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Research Note

Comparing Real-Time and Conventional PCR to Culture-Based Methods for Detecting and Quantifying *Escherichia coli* O157 in Cattle Feces

M. E. JACOB,[1]* J. BAI,[2] D. G. RENTER,[2] A. T. ROGERS,[1] X. SHI,[2] AND T. G. NAGARAJA[2]

[1]*Department of Population Health and Pathobiology, North Carolina State University, 1060 William Moore Drive, College of Veterinary Medicine, Raleigh, North Carolina 27607; and* [2]*Department of Diagnostic Medicine/Pathobiology, Kansas State University, 1800 Denison Avenue, Manhattan, Kansas 66506, USA*

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ABSTRACT

Detection of *Escherichia coli* O157 in cattle feces has traditionally used culture-based methods; PCR-based methods have been suggested as an alternative. We aimed to determine if multiplex real-time (mq) or conventional PCR methods could reliably detect cattle naturally shedding high ($\geq 10^4$ CFU/g of feces) and low ($\sim 10^2$ CFU/g of feces) concentrations of *E. coli* O157. Feces were collected from pens of feedlot cattle and evaluated for *E. coli* O157 by culture methods. Samples were categorized as (i) high shedders, (ii) immunomagnetic separation (IMS) positive after enrichment, or (iii) culture negative. DNA was extracted pre- and postenrichment from 100 fecal samples from each category (high shedder, IMS positive, culture negative) and subjected to mqPCR and conventional PCR assays based on detecting three genes, *rfbE*, stx_1, and stx_2. In feces from cattle determined to be *E. coli* O157 high shedders by culture, 37% were positive by mqPCR prior to enrichment; 85% of samples were positive after enrichment. In IMS-positive samples, 4% were positive by mqPCR prior to enrichment, while 43% were positive after enrichment. In culture-negative feces, 7% were positive by mqPCR prior to enrichment, and 40% were positive after enrichment. The proportion of high shedder–positive and culture-positive (high shedder and IMS) samples were significantly different from mqPCR-positive samples before and after enrichment ($P < 0.01$). Similar results were observed for conventional PCR. Our data suggest that mqPCR and conventional PCR are most useful in identifying high shedder animals and may not be an appropriate substitute to culture-based methods for detection of *E. coli* O157 in cattle feces.

Ruminants are reservoirs for *Escherichia coli* O157, a foodborne pathogen that can colonize the hindgut and be shed in the feces *(17, 24)*. Typically, cattle shed *E. coli* O157 concentrations at or below 10^2 CFU/g of feces; however, cattle referred to as high shedders (or super shedders) can shed greater than 10^4 CFU/g of feces *(15, 18)*. The presence and concentration of *E. coli* O157 in cattle feces affects the transmission of the organism between animals, within the environment, and eventually onto carcasses at slaughter *(1, 8, 11, 16)*. Detection of *E. coli* O157 in cattle feces has allowed for an improved understanding of the organism's epidemiology and ecology and has contributed to our understanding of preharvest interventions strategies *(4, 5, 23)*.

Traditionally, culture-based methods have been used to detect *E. coli* O157 in cattle feces. Culture methods to detect *E. coli* O157 commonly include selective enrichment, immunomagnetic bead separation (IMS), and plating onto selective and differential media that contain antibiotics or chromogenic substrates *(4, 14, 18, 23, 24)*. Culture methods used to quantify *E. coli* O157 in feces, or detect high shedders, have included IMS with the most-probable-number technique *(9, 22)*, spiral plating on selective or chromogenic media *(9, 19)*, and a technique of directly plating a fecal sample onto a selective medium *(8, 11, 20)*. Isolates obtained after culture are confirmed by biochemical tests, antigen agglutination, and/or PCR detection of virulence genes, including those that encode O157, H7, and Shiga toxins. Although culture-based methods are common and highly specific, they are time consuming and labor intensive.

Molecular-based *E. coli* O157 detection methods potentially offer advantages, including a faster turnaround time and high throughput capacity. Molecular methods designed to detect and quantify *E. coli* O157 in cattle feces by targeting various combinations of *rfbE*, *eae*, and Shiga toxigenic genes have been previously described *(7, 10, 12, 21)*. We have developed a multiplex real-time PCR (mqPCR) that targets *rfbE*, stx_1, and stx_2 for quantification of *E. coli* O157 in cattle feces *(12)*. However, a large-scale evaluation comparing the culture and molecular-based detection techniques for *E. coli* O157 has not been done in cattle naturally shedding the organism in feces. Therefore, we conducted a study to compare detection of *E. coli* O157

* Author for correspondence. Tel: 919-513-6236; Fax: 919-513-6464; E-mail: megan_jacob@ncsu.edu.

by mqPCR and conventional PCR, both targeting *rfbE,* stx_1, and stx_2 to cattle feces that were culture negative (IMS negative after enrichment), IMS positive (positive after enrichment and IMS), and high shedders (culture positive by direct plating before enrichment). Our objectives were to (i) determine if the mqPCR assay could be used to detect high shedders in cattle feces and (ii) determine if, after enrichment, the assay could reliably detect cattle naturally shedding low concentrations of *E. coli* O157.

MATERIALS AND METHODS

Study design. An epidemiologic preharvest *E. coli* O157 intervention trial that used culture-based detection methods *(5)* were the source of cattle naturally shedding *E. coli* O157 in their feces for this study. Prior to and after incubation, an aliquot of the fecal enrichment suspension, used in the culture-based detection of *E. coli* O157, was collected for each sample (animal) and stored for further analysis. After culture-based methods indicated samples were from high shedder animals, IMS-positive animals, or were culture negative, 100 samples from each group were identified, and the DNA from stored samples (pre- and postenrichment) was extracted and evaluated by mqPCR and conventional PCR for detection of *E. coli* O157.

Culture-based detection of *E. coli* O157 in cattle feces. Fresh pen-floor fecal samples ($n = 4{,}800$) were collected from a cattle feedlot in the midwestern United States *(5).* Briefly, feces were collected from animals observed defecating throughout a feedlot pen, and care was taken to avoid ground contamination. Samples were placed in sterile bags and transported to the laboratory on ice for processing within 24 h. Samples were cultured for *E. coli* O157 using direct plating methods and enrichment with IMS as previously described *(5).* The direct plating procedure used to classify animals as high shedders has been previously validated *(20).* Approximately 1 g of feces was placed into 9 ml of gram-negative broth (BD, Franklin Lakes, NJ) with cefixime (0.5 mg/liter), cefsoludin (10 mg/liter), and vancomycin (GNccv; 8 mg/liter), and tubes were vortexed. A swab of fecal suspension in GNccv broth was plated onto the top quadrant of sorbitol MacConkey agar supplemented with cefixime (0.05 mg/liter) and potassium tellurite (2.5 mg/liter; ct-SMAC). A loop was used to streak for isolation, and plates were incubated for 20 h at 37°C. The GNccv tubes were incubated for 5.5 h at 37°C, after which IMS (Dynabeads; Invitrogen Corp., Carlsbad, CA) was performed on 1 ml of enrichment. Prior to and after incubation in GNccv, 1-ml aliquots of vortexed fecal homogenate were removed from each sample and frozen at −80°C. Following IMS, bead suspensions were plated onto ct-SMAC plates and incubated overnight at 37°C. Up to six sorbitol-negative colonies from direct and enrichment ct-SMAC plates were streaked onto blood agar plates (Remel, Lenexa, KS) and incubated overnight at 37°C. Colonies were subsequently tested for indole production and latex agglutination for the O157 antigen (Oxoid, Remel). Isolates were confirmed to be *E. coli* O157:H7 with a multiplex PCR detecting the *rfbE, eae,* stx_1, stx_2, *ehxA,* and *fliC* genes *(2).* Fecal samples were classified as high shedders if positive for *E. coli* Ol57 on the direct plates, IMS positive if positive after enrichment and IMS only (not positive on direct plates), and culture negative if *E. coli* O157 was not obtained from either the direct plate or after enrichment and IMS plating.

Detection of *E. coli* O157 in cattle feces by mqPCR. Three hundred of the 4,800 collected fecal samples with a known culture status were identified from all fecal samples evaluated by culture-based methods. One hundred samples were randomly selected from each culture group (high shedder, IMS positive, culture negative) using a spreadsheet-based randomization table. A 100-μl aliquot of fecal suspension from each sample was subject to DNA extraction using a commercial kit (GeneClean, MP Biomedicals, Solon, OH) before and after enrichment in GNccv broth (pre- and postenrichment). Extracted DNA from all samples (299 preenrichment and 300 postenrichment) was subject to mqPCR and conventional PCR as described below; personnel responsible for mqPCR and conventional PCR reactions were blinded to the culture status of each sample. One preenrichment sample from the IMS-positive culture group failed to yield product after DNA extraction and was not included in the study.

The mqPCR used in this study has been previously described and validated for use on cattle feces *(12).* Briefly, all primers (stx_1 forward/reverse; stx_2 forward/reverse; and *rfbE* forward/reverse) and probes (stx_1, Texas Red, BHQ-2; stx_2, Cy5, BHQ-2; *rfbE,* FAM, BHQ-1) were diluted to a working stock concentration of 10 μM. Each reaction contained 2.5 μl of distilled (d) H_2O, 1 μl of forward primer, reverse primer, and probe for stx_1, stx_2, and *rfbE,* 12.5 μl of BioRad iQ multiplex powermix, and 1 μl of DNA template for a total reaction of 25 μl. The running conditions were an initial denaturation of 95°C for 10 min, followed by 45 cycles of 95°C for 15 s, 56°C for 20 s, and 72°C for 40 s. All reactions were run using the BioRad CFX96 Touch Real-Time PCR Detection System. Each sample was run in triplicate, and for most analyses the average cycle threshold (C_T) value for each gene was used (except where noted). Based on previous estimates of analytical sensitivity *(12),* a sample was considered positive if the *rfbE* (O157 side chain) gene and either stx_1 (Shiga toxigenic gene 1) or stx_2 (Shiga toxigenic gene 2) had an average C_T value of ≤36 (except where noted). DNA extracted from *E. coli* O157:H7 ATCC 43894 was used as a positive control in all reactions in this study.

Twenty-five postenrichment samples that were culture negative but were considered positive by mqPCR were subjected to an additional mqPCR reaction with SYBR Green to develop a stepwise melting temperature curve. Melt curve analysis was used to determine if the gene products amplified represented expected products. Additionally, mqPCR products from nine samples positive for at least one virulence gene (three each of stx_1, stx_2, and *rfbE*) and culture negative were further amplified using gene-specific primers. Resulting PCR amplicons were cleaned (QIAquick PCR Purification Kit, Qiagen, Germantown, MD) and sent for sequence analysis at the North Carolina State University Genome Sciences Laboratory. Sequences were analyzed using BLAST. Finally, 30 mqPCR reactions with C_T values between 21 and 45 were randomly chosen and imaged (QIAxcel, QIAgen) to evaluate if gene products were visually representative of the intended targets.

Identification of *E. coli* O157 in cattle feces by conventional PCR. In addition to mqPCR detection of *E. coli* O157, extracted DNA from pre- and postenrichment samples were directly subjected to a multiplex conventional PCR to detect *rfbE,* stx_1, and stx_2 genes *(2).* Briefly, the stock concentration of all primers was 100 μM. The conventional PCR reaction contained 8 μl of dH_2O, 10 μl of BioRad iQ multiplex powermix, 1 μl of the primer mix (equal volume of each stock primer), and 1 μl of DNA template to make a total reaction volume of 20 μl. The running conditions were an initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 65°C for 20 s, and 68°C for 75 s. A final extension of 68°C for 7 min was performed. Assays were run using the BioRad C1000. After the reaction, images were obtained on the QIAxcel (QIAgen) using a DNA screening cartridge, with

an alignment marker range of 15 bp to 3 kb and a DNA size marker from 50 to 1,500 bp. Samples were considered positive if there was visible product for *rfbE* and either stx_1 or stx_2. For three samples from the preenrichment subset, there was not enough remaining DNA to complete the conventional PCR assay.

Statistical analysis. To assess the level of agreement between mqPCR or conventional PCR and culture-based methods for *E. coli* O157 in cattle feces, Cohen's kappa statistics (and 95% confidence intervals [CI]) were estimated *(6)* using an online statistical program (http://www.vassarstats.net/index.html); interpretations were based on an accepted scale proposed by Landis and Koch *(13).* These statistics are useful when no perfect ''gold standard'' diagnostic test exists, and they enabled us to determine how well culture-based methods and PCR-based methods agreed in categorizing positive or negative samples. McNemar's chi-square test was used to compare the proportion of samples identified by mqPCR or conventional PCR as positive compared with either culture-positive (high shedder and IMS) or high shedder–positive samples. A significant difference in the proportion of positive samples by culture- and molecular-based methods suggests serious disagreement between testing methods and may bias the interpretative scale of the kappa statistic.

RESULTS AND DISCUSSION

We compared culture-based *E. coli* O157 detection methods to mqPCR and conventional PCR-based assays designed to detect genes common to *E. coli* O157 in feces of naturally shedding cattle. Samples were compared prior to and after a selective enrichment of the feces to evaluate the assays utility in identifying cattle shedding high ($>10^4$ CFU/g of feces) and low ($\leq 10^2$ CFU/g of feces) concentrations of the organism. Previously, we have shown the analytical sensitivity for an mqPCR assay detecting *rfbE,* stx_1, and stx_2 genes to be approximately 10^4 CFU/g of spiked cattle feces *(12),* 100-fold above the 10^2 CFU/g reported for culture-based techniques using IMS *(14).* However, the analytical sensitivity could be increased to a detection limit near 10 CFU/g of feces if an enrichment step was used prior to mqPCR *(12).* The aim of this study was not to revalidate the mqPCR assay, but instead, to determine if it could be practically and reliably adapted for large-scale field use.

In feces from cattle classified as *E. coli* O157 high shedders based upon direct plating, 37% were positive by mqPCR prior to fecal sample enrichment; 85% of samples were positive by mqPCR after selective enrichment (Fig. 1). McNemar's chi-square test indicated that the proportion of high shedder–positive samples detected by culture and mqPCR methods differed significantly both before ($P < 0.01$) and after ($P < 0.01$) enrichment. Therefore, kappa statistics are potentially biased and provided for reference only. Kappa statistics indicated fair agreement ($\kappa = 0.3$; 95% CI = 0.17 to 0.43) between identification of high shedders by culture and mqPCR prior to enrichment, and moderate agreement ($\kappa = 0.45$; 95% CI = 0.33 to 0.57) after 6-h enrichment. In samples considered IMS positive, 4% were positive by mqPCR prior to enrichment, while 43% were positive after enrichment. In total, after enrichment, 64% (128 of 200) culture-positive (high shedders and IMS) fecal

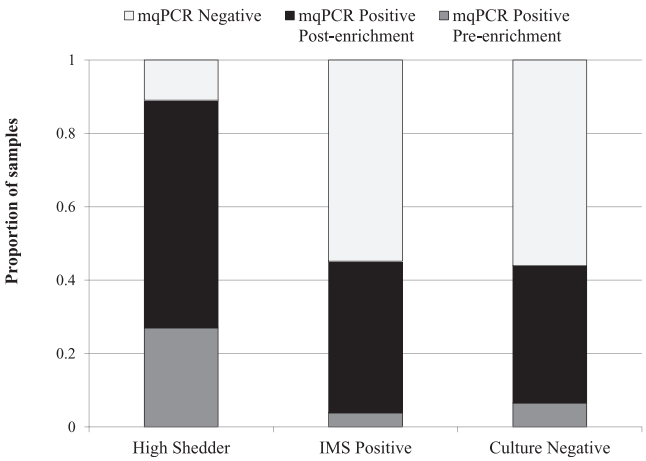

FIGURE 1. *The proportion of samples classified positive for* Escherichia coli *O157 by multiplex real-time (mq) PCR prior to or after selective enrichment for samples originating from high shedder, immunomagnetic separation (IMS)–positive or culture-negative cattle feces.*

samples were identified positive by mqPCR, with 20.6% (41 of 199) positive prior to enrichment. Finally, 7% of culture-negative feces were positive by mqPCR prior to fecal sample enrichment, and 40% were positive after enrichment. The proportions of samples considered culture positive (IMS and high shedder) and mqPCR positive were different both before ($P < 0.01$) and after ($P < 0.01$) enrichment. Therefore, the kappa measures of agreement may be biased; the agreement between the culture and mqPCR methods was slight ($\kappa = 0.1$; 95% CI = 0.006 to 0.19) prior to enrichment and fair ($\kappa = 0.23$; 95% CI = 0.11 to 0.34) postenrichment.

The conventional PCR assay also used to detect *E. coli* O157 from extracted fecal DNA performed similarly to the mqPCR assay. In fecal samples classified as a high shedder, 28% were determined to be positive by conventional PCR prior to fecal sample enrichment and 75% were positive after enrichment. The McNemar's chi-square test indicated the proportion of high shedder–positive and conventional PCR-positive samples was different prior to enrichment ($P < 0.01$), with kappa statistic (potentially biased) indicating fair agreement ($\kappa = 0.23$; 95% CI = 0.1 to 0.37). The proportions of high shedder and conventional PCR-positive samples postenrichment were not different ($P > 0.1$) and displayed moderate agreement ($\kappa = 0.46$; 95% CI = 0.34 to 0.58). In samples considered IMS positive, 3% were considered positive by conventional PCR prior to enrichment, while 33% were positive after enrichment. In samples culture positive, 54% were identified as positive by conventional PCR. In 5% of culture negative samples, a positive by conventional PCR was obtained prior to enrichment, and 29% were positive after enrichment. The proportions of culture-positive (high shedder and IMS) and conventional PCR-positive samples were significantly different both before and after enrichment ($P < 0.01$). The agreement between culture and conventional PCR was slight ($\kappa = 0.07$; 95% CI = 0 to 0.16) prior to enrichment and fair ($\kappa = 0.22$; 95% CI = 0.11 to 0.32) after enrichment. Finally, although similar, the results from the

mqPCR and conventional PCR detection methods for *E. coli* O157 were not always identical. In high shedder samples evaluated after enrichment, 84.7% (72 of 85) of the mqPCR-positive samples agreed with conventional PCR results. This was less for IMS-positive samples (51.2% or 22 of 43). Fifty-three percent of culture-negative samples were considered *E. coli* O157 positive by mqPCR and conventional PCR. Previous research has established that quantitative PCR is known to be more sensitive than conventional PCR *(3),* so these results were not surprising.

Analytical and diagnostic sensitivity and specificity should both be considered when selecting an appropriate diagnostic test. Although analytical estimates of sensitivity and specificity are easily obtainable when designing mqPCR for *E. coli* O157 and validating in *E. coli* O157–spiked cattle feces, few studies have evaluated the diagnostic sensitivity and specificity of the assay by comparing results to culture-based methods. We found evidence for misclassification of samples using both culture- and molecular-based detection methods; 36 and 46% of *E. coli* O157 culture-positive samples were not detected by mqPCR or conventional PCR methods, respectively. More specifically, 15% of high shedder fecal samples were still not detected as positive by mqPCR, even following enrichment. Although we do not know why the remaining samples were not positive, there are several possible explanations, including limitations of the DNA extraction procedure, PCR inhibitory substances in the feces, misclassification of high shedder–positive samples by the direct plating methods, samples that approach the minimum concentration requirement of 10^4 CFU/g of feces, or the diagnostic sensitivity of mqPCR methods.

In addition, 29 and 40% of culture-negative samples were considered positive by conventional and mqPCR. Melt-curve analyses on samples determined to be culture negative and mqPCR positive did show the potential for misclassification as mqPCR positive due to a lack of gene specific amplification; however, the majority of samples evaluated had product with an expected melting temperature and matching that of the positive control strain (data not shown). The range in melt-curve temperature was widest for the *rfbE* gene detected in different fecal samples; 52% (13 of 25 samples) had a melt-curve temperature within 0.5°C of the positive control. Sequence results from samples representing each of the three gene targets were representative of the target of interest in all but one instance. Based on these data, there is evidence that samples classified as culture negative in this study likely contained *E. coli* O157. It was not the goal of this study to further establish the diagnostic sensitivity of culture-based methods for detecting *E. coli* O157 in cattle feces, so the total number of culture-negative samples that were misclassified was not determined. There are, however, several additional opportunities for misclassification of the true *E. coli* O157 status of each sample. Examples include false-positive mqPCR samples due to nonspecific amplification of *rfbE* and *stx* gene detection from non-O157 Shiga toxin–producing *E. coli* presence in the sample or amplification of dead *E. coli* O157 cells. Although we could not evaluate these factors individually, these potential influences need consideration before using molecular-based methods as a primary detection tool for *E. coli* O157.

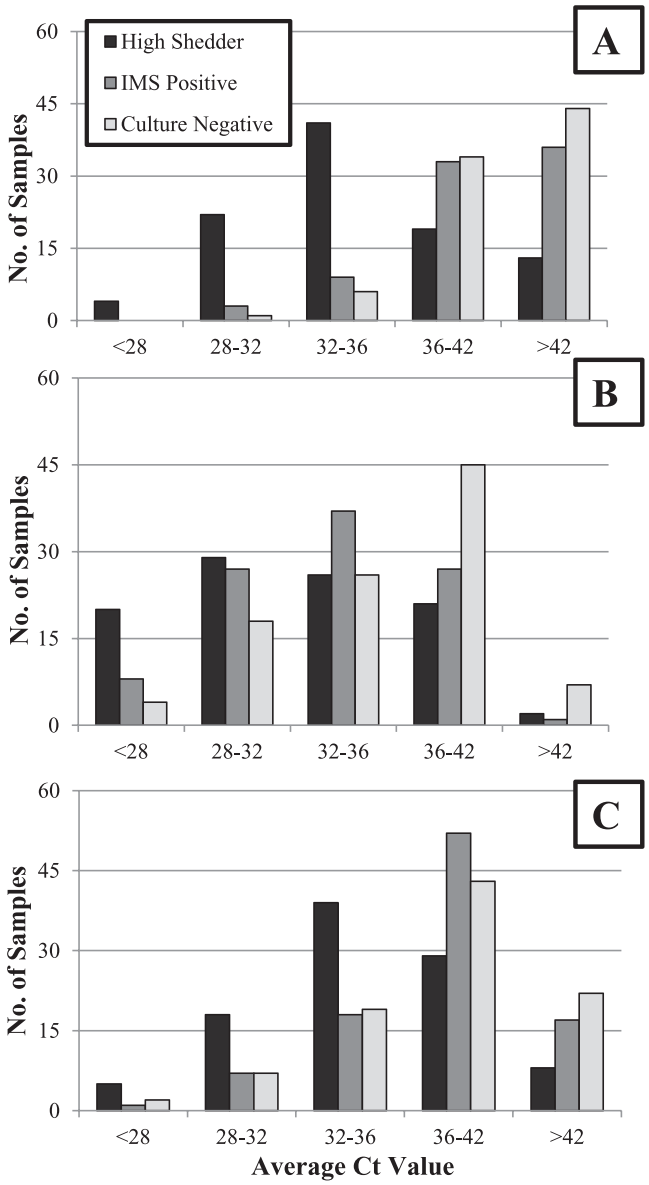

FIGURE 2. *The distribution of* C_T *values for (A)* rfbE *gene, (B)* stx_1 *gene, and (C)* stx_2 *gene obtained from enriched feces classified by culture as an* E. coli *O157 high shedder, as IMS positive, or as culture negative.*

Another mechanism by which we evaluated the diagnostic sensitivity and specificity of the mqPCR assay was to determine the impact of changing the C_T cutoff value for identifying *E. coli* O157–positive samples. Upon validating the assay *(12),* we established criteria of at least one Shiga toxin–producing gene and the *rfbE* gene having an average (triplicate runs) C_T value of $\leq$36 to be considered positive for *E. coli* O157. However, bands representing PCR amplification of an expected size for the three gene products was shown here to occur at C_T values $>$36 (maximum 36.7). The distribution of the average C_T value for each gene in enriched feces and the culture classification are shown in Figure 2. There are apparent differences in the distribution of C_T values for samples

TABLE 1. *The number of postenrichment fecal samples, categorized by culture status, determined to be* E. coli *O157 positive by multiplex real-time (mq) PCR when the minimum cycle number was varied*

		Minimum cycle number (C_T value)				
	No. of samples	≤34	≤35	≤36	≤37	≤38
High shedder	100	75	79	85	91	95
IMS positive	100	28	34	43	50	58
Culture negative	100	15	26	40	52	63

classified as high shedder, IMS positive, or culture negative, and this difference may be gene (*stx*$_1$, *stx*$_2$, or *rfbE*) dependent. Based on our results, the diagnostic sensitivity and specificity are dramatically influenced by the accepted C_T cutoff value (Table 1).

Finally, for the above comparisons of culture and mqPCR methods, we evaluated the average of three mqPCR runs (triplicate) for each gene when classifying a sample as positive or negative. To determine what effect the number of mqPCR runs has on the diagnostic sensitivity and specificity of the reaction, we determined the number of mqPCR-positive or -negative samples that would have been obtained if we chose to use single or duplicate runs. When a single mqPCR run was used after enrichment of the feces, 88% of high shedders, 59% of IMS-positive samples, and 59% of culture-negative samples were considered positive. Ninety percent of high shedders, 56% of IMS positives, and 53% of culture negatives were mqPCR positive when the assay was run in duplicate. In each instance, the number of presumptive positives was higher than those reported when the assay was run in triplicate. This comparison highlights the importance of technical repeatability of the PCR assay on the result outcome. Although one potential advantage to mqPCR for detection of *E. coli* O157 in cattle feces is the high throughput capacity, the number of replicates needed to provide an accurate outcome may add to expense and labor. We did not evaluate the potential repeatability associated with DNA extraction techniques or the use of a different aliquot of fecal sample; however, we would expect these considerations also would impact the reliability of an mqPCR assay.

In conclusion, the culture- and molecular-based methods used to detect *E. coli* O157 in naturally shedding cattle feces had increased agreement after feces were enriched in a selective broth. The utility of mqPCR or conventional PCR assays as a primary detection tool without enrichment seems minimal. In addition, for feces likely to contain a concentration of *E. coli* O157 less than 10^4 CFU/g (IMS positive only), the diagnostic sensitivity of the mqPCR assay appears poor. This strongly suggests that this diagnostic assay is most useful in identifying high shedder animals and is not be appropriate to replace culture-based methods for identifying the majority of cattle shedding *E. coli* O157. Our data indicates misclassification of some culture-negative cattle feces as *E. coli* O157 positive, although the true sensitivity was not determined and the concentration of *E. coli* O157 that animals may have been shedding was unknown. Although mqPCR and conventional PCR techniques have the potential for a high throughput capacity, careful consideration should be given when designing this methodology and interpreting data. Work to compare the utility of mqPCR and culture-based methods in other sample matrices may be warranted.

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