5-11-2022

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Food Control 139 (2022) 109095

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Development and validation of high-resolution melting assays for the detection of potentially virulent strains of *Escherichia coli* O103 and O121

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ARTICLE INFO

Keywords:
Adulterants
Beef
O103
O121
Pork
Real-time PCR
Shiga toxin-producing *Escherichia coli* (STEC)
USDA

ABSTRACT

Virulent strains of Shiga toxin-producing *Escherichia coli* (STEC) serogroups O103 and O121 are considered adulterants in beef. Two high-resolution melting (HRM) real-time PCR assays were standardized for the specific detection and discrimination of potentially virulent and avirulent strains of *E. coli* O103 and O121. The O103 HRM assay offered the possibility to distinguish clearly STEC O103:H2 from STEC O103:H25. The two standardized assays were extensively validated using 215 pure culture strains, laboratory inoculated food samples, and naturally contaminated beef (n = 84) and pork (n = 84) enrichments collected from the red meat surveillance program. Both HRM assays showed 100% inclusivity and exclusivity using pure culture strains and enriched spiked food samples. Data from this study shows the ability of the standardized assays to specifically detect the strains of each target serogroup and, most importantly, to differentiate the strains present into potentially virulent or avirulent groups. The assays standardized in this study can be helpful for food surveillance programs and help mitigate product loss due to the presence of avirulent strains lacking crucial virulence genes (*stx* and *eae*).

1. Introduction

The beef industry is an important component of US agriculture. In 2019, the United States had an inventory of 94.8 million head of cattle and calves, with a cash receipt value of 66.2 billion dollars (USDA, ERS, 2021). Contamination with foodborne pathogens is a major cause of concern for the beef industry, resulting in costly product recalls. Cattle and other ruminants are considered reservoirs for Shiga toxin (*stx*)-producing *E. coli* (STEC) that are responsible for causing disease of varying severity ranging from bloody diarrhea to renal failure in humans (Dewsbury et al., 2015; Fratamico, 2013; Shridhar et al., 2017).

STECs, especially those that possess the adherence factor intimin (*eae*), are significant foodborne pathogens commonly associated with beef products. STEC serogroups cause human disease of varying severity and are broadly divided into two groups (O157 and non-O157 STEC) (USDA, 2020). In the US, STEC strains of the O157:H7 serotype and the "big 6" (i.e., O26, O45, O103, O111, O121, and O145) serogroups are considered adulterants in non-intact beef (USDA, 2020) and are often referred to as the top 7 STEC serogroups. However, recent studies have indicated the presence of STEC strains in pork products, and these STEC are considered an emerging concern for pork products (Nastasijevic et al., 2020; Zhang et al., 2021; Zhang, Yamamoto, et al., 2021).

Based on the genetic makeup, these abovementioned serogroups can be further subdivided into virulent and avirulent strains. The virulent strains harbor a combination of *stx*1, *stx*2, and *eae*, which are considered crucial for causing human disease (NACMCF, 2019) and are used to define the adulterant STEC. In contrast, the avirulent or non-STEC strains lacking these crucial virulence genes are not a cause of concern for the food industry. The current United States Department of Agriculture - Food Safety and Inspection Service (USDA-FSIS) STEC testing workflow considers any samples testing negative for either *stx* or *eae* as negative (USDA, FSIS, 2021). However, samples that test positive for *stx*, *eae*, and any serogroup-specific gene(s) may include multiple strains in a sample carrying these genes. Thus, a positive signal from the PCR assay for the presence of adulterant STEC serogroups can be likely from a combination of avirulent STEC strains, with other bacterial cells

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https://doi.org/10.1016/j.foodcont.2022.109095
Received 6 January 2022; Received in revised form 6 May 2022; Accepted 7 May 2022
Available online 11 May 2022

harboring the *stx* and/or *eae* genes. Interestingly, these avirulent strains of the top-seven serogroups have been reported to be highly prevalent in raw meat samples (Bosilevac & Koohmaraie, 2012), posing a significant challenge for the food testing laboratories, regulatory agencies, and the beef industry. These avirulent strains interfere with the currently available STEC detection assays data interpretation, resulting in a higher number of potential-positive enrichment broths, consequentially causing product hold-up for further confirmation by a culture-based method (Bosilevac & Koohmaraie, 2012; Singh et al., 2020; Velez et al., 2021).

In the United States, the Microbiology Laboratory Guidebook (MLG 5C.01) describes the standard test method for detecting and isolating strains belonging to the top seven STEC serogroups. Based on a USDA, FSIS cost-benefit analysis, the currently available STEC detection method has a false-positive rate of 81–100% (93% for beef manufacturing trimming, 81% for ground beef, 100% for bench trim, and 94% for other components), which results in a loss of around $47 million annually for raw beef products (USDA, FSIS, 2021). Additionally, the culture-based method using chromogenic culture media for the isolation of non-O157 lacks the ability to specifically isolate virulent non-O157 strains. Therefore, there is a need for assays that can accurately detect virulent strains of the targeted STEC serogroups, avoid assay interference caused by the presence of virulence genes present in other Enterobacteriaceae, and bacteriophages, and differentiate enrichments testing positive into potentially virulent and avirulent strains.

In our previous work, we described a high-resolution melt (HRM) assay for the specific identification of potentially virulent strains of *E. coli* O26 and O111 (Singh et al., 2020; Velez et al., 2021). As an extension, we present HRM assays for the specific detection of potentially virulent strains of STEC O103 and O121 and its comparison with the iQ-Check STEC SerO PCR Detection Kit (Bio-Rad Laboratories, Hercules CA), which is currently part of USDA-FSIS standard method MLG 5C.01. We hypothesize that the performance of two HRM assays standardized in this study is either equivalent or superior to the current standard method.

2. Materials and methods

2.1. Bacterial strains

Strains and DNA samples for this study were obtained from Center for Food Safety (University of Georgia, GA, USA), Roman L. Hruska U.S. Meat Animal Research Center (Clay Center, NE, USA), Michigan State University STEC Center (East Lansing, Michigan, USA), and ANSES, Laboratory for Food Safety (Maisons-Alfort, France). The two HRM assays were validated using 112 pure culture bacterial DNA samples, which consisted of twenty-six O103 (Supplementary Table 1), nineteen O121 strains (Supplementary Tables 2) and 67 non-target strains for exclusivity testing (Supplementary Table 3).

2.2. Primer design

The serogroup-specific primer-pairs flanking target single nucleotide polymorphism (SNP) were designed using the Primer3 software (Untergasser et al., 2012). The specificity of designed primer pairs was tested using the NCBI Primer-BLAST tool. The designed oligonucleotides were synthesized by IDT (Coralville, IA, USA). The primer-pairs for O103 were designed targeting the serogroup-specific *wbt*D gene SNP at the 937 position (C to T). The O121 primer pair targeted the serogroup-specific *vio*A gene SNP at the 313 position (C to T). These SNPs were previously identified to differentiate potentially virulent and avirulent strains (Norman et al., 2012). Multiple primer pairs for each target were designed and tested for their applicability to accurately identify the target SNP. Selected primer pairs were optimized for $MgCl_2$ concentration, annealing temperature, primer concentrations, and HRM master mix. The final sets of oligonucleotides used for this study are listed in Table 1.

2.3. Real-time PCR

Real-time PCR assays were performed on a LightCycler® 96 real-time PCR instrument (Roche Diagnostics, Indianapolis, USA). A 2x Apex Green Master Mix (Genesee Scientific, California, USA) was used for standardizing the O103 HRM assay. The 10 μl O103 PCR reaction mixtures consisted of 20 ng of DNA, 50 nM of forward and reverse primers, 2.5 mM $MgCl_2$, and 1.25 μM EvaGreen (Biotium, California, USA). A three-step PCR amplification protocol was used for the amplification of the O103 assay. The amplification protocol entailed an initial denaturation step at 95 °C for 900s, followed by 45 cycles of denaturation at 95 °C for 15s, with annealing at 60 °C for 30s, and extension at 72 °C for 25s. Finally, a HRM step was added at the end of all amplification cycles. The HRM step consisted of a gradual temperature increase of 0.04 °C/s from 65 to 97 °C. Fluorescence data from amplification and HRM was collected in channel 1 (FAM λ 470/514) of the instrument. The HRM data for O103 was analyzed with a pre-melt region of 71.5–72.5 °C and a post-melt region of 76.5–77.5 °C.

A 2x LightCycler® 480 High-Resolution Melting Master Mix (Roche Diagnostics, Indianapolis, USA) was used for the O121 assay. The O121 reaction mixture consisted of 20 ng of DNA, 60 nM of forward and reverse primer-pair, and 2.5 mM $MgCl_2$. A three-step PCR protocol was used for the amplification of the O121 target sequence. The amplification protocol entailed an initial denaturation step at 95 °C for 600s, followed by 45 cycles of denaturation at 95 °C for 15s, with annealing at 60 °C for 30s, and extension at 72 °C for 10s. A HRM step was added at the end of amplification as described above. The HRM data for O121 was performed with a pre-melt region of 75.5–76.5 °C and a post-melt region of 79.5–80.5 °C. HRM analysis for O103 and O121 assays was performed only for the samples testing positive in the absolute quantification analysis. Considering that the baseline signal from samples showing no amplification can interfere with HRM analysis, we removed the negative control, the uninoculated samples, and samples testing negative from the HRM analysis.

2.4. Laboratory enriched food samples

Assay validation was performed using laboratory inoculated food samples as previously described (Singh et al., 2020). Twelve strains comprised of three virulent (*stx*+, *eae*+) and avirulent strains *(stx*-, *eae*- or *stx*+, *eae*-) each of O103 and O121 (Table 2), were used to inoculate ground beef, pot roast, and spinach.

2.5. Inoculum preparation and plate count

Each test strain was individually aerobically cultured overnight in 10 mL of tryptic soy broth (TSB) (Hardy Diagnostics, Santa Maria, Calif., USA) at 37 °C overnight. After the incubation, the cultures were serially diluted and spread plated on plate count agar (PCA) (Hardy Diagnostics, Santa Maria, Calif., USA). The cultures awaiting enumeration were kept in the refrigerator at 4 °C, while the PCA plates were incubated at 37 °C overnight. Counts from the PCA plates were used to calculate the appropriate dilution and inoculation volume to achieve 10 CFU inoculation levels. The calculated volume for each strain was used to spike the food samples, and the same volume was spread plated onto PCA plates to enumerate the inoculation load. Beef samples were inoculated at 10 CFU/325 g, and spinach samples were inoculated as 10 CFU/25 g.

2.6. Food sample preparation

Ground beef (12% fat, 88% lean), beef roast, and spinach were purchased from the local grocery store (Tallahassee, Florida). The food samples were inoculated and enriched as described previously (Singh et al., 2020). Briefly, food samples testing negative for O103 and

Table 1
Oligonucleotides used in this study.

NAME	PRIMER SEQUENCE	TARGET GENE	PRODUCT SIZE	REFERENCE
O103–850F	5′-GATGAAACAAACGGTAAAT-3′	*wbtD*	146	This study
O103–995R	5′-TTTCATATTTAGCTAACAAGTTT-3′			
Wzx O103–F	5′-TTGGAGCGTTAACTGGACCT-3′	*wzx*	191	Wasilenko et al. (2012)
Wzx O103-R	5′-ATATTCGCTATATCTTCTTGCGGC-3′			
O103 PROBE	5′-CAL Fluor Red 610/AGGCTTATCTGG CTGTTCTTACTACGGC-IABkFQ-3′	*wzx*		Wasilenko et al. (2012)
O121–257F	5′-CAACTGCACACTCCTTGGTC-3′	*vioA*	98	This study
O121–354R	5′-CGCCTCTTCAATTCTTCTCG-3′			
Wzx O145–F	5′-AAACTGGGATTGGACGTGG-3′	*wzx*	132	Wasilenko et al. (2012)
Wzx O145-R	5′-CCCAAAACTTCTAGGCCCG-3′			
P1285	5′-ATGGCACAAGTCATTAATAC-3′	*fliC*	1263	Beutin et al. (2015)
P1286	5′-TTAACCCTGCAGTAGAGACA-3′			

Table 2
E. coli strains used for inoculating food samples.

E. coli	Strain	Source	Virulence Gene
O103: H16	33		*stx*-, *eae*-
O103: H38	75.2		*stx*-, *eae*-
O103:H2	302.1		*stx*-, *eae*-
O103:H2	−1 hSTEC 05	human	*stx*1+, *eae*+, *hylA*+
O103:H2	−2 MDR 0089 (USMARC_GB_STEC 045)	beef	*stx*1+, *eae*+, *hylA*+
O103:H2	−3 Mar 125 B (USMARC_GB_STEC 046)	beef	*stx*1+, *eae*+, *hylA*+
O121	75.3		*stx*+, *eae*-
O121	219.5		*stx*-, *eae*-
O121	508.3		*stx*-, *eae*-
O121	−2′ nphl_12,738	human	*stx*+, *eae*+
O121	−3′ C4-63-1_3218	beef	*stx*+, *eae*+
O121	−4 V2-G2 1-C 16.3	beef	*stx*+, *eae*+

O121were spiked with test strains. Inoculated samples were stressed by storing the food samples at 4 °C for 24 h. Inoculated samples were enriched for 15-h at 42 °C following the FSIS MLG 5C.01 reference method. After 15 h, 1.8 mL of broth was taken from the enrichment bags and transferred to a 2 mL Eppendorf tube. DNA from each 1.8 mL portion was isolated using the DNeasy® Power Food Microbial Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. All experiments were performed in duplicates.

2.7. Assay validation with naturally contaminated beef and pork samples

The standardized assays were further validated using DNA isolated from 168 naturally contaminated beef (n = 84) and pork (n = 84) enrichments. These samples were initially tested using the USDA, FSIS reference method (MLG 5C.01) for the presence of STEC. The enrichments were selected based on the results of screening for *stx*, *eae*, O121, and O103, as well as the results of culture confirmation. All enrichments were *stx-eae* positive with varying results for serogroups present. Nine beef enrichments had been confirmed to contain a STEC-O103, while two pork enrichments were culture-confirmed to contain a STEC-O121. The DNA samples were prepared from 1 mL of frozen archived (17% glycerol; −20 °C) broth using a 96 well Bacteria DNA Kit (IBI Scientific, Dubuque, IA) according to the manufacturer's instructions, then quantified using a Nanodrop One Spectrophotometer (Thermo Fisher, DE, USA), diluted to 10 ng/μl working stock, and used for the two HRM assays. All naturally contaminated beef and pork sample DNA were tested using the O103 specific primer and probe (Table 1) as described in the MLG 5C Appendix 4.00. The 5′-nuclease assay was performed using Apex probe mix (Genesee Scientific, California, USA). Additionally, selected few samples (n = 10) were retested using the new version of iQ-Check STEC SerO PCR Detection Kit, which detects O103 and O145 individual detection channels.

2.8. Sequencing

Samples that tested positive in either the O103 or O121 HRM assays were confirmed by Sanger sequencing of the purified HRM PCR product. In addition, the *fliC* gene of O103 strains was sequenced using our previously published primer pair (Beutin et al., 2015). The PCR amplicons generated were purified using Omega Bio-tek E.Z.N.A® Cycle Pure Kit (Omega Bio-Tek, GE, USA). Each purified amplicon was diluted to a concentration of 10 ng/μl and 3.2 μM of the appropriate forward primer and sequenced at the Florida State University molecular cloning facility (Tallahassee, FL, USA). Chromatograms of obtained sequences were edited using Chromas Lite v2.1.1 (Technelysium, Brisbane, Australia), and an NCBI BLAST analysis was used to confirm the identity of sequences.

2.9. Statistical analysis

The proportion of target serogroups (i.e., O103, O121) detected by the Reference method (MLG Chapter 5C.01) versus the O103 and O121 HRM assays were compared using the two-tailed Fisher's exact test. Differences were considered significant at $P < .05$.

3. Results

3.1. Detection of serogroup O103 and O121 E. coli strains

Two real-time PCR HRM assays were standardized for the specific detection of O103 and O121 strains. The O103 and O121 assays were initially standardized using pure culture DNA samples. The O103 assay accurately identified all the pure culture O103 strains (n = 26) (hSTEC 05, USMARC_GB_STEC 045, USMARC_GB_STEC 046, MT#80, TB154A, 302.1, 33, 612.1, 621.2, 745.1, 75.2, 802.1, 8417 PT91-24, ATCC - 2199, ATCC - 2207, ATCC - 2210, ATCC - 2213, 06QMA137.4, 06QMA221.E, 07QMA185.8, PMK5, UTI, VTH10, ED172, EC146, and CH-087) (Supplementary Table 1). The O103 primer pair showed 100% inclusivity and exclusivity for detecting the specific serogroup using the pure culture strains. Further, the O103 HRM assay correctly differentiated between avirulent and virulent pure culture O103 strains (Fig. 1a and b) with two virulent O103 strains (ATCC 2199 and 2213) forming a separate and distinct melt curve group (Supplement Figure 1). The *fliC* gene sequencing of ATCC 2199 and 2213 strains showed these two virulent strains belonged to the O103:H25 serotype (Supplement Figure 3), which is distinct from commonly isolated O103:H2 virulent strains.

Similarly, using the pure culture strains, the O121 HRM assay showed 100% inclusivity and exclusivity. The O121 primer-pair identified all the O121 isolates tested in the study (n = 19) (3377–85, MT#2, MT#18, DA-5, 2′ nphl_12,738, 211–1, 219.5, 256–1, 3′ C4-63-1_3218, 4 V2-G2 1-C 16.3, 508.3, 75.3, 785.2, 967.1, ATCC - 2187, ATCC - 2203, ATCC - 2219, ATCC - 2220, and ATCC - 2221) (Supplementary Table 2). The O121 HRM assay generated a distinct melt curve profile for the

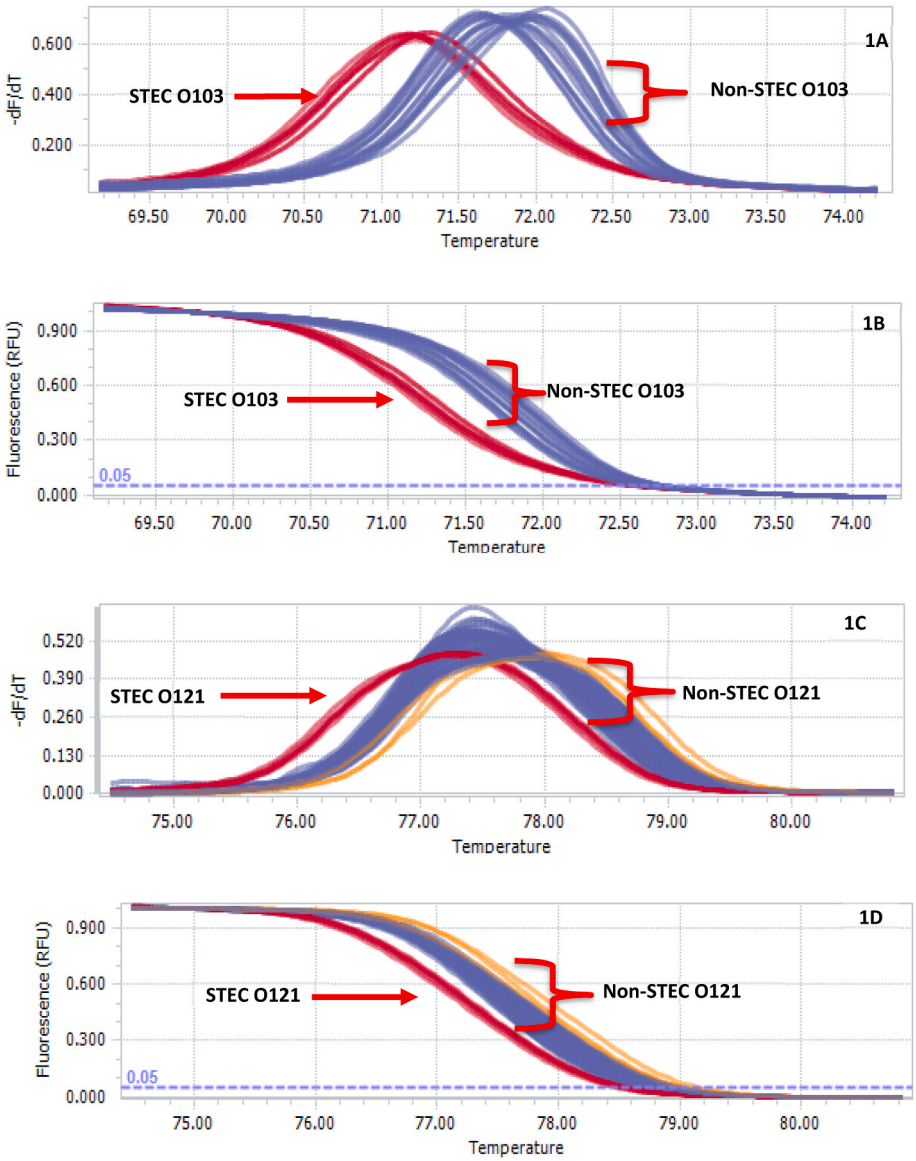


Fig. 1. High-resolution melting of assays for the detection and differentiation of STEC strains of O103 and O121. The cluster grouped in red indicates the presence of STEC strains. In contrast, cluster in blue and yellow indicates the presence of avirulent non-STEC strains. Fig. 1A. Normalized O103 Melting Peaks; Fig. 1B. Normalized O103 Melting Curves; Fig. 1C. Normalized O121 Melting Peaks, and 1D. Normalized O121 Melting Curves.

avirulent and virulent strains (Fig. 1c and d) that accurately identified all the virulent O121 strains tested in this study. Both HRM assays showed no cross-amplification with any non-target bacterial strains tested (n = 67) (Supplementary Table 3).

3.2. Inoculation studies

The inocula used for the spiking of the food samples ranged from 2 to 11 CFU/test portions. The two HRM assays accurately detected the inoculated strains in the spiked food samples (O103 33, O103 75.2, O103 302.1, O103-1, O103-2, O103-3, O121 75.3, O121 219.5, O121 508.3, O121-2, O121-3, O121-4) following a 15 h enrichment period. The two HRM assays correctly distinguished between the virulent and avirulent strains based on their HRM profile amongst the DNA isolated from the food enrichments.

3.3. Validation with naturally contaminated beef and pork samples

Out of the 168 naturally contaminated beef and pork samples, 38 beef and 61 pork samples were found to be positive for the presence of *stx*, *eae*, and either O103 or O145 by the iQ-Check STEC VirX and SerO PCR Detection Kits (Bio-Rad Laboratories, Hercules CA) which are used as the MLG 5C.01 method. Since the previous version of iQ-Check SerO test identifies O103 and O145 in the same detection channel, we wished to distinguish the two serogroups from one another for our analysis. Therefore, further testing of these samples with O103 specific primer and probe (Table 1) (Wasilenko et al., 2012) was performed, and 16 (beef: 6; pork: 10) samples, which initially tested positive for O103 by iQ-Check STEC SerO PCR Detection Kit, tested negative and, these 16 samples were considered negative for O103 during assay comparison.

The O103 HRM assay standardized in this study matched with 25/32 beef samples and 48/51 pork samples. An additional four beef (n = 25 + 4) and five pork (n = 48 + 5) samples tested positive only by the HRM assay and were confirmed by Sanger sequencing. Among the beef (n = 29) and pork (n = 53) samples that were positive by the O103 HRM assay, 73 samples grouped with avirulent non-STEC clusters, while the remaining nine samples were grouped with the potentially virulent STEC strains. Three of these nine potentially virulent samples had a

culture-confirmed isolate, and no isolates were obtained for the remaining six samples. Additionally, the HRM assay failed to detect three samples from which an O103 isolate was previously obtained. Failure of the HRM to detect these three samples can be attributed to several causes elaborated in the discussion section below.

Out of the 168 naturally contaminated samples, 22 beef and 57 pork samples were found to be positive for O121 STEC by the iQ-Check STEC VirX and SerO tests. The O121 HRM assay identified 29 beef samples; 22 directly correlated with iQ-Check STEC SerO screen results, with an additional seven positive samples identified among iQ-Check STEC SerO screen negative enrichments. The seven HRM O121 positive enrichments were later confirmed by Sanger sequencing of the amplicons. The assay matched all 57 pork samples with an additional seven samples positive, which were confirmed by Sanger sequencing. Of all the beef and pork samples that tested positive for O121 by the HRM assay, only two samples clustered with the virulent STEC strains, while the remaining samples clustered with the avirulent non-STEC strains. These two STEC-O121 enrichments were culture-confirmed for the presence of virulent STEC-O121 strains.

Our O103 and O121 assays yielded a comparable detection rate compared to the currently used STEC detection assay (iQ-Check STEC SerO PCR Detection Kit) used by FSIS. Fisher exact test values for O103 detection among beef and pork enrichments were 0.75 and 0.41 (Fig. 2A and B), respectively. Similarly, Fisher exact test values for O121 detection among beef and pork enrichments were 0.31 and 0.22, respectively (Fig. 2C and D).

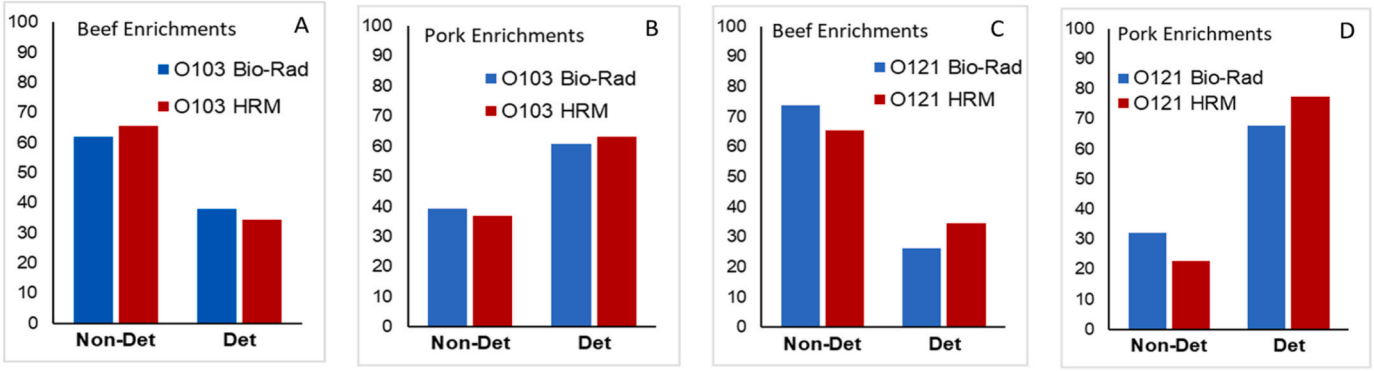
4. Discussion

Multiplex real-time PCR assays are commonly used for the detection of foodborne pathogens. In the United States, the MLG 5C.01 is the standard method used for detecting the seven STEC serogroups declared adulterants in beef. This method relies on using 9 primer pairs and dual-labeled probes in four multiplex formats to detect the seven STEC serogroups. This two-tiered approach relies on an initial virulence gene screening, followed by testing for the presence of the adulterant serogroups. At times, this testing approach results in a large proportion of positive results due to the presence of mixed populations of avirulent strains in the enrichment. Enteropathogenic *E. coli* strains (EPEC), bacteriophages, and other interfering bacterial genera are known to harbor the virulence genes (*eae*, *stx*) which are targeted for the screening of STEC (Margot et al., 2013; Quirós et al., 2015; Gassama et al., 2001; Hyma et al., 2005). Therefore, in this study, we developed and validated a highly specific SNP-based approach that directly detects potentially virulent strains of O103 and O121 and does not rely on the two-tiered approach.

High-resolution melting real-time PCR assays for the detection of specific SNP have been used for the development of pathogen detection assays with higher accuracy, i.e., *E. coli* O157:H7 (Bosilevac et al., 2019; Liu et al., 2018), and identification of potentially virulent strains of O26 and O111 (Singh et al., 2020; Velez et al., 2021). The HRM assays are a low-cost alternative to dual-labeled probe assay for SNP identification. As HRM assays are performed in singleplex, they are easy to standardize, and they do not suffer from fluorescent signal bleed-through problems, which is the common challenge associated with the multiplex dual-labeled probe assay.

The O103 HRM assay in this study was standardized using 2x Apex Green Mix. Whereas the O121 HRM assay was performed using the 2x LightCycler® 480 High-Resolution Melting Master Mix. Initial efforts were made to standardize both assays using 2x LightCycler® 480 High-Resolution Melting Master Mix. However, the O103 assay using the 2x LightCycler® 480 High-Resolution Melting Master Mix showed a poor amplification (Supplementary Fig. 2a), evidenced by a higher Cq value, making it challenging to distinguish positive from negative amplification plots. Every possible attempt made to improve O103 amplification efficiency (i.e., optimization of primer concentration, annealing temperature, $MgCl_2$ concentration, primer with locked nucleic acid bases, use of other high-resolution melting mixes) failed to generate a good sigmoid amplification plot. Eventually, the use of 2x Apex Green Mix showed a sigmoid amplification plot (Supplementary Fig. 2b), and the master mix's HRM capability to identify the target mutation was improved by the addition of an optimum concentration of EvaGreen dye. A similar challenge with 2x LightCycler® 480 High-Resolution Melting Master Mix was observed in our previous study, where the use of the same master mix showed a higher Cq-value (Sharma et al., 2020). The HRM master mixes are commonly optimized for superior genotyping during HRM analysis and may suffer from low PCR amplification efficiency. This may be a possible cause for high Cq-values observed with 2x LightCycler® 480 High-Resolution Melting Master Mix.

The previous version of the iQ-Check STEC SerO assay, which was



A	HRM	Bio-Rad
Sensitivity	0.81	0.89
Specificity	1.00	0.88
False positive rate	0.00	0.00
False negative rate	0.19	0.11
Positive predictive value	1.00	0.84
Negative predictive value	0.87	0.91
Test accuracy	0.92	0.88

B	HRM	Bio-Rad
Sensitivity	0.95	0.91
Specificity	1.00	0.64
False positive rate	0.00	1.00
False negative rate	0.05	0.09
Positive predictive value	1.00	0.84
Negative predictive value	0.90	0.78
Test accuracy	0.96	0.82

C	HRM	Bio-Rad
Sensitivity	1.00	0.76
Specificity	1.00	1.00
False positive rate	0.00	1.00
False negative rate	0.00	0.24
Positive predictive value	1.00	1.00
Negative predictive value	1.00	0.89
Test accuracy	1.00	0.92

D	HRM	Bio-Rad
Sensitivity	1.00	0.88
Specificity	1.00	1.00
False positive rate	0.00	1.00
False negative rate	0.00	0.12
Positive predictive value	1.00	1.00
Negative predictive value	1.00	0.70
Test accuracy	1.00	0.90

Fig. 2. (A) The Fisher exact test statistic value is 0.75. The result is not significant at $p < .05$. (B) The Fisher exact test statistic value is 0.87. The result is not significant at $p < .05$. (C) The Fisher exact test statistic value is 0.31. The result is not significant at $p < .05$. (D) The Fisher exact test statistic value is 0.22. The result is not significant at $p < .05$.

commercially available at the time the current study was conducted, has O103 and O145 results in the same detection channel, making it difficult to specifically detect O103. Therefore, all samples were tested with O103-specific primers and probe designed by Wasilenko et al. (2012) to differentiate if the positive signal from the iQ-Check STEC SerO assay was either for O103 or O145. The determination of positive and negative samples after the 5′-nuclease assay was challenging as many samples generated poor, non-sigmoid amplification plots. Hence, the 5′-nuclease assays for all 168 samples were repeated twice, and 16 samples were determined to be negative for O103 and excluded from the analysis. In the end, only 10 samples with a Cq value less than 30 using the O103 primers and probe designed by Wasilenko et al. (2012) were not detected with our O103 HRM assay. These 10 samples were retested using a newer version of iQ-Check STEC SerO assay, which detects O103 and O145 in separate detection channels. Data from retesting showed only five were positive for the presence of O145, two positives for O26, two samples were negative for all 7 STEC serogroups, while the only sample to show the presence of O103 was also positive for the combination O26 and O145 serogroups. The lack of detection of this one sample can be attributed to the presence of lower CFUs in the archived enrichments that were diluted with glycerol and stored at −20 °C before thawing and preparing DNA templates for these studies. The freeze-thawing of archived samples can result in a reduction of viable cells.

Interestingly, the standardized O103 HRM assay generated a distinct melt curve group for two virulent strains (i.e., ATCC 2199 and 2213). These two O103 strains belonged to the O103:H25 serotype and were positive for *stx*1 and *eae* virulence genes. The virulent O103:H25 are distinct from the commonly isolated virulent O103:H2 serotype. Sanger sequencing of the PCR amplicons generated for ATCC 2199 and 2213 DNA samples showed a T to C SNP at the 927 position in the *wbt*D gene (reference GenBank AY532664). Although outbreaks associated with STEC O103:H25 are rare, virulent O103:H25 strains were previously isolated from a cured mutton sausages outbreak in Norway (Schimmer et al., 2008), which had a high hemolytic uremic syndrome rate (60%). The presence of an H25 associated SNP hints towards divergent evolution from the H2 serotype, and this O103 HRM assay can be a useful tool for discriminating amongst these pathogen serotypes if needed.

The standardized HRM assays were further validated with naturally contaminated beef (n = 84) and pork (n = 84) samples. When following the MLG 5C.01 protocol, FSIS, by their own admission, can only culture-confirm about 10% of beef samples that contain *stx*, *eae,* and O-group gene; this confirmation rate drops to about 1% when considering pork samples. Between 2014 and 2020, the FSIS tested 18,339 beef samples, and among those samples, 1008 tested positive for the presence of STEC serogroup-specific genes. Of these 1008 samples, only 144 were culture-confirmed to contain a STEC strain (Dr. J. Emilio Esteban, USDA, FSIS, personal communication, 2020). In their National Pork Baseline study, FSIS reported 1395 pork samples tested for the presence of STEC, 309 screened positive, of which only three were confirmed by the isolation of two O103 and one O157:H7 STEC strains (Scott et al., 2020). Other groups have reported similar disappointing rates of confirming PCR screens for STEC. Despite a high PCR detection rate of STEC serogroups in the pork samples collected from Italy, culture-based methods failed to isolate any STEC strain belonging to the top seven STEC serogroups (Bardasi et al., 2017). Parallel findings were reported from a Canadian study where all non-O157 strains isolated from the retail raw ground pork lacked the *eae* gene (Zhang, Yamamoto, et al., 2021). This difference (≈85%) between screen positive and confirmed STEC positive samples signifies interference caused by the presence of avirulent strains that must be overcome to make non-O157 STEC testing acceptable by end-users.

Strains belonging to Top7 STEC serogroups are considered adulterants in beef products. Meat samples testing positive due to the presence of avirulent strains results in product loss and economic loss for the processors and stakeholders. Similarly, samples testing positive due to the presence of avirulent strains result in significant amounts of labor and materials invested in futile culture isolation attempts by regulators. Compared to commercially available methods, the two HRM assays standardized in this study groups all samples testing positive into two distinct groups (i.e., potentially virulent and avirulent), based on their high-resolution melt profile. Thus, facilitating the differentiation of samples contaminated by a potentially virulent STEC strain from avirulent non-STEC strains, resulting in higher assay accuracy for the detection of potentially virulent STEC-O103 and O121 strains.

The HRM approach described here provides reliable results for the presence of a virulent STEC without the need for a multistep screening protocol as described in the MLG. Further, the HRM assays described here can be used as a molecular confirmation test for the enrichments identified as potentially positive by the MLG or other assays, quickly distinguishing between safe and adulterated beef.

5. Conclusion

In conclusion, this study standardized two HRM assays to detect potentially virulent O103 and O121 STEC strains with high accuracy. The O103 and O121 HRM assays are low-cost methods that distinguish between potentially virulent strains that are adulterants in beef from avirulent non-STEC background strains that interfere with current STEC detection methods. These two assays can be incorporated into the current red meat testing protocols and surveillance programs to accurately identify adulterant strains and help reduce meat product loss. Their use in standard practice will reduce the amounts of product diverted to lethality steps and increase the accuracy of regulatory monitoring for these adulterant STEC serogroups.

CRediT authorship contribution statement

Frank J. Velez: Investigation, Writing – original draft. **Joseph M. Bosilevac:** Resources. **Sabine Delannoy:** Provided DNA samples for the assay validation, Manuscript - Review & Editing. **Patrick Fach:** Provided DNA samples for the assay validation, Manuscript - Review & Editing. **Ravinder Nagpal:** Statistical analysis. **Prashant Singh:** and DNA samples for the assay validation, Writing – review & editing, Conceptualization, Methodology, Validation, Data curation, Supervision, Project administration.

Acknowledgments

The study was supported by the Florida State University startup funds, GAP funds and Foundation for Meat and Poultry Research and Education grant. Product names are necessary to report factually on available data; however, their mentioning by a USDA author neither guarantees nor warrants the standard of the product, and the use of the name implies no approval of the product to the exclusion of others that may also be suitable. The USDA is an equal opportunity provider and employer. We thank Greg Smith for technical assistance and Jody Gallagher for administrative support

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2022.109095.

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