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Detection of Shiga toxin-producing *Escherichia coli*, stx_1, stx_2 and *Salmonella* by two high resolution melt curve multiplex real-time PCR

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ABSTRACT

In the United States, Shiga toxin-producing *Escherichia coli* (STEC) O157 and six non-O157 serogroups O26, O45, O103, O111, O121 and O145 are considered adulterants in non-intact beef. Further, *Salmonella* is responsible for one of the highest numbers of foodborne infections worldwide. Multiple foods, especially meats, are routinely tested for these pathogens using methods like PCR. However, with such a large group of organisms, multiplexing using probe-based PCR assays is expensive due to the need for differently labeled oligonucleotide probes and sophisticated instrumentation. The aim of this study was to design low-cost multiplex real-time PCR assays for the detection of seven STEC serogroups, stx_1, stx_2 genes and virulent *Salmonella*. Two multiplex real-time PCR melt curve assays with internal amplification controls (IAC) were standardized. The first assay detected *E. coli* O121, *E. coli* O145, *E. coli* O157, stx_1, and stx_2. The second assay targeted *E. coli* O26, *E. coli* O111, *E. coli* O103, *E. coli* O45, and *Salmonella*. Ground beef and beef trim inoculated with 5–27 CFU/325 g of STEC and 9–36 CFU/325 g of *Salmonella* could be detected following an 8–10 h enrichment at 40 °C ± 2 °C in buffered peptone water containing 8 mg/L vancomycin. The assays showed reproducible results for beef products with different fat contents. These assays do not rely on fluorescent-labeled probes or immunomagnetic beads, yet accurately detect seven STEC serogroups, seven *stx* gene subtypes and *Salmonella*, making them suitable for routine testing of STEC and *Salmonella* in beef.

1. Introduction

Shiga toxin producing *Escherichia coli* (STEC) are a group of *E. coli* strains that can produce Shiga toxins and cause debilitating and fatal human diseases. The ability to produce Shiga toxins in *E. coli* is conferred by the stx_1 and stx_2 genes (Melton-Celsa, 1998). These STEC are also referred to as enterohemorrhagic *E. coli* (EHEC) and often possess the adherence factor intimin (*eae*), which is another important virulence factor in these organisms. According to a recent report, STEC annually leads to 2,801,000 cases of acute illness, 3890 HUS cases, 270 permanent end-stage renal disease and 270 deaths worldwide (Majowicz et al., 2014).

Further, STEC infection has been traced to ruminants, water contaminated with ruminant manure, direct contact with infected animals at farms or petting zoos and person-to-person transmission (Duffy, Burgess, & Bolton, 2014). Food products of cattle origin, such as ground or minced beef (especially undercooked ground beef) and raw milk are at a greater risk of STEC contamination (Smith, Fratamico, & Gunther IV, 2014). Unpasteurized fruit juices, salads and sprouts are other food commodities that have been involved in previous outbreaks (Smith et al., 2014).

Similarly, *Salmonella* is also one of the most important foodborne pathogens. According to the Centers for Disease Control and Prevention (CDC) FoodNet recent data (2013), *Salmonella* was responsible for the highest number of foodborne infections, accounting for 38% of all reported infections (15.19 per 100,000 population) (CDC, 2013). In the years 2014–2015, a total of 17 *Salmonella* related outbreaks has been reported (CDC, 2015).

Food processors, and especially meat and beef processors, routinely test their non-intact products for pathogens such as STEC and *Salmonella* to ensure their antimicrobial control measures are performed as desired (Arthur, Bosilevac, Nou, & Koohmaraie, 2005). Detection tests must be rapid due to the perishable nature of meat products and also exhibit high specificity and sensitivity. Most rapid

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pathogen screening tests are antibody- (lateral flow device) or molecular (PCR)-based. There are advantages and disadvantages with either approach. Antibody methods are rapid and simple but can lack the sensitivity and specificity that processors require to make immediate product disposition decisions. Molecular methods, on the other hand, can have high running costs, especially multiplex real time PCR assays requiring fluorescent labeled probes that can degrade with a longer storage time. A simpler and less expensive approach for real time PCR involves melt peak analysis of the real time PCR product(s), wherein multiple molecular targets can be distinguished from one another by the temperature at which their DNA strands melt. Melt-curve based multiplex real-time PCR assays are sensitive and economical alternatives to currently used fluorescent labeled probe-based assays.

The aim of this study was to develop high resolution melt (HRM) curve real-time PCR assays for the detection of seven STEC serogroups (*E. coli* O145, O121, O157, O26, O45, O103, and O111), virulence genes (stx_1 and stx_2) and *Salmonella.* The optimized assays were validated in 325 g of ground beef and beef trims of different fat contents to comply with the United States Department of Agriculture (USDA) Food Safety Inspection Service (FSIS) 2013 guidelines.

2. Materials and methods

2.1. Bacterial strains

Non-O157 STEC and *E. coli* O157 strains were procured from the STEC Center at Michigan State University (USA) (Table 1). Five *E coli* O157 strains and *Salmonella* strains were obtained from the University of Missouri, Columbia, Food Microbiology Lab (USA) culture collection. All cultures used in this study were grown at 37 °C in Tryptic Soy broth (TSB) (Difco Labs, BD Diagnostics Systems, Sparks, MD, USA). All strains were preserved as glycerol stocks (30% v/v) and maintained at −50 °C.

2.2. Bacterial DNA extraction

Genomic DNA from all bacterial strains and enriched food samples was isolated using PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The concentration and purity of the obtained DNA samples were determined using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

2.3. Primer design

PCR primers used in this study were designed using the Primer3 software (Untergasser et al., 2012). A serogroup-specific *wzx* gene, which encodes the O-antigen flippase, and Shiga-toxin producing virulence genes (stx_1 and stx_2) were targeted. The previously designed *uid*A gene primers for the identification of *E. coli* O157:H7 (Cebula, Payne, & Feng, 1995; Wang, Li, & Mustapha, 2009) were used for the detection of *E. coli* O157. *Salmonella* virulence gene (*inv*A) was targeted for the amplification of virulent strains of *Salmonella*. The specificity of the designed PCR primers was initially tested using the NCBI Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and validated using the STEC and *Salmonella* DNA samples used in this study. Shiga toxin gene primers designed in this study were evaluated for their ability to amplify various *stx* subtypes using reference DNA samples of various stx_1 and stx_2 subtypes (AA1-stx_{1c}, stx_{2b}; BB2 - stx_{1a}; CC3 - stx_{2f}; DD4 - stx_{2c}, stx_{2d}; EE5 - stx_{1a}, stx_{2a}; II9 - stx_{1d}; D3509 - stx_{2g}; 05622 - stx_{2e}; JJ10 - stx_{2c} and B2F1- stx_{2d}) which were generously donated by Dr. Peter C. H. Feng (FDA, College Park, MD, USA; Feng, Jinneman, Scheutz, & Monday, 2011). All designed oligonucleotides were commercially synthesized (IDT, Coralville, IA, USA).

Table 1
STEC strains used to validate the multiplex real-time PCR assays.

STEC	Strain	Origin
E. coli O157:H7	3178–85	Human
E. coli O157:H7	C7927	Human
E. coli O157	93–111	Human
E. coli O157	EDL-933	Hamburger
E. coli O157	OK-1	Human
E. coli O157	2886–75	Human
E. coli O157	86–24	Human
E. coli O157	G5101	Human
E. coli O26:H11	DEC10B	Human
E. coli O26:H	MT#10	Human
E. coli O26	H19	Human
E. coli O26	DEC10C	Human
E. coli O26	DEC9F	Human
E. coli O26	TB285C	Human
E. coli O26	VP30	Human
E. coli O26	DEC9A	Human
E. coli O45:H2	M103-19	Human
E. coli O45:H NM	DA-21	Human
E. coli O45	DEC11C	Human
E. coli O45	5431–72	Human
E. coli O45	4309–65	Human
E. coli O45	88–4110-H	Cow
E. coli O45	D88-28058	Cow
E. coli O45	2566–58	Pig
E. coli O103:H2	MT#80	Human
E. coli O103:H N	PT91-24	Human
E. coli O103	DA-41	Human
E. coli O103	6:38	Human
E. coli O103	PT91-24	Human
E. coli O103	DA-55	Human
E. coli O103	87–2931	Human
E. coli O103	GS G5550637	Human
E. coli O111:H2	RD8	Human
E. coli O111:H NM	3007–85	Human
E. coli O111	CL-37	Human
E. coli O111	DEC8B	Human
E. coli O111	TB226A	Human
E. coli O111	928/91	Human
E. coli O111	412/55	Human
E. coli O111	DEC8C	Cow
E. coli O121:H [19]	DA-5	Human
E. coli O121	87–2914	Human
E. coli O121	DA-1	Human
E. coli O121	7927 + + +	Not known
E. coli O121	5518	Not known
E. coli O121	O121 standard	Not known
E. coli O121	PT91-4	Not known
E. coli O145:H NT	D177	Human
E. coli O145:H NT	IH 16	Human
E. coli O145	70300885	Not known
E. coli O145	MT#66	Human
E. coli O145	6940	Not known
E. coli O145	BCL73	Cow
E. coli O145	B6820−C1	Cow
Salmonella Agona	LJH1132	Not known
Salmonella Agona	LJH1122	Not known
Salmonella Newport	LJH692	Not known
Salmonella Typhimurium	14028	Not known
Salmonella Typhimurium	788	Not known
Salmonella Typhimurium	LJH666	Not known
Salmonella Thompson	B&B3	Not known
Salmonella Enteritidis		Not known

2.4. Internal amplification control (IAC) design

Two single-stranded 83 bp long oligonucleotides were designed that could be amplified using one of the primer pairs of the multiplex assays. In the first reaction, the primer pair O121-F-716 and O121-R-865 co-amplified IAC-O157-set-121pp and O121 serogroup-specific flippase gene. In the second multiplex reaction, the primer pair O103-F-752 and O103-R-920 co-amplified IAC-Sal-set-103pp and O103 serogroup-specific flippase gene. The co-amplified products for each multiplex