Detection of Enterohemorrhagic *Escherichia coli* in Beef Products Using Droplet Digital PCR

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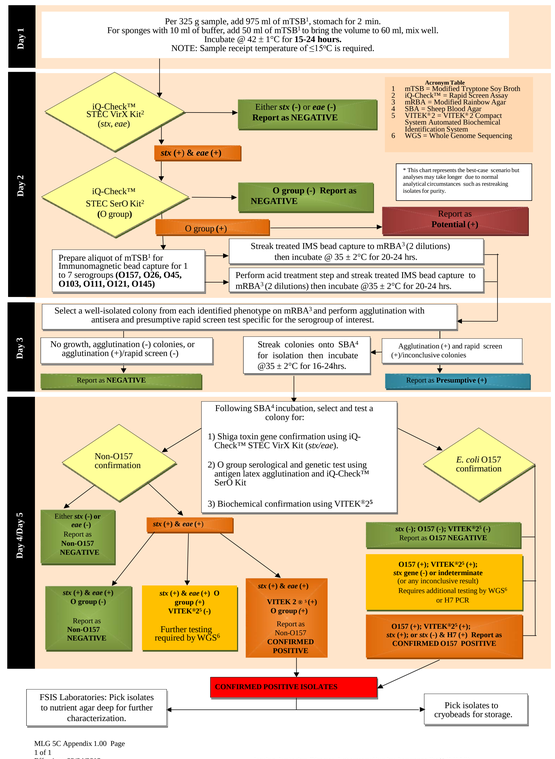
Abstract

Introduction: The currently validated microbiological methods for the detection of enterohemorrhagic *Escherichia coli* (EHEC) from foods relies on a PCR-based screen for the pathotype-specific genetic markers *stx* and *eae*. In comparison to culture confirmation this screening method suffers from a very high rate of false positives (up to 92.5%) that is partly due to the inability of current PCR-based methods to determine if both *stx* and *eae* are within the same organism.
Purpose: This study was undertaken to reduce the false positive rate associated with current EHEC screening methods by confirming the presence of *stx* and *eae* from the same cell.
Methods: The ddPCR system used in this study works by partitioning intact cells into emulsion droplets, which subsequently undergo multiplexed endpoint PCR. This allows the differentiation of samples where a single organism contains both *stx* and *eae* from samples in which *stx* and *eae* reside in different organisms. A study which compared the response of ddPCR to commercial real time PCR assays was conducted using over thirty (30) unique simulations of EHEC contamination in ground beef.

Current FSIS Detection Protocol (MLG 5C)

Flow Chart Specific for FSIS Laboratory Isolation and Identification of Top Seven Shiga Toxin-Producing *Escherichia coli* (STEC)



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Issuing Authority: Director, Laboratory Quality Assurance Staff (LQAS)

Challenges and Opportunity

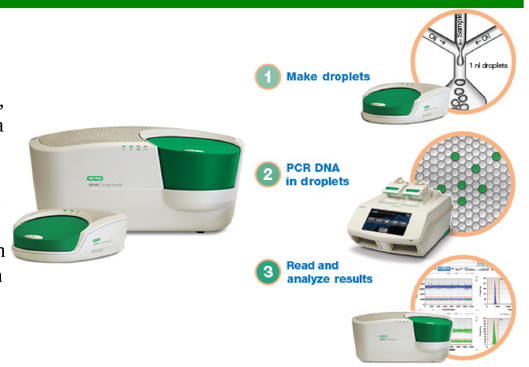
EHEC screening systems detect genetic markers to identify potential positive (PP) samples. Conversion rates of PP's to confirmed positives are low for non-O157 EHEC.

A survey of EHEC in Australian cattle feces conducted in 2013 had a low conversion rate of PP to confirmed positives. 1,500 samples tested, 44.5% were PP for non-O157 EHEC but only 1.3% were culture confirmed as non-O157 EHEC.

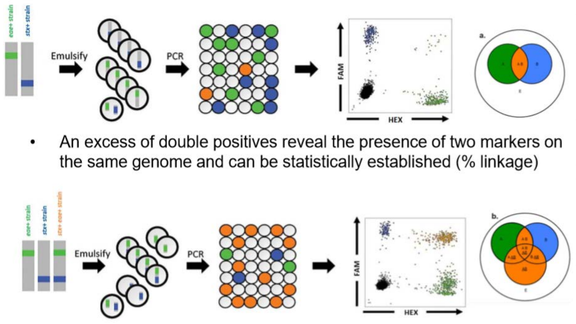
Culture confirmation of non-O157 EHEC is a laborious and lengthy procedure as it attempts to identify a small group of pathogenic *E. coli* that appear similar to harmless *E. coli*.

Principle of Droplet Digital PCR

ddPCR technology uses a combination of microfluidics and proprietary surfactant chemistries to divide PCR samples into water-in-oil droplets. The droplets serve essentially the same function as individual test tubes or wells in a plate in which the PCR takes place, albeit in a much smaller format. The massive sample partitioning is a key aspect of the ddPCR technique. Partitioning facilitates absolute quantification without the requirement for calibration or reference standards. Further, high-copy templates and background are diluted; effectively enriching template concentration in target-positive partitions, allowing for the sensitive, precise and repeatable detection of rare targets. Error rates are reduced by removing the amplification efficiency reliance. By increasing the signal to noise ratio, reliable measurements of small fold differences in target DNA sequence copy numbers among samples can be made.



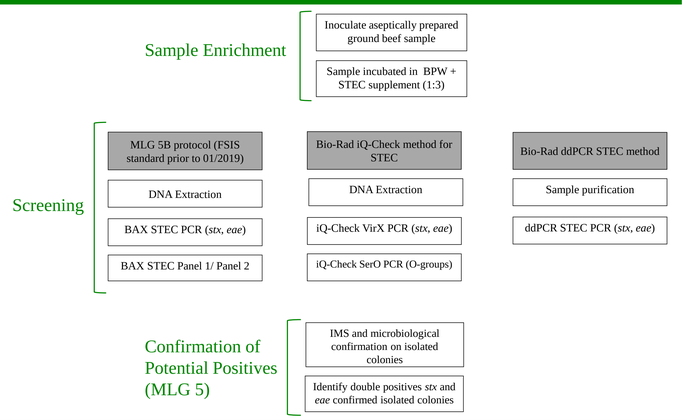
Principle of Co-localization



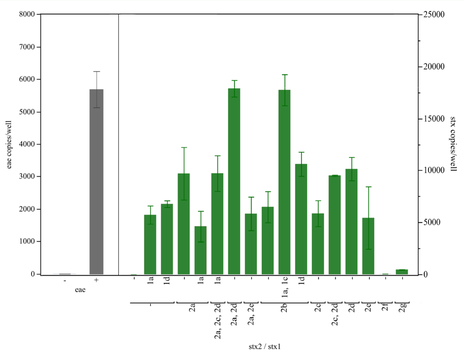
The technique can be applied to partitioning individual intact bacterial cells within a single droplet within complex mixtures. Multiplexed endpoint PCR is conducted and the fluorescence intensity for each droplet is plotted. The 2D graph is used to generate a binary response for *stx* and *eae* within each droplet. The software uses the droplet counts to differentiate between samples that contain coincidental co-localization of *stx* and *eae* from a mixture of bacteria from a single bacteria which displays *stx*/*eae* genotype (Regan et al., 2015) For illustrative purposes, droplets are color coded to indicate those which contain, *eae*, *stx* or both genes in green, blue and orange respectively. The Venn diagrams show the droplet species expected when the two detected genes (*stx* and *eae*) are unlinked (panel a) or linked (panel b).

Experimental Design

The MLG 5, published by FSIS was selected to serve as a guideline in this study. EHEC contaminated food was simulated by inoculating beef samples using a panel of 58 unique strains (26 O-groups, including all 7 regulated). Isolates of strains and mixtures of strains were used to simulate a wide range of potential contamination, The samples were assessed using three different screening methodologies. , and their outcome at the screening and confirmation stages were compared for accuracy. The objective was to test the hypothesis that the ddPCR system could more efficiently identify samples containing individual bacteria that possses both *stx* and *eae* from a mixture of bacteria that collectively contain the genes.

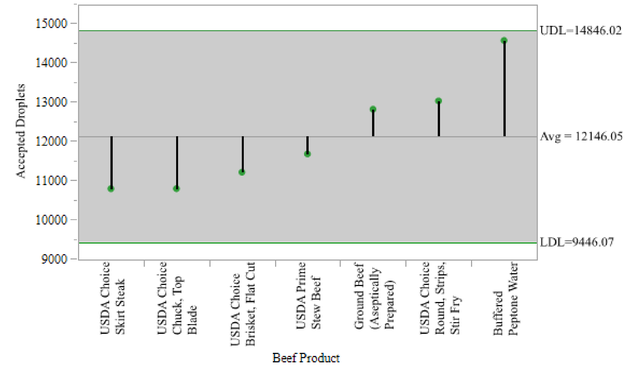


Inclusivity



Detection of *eae* and the various *stx* subtypes of EHEC by ddPCR. The number of *stx* and *eae* copies/well measured by the ddPCR is plotted on the y-axis. The presence/absence of *eae* and *stx* subtype, defined via the WHO standard primer sets, are noted on the x-axis. Individual Student's t-tests indicate that the response measured for samples that were determined to be *eae* negative were statistically different from strains contains *eae*. Further, the response measured for samples that were determined to be *stx* negative were statistically different from strains containing *stx*, with the exception of 2f and 2g variants (α=0.05). Note, while the measurement for 2g is not statistically significant from the negative control, the value for number of copies *stx* is 76 times higher than the average value for the negative control.

Principle of Droplet Digital PCR



Accepted droplets produced with different beef matrices. To ensure the minimum threshold necessary for linkage analysis (8,000 droplets) could be obtained, the total number of accepted droplets created using different beef matrices were measured. The y-axis depicts the number of accepted droplets for each beef matrix presented on the x-axis. The grand mean of the data is identified by the horizontal line at approximately 12, 000 droplets, and the upper and lower decision limits are marked at their respective values.

Results

	BAX		iQ-Check		dd PCR	
Sample	Potential Positive	Confirmed Positive	Potential Positive	Confirmed Positive	Potential Positive	Confirmed Positive
Linked (eae, stx) Regulated O- group	15	15	15	15	15	15
Linked (eae, stx) nonregulated O-group	**1**	**0**	1	**0**	10	**10**
Unlinked (eae, stx) nonregulated O-group	**10**	0	**12**	0	**0**	0
Negative	0	0	0	0	0	0

No false negative responses were observed for any of the three screening assays. All of the samples which were identified as positive for *stx*, *eae*, and one of the regulated O-groups, were subjected to further investigation as per the FSIS MLG 5 protocol. Briefly, immunomagnetic separation was used to isolate the *E. coli* from the enriched ground beef homogenate according to the O-group identified in the screening assay. Single colony isolates were grown on differential/selective agar, and tested via latex agglutination for the presence of the specific O-antigens. Colonies identified on chromogenic agar and confirmed positive through agglutination were subjected to a follow-up evaluation using the initial PCR screening assay. The response of the assay on colonies isolated in this study confirmed the co-localization assessment of the ddPCR. The ddPCR system was able to accurately differentiate between samples that contain coincidental co-localization of *stx* and *eae* from a mixture of bacteria from a single bacteria which displays *stx*/*eae* genotype. Further the ddPCR system displayed the capability to identify pathogenic EHEC in a manner that is independent of O-group.

Conclusions

Results: In this comparative study the ddPCR assay demonstrated equivalent sensitivity to the established screening techniques. Further the results indicate the ddPCR has the potential to reduce the number of false positives identified in an EHEC screening.
Significance: This study demonstrates the ability of the ddPCR system to confirm the co-existence of multiple genes within the same cell in a mixed microbial population; specifically, *stx* and *eae*. Further the technology does not rely on O-group. Ultimately, this system will result in cost-savings by reducing the man-hours and testing expenses associated with the evaluation of false-positive samples. Furthermore, this would enable more samples to be analyzed, which could reduce the probability of contaminated foods reaching consumers.