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Detection of five Shiga toxin-producing *Escherichia coli* genes with multiplex PCR

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ABSTRACT

Escherichia coli serogroup O157 is the pathogen most commonly associated with foodborne disease outbreaks, but epidemiological studies suggest that non-O157 Shiga toxin-producing *E. coli* (STEC) is a major player as well. The ten most clinically relevant STECs belong to serogroups O26, O103, O111, O145, O157, O91, O113, O128, O45, and O121; but emerging strains, such as O104:H4 that was identified with the 2011 German outbreak, could become more prevalent in the future. A 75-min conventional multiplex PCR assay, IS-5P, targeting the four virulence factors *stx*1, *stx*2, *eae*, and *ehxA* plus the O157:H7-specific +93 *uidA* single nucleotide polymorphism was developed to better assess the potential pathogenicity of STEC isolates. All 212 STEC DNAs showed one to five amplification products, while the non-*E. coli* DNA did not react to this multiplex PCR assay. Enrichment broths obtained from baby spinach, alfalfa sprouts, and cilantro artificially inoculated with O26, O103, and O121 STECs reacted positively to the multiplex assay. Unlike the current FDA BAM 5P PCR, designed for the specific detection of O157:H7, IS-5P will identify potentially harmful O157:H7 and non-O157 STECs so they can be removed from the nation's food supply.

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) strains are foodborne infectious agents that cause a number of life-threatening diseases, including hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Gyles, 2007). Grant et al. (2011) had reported twenty-three outbreaks of non-O157 STEC illnesses between 1990 and 2007 in the U.S. alone. Because non-O157 STECs have no unique or distinguishing physiological features or phenotypic characteristics to distinguish them from other *E. coli* strains, the burden of human illness from non-O157 STEC is probably much greater than currently reported (Grant et al., 2011). Epidemiological studies suggest that non-O157 strains cause 20–50% of STEC infections, which cause approximately 169,000 illnesses annually in the U.S. (Scallan et al., 2011). Over 70% of non-O157 STEC infections are caused by serogroups O26, O103, O111, O145, O157, O91, O113, O128, O45, and O121 (CDC, 2006). Emerging strains, such as O104:H4 that was identified with the 2011 German outbreak, could also become more prevalent. Out of a concern for public health, US regulatory agencies like the U.S. Department of Agriculture (USDA) have started to pay attention to the prevalence of STEC in food. Since June 4, 2012, the USDA Food Safety and Inspection Service (FSIS) has implemented verification testing for six STECs (O26, O45, O103, O111, O121, and O145) in raw beef manufacturing trimmings (FSIS USDA, 2012).

STEC O serogroup determination is an important focus of pathogen identification, but complementary assays that detect the virulence factors associated with pathogenicity have also been developed (Fujioka et al., 2013; Clotilde et al., 2012). Over 100 STEC serotypes possess the Shiga-toxin type 1 (*stx*1) gene, one of several Shiga-toxin type 2 *stx*2 variants, or a combination of these genes (Beutin et al., 2007). The *stx*1 toxin is relatively homogeneous in genetic composition, but seven subtypes of *stx*2 have been identified: *stx*2a, *stx*2b (Díaz-Sánchez et al., 2012), *stx*2c (Ito et al., 1990; Lindgren et al., 1994), *stx*2d (Paton et al., 1992, 1993; Pierard

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et al., 1998), *stx*2e (Gyles et al., 1988; Marques et al., 1987), *stx*2f (Gannon et al., 1990; Schmidt et al., 2000) and *stx*2g (Díaz-Sánchez et al., 2012).

Intimin, encoded by the *eae* gene, is a highly polymorphic outer membrane protein responsible for the intimate attachment of bacteria to the enterocyte membrane and the effacement of the microvilli of the enterocyte (Kaper et al., 1998). Blanco et al. (2004) and Lacher et al. (2006) classified intimin genes into the *eae* types $\alpha 1$, $\alpha 2$, β, $\beta 1$, $\beta 2$, $\gamma 1$, $\gamma 2/\theta$, δ/κ, ε, ζ, η, ι, λ, μ, ν, and ξ. Intimin plays an important role in the ability of enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) to attach-efface. Several studies have associated the *eae* gene with the capacity of STEC strains to cause severe human disease, especially HC and HUS (Adu-Bobie et al., 1998; Oswald et al., 2000; Paton and Paton, 1998); but the presence of intimin is not necessary for pathogenesis because many sporadic cases of HUS were caused by *eae*-negative non-O157 STEC strains (Paton and Paton, 2002), such as the deadly German outbreak strain O104:H4 associated with the consumption of fenugreek sprouts. Interestingly, *stx*2a, *stx*2c, and *stx*2d in combination with *eae* genes are frequently found in STEC strains from HUS patients (Persson et al., 2007).

Another STEC virulence factor is a plasmid-encoded enterohemolysin (*ehxA*), which is often associated with severe clinical disease in humans (Schmidt et al., 1995; Law, 2000) and has been used as a possible epidemiological marker for pathogenic STEC. Six genetically distinct *ehxA* subtypes (A–F) have been characterized by Cookson et al. (2007). Like intimin, researchers think enterohemolysin plays a role in non-O157 STEC pathogenicity (Law, 2000). Brooks et al. (2005) reported that, between 1983 and 2002, 61% of human non-O157 STEC isolates contained *stx*1 alone, 21% had *stx*2 alone, 18% carried *stx*1 and *stx*2, and 84% of those human isolates harbored *eae*.

Table 1
Target genes and primer sequences used in this study.

Primer	Sequence (5′–3′)	Target gene	Size of PCR amplicon (bp)	Reference
*stx*1-F	GACTTCTCGACTGCAAAGAC	*stx*1	306 bp	This study
*stx*1-R	TGTAACCGCTGTTGTACCTG			This study
*stx*2-F	CCCGGGAGTTTACGATAGAC	*stx*2	482 bp	This study
*stx*2-R	ACGCAGAACTGCTCTGGATG			This study
eae-F	GCGCGTTACATTGACTCCCG	*eae*	245 bp	This study
eae-R	CCATTTGCTGGGCGCTCATC			This study
ehxA-F	TCTGTATCTGCGGGAGTTAG	*ehxA*	136 bp	This study
ehxA-R	CAACGTGCTCAAACATAGCC			This study
PT-2	GCGAAAACTGTGGAATTGGG	+93 *uidA*	382 bp	Cebula et al. (1995)
uidA-R	TCGTCGGTAATCACCATTCC			This study

The current U.S. Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM) five-gene (5P) multiplex PCR (Feng and Monday, 2000; Feng et al., 2011a,b), called PF-5P in this paper, was developed to identify the genetic markers *stx*1, *stx*2, γ-*eae*, and *ehxA* found in *E. coli* O157:H7 strains. PF-5P also included primers that specifically recognized the O157:H7 single-nucleotide polymorphism (SNP) at position +93 of the *uidA* housekeeping gene. This +93 *uidA* SNP is conserved in O157:H7 and non-motile O157 strains, including atypical O157:H- clones (Cebula et al., 1995; Feng, 1993; Feng et al., 1998). It is important to note that the PF-5P does not detect all *stx*, *eae* and *ehxA* variants found in other STECs (Feng et al., 2011a,b).

Table 2
The inclusivity test with *stx*2, *eae*, *ehx*A variants.

Virulence genes	Strain	Serotype	Toxin	IS-5P PCR					BAM PF-5P PCR				
				*stx*1	*stx*2	*eae*	*ehxA*	+93 *uidA*	*stx*1	*stx*2	*eae*	*ehxA*	+93 *uidA*
*stx*2	EH250	O118:H12	2b	–	+	–	–	–	–	+	–	–	–
	D2587	O174:H21	2c	–	+	–	–	–	–	+	–	–	–
	D3435	O73:H18	2d	–	+	–	–	–	–	+	–	–	–
	B2F1	O91:H21	2d	–	+	–	+	–	–	+	–	+	–
	S1191	O139:K12:H1	2e	–	+	–	–	–	–	+	–	–	–
	7VD3509	O2:H25	2g	–	+	–	+	–	–	+	–	+	–
eae	TW06375	O127:H6	α	–	–	+[a]	–	–	–	–	–	–	–
	TW01270	O125:H6	α2	–	–	+	–	–	–	–	–	–	–
	TW07862	O26:[h11]	β	+	–	+	–	–	+	–	–	–	–
	RDEC-1	O15:H-	β	–	–	+	–	–	–	–	–	–	–
	EDL933	O157:H7	γ	+	+	+	+	+	+	+	+	+	+
	TW08101	O103:H2	ε	+	–	+	+	–	+	–	–	+	–
	TW08023	O121:H19	ε	–	+	+	+	–	–	+	–	+	–
	TW10363	O-:[h8]	ε	–	–	+	–	–	–	–	–	–	–
	TW07920	O103:H2	ε	+	–	+	+	–	+	–	–	+	–
	TW10371	O116:[h9]	ε2	–	–	+	–	–	–	–	–	–	–
	TW07863	O84:[h2]	ζ	+	–	+	+	–	+	–	–	+	–
	TW04892	O111:H9	ζ	–	–	+	–	–	–	–	+	–	–
	TW07892	O142:[h21]	η	–	–	+	–	–	–	–	–	–	–
	TW01387	O111:H8	θ	+	–	+	+	–	+	–	–	+	–
	TW01933	O55:[h34]	ι	–	–	+	–	–	–	–	+	–	–
	TW04174	O86:[h8]	ι	–	–	+	–	–	–	–	–	–	–
	TW06584	O86:H34	κ	–	–	+	–	–	–	–	–	–	–
	TW10337	O49:[h10]	κ	–	–	+	–	–	–	–	–	–	–
	TW10327	O33:[h34]	λ	–	–	+	–	–	–	–	–	–	–
	TW08260	O55:[h51]	μ	–	–	+	–	–	–	–	–	–	–
	TW10334	O5:[2]	ξ	–	–	+	–	–	–	–	–	–	–
	TW10366	O21:[h5]	ρ	–	–	+	–	–	–	–	–	–	–
*ehx*A	12-00049		A	–	+	–	+	–	–	+	–	+	–
	11-00064	O157:H7	B	–	+	+	+	+	–	+	+	+	+
	12-00004	O26:H11	C	+	–	+	+	–	+	–	–	+	–
	09-00049	O168:H-	D	–	+	–	+	–	–	+	–	+	–
	03-3375	O145:H25	E	–	+	+	+	–	–	+	–	+	–
	GB046	O103:H2	F	+	–	+	+	–	+	–	–	+	–

[a] Gray cells show the difference between IS-5P PCR and BAM PF-5P PCR.

Table 3
Comparative results of some STECs from Feng and Monday (2000) in IS-5P PCR and BAM PF-5P PCR.

Strain	Serotype	IS-5P PCR					BAM PF-5P PCR				
		*stx*1	*stx*2	*eae*	*ehxA*	+93 *uidA*	*stx*1	*stx*2	*eae*	*ehxA*	+93 *uidA*
MA6	O rough:H7	−	+	+	+	+	−	+	+	+	+
G5101	O157:H7	+	+	+	+	+	+	+	+	+	+
493/89	O157:H$^-$	−	+	+	+	+	−	+	+	+	+
5A	O55:H7	−	−	+	−	−	−	−	+	−	−
5905	O55:H7	−	+	+	−	−	−	+	+	−	−
TT4	O165:H25	−	+	+[a]	+	−	−	+	−	+	−

[a] Gray cells show the difference between IS-5P PCR and BAM PF-5P PCR.

The objective of this study was to develop a multiplex PCR (IS-5P PCR) that confirms the presence of *stx*1, *stx*2, *eae*, and *ehxA* variants as well as +93 *uidA* targets in clinically relevant STEC serogroups, including O26, O103, O111, O145, O157, O91, O113, O128, O45, and O121. The effectiveness of our IS-5P PCR and the reference PF-5P PCR was compared using pure culture isolates and enrichment samples from baby spinach, alfalfa sprouts, and cilantro artificially contaminated with various STEC strains.

2. Materials and methods

2.1. PCR conditions

The oligonucleotide primers used in this study are listed in Table 1. All primers with the exception of PT-2 (Feng and Monday, 2000) were designed by Clone Manager 9 (Scientific & Educational Software, NC, USA) using the *stx*1, *stx*2, *eae*, *ehxA*, and *uidA* sequences available in public databases for various STECs strains. A primer master mix containing a final concentration of 100 nM for each of ten primers (Table 1) was prepared and stored at −20 °C until use. Each 20 μl PCR reaction mixture contained 10 μl of the 2X Fast Cycling PCR master mix (Qiagen Fast Cycling PCR Kit, Qiagen, Valencia, CA); 5 μl of the primer master mix (*stx*1, *stx*2, *eae*, *ehxA*, and +93 *uidA*); 4 μl of DNase, RNase-free water; and 1 μl of template DNA (200–900 ng/μl). PCRs were performed on a DNA Engine® System (Bio-Rad Laboratories Inc., Hercules, CA) under the following conditions: 95 °C for 5 min, 25 cycles, each cycle consisting of 96 °C for 5 s, 54 °C for 10 s and 68 °C for 15 s, plus a final extension step at 72 °C for 1 min. The expected size of PCR amplicons was 306 bp for *stx*1, 482 bp for *stx*2, 245 bp for *eae*,

Table 4
Comparative results of Microbiological Data Program (MDP) STECs in IS-5P PCR and BAM PF-5P PCR.

Original ID	State	Commodity	Serotype	IS-5P PCR					BAM PF-5P PCR				
				*stx*1	*stx*2	*eae*	*ehxA*	+93 *uidA*	*stx*1	*stx*2	*eae*	*ehxA*	+93 *uidA*
MDP-05-00697	Michigan	Parsley	O-:H38	−	−	−	−	−	−	−	−	−	−
MDP-06-00074	Michigan	Lettuce	O-:H2	−	+	−	−	−	−	+	−	−	−
MDP-06-00236	Minnesota	Lettuce	O8:H28	−	+	−	+	−	−	+	−	+	−
MDP-07-00004	New York	Cantaloupe	O88:H38	+	−	−	−	−	+	−	−	−	−
MDP-07-00006	Ohio	Bagged lettuce	Unknown	−	+	−	+	−	−	+	−	+	−
MDP-08-00007	California	Spinach	O130:H11	−	+	−	+[a]	−	−	+	−	+	−
MDP-08-00015	Michigan	Spinach	Unknown	+	+	−	−	−	+	+	−	−	−
MDP-08-00017	California	Spinach	Unknown	+	−	−	−	−	+	−	−	−	−
MDP-08-00021	California	Spinach	Unknown	−	+	−	−	−	−	+	−	−	−
MDP-08-00022	Washington	Bagged lettuce	O136:H16	+	−	−	+	−	+	−	−	+	−
MDP-08-00024	California	Bagged lettuce	O8:H28	−	+	−	+	−	−	+	−	+	−
MDP-08-00025	Texas	Spinach	O73:H12	+	+	−	+	−	+	+	−	+	−
MDP-04-01392	Maryland	Cantaloupe	Unknown	+	−	−	−	−	+	−	−	−	−
MDP-04-02111	Michigan	Cilantro	Unknown	+	−	−	−	−	+	−	−	−	−
MDP-04-02307	California	Cilantro	Unknown	−	+	−	+	−	−	+	−	+	−
MDP-04-02745	Texas	Leaf lettuce	O121:H19	−	+	+[a]	−	−	−	+	−	−	−
MDP-05-00613	California	Lettuce	O174:H36	−	+	−	−	−	−	+	−	−	−
MDP-06-00048	California	Alfalfa sprouts	O36:H14	−	+	−	+	−	−	+	−	+	−
MDP-09-00002	Maryland	Spinach	OX25	−	+	−	+	−	−	+	−	+	−
MDP-09-00009	Wisconsin	Spinach	O172:H2	−	+	−	−	−	−	+	−	−	−
MDP-09-00024	Michigan	Spinach	O116:H21	−	+	−	+	−	−	+	−	+	−
MDP-09-00025	California	Spinach	O116:H21	−	+	−	+	−	−	+	−	+	−
MDP-09-00027	Florida	Spinach	O113:H21	−	+	−	+	−	−	+	−	+	−
MDP-09-00028	Texas	Spinach	O8	−	+	−	+	−	−	+	−	+	−
MDP-09-00031	Michigan	Spinach	Unknown	−	+	−	+	−	−	+	−	+	−
MDP-09-00039	Florida	Spinach	O168:H8	−	+	−	+	−	−	+	−	+	−
MDP-09-00043	New York	Spinach	Unknown	−	+	−	−	−	−	+	−	−	−
MDP-09-00047	Maryland	Spinach	O113w:H21	−	+	−	+	−	−	+	−	+	−
MDP-09-00049	Michigan	Lettuce	O168	−	+	−	+	−	−	+	−	+	−
MDP-10-00001	Washington	Spinach	Unknown	+	−	−	−	−	+	−	−	−	−
MDP-10-00006	New York	Cilantro	O79W	−	+	−	−	−	−	+	−	−	−
MDP-10-00007	New York	Cilantro	O79W	−	+	−	−	−	−	+	−	−	−
MDP-10-00013	Texas	Cilantro	Unknown	−	+	−	−	−	−	+	−	−	−
MDP-10-00018	Florida	Spinach	O4:H7	−	+	−	−	−	−	+	−	−	−
MDP-10-00025	Ohio	Spinach	O88W	+	+	−	+	−	+	+	−	+	−
MDP-10-00031	Texas	Lettuce	O88:H21	+	−	−	+	−	+	−	−	+	−
MDP-10-00032	Texas	Spinach	O8:H49	+	−	−	+	−	+	−	−	+	−
MDP-10-00033	Wisconsin	Sprouts	O79:H2	+	−	−	−	−	+	−	−	−	−

[a] Gray cell show the difference between IS-5P PCR and BAM PF-5P PCR.

Table 5
Comparative results of STEC strains in IS-5P PCR and BAM PF-5P PCR.

Original ID	Serotype	IS-5P PCR					BAM PF-5P PCR				
		*stx*1	*stx*2	*eae*	*ehxA*	+93 *uidA*	*stx*1	*stx*2	*eae*	*ehxA*	+93 *uidA*
08-00011[b]	O1	−	−	−	−	−	−	−	−	−	−
08-00016[b]	O1	−	−	−	−	−	−	−	−	−	−
TB157A	O103	+	−	+[a]	+	−	+	−	−	+	−
87-2931	O103	+	−	+	+	−	+	−	−	+	−
MT#82	O103	+	−	+	+	−	+	−	−	+	−
MT#80	O103	+	−	+	+	−	+	−	−	+	−
EK30	O103	+	−	+	+	−	+	−	−	+	−
EK31	O103	+	−	+	+	−	+	−	−	+	−
EK32	O103	+	−	+	+	−	+	−	−	+	−
109-494	O103	+	−	+	+	−	+	−	−	+	−
107-226	O103	+	−	+	+	−	+	−	−	+	−
PMK5	O103	+	−	+	+	−	+	−	−	+	−
RW1372	O103	+	−	+	+	−	+	−	−	+	−
RW1374	O103	+	−	+	+	−	+	−	−	+	−
DA40	O103	+	−	+	+	−	+	−	−	+	−
D55	O103	+	−	−	+	−	+	−	−	+	−
G5506	O104	−	+	−	+	−	−	+	−	+	−
13i51	O104:H4	−	+	−	−	−	−	+	−	−	−
CL-37	O111	+	−	+	+	−	+	−	−	+	−
DEC8B	O111	+	+	+	+	−	+	+	−	+	−
3007-85	O111	+	+	+	+	−	+	+	−	+	−
TB226A	O111	+	+	+	+	−	+	+	−	+	−
928/91	O111	+	+	+	+	−	+	+	−	+	−
412/55	O111	+	−	+	−	−	+	−	−	−	−
DEC8C	O111	+	−	+	+	−	+	−	−	+	−
C412	O111	+	−	+	+	−	+	−	−	+	−
BCL19	O111	−	+	+	−	−	−	+	−	−	−
ED-31	O111	+	+	+	−	−	+	+	−	−	−
BCL17	O111	+	−	+	+	−	+	−	−	+	−
EK35	O111	+	−	+	+	−	+	−	−	+	−
3215-99	O111	+	+	+	+	−	+	+	−	+	−
DA-18	O111	+	−	−	+	−	+	−	−	+	−
88-4110H	O111	+	−	+	+	−	+	+	−	+	−
CL3	O113	−	+	−	+	−	−	+	−	+	−
DEC16A	O113	+	+	−	+	−	+	+	−	+	−
90-1787	O113	−	+	−	+	−	−	+	−	+	−
CL-15	O113	−	+	−	+	−	−	+	−	+	−
MDCH-4	O113	−	+	−	+	−	−	+	−	+	−
87-307	O113	−	+	−	+	−	−	+	−	+	−
24-35	O113	−	+	−	+	−	−	+	−	+	−
DEC16C	O113	−	+	−	+	−	−	+	−	+	−
M2101	O113	−	+	−	+	−	−	+	−	+	−
M2415	O113	−	+	−	−	−	−	+	−	−	−
M2443	O113	−	+	−	−	−	−	+	−	−	−
D167	O113	−	+	−	+	−	−	+	−	+	−
ECOR-30	O113	−	+	−	−	−	−	−	−	−	−
DA-69	O121	−	+	+	+	−	−	+	−	+	−
MT42	O121	−	+	+	+	−	−	+	−	+	−
403-3	O121	−	+	−	−	−	−	+	−	−	−
3-524	O121	−	+	+	−	−	−	+	−	−	−
F6173	O121	−	+	+	+	−	−	+	−	+	−
DA-1	O121	−	+	+	+	−	−	+	−	+	−
DA-5	O121	−	+	+	+	−	−	+	−	+	−
DA-37	O121	−	+	+	+	−	−	+	−	+	−
MT#2	O121	−	+	+	+	−	−	+	−	+	−
87-2914	O121	−	−	−	−	−	−	−	−	−	−
3377-85	O121	+	+	+	+	−	+	+	−	+	−
MT#11	O121	+	+	+	−	−	+	+	−	−	−
MT#22	O121	−	+	+	+	−	−	+	−	+	−
8153B-86	O125	+	−	+	+	−	+	−	−	+	−
BM2018	O128	−	−	+	−	−	−	−	−	−	−
BM2020	O128	−	−	+	−	−	−	−	−	−	−
BM2021[c]	O128	−	−	−	−	−	−	−	−	−	−
DEC11D	O128	−	−	+	−	−	−	−	−	−	−
DEC13E	O128	−	−	+	−	−	−	−	−	−	−
DEC14D	O128	−	+	−	+	−	−	+	−	+	−
DEC13D	O128	−	−	−	−	−	−	−	−	−	−
DEC14E	O128	+	+	+	+	−	+	+	−	−	−
DEC13C	O128	−	−	−	−	−	−	−	−	−	−
DEC13A	O128	−	−	−	−	−	−	−	−	−	−
DEC14B	O128	−	−	−	−	−	−	−	−	−	−
DEC14A	O128	−	−	−	−	−	−	−	−	−	−

Table 5 (*continued*)

Original ID	Serotype	IS-5P PCR					BAM PF-5P PCR				
		*stx*1	*stx*2	*eae*	*ehxA*	+93 *uidA*	*stx*1	*stx*2	*eae*	*ehxA*	+93 *uidA*
08-00007	O130	−	+	−	+	−	−	+	−	+	−
08-00022[b]	O136	+	−	−	+	−	+	−	−	+	−
GS-G5578620	O145	+	−	+	+	−	+	−	+	+	−
DEC101	O145	+	−	+	−	−	+	−	−	−	−
IHIT0304	O145	−	+	+	+	−	−	+	+	+	−
TB269C	O145	−	−	+	−	−	−	−	−	−	−
75-83	O145	+	−	+	+	−	+	−	−	+	−
MT#66	O145	+	−	+	+	−	+	−	+	+	−
BCL73	O145	−	−	+	−	−	−	−	−	−	−
314-S	O145	+	−	+	−	−	+	−	−	−	−
IH16	O145	−	+	+	+	−	−	+	−	+	−
02-3422	O145	−	−	+	−	−	−	−	−	−	−
4865/96	O145	+	+	+	+	+	+	+	+	+	+
DEC16E	O146	+	+	−	−	−	+	+	−	−	−
RDEC-1	O15	−	−	+	−	−	−	−	−	−	−
88-1509	O15	+	+	−	−	−	+	+	−	−	−
M2113	O156	+	+	+	+	−	+	+	−	+	−
S27a	O157	−	+	+	+	+	−	+	+	+	+
S103a	O157	+	+	+	+	+	+	+	+	+	+
S111a	O157	−	−	+	+	+	−	−	+	+	+
H19	O26	+	−	+	+	−	+	−	−	+	−
DEC10B	O26	+	−	+	+	−	+	−	−	+	−
DEC10C	O26	−	−	+	+	−	−	−	−	+	−
DEC9F	O26	−	−	+	−	−	−	−	−	−	−
TB285C	O26	+	−	+	+	−	+	−	−	+	−
VP30	O26	−	−	+	+	−	−	−	−	+	−
TB206A	O26	−	−	−	−	−	−	−	−	−	−
TB285A	O26	+	−	+	+	−	+	−	−	+	−
TB352A	O26	+	−	+	+	−	+	−	−	+	−
EK29	O26	+	−	+	+	−	+	−	−	+	−
97-3250	O26	+	+	+	+	−	+	+	+	+	−
B8026-C1	O45	+	−	+	+	−	+	−	−	+	−
B8227-C8	O45	+	−	+	+	−	+	−	−	+	−
B8828-C2	O45	+	−	+	+	−	+	−	−	+	−
08-00017[b]	O45	+	−	−	−	−	+	−	−	−	−
D88-28058	O45	+	−	+	+	−	+	−	−	+	−
DEC11C	O45	+	−	+	+	−	+	−	−	+	−
5431-72	O45	−	−	−	−	−	−	−	−	−	−
4309-65	O45	+	−	+	+	−	+	−	−	+	−
2566-58	O45	−	−	+	−	−	−	−	−	−	−
B8227-C8	O45	+	−	+	+	−	+	−	−	+	−
E-D-371	O45	−	−	+	+	+	−	−	+	+	+
DEC10J	O70	+	−	+	+	−	+	−	−	+	−
08-00025	O73	+	+	−	+	−	+	+	−	+	−
06-00236	O8	−	+	−	+	−	−	+	−	+	−
08-00024[b]	O8	−	+	−	+	−	−	+	−	+	−
B2F1	O91	−	+	−	+	−	−	+	−	+	−
23/67	O91	−	−	+	−	−	−	−	−	−	−
87-2927	O91	+	+	−	+	−	+	+	−	+	−
M710	O91	+	+	−	+	−	+	+	−	+	−
988/2	O91	+	+	−	−	−	+	+	−	−	−
1120/3	O91	+	+	−	−	−	+	+	−	−	−
852/3	O91	−	+	−	+	−	−	+	−	+	−
848/1	O91	−	+	−	+	−	−	+	−	+	−
907/1	O91	−	+	+	+	−	−	+	−	+	−
226-1	O91	+	+	−	−	−	+	+	−	−	−
68-ll-38	O91	−	+	−	−	−	−	+	−	−	−
07-00006	−	−	+	−	+	−	−	+	−	+	−
08-00015	−	+	+	−	−	−	+	+	−	−	−
08-00021[b]	−	−	+	−	−	−	−	+	−	−	−
AD4001-1B	−	+	+	−	−	−	+	+	−	−	−
AD4001-4B[d]	−	+	+	−	−	−	+	+	−	−	−

Except for the three strains above, all strains were provided by Michigan State University.

[a] Gray cells show the difference between IS-5P PCR and BAM PF-5P PCR.

[b] Ohio State Department of Agriculture, Reynoldsburg, OH

[c] Robert Mandrell, USDA-Agricultural Research Services, Albany, NY

[d] San Francisco-DO

136 bp for *ehxA*, and 382 bp for +93 *uidA*. PCR amplification products were separated by electrophoresis in an E-Gel® 2% agarose gel (Invitrogen, Grand Island, NY) and visualized on a UV transilluminator (G:Box, Imgen Technologies, Alexandria, VA). A molecular weight marker, TrackIt™ 100-bp DNA ladder (Invitrogen Life Technologies), was included in each gel.

The current FDA BAM PF-5P PCR methodology used a final concentration of 20 nM of each primer and the HotStar *Taq* enzyme

Table 6
The organisms in the 46-strain exclusivity test.

Organisms	ATCC
Enterobacter cloacae	13047
Enterobacter aerogenes	13048
Klebsiella oxytoca	13182
Serratia marcescens	13880
Streptococcus thermophilus	14485
Bacillus subtilis	14807
Edwardsiella tarda	15469
Leclercia adecarboxylata	23216
Citrobacter koseri	27028
Klebsiella pneumonia	29013
Providencia rettgeri	29944
Serratia ficaria	33105
Enterococcus faecalis	29212
Buttiauzella noakiae	51713
Citrobacter freundii	8090
Bacillus licheniformis	9789
Enterobacter helveticus sp. Nov	E440
Enterobacter novel species	E441
Enterobacter cloacae	E644
Enterobacter homaechei	E904
Enterobacter asburiae	E883
Enterobacter hormaechei	E890
Enterobacter turicensis, sp. Nov	E910
Enterobacter helveticus, sp. Nov	E912
Enterobacter novel species	E908
Enterobacter sakazakii	FDA
Yersinia pseudotuberculosis	Yp 1313
Yersinia enterocolitica	Ye 37
Salmonella enterica Typhimurium	14028
Salmonella enterica Enteritidis	FDA
Hafnia alvei	FDA
Morganella morganii	FDA
Edwardsiella tarda	FDA
Klebsiella pneumonia	FDA
Proteus hauseri	FDA
Pseudomonas aeruginosa	FDA
Serratia marcescens	FDA
Aeromonas hydrophila	FDA
Staphylococcus aureus	FDA
Streptococcus faecalis	FDA
Bacillus subtilis	FDA
Bacillus cereus	FDA
Listeria monocytogenes	FDA
Listeria innocua	FDA
Shigella flexneri	FDA
Shigella sonnei	FDA

(Qiagen) as a polymerase (Feng and Monday, 2000). The PCR conditions were 95 °C for 15 min, then 25 cycles at 95 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min, plus a 72 °C 5-min final extension step. The current FDA BAM PF-5P PCR was performed with the Qiagen Fast Cycling PCR master mix using PCR conditions identical to the ones used for IS-5P (see above) and therefore named modified PF-5P. This shortened the FDA BAM PF-5P PCR from 145 min to 75 min. In addition, the modified PF-5P PCR contained twice the concentration of the two *ehxA* primers to increase the intensity level of the *ehxA* amplification product (data not shown).

2.2. Bacterial strains and DNA preparation

A total of 212 STEC strains from various sources and geographical locations were collected to test the inclusivity of the IS-5P PCR. This strain collection included six *stx*2 variants, 22 *eae* alleles, and six *ehxA* subtypes (Table 2).

Six STECs that were previously tested when PF-5P was developed were provided by Dr. Peter Feng, FDA, College Park, MD (Table 3). Thirty-eight STEC food isolates obtained during the Microbiological Data Program (MDP), a national food-borne pathogen monitoring program conducted from 2001 to 2012 (http://www.ams.usda.gov/mdp), were provided by Dr. Shanker Reddy, USDA, Washington D.C. (Table 4). One hundred thirty-four STEC strains (Table 5) were obtained from the STEC Center at Michigan State University's National Food Safety and Toxicology Center, East Lansing, MI; the Ohio State Department of Agriculture; Dr. Robert Mandrell, USDA-ARS, Albany, CA; and the FDA Pacific Regional Lab-Northwest (PRL-NW), Applied Technology Center (ATC), Bothell, WA. *E. coli* strain EDL933 (ATCC® 43895™, O157:H7, *stx*1, *stx*2, *eae*-γ1, *ehxA*, +93 *uidA*) was used as a positive control. Forty-six non-*E. coli* isolates were used to test for false positive results (Table 6).

All isolates were grown on tryptic soy agar at 37 °C for 24 h. Bacterial DNA was extracted from colonies using the InstaGene™ Matrix (Bio-Rad, Hercules, CA) as described by the manufacturer. The DNA preparations were stored at −20 °C until use.

2.3. Preparation of inocula for enrichment procedure

Three STEC strains of serotypes O26, O103, and O121 (Table 7) were inoculated into baby spinach, alfalfa sprouts, and cilantro. Approximately 35 mL of Tryptic Soy Broth was inoculated with a single colony of *E. coli* and incubated with agitation for 24–28 h at 35 °C. The culture was centrifuged at 3000 × g and the pellet was suspended in 10 mL of Butterfield's phosphate buffer (BPB, pH 6.8–7.2). The bacterial cells were washed twice with 10 mL of BPB and resuspended in 25 mL of BPB. Dilutions were made to reach an inoculum level of 0.035 CFU/g (baby spinach), 0.05 CFU/g (cilantro), and 0.06 CFU/g (alfalfa sprouts); levels that would provide fractionally positive results when empirically determined. Results are considered fractionally positive when some of the test results are positive and some are negative. Preferably 50% of the total tests are positive per experiment.

2.4. Surface inoculation of fresh produce

Bagged baby spinach, alfalfa sprouts, and bunches of cilantro were purchased from local supermarkets in the Washington, D.C., metropolitan area. Approximately 300 g of each product (baby spinach, cilantro, and alfalfa sprouts) were spray inoculated with 8 mL of inocula at levels that would provide fractionally positive results. After spray inoculation, the inoculated produce was mixed gently but thoroughly with either sterile tongs or by hand wearing sterile gloves for 15 min. The inoculated produce was placed in clean, sterile laboratory totes for 48 h at 4 °C, loosely covered with aluminum foil. Each experiment per product and per strain was replicated four times independently.

2.5. Enrichment procedure for fresh produce

Each fresh produce sample was divided into four 25 g test portions. Two hundred and twenty-five mL of single-strength (1×) Modified Buffered Peptone water with pyruvate (mBPWp) was added to each test portion in a sterile resealable plastic bag, agitated gently by hand for 15 s, and incubated at 37 °C ± 1 °C for 5 h (Kase et al., 2012). Then 1 mL of Acriflavine, Cefsulodin, and Vancomycin Supplements was added as described in the BAM (Feng et al., 2011a,b). All test portions were incubated at 42 °C ± 1 °C static for 18 h. Uninoculated fresh produce portions were processed alongside the inoculated samples and enriched, as described above, to demonstrate the absence of cross-contamination when the inoculated material was processed.

Table 7
Comparative results of IS-5P PCR and BAM PF-5P PCR in *E. coli* non-O157:H7 strains from three inoculated fresh produces with enrichment broth.

E. coli Strain	Inoculation level (CFU/g)	Produce type	IS-5P PCR					BAM PF-5P PCR				
			*stx*1	*stx*2	*eae*	*ehxA*	+93 *uidA*	*stx*1	*stx*2	*eae*	*ehxA*	+93 *uidA*
O26	0.043	Baby spinach	+	–	+[a]	+	–	+	–	–	+	–
			+	–	+	+	–	+	–	–	+	–
			+	–	+	+	–	+	–	–	+	–
			+	–	+	+	–	+	–	–	+	–
		Result	Pos[b]	Neg[c]	Pos	Pos	Neg	Pos	Neg	Neg	Pos	Neg
	0.046	Cilantro	–	–	–	–	–	–	–	–	–	–
			–	–	–	–	–	–	–	–	–	–
			+	–	+	+	–	+	–	–	+	–
			–	–	–	–	–	–	–	–	–	–
		Result	Pos	Neg	Pos	Pos	Neg	Pos	Neg	Neg	Pos	Neg
	0.048	Alfalfa sprouts	+	–	+	+	–	+	–	–	+	–
			–	–	–	–	–	–	–	–	–	–
			+	–	+	+	–	+	–	–	+	–
			+	–	+	+	–	+	–	–	+	–
		Result	Pos	Neg	Pos	Pos	Neg	Pos	Neg	Neg	Pos	Neg
O103	0.035	Baby spinach	+	–	+	+	–	+	–	–	+	–
			–	–	–	–	–	–	–	–	–	–
			+	–	+	+	–	–	–	–	–	–
			–	–	–	–	–	–	–	–	–	–
		Result	Pos	Neg	Pos	Pos	Neg	Pos	Neg	Neg	Pos	Neg
	0.043	Cilantro	+	–	+	+	–	+	–	–	+	–
			+	–	+	+	–	+	–	–	+	–
			+	–	+	+	–	+	–	–	+	–
			–	–	–	–	–	–	–	–	–	–
		Result	Pos	Neg	Pos	Pos	Neg	Pos	Neg	Neg	Pos	Neg
	0.044	Alfalfa sprouts	–	–	–	–	–	–	–	–	–	–
			–	–	–	–	–	–	–	–	–	–
			+	–	+	+	–	+	–	–	+	–
			+	–	+	+	–	+	–	–	+	–
		Result	Pos	Neg	Pos	Pos	Neg	Pos	Neg	Neg	Pos	Neg
O121	0.035	Baby spinach	–	+	+	+	–	–	–	–	–	–
			–	+	+	+	–	–	+	–	+	–
			–	+	+	+	–	–	+	–	+	–
			–	–	–	–	–	–	–	–	–	–
		Result	Neg	Pos	Pos	Pos	Neg	Neg	Pos	Neg	Pos	Neg
	0.038	Cilantro	–	+	+	+	–	–	+	–	+	–
			–	+	+	+	–	–	+	–	+	–
			–	+	+	+	–	–	+	–	–	–
			–	+	+	+	–	–	+	–	+	–
		Result	Neg	Pos	Pos	Pos	Neg	Neg	Pos	Neg	Pos	Neg
	0.054	Alfalfa sprouts	–	+	+	+	–	–	+	–	+	–
			–	+	+	+	–	–	+	–	+	–
			–	+	+	+	–	–	+	–	+	–
			–	+	+	+	–	–	+	–	+	–
		Result	Neg	Pos	Pos	Pos	Neg	Neg	Pos	Neg	Pos	Neg

[a] Gray cells show the difference between IS-5P PCR and BAM PF-5P PCR.
[b] If there is one positive in four replications, the final result was considered as a positive in targeted gene.
[c] If there is no positive in four replications, the final result was considered as a negative in targeted gene.

2.6. Template DNA preparation after enrichment

One mL of overnight culture was transferred to a micro-centrifuge tube and centrifuged 12,000 × g for 3 min. The supernatant was removed and the pellet was resuspended in 1 mL of 0.85% NaCl. After another centrifugation at 12,000 × g for 3 min, the supernatant was removed and the pellet was resuspended in 1 mL of sterile water. After boiling at 100 °C for 10 min and being centrifuged at 12,000 × g for 1 min, the supernatant was saved as a template DNA for PCR analysis.

3. Results and discussion

More than 100 STEC serotypes have been associated with human diseases (Johnson et al., 2006; Bettelheim, 2000), including mild to bloody diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS) (Mead and Griffin, 1998; Besser et al., 1999). The nine most clinically relevant non-O157 STECs belong to serogroups O26, O103, O111, O145, O91, O113, O128, O45, and O121 (CDC, 2006). These O serogroups were targeted when we designed the 10 primers to detect *stx*1, *stx*2, *eae*, *ehxA*, and +93 *uidA* SNP (O157). The primers directed against *stx*1, *stx*2, *ehxA*, and *uidA* were located in conserved areas of the respective genes in both the IS-5P and PF-5P PCR method (Supplemental Fig. S1), but only the *eae* primers in IS-5P recognized the 21 different *eae* alleles subtypes (Fig. 1).

When used with the Qiagen Fast-Cycling PCR kit, the 10 primers designed in this study detected the five targeted genes in 75 min from start to finish. The EDL933 O157:H7 (*stx*2a)-positive control strain exhibited five well-separated bands, as expected, and all of the STEC DNAs had one to five amplification products (Fig. 2 and Tables 2–5). None of the strains listed in our exclusivity panel (Table 6) reacted with the IS-5P, showing the specificity of our multiplex assay (data not shown). However, *Shigella dysenteriae* serotype 1 also produced Shiga toxin (*stx*1) (Leyva-Llades et al., 2012). Seven *S. dysenteriae* serotype 1 (CDC#84-305, CDC#87-3334, CDC#95-3140, CDC#97-3005, CDC#99-3110, ATCC29026, ATCC9361) tested with the IS-5P showed to be positive to *stx*1 (data not shown).

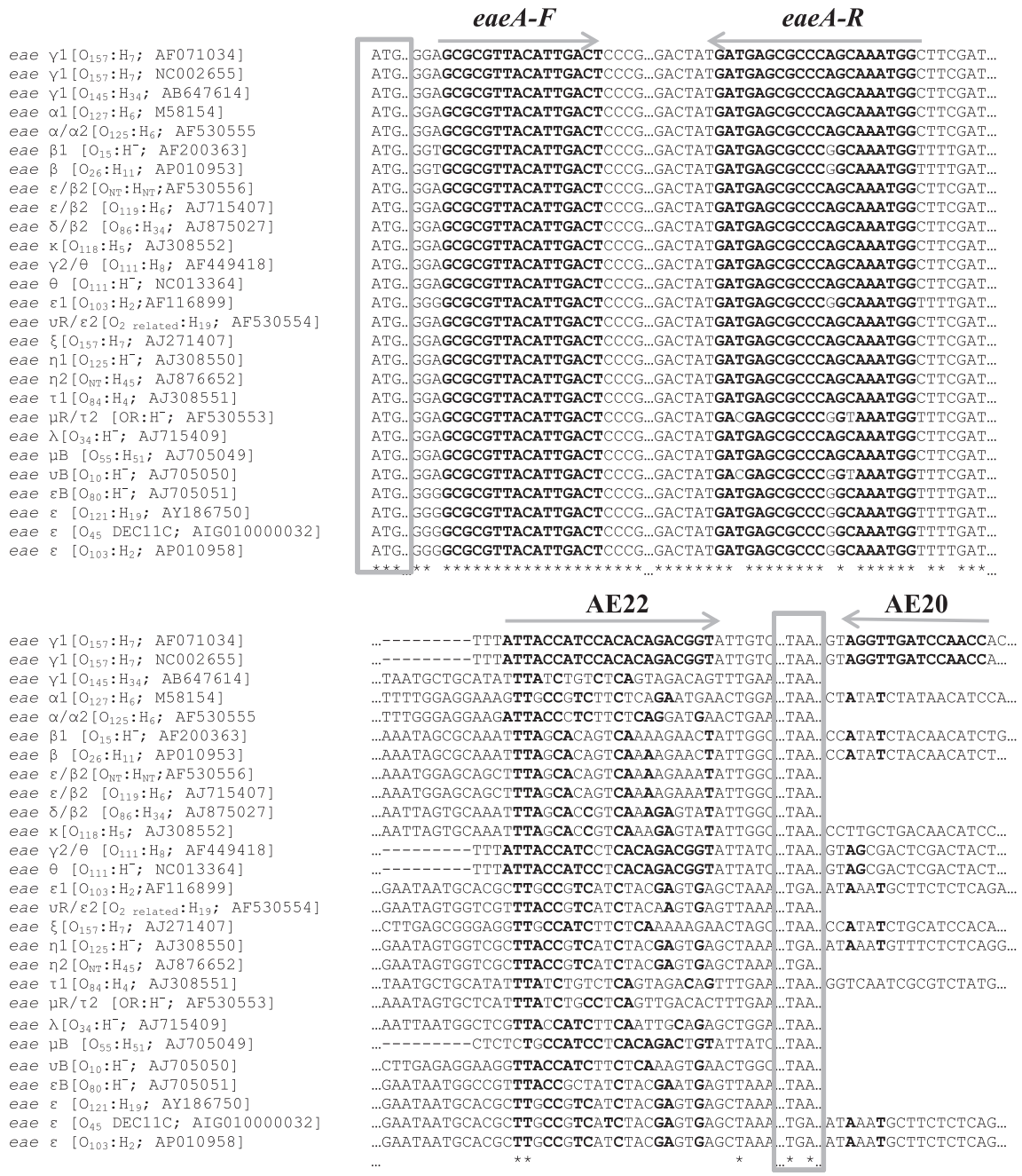

Fig. 1. Position of primers used in IS-5P and BAM PF-5P on partial sequence alignment of 27 *eae* genes belonging to 21 variant groups (Blanco et al., 2005). Alignments were generated using Clustal w (www.genome.jp/tools/clustalw/) (Thompson et al., 1994). Each sequence is identified by the accession number, preceded by the serotype of the *E. coli* strain, when appropriate. The start codons and the stop codons are boxed, and identical nucleotides are identified with the mark "*".

The pathogenesis of STEC infections in humans is not fully understood; but the main pathogenetic entities of HUS are thought to be the presence of *stx* genes (*stx*1 and *stx*2) (Eklund et al., 2002). Also, some STEC serotypes as known EHEC with non-Shiga toxin virulence gene were associated with bloody diarrhea, which progressed to HUS (Bielaszewska et al., 2008). New nomenclatures describe the *stx*1 group as the *stx*1a, *stx*1c, and *stx*1d subtypes (Margot et al., 2013). The subtyping nomenclature proposals and discussions held in 2009 at the 7th International Symposium on Shiga Toxin (Verocytotoxin)-Producing *E. coli* Infections in Buenos Aires reported seven *stx*2 subtypes (2a, 2b, 2c, 2d, 2e, 2f, and 2g) (Feng et al., 2011a,b). Our IS-5P PCR detected six *stx*2 variants (2a, 2b, 2c, 2d, 2e, 2g), similar to the current BAM PF-5P (Table 2). Moreover, ECOR-30 (O113; Table 5) was *stx*2-positive in our IS-5P PCR, but negative in the BAM PF-5P PCR. Neither PCR method can capture *stx*2f-positive STECs (data not shown), but previous studies showed that *stx*2f has only been found in the stools of feral pigeons (Schmidt et al., 2000) and has rarely caused severe human illness (van Duynhoven, 2008).

Descriptions and classifications of *eae* (intimin) gene variants have been published previously (Blanco et al., 2003, 2004; Mora et al., 2007). The *eae* primers in our IS-5P PCR were designed to detect *eae* universally, and indeed all 22 *eae* variants showed a 245 bp amplification product (Table 2). This is an improvement over

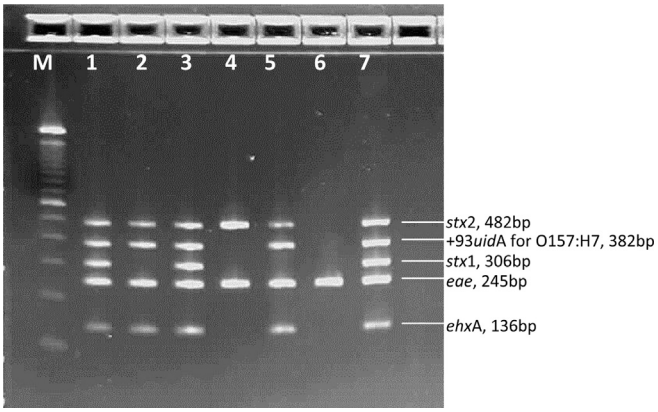

Fig. 2. An E-Gel 2% agarose gel electrophoresis of DNA fragments amplified by IS-5P PCR. Lanes, M; Molecular weight marker, 1; EDL933 (O157:H7); positive control, 2; 493/89 (O157:H$^-$); 3, G5101 (O157:H7); 4, 5905 (O55:H7); 5, MA6 (O rough:H7); 6, 5A (O55:H7); 7, EDL933. The amplified products (size: base pairs) from EDL933 as a positive control are shown in Lanes 1 and 7 and consist of *stx*2 (482 bp), +93 *uidA* (382 bp), *stx*1 (306 bp), *eae* (245 bp), and *ehxA* (245 bp).

the FDA BAM PF-5P PCR that can only detect the O157:H7 γ-*eae* gene (Feng and Monday, 2000) and the *eae* variants γ, ζ, and ι.

In Table 3, the data on six STEC strains tested in a previous study (Feng and Monday, 2000) using the BAM PF-5P PCR were examined and compared to the data from our PCR assay and the modified PF-5P PCR. Both 5P PCRs showed the same amplification patterns. The modified PF-5P PCR failed to detect *eae* in strain TT4 (O165:H25), in contrast to the IS-5P PCR, which did detect *eae* in this strain. Overall, the amplification results for *eae* in both PCR methods demonstrate that IS-5P PCR can detect more *eae* variants (Tables 2and 3).

The IS-5P PCR was also evaluated using foodborne STEC strains collected through the USDA MDP program that surveyed selected agricultural commodities for the presence of food-borne pathogens, including STEC (http://www.ams.usda.gov/mdp). All MDP STEC strains reacted comparably with both 5P PCR methods except for strain MDP-04-02745 that showed the presence of *eae* only when the IS-5P PCR assay was used (Table 4). The most substantial differences between the IS-5P and BAM PF-5P multiplex assays were seen in the examination of 134 STEC strains collected from various sources (Table 5). IS-5P PCR detected *eae* in 73 more strains than BAM PF-5P PCR in this setting. This can be explained by the difference in the detection of *eae* variants shown in Table 2.

Enterohemolysin, encoded by the *ehxA* gene, is present in most O157:H7 strains and is located on the EHEC large virulence plasmid. The *ehxA* gene is highly conserved in STEC strains (Boerlin et al., 1998). All six *ehxA* subtypes were detected by both 5P PCR assays (Table 2), but DEC14E (O128) was *ehxA*-positive in our IS-5P PCR (Table 5) and *ehxA*-negative in the BAM PF-5P PCR.

The specificity of IS-5P PCR was also tested using non-STEC bacteria strains. A total of 46 strains were examined and showed no false positive results (Table 6).

The polysaccharides and phenolic compounds in plant-based material contain PCR inhibitors that can be released into enrichment media and interfere with target DNA amplification (Wilson, 1997). To determine the detection efficiency of the IS-5P PCR and modified PF-5P PCR, baby spinach, cilantro, and alfalfa sprouts were artificially inoculated with three different STEC strains (O26, O103, and O121) and both assays were run on enrichment broths from each sample. Overall, both 5P PCR methods led to identical results with a few exceptions (Table 7). For instance, the *eae* gene in all tested produces and strains was detected using IS-5P PCR, but PF-5P PCR did not detect it. Amplification product(s) corresponding to *stx*1 and *ehxA*, *stx*2 and *ehxA*, or *ehxA* alone were detected in enrichment broths from baby spinach inoculated with an O103 STEC strain, baby spinach inoculated with an O121 strain, and cilantro inoculated with another O121 STEC strain, respectively.

In conclusion, EHEC are a subset of STEC and comprised of human pathogenic strains. Many public health agencies have focused only on O157:H7. However, the presence of potential pathogenic STEC in food products intended for human consumption is of concern. The fast and accurate detection of certain *E. coli* genes can identify the presence of harmful STEC strains so that the contaminated food can be quickly identified and proper action taken to prevent illness. Our study showed that our IS-5P multiplex PCR assay can detect five different genes – *stx*1, *stx*2, *eae*, *ehxA* and +93 *uidA* – in various STEC strains in a little over an hour. Importantly, this assay can also detect virulence factors in the nine most clinically relevant STEC O serogroups. If one of five bands in the IS-5P multiplex PCR is positive, STEC molecular serotyping (Lin et al., 2011) suggests being performed. The IS-5P PCR offers several important advantages over the current BAM PF-5P PCR. First, it is more rapid, taking only 75 min compared to 2hr 25 min for the BAM PF-5P, although we cut the time for the BAM PF-5P down to 75 min when we adapted it to the Qiagen Fast Cycling PCR kit. Second, although both assays target the same virulence genes, *ehxA* was only detected by the IS-5P assay (Table 5). Third, only IS-5P consistently recognized all the *eae* gene variants. The superior detection ability of the IS-5P assay means that more contaminated produce will be identified and it will be removed from the food supply as soon as possible, preventing outbreaks of human disease.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2013.11.016.

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