1} while 27.9% (n=34) of samples were 10^4-10^5 CFU g^{-1}. These results are consistent with other studies reporting *E. coli* O157 MPN concentration

between 10^2-10^6 $CFUg^{-1}$ (Omisakin *et al.,* 2003; Robinson *et al.,* 2004a). Berry and Wells (2008) used spiral plating method with CHROMagar O157 medium, a commercially available, selective medium for *E. coli* O157, and reported a LOD of 200 CFU/g of *E. coli* O157:H7 in aged manure and manure-based compost (Table 1.1).

Spiral plate method has also been used to quantify *E. coli* O157 in feces from cattle inoculated with the organism. Fox *et al.* (2007) used spiral plate technique to quantify the organism from pre-enriched feces (n=150). One-hundred microliter aliquots of fecal slurry were dispensed onto CT-SMAC and CT-SMAC supplemented with nalidixic acid (50 μg/ml; CT-SMACNal^{50}). Spiral plate technique successfully identified samples with high *E. coli* O157 concentrations (> 3.0 $\log_{10}$ CFU/g) in 79.0% and 63.2% of samples assayed with CT-SMAC and CT-SMACNal^{50}, respectively.

As mentioned before, direct plating technique without serial dilutions suffers from variability in the concentration of background flora present in any given sample, which can potentially overcrowd target colonies. Alternatively, if bacteria (including the target organism) are present at low concentrations in the feces, there is a risk in aliquoting too little of the sample for adequate quantification. The spiral plate method has the unique advantage of being able to guard against variability of concentration of background flora present in the sample, as it dispenses a sample aliquot in an ever decreasing volume from the center of the agar surface outward. The resulting dilution effect avoids the need to prepare serial dilutions of the sample, eliminating expense and reducing time spent processing samples. Brichta-Harhay *et al.* (2007) reported that the spiral plate technique can be used to enumerate *E. coli* O157 in cattle feces at one fourteenth the cost of the traditional MPN method. Compared to MPN method, the spiral plating method is capable of processing a large number of samples (Berry and Wells 2008). The

spiral plate method requires less sample preparation compared to MPN method, likely resulting in less risk for laboratory error. However, the relatively low minimum detection limit (10^2 CFU g^{-1}) could be considered a major disadvantage as the minimum infectious dose of *E. coli* O157 has been estimated to be as low as 10-100 viable cells (Yoon and Hovde, 2008). Furthermore, a large number of samples and/or high prevalence of *E. coli* O157 in samples will result in increased time and labor spent manually counting inoculated plates, limiting through-put capabilities of the technique (Robinson *et al.*, 2004a).

Immunomagnetic separation (IMS)

Immunomagnetic separation (IMS) has allowed for a considerable increase in diagnostic sensitivity when detecting *E. coli* O157 from cattle feces (Chapman *et al.*, 1994), although debate remains about the degree of increased sensitivity. Stephens *et al.* (2007) reports that IMS technique can require as few as 100 cells in a sample for detection, whereas LeJeune *et al.* (2006) reported that >100 cells are required for detection. Omisakin *et al.* (2003) demonstrated that the IMS technique had a LOD of around 5 CFU g^{-1} feces. Regardless, the IMS step can increase sensitivity by 100 fold compared to direct plating method (Widiasih *et al.*, 2004). Although sensitive, the IMS procedure does not allow for quantification of *E. coli* O157. However, IMS has been used *in combination* with other quantification methods in an effort to enumerate *E. coli* O157 from cattle feces (Fegan *et al.*, 2004; Fox *et al.*, 2007; Stephens *et al.*, 2007; Widiasih *et al.*, 2004). Direct plating is not a recommended method for detecting *E. coli* O157 in feces at concentrations $<10^2$ CFU g^{-1} feces (Chapman *et al.*, 1997). Therefore, enrichment and IMS procedures have been employed prior to enumeration of *E. coli* O157 in feces (Omisakin *et al.*, 2003; Parham *et al.*, 2003). As previously mentioned, differing micro-environments between samples during the enrichment period may lead to a bias in quantification

of target bacteria. Furthermore, enumeration of *E. coli* O157 from enriched feces is not representative of the true concentration of *E. coli* O157 being shed in the feces.

PCR-based quantification techniques

Quantitative PCR (qPCR)

Higuchi *et al.* (1993) were the first to report an assay to quantify amplified DNA in a sample. Since then, qPCR has been used for a wide variety of applications, including quantification of *E. coli* in cattle feces. To make quantification possible, first, serial dilutions of the target organism are performed and then aliquots of each dilution are plated on a culture medium to determine viable cell counts. Extracted DNA from each dilution is subjected to qPCR. The qPCR allows for quantification of a target organism by converting the cycle threshold (Ct) value of a fluorescent signal, specific to the target organism, into a target cell density that is determined by the cultured organism. Therefore, quantifiable DNA can be directly related to colony counts (CFU/mL or CFU/g; (Ibekwe and Grieve, 2003). The *rfb* gene cluster is a common O157 specific target for many qPCR assays, with gene targets including the *rfb*E (Anklam *et al.*, 2012; Fortin *et al.*, 2001; Hsu *et al.*, 2005; Jacob *et al.*, 2012) and perosamine synthetase genes (*per*; Guy *et al.*, 2014). The *uid*A gene encoding b-glucuronidase specific for *E. coli* (Pavlovic *et al.*, 2010) or a unique conserved single-nucleotide polymorphism of the *uid*A gene specific to *E. coli* O157:H7/H^- (Jinneman *et al.*, 2003; Wang *et al.*, 2007) have also been targeted. Luedtke *et al.* (2014) targeted an EHEC attaching and effacing gene-positive conserved fragment 1 (*ecf*1) from virulence plasmid pO157 and pO157 like plasmids, a marker common to EHEC bacteria, including *E. coli* O157.

In addition to serogroup identification, qPCR can quantify a genome copy of virulence genes associated with Shiga toxin-producing *E. coli*, most commonly, Shiga toxin genes (*stx*1 and

*stx*2), the intimin gene for attaching and effacing (*eae*), and the enterohemolysin gene (*ehx*A) (Anklam *et al.,* 2012; Fitzmaurice *et al.,* 2004; Guy *et al.,* 2014; Jacob *et al.,* 2012; Pavlovic *et al.,* 2010; Reischl *et al.,* 2002). After all, confirmation of the O157 serogroup alone is not enough to indicate that a strain is pathogenic and the use of the *rfb* cluster as a qPCR target for O157-specific amplification can result in quantification of both pathogenic and non-pathogenic *E. coli* O157 (Guy *et al.,* 2014). Pathogenicity must be confirmed by associated virulence genes. However, feces can contain a number of *E. coli* serogroups. It is possible that virulence genes amplified in a sample could be from non-O157 *E. coli* present in the feces. Therefore, although qPCR allows for quantification of virulence genes, it is still not possible to associate those genes with a particular species or serotype present in a sample, without an intermediary culture-based isolation step and confirmation by PCR. Some qPCR assays have reported successful amplification of *E. coli* O157 specific virulence genes, including *uid*A and *eae* (Jinneman *et al.,* 2003; Sharma and Nystrom, 2003; Wang *et al.,* 2007). However, there have been no assays developed that can amplify serogroup specific Shiga toxin genes. This is a critical limitation of current qPCR assays, as Shiga toxin production is the major component of STEC pathogenesis (Karmali, 2004). Although, the qPCR procedure cannot associate Shiga toxin genes with a particular serogroup, it can be used to accurately quantify virulence gene copies, and as a result quantify total STEC directly from feces (Guy *et al.,* 2014). This makes qPCR a valuable screening tool for quantifying Shiga toxin present in cattle feces. Feces positive for Shiga toxin markers by qPCR can then be plated on selective media to isolate STEC and associate the Shiga toxin with a certain *E. coli* serogroup (Guy *et al.,* 2014).

Anklam *et al.* (2012) developed qPCR assays targeting O157 (*rfb*E*;* assay 1) and STEC-associated virulence genes (assay 2) including: Shiga toxin (*stx*1 and *stx*2), intimin (*eae*) and

enterohemolysin (*ehx*A). The analytical sensitivity of the assays was determined by testing cattle feces (1 g) suspended in 50 mL of modified *E. coli* medium and then spiked with ten-fold serially-diluted pure cultures of *E. coli* O157 FRIK 47, before and after six hour incubation at 37ºC. Before enrichment, researchers reported a LOD of 10^3 CFU/mL for *E. coli* O157 (assay 1) and the three virulence genes (assay 2; Table 1.2). After a six hour enrichment, the LOD decreased to 10^0 CFU/mL for gene targets from both assays. Guy *et al.* (2014) reported a similar LOD for pre-enriched feces (10^3 gene copies/g; Table 1.2) of the qPCR assay they developed for quantification of the O157 somatic antigen (*per*) and Shiga toxin genes (*stx*1 and *stx*2) in cattle feces. Luedtke *et al.* (2014) reported a LOD of 10^3 CFU/mL directly from feces (Table 1.2) for their qPCR assay developed for detection and quantification of EHEC, including *E. coli* O157. However, many qPCR procedures report a LOD of 10^4 CFU/g directly from feces (Hsu *et al.*, 2005; Ibekwe and Grieve 2003; Jacob *et al.*, 2012; Sharma & Nystrom, 2003; Table 1.2) which still allows for accurate detection and quantification of *E. coli* O157 from the super-shedder population. However, the majority of qPCR assays may not be applicable for quantification of *E. coli* O157 from cattle shedding the organism at lower concentrations (10^2 to 10^3 CFU/g) that still pose a pathogenic risk.

It has been reported that a six hour enrichment period can increase sensitivity of qPCR by approximately 1,000 times (Jacob *et al.*, 2012). Although this enrichment period may increase detection sensitivity of the qPCR assays, it will invalidate efforts at quantification of target genes. Bile salts, present at different concentrations in fecal samples, have been shown to significantly influence growth of *E. coli* O157 as well as transcription rates of many associated virulence genes (Hamner *et al.*, 2013). Competition with non-target bacteria and or phage-killing bacteria during enrichment can also confound growth and quantification of target bacteria

(Guy *et al.*, 2014). In addition, Imamovic *et al.* (2010) reported an abundance of *stx*-converting bacteriophages in cattle feces, ranging from 10 to 10^5 gene copies per g. Therefore, differing microenvironments between fecal samples that favor or inhibit bacterial growth and gene expression during prolonged incubation may complicate efforts to truly quantify target genes post-enrichment.

The qPCR method has many advantages, including the potential for automation, making the method conducive to screening large number of samples in a short period of time (Ibekwe and Grieve, 2003). The qPCR procedure also allows for enumeration across a broad dynamic range, allowing for easy quantification of super-shedders (Guy *et al.*, 2014). This wide range of quantification is especially important when considering that cattle shed *E. coli* O157 in feces with concentrations ranging from $<10^2$ CFU/g in most cattle to 10^7 CFU/g in small populations (Chase-Topping *et al.*, 2008; Omisakin *et al.*, 2003; Robinson *et al.*, 2004b). The qPCR method is at least one hundred fold more sensitive than traditional end-point PCR detection and highly specific as well (Bianssoni and Raso, 2014). The increased sensitivity and specificity of this procedure is important in a sample matrix such as feces, which can be replete with DNA and PCR inhibitors (Hamner *et al.*, 2013; Hsu *et al.*, 2005). Compared to end-point PCR detection, qPCR has reduced cycle times and does not require time-consuming post-amplification detection procedures (Jinneman *et al.*, 2003; Mackay 2002). Above all else, qPCR has allowed for a dramatic increase in speed at which quantification results can be obtained. Unlike other quantification methods that rely on phenotypic identification of *E. coli* O157 after an overnight incubation in culture media, qPCR can quantify O157 specific genes directly from fecal DNA.

For all of its obvious advantages, the qPCR method is not without its drawbacks. A technical expertise of the procedure and of the specialized instrumentation required for qPCR is

necessary. Compared to other quantification methods, there is extra expense associated with PCR reagents required for each reaction (Biassoni & Raso, 2014). However, in a high-throughput setting, qPCR has proven to be cost-effective (Martell *et al.,* 1999). Similar to any enumeration procedure, quantification data from qPCR can also suffer from an unequal distribution of the target organism in the feces. There is potential for heterogenic distribution of *E. coli* O157 in feces samples (Pearce *et al.,* 2004; Robinson *et al.,* 2005) and the portion of the sample subjected to either culture or qPCR testing may not truly reflect the larger sample (Jacob *et al.*, 2012). Although qPCR assays can allow for accurate quantification of *E. coli* O157 from feces, there is no isolate obtained from the procedure for follow-up studies unless the sample is also subjected to culture-based methods of quantification or detection (Jacob *et al.,* 2012). Furthermore, unlike culture method qPCR does not discriminate based on viability of cells, which can result in amplification of DNA from dead cells. (Wang and Mustapha, 2010). Perhaps most importantly, there are no qPCR assays currently developed that can amplify O157 specific Shiga toxin genes, complicating efforts to obtain STEC O157 quantification data.

Conclusion

Although there are numerous pre- and post-harvest intervention strategies along the chain of beef production, not all *E. coli* O157 are eliminated by these strategies, which highlights the importance of diagnostic assays that can accurately identify the organism. However, mere detection of the pathogen at any particular stage is not sufficient for determining transmission risk. In order to effectively implement Hazardous Analysis and Critical Control Points procedures along the beef production chain, it is necessary to have validated assays that not only detect the presence but quantify *E. coli* O157. Traditionally, qualitative data have been used to compare effectiveness of various intervention strategies that occur along the beef production

chain. However, it is possible for an intervention to still be effective by reducing rather than eliminating the total bacterial load. Although many prevalence data are reported as presence-absence of *E. coli* O157 in cattle feces, there is an increased need to produce prevalence estimates based on enumeration data (Low *et al.*, 2005). Nonetheless, there remains a lack of overall quantification data, compared to the abundance of quantitative prevalence data available for *E. coli* O157 along the beef production chain (Brichta-Harhay *et al.*, 2007). More research is needed to identify factors that determine variation in *E. coli* O157 shedding, but quantification of *E. coli* O157 in cattle feces is an important indicator of relative risk of carcass contamination at harvest (Robinson *et al.*, 2004b, Stephens *et al.*, 2007).

It has been shown that animals shedding *E. coli* O157 at much lower concentrations ($<10^2$ CFU/g) are not contributing to environmental or food contamination nearly as much, relative to super-shedders (LeJeune *et al.*, 2006). Therefore, in order to minimize the public health risk, it may be more important to concentrate on pre-harvest intervention strategies aimed at super-shedders (Omisakin *et al.*, 2003). Consequently, mere prevalence of *E. coli* O157 in cattle may not be as important as the magnitude at which cattle are shedding the organism in their feces (Robinson *et al.*, 2004b), highlighting the increasing importance of various quantification methods used to enumerate the organism in cattle feces.

There are several quantification assays for enumeration of O157 from cattle feces, each with advantages and at the same time suffering from certain disadvantages. Direct plating methods can yield data without the extra cost associated with other enumeration techniques. However, the variable concentration of background flora present in any given sample can complicate efforts to enumerate *E. coli* O157 from feces. Culture-based direct plating methods, such as MPN and spiral plating technique, address this issue by diluting the fecal sample. However, both

methods can be time-consuming, labor intensive and relatively low-throughput. The qPCR method is novel in that it allows for quantification of not only O157-specific genes (*rfb*E, *uid*A, *per*) but also virulence genes (*stx*1, *stx*2, *eae*, *ehx*A) typical of most EHEC. However, it is not possible to associate Shiga-toxin genes with the O157 serogroup in the same reaction. Nonetheless, the qPCR method still has many advantages relative to other quantification techniques, including high sensitivity and specificity, high-throughput and rapid turn-around for results. However, culture-based techniques still have the advantage of allowing for discrimination between dead and live cells as well as providing an isolate for further characterization. Furthermore, minimum detection sensitivity for most culture-based quantification assays is ~10^2 CFU/g, whereas the majority of qPCR assays have a LOD of between 10^3-10^4 CFU/g directly from feces.

It is estimated that 61-85% of adult cattle shed *E. coli* O157 at concentrations less than 10^2 CFU/g (Lahti *et al.,* 2003; Omisakin *et al.,* 2003; Pearce *et al.,* 2004), less than the minimum detection capabilities of most quantification assays (LeJeune *et al.,* 2006). The minimum detection limit of a given assay will not only influence the isolation rate of *E. coli* O157 (Omisakin *et al.,* 2003), but will likely impact quantification of the organism when shed at low levels. Therefore, low concentrations of *E. coli* O157 ($\leq 10^2$ CFU/g) are likely under-reported in the cattle population. This is of significance when considering that the minimum infectious dose of *E. coli* O157 is considered to be as low as 10-100 viable cells (Yoon and Hovde, 2008). Therefore, improvements in minimum detection for most quantification assays may be necessary in order to satisfy risk assessment models.

However, enumeration data can be confounded by a variety of factors. The assumption of equal pathogen distribution in a sample matrix can have a significant impact on the resulting data

from any quantification assay. It has been shown that *E. coli* O157:H7 can be unevenly distributed in feces (Pearce *et al.*, 2004; Robinson *et al.*, 2005). The choice of sample matrix (RAMS vs. fecal culture) may confound efforts to accurately describe concentration of *E. coli* O157 in the cattle population. Many studies have shown that RAMS provide an increased sensitivity of *E. coli* O157 detection compared to voided fecal samples (Davis *et al.*, 2006; Greenquist *et al.*, 2005; Rice *et al.*, 2003), which likely impacts enumeration data (Davis *et al.*, 2006). Moreover, most quantification assays utilize only a portion of the collected sample, which may not be representative of the entire sample. A thorough homogenization of the sample followed by multiple sampling from the same sample may adequately address this issue. Assay sensitivity can even increase by taking multiple measurements from each sample (Brichta-Harhay *et al.*, 2007; Guy *et al.*, 2014). Increasing the amount of feces subjected to processing (≥10 g) may also increase detection sensitivity (LeJeune *et al.*, 2006). Many epidemiological studies have enriched large volumes (≥10 g) of feces and then subjected fecal suspensions to IMS to increase sensitivity of detection (LeJeune *et al.*, 2004; Omisakin *et al.*, 2003). In order to optimize sensitivity of detection and quantification, a sub-sample of homogenized feces could be subjected to the IMS procedure before and after enrichment. An aliquot of the pre-enriched sample after IMS could be subjected to qPCR and an additional aliquot of the pre and post-enriched sample after IMS could be spread-plated onto a selective medium. Subjecting pre-enriched samples to IMS would ensure recovery of *E. coli* O157 from feces, even when present at low concentrations (5-100 CFU/g). The qPCR could allow for quantification of *E. coli* O157 across a wide dynamic range (10^3-10^9 CFU/g), producing enumeration data for the majority of samples on the same day of processing. The culture-based method, in addition to providing enumeration data for samples with *E. coli* O157 concentration less than the qPCR LOD (10^3 –

10^4 CFU/g), would provide a potential *E. coli* O157 isolate from each sample for confirmation of virulence genes. This approach would optimize detection sensitivity through IMS of enriched feces, but also allow for enumeration of *E. coli* O157 present in pre-enriched feces at very low concentrations (5-100 CFU/g).

In conclusion, concentration data of *E. coli* O157 in cattle feces are an important component of microbial risk assessments and further generation of such data will serve to enhance assessment of transmission risks associated with this organism. There are numerous advantages and disadvantages to the various quantification procedures discussed here and perhaps a combination of PCR- and culture-based techniques is optimal.

Table 1.1 Culture-based methods of quantification of *Escherichia coli* O157 in cattle feces

Quantification Method	Concept of Method	Reported Limit of Detection or Quantification Range of O157	Reference
Direct plating technique	Feces were serially diluted and spread plated onto CT-SMAC	$< 10^2$ to 10^5 $\log_{10}$ CFU/g	Omisakin *et al.*, 2003
	Feces from challenged cattle were serially diluted and spread plated onto CT-SMAC	10^2 $\log_{10}$ CFU/g	LeJeune *et al.*, 2006
	Feces from challenged cattle were serially diluted and spread plated onto CT-SMAC	10^1 to 10^6 $\log_{10}$ CFU/g	Fox *et al.*, 2007
Most-probable-number technique (MPN)	Enriched feces were subjected to IMS and MPN technique was applied to O157 bead suspensions.	$< 10^1$ to 10^4 $\log_{10}$ MPN/g	Fegan *et al.*, 2003
	MPN technique was applied to enriched feces	$< 10^0$ to $\geq 10^5$ $\log_{10}$ MPN/g	Lahti *et al.*, 2003
	Enriched feces were subjected to IMS and MPN technique was applied to O157 bead suspensions.	$< 10^0$ to $\geq 10^5$ $\log_{10}$ MPN/10 g	Widiasih *et al.*, 2004
	Cattle were challenged with O157 and MPN technique was applied to enriched feces before and after IMS.	10^3 $\log_{10}$ MPN/g	Fox *et al.*, 2007
	Inoculated feces were enriched and subjected to IMS, then MPN technique was applied to O157 bead suspensions.	10^1 to 10^4 $\log_{10}$ MPN/g	Stephens *et al.*, 2007
	Enriched feces were subjected to IMS and MPN technique was applied to O157 bead suspensions.	10^0 $\log_{10}$ CFU/g	Guy *et al.*, 2014
Spiral plate count method	Used to quantify serial diluted *E. coli* spiked in cattle feces.	10^2 to 10^8 $\log_{10}$ CFU/g	Robinson *et al.*, 2004(a)
	Used to quantify *E. coli* in cattle feces.	10^2 to $> 10^5$ $\log_{10}$ CFU/g	Brichta-Harhay *et al.*, 2007
	Used to quantify O157 in feces from challenged cattle.	10^2 to 10^6 $\log_{10}$ CFU/g	Fox *et al.*, 2007
	Used to quantify *E. coli* in aged manure and manure-based compost.	10^2 $\log_{10}$ CFU/g	Berry and Wells, 2008

Table 1.2 Real-time PCR assays used for quantification of *Escherichia coli* O157 in cattle feces

*Genes Targeted	Validation Method	Limit of Detection in Feces		Reference
		Before Enrichment	After Enrichment	
*stx*1 and *eae*	Spiked feces	10^4 $\log_{10}$ CFU/g	< 10^1 $\log_{10}$ CFU/g	Ibekwe and Grieve, 2003
*uid*A, *stx*1, *stx*2	Pure culture	< 10^1 $\log_{10}$ CFU/reaction	Not performed	Jinneman *et al.*, 2003
eae, *stx*1, *stx*2	Spiked feces	10^4 $\log_{10}$ CFU/g	10^0 $\log_{10}$ CFU/g	Sharma & Nystrom, 2003
*uid*A, *stx*1, *stx*2, *eae*	Pure culture	<10^1 to <10^2 $\log_{10}$ CFU/reaction	Not performed	Pavlovic *et al.*, 2010
*rfb*E (assay 1) *stx*1, *stx*2, *eae*, *ehx*A (assay 2)	Spiked feces	10^3 $\log_{10}$ CFU/g	10^0 $\log_{10}$ CFU/g	Anklam *et al.*, 2012
*rfb*E, *stx*1, *stx*2	Spiked feces	10^4 $\log_{10}$ CFU/g	10^1 $\log_{10}$ CFU/g	Jacob *et al.*, 2012
per , *stx*1, *stx*2	Spiked feces	10^3 $\log_{10}$ CFU/g	10^1 $\log_{10}$ CFU/g	Guy *et al.*, 2014
*ecf*1, *stx*1, *stx*2, *eae*	Spiked feces	10^3 $\log_{10}$ CFU/g	Not performed	Luedtke *et al.*, 2014

*Genes targets include: *rfb*E (O157 somatic antigen), *uid*A (ß-glucuronidase or unique conserved single-nucleotide polymorphism of O157 ß-glucuronidase), *per* (O157 somatic antigen), *ecf*1 (EHEC attaching and effacing gene-positive conserved fragment 1), *eae* (intimin or EHEC O157:H7-specific intimin), *stx*1 (Shiga toxin 1), *stx*2 (Shiga toxin 2), and *ehx*A (enterohemolysin).

Chapter 2 - A Four-Plex Real-Time PCR Assay, Based on *rfb*E, *stx*1, *stx*2, and *eae* Genes, for the Detection and Quantification of Shiga toxin-producing *Escherichia coli* O157 in Cattle Feces

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) O157 reside in the hindgut of cattle and are shed in the feces, which can be a source of foodborne infections in humans (Rangel *et al.*, 2005; Gyles, 2007). Cattle generally shed *E. coli* O157:H7 in feces at concentrations of 10^2 CFU or below per gram of feces, which is below the detection limit of most assays, thereby necessitating an enrichment step (Chapman, 2000). High concentrations (> 10^4 CFU/g) of *E. coli* O157 are shed in a subset of cattle population known as supper-shedders (Matthews *et al.*, 2005; Arthur *et al.*, 2010; Stanford *et al.*, 2010). Super shedders are responsible for increased transmission within pens, and during transportation and lairage, likely resulting in a significant increase in the risk of hide and carcass contaminations at slaughter (Omisakin *et al.*, 2003; Fox *et al.*, 2008; Jacob *et al.*, 2010b). Therefore, it is important to develop diagnostic procedures for STEC O157 in feces with an improved level of detection and quantification.

Although culture-based procedures (direct plating, spiral plating, most-probable number method) have been developed to quantify STEC O157 in feces, they are time-consuming and labor-intensive, therefore not practical in studies that require testing a large number of samples (Omisakin *et al.*, 2003; Fegan *et al.*, 2004; Robinson *et al.*, 2004; LeJeune *et al.*, 2006; Brichta-Harhay *et al.*, 2007; Fox *et al.*, 2007; Stephens *et al.*, 2007). Real-time quantitative PCR to detect and quantify STEC O157 is a sensitive and rapid method, with the added advantages of high-throughput capability and potential for automation. Several multiplex qPCR assays targeting different combinations of major genes of STEC O157, including *rfb*E (O157 antigen),

per (O157 antigen), *ecf1* (attaching and effacing gene conserved fragment 1), *uidA* (b-glucronidase), *stx*1 (Shiga toxin 1), *stx*2 (Shiga toxin 2), *eae* (intimin) and e*hx*A (enterohemolysin) have been developed (Fortin *et al.*, 2001; Reischl *et al.*, 2002; Ibekwe and Grieve, 2003; Sharma and Dean-Nystrom, 2003; Fitzmaurice *et al.*, 2004; Hsu *et al.*, 2005; Wang *et al.*, 2007; Jacob *et al.*, 2012; Guy *et al.*, 2014; Luedtke *et al.*, 2014). However, these assays have targeted only up to three genes concurrently, and none have included antigen specific O157 gene (*rfb*E) in combination with the three major virulence genes (*stx*1, *stx*2 and *eae*). Our group has developed a multiplex real-time PCR assay that targeted *rfb*E, *stx*1 and *stx*2 to quantify STEC O157 in cattle feces (Jacob *et al.*, 2012). Because *eae* is critical for bacterial attachment and a certain proportion of STEC O157 contain *stx*1 alone or in association with *stx*2, all four genes (*rfb*E, *stx*1, *stx*2 and *eae*) are better targets to detect and quantify STEC O157 in cattle feces. Optimization of a four-plex PCR reaction is challenging because of potential interactions among primers and probes in the reaction. Multiple primer pairs and probes can often result in the formation of secondary structures, which can then be amplified in lieu of target DNA. Dimer interactions become increasingly problematic for optimization of mqPCR, especially when more than three genes are targeted (Elnifro *et al.*, 2000); therefore, very few mqPCR assays have been developed that target four genes simultaneously (Pavlovic *et al.*, 2010; Anklam *et al.*, 2012; Luedtke *et al.*, 2014). The objectives of this study were to develop a multiplex (four-plex) qPCR (mqPCR) assay that targeted *rfb*E$_{O157}$, *stx*1, *stx*2 and *eae* genes for quantification of STEC O157 and validate the assay with pure cultures and cattle feces spiked with pure cultures. In addition, the applicability of the assay to detect STEC O157 in feedlot cattle feces was determined and compared with conventional PCR targeting the same four genes and a culture method of detection.

Materials and Methods

STEC Strains

A strain of STEC O157 (CDC EDL932; ATCC 43894, American Type Culture Collection, Manassas, VA) that is positive for the four target genes (*rfb*E, *stx*1, *stx*2 and *eae*) was used in the initial development and optimization of the assay conditions. The strain was grown overnight at 37°C on a blood agar plate (Remel, Lenexa, KS). DNA extraction from colonies was performed with Qiagen DNeasy blood and tissue kit (Qiagen, Valencia, CA). Ten-fold serial dilutions of the extracted DNA were performed in ddH_20 for the mqPCR assay. Subsequent validation of the assay included additional strains of STEC O157 with different combinations of the target genes, and strains of non-O157 STEC (Table 2.1). Analytical specificity of the mqPCR assay was tested with 25 strains of STEC O157, 26 strains of non-O157 STEC that included O26, O45, O103, O111, O121 and O145 serogroups and four strains of non-*E. coli* bacteria (*Klebsiella pneumoniae*, *Morganella morganii*, *Salmonella enterica* [serogroup C1] and *Serratia marcescens*).

Primers and Probes

The primers and probes for *rfb*E_{O157}, *stx*1 and *stx*2 were from a previous study (Jacob *et al.*, 2012). Primers and probe for *eae* were designed based on evaluations of 315 sequences from the GenBank database available at the time. Sequences from the database were aligned using ClustalX version 2.1, and viewed in BioEdit version 7.1.3.0 for primers and probe selections. Primer and probe candidates with the greatest number of matched sequences were chosen for further analyses. Primers and probe for *eae* were chosen to have melting temperatures similar to the other three genes in the same mqPCR reaction (Table 2.2).

mqPCR running conditions

The working concentrations of all primers in a primer mix were 10 pM/µL. The working concentrations of probes were as follows: *rfb*E (2.5 pM/µL), *stx*1 (1.0 pM/µL), *stx*2 (10.0 pM/µL), and *eae* (10.0 pM/µL). The reaction consisted of 1 µL of primer mix and each probe, 10 µL of BioRad iQ multiplex powermix, 4 µL of sterile PCR grade water, and 1 µL of DNA template (total reaction volume = 20 µL). The assay running conditions consisted of 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec, 56°C for 20 sec, and 72°C for 40 sec. Probe concentrations were optimized by comparing different dilutions of each probe for each target gene. All samples used to generate standard curve data, PCR efficiency, and R^2 values were run in triplicate. Assays were performed with the BioRad CFX96 Real-Time System.

Analytical sensitivity of the mqPCR assay

The analytical sensitivity of the assay was assessed with five strains of *E. coli* O157 with variable target genes (four STEC and one non-STEC) and six strains of non-O157 STEC belonging to O26, O45, O103, O111, O121, and O145 serogroups (Table 2.1). Single colonies of *E. coli* strains grown on blood agar plates were inoculated into 10 mL Luria-Bertani (LB; Becton Dickinson Co., Sparks, MD) broth and incubated at 37°C for 16 h. Then, 100 µL was inoculated into 10 mL LB and incubated at 37°C until an absorbance of 0.4 (600 nm) was achieved (approximately 3 h and 10^8 CFU/mL). Serial ten-fold dilutions in LB broth were prepared and 1 mL from each of 10^{-1} to 10^{-7} dilutions was boiled for 10 min and centrifuged at 9,000 RCF for 5 min to obtain a crude DNA preparation. Extracted DNA (1 µL) was then subjected to the mqPCR assay. To determine viable cell counts (CFU/mL), 100 µL of the serially diluted LB broth (dilutions 10^{-5}, 10^{-6} and 10^7) were spread-plated on blood agar plates

and incubated overnight at 37°C, then colonies were counted. Two replications of the assay with pure cultures of STEC/non-STEC O157 and non-O157 STEC strains were performed.

Cattle feces that were spiked with pure cultures of STEC O157 ATCC 43894 and ATCC 43889 strains with variable target genes also were tested to determine the analytical sensitivity of the assay (Table 2.1). Fecal samples were obtained from pen-floor fecal pats from the university dairy farm. Fecal samples confirmed as negative for the four target genes by the mqPCR assay were used. Serial ten-fold dilutions of STEC ATCC 43894 and 43889 were prepared in LB broth as previously described and DNA was extracted from each dilution. One milliliter from each serial dilution of LB was added to 10 g of feces and mixed thoroughly. Then, 1 g of spiked fecal mix was added to 9 mL of Gram-negative broth (Becton Dickinson Co.) amended with cefixime (0.05 mg/liter), cefsulodin (10 mg/liter), and vancomycin (8 mg/liter; GNccv) and vortexed. One milliliter of spiked fecal sample suspended in GNccv broth was removed before and after a 6-h enrichment at 37^{o} C, boiled for 10 min and centrifuged at 9,000 RCF for 5 min to obtain a crude DNA template. Extracted DNA was purified with a GeneClean® Turbo Kit (MP Biomedicals LLC, Solon, OH) and subjected to the mqPCR assay. Two replications of the spiked fecal sample experiment were performed for each strain of STEC O157 (ATCC 43894 and 43889). Minimum detection limits, based on Ct values from the mqPCR assay, were determined for pure cultures, as well as for spiked fecal samples before and after 6-hour enrichment.

Application of mqPCR assay to detect and quantify STEC O157 in fecal samples from naturally shedding feedlot cattle

DNA extracted from cattle fecal samples, before and after a 6-h enrichment, was utilized from a prior study (Cull *et al.*, 2012) on fecal shedding of STEC O157:H7. Extracted DNA was

from a subset of fecal samples (n=278), randomly selected from a total of 1,200 samples from the control group. Of the samples that were positive (136/278) and negative by mqPCR (142/278) for STEC O157, 100 randomly selected (using the RAND and SORT functions in Microsoft Excel) samples of each were tested by a conventional PCR assay (Bai *et al.*, 2010) that targeted the same four genes *rfb*E_{O157}, *stx*1, *stx*2 and *eae*. Multiplex qPCR and conventional PCR results were compared with a culture-based method of detection, which was based on immunomagnetic bead separation, plating on a selective medium, and PCR confirmation of the putative isolates. The culture data were obtained from the previous study (Cull *et al.*, 2012).

In a separate experiment, fecal samples (n=576) of crossbred finishing cattle were collected from pens in a commercial feedlot in the central US during a 12-week period from June through August 2013. Approximately 2 g of feces were added to 18 ml of *Escherichia coli* broth (EC medium; Difco™, Becton, Dickinson Co., Sparks, MD), vortexed for 30 s and incubated at 40° C for 6 h. One hundred microliters of a 1:10 dilution of pre-enriched fecal suspension was subjected to spiral-plate method for quantification of *E. coli* O157. One milliliter aliquots of the fecal suspension in EC broth before (pre-enrichment) and after incubation (post-enrichment) were boiled for 10 min, then centrifuged at 9,300 x g for 5 min. One hundred µl of crude DNA, from pre- and post-enriched samples, were then purified with GeneClean® Turbo Kits (MP Biomedicals, Solon, OH) and subjected to the 4-plex qPCR assay and a cPCR assay (Bai *et al.*, 2012) to detect the O157 serogroup and four virulence genes (*stx*1, *stx*2, *eae* and *ehx*A). Enriched fecal suspensions were subjected to IMS procedure and plated on selective medium. Nine-hundred and eighty microliters of enriched sample were mixed with 20 µl of serogroup-specific IMS beads (Abraxis, Warminster, PA) in 96-well microtiter plates. The IMS procedure was carried out in a Kingfisher™ Flex Magnetic Particle Processor (Thermo Scientific,

Waltham, MA) according to the protocol provided by the manufacturer. Then, 50 µl of the sample bead suspensions were spread-plated onto sorbitol MacConkey agar containing cefixime (0.05 mg/l) and potassium tellurite (2.5 mg/l; CT SMAC). Plates were incubated for 20 to 24 h at 37° C. Six sorbitol-negative colonies from CT-SMAC were picked, inoculated onto blood agar (Remel, Lenexa, KS) plates, and incubated at 37° C for 24 h. The non-sorbitol-fermenting colonies from the CT-SMAC plates were tested for the O157 antigen by latex agglutination (Oxoid, Basingstoke, United Kingdom); if positive, a spot-indole test was performed. Colonies positive for agglutination and indole production were tested by a 6-plex PCR (Bai *et al.*, 2010) that targeted the *rfb*E, *fliC*$_{H7}$, *eae*, *stx*1, *stx*2, and *ehx*A genes.

Statistical analysis

Cohen's Kappa statistic and 95% confidence intervals were computed to determine the agreement beyond that due to chance between mqPCR, cPCR, and culture methods for detection of STEC O157 in fecal samples using STATA MP 12.0 (StataCorp, College Station, TX).. Interpretations of the Kappa statistic were based on the scale proposed by Landis and Koch (Landis and Koch, 1977). In addition, the McNemar's chi-square test (McNamara, 1947) was used to compare the proportion of positives identified by the three detection methods. When McNemar's tests were significant, Kappa statistics were provided for reference only.

Receiver operating characteristic (ROC) curve analysis was used to analyze average Ct values of 100 mqPCR positive samples against positive or negative detection values of cPCR. Given that Ct values are negatively correlated with a positive outcome (e.g., lower Ct values indicate a positive reaction, thus a sample is more likely to be classified as positive), a reciprocal transformation of the Ct values was performed. Reciprocal Ct values were used as

discrimination thresholds to determine diagnostic sensitivity and specificity of the cPCR assay as it relates to the mqPCR.

Results

The optimization of the mqPCR assay was achieved with DNA from the STEC O157 ATCC 43894 strain, which was positive for all four target genes. The end-point threshold cycle (Ct) values for each of the four genes were determined and standard curve data were generated from serially-diluted (ten-fold dilutions) DNA. An average end-point Ct value of 37.4 was obtained for the four genes. Correlation coefficients were > 0.99, and PCR efficiency was 91-108%. Based on colony count data, the average minimum detection limit was 3.1 x 10^3 CFU/mL for STEC *E. coli* ATCC 43894 (Table 2.3).

Analytical specificity

Specificity of the mqPCR assay was tested with strains of STEC O157 (n=25), non-O157 STEC (n=26) and non-*E. coli* bacterial species (n=4) and results indicated that the assay correctly detected the presence or absence of the four genes, *rfb*E_{O157}, *stx*1, *stx*2 and *eae* (data not shown) in all strains (n=55). In the non-O157 STEC serogroups, the assay detected only the virulence genes and not the *rfb*E gene (Table 2.3).

Analytical sensitivity with pure cultures

Two replications of the pure culture assay were performed on STEC/non-STEC O157 and non-O157 STEC strains with variable target genes (Table 2.3). Initial concentrations of all STEC/non-STEC O157 subjected to ten-fold serial dilutions were from 2.6 to 4.3 x 10^8 CFU/mL. The average minimum detection limits of the assay for all STEC/non-STEC O157 strains used in the study were from 3.1 to 3.5 x 10^3 CFU/mL with average Ct values of 37.6 to 38.8. Correlation coefficients were > 0.99 for all pure cultures of STEC/non-STEC O157 and PCR

efficiencies were 90 to 110%. Initial concentrations of pure cultures of non-O157 STEC strains subjected to ten-fold serial dilutions were from 1.7 to 2.9 x 10^8 CFU/mL with average Ct values of 37.6 to 38.8. As expected, none of the six non-O157 STEC showed amplification of the *rfb*E_{O157} gene. For non-O157 STEC with variable *stx*1, *stx*2 and *eae* genes, the average minimum detection limit of the assay was from 2.2 to 2.7 x 10^3 CFU/mL. Correlation coefficients for all non-O157 STEC pure cultures, determined from the standard curve data, were > 0.99 and PCR efficiencies were 91 to 112% (Table 2.3).

Analytical sensitivity with cattle feces spiked with STEC O157

Before enrichment, the minimum detection limits of the assay with feces spiked with serially diluted cultures (ten-fold) of *E. coli* ATCC 43894 (positive for all four genes) or ATCC 43889 (positive for *rfb*E_{O157}, *stx*2 and *eae*) were 2.8 x 10^4 and 2.9 x 10^4 CFU/g, respectively. After 6-h of enrichment, detection limit was improved to 2.8 x 10^0 and 2.9 x 10^0 CFU/g for *E. coli* ATCC 43894 and ATCC 43889, respectively (Table 2.4).

Application of mqPCR assay and comparison with cPCR and a culture method for detection and quantification of STEC O157 in fecal samples from feedlot cattle

In the assay of fecal samples (n=278) by mqPCR assay, a sample positive for *rfb*E, *eae* and at least one *stx* gene was considered as positive and a sample negative for *rfb*E was considered negative for STEC O157. Of the 278 fecal samples subjected to mqPCR, 136 were positive and had Ct values that were below the designated maximum threshold for *rfb*E (37.6), *eae* (37.1) and either or both *stx*1 (37.6) and *stx*2 (37.2) genes. Of the 100 samples that were randomly picked from the 136 positive samples, 35 tested positive by cPCR and 48 were positive by culture-based detection (Table 2.5). Of the 100 samples randomly chosen from the 142 that were negative by mqPCR, none were positive by cPCR, but 21 samples tested positive by the culture-method

(Table 2.5). The Cohen's Kappa statistics indicated a slight agreement beyond that due to chance between the mqPCR and cPCR tests (Kappa = 0.35) and between mqPCR and the culture method (Kappa = 0.27). The McNemar's chi-square tests for these comparisons were statistically significant ($P < 0.05$), indicating a disagreement between the proportions of positive samples detected by these methods (Table 2.5).

Analysis of the Ct values of the 35 samples that tested positive by the cPCR assay indicated that 30 samples (86%) had average Ct values less than 31.0 for all four target genes (Figure 1A). For the Ct values of the 65 samples that tested negative by the cPCR assay, 63 samples (97%) had average Ct values greater than 31.0 (Figure 1B). The ROC curve analysis determined that a Ct value of 31.0 (reciprocal value = 0.0322) yielded optimum sensitivity (85.7%) and specificity (96.9%) as well as the highest number of samples correctly classified (93%) by the cPCR assay. Diagnostic sensitivity, false positive rate (1- specificity) and area under the curve (AUC) for all observations are depicted in the ROC curve in Figure 2.

In the assay of fecal samples (n=576) from a commercial feedlot, the mqPCR detected more samples as positive for O157 than cPCR or culture method. The mqPCR assay quantified O157 in a higher proportion (62/576; 10.8%) of pre-enriched samples than the spiral-plate method (26/576; 4.5%). Fecal samples positive by mqPCR were at concentrations of 10^6 (3/62), 10^5 (24/62) and 10^4 (35/62) CFU/g and by spiral-plate method concentrations were at 10^5 (2/26), 10^4 (5/26), 10^3 (2/26) and $< 10^3$ (17/26) CFU/g. A higher proportion of enriched samples were positive for O157 by mqPCR (517/576; 89.8%) than by cPCR (315/576; 54.7%) or culture-based method (247/576; 42.9%).

Discussion

Our multiplex, real-time PCR assay is novel in that it targets the O157-specific gene and the three major virulence genes concurrently. Many multiplex qPCR assays that target different combinations of major genes of STEC O157, generally *rfb*E$_{O157}$, *per, ecf1, uid*A, *fliC*$_{H7}$, *stx*1, *stx*2, *eae*, and *ehx*A have been developed (Fortin *et al.*, 2001; Ibekwe and Grieve, 2003; Sharma and Dean-Nystrom, 2003; Fitzmaurice *et al.*, 2004; Hsu *et al.*, 2005; Bertrand and Roig, 2007; Wang *et al.*, 2007; Madic *et al.*, 2011; Anklam *et al.*, 2012; Jacob *et al.*, 2012; Leudtke *et al.*, 2014; Mancusi and Trevisani 2014; Russo *et al.*, 2014). In addition, a number of real-time PCR-based commercial detection systems with undisclosed gene targets have been used to detect and quantify STEC O157 (Burns *et al.*, 2011; Fratamico *et al.*, 2014; Wasilenko *et al.*, 2014). These assays have been used to characterize isolates, and to detect and or quantify STEC O157 in a variety of sample matrices, including feces, ground beef, dairy products, produce, and wastewater. However, only a few of the assays have been applied for the detection and quantification of STEC O157 in fecal samples (Ibekwe and Grieve, 2003; Sharma and Dean-Nystrom, 2003; Fitzmaurice *et al.*, 2004; Hsu *et al.*, 2005; Wang *et al.*, 2007; Anklam *et al.*, 2012; Jacob *et al.*, 2012; Leudtke *et al.*, 2014). Only the assay developed by Jacob *et al.* (2012) targeted the combination of the serogroup-specific *rfb*E gene with the *stx*1 and *stx*2 genes, but the assay did not include *eae*. Although *stx*1 and *stx*2 genes share nucleotide (58%) and amino acid (56%) sequence homologies (Jackson *et al.*, 1987), there is no consensus region (65-200 bp) that can be used to design a single qPCR test for both genes. Because intimin is a critical virulence factor for attachment to enterocytes, we chose to include *eae* gene to make it a four-plex assay. According to the FSIS beef testing protocol that uses a two-stage PCR screening test, a beef sample would be considered positive (adulterated) when it tested positive for at least one Shiga toxin (*stx*1 or *stx*2), *eae* gene and an O-group gene USDA-FSIS). Although this definition

is not applicable to feces, inclusion of *eae* distinguishes EHEC from STEC. However, based on our experience, the occurrence of STEC O157 in cattle feces that is negative for *eae* is rare. Anklam *et al.* (2012) developed two sets of multiplex qPCR assays; one targeting *rfb*E with *uidA* as an internal *E. coli* control and the other targeting the four virulence genes (*stx*1, *stx*2, *eae,* and *ehx*A). Leudtke *et al.* (2014) targeted the enterohemorrhagic *E. coli* (EHEC)-specific target gene, *ecf1,* in combination with the three virulence genes (*stx*1, *stx*2, and *eae*). The advantage of targeting the *ecf1* gene is that it identifies all EHEC (O157 and other serogroups). Our assay is designed to detect and quantify only STEC O157 serogroup. None of the previous studies with the exception of Jacob *et al.* (2014) have compared the real-time assay with conventional PCR and culture method of detection of STEC O157 in the feces of naturally shedding cattle.

The results obtained in this study indicate that the mqPCR assay is a sensitive method to detect and quantify STEC O157 in cattle feces. The validation of the quantification was done with pure cultures or feces spiked with pure cultures of STEC O157. The assay also may be useful in identifying cattle that shed very high concentrations of STEC O157 ($\geq 10^4$ CFU/g). The application of mqPCR in determining fecal concentration of STEC O157 in naturally-shedding cattle and a comparison with the culture-based spiral-plate method was evaluated. Our data indicate that mqPCR was more sensitive than spiral-plate method in detecting samples positive for *E. coli* O157 at $\geq 10^4$ CFU/g. However, the spiral-plate method was more sensitive in detecting samples positive for O157 at $\leq 10^4$ CFU/g. Based on the assay with pure cultures of strains of STEC O157, the minimum detection limit was 10^3 CFU/mL, which is in agreement with a previous study (Jacob *et al.*, 2012). A positive fluorescence signal for *rfb*E was absent for all non-O157 STEC, indicating the serogroup specificity of the mqPCR assay for STEC O157.

Average minimum detection limits for remaining target genes with non-O157 STEC, subjected to ten-fold serial dilutions, were slightly higher compared to STEC O157 strains.

DNA extracted directly from cattle feces spiked with two strains of STEC O157 (ATCC 43894 and ATCC 43889) generated a minimum detection limit of ~10^4 CFU/g for the mqPCR assay, which is also in agreement with the previous study (Jacob *et al.*, 2012). However, detection limits improved to 10^0 CFU/g when an enrichment step was included, one log lower detection than reported by Jacob *et al.* (2012). Minimum detection limits of the mqPCR assay for both pre-enriched and enriched samples were similar between the strains tested, indicating precise detection of STEC O157 strains variable for the target genes. Although the assay can accurately detect the presence of STEC O157 serogroup and the three virulence genes, it is possible that virulence genes amplified in a sample could be from non-O157 STEC present in the feces.

When comparing agreement among tests, McNemar's chi-square tests ($P < 0.05$) indicated disagreement between the proportion of positive samples detected by mqPCR, cPCR and the culture method, indicating possible differences in sensitivity among the three methods. Based on ROC curve analysis of Ct values, the cPCR was less sensitive than mqPCR in detecting *rfb*E, *stx*1, *stx*2 and *eae* genes. Overall, the mqPCR assay detected a higher proportion of positive samples than the cPCR assay or culture method. However, 21% of samples that were positive by culture method were negative by the mqPCR, which indicates that the use of real-time PCR to screen samples before subjecting positive samples to a culture method may underestimate the presence of STEC O157 in fecal samples. The reason for the misidentification of culture positive samples by mqPCR is not known but is likely reflective of the difference in detection limit between the two methods. The mqPCR requires a concentration approaching 10^4 CFU per

g for detection; whereas the immunomagnetic bead-based culture method may be able to capture STEC O157 cells at lower concentrations. As with all PCR assays, there is also a possibility for false positives because of amplification of DNA from non-viable cells in the feces.

In conclusion, the validated mqPCR assay is novel in that it targets four major genes (*rfb*E, *stx*1, *stx*2 and *eae*) of STEC O157. Although mqPCR is a more sensitive method of detection, the use of real-time PCR as a screening method to identify positive samples and then subjecting only positive samples to a culture method may underestimate the presence of STEC O157 in fecal samples. Therefore, subjecting fecal samples to culture-based methods may remain necessary, in addition to real-time PCR, to obtain a more accurate estimate of the presence of STEC O157 in cattle feces.

Figure 2.1 Percentages of cycle threshold (Ct) values of the 100 multiplex quantitative PCR positive fecal samples from feedlot cattle that were greater (dark gray) or lower (light gray) than Ct 31 in positive (n=35; A) or negative (n=65; B) by conventional PCR (cPCR)

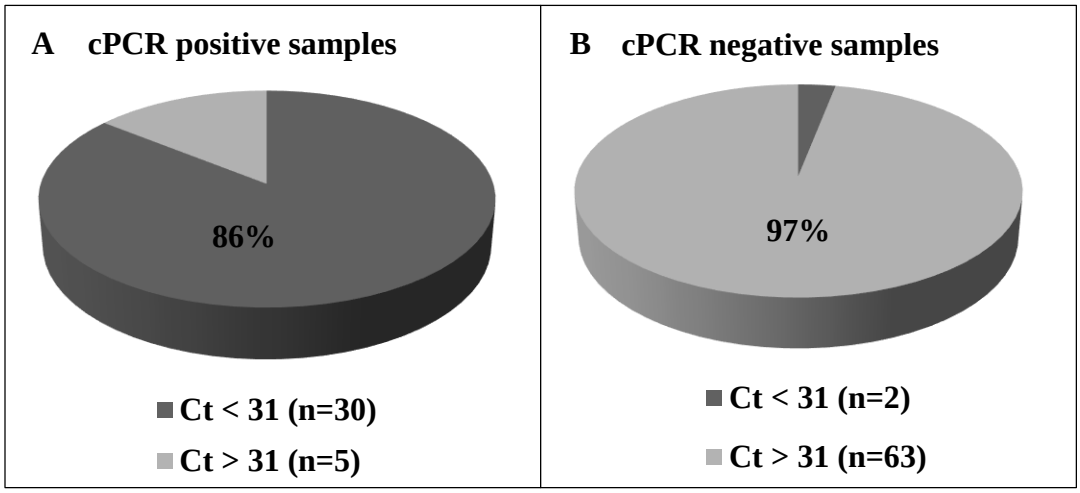
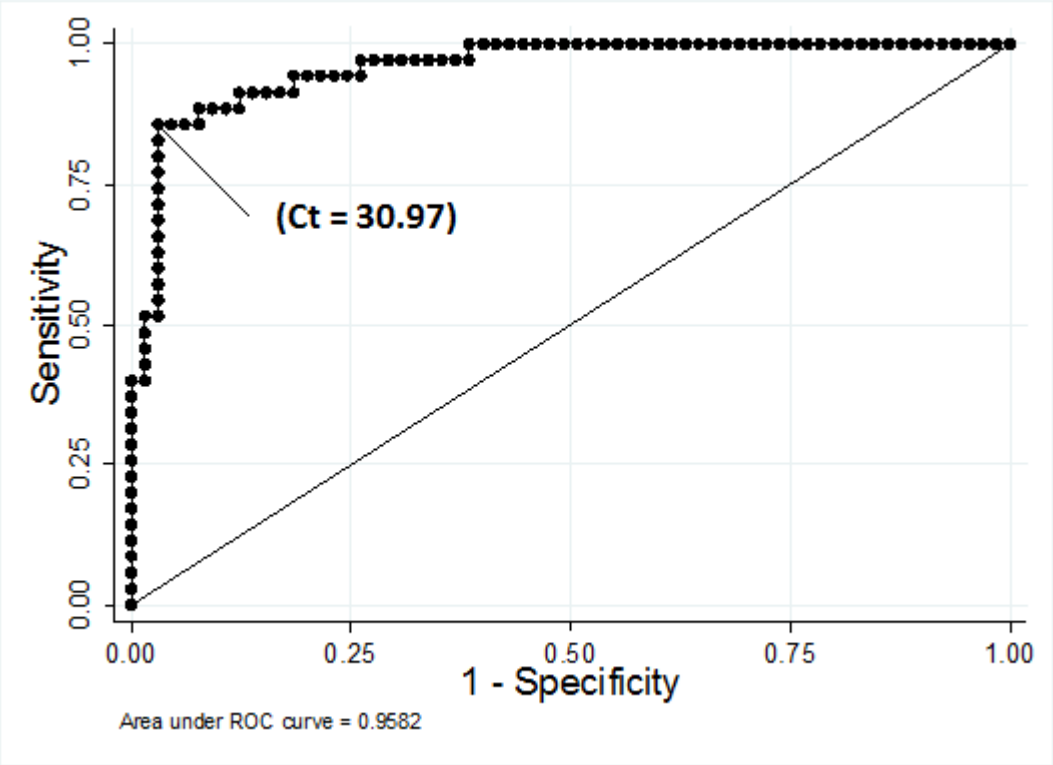


Figure 2.2 Receiver Operating Characteristic (ROC) Graph of Conventional PCR for 100 Feedlot Cattle Fecal Samples Positive for Shiga toxin-producing *Escherichia coli* O157 by Multiplex Quantitative PCR



95% Confidence Interval (0.90074-0.98900)
Standard Error = 0.0184

Table 2.1 Virulence gene profiles for Shiga toxin-producing and non-Shiga toxin-producing *Escherichia coli* (STEC) used in the development and validation of the multiplex quantitative PCR assay

Serogroup	*Strain*	*Source*	*rfb*E	*stx*1	*stx*2	*eae*
E. coli O157	ATCC 43894	Human	+	+	+	+
E. coli O157	ATCC 43888	Human	+	-	-	+
E. coli O157	ATCC 43889	Human	+	-	+	+
E. coli O157	ATCC 43890	Human	+	+	-	+
E. coli O157	08-4-553-F	Bovine	+	-	-	-
E. coli O26	TW 8569	Human	-	-	+	+
E. coli O45	KDHE 22	Human	-	+	-	+
E. coli O103	TW 8103	Human	-	+	-	+
E. coli O111	7726-1	Bovine	-	+	+	+
E. coli O121	KDHE 48	Human	-	-	+	+
E. coli O145	KDHE 53	Human	-	+	+	+

Table 2.2 Primers and probes used in the multiplex quantitative PCR assay for the detection and quantification of Shiga toxin-producing *Escherichia coli* O157

Gene	*Primer/probe*	*Sequence*	*Fluorescent dye*	*Quencher*	*Reference*
rfbE_{O157}	Probe	TTAATTCCACGCCAACCAAGATCCTCA	FAM	Iowa Black FQ	Jacob *et al.* (2012)
	Forward Primer	CTGTCCACACGATGCCAATG			
	Reverse Primer	CGATAGGCTGGGGAAACTAGG			
*stx*1	Probe	ACATAAGAACGCCCACTGAGATCATCCA	Texas Red	BHQ-2	Jacob *et al.* (2012)
	Forward Primer	CAAGAGCGATGTTACGGTTTG			
	Reverse Primer	GTAAGATCAACATCTTCAGCAGTC			
*stx*2	Probe	TGTCACTGTCACAGCAGAAGCCTTACG	Cy5	BHQ-2	Jacob *et al.* (2012)
	Forward Primer	GCATCCAGAGCAGTTCTGC			
	Reverse Primer	GCGTCATCGTATACACAGGAG			
eae	Probe	CTCTGCAGATTAACCTCTGCCG	VIC	ZEN	This study
	Forward Primer	AAAGCGGGAGTCAATGTAACG			
	Reverse Primer	GGCGATTACGCGAAAGATAC			

Table 2.3 Average detection limits, correlation coefficients and PCR amplification efficiencies of multiplex quantitative PCR of pure cultures of Shiga toxin-producing *Escherichia coli* (STEC)/non-STEC O157 and non-O157 STEC serogroups cultured in Luria Bertani broth

E. coli serogroup	*Strain (gene profile)*	**Average end-point threshold cycle (Ct) of gene*				*Total average Ct**	*Detection limit (CFU/mL)*	*Correlation coefficients*	*PCR efficiency (%)*
		*rfb*E	*stx*1	*stx*2	*eae*				
O157	ATCC 43894 (*rfb*E$^+$, *stx*1$^+$, *stx*2$^+$, *eae*$^+$)	37.0	38.0	39.2	39.6	38.5	3.1x10^3	> 0.99	90-110
O157	ATCC 43888 (*rfb*E$^+$, *stx*1$^-$, *stx*2$^-$, *eae*$^+$)	38.9	-	-	38.2	38.6	3.2x10^3	> 0.99	92-104
O157	ATCC 43889 (*rfb*E$^+$, *stx*1$^-$, *stx*2$^+$, *eae*$^+$)	37.7	-	39.0	39.6	38.8	3.4x10^3	> 0.99	96-105
O157	ATCC 43890 (*rfb*E$^+$, *stx*1$^+$, *stx*2$^-$, *eae*$^+$)	37.7	37.9	-	39.4	38.3	3.1x10^3	> 0.99	96-110
O157	08-4-553-F (*rfb*E$^+$, *stx*1$^-$, *stx*2$^-$, *eae*$^-$)	37.6	-	-	-	37.6	3.5x10^3	> 0.99	105-107
O26	TW 8569 (*rfb*E$^-$, *stx*1$^-$, *stx*2$^+$, *eae*$^+$)	-	-	39.5	37.5	38.5	2.5x10^3	> 0.99	101-112
O45	KDHE 22 (*rfb*E$^-$, *stx*1$^+$, *stx*2$^-$, *eae*$^+$)	-	38.5	-	38.1	38.3	2.3x10^3	> 0.99	107-112
O103	TW 8103 (*rfb*E$^-$, *stx*1$^+$, *stx*2$^-$, *eae*$^+$)	-	38.7	-	38.9	38.8	2.3x10^3	> 0.99	91-110
O111	7726-1 (*rfb*E$^-$, *stx*1$^+$, *stx*2$^+$, *eae*$^+$)	-	38.0	37.4	38.5	38.0	2.7x10^3	> 0.99	92-98
O121	KDHE 48 (*rfb*E$^-$, *stx*1$^-$, *stx*2$^+$, *eae*$^+$)	-	-	37.1	38.1	37.6	2.2x10^3	> 0.99	97-104
O145	KDHE 53 (*rfb*E$^-$, *stx*1$^+$, *stx*2$^+$, *eae*$^+$)	-	37.6	38.1	37.6	37.8	2.5x10^3	> 0.99	92-106

*Data are shown as means from two independent experiments

Table 2.4 Detection limits, correlation coefficients and PCR amplification efficiencies of multiplex quantitative PCR of feces spiked with Shiga toxin-producing *Escherichia coli* O157 strains

Experiment	**Average end-point threshold cycle (Ct) of gene*				Total average Ct*	*Detection limit* (CFU/g)*	*Correlation coefficients*	*PCR efficiency (%)*
	*rfb*E	*stx*1	*stx*2	*eae*				
E. coli O157 ATCC 43894 in Luria Bertani broth	37.4	37.1	37.5	37.3	37.3	$2.8x10^3$	> 0.99	96-102
Feces spiked with *E. coli* O157 ATCC 43894 (*rfb*E^+, *stx*1^+, *stx*2^+, *eae*$^+$)								
Before enrichment	37.8	38.2	38.4	38.8	38.3	$2.8x10^4$	> 0.99	91-110
After enrichment	38.1	37.8	37.9	37.9	37.9	$2.8x10^0$	> 0.98	97-111
E. coli O157 ATCC 43889 in Luria Bertani broth	37.0	-	37.2	37.4	37.2	$2.9x10^3$	> 0.99	90-101
Feces spiked with *E. coli* O157 ATCC 43889 (*rfb*E^+, *stx*1^-, *stx*2^+, *eae*$^+$)								
Before enrichment	38.3	-	38.1	38.0	38.1	$2.9x10^4$	> 0.95	88-110
After enrichment	38.1	-	38.1	38.1	38.1	$2.9x10^0$	> 0.99	92-112

*Data are shown as means from two independent experiments

Table 2.5 Comparison of multiplex quantitative PCR, conventional PCR and culture method for detection of Shiga toxin-producing *Escherichia coli* O157 in cattle feces (n=200) enriched in Gram negative broth

Methods		*Quantitative PCR*			*Kappa*			*McNemar's* χ^2	
		Positive (n=100)	Negative (n=100)	Total	Statistic (95% CI)	SE	*P*-value	Statistic	*P*-value
Conventional PCR[a]	Positive	35	0	35	0.35	0.05	< 0.01	65.0	< 0.01
	Negative	65	100	165	(0.25 – 0.45)				
Culture method[b]	Positive	48	21	69	0.27	0.07	< 0.01	13.2	< 0.01
	Negative	52	79	131	(0.13 – 0.41)				

[a]Based on the detection of the genes that code for O157 antigen, *rfb*E, and the three virulence genes, *stx*1, *stx*2 and *eae* in feces enriched in Gram negative broth amended with cefixime (0.05 mg/liter), cefsulodin (10 mg/liter), and vancomycin (8 mg/liter) at 37 C for 6 h (Bai *et al.*, 2012).

[b] Based on immunomagnetic separation, plating on selective medium and confirmation of the isolate by a multiplex PCR that targeted *rfb*E, *stx*1, *stx*2 and *eae* genes (Cull *et al.*, 2012).

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